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# Multiclonal study of *Daphnia magna* with respect to adaptation to toxic cyanobacteria

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## *Abstract*

Mass developments of toxic cyanobacteria have increased in frequency due to global warming and eutrophication. Such cyanobacterial blooms impact whole freshwater ecosystems, especially reducing the abundance of herbivory species of the genus *Daphnia*. These negative effects on *Daphnia* have frequently been attributed to cyanobacterial secondary metabolites, among them hepatotoxic microcystins and protease inhibitors. Protease inhibitors inhibit major digestive proteases in the gut of *Daphnia* which results in reduced fitness, that is, population growth. To date evidence for local adaptation of *Daphnia* to cyanobacteria is confined to microcystin-producing cyanobacteria and based on comparison of individual clones from different populations but lacks evidence from multiclone microcosm experiments. In the present study, *D. magna* clones from a Swedish lake where they coexist with the microcystin-free *Microcystis* sp. strain BM25 were compared to clones from a Polish population without cyanobacteria, first in single-clone experiments and subsequently in a multiclonal experimental population. The Swedish clones were assumed to be locally adapted to this protease inhibitor-producing cyanobacterium and indeed showed higher population growth rates, a proxy for fitness, and dominated the population in the presence of dietary *Microcystis* sp. BM25, but not in the absence of this cyanobacterium. The results indicate an adaptive tolerance of the Swedish population and point at local adaptation to locally co-occurring protease inhibitor-producing cyanobacteria.

Cyanobacterial mass developments have increased in frequency over the last decades, due to eutrophication of waterbodies and climate change, which comes along with high levels of $CO_2$ and rising temperatures (Lampert and Sommer 1999; Paerl and Huisman 2008). Such cyanobacterial blooms are described as harmful for human and livestock (Falconer 1996), as many cyanobacterial strains produce toxins (Sivonen 1996). Furthermore, cyanobacterial blooms influence the aquatic ecosystem in terms of abiotic conditions, like light and turbidity (Paerl and Huisman 2008). Moreover, cyanobacteria are known to negatively impact the fitness of planktivorous zooplankton, like for example the freshwater cladoceran *Daphnia* sp. (reviewed by Ger et al. 2016), which is an important link between primary producers and higher trophic levels. Thus, cyanobacterial blooms reduce the flow of energy and material within the food web (Müller-Navarra et al. 2000). However, there are several main reasons why a cyanobacterial diet leads to reduced growth and reproduction rates of the non-selective filter-feeder *Daphnia* (Hansson et al. 2007; Ger et al. 2016). Besides low ingestibility of cyanobacterial colonies and filaments (Porter and McDonough 1984), deficiency of lipids in cyanobacteria that are essential for *Daphnia* (von Elert et al. 2003) and resultant changes in *Daphnia* behavior and anatomy (Bednarska and Dawidowicz 2007), the negative effects of cyanobacteria on *Daphnia* can at least partly be assigned to cyanobacterial secondary metabolites. Cyanobacteria produce a variety of biologically active secondary metabolites (Gademann and Portmann 2008; Janssen 2019) like microcystins and protease inhibitors, which have been shown to cause reduced growth and reproduction rates and increased mortality of *Daphnia* (Rohrlack et al. 2001; Lürling 2003; Gademann and Portmann 2008). Microcystins are a group of widely described cyanobacterial metabolites, but Lürling (2003) was the first who demonstrated that a microcystin-free cyanobacterial strain, *Microcystis aeruginosa* NIVA Cya 43, led to growth reduction and he suggested other secondary metabolites to impair the fitness of *Daphnia*. As a first case for negative effects of such other cyanobacterial secondary metabolites,

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Additional Supporting Information may be found in the online version of this article.

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Rohrlack et al. (2004) demonstrated molt inhibition in *Daphnia* by a cyanobacterial protease inhibitor. Von Elert et al. (2005) identified two major protease inhibitors in *M. aeruginosa* NIVA Cya 43 and demonstrated that these two protease inhibitors reduced juvenile growth of *Daphnia*, when these protease inhibitors were fed via liposomes (von Elert et al. 2012). Hence, other unknown metabolites could be excluded as cause for the reduction of growth (von Elert et al. 2012). Similarly, Czarnecki et al. (2006) identified cyanobacterial inhibitors of *Daphnia* trypsins. Another microcystin-free strain is *Microcystis* sp. BM25 (Schwarzenberger et al. 2013*b*), which contains the three protease inhibitors micropeptins DR1056, DR1006, and MM978 (Schwarzenberger et al. 2010). These protease inhibitors are depsipeptides that inhibit digestive proteases in the gut of *Daphnia*, which is also known to result in reduced somatic growth and population growth rate, decreased ingestion rates and increased mortality of *Daphnia* (Lürling 2003; Schwarzenberger et al. 2010).

From the perspective of water management an increased biomass of *Daphnia* constitutes a means to suppress cyanobacterial blooms (Wright and Shapiro 1984; Leibold 1989). However, it is controversial in how far *Daphnia* are able to suppress cyanobacterial blooms. Several studies demonstrated that bloom forming cyanobacteria have a negative impact on the abundance of *Daphnia* (Threlkeld 1979; Hansson et al. 2007) and that they cannot be controlled by grazing zooplankton (Ghadouani et al. 2003). These results are in contrast to other studies that depict that in particular *Daphnia* plays an important role in controlling the development of bloom forming cyanobacteria: Chislock et al. (2013) demonstrated, that *Daphnia pulicaria* was able to decimate a phytoplankton community consisting of 96% microcystin-producing *Microcystis* sp. and *Anabena* sp. by over 70%. In line with this, Sarnelle (2007) depicted that *D. pulicaria* were able to suppress an already developed cyanobacterial bloom consisting of 90% toxic *M. aeruginosa*. These partly contradictory results may be explained by the finding that *Daphnia* coexisting with cyanobacteria can evolve increased tolerance to toxic cyanobacteria both in time (Hairston et al. 1999; Isanta-Navarro et al. 2021) and space (Sarnelle and Wilson 2005; Wojtal-Frankiewicz et al. 2013; Schwarzenberger et al. 2017) the latter resulting in local adaptation. From the perspective of lake management, the identification of *Daphnia* with elevated tolerance to protease inhibitor-producing cyanobacteria would be of high interest, as these *Daphnia* could possibly be used as a tool to suppress and control protease inhibitor-containing cyanobacterial blooms. However, as even single cyanobacterial strains may contain more than one bioactive secondary metabolite, it is unclear which of these cyanobacterial metabolites has become more tolerated in locally adapted *Daphnia*. Recently evidences for locus-specific positive selection underlying evolutionary adaptation of a Swedish *Daphnia magna* population to cyanobacterial protease inhibitors have been presented (Schwarzenberger et al. 2020).

Despite these strong molecular evidences for positive selection due to cyanobacterial protease inhibitors in the Swedish *D. magna* population, evidence for local adaptation of the Swedish population is confined to two approaches: (1) effects of cyanobacterial extracts on proteases in body homogenates of *D. magna* clones (Schwarzenberger et al. 2013*a*, 2017) and (2) effects of cyanobacterial strains on somatic growth and clutch size of various single *D. magna* clones from this experienced population (Schwarzenberger et al. 2021). However, it still remains to be tested if this local adaptation can be demonstrated on the level of fitness of various single *D. magna* clones and if these evidences for local adaptation can be confirmed in multiclonal experiments.

