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Original article

Growth and microcystin production of a Brazilian *Microcystis aeruginosa* strain (LTPNA 02) under different nutrient conditions

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ABSTRACT

Cyanobacteria are prokaryotic and photosynthetic organisms, which can produce a wide range of bioactive compounds with different properties; including a variety of toxic compounds, also known as cyanotoxins. In this work, we describe the isolation of seven cyanobacterial strains from two reservoirs in São Paulo State, Brazil. Seven different chemical variants of microcystins (MC-RR, MC-LR, MC-YR, MC-LF, MC-LW, and two demethylated variants, dm-MC-RR and dm-MC-LR) were detected in three of the ten isolated strains. One particular *Microcystis aeruginosa* strain (LTPNA 02) was chosen to evaluate its growth by cell count, and its toxin production under seven different nutritional regimes. We observed different growth behaviors in the logarithmic growth period for only three experiments ($p < 0.05$). The total growth analysis identified four experiments as different from the control ($p < 0.01$). Three microcystin variants (MC-RR, MC-LR and MC-YR) were quantified by liquid chromatography-tandem mass spectrometry. At the experimental end, the toxin content was unchanged when comparing cell growth in ASM-1 (N:P = 1), MLA and BG-11 (N:P = 10) medium. In all other experiments, the lowest microcystin production was observed from cells grown in Bold 3N medium during the exponential growth phase. The highest microcystin content was observed in cultures using BG-11(N:P = 100) medium.

Introduction

Cyanobacteria are aerobic prokaryotes and photoautotrophic microorganisms, found at a wide range of habitats across the world (Chorus and Bartram, 1999; Herrero et al., 2001). The planktonic species are classified into four orders: *Chroococcales*, *Oscillatoriales*, *Nostocales* and *Stigonematales* (Azevedo and Sant'Anna, 2006). Cyanobacteria produce a variety of secondary metabolites and bioactive compounds, some of which exhibit toxic properties and are referred to as cyanotoxins. Cyanotoxins

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vary widely in their chemical structures; they are divided into three classes: (i) cyclic peptides, nodularins and microcystins, composed of five and seven aminoacids respectively; (ii) alkaloids such as cylindrospermopsin, anatoxin-a, homoanatoxin, anatoxin-a (S) and saxitoxins; and (iii) lipopolysaccharides (van Apeldoorn et al., 2007). The structural diversity of cyanotoxins reflects on the different mechanisms of toxicity described: hepatotoxic, neurotoxic, dermatotoxic cyanotoxins, as well as cyanotoxins capable of inhibiting protein synthesis (Carmichael et al., 2001; Rodríguez et al., 2012). Eutrophication of water bodies can increase cyanobacterial growth and trigger cyanotoxin release (De Figueredo et al., 2004; dos Anjos et al., 2006; Dörr et al., 2010). Microcystins (MC) are cyanotoxins frequently observed in the environment as consequence of the occurrence of cyanobacterial blooms under eutrophic conditions in lakes, ponds and water reservoirs (Codd, 1995; Wiegand and Pflugmacher, 2005; Frias et al., 2006; Cardozo et al., 2007; Moschini-Carlos et al., 2009). Microcystins are potent hepatotoxins, which have been linked to many cases of livestock and wildlife poisoning incidents (Carbis et al., 1994; Kuiper-Goodman et al., 1999; Kujbida et al., 2006) as well as human fatalities (Jochimsen et al., 1998; Carmichael et al., 2001). Their structures consist of a cyclic peptide element, cyclo (D-Ala-L-*X*-D-MeAsp-L-*Z*-Adda-D-Glu-Mdha), were *X* and *Z* are variable L-amino acids: MeAsp is D-erythro-*b*-methylaspartic acid; Mdha is *N*-methyldehydroalanine; and Adda is 2*S*, 3*S*, 8*S*, 9*S*-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4*E*, 6*E*-dienoic acid (Carmichael et al., 1988). These molecules also show other variations such as demethylations and amino acid substitutions at different positions (Dörr et al., 2010). So far, 94 variants have been described (Bortoli and Volmer, 2014) and modifications of the general structure directly correlate with the toxicity levels, varying from 50 to > 1.200 $\mu g.kg^{-1}$ administered intra-peritoneal (*i.p.*) into mice. Also, rare non-toxic microcystins species have been observed (Chorus and Bartram, 1999; Furey et al., 2008).

