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Effects on DNA and cell viability of treated water contaminated with *Cylindrospermopsis raciborskii* extract including cylindrospermopsin

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Abstract

It is well known that chlorine can oxidize cyanotoxins, thus increasing water potability. Considering that the first steps of conventional treatment do not remove the toxins, the aim of this study was to investigate the toxicity of treated water containing cylindrospermopsin after chlorine addiction. It was analyzed DNA damage and viability of HepG2 cells exposed to the following treatments: cylindrospermopsin (CYN) containing extract of *Cylindrospermopsis raciborskii*; this same extract added to treated water; non toxic *C. raciborskii* (all extracts at concentration of 0.1, 0.5 and 1 µg of dry material mL^{-1}), and treated water only. Cells were exposed for 24, 48 and 72 hours. A decrease in cell viability of HepG2 cells was observed after the treatment with toxic *C. raciborskii* extract (at 0.5 and 1 µg mL^{-1} for all times of exposure) and the toxic extract with treated water at the two higher concentrations at 48 and 72 hours. Comet assays also revealed DNA damage in HepG2 cells under toxic *C. raciborskii* extract. Data indicated that chlorine can prevent damage to DNA and to cell viability in most of conditions. In conclusion, chlorine addiction in conventional water treatment has a potential to provide protection or reduce toxic effects of CYN.

Keywords: cell viability, cylindrospermopsin, chlorination, DNA damage.

Efeito ao DNA e viabilidade celular de água tratada contaminada com extrato de *Cylindrospermopsis raciborskii* produtor de cylindrospermopsina

Resumo

É bem conhecida a ação do cloro na oxidação de cianotoxinas, permitindo a potabilidade da água. Considerando que as etapas do tratamento convencional de água não removem as cianotoxinas, o objetivo deste estudo foi investigar a toxicidade da água tratada com cloro acrescentada da cianotoxina cilindrospermopsina. Neste sentido foi analisado o dano ao DNA e a viabilidade celular em células HepG2 expostas aos seguintes tratamentos: extrato de *Cylindrospermopsis raciborskii* produtor de cilindrospermopsina (CYN); este mesmo extrato adicionado a água tratada; extrato não tóxico de *C. raciborskii* (Todos os extratos continham as concentrações de 0,1, 0,5 e 1 µg de material seco. mL^{-1}) e somente água tratada. As células foram expostas por 24, 48 e 72 horas. Foi observado decréscimo na viabilidade celular em células HepG2 após tratamento com o extrato tóxico de *C. raciborskii* nas concentrações de 0.5 e 1 µg mL^{-1} em todos os tempos de exposição e com água tratada acrescida do extrato tóxico nas duas concentrações mais altas em 48 e 72 horas de exposição. Ensaio cometa revelou dano ao DNA em células HepG2 expostas ao extrato tóxico de *C. raciborskii*. Dados indicaram que o cloro pode prevenir o dano ao DNA bem como a viabilidade celular na maioria das condições testadas. Como conclusão, a adição do cloro no tratamento de água convencional tem o potencial de proteger ou reduzir os efeitos tóxicos da CYN.

*Palavras-chaves***:** viabilidade celular, cilindrospermopsina, cloração, Dano ao DNA.

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INTRODUCTION

The presence of toxic cyanobacterial blooms in natural waters used for drinking or for recreational purposes may present serious risks to the human health. The cyanotoxin cylindropermopsin (CYN) is produced by the cyanobacteria *Cylindrospermopsis raciborskii, Anabaena, Umezakia* and *Aphanizomenon* (Falconer, 2005). Of these species, the most widely distributed is *Cylindrospermopsis raciborskii* (Briand *et al*. 2004; Bouvy, *et al*. 2000). CYN has been detected in water bodies in Australia (Shaw *et al.* 1999; Schembri *et al.* 2001; Seifert *et al*. 2007), New Zealand (Stirling & Quilliam, 2001) Europe (Preussel *et al*. 2006; Quesada *et al*. 2006; Spoof *et al.* 2006; Brient *et al.* 2009), Asia (Harada *et al.* 1994; Banker *et al.* 1997; Li *et al.* 2001) and the Americas (Azevedo, 2002; Bittencourt, 2011).

