Aquat Ecol (2016) 50:103–119
DOI 10.1007/s10452-015-9559-7

Microcystin production in *Microcystis aeruginosa*: effect of type of strain, environmental factors, nutrient concentrations, and N:P ratio on *mcyA* gene expression

Rosa María Pineda-Mendoza · Gerardo Zúñiga · Fernando Martínez-Jerónimo

Received: 24 March 2015 / Accepted: 19 November 2015 / Published online: 28 November 2015
© Springer Science+Business Media Dordrecht 2015

Abstract Blooms affect water quality mainly due to the release of microcystins (MCs) by cyanobacteria. The synthesis of MCs is influenced by factors such as nutrient concentration, temperature, pH, light intensity, salinity, turbidity, and the presence of xenobiotics. In this study, we evaluated the effect of environmental factors (temperature and light intensity), the concentration of three nutrients ($NaNO_3$, K_2HPO_4, and $FeCl_3$), and the N:P ratio on the growth of two *Microcystis aeruginosa* strains (Ch10 and UTEX LB2385), as well as on *mcyA* gene expression and intracellular MC concentration. Under similar conditions, the population growth and chlorophyll *a* concentration per cell of both strains were different. The *mcyA* gene was significantly up-regulated from the early growth phase (5 days) to the stationary phase (15 days) in most cases, whereas intracellular MC concentrations varied depending on the assessed factor. The N:P ratio affected the development of both strains and MCs production differently. High concentration of intracellular MCs was recorded at low nitrogen and iron concentrations, low temperature, and high light intensity. The response in *mcyA* gene expression, related to the incubation time, of both strains was different, because strain Ch10 responded in most cases starting at 5 days of growth, whereas UTEX LB2385 responded until 10 and 15 days. This difference reflects physiological plasticity that could help to understand the permanence and dominance of *Microcystis* genus blooms in eutrophic freshwaters. The variability in response to the tested environmental factors confirms that population growth, genetic expression, and microcystin production are not related to a single factor but to an array of conditions that, when combined, stimulate MCs production. These conditions can be both stress-causing and favorable; hence, monitoring of environmental factors aimed at alerting against health risks provoked by cyanotoxins is a very complex task.

Keywords Eutrophication · Cyanobacteria · Cyanotoxin · Microcystin · qPCR

Handling Editor: Bas W. Ibelings.

Electronic supplementary material The online version of this article (doi:10.1007/s10452-015-9559-7) contains supplementary material, which is available to authorized users.

R. M. Pineda-Mendoza · F. Martínez-Jerónimo (✉)
Laboratorio de Hidrobiología Experimental, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Prol. Carpio esq. Plan de Ayala s/n, Col. Sto. Tomás, Mexico, DF 11340, Mexico
e-mail: ferjeronimo@hotmail.com

R. M. Pineda-Mendoza · G. Zúñiga
Laboratorio de Variación Biológica y Evolución, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Prol. Carpio esq. Plan de Ayala s/n, Col. Sto. Tomás, Mexico, DF 11340, Mexico

Introduction

Cyanobacterial blooms occur in eutrophic lakes, ponds, and water reservoirs worldwide, causing an environmental problem (Carmichael 1992) that affects water quality by the production and release of cyanotoxins. These compounds are secondary metabolites that can produce toxic effects on microalgae, invertebrates, fish, birds, plants, and mammals (Neilan et al. 1999; Briand et al. 2008) and disturb the community structure in aquatic environments (Giaramida et al. 2013).

Microcystins (MCs) are the most frequently reported cyanotoxins in the freshwater species of the genera *Microcystis*, *Planktothrix, Anabaena, Nostoc, Umezakia*, and *Synechocystis* (Carmichael 1992; Codd et al. 1999; Pflugmacher et al. 1999; Pflugmacher and Wiegand 2001). These toxins are inhibitors of ser/thr protein phosphatases (PP-1 and PP-2) in eukaryotes and promote hepatic and gastric cancer in mammals (Falconer and Humpage 1996; Carmichael et al. 2001; Dittmann and Wiegand 2006; Moreira et al. 2013).

MCs are synthesized by a non-ribosomal pathway through a multienzyme complex known as microcystin synthetase (*mcy*), integrated by 10 genes arranged in two different operons (*mcyA-C* and *mcyD-J*). Transcription of the *mcy* gene in *M. aeruginosa* occurs through a central promoter located between the *mcyA* and *mcyD* genes (Tillett et al. 2000).

Numerous *Microcystis* species are the main producers of MCs in toxic blooms (Carmichael 1992; Sivonen and Jones 1999; Bittencourt-Oliviera et al. 2011). Various studies have demonstrated that environmental factors, such as temperature, light intensity, pH, nutrient concentration (NO^-, NH_4^+, PO_4^{3-}, Fe^{2+}), salinity, CO_2 concentration, turbidity, consumers (e.g., daphnids), and xenobiotics, affect the permanence of many species of cyanobacteria in blooms, as well as the dominance of toxigenic strains and the increase in MCs production (Wicks and Thiel 1990; Rapala et al. 1997; Rapala and Sivonen 1998; Chorus and Bartram 1999; Kaebernick et al. 2000; Oh et al. 2000; Kaebernick and Neilan 2001; Long et al. 2001; Amé et al. 2003; van Gremberghe et al. 2009).

Despite the efforts to understand the possible ecological role of MCs and how the environmental factors affect their production, the available information is contradictory (Pearson et al. 2010; Sevilla et al. 2011), or ambiguous (Wood et al. 2011). Currently available information is not conclusive about the factors that affect MCs synthesis (Boopathi and Ki 2014); this could be explained, at least partially, by the fact that MCs are usually quantified using enzymatic (enzyme-linked immunosorbent assay—ELISA—or inhibition of protein phosphatases 1 and 2A) or analytical (HPLC, MS, MALDI-TOF) methods, which hinder the transcriptional evaluation of these toxins. Additionally, other studies have indicated that MCs synthesis occurs in the stationary phase of population growth (Kaebernick et al. 2000; Lyck and Christoffersen 2003). At this stage, high cell density and environmental conditions related to scum formation increase the synthesis and release of MCs, as observed by Wood et al. (2011, 2012), but we hypothesize that synthesis may occur also at the beginning of the cyanobacteria population growth and not until cell concentrations are high and scums can be formed. MCs synthesis can vary depending on the rate of population growth (Orr and Jones 1998), the studied strain (Blackburn et al. 1996; Carmichael 1997), and the differences in critical environmental factors. Moreover, Wood et al. (2012) documented responses few hours after inducing an experimental increase in cell density in a mesocosm system.

In view of the disperse and, in some cases, opposing results in the investigation of the effects of environmental factors involved in MCs production in cyanobacteria, this study was aimed at evaluating, in two strains of *M. aeruginosa* (one reference strain and one locally obtained through isolation), the effects of different environmental factors frequently recognized as important in cyanotoxins production, i.e. temperature, light intensity, and nutrient concentration ($NaNO_3$, K_2PO_4, and $FeCl_3$). In addition, we assessed the effect of varying the proportion of two important macronutrients (nitrogen and phosphorus), expressed as the N:P ratio, on the *mcyA* gene expression at 5, 10, and 15 days of growth. The purpose was to determine whether one of the tested treatments had a greater influence on the assessed endpoints and whether it could be identified as either a stress-causing or a favorable condition to induce cyanotoxins production in *Microcystis*.

Materials and methods

Test organisms and culture conditions

We used two MCs-producing strains of *Microcystis aeruginosa* (Arzate-Cárdenas et al. 2010): the wild

strain Ch10 isolated from *Lago Menor* of the *Bosque de Chapultepec* (urban lake) in Mexico City, Mexico, and the reference strain UTEX LB2385, a microcystin producer strain frequently used as reference strain. When *M. aeruginosa* Ch10 was isolated from bloom samples, it was growing as dense colonies, but the growth under laboratory conditions is as single cells; the strain UTEX LB2385 grows as small colonies (without mucilage) and also as single cells.

Both strains were grown in 150-mL Erlenmeyer flasks with Z8 medium (5 mM $NaNO_3$, 0.18 mM K_2HPO_4, 0.1 mM $MgSO_4$, 0.25 mM $CaCl_2$, 0.2 mM Na_2CO_3, 0.01 mM disodium EDTA, 0.01 mM $FeCl_3$, and micronutrient solution at pH 10) (Kotai 1972). All cultures were incubated in an environmental chamber at 25 ± 1 °C with constant aeration and illuminated with fluorescent “daylight” lamps (25 µmol of photons m^{-2} s^{-1} and a photoperiod of 16 h:8 h (light/darkness)). These nutrient concentrations and incubation settings are termed as the normal conditions.

Two independent experiments were performed to evaluate the effect of different environmental factors and nutrient concentrations on the *mcyA* gene expression. Additionally, we evaluated the effect of these factors on the growth of *M. aeruginosa* and the MCs production as control measures to associate the gene expression with cellular growth and toxin synthesis.

Effect of temperature, illumination, and nutrient (N, P, and Fe) concentrations

Based on the N, P, and Fe concentrations of the Z8 medium and the temperature and illumination conditions of the control treatment (C), a lower and a higher value for each factor were tested. For nutrients, two concentrations were assessed: one corresponding to 0.25 (low) and the other to four times the one contained in the Z8 medium. Regarding temperature, we tested two temperatures, 20 and 30 °C, that is, one was 5 °C less and the other was 5 °C more than the normal incubation temperature (25 °C). For light, half or double the normal value of the incubation illumination was applied. The factors were changed one at a time, keeping constant the other conditions (the same as for the control), with a total of 10 treatments plus the control. Table 1 shows the values of treatments and the symbols that are used to identify them henceforth.

