Aquat Ecol
DOI 10.1007/s10452-016-9603-2

Competition between a toxic and a non-toxic *Microcystis* strain under constant and pulsed nitrogen and phosphorus supply

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Received: 15 March 2016 / Accepted: 30 September 2016

Abstract The toxicity of a harmful algal bloom is strongly determined by the relative abundance of non-toxic and toxic genotypes and might therefore be regulated by competition for growth-limiting resources. Here, we studied how the toxic *Microcystis aeruginosa* strain PCC 7806 and a non-toxic mutant compete for nitrogen and phosphorus under constant and pulsed nutrient supply. Our monoculture and competition experiments show that these closely related genotypes have distinct nutrient physiologies and that they differ in their ability to compete for nitrogen and phosphorus. The toxic wild type won the competition under nitrogen limitation, while the non-toxic mutant dominated under phosphorus limitation. Pulses of both nitrogen and phosphorus increased the dominance of the toxic genotype, which lead to an even faster competitive exclusion of the non-toxic genotype under nitrogen pulses and to coexistence of both genotypes under phosphorus pulses. Our findings indicate that the genotype level dynamics driven by resource competition can be an important factor in determining cyanobacterial bloom toxicity.

Keywords Algal toxins · Droop model · Harmful cyanobacteria · Nutrient limitation · Microcosm · *Microcystis aeruginosa* · Plankton dynamics · Resource pulses · Quantitative PCR

Handling Editor: Miquel Lurling.

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Electronic supplementary material The online version of this article (doi:10.1007/s10452-016-9603-2) contains supplementary material, which is available to authorized users.

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Introduction

The harmful blooms of microalgae and cyanobacteria (harmful algal blooms, HABs) that often form in

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Published online: 15 October 2016

eutrophic freshwater and estuaries are among the most eye-catching and problematic microbial populations found in nature (Paerl and Otten 2013; Brooks et al. 2015). These microbial mass occurrences are of great societal relevance because they threaten the production of drinking water and the recreational use of water bodies. It has been predicted that global change will further increase the risk and intensity of HABs due to human-induced eutrophication (O'Neil et al. 2012; Mantzouki et al. 2015), global warming (Paerl and Huisman 2008, 2009), increased water column stratification (Joehnk et al. 2008) and rising CO_2 levels (Verspagen et al. 2014).

Although HABs are often dominated by a single species, they harbour substantial genetic diversity and can vary considerably in toxicity. It has been shown that the toxicity of a bloom depends to a large extent on the relative proportions of toxic versus non-toxic or less toxic strains or genotypes of the same species (e.g. Park 1998; Chorus 2001; Briand et al. 2002; Wiedner et al. 2002; Welker et al. 2003; Huisman et al. 2005). Investigating the relative genotype frequencies might be a useful tool but only when they take biomass effects into account. Pacheco et al. (2016) argued that it is difficult to predict the toxin concentrations by assessing the potentially toxic cells, and the further technical advancements are still needed. The difference in toxin production level between genotypes can cover several orders of magnitude and exceeds by far the within-genotype variability in toxin production caused by environmental factors (Sivonen and Jones 1999; Rohrlack et al. 2001; Carrillo et al. 2003). Several studies have indicated that the temporal and spatial distribution of toxic and non-toxic genotypes within natural blooms could be explained as the result of resource competition. For instance, Kardinaal et al. (2007) demonstrated in laboratory experiments that a toxic genotype of the harmful cyanobacterium *Microcystis aeruginosa* was displaced by a non-toxic type under light limitation. The authors suggested that the transition from toxic to non-toxic *Microcystis* genotypes during the duration of a bloom could be explained by the development of high biomass concentrations towards the end of the bloom, which increases self-shading and enhances competition for light. Furthermore, van de Waal et al. (2011) demonstrated that the same non-toxic strain of *Microcystis* outcompeted the toxic strain in CO_2-limited conditions.

Yet, HABs are also known to be affected by limitation of nutrients, in particular, nitrogen and phosphorus. If toxic and non-toxic genotypes differ in their competitive ability for these two nutrients, the type of nutrient limitation that arises might strongly influence the toxicity of a bloom. Nitrogen and phosphorus limitation may occur towards the end of blooms, but these have also been observed during earlier phases (Moisander et al. 2003; Walve and Larsson 2007; Davis et al. 2009). Earlier periods of nutrient limitation may be interrupted by pulses of nutrient supply, for example, when wind-induced mixing of the water column enhances the diffusive inflow of nutrients from deeper water layers (Kononen et al. 1996; Stal et al. 1999; Davis et al. 2009). In fact, it has been argued that in natural systems such fluctuations in nutrient availability are the rule rather than the exception (e.g. Grover 1988, 1991; Padisák et al. 1993; Sommer 2002). Thus, if toxic and non-toxic genotypes also differ in their abilities to exploit nutrient pulses, the intensity of nutrient fluctuations might represent an additional important factor that influences how the toxin content of the bloom develops.

In this study, we used controlled microcosm experiments to investigate the competition between a toxic and a non-toxic strain (hereafter interchangeably also referred to as genotypes) of the harmful cyanobacterium *Microcystis aeruginosa* for nitrogen and phosphorus under both constant and pulsed supply. First, we measured several growth parameters for each genotype in replicated monocultures. Second, we investigated the competition between both genotypes under constant nutrient supply and under different kinds of nutrient pulses in replicated continuous and semi-continuous cultures. We also applied a parameterized Droop model to study the competition for phosphorus under constant and pulsed conditions in more detail with the help of numerical computer simulations.

