In vivo genotoxicity of treated water containing the cylindrospermopsin-producer *Cylindrospermopsis raciborskii*

A. L. Fonseca, J. Da Silva, E. A. Nunes, S. M. F. O. Azevedo and R. M. Soares

ABSTRACT

Cylindrospermopsin (CYN) is an alkaloid commonly produced by some cyanobacteria that has been implicated in outbreaks of human illness. The aim of this study was to investigate the genotoxicity of *Cylindrospermopsis raciborskii* cellular content (including CYN) and its byproducts resulting from chlorination during water treatment. DNA damage in blood and liver cells was analysed by the comet assay and micronucleus test (MN). Mice were injected intraperitoneally with the following treatments: (a) physiological saline, (b) treated water, (c) treated water plus *C. raciborskii* extract (CYN producer strain, CYPO-011 K), (d) *C. raciborskii* extract (CYN producer strain, CYPO-011 K), (e) *C. raciborskii* extract (CYN non producer strain), and (f) treated water plus *C. raciborskii* extract (CYN non producer strain) extract. After 48 h, samples were taken to perform tests (blood and liver cells to the comet assay and bone marrow to MN test). The CYPO-011 K had a genotoxic and mutagenic effects on liver and bone marrow cells. The group that received chlorine-treated water plus CYPO-011 K also exhibited genotoxic effects in the liver, as well as in the blood, and a mutagenic effect in blood marrow cells. The results emphasise the need of improving CYN monitoring in waters bodies in order to reduce the risk of human exposure.

Key words | chlorination, comet assay, cylindrospermopsin, drinking water, micronucleus test

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INTRODUCTION

High densities of toxic cyanobacteria in natural waters that are used for drinking or for recreational purposes may present serious risks to human health (Chorus 2005).

Among the 150 known genera of cyanobacteria, 40 include toxin-producing species. *Cylindrospermopsis raciborskii* (Woloszynska 1912; Seenaya & Subba Raju 1972) is widely distributed worldwide and is a potential toxin producer (Bouvy *et al.* 2000; Briand *et al.* 2004). One of the toxins produced by *C. raciborskii* is cylindrospermopsin (CYN), which has previously 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 South America (Azevedo *et al.* 2002; Bittencourt-Oliveira *et al.* 2011).

CYN is frequently categorised as a cytotoxin that mainly targets the liver in mammals. Several other organs, such as the kidneys, lungs, and intestines, can also be affected (Codd *et al.* 2005; Wormer *et al.* 2008) by this guanidine alkaloid that acts as a potent protein synthesis inhibitor (Terao *et al.* 1994). Using both the micronucleus test (MN) and

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the alkaline, the comet assay, Humpage *et al.* (2000, 2005), Štraser *et al.* (2011), Zegura *et al.* (2011a), demonstrated that CYN induces genotoxicity *in vitro*.

Other authors have suggested that *in vivo* genotoxic effects depend on CYN metabolisation (Fessard & Bernard 2003; Bazin *et al.* 2012). *In vivo* genotoxicity studies by Shen *et al.* (2002) and Shaw *et al.* (2000) reported DNA strand breakage and the induction of DNA adducts in mouse livers. Similarly, Bazin *et al.* (2012) observed genotoxicity of CYN (by the comet assay) in mice, in which the main effects occurred in the colon. A revision about the genotoxic effects and potential carcinogenicity of CYN for *in vitro* and *in vivo* studies is summarized in Zegura *et al.* (2011b).

Due to its high water solubility and low rate of bio and photodegradation, significant amounts of CYN can likely be found in the water column (Wormer *et al.* 2008, 2010). The guideline value recommended by the WHO is 15 $\mu g.L^{-1}$. However, the value of 1 $\mu g.L^{-1}$ proposed by Humpage & Falconer (2003) is used in Australia and has been incorporated as a guideline in New Zealand legislation (Chorus 2005) and recommended in Brazilian legislation (MS 2.914/2011 for drinking water). A CYN concentration of 1 $\mu g.L^{-1}$ is generally associated with cell densities of approximately 20,000 cells mL^{-1}, this cell number threshold has been adopted in monitoring programs in some countries (McGregor & Fabbro 2000; MS 2.914/2011).