A variety of studies have shown that environmental factors such as light, temperature, pH, irradiance, nutrient concentration and metals have a direct influence on growth and toxin production under different cultivation conditions (Sivonen, 1990; Wiedner et al., 2003; Ame and Wunderlin, 2005; Bittencourt-Oliveira et al., 2005; Marinho and Azevedo, 2007; Carneiro et al., 2009). Nitrogen and phosphorus content have been widely used to study variation of the nutrient composition of medium components. The N:P ratio is used as a prime indicator, as well as a predictor, of cyanobacterial and algal blooms, composition and seasonal succession (Kim et al., 2007). Research of the influence of N, P and their concentration ratio on the growth of *Microcystis* sp. strains and microcystin synthesis has shown contradictory results. Lee and co-workers report N:P ratios of 10 to 16 as optima for bloom development (Lee et al., 2000); however, Downing and co-workers found the highest microcystin concentration at N:P = 31.1 (Downing et al., 2005). Vezie and co-workers determined this ratio to be much higher, 237 < N:P < 753 (Vezie et al., 2002) but other studies did not observe any differences on growth for N:P ratios of 3, 10 and 15 (Marinho and Azevedo, 2007). Nevertheless, the growth of *Microcystis* sp. in a hypertrophic reservoir showed a rapid growth rate for N:P ratios of < 30 (Kim et al., 2007).

The present study systematically investigates the influence of different N:P ratios in different culturing media for a Brazilian *Microcystis aeruginosa* strain over growth and the resulting microcystin expression.

Materials and methods

Chemicals and reagents

Microcystins standards were obtained from Abraxis (Warminster, PA, USA). J.T. Barker (Xalostoc, Mexico) supplied HPLC-grade methanol and acetonitrile. Purified water was obtained from a Direct Q8 purification system (Millipore, Milford, MA, USA).

Isolation of strains and morphological identification

Salto Grande Reservoir is located at the Americana city at coordinates 22° 44'S e 47° 15'W, 530 m above sea level. It is a part of the Atibaia River in the hydrographic basin of Piracicaba River (Leite, 2002). Samples were collected at two different seasons, during bloom events, in July and September 2007. The above-mentioned reservoir is extensively used for recreational purposes by the local population (Bittencourt-Oliveira, 2003; Cetesb, 2007). Taquacetuba Reservoir is part of Billings Reservoir located at coordinates 23°42'S e 46°42'W (Rocha et al., 1985) between São Paulo and São Bernardo do Campo, São Paulo State, and is used as a public water supply. Samples were collected during a bloom event in March 2007. A preliminary observation of the samples was carried out using a light microscope (Zeiss Primo Star, Jena, Germany) fitted with achromatic lenses at 4 to 100 magnification levels.

Unicellular strains were isolated on ASM-1 medium supplemented with 1% agar (Gorham et al., 1964). The dishes were observed daily until the first colonies' growth. Confirmation of colony growth was conducted by microscopic analysis and bacteria were isolated into tubes containing 15 ml of ASM-1 medium. The strains were grown at 22 $\mu E.m^{-2}.s^{-1}$, 12:12 h (L:D) photoperiod at 25°C (± 1°C). Another isolation method was based on mechanical isolation of the filamentous species and was carried out under a microscope using sterilized Pasteur pipettes to collect single filaments from a small droplet of the sample. This coarse isolation procedure was repeated several times until there was only a single filament in the surrounding medium. These single filaments were isolated into tubes containing 15 ml of ASM-1 and their growth was monitored daily. After a growing period of the strains, they were morphologically characterized. Cyanobacterial genera and species were identified by microscopic observation and by identification of morphological characters (Anagnostidis and Komárek, 1988; Komárek and Anagnostidis, 1999).

Toxin analysis and microcystin quantification of isolated strains

The lyophilized material of each cyanobacteria strain (50 mg) was added methanol and acetic acid 0.1M (1:1) (1 ml), the mixture homogenized and sonicated with an ultrasonic

probe for 1 min. After centrifugation at 2500× *g* for 10 min, the supernatant was filtered through 0.22 µm nylon syringe filters (Millex, Millipore), as described previously by Hiller and coworkers (Hiller et al., 2007).

