ECOTOX - BRASIL

Ecotoxicol. Environ. Contam., v. 09, n. 1, 2014, 93-98
doi: 10.5132/eec.2014.01.012

EEC

Acute toxicity of *Microcystis* spp. (Cyanobacteria) bloom on *Moina minuta* (Cladocera) in a tropical reservoir, Northeastern Brazil

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(Received May 7, 2014; Accept October 14, 2014)

Abstract

Worldwide cyanobacterial blooms have been registered, where harmful species dominance is associated to producing of toxic compounds (cyanotoxins) with adverse effects on several organisms. Acute toxicity of crude extracts from *Microcystis* bloom occurring in Mundaú reservoir was evaluated by bioassays with the neotropical Cladocera *Moina minuta*. Samples were taken in the reservoir during the rainy (April/2012) and dry season (September/2012). Cyanobacterial analyses were performed by identification on optical microscopy and direct counting using an inverted microscope. Bloom samples were frozen, lyophilized and re-suspended in deionized water for preparation of extracts. Tests with the cladoceran were carried out in test tubes with different concentrations of the crude extract, diluted in 10 mL reconstituted water. In both rainy and dry periods, densities of *Microcystis* spp. were above $15x10^3$ ind mL^{-1}. Microcystin concentrations in the extracts were 0.70 (± 0,009) (rainy season) and 0.69 (± 0,005) (dry season) μg g^{-1}. The LC_{50} (48h) of crude extract for both rainy and dry periods was 160 (100 – 255) and 72 (4 – 1113) mg L^{-1}, respectively. These results indicated that extracts of *Microcystis* spp. were acutely toxic to *M. minuta* population with suggesting that such events represent potential toxicity to zooplankton.

Keywords: Eutrophication, microcystin, bioassay, zooplankton.

INTRODUCTION

Artificial eutrophication has been one of the main factors promoting cyanobacterial blooms in inland waters. The massive proliferation of these prokaryotic photosynthetic microorganisms, in most cases, is associated with production of toxins, which compromise the ecological stability of aquatic environments. Several genera are involved in the formation of blooms, especially *Microcystis*, a potential producer of microcystins, whose blooms form a dense algal biomass on the surface of the water body, consisting of colony aggregations, mainly in periods of high water residence time and of thermal stratification (Paerl, 1988).

The toxicity of *Microcystis* species has been widely reported in the literature, with records of animal (Chellapa *et al*., 2008; Nasri *et al*., 2008) and human poisoning, such as in Caruaru, state of Pernambuco (Northeastern Brazil), where 76 dialysis patients died after intravenous contamination by microcystin (Jochimsen *et al*., 1998).

Toxins synthesized by cyanobacteria can be characterized with regards to their biological activity, such as: hepatotoxins, cytotoxins (peptides and alkaloids), neurotoxins (alkaloids) and dermatotoxins (lipopolysaccharides, alkaloids and phenolic bislactones) (Chorus & Bartram, 1999; Dittmann *et al.*, 2013). The hepatotoxins are the most studied, especially the microcystins (MCs), which are cyclic heptapeptides distributed over 80 isoforms that vary according to the amino acids allocation in the side chains, methylations and double bonds, which provide different toxicity and polarity, being MC-LR the isoform of greatest toxicity, with an LD_{50} of 50 μg

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kg^{-1} in mice bioassays (Nidhi-Gupta *et al.*, 2003; McElhiney & Lawton, 2005; Dittmann & Wiegand, 2006).

The availability of MCs in the water column can directly limit the survival of other individuals such as zooplankton due to potential toxicity. This has been discussed in the evolutionary perspective, regarding the role of each toxin as a chemical defense against herbivory (Lampert, 1981). Thus, these organisms can be excellent indicators of toxicity and bioaccumulation of cyanotoxins in aquatic systems (Ferrão-Filho *et al*., 2009; Ferrão-Filho & Kozlowsky-Suzuky, 2011).

Cladocerans are the most used group in ecotoxicological studies, especially the daphnid species: *Daphnia similis* Claus 1876, *D. magna* Straus, *Ceriodaphnia silvestrii* Daday and *C. dubia* Richard, which have been incorporated into protocols for toxicity testing in Brazil (ABNT, 2004; 2005). However, only *C. silvestrii* is a tropical species.

Investigations on the toxicity of cyanobacterial blooms in Brazilian reservoirs have been conducted with native species, such as *C. silvestrii* (Sotero-Santos *et al*., 2006; Okumura *et al*., 2007) which appear to be sensitive to the toxins. Species of the genus *Moina* Baird are also being used in toxicity experiments, due to their wide distribution in tropical waters, such as *M. macropora* Straus (Agrawal *et al*., 2001; Alva-Martínez *et al*., 2007) and *M. micrura* Kurz (Ferrão-Filho *et al.*, 2014). These finds have consolidated the hypothesis of Ferrão-Filho *et al*. (2009) which emphasizes the importance of developing protocols with tropical cladocerans common to these water bodies.

