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High local trait variability in a globally invasive cyanobacterium

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Abstract

During the last couple of decades, invasive species have become a worldwide problem in many freshwater systems. Besides higher plants and animals, microbes, in particular the potentially toxic cyanobacterium *Cylindrospermopsis raciborskii*, has attracted increasing attention, due to its spread towards temperate zones of the northern and southern hemisphere. A number of advantageous functional traits and a high intraspecific plasticity have been suggested to explain its invasion success. The aim of this study was to examine intraspecific functional trait variability of 12 different isolates of *C. raciborskii* originating from different lakes in an invaded region in Northeast Germany. We measured growth rate, C:N:P ratios, chlorophyll-*a* content and the abundance of heterocysts under nutrient-replete and phosphorus-limited conditions. Moreover, the isolate-specific morphology and grazing losses by an herbivorous rotifer, as a top-down force, were studied. DNA fingerprinting revealed that all isolates were genetically different. *C. raciborskii* exhibited a large variability in all measured traits among isolates. The C:P, N:P and chl-*a*:C ratios differed by a factor of two or more. The trait variability among isolates was higher under nutrient-replete conditions, except for the C:P ratio, which varied most during phosphorus limitation. The susceptibility to grazing, calculated as maximum ingestion rates of *Brachionus calyciflorus* on *C. raciborskii,* varied most among isolates, but was not related to any of the measured physiological or morphological traits i.e. no trade-off was found. Ecological and genetic clustering did not match, indicating that the genetic relationship based on DNA fingerprinting did not cover ecological differences. Our results show a high trait variability within locally occurring and partly co-occurring *C. raciborskii* isolates. No overall trade-offs between the measured functional traits were found. This demonstrates the ecological relevance of linking multiple traits e.g. competitive and consumptive.

Furthermore, this study emphasises the importance of analysing more than one strain of a species, as different strains show different trait values potentially relevant for their invasibility and the field of general trait-based ecology.

Introduction

Biological invasions in freshwater systems are one of the biggest threats to biodiversity (Sala *et al.*, 2000) and invasive species occur at all taxonomic levels from animals and plants to bacteria. However, invasions of microorganisms are often overlooked due to their small size and therefore our knowledge of invasions of aquatic free-living microorganisms is still limited compared to that from larger aquatic plants or animals (Litchman, 2010). One of the few invasive microorganisms that has raised attention is the invasive, bloom forming tropical cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju, which has expanded its global distribution during recent decades. Now it is a common, sometimes dominating species, in the temperate zones of Europe and North America (Padisák, 1997; Hamilton *et al.*, 2005; Wiedner *et al.*, 2007; Sinha *et al.*, 2012; Antunes, Leão & Vasconcelos, 2015; Kokociński *et al.*, 2017).

One explanation for the success of invasive species is their advantage through particular functional traits that promote invasions into new communities and habitats. However, *C. raciborskii* has similar traits as, for example *Anabaena*, *Aphanizomenon* and *Nostoc* - other common cyanobacteria species in the temperate zones (Padisák, 1997; Wu, Shi & Li, 2009; Mehnert *et al.*, 2010; Plominsky *et al.*, 2013). These traits include the ability to grow in warmer waters, the storage capacity of phosphorus, the formation of resting stages (= akinetes) and dinitrogen fixation (Istvánovics *et al.*, 2000; Carey *et al.*, 2012; Sukenik *et al.*, 2012; Thomas & Litchman, 2016). Therefore, one crucial trait for *C. raciborskii* might be a better resource use under nutrient limitation. In this case, a lower nutrient to carbon ratio (cell quota) is expected to lead to a comparably higher gain of biomass per unit of nutrient in limiting environments. It is also known that *C. raciborskii* has a high plasticity and a wide intraspecific variety in those resource use traits, like photosynthesis performance and growth rate facilitating invasions in new, variable habitats (Saker & Neilan, 2001; Briand *et al.*, 2004; Wu *et al.*, 2009; Piccini *et al.*, 2011; Bonilla *et al.*, 2012; Pierangelini *et al.*, 2014).

Besides these bottom-up traits, top-down control also drives the invasiveness of species. For freshwater systems, it has been found that the consumptive resistance is often higher than the competitive resistance (Alofs & Jackson, 2014). *C. raciborskii* is typically considered of poor food quality for herbivores and hardly edible due to its shape (Panosso & Lürling, 2010) similar to filamentous cyanobacteria in general (e.g. Lampert,

1987). Nevertheless, some zooplankton species such as *Brachionus calyciflorus* have exhibited intermediate growth with *C. raciborskii* as the only food source (Soares, Lürling & Huszar, 2010). However, contrasting results have also been found where *C. raciborskii* was toxic to zooplankton (e.g. Nogueira *et al.,* 2004). Overall, the grazing loss of *C. raciborskii* appears to be driven by the interplay of morphology and toxicity, which are both very flexible traits (Neilan *et al.*, 2003; Vidal & Kruk, 2008; Rangel *et al.*, 2016; Burford *et al.*, 2016). From a population point of view, flexible traits and intraspecific trait variations are regarded as key factors for invasion success (Smith, 2009; Engel, Tollrian & Jeschke, 2011). Only recently, trait variations among isolates were studied among an Australian *C. raciborskii* population (Willis *et al.*, 2016b), considered as native rather than invasive. To understand the role of trait variation for successful invasions, a comprehensive analysis of both, bottom-up and top-down traits of different genotypes is needed from an invaded region.

The aim of this study was to analyse the variability of functional traits of *C. raciborskii* that potentially influence its invasion success. Traits considered in this study were: growth rate, nutrient to carbon ratios and phosphorus-uptake under low and high nutrient conditions as well as grazing loss. To account for the variability of *C. raciborskii*, the experiments were conducted with twelve isolates from one local region in Northeast Germany where it is frequently found (Mischke, 2003; Fastner *et al.*, 2003; Stüken *et al.*, 2006). Short tandem repetitive DNA fingerprinting was used to estimate the genetic distance among isolates (Mazel *et al.*, 1990; Rasmussen & Svenning, 1998).

Methods

Twelve isolates of *C. raciborskii,* all with straight filaments, were obtained from six lakes in Northeast Germany. The year of isolation ranged from 2004 to 2010 (Table 1) and since then, they were kept in stock cultures under the same conditions.

Table 1: List of used German *Cylindrospermopsis raciborskii* isolates with sampling date and point with federal state and coordinates. BB = Brandenburg, MWP = Mecklenburg-West Pomerania; N = North, E = East. Indexed numbers indicate published studies with isolates: Haande *et al.,* 2008[1], Mehnert *et al.,* 2010[2], Mehnert, Rücker & Wiedner 2014[3], Ramm *et al.,* 2012[4], Sinha *et al.,* 2012[5], Sperfeld *et al.,* 2010[6], Weithoff, Taube & Bolius 2017[7].

Isolate	Isolation date	Lake, State, Location
19F6 [2, 3, 4]	17.08.2004	Melangsee, BB, 52°09'40"N 13°59'18"E
22F8	22.08.2004	Heiliger See, BB, 52°24'44"N 13°04'18"E
26D9 [2, 4, 7]	09.08.2004	Rangsdorfer See, BB, 52°17'19"N 13°24'14"E
27F11	31.08.2004	Melangsee, BB, 52°09'40"N 13°59'18"E
AB2008/71	25.08.2008	Melangsee, BB, 52°09'40"N 13°59'18"E
MEL07[7]	10.03.2010	Melangsee, BB, 52°09'40"N 13°59'18"E
Peter07_149	25.07.2007	Petersdorfer See, BB, 52°18'52"N 14°04'27"E
Peter07_163	25.07.2007	Petersdorfer See, BB, 52°18'52"N, 14°04'27"E
Peter09.1	25.07.2007	Petersdorfer See, BB, 52°18'52"N 14°04'27"E
SP08_4	06.08.2008	Stolpsee, BB, 53°10'31"N 13°12'28"E
ZIE05 [1, 5]	07.09.2005	Zierker See, MWP, 53°21'44"N 13°02'17"E
ZIE11 [1 - 4, 6, 7]	07.09.2005	Zierker See, MWP, 53°21'44"N 13°02'17"E

These isolates were examined for their genetic variability by DNA fingerprinting and their ecophysiological flexibility in top-down and bottom-up traits in laboratory experiments.