It has been demonstrated in recent studies that the outcome of single-clone experiments does not necessarily predict the outcome of multiclonal experiments (Weider et al. 2008; Drugă et al. 2016). In this study, we performed multiclonal microcosm experiments with an experienced Swedish and with a naïve Polish experimental *Daphnia* population in the presence and absence of a protease inhibitor-producing cyanobacterium that had been isolated from the environment of the experienced *Daphnia* population to determine the fitness of the experienced *Daphnia* population, which has undergone positive selection. We used microsatellite analyses to track changes in genotype frequencies over a time period of 51 d in order to allow for several rounds of reproduction thereby assessing effects of the dietary cyanobacterium on *Daphnia* fitness.

## Methods

### Organisms and cultivation

The green alga *Chlamydomonas klinobasis* (strain 56, culture collection of the Limnological Institute, University of Konstanz, Konstanz, Germany) was cultivated semi-continuously in cyanophycean medium (von Elert and Jüttner 1997) at 20°C at 130 $\mu$E m$^{-2}$ s$^{-1}$, with 20% of the medium exchanged daily. The cyanobacterium *Microcystis* sp. strain BM25 (kindly provided by Ineke van Gremberghe, Ghent University, Ghent, Belgium) originates from Lake Bysjön in Southern Skania, Sweden. Strain BM25 has been shown to inhibit *D. magna* chymotrypsins, but not trypsins, in vitro (Schwarzenberger et al. 2013*a*) and does not contain microcystins (Schwarzenberger et al. 2013*b*). The cyanobacterium *M. aeruginosa* NIVA Cya 43 (culture collection of the Norwegian Institute for Water Research) does not produce microcystins, but the two protease inhibitors cyanopeptolin 954 and nostopeptin 920 (von Elert et al. 2005). Both cyanobacteria were cultivated in chemostats on cyanophycean medium (von Elert and Jüttner 1997) with a light intensity of 50 $\mu$E m$^{-2}$ s$^{-1}$, at a temperature of 20°C and constant air supply and a dilution rate of 0.1 d$^{-1}$. The concentration of particular organic carbon (POC) in the food suspensions was estimated regularly by measuring the extinction of the cultures at 470 nm. Carbon concentrations were calculated by a previously determined food-specific calibration curve to estimate the required volume of the food suspension for *Daphnia* growth experiments.

The *D. magna* clones used in this study (Table 1) were isolated from Lake Bysjön, Sweden from May to July 2010 (Schwarzenberger et al. 2013*a*) and in August 2010 from a Polish pond near Warsaw and cultivated in the lab for 9 yr prior to the experiments. The Swedish population experienced frequently cyanobacterial blooms whereas the Polish population did not (Schwarzenberger et al. 2017). For regular cultivation, 15 *Daphnia* originating from one clutch of a single mother were cultured in 800 mL membrane-filtered aged tap water, at 19°C, at low light conditions with a day and night rhythm of 16 : 8 h. Every second day all animals were transferred into freshwater and fed with 2 mg POC $L^{-1}$ of *C. klinobasis* as food alga. Third clutch neonates of one mother were kept for constant culture extension.

### Growth experiments with single Swedish and Polish *D. magna* clones

Each *D. magna* clone (Table 1) of either population was exposed to either *Microcystis* strain NIVA or strain BM25 in separate single-clone growth experiments. Each clone was grown in 0% (control), 10%, or 20% of POC derived from NIVA or BM25 and 100%, 90%, or 80% POC from *C. klinobasis*, respectively. All experiments were performed at low light, 19°C, 16 : 8 D : N rhythm and a total food concentration was 2 mg POC $L^{-1}$. For the exposure, newborns from the 3rd and 4th clutch from a cohort of synchronized *D. magna* mothers were used. They were randomly distributed at 5 ind/glass into 250 mL aged tap water containing the five food treatments with each food treatment being run in triplicate. The experimental animals were transferred every second day into freshwater and food until the first clutch of the test animals hatched. The number of hatched neonates and the number of females which had released them were counted to calculate the population growth rate $r$ (Eq. 1).

$$r = \sum l_x \times mx \times e^{-rx}. \quad (1)$$

Euler–Lotka equation for the calculation of the population growth rate $r$ ($d^{-1}$). $l_x$ is age-specific survivorship, $m_x$ is number of neonates at day $x$, $x$ is age in days.

**Table 1.** List of *Daphnia magna* clones used for the growth experiments according to Schwarzenberger et al. (2017).

| *D. magna* clones | Sampling year | Origin; geographical coordinates |
|---|---|---|
| Mai7/M7, Mai17/M17, Mai20/M20, Mai24/M24, Jun6/J6, Jun17/J17, Jul8/J8 | 2010 | Lake Bysjön, Sweden; lat 55.675448, lon 13.545070 |
| P2, P4, P12, P13, P21, P27, P31 | 2010 | Pond near Warsaw; lat 52.322722, lon 20.730515 |

Mean and standard deviation of the population growth rates were calculated for each clone. In order to illustrate the effect of 20% BM25 on the population growth rate $r$ of each clone, tolerance was calculated as the difference between $r$ on the BM25 treatment and $r$ on control food normalized to $r$ on control food (Eq. 2).

$$\text{tolerance}\,(\%) = \left(\frac{r_{\text{mean(treat)}} - r_{\text{mean(ctrl)}}}{r_{\text{mean(ctrl)}}}\right) \times 100, \quad (2)$$

Tolerance (%): $r_{\text{mean(treat)}}$ is average population growth rate of the clone in the BM25 treatment, $r_{\text{mean(ctrl)}}$ is average population growth rate of the clone in the control treatment (Brzeziński and von Elert 2007).

The single-clone growth experiments could not all be performed at the same time but were performed in four different blocks. To account for possible changes in conditions across experiments, the clone M17 was included in all four blocks of these single-clone growth experiments as a reference clone. Populations growth rates of M17 neither differed under control conditions (100% green alga, one-way ANOVA, $F = 0.608$, $p > 0.05$) nor in food mixtures with 10% of the cyanobacterium strain BM25 (one-way ANOVA, $F = 3.263$, $p > 0.05$) nor with 20% of BM25 (one-way ANOVA, $F = 0.434$, $p > 0.05$) across the four experimental blocks (Table S2). The treatments 10% and 20% of the cyanobacterium NIVA were performed in block 3 and 4 only, and the respective population growth rates of M17 for these treatments were neither statistically distinguishable (Welch's $t$-test, for 10% NIVA: $t = -0.235$, $p > 0.05$; for 20% NIVA: $t = -0.676$, $p > 0.05$). We conclude that for neither food treatment block effects were detectable, and we hence assumed the absence of block effects with respect to food quality. This assumption is corroborated by a principal component analysis of population growth rates of all clones across all treatments in which no separation of data according to experimental blocks was visible (Fig. S1).

Hence, data from all experimental blocks were pooled. To compare the effect of both cyanobacterial strains on the growth rates of the two *Daphnia* populations, for each clone a mean population growth rate $r$ on the control, the 10% and 20% BM25 treatment was calculated. These mean values ($n = 7$) from either population were used to test for statistical differences among the Swedish and the Polish *Daphnia* population. Statistical differences were calculated with an ANOVA and post hoc test (Tukey's HSD) after testing for normal distribution (Shapiro–Wilk test) and homogeneity of the data (Levene's test).

