Full Length Research Paper

Effects of toxic and non-toxic crude extracts on different *Microcystis* species (Cyanobacteria)

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Our objective was to investigate crude extracts toxic (MC+) and non-toxic (MC-) effects on the growth of *Microcystis panniformis*, *Microcystis aeruginosa* and *Microcystis wesenbergii*. Cultures were submitted to extracts MC+ at 5 and 10 MC µg.L^{-1}, and to MC- equivalent weight of these concentrations. Both crude extracts worked toward reducing the growth of *M. wesenbergii* and stimulating the growth of *M. panniformis*. Likewise, *M. aeruginosa* was inhibited by addition of crude MC- extract. The action of microcystins at concentrations commonly found in natural environments, plus the presence of other substances in intracellular *M. aeruginosa* produced differentiated effects in *Microcystis* spp.

Key words: *Microcystis aeruginosa*, *Microcystis panniformis*, *Microcystis wesenbergii*, allelopathy, crude extract, microcystin, phytoplankton, toxin.

INTRODUCTION

Microcystins (MCs) are known as highly toxic secondary metabolites which may be produced by cyanobacteria that commonly form blooms (Sivonen and Jones, 1999). Experimental studies show that substances like MC have allelopathic effects on direct competitors of cyanobacteria such as microalgae and aquatic plants (Kearns and Hunter, 2001; Pflugmacher, 2004). Allelopathy is a set of biochemical interactions, stimulating or inhibitory, bet-ween bacteria, fungi, algae or plants in a target organism (Rice, 1984). On the other hand, there are a few studies indicating that MCs has allelopathic effects on aquatic macrophytes (Pflugmacher, 2002, 2004), terrestrial plants

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(Peuthert et al., 2008), zooplankton (Ferrão-Filho and Azevedo, 2003), phytoplankton (Suikkanen et al., 2005; Sedmak and Elersek, 2005; Babica et al., 2007) and cyanobacteria (Sedmak and Kosi, 1998; El Sheekh et al., 2010). Overall, allelopathic effects encompass biochemical interactions, involved both in stimuli and growth inhibition, among diverse primary producers or among these and microorganisms (Rice, 1984). Zilliges et al. (2011) shown that MC could be attached to the intracellular proteins of cyanobacteria increasing, in this way, the life time of these molecules. In this sense, it might well be possible that MC is able to stimulate the enhancement of cyanobacteria cellular density, which could favor the reproduction of certain *Microcystis* strains (MC producing or not). Moreover, growth inhibition of other phytoplanktonic organisms could take place, a circumstance that could help dominance enforcement of *Microcystis* blooms.

There are few studies revealing the effects of microcystins at concentrations typically found in natural environments (Kearns and Hunter, 2001). However, a greater number of studies showed altered growth of phytoplankton species caused by microcystins when subjected to high concentrations not usually found in the environment (Sedmak and Kosi, 1998; Suikkanen et al., 2004; Babica et al., 2007; El Sheekh et al., 2010). The concentration of dissolved microcystins in aquatic ecosystems is in general less than 10 µg.L^{-1}, except during the break down of blooms (Sivonen and Jones, 1999). Therefore, our objective was to investigate crude extracts toxic (MC+) and non-toxic (MC-) effects, on the growth of *Microcystis panniformis* Komárek, Komárková-Legnerová, Sant'Anna, Azevedo and Senna, *Microcystis aeruginosa* (Kützing) Kützing and *Microcystis wesenbergii* (Komárek) Komárek, at concentrations similar to those found in natural environments.

