Comparison of *Microcystis aeruginosa* (PCC7820 and PCC7806) growth and intracellular microcystins content determined by liquid chromatography–mass spectrometry, enzyme-linked immunosorbent assay anti-Adda and phosphatase bioassay

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ABSTRACT

Cyanobacteria are able to produce several metabolites that have toxic effects on humans and animals. Among these cyanotoxins, the hepatotoxic microcystins (MC) occur frequently. The intracellular MC content produced by two strains of *Microcystis aeruginosa*, PCC7806 and PCC7820, and its production kinetics during the culture time were studied in order to elucidate the conditions that favour the growth and proliferation of these toxic strains. Intracellular MC concentrations measured by liquid chromatography (LC) coupled to electrospray ionization mass spectrometer (MS) were compared with those obtained by enzyme-linked immunosorbent assay (ELISA) anti-Adda and protein phosphatase 2A (PP2A) inhibition assays. It has been demonstrated there are discrepancies in the quantification of MC content when comparing ELISA and LC-MS results. However, a good correlation has been obtained between PP2A inhibition assay and LC-MS. Three MC were identified using LC-MS in the PCC7806 strain: MC-LR, demethylated MC-LR and a new variant detected for the first time in this strain, [MeSer[7]] MC-LR. In PCC7820, MC-LR, D-Asp[3]-MCLR, Dglu(OCH$_3$)-MCLR, MC-LY, MC-LW and MC-LF were identified. The major one was MC-LR in both strains, representing 81 and 79% of total MC, respectively. The total MC content in *M. aeruginosa* PCC7820 was almost three-fold higher than in PCC7806 extracts.

Key words | ELISA, LC-MS, *Microcystis aeruginosa*, PCC7820, PCC7806, PP2A inhibition assay

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INTRODUCTION

Cyanobacteria blooms have been posing a worldwide environmental issue in recent decades (Shao *et al.* 2009). Not all cyanobacterial blooms are toxic, with the mean frequency of toxic bloom occurrence being 59%. The potency of blooms can vary substantially according to sites, seasons, weeks, or even days. These variations could be due to changes in species composition, to the production of different toxins with varying toxicity for each clone, and to the influence of environmental factors (Robillot *et al.* 2000). There is a risk that blooms of these cyanobacteria contaminate water supplies that are used for recreation, fisheries,

agriculture, livestock-watering and for drinking water (Gurbuz *et al.* 2009).

Microcystis is one of the most common and widespread genus of freshwater cyanobacteria throughout the world. In most cases, it is the dominant component of phytoplankton in eutrophic lakes. In temperate regions, *Microcystis* starts to grow in spring and, after growing exponentially during summer months, it moves into the stationary phase in late summer (Vasas *et al.* 2010). When a *Microcystis* bloom occurs, a lot of biomass accumulates at the water surface for several days. The smallest *Microcystis* colonies may be

composed of only a few individual cells, whereas the largest ones may be composed of thousands of cells (Wu & Kong 2009). *Microcystis aeruginosa* is the most common and best-known bloom forming *Microcystis* and it is the most prominent producer of cyanotoxins called microcystins (MC). These toxins are contained in *Microcystis* cells and are released when these cells die. Usually, cell-bound concentrations of MC are several orders of magnitude higher than extracellular toxins, whose concentrations vary from being not detectable to about 10% of the total (Jüttner & Lüthi 2008). The concentration of dissolved MC may be much higher in ageing or declining blooms when cell lysis is triggered and the toxins are released to the environment (Jähnichen *et al.* 2007; Jüttner & Lüthi 2008; Shao *et al.*

Q1 2009; Sakai *et al.* 2009; Zegura *et al.* 2011). Currently, 80 variants of MC are known and they differ widely in their abundance and toxicity (Gurbuz *et al.* 2009). These toxins have a structure cyclo-(D-alanine-X-D-MeAsp-Z-Adda-D-glutamate-Mdha) where Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic   acid. The toxins are named according to the two variable L-amino acids at positions X and Z. Minor modifications can occur on the other amino acids, such as demethylation on MeAsp or Mdha, or esterification of glutamic acid. The most frequently found MC include the leucine-arginine (MC-LR), arginine-arginine (MC-RR), tyrosine-arginine (MC-YR) and leucine-alanine (MC-LA) variants (Gurbuz *et al.* 2009; Vasas *et al.* 2010). MC-LR is the most toxic MC variant and the World Health Organization (WHO) has established a guideline value of 1 μg/L for drinking water (WHO 1998).

MC inhibit eukaryotic protein phosphatases 1 and 2A, and are recognized as potential tumour promoters and carcinogens: in this sense, the International Agency for Research on Cancer classified MC-LR as a possible human carcinogen, group 2B (http://www.iarc.fr/). In the last decade, evidence has been accumulating showing that MC induce damage to the DNA, which means that they are genotoxic and can act as tumour initiators. Hence, it is important to identify the production or potential synthesis of these toxins in the environment to avoid even low-level exposure to humans (Zegura *et al.* 2011).

To date, several screening and analytical methods have been developed for cyanotoxin detection and quantification. Enzyme-linked immunosorbent assays (ELISA) developed for MC are highly specific, sensitive, quick to perform, inexpensive and require minimum sample processing (Gurbuz *et al.* 2009). The most common ELISA technique used (ELISA anti-MC-LR) is based on molecular recognition by polyclonal antibodies that have good cross-reactivity with MC-LR (MC-LR), MC-RR and MC-YR, but less reactivity with the variants MC-LY and MC-LA (Ueno *et al.* 1996). This technique has been applied to detect MC in waters, freshwaters sediments and cyanobacteria cultures (Moreno *et al.* 2003, 2004; Babica *et al.* 2006). In this ELISA technique, several physicochemical variables (salinity, pH, etc.) can also produce false positive MC results (Metcalf *et al.* 2000). Moreover, an indirect competitive ELISA anti-Adda has enabled the congener-independent detection of MC, which is based on recognizing *Microcystis* by specific antibodies and has been used to determine these in water (Zeck *et al.* 2001; Fischer *et al.* 2001) and in fish liver tissues (Ernst *et al.* 2005; Moreno *et al.* 2011).

The protein phosphatase inhibition assay (PPI assay) was developed on the basis that MC have the ability to inhibit serine-threonine protein phosphatase enzymes. Not all MC variants react with protein phosphatase enzymes to a similar extent and the assay is sensitive to protein phosphatase inhibitors other than MC, such as okadaic acid (Rapala *et al.* 2002; Sevilla *et al.* 2010).