DNA fingerprinting

We used DNA fingerprinting to search for intraspecific heterogeneity and to distinguish between isolates (Wilson *et al.*, 2000; Saker & Neilan, 2001). DNA from all isolates was extracted from live cells (Metagenomic DNA Isolation Kit for Water epicentre, Madison, Wisconsin, USA) and a short tandemly repeated repetitive (STRR) sequence was amplified with the STRR1A primer (5'-CCAATCCCCAATCCCC-3'; Rasmussen & Svenning 1998). For the amplification a polymerase chain reaction (PCR) was run in 20 µl aqueous reaction volume containing 1x PCR buffer**,** 200 µM dNTPs, 0.5 M primer, 0.02 U μl^{-1} *Phusion* High-Fidelity DNA polymerase (Thermo Fisher Scientific GmbH, Dreieich, Germany) and approx. 10 ng of DNA. Initial denaturation was at 98 °C for 1 minute, followed by 35 cycles of denaturation at 98 °C for 1 minute, annealing at 56 °C for 1 minute, extension at 65 °C for 5 minutes and a final extension at 65 °C for 15 minutes with the final step at 4 °C (Rasmussen & Svenning, 1998). PCR products and size markers (Lambda DNA-*Pst*I digest, Fermentas Life Sciences/Thermo Fisher; O'GeneRuler Low Range DNA Ladder, ready-to-use, Thermo Fisher) were visualized on a 1 % agarose gel stained with

ethidium bromide and documented under UV light on ChemiDoc™XRS+ Imager and software (Bio-Rad Laboratories, Hercules, California, USA). The band patterns, generated for each isolate by STRR1A primer, were transformed into binary (presence/absence) data. From this data, the Jaccard similarity index between isolates was calculated and a cluster constructed using the Unweighted Pair Group Method with Arithmetic Average (UPGMA, in R (R Development Core Team, 2008; RStudio 0.99.486) with packages "vegan" (Oksanen *et al.*, 2015) and "ape" (Paradis, Claude & Strimmer, 2004)).

Resource use experiments

Two experiments were conducted to analyse the intraspecific variability of relevant physiological traits of *C. raciborskii*. The first experiment was run under semi-continuous conditions at low cell densities to enable exponential growth. This reflects growth conditions in a eutrophic lake, before seasonal nutrient limitation. A second experiment was run in a batch system mimicking conditions under increasing nutrient depletion within a cyanobacterial bloom. Both experiments were carried out in phosphorus (P)-reduced Woods Hole (WC) medium (after Nichols, 1973; 2 mM HEPES buffer, pH 8, 80 µg P L^{-1}, nitrogen (N):P = 20:1). A concentration of 80 µg L^{-1}/2.581 µmol P was chosen to mimic typical eutrophic temperate lake conditions. Experiments were run at 20 °C at a 16:8 h light:dark cycle and an average light intensity of 130 µmol photons m^{-1} s^{-1} measured in water with a spherical light sensor (Li-Cor, SQSA 0107, WALZ Mess-& Regeltechnik, Effeltrich, Germany). Experimental flasks were gently shaken 15 min h^{-1} to keep cyanobacteria in suspension.

Semi-continuous experiment

A pre-culture of 650 ml of each isolate was set up at an optical density (OD at 800 nm, 5 cm cuvette, spectrophotometer UV Mini 1240 UV-VIS, Shimadzu, Kyoto, Japan) of 0.1, which is far below the carrying capacity under these conditions (see below). From these pre-cultures, 150 ml suspension was transferred into 300 ml Erlenmeyer flasks and kept as "semi-continuous turbidostat" cultures at an OD_{800} of 0.1, in quadruplicate for each isolate. Every 24 h the OD_{800} of a subsample was measured and cultures were diluted to the target OD of 0.1 with fresh medium. After 6 days of acclimation, the growth rates (µ) were calculated for six consecutive days according to:

$$\mu = \frac{\ln\left(\frac{OD_1}{OD_0}\right)}{t_1 - t_0} \qquad (1)$$

where OD_1 is the OD_{800} of the culture at t_1 and OD_0 the initial OD_{800} of 0.1 at t_0; i.e. the dilution rate equals the growth rate per day. At the final day, samples were taken for the

analysis of particulate carbon (C) and nitrogen on pre-combusted glass fibre filters (GF/C, 25 mm, Whatman International Ltd, Maidstone, UK), for particulate phosphate on 0.45 µm membrane filters (25 mm, PALL Cooperation, Port Washington, New York, USA) and for chlorophyll-*a* (chl-*a*) on GF/C filters by gentle vacuum-filtrations.

Batch experiment

With the batch experiment we analysed the temporal ecophysiological changes of *C. raciborskii* during 20 days without any exchange of medium, which led to severe nutrient limitation towards the end of the experiment. Pre-cultures in P-reduced WC medium (as above) were kept at an OD_{800} of 0.1 for 4 days before starting the experiment (day 0) with 400 ml suspension of approx. OD_{800} of 0.1 in 500 ml Erlenmeyer flasks in quadruplicate. At days 0, 2, 5, 8, 12, 16 and 20 samples were taken for the analysis of particulate carbon, nitrogen and phosphorus and chl-*a* (as above). After 20 days, all isolates had reached or were very close to their carrying capacity (Supplementary Material Fig. S1), thus we regard the cell quotas at day 20 as minimal cell quotas. Additionally, we calculated the number of vegetative cells per heterocyst as an indicator of the response of *C. raciborskii* to nitrogen limitation, a second measure, the number of heterocysts per unit of particulate carbon, was determined to relate nitrogen fixation to carbon fixation. At day 20, the cultures received a phosphorus-pulse (+ modified WC medium with 4 mg P L^{-1}) overnight (15 h) to determine their phosphate uptake and storage capacity (day 21).

We compared the ecological trait values from the semi-continuous experiment with the values from day 20 of the batch experiment - representing “good” nutrient conditions with exponential growth and “bad” conditions of severe nutrient limitation respectively.

Grazing losses

We calculated the grazing losses of *C. raciborskii* as the ingestion rate of the generalist, herbivorous rotifer *B. calyciflorus* following the presence/absence method as in Weithoff (2005). Prior to the experiment, *B. calyciflorus* was pre-cultured for two days with the green algae *Monoraphidium minutum* (SAG 243-1, Culture Collection of Algae, Göttingen, Germany) at a saturating food density of approx. 2.5 mg C L^{-1} to ensure good physiological state (Fussmann, Weithoff & Yoshida, 2005). 20 individuals of *B. calyciflorus* were transferred to 3.5 ml glass vials with 6-7 concentrations of *C. raciborskii* (4 - 6 replicates each), pre-cultured in P-replete medium. Glass vials were kept at 20 °C in darkness to prevent *C. raciborskii* growth and were gently shaken to avoid sedimentation. After 24 h, the experiment was terminated by adding Lugol’s iodine solution. Samples without *B. calyciflorus* served as a control to include the (possible) cyanobacteria growth in the calculation. The biovolume of *C. raciborskii* was measured at the start and after 24 h to

calculate *C. raciborskii* growth/decline. Filtration rate (f) of the herbivore was calculated as:

$$f = \frac{V(\ln C_{t0} - \ln C_{t1})}{NDt} \quad (2)$$

where V is the experimental volume, C_{t0} and C_{t1} are the densities of *C. raciborskii* at the start (t0) and after 24 h (t1) and N is the abundance of *B. calyciflorus* in the experiment. Dt is the duration of the experiment. Ingestion rate (I) is filtration rate multiplied by *C. raciborskii* density. The data were fitted to the Holling type II functional response. I_{max} (maximal ingestion per individual and time) and k_s (resource concentration at $I_{max}/2$) were calculated by least square regression using IBM SPSS Statistics (24.0). For the isolate Peter09.1 a unimodal relationship was found and I_{max} was set fixed at the maximum of the curve and the k_s value was calculated from that. The isolate ZIE11 exhibited no saturation and the results were discarded from further analysis.

Analyses

The particulate carbon and nitrogen content of the cells were determined in triplicate using an elementary analyser (EA 3000, EuroVector S.p.A., Milan, Italy). Particulate phosphorus was measured photometrically (also in triplicate) after digestion with H_2SO_4, $K_2S_2O_8$ and 1 h autoclaving at 121 °C using the molybdate blue method according to Murphy & Riley (1962) at 880 nm (UV Mini 1240 UV-VIS spectrophotometer, Shimadzu). Chl-*a* was extracted from filters in hot, 60 °C, 90 % ethanol overnight, followed by fluorometric measurement (Welschmeyer, 1994) with chl-*a* standards in a fluorometer (TD 700, Turner Designs, Sunnyvale, California, USA).

The abundance of *C. raciborskii* was determined by counting the filaments according to the Utermöhl technique after fixation with Lugol´s iodine (Utermöhl 1958; Axio Observer, Carl Zeiss, Jena, Germany; TSO Thalheim Spezialoptik GmbH, Pulsnitz, Germany). The length and width of *C. raciborskii* filaments were measured (AxioVision Software Zen 2; Carl Zeiss; TSO-VJD-MESS-HY Version 3.4) and the biovolume was calculated assuming a cylindrical shape (Hillebrand *et al*., 1999). The heterocysts and the vegetative cells of *C. raciborskii* and the abundance of *B. calyciflorus* were counted accordingly at an inverted light microscope (Axio Observer; TSO).