Each experiment was performed in duplicate, and six flasks were prepared per treatment, testing two of them at 5 days (incipient growth phase), two at 10 days (logarithmic phase), and two at 15 days of growth (stationary phase). The sampling days were determined based on the growth kinetics, previously calculated for each strain (data not shown). The biomass of these cultures was used to obtain total RNA and to determine population growth parameters (cell count and chlorophyll *a* concentration); the samples used for cell counting were fixed in 1 % Lugol’s iodine. Furthermore, MCs concentrations in the 15-day cultures were determined, using a commercial ELISA kit, as described below; this time was selected based on many published studies that report the stationary phase of growth as that when MCs are synthesized.

The control and treatments were inoculated with 2×10^5 *M. aeruginosa* cells mL^{-1}, in a test volume of 80 mL, incubated at 25 ± 1 °C, with constant aeration, and illuminated with fluorescent “daylight” lamps (25 µmol of photons m^{-2} s^{-1} and a photoperiod of 16 h:8 h (light/darkness)).

Table 1 Values of environmental factors and nutrient concentrations tested in strains Ch10 and UTEX LB2385 of *Microcystis aeruginosa*. Treatments consisted of lower (−) and higher (+) values, taking as reference the magnitudes given in the column of the control (C) series

Parameter	Concentration/value				
	Control (C)	Lower	Symbol	Higher	Symbol
K_2HPO_4 (mM)	0.18	0.045	**−P**	0.72	**+P**
$NaNO_3$ (mM)	5	1.25	**−N**	20	**+N**
$FeCl_3$ (mM)	0.1	0.0025	**−Fe**	0.04	**+Fe**
Temperature (°C)	25	20	**−T**	30	**+T**
Light (µmol m^{-2} s^{-1})	25	12.5	**−L**	50	**+L**

Effect of N:P ratio

Three $NaNO_3$ and three K_2HPO_4 concentrations were assayed, in all possible combinations of both nutrients according to a bi-factorial design, to obtain a total of nine treatments, whose values and nomenclature are shown in Table 2. The test values were established based on the N and P concentrations of the Z8 medium (5 mM $NaNO_3$ and 0.18 mM K_2HPO_4), which has a normal ratio of 28:1 (control treatment), without changing the concentration of the other nutrients of the Z8 medium. One higher concentration, equivalent to four times the Z8 value, and one lower concentration, equivalent to one-fourth of the normal concentration, of both nutrients were selected. According to this design, the normal ratio (28:1) was maintained in one treatment having four times the concentration and the other with 0.25 of the normal concentration. To differentiate from the normal concentrations in all the ratios, the sign (+) or (−) was added to indicate proportions with high or low N and P concentrations, respectively. The number of replicates per treatment and the processing of samples were as described in the previous experiment.

Growth of *M. aeruginosa*

Cell density was determined at 5, 10, and 15 incubation days counting in a Neubauer chamber, after breaking up the *M. aeruginosa* colonies by heating at 60 °C for 20 min according to Bernard et al. (2004). In addition, we determined the amount of chlorophyll *a* (Chl *a*) per cell through extraction with 90 % acetone using a tissue homogenizer to break the cells, maintaining the homogenates at 4 °C for 24 h protected from light. Afterward, the samples were centrifuged at 5800*g* to eliminate cell debris and the absorbance of the extracts was measured at 664, 645, and 630 nm. The Chl *a* concentration was calculated according to Strickland and Parsons (1972); resulting values were divided by the cell density to determine the content per cell.

Table 2 Nitrogen ($NaNO_3$) and phosphorus (K_2HPO_4) concentrations and N:P ratios evaluated in *Microcystis aeruginosa* strains Ch10 and UTEX LB2385

K_2HPO_4 (mM)	$NaNO_3$ (mM)		
	0.045	0.18	0.72
1.25	28:1 (−)	7:1 (−)	1.7:1
5	111:1 (−)	28:1 (Control)	7:1 (+)
20	444:1	111:1 (+)	28:1 (+)

The concentrations of both nutrients in Z8 medium are 5 mM $NaNO_3$ and 0.18 mM K_2HPO_4 (for a 28:1 ratio), designated as the control values; treatments were combinations of four times and/or a quarter of the normal N and P concentrations. Minus signs are for ratios with N and/or P concentrations lower than the control, whereas plus signs are for ratios with N and/or P concentrations higher than the control

RNA extraction and cDNA synthesis

The biomass contained in 20 mL of culture in each flask per treatment was separated by centrifugation and handled as a compound sample for total RNA extraction. The pellet was macerated with 200 µL of TRI Reagent® solution (Applied Biosystems, Foster City, CA, USA) with a Teflon pestle in 1.5-mL vials; afterward, 800 µL of TRI Reagent® solution was added, and three cycles of freezing with liquid nitrogen, and thawing at room temperature were performed to disrupt the cells. Finally, complete RNA extraction was accomplished with the RiboPure™ kit (Ambion® by Life Technologies, Austin, TX, USA). RNA integrity and non-DNA contamination were verified through electrophoresis in a 1.5 % agarose gel; the RNA concentration was quantified by means of the A_{260}/A_{280} ratio. Synthesis of the RNA complementary chain (cDNA) was achieved with the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol and using 2 µg of total RNA in a 20-µL reaction. RNA and cDNA samples were stored at −70 °C until their further processing.

RT-qPCR

The primers and the hydrolysis probe (TaqMan®) of the genes *mcyA* (Pineda-Mendoza et al. 2014) and PC (reference gene) (Kurmayer and Kutzenberger, 2003) are shown in Table 3.

The reaction mixture was set to a final volume of 20 µL, containing 10 µL of TaqMan® universal PCR Master Mix II, no UNG (Applied Biosystems) and 5 µL of the diluted cDNA. For the reference gene, the primers were adjusted to a concentration of 300 nM and the probe to 100 nM, whereas for the target gene (*mcyA*) adjustments were 900 and 250 nM for the

Table 3 Primers and TaqMan® probes used in qPCR

Gene	Primer/probe	Sequence 5′–3′	Size	References
mcyA	AIX0ZUQ-F	AGGCCTCCAGATAACTCTAAACGTA	114 bp	Pineda-Mendoza et al. (2014)
	AIX0ZUQ-R	AGGGATATTGTTACGGGTTTAGTCTCT		
	AIX0ZUQ-M	CCCCATCGG CTGCTTC		
PC	188F	GCTACTTCGACCGCGCC	66 bp	Kurmayer and Kutzenberger (2003)
	254R	TCCTACGGTTTAATTGAGACTAGCC		
	Probe	CCGCTGCTGTCGCCTAGTCCCTG		

primers and the probe, respectively. RT-qPCR for each treatment was performed in triplicate.

The qPCR were performed in a StepOne® real-time thermocycler using StepOne version 2.1 software (Applied Biosystems) (Supplementary data S1) and the standard ramp speed.

The calibration curves for the reference and target genes were constructed with five cDNA dilutions (1.0, 0.33, 0.11, 0.037, and 0.012 µg μL^{-1}) starting from the initial concentration with a dilution factor of 3. The efficiency of RT-qPCR and the validation of both genes were assessed using a linear regression analysis from the average values obtained in three replicates of the quantification cycles (Cq or C_T). The efficiency value was −3.33, and R^2 (determination coefficient) was 0.99, indicating an almost perfect fit in the regression line. To assess the specificity of the primers, the amplicons were analyzed by electrophoresis on 1.5 % agarose gels.

Relative gene expression values for each treatment were determined with the C_T ($2^{-\Delta\Delta C_T}$) method (Livak and Schmittgen 2001).

Quantification of microcystins

Cyanotoxins were extracted from 40 mL samples obtained from the 15-day growth cultures, through centrifugation at 12,000*g* for 15 min; 10 mg of the pellet was weighed, macerated on dry ice, resuspended in 1 mL of 70 % methanol, vigorously vortexed for 1 min, and centrifuged at 13,000*g* for 5 min to eliminate cellular debris. This procedure was repeated five times for each sample; finally, methanol was evaporated and the extracts were resuspended in deionized water to obtain the MCs concentration contained in 1 mg biomass per mL.

The MC concentrations in each treatment were determined in the extracts by means of ELISA with the QuantiPlate kit for Microcystins (Envirologix™), following the manufacturer's instructions. This method quantifies five different cyanotoxins (microcystin −LR, −LA, −RR, and −YR, and nodularin), but results are expressed as MC–LR equivalents. The intracellular MCs concentration was divided by the cell density to express results as amount of MC–LR per cell (fg $cell^{-1}$).

Statistical analyses

To determine significant differences in *mcyA* gene expression, two-way analysis of variance (ANOVA) was applied, using as factors the treatments and the time of exposure. When ANOVA was significant, Dunnett's and Tukey's post hoc comparisons were performed (Supplementary data S2). Values of $2^{-\Delta\Delta C_T}$ were transformed to $\log_2$ for their graphical representation and for statistical analysis. SigmaPlot software version 12.0 was used for all the analyses.

Results

Effect of temperature, illumination, and nutrient (N, P, and Fe) concentrations

The cell densities of both *M. aeruginosa* strains, recorded at days 5, 10, and 15, are shown in Supplementary data S3; with these data, population growth curves were constructed. Ascending trends are observed in most cases, but in some treatments stabilization was observed on day 15. Furthermore, differences in the response of both strains to the same

factors are observed. The greatest growth was observed in the UTEX LB2385 strain with treatment −L (72,750,000 cell mL^{-1}) and was higher than the maximum achieved in strain Ch10 (treatment −P: 54,500,000 cell mL^{-1}). Although no clear trends in population growth related to the tested environmental factors are depicted, differences in the response of both strains to the same factor were observed. Notwithstanding the changes in nutrients concentration were not measured during the experiment, the comparatively reduced growth in the lower N treatments (−N) was not provoked by N depletion, because formulation of the Z8 medium has an excess of nutrients; rather, this result could be explained as an unbalance in the nutrient proportions.