Confirmatory techniques including liquid chromatography (LC) with different detectors can be used, which allow the identification and quantification of individual MC variants (Gurbuz *et al.* 2009). LC-mass spectrometry (LC-MS) is the preferred technique to identify and quantify MC, electrospray (ES) interface being the most applied interface for this type of compound in environmental samples. This technique makes it possible to monitor precisely and simultaneously various algae toxins in extracts from laboratory cultures or algal blooms present in eutrophic waters (Dahlmann *et al.* 2003; Cameán *et al.* 2004).   **Q2**

M. aeruginosa PCC7806 has been frequently used for different purposes, such as to study how some parameters can affect the transcriptional response of some genes or the MC-LR production (Dahlmann *et al.* 2003; Wiedner *et al.* 2003; Jähnichen *et al.* 2007; Tonk *et al.* 2009), topology and toxicity of this strain (Jüttner & Lüthi 2008), or physiological response to some chemicals (Shao *et al.* 2009). In these studies different analytical techniques have been

applied (LC coupled with photodiode-array detection LC-DAD, ELISA, PPI assay, etc.) in order to detect cyanotoxins. However, no information is available on the MC production kinetics of this strain determined by LC-MS. Robillot *et al.* (2000) carried out a hepatotoxin production kinetic study of *M. aeruginosa* PCC7820 employing PP2A inihibition assay and LC-MS technique.

The aim of this work was to study MC production kinetics of two *M. aeruginosa* strains (PCC7806 and PCC7820) during the growth period. Biomass and toxins production were measured along with growth in controlled cultures. LC-MS was used to identify and quantify the intracellular MC production of both strains. In the present study, it has been detected for the first time in *M. aeruginosa* PCC7806, a MC identified as [L-MeSer[7]] MC-LR. Moreover, intracellular MC concentrations were measured by three techniques: LC-MS, ELISA anti-Adda and PP2A inhibition assay in order to compare them.

MATERIALS AND METHODS

Cyanobacteria culture

M. aeruginosa PCC7806 was used in this study in order to establish for the first time its MC production kinetics determined by LC-MS since no information has been available. *M. aeruginosa* PCC7820 was used to compare the MC production between strains of the same cyanobacteria species cultivated under the same conditions.

Axenic cultures of both planktonic cyanobacteria strains (*M. aeruginosa* PCC7820 and PCC7806) were obtained from the Pasteur Culture Collection. Both of them were maintained in sterilized 250 mL Erlenmeyer flasks containing 100 mL of BG11 medium ($+1$ M $K_2HPO_4 \cdot 3H_2O + 5$ mM $NaNO_3 + 12$ mM $NaHCO_3$) at 30 °C under continuous illumination with an intensity of 28 μmol photons $m^{-2} s^{-1}$ provided by cool white fluorescent tubes. Sterile air was bubbled through the culture medium. Once cultures reached 1.5 ± 0.2 units of optical density (OD), they were transferred to bottles containing 20 L of BG11 medium. An OD $= 0.1$ was set (time 0) as the initial culture density for each growth period, according to Preußel *et al.* (2009).

The *M. aeruginosa* density of both cultures was obtained by microscopic counting of samples collected at intervals of 72 h for 21 days. Aliquots were taken and preserved in Lugol's solution for cell counting. Cell density was measured on 1 mL samples using Sedgewick-Rafter chambers and an inverted microscope (Olympus CKX41) according to the Throndsen (1995) method. After the period of 21 days, cultures were harvested and concentrated by continuous centrifugation (23,428 g). The concentrated biomass was preserved at -80 °C until lyophilization. The cyanobacterial biovolume of the samples was used to estimate the biovolume related to toxin concentrations of the different cultures.

In addition, growth was monitored by measuring optical density at 680 nm (Wu *et al.* 2009) and chlorophyll *a*. Measures of chlorophyll *a* were made following the MacKinney (1941) method. Growth rates were calculated from OD values of the cultures measured photometrically at 680 nm. The OD was determined in 1.5 mL aliquots of culture taken at intervals of 72 h throughout the study. Specific growth rates were calculated according to the equation (Wu *et al.* 2009):

$$\mu = (\ln OD_2 - \ln OD_1)/t_2 - t_1$$

Intracellular MC extraction

A subsample of 100 mL of a cell suspension was removed for analysis at each sampling time (72 h). Cells and culture media were separated by filtration through glass fibre APFC 47 mm discs (Millipore). Discs obtained at each sampling time and the concentrated biomass obtained at the end of the study were lyophilized prior to the extraction of the MC, which was carried out according to Moreno *et al.* (2004), using 0.1 M acetic acid and a mixture of methanol/chloroform (1:1 v/v) solution. After sonication in an ultrasound bath for 15 min and stirring for 30 min at room temperature, the cell suspensions were centrifuged. For a quantitative extraction of the toxins, this procedure was performed twice. The toxin-containing fractions were eluted in 0.5 mL of methanol. The final extract was divided into three aliquots, one of them was directly injected into the LC-MS to identify and quantify the MC present in the samples, and the

other two were dried and resuspended in water in order to analyse the intracellular MC content by ELISA anti-Adda and PP2A inhibition assay.

ELISA anti-Adda assay

For determination of the intracellular MC by ELISA, a commercially available competitive anti-Adda ELISA kit (Abraxis LLC, USA) was used. This assay uses an antiserum raised against the unique C20 amino acid Adda, which is common to all MC variants. The main reason to employ this anti-Adda ELISA assay to detect MC is because of the high variability of compounds that might be found in the cultures. Detection based on ELISA anti-MC-LR is limited to the hepatotoxins MC-LR, MC-LA, MC-RR, MC-YR and nodularin, which are known to have cross-reactivity (Wiedner *et al.* 2003). Samples were analysed according to the manufacturer's instructions, previous calibration and verification with a control standard. The negative control, the standards and samples were all analysed in duplicate. Microtitre plates were read at 450 nm and B_0 values (%) calculated. Results were expressed as micrograms of MC/L.

Protein phosphatase 2A inhibition assay

PP2A inhibition assay was made using a commercially available kit (MicroCystest, Zeu-Inmunotec S.L.) following the Sevilla *et al.* (2010) method. The assay is based on the degradation of p-nitrophenyl phosphate into p-nitrophenol. Inhibition rate is directly related to the absorbance at 405 nm measured by a plate reader (Titertek Multiskan). MC concentration was obtained by reporting the inhibition rate on a standard MC-LR calibration curve. The calibration curve was determined using inhibition percentages and log-transformed concentrations of MC-LR standards, and used to calculate toxin concentrations (MC-LR equivalents, μg/L) of unknown samples.

Chromatographic conditions

Chromatographic separation was performed using a Perkin-Elmer Series 200 LC system coupled to an Applied Biosystems QTRAP LC-MS/MS system consisting of a quadrupole-linear ion trap mass spectrometer equipped with an electrospray ion source. Analytical column was a LiChroCART 250-4 reversed-phase column with a particle size of 5 μm (Merck kGaA). The flow rate was 0.4 mL/min. Chromatographic separation was performed using a binary gradient consisting of (A) water, and (B) Acetonitrile. Both components contained 0.05% trifluoroacetic acid (v/v). The elution profile was: 35% B (2 min), 35% up to 65% B (15 min), 65% B (5 min), 100% B (3 min). Injection volume was 10 μL. Single ion monitoring (SIM) experiment was applied for the detection of MC, where selected ions were monitored at Q1 (m/z 995.6 for MC-LR, m/z 981.5 for [D-Asp^3]MC-LR, m/z 1013.6 for [MeSer^7]MC-LR, m/z 1009 for Dglu(OCH$_3$)MC-LR, m/z 1002.5 for MC-LY, m/z 1025.5 for MC-LW and m/z 986.5 for MC-LF). The ion source was operated in positive mode. The capillary voltage and declustering potential were set to 5,500 and 105 V, respectively. Curtain gas, nebulizer gas (gas 1) and heater gas (gas 2) were 35 psi, 60 psi and 60 psi, respectively, and the Turboprobe temperature was maintained at 350 °C.