Chlorination is perhaps the most common treatment for the oxidation of dissolved algal toxins (Svrcek & Smith 2004; Acero *et al.* 2005; Rodriguez *et al.* 2007a; Merel *et al.* 2010b). This process is normally used as a disinfection step in the final part of the drinking water treatment plant. It typically involves exposing the water to variable doses of chlorine for 30 min at an alkaline pH, until break point concept (Chorus & Bartram 1999; Senogles *et al.* 2000; Rodriguez *et al.* 2007a). Among cyanotoxins, chlorine effectively removes microcystin, nodularin, saxitoxins, and CYN (Merel *et al.* 2010b). However, characterization of the by-products of chlorination, which may also be toxic, has been limited (Svrcek & Smith 2004). Chlorine has been shown to provide significant CYN transformation. In one study, by-products such as 5-chloro-cylindrospermopsin and cylindrospermopsic acid were identified (Rodriguez *et al.* 2007a). In another report, Merel *et al.* (2010a) indicated the formation of a third, unidentified cylindrospermopsin by-product.

It is important to consider that, at the final step of the disinfection process, it is still possible to find remaining cells of cyanobacteria in the water. Certainly, with the action of chlorine those would be lysed and would release the cellular content which may include cyanotoxin(s). In this context, the main objective of this study was to investigate genotoxicity effects of treated water contaminated with *C. raciborskii* (CYN producer) cellular contents and its by-products (potential total trihalomethanes – THMs) originating from chlorination, in a murine model *in vivo*.

MATERIAL AND METHODS

Treated water sampling

Treated water samples were collected at a rate of 50 mL per hour to generate final integrated samples of drinking water that were representative of 24 h of treatment at the Boa Esperança City water treatment plant in Minas Gerais State, Brazil. The conventional water treatment process at this treatment plant includes the following steps: coagulation, flocculation, decantation, filtration, and disinfection. In this final step, after reaching the breaking point, an extra dose of chlorine is added with 30 min of contact time in order to reach 0.5 $mg.L^{-1}$ of free (residual) chlorine in the drinking water before distribution (information provided by water treatment plant managers). This procedure was necessary in order to restore the free chlorine concentration in treated water lost by volatilization during transport to the laboratory.

The treatments containing CYN were prepared mixing water treated (sample with 0.5 $mg.L^{-1}$ of free chlorine) with a dose of the extract of CYPO-011 K (CYN producer) equivalent to a cell density of 10^6 $cell.mL^{-1}$. The time of mixture was 30 min. Both extracts (CYN producer and non-producer) were diluted using ultrapure water containing 5% of the phosphate buffered saline (PBS) solution.

The final CYN concentration, either in treated water with CYP-011 K extract and/or in diluted CYP-011 K extract, was the equivalent to a cell density of 10^6 $cell.mL^{-1}$ (maximum bloom in field conditions).

The treated water from the Boa Esperança water plant was also previously analysed for the presence of CYN, and the results indicated that the CYN concentration was

below the limit of detection (0.3 ng.mL^{-1}). This result suggested that CYN was absent mainly because there were no potential CYN-producing cyanobacterial species in the water body and consequently there were not CYN by-products generated by chlorination in the water samples.

Chemical analyses

To determine the CYN concentration in the treatment solutions, analyses were conducted by liquid chromatography coupled to electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) in positive mode. Following modified procedures recommended by Eaglesham *et al.* (1999), a 3,200 Q trap manufactured by Applied Biosystems was used to complete the LC/ESI-MS/MS analyses.

For the chromatography analysis, a Kinetex 2.6 μm 100A C18 column was used. The mobile phase was isocratic conditions using 60% of ammonium acetate (2 mM +0.1% formic acid) as phase A, and 40% of methanol as phase B for 6 min of the chromatographic run. The injected volume was 10 μL with a flow rate of 800 μL/min. The following transitions were analysed: m/z 416 $(M + H)^+$ > m/z 194 for quantification and m/z 416 $(M + H)^+$ > m/z 336 for confirmation. For sample quantification, a calibration curve ranging from 0.625 to 20 ng/ml, constructed with a standard stock solution at 30 μM, purchased from the Institute for Marine Bioscience (NRC-CNRC – number 20050531) was used.