Chl *a* content per cell is shown in Fig. 1; average values for the control (±standard error limits) are shown as horizontal lines to ease comparisons. In general, values were variable for both the treatments and the control at the three times observed, so no clear tendencies can be recognized. For example, compared to the control, Ch10 strain cells showed higher Chl *a* concentrations per cell on day 5 in treatment −Fe; on day 10, in +P and −L, Chl *a* concentrations were higher; whereas, on day 15, in +N and +F, Chl *a* concentrations were higher than in the control. Noteworthy was the gradual reduction in Chl *a* content per cell as the cultures matured.

For the UTEX LB2385 strain, higher concentration of Chl *a* per cell as compared to the control was attained on days 5 for +P: At day 10, all the treatments had lower values, and at day 15, all the treatments induced higher Chl *a* concentrations (Fig. 1). Results indicate that no correlation between cell density and Chl *a* content can be established for both strains.

Responses of both *M. aeruginosa* strains were greatly variable regarding population growth and chlorophyll content per cell. This can be explained as a consequence of the intrinsic variability of the measured endpoints, but sampling at only three times did not enable us to determine variations along growth kinetics. Because these results are not conclusive, they are important and were obtained as additional support to the main aim of the present study, which was to determine how these factors could influence the expression of one of the main genes involved in microcystins synthesis. These results also support the assumption that different strains of *M. aeruginosa* are affected in different ways by the same environmental factors.

Expression of the mcyA gene and microcystin concentrations

The relative expression of *mcyA* gene of strain Ch10 (Fig. 2) revealed up-regulation in all the treatments in which illumination conditions and nutrient concentrations were varied at all the observed times (except for +F at day 10), whereas down-regulation was produced by both temperature conditions. Two-way ANOVA revealed that *mcyA* gene expression was significantly affected by the different environmental factors tested ($F_{(10,346)} = 5563$, $P < 0.001$) and also by the incubation times ($F_{(3,346)} = 4500$, $P < 0.001$), but because the interaction between both factors was also significant ($F_{(30,346)} = 1076$, $P < 0.001$), no multiple comparison was possible.

The *mcyA* gene of strain UTEX LB2385 was also up-regulated in most of the treatments, mainly at 10 and 15 days incubation, as shown in Fig. 2. Down-regulation was observed in some treatments; specifically for temperature, no clear pattern is depicted, and this situation is quite different from that observed for strain Ch10. Two-way ANOVA evidenced significant differences in *mcyA* gene expression caused by environmental factors ($F_{(10,346)} = 145$, $P < 0.001$) and also by incubation periods ($F_{(3,346)} = 1176$, $P < 0.001$), but significant interaction between both factors was detected also ($F_{(30,346)} = 551$, $P < 0.001$) so no multiple comparison analysis was possible.

As shown in Table 4, MCs concentration in the control treatments was significantly higher in Ch10 (62.8 fg $cell^{-1}$), and in this strain, values ranged from 27.2 to 122.2 fg $cell^{-1}$; only in treatment −L, concentration was significantly lower than the respective control. In strain UTEX LB2385, MC values ranged from 26.6 to 138.6 fg $cell^{-1}$, but only in the treatments –N, +L, and –T, concentrations higher than the control were recorded. No pattern was detected, but varying conditions from that established for the controls modify MCs production, and differences in strain responses were also observed. Furthermore, *mcyA* gene expression at day 15 was related to MCs production, but exceptions were documented.

Effect of N:P ratio

The cell density values are shown in Supplementary data S3. Results show again that the strains responded differently to the same treatments, but in most cases,

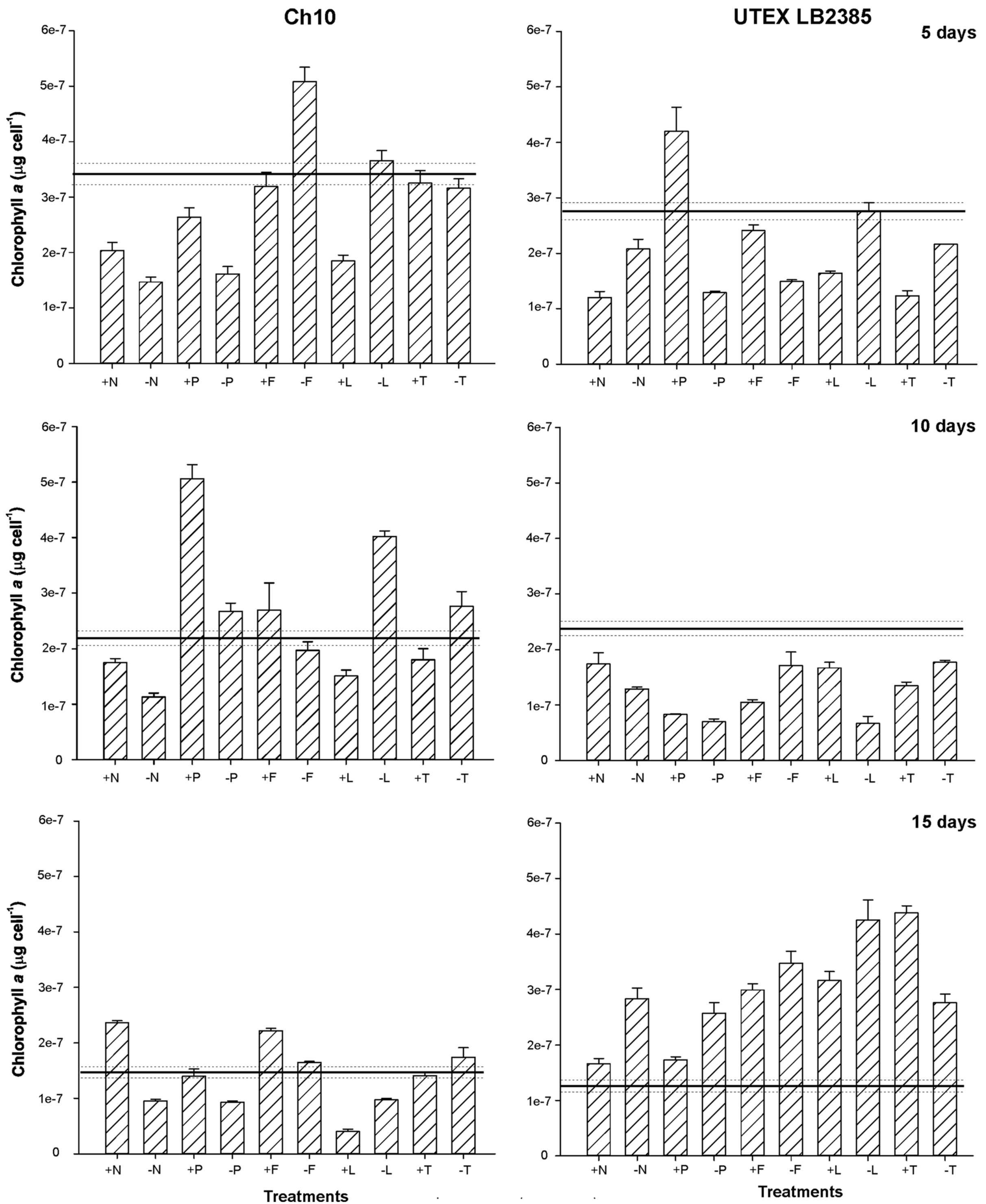


Fig. 1 Chlorophyll *a* determined at 0, 5, 10, and 15 days of incubation in strains Ch10 and UTEX LB2385 of *Microcystis aeruginosa* under different values of some environmental conditions and nutrient concentrations. Average values ± standard error limits are shown. The average value of the control treatment is shown as solid line, and the ± standard error bars are shown as *dotted line*

