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Competition between toxic and non-toxic *Microcystis aeruginosa* and its ecological implication

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Abstract The frequency of toxic cyanobacterial blooms has increased in recent decades, but the factors that regulate the dominance of toxin-producing cyanobacteria over non-toxin-producing strains of one species are still obscure. This study examined the effects of temperature, light intensity, nitrate and phosphate on the dominance of MC-producing and non-MC-producing strains of *Microcystis aeruginosa* in monoculture and co-culture experiments. In the monoculture experiments, growth rates of the non-MC-producing strain were higher than those of the MC-producing strain under the same growth conditions. However, at the end of the co-culture experiments, the MC-producing strain became surprisingly dominant in all treatments except when treated with extreme low phosphate concentrations. Higher temperatures and nutrient levels can shift the dominance more quickly towards the toxic strain. The dominance may be explained by allelopathic interactions through allelochemicals and other secondary metabolites, but not MC. Environmental factors such as extremely low phosphate content may exert an indirect effect on strain dominance by changing the production of allelochemicals. Our findings highlight the complications in predicting competitive outcome for cyanobacterial strains in natural environments.

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Introduction

The frequency of cyanobacterial blooms associated with eutrophication, and global warming has increased in recent decades and is expected to continue to rise (Carey et al. 2012; O'Neil et al. 2012). A major bloom-forming component is *Microcystis* spp., which can proliferate quickly in eutrophic freshwaters and produce hepatotoxic microcystins (MC) (Watanabe et al. 1996; de Figueiredo et al. 2004). The MC content in water usually fluctuates, making it difficult to predict their health risk. Some studies attributed this fluctuation partially to changes in the ratio of MC-producing (toxic) and non-MC-producing (non-toxic) cells in *Microcystis* populations (Kardinaal et al. 2007a; Welker et al. 2007).

The discovery of the MC synthetase gene cluster (Tillett et al. 2000) has paved the way for quantitative PCR methods for studying the dynamics of toxigenic cells in natural bloom samples (Kurmayer and Kutzenberger 2003), such as clarification of possible environmental factors affecting the ratio of toxic and non-toxic cells in *Microcystis* populations (Yoshida et al. 2007; Sabart et al. 2010). However, current conclusions based on such molecular methods are inconsistent. For example, temperature and ortho-phosphate were found to be uncorrelated with the presence of MC-producing *Microcystis* cells in a Japanese lake (Yoshida et al. 2007), whereas a positive relationship was observed in four lakes across Northeast USA (Davis et al. 2009). Kardinaal et al. (2007a) proposed that a seasonal succession of toxic and non-toxic *Microcystis* populations could be a key factor in determining MC

contents. However, data from a French reservoir and from Lake Erie suggested no such relationship exists between MC concentrations and the number of toxigenic *Microcystis* cells (Rinta-Kanto et al. 2009; Sabart et al. 2010).

Numerous monoculture and co-culture investigations using MC-producing and non-MC-producing strains have been carried out to explain the selection of toxic and non-toxic cells under different growth conditions (Vézie et al. 2002; Kardinaal et al. 2007a, b; Briand et al. 2008a, b, 2012; Dziallas and Grossart 2011; Fujii et al. 2011; Leblanc Renaud et al. 2011; Van de Waal et al. 2011). The general consensus is that toxic cells dominate in growth-limiting conditions, such as in the presence of high temperatures, high concentrations of oxygen radicals, higher or lower levels of nitrate and phosphate, and lower levels of CO_2. However, results regarding the effect of light intensity on *Microcystis* population dynamics remain highly inconsistent (Kardinaal et al. 2007a, b; Briand et al. 2008a, b, 2012; Leblanc Renaud et al. 2011). Most co-culture experiments tend to support the putative role and metabolic cost of MC production, but the conclusions provided are conflicting. For instance, Kardinaal et al. (2007a, b) hypothesized a possible high energetic cost of MC synthesis to explain why non-toxic strains are generally better competitors. However, this hypothesis was not supported by Van de Waal et al. (2011) and Leblanc Renaud et al. (2011), who showed that the cost of MC production may be overstated.