Chemicals

All reagents were of LC grade or analytical grade. Methanol, acetonitrile and trifluoracetic acid were purchased from Merck. LC-quality water was obtained by purifying demineralized water in a Milli-Q filtration system (Millipore). MC-LR, MC-RR and MC-YR standards were supplied by Alexis with a purity of 99%.

RESULTS AND DISCUSSION

Cyanobacterial growth

Triplicate measures of cellular biomass, chlorophyll *a* and OD were performed in order to study the cyanobacterial growth. The growth dynamics were different between both strains (Figure 1(a)). The stationary phase of PCC7806 was not observed after 21 days, whereas PCC7820 reached this point after 12 days. The growth during the culture time of PCC7806 was higher than PCC7820 (1.7-fold) with a maximum cellular concentration of 6.22×10^7 cells/mL and 3.68×10^7 cells/mL, respectively. Robillot *et al.* (2000) also observed that PCC7820 achieved the stationary phase after
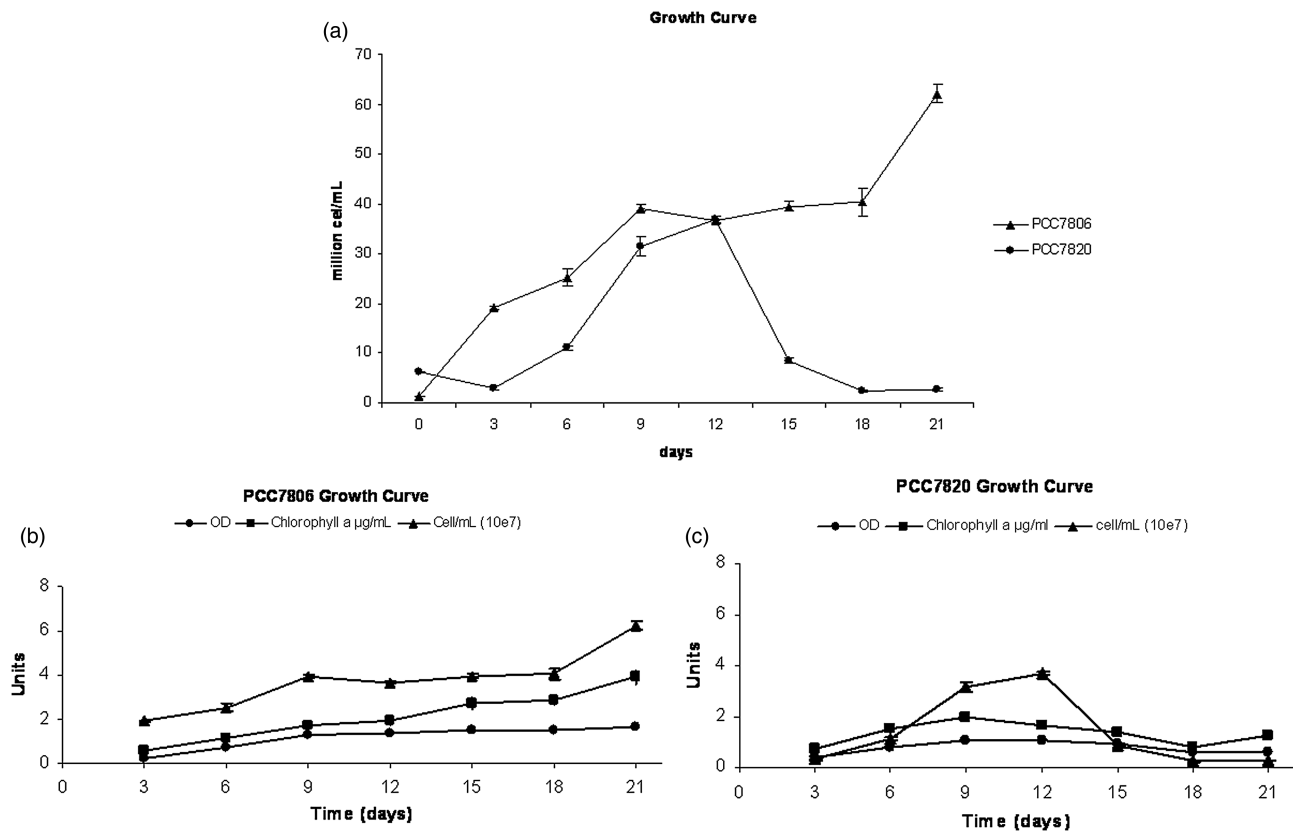
Figure 1 │ Comparison of the cell growth of both strains (PCC7806 and PCC7820) during 21 days of culture (a) and quantification of number of cells, levels of chlorophyll *a* and OD of both **Q4** strains (b) PCC7806 and (c) PCC7806. Values are given as means ± standard deviation (*n* = 3).

12 days reaching a maximum cellular dry weight of 60 mg, corresponding to 600 mg/L. On the other hand, the growth of PCC7806 was studied by Shao *et al.* (2009) during 10 days of culture, obtaining a maximum level of cells of 4×10^7 cells/mL. Sevilla *et al.* (2010) also studied the growth of this strain obtaining different growth curves depending on the nitrogen availability. Thus, with 2 mM of nitrate (Pasteur Institute recommendations) the maximum concentration of cells was $\approx 4 \times 10^7$ cells/mL and with 20 mM (nitrate excess) an outstanding growth of the cells ($\approx 6 \times 10^7$ cells/mL) was observed after 15 days of culture. In our study, at 5 mM $NaNO_3$ the quantification of cells after 15 days of culture was 4×10^7 cells/mL for PCC7806.

Culture growth of *M. aeruginosa* PCC7806 and PCC7820 was followed using three different parameters: quantification of number of cells, levels of chlorophyll *a* and OD. The variation of *M. aeruginosa* PCC7806 growth is synchronous with chlorophyll *a* levels (Figure 1(b)), which could be useful for

the growth estimation as previously stated by Wang *et al.* (2010). In the present study, we observed that chlorophyll *a* content showed an increasing trend with increasing growth (number cells/mL) of the strain PCC7806 in agreement with the results observed by Downing *et al.* (2005). However, this trend was not clear in *M. aeruginosa* PCC7820 in our culture conditions. The maximum quantification of cell numbers for this strain did not correspond to the highest chlorophyll *a* content (Figure 1(c)).

