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Evaluation of the Ecotoxicological Effects of *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii* on *Ceriodaphnia dubia* Before and After Treatment with Ultrasound

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Abstract Bodies of water contaminated by cyanobacteria and their neuro- and hepatotoxins have caused environmental and public health issues all over the world. Therefore, determining safe concentrations in water for multiple uses to protect aquatic biota and identify forms of remediation are of broad interest. In this study, we isolated strains of the cyanobacteria *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii*, which produce microcystin (MC) and saxitoxin (STX), respectively. Ecotoxicological tests using suspensions of lysed lyophilizated cells with concentrations of toxins equivalent to those permitted by legislation for potability (1 μg L^{-1} for MC and 3 μg L^{-1} for STX) did not result in significant mortality of the model organism, *Ceriodaphnia dubia*, where as concentrations five times greater resulted in decreased survival for both toxins. However, reproduction was significantly reduced even in the lower concentrations, indicating that the currently permitted standards are not safe for environmental protection. When cyanotoxins were treated with ultrasound, mortalities were no longer significant, independent of concentrations. Although reproduction was still lower in relation to the control, it was significantly higher when compared to the results obtained before ultrasound. Ultrasound has been previously applied to cyanobacteria cell lysis, but this is the first study to investigate the ecotoxicological effects of ultrasound on cyanotoxins. Using new test organisms and different times and potency of sonication will permit the development of more efficient techniques for the remediation of these toxins and the development of more adequate parameters for the protection of aquatic life.

Keywords Microcystin · Saxitoxin · *Cylindrospermopsis raciborskii* · *Microcystis aeruginosa* · *Ceriodaphnia dubia* · Ultrasound

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1 Introduction

Multiple sources of punctual and diffuse pollution can cause several types of injuries when they reach surface waters, including the eutrophication and consequent proliferation of potentially toxic microalgae that are responsible for environmental and public health issues. These issues have gained attention in the literature, and the presence of cyanobacteria strains with their neuro- and hepatotoxins have been of special concern (Dörr et al. 2010; Rastogi et al. 2014; Mowe et al. 2015). In

tropical regions, the predominant genera are *Microcystis* and *Cylindrospermopsis* (Mowe et al. 2015), both of which are able to produce a variety of cyanotoxins. Among these cyanotoxins, the microcystin variations (e.g., LR, RR, YR, LA, 7dmLR, WR, LF, LY, and LW), saxitoxins, neosaxitoxins, gonyautoxins (GTX1-4), and other analogues are particularly harmful.

The World Health Organization (WHO 2011) established the concentration of 1 μg L^{-1} microcystins (MC-LR) as the maximum daily concentration permitted in water appropriate for human consumption, and this amount has been used to standardize guidelines all over the world (NWQMS 2011; Health Canada 2014; US EPA 2015; Ordinance no. 2914/11 BRASIL 2015). However, the available information of the research with saxitoxins until now is not sufficient to generate limit for human consumption. Based on an event of human intoxication, Fitzgerald et al. (1999) proposed 3 μg L^{-1} as the maximum limited acceptable in potable water, and this value was adopted, for instance, in Brazilian, Australian, and New Zealand regulations (Merel et al. 2013). Nevertheless, it is important to mention that the standards established for human life are not necessarily appropriate for the protection of aquatic life.

Since this is a subject of broad interest, it is crucial to identify forms of remediation to maintain the multiple uses of freshwater. In this context, the use of ultrasound seems to be an efficient method, since it reduces the number of algal cells and cyanobacteria and, as a consequence, limits the amount of toxins produced (Wu et al. 2011; Yamamoto et al. 2015). Currently, there is no information in the literature about ecotoxicological conditions or relevant information to identify the chemical and ecotoxicological efficiency of this method. Given that, this study was based on sonicating samples from a reservoir of isolated cyanobacteria in the lab and further conducting toxicity tests after and before the sonication.