Toxin determination of all isolated strains was performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a series 200 HPLC system (Perkin Elmer, Waltham, MA, USA) coupled to an electrospray ionization (ESI) mass spectrometer. Chromatographic separations were carried out on a Luna C18 column (150×2 mm; 5 µm particles, Phenomenex, Torrance, CA, USA). The mobile phases consisted of 5 mM ammonium formate and 53 mM formic acid (eluent A) in water and acetonitrile/water (90/10; v/v, eluent B). Gradient elution was applied using the following program: linear increase from 10 to 90% B within 15 min, hold at 90% B for 2 min, and subsequently decrease to 10% B within 12 min. A flow rate of 300 µl.min^{-1} was used. MS/MS experiments were performed using an API 365 triple quadrupole (QqQ) mass spectrometer (AB Sciex, Concord, ON, Canada) equipped with a turbo ionspray source. Nitrogen at 400°C (12 l.min^{-1}) was used as curtain gas and the nebulizer gas flow rate was set to 10 l.min^{-1}. An electrospray voltage of 5 kV, declustering potential of 30 V, focusing potential of 150 V, and entrance potential of 9 V were used; the collision gas flow rate was set to 5 l.min^{-1}, collision energy to 30 V, and collision cell exit potential to 10 V. The instrument was operated in selected reaction monitoring (SRM) mode, with specific *m/z* transitions selected for highest sensitivity and selectivity. Single and double-charged ions were monitored in positive ion mode. Characteristic precursor ions for SRM were *m/z* 166 (anatoxin-a), 416 (cylindrospermopsin), 825 (nodularin), 519 (MC-RR), 910 (MC-LA), 986 (MC-LF), 995 (MC-LR), 1025 (MC-LW), 1045 (MC-YR), 512 (dm-MC-RR) and 981 (dm-MC-LR). Because only microcystins were detected in the studied samples, quantification of MC in SRM mode was based on the characteristic Adda fragment at *m/z* 135. Quantification of the demethylated microcystin structures was performed by relative quantification using the corresponding non-demethylated microcystin as analytical standards.

Toxin quantification during nutritional variation experiments

Every 72 h, 5 ml of the cultures were sampled and ultrasonicated (Virsonic Digital 550, Pittsburg, PA, USA) for 1 min to disrupt the cells; samples were then centrifuged at 3.214× *g* for 10 min at 4°C. The supernatant was removed and filtered using Millipore micro-filters (Millex PVDP 0.45 µm). The resulting material was stored at -20°C until LC-MS/MS analysis.

Quantification of MC-RR, MC-LR and MC-YR was performed on Agilent (Waldbronn, Germany) 1200 HPLC and AB Sciex 3200 QTRAP linear ion trap (QqLIT) instruments. Chromatographic separations were achieved using a Phenomenex Synergi MAX-RP column (150×2 mm; 4 µm particles), with the following mobile phase: (A) water, formic acid 0.1% and 5 mM ammonium formate, and (B) acetonitrile and formic acid 0.1% at 300 µl.min^{-1} flow rate. The mobile phase gradient started at 25% B and linearly increased to 90% within 3 min, followed by a return to 35% B within 5 min. The temperature of the ESI source was set to 650°C; the positive ion mode was used for detection at a curtain gas flow of 10 (arbitrary units); ion spray voltage of 5.5 kV; GS1 flow of 60; GS2 flow of 50, and collision gas pressure set to medium. Detection was performed in SRM mode using the following transitions: *m/z* 519→135(MC-RR); *m/z* 995→135 (MC-LR) and *m/z* 1045→135 (MC-YR).

Experimental conditions for growth experiments

The *Microcystis aeruginosa* strain (LTPNA 02) was grown in 250 ml flasks for one week as inoculum preparation. Subsequent experiments were prepared in 1 l flasks, which contained 800 ml of medium. The culture took place at 22 µE·m^{-2}·s^{-1}, 12:12h (L:D) photoperiod at 25°C (±1°C) under continuous aeration. The initial cell concentration was 5×10^5 cell·ml^{-1}. All procedures were performed in a sterilized environment in triplicate. The experiments were performed using seven different nutritional medium concentrations:(i) ASM-1 medium (Gorham et al., 1964) at three different N:P ratios: (N:P) = 1, 10 (unmodified media) and 20; (ii) MLA medium (Bolch and Blackburn, 1996) at (N:P) =10; (iii) BOLD 3N medium (Nichols and Bold, 1965) at (N:P) =16; and (iv) BG-11 medium (Rippka et al., 1979; Bittencourt-Oliveira, 2003) at (N:P) =10 and 100. The media components' concentrations are summarized in Table 1.

Growth evaluation

Every 72 h, 2 ml of each culture were sampled under sterilized environmental conditions, to evaluate cell growth using a Neubauer's chamber under a light microscopy (Zeiss Primo Star). Exponential regressions were applied to the cell data and sampling times to calculate the exponential growth period, determined for data adherence > 95% (r^2 = 0.95). Growth rates were calculated according to the exponential regression formula $N = N_0^{rn\text{-}t}$, where N is the number of cells at time t, N0 the initial number of cells and *rn* the growth rate (Reynolds, 2006).

Cellular microcystin content

A microcystin production quotient (QMC) was calculated to estimate the cellular microcystin production, assuming the extracellular content of microcystin as zero. The toxin concentration (ng·ml^{-1}) was divided by the number of cells (cell·ml^{-1}) for each sampling day and for each of the three microcystin variants quantified, generating the Q_{MC} values: $Q_{MC\text{-}RR}$; $Q_{MC\text{-}LR}$ and $Q_{MC\text{-}YR}$.