During the study period (21 days) the maximum chlorophyll *a* level in PCC7806 culture was reached after 21 days (4 μg/mL), together with the highest cell number. In PCC7820, the highest level was reached at day 9 (2 μg/ mL), whereas the maximum cell number was reached after 12 days. In our culture conditions, both strains reached lower levels of chlorophyll *a* at 15 days (2.7 μg/mL PCC7806, 1.37 μg/mL PCC7820) than those reported by Sevilla *et al.* (2010) with PCC7806 (~5.3 μg/mL). However,

our results agree with the levels obtained by Wu *et al.* (2009) who observed that the chlorophyll *a* concentration of PCC7806 increased with time ($\approx$4 μg/mL at 20 days).

Specific growth rates under the given conditions ranged from 0.21 to 0.033 d^{-1} and 0.25 to 0.004 d^{-1} for PCC7806 and PCC7820 strains, respectively. The growth rates of both strains decreased during the cultivated period and were similar to those calculated by Wu *et al.* (2009) for *Microcystis* PCC7806 after 20 days of culture.

Comparing the three parameters measured, cell numbers, chlorophyll *a* and OD, we found that chlorophyll *a* correlated well with OD and with cell number in the PCC7806 strain. However, for the PCC7820 strain, chlorophyll *a* correlated better with OD rather than with cell number. Thus, under our conditions, the results for these three parameters are not always consistent with each other.

Identification and quantification of MC produced by *M. aeruginosa* PCC7806 versus PCC7820

LC-MS was used to separate and identify MC in the cellular extracts of both strains. Since MC are mainly accumulated within the cell and extracellular toxins only contributed marginally to the total MC concentration (Jänichen *et al.* 2007; Jüttner & Lüthi 2008; Shao *et al.* 2009), we determined intracellular MC content.

The chromatogram of the *Microcystis* PCC7806 extract shows three MC (Figure 2(a)), one of them being identified for the first time in this strain. The assignation of the ions obtained from the fragmentation of MC is presented in Table 1. The majority was MC-LR at *m/z* 995.6, representing 81% of total MC (Figure 2(a)). This toxin was identified using its respective commercial reference. The compound detected at 981.5 Da corresponds to a demethylated MC-LR (D-Asp^3-MCLR). The presence of both MC in extracts from *Microcystis* PCC7806 has already been described in previous studies; however, their objective was to study the impact of some parameters in the MC production (Downing *et al.* 2005; Jähnichen *et al.* 2007; Sevilla *et al.* 2010), but no MC production kinetic during growth was described. The third compound was detected at *m/z* 1013.6, which has been detected for the first time in this strain, and could correspond to a MC-LR variant with methyl serine (MeSer) at position 7 ([L-MeSer^7] MC-LR), which has been identified

previously by del Campo & Ouahid (2010) in *M. aeruginosa* **Q3** UAM 1303 strain. It should be noted that serine is the biosynthetic precursor of dehydroalanine, so this toxin together with the desmethylated MC-LR could be biogenetic precursors of LR (Namikoshi *et al.* 1992).

Moreover, the complete identification of six different MC in *Microcystis* PCC7820 has been performed (Figure 2 (b)). This analysis confirmed the presence of MC-LR, D-Asp^3-MCLR, Dglu(OCH$_3$)-MCLR, MC-LY, MC-LW and MC-LF. Table 1 shows the monoisotopic *m/z* ratios of [M + H]$^+$ ions for all these MC. The MC profile of *Microcystis* PC7820 observed in this study was similar but not identical to that described by Lawton *et al.* (1994). In this sense, MC-LM detected previously by these authors was not identified during the present work and D-Asp^3-MCLR observed in the present study was not detected by these authors. The relative abundance of MC-LR (79%), the major variant detected, agrees with the percentage previously found by Lawton *et al.* (1994). Moreover, Bateman *et al.* (1995) identified seven MC variants, MC-LR being predominant; MC-VF and MC-AR were also detected, but D-Asp^3-MCLR was not identified. Robillot *et al.* (2000) have reported the presence of three new variants, desmethylated MC-LW, desmethylated MC-LF and MC-LL, which we did not observed. According to these authors, MC-LR, MC-LW and MC-LF were the major toxins, whereas in the present study the main toxins were MC-LR, D-Asp^3-MCLR and Dglu(OCH$_3$)-MCLR. The discrepancy between the MC profiles could be due to differences in the methods used for MC extraction, purification, LC-MS determination, as well as in culture conditions (del Campo & Ouahid 2010).

The total amount of MC was estimated by measuring the area of the highest UV absorption peaks at 238 nm. However, only the commercial standard of MC-LR was available implying that, theoretically, only this toxin could be quantified. Consequently, according to Robillot *et al.* (2000), we approximated that UV absorbance coefficient at 238 nm is identical for all MC, as it has been verified for the three available standards (MC-LR and MC-RR/MC-YR not detected in this study). Thus, quantifications were made using MC-LR calibration curves, admitting a systematic error in the case of tryptophan and tyrosine containing MC.