### Multiclonal microcosm experiment

A microcosm experiment with Swedish and Polish clones was conducted in the presence and absence of 20% *Microcystis* sp. BM25 in the diet. In the control treatment the feeding suspension consisted of 100% *C. klinobasis*, while the BM25 treatment consisted of 80% *C. klinobasis* and 20% BM25 with a

total food concentration of 2 mg POC $L^{-1}$. These two treatments were conducted with five replicates each. The experiment was performed in 10 L buckets filled with 5 L water and feeding suspension. Within each replicate three Swedish (M7, M17, M24) and three Polish (P4, P27, P31) *D. magna* clones were inoculated together. In order to prevent a clone-depended bias of the results, M7 and P4 were chosen as low performing clones, M17 and P31 as moderately performing clones and M24 and P27 as good performing clones in the presence of 20% BM25, based on the single-clone experiments. All replicates were inoculated with four neonates per clone (initial relative abundance of each clone 16.7% in each replicate). Once a week, the water of each replicate was replaced by new aged tap water. The replicates were fed with 2 mg POC $L^{-1}$ every other day and it was assured that the food concentration never fell below 0.4 mg POC $L^{-1}$ within a replicate in order to exclude quantitative food limitation. The experiment took place at 19°C and low light conditions with a day and night rhythm of 16 : 8. Based on developmental times observed in the single-clone experiments and to make sure that at least a first generation of each clone had hatched, the first sampling was performed on day 16. The population within a replicate was thoroughly mixed before sampling and 10% of the total volume, that is, 500 mL were removed from the bucket and the *Daphnia* within these 500 mL were counted and collected in PCR tubes (two animals per tube). If the overall population within a bucket exceeded a number of 250 animals in total, the population was reduced to 250 animals, to avoid crowding effects. The experiment was stopped after 51 d. To analyze the abundances of the clones over the timespan of 7 weeks, the relative abundances of the clones were determined at day 16, 37, and 51.

### Microsatellite analysis

The animals in the PCR tubes were frozen and stored (−20°C) until DNA extraction. For DNA extraction the animals were squished with a pipette tip in 15 $\mu$L squish buffer, containing 10 mmol $L^{-1}$ Tris pH 8.0, 1 mmol $L^{-1}$ EDTA pH 8.0, and 24 mmol $L^{-1}$ NaCl. The squished *Daphnia* were lysed with 5 $\mu$L proteinase K (20 mg $mL^{-1}$, VWR International Radnor) and incubated at 37°C for 30 min with a termination step at 95°C for 3 min. Subsequently, the samples were centrifuged for 5 min at 13,000 rpm to clear the supernatant, which contained the DNA for the multiplex PCR. The QIAGEN Multiplex PCR Kit 1000 (Qiagen GmbH) was used according to the manual and (Brede et al. 2006) with the following primers (Table 2) and PCR reaction: An initial temperature step of 95°C for 15 min was followed by 40 cycles of 30 s at 94°C and 90 s at 56°C (primer temperature) and extension for 90 s at 72°C, and a final step at 95°C for 10 min. The multiplex PCR products were diluted 1 : 150 and analyzed at the Cologne Center of Genomics with a fluorescence-based-DNA-electrophorese-analysis (GeneScan 500 ROX dye Size Standard, Thermo Fisher Scientific).

The resulting electropherograms were analyzed with the software Geneious (Version 6.1.8, Biomatters Ltd). Multiplex primers were used as according to Brede et al. (2006), and within the loci (Table S1) the alleles of the six *D. magna* clones were determined by calling the peaks and setting bins, according to the user manual of the microsatellite plugin of Geneious. In prior experiments it had been possible to distinguish the six clones of the multiclonal microcosm samples via this analytical procedure. The resulting data were then analyzed with Microsoft Excel (Version 2016) and subsequently with R (Version 3.6.1). First, relative abundances of each clone and each replicate were calculated. Second, means and standard deviations were determined. The data of at all three time points passed the normality test and the equal variance test ($p > 0.05$). Thereafter, a repeated measures three-way ANOVA was performed with the factors "population," "treatment," and the repeated factor "day." Pairwise comparisons were run between the two *Daphnia* populations for "day" (days 16, 37, 51) and the two treatments (control food and 20% BM25) with subsequent Bonferroni adjustment.

### Statistical analyses

Statistical analyses were conducted in R (version 3.5.3, R Core Team 2019) and RStudio (version 1.2.5033, RStudio Team 2019). We tested for normal distribution of the data using a Shapiro–Wilk test and used a Levene's test to test for homogeneity of variance.

## Results

For each of the seven clones from the Swedish or Polish *D. magnas* (Table 1) population growth rates were determined on pure *C. klinobasis* and on mixtures of *C. klinobasis* with 10% or 20% of *Microcystis* strain NIVA or strain BM25. The population growth rates *r* for the Swedish and the Polish population represent means calculated from the single clonal means ($n = 7$) (Fig. 1). Interestingly, the presence of strain NIVA did not affect *r* of either the Swedish or the Polish population (Fig. 1a), whereas a significant interaction of population and strain BM25, a cyanobacterium that originated from the same lake as the Swedish *D. magna* clones, was observed: The 20% BM25 treatment negatively affected the Polish population growth rate compared to the control (Fig. 1b), whereas the Swedish population was not affected by BM25. In the presence of 20% BM25, the Polish population growth rate *r* was significantly lower than that of the Swedish population (Fig. 1b, Tukey HSD after two-way ANOVA, $p < 0.05$; Table S3). In conclusion, effects of NIVA did not differ among the *Daphnia* populations, whereas effects of BM25 on the population growth rate were affected by the population origin such that 20% BM25 had no effect on the Swedish population but significantly reduced *r* in the Polish population.

In single-clone experiments we determined the tolerance to either strain BM25 or strain NIVA. Within the Swedish and

**Table 2.** Multiplex primers used for the multiplex PCR. Tm: annealing temperature (°C). AN: NCBI accession number. F: forward primer. R: reverse primer. Dye label was used to label the forward primers (Brede et al. 2006).

| Locus | AN | Size range (bp) | Primers (5′–3′) | Dye label | Tm (°C) |
|---|---|---|---|---|---|
| B045 | HQ234168 | 118–126 | F: GCTCATCATCCCTCTGCTTC | FAM | 56.0 |
| | | | R: ATAGTTTCAGCAACGCGTCA | | |
| B074 | HQ234174 | 194–204 | F: TCTTTCAGCGCACAATGAAT | FAM | 56.0 |
| | | | R: TGTGTTCCTTGTCAACTGTCG | | |
| B050 | HQ234170 | 234–248 | F: TTTCAAAAATCGCTCCCATC | HEX | 56.0 |
| | | | R: TATGGCGTGGAATGTTTCAG | | |

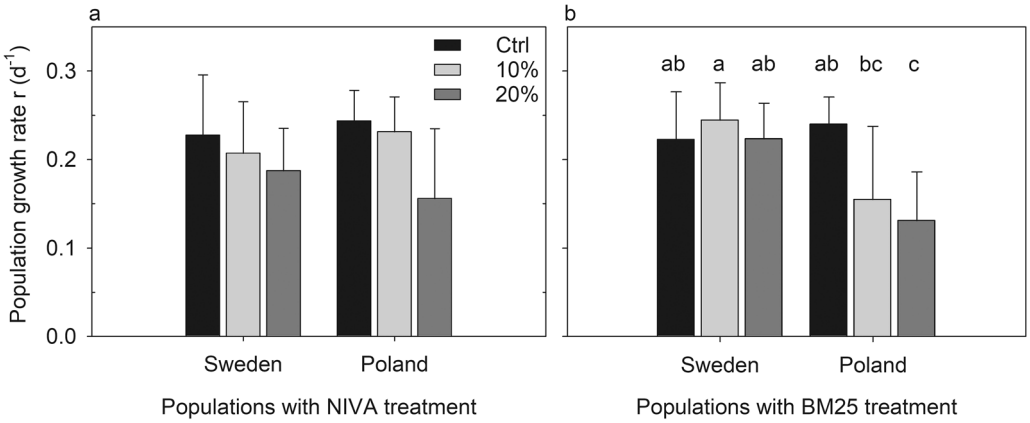


**Fig. 1.** Population growth rates of Swedish and Polish *Daphnia magna* clones exposed to different *Microcystis* strains. Population growth rates represent means of clonal means determined for each clone individually. (**a**) Both populations were fed with the green alga *Chlamydomonas klinobasis* and either 0% (ctrl), 10% or 20% of *Microcystis aeruginosa* NIVA Cya43 and (**b**) both populations were fed with the green alga *C. klinobasis* and either 0% (ctrl), 10% or 20% of *Microcystis* sp. BM25. Shown are means ± SD ($n = 7$) for each population. Tukey's HSD post hoc test after two-way ANOVA for A or B, $p < 0.05$. Letters indicate statistical differences between treatments within both populations.