Statistical analysis

Statistical analyses were performed for cell numbers and Q_{MC} for all different nutritional cultivation groups and were described as mean ± standard deviation. Brown-Forsythe and Shapiro-WilkW tests were used to verify the homogeneity of variance and normality of the data. According to previous results *one-way* ANOVA test was used followed by Tukey. The tests were performed with a significance of 95% ($p < 0.05$). For statistical analysis Statistica (v 8.0 Stat Soft Inc, Tulsa, OK, USA) software was used.

Table 1
Media composition (l^{-1}).

Composition	ASM-1[a] [N:P=10]	ASM-1 [N:P=1]	ASM-1 [N:P=20]	MLA[a] [N:P=10]	Bold 3N[a] [N:P=10]	BG11[a] [N:P=100]	BG11 [N:P=10]
$NaNO_3$	0.17 g	0.017 g	0.34 g	0.17 g	0.75 g	1.5 g	0.15 g
$MgCl_2$. 6 H_2O	41 mg	41 mg	41 mg	-	-	-	-
Mg SO_4 . 7 H_2O	49 mg	49 mg	49 mg	49.4 mg	75 mg	75 mg	75 mg
$CaCl_2$. 2 H_2O	29 mg	29 mg	29 mg	29.4 mg	25 mg	36 mg	36 mg
KH_2PO_4	13.6 mg	13.6 mg	13.6 mg	-	175 mg	-	-
Na_2HPO_4. 7 H_2O	26.8 mg	26.8 mg	26.8 mg	-	-	-	-
H_3BO_3	2.48 mg	2.48 mg	2.48 mg	12.3 mg	-	2.86 mg	2.86 mg
K_2HPO_4	-	-	-	-	75 mg	-	-
K_2HPO_4. 3 H_2O	-	-	-	43.8 mg	-	40 mg	40 mg
$MnCl_2$. 4 H_2O	1.39 mg	1.39 mg	1.39 mg	0.36 mg	41 µg	1.81 mg	1.81 mg
$FeCl_3$. 6 H_2O	1.08 mg	1.08 mg	1.08 mg	1.58 mg	97 µg	6 mg	6 mg
Zn Cl_2	3.35 mg	3.35 mg	3.35 mg	-	5 µg	-	-
$CoCl_2$. 6 H_2O	0.19 mg	0.19 mg	0.19 mg	10 µg	2 µg	-	-
$CuCl_2$	1.4 µg	1.4 µg	1.4 µg	-	-	-	-
Edta Na_2	7.44 mg	7.44 mg	7.44 mg	4.36 mg	0.75 mg	1 mg	1 mg
NaCl	-	-	-	-	25 mg	-	-
$CaCO_3$	-	-	-	-	5 mg	-	-
Biotin[b]	-	-	-	1 µg	50 ng	-	-
Cobalamin[b]	-	-	-	1 µg	50 ng	-	-
Tiamin Hcl[b]	-	-	-	2 mg	0.1 mg	-	-
$Na_2MoO_4.2H_2O$	-	-	-	6 µg	4 µg	0.39 mg	0.39 mg
$NaHCO_3$	-	-	-	169.6 mg	-	-	-
$CuSO_4.5H_2O$	-	-	-	10 µg	-	79 µg	79 µg
$ZnSO_4.7H_2O$	-	-	-	22 µg	-	0.222 mg	0.222 mg
Citric acid	-	-	-	-	-	6 mg	6 mg
Na_2CO_3	-	-	-	-	-	20 mg	20 mg
$Co(NO_3)_2.6H_2O$	-	-	-	-	-	49.4 µg	49.4 µg

[a]Culture media as referred in literature.
[b]Biotin, $C_{10}H_{16}N_2O_3S$; Cobalaminn, $C_{63}H_{88}N_{14}O_{14}P$; Tiamin Hcl, $C_{12}H_{17}ClN_4OS$. HCl; Citric acid, $C_6H_8O_7$.

Results

In this study, all isolated strains were identified by genera. For one strain, it was possible to identify the species according to morphological characteristics by light microscopy (Anagnostidis and Komárek, 1988; Komárek and Anagnostidis, 1999; Via-Ordorika et al., 2004). The strains were named LTPNA (Laboratory of Toxins and Natural Products of Algae) followed by numbers from 01 to 10. Of the seven strains isolated from Salto Grande Reservoir, six were identified as *Microcystis* sp. and one as *Microcystis aeruginosa*. From the Taquacetuba reservoir, only three filamentous strains were isolated, two *Pseudoanabaena* sp. strains and an *Oscillatoria* sp. strain. The strains were incorporated into the culture collection of the LTPNA at University of São Paulo, Brazil. The genera and origin are summarized in Table 2. One particular *Microcystis aeruginosa* strain (LTPNA 02) was chosen to study its growth and microcystin production under different nutritional conditions.