Methods

Microcystis strains and culture maintenance

We investigated the competition between two axenic strains (hereafter genotypes) of *M. aeruginosa*: the toxic (microcystin-producing) wild type (WT) PCC

7806, obtained from the Pasteur Culture Collection in Paris, and the non-toxic mutant genotype (M), provided by Prof. E. Dittmann. The mutant genotype was originally generated from the PCC 7806 by inserting a chloramphenicol resistance cartridge into the *mcyB* gene involved in microcystin biosynthesis (Dittmann et al. 1997). Axenic pre-cultures of both genotypes were grown at 25 ± 1 °C in a 250-mL Erlenmeyer flask containing 40 mL of Z8 growth medium (Kotai 1972) at a constant light intensity of 90–100 µmol photons m^{-2} s^{-1}. Cultures were renewed weekly. Z8 growth medium was prepared with analytical grade water (ELIX®, Merck Millipore, Billerica, MA, USA). Stock cultures of the mutant genotype also contained 5 µg chloramphenicol mL^{-1} (Dittmann et al. 1997).

Total *Microcystis* cell densities

The *Microcystis* populations in our study exhibited a linear relationship between cell density and optical density (OD), as shown previously (Briand et al. 2012). We therefore established a standard conversion formula to estimate cell density as a function of OD. To achieve this, cell numbers of live samples from a chemostat culture at steady state were counted with a haemocytometer counting chamber using a compound microscope (Zeiss Axioskop 2 plus, Oberkochen, Germany). OD was measured at the wavelength 750 nm (UV-1800 spectrophotometer, Shimadzu, Japan). In the range of OD values that we observed in our experiments (0.01–0.25), the cell density of *Microcystis* (cells mL^{-1}) could be reliably estimated according to the following linear regression equation: density (cells mL^{-1}) = $OD_{750} \times 4 \times 10^7$ ($N = 7$; $R^2 = 0.949$).

Relative genotype proportions

We sampled our microcosms every 1–3 days, depending on the experiment, with sterile syringes (chemostats) or pipettes (batch cultures). Note that not all the samples were analysed in the end. We used quantitative real-time PCR (qPCR) for quantifying genotype proportions in our experimental samples. We followed previously published general protocols for the *Microcystis* system (Briand et al. 2009, 2012; Kurmayer and Kutzenberger 2003). In our study, DNA was extracted using the DNeasy Plant Mini Kit (Qiagen). Extractions were diluted tenfold for qPCR, which was used to estimate the proportion of the mutant genotype. All cells in the culture were quantified with previously described primers and a probe for an intergenic spacer in the phycocyanin (PC) gene (Briand et al. 2009, 2012; Kurmayer and Kutzenberger 2003). The primers and the probe used to quantify the mutant strain targeted the inserted chloramphenicol resistance cassette in the *mcyB* gene (Briand et al. 2012). The probes were labelled with the fluorescent reporter dye FAM (6-carboxyfluorescein) at the 5′ end and the amidite Black Hole Quencher-1 (BHQ-1) at the 3′ end. qPCR was performed with Applied Biosystems 7300 Real-Time PCR Systems (Life Technologies). The PCR reactions contained 10 µL of the PerfeCta FastMix® II supermix with ROX™ (Quanta Biosciences), 200 nM of forward and reverse primers, 100 nM of probe and 5 µL of template DNA in a total volume of 20 µL. The PCR was performed with a 2-step PCR program. The PCR program included initial denaturation for 3 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All PCR reactions were performed in triplicates, including negative reactions not containing DNA template and a positive control containing 50 % mutant cells, which was used to normalize results. The fluorescence threshold value was manually set to 0.15 in all qPCR runs, while baseline values were determined automatically. The proportion of mutant strain related to all cells was determined using the ΔCt method first developed by Briand et al. (2008) for *Planktothrix agardhii* and subsequently used for *M. aeruginosa* (Briand et al. 2009, 2012; Sabart et al. 2010). Further details of the qPCR protocol can be found from the supplementary information file.

Monoculture parameters

As a first step, we used monoculture experiments to determine several growth parameters for each genotype. These parameters allow to make predictions about the outcome of competition in mixed culture, which can then be compared to the results from the competition experiments. The monoculture parameters were also used to parameterize a variable-internal-stores model of competition.

Realized maximum specific growth rate

For each genotype, the realized maximum specific growth rate, μ_{max}, was determined in semi-continuous

cultures ($n = 6$) on N-limited and P-limited growth medium, respectively (Table 1). Cultures of 100 mL were inoculated with very low cell density (<1 % of the carrying capacity, cells pre-cultured in the same conditions for several days) and grown in non-agitated 250-mL Erlenmeyer flasks for 3 days with saturating 90–100 µmol photons m^{-2} s^{-1} light intensity at 25 ± 1 °C. Every day, 10 % of the culture was replaced with fresh growth medium, and the cell density, N_t, at time point t was determined. The realized maximum specific growth rates in N-limited and P-limited growth medium, $\mu_{max,N}$ and $\mu_{max,P}$, were calculated as the mean of the two growth rates calculated between the two time intervals as $\ln(N_{t+1}/N_t)/(t) + d$, where d represents the dilution rate of 0.1 day^{-1}. Time points were chosen so that population was growing exponentially at that time.