Thus, the present study aimed to determine the potential of using the native cladoceran *Moina minuta* Hansen in assessing the effect of cyanobacterial blooms in a tropical reservoir with predominance of *Microcystis* species, through acute toxicity tests with crude extract of microcystin.

MATERIALS AND METHODS

Sampling and qualitative and quantitative analysis

The Mundaú Reservoir (8º57'17'' S, 36º29'55' W) is an eutrophic and shallow water body, which is located in the city of Garanhuns, Pernambuco (Brazil); sited in the Mundaú River watershed at an altitude of 716 m, with an accumulation capacity of 1.968.000 m^3 and water retention time occurring during the entire dry period (Moura *et al.*, 2007; Dantas *et al.*, 2008). The reservoir is intended for the public water supply, however currently receives part of the urban drainage and industrial effluent.

Samples of bloom were collected in April/2012 (rainy season) and September/2012 (dry season) using 20µm mesh. These months showed a historical average rainfall (last 10 years) for the area of 97 and 62 mm, respectively. Cyanobacteria of the genus *Microcystis* prevailed during the sampling period as recorded for other studies as Bittencourt-Oliveira *et al*. (2010; 2014).

Samples for taxonomic analysis were stored in polyethylene vials (100 mL) and preserved with 4% formalin. Cyanobacteria taxa were identified to the infrageneric level using specialized bibliography (Komárek & Anagnostidis, 1989; 2000; 2005). For the quantitative analysis, subsurface samples were collected, stored in amber vials (100 mL) and preserved with 1% acetic Lugol solution. The taxa of cyanobacteria were expressed as a density (ind mL^{-1}) using the method of Utermöhl (1958).

Preparation of crude bloom extracts

The bloom samples were centrifuged and lyophilized at -80°C, until complete dehydration to obtain dry biomass, which was stored at -20°C until preparation of the extract. The lyophilized material was weighed on a precision balance, re-suspended in Milli-Q deionized water and homogenized in a vortex mixer for 2 minutes to obtain the extract at a concentration of 1000 mg L^{-1}. The cells were then lysed in a Sonoplus HD 2070 sonicator for two 5-minute cycles at a frequency of 20 kHz, aiming to disrupt the cells and expose the intracellular content. Slides were prepared with debris to observe if all cells had been broken. The material was then centrifuged at 3,500 rpm for 10 minutes, which removed the supernatant, for preparation of different concentrations of crude extract to be tested, by dilution in reconstituted water (adapted from Okumura *et al.*, 2007).

Analysis of microcystin

The content of microcystin in the extracts was quantified by ELISA. The results were expressed as total microcystin (µg microcystin per g of dry biomass). The analyses were performed following the protocol of the kit for microcystin (Beacon Analytical Systems, Inc., Portland, ME, USA). The procedures were performed in triplicate in a microplate reader (ASYS Hitech GmbH, Nordstrasse 171 4 Model A - 5301 Eugendorf, Austria).

Cultivation and maintenance of cladocerans

Clonal individuals of *Moina minuta* were obtained from cultures of the Laboratory of Aquatic Ecology at the Federal University of Paraiba. Cladocerans were maintained in reconstituted water under pH 7-8, average temperature 25±2°C and 12h photoperiod. Cladocerans were fed on a suspension of green algae: *Desmodesmus quadricauda* Turpin and *Chlorella vulgaris* Beyjerink [Beijerink] *ad libitum.*

Acute toxicity tests

Toxicity tests consisted of exposure of 10 neonates (<24h) of *M. minuta* in test tubes containing 10 mL of different concentrations of crude extract, in triplicate. The concentrations used were: 0, 62, 125, 250 and 500 mg L^{-1} for the sample of the rainy season and 0, 15, 31, 62 and 125 mg L^{-1} for the dry season. The crude extract was diluted in reconstituted water and test organisms were exposed without

feeding under average temperature 25±2°C and a photoperiod of 12h, for 48 hours. After the exposure period, the numbers of individuals killed at each concentration and in the control were recorded for calculation of LC_{50} 48h.

Statistical analysis

Data from the acute toxicity tests were analyzed using the Trimmed Spearman-Karber method for estimating median lethal concentration (Hamilton *et al*., 1977). To identify significant differences between the tested concentrations, the univariate analysis of variance (one-way ANOVA) was used, after examination of data normality and homoscedasticity of variances. If a difference was detected, the Dunnett test was used *a posteriori*. Statistical tests were carried out using SigmaPlot 11.0.

