Microcystins production in *Microcystis* induced by *Daphnia pulex* (Cladocera) and *Brachionus calyciflorus* (Rotifera)

Producción de microcistinas en *Microcystis* inducida por *Daphnia pulex* (Cladocera) y *Brachionus calyciflorus* (Rotifera)

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ABSTRACT

Freshwater cyanobacteria often are the predominant division of phytoplankton in eutrophic environments. *Microcystis* is a bloom-forming cyanobacterium, commonly found in urban lakes of central Mexico. Several biotic factors including the presence of zooplankton induce the production of toxins (microcystins, MCs) in *Microcystis*. Here, it present data on the effect of the presence of the cladoceran *Daphnia pulex* (Leydig, 1860) and the rotifer *Brachionus calyciflorus* (Pallas, 1776) on total MCs production in *Microcystis* spp. Results indicate that *Microcystis* spp. with or without the presence of zooplankton (controls), contained certain level of MCs (1.32 to 3.98 x 10^{-4} ng/cell). At low cell density of *Microcystis* spp. (0.5 x 10^{6} cells/mL) and, in the presence of high zooplankton abundance (25 cladocerans or 250 rotifers, in 50 mL), the MCs concentration was significantly higher as compared to controls. Moreover, under low zooplankton density and at low density of *Microcystis* spp., the MCs levels did not vary significantly. Compared to rotifers, the presence of cladocerans resulted in higher MCs levels. This work demonstrates that zooplankton presence may induce microcystins production in *Microcystis* spp.

Key words: Cyanobacteria, Mexico, microcystins detection, microcystins production, zooplankton.

RESUMEN

Las cianobacterias de agua dulce regularmente son la división predominante del fitoplancton en ambientes eutrofizados. *Microcystis* es una cianobacteria formadora de florecimientos, comúnmente encontrada en lagos urbanos del centro de México. Diversos factores bióticos incluyendo la presencia de zooplancton inducen la producción de toxinas (microcistinas, MCs) en *Microcystis*. En este trabajo se presentan datos sobre el efecto de la presencia del cladócero *Daphnia pulex* (Leydig, 1860) y del rotífero *Brachionus calyciflorus* (Pallas, 1776) en la producción total de MCs en *Microcystis* spp. Los resultados indicaron que *Microcystis* spp. con o sin presencia de zooplancton (controles), contiene ciertos niveles de MCs (1.32 a 3.98 x 10^{-4} ng/cél). A baja densidad celular de *Microcystis* spp. (0.5 x 10^{6} cél/mL) y con la presencia de altas abundancias de zooplancton (25 cladóceros o 250 rotíferos, en 50 mL), la concentración de MCs fue significativamente mayor al compararla con los controles. Además, con una baja densidad de zooplancton y una baja densidad de *Microcystis* spp., el nivel de MCs no varió significativamente. En comparación con rotíferos, la presencia de cladóceros resultó en niveles altos de MCs. Este trabajo demostró que la presencia de zooplancton puede inducir la producción de microcistinas en *Microcystis* spp.

Palabras clave: Cianobacterias, detección de microcistinas, México, producción de microcistinas, zooplancton.

INTRODUCTION

Freshwater cyanobacteria form blooms and scums containing millions of cells per liter and often are the predominant division of phytoplankton in eutrophic environments. It is a world-wide problem because may cause anoxic conditions and frequently high mortalities among aquatic animal populations, furthermore these blooms affect directly the abundance and distribution of phytoplankton and zooplankton in freshwater environments; some of these cyanobacteria form colonies and synthesize numerous secondary metabolites with toxic properties (WHO, 1999; Pietsch *et al*., 2001; Hudnell, 2008).

In Mexico, *Microcystis* is one of the most common cyanobacterium that inhabit tropical and temperate waterbodies, also is the most abundant bloom-forming throughout the year (Ramírez-García *et al*., 2002; Arzate-Cardenas *et al*., 2010; Vasconcelos *et al*., 2010). The principal secondary metabolites produced by *Microcystis* are a family of more than 80 cyclic peptide toxins, collectively known as microcystins (MCs). In freshwater environments, MCs are the most frequently reported and monitored toxins; these are characterized based on the arrangement of seven amino acids in a ring structure containing five fixed D-amino acids and two variable L-amino acids which differ mainly at positions 2 and 4 (fig. 1), the most common MCs isoforms are MC-RR, MC-YR, MC-LR and MC-LA (Harada *et al*., 1996; WHO, 1999; Metcalf *et al*., 2009).