Environmental factors affecting the selection of MC-producing and non-MC-producing *Microcystis* genotypes have been identified in a relatively small number of strains. Clearly, further work is required to address this globally relevant issue on the relationship between genotypic dominance and MC concentration, and of the effects of environmental factors. In the present study, we examined the dynamics of toxic and non-toxic strains of *Microcystis aeruginosa* isolated from freshwater in China under gradients of temperatures, light intensities, and nitrate and phosphate concentrations in monoculture and co-cultures. The results are discussed with respect to the role of MC production in genotypic dominance.

Materials and methods

Strains and culture conditions

One MC-producing strain (*M. aeruginosa*-FACHB905) and one non-MC-producing strain (*M. aeruginosa*-FACHB469) were provided by the culture collection of Chinese Academy of Science in Wuhan. They were isolated from shallow freshwater in China, and have the chance to meet and compete each other in one lake. FACHB905 was chosen as it has a simple MC profile, producing mainly MC-LR. The two strains were non-axenic and grew as single-cell populations. A pre-culture of each strain was grown at 25 °C in a 1 L Pyrex Erlenmeyer flasks containing 500 mL BG11 medium with an adjusted pH of 8.2 (Rippka et al. 1979). Erlenmeyer flasks were placed in a culture chamber with the appropriate light intensity using cool white fluorescent lights. Light intensities were measured in empty culture chambers using a QSL-2101 Scalar PAR Irradiance Sensor (Biospherical Instruments Inc., USA). A 16/8 h light/dark cycle was systematically applied.

Monoculture experiments

Two experiments involving each *M. aeruginosa* strain were conducted. The first such monoculture experiment was conducted using a crossed factorial design with three temperature levels (LT = 16 °C, MT = 24 °C, HT = 32 °C) and three light levels (LL = 25 μmol m^{-2} s^{-1}, ML = 60 μmol m^{-2} s^{-1}, HL = 120 μmol m^{-2} s^{-1}), giving a total of nine combinations (LTLL, LTML, LTHL, MTLL, MTML, MTHL, HTLL, HTML and HTHL). A second experiment was conducted using three nitrate concentrations (N1 = 15 mg L^{-1}, N2 = 150 mg L^{-1}, N3 = 7500 mg L^{-1}) or three phosphate concentrations (P1 = 0.4 mg L^{-1}, P2 = 4 mg L^{-1}, P3 = 200 mg L^{-1}) under constant temperature (28 °C) and light intensity (about 35 μmol m^{-2} s^{-1}). Cells cultured in complete BG11 medium with nitrate concentration of 1500 mg L^{-1} and phosphate concentration of 40 mg L^{-1} were used as controls. Essential potassium and sodium media components were provided in the form of KCl and NaCl.

All cultures were inoculated with 10^5 cells mL^{-1} in three identical 50 mL capped test tubes (25 mm × 150 mm) containing 35 mL of the medium and were gently mixed twice daily. Chlorophyll-a concentrations were measured daily with a TD-700 laboratory Fluorometer with 436 nm excitation filter and 680 nm emission filter (Turner Designs, California, USA). The specific growth rates (SGR, d^{-1}) of samples in the exponential growth phase were calculated according to the method of Guillard (1973) by a least squares fit of a straight line to the data after logarithmic transformation. The similarity of the SGR was tested by covariance analysis using statistical software package SPSS 18.0.

Co-culture experiments

Co-culture experiments involved growing of both MC-producing FACHB905 and non-MC-producing FACHB469 strains in the same culture. They were conducted in a similar fashion to the monoculture experiments. In the first experiment, however, two light levels (LL = 35 μmol m^{-2} s^{-1} and HL = 120 μmol m^{-2} s^{-1}) were used instead of three

light levels, resulting in a crossed factorial design with a total of six combinations (LTLL, LTHL, MTLL, MTHL, HTLL and HTHL) instead of nine combinations. There were three temperature levels (LT, MT and HT) as in the monoculture experiment. The second experiment was conducted with three nitrate (N1, N2 and N3) and phosphate (P1, P2 and P3) concentrations as in the monoculture experiment. The flasks were randomly repositioned each day relative to the light source.