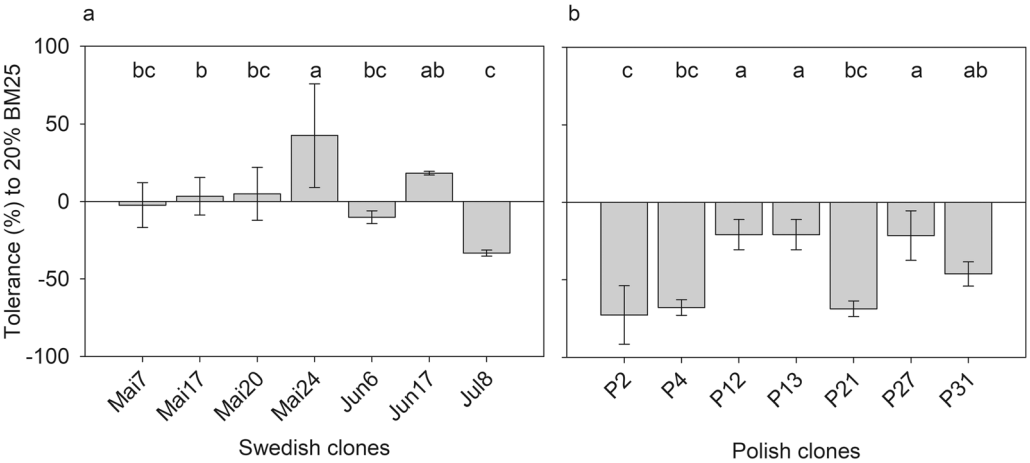


**Fig. 2.** Tolerance of the Swedish and the Polish population of *Daphnia magna* clones to *Microcystis* sp. BM25: (**a**) Swedish clones Mai7, Mai17, Mai20, Mai24, Jun6, Jun17, Jul8 and (**b**) Polish clones P2, P4, P12, P13, P21, P27, P31. Clones were grown on pure *Chlamydomonas klinobasis* ("control") and on a mixture of 20% *Microcystis* sp. BM25 + 80% *C. klinobasis* ("20% BM25"). For each clone tolerance was calculated as the difference between population growth on 20% BM25 and population growth on the control. Positive values indicate increased, negative values indicate decreased growth on 20% BM25. Shown are means ± SD ($n = 3$) for each clone. Letters indicate differences between clones within the same population (Tukey's HSD post hoc test after one-way ANOVA $p < 0.05$).

within the Polish *D. magna* population clones differed significantly in tolerance to 20% of strain NIVA (Fig. S2; Table S5), although tolerance was not distinguishable on the population level (Fig. 1). With respect to 20% strain BM25, significant clonal differences were apparent in both the Swedish and the Polish population (Fig. 2) with an overall reduced tolerance of all Polish population (Table S8). Three clones of each population were chosen for high, medium and low tolerance to strain BM25 to ensure a balanced population for the subsequent multiclonal experiment with 20% BM25. Clone Mai24 was significantly more tolerant to BM25 than the other tested clones (Fig. 2a; Tukey HSD after one-way ANOVA, $p < 0.05$; Table S4). Differences in tolerance were as well visible between the Polish clones. Here, P13, P12, and P27 were significantly more tolerant to BM25 and performed the best (Fig. 2b; Tukey HSD after one-way ANOVA, $p < 0.05$; Table S4). Therefore, for the Swedish population Mai24 was chosen as high performing, Mai17 as medium and Mai7 as low permforming clone, and for the Polish population clone P27 represented a high performing clone, followed by P31 as medium and P4 as low perfoming clone. When considering population growth rates of these clones, the Swedish population, consisting of clones M7, M17, and M24, showed a significantly lower population growth rate *r* than the Polish population (clones P4, P27, and P31) under control conditions. This pattern was reversed when 20% BM25 was present, thus the Polish population was significantly less tolerant against the cyanobacterium (Fig. S3).

In a subsequent microcosm experiment four synchronized neonates of each of these chosen Swedish and Polish clones were used to examine their performance within a mixed population with and without *Microcystis* sp. BM25 over a period of 51 d. Microsatellite analyses were conducted with samples from day 16, day 37, and day 51. Ten percent of the population was sampled and analyzed to determine the abundance of the Swedish and Polish *D. magna* clones (Fig. 3). A repeated measures three-way ANOVA showed significant interaction of the factors population, day and treatment ($F(2,8) = 11.985$, $p = 0.004$) and of the factor population, $F(1,4) = 146.429$, $p = 0.000268$. Subsequently pairwise comparisons were run between the two *Daphnia* populations for "Day: 16, 37, 51" and the two treatments control and 20% BM25 with subsequent Bonferroni adjustment. The abundances of the experimental Swedish and Polish population were significantly different in the control treatment on day 16 and 37 ($p < 0.05$)

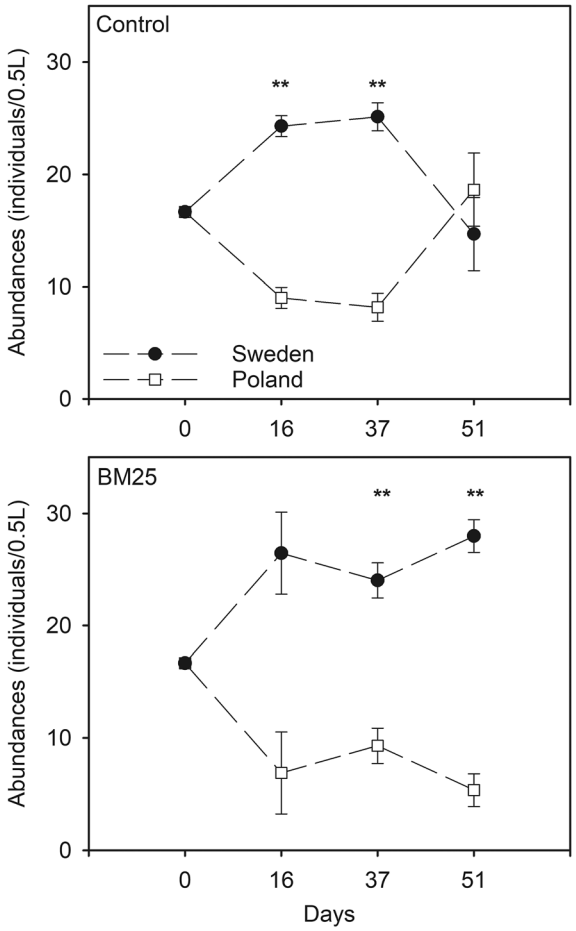


**Fig. 3.** Abundances over time of *Daphnia magna* from the Swedish and Polish population during the microcosm experiment. Clones were distinguished by microsatellite analyses and pooled according to their population. Clones were tested in mixture with equal number of individuals in the beginning and exposed to two treatments: "Control": 100% *Chlamydomonas klinobasis* and "20%; BM25": 20% *Microcystis* sp. BM25 + 80% *C. klinobasis*. Shown are mean ± SE ($n = 5$) for all time points. Pairwise comparison after repeated measures three-way ANOVA, ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