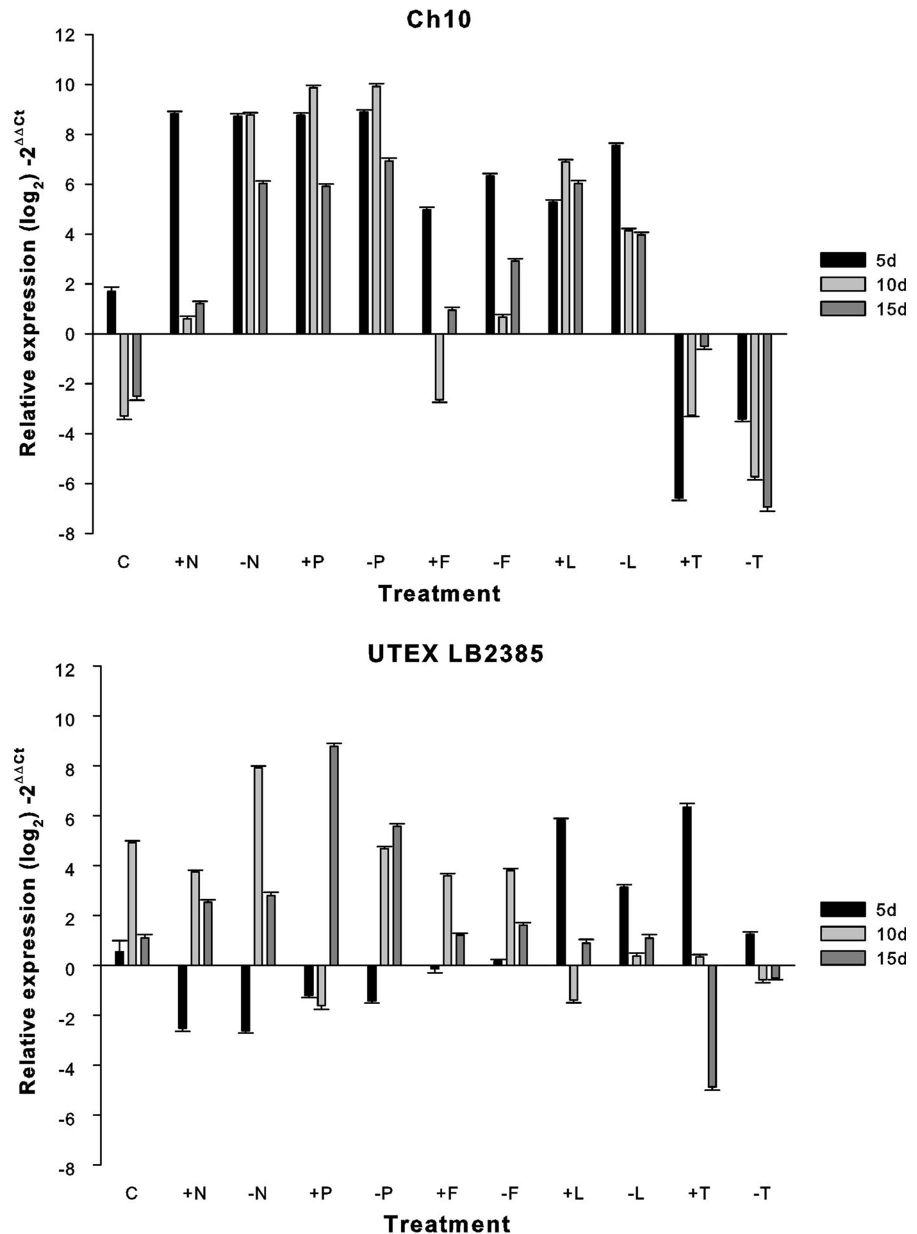


Fig. 2 *mcyA* gene expression (mean ± SE) recorded on days 0, 5, 10, and 15 of incubation of *Microcystis aeruginosa* (Ch10 and UTEX LB2385 strains), under different environmental conditions and nutrient concentrations. *mcyA* gene expression was normalized using the reference gene *PC*

cell density at day 15 was lower than that recorded for the control. Maximum growth was promoted by different N:P ratios, involving increment or reduction of phosphorus. For example, in strain Ch10, the maximum cell density was observed when P concentration was reduced to a quarter of that contained in the control [treatment 111:1 (−)]; whereas for strain UTEX LB2385, the highest value was for treatment containing four times the P concentration in the control [treatment 7:1 (+)].

Cellular chlorophyll *a* concentration reduced from day 5 to 15 in both strains as shown in Fig. 3; this condition could indicate aging in cultures, certainly not related to nutrients consumption but to limits in growth imposed by the culture system (batch). As a general trend, Chl *a* concentration was significantly lower than in the control in most of the N:P ratios at the three observed times; but, noteworthily, the effects of treatments were different in both strains (Fig. 3).

Table 4 Intracellular content of microcystins (fg cell^{-1} as MC–LR equivalent) quantified in *Microcystis aeruginosa* (strains Ch10 and UTEX LB2385) at 15-day exposure to different values of environmental factors and nutrient concentrations

Treatment	Ch10 (fg cell^{-1})	UTEX LB2385 (fg cell^{-1})
Control	62.8^{abcd}	41.8^{ac}
+N	66.6^{abcd}	56.2^{ac}
−N	74.6^{abcd}	108.8*^{b}
+P	38.3^{abcd}	69.2^{ac}
−P	27.2^{abd}	44.0*^{a}
+Fe	41.7^{abcd}	52.9^{ac}
−Fe	107.8^{a}	79.3^{ac}
+L	122.2^{a}	138.6*^{bc}
−L	34.6*^{abcd}	26.6^{a}
+T	35.8^{abcd}	60.4^{ac}
−T	97.4^{abd}	86.6*^{ac}

* Indicates significant differences ($P < 0.05$) compared with the control (Dunnett's test). Different letters indicate significant differences between treatments and control ($P < 0.05$) after Tukey's pairwise comparisons

Expression of the mcyA gene and microcystins concentration

The relative expression graph shows that the *mcyA* gene in strain Ch10 was up-regulated at the three exposure times in most of the treatments (Fig. 4); in the control (28:1), the *mcyA* gene expression was up-regulated at day 5, but down-regulated at days 10 and 15. Two-way ANOVA revealed significant differences in expression levels between treatments ($F_{(8,285)} = 1487$, $P < 0.001$) and incubation times ($F_{(3,285)} = 302$, $P < 0.001$), but the interaction between these two factors was also significant ($F_{(24,285)} = 73$, $P < 0.001$) so no multiple comparisons test was possible.

The expression of *mcyA* gene of the UTEX LB2385 strain was up-regulated at all exposure times in most of the treatments and in the control; down-regulated expression was only observed at day 5 in some N:P ratios (Fig. 4). Two-way ANOVA revealed significant differences between treatments ($F_{(8,285)} = 524$, $P < 0.001$) and observation times ($F_{(3,285)} = 5405$, $P < 0.001$), as well as in the interaction between these two factors ($F_{(24,285)} = 418$, $P < 0.001$).

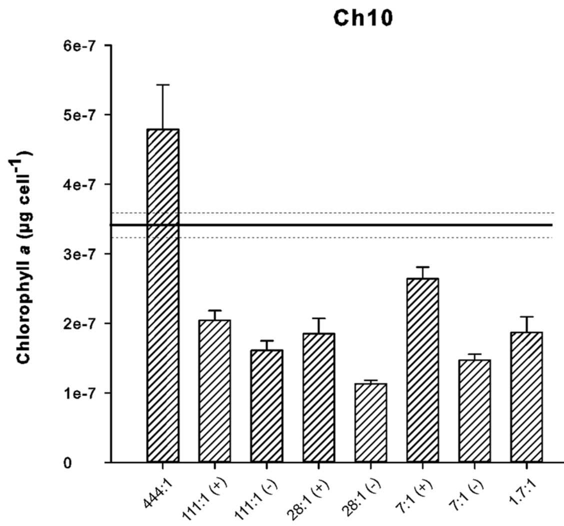
In strain Ch10, the MC concentrations ranged from 27.2 to 220.9 fg cell^{-1} (Table 5); significantly different concentrations of MCs with respect to the control were recorded only in the treatments 28:1 (−) and 111:1 (−). In strain UTEX LB2385, the values ranged from 41.8 to 195.9 fg cell^{-1}, but the MCs concentration was significantly higher with respect to the control only in the ratio 28:1 (−). Again, strain-specific differences were observed.

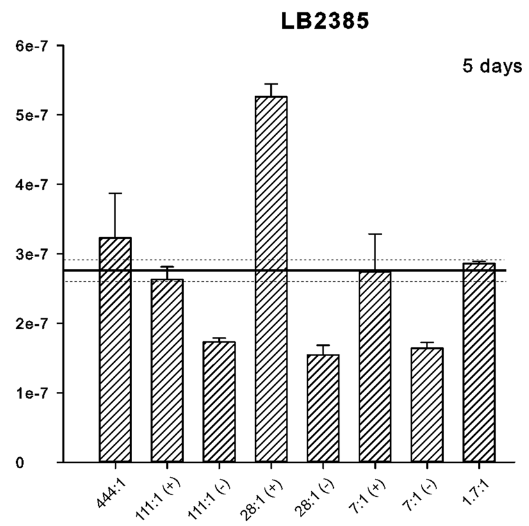
Discussion

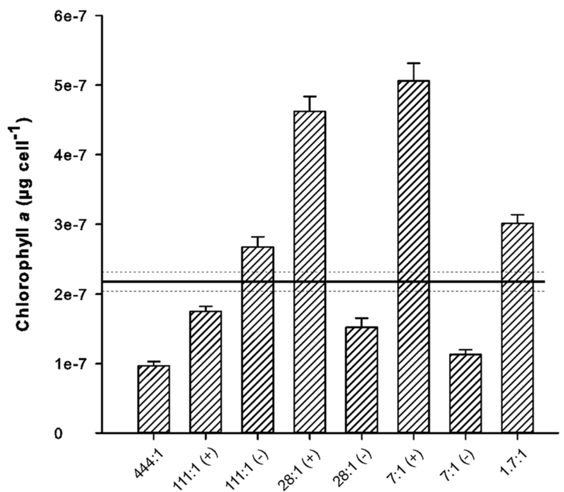
MCs production by cyanobacteria dates back to their phylogenetic origin and could be recognized as a response that has allowed them to persist and dominate in different environments (Rantala et al. 2004). Many efforts have been made to understand the intra- and inter-cellular mechanisms associated with the production of these metabolites, but no clear results have been obtained. From an ecological point of view, it has been noted that diverse environmental, nutritional, and biological factors can regulate and positively or negatively control the production of these toxins (Kotak et al. 2000). Our results indicate that variation in environmental factors, nutrient concentrations, and N:P ratios affects differently the *mcyA* gene expression and microcystins production, depending on the *M. aeruginosa* strain and the incubation time. Despite that no general trends could be established, all the variables here tested produced changes in the response of the tested toxigenic strains.