The toxin is an alkaloid that acts as a potent protein synthesis inhibitor. The main target of CYN in vertebrates is the liver, but other organs such as the kidney, thymus, adrenal glands, lungs, intestinal tract and heart are also affected (Wormer *et al.* 2008).

Humpage *et al.* (2000, 2005) and Shen *et al.* (2002) showed that CYN induced genotoxicity, and Falconer & Humpage (2001) demonstrated a carcinogenic activity. Due to all of these effects, along with observed hepatotoxic and cytotoxic damage, a debate recently arose at the World Health Organization (WHO) regarding whether or not the published information on CYN is sufficient to establish a guideline value for drinking water (Rodriguez *et al.* 2007). In this context, the guideline value recommended by the WHO is 15µg L^{-1}. However, the value of 1 µg L^{-1} proposed by Humpage & Falconer (2003) is used in some locations in Australian and was incorporated as guideline into New Zealand legislation (Chorus, 2005).

Due to its high solubility in water and low rate of biodegradation and photo degradation, significant amounts of the toxin can be expected to occur in the water column (Wormer *et al.* 2008, 2010). This was first observed by Norris *et al*. (2001) in an experimental study with toxic *C. raciborskii* cultures where it was found a maximum amount of 556 µg L^{-1} of CYN in the extracellular medium.

CYN can be eliminated from natural waters with some of the oxidants and disinfectants typically applied during the treatment of water. There are some data that show the efficiency of chlorine in the oxidation of cyanotoxins, mainly with all types of microcystins, thus increasing water potability. However, the inactivation of saxitoxins, CYN and anatoxin-a by chlorination has not been well studied (Westrick, 2008).

Disinfection by chlorination has been evaluated as a feasible process to remove CYN from water at pH values of six to nine and with low concentrations of total organic carbon; a residual chlorine concentration of 0.5 mg L^{-1} would be sufficient to degrade 99% of this cyanotoxin (Senogles et al. 2000). However, some studies have shown that cyanobacteria and cyanotoxins are precursors to the production of chlorinated byproducts (Di Bernardo, 1995; Sales, 2005; Kuroda, 2006).

In many countries, chlorine is commonly used at water treatment plants, and the values of dissolved organic carbon (DOC) in reservoir waters are considered high (4 -11 mg L^{-1}). Therefore, the potential for producing toxic byproducts might be increased, and the toxicities of most arising substances have been poorly evaluated.

Ninety-day and 170-day oral toxicity studies of chlorinated solutions with microcystin, saxitoxin and CYN byproducts produced ill effects such as fatty vacuolations of liver cells in mice (Senogles-Derham, 2003).

Considering this, the aim of our study was to determine whether DNA damage, which is a sensitive and early toxicity response, occur *in vitro* in human hepatocytes (HepG2 cells) exposed to dissolved cellular content of *C.raciborskii* (containing also CYN) in water after conventional treatment.

MATERIALS AND METHODS

Treated water sampling

The treated water samples were collected at a rate of 50 mL per hour to generate final integrated samples (drinking water) representative of 24h of treatment at the Boa Esperança City water treatment in Minas Gerais State/Brazil. The conventional process at this treatment plant has the following steps: coagulation, flocculation, decantation, filtration and disinfection. As the final step of treatment, 0.8 mg L^{-1} of free chlorine is added to water for 30 minutes of contact time (the time required by Brazilian Legislation MS 2.914/2011 for drinking water). In this study, the same final step was repeated in the laboratory due to chlorine evaporation during the transportation of water samples.