RESULTS

During the two sampling periods, the phytoplankton community showed predominance of the cyanobacteria *Microcystis aeruginosa* and *M. panniformis*, forming dense multispecies blooms, and co-occurring at densities of $59x10^3$ and $14x10^2$ ind mL^{-1} in the rainy season, and $96x10^2$ and $56x10^2$ ind mL^{-1} in the dry season, respectively. We also recorded the occurrence of the cyanobacteria *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya & Subba Raju, *Geitlerinema amphibium* (C. Agardh) Anagnostidis and *Merismopedia tenuissima* Lemmermann, which showed total densities of less than $66x10^2$ ind mL^{-1} in both study periods.

Figure 1 shows the mortality of *Moina minuta* after 48h exposure to various concentrations of crude extract of cyanobacterial bloom. Mortality was greater for the different periods in the higher concentration of crude extract analyzed. We observed a significant increase in the percentage of mortality with increased concentration of the extract in the rainy season ($F = 10.769$, $p < 0.01$) and dry season ($F = 5.18$, $p < 0.05$), with the observed effect concentration (OEC) equal to or above 125 mg L^{-1} (Dunnett, $p < 0.05$) and 62 mg L^{-1} (Dunnett, $p < 0.05$) for these periods, respectively.

The results show that the content of microcystin in the bloom extract from Mundaú reservoir was similar in both rainy ad dry season, however there were differences in the LC_{50} (48h) to *M. minuta* **(Table 1)**, with greater toxicity in the September/2012 (dry season) sample.

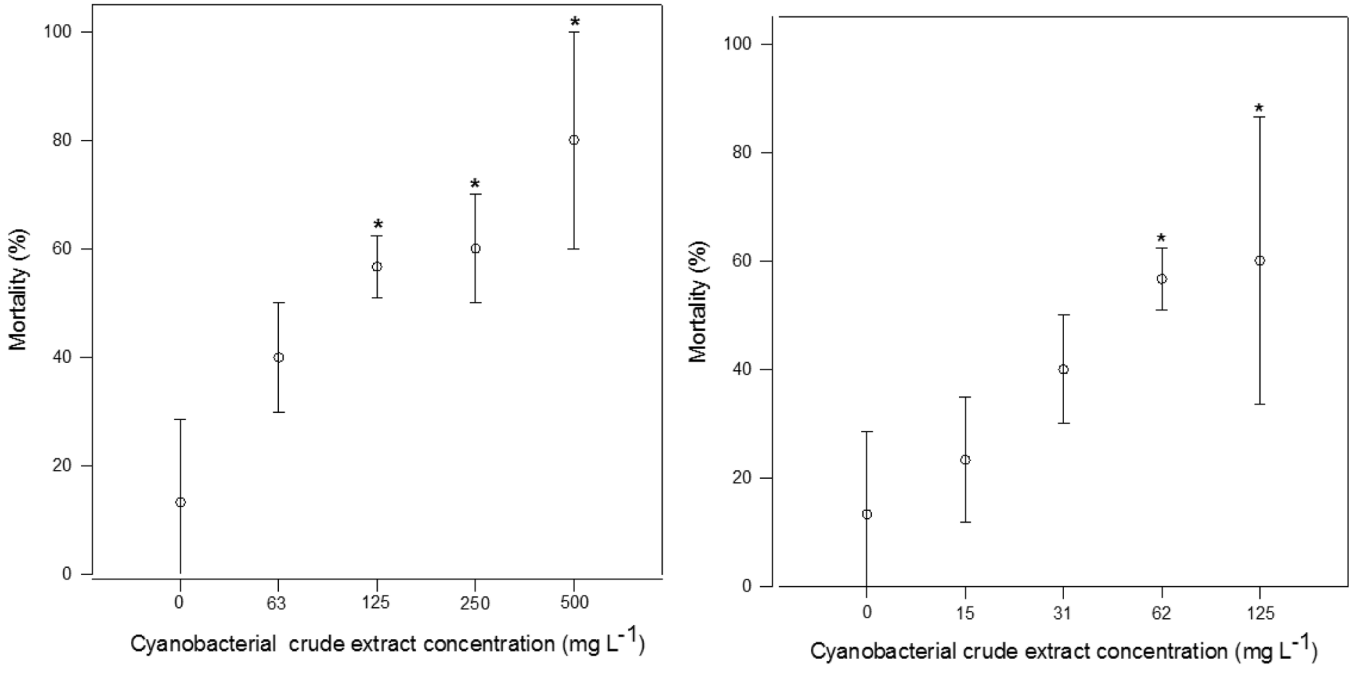
DISCUSSION

This record of prevalent and multispecies blooms of *Microcystis* is evidence of the high stage of eutrophication of the Mundaú Reservoir. Soares *et al*. (2013) suggest that the high flexibility of these taxa in adapting to seasonal environmental changes may have promoted an additional advantage for the increase of persistent blooms, particularly in tropical regions.