**Table 3.** Results of pairwise comparison after repeated measures three-way ANOVA for the microcosm experiment. The data were grouped by "day" and "treatment", and pairwise comparisons were performed between the two *Daphnia* "populations" ($n = 5$) with Bonferroni adjustment. The treatments consisted of the pure green alga *Chlamydomonas klinobasis* ("control") or a mixture of 80% *C. klinobasis* with 20% *Microcystis aeruginosa* BM25 ("BM25").

| Day | Treatment | Population | *p* Value (adjusted) | Significance |
|---|---|---|---|---|
| 16 | BM25 | Sweden, Poland | 0.055 | n.s. |
| 16 | Ctrl | Sweden, Poland | 0.001 | ** |
| 37 | BM25 | Sweden, Poland | 0.009 | ** |
| 37 | Ctrl | Sweden, Poland | 0.002 | ** |
| 51 | BM25 | Sweden, Poland | 0.001 | ** |
| 51 | Ctrl | Sweden, Poland | 0.577 | n.s. |

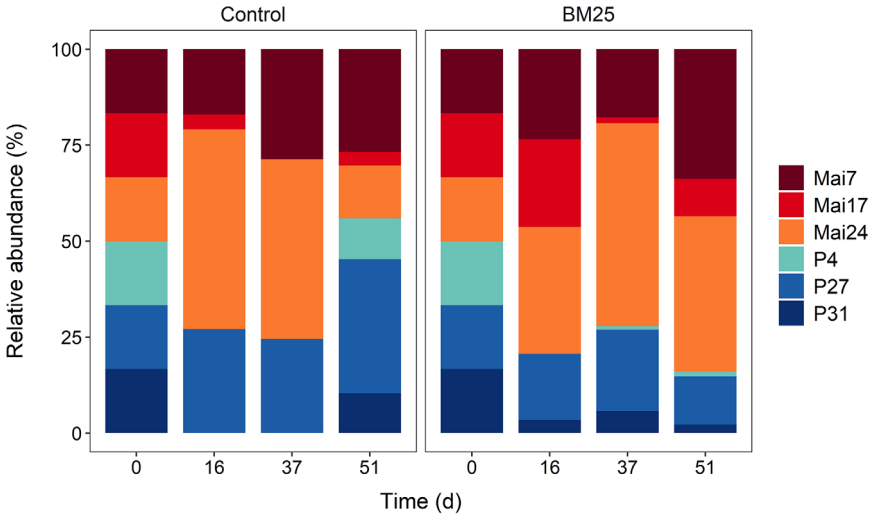


**Fig. 4.** Community composition (relative abundances in %) of *Daphnia magna* clones Mai7, Mai17, Mai24, P4, P27, and P31 in a microcosm experiment over a duration of 51 d. Clones were inoculated at identical relative abundances (16.4%) and exposed to a control (100% *Chlamydomonas klinobasis*) and a BM25 treatment (20% *Microcystis* sp. BM25 + 80% *C. klinobasis*). Shown are mean values ($n = 5$) for all time points.

but not 51 ($p > 0.05$), whereas there was a significant difference between the two populations on days 37 and 51 ($p < 0.05$) in the 20% BM25 treatment (Table 3). In the control treatment the abundacne of the Polish population increased toward the end of the experiment (day 51) while that of the Swedish population decreased, so that the abundance of both populations was not different. This pattern differed from that in the BM25 treatment where the Swedish population dominated the community in the latter part of the experiment (Fig. 3; Table 3). Especially clone Mai24 was fairly abundant in both treatments (Fig. 4, for further details *see* Fig. S4), corroborating its high tolerance to BM25 in the single-clone experiments. In accordance with its relatively high tolerance to BM25 in the single-clone experiments, the Polish clone P27 had a higher abundance (35% of the population) than the two other Polish clones (P4: 11%, P31: 10%) in the BM25 treatment on day 51. The supposed disappearance of clones and their subsequent reappearance can be attributed to the low relative abundance of these clones which may lead to accidental absence in the subsamples taken for determination of clonal abundances. The strain *Microcystis* sp. BM25 originates from the same lake as the Swedish *D. magna* population. Hence, our results show that the experimental Swedish population had a higher fitness in the presence of *Microcystis* sp. BM25 but could not obtain dominance over the experimental Polish population under control conditions over time. The long-term dominance of the Swedish population in the presence of a cyanobacterium with the same origin indicates local adaptation to this cyanobacterial strain.

## Discussion

Our results demonstrate local adaptation of a Swedish *D. magna* population to a *Microcystis* strain isolated from the same location and dominance over a naïve *Daphnia* population in the presence of this specific *Microcystis* strain. We showed that the fitness of the Swedish population was not negatively affected by *Microcystis* sp. BM25 whereas the Polish population was, when 20% BM25 was present in the diet. This finding points at local adaptation of the Swedish population to a co-occurring cyanobacterium as it has been suggested in previous studies (Schwarzenberger et al. 2017, 2020, 2021). Such adaptations of *Daphnia* originating from lakes with annual exposure to toxic cyanobacteria are often assigned to microevolution (Hairston et al. 1999; Sarnelle 2007). Here, we used the two *M. aeruginosa* strains NIVA Cya43 and BM25. Neither cyanobacterial strain produces microcystins, and both produce inhibitors of *Daphnia* digestive proteases, which have been shown to be inhibitors of digestive chymotrypsins. However, the strains synthesize chemically different inhibitors: The strain NIVA Cya43 produces two inhibitory cyanopeptolines (von Elert et al. 2005), whereas strain BM25, which was isolated from the Swedish lake, produces three inhibitory micropeptins (Schwarzenberger et al. 2013*b*). Secondary metabolites in *M. aeruginosa* strain BM25 and strain NIVA have only been characterized with respect to known protease inhibitors (von Elert et al. 2005; Schwarzenberger et al. 2013*b*). As single cyanobacterial strains may synthesize several classes of bioactive secondary metabolites (Gademann and Portmann 2008; Huang and Zimba 2019; Janssen 2019), we cannot rule out the possibility that the higher fitness of the Swedish *D. magna* population in the presence of BM25 is caused by substances other than protease inhibitors in BM25. However, due to the detailed investigation of *Daphnia*'s response to BM25 ranging from seasonal succession of *Daphnia* genotypes (Schwarzenberger et al. 2013*a*) over effects on growth and reproduction (Schwarzenberger et al. 2021) of

varying inhibitor content (Schwarzenberger et al. 2013*b*) and sensitivity of gut proteases to protease gene copy numbers (Schwarzenberger et al. 2017) and evidence for site-specific selection on protease alleles (Schwarzenberger et al. 2020), we are confident that the observed higher fitness of the Swedish *D. magna* population may be attributed to the protease inhibitors present in strain BM25.