MATERIALS AND METHODS

Strains and culture conditions

The *M. aeruginosa* BCCUSP232 strain produces MC-LR and MC-RR according to Bittencourt-Oliveira et al. (2011). On the other hand, *M. aeruginosa* BCCUSP03, *M. panniformis* BCCUSP200 and *M. wesenbergii* BCCUSP11 are non microcystin-producing (Bittencourt-Oliveira, 2003). All strains were clonal and non-axenic, belonging to the Brazilian Cyanobacteria Collection of the University of São Paulo. All cultures were grown as batch cultures maintained in BG-11 or ASM-1 medium, pH 7.4, according to Rippka et al. (1979) and Gorham et al. (1964), respectively, in climatic chambers with controlled conditions of light (40 µmol.m^{-2}.s^{-1}, using photometer with spheric subaquatic sensor LI-COR, mod. LI-250), photoperiod (14:10 h light:dark) and temperature (24 ± 0.5°C). The irradiance was measured in the center of the incubating flasks. Flasks positions inside the climatic chamber were intertwined in order to smooth out data fluctuations.

Obtaining crude extracts and quantification of microcystins

Twenty liters (20 L), approximately of M. aeruginosa BCCUSP232

(microcystin-producing strain) and *M. aeruginosa* BCCUSP03 (no microcystin-producing strain) cultures were cultivated. The resulting culture media were centrifuged, frozen in liquid nitrogen, lyophilized and stored in a -80ºC freezer until further use. The lyophilized biomass (0.045 to 10 and 0.022 g to 5 MC µg.L^{-1} was resuspended in approximately 1 mL of deionized water and ultrasonicated (Microson Ultrasonic Cell Disruptor, Misonix, USA) for 5 min (15 W and 22.5 kHz) for the disruption of cells. From crude extracts of BCCUSP232 (MC+) strain, concentrations of 5 and 10 MC µg.L^{-1} were obtained and used in the treatments. The BCCUSP03 strain lyophilized biomass has the equivalent weight used with the BCCUSP232. The MCs were quantified using a commercial Enzyme-Linked Immuno Sorbent Assays (ELISA) kit (Beacon Analytical Systems, Inc., Portland, ME, USA) and analyses were carried out in triplicate.

Experiments

All experiments and controls were prepared in 1000 mL Erlenmeyer - flasks with 600 mL of culture medium in triplicate, and maintained in the conditions aforementioned. For each experiment, 1.0×10^5 cell.mL^{-1} were used as the initial cellular density (day 0). The crude extracts at concentrations of 5 and 10 MC µg.L^{-1} for BCCUSP232 (MC+) and their equivalent biomass for BCCUSP03 (MC-) were added at the fourth day of experiments. A 2 mL sample was picked up from all cultures (control and treatments) every 2 days and preserved with acetic lugol 10%. The cellular densities were estimated by counting using a Fuchs Rosenthal hemocytometer and a binocular optical microscope (Nikon E200, Melville, NY, USA) at 400x magnification, according to Lund et al. (1958) to a 5% error level.

Statistical analyses

The cell density data were tested for normality and homogeneity of variances and, afterward, to an analysis of variance (ANOVA) using repeated measurements as to determine significant differences between target cultures strains treated with crude extracts (MC+ and MC-) and controls, along all experiments. Once differences were observed, we carried out a Tukey test to determine which treatments were significantly different, among them and relatively to the control. The significance was 5% for all tests and all data. All data were presented as averages with their corresponding standard deviations.