Biotic and abiotic factors control the production of MCs, further some authors have reported differences in the production of MCs at strain-level for the same species of *Microcystis* (Hisbergues *et al*., 2003; Metcalf *et al*., 2009); among biotic factors, competition from other phytoplankton species is one of the possible factors responsible for the induction of MCs in *Microcystis*, but the most documented is the impact of zooplankton grazing on *Microcystis* (Jang *et al*., 2003; 2007; 2008; Reynolds, 2006; Hudnell, 2008).

Several species of zooplankton, including copepods, cladocerans, rotifers, and protozoans are reported from waterbodies containing bloom-forming *Microcystis* (Ramírez-García *et al*., 2002). Available literature also suggests that copepods are selective grazers and hence probably avoid *Microcystis*, large cladocerans are usually absent in *Microcystis*-blooms and, rotifers and protozoans are generally too small to consume colonial *Microcystis* (Kâ *et al*., 2012). Therefore, it is not clear if the eventual extinction of large sized (>1000 µm) cladocerans in eutrophic waterbodies is the result of toxin-induction in cyanobacteria through zooplankton grazing or feeding difficulties associated with mechanical manipulation of large colonies. Feeding studies tend to suggest, cladocerans including *Daphnia pulex* and rotifers including *Brachionus calyciflorus* are capable of feed on sonicated cyanobacterial cells (Pérez-Morales *et al*., 2014). Laboratory studies also show that MCs from ingested cyanobacterial cells cause reduction in somatic and population growth rates of both cladocerans and rotifers (Nandini & Rao, 1998). It is not known if the production as well as the quantity of MCs in natural populations of *Microcystis* is mainly due to the presence of cladocerans or rotifers.

Therefore, the aim of this work was to determine the effect of the presence of the cladoceran *Daphnia pulex* and the rotifer *Brachionus calyciflorus* on the production of MCs in the sonicated cells of *Microcystis* spp.

MATERIALS AND METHODS

Zooplankton species *Daphnia pulex* (1500 µm) was originally isolated from Espejo de Los Lirios Lake, while *Brachionus calyciflorus* (175 µm) was from Xochimilco Lake (both Lakes in Mexico City). Clonal cultures were separately established for each zooplankton species starting from a single parthenogenetic female. The zooplankton species were cultu-

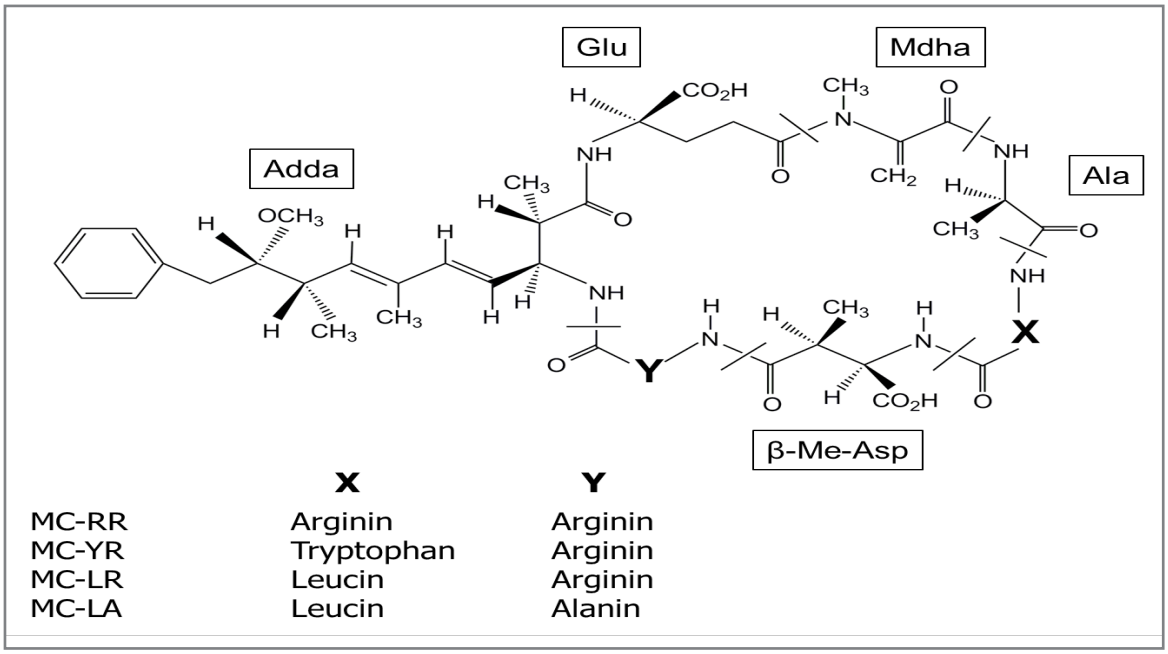

Figure 1. General structure of microcystins (MCs). X and Y are variable L-amino acids (Harada *et al*., 1996).