Most assays using ultrasound and cyanobacteria have often been verifications for this method in cell lysis (Pietsch et al. 2001; Okumura et al. 2007; Zagatto et al. 2012; Barrios et al. 2015; Herrera et al. 2015). Zagatto et al. (2012) is the only report for performing ecotoxicological tests after cell lysis and found increased levels of toxicity in the test organism *Ceriodaphnia dubia*, possibly due to released cyanotoxins. However, the effects of the ultrasound on the toxins are still unknown, and it remains unclear whether this treatment can also be used as an alternative to remediation. Therefore, an integrated approach of chemical, biological, and ecotoxicological aspects of these toxins may elucidate some of the influences that have compromised the multiple uses of aquatic environmental systems. In this study, we isolated lineages of cyanobacteria to answer the following questions: 1—Do the isolated lineages of cyanobacteria produce cyanotoxins? 2—Are the cyanotoxins toxic to *Ceriodaphnia dubia* in concentrations permitted worldwide and five times greater? 3—Are the amounts established for human consumption also adequate for protecting aquatic organisms? 4—Can ultrasound remediate toxicity? This is the first study to investigate the effects of cyanotoxins after sonication in biological systems.

2 Methods

2.1 Study Area and Sampling

The cyanobacteria *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii* were isolated from samples of surface water collected in November 2013 at the Itupararanga reservoir, São Paulo State, Brazil (23° 34′ 49″–23° 40′ 12″ S, 47° 13′ 11″–47° 24′ 34″ W), using nets with 20-μm pores (Fig. 1).

2.2 Isolation and Cultivation of Cyanobacteria Lineages

Isolation and axenization of the cyanobacteria were performed using the microcapillary technique (Anderson 2005). Organisms were grown in 8 L of ASM-1 medium at pH 7.0 (Gorham et al. 1964) in polycarbonate bottles with a 9-L capacity. After 5 days of culture (exponential phase), the cells were concentrated by continuous flow-refrigerated centrifuge (Heraeus model Contifuge Stratos with bomb Watson Marlow 520U) at 6000 rpm and a flux of 25 mL min^{-1}. The biomass obtained was frozen in liquid nitrogen and lyophilized (Heto Drywinner).

2.3 Detection and Quantification of the Cyanotoxins

The presence of cyanotoxins and their concentrations per milligram of lyophilized cells were determined by HPLC-MS/MS according to the methods described by Ferrão-Filho et al. (2009), which follows the recommendations of Spoof et al. (2003) for microcystins,

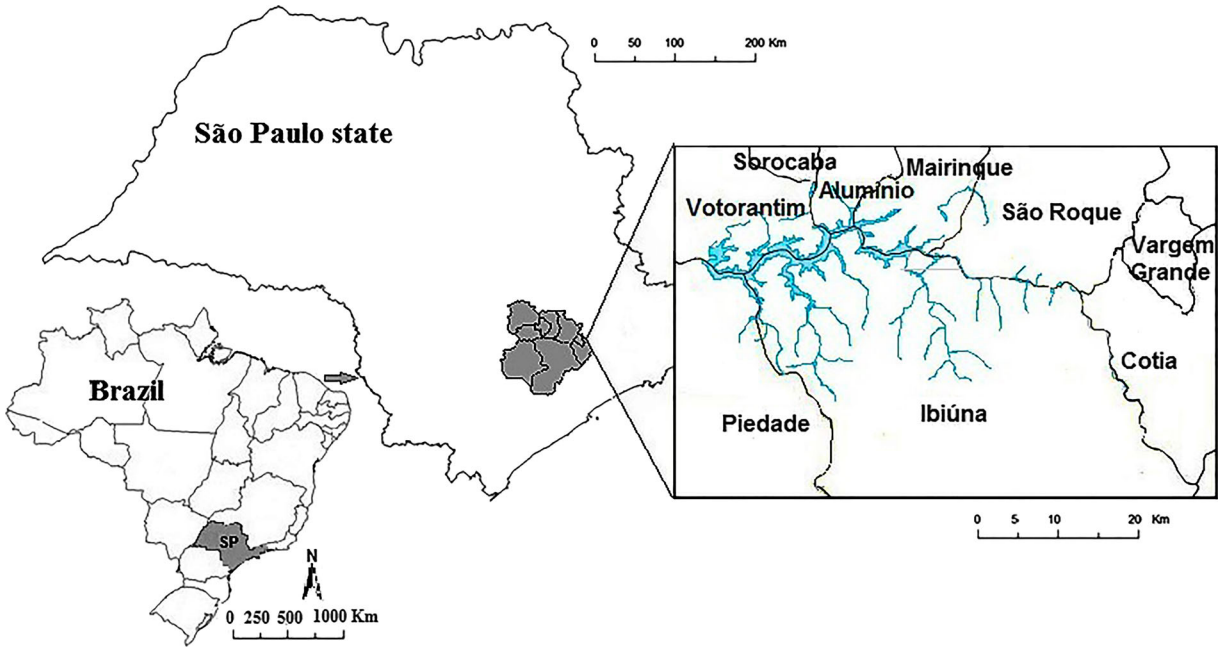

Fig. 1 Localization of the Itupararanga reservoir. Emphasis is given to the municipalities surrounding the reservoir. Source: modified from IBGE (Brazilian Institute of Geography and Statistics. Available in: http://www.ibge.gov.br/english/)

Oshima (1995) for saxitoxins, and Eaglesham et al. (1999) for cilindrospermopsins.