We as well observed an intra-population variance among of the *Daphnia* clones to the two cyanobacterial strains as Schwarzenberger et al. (2021) reported previously. The idea that this variance within the Swedish *D. magna* population results from a strong punctual selection caused by the seasonal occurrence of cyanobacterial blooms could not be supported: Although a strong bloom of protease inhibitor-containing cyanobacteria occurred in May in the Swedish lake, no difference in tolerance, measured as $IC_{50}$ values, to inhibition by natural lake seston from May was found between the clones from before and after the bloom (Schwarzenberger et al. 2013*a*), which corroborates findings obtained for a hypertrophic pond (Küster et al. 2013). However, in another case study, increasing tolerance to cyanobacteria over the season has been demonstrated in a *Daphnia* population (Schaffner et al. 2019).

It has been shown repeatedly that single-clone experiments cannot predict the outcome of complex multiclonal competition experiments (Weider et al. 2008; Drugă et al. 2016). Thus, it was necessary to test both populations in a microcosm experiment in order to verify the results of the single-clone experiments in a community context. Similar to other multiclonal experiments, in which population dynamic effects were reported after 30–50 d (Engel and Tollrian 2009; Jeyasingh et al. 2009), our experiment lasted 51 d. The Swedish clones dominated the community in the 20% BM25 treatment over the second half of the experimental time, but were not dominating in the control treatment after 51 d. This demonstrates that under non-blooming conditions the Swedish clones do not show higher fitness over a long period.

Chymotrypsin and trypsin, which belong to the S1 family of serine proteases, are the most important digestive enzymes in the gut of *D. magna* as they account for 80% of the whole proteolytic activity (von Elert et al. 2004). More than 20 protease inhibitors, which specifically inhibit chymotrypsins and trypsins, have been found in different marine and freshwater cyanobacteria (Gademann and Portmann 2008; Köcher et al. 2020). Protease inhibitor-producing cyanobacteria have a negative impact on *Daphnia* and thus on the aquatic ecosystem (Baumann and Jüttner 2008). Schwarzenberger et al. (2020) showed that the digestive proteases in *D. magna* of the Swedish population have undergone positive selection on the loci of the serine proteases CT448 and CT802. Especially, CT448 harbors a non-synonymous mutation in the Swedish clones, which might have altered the protein structure such that it resulted in higher tolerance to protease inhibitors, which in turn would require lower expression levels of CT448 to obtain the same protease activity in the presence of protease inhibitors. Given that this assumption is correct, it provides an explanation for the finding that increased tolerance to protease inhibitors in the Swedish population is associated with lower copy numbers of the CT448 that harbors this non-synonymous mutation (Schwarzenberger et al. 2017). Although elevated tolerance to protease inhibitors still remains to be shown for CT448 itself, gut homogenate of the Swedish *Daphnia* population proved to be more tolerant to natural protease inhibitors (based on $IC_{50}$ values) than the Polish *Daphnia* population (Schwarzenberger et al. 2017). The most abundant clones in our microcosm experiment (clones M24 and M7) have low copy numbers of CT448 (M24; two copies; M7: two copies; M17: three copies; P27: three copies; P4: three copies; P31: four copies) whereas the copy numbers of the other serine proteases CT383 and CT802 did not differ between the populations (Schwarzenberger et al. 2020). This suggests that a low copy number of CT448 might result in a reduced susceptibility against protease inhibitors likewise in the most dominant Swedish clones in our experiment.

Further studies have shown that CT448 is upregulated when cyanobacteria with protease inhibitors are present in the diet. Drugă et al. (2016) conducted a competition experiment with *D. galeata* in which CT448 was by far the most upregulated gene of all tested candidates when *M. aeruginosa* was present. Although Drugă et al. (2016) could not correlate the tolerance of the single clones with an upregulation of this gene, they suggested that CT448 strongly reacts to food quality changes. Still, we suspect that CT448 might play an important role in the tolerance to cyanobacteria and in local adaptation. Here we have compared two *D. magna* populations in the presence of 20% of a cyanobacterium that does not form colonies in a mixture with a high-quality food alga with the aim to limit dietary effects on *Daphnia* exclusively to the secondary metabolites of the *Microcystis* strains. This experimental setting may not reflect the environmental scenario during cyanobacterial mass developments which may be characterized by the occurrence of filamentous and colonial cyanobacteria comprising considerably higher shares of phytoplankton, in particular during cyanobacterial mass developments. With respect to the control of cyanobacterial blooms, *Daphnia* biomass has been shown to be important for food chain manipulation in terms of lake management (Leibold 1989; Carmichael et al. 2001; Codd et al. 2005; Sarnelle 2007). However, the degree of suppression of cyanobacterial blooms is largely dependent on the initial conditions, that is, the relative abundances of cyanobacteria and *Daphnia* in a complex way (Sarnelle 2007; for more details *see* reviews by Ger et al. 2016) and Moustaka-Gouni and Sommer (2020). Although progress has been made with respect to understanding the molecular basis of local adaptation of *Daphnia* to protease inhibitors in cyanobacteria (Schwarzenberger et al. 2020), the significance of these findings with respect to the control of cyanobacteria by *Daphnia* has yet to be determined. Here, we have demonstrated superiority of the adapted

Swedish *D. magna* population in the presence of 20% of cyanobacteria, which rather mimics prebloom conditions.

In this context, it has to be noted that the protease inhibitor content of a cyanobacterial strain may vary. As shown for strain NIVA Cya 43 (Burberg et al. 2019, 2020), Schwarzenberger et al. (2013*b*) demonstrated that the protease inhibitor content of *M. aeruginosa* strain BM25 was strongly affected by available nutrient ratios. In accordance with the carbon-nutrient-balance hypothesis (van de Waal et al. 2014) the content of the N-containing protease inhibitors in BM25 decreased under N-limitation (C : N = 13) and increased under P-limitation (C : P = 234).

In the few cases in which adaptation of *Daphnia* populations to cyanobacteria has been demonstrated in time and space, evidence for adaptation is based on effects of dietary cyanobacteria on somatic growth rate (and partly on clutch size) in experiments with individual *Daphnia* genotypes (Hairston et al. 1999; Sarnelle and Wilson 2005; Schwarzenberger et al. 2017, 2020). However, somatic growth rate is not a good predictor of fitness in case of xenobiotic stressor, which include toxic cyanobacteria (Trubetskova and Lampert 2002). Here, for the first time we used increased fitness as a proxy for adaptation of populations, here and show local adaptation to cyanobacteria of the Swedish *D. magna* population, which represents the only case in which the molecular basis of local adaptation of *Daphnia* to cyanobacteria has been understood (Schwarzenberger et al. 2017, 2020). By calculating *F*ST values in neutral loci (microsatellites, exon sequences of selected single copy genes), Schwarzenberger et al. (2020) found evidences for genetic isolation of the Swedish and the Polish *D. magna* populations. Furthermore, these two populations were genetically more distant in three protease loci (three chymotrypsin genes) than in the neutral loci, which suggested that the evolution of these protease genes has been driven by adaptive selection by cyanobacterial protease inhibitors present in the Swedish lake. This suggestion was further confirmed by population genetic tests applied to the tolerant Swedish *D. magna* population (Schwarzenberger et al. 2020).