Statistical analyses

Data were statistically analysed by using SigmaPlot (13.0) and IBM SPSS Statistics (24.0). For the difference between experiments a two-tailed t-test or Mann-Whitney Rank Sum Test, in case of unequal variance, were carried out. We applied linear regression models to test for correlations between traits. Principal component analysis was run using R. To

compare the variation of the different trait values we calculated the coefficient of variation (CV) as the standard deviation divided by the mean.

Results

DNA fingerprinting

The DNA fingerprinting displayed a unique band pattern for each *C. raciborskii* isolate (Fig. 1 A), confirming that all isolates were genetically different. While some PCR products were found in all isolates, others were diagnostic for the differentiation between isolates. The dendogram based on the specific band patterns revealed two major clusters (Fig. 1 B), one containing the isolates MEL07, 26D9, Peter09.1 and 19F6, the second cluster comprised the remaining isolates (ZIE11, ZIE05, 27F11, Peter07_163, AB2008/71, SP08_4, 22F8 and Peter07_149). This cluster split into two further clusters with the isolates SP08_4, 22F8 and Peter07_149 and the remaining five. No clear relationship between genetic clustering and lake of origin of isolates was found.

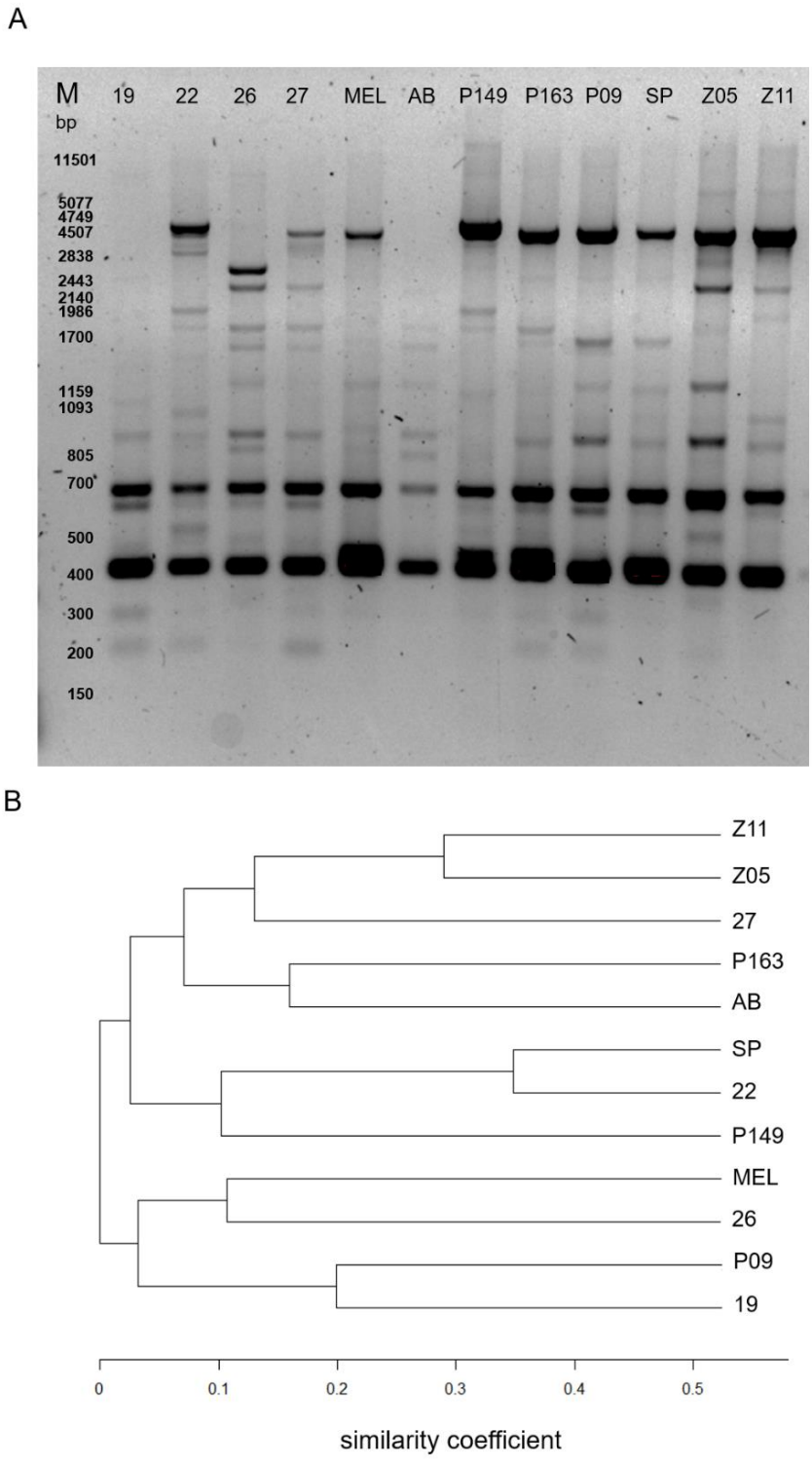

Figure 1: (A) DNA fingerprinting pattern of *Cylindrospermopsis raciborskii* isolates with STRR1A primer. Lane M represents base pair marker, lanes 1–12 the isolates: 19—19F6, 22—22F8, 26—26D9, 27—27F11, AB—AB2008/71, MEL—MEL07, P149—Peter07_149, P163—Peter07_163, P09—Peter09.1, SP—SP08_4, Z05—ZIE05, Z11—ZIE11. (B) Dendogram with Jaccard similarity clustered by UPGMA. Abbreviation for isolates as in (A).

Growth rate and resource use

The isolates of *C. raciborskii* under semi-continuous conditions had an average growth rate of 0.31 day^{-1}, ranging from 0.11 (22F8) to 0.40 day^{-1} (Peter07_149) with a coefficient of variation (CV) of 0.26 (Fig. 2 A).

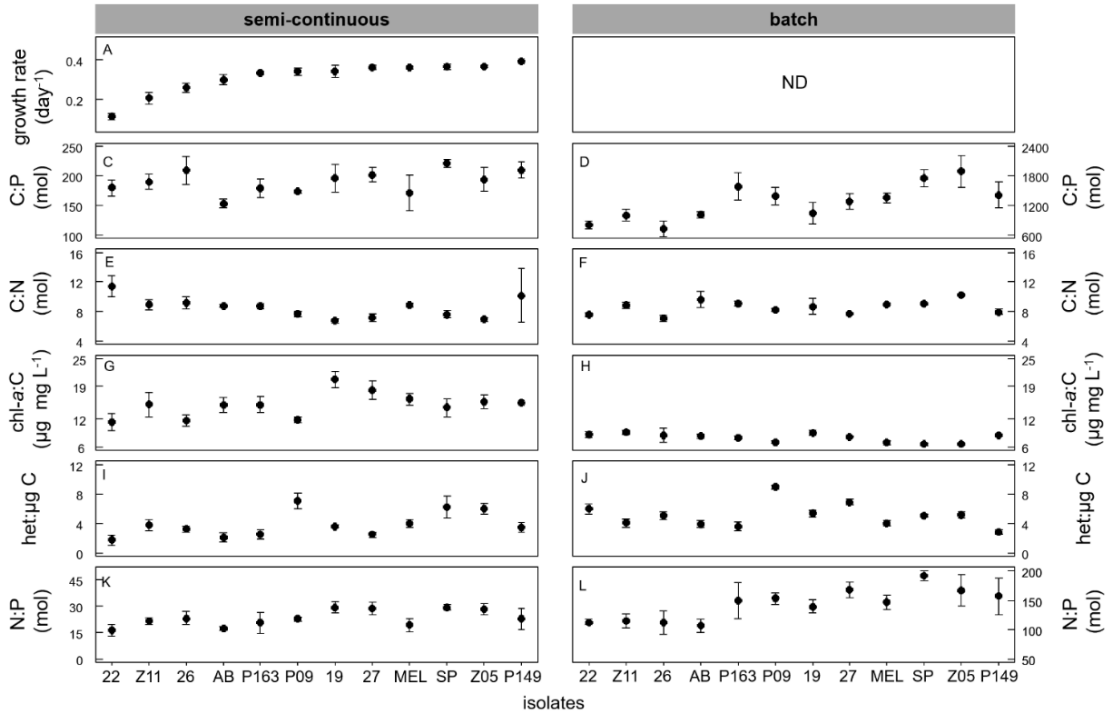

Figure 2: Traits of *Cylindrospermopsis raciborskii* from semi-continuous and batch (day 20) culturing, plotted by increasing growth rate. (a) Growth rate under semi-continuous culturing—intracellular C:P (mol) ratio under (c) semi-continuous and (d) batch conditions—intracellular C:N (mol) ratio under (e) semicontinuous and (f) batch conditions—intracellular Chl-*a*:C (µg mg L^{-1}) under (g) semi-continuous and (h) batch conditions—heterocysts (het):µg C under (i) semi-continuous and (j) batch conditions—N:P (mol) ratio under (k) semi-continuous and (l) batch conditions. Plotted is the mean ± *SD* for each isolate, *n* = 4. Abbreviation used for isolates: 19—19F6, 22—22F8, 26—26D9, 27—27F11, AB—AB2008/71, MEL—MEL07, P149—Peter07_149, P163—Peter07_163, P09—Peter09.1, SP—SP08_4, Z05—ZIE05, Z11—ZIE11. For C:P batch not day 20 but mean of day 16 and day 20, as both days have similar high ratios. ND - data not determined.