The disinfection by-products (DBPs) commonly present in drinking water comprise four main types of chlorinated: bromodichloromethane, bromoform, chloroform, and dibromochloromethane. This group of DBPs is referred to as total trihalomethanes (THMs) and in the present study was analysed following the procedure described by the USEPA (1994) by Companhia de Saneamento de Minas Gerais (COPASA).

Calcium hypochlorite (Ca $(OCl)_2$) is normally used as the chemical disinfectant. A stock solution of 6 g.L^{-1} of available chlorine (approximately 65%) was prepared from $Ca(OCl)_2$ and ultrapure water (Milli-Q) and used to restore the free chlorine concentration in the water treated. The concentration of free chlorine in the solutions was determined with a spectrophotometer model HACH, DR 2000.

A test was conducted to determine the oxidation efficiency of $Ca(OCl)_2$ using an extract generated from a lower cell concentration (lower organic matter load). A treated water sample from the Boa Esperança City water plant was contaminated with a CYP-011 K extract equivalent to 10^4 cell.mL^{-1} and 19.6 μg of CYN. The procedures and the addition of chlorine followed the main experiment as described in treated water sampling section.

Culture conditions and preparation of toxic and non-toxic *C. raciborskii* strain extracts

The CYP-011 K *C. raciborskii* strain (CYN producer) was kindly provided by Dr Andrew Humpage of the Australian Water Quality Centre (Adelaide, Australia), and the non-toxic (CYN non producer) NPLP *C. raciborskii* strain was isolated from Paranoá Lake in Brazil (15°46′14″S and 47°49′21″O). The cultures were maintained at 20 ± 3 °C in ASM-1 medium (Gorham *et al.* 1964) with pH adjusted to 8.0, under continuous aeration, with 40 μmol photons/m^2/s of light intensity and a photoperiod of 12 h. Cyanobacterial cells were harvested by centrifugation and repeatedly frozen/thawed to disrupt the cells. The extract was then filtered using a 0.22-μm membrane to remove cell debris.

The CYN concentrations in the final extract of CYP-011 K and in the treatments were determined by LC/MS/MS, as described below.

Animals and treatments

In total, 20 male, 6-week-old Swiss mice were used in this study. The mice weighed 32.76 ± 2.65 g and were provided by the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil). The animals were kept in plastic cages for 1 week before the experiments started and had free access to tap water and a rodent pellet diet. The animals were maintained under a 12 h light/dark cycle at a constant temperature of 25 ± 2 °C and a relative humidity of approximately 60%. The experiments were approved by the Ethics Committee of the Health Sciences Center at the Federal University of Rio de Janeiro.

Animals were exposed to six treatments: (a) physiological saline (negative control, 0.9% NaCl); (b) treated water (from water treatment plant); (c) treated water plus CYP-011 K extract (CYN concentration of 832 μg.L^{-1} – equivalent to a cell density of 10^6 cell.mL^{-1}); (d) CYP-011 K extract

(CYN concentration of 870 μg.L^{-1} – equivalent to a cell density of 10^6 cell.mL^{-1}); (e) NPLP extract; and (f) treated water plus NPLP extract.

The animals (four per group) were intraperitoneally injected (i.p.) once with each treatment solution at a volume of 1 mL/30 g of body weight. The toxin concentration of solutions containing CYN generated a dose of 28 μg/Kg of body weight of injected animal. This dose was below the value of CYN No-Observed-Adverse-Effect Level (NOAEL) which is 30 μg/kg/day, established by Humpage & Falconer (2003). However, considering that the mentioned study did not evaluate genotoxic effects and these normally rise under lower doses, we consider that it would be important to investigate DNA damage in animals subject to such realist and low dose.

The mice were euthanized by cervical dislocation after 48 h, and blood samples were taken for the micronucleus test and the comet assay. The liver was also removed and analysed for DNA damage by the comet assay.