The quantification of the intracellular MC identified in both strains at the end of the period of culture is shown in
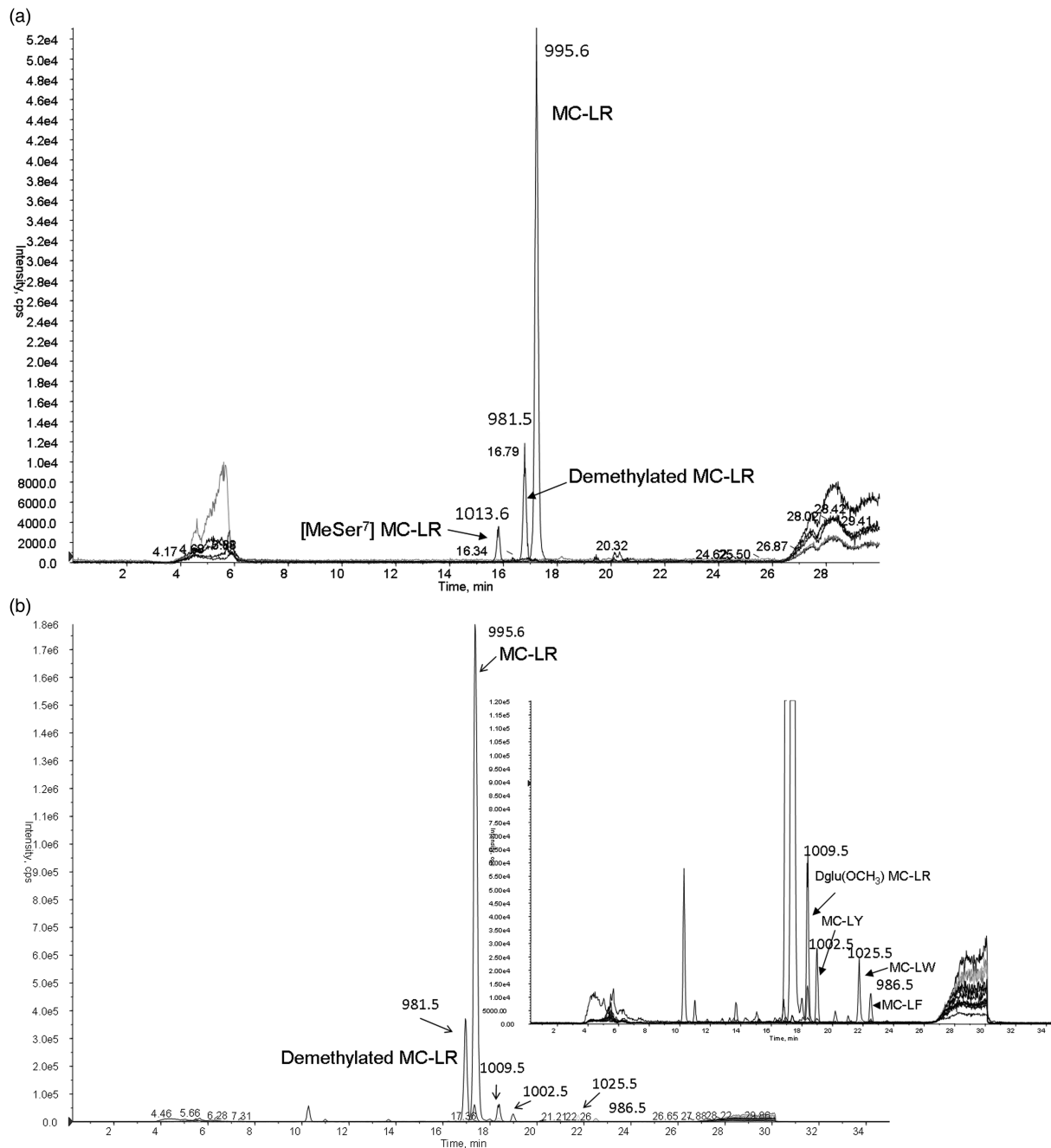
Q5 **Figure 2** │ LC-MS chromatogram of *M. aeruginosa*. (a) PCC7806 cellular extracts. Electrospray mass spectra were associated with MC-LR, demethylated MC-LR and [MeSer7] MC-LR. (b) PCC7820 cellular extracts. Electrospray mass spectra were associated with MC-LR, demethylated MC-LR, Dglu(OCH3) MC-LR, MC-LY, MC-LW and MC-LF.

Table 2. The total MC content detected in *M. aeruginosa* PCC7820 was higher than in PCC7806 extracts (~3-fold). When MC-LR levels in both extracts are compared this proportion, $498,000 \pm 54,780$ ng MC-LR/g and $166,000 \pm 21,580$ ng MC-LR/g, respectively is confirmed. However, this

proportion increased significantly (~9-fold) in D-Asp^3-MC-LR levels ($92,000 \pm 10,120$ ng/g versus $10,600 \pm 1,378$ ng/g), the other common MC in both strains. The differences in the MC production between strains of the same cyanobacteria species have been previously observed by other

Table 1 │ Monoisotopic *m/z* ratios of $[M + H]^+$ ions for different MC

MC ascription	Molecular mass	*m/z* values
M. aeruginosa PCC7806		
MC-LR	994	995.6
D-Asp³-MC-LR	980.5	981.5
[MeSer⁷]MC-LR	1,012	1,013.6
M. aeruginosa PCC7820		
MC-LR	994	995.6
D-Asp³-MC-LR	980.5	981.5
Dglu(OCH₃)-MC-LR	1,008.5	1,009.5
MC-LY	1,001.5	1,002.5
MC-LW	1,024.5	1,025.5
MC-LF	985.5	986.5

Table 2 │ Quantification by LC-MS of intracellular MC identified in PCC7806 and PCC7820 at the end of the study (21 days)

MC	Intracellular concentration (ng/g)	
	PCC7806	PCC7820
MC-LR	166,000 (21580)[a]	498,000 (54,780)[a]
MC-LF		3,600 (396)[a]
MC-LW		6,580 (724)[a]
MC-LY		10,000 (1,100)[a]
[MeSer⁷]MC-LR	33,600 (43,68)[a]	
D-Asp³-MC-LR	10,600 (1,378)[a]	92,000 (10,120)[a]
Dglu(OCH₃)LR		16,460 (1,811)[a]
Total MC	**210,200**	**626,640**

[a]Standard deviation (*n* = 3).

authors in cultures of different strains from *M. aeruginosa* (Ohtake *et al.* 1989).

Toxin production kinetics

Intracellular MC concentration was studied by LC-MS, ELISA anti-Adda technique and the PP2A inhibition assay during the study period (21 days). For the strain samples of PCC7806, analysis of MC by LC-MS and PP2A inhibition assay gave lower values than ELISA anti-Adda (Figure 3(a)). The maximum levels of MC were reached at day 15 (400.6 ± 52.1 μg/L by PP2A inhibition assay; 356.4 ± 46.3 μg/L by LC-MS) and 21 (417.3 ± 54.2 μg/L by PP2A inhibition
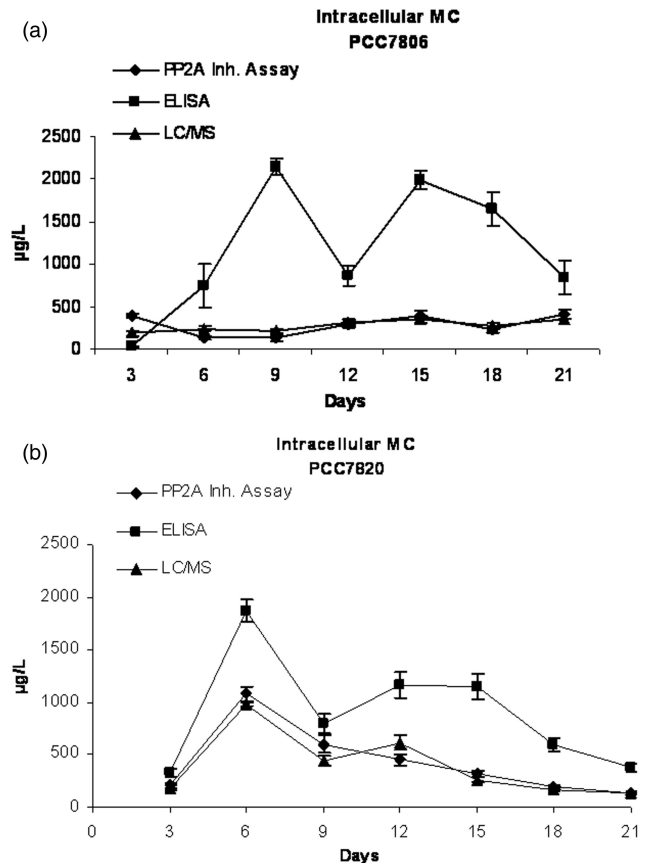
Figure 3 │ Levels of MC detected by LC-MS, ELISA anti-Adda and PP2A inhibition assay in samples from (a) PCC7806 strain and (b) PCC7820 strain. Values are given as means ± standard deviation (*n* = 3). **Q6**

assay; 347.5 ± 36.2 μg/L by LC-MS). The levels of MC calculated by ELISA anti-Adda were higher than MC detected by the other two techniques, with maximum concentrations observed at day 9 (2151.6 ± 97.4 μg/L) and at day 15 (1997.7 ± 110.9 μg/L), and showing significant decreases at days 12 (866 ± 112.65 μg/L) and 21 (853.0 ± 200.0 μg/L).