Toxin production from the investigated strains

We analyzed all isolated strains for different classes of cyanotoxins content such as anatoxin-a, cylindrospermopsin, nodularin and microcystins. The analysis revealed that none of the filamentous strains produced any of the studied cyanotoxins and 50% of the *Microcystis* sp. strains were microcystin producers. Importantly, toxic and non-toxic strains were isolated from the Salto Grande reservoir during the same bloom event.

The microcystin analysis of our samples revealed that the three microcystin producer strains generated seven different congeners. Based on tandem mass spectrometry

Table 2
Cyanobacterial strains isolated from the Salto Grande and Taquacetuba Reservoirs.

Strain	Microorganism	Isolation technic	Reservoir	Toxin
LTPNA 01	*Microcystis* sp.	Plating	Salto Grande	Non producer[a]
LTPNA 02	*Microcystis aeruginosa*	Plating	Salto Grande	MC-RR, MC-YR, MC-LR, MC-Dmt
LTPNA 03	*Microcystis* sp.	Plating	Salto Grande	Non producer[a]
LTPNA 04	*Aphanocapsa* sp.	Plating	Salto Grande	Non producer[a]
LTPNA 05	*Microcystis* sp.	Plating	Salto Grande	Non producer[a]
LTPNA 06	*Pseudoanabaena* sp.	Mechanic isolation	Taquacetuba	Non producer[a]
LTPNA 07	*Pseudoanabaena* sp.	Mechanic isolation	Taquacetuba	Non producer[a]
LTPNA 08	*Microcystis* sp.	Plating	Salto Grande	MC-RR, MC-YR, MC-LR, MC-Dmt
LTPNA 09	*Microcystis* sp.	Plating	Salto Grande	MC-RR, MC-YR, MC-LR, MC-Dmt
LTPNA 10	*Oscillatoria* sp.	Plating	Taquacetuba	Non producer[a]

[a]Anatoxin-a(S), Cillindrospermiopsin e Nodularin non producer.

analysis, we confirmed the presence of MC-RR, MC-LR, MC-YR, MC-LF, MC-LW and two demethylated forms, dm-MC-RR and dm-MC-LR. It was not possible, however, to determine the specific amino acids that were demethylated for these dm-MC species.

For the experiments performed under different nutrient condition, we measured three microcystins homologues: MC-RR, MC-LR and MC-YR. Based on the quantitative data, it was possible to calculate the intracellular microcystin content, by generating the quotients $Q_{MC\text{-}RR}$, $Q_{MC\text{-}LR}$, $Q_{MC\text{-}YR}$. Our data did not show differences between production levels of these variants at any of the sampling times, and therefore we decided to always use the intracellular microcystin content as sum of the three variants, $Q_{MC\text{-}TOTAL}$.

Growth behavior and microcystin production in different media

The growth and microcystin expression of *Microcystis aeruginosa* strain (LTPNA 02) under different nutritional conditions was studied. Four different media were used in these experiments, ASM-1 at three N:P ratios (1, 10, 20); Bold 3N (N:P = 16); MLA (N:P = 10) and BG-11 at two N:P ratios (10, 100).

It was possible to compare the growth rate at the exponential growth period, the duration in days, and also the total growth period, which was 21 days, as demonstrated in Fig. 1.The growth values in Table 3 represent the mean values of triplicates of each experiment. Using the experiment in ASM-1 (N:P = 10) as control, we observed that exponential growth was lower for experiments in ASM-1 (N:P=1) ($p < 0.03$) and BG-11 (N:P = 100) ($p < 0.05$). Only the culture maintained in BG-11 (N:P = 10) had an exponential growth higher than the control ($p < 0.0002$). After 21 days, it was possible to compare the total growth. The experiments in ASM-1 (N:P=1) ($p < 0.0002$); Bold 3N ($p < 0.0002$) and in BG-11 (N:P 10 and 100) ($p < 0.0002$ and $p < 0.02$ respectively) showed a lower total growth than the control group.

In our study, growth and intracellular microcystin concentration did not correlate (Fig. 2). The maximum microcystin content was found at the highest and the lowest growth. During the three experiments in different media ASM-1, MLA and BG-11 (but identical N:P =10 ratios), we did not observe statistical differences between exponential and total growth between ASM-1 and MLA media. The cultures in BG-11 displayed a higher exponential growth than ASM-1 ($p < 0.0002$) and MLA ($p < 0.01$). Total growth between ASM-1 and MLA, and MLA and BG-11 was similar, but we found a statistical difference between ASM-1 and BG-11. Total growth for BG-11 was lower than in ASM-1 ($p < 0.02$).

The intracellular microcystin content varied over time. On sampling days 0, 3 and 6, there were differences between the intracellular microcystin concentrations. After the day 9, the three experiments did not show differences in microcystin production. Within these results, we can confirm that the tested media were suitable for cultivation of this specific LTPNA 02 *Microcystis aeruginosa* strain and provide a stable microcystin production.