FACHB905 and FACHB469 were inoculated at a cell ratio of 1:1 (cell density for each strain of 5×10^4 - cells mL^{-1}) in three 500 mL Pyrex Erlenmeyer flasks containing 400 mL of the medium and were gently mixed twice daily. The culture samples were taken every 3–4 days for OD measurement and total MC quantification, as well as for subsequent real-time PCR. *Microcystis* total cell density was estimated by converting the OD at 750 nm to cell density (cells mL^{-1}) based on the positive correlation between these two parameters ($R^2 = 0.9926$, $N = 40$; $P < 0.01$) (Briand et al. 2008a, b).

DNA extraction and real-time PCR

The proportion of MC-producing cells was determined by real-time PCR analysis in the co-culture experiments. Depending on the cell density, between 5 and 10 ml of culture was taken and filtered through Whatman GF/C filter paper. Total DNA was extracted from filtered samples using a DNeasy Plant Mini Kits (Qiagen) according to the manufacturer's protocol. The quantity and quality of extracted DNA were assessed with a BioPhotometer plus spectrophotometer (Eppendorf AG, Germany).

Two *Microcystis*-specific genetic targets were used: the phycocyanin (PC) operon and the microcystin synthetase (*mcy*B) region. The primers and probes used in this study (listed in Table 1) have been described previously by Kurmayer and Kutzenberger (2003).The PC operon primers (118F and 254R) were designed to amplify the PC gene exclusively from *Microcystis* species, but not other cyanobacteria, which allowed us to quantify the abundance of the *Microcystis* population. The *mcy*B primers (30F and 108R) were designed to amplify part of the *mcy*B gene, which allowed us to quantify the abundance of the toxic *Microcystis* population.

The external standards were prepared using genomic DNA of *M. aeruginosa* FACHB915. To establish a standard curve, genomic DNA was serially diluted (10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) with sterile water, and the different concentrations of DNA were used for real-time PCR to quantify the two genes (PC operon and *mcy*B). The concentration of DNA in the template (expressed in cell equivalents) was related to the threshold cycle (Ct) value (defined as the value at which the fluorescence first exceeds the threshold). The fluorescence threshold of all samples was set manually to 0.1 (relative fluorescence) for PC and *mcy*B gene amplifications. Linear regression equations for obtained cycle threshold values were calculated as a function of known gene copy numbers. The obtained Ct values were converted into PC copy numbers and *mcy*B copy numbers according to the regression equations of the external standards. The proportion of toxic *M. aeruginosa* cells in the co-culture experiments was determined using the copy numbers of *mcy*B and PC.

For real-time PCR analysis, each reaction was processed in duplicate on an ABI 7500 thermal real-time PCR cycler. All multiplex reactions were performed with a volume that contained 2.5 μL 10 × buffer, 3 mmol of 1 × dNTP, 10 pmol of each primer, 10 pmol of each Taqman probe, 0.5U of DNA polymerase, and 3 μL (20–30 ng μL^{-1}) of DNA from a standard strain or culture sample and made up to 25 μl with sterile Millipore water. Real-time PCR conditions were as follows: 50 °C for 2 min, 95 °C for 15 min and 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. Negative controls without DNA were included in each real-time PCR.

MC analysis

The culture samples were frozen at −20 °C and the cells were lysed by the freeze–thaw cycle prior to measurement.

Table 1 Real-time PCR primers and probes used to amplify PC and *mcy*B genes

Gene region and primer or probe	Sequence (5′-3′)
PC operon	
118F	GCTACTTCGACCGCGCC
254R	TCCTACGGTTTAATTGAGACTAGCC
TaqMan probe	FAM-CCGCTGCTGTCGCCTAGTCCCTG-BHQ1
*mcy*B	
30F	CCTACCGAGCGCTTGGG
108R	GAAAATCCCCTAAAGATTCCTGAGT
TaqMan probe	VIC-CACCAAAGAAACACCCGAATCTGAGAGG-BHQ1

FAM 6-carboxyfluorescein, *BHQ1* black hole quencher-1, *VIC* Victoria

Insoluble cell debris was removed by centrifugation (15,000 rpm × 10 min) and the supernatant was submitted for total MC determination by a microcystin-specific enzyme-linked immunoabsorbent assay (ELISA) test (Beacon Analytical Systems Inc., USA) in accordance with the manufacturer's specifications. Absorbance was measured using a BIO-RAD iMARK Microplate Reader. The MC contents were calculated from a standard calibration curve and the results were expressed as microgram equivalents of MC-LR per liter. The lower detection limit for MC was calculated at 0.1 μg L^{-1} of water. The calculated MC quotas corresponded to the ratio between total MC concentration and cell density of the MC-producing strain as determined by real-time PCR.