The cell quotas, measured as nutrient ratios, under semi-continuous and batch conditions also showed a high overall variability. The average molar C:P ratio was 190:1 ranging from 153:1 (AB2008/71) to 220:1 (SP08_4) under exponential growth to a mean of 1259:1,

ranging from 724:1 (26D9) to 1890:1 (ZIE05), under batch conditions (Fig. 2 D, Mann-Whitney Rank Sum Test, T = 78, $p < 0.001$). After the 15 h P-pulse at day 21, the mean C:P ratio dropped down to 13:1 (range: 8.5:1 Peter09.1 and 20:1 in 19F6 and AB2008/71, Fig. 4 day 21). The daily growth rate (see above and Fig. 2 A) was positively correlated to the maximum C:P under batch conditions (mean from day 16 and 20, see Fig. 2 C D; linear regression, $R^2 = 0.504$, $p = 0.01$), i.e. the fast growing isolates are the ones that can also shift their cell quota to extreme values.

The mean C:N ratios were fairly similar between the two experiments (two-tailed t-test, df = 22, t = -0.139, $p = 0.891$) with a mean of 8.50:1 (exponential growth; CV 0.16) and 8.57:1 (batch conditions; CV 0.11). The mean N:P ratios were 23:1 (CV 0.20) under exponential growth (16:1 22F8 and 29:1 19F6, 27F11 and SP08) and 143:1 (CV 0.19) under batch conditions, ranging from 106:1 (AB2008/71) to 191:1 in SP08_4 (Fig. 2 K L; Mann-Whitney Rank Sum Test, U = 78, $p < 0.001$). These values indicate a P-limitation in both experiments (N:P > 16:1) with a higher degree of P-limitation at the end of the batch experiment.

The cell quota of chl-*a* (µg chl-*a* mg carbon^{-1}) ranged from 15 (CV 0.18) under semi-continuous culture conditions to 8 (CV 0.12) under batch conditions (Fig. 2 G H; significantly different, Mann-Whitney Rank Sum Test, U = 222, $p < 0.001$).

In comparison, the abundance of heterocysts under semi-continuous and batch conditions (Fig. 1 I J) showed no significant differences (two-tailed t-test, df = 22, t = -1.776, $p = 0.0895$). On average, there were 3.87 heterocysts:µg C in the semi-continuous cultures and 5.09 heterocysts:µg C in the batch cultures. No correlation was found between the ratios of heterocysts:µg C in both experiments (linear regression, $R^2 = 0.172$, $p = 0.180$). The vegetative cells per heterocyst did not vary between culture conditions (two-tailed paired t-test, df = 11, t = 0.0397, $p = 0.97$; Supplementary Material Fig. S2). On average, there were 19 vegetative cells per heterocyst in the semi-continuous (± 5.59) and in the batch (± 8.19) cultures.

Under batch conditions, the amount of particulate, intracellular nitrogen and the abundance of heterocysts were positively correlated for each day (linear regression between each sampling day) with the highest explanatory power ($R^2 > 0.8$) from day 5 to day 20. The ratio of vegetative cells:heterocyst is positively correlated with increasing intracellular nitrogen under batch conditions and negatively under exponential growth conditions (Supplementary Material Fig. S3). Under batch conditions, isolates with a lower nitrogen content had more heterocysts, whereas under exponential growth isolates with a higher nitrogen content had fewer heterocysts.

Comparing the CV of physiological traits between exponential growth and severe nutrient limitation (end of batch experiment), we found that the CV among isolates was

higher at exponential growth for the C:N (0.16 vs. 0.11), heterocysts:µg C (0.45 vs. 0.32) and chl-*a*:C (0.18 vs. 0.12) ratios, but lower or equal for the ratios that include phosphorus: C:P (0.10 vs. 0.29) and N:P (0.20 vs. 0.19). Resource use traits did not correlate between semi-continuous and batch (day 20) culture conditions indicating the absence of trade-offs or general superiority of some isolates (linear regression, R^2 ranged from 0.007 to 0.185, *p* from 0.16 to 0.79).

Temporal changes in batch experiment

The C:N, C:P, chl-*a*:C and heterocysts:µg C ratios exhibited pronounced dynamics in the batch experiment (Fig. 4). From day 0 to day 20, the cyanobacteria biomass increased on average 38-fold in terms of carbon, with differences of a factor of 2.2 between isolates. Final cyanobacteria biomass was positively correlated with the growth rate during exponential growth (linear regression, $R^2 = 0.342$, $p = 0.046$). After 20 days, half of the isolates had reached their stationary growth phase (22F8, AB2008/71, Peter07_163, Peter09.1, 19F6, 27F11) and started to decline, the other isolates were very close to the stationary phase (Supplementary Material Fig. S1). The nutrient conditions at the beginning of the experiment resembled these of the semi-continuous experiment, but from day 2 onwards the concentration of particulate intracellular phosphorus remained constant and intracellular nitrogen exceeded the originally supplied inorganic nitrogen concentration and continuously increased along with intracellular carbon (Fig. 3).

The increase of carbon and nitrogen was similar over the time period, however, a difference was found before and after the complete exploitation of inorganic nitrogen provided in the medium. When dissolved inorganic nitrogen was still available *C. raciborskii* fixed less carbon per nitrogen than under nitrogen-depleted conditions, indicated by different slopes of the regression lines of particulate, intracellular carbon vs. nitrogen (Fig. 3). We found a lower slope of the regression until day 2 compared to that from day 5 onwards (Fig. 3) when the provided nitrogen was exploited and *C. raciborskii* fixed nitrogen. The overall C:N ratios among all isolates were relatively constant despite large differences in carbon biomass. The heterocysts:µg C ratio did not change much over the time course but declined when *C. raciborskii* had reached its stationary phase (from day 16).