Studies in tropical eutrophic reservoirs have recorded the occurrence of multispecies algal blooms formed by potentially toxic cyanobacteria (Costa *et al.*, 2006; Moura *et al*., 2011),

Table 1. Median lethal values (LC_{50} – 48h) and 95% confidence intervals (CI) estimated for *Moina minuta* and concentrations of microcystin with its standard deviation (SD) for the crude extracts tested. Values represented as dry weight (DW) and as microcystin (MC) content in crude extract.

Sample	LC_{50} – 48h (95% CI)	Microcystin concentration (SD)
	mg DW L^{-1}	µg MC g^{-1}
April/2012 (rainy season)	160 (100 – 255)	0.70 (± 0.009)
September/2012 (dry season)	72 (4 – 1104)	0.69 (± 0.005)



mainly in Northeastern Brazil where high densities of these microorganisms are a recurring feature in public water supply reservoirs (Moura *et al*., 2007; Chellappa *et al.*, 2008; Dantas *et al*., 2010; Lira *et al*., 2011). Furthermore, there are frequent reports of cyanotoxins to the reservoir sampled, as Bittencourt-Oliveira *et al.* (2010; 2014).

In this study, the 48h LC_{50} values to *Moina minuta* (160 and 72 mg L^{-1}) are in agreement with those observed in previous studies as Sotero-Santos *et al*. (2008; 2006) and Takenaka *et al.* (2007). Most have investigated sensibility of native cladocerans to cyanobacteria as Ferrão-Filho *et al.* (2014) that reported greater 48h LC_{50} values to *M. micrura* (413 and 853 mg L^{-1}) and *Daphnia laevis* (413 and 853 mg L^{-1}) exposed to microcystin-producing cyanobacterial blooms, which was less sensitive in comparing to our findings. However, Sotero-Santos *et al*. (2008) have pointed out that a cyanobacterial crude extract may contain others metabolites which can exert synergistic, additive or antagonistic effects.

Although most ecotoxicological studies have been performed with species of daphnids (Okumura *et al*., 2007; Ferrão-Filho *et al*., 2010), toxicity tests with *Moina* spp., especially with tropical species, have demonstrated the sensitivity of these organisms that enables them to be used as indicators of the toxic effects of cyanobacteria (Yasuno & Sugaya, 1991; Ferrão-Filho *et al.*, 2000; Agrawal *et al*., 2001). Furthermore, although there is a trend in the use of cladocerans in ecotoxicological tests, studies with other microcrustaceans, like the tanaidacea *Kalliapseudes schubartii*, have also showed the cyanobacterial toxicity (Montagnolli *et al*., 2004).

A difference in toxicity between the different extracts tested was observed, although similar concentrations of microcystin were recorded **(Table 1)**. Other studies, as Takenaka *et al*. (2007), have reported an indirect relationship between the concentration of microcystins and the toxicity of the crude extracts of cyanobacteria to the cladocerans *C. dubia* and *C. silvestrii*.

Furthermore, sometimes cyanobacterial blooms can be composed by several cyanotoxin-producing species, which can result in a mixture of different bioactive compounds. Exposition experiments of *D. magna* to hepatotoxic and neurotoxic cyanobacterial extracts have shown additive and synergistic toxic effects on its feeding rate (Freitas *et al*., 2014).

Blooms in the same water body may vary in toxicity over a short period of time depending on the composition of toxic and non toxic strains (Yéprémian *et al*., 2007; Molica & Azevedo, 2009). Znachor *et al.* (2006) recorded monthly changes on the qualitative and quantitative content of microcystin (MC–LR; –RR and –YR) in *Microcystis* spp. bloom samples of 18 reservoirs in the Czech Republic. The authors observed different concentrations of MC–LR among months. This dynamic on microcystin isoforms may produce different toxicity, as reported by Nidhi-Gupta *et al.* (2003) which studied the comparative toxicity of microcystin–LR, –RR and –YR in mice, where the MC–LR was the most toxic.

Taking into consideration our findings regarding the toxicity of microcystin containing extract to *M. minuta,* as well as the possibility that high concentrations of extracellular cyanotoxins can be reached in the water body during bloom senescence, we can expect some negative effects of the toxin on aquatic ecosystems.

In conclusion, the data obtained in this acute toxicity test showed that *M. minuta* was sensitive to cell-bound microcystin released from bloom of *Microcystis* spp. sample, reinforcing the importance of using indigenous organisms for biomonitoring and predicting ecological impacts from cyanotoxins in tropical freshwater reservoirs.

AKNOWLEDGEMENTS

We would like to thank CNPq for scholarship, Laboratory of Biotechnology/CENAPesq/UFRPE (Center for Research Support/Federal Rural University of Pernambuco) for the samples processing, Laboratory of Aquatic Ecology from the Federal University of Paraiba, for the cladoceran clones and the anonymous reviewers for the comments on improving this paper.

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