Results

Microcystis growth in the monoculture experiments

During the exponential phase, increasing light intensity seemed to reduce the growth rates of both toxic and non-toxic strains. Overall growth rates under low light intensity were higher than those under medium and high light intensities at the same growth temperatures (Fig. 1). Growth rates for non-toxic FACHB469 cells cultured under low light intensity were significantly higher ($P < 0.01$) at all three temperature levels (Fig. 1a). Growth rates for toxic FACHB905 cells cultured under low light were significantly higher ($P < 0.01$) at 16 °C (Fig. 1b). Temperature had a positive effect on the growth rates of both strains ($P < 0.01$), with HTLL (high temperature of 32 °C and low light intensity of 35 μmol m^{-2} s^{-1}) yielding the highest growth rates (0.343 ± 0.0028 for toxic strain and 0.482 ± 0.0187 for non-toxic strain) and low temperature of 16 °C under higher light intensity yielding the lowest growth rates (0.0822 ± 0.0077 for toxic strain and 0.142 ± 0.0078 for non-toxic strain). The growth rate of non-toxic strain FACHB469 was significantly higher than that of the toxic strain FACHB905 in all nine treatments ($P < 0.01$, Fig. 1).

During the exponential phase, with the exception of P1 treatment for the non-toxic strain ($P > 0.05$), nitrate and phosphate treatments resulted in significantly lower growth rates than those observed under the control condition ($P < 0.01$, Fig. 2). At any given nitrate or phosphate regime, non-toxic FACHB469 grew faster than the toxic strain FACHB905 ($P < 0.01$), the exception being the N3 condition (Fig. 2).

Microcystis growth and competition in the co-culture experiments

The mixed *Microcystis* population shifted towards dominance by the toxic strain from day 4 (above 58 %) until the end (above 80 %) of the experiment in the MTLL, MTHL and HTHL treatments (Fig. 3a). Cell growth in these three treatments showed similar profiles, and the toxic cell

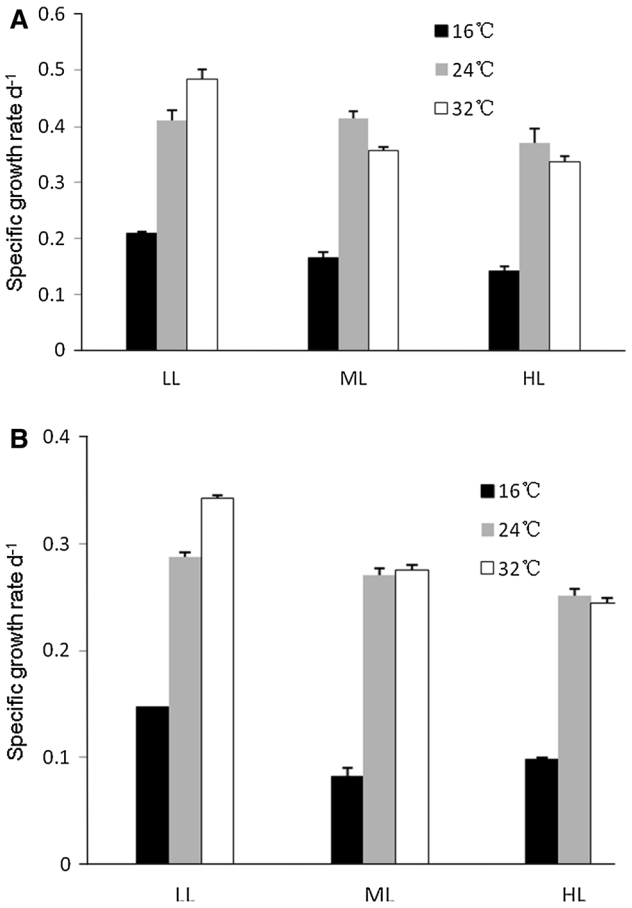

Fig. 1 Growth rates of non-MC-producing strain *M. aeruginosa*-FACHB469 (**a**) and MC-producing strain *M. aeruginosa*-FACHB905 (**b**) in monoculture under different light intensity and temperature conditions