Critical nutrient concentrations

In order to measure the critical resource concentration for nitrogen, R_N^*, and phosphorus, R_P^*, each genotype was grown separately in chemostats (continuous cultures) of 470 mL culture volume with a constant dilution rate of 0.1 day^{-1} ($n = 3$, please see Table 1 for culture media details) for approximately 3 weeks until it reached a steady state. Light was unidirectional, with 90–100 µmol photons m^{-2} s^{-1} intensity. The R_N^* and R_p^* values of each genotype were then determined as the residual nutrient concentration in the nitrogen- and phosphorus-limited steady state, respectively. Nutrient analysis was based on triplicate samples of 50 mL that were taken from the steady state cultures and centrifuged at 8000×g and 4 °C for 5 min in order to remove *Microcystis* cells. The supernatant was then filtered through a 0.22 µm filter with *Supor*® membrane (Merck Millipore, Billerica, MA, USA) and stored at −20 °C. Analyses of N and P were conducted at Lammi Biological Station (University of Helsinki) with the following methodology: total dissolved nitrogen and phosphorus were analysed by flow injection analysis (FIA) colorimetry (Quikchem8000, Lachat Instruments, Hach Company, Loveland, CO, USA) using the alkaline persulfate oxidation method (QuikChem®METHOD 10-107-04-1-I, Lachat Instruments, Hach Company, Loveland, CO, USA) for nitrogen concentrations and the alkaline persulfate digestion method (QuikChem®METHOD 10-115-01-1-S, Lachat Instruments, Hach Company, Loveland, CO, USA) for phosphorus concentrations, respectively.

Minimum nutrient quota

The minimum quotas for nitrogen and phosphorus, $Q_{min,N}$ and $Q_{min,P}$, of each genotype were determined as the intracellular nutrient concentration measured from cells obtained from each chemostat on two separate days, 1 week apart, when the culture had reached steady state. 50 mL of culture was collected and centrifuged at 8000×g and 4 °C for 5 min. The supernatant was removed from the

Table 1 Nutrient supply concentrations and supply regimes of the different competition experiments

Type of experiment	N-supply concentration, $R_{in,N}$ (µmol/L)	P-supply concentration, $R_{in,P}$ (µmol/L)	N:P-supply ratio	Pulse height (µmol N or P)	Pulse period (days)
Continuous supply (chemostat and parameter estimation)					
N-limited	105	26.25	4	–	–
P-limited	192	3	64	–	–
3-day pulse (semi-continuous culture)					
N-limited	105	26.25	4	10.5	3
P-limited	160	2.5	64	0.25	3
Single pulse (semi-continuous culture)					
N-limited	1050[a]	26.25	40[a]	105	28
P-limited	160	25[a]	6.4[a]	2.5	28

[a] Please note that these supply rates of the limiting nutrient are only for the beginning of the experiment. After the start of the experiment, we did not provide any of the limiting nutrient (N or P)

cells, and 3 mL of type-2 analytical grade water (ELIX®, Merck Millipore, Billerica, MA, USA) was used to resuspend the cells. A volume of 1.5 mL of the cell solution was collected into a 2 mL tube and stored at −20 °C. Nutrients were analysed at a later stage as described in the paragraph above on critical nutrient concentrations.

Maximum nutrient uptake rates

To measure the maximum phosphorus uptake rate, 50 mL of a P-depleted (cells cultured without P for 4 days) culture was added in 500 mL of growth medium containing 25 µmol L^{-1} of phosphate. The reduction in dissolved phosphorus concentration was then monitored after 0, 2, 4, 6 and 24 h. Plotting the external phosphorus concentrations versus time revealed an initial linear phase so that the maximum P-uptake rate was estimated by linear regression as the absolute value of the slope. Unfortunately, due to technical problems, the experiments to measure the maximum uptake rate of nitrogen failed so that this rate could not be determined.

Maximum nutrient quota

Maximum intracellular nitrogen and phosphorus quotas, $Q_{\max,N}$ and $Q_{\max,P}$, were determined by culturing cells in 250-mL Erlenmeyer flasks containing Z8 culture medium with nitrogen and phosphorus in non-limiting concentrations (Kotai 1972). Cultures were grown for 3 days in the same conditions as in growth test before cells were collected for nutrient measurements. 50 mL of the sample was centrifuged at 8000×*g* and 4 °C for 5 min. The supernatant was removed as precisely as possible, cells were washed three times with 50 mL of type-2 analytical grade water, and the sample was stored frozen in −20 °C. Nitrogen and phosphorus concentrations were analysed at a later stage as described for critical nutrient concentrations. Uptake rate and nutrient quota parameters are based on values obtained from a single microcosm.

Half-saturation constant

The half-saturation constant for phosphorus uptake for each genotype, *K*, was estimated by optimizing the numerical solution to the model described in the methods section further below (Eqs. 1–3). The model was first solved by using the function lsoda of the R-package deSolve, which automatically switches between stiff and non-stiff integration methods (Soetaert et al. 2010). The model predictions were then fit to the data from the monoculture experiments by minimizing the residual sum of squares with the help of the Broyden-Fletcher-Goldfarb-Shanno algorithm of the function optim in R (version 3.0.3). Half-saturation constants for nitrogen uptake could not be determined because of the missing estimates of the maximum nitrogen uptake rate.