2.4 Experimental Design

The ecotoxicological experiments used two sets of stock solutions containing lyophilized cultures with equivalent concentrations of 0.02 mg L^{-1} microcystin (MC) and 0.04 mg L^{-1} saxitoxin (STX). The cyanobacterial suspensions were exposed to a cycle of freezing and thawing three times to rupture the cells, followed by their observation under an optical microscope (Zeiss Axiovert 40C) with ×40 magnification to verify that the cell disruption was effective. Then, one set was sonicated for 5 min with 100 W power and 19 kHz, 2 W mL^{-1} (probe sonicator Unique, model DES100; Fig. 2).

The tests were performed by exposing the model organism, *Ceriodaphnia dubia*, to concentrations allowed by legislations (1 μg L^{-1} MC and 3 μg L^{-1} STX) and arbitrarily exceeding five times the concentrations (5 μg L^{-1} MC and 15 μg L^{-1} STX), using disrupted cells before and after sonication. The tests used cultivation water, which was prepared with reconstituted mineral water, pH varying from 7.2 to 7.6, and hardness ranging from 40 to 48 mg $CaCO_3$ L^{-1}. Control groups only used the cultivation water, and

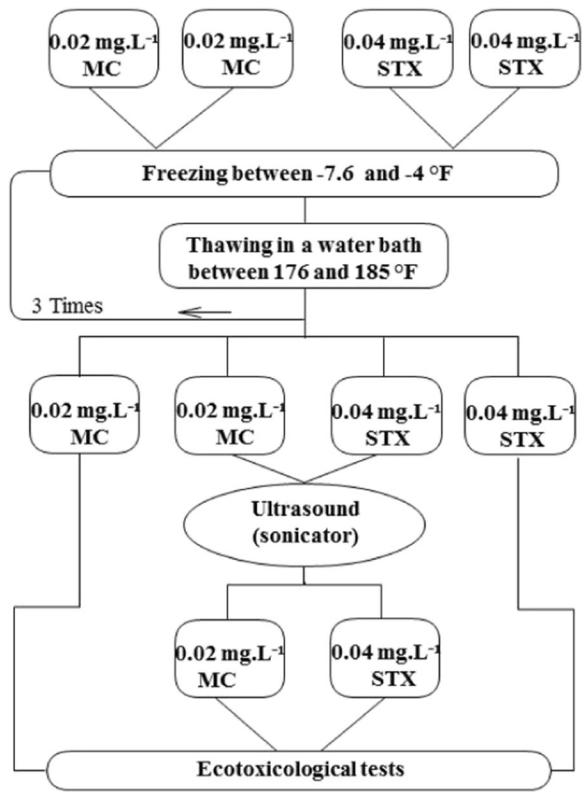

Fig. 2 Schematic of solutions preparation for ecotoxicological tests

each treatment consisted of ten replicates containing one organism. These tests followed the recommendations from the US EPA method 1002 (2002), where test organisms with 6- to 24-h lifecycles (neonates) are used at the beginning of the experiment, and they are 8 days old at the end, as adults. The endpoints analyzed were survival and reproduction (number of neonates).

Statistical analyses were performed using the software Toxstat 3.5 (West and Gulley 1996), with the Kruskal-Wallis test to assess the potential toxicity effects in the experiments.

3 Results and Discussion

The cyanobacteria *M. aeruginosa* produced microcystin at a concentration of 440 ng mg^{-1}, and *C. raciborskii* produced a concentration of 48 ng mg^{-1} saxitoxin. The presence of these toxins is similar to previous studies with these cyanobacteria, and the concentrations are within the value range found in other strains originating from Brazilian locations (Table 1), although they are not directly comparable due to the use of different methods for quantification.