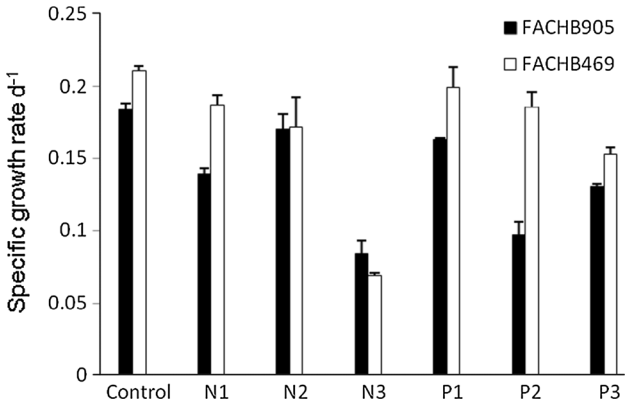

Fig. 2 Growth rates of non-MC-producing strain *M. aeruginosa*-FACHB469 and MC-producing strain *M. aeruginosa*-FACHB905 in monoculture under different nitrate and phosphate concentrations

numbers increased to a similar abundance at the end of the experiment. In the LTLL and HTLL treatments, the toxic strain became dominant from day 15 and outcompeted the non-toxic strain at the end of test (80 and 65 %, respectively). In the LTHL treatment, the toxic and non-toxic strains appeared to increase with a similar growth rate and their relative proportion was around 50 % most of the time (Fig. 3a), although the toxic strain became dominant at the end of the experiment (62 %). Toxic cell densities increased slowly in the LTLL, HTLL and especially LTHL treatments (Fig. 3b), and the final cell densities observed were distinctly lower than those in the MTLL, MTHL and HTHL treatments. This experiment was repeated with a cell ratio of 9:1 to give the non-toxic strain an initial advantage in abundance, and a similar outcome of a toxic strain dominance was found after 18 days.

The mixed population had an obvious shift towards dominance by the toxic strain from day 4 (above 78 %) until the end (above 98 %) of the experiment in the control, and also in N3, P2 and P3 treatments (Fig. 4a). Growth curves of these three treatments and of the control were similar (Fig. 4a), and their toxic cell densities were obviously higher at the end of the experiment than those in the N1, N2 and especially P1 treatments. Toxic and non-toxic strains in N1 and N2 treatments appeared to increase with a similar growth rate and the proportion of the strains was maintained at around 50 % throughout (Fig. 4a). Only under P1 condition did the toxic strain lose dominance from the beginning of the experiment and its proportion fluctuated between 21 and 41 %. The lowest toxic cell numbers observed at the end of the experiment was also found in the P1 treatment (Fig. 4b).

MC production and MC quota

The MC quota in the first co-culture experiment was highest in the LTHL treatment. It was statistically-significantly higher than in all other conditions except in the HTLL treatment, even though the mean MC quota for LTHL (137.04 fg cell^{-1}) was almost double that in HTLL