red in EPA medium (Weber, 1993) and fed using the laboratory-grown green alga *Scenedesmus acutus* (Meyen, 1829) for several months before experimentation. EPA medium is moderately hard water; this was prepared by dissolving $NaHCO_3$, $CaSO_4$, $MgSO_4$ and KCl (96, 60, 60 and 4 mg, respectively) in one liter of distilled water. *Scenedesmus acutus* was batch-cultured using Bold's basal medium supplemented with $NaHCO_3$ (0.25 g/L every third day) (Borowitzka & Borowitzka, 1988).

Colonies of *Microcystis* spp. were freshly collected from Chapultepec Lake (Mexico City) during summer season. Once in the laboratory, selected colonies were sonicated using an ultrasonicator (ColePalmer Instruments Co., USA) at 20 kHz 50 Watts for 1 min to release unbroken *Microcystis* cells from mucilage and quantify. Then unicellular *Microcystis* were maintained at three cell densities (0.5, 1.5 and 4.5 x 10^6 cells/mL, adding EPA medium to achieve the desired densities) in the presence of *D. pulex* (25, 5 and 0 (control), individuals) or *B. calyciflorus* (250, 50 and 0 (control), individuals). Experiments were conducted in 50 mL in transparent jars. Treatments were replicated 3 times and incubated at 25 ± 2 °C. The experiments were finished at day 4. Thus, the experimental design was as follow, for each zooplankton species 27 test jars were used (3 *Microcystis* cell densities x 3 zooplankton densities (including control) x 3 replicates). Loss of *Microcystis* cells due to possible feeding by the tested zooplankton was taken into account (Pérez-Morales *et al.*, 2014).

Once finished the experiments, zooplankton species were filtered and, *Microcystis* cells were frozen at -80 °C for two days and then thawed at room temperature for 1 day. The material was then sonicated in an ultrasonicator (ColePalmer Instruments Co., USA) at 20 kHz 50 Watts for 5 min. The freeze/thaw cycle was repeated 5 times (including sonication) to disrupt the cells and release toxins, following Pietsch *et al.* (2001). Worth noting that previous test on laboratory shown that 1 min of *Microcystis* colony sonication release *Microcystis* cells from mucilage with no lysis of individual cells (Pérez-Morales *et al*., 2014); in this study it was found that 5 min of sonication disrupt *Microcystis* cells, then MCs inside the cells may be released.

Samples were filtered to obtain cellular extracts under low vacuum, through nitrocellulose membrane filters (Ø 47 mm and nominal pore size of 0.45 µm; Whatman International®, Kent, UK) to remove broken cells. Cell extracts were stored in darkness and frozen until evaluation. Then quantification of MCs concentration in the cell extracts was performed with ELISA test, based on specifications of the QuantiPlate™ kit for MCs detection (Envirologix™). The test quantified total MCs in water samples from which we derived the MCs concentration per cell. The same procedure was followed for controls (sonicated *Microcystis* cells but without the presence of zooplankton).

Data on MCs production for each zooplankton species were statistically tested using two-way statistical analysis of variance (ANOVA), and Tukey post-hoc multiple comparisons test. The minimum level of statistic significance was set at $p<0.05$ (SigmaPlot ver. 11, Systat Software, San Jose, CA).

RESULTS

In controls, the MCs levels decreased (3.98 to 1.32 x 10^{-4} ng/cell) with the increase of cell density (table 1). At low cell density and in the presence of high zooplankton density, the MCs concentration was significantly higher as compared to controls ($p<0.001$, F-test, table 2). However, under low zooplankton density and at low cell density (0.5 x 10^6 cell/mL), the MCs levels did not vary significantly. At intermediate cell density (1.5 x 10^6 cell/mL) MCs content not showed significant differences among zooplankton densities compared to control, excepting the interaction with the higher density of *Daphnia*, with a MCs content of ~4.71 x 10^{-4} ng/cell. Moreover, interaction among low zooplankton density and intermediate cell density (1.5 x 10^6 cell/mL) induce higher MCs production than interaction among low zooplankton density and high cell density (4.5 x 10^6 cell/mL) for both rotifers and cladocerans; these results showed significant differences. Differences in *Microcystis* cell densities among treatments (including control) before and after of the experiments were not significant.