Design of competition experiments

We investigated the competition between the toxic and non-toxic strain under N- and P-limitation and three different nutrient supply regimes: (1) Competition experiments under constant supply were conducted in chemostats with constant dilution rate of 0.1 day^{-1} ("continuous supply"). The set up and maintenance of the chemostats followed the same methodology as described previously (Fussmann et al. 2000; Hiltunen et al. 2013), and all containers used during the experimental procedures were washed with 2 % HCl. Chemostat cultures were started as follows: both genotypes were grown in monocultures into steady state, cell densities were enumerated, and then both genotypes were mixed in equal proportion. (2) Competition experiments under pulsed nutrient supply were run as semi-continuous cultures, using 1000-mL Erlenmeyer flasks that contained 500 mL of culture. For the experiments with short pulse intervals, 10 % of the culture was replaced every third day with fresh growth medium ("3-day-interval-pulse"). (3) For the experiments with a single large pulse, 10 % of the mixed culture was replaced on the first day with fresh growth medium ("single pulse"). Subsequent replacements occurred every third day, where 10 % of the mixed culture was replaced with fresh growth medium devoid of nitrate or phosphate, respectively. Experiments were carried out in Z8 culture medium without chloramphenicol and with varying nitrogen (N) and phosphorus (P) concentrations, depending on the type of nutrient limitation and supply regime. An overview of the different types of experiments and their corresponding nutrient supply concentrations and supply regime is given in Table 1. In the two different pulsed experiments, the total amount of limiting

nutrient provided during the course of the experiment was the same. All experiments were replicated at least three times and were initially inoculated at a 1:1 ratio of toxic versus non-toxic strain cells. Light was unidirectional, with 90–100 µmol photons m^{-2} s^{-1} intensity. Furthermore, in all experiments, the light intensity measured at the backside of the culture vessels was 35 µmol photons m^{-2} s^{-1} or higher, which indicates that light availability within the culture was growth-saturating (Hesse et al. 2001; Huisman et al. 2002). Light intensity was measured with a model LI-250 light metre (LI-COR, Lincoln, NE, USA).

Statistics

We used Student's *t*-test to investigate the effect of genotype identity on the different nitrogen and phosphorus parameters, and we used a repeated measures ANOVA (RMANOVA) to investigate the effects of growth medium and supply regime on *Microcystis* cell density. All analyses were performed with PASW statistics (SPSS Inc. Chicago IL, v. 20.0) software.

Mathematical model and numerical simulations

Many phytoplankton species take up nutrients such as nitrogen and phosphorus in excess when these resources are abundant and store them intracellularly until periods of low nutrient availability arise. Therefore, we use a variable-internal-stores model ("Droop model") in order to investigate the effect of nutrient fluctuations on the competition between two *Microcystis* genotypes (Droop 1973). This model has been analysed previously by Grover (1991) and will therefore be explained here only in brief. The model assumes a spatially homogeneous habitat, such as a well-mixed chemostat. The population density of a specific genotype, N_i, increases with a certain specific growth rate, μ_i, and it decreases with a constant rate, D, the dilution rate. The specific growth rate of a genotype depends on its intracellular phosphorus pool, Q_i, which increases with the specific uptake rate, v_i, and decreases due to cell division.

The phosphorus concentration in the water, R, increases due to a constant inflow of phosphorus from the supply vessel and decreases due to dilution and uptake by phytoplankton cells. The dynamics of N_i, Q_i and R in the chemostat are then given by

$$\frac{dN_i}{dt} = \mu_i(Q_i)N_i - DN_i \quad (1)$$

$$\frac{dQ_i}{dt} = v_i(R, Q_i) - \mu_i(Q_i)Q_i \quad (2)$$

$$\frac{dR}{dt} = D(R_{in} - R) - \sum_{i=1}^{2} v_i(R, Q_i)N_i \quad (3)$$

Here, R_{in} defines the supply concentration of phosphorus. The specific growth rate of strain i, μ_i, is a saturating function of the internal phosphorus quota and is given by

$$\mu_i = \mu'_{max,i}\left(1 - \frac{Q_{min,i}}{Q_i}\right), \quad (4)$$

where $\mu'_{max,i}$ denotes the maximum specific growth rate of strain i and $Q_{min,i}$ denotes the persistence quota, i.e. the phosphorus concentration inside the cell of strain i at which growth ceases [i.e. where $\mu(Q_{min}) = 0$]. Note that the maximum specific growth rate, $\mu'_{max,i}$, is never reached because phosphorus quotas do not increase infinitely. Instead, the realized maximum specific growth rate, $\mu_{max,i}$, which is reached at the point where the phosphorus quota is at its maximum, $Q_{max,i}$, is always lower. The phosphorus uptake rate of strain i, v_i, is given by

$$v_i = v_{max,i}\left(\frac{R}{K_i + R}\right)\left(\frac{Q_{max,i} - Q_i}{Q_{max,i} - Q_{min,i}}\right), \quad (5)$$

where K_i describes the half-saturation constant of genotype i for phosphorus uptake. Thus, v increases with the external phosphorus concentrations as described by the Michaelis–Menten-function, and it decreases with increasing intracellular phosphorus quota. For each genotype, the model parameters were measured in monocultures, except for the half-saturation constants for phosphorus uptake, K_i, which were estimated by the curve-fitting method as describe above. The parameterized model was then used to investigate the competition for a large number of different phosphorus pulse heights and pulse frequencies numerically. In total, simulations were run for $251 \times 351 = 88{,}101$ different combinations of pulse height and pulse period in the range of 10^{-2} (=0.01) and $10^{0.5}$ (=3.16) µmol P L^{-1} and in the range of 10^{-2} (=0.01) and $10^{1.5}$ (=31.6) days. Note that to be comparable with the experimental results, the model simulations were not run until the final outcome of

competition was observed, but they were stopped after 40 days. The observed genotype frequencies at this time point were categorized, and the result was drawn on the plane of pulse height and frequency (Fig. 2).