RESULTS AND DISCUSSION

Crude extracts of microcystins act differently in the three species of *Microcystis* (Figure 1a to f). The MC+ and MC- extracts reduced the cellular density of *M. wesenbergii* (BCCUSP11) but increased in *M. panniformis* (BCCUSP200). However, *M. aeruginosa* (BCCUSP03) strain was inhibited by the MC- extract and was stimulited by the MC+ one. Both crude extracts, MC+ and MC- worked toward reducing the cellular densities of *M. wesenbergii* (BCCUSP11) after addition of extracts in the fourth day, being significant from experiment day 10 on p <0.05. The concentrations of 5 and 10 MC µg.L^{-1} showed similar results (Figure 1a to b). Likewise, *M. aeruginosa* (BCCUSP03) was inhibited by addition of crude extract MC-, being significant from 8th day on p <0.05 and more evident at 10 MC µg.L^{-1}. With the extract MC- at 5 MC
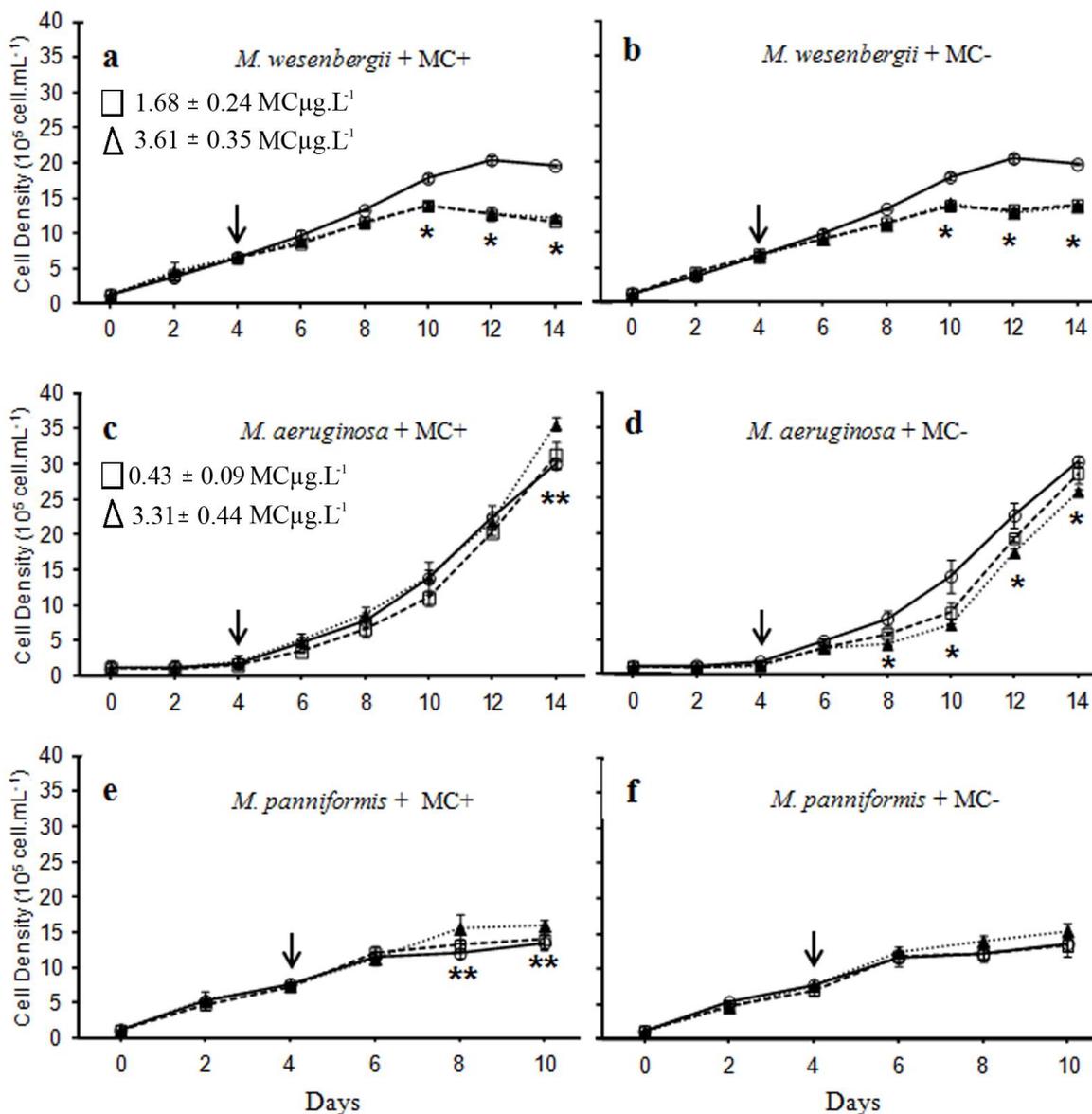
Figure 1. *Microcystis* spp. growth with addition of crude extracts of *Microcystis aeruginosa* BCCUSP232 (MC+) and *M. aeruginosa* BCCUSP03 (MC-). (a-b) *M. wesenbergii* BCCUSP11; (c-d) *M. aeruginosa* (BCCUSP03); (e-f) *M. panniformis* BCCUSP200. (*) Significant statistical difference between the treatments 5 and 10 MC μg.L^{-1} and control. (**) Significant statistical difference between treatment with 10 MC μg.L^{-1} and control. The arrow indicates the day of crude extracts addition. MC concentrations used: 5 MC μg.L^{-1} (--□--) and 10 MC μg.L^{-1} (···▲···) and Control (—O—). Error bars refer to standard deviation (n = 3). ( ) Average and standard deviation of microcystin concentrations at the 14th day of the experiment with treatment with 5 MC μg.L^{-1}. ( ) Average and standard deviation of microcystin concentrations at the 14th day of the experiment with treatment with 10 MC μg.L^{-1}.