Extraction of toxic and non-toxic C. raciborskii strains

The strain CYP-011K (CYN producer) was kindly provided by Dr. Andrew Humpage (Australia) and the non-toxic (non-CYN producer) strain NPLP was isolated from the Paranoá lake in Brazil ($15^0$46'- 14"S and $47^0$49'- 21"O). The cultures were maintained at 20 ± 3ºC in the Laboratory of Ecophysiology and Toxicology of Cyanobacteria (Federal University of Rio de Janeiro) in ASM1 medium (pH 8.0) under continuous aeration, with 40 µmoles photons/m^2/s of light intensity, and with a 12/12h photoperiod. During the end of exponential phase (approximately 15 days), the cultures were centrifuged, lysed by freeze–thawing and filtered through 0.45µm glass fiber filters (Millipore) to remove the debris. The *C.raciborskii* extracts were produced with a cell density equivalent to bloom conditions (10^6cells mL^{-1}).

Treatments

C. raciborskii extract containing cylindrospermopsin (Cyl toxic extract) was added to treated water reaching a toxin concentration of 990 µg L^{-1} (cell density equivalent to 10^6cells mL, bloom condition). Chlorine was added to a final

concentration of 0.8 mg L^{-1} and 30 minutes of contact time in order to simulate that step of the water treatment and the oxidation process that could occur in treatment of the water containing dissolved substances of a senescent bloom of *C. raciborskii* (represented by the toxic extract).

The four treatments were: treated water; treated water + *C. raciborskii* (Cyl toxic extract); Cyl toxic extract and Cyl non-toxic extract. After 30 minutes of contact time, 70 mL of each treatment was lyophilized, and the obtained mass was used for preparing the solutions for the comet and viability assays with the cells. At the same time, samples were collected for quantification of remaining CYN and some of the chlorination byproducts. It is worthy to say that the treated water was previously analyzed for CYN presence by ELISA immunoassay and the result indicated that the concentration was below the limit of detection which confirms the absence of this toxin, once there were no potentially CYN producer cyanobacteria species in the water body.

HepG2 cell culture

HepG2 cells were cultured at 37°C in a humidified 5% CO_2 atmosphere in plastic dishes in Dulbecco's modified Eagle's medium, supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine and antibiotics (100 units mL penicillin and 100 µg mL^{-1} streptomycin). Exponentially growing cells were used for all experiments.

HepG2 cells were previously treated with the original solutions of the 4 treatments after the 30 minutes of contact time. The results showed the need for additional dilutions. That was done by lyophilizing the solutions and diluting the dry material into ultrapure water, following centrifugation, to the final concentrations of 0, 0.1, 0.5, 1 µg mL^{-1}. The final diluted solutions eventually reached toxin concentrations corresponding to 0, 30, 150 and 300 µg L^{-1} of CYN, respectively, and the cells were exposed in the experiment for 24, 48 and 72 hours.

Propidium iodide cell viability assay

After the time of the exposure mentioned above, viability of HepG2 cells was determined by the propidium iodide cell viability assay. This method uses propidium iodide that does not enter cells with intact cell membranes, but can penetrate the membranes of dying/dead cells. Once inside the cell, the dye binds to intracellular structures, producing highly fluorescent adducts that identify the cell as non-viable. The cells were washed twice with PBS, trypsinized, centrifuged and resuspended at a concentration of 1 $x10^6$ cells mL in a staining buffer (10 mg mL propidium iodide in PBS). The cell suspension was incubated for 15 min in the dark at room temperature. Thereafter, the samples were analyzed using a FACS can flow cytometer (Becton Dickinson). A computer system (CellQuest Pro, Becton Dickinson) was used for data acquisition and analysis. Data from 20,000 events were stored. A cell gate containing HepG2 cells was established on the basis of the forward and side light scatters.