Results

Monocultures

Nitrogen parameters

The monoculture experiments revealed that the toxic and the non-toxic *Microcystis* genotype differed in their growth and resource utilization traits. Under nitrogen-limited conditions, the critical nitrogen concentration, R_N^* , of the toxic genotype measured in chemostats at steady state was clearly lower than that of the non-toxic strain (*t*-test: $t(6) = 7.56, p < 0.001$). The toxic genotype also exhibited a higher maximum growth rate, $\mu_{\max}$ (*t*-test: $t(10) = -3.81$, $p = 0.003$). Even though we do not have replication required for statistical testing, it appears that the WT also had a lower minimum nitrogen quota, $Q_{\min,N}$, and a higher maximum nitrogen quota, $Q_{\max,N}$, compared to the non-toxic genotype (Table 2).

Phosphorus parameters

Under phosphorus-limited conditions, the critical phosphorus concentration, R_P^*, of the toxic genotype measured in chemostats at steady state was higher than of the non-toxic genotype [*t* test: $t(3) = -5.0$, $p = 0.015$]. The toxic genotype also had a higher maximum specific growth rate than the non-toxic genotype (Grover 1990, 1991; *t*-test: $t(10) = -2.56$, $p = 0.028$). In contrast, the non-toxic strain had a higher maximum uptake rate for phosphorus, higher maximum phosphorus quota and a slightly lower minimum phosphorus quota (Table 2).

Competition dynamics

Nitrogen limitation

The replicated competition experiments under nitrogen limitation revealed very consistent results (dynamics are presented in Fig. 1, and statistics for competitive exclusion in Table 3). Under constant supply, the total cell density remained constant throughout the experiment (Fig. 1a). Yet, the relative frequency of the toxic genotype gradually increased until it reached 1 after approximately 40 days (Fig. 1a;

Table 2 Nitrogen and phosphorus parameters of the toxic and the non-toxic *Microcystis* genotypes

Parameter	Description	Unit	WT	M
Nitrogen				
$R_N{}^*$	Critical resource concentration	µmol L^{-1}	32.08	37.08
$\mu_{\max,N}$	Realized maximum specific growth rate	day^{-1}	0.549 ± 0.039	0.472 ± 0.036
$v_{\max,N}$	Maximum nitrogen uptake rate	fmol $cell^{-1}$ day^{-1}	n.d.	n.d.
K_N	Half-saturation constant for nitrogen uptake	µmol L^{-1}	n.d.	n.d.
$Q_{\min,N}$	Minimum intracellular nitrogen content	fmol $cell^{-1}$	8.019	8.506
$Q_{\max,N}$	Maximum intracellular nitrogen content	fmol $cell^{-1}$	18.92	16.36
Phosphorus				
$Rp*$	Critical resource concentration	µmol L^{-1}	0.073 ± 0.016	0.03 ± 0.0
$\mu_{\max,P}$	Maximum specific growth rate	day^{-1}	0.448 ± 0.040	0.398 ± 0.026
$v_{\max,P}$	Maximum phosphorus uptake rate	fmol $cell^{-1}$ day^{-1}	1.853	2.272
K_P	Half-saturation constant for phosphorus uptake	µmol L^{-1}	3.004[a]	3.004[a]
$Q_{\min,P}$	Minimum intracellular phosphorus content	fmol $cell^{-1}$	0.285	0.273
$Q_{\max,P}$	Maximum intracellular phosphorus content	fmol $cell^{-1}$	1.880	1.210

Values marked with [a]were estimated by curve fitting. The maximum nitrogen uptake rate, $v_{\max,N}$, and the corresponding half-saturation constant, K_N, were not determined due to technical problems

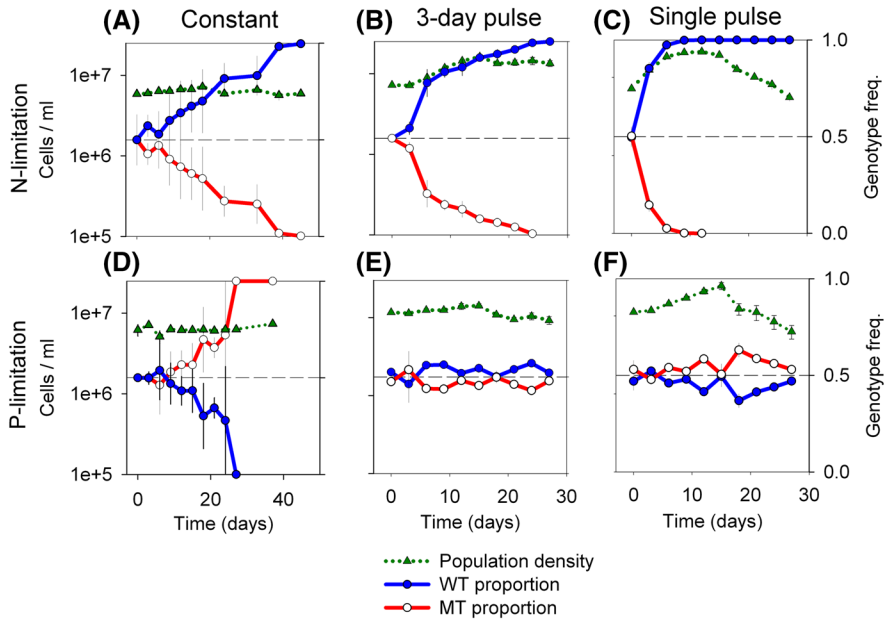

Fig. 1 Relative frequencies of the non-toxic wild-type strain (*red line*, *filled symbols*) and the non-toxic mutant (*blue line*, *closed symbols*) during competition for nitrogen (**a**–**c**) and phosphorus (**d**-**f**) at different levels of nutrient fluctuations: (**a**, **d**) nutrients supplied continuously, (**b**, **e**) nutrients pulsed at 3-day-intervals, (**c**, **f**) single large nutrient pulse at the beginning of the experiments. *Triangles with green dotted line* show total *Microcystis* densities (cells mL^{-1}). *Symbols* represent means of four replicate microcosms (except in A, $n = 5$), and *vertical bars* show standard error of the mean. (Color figure online)