The comet assay

DNA damage was assessed by the alkaline the comet assay, which was performed according to Tice *et al.* (2000) and Da Silva *et al.* (2000) with heparinised blood and homogenised liver samples. Liver cells were obtained according to the method described by Tice *et al.* (2000). Two replicates of 10 μL cell sample aliquots were embedded in 95 μL of 0.75% (w/v) low melting point agarose (LMA-Invitrogen) at 36 °C. The mixture was added to a microscope slide that was pre-coated with 1.5% (w/v) normal melting point agarose and covered with a cover slip.

The slides were immersed overnight at 4 °C in the dark in a lysis solution (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris buffer, pH 10.0–10.5) containing freshly added 1% Triton X-100 (Sigma-Aldrich) and 10% dimethyl sulphoxide (DMSO). After washing, the slides were placed in a horizontal gel electrophoresis chamber in an alkaline buffer (300 mM NaOH and 1 mM EDTA, pH >13) for 20 min for DNA unwinding. Electrophoresis was performed for 15 min at 300 mA and 25 V (0.7 V/cm). All of these steps occurred under indirect light to prevent additional DNA damage.

The slides were neutralised in 400 mM Tris buffer (pH 7.5), fixed (15% w/v trichloroacetic acid, 5% w/v zinc sulphate, 5% glycerol), washed and air-dried overnight. After rehydration, the gels were stained for 15 min (37 °C) with the following: 34 mL of a solution of 0.2% ammonium nitrate (w/v), 0.2% silver nitrate (w/v), 0.5% tungstosilicic acid (w/v), 0.15% formaldehyde (v/v), and 5% sodium carbonate (w/v); and 66 mL of 5% sodium carbonate (w/v). Staining was stopped with 1% acetic acid, and the gels were air-dried (Nadin *et al.* 2001) before analysis by optical microscopy (Olympus).

Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analysed from each animal; all slides were coded for blind analysis. To calculate the damage index (DI), cells were visually grouped into five classes according to tail size (0 = no tail and 4 = maximum-length tail), which resulted in a single DNA damage score for each sample and, consequently, for each group studied. Thus, the DI of the group could range from 0 (completely undamaged = 100 cells × 0) to 400 (maximum damage = 100 cells × 4). The damage frequency (DF in %) was calculated for each sample based on the number of cells with a tail versus those without a tail (Da Silva *et al.* 2000).

Micronucleus test

The MN test was also performed to verify DNA damage, but in this study, this assay was used as an indirect measure of structural or numerical chromosomal aberration induction. The assay was performed according to the US Environmental Protection Agency Gene – Tox Program (Mavournin *et al.* 1990) and recommendations from Hayashi *et al.* (1990). Immediately following sacrifice, two whole blood smears per animal were taken and prepared on slides. The slides were stained with 5% Giemsa, air-dried and coded for blind analysis. To avoid false negative results and as a measure of toxicity, the ratio of polychromatic erythrocytes: normochromatic erythrocytes (PCE/NCE) was scored in 1,000 cells. The incidence of MN was observed in 2,000 cells for each animal (i.e. 1,000 from each slide) by bright-field optical microscopy at a magnification of ×200–1,000.

Data analysis

The results are expressed as the means ± standard deviation. Significant statistical differences ($P < 0.05$) between the six

groups were determined by one-way analysis of variance (ANOVA, Tukey's test).

RESULTS

The levels of total THMs were below the guideline values of USEPA (2004) (80 μg.L^{-1}) and Canadian Drinking Water (2006) and Brazilian Legislation MS 2.914/2011 (100 μg.L^{-1}) for drinking water. None of the four analysed by-products was detected at levels above these guidelines. However, as expected, *C. raciborskii* extracts generated an increase in the dissolved organic carbon (DOC) values in treated water (data not shown). Thus, the increase in DOC does not lead to the generation of more THMs, but the increased DOC may still be a target for free chlorine. This increase in DOC could explain why 0.5 mg.L^{-1} of free chlorine and 30 min of contact time were not enough to destroy the toxin (Table 1). In the test performed to analyse the oxidative potential of chlorine for CYN, 0.5 mg.L^{-1} of free chlorine was able to partially destroy CYN (only a 27% reduction) at a low concentration (19.6 μg.L^{-1}) for the same 30 min of contact time.