Comet assay

After 24, 48 and 72 hours, the comet assay was performed according to Singh et al. (1988) with slight modifications as follows. In brief, 200 µL of cell suspension containing about 100,000 cells was mixed with 200 µL of 2% low-melting temperature agarose at 37˚C and then placed on a slide pre-coated with a dried thin layer of 0.5% normal-melting agarose. The cell suspension was covered with a cover slip, and the slides were kept at 4˚C for 5 min to allow for solidification of the agarose. After removing the cover slip, the cells were lysed in a lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, and pH10) for 1 hour. After washing in re-distilled water, the slides were placed on a horizontal gel electrophoresis chamber. The chamber was filled with electrophoretic buffer (1mM EDTA, 300 mM NaOH, pH 13), and the slides were maintained in this buffer at 4˚C for 20 minutes to allow the DNA to unwind. Electrophoresis was performed for 20 minutes (0.9V/cm, 300 mA). After electrophoresis, the slides were washed three times with a neutralization buffer (0.4 M Tris, pH 7.5). All preparative steps were conducted in the dark to prevent additional DNA damage. The slides were stained with 1 µM DAPI for 24 hours and analyzed with a fluorescence microscope (NIKON Eclipse 400) equipped with a CCD-4230 A video camera. Digital images were obtained using MultiScan software (Poland). At least 100 images per dose and treatment time were analyzed, and the % of Tail DNA was determined using CASP software (Konca *et al.* 2003). Standardization of the comet assay results was adopted from Bergquist *et al*. (1998) and was made by dividing the actual value of % of Tail DNA obtained from the treated cells by the value obtained from the non-treated control cells included in the same electrophoresis run. The results are presented as the average values (± S.D.) from four independent experiments.

Chemical analyses

The CYN concentration in the treatments solutions was determined with ELISA immunoassay commercial kits (Beacon® Analytical Systems, Portland-ME, USA) following the procedure recommended by the manufacturer.

Only four main types of chlorinated byproducts (bromodichloromethane, bromoform, chloroform and dibromochloromethane) were analyzed. The analyses followed the procedure described at USEPA (1994). Before submit samples to the lyofilization as mentioned above, a test was done to verify the potential volatilization of those byproducts by that process. The results indicated that lyofilization did not alter the concentration of those substances.

In Brazil, particularly at the Boa Esperança City water treatment plant, calcium hypochlorite ($Ca(OCl)_2$) is used as the chemical disinfectant. For this experiment, a stock solution of 6 gL^{-1} of available chlorine (approximately 65%) was prepared with ultrapure water (Milli-Q). The free chlorine was determined colorimetrically by the DPD procedure (HACH, 1993) using a HACH DR/2000 spectrophotometer.

Statistical analysis

The results are presented as mean values and standard deviations obtained from four independent experiments. Data were analyzed by ANOVA followed by Mann-Whitney Rank Sum test. Statistical significance was tested at $p < 0.05$ as the critical value.

RESULTS

In the treatments of treated water only, treated water plus Cyl toxic extract and Cyl non-toxic extract, no DNA damage effects were observed (Fig. 1). The DNA damage caused by Cyl toxic extract was detected at the concentration of 1 µg mL^{-1} (=300 µg of CYN L^{-1}) from 24h of exposure and at 0.5 µg mL^{-1} (=150 µg of CYN L^{-1}) after 48 and 72 h of exposure (Fig.1).

In the treatment of Cyl toxic extract, the cell viability was reduced in all concentrations tested and in all times of exposure with exception for the lowest CYN concentration, where cell viability decline was observed only after 72h (Fig. 2). That response could be attributed to toxin effects, such as the inhibition of the protein synthesis. However, the chlorine present in treated water seemed to prevent the toxic effects of CYN, since that in the treatments where both were present, the cells had viability reduced only in the highest concentration of the toxin and after longer times of exposure (48 and 72h). Interestingly, the ELISA analysis detected the same concentration of CYN before and after chlorine addiction, indicating that the oxidant, at concentration of 0.8 mg L^{-1}, was not effective in destroy the molecule, but, some how interfered in the biologic activity.

The values of total trihalomethanes (THMs) were below the guideline value (100 µg.L^{-1}) required by Brazilian Legislation MS 2.914/2011 for drinking water. Any of the four types of byproducts were detected above the mentioned guideline. However, CYN extract did generate an increase in the DOC values in treated water (data not shown), and in this context, this may be the reason why 0.8 mg L^{-1} of free chlorine and 30 minutes of contact time were not enough to destroy the toxin (Table 1).