DISCUSSION

Microcystins produced by cyanobacteria are a global problem because they limit the use of potable water, recreational area, or agricultural irrigation; the maximum permissible value of MCs for drinking water

Table 1. Microcystins production (ng/cell. • 10^{-4}) in *Microcystis* spp. induced by zooplankton (*Daphnia pulex* and *Brachionus calyciflorus*).

Zooplankton		*Microcystis* spp. (cell density)		
Species	Ind./ 50 mL	0.5 x 10^6	1.5 x 10^6	4.5 x 10^6
Daphnia pulex	25	7.22 ± 0.9^{a}	4.71 ± 1.2^{b}	6.49 ± 1.3^{a}
	5	2.76 ± 0.5^{c,d}	4.01 ± 0.4^{b,c}	1.54 ± 0.5^{d}
	0	3.98 ± 0.9^{c,e}	2.73 ± 0.5^{c,d}	1.32 ± 0.2^{d,f}
Brachionus calyciflorus	250	6.66 ± 1^{a}	3.71 ± 0.7^{b}	1.80 ± 0.2^{c,d}
	50	2.86 ± 0.4^{d,e}	3.22 ± 0.2^{b,e}	1.92 ± 0.4^{c,d}
	0	3.98 ± 0.9^{e,f}	2.73 ± 0.5^{b,g}	1.32 ± 0.2^{d,h}

Data show the mean ± standard deviation based on 3 replicates. For each variable, data carrying similar alphabet are not statistically significant ($p>0.05$, Tukey test).

Table 2. Results of two-way analysis of variance performed on microcystins production (ng/cell), interaction among *Microcystis* spp. and zooplankton (*Daphnia pulex* and *Brachionus calyciflorus*).

Source of variation	DF	SS	MS	F	P
Microcystins production					
Daphnia pulex (A)	2	70×10^{-8}	35.0×10^{-8}	54.38	<0.001
Microcystis spp. (B)	2	10.7×10^{-8}	5.33×10^{-8}	8.28	0.003
Interaction A x B	4	19.1×10^{-8}	4.77×10^{-8}	7.42	0.001
Residual	18	11.6×10^{-8}	0.64×10^{-8}		
Error	26	111×10^{-8}	4.28×10^{-8}		
Microcystins production					
Brachionus calyciflorus (A)	2	11.5×10^{-8}	5.74×10^{-8}	16.58	<0.001
Microcystis spp. (B)	2	35.9×10^{-8}	18.0×10^{-8}	51.91	<0.001
Interaction A x B	4	13.5×10^{-8}	3.36×10^{-8}	9.72	<0.001
Residual	18	6.23×10^{-8}	0.35×10^{-8}		
Error	26	67.1×10^{-8}	2.58×10^{-8}		

DF= degrees of freedom; SS= sum of squares; MS= mean square; F= *F*-ratio.

sources is set to 1 ng/mL (WHO, 1999). In Mexican freshwater bodies used for drinking-water or irrigational purposes, the MCs levels in certain months occasionally reach values much higher than the maximum permissible limits (Vasconcelos *et al*., 2010; Pineda-Mendoza *et al*., 2012). For example, recently Alillo-Sánchez *et al*. (2014) have reported higher than 5 ng/mL MCs levels in Valle de Bravo, a drinking water reservoir in Central Mexico.

It is well known that abiotic factors such as light intensity, temperature and nutrients (nitrogen and phosphorus compounds, mainly) are among the main triggers to induce MCs production; otherwise, fewer studies are focused in biotic factor as triggers of MCs in *Microcystis*, mainly phytoplankton competition and zooplankton grazing (Izydorczyk *et al*., 2008; Deblois & Juneau, 2012).

Under natural conditions, *Microcystis* cells from Chapultepec Lake contain certain level of MCs (Arzate-Cárdenas *et al*., 2010; Vasconcelos *et al*., 2010), which was corroborated in control test (without the zooplankton presence). Here it was detected an inverse relation between MCs concentration and cell density, which could be generated as a response for resources competition, then may increase their cell density and grow faster may possibly increase at the expense of toxin production (Reynolds, 2006; Hudnell, 2008).