In addition, the maximum concentrations obtained from samples of the PCC7820 strain were similar with the PP2A inhibition assay (1086.0 ± 66.4 μg/L) and LC-MS (981.0 ± 17.9 μg/L) techniques, and an overestimated MC concentration was detected with ELISA anti-Adda assay (1879.0 ± 106.2 μg/L) (Figure 3(b)). The intracellular MC concentrations during the study period showed the same trend with the three techniques employed.

The mean levels of intracellular MC detected by ELISA were higher than those obtained by LC-MS; these differences were more marked in PCC7806 samples (4-fold)

than in the case of PCC7820 (2-fold). An overestimation in the amounts of MC detected by ELISA in comparison with LC-UV was previously reported in our laboratory in natural blooms from the Guadiana River (Moreno *et al.* 2005). Similarly, the comparison of LC-MS, ELISA and phosphatase assay for the determination of MC in cyanobacteria products revealed that the LC-MS/MS results were significantly lower than biochemical assays (Lawrence *et al.* 2001). Rapala *et al.* (2002) compared the suitability of ELISA and LC-UV to detect different toxin variants using several matrices (pure toxins, laboratory cultures, water and bloom samples of toxic cyanobacteria). According to these authors, despite the possible overestimation of toxin concentrations with LC in standard toxins, the analyses of the field samples gave lower values than ELISA assay. All these results are in agreement with those obtained by Tillmanns *et al.* (2007) who reported that results measured by ELISA were higher than those obtained by LC-DAD (4-fold). They suggested that this difference may depend on the number of MC variants being produced *in situ*. The overestimation in MC concentrations observed with the ELISA assay cannot be only due to the matrix effects, because when using extracts the organic compounds are concentrated and may interfere with the results. On the contrary, other authors, such as Gurbuz *et al.* (2009), have found a positive correlation between ELISA and LC-DAD results for MC-RR and MC-LR, although the ELISA results for MC-LF and LW were almost 6-fold lower than the LC results. In this sense, Mikhailov *et al.* (2000) also detected lower values of MC by ELISA than those determined by LC-DAD. The correlation between the ELISA and LC-DAD analyses of the MC in sediment samples was studied by Babica *et al.* (2006). Although the results from both methods were in agreement for higher MC concentrations, only weak correlation was observed below 0.1 µg/g, which may reflect the generally higher variability in the ELISA results. In conclusion, we can say that the ELISA assay is not a suitable method for quantification of MC due to the larger variation observed when it is compared with another technique such as LC-DAD or LC-MS. Indeed, it would be necessary to validate the ELISA assay in order to set up a reliable cut-off for detecting MC in culture extracts to discriminate between false positives, false negatives, true positives and true negatives (Moreno *et al.* 2011).

Intracellular MC production measured by PP2A inhibition assay did not show variations as consistent as the above relating between ELISA and LC. The maximum concentration calculated by the PP2A inhibition assay in both strains is slightly higher than that measured by LC (417.3 ± 54.2 µg/L versus 347.5 ± 36.2 µg/L in PCC7806 and 1086.0 ± 66.4 µg/L versus 981.0 ± 17.9 µg/L in PCC7820). To understand these slight differences, a correlation diagram was established, displaying the MC content from the PP2A inhibition assay as a function of the MC concentration from the LC-MS analysis for both strains (Figure 4). The diagrams have a linear trend and the slopes correspond to the ratio α calculated following this formula (Robillot *et al.* 2000):

$$\alpha = \frac{[\text{equivalent MC-LR}] \text{ by PP2A inh. assay}}{[\text{total MC}] \text{ by LC}}$$

Its value would theoretically be equal to 1 if the sample contained only hepatotoxins that inhibit PP2A as much as MC-LR (Robillot *et al.* 2000). In our study, the calculated values are 1.06 ± 0.50 and 1.10 ± 0.19 for PCC7806 and PCC7820, respectively. Consequently, such linear trends suggest that α is not time-dependent, indicating that MC composition might be stable over the growth period in both strains. LC-MS analysis showed that the proportion
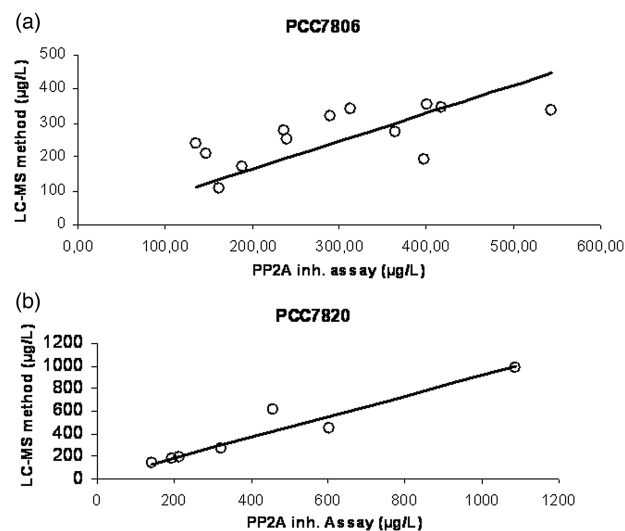

Figure 4 | Correlation between intracellular MC concentrations measured by LC-MS and by the PP2A inhibition assay in samples from (a) PCC7806 and (b) PCC7820 strains ($n = 3$).   **Q7**

of MC-LR in PCC7806 varied between 70.5% and 88.2% during growth, and in PCC7820 the range was 79.4–80.1%.

Our results have demonstrated that the choice of analytical procedures to measure MC content can cause discrepancies in the quantification of these cyanotoxins when comparing ELISA and LC-MS results. However, a good correlation has been obtained between PP2A inhibition assay and LC-MS. All these methods are based on different principles. The ELISA test measures the total toxin concentration of the sample, while the PP2A inhibition assay assesses its total potential toxicity. In the case of LC-MS, individual MC can be separated and recognized on the basis of their retention times and mass spectra (Rapala *et al.* 2002).

Comparison of intracellular toxin production between both strains during culture

Intracellular MC concentrations were not constant during the culture period in both strains (Figures 5(a) and 5(b)). This fact was mainly observed in the *M. aeruginosa*

(a)

(b)

Q8 **Figure 5** │ Levels of MC per cell (fentogram/cell) in samples from (a) PCC7806 and (b) PCC7820 strains during the studied period. Values are given as means ± standard deviation ($n = 3$).