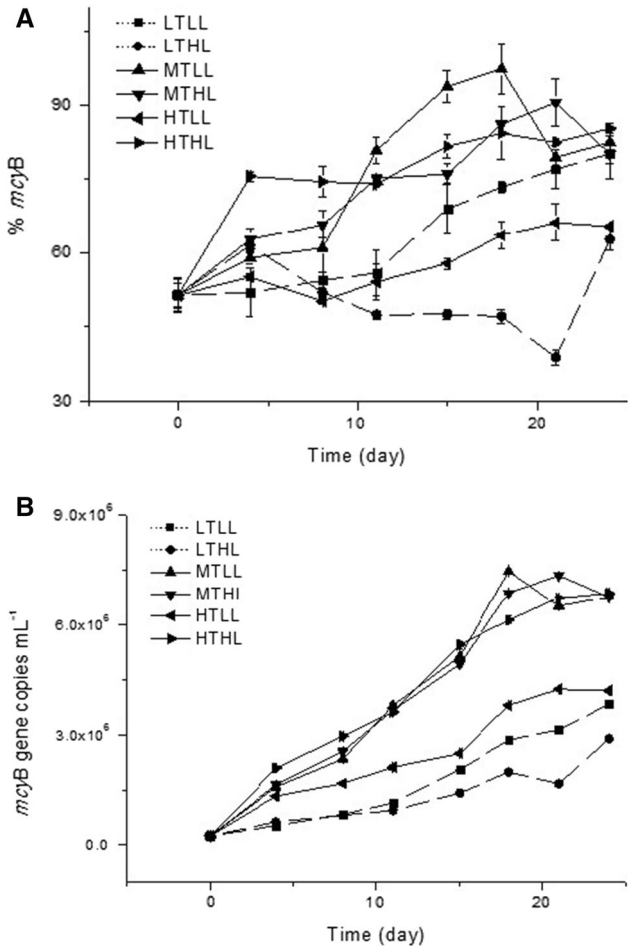

Fig. 3 Ratio of MC-producing strain *M. aeruginosa*-FACHB905 (**a**) and toxic gene copies (**b**) under different light intensities and temperatures in co-culture experiments

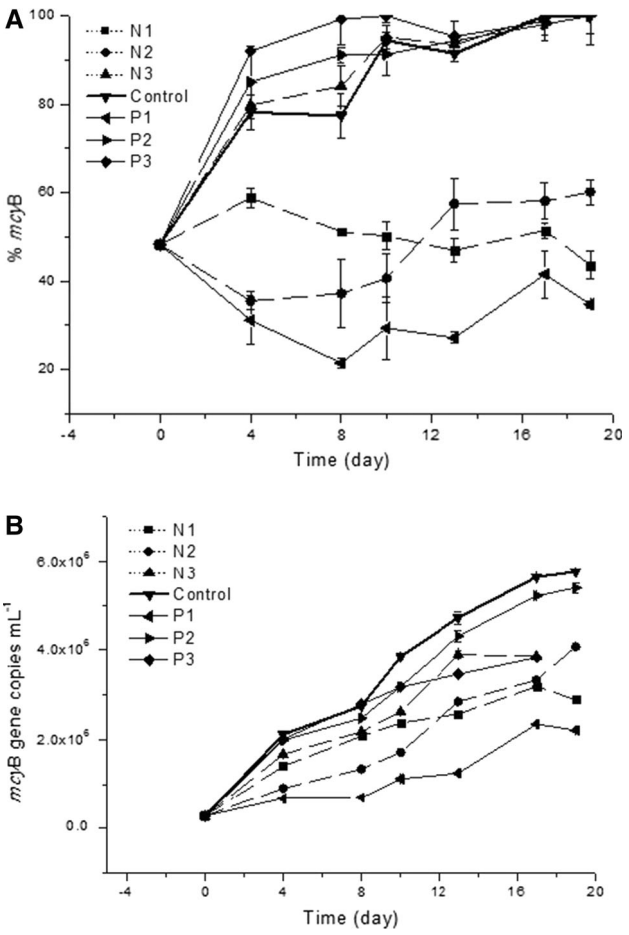

Fig. 4 Ratio of MC-producing strain *M. aeruginosa*-FACHB905 (**a**) and toxic gene copies (**b**) under different nitrate and phosphate concentrations in co-culture experiments

(74.81 fg cell^{-1})(Fig. 5a).The MC quota in the second co-culture experiment was significantly higher in the P1 treatment than the control and when compared to all other treatments. Generally, nitrate deficiency (N1 and N2 treatments) decreased the MC quota (Fig. 5b).

Discussion

When grown in monocultures, both MC-producing FACHB905 and non-MC-producing FACHB469 *M. aeruginosa* strains responded similarly to environmental temperature, light intensity, and nitrate and phosphate concentrations. However, growth rates of the non-MC-producing strain were always higher than those of the MC-producing strain. It has been suggested that the high cost of producing MC led to a slower growth rate for the toxic strain (Briand et al. 2012). If so, it might be expected that