DISCUSSION

The presence of cyanobacteria causes serious water-quality problems by producing taste, odor and toxic effects through several compounds and toxins, and chlorination is an effective method to destroy these cyanotoxins. The efficiency seems to depend largely on the chlorine compounds and concentrations used (Hitzfeld *et al*. 2000). Aqueous chlorine and calcium hypochlorite at 1 mg L^{-1} remove more than 95% of microcystins or nodularin, while sodium hypochlorite at the same dose or chloramine achieve 40-80% removal of most microcystins (Nicholson *et al*.1994).

Cyanobacteria toxins can be found intracellularly and extracellularly in water, and the efficiency of its removal depends on that. Moreover, the total concentration of the toxins is also determinant (Westrick, 2008). In contrast to

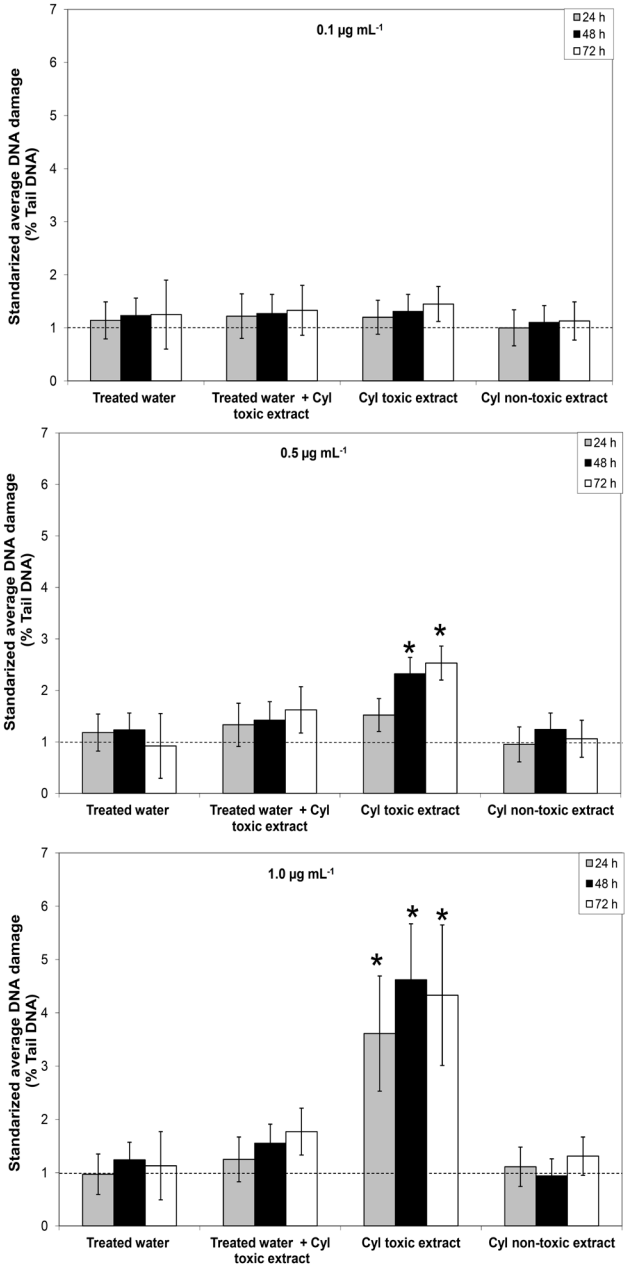

Fig.1 - Standardized average DNA damage [% Tail DNA] in HepG2 cells exposed *in vitro* to treated water, treated water plus *Cylindrospermopsis raciborskii* (Cyl) toxic extract, Cyl toxic extract and Cyl non-toxic extract at concentrations of 0.1; 0.5 and 1 µg of liophylized material mL for 24, 48 and 72 hours. Data are presented as mean values ± SD from four independent experiments. Dotted horizontal line represents the control average value.

other cyanotoxins, a high proportion of CYN in growing *C. raciborskii* blooms may be found free in the water (Chiswell *et al*. 1999). Depending on the circumstances, only the proportion of CYN that is cell-bound can be removed by coagulation and filtration in a conventional treatment plant (Chorus & Bartram, 1999) and the major extracellular fraction can persist in the water for weeks without degradation (Wormer *et al*. 2008, 2010).