Table 3 Rates of competitive exclusion in the six different competition experiments

Resource supply regime	Limiting nutrient	*n*	Slope*	S.E.	*t* value	*p* value	Outcome of competition	Figures
Constant	N	5	**−0.11**	0.001	−12.8	<0.0001	WT wins	1a
Constant	P	4	**0.012**	0.004	3.14	0.005	MT wins	1d
3-day pulse	N	4	**−0.144**	0.015	−9.80	<0.0001	WT wins	1b
3-day pulse	P	4	−0.001	0.002	−0.59	0.559	Co-existence	1e
Single pulse	N	4	**−0.541**	0.038	−14.1	<0.0001	WT wins	1c
Single pulse	P	4	0.004	0.002	1.77	0.087	Co-existence	1f

Rates (cells mL^{-1} day^{-1}) are calculated as the slope of the linear regression of the relative frequency of the non-toxic mutant versus time. Significant positive slopes indicate competitive exclusion of the wild type by the mutant; significant negative slopes indicate competitive exclusion of the mutant by the wild type. Non-significant slopes indicate coexistence. Significant slopes values ($p < 0.05$) are marked in bold

Table 3). Correspondingly, the relative frequency of the non-toxic genotype decreased until it was washed out within the same time period. Thus, under constant nitrogen supply, the toxic genotype clearly outcompeted the non-toxic genotype. When nitrogen was supplied in pulses every 3 days, the total cell density increased in the beginning of the experiments and reached a constant density after 15 days. The relative frequency of the toxic genotype showed a steep increase within the first 6 days of the experiment, followed by a more moderate rise until it completely dominated the culture after 24 days. By definition, the relative frequency of the non-toxic genotype exhibited the opposite pattern. When nitrogen was supplied as a single large pulse at the beginning of the experiments, the dynamics of the total cell density showed a hump-shaped curve (Fig. 1c). The relative frequency of the toxic genotype rose steeply in the beginning and

reached a value of 1 after 9 days, while the non-toxic genotype was washed out (Table 3).

Phosphorus limitation

The total cell density in the experiments with constant phosphorus supply remained constant throughout the experiments and did not differ from the experiments with constant nitrogen supply (Fig. 1a, d; average cell density during the experiment, N-limitation: $6.43 \pm 0.49 \times 10^6$ cells mL^{-1}; Fig. 1d; P-limitation $6.31 \pm 0.39 \times 10^6$ cells mL^{-1}; identity of the limiting nutrient: *t* test, $t(5) = 0.185$, $p = 0.860$). Yet, the relative genotype frequencies showed considerable variation between replicates. Although the critical phosphorus concentrations measured in monocultures suggested that the non-toxic genotype should win the competition, three competition experiments showed that the non-toxic genotype increased in relative frequency or outcompeted the toxic mutant, while two competition experiments showed coexistence of both genotypes at approximately equal frequencies. Yet, the replicate experiments showed consistent results in the pulsed experiments. In the 3-day-interval-pulse experiments, the total cell density remained constant throughout the experiment and did not differ from the constant supply experiment. Both genotypes coexisted at approximately equal densities for 27 days. (Fig. 1e). In the single-pulse experiments, the total cell density showed a hump-shaped curve, and both genotypes coexisted again at approximately equal densities (Fig. 1f).

Rate of competitive exclusion

As described in the previous section, the nutrient supply regime affected the rate of competitive exclusion, whereby the effect depended on the type of nutrient limitation. We therefore plotted the natural logarithm of the ratio of mutant to wild-type relative frequencies versus time and calculated the rate of competitive exclusion from the slope of the linear regression line (see, e.g. van de Waal et al. 2011). In the nitrogen-limited experiments, the rate of competitive exclusion increased with pulse intensity, being slowest under constant supply and fastest when a single large nitrogen pulse was given (Table 3; Fig. 1a–c). In the phosphorus-limited experiments, competitive exclusion was reversed and was fastest under constant supply, while pulsed phosphorus supply considerably slowed down competitive exclusion (Table 3; Fig. 1d–f).

Model predictions concerning competition for phosphorus

Because the phosphorus-limited experiments did not provide very clear results, we also investigated competition under phosphorus limitation with the help of numerical simulations using the growth parameters for each genotype measured in monoculture. The results observed after 40 days show that the non-toxic genotype wins the competition under very short pulse intervals and low pulse height in the lower left corner of Fig. 2. Because the results are visualized in a log–log plot, the dominance of the non-toxic genotype is actually confined to a rather small parameter space of nearly constant phosphorus supply. The dominance region of the non-toxic genotype extends further as a fine, narrowing, diagonal band towards the upper right corner of the graph, but then bends off beforehand and continues rising more slowly with increasing oscillations. To the upper left side of this stripe extends a large region of longer pulse intervals but relatively high pulses, where both genotypes are able to coexist for the period of 40 days. In the lower right corner of the graph, the pulses are too seldom and too low so that both genotypes are washed out before the end of the experiments.

Discussion

Our study demonstrates that closely related genotypes of one species can have distinct nutrient physiologies. Our study also shows that these physiological differences can have consequences for the growth and competition between these genotypes, which eventually may have a profound impact on the functioning and the toxicity of blooms in nature. We here discuss the physiological and theoretical background and the ecological implications of these results.