μg.L^{-1}, the strain only recovered its growth on the 14th day of the experiment. Intriguing however, for the crude extract MC+, there was an increasing tendency of the cellular density with 10 MC μg.L^{-1}, turning out to be evident on day 14th of the experiment (Figure 1c to d) and

with a significant difference. On the other hand, *M. panniformis* (BCCUSP200) behaved differently from the other two species, putting in evidence cellular density increasing stimulation from both MC+ and MC- crude extracts, and showing significant cellular density differen-

ces at the 8th day of the experiment (p <0.05) at 10 MC µg.L^{-1} (Figure 1e to f). Although, extracts do act on the cellular density a few days after their application, one could not state that earlier physiologic effects driven by cellular metabolism did not take place, since these were not scrutinized in this work.

Cellular density variations (increase or decrease) in the three species of *Microcystis* following addition of MC-crude extracts indicated that there are other substances with presumable allelopathic effects acting on the growth of these species. This fact confirms the results found by El-Sheekh et al. (2010), where a cellular density increase of the cyanobacteria *Oscillatoria angutissima* West and West and *Anabaena* sp., and of the green microalgae *Chlorella vulgaris* Beijerinck, was reported. In this case, a cell-free filtrate from a culture of MC producing *M. aeruginosa* was used. However, this cell-free filtrate inhibited the *Scenedesmus obliquus* (Türpin) Kützing cellular density. Schagerl et al. (2002) and Gross (2003) put in evidence the possibility that cyanobacteria could produce compounds able to stimulate their own growth, and/or inhibit growth of other species. In this sense, by orchestrating acting with abiotic factors, the production of these compounds (MCs included) by *Microcystis* would work synergistically as a "trigger", such that one or more strains could reach dominance over the phytoplanktonic community. Although, Babica et al. (2007) and B-Beres et al. (2012) stated that an allelopathic character of the MCs seem to be lacking; Sedmak and Elersek (2005) pointed out that the ecologic role of the MCs could be different for the producing individuals and for those having contact with the toxin in the aquatic environment. Under this circumstance, it is possible that the MCs be an element of unknown communication routes among diverse toxic and/or non toxic strains of *Microcystis* (Vassilakaki and Pflugmacher, 2008) as well as for other organisms of the phytoplanktonic community. Also, by interacting with other substances and/or other abiotic factors (B-Beres et al., 2012), the MCs favor dominance of certain strains.

The process of allelopathy in cyanobacteria may be less frequent with low cell densities, which is the opposite of what occurs in blooms with high biomass (Leão et al., 2009). The suggestion that some cyanobacteria release autostimulatory (Monahan and Trainor, 1970; Suikkanen et al., 2005) or quorum-sensing compounds (Swift et al., 1994), which accelerated the growth of the same and related species was not confirmed in our study. The hypothesis that natural concentrations of cyanotoxins have allelopathic effects has not yet been confirmed, since most studies have used high concentrations that are unusual in aquatic ecosystems (Sedmak and Kosi, 1998; Babica et al., 2006). In conclusion, the action of microcystins and other substances present in intracellular *M. aeruginosa* (BCCUSP232 and BCCUSP03), in concen-

trations normally found in natural environments, produced differentiated effects in species of *Microcystis.*

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