Both *C. raciborskii* and *M. aeruginosa* have been frequently identified at the Itupararanga reservoir (Moschini-Carlos et al. 2007; Cunha and Calijuri 2011; Vargas 2012; Cetesb 2014, 2015), and between 2009 and 2010, they were among the phytoplanktonic organisms with higher relative densities (Cunha & Calijuri op cit).

Without sonication, both higher concentrations of MC (5 μg L^{-1}) and STX (15 μg L^{-1}) significantly reduced the survival of the model organisms (Fig. 3), while lower concentrations of both substances resulted in a reduction of neonate production compared to the control (Fig. 4). According to the current Brazilian legislation (ordinance no. 2914/11 BRASIL 2015) and the WHO, the maximum permitted concentration of microcystin in potable water is 1 μg L^{-1}. However, our data revealed that this concentration can cause adverse effects on aquatic organisms, which means this concentration is not adequate to protect aquatic life.

Sotero-Santos et al. (2008) performed acute assays with *C. dubia* using natural phytoplanktonic extracts and obtained CL50 in 48 h of exposition with concentrations of MC varying from 18.5 to 90 μg L^{-1}. Negative effects of the *M. aeruginosa* lineages that produce microcystin were also detected for the development and reproduction of *Daphnia* (*D. similoides* and *D. pulex*), which was confirmed by Li et al. (2014).

When using lower microcystin concentrations (0.41 to 0.44 μg L^{-1}), Barrios et al. (2015) observed a reduction in the reproduction and survival of *Ceriodaphnia cornuta*. Conversely, Dao et al. (2010) did not find negative impacts in chronic assays with *D. magna* using MC-LR (5 μg L^{-1}), and Okumura et al. (2007) also did not find interference to their production or survival of *C. silvestrii* using concentrations of MC varying from 60 to 480 μg g^{-1} obtained from lyophilized cultures of *M. aeruginosa*.

Herrera et al. (2015) suggested that microcystin-LR obtained from natural phytoplankton extracts were probably responsible for the toxicity of *D. similis*, indicating that 83.5 μg L^{-1} MC-LR was lethal for 50% of these cladocerans after a 48-h exposure. Dao et al. (2010) showed that 50 μg L^{-1} MC-LR did not affect survival in the parental generation of *D. magna*, but after 2 months of exposure, it caused a 50% reduction in the population represented by the following generations. Therefore, distinct strains of cyanobacteria can present different levels of toxicity, produce different forms of microcystins or other metabolites, and produce toxic elements during the degradation of these organisms (Yang et al. 2012).

STX often reduce survival by compromising natatory movements and consequently the feeding capacity, which is typical of neurotoxins. For instance, Restani and Fonseca (2014) observed that *D. laevis* were immobilized after a 3-h exposure in the range of 0.14 to 1.4 ng L^{-1} saxitoxin. Ferrão-Filho et al. (2010) also observed toxic effects in the motility of *D. pulex* exposed for 3 h to intact cells of *C. raciborskii*, which corresponded to approximately 2.6 ng L^{-1} STX.

Previous reports identified that a concentration of 1.37 ng L^{-1} STX caused a 50% reduction in the motility of *D. similis* after a 2-h exposure (Ferrão-Filho et al. 2014). Even smaller concentrations (0.24 ng L^{-1}) inhibited natatory movements of *D. pulex* (Costa et al. 2013).

Zagatto et al. (2012) exposed *C. dubia* to different concentrations of intact and lysed cells from two lineages of *C. raciborskii* and saw a 50% reduction in fertility with lower concentrations (244 cells mL^{-1}) and a 100% mortality with higher concentration (2.44×10^4 cells mL^{-1}). The toxic effects of *C. raciborskii* producing saxitoxins on cladocerans have also been demonstrated by Ferrão-Filho et al. (2008) and Soares et al. (2009).

In this study, we found that after ultrasound treatments on the lyophilized cells, none of the substances had an effect on survival for both concentrations. However, the significant differences in reproduction compared to the control persisted in both concentrations of saxitoxin and

Table 1 Strains of cyanobacteria isolated from Brazilian bodies of water and the cyanotoxins analyzed