Physiological differences of toxic and non-toxic genotypes

Genetically, the non-toxic mutant genotype used in our study differed from the toxic wild-type genotype

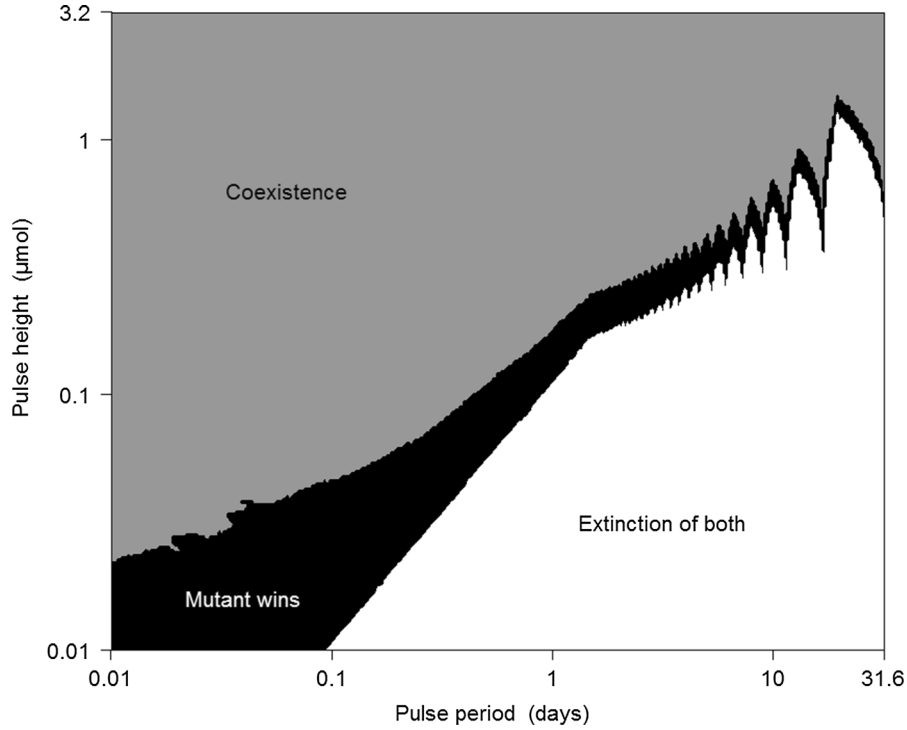

Fig. 2 Simulation results showing the outcome of competition between the toxic *Microcystis* wild-type PCC 7806 and the non-toxic *mcyB*-deficient mutant genotype for different periods and heights of phosphorus pulses. Note that to be comparable with the experimental results, the graph shows the state of competition after 40 days rather than the equilibrium outcome

only due to the deletion of the *mcyB* gene. Transcription of *mcy*-genes has been shown to be stimulated by light (Kaebernick et al. 2000; Tonk et al. 2005), and a number of recent studies suggest that adaptation to high light conditions and oxidative stress protection might be the primary role of microcystin (Zilliges et al. 2011; Makower et al. 2015; Meissner et al. 2015), though also other functions have been suggested (Kaplan et al. 2012). Interestingly, however, our monoculture experiments reveal that the toxic and the non-toxic genotype show significant differences also in other traits, which do not seem to be directly linked to the cellular functions of microcystins that have so far been described. The toxic genotype appeared to have a higher maximum specific growth rate, μ_{max}, than the non-toxic genotype in both types of growth media (see Table 1). Yet, this is in disagreement with Hesse et al. (2001) and Briand et al. (2012), who did not find any difference between the two genotypes. The toxic genotype also had higher maximum quotas for nitrogen, $Q_{max,N}$, and phosphorus, $Q_{max,P}$, which suggest that it can store more nutrients and that it can maintain high uptake rates over a longer time period (Table 1; Grover 1991). The non-toxic genotype had a lower critical phosphorus concentration, R_P^*, a lower minimum phosphorus requirement, $Q_{min,P}$, and a higher maximum phosphorus uptake rate, $v_{max,P}$, which suggest that it can deal better with constantly low phosphorus concentrations and that it can better exploit small phosphorus pulses. One possible explanation for this result is that microcystin production is costly in terms of ATP, and thus also in terms of phosphorus, because the corresponding gene cluster and enzyme complex need to be maintained active (Bickel et al. 2000). However, Briand et al. (2016) found in their experiments with the same strains (WT and mutant) under monoculture and co-culture conditions that the mutant, which is not able to produce microcystin, produces higher concentrations of the remaining cyanopeptides. In the light of this finding, interpreting the cost of microcystin production is not so straight forward.

It is surprising that the toxic genotype also had a lower minimum nitrogen quota, $Q_{min,N}$, and a lower critical nitrogen concentration, R_N^*, than the non-toxic genotype. Yet, this result has to be taken with care, because the very high R_N^* - values compared to other studies (Brussaard and Riegman 1998; Wilken et al. 2013) indicate that something else might have been limiting growth during these measurements as well. In fact, one would expect the toxic genotype to have a higher nitrogen demand because the production of

microcystins is costly in terms of nitrogen (e.g. Long et al. 2001; Downing et al. 2005; van de Waal et al. 2009, 2010).

We do not yet have a clear explanation for the physiological differences found in our study. Yet, it is known that the deletion of the *mcyB* gene has various consequences for the cell, e.g. for pigmentation and cellular structure (Hesse et al. 2001), but also for the expression of other genes and proteins, which are related to photosynthesis and respiration, energy-metabolism or carbon–nitrogen metabolism, to name a few (Alexova et al. 2011; Zilliges et al. 2011; Makower et al. 2015). Thus, the intracellular effects of microcystin production seem manifold, and it is therefore likely that it has side-effects on other phenotypic traits as well.