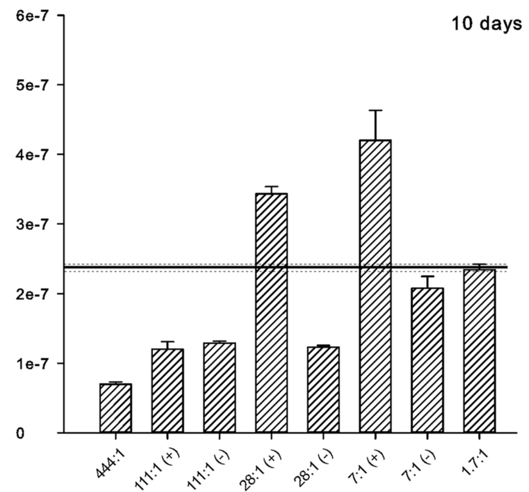
Previous studies suggested that an increase in nitrogen concentration promotes MCs production during blooms (Gobler et al. 2007) and that reduced concentrations of this nutrient limit the population growth of cyanobacteria (Sevilla et al. 2010), as well as microcystins production (Gobler et al. 2007). The latter was partially observed in the present study. The growth of *M. aeruginosa* Ch10 strain in low nitrogen concentration was lower than in the control, but up-regulation of the *mcyA* gene and the MCs concentration per cell were higher in the Ch10 and UTEX LB2385 strains, suggesting that this acted as a stimulatory stress factor to produce toxins. These results are contrary to those reported by Gobler et al. (2007), but agree with the observation by Ginn et al. (2010), who found that transcription of the *mcyB* and *ntcA* genes increases in nitrogen stress conditions.

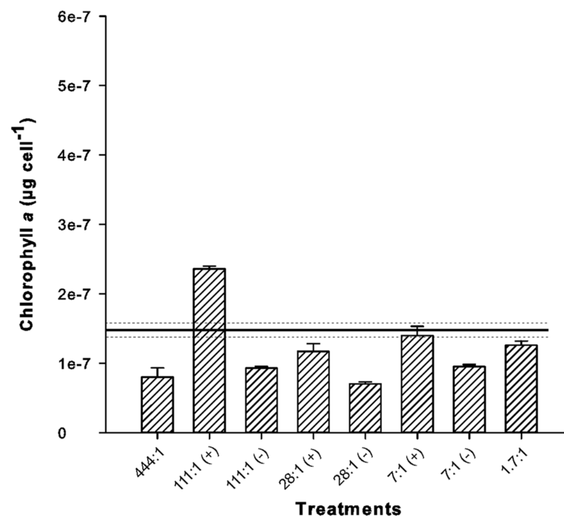
Nitrogen limitation in N:P ratios affected the growth of this cyanobacterium, but did not affect

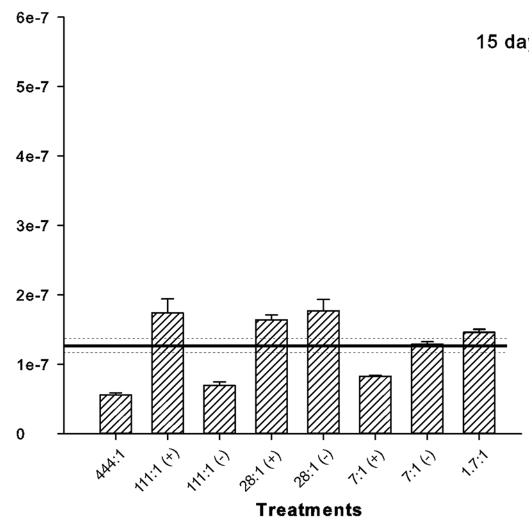

Ch10
Chlorophyll a (µg cell-1)
6e-7
5e-7
4e-7
3e-7
2e-7
1e-7
0
444:1
111:1 (+)
111:1 (-)
28:1 (+)
28:1 (-)
7:1 (+)
7:1 (-)
1.7:1


LB2385
5 days
6e-7
5e-7
4e-7
3e-7
2e-7
1e-7
0
444:1
111:1 (+)
111:1 (-)
28:1 (+)
28:1 (-)
7:1 (+)
7:1 (-)
1.7:1


Chlorophyll a (µg cell-1)
6e-7
5e-7
4e-7
3e-7
2e-7
1e-7
0
444:1
111:1 (+)
111:1 (-)
28:1 (+)
28:1 (-)
7:1 (+)
7:1 (-)
1.7:1


10 days
6e-7
5e-7
4e-7
3e-7
2e-7
1e-7
0
444:1
111:1 (+)
111:1 (-)
28:1 (+)
28:1 (-)
7:1 (+)
7:1 (-)
1.7:1


Chlorophyll a (µg cell-1)
6e-7
5e-7
4e-7
3e-7
2e-7
1e-7
0
444:1
111:1 (+)
111:1 (-)
28:1 (+)
28:1 (-)
7:1 (+)
7:1 (-)
1.7:1
Treatments


15 days
6e-7
5e-7
4e-7
3e-7
2e-7
1e-7
0
444:1
111:1 (+)
111:1 (-)
28:1 (+)
28:1 (-)
7:1 (+)
7:1 (-)
1.7:1
Treatments

◀ **Fig. 3** Chlorophyll *a* concentration determined at 0, 5, 10, and 15 days of incubation in Ch10 and UTEX LB2385 strains of *Microcystis aeruginosa* at different $NaNO_3$ and K_2HPO_4 concentrations (N:P ratio). Average values ± standard error bars are shown. The average value of the control treatment is shown as *solid line*, and the ± standard error bars are shown as *dotted line*

MCs synthesis. These results are in agreement with Smith (1983) and Fujimoto et al. (1997), who reported that N availability does not affect MCs synthesis, and with Sevilla et al. (2010), who reported that the transcription of *mcyD* gene in *M. aeruginosa* is not disturbed. Differences observed between strains in this

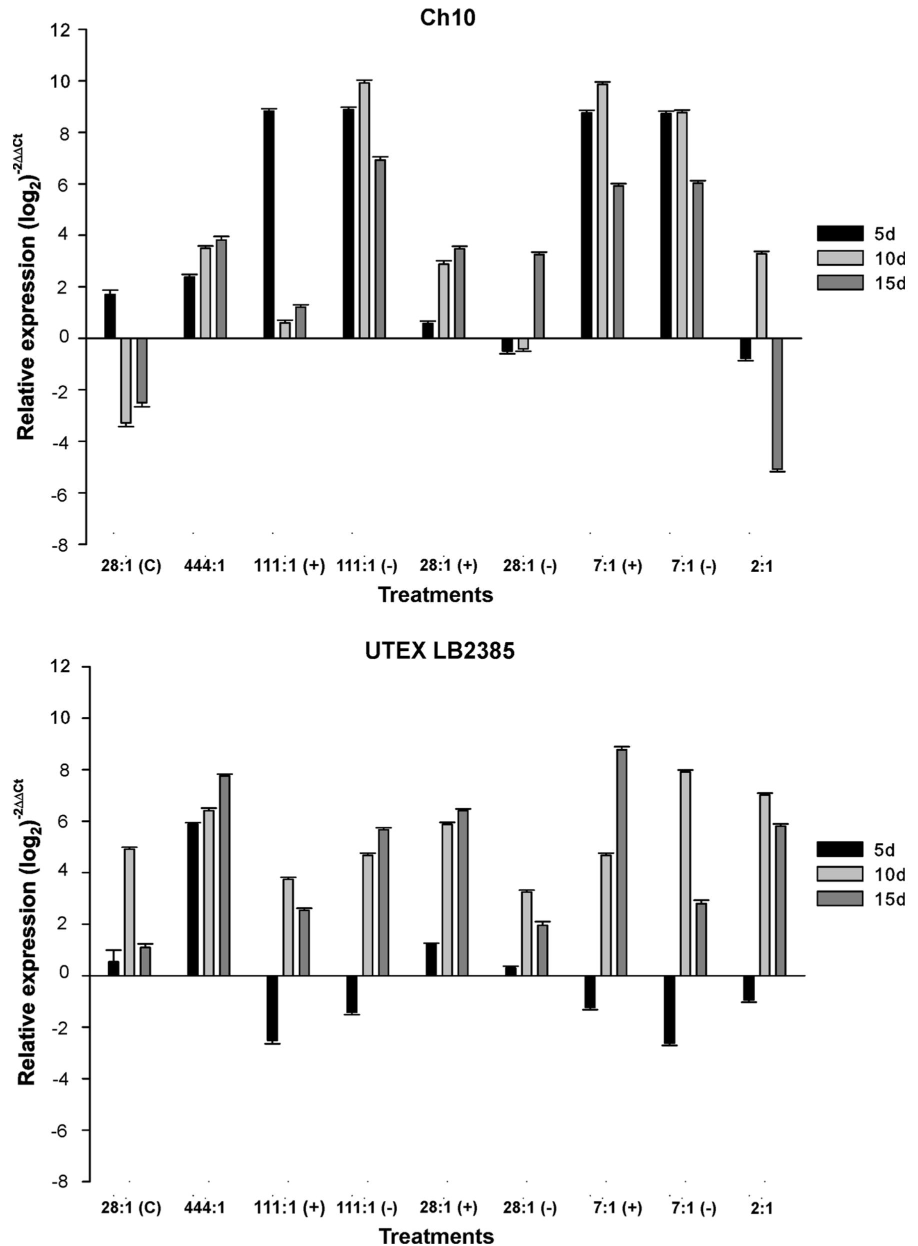


Fig. 4 *mcyA* gene expression (mean ± SE) recorded on days 0, 5, 10, and 15 during development of Ch10 and UTEX LB2385 strains of *Microcystis aeruginosa* under different $NaNO_3$ and K_2HPO_4 concentrations and N:P ratios. *mcyA* gene expression was normalized using the reference gene *PC*

Table 5 Intracellular content of microcystins (fg cell^{-1} as MC–LR equivalent) quantified in *Microcystis aeruginosa* (strains Ch10 and UTEX LB2385) at 15-day exposure to different nitrogen/phosphorus ratios

Treatment	Ch10 (fg cell^{-1})	UTEX LB2385 (fg cell^{-1})
Control (28:1)	62.8^{acd}	41.8^{a}
28:1 (−)	220.9*^{b}	195.9*^{b}
7:1 (−)	74.6^{ac}	108.8^{b}
1.7:1	87.1^{a}	115.8^{b}
111:1 (−)	27.2*^{acd}	44.0^{a}
7:1 (+)	38.3^{acd}	69.2^{a}
444:1	47.5^{acd}	57.2^{a}
111:1 (+)	65.6^{acd}	56.2^{a}
28:1 (+)	49.2^{acd}	49.1^{a}

The concentrations of both nutrients in Z8 medium are 5 mM $NaNO_3$ and 0.18 mM K_2HPO_4 (for a 28:1 ratio), designated as the control. For tested values, see Table 2

* Indicates significant differences ($P < 0.05$) compared with the control (Dunnett's test). Different letters indicate significant differences between treatments and control ($P < 0.05$) after Tukey's pairwise comparisons

study could be explained as a strain-specific response, indicating also the high plasticity observed in this cyanobacterium under diverse conditions.