Table 2 summarises the comet assay data expressed as the damage index (DI) and damage frequency (DF) in mouse blood and liver cells after 48 h of exposure to treated water and *C. raciborskii* extracts. The group that received treated water plus CYP-011 K extract exhibited significant increases in DI and DF in both blood and liver cells when compared to all other groups. The group that received only the CYP-011 K extract exhibited a significant increase in mean values of DI and DF in liver cells only when compared to the negative control. No DNA damage was observed in the groups that received the treatments containing NPLP extract.

Table 3 shows the results of the MN test. The erythrocytes of mice exposed to CYP-O11 K extract exhibit significant increase in MN when compared with the control and chlorine-treated water groups, except to the group that received only the NPLP extract. In addition, the group treated with water plus CYP-O11 K extract showed increases in MN when compared to the control. There was no significant difference in the PCE/NCE ratios (no toxicity).

Table 1 | Mean values of cylindrospermopsin by MS/MS

Treatments	CYN (μg. L^{-1})
CYP-O11 K extract	870 ± 19
Treated water plus CYP-O11 K extract	832 ± 32

DISCUSSION

In Brazil, the conventional water treatment process consists of coagulation, flocculation, sedimentation, filtration, and

Table 2 | Damage index and damage frequency (comet assay) (mean ± standard deviation) in peripheral blood and liver cells of mice exposed to different treatments for 48 h ($n = 4$ male mice per group)

	Comet assay parameters for exposure period			
	Damage index		Damage frequency	
Groups	Peripheral blood	Liver	Peripheral blood	Liver
Negative control	7.50 ± 3.79	5.25 ± 4.50	7.25 ± 3.30	2.75 ± 2.22
Treated water	6.50 ± 1.29	7.26 ± 6.22	6.00 ± 0.81	9.25 ± 5.56
Treated water plus CYP-O11 K extract	22.50 ± 13.30[a,c,e]	54.00 ± 9.87[b,d,e,f]	18.25 ± 10.28[a,c,e]	37.00 ± 4.55[b,d,e,f]
CYP-O11 K extract	7.50 ± 2.08	14.93 ± 14.63	6.75 ± 2.50	18.75 ± 8.34[a]
NPLP extract	9.50 ± 1.73	15.25 ± 8.02	8.50 ± 1.29	14.00 ± 6.98
Treated water plus NPLP extract	9.50 ± 4.12	16.50 ± 10.21	7.25 ± 2.87	12.00 ± 5.72

[a]Data significant relative to the negative control group at $P < 0.05$.
[b]At $P < 0.001$.
[c]Data significant relative to the treated water group at $P < 0.05$.
[d]At $P < 0.001$.
[e]Data significant relative to the CYP-O11 K extract (CYN producer) group at $P < 0.05$.
[f]Data significant relative to the NPLP extract (CYN non-producer) and treated water plus NPLP extract groups at $P < 0.05$ (ANOVA; Tukey's Test). 100 cells/animal.

Table 3 | Detection of micronuclei (mean ± standard deviation) in erythrocytes of mice exposed to different treatments for 48 h ($n = 4$ male mice per group)

Groups	MN/2,000 cells per animal	Ratio (PCE: NCE)
Negative control	3.00 ± 1.41	0.35 ± 0.11
Treated water	5.33 ± 1.53	0.32 ± 0.10
Treated water plus CYP-O11 K extract	6.75 ± 1.26[a]	0.27 ± 0.10
CYP-O11 K extract (CYN producer)	11.67 ± 0.58[b,c,d,e]	0.44 ± 0.10
NPLP extract (CYN non producer)	5.25 ± 2.22	0.40 ± 0.11
Treated water plus NPLP extract	5.75 ± 2.50	0.30 ± 0.14

[a]Data significant relative to the negative control group at $P < 0.05$.
[b]At $P < 0.001$.
[c]Data significant relative to the treated water group at $P < 0.01$.
[d]Data significant relative to the treated water plus CYP-O11 K extract group at $P < 0.01$.
[e]Data significant relative to the treated water plus NPLP extract (CYN non-producer) group at $P < 0.001$ (ANOVA; Tukey's Test).

disinfection. Chorus & Bartram (1999) recommend that the best treatment process for removal of CYN would include this conventional treatment followed by an oxidation step. Chlorination is a common drinking water disinfection process (Merel *et al.* 2010b).