Comparing the experiments conducted on BG-11 and the control in ASM-1, we observed a statistical difference for exponential and total growth. Exponential growth and total growth were lower for BG-11 N:P = 100 ($p < 0.05$ and $p < 0.0002$ respectively), and for BG-11 N:P =10, exponential growth was higher than control ($p < 0.0002$) and lower for total growth ($p < 0.02$). The toxin content was also statistically different. For both experiments in BG-11, the initial QMC-TOTAL was higher than the control on days 0 and 3. After this, the BG-11 N:P=10 culture was similar to the control, but the experiment for BG-11 N:P=100, even with the lowest growth, showed the highest intracellular microcystins concentration, statistically higher than the control and all the other experiments at the last day of the experiment for all microcystin homologues quantified ($p < 0.0002$).

Table 3
Growth rates (exponential and total) in different nutrient condition experiments. Control experiment was ASM-1 [N:P = 10]. Variance analyses were performed with *one-way* ANOVA followed by *Tukey's test*.

Medium	N:P	Exponential growth	Reservoir	Toxin
ASM-1	10	15	0.2126	0.1661
ASM-1	1	6	0.1599[a]	0.0425[b]
ASM-1	20	18	0.2030	0.1731
MLA	16	12	0.2474	0.1588
Bold-3N	10	9	0.1882	0.0979[b]
BG-11	100	9	0.1659[a]	0.0682[b]
BG-11	10	9	0.3065[b]	0.1484[a]

[a]Significantly different from control; $p < 0.05$.
[b]Significantly different from control; $p < 0.0002$.

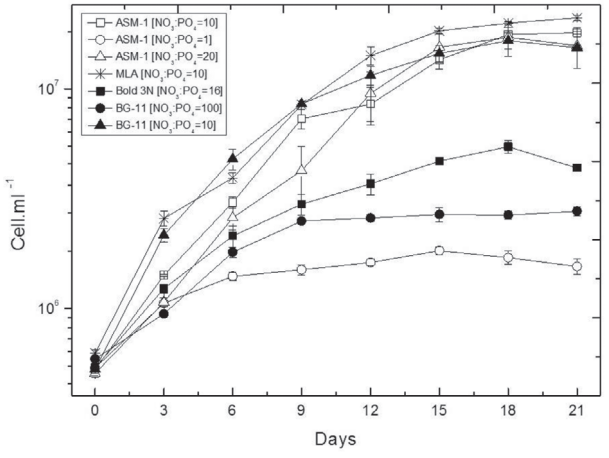

Figure 1 – Growth curves of *Microcystis aeruginosa* LTPNA 02 under different medium cultivation conditions. *Error bars* denote standard deviation (SD).

The Bold 3N experiment exhibited exponential and total growth significantly lower than control ($p < 0.0002$). Intracellular microcystin production was also severely compromised. The microcystin homologues MC-LR and MC-YR were below the limit of quantification and MC-RR exhibited the lowest concentration values observed, reflecting the lowest QMC-TOTAL observed in this study.

Growth behavior and microcystin production at different N:P ratios

Comparing experiments conducted in the same media (ASM-1), we observed that for a low N:P (=1) ratio, growth and toxin production were lower than in the control (N:P = 10) during the exponential growth phase, being statistically different for all microcystin homologues quantified and resuming a lower $Q_{MC\text{-}TOTAL}$ ($p < 0.005$). Also, in our experiments, the intracellular microcystin content was not different from the control during the stationary phase. An N:P ratio of 20 showed no influence on growth, but it was possible to observe a reduction of the intracellular microcystin content during the earlier exponential growth, significantly different from the control $Q_{MC\text{-}TOTAL}$ ($p < 0.05$) and an enhancement of the production during the stationary phase $Q_{MC\text{-}TOTAL}$ ($p < 0.05$).

By comparing the BG-11 experiments, it was possible to observe that for a ratio of N:P = 100, the exponential growth and total growth were statistically lower than for the ratio of N:P = 10 ($p < 0.0002$). The intracellular microcystin content was also different. The $Q_{MC\text{-}TOTAL}$ was higher for BG-11 N:P = 100 on day 0 ($p < 0.002$), then it remained unchanged for N:P = 10 for the next 15 days, and finally increased at days 18 and 21 ($p < 0.03$).