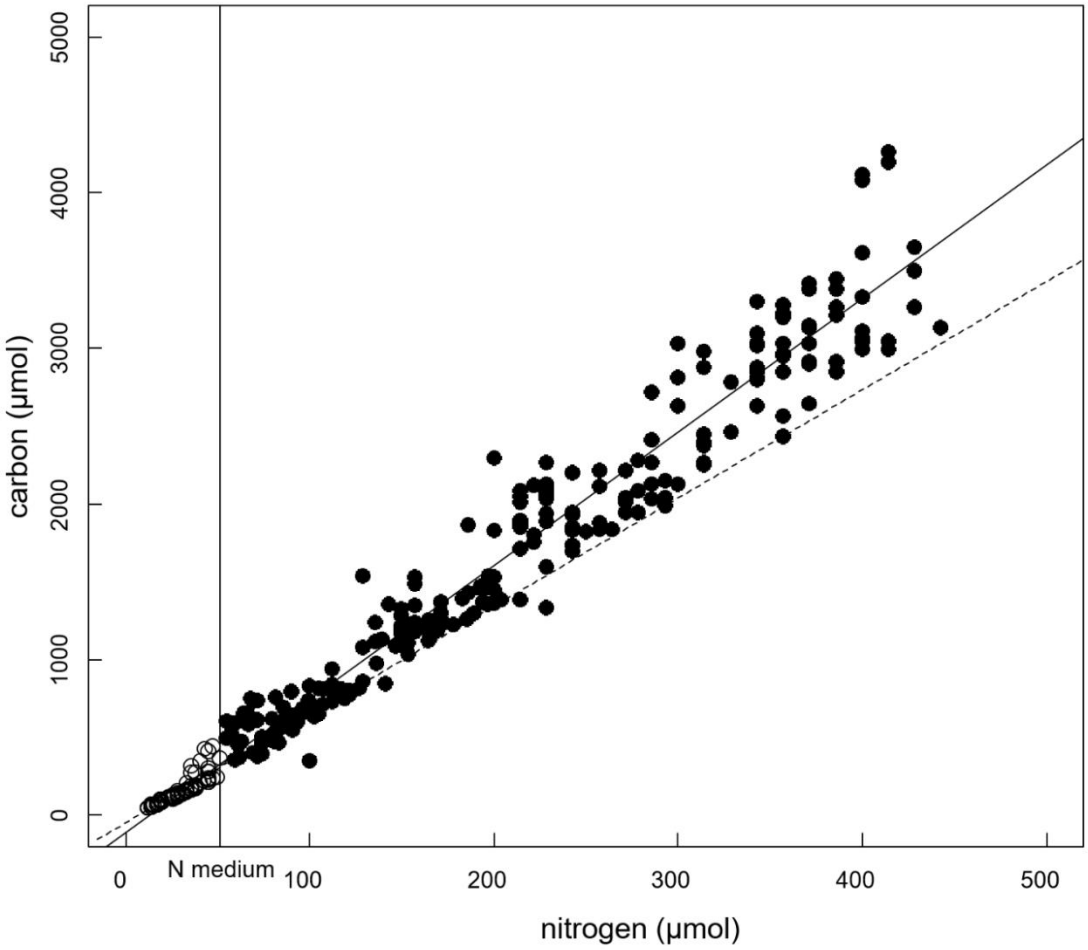

Figure 3: Particulate intracellular carbon versus nitrogen (µmol) values for day 0–20 of the batch experiment. *Cylindrospermopsis raciborskii* with available N in the medium (mainly day 0–2; white circles, linear regression $R^2 = 0.337$, $p < .001$—dashed line) and after N is depleted and N_2 fixation occurred (black circles, linear regression $R^2 = 0.941$, $p < .001$—solid line). Slopes of regression lines are significantly different ($t = 4.257$, $p < .001$).

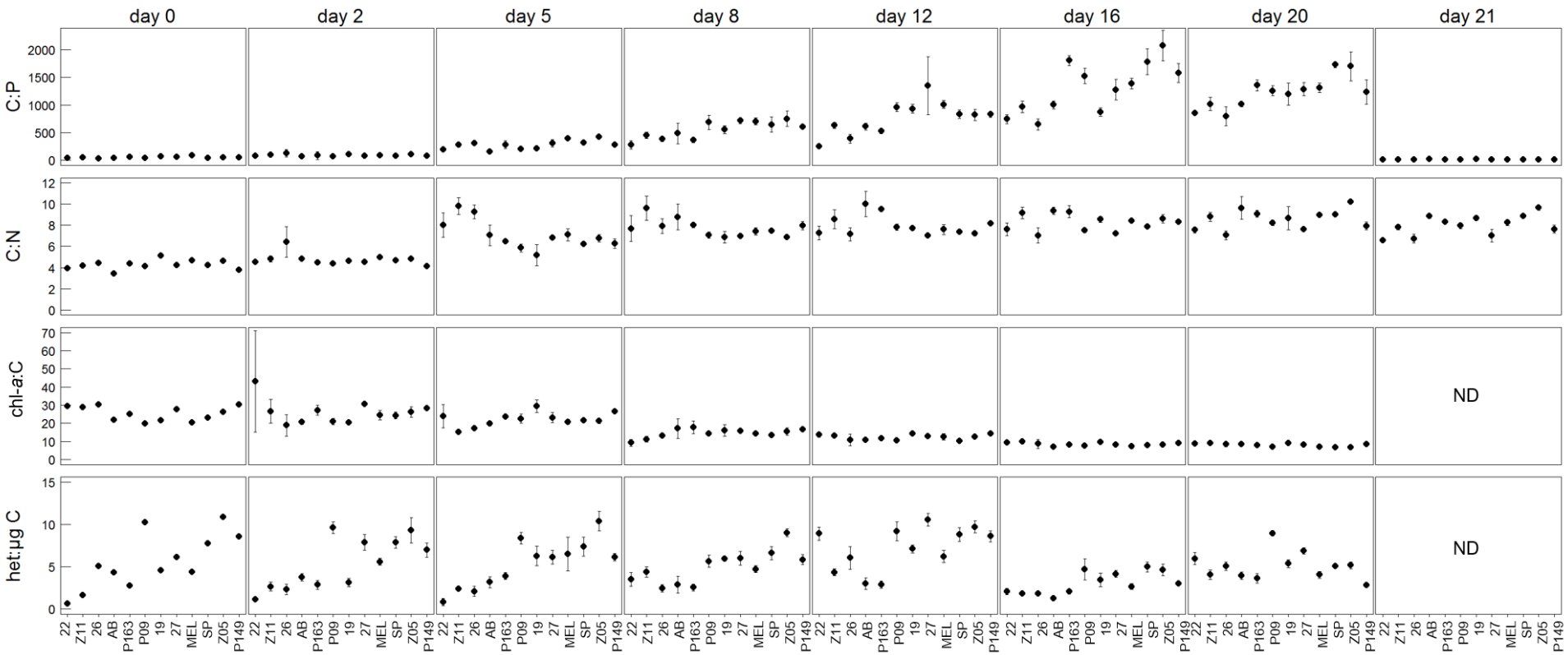

Figure 4: Temporal changes in C:P (mol), C:N (mol), heterocysts (het):µg C and Chl-*a*:C (µg mg L^{-1}) of *Cylindrospermopsis raciborskii*) from day 0 to day 20 of batch experiment. Additional C:P and C:N for day 21, after P-pulse. Mean ± *SD*, *n* = 4. Abbreviation used for isolates: 19—19F6, 22—22F8, 26—26D9, 27—27F11, AB—AB2008/71, MEL—MEL07, P149—Peter07_149, P163—Peter07_163, P09—Peter09.1, SP—SP08_4, Z05—ZIE05, Z11—ZIE11. ND - data not determined.

Morphology and grazing loss

The mean filament lengths of the isolates were highly variable, by a factor of 8, and ranged from 62 µm (ZIE11) to 496 µm (Peter07_163). The width of the filaments varied only 1.7 fold (Table 2).

Table 1: Maximum ingestion rate (I_{max}) and half-saturation constant (k_S) of *Brachionus calyciflorus* (ind) on *Cylindrospermopsis raciborskii*, length and width of C. raciborskii filaments. Mean ± standard deviation.

Isolate	Ingestion rate of *B. calyciflorus*			Filaments *C. raciborskii*	
	I_{max} (10^3 µm^3 ind^{-1} h^{-1})	k_S (10^3 µm^3 ml^{-1} *C. raciborskii)*		length (µm)	width (µm)
19F6	17.43 ± 9.67	2.80	no saturation	181 ± 138	2.27 ± 0.32
22F8	38.05 ± 11.50	2.17 ± 2.04		333 ± 208	1.39 ± 0.25
26D9	15.63 ± 4.41	3.28 ± 2.98		99 ± 36	1.89 ± 0.26
27F11	5.14 ± 1.18	0.08 ± 0.19		273 ± 134	1.81 ± 0.26
AB2008/71	3.11 ± 1.41	0.13 ± 0.40		439 ± 305	2.17 ± 0.27
MEL07	15.03 ± 5.35	0.74 ± 1.02		266 ± 163	1.84 ± 0.20
Peter07_149	35.21 ± 13.84	0.80 ± 2.80		116 ± 49	1.84 ± 0.22
Peter07_163	8.32 ± 2.38	2.88 ± 1.28		496 ± 260	1.73 ± 0.21
Peter09.1	17.00 ± 3.60	0.68	unimodal	123 ± 31	1.55 ± 0.21
SP08_4	31.09 ± 9.94	3.89 ± 3.19		74 ± 37	2.23 ± 0.31
ZIE05	13.27 ± 4.67	1.20		79 ± 33	1.97 ± 0.26
ZIE11	-	-	no saturation	62 ± 41	1.56 ± 0.18

The maximal ingestion rates by *B. calyciflorus* varied among isolates by a factor of 12 (Table 2, Fig. 5 A, top), the highest maximum ingestion rate was found for the isolate 22F8, and the lowest for AB2008/71. The half-saturation constant (k_s) varied as well between isolates. Morphology was no predictor for the ingestion rate of *C. raciborskii* (Table 2; linear regression: filament volume $R^2 = 0.196$, $p = 0.272$; filament length $R^2 = 0.166$, $p = 0.214$; filament radius $R^2 = 0.065$, $p = 0.448$). We found a positive linear relationship between I_{max} vs. k_s ($R^2 = 0.462$, $p = 0.013$, Fig. 5 B), however, due the large amount of variation of k_s at low food densities, this relationship should be treated with care (Fig. 5 A, bottom).