Cyanobacteria	Concentrations of cyanotoxins analyzed	Methodology for quantification	Toxicity assays performed	References
M. aeruginosa *C. raciborskii*	440 ng mg^{-1} of MC 48 ng mg^{-1} of STX	HPLC (lyophilized culture)	Chronic assays (*C. dubia*)	Present study
M. aeruginosa	936 to 15,000 μg g^{-1} of MC	HPLC (lyophilized culture)	Acute assays (*Daphnia similis*, *C. silvestrii*, and *C. dubia)*	Okumura et al. (2007)
M. aeruginosa	674.8 to 1745.5 μg L^{-1} of MC	ELISA (liquid culture)	Acute and chronic assays (*C. dubia* and *C. silvestrii*)	Takenaka (2007)
C. raciborskii	0.000846 to 0.846 ng L^{-1} of STX 0.0937 to 9.37 ng L^{-1} of STX	HPLC (liquid culture)	Acute assays with intact cells (*D. pulex*, *D. gessneri*, and *Moina micrura*)	Ferrão-Filho et al. (2008)
C. raciborskii	32.0 and 52.0 mg g^{-1} of STX 47.7 and 55.0 mg g^{-1} of GTX1	HPLC (lyophilized culture)	Acute assays with intact cells (*Daphnia pulex* and *Moina micrura*)	Ferrão-Filho et al. (2010)
C. raciborskii	0.25 to 2.05 μg L^{-1} of STX	ELISA (liquid culture)	*	Vargas (2012)
M. aeruginosa	Presence of variations of MC (MC-RR, MC-YR, MC-LR, and MC Dmt)	HPLC (lyophilized culture)	*	Bortoli et al. (2014)
C. raciborskii	0.05 to 8.94 ng L^{-1} of STX	HPLC (liquid culture)	Chronic assays (*Daphnia pulex*, *Daphnia gessneri*, and *Moina micrura*)	Costa et al. (2013)
C. raciborskii	1.37 to 10.97 ng L^{-1} of STX	HPLC (lyophilized culture)	Acute assays (*D. similis*)	Ferrão-Filho et al. (2014)
C. raciborskii	0.0014 to 14 ng L^{-1} of STX	HPLC (lyophilized culture)	Acute assays (*D. laevis*)	Restani and Fonseca (2014)

MC microcystin, *STX* saxitoxin, * not performed

in the lower concentration of microcystin (Fig. 5). Despite the negative effect in three of the sonicated treatments (Fig. 5), the average number of neonates was higher than the assays where sonication was not used.

Several studies have addressed alternative forms of treatment for the removal and control of algae, in particular, the cyanobacteria, and techniques using ultrasound are the most efficient for controlling blooms of cells compared to other conventional techniques (Zhang et al. 2006b, 2009; Ahn et al. 2007; Wu et al. 2011; Rajasekhar et al. 2012a; Purcell et al. 2013). Zhang et al. (2009) investigated the use of sonication in the removal of *M. aeruginosa* by coagulation and confirmed the reduction of algae cells and chlorophyll *a*, without augmenting the concentration of microcystin in the water. This finding was demonstrated by Srisuksomwong et al.

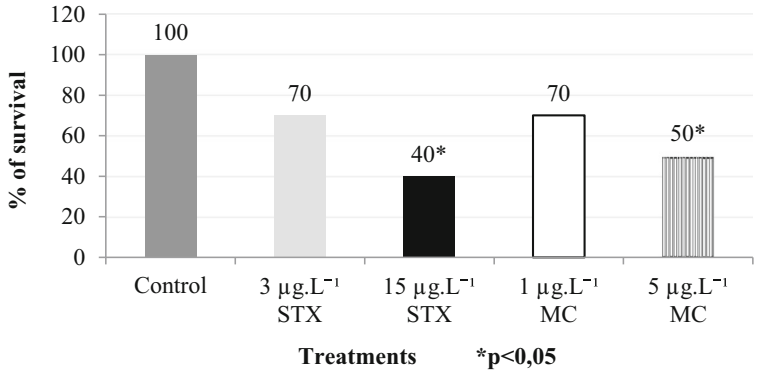

Fig. 3 Survival of *Ceriodaphnia dubia* after 8 days of exposure to saxitoxin (STX) and microcystin (MC) solutions before sonication; significant differences ($p < 0.05$) compared to the control at the higher concentrations