Discussion

Previous studies have demonstrated that the rates between microcystin producer and non-producer strains isolated from

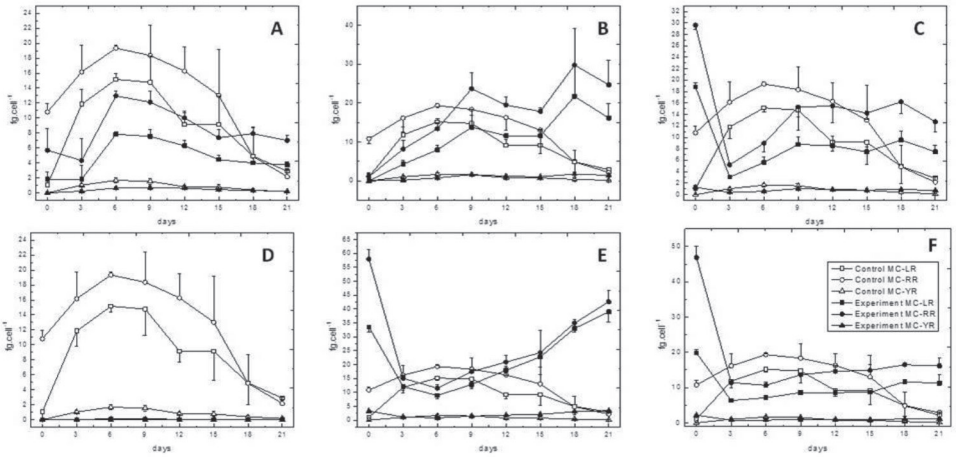

Figure 2 – Intracellular microcystin content (fg.cell^{-1}) of *Microcystis aeruginosa* LTPNA02 for the three microcystin variants (MC-LR, MC-RR and MC-YR) in each of the different media. A ASM-1 (N:P = 1); B ASM-1 (N:P=20); C MLA; D Bold 3N, E BG-11 (N:P = 100) and F BG-11 (N:P = 10). *Error bars* denote SD.

the same bloom event can vary (Vezie et al., 1998; Saker et al., 2005; Haande et al., 2007; Martins et al., 2009). In Portugal, two different studies demonstrated that more than half of the isolated strains were microcystin producers (55%) (Martins et al., 2009). In another study, a similar situation was observed for 62% of the isolated strains (Saker et al., 2005). Contrary to these results was a study in East Africa, where only 17% of the isolated strains were microcystin producers (Haande et al., 2007). Microcystins analogues identified in this study for the three strains LTPNA 02, LTPNA 08 and LTPNA 09 have been previously described as a product of Brazilian *Microcystis* sp. strains; MC-RR, MC-LR and MC-YR were reported in natural bloom samples collected in different Brazilian reservoirs (Frias et al., 2006; Moschini-Carlos et al., 2009) as well as the demethylated forms of MC-LR and MC-RR (Matthiensen et al., 2000; dos Anjos et al., 2006; Bittencourt-Oliveira et al., 2011), and MC-LW and MC-LF (Azevedo et al., 1994; Bittencourt-Oliveira et al., 2011).

Growth and intracellular microcystin production are influenced by different N:P ratios, which highlight the main effects these nutrients have on the modulation of microcystin biosynthesis. Older studies concluded that higher microcystin content was related to favorable growing conditions and increased nitrogen supply (Orr and Jones, 1998; Lee et al., 2000), whereas more recent studies show no correlation (Kruger et al., 2012; Lyck, 2004). Our study corroborates the newer findings. It is important to point out that differences between the investigated media go further than simply the N:P ratio and that there are other important parameters. The media differ in nutrients concentration, and in the presence or absence of vitamins and metals, which act as micronutrients.

Orr and Jones (1998) observed that the maximum cell yield was directly proportional to the initial nitrate concentration; therefore, in their experiments the authors demonstrated that nitrate was the limiting nutrient. Some of our data showed similar behavior. For a N:P ratio of 1 in ASM-1 media, a lower exponential growth and also a lower total growth were observed. The same was seen by Lee et al. for N:P = 1 (Lee et al., 2000), confirming that under nitrogen limitation there is also a significant reduction of growth. On the other hand, for the experiment using two-fold concentration of nitrogen in ASM-1 (N:P = 20), we did not observe significant differences as compared to the control in ASM-1 (N:P = 10). This confirms the findings of Krüger et al. (2012), who found no significant increase in *Microcystis* sp. biomass at N:P ratios of 6.8; 13.7 and 20.6.

The toxin cell production as a constant has been a hypothesis defended by many authors in the past (Sivonen, 1990; Vezie et al., 2002). A complex relation between growth and toxin production was observed by Lyck (2004). The linear correlation between microcystin production and growth reported by Orr and Jones (1998) was also observed by Lyck in certain occasions. In this work, we observed this behavior under some culture conditions, but the variation of the toxin concentration per cell does not allow the verification of a constant microcystin production. Orr and Jones also observed a strong correlation between microcystin production and cell division. The authors observed that microcystin production is influenced by the environmental effects on growth of the organism but not by effects of the metabolic pathway of toxin production (Orr and Jones, 1998). Vézie et al. (2002) reported a significant influence of nutrients on microcystin production; they found the highest intracellular microcystin content at the highest and lowest growth rates within their experiments. Lee and coworkers (2000) observed changes of microcystin production under different ratios of N:P. It was found that N:P = 16 was the optimum condition for growth and microcystin production by a *Microcystis aeruginosa* strain. A different optimum rate was reported by Downing and coworkers (2005); they found higher microcystin concentrations for a range of N:P values, between 18 and 51, with N:P=31.1 described as the optimum value. There were no significant associations between *Microcystis aeruginosa* cellular constituents such as microcystin content, chlorophyll, proteins and strain growth, even though the authors observed a strong correlation between growth and microcystin production. They emphasized that microcystin production depends on intracellular nitrogen availability and that discrepancies between studies are due to the modulation of elements that control the entrance of nitrogen into the cells.