Ecological clustering

Combining data from growth rate, ingestion rate and all nutrient related traits for a principal component analysis (PCA), no clear clustered ordination of isolates according to the lake of origin was found (Fig. 6 A). Isolates from Melangsee (MEL07, 27F11, 19F6, AB2008/71) ordinated all in the bottom area, though not closely together. Other isolates from the same lake (Petersdorfer See or Zierker See) did not exhibit close ecological similarities. These results were mostly driven by variables from the batch experiment (C:P, P:C day 21, heterocysts:µg C) and I_{max}, explaining around 54 % of the variation, whereas the first axis is also driven by the

growth rate and C:N ratio under conditions of exponential growth. A cluster analysis revealed two main clusters and the separated isolate 22F8 (Fig. 6 B). As the PCA indicated, isolates from Melangsee are in one major cluster, but not the isolates from Zierker See despite being genetically very close (see Fig. 1).

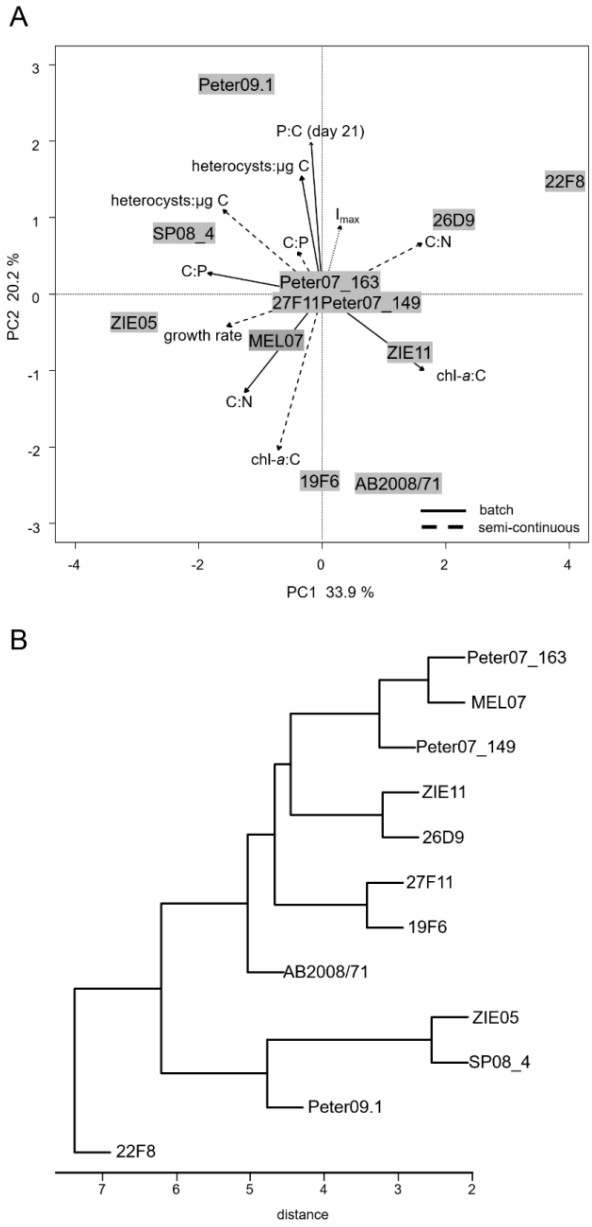

Figure 6: (A) Axis one (PC 1) and two (PC 2) of principal component analysis based on data of growth rates and C:P, C:N, Chl-*a*:C, heterocysts:lg C for the semi-continuous (solid lines) and batch (dashed lines) experiments, P:C ratio after P-pulse in batch experiment (day 21) and ingestion rate of herbivore on *Cylindrospermopsis raciborskii* (dotted line). Grey labels show ordination of isolates. (B) Hierarchical clustering of the ecophysiological data with Euclidean distance.

Discussion

Our aim was to study ecological trait variation among 12 isolates of *C. raciborskii* to better understand their invasion success and potential for further expansion.

DNA fingerprinting

DNA fingerprinting revealed that all isolates were genetically different and they can be referred to as strains. Remarkably, isolates from the same lake were either very similar e.g. from Zierker See or different as the three isolates from Petersdorfer See and the four isolates from Melangsee. The isolates from these lakes were all sampled on the same day (see Table 1) suggesting that several genotypes might co-occur in one lake. However, since the isolates were in cultures for, on average, ten years, some genetic changes might have occurred, although culture conditions were constantly beneficial without imposing a directed selective pressure. For green algae for example, laboratory selection experiments demonstrated evolutionary responses to elevated CO_2 levels on relatively short time scales (Collins & Bell, 2004; Bell & Collins, 2008). In natural populations, co-occurrence of different geno- or ecotypes have been found for *Microcystis* (Kardinaal *et al.*, 2007; Welker *et al.*, 2007), whereas Willis *et al.* (2016b) found for 24 isolates of *C. raciborskii* almost no genetic variation despite substantial trait variation.

Growth rate and resource use

The average growth rate of the investigated *C. raciborskii* (0.31 day^{-1}) is at the lower end of the spectrum found in previous studies (0.2 – 1.0 day^{-1}; e.g. Isvánovics *et al.,* 2000; Briand *et al.,* 2004; Mehnert *et al.,* 2010; Bonilla *et al.,* 2012; Willis *et al.,* 2015; Thomas & Litchman 2016). One likely reason is that growth conditions, even in the semi-continuous experiment, were sub-optimal because of moderate P-limitation (80 µg L^{-1}) and a temperature (20 °C) below the optimal growth temperature for isolates originating from temperate regions (Briand *et al.*, 2004; Mehnert *et al.*, 2010). The overall variability between isolates (CV 0.26) was similar as in the study by Briand *et al.*, (2004), who tested isolates from worldwide regions, but higher than in isolates obtained from one small-volume sample in an Australian lake (Willis *et al.,* 2016b; CV of growth rate 0.19). Compared with growth rates of eukaryotic algae from temperate regions, the rates of *C. raciborskii* are rather low and they alone do not explain their high invasive potential.

The molar C:P ratio under semi-continuous conditions ranged from 153:1 to 220:1 suggesting low to moderate P-limitation assuming a balanced nutrient content at a C:N:P ratio of 106:16:1 (Redfield ratio, Redfield, Ketchum & Richards, 1963). Under similar C:P ratios, a Hungarian strain exhibited higher growth rates (Istvánovics *et al.*, 2000) than all isolates from this study. A low cell quota (i.e. a high C:P ratio) is competitively advantageous because more

biomass is available when nutrient pulses occur and phosphorus is taken up. In shallow eutrophic lakes short nutrient pulses often occur, when mixing events transport nutrient-rich water from above the sediment surface into the water column (Weithoff, Walz & Gaedke, 2001). A high biomass with high uptake rates has then the potential to monopolize the nutrients and to outcompete other species despite relatively low growth rates (Schmidtke, Gaedke & Weithoff, 2010). After the P-pulse the C:P ratio dropped rapidly, on average by a factor of 100 within 15 hours (7 h light, 8 h dark) with a P-uptake rate of 0.0147 µg P µg C^{-1} (µg L^{-1}) h^{-1}. During this period, the biomass remained fairly constant, underlining the priority for excess P-uptake und postponing growth (Spijkerman & Coesel, 1998).

Under semi-continuous conditions the N:P (up to 23:1) and C:N (up to 9:1) ratios were only slightly higher than the Redfield ratio (16:1 and 6.625:1, respectively) indicating no severe N-limitation. The variation of the C:N ratio under both conditions was lower than the C:P ratio, indicating a correlated uptake of nitrogen and carbon (see Fig. 3) under P-limitation, which seems to be a relatively fixed trait among isolates. Under severe phosphorus-limitation in the batch experiment (day 5: N:P 40:1, C:P 281:1; P-limitation if N:P > 22:1 and severe if C:P > 258:1 from North *et al.*, 2007), the abundance of heterocysts in relation to carbon decreased towards days 16 – 20 (see Fig. 4), assuming growth saturation under the P-limitation was reached and further N-fixation was no longer of use. This view is reinforced by the reduced intracellular nitrogen increase per heterocyst at day 20 (Supplementary Material Fig. S4) and the similar ratio of vegetative cells:heterocyst between batch and semi-continuous cultures (Supplementary Material Fig. S2 and S3). Willis, Chuang & Burford (2016a) proposed that *C. raciborskii* can downregulate their N-fixation by discarding heterocysts and that heterocysts age and drop off. Along with the increasing limitation, the chl-*a*:C ratio has also decreased either by a stop of chlorophyll-*a* synthesis or dilution by growth (Collier & Grossman, 1992).