Phosphorus is the limiting nutrient in primary productivity in freshwater lakes (Kaebernick and Neilan 2001); hence, small changes in the concentration of this nutrient influence significantly the growth of cyanobacteria, as it has been observed in blooms dominated by *Microcystis* (Vézie et al. 2002). High P concentrations induced an increase in MC's synthesis in *Microcystis* strains under laboratory (Sivonen 1990; Vézie et al. 2002) and natural (Rinta-Kanto et al. 2009) conditions. Our results show that the *mcyA* gene expression was up-regulated independently from the P concentration; however, the highest MC values in both strains were recorded in the treatments with the lowest P and N concentrations. Oh et al. (2000) reported that phosphorus limitation decreases the growth of *M. aeruginosa*, but increases the MCs content. Certainly, P deficiency affects the growth of any photosynthesizing organisms (Dai et al. 2008); however, its effect on MCs production is not clear.

Apparently, MCs production is not regulated by the environmental concentration of this nutrient, because these toxins can be produced in different strains of *M. aeruginosa, Anabaena*, and *Oscillatoria*, in both high and low P concentrations (Utkilen and Gjølme 1995; Chorus and Bartram 1999; Jacoby et al. 2000; Vézie et al. 2002; Kameyama et al. 2002). Furthermore, no clear association between *mcy*-cluster genes expression and toxins production has been established; however, our results show that higher MCs production is related to an increase in *mcyA* gene expression. This is a partial result because we did not analyze the production of toxins at all expression times, and only one gene of the *mcy*-cluster was assessed. Jonasson et al. (2008) demonstrated in the *Nodularia spumigena* strain AV1 that phosphate starvation has a differential effect on the expression of the nine genes of the *nda* cluster, but not on the intracellular and extracellular concentration of nodularin. Hence, further studies should be done to evaluate the expression of other *mcy*-cluster genes and the MCs production in order to establish any association between them in cyanobacteria.

Regarding the influence of micronutrients, Kosakowska et al. (2007) reported that 10 µM of iron is required to maintain Chl *a* synthesis and a positive growth rate in *M. aeruginosa*. Nevertheless, we observed that the strains Ch10 and UTEX LB2385 grew adequately under lower Fe concentrations. In addition, the induction of the *mcyA* gene and intracellular MC values were higher in low Fe concentrations than in high concentration. This result agrees with previous studies indicating that Fe-limiting conditions increase the transcription rate of *mcy* genes and the production of cyanotoxins (Lukaĉ and Aegerter 1993; Lyck et al. 1996; Amé et al. 2003; Alexova et al. 2011a), resulting from the dissociation of the FurA protein, whose function is to repress the expression of *mcy* genes in the presence of Fe (Martin-Luna et al. 2006, 2011). Notwithstanding, other studies report an increase in MCs synthesis associated with a high Fe content in the medium (Utkilen and Gjølme 1995; Bickel and Lyck 2001; Amé and Wunderlin 2005); but, for a complete comparison, the specific condition of the other environmental factors should be reviewed.

Our results also indicate that expression of the *mcyA* gene can change during population growth. Whereas the Ch10 strain showed a marked up-regulation from the start of the exponential phase (5 days), similar to that observed by Rueckert and Cary (2009) and Alexova et al. (2011b) for the *mcyE* gene, in strain UTEX LB2385, the *mcyA* gene was up-regulated in the late exponential phase (10 days), similar to the results reported by Welker et al. (2006).

Likewise, the difference in these results can be attributed to the metabolic plasticity of *Microcystis* strains documented by Chorus (2001).

It is not known whether temperature activates or deactivates the genes or proteins that regulate microcystin synthetase activity; however, our results suggest that the effect of temperature is not tightly related to *mcyA* gene expression, intracellular MCs production, or the population growth of both strains. Gene expression was low at the two temperatures studied, whereas the intracellular MCs concentration was comparatively higher at the lower temperature, which could be expected as a less favorable condition for the growth of both strains, at least in tropical and subtropical latitudes. At low temperature, Sivonen (1990), Watanabe (1996), and Lehman et al. (2008) documented high MC concentrations in different cyanobacteria. These results coincide with observations in *Microcystis* spp. (Wang et al. 2010) and *Oscillatoria agardhii* (Sivonen 1990), for which the optimal temperature for MCs production was below 23 °C, but the optimal temperature for growth was above 25 °C (Sivonen 1990; Lehman et al. 2008); thus, optimal population growth might not necessarily be related to the synthesis of these secondary metabolites. Despite the aforementioned, and unlike our results, other studies report that relatively high temperatures (26 and 32 °C) favor an increase in the MCs concentration (Amé and Wunderlin 2005; Dziallas and Grossart 2011).

Temperature variations have been related to changes in the composition of MCs; at temperatures below 25 °C, the *Anabaena* sp. strains studied by Rapala et al. (1997) and Msagati et al. (2006) produced MC–LR, whereas above 25 °C, they produced MC–RR. In contrast, Amé and Wunderlin (2005) observed a higher proportion of MC–RR at 20 °C and of MC–LR at 28 °C in cultures of concentrated, natural *M. aeruginosa* populations. In our study, we did not detect different MCs because the quantification method used could not discriminate MC isoforms; thus, no comparison is possible. Additional assays in a wide range of temperatures are necessary to positively conclude on the effect of this factor.

The growth diminution of *M. aeruginosa* observed in this study at the highest light intensity agrees with observations made in *Oscillatoria agardhii* (Sivonen 1990). This result could be explained by the saturation of the light uptake complex, inducing the generation of free radicals and photo-inhibition (Walsh et al. 1997; Zilliges et al. 2011), which would lead to a reduction in cell growth. We also observed that the highest light intensity induced up-regulation of the gene *mcyA* in both *M. aeruginosa* strains, which agrees with previous studies reporting high transcription levels for *mcyB* (16–68 μmol photons m^{-2} s^{-1}) (Kaebernick et al. 2000) and *mcyD* (16–400 μmol photons m^{-2} s^{-1}) (Kaebernick et al. 2000; Sevilla et al. 2012). It has been suggested that microcystin synthetase is controlled by the amount of light through regulating sequences located in the promoter region between operons *mcyA-C* and *mcyD-J* (Kaebernick et al. 2000). The *mcyA* gene induction here observed explains the high intracellular MC concentrations recorded in both strains with the high light intensity treatment. These results agree with those reported in *Microcystis* (Utkilen and Gjølme 1995; Rapala et al. 1997; Kaebernick et al. 2000) and *Planktothrix agardhii* (Tonk et al. 2005), but differ from those obtained with *Anabaena* and *Oscillatoria* (Sivonen 1990; Rapala et al. 1997). Additional assays could be required to test whether the light effect may also be a species-specific trait in cyanobacteria.

The effect of the N:P ratio on the growth of cyanobacteria and the synthesis of MCs (mainly in *Microcystis*) is not conclusive; in some studies, high ratios increase the synthesis of cyanotoxins (Van der Westhuizen and Eloff 1985; Vézie et al. 2002; Downing et al. 2005), whereas other authors report the opposite (Bulgakov and Levich 1999; Jacoby et al. 2000; Havens et al. 2003). The N:P ratio in natural environments can induce a higher production of MCs and, according to Downing and McCauley (1992), the low N:P ratio could be the consequence and not the cause of bloom formation. Here, the *mcyA* gene expression and the intracellular MCs concentration did not show a clear correlating tendency with the N:P ratio in the two *M. aeruginosa* strains, concordant with observations by Marinho and Azevedo (2007) on *M. aeruginosa* and *Aulacoseira distans*. The higher MCs synthesis recorded in UTEX LB2385, when nitrogen was reduced [28:1 (−) N:P ratio], agrees with the observations of Downing et al. (2005), suggesting that MC synthesis depends on nitrogen concentration. Similar results were reported by Dai et al. (2008) for other strains of *M. aeruginosa*; this could indicate that N but not P has a major influence on MCs synthesis for some cyanobacterial strains.

The effects on population growth and MCs production in both *M. aeruginosa* strains observed in the treatments with different nutrients concentrations and N:P ratios led us to conclude that this cyanobacterium displays stress responses at limiting N or P concentration, whereas in other ratios, the effect is clearly stimulatory, depending on the strain. We could expect that different species and different strains of the same species could react in a different manner, confirming the variability of responses to the effects of important environmental factors.