In Brazil, specifically in the Minas Gerais State, the conventional treatment is common and used extensively. Therefore, there is a significant possibility that this process does

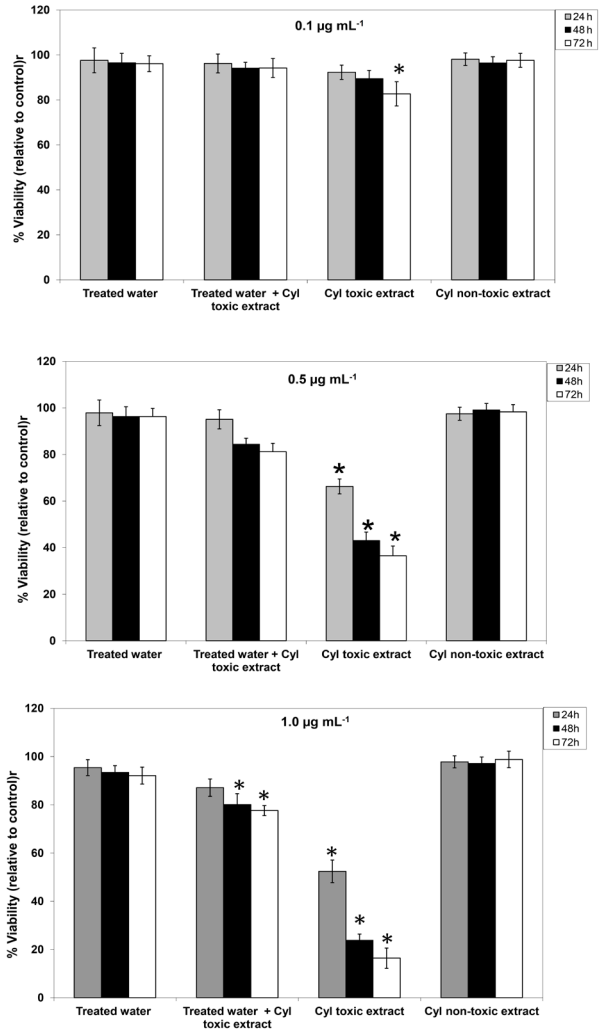

Fig. 2 - Viability of HepG2 cells exposed *in vitro* to treated water, treated water, treated water plus *Cylindrospermopsis raciborskii* (Cyl) toxic extract, Cyl toxic extract and Cyl non-toxic extract at concentrations of 0.1; 0.5 and 1 µg of liophylized material mL for 24, 48 and 72 hours. Data are presented as mean values ± SD from four independent experiments.

not remove CYN. Chorus & Bartram (1999) recommends that the best-practice treatment for removal of CYN would include conventional treatment followed by an oxidation step.

Rodriguez *et al*. (2007) found that a concentration of 1.5 mg L^{-1} of free chlorine was enough to oxidize CYN in surface raw water almost completely, and produced around 82 µg L^{-1} of THM, a level well below the standard value for drinking water according to international legislation (100 µg L^{-1}). The initial concentration of CYN in the mentioned study above was 9.4 µg L^{-1} a value significantly below that used in the present work. And in our case, there was no CYN destruction, or at least a light degradation, at the 0.8 mg L^{-1} of free chlorine, since the ELISA antibodies were able to detect the molecule. DOC concentration in our samples was low (data not shown)

Table 1 – Mean values of cylindrospermopsin by immunoassay (ELISA)

Treatments	µg CYN L^{-1}
Cyl toxic extract	945 ± 39.5
Treated water + Cyl toxic extract	959 ± 29.1

e could not compete with the toxin in the oxidation process. Therefore, those observations suggest that when a higher CYN concentration is present in the water, a higher chlorine concentration is necessary to destroy the toxin.