In this work, the results showed a clear effect of zooplankton species on *Microcystis*, because MCs production in *Microcystis* was higher in presence of zooplankton as compared with controls; specially, the presence of *D. pulex* induced significantly higher MCs levels in *Microcystis* than *B. calyciflorus*. In this regard, Jang *et al*. (2003; 2007) have examined the feeding activity of zooplankton (*Moina macrocopa* (Straus, 1820), *Daphnia magna* (Straus, 1820) or *D. pulex*) and quantified the direct (*Microcystis* cultured with zooplankton) and the indirect (*Microcystis* cultured using zooplankton-conditioned medium) effects on the production of MCs in four *Microcystis aeruginosa* ((Kützing) Kützing, 1846) strains. Moreover, they showed that the MCs production was 5 times higher when exposed to direct zooplankton grazing pressure and, indicated that all strains of *M. aeruginosa* tested increase toxin production when exposed to herbivorous zooplankton; also they found that large zooplankton (*D. magna*) caused more MCs production in *M aeruginosa* strains (included one strain previously reported as non-toxic) than small zooplankton (*M. macrocopa*) over the time exposure. Similar results were reported by Izydorczyk *et al.* (2008), they found sufficient evidence that shows not only the abiotic factors such as temperature and nutrients but also the presence of herbivorous zooplankton such as large cladocerans (*Daphnia cucullata* (G.O. Sars, 1862) and *D. longispina* (O.F. Müller, 1875)) and small cladocerans (*Bosmina* sp., *Alona* sp. and *Chydorus* sp.), may influence strongly in MCs content of *Microcystis*.

In addition to the direct grazing, mere presence of zooplankton is sufficient to induce defense mechanisms such as colony formation, reduction of growth rate and toxin production in cyanobacteria (Jang *et al*., 2003). Some authors have documented that the release of infochemicals from zooplankton is an important factor to induce MCs production in cyanobacteria; in turn, it has been documented that each stage of the life cycle of zooplankton may induce different levels of MCs (Izydorczyk *et al*., 2008; Jang *et al*., 2008). For example, some studies document that adult zooplankton induce higher levels of MCs in *Microcystis* rather than the neonates or juveniles. Regarding this, Jang *et al*. (2008) suggested that adult zooplankton possibly produced more infochemical signals than the equal numbers of juveniles and neonates. Thus, it appears that the MCs production in cyanobacteria may also be influenced by different developmental stages of zooplankton and/or physiological characteristics such as body size, and feeding habits of herbivores. In this study, was used only adult zooplankton and hence the differences in the MCs production were probably not related to the physiological factors or to the developmental stages of cladocerans and rotifers.

In summary, it was determined that the presence of cladocerans triggered higher MCs levels compared to rotifers in *Microcystis* spp. from Chapultepec Lake. Worth noting that several colonial and filamentous cyanobacteria inhabit Chapultepec Lake, and at least two *Microcystis* species (MCs producers confirmed) are present, of which *M. aeruginosa*

is strongly dominant. Besides, some filamentous cyanobacteria species has been associated to *Microcystis* colonies; however, these do not produce MCs (Arzate-Cárdenas *et al*., 2010; Pineda-Mendoza *et al*., 2012).

Lastly, this work confirms that zooplankton may induce MCs production in *Microcystis*. Whether other zooplankton species (copepods, other cladocerans or rotifers) or zooplankton densities may increase MCs production in *Microcystis* spp. remains to be tested. Further researches are needed to determine the specific role of zooplankton in the stimulation of cyanobacteria to produce toxins.

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REFERENCES

Alillo-Sánchez, .J. L., M. L. Gaytán-Herrera, V. M. Martínez-Almeida & P. Ramírez-García. 2014. Microcystin-LR equivalents and their correlation with *Anabaena* spp. (*Dolichospermum* spp.) in the main reservoir of a Hydraulic System of Central Mexico. *Inland Waters* 4: 327-336.

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

Borowitzka, M. A. & L. J. Borowitzka. 1988. *Micro-algal biotechnology*. Cambridge University Press, United Kingdom. 477 p.

Deblois, C. P. & P. Juneau. 2012. Comparison of resistance to light stress in toxic and non-toxic strains of *Microcystis aeruginosa* (Cyanophyta). *Journal of Phycology* 48: 1002-1011.