Based on the experimental results here observed and comparing with those obtained by Wood et al. (2011), in natural conditions, the regulation of genes in the cluster *mcy* is more complex than frequently assumed and cannot be attributed only to one environmental variable. Future research should be aimed at evaluating the expression of the *mcy*-cluster genes related to the use of MCs as siderophores, quorum sensing, and in the photosynthesis process, and not just as allelopathic substances or as a defense mechanism against predators. The results observed in a recently isolated strain from an urban lake with high predominance of *Microcystis* spp. blooms (Ch10), compared to the reference strain (UTEX LB2385), which was isolated more than 50 years ago, indicate that toxigenic strains kept their capability to synthesize MCs, despite the general thought that these secondary metabolites are only produced under selective pressure. This fact indicates that cyanotoxins could be involved in other cell metabolic functions.

Conclusions

Under the conditions here studied, the *mcyA* gene expression in *M. aeruginosa* responded to light, temperature, and the concentration of some nutrients (N, P, and Fe), but not to N:P ratios. The differential gene expression observed in the two strains suggests that their adaptive strategies to the environment are complex, because, in general, some strains can respond immediately to environmental changes (Ch10) and others do more slowly (UTEX LB2385). This strategy allows understanding, at least partially, the successful permanence and dominance of the genus *Microcystis* in blooms, because its species can tolerate environmental variability and react differently to the changes in the aquatic environment.

This strategy could be a consequence of the large physiological plasticity developed among the species of this genus. In addition, *mcyA* gene expression in early growth stages suggests that MCs production could be important for the development of cyanobacteria. The capability of producing cyanotoxins and the way that this synthesis is promoted by environmental factors were different for both strains, which display variable responses under the same culture conditions. In general, reduced concentrations of nitrogen and iron, low temperature, and high light intensity were the conditions in which a significant increase in MCs concentration was observed. Furthermore, it is important to study the factors that affect the microcystins synthesis to elucidate the molecular mechanisms by which these secondary metabolites are regulated and produced.

Acknowledgments We are grateful to two anonymous reviewers for their comments and valuable suggestions regarding the manuscript. Pineda-Mendoza was a fellow of *Consejo Nacional de Ciencia y Tecnología (CONACYT)* (No. 206867) and *Programa Institucional de Formación de Investigadores of the IPN (PIFI-IPN).* Fernando Martínez-Jerónimo is a fellow of the *Sistema de Estímulo al Desempeño de los Investigadores (EDI) and the Comisión de Operación y Fomento de Actividades Académicas (COFAA)* of the Instituto Politécnico Nacional.

References

Alexova R, Fujii M, Birch D, Cheng J, Waite TD, Ferrari BC, Neilan BA (2011a) Iron uptake and toxin synthesis in the bloom-forming *Microcystis aeruginosa* under iron limitation. Environ Microbiol 13:1064–1077

Alexova R, Haynes PA, Ferrari BC, Neilan BA (2011b) Comparative protein expression in different strains of the bloom-forming cyanobacterium *Microcystis aeruginosa*. Mol Cell Proteomics 10:1–16

Amé MV, Wunderlin DA (2005) Effects of iron, ammonium and temperature on microcystin content by a natural concentrated *Microcystis aeruginosa* population. Water Air Soil Pollut 168:235–248

Amé MV, Díaz MP, Wunderlin DA (2003) Occurrence of toxic cyanobacterial blooms in San Roque Dam (Córdoba—Argentina): a field and chemometric study. Environ Toxicol 18:192–201

Arzate-Cárdenas M, Olvera-Ramírez R, Martínez-Jerónimo F (2010) *Microcystis* toxigenic strains in urban lakes: a case of study in Mexico City. Ecotoxicology 19:1157–1165

Bernard C, Monis P, Baker P (2004) Disaggregation of colonies of *Microcystis* (Cyanobacteria): efficiency of two techniques assessed using an image analysis system. J Appl Phycol 16:117–125

Bickel H, Lyck S (2001) Importance of energy charge for microcystin production. In: Chorus I (ed) Cyanotoxins—occurrence, causes, consequences. Springer, Berlin, pp 133–141

Bittencourt-Oliviera MC, Oliviera MC, Pinto E (2011) Diversity of microcystin-producing genotypes in Brazilian strains of *Microcystis* (Cyanobacteria). Braz J Biol 71:209–216

Blackburn SI, McCausland MA, Bolch CJS, Newman SJ, Jones GJ (1996) Effect of salinity on growth and toxin production in cultures of the bloom-forming cyanobacteria *Nodularia spumigena* from Australian waters. Phycologia 35:511–522

Boopathi T, Ki JS (2014) Impact of environmental factors on the regulation of cyanotoxin production. Toxins 6:1951–1978

Briand E, Gugger M, François JC, Bernard C, Humbert JF, Quiblier C (2008) Temporal variations in the dynamics of potentially microcystin-producing strains in a bloom-forming *Planktothrix agardhii* (Cyanobacterium) population. Appl Environ Microbiol 74:3839–3848

Bulgakov NG, Levich AP (1999) Ammonium-nitrogen: a key regulatory factor causing dominance of non-nitrogen fixing cyanobacteria in aquatic systems. Arch Hidrobiol 132:141–164

Carmichael WW (1992) Cyanobacteria secondary metabolites—the cyanotoxins. J Appl Bacteriol 72:445–459

Carmichael WW (1997) The cyanotoxins. Adv Bot Res 27:211–240

Carmichael WW, Azevedo SM, An SI, Molica RJ, Jochimsen EM, Lau S, Rinehart KL, Shaw GR, Eaglesham GK (2001) Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. Environ Health Perspect 109:663–668

Chorus I (2001) Cyanotoxin occurrence in freshwaters—a summary of survey results from different countries. In: Chorus I (ed) Cyanotoxins. Springer, Berlin, pp 75–82

Chorus I, Bartram J (1999) Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. WHO, London

Codd GA, Bell SG, Kaya K, Ward CJ, Beattie KA, Metcalf JS (1999) Cyanobacterial toxins, exposure routes and human health. Eur J Phycol 34:405–415

Dai GZ, Deblois CP, Liu SW, Juneau P, Qiu BS (2008) Differential sensitivity of five cyanobacterial strains to ammonium toxicity and its inhibitory mechanism on the photosynthesis of rice-field cyanobacterium Ge-Xian Mi (*Nostoc*). Aquat Toxicol 89:113–121

Dittmann E, Wiegand C (2006) Cyanobacterial toxins—occurrence, biosynthesis and impact on human affairs. Mol Nutr Food Res 50:7–17

Downing JA, McCauley E (1992) The nitrogen:phosphorus relationship in lakes. Limnol Oceanogr 37:936–945

Downing TG, Sember CS, Gehringer MM, Leukes W (2005) Medium N:P ratios and specific growth rate comodulate microcystin and protein content in *Microcystis aeruginosa* PCC7806 and *M. aeruginosa* UV027. Microb Ecol 49:468–473

Dziallas C, Grossart HP (2011) Increasing oxygen radicals and water temperature select for toxic *Microcystis* sp. PLoS ONE 6:e25569

Falconer IR, Humpage AR (1996) Tumour promotion by cyanobacteria. Phycologia 35:74–79

Fujimoto N, Sudo R, Sugiura N, Inamori Y (1997) Nutrient-limited growth of *Microcystis aeruginosa* and *Phormidium tenue* ad competition under various N:P ratios and temperatures. Limnol Oceanogr 42:250–256

Giaramida L, Manage PM, Edwards C, Singh BK, Lawton LA (2013) Bacterial communities' response to microcystins exposure and nutrient availability: linking degradation capacity to community structure. Int Biodeter Biodegr 84:111–117

Ginn HP, Pearson LA, Neilan BA (2010) NtcA from *Microcystis aeruginosa* PCC 7806 is autoregulatory and binds to the microcystin promoter. Appl Environ Mibrobiol 76:4362–4368

Gobler CJ, Davis TW, Coyne KJ, Boyer GL (2007) Interactive influences of nutrient loading, zooplankton grazing, and microcystin synthetase gene expression on cyanobacterial bloom dynamics in a eutrophic New York lake. Harmful Algae 6:119–133

Havens K, James RT, East TL, Smith VH (2003) N:P ratios, light limitation, and cyanobacterial dominance in a subtropical lake impacted by non-point source nutrient pollution. Environ Pollut 122:379–390

Jacoby JM, Collier DC, Welch EB, Hardy FJ, Crayton M (2000) Environmental factors associated with a toxic bloom of *Microcystis aeruginosa*. Can J Fish Aquat Sci 57:231–240

Jonasson S, Vintila S, Sivonen K, El-Shehawy R (2008) Expression of the nodularin synthetase genes in the Baltic Sea bloom-former cyanobacterium *Nodularia spumigena* strain AV1. FEMS Microbiol Ecol 65:31–39

Kaebernick M, Neilan BA (2001) Ecological and molecular investigations of cyanotoxin production. FEMS Microbiol Ecol 35:1–9

Kaebernick M, Neilan BA, Börner T, Dittmann E (2000) Light and the transcriptional response of the microcystin biosynthesis gene cluster. Appl Environ Microbiol 66:3387–3392

Kameyama K, Sugiura N, Isoda H, Inamori Y, Maekawa T (2002) Effect of nitrate and phosphate concentration on production of microcystins by *Microcystis viridis* NIES 102. Aquat Ecosyst Health Manag 5:443–449

Kosakowska A, Nedzi M, Pempkowiak J (2007) Responses of the toxic cyanobacterium *Microcystis aeruginosa* to iron and humic substances. Plant Physiol Biochem 45:365–370

Kotai J (1972) Instructions for preparation of modified Z8 for algae, B-11/69'. Norwegian Institute for Water Research Publication, Blinderan, Oslo

Kotak BG, Lam AK-Y, Prepas EE, Hrudey SE (2000) Role of chemical and physical variables in regulating microcystin-LR concentration in phytoplankton of eutrophic lakes. Can J Fish Aquat Sci 57:1584–1593