Here, we determined population growth rates as a proxy for fitness of individual *Daphnia* genotypes and corroborate the evidence for local adaptation of this Swedish *D. magna* population to cyanobacteria. In a microcosm experiment with the absence/presence of a local Swedish cyanobacterium we demonstrate that the presence of cyanobacteria impacts the genetic composition of *Daphnia* and show that the Swedish *D. magna* population is capable of dominating a mixed population over a long period in the presence of cyanobacteria. Under control conditions this fitness advantage disappears, but there is no evidence that the Swedish *D. magna* population underperforms relative to the Polish population. Hence, we cannot provide evidences for a cost associated with higher tolerance to *M. aeruginosa* strain BM25, so that there is no evidence for local adaptation in the strict definition given by Kawecki and Ebert (2004); still the higher fitness of the Swedish *D. magna* population in the presence of the cyanobacterium and evidences for adaptive selection on the chymotrypsin loci in this *D. magna* population (Schwarzenberger et al. 2020) point at local adaptation to co-occurring cyanobacterial protease inhibitors.

**Data availability statement**

Research data are not shared.

## References


Baumann, H. I., and F. Jüttner. 2008. Inter-annual stability of oligopeptide patterns of *Planktothrix rubescens* blooms and mass mortality of *Daphnia* in Lake Hallwilersee. Limnologica **38**: 350–359. doi:10.1016/j.limno.2008.05.010

Bednarska, A., and P. Dawidowicz. 2007. Change in filter-screen morphology and depth selection: Uncoupled responses of *Daphnia* to the presence of filamentous cyanobacteria. Limnol. Oceanogr. **52**: 2358–2363. doi:10.4319/lo.2007.52.6.2358

Brede, N., A. Thielsch, C. Sandrock, P. Spaak, B. Keller, B. Streit, and K. Schwenk. 2006. Microsatellite markers for European *Daphnia*. Mol. Ecol. Notes **6**: 536–539. doi:10.1111/j.1471-8286.2005.01218.x

Brzeziński, T., and E. von Elert. 2007. Biochemical food quality effects on a *Daphnia* hybrid complex. Limnol. Oceanogr. **52**: 2350–2357. doi:10.2307/4502384

Burberg, C., M. Ilić, T. Petzoldt, and E. von Elert. 2019. Nitrate determines growth and protease inhibitor content of the cyanobacterium *Microcystis aeruginosa*. J. Appl. Phycol. **31**: 1697–1707. doi:10.1007/s10811-018-1674-0

Burberg, C., T. Petzoldt, and E. von Elert. 2020. Phosphate limitation increases content of protease inhibitors in the cyanobacterium *Microcystis aeruginosa*. Toxins **12**: 33. doi:10.3390/toxins12010033

Carmichael, W. W., S. M. Azevedo, J. S. An, R. J. Molica, E. M. Jochimsen, S. Lau, K. L. Rinehart, G. R. Shaw, and G. K. Eaglesham. 2001. Human fatalities from cyanobacteria: Chemical and biological evidence for cyanotoxins. Environ. Health Perspect. **109**: 663–668. doi:10.1289/ehp.01109663

Chislock, M. F., O. Sarnelle, L. M. Jernigan, and A. E. Wilson. 2013. Do high concentrations of microcystin prevent *Daphnia* control of phytoplankton? Water Res. **47**: 1961–1970. doi:10.1016/j.watres.2012.12.038

Codd, G. A., L. F. Morrison, and J. S. Metcalf. 2005. Cyanobacterial toxins: Risk management for health protection. Toxicol. Appl. Pharmacol. **203**: 264–272. doi:10.1016/j.taap.2004.02.016

Czarnecki, O., M. Henning, I. Lippert, and M. Welker. 2006. Identification of peptide metabolites of *Microcystis* (cyanobacteria) that inhibit trypsin-like activity in planktonic herbivorous *Daphnia* (Cladocera). Environ. Microbiol. **8**: 77–87. doi:10.1111/j.1462-2920.2005.00870.x

Drugă, B., P. Turko, P. Spaak, and F. Pomati. 2016. Cyanobacteria affect fitness and genetic structure of experimental *Daphnia* populations. Environ. Sci. Technol. **50**: 3416–3424. doi:10.1021/acs.est.5b05973

Engel, K., and R. Tollrian. 2009. Inducible defences as key adaptations for the successful invasion of *Daphnia lumholtzi* in North America? Proc. Biol. Sci. **276**: 1865–1873. doi:10.1098/rspb.2008.1861

Falconer, I. R. 1996. Potential impact on human health of toxic cyanobacteria. Phycologia **35**: 6–11. doi:10.2216/i0031-8884-35-6S-6.1

Gademann, K., and C. Portmann. 2008. Secondary metabolites from cyanobacteria: Complex structures and powerful bioactivities. Curr. Org. Chem. **12**: 326–341. doi:10.2174/138527208783743750

Ger, K. A., P. Urrutia-Cordero, P. C. Frost, L.-A. Hansson, O. Sarnelle, A. E. Wilson, and M. Lürling. 2016. The interaction between cyanobacteria and zooplankton in a more eutrophic world. Harmful Algae **54**: 128–144. doi:10.1016/j.hal.2015.12.005

Ghadouani, A., B. Pinel-Alloul, and E. E. Prepas. 2003. Effects of experimentally induced cyanobacterial blooms on crustacean zooplankton communities. Freshw. Biol. **48**: 363–381. doi:10.1046/j.1365-2427.2003.01010.x

Hairston, N. G., and others. 1999. Lake ecosystems: Rapid evolution revealed by dormant eggs. Nature **401**: 446. doi:10.1038/46731

Hansson, L.-A., S. Gustafsson, K. Rengefors, and L. Bomark. 2007. Cyanobacterial chemical warfare affects zooplankton community composition. Freshw. Biol. **52**: 1290–1301. doi:10.1111/j.1365-2427.2007.01765.x

Huang, I. S., and P. V. Zimba. 2019. Cyanobacterial bioactive metabolites—A review of their chemistry and biology. Harmful Algae **86**: 139–209. doi:10.1016/j.hal.2019.05.001

Isanta-Navarro, J., N. G. Hairston, J. Beninde, A. Meyer, D. Straile, M. Möst, and D. Martin-Creuzburg. 2021. Reversed evolution of grazer resistance to cyanobacteria. Nat. Commun. **12**: 1945. doi:10.1038/s41467-021-22226-9

Janssen, E. M.-L. 2019. Cyanobacterial peptides beyond microcystins—A review on co-occurrence, toxicity, and challenges for risk assessment. Water Res. **151**: 488–499. doi:10.1016/j.watres.2018.12.048

Jeyasingh, P. D., L. J. Weider, and R. W. Sterner. 2009. Genetically-based trade-offs in response to stoichiometric food quality influence competition in a keystone aquatic herbivore. Ecol. Lett. **12**: 1229–1237. doi:10.1111/j.1461-0248.2009.01368.x

Kawecki, T. J., and D. Ebert. 2004. Conceptual issues in local adaptation. Ecol. Lett. **7**: 1225–1241. doi:10.1111/j.1461-0248.2009.01368.x

Köcher, S., S. Resch, T. Kessenbrock, L. Schrapp, M. Ehrmann, and M. Kaiser. 2020. From dolastatin 13 to cyanopeptolins, micropeptins, and lyngbyastatins: The chemical biology of Ahp-cyclodepsipeptides. Nat. Prod. Rep. **37**: 163–174. doi:10.1039/c9np00033j

Küster, C. J., A. Schwarzenberger, and E. von Elert. 2013. Seasonal dynamics of sestonic protease inhibition: Impact on *Daphnia* populationsImpact on *Daphnia* populations. Hydrobiologia **715**: 37–50. doi:10.1007/s10750-012-1303-x

Lampert, W., and U. Sommer. 1999. Limnoökologie. Georg Thieme Verlag.