Competition in pulsed habitats

Our competition experiments show that the distinct nutrient physiologies of the toxic and the non-toxic genotype discovered in the monoculture experiments also have important consequences for their population dynamics. Our results are largely in agreement with common resource competition models (Tilman 1982; Grover 1991, 1997). Under constant nutrient inflow, competition was won by the affinity-specialist (sensu Sommer 1984), i.e. by the genotype which had the lowest R^* value for the limiting nutrient (Table 1; Fig. 1a, d). Interestingly, the toxic and the non-toxic genotype exhibited a trade-off in the competitive ability for nitrogen and phosphorus, which is also commonly observed between phytoplankton species (Edwards et al. 2011). On the one hand, our results are consistent with Vézie et al. (2002) and Lei et al. (2015) who found higher growth rates of the mutant genotype and a non-toxic strain under phosphorus limitation. On the other hand, our results are inconsistent with Vézie et al. (2002) and Briand et al. (2012) who found that the mutant genotype, rather than the wild type, was growing faster under N-limitation and Lei et al. (2015) did not observe a clear difference. One reason for this discrepancy might be that these studies used batch cultures rather than continuous or semi-continuous cultures, where the dominant species or genotype in the competition does not need to be the affinity-specialist. Another reason might be that all three experiments differed in the degree of N-limitation and that we cannot assure that N-limitation in our experiments was absolute.

Under pulsed nutrient inflow, resource competition theory predicts that the growth-specialist (sensu Sommer 1984), i.e. the species with the highest maximum growth rate, μ_{max}, wins the competition under small (and frequent) nutrient pulses, while the storage specialist (sensu Sommer 1984), i.e. species with lowest Q_{min}, highest Q_{max} and highest v_{max}, wins the competition under large (and unfrequent) nutrient pulses (Grover 1991, 1997). Our monoculture and competition experiments together suggest that the toxic genotype was the growth as well as the storage specialist for both nitrogen and phosphorus (Table 1; Fig. 1b, c, e, f). Thus, under nitrogen limitation, we did not observe a trade-off in the competitive ability under constant and pulsed nutrient inflow. Under phosphorus limitation, however, this trade-off seemed to be existent. Here, the non-toxic genotype was the superior competitor under constant phosphorus inflow (Table 1; Fig. 1d). Under pulsed phosphorus inflow, we did not observed a full reversal of competitive dominance, but the non-toxic genotype no longer outcompeted the toxic genotype. Instead, both genotypes coexisted for the duration of the experiment (Fig. 1e, f). Moreover, our simulation results indicate that the dominance of the toxic genotype would further increase with higher phosphorus pulse intensity (results not shown). This result is in agreement with the general observation that phytoplankton species show a trade-off between the competitive ability under constant and variable phosphorus limitation (Edwards et al. 2013).

Previous studies with toxic and non-toxic *Microcystis* strains have demonstrated distinct competitive abilities under light carbon, nitrogen and phosphorus limitation (Kardinaal et al. 2007; Van de Waal et al. 2011; LeBlanc Renaud et al. 2011; Briand et al. 2012; Lei et al. 2015). Yet, as Briand et al. (2012) and Lei et al. (2015) conducted their studies in batch cultures competition for nitrogen and phosphorus in continuous or semi-continuous culture has not been investigated before. At first sight, our experiments suggest that phosphorus limitation might favour the dominance of non-toxic genotypes and that microcystin production does not provide a clear benefit under P-limitation. As P-limitation should not affect the reducing power of cells to the same extent as C- or N-limitation (Sterner and Elser 2002), this results is in line with the hypothesis that microcystins are beneficial under oxidative stress (Zilliges et al. 2011;

Makower et al. 2015; Meissner et al. 2015). Yet, it has to be pointed out that the competitive dominance of the mutant genotype under constant phosphorus limitation was rather fragile and seemed to be limited to a very low parameter range of nearly constant conditions, as rather small nutrient pulses already enabled the toxic genotype to coexist. (Figs. 1d, 2). It therefore remains an open question whether such highly constant conditions occur in natural freshwater systems and whether phosphorus limitation can really mitigate bloom toxicity.

Nutrient limitation in natural blooms

Our experiments suggest that toxic *Microcystis* genotypes might be better competitors under nitrogen limitation than non-toxic genotypes, while (constant) phosphorus limitation favours non-toxic genotypes. Results that we present in this study are obtained in simplified laboratory conditions and not directly comparable with natural systems. However, if some comparison is allowed, our result on genotype dominance under N-limitation is consistent with several field studies showing that low N:P ratios seem to favour toxic *Microcystis* genotypes over their non-toxic counterparts (Davis et al. 2009; Rinta-Kanto et al. 2009; Gobler et al. 2016). Furthermore, higher microcystin concentrations have been linked to low N:P ratios in four reservoirs in the USA (Harris et al. 2016), indirectly indicating higher frequencies of toxic genotypes. However, our results do not explain why the relative frequency of toxic genotypes has been found to increase with increasing N concentrations (Yoshida et al. 2005; Davis et al. 2010).

Acknowledgments We thank Lyudmila Saari and Matti Wahlsten for technical assistance, Prof. Elke Dittmann for providing the mutant *Microcystis* strain and Riitta Ilola and Jaakko Vaininpää for nutrient analysis and Jouni Jokela for advising with the analysis. We also thank Johannes Cairns for editing the language and two anonymous reviewers and editor for helpful comments. This work was funded by University of Helsinki 3-year Grant (2015–2018) to T.H. and Academy of Finland (project #106993 to T.H.).

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