Kurmayer R, Kutzenberger T (2003) Application of real-time PCR for quantification of microcystin genotypes in a population of the toxic cyanobacterium *Microcystis* sp. Appl Environ Microbiol 69:6723–6730

Lehman P, Boyer GL, Satchwell MF, Waller S (2008) The influence of environmental conditions on the seasonal variation of *Microcystis* cell density and microcystins concentration in San Francisco Estuary. Hydrobiologia 600:187–204

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods 25:402–408

Long BM, Jones GJ, Orr PT (2001) Cellular microcystin content in N-limited *Microcystis aeruginosa* can be predicted from growth rate. Appl Environ Microbiol 67:278–283

Lukaĉ M, Aegerter R (1993) Influence of trace metals on growth and toxin production of *Microcystis aeruginosa*. Toxicon 31:293–305

Lyck S, Christoffersen K (2003) Microcystin quota, cell division and microcystin net production of precultured *Microcystis aeruginosa* CYA288 (Chroococcales: Cyanophyceae) under field conditions. Phycologia 42:667–674

Lyck S, Gjølme N, Utkilen H (1996) Iron starvation increases toxicity of *Microcystis aeruginosa* CYA 228/1 (Chroococcales, Cyanophyceae). Phycologia 35:120–124

Marinho MM, Azevedo SMFO (2007) Influence of N/P ratio on competitive abilities for nitrogen and phosphorus by *Microcystis aeruginosa* and *Aulacoseira distans*. Aquatic Ecol 41:525–533

Martin-Luna B, Sevilla E, Hernandez JA, Bes MT, Fillat MF, Peleato ML (2006) Fur from *Microcystis aeruginosa* binds in vitro promoter regions of the microcystin biosynthesis gene cluster. Phytochemistry 67:876–881

Martin-Luna B, Sevilla E, Fillat MF, Peleato ML, Gonzalez A, Bes MT (2011) Expression of *fur* and its antisense *α-fur* from *Microcystis aeruginosa* PCC7806 as response to light and oxidative stress. J Plant Physiol 168:2244–2250

Moreira C, Vasconcelos V, Antunes A (2013) Phylogeny of microcystins: evidence of a biogeographical trend? Curr Microbiol 66:214–221

Msagati TAM, Siame BA, Shushu DD (2006) Evaluation of methods for the isolation, detection and quantification of cyanobacterial hepatotoxins. Aquat Toxicol 78:382–397

Neilan BA, Dittmann E, Rouhiainen L, Bass RA, Schaub V, Sivonen K, Börner T (1999) Nonribosomal peptide synthesis and toxigenicity of cyanobacteria. J Bacteriol 181:4089–4097

Oh HM, Lee S, Jang MH, Yoon BD (2000) Microcystin production by *Microcystis aeruginosa* in a phosphorus-limited chemostat. Appl Environ Microbiol 66:176–179

Orr PT, Jones GJ (1998) Relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures. Limnol Oceanogr 43:1604–1614

Pearson L, Mihali T, Moffitt M, Kellmann R, Neilan B (2010) On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. Mar Drugs 8:1650–1680

Pflugmacher S, Wiegand C (2001) Metabolism of microcystin-LR in aquatic organism. In: Chorus I (ed) Cyanotoxins. Springer, Berlin, pp 257–260

Pflugmacher S, Codd GA, Steinberg CEW (1999) Effects of the cyanobacterial toxin microcystin-LR on detoxication enzymes in aquatic plants. Environ Toxicol 14:111–115

Pineda-Mendoza R, Zúñiga G, Martínez Jerónimo F (2014) Infochemicals released by *Daphnia magna* fed on *Microcystis aeruginosa* affect *mcyA* gene expression. Toxicon 80:78–86

Rantala A, Fewer P, Hisbergues M, Rouhiainen L, Vaitomaa J, Börner T, Sivonen K (2004) Phylogenetic evidence for the early evolution of microcystin synthesis. Proc Natl Acad Sci USA 101:568–573

Rapala J, Sivonen K (1998) Assessment of environmental conditions that favour hepatotoxic and neurotoxic *Anabaena* spp. Strains in cultured under light-limitation at different temperatures. Microbial Ecol 36:181–192

Rapala J, Sivonen K, Lyra C, Niemelä SI (1997) Variation of microcystins, cyanobacterial hepatotoxins in *Anabaena* spp. as a function of growth stimuli. Appl Environ Microbiol 63:2206–2212

Rinta-Kanto JM, Konopko EA, DeBruyn JM, Bourbonniere RA, Boyer GL, Wilhelm SW (2009) Lake Erie *Microcystis*: relationship between microcystin production, dynamics of genotypes and environmental parameters in a large lake. Harmful Algae 8:665–673

Rueckert A, Cary SC (2009) Use of an armored RNA standard to measure microcystin synthetase E gene expression in toxic *Microcystis* sp. by reverse transcription QPCR. Limnol Oceanogr Methods 7:509–520

Sevilla E, Martin-Luna B, Vela L, Bes MT, Peleato ML, Fillat MF (2010) Microcystin-LR synthesis as response to nitrogen: transcriptional analysis of the *mcyD* gene in *Microcystis aeruginosa* PCC7806. Ecotoxicology 19:1167–1173

Sevilla E, Martín-Luna B, González A, Gonzalo-Asensio JA, Peleato ML, Fillat MF (2011) Identification of three novel antisense RNAs in the *fur* locus from unicellular cyanobacteria. Microbiology 157:3398–3404

Sevilla E, Martin-Luna B, Bes MT, Fillat MF, Peleato ML (2012) An active photosynthetic electron transfer chain required for *mcyD* transcription and microcystin synthesis in *Microcystis aeruginosa* PCC7806. Ecotoxicology 21:811–819

Sivonen K (1990) Effects of light, temperature, nitrate, orthophosphate, and bacteria on growth of and hepatotoxin production by *Oscillatoria agardhii* strains. Appl Environ Microbiol 56:2658–2666

Sivonen K, Jones G (1999) Cyanobacterial toxins. In: Chorus I, Bartram J (eds) Toxic cyanobacteria in water. WHO, London, pp 41–111

Smith V (1983) Low nitrogen to phosphorus ratios favor dominance by blue-green algae in lake phytoplankton. Science 221:669–671

Strickland JDH, Parsons TR (1972) A practical handbook of seawater analysis, second ed. Fish Res Bd Can Bull 167:1–310

Tillett D, Dittmann E, Erhard M, von Döhren H, Börner T, Neilan BA (2000) Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC 7806: an integrated peptide-polyketide synthase system. Chem Biol 7:753–764

Tonk L, Visser PM, Christiansen G, Dittmann E, Snelder EOFM, Wiedner C, Mur LR, Huisman J (2005) The microcystin composition of the cyanobacterium *Planktothrix agardhii* changes toward a more toxic variant with increasing light intensity. Appl Environ Microbiol 71:5177–5181

Utkilen H, Gjølme N (1995) Iron-stimulated toxin production in *Microcystis aeruginosa*. Appl Environ Microbiol 61:797–800

Van der Westhuizen AJ, Eloff JN (1985) Effect of temperature and light on the toxicity and growth of blue–green alga *M. aeruginosa* (UV-006). Planta 163:55–59

van Gremberghe I, Vanormelingen P, van der Gucht K, Mancheva A, D'hondt S, De Meester L, Vyverman W (2009) Influence of *Daphnia* infochemicals on functional traits of *Microcystis* strains (Cyanobacteria). Hydrobiologia 635:147–155

Vézie C, Rapala J, Vaitomaa J, Seitsonen J, Sivonen K (2002) Effect of nitrogen and phosphorus on growth of toxic and nontoxic *Microcystis* strains and on intracellular microcystin concentrations. Microb Ecol 43:443–454

Walsh K, Jones GJ, Dunstan RH (1997) Effect of irradiance on fatty acid, carotenoid, total protein composition and growth of *Microcystis aeruginosa*. Phytochemistry 44:817–824

Wang Q, Niu Y, Xie P, Chen J, Ma Z, Tao M, Qi M, Wu L, Guo L (2010) Variations of microcystins in Gonghu Bay of Lake Taihu, with potential risk of microcystin contamination to human health. Sci World J 10:1795–1809

Watanabe MF (1996) Production of microcystin. In: Watanabe MF, Harada K, Carmichael WW, Fujiki H (eds) Toxic microcystis. CRC Press Inc., Boca Raton, pp 35–56

Welker M, Malek B, Sejnohova L, Dohren H (2006) Detection and identification of oligopeptides in *Microcystis* (cyanobacteria) colonies: toward an understanding of metabolic diversity. Peptides 27:2090–2103

Wicks RJ, Thiel PG (1990) Environmental factors affecting the production of peptide toxins in floating scums of the cyanobacterium *Microcystis aeruginosa* in a hypertrophic African reservoir. Environ Sci Technol 24:1413–1418

Wood SA, Rueckert A, Hamilton DP, Cary SC, Dietrich DR (2011) Switching toxin production on and off: intermittent microcystin synthesis in a microcystis bloom. Environ Microbiol Rep 3:118–124

Wood SA, Dietrich DR, Cary SC, Hamilton DP (2012) Increasing microcystis cell density enhances microcystin synthesis: a mesocosm study. Inland Waters 2:17–22

Zilliges Y, Kehr JC, Meissner S, Ishida K, Mikkat S, Hagemann M, Kaplan A, Börner T, Dittmann E (2011) The cyanobacterial hepatotoxin microcystin binds to proteins and increases the fitness of *Microcystis* under oxidative stress conditions. PLoS One 6:e17615