PCC7820 strain (Figure 5(b)). The cell quota (fentogram/cell) varied within this strain during culture growth. The highest concentration of toxins by cell was reached at the beginning of the exponential growth phase (6 days) with a value of 87 ± 6.5 fgMC/cell measured by LC-MS and 97 ± 6.7 fgMC/cell with PP2A inhibition assay. During the stationary phase the MC levels by cell decreased considerably down to levels of approximately 17 fgMC/cell detected by both techniques. In the decline growth curve phase (12–21 days), an increased in the MC concentration by cells was observed, reaching 70 ± 6.3 to 78 ± 7.6 fgMC/cell after 18 days of culture.

Concerning the results obtained for PCC7806, slight variations in the MC levels by cell overall by PP2A inhibition assay have been detected (Figure 5(a)). The maximum concentration of fgMC/cell detected by LC-MS was ~10 fgMC/cell during the exponential growth phase, and the minimum level was 5.3 ± 0.9 fgMC/cell at day 9, just at the end of the exponential phase and at the beginning of the stationary phase. The MC cell quota in *M. aeruginosa* PCC7806 is lower than in PCC7820 (~9-fold). In both strains, the maximum MC production took place at the beginning of the exponential growth curve in our conditions. Robillot *et al.* (2000) studied the MC production kinetics of *M. aeruginosa* PCC7820, observing that the maximum intracellular MC concentration was reached at day 7. In this study, the stationary phase was reached after 12 days of culture.

Considering these results, it can be confirmed that the toxicity of toxic *Microcystis* cells is variable and dependent on the strains used and the culture conditions. It implies that the comparison of toxin production between different species or strains should consider their production kinetics.
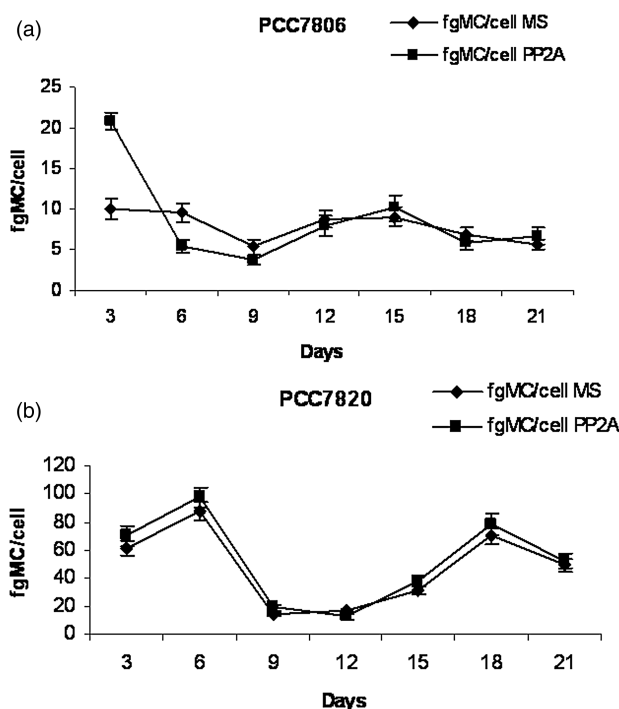
CONCLUSION

The comparative study of MC production during the culture period of two strains of *M. aeruginosa* (PCC7806 and PCC7820) has shown that the majority of intracellular MC production was observed during the exponential growth phase. In the present study, for the first time a new MC has been identified ([MeSer[7]]) in the *M. aeruginosa* PCC7806 strain. The MC production and the MC cell

quota during the culture period in PCC7806 were lower than in PCC7820 (3-fold and ∼9-fold, respectively). In both cases, MC-LR was the predominant toxin detected. In both strains, the maximum MC production was observed at the beginning of the exponential growth curve under our conditions. In this study, a good correlation has been demonstrated in MC levels determined by PP2A inhibition assay and LC-MS in both strains that were considered during the culture period.

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REFERENCES

Babica, P., Kohoutek, J., Blaha, L., Adamovsky, O. & Marsalek, B. 2006 Evaluation of extraction approaches linked to ELISA and HPLC for analyses of microcystin-LR, -RR and -YR in freshwater sediments with different organic material contents. *Anal. Bioanal. Chem.* **385**, 1545–1551.

Bateman, K. P., Thibault, P., Douglas, D. J. & White, R. L. 1995 Mass spectral analyses of Microcystins from toxic cyanobacteria using on-line chromatographic and electrophoretic separations. *J. Chrom. A* **712**, 253–268.

Cameán, A., Moreno, I. M., Ruiz, M. J. & Picó, Y. 2004 Determination of microcystins in natural blooms and cyanobacterial strain cultures by matrix solid-phase dispersion and liquid chromatography-mass spectrometry. *Anal. Bioanal. Chem.* **380**, 537–544.

Dahlmann, J., Budakowski, W. R. & Luckas, B. 2003 Liquid chromatography–electrospray ionisation-mass spectrometry based method for the simultaneous determination of algal and cyanobacterial toxins in phytoplankton from marine waters and lakes followed by tentative structural elucidation of microcystins. *J. Chromat. A* **994**, 45–57.

del Campo, F. F. & Ouahid, Y. 2010 Identification of microcystins from three collection strains of *Microcystis aeruginosa*. *Environ. Pollut.* **158**, 2906–2914.

Downing, T. G., Sember, C. S., Gehringer, M. M. & Leukes, W. 2005 Medium N:P ratios and specific growth rate comodulate microcystin and protein content in *Microcystis aeruginosa* PCC7806 and M. aeruginosa UV027. *Microbiol. Ecol.* **49**, 468–473.

Ernst, B., Dietz, L., Hoege, S. J. & Dietrich, D. R. 2005 Recovery of MC-LR in fish liver. *Environ. Toxicol.* **20**, 449–458.

Fischer, W. J., Garthwaite, I., Miles, C., Ross, K. M., Aggen, L. B., Chamberlin, A. R., Towers, N. R. & Dietrich, D. R. 2001 Congener-independent immunoassay for microcystins and nodularins. *Environ. Sci. Technol.* **35**, 4849–4856.

Gurbuz, F., Metcalf, J. S., Karahan, A. G. & Codd, G. A. 2009 Analysis of dissolved microcystins in surface water samples from Kovada Lake, Turkey. *Sci. Total Environ.* **407**, 4038–4046.

IARC (International Agency for Research on Cancer) World Cancer Report 2014. Available at: http://www.iarc.fr/.

Jähnichen, S., Ihle, T., Petzoldt, T. & Benndorf, J. 2007 Impact of inorganic carbon availability on microcystin production by *Microcystis aeruginosa* PCC 7806. *Appl. Environ. Microbiol.* **73**, 6994–7002.

Jüttner, F. & Lüthi, H. 2008 Topology and enhanced toxicity of bound Microcystins in Microcystis PCC 7806. *Toxicon* **51**, 388–397.