Morphology and grazing loss

The morphology of the *C. raciborskii* filaments was highly variable among isolates and the length varied more than the width. The width correlated with the P-uptake: thinner filaments exhibited a higher uptake rate per unit of carbon. Such an effect likely results from a higher surface to volume ratio, facilitating the nutrient influx per volume (Friebele, Correll & Faust, 1978). As expected, *B. calyciflorus* was able to ingest all isolates at variable rates, however the maximal grazing loss I_{max} of *C. raciborskii* was not correlated with the filament morphology. Although filamentous cyanobacteria are typically regarded as poorly edible, several studies demonstrated that *C. raciborskii* was ingested by different zooplankton species. For example, *Daphnia magna* ingested *C. raciborskii* independent of the filament length (Panosso & Lürling, 2010), whereas the clearance rate of the copepod *Eudiaptomus gracilis* was higher on shorter filaments (Rangel *et al.*, 2016). None of the isolates produces the toxins saxitoxin or

cylindrospermopsin, so that a direct toxin effect is unlikely. Nevertheless, some other not determined biochemical compounds such as allelochemicals or secondary metabolites might have influenced the ingestion.

Higher variability under nutrient sufficient conditions

Comparable studies are rare, but we consider the variation in multiple traits among the 12 *C. raciborskii* isolates as high and comparable to that of Australian *C. raciborskii* isolates (Xiao, Willis & Burford, 2017). Remarkably, the variability between traits among isolates was higher under semi-continuous conditions than at phosphorus-limiting batch conditions except for the C:P ratio, measured as the CV (0.29 vs. 0.10). Thus, imposing a stressor (here P-limitation) led to a higher variability in the physiological response to that stressor but trait values not directly related to that stressor became more similar. In experiments with 7 *C*. *raciborskii* isolates from Australia the growth rates differed most under nitrate-replete conditions and became more similar at nitrogen depletion (Saker & Neilan, 2001). This suggests that general functional traits separate isolates under 'good' conditions and stressor-related traits under stress conditions.

Lack of trade-offs

We neither found a clear trade-off in the physiological traits between the two experiments nor a generally superior isolate. However, single isolates performed on average better than others such as Peter07_149, but its maximal ingestion through *B. calyciflorus* was also comparatively high. The k_s values suggest a trade-off between isolates, which are grazed more at low or high concentrations respectively (Fig. 5).

Linkage of ecological and genetic clustering

The ordination of the isolates by PCA (see Fig. 6) supports the conclusions drawn from the ecological data. We neither found similarities between isolates of certain lakes nor were isolates from the same lake more similar than isolates from different lakes. However, for this comparison, a higher number of isolates would be desirable. Overall, the isolates ordinated relatively evenly along the two PCA axes without distinct clusters or obvious trade-offs. Only the isolates from Melangsee appear to be a bit closer to each other than expected from random ordination. This view was supported from the ecological cluster analysis where all four isolates belong to the upper large branch. Comparing the ecological clustering with the genetic clustering, we found two very different clusters, demonstrating no relation between the genetic relationship and ecological traits. Since the DNA fingerprinting displays only some random genetic distance, a perfect match cannot be expected. A deeper genetic analysis of functional genes would be more appropriate for further comparisons.

Furthermore, this study linked top-down and bottom-up traits and the genetic relationship for several strains of one invasive species. The results allow for a better insight into the possible invasibility of that species. Based on our analysis of the 12 selected isolates, we conclude that *C. raciborskii* is very flexible and therefore it is important for future experiments, which strain or set of strains is used under selected experimental conditions. Moreover, the plasticity of the cyanobacterium in Germany, an invaded region, was clearly shown and a larger number of analysed strains would likely result in an even higher degree of variation.

Relevance of traits for invasion success

C. raciborskii has spread over large geographic regions and has become a dominant species in some newly invaded lakes and in others, it is a member of minor importance in the phytoplankton community, without high abundances or regular blooms (e.g. Kokociński *et al.,* 2017). A large number of hypotheses or factors have been described to explain the invasive success of species. In some cases, species are successful because they are different from local species and the new environment is predator-free, for example the introduction of rats to isolated tropical islands (e.g. Thorsen *et al.*, 2000). In other cases, invasive species are very similar to native ones (and belong to the same genus) suggesting that slight differences in key life history traits are sufficient to replace native species (as e.g. in trout or gammarids). *C. raciborskii* exhibits no features that are unique within the order of Nostocales. Many traits of *C. raciborskii* are competitively advantageous such as N-fixation, forming of akinetes, high P-uptake rates and storage capacity and a low light requirement, however, these traits or trait values are also characteristic of other species of this order that are typical competitors of *C. raciborskii* like *Anabaena* spp. or *Aphanizomenon* spp. Considering the high trait variability, we propose that, within a community context, the invasion success of *C. raciborskii* depends on the specific environmental conditions and the trait (trait value) combination of the invading genotypes (Weithoff, Taube & Bolius 2017). This might explain why *C. raciborskii* is dominant in some lakes and failed to invade other lakes of the same geographic region. Although grazing losses of cyanobacteria are low compared to those of e.g. small flagellates and diatoms, grazing might influence the initial phase of the seasonal development of *C. raciborskii* (Weithoff *et al*., 2017).

Many studies in the past addressed only one or few ecological traits and/or few strains so that a comprehensive view about the intraspecific variability, especially of the genotypes within the invaded regions, is still lacking. Including further factors such as temperature, light and more invaded isolates will likely improve our understanding of the invasion processes of *C. raciborskii*. Furthermore, our results also have general implications for the field of trait-based ecology. In most studies, traits are treated as fixed characteristics, because data on within-species trait value variation is lacking for almost all species. Our results provide valuable

information for the variation of traits both bottom-up, such as resource use and growth rates, and top-down, such as grazing losses, as well as morphological variation.

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Supplementary Material

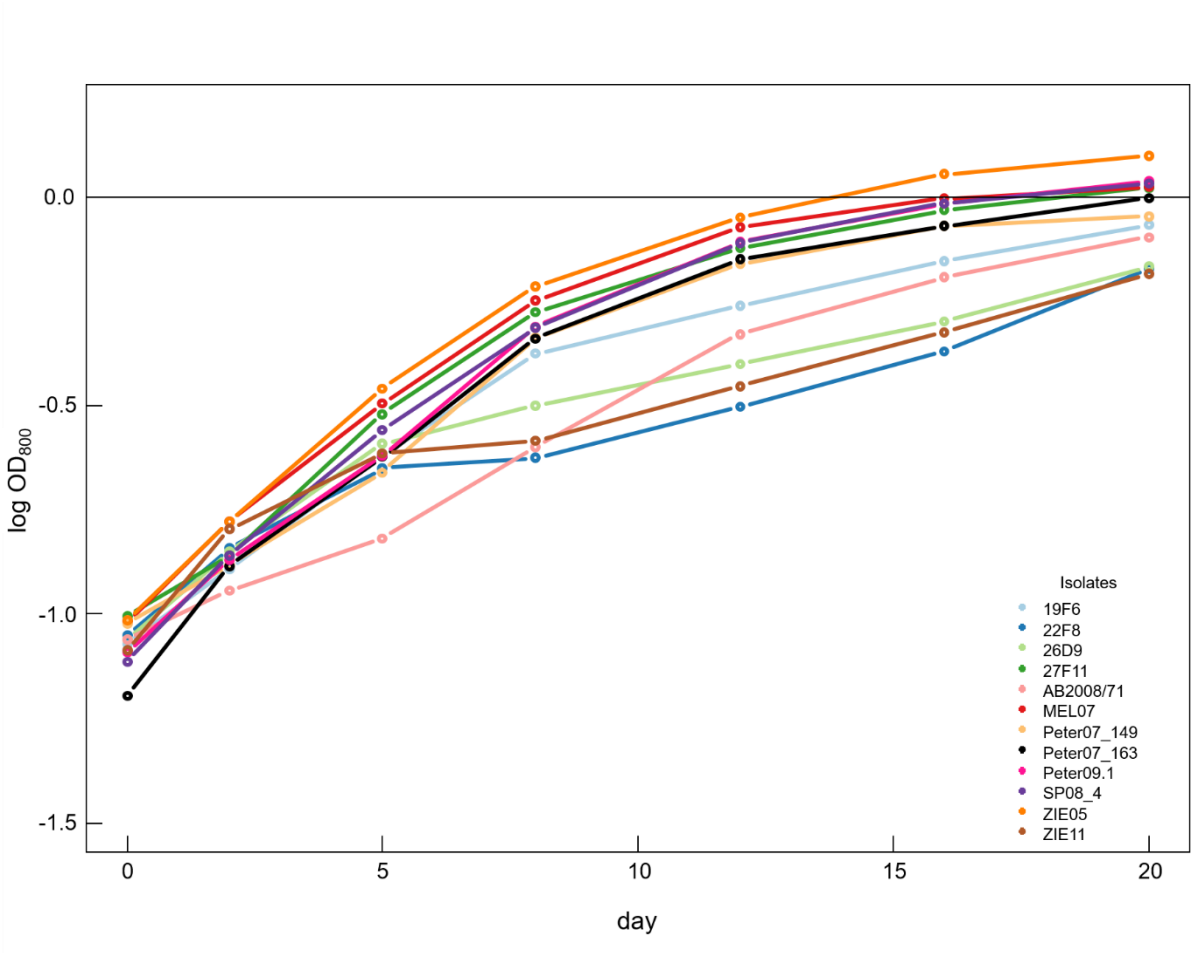

Figure S1: Logarithmical OD_{800} of isolates in the batch-culturing, day 0 to 20.