Harada, K. I., K. Fujii, K. Hayashi & M. Suzuki. 1996. Application of D, L-FDLA derivatization to determination of absolute configuration of constituent amino acids in peptide by advanved Marfey's method. *Tetrahedron Letter* 37(17): 3001-3004.

Hisbergues, M., G. Chriastiansen, L. Rouhiainen, K. Sivonen & T. Börner. 2003. PCR-based identification of microcystin-producing genotypes of different cyanobacterial genera. *Archives of Microbiology* 180: 402-410.

Hudnell, H. K. 2008. *Advances in experimental medicine and biology. Volume 619 – Cyanobacterial harmful algal blooms: state of the science and research needs*. Springer, New York, USA, 949 p.

Izydorczyk, K., T. Jurczak, A. Wojtal-Frankiewicz, A. Skowron, J. Mankiewicz-Boczek & M. Tarczynska. 2008. Influence of abiotic and biotic factors on microcystin content in *Microcystis aeruginosa* cells in a eutrophic temperate reservoir. *Journal of Plankton Research* 30(4): 393-400.

Jang, M. H., K. Ha, G. J. Joo & N. Takamura. 2003. Toxin production of cyanobacteria is increased by exposure to zooplankton. *Freshwater Biology* 48: 1540-1550.

Jang, M. H., J. M. Jung & N. Takamura. 2007. Changes in microcystin production in cyanobacteria exposed to zooplankton at different population densities and infochemical concentrations. *Limnology and Oceanography* 52 (4): 1454-1466.

Jang, M. H., K. Ha & N. Takamura. 2008. Microcystin production by *Microcystis aeruginosa* exposed to different stages of herbivorous zooplankton. *Toxicon* 51: 882-889.

Kâ, S., J. M. Mendoza-Vera, M. Bouvy, G. Champalbert, R. N'Gom-Kâ and M. Pagano. 2012. Can tropical freshwater zooplankton graze efficiently on cyanobacteria?. *Hydrobiologia* 679: 119-138.

Metcalf, J. S., M. Reilly, F. M. Young & G. A. Codd. 2009. Localization of microcystin synthetase genes in colonies of the cyanobacterium *Microcystis* using fluorescence in situ hybridization. *Journal of Phycology* 45 (6): 1400-1404.

Nandini, S. & T. R. Rao. 1998. Somatic and population growth in selected cladoceran and rotifer species offered the cyanobacterium *Microcystis aeruginosa* as food. *Aquatic Ecology* 31: 283-298.

Pérez-Morales, A., S. S. S. Sarma & S. Nandini. 2014. Feeding and filtration rates of zooplankton (rotifers and cladocerans) fed toxic cyanobacterium (*Microcystis aeruginosa*). *Journal of Environmental Biology* 35: 1013-1020.

Pietsch, C., C. Wiegand, M. V. Amé, A. Nicklisch, D. Wunderlin & S. Pflugmacher. 2001. The effects of a cyanobacterial crude extract on different aquatic organisms: Evidence for cyanobacterial toxin modulating factors. *Environmental Toxicology* 16: 535-542.

Pineda-Mendoza, R. M., R. Olvera-Ramírez & F. Martínez-Jerónimo. 2012. Microcystins produced by filamentous cyanobacteria in urban lakes. A case study in Mexico City. *Hidrobiológica* 22 (3): 290-298.

Ramírez-García, P., S. Nandini, S. S. S. Sarma, E. Robles-Valderrama, I. Cuesta & D. Hurtado-Maria. 2002. Seasonal variations of zooplankton abundance in the freshwater reservoir Valle de Bravo (Mexico). *Hydrobiologia* 467: 99-108.

Reynolds, C. S. 2006. *The ecology of phytoplankton*. Cambridge University Press, New York, USA, 535 p.

Vasconcelos, V., A. Martins, M. Vale, A. Antunes, J. Azevedo, M. Welker, O. Lopez & G. Montejano. 2010. First report on the occurrence of microcystins in planktonic cyanobacteria from Central Mexico. *Toxicon* 56: 425-431.

Weber, C. I. 1993. *Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms*. 4th ed. United States Environmental Protection Agency, Cincinnati, Ohio, EPA/600/4-90/027F, 266 p.

WHO (World Health Organization). 1999. *Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management*. (eds.: I. Chorus and J. Bartram). St Edmundsbury Press, Great Britain, 400 p.

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