Krüger et al. (2012) studied the influence of a broad range of Nitrate-N concentrations on a *Microcystis aeruginosa* strain and found no significant influence on cell growth and microcystin production. They also observed a growth decrease under N-starvation conditions. In the same study, they proposed an adequate ratio of N:P that would favor the growth and microcystin production. In this scenario, a phosphate limitation leads to an excess of nitrogen stimulating the synthesis of nitrogen rich compounds, such as microcystins, in particular MC-RR and dm-MC-RR, which are nitrogen-rich microcystin homologues. On the other hand, nitrate excess can lead to the enhancement of intracellular nitrite concentration. Generally, nitrate excess can be stored in the cell, but once converted to nitrite; the nitrite excess can cause deleterious effects to the cell, mainly by affecting the photosynthetic process through the inhibition of electron transport via modification of the intracellular pH, causing direct damage to cell membranes (Chen et al., 2009). This is the most probable explanation for the lowest growth rates observed in our study during the experiment with BG-11 N:P = 100.

It is important to point out that the culture media used in this work have different nutrient compositions, in particular regarding to metals and vitamins. One example is iron. This element is essential to several metabolic processes for cyanobacteria such as chlorophyll-a synthesis, respiration, photosynthesis and nitrogen fixation (Alexova et al., 2011). The concentration in Bold 3N medium is the lowest of all the investigated media. It is well known that iron also has direct influence on *Microcystis* sp. growth. When modified BG-11 medium is completely devoid of iron, a 32-50% decrease in growth rate was seen (Lukac and Aegerter, 1993). This is a possible explanation for the lowest total growth seen in Bold 3N, which has the lowest iron concentration per liter (97 µg of $FeCl_3$.6 H_2O, while ASM-1 has 1.08 mg; MLA 1.58 mg, and BG-11 6 mg). In contrast to Lukac and Aegerter, however, we did not find any increase in toxin production. More recently, Alexova et al. (2011) also reported on severe growth limitations under iron-limiting conditions for a concentration of 10 nM Fe. Nevertheless, they observed an increase in microcystin production. Therefore, the N:P

= 16 ratio in Bold 3N medium by itself was probably not influencing the growth, but rather the low iron concentration was the growth limiting factor.

Many studies have tried to elucidate the influence of environmental parameters, especially nutrients such as nitrogen and phosphorus and their proportions, on growth and microcystin production by *Microcystis* sp. strains. In this study, we investigated the effect of different media and N:P ratios on growth and microcystin production of a *Microcystis aeruginosa* strain. We observed different growth behavior at experiments in the same media, but with different N:P ratios. This reinforces the fact that these nutrients are essential for the development of these organisms. Moreover, unlike other studies, we observed no linearity between growth and toxin production for all the experiments. It was possible to detect high concentrations of intracellular microcystin at experiments with the highest and lowest growth rates. We also conclude that in addition to the ratio N:P, other elements are essential for development, growth and production of microcystins by these organisms. The presence of iron and a total nitrate concentration are important factors for the development of the particular *Microcystis aeruginosa* strain that we evaluate.

Another important issue to consider is that many authors use different parameters to express microcystin production, such as protein content, chlorophyll a and dry weight. This makes attempts of a systematic comparison of intracellular microcystin production very difficult and may lead to results that are not equivalent. Protein content and chlorophyll a depend on cellular processes and are expected to change under different exposure conditions. Finally, there remains the unanswered question of whether *Microcystis* strains isolated in different parts of the world can be compared regarding differences of environmental factors and microcystin production.

Authors' contributions

SB contributed in collecting environmental material, identification, isolation and strain culture, study design, ran the laboratory work, analysis of the data and drafted the paper. DO-S contributed with the LC-MS microcystins quantification on the nutrient conditions experiments. TK contributed with the LC-MS microcystins identification of the isolated strains and the critical reading of the manuscript. FAD supervised the laboratory work. PC and DAV contributed to the critical reading of the manuscript. EP designed the study and supervised the laboratory work.

Conflicts of interest

The authors declare no conflicts of interest.

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