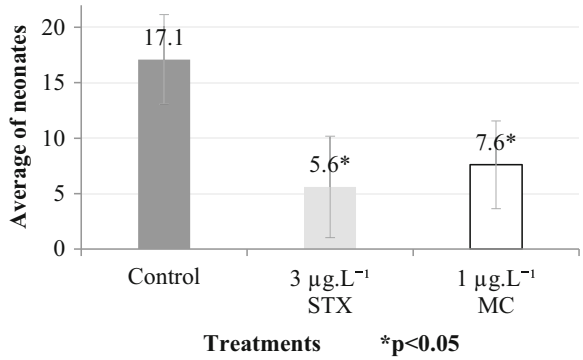

Fig. 4 Toxicity tests indicating the average number of neonates per treatment for 8 days of exposure. *Bars* represent standard deviations

(2011), showing a significant reduction in the amount of MC in natural samples of cyanobacteria that were sonicated for 10 min (3 W) at 108 and 200 kHz, with reductions of 72.3 and 80%, respectively. Rajasekhar et al. (2012b) identified a reduction of more than 60% in the number of suspended cells when exposed to 0.32 W mL^{-1} (20 kHz) for 5 min. Similarly, Zhang et al. (2006a) showed that exposure to 0.32 W mL^{-1} (25 kHz) for 5 min was also efficient, since after 2 weeks, the cell concentrations were only 14.1% compared to non-sonicated cultures.

Although the works cited above have not evaluated the toxic effects after treatment, they reinforce the results of the present research by demonstrating the efficiency of ultrasound in the reduction of cells and their associated toxins. Regarding the ultrasonic frequency, Wu et al. (2012) obtained the best results showing that the lower frequency (20 kHz) inactivates *M. aeruginosa* cells.

This study demonstrated a higher toxicity for the lower concentration of MC on the reproduction of the organisms. A similar result was reported by Takenaka (2007), after performing acute and chronic tests with microcystins derived from *M. aeruginosa* using *C. dubia* and *C. silvestrii*. We found that the lower concentrations of MC (191.87 µg L^{-1}) were more toxic than higher concentrations (1194.84 µg L^{-1}). The present work did not consider other metabolites produced by cyanobacteria, such as protease inhibitors, and structural variations of the MCs, such as MC-RR, MC-LR, MC-YR, MC-LF, MC-LW (Dörr et al. 2010; Bortoli et al. 2014; Rastogi et al. 2014), STXs, and GTXs (Ferrão-Filho et al. 2010; Wiese et al. 2010; Carneiro et al. 2013). Kuster and Elert (2013) verified a negative effect in the development of *D. pulex* after exposure to different concentrations of the two lineages of non-MC producing *M. aeruginosa*, which contained the inhibitor chymotrypsin or trypsin.

4 Conclusion

The cyanobacteria isolated from the reservoir can indeed produce toxins. Even low concentrations of microcystin and saxitoxin caused negative effects, mainly on the reproduction of our model organism, *C. dubia*, suggesting that the concentrations permitted for potability are not recommended for the protection of aquatic life. However, sonication of lysed cyanobacteria was effective in reducing toxicity, which indicates that this treatment can neutralize the toxins. In water treatment, ultrasound may first cause cell lysis, releasing cyanotoxins and increasing toxicity. Thus, remediation using ultrasound relies on determining the time exposure that may be enough to cause both cell and cyanotoxin lysis. These data may have broad utility since cyanobacteria contamination is a growing problem all over the world. The use of new model organisms

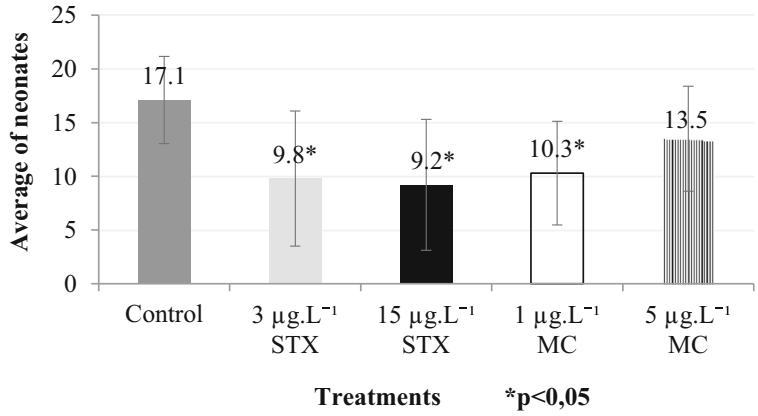

Fig. 5 Average neonates by treatment after 8 days of *C. dubia* exposure to cyanotoxin solutions following treatment with ultrasound. *Bars* represent standard deviation

with different times and potency of sonication will further develop more efficient techniques for the remediation of these toxins.

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