Lawrence, J. F., Niedzwiadek, B., Menard, C., Lau, B. P., Lewis, D., Kuper-Goodman, T., Carbone, S. & Holmes, C. 2001 Comparison of liquid chromatography/mass spectrometry, ELISA and phosphatase assay for the determination of microcystins in blue green algae products. *J. AOAC Int.* **84**, 1035–1044.

Lawton, L. A., Edwards, C. & Codd, G. A. 1994 Extraction and high-performance liquid chromatographic method for the determination of Microcystins in raw and treated waters. *Analyst* **119**, 1525–1530.

MacKinney, G. 1941 Absorption of light by chlorophyll solutions. *J. Biol. Chem.* **140**, 315–322.

Metcalf, J. S., Beattie, K. A., Pflugmacher, S. & Codd, G. A. 2000 Immuno-cross reactivity and toxicity assessment of conjugation products of the cyanobacterial toxin, microcystin-LR. *FEMS Microbiol. Lett.* **189**, 155–158.

Mikhailov, A., Harmala-Brasken, A.-S., Meriluoto, J., Sorokina, Y., Dietrich, D. & Eriksson, J. E. 2000 Production and specificity of mono and polyclonal antibodies against microcystins conjugated through N-methyldehydroalanine. *Toxicon* **39**, 477–483.

Moreno, I., Cameán, A., Tavares, M. J., Pereira, P. & Franca, S. 2003 Toxicity of cyanobacteria isolated from the Guadiana River. *Aquat. Ecosys. Health Manage.* **6**, 1–5.

Moreno, I., Herrador, M. A., Atencio, L., Puerto, M., González, A. G. & Cameán, A. M. 2011 Differentiation between microcystin contaminated and uncontaminated fish by determination of unconjugated MCs using an ELISA anti-Adda test based on receiver-operating characteristic curves threshold values: application to Tinca tinca from natural ponds. *Environ. Toxicol.* **26**, 45–56.

Moreno, I., Pereira, P., Franca, S. & Cameán, A. 2004 Toxic cyanobacteria strains isolated from blooms in the Guadiana River (Southwest of Spain). *Biol. Res.* **37**, 405–417.

Moreno, I., Repetto, G., Carballal, E., Gago, A. & Cameán, A. M. 2005 Cyanobacteria and microcystins occurrence in the Guadiana river (SW Spain). *Int. J. Environ. Anal. Chem.* **85**, 461–474.

Namikoshi, M., Rinehart, K. L. & Sakai, R. 1992 Identification of 12 hepatotoxins from a Homer Lake bloom of the cyanobacteria *Microcystis aeruginosa, Microcystis viridis, and Microcystis wesenbergii*: nine new microcystins. *J. Org. Chem.* **57**, 866–872.

Ohtake, A., Shirai, M., Aida, T., Mori, N., Harada, K., Matsuura, K., Suzuki, M. & Nakano, M. 1989 Toxicity of *Microcystis* species isolated from natural blooms and purification of the toxin. *Appl. Environ. Microbiol.* **55**, 3202–3207.

Preußel, K., Wessel, G., Fastner, J. & Chorus, I. 2009 Response of cylindrospermopsin production and release in *Aphanizomenon flos-aquae* (Cyanobacteria) varying light and temperate conditions. *Harmful Algae* **8**, 645–650.

Rapala, J., Erkomaa, K., Kukkonen, J., Sivonen, K. & Lahti, K. 2002 Detection of Microcystins with protein phosphatase inhibition assay, high-performance liquid chromatography-UV detection and enzyme-linked immunosorbent assay: comparison of methods. *Anal. Chim. Acta* **466**, 213–231.

Robillot, C., Vinh, J., Puiseux-Dao, S. & Hennion, M. C. 2000 Hepatotoxin production kinetics of the cyanobacterium *Microcystis aeruginosa* PCC7820, as determined by HPLC-Mass Spectrometry and Protein Phosphatase bioassay. *Environ. Sci. Technol.* **34**, 3372–3378.

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

Shao, J. H., Wu, X. Q. & Li, R. H. 2009 Physiological responses of *Microcystis aeruginosa* PCC7806 to nonanoic acid stress. *Environ. Toxicol.* **24**, 610–617.

Throndsen, J. 1995 Estimating cell numbers. In: *Manual on Harmful Marine Microalgae* (G. M. Hallegraeff, D. M. Anderson & A. D. Cembela, eds). IOC UNESCO, Paris, pp. 63–80.

Tillmanns, A. R., Pick, F. R. & Aranda-Rodriguez, R. 2007 Sampling and analysis of microcystins: implications for the development of standarized methods. *Environ. Toxicol.* **22**, 132–143.

Tonk, L., Welker, M., Huisman, J. & Visser, P. M. 2009 Production of cyanopeptolins, anabaenopeptins, and microcystins by the harmful cyanobacteria Anabaena 90 and Microcystis PCC 7806. *Harmful Algae* **8**, 219–224.

Ueno, Y., Nagata, S., Tsutsumi, T., Hasegawa, A., Yoshida, F., Suttajit, M., Mebs, D., Pütsch, M. & Vasconcelos, V. 1996 Survey of microcystins in environmental water by a highly sensitive immunoassay based on monoclonal antibody. *Nat. Toxins* **4**, 271–276.

Vasas, G., Bacsi, I., Suranyi, G., Hamvas, M. M., Mathe, C., Nagy, S. A. & Borbely, G. 2010 Isolation of viable cell mass from frozen *Microcystis viridis* bloom containing microcystin-RR. *Phytoplankton* **639**, 147–151.

Wang, C., Kong, H. N., Wang, X. Z., Wu, H. D., Lin, Y. & He, S. B. 2010 Effects of iron on growth and intracellular chemical contents of *Microcystis aeruginosa*. *Biomed. Environ. Sci.* **23**, 48–52.

WHO 1998 *Guidelines for Drinking Water Quality*, 2nd edn., (addendum to Vol. 2). World Health Organization, Geneva, Switzerland.

Wiedner, C., Visser, P. M., Fastner, J., Metcalf, J. S., Codd, G. A. & Mur, L. R. 2003 Effects of light on the microcystin content of *Mirocystis* strain PCC 7806. *Appl. Environ. Microbiol.* **69**, 1475–1481.

Wu, X. & Kong, F. 2009 Effects of light and wind speed on the vertical distribution of *Microcystis aeruginosa* colonies of different sizes during a summer bloom. *Int. Rev. Hydrobiol.* **3**, 258–266.

Wu, Z., Shi, J. & Li, R. 2009 Comparative studies on photosynthesis and phosphate metabolism of *Cylindrospermopsis raciborskii* with *Microcystis aeruginosa* and *Aphanizomenon flos-aquae*. *Harmful Algae* **8**, 910–915.

Zeck, A., Weller, M. G., Bursill, D. & Niessner, R. 2001 Generic microcystin immunoassay based on monoclonal antibodies against Adda. *Analyst* **126**, 2002–2007.

Zegura, B., Straser, A. & Filipic, M. 2011 Genotoxicity and potential carcinogenicity of cyanobacterial toxins – a review. *Mutat. Res.* **727**, 16–41.

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