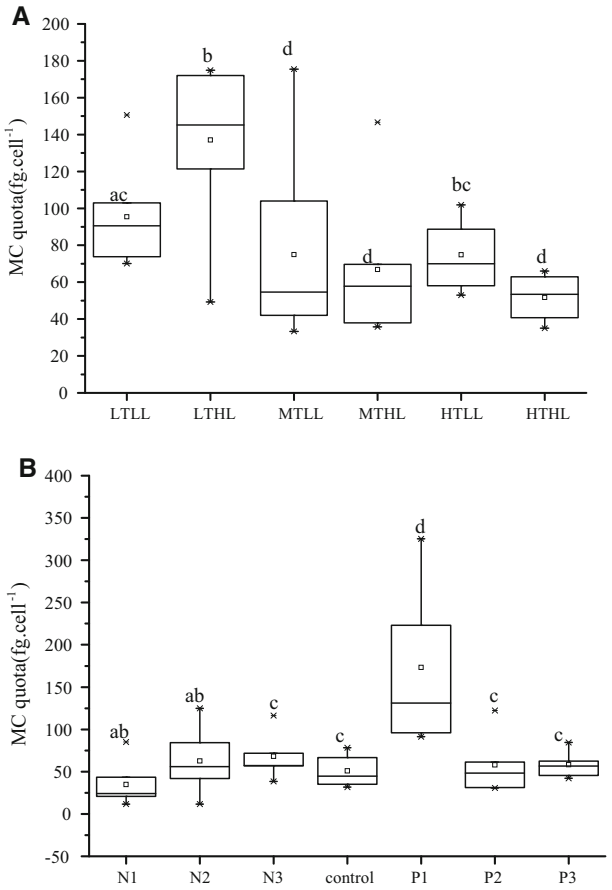

Fig. 5 MC quota under different culture conditions: **a** different light intensities and temperatures; **b** different nitrate and phosphate concentrations. *Boxes* represent 25th to 75th percentiles, *horizontal line* within box marks the median, and *open square* within box indicates the mean. *Whiskers* below and above the boxes indicated 10th and 90th percentiles, and *asterisks* represent 5th and 95th percentiles. Treatments with common letters are not statistically different from each other (ANOVA/Newman–Keuls test, $P > 0.05$)

the mixed population in the co-culture experiments would result in dominance by the non-toxic strain. However, with the exception of the P1 treatment (low phosphate treatment), our co-culture experiments produced a lack of dominance by the non-toxic strain.

The contrasting growth profiles from the monoculture and co-culture experiments may be explained by allelopathic interactions (Figueredo et al. 2007; Leão et al. 2009). Schatz et al. (2005) showed that toxic *Microcystis* cells can severely inhibit the growth of the non-toxic cells. However, Kardinaal et al. (2007a, b) and Briand et al. (2008a, b, 2012) did not find any allelopathic interactions between the strains used in their experiments. In our study, we added some crude cell extracts of toxic FACHB905 to the culture media of FACHB905 and non-toxic FACHB469, and found that growth of FACHB469 was significantly suppressed while FACHB905 grew well. Our observations clearly indicated that FACHB905 produces metabolites that function as growth inhibitors, and this is consistent with the findings of Schatz et al. (2005). Further evidence for a possible allelopathic effect was obtained from the co-culture experiment, which commenced with a starting cell inoculum ratio of 9:1. Despite this advantage to the non-toxic strain, the toxic strain ultimately outgrew the non-toxic strain under the different temperature and light intensity conditions (data not shown). The evidence showed that the toxic strain can suppress the growth of the non-toxic strain, and the final dominance of the toxic strain was unrelated to a change in any particular environmental factor.

MC was suggested to act as allelopathic substances that allow some cyanobacterial strains to inhibit the growth of their competitors in phytoplanktonic communities (Sedmak and Elersek 2005; Leão et al. 2009), although Babica et al. (2006) underlined the unlikelihood of MC acting as an allelochemical. In the present study, the MC-producing strain was out-competed by the non-toxic strain only under low phosphate (P1) conditions, when the MC quota was high. A similar phenomenon was observed in the low temperature and high light intensity (LTHL) treatment. Therefore, the results of our co-culture experiments do not support the role of MC in allelopathy, and are consistent with the findings of other competition studies (Schatz et al. 2005; Kardinaal et al. 2007a, b; Briand et al. 2012). The genus *Microcystis* has been recognized as a producer of a high number of secondary metabolites (Welker et al. 2004; Haande et al. 2007). Other secondary metabolites such as kasumigamide (Ishida and Murakami, 2000) and microcin (Wiegand et al. 2002) may also be responsible for the observed growth inhibition of the non-toxic strain, although further research is required to test this theory.