It is important to understand the processes by which CYN may be removed from water because, compared to other cyanotoxins in *C. raciborskii* blooms, CYN may be dissolved in the water column at higher concentrations (Chiswell *et al.* 1997) and can persist for weeks without degradation (Wormer *et al.* 2008, 2010). Depending on the circumstances, the proportion of CYN that is cell-bound can be removed by coagulation and filtration (Chorus & Bartram 1999). However, such treatments are ineffective for the removal of dissolved extracellular CYN (Himberg *et al.* 1989; Newcombe & Nilcholson 2004; Ho *et al.* 2011).

CYN is vulnerable to chlorine (Senogles *et al.* 2000; Banker *et al.* 2001; Newcombe & Nilcholson 2004; Rodriguez *et al.* 2007a, b), although different effects were obtained depending on the chlorine source, pH values, and temperature. Rodriguez *et al.* (2007a) observed a 1.7 min half-life for CYN when the toxin was exposed to hypochlorous acid and a 14.4 min half-life upon exposure to chlorine dioxide. After chlorinating a cell-free extract of *C. raciborskii*, Senogles *et al.* (2000) concluded that degradation of CYN is reduced at pH values below 6.0. Other studies suggest that the pH should be maintained at 7.0 because higher pH values would decrease the toxin transformation rate (Senogles-Derham *et al.* 2003; Rodriguez *et al.* 2007a, b).

In the present study, at pH 7.7, 0.5 $mg.L^{-1}$ of free chlorine was sufficient to destroy a small percentage of low concentrations of CYN (19 $\mu g.L^{-1}$). These same conditions were not sufficient for the oxidation of a higher toxin concentration (832 $\mu g.L^{-1}$). These results suggest that the chlorine oxidation efficiency is more closely related to the CYN concentration in the water and the DOC) amount than to pH. An increase in DOC was observed in treated water (original concentration, 2.5 $mg.L^{-1}$) when CYP-011 K extract was added (final concentration, 7.6 $mg.L^{-1}$). It can be considered that the competition between chlorine and organic matter (OM) should be taken into account to achieve successful CYN removal. In fact, the occurrence of OM increases the chlorine dose necessary for CYN destruction (Senogles *et al.* 2000; Rodriguez *et al.* 2007a, b) because an additional chlorine demand must be satisfied. For example, Rodriguez *et al.* (2007b) reported that, at pH 7.3 and 20 °C, 1.5 $mg.L^{-1}$ free chlorine was necessary to remove more than 98% of the toxin (415 $\mu g.L^{-1}$) in water containing dissolved organic carbon (6.7 mg/L), ammonia (2.3 μM), and bicarbonate (0.6 mM).

As mentioned in the results, the main DBPs were not detected (in all treatments) above important drinking water guidelines. However, the addition of CYP-011 K extract in the treated water did cause a small increase in the levels of chloroform (data not shown). Although, according to the results of *in vitro* experiments described by Richardson *et al.* (2007), chloroform is not considered genotoxic. The same authors reported up to 600 types of DBPs, including haloacetic acids (HAAs), as products of organic matter oxidation by chlorine. In addition, DNA damage caused by the main HAA has been observed in mammalian cells (analysed by the comet assay) (Fuscoe *et al.* 1996; Plewa *et al.* 2004; Cemeli *et al.* 2006). Thus, it is possible that the genotoxic effects observed in the present work by the comet assay may also have had an influence from other toxic DBPs formed (but not quantified) in chlorinated water in the presence of the extract of *C. raciborskii* due to the increased OM concentration. These substances in

combination with CYN may have promoted a stronger genotoxic effect.

In our study animals that received treated water plus CYP-011 K extract exhibited genotoxic responses in blood and liver cells (DNA damage), and mutagenicity effect in bone marrow cells, but no toxicity occurred (micronucleus test evaluation).