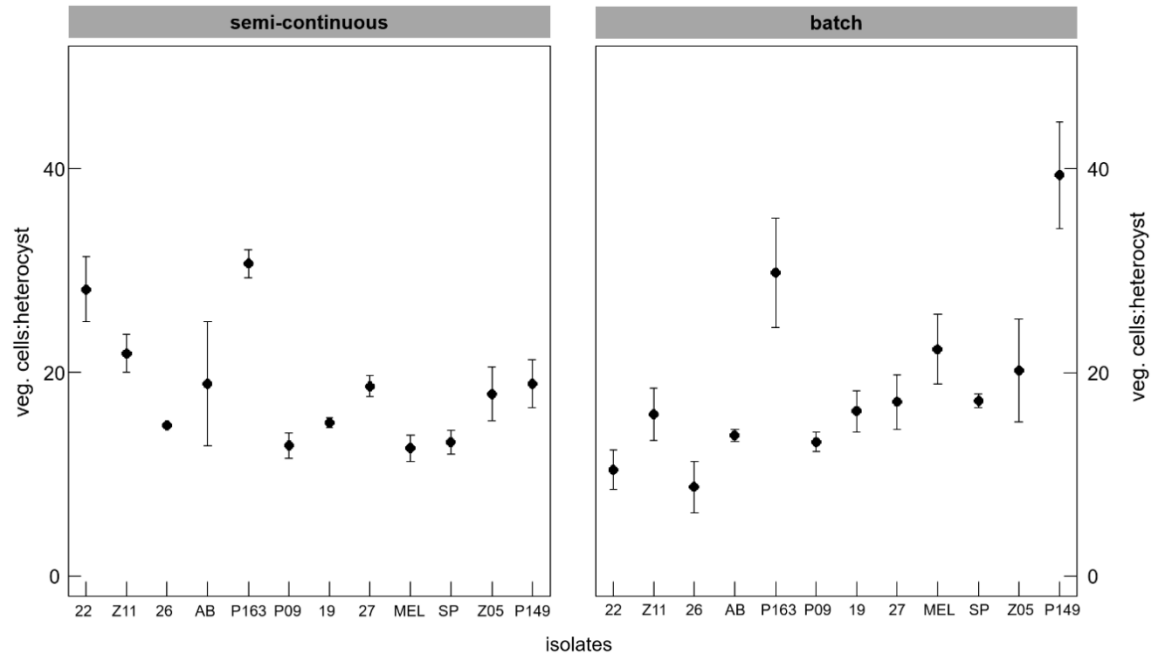

Figure S2: Vegetative cells per heterocyst of C. raciborskii, under semi-continuous and batch (day 20) culturing. Plotted is the mean ± standard deviation for each isolate, n = 4. Abbreviation used for isolates: 19 - 19F6, 22 - 22F8, 26 - 26D9, 27 - 27F11, AB - AB2008/71, M - MEL07, P149 - Peter07_149, P163 - Peter07_163, P09 - Peter09.1, SP - SP08_4, Z05 - ZIE05, Z11 - ZIE11.

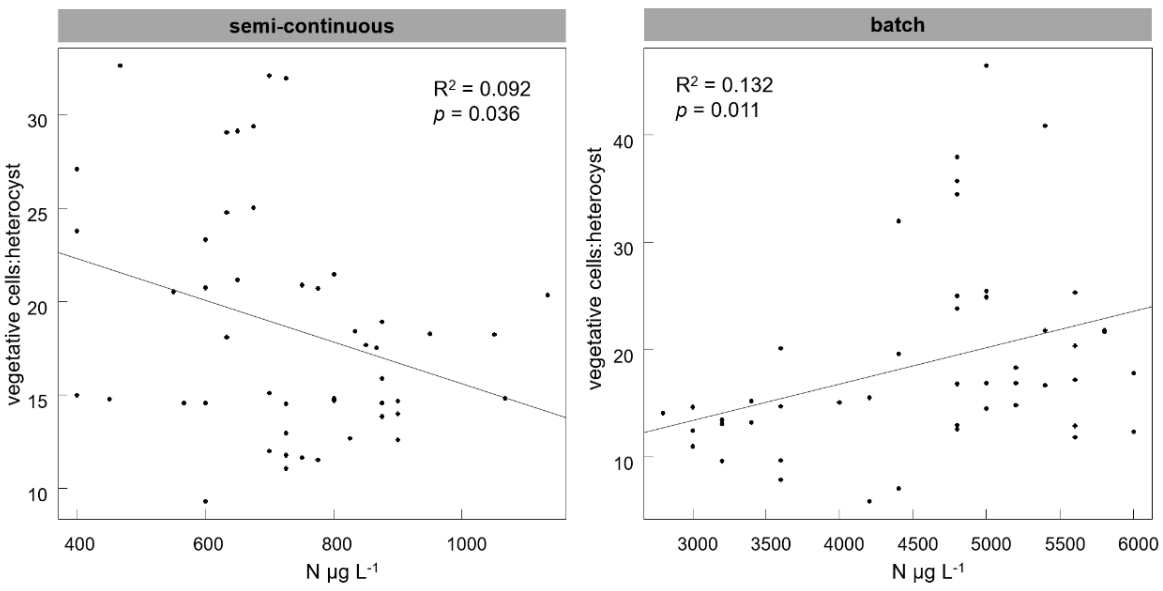

Figure S3: Linear regression among vegetative cells:heterocyst and intracellular N (µg L^{-1}) under semi-continuous and batch (day 20) conditions.

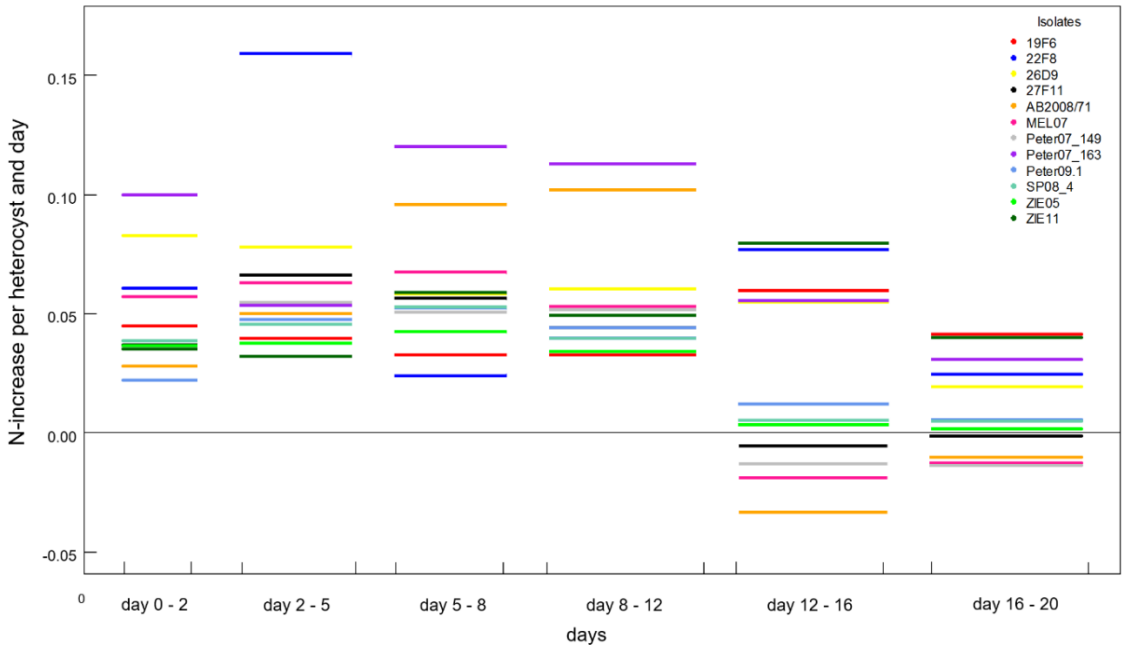

Figure S4: The daily N-increase per heterocyst (µg L^{-1}) for the isolates of *C. raciborskii* between the sampling points of the batch experiment. Plotted is the mean.