Leibold, M. A. 1989. Resource edibility and the effects of predators and productivity on the outcome of trophic interactions. Am. Nat. **134**: 922–949. doi:10.1086/285022

Lürling, M. 2003. Effects of microcystin-free and microcystin-containing strains of the cyanobacterium *Microcystis aeruginosa* on growth of the grazer *Daphnia magna*. Environ. Toxicol. **18**: 202–210. doi:10.1002/tox.10115

Moustaka-Gouni, M., and U. Sommer. 2020. Effects of harmful blooms of large-sized and colonial cyanobacteria on aquatic food webs. Water **12**: 1587. doi:10.3390/w12061587

Müller-Navarra, D. C., M. T. Brett, A. M. Liston, and C. R. Goldman. 2000. A highly unsaturated fatty acid predicts carbon transfer between primary producers and consumers. Nature **403**: 74–77. doi:10.1038/47469

Paerl, H. W., and J. Huisman. 2008. Blooms like it hot. Science **320**: 57–58. doi:10.1126/science.1155398

Porter, K. G., and R. McDonough. 1984. The energetic cost of response to blue-green algal filaments by cladocerans. Limnol. Oceanogr. **29**: 365–369. doi:10.4319/lo.1984.29.2.0365

Rohrlack, T., E. Dittmann, T. Börner, and K. Christoffersen. 2001. Effects of cell-bound microcystins on survival and feeding of *Daphnia* spp. Appl. Environ. Microbiol. **67**: 3523–3529. doi:10.1128/AEM.67.8.3523-3529.2001

Rohrlack, T., K. Christoffersen, M. Kaebernick, and B. A. Neilan. 2004. Cyanobacterial protease inhibitor microviridin J causes a lethal molting disruption in *Daphnia pulicaria*. Appl. Environ. Microbiol. **70**: 5047–5050. doi:10.1128/AEM.70.8.5047-5050.2004

Sarnelle, O. 2007. Initial conditions mediate the interaction between *Daphnia* and bloom-forming cyanobacteria. Limnol. Oceanogr. **52**: 2120–2127. doi:10.4319/lo.2007.52.5.2120

Sarnelle, O., and A. E. Wilson. 2005. Local adaptation of *Daphnia pulicaria* to toxic cyanobacteria. Limnol. Oceanogr. **50**: 1565–1570. doi:10.4319/lo.2005.50.5.1565

Schaffner, L. R., and others. 2019. Consumer-resource dynamics is an eco-evolutionary process in a natural plankton community. Nat. Ecol. Evol. **3**: 1351–1358. doi:10.1038/s41559-019-0960-9

Schwarzenberger, A., A. Zitt, P. Kroth, S. Mueller, and E. von Elert. 2010. Gene expression and activity of digestive proteases in *daphnia*: Effects of cyanobacterial protease inhibitors. BMC Physiol. **10**: 6. doi:10.1186/1472-6793-10-6

Schwarzenberger, A., S. D'Hondt, W. Vyverman, and E. von Elert. 2013*a*. Seasonal succession of cyanobacterial protease inhibitors and *Daphnia magna* genotypes in a eutrophic

Swedish lake. Aquat. Sci. **75**: 433–445. doi:10.1007/s00027-013-0290-y

Schwarzenberger, A., T. Sadler, and E. von Elert. 2013*b*. Effect of nutrient limitation of cyanobacteria on protease inhibitor production and fitness of *Daphnia magna*. J. Exp. Biol. **216**: 3649–3655. doi:10.1242/jeb.088849

Schwarzenberger, A., N. R. Keith, C. E. Jackson, and E. von Elert. 2017. Copy number variation of a protease gene of *Daphnia*. Its role in population tolerance. J. Exp. Zool. **327**: 119–126. doi:10.1002/jez.2077

Schwarzenberger, A., M. Hasselmann, and E. von Elert. 2020. Positive selection of digestive proteases in daphnia: A mechanism for local adaptation to cyanobacterial protease inhibitors. Mol. Ecol. **29**: 912–919. doi:10.1111/mec.15375

Schwarzenberger, A., M. Ilić, and E. von Elert. 2021. *Daphnia* populations are similar but not identical in tolerance to different protease inhibitors. Harmful Algae **106**: 102062. doi:10.1016/j.hal.2021.102062

Sivonen, K. 1996. Cyanobacterial toxins and toxin production. Phycologia **35**: 12–24. doi:10.2216/i0031-8884-35-6S-12.1

Threlkeld, S. T. 1979. The midsummer dynamics of two *Daphnia* species in wintergreen Lake, Michigan. Ecology **60**: 165–179. doi:10.2307/1936478

Trubetskova, I., and W. Lampert. 2002. The juvenile growth rate of *Daphnia*: A short-term alternative to measuring the per capita rate of increase in ecotoxicology? Arch. Environ. Contam. Toxicol. **42**: 193–198. doi:10.1007/s00244-001-0010-9

Van de Waal, D. B., V. H. Smith, S. A. J. Declerck, E. C. M. Stam, and J. J. Elser. 2014. Stoichiometric regulation of phytoplankton toxins. Ecol. Lett. **17**: 736–742. doi:10.1111/ele.12280

von Elert, E., and F. Jüttner. 1997. Phosphorus limitation and not light controls the extracellular release of allelopathic compounds by *Trichormus doliolum* (cyanobacteria). Limnol. Oceanogr. **42**: 1796–1802. doi:10.4319/lo.1997.42.8.1796

von Elert, E., D. Martin-Creuzburg, and J. R. Le Coz. 2003. Absence of sterols constrains carbon transfer between cyanobacteria and a freshwater herbivore (*Daphnia galeata*). Proc. Biol. Sci. **270**: 1209–1214. doi:10.1098/rspb.2003.2357

von Elert, E., M. K. Agrawal, C. Gebauer, H. Jaensch, U. Bauer, and A. Zitt. 2004. Protease activity in gut of *Daphnia magna*: Evidence for trypsin and chymotrypsin enzymes. Comp. Biochem. Physiol. B Biochem. Mol. Biol. **137**: 287–296. doi:10.1016/j.cbpc.2003.11.008

von Elert, E., L. Oberer, P. Merkel, T. Huhn, and J. F. Blom. 2005. Cyanopeptolin 954, a chlorine-containing chymotrypsin inhibitor of *Microcystis aeruginosa* NIVA Cya 43. J. Nat. Prod. **68**: 1324–1327. doi:10.1021/np050079r

von Elert, E., A. Zitt, and A. Schwarzenberger. 2012. Inducible tolerance to dietary protease inhibitors in *Daphnia magna*. J. Exp. Biol. **215**: 2051–2059. doi:10.1242/jeb.068742

Weider, L. J., P. D. Jeyasingh, and K. G. Looper. 2008. Stoichiometric differences in food quality: Impacts on genetic diversity and the coexistence of aquatic herbivores in a *Daphnia* hybrid complex. Oecologia **158**: 47–55. doi:10.1007/s00442-008-1126-7

Wojtal-Frankiewicz, A., J. Bernasińska, T. Jurczak, K. Gwoździński, P. Frankiewicz, and M. Wielanek. 2013. Microcystin assimilation and detoxification by *Daphnia* spp. in two ecosystems of different cyanotoxin concentrations. J. Limnol. **72**: 13. doi:10.4081/jlimnol.2013.e13

Wright, D. I., and J. Shapiro. 1984. Nutrient reduction by biomanipulation: An unexpected phenomenon and its possible cause. Int. Ver. Theor. Angew. Limnol. Verh. **22**: 518–524. doi:10.1080/03680770.1983.11897338

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## Conflict of Interest

None declared.

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