The finding that FACHB905 always dominated mixed cultures, even under control conditions, suggests that the

chemophysical factors examined did not directly influence this outcome. A similar observation was made by Briand et al. (2008a, b), who carried out monoculture and competition experiments to compare the fitness of MC-producing and non-MC-producing *Planktothrix agardhii*. However, the indirect effect of chemophysical parameters on differential response through the regulation of secondary metabolite production cannot be excluded. Some abiotic factors such as pH, light intensity and nutrient levels have been shown to influence allelopathic interactions (De Nobel et al. 1998; Ray and Bagchi 2001). Here we found that phosphate limitation (P1 treatment) and high light intensity (HL) combined with low growth temperature (16 °C) stimulated MC production, but could also simultaneously decrease the production of other secondary metabolites that inhibit growth of the non-toxic strain. Another possibility could be that the interaction between MC and other secondary metabolite led to a significant decrease or even disappearance in growth suppression. However, our results showed that lower nitrogen concentrations decreased both MC yields and the suppressive effect of the toxic strain. Thus it is unlikely that MC could interact with some other secondary metabolite. Instead, nitrogen limitation (N1 and N2), extreme low phosphate concentration (P1) and high light intensity (HL) under low temperature (16 °C) may have a negative effect on the production of some allelochemicals that prevented toxic FACHB905 dominance.

Although different temperature and light intensity treatments did not change the final dominance of the toxic strain in co-culture experiments in most cases, they can affect the growth and the maximum cell densities of the strain. Cell densities under 16 °C were much lower than those under higher temperatures (24 and 32 °C), and the lowest cell densities were found at the end of the experiment in the N1 and P1 treatments. Our findings were in accordance with the widespread expectation that increasing temperature and eutrophication will switch dominance towards toxin-producing cells within cyanobacterial blooms (Davis et al. 2010; Dziallas and Grossart 2011; Joung et al. 2011).

Several competition studies associated the changes of MC production with the benefits and physiological roles of MC (Kardinaal et al. 2007a, b; Briand et al. 2008a, b, 2012; Dziallas and Grossart 2011; Leblanc Renaud et al. 2011; Van de Waal et al. 2011). Here we found MC concentrations in the LTHL and P1 treatment were relatively high, but the toxic strain lost its dominance, thus MC production may be energetically costly for the toxic strain, making it difficult to maintain its dominance. Our results tend to support Kardinaal et al.'s (2007a, b) suggestion that energetic costs of MC synthesis might be high so that toxic strains are inferior competitors. However, the nitrogen-limited treatment appeared to be inconsistent with the above hypothesis. Indeed, the relatively low MC concentration only led to the nearly neutral coexistence of toxic and non-toxic strains, instead of dominance of the non-toxic strain. This is a further indication that FACHB905 may have been producing some other compounds that inhibited the growth of FACHB469.

Microcystis species are diverse in morphology, genetics and chemical production in natural waters (Welker et al. 2004; Haande et al. 2007; Yoshida et al. 2008). Isolated *Microcystis* strains maintain considerable variation in growth rate and physiological response to environmental factors (Vézie et al. 2002; Wilson et al. 2006, 2010). Theoretically, the co-culture experiments with two strains of random origin present a differentiated mixed population. Several studies (Briand et al. 2008a, b, 2012; Leblanc Renaud et al. 2011; Van de Waal et al. 2011) partially contributed the differentiation in co-culture experiments to strain particularity. The toxic strain FACHB905 used in our experiments seems to be able to produce allelochemicals, which may have facilitated its dominance in most cases. In natural waters different *Microcystis* populations that include toxic and non-toxic strains always coexist, and their compositions typically show temporal variability (Yoshida et al. 2007; Rinta-Kanto et al. 2009; Sabart et al. 2010). Our findings, coupled with strain particularity, highlight the difficulties to precisely predict the ecological outcome between any competitive pair of toxic and non-toxic strains. More toxic and non-toxic strains of *Microcystis* and other cyanobacterial species have to be systematically experimented for a clear conclusion.

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Conflict of interest The authors declare that they have no conflict of interest.

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