The viability of HepG2 cells exposed to treated water plus Cyl toxic extract was less pronounced than in the treatment with Cyl toxic extract alone. That is in accordance to some reports showing that chlorine can act on CYN to form byproducts as 5-chloro-cylindrospermopsin and cylindrospermopsic acid (Senogles-Derham, 2003; Rodriguez *et al.* 2007) and to i*n vivo* studies showing that these by products are less toxic than original CYN (Banker *et al*. 2001). Merel *et al.* (2010) also showed a protective effect of chlorine, once it reduced the citotoxicity of CYN to human CaCo-2 cells.

Our results are in agreement with data presented by Humpage *et al.* (2005), which demonstrate the ability of CYN (0.05-0.5 µM) to induce DNA strand breaks in primary mouse hepatocytes. Straser *et al*. (2011) also showed DNA damage evaluated by comet assay (24h) on HepG2 cells exposed to 50 µg L^{-1} and 500 µg L^{-1} of CYN (a similar range to the present study). Our data suggest that the effects caused by CYN extract only treatment is mainly related to CYN since the non-toxic *C. raciborskii* extract did not induce any damage in HepG2 cells.

Taking into consideration all data (Figs. 1 and 2), we cannot exclude the possibility that the observed DNA damage could be related to cytotoxic rather than genotoxic effects of CYN and CYN-related byproducts. The comet assay is widely used to investigate DNA damage induced by genotoxins, but the data produced can be subject to misinterpretation and bias due to comets formed from cells undergoing apoptosis and necrosis. This is because it has not been possible to distinguish comets formed by viable cells from those formed by non-viable (apoptotic or necrotic) cells (Morley *et al*. 2006).

It is likely that the observed toxic effects were not produced by the four main types of chlorination byproducts analyzed, once they were present at very low concentrations. However, Sales (2005) confirmed the possibility that members of *Cylindrospermopsis* genus could produce haloacetic acids (HAAs) and TMHs. This author found experimentally that *C.raciborskii* at 4.4x10^5cells mL with 2 mg L^{-1} of free chlorine produced 187 µg L^{-1} of those byproducts, a value above of the international guideline.

In this study, the concentration of CYN in the toxic extract - 300, 150, 30 µg mL^{-1} corresponded to 10.000, 5.000 and 1.000 cells mL, respectively. When the water body presents cell counts above 10^4 cells mL, it is considered that an initial bloom is taking place (Chorus & Bartram, 1999). Our data indicate that when a bloom condition is effectively established, and consequently the concentration of toxins can reach higher levels, 0.8 mg L^{-1} of free chlorine could not completely prevent the toxic effects of CYN.

In Brazil, toxic *C.raciborskii* is found frequently producing saxitoxins (Sant'Anna *et al.* 2008). However, the presence of CYN was detected (along with microcystins) in the activated carbon used for water treatment in the Caruaru

tragedy (Pernambuco State, in 1996). In that episode, 52 renal patients died after hemodialysis treatment with contaminated water, but unfortunately the cyanobacteria genus responsible for that toxin production is still unknown (Azevedo *et al.* 2002). Recently, cells of *Cylindrospermopsis* were detected producing CYN in a reservoir from Minas Gerais State (Moreira & Bizi, 2007 and by Bittencourt, 2011). This fact represents a growing potential risk to public health through drinking water chronic and sub lethal exposures.

CONCLUSIONS

The present work confirmed the importance of *Cylindrospermopsis* blooms monitoring since cylindrospermopsin, potentially produced by that cyanobacteria genus, can induce DNA damage and diminish cell viability. Our data showed that, in a bloom condition, the usual chlorine addiction in conventional water treatment plants has a potential to reduce but do not completely eliminates toxic effects of CYN.

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