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Genotoxicity detected during cyanobacteria bloom in a water supply reservoir

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Abstract

The aquatic ecosystems are susceptible to cyanobacterial blooms due to the eutrophication of water bodies caused by human activities. In this study, phytoplankton and cyanotoxins analysis, as well as cellular and genetic biomarkers of toxicity (*Allium cepa* test - higher plant test system), were evaluated in water samples of Alagados Reservoir during a cyanobacterial bloom in South Brazil. The water samples were collected during the wet season at two sites in the Reservoir. Paralytic shellfish toxins (PSTs) were detected in both samples (sites 1 and 2); however, the levels of PSTs were higher in site 1. Gonyautoxin 2 was the major cyanotoxin found in the Reservoir. Both samples were able to induce cytotoxic effects (reduced Mitotic Index) and damage the genetic material (i.e., increased frequencies of chromosome aberration and micronuclei) of meristematic cells of *A. cepa*. The cellular and genetic damages were higher in the sample site 1, wherein high levels of PSTs were verified. Thus, our findings suggested that cyanotoxins-contaminated waters may damage the genetic material of living organisms, and therefore this group of contaminants should be assessed for their potential genotoxicity.

Keywords: *Allium cepa*