The effect of the CYP-011 K extract alone was observed only in liver cells (DNA damage by the comet assay), confirming that hepatocytes are important targets of CYN genotoxicity (Shen *et al.* 2002; Humpage *et al.* 2005; Štraser *et al.* 2011). Mutagenicity was also observed by increase of micronucleated erythrocytes.

In addition, the MN test reveals significant increases in MN in groups that received CYP-011 K extracts (alone and plus treated water). Different authors demonstrated genotoxic and mutagenic effects of CYN *in vitro* (Humpage *et al.* 2000, 2005; Bazin *et al.* 2010; Štraser *et al.* 2011; Zegura *et al.* 2011a) and *in vivo* (Shaw *et al.* 2000; Shen *et al.* 2002; Bernard *et al.* 2003; Bazin *et al.* 2012). In an *in vivo* study, Bazin *et al.* (2012) observed MN results at 24 h that were similar to those observed at 48 h in the current study. The authors suggested that the formation of MN likely occurs over a longer period of exposure because its likely cause, inhibition of protein synthesis, occurs in a slower cycle. In addition, Štraser *et al.* (2013) demonstrated that MN can also be induced due to formation of DNA double strand breaks (DSBs), after prolonged exposure (e.g. 72 h), in human hepatoma cells, HepG2. In our study toxicity was not observed, that was confirmed by no significant alterations in PCE/NCE ratios, this result is important because avoid false negative results.

The comet assay detects recent lesions that can be repaired, such as breaks and alkali-labile sites, while the MN test can indicate non-repairable damage, such as clastogenic and aneugenic lesions (Da Silva *et al.* 2000; Dhawan *et al.* 2009; Groff *et al.* 2010). A limitation of the Comet assay is that aneugenic effects, which may be a possible mechanism for carcinogenicity, and epigenetic mechanisms (indirect) of DNA damage, such as effects on cell-cycle checkpoints, are not detected (Dhawan *et al.* 2009). Based on this observation it is possible to conclude that CYN extract could induce aneugenic lesions (DNA damage observed only in MN test in relation to the comet assay effect). Similar to our results, Humpage *et al.* (2000) suggest the aneugenic effect of CYN using a specific stain for centromeres that reveal the presence of whole chromosomes within MN.

Some studies of drinking water have suggested that alternative disinfection methods yield considerably fewer mutagenic effects when compared to chlorination processes (for a review, see Richardson *et al.* (2007)). Thus, additional genotoxicity studies would provide new insights into the toxicological effects associated with conventional drinking water production.

The results of the present study contribute to knowledge of the genotoxicity effects of CYN and/or its metabolite(s), as observed in *in vitro* (Humpage *et al.* 2000, 2005) and *in vivo* (Shaw *et al.* 2000; Shen *et al.* 2002; Bazin *et al.* 2012) studies. Some *in vivo* (Norris *et al.* 2002) and *in vitro* (Froscio *et al.* 2003; Humpage *et al.* 2005; Bazin *et al.* 2010) studies have concluded that CYN toxicity is possibly related to cytochrome P450 CYN metabolite(s), suggesting that CYN is a progenotoxin and that its genotoxic effects could be potentiated by these metabolites. Although this enzyme complex was not analysed in our study, these metabolites may have contributed to the observed genotoxic effects. However, this assumption requires further investigation.

CONCLUSIONS

The results obtained in this study confirm that CYN, originating from lysed cells at the density of 10^6 cell.mL^{-1} (bloom density), was not completely destroyed by free chlorine at the concentration normally found at the final step of disinfection after the breaking point in water treatment processes (0.5 mg.L^{-1}). Therefore, in our simulation the treated water to be distributed to population would still be contaminated with that CYN.

The genotoxity and mutagenic effects were observed after a single injection of solutions containing CYN (CYPO-011 K) and CYPO-011 K plus chlorine-treated water (dose below the value of cylindrospermopsin NOAEL established by Humpage & Falconer (2003)).

The present investigation emphasises the need to improve the monitoring of CYN in water bodies used for public supply in order to reduce the risk of human exposure to this toxin.

The authors declare that there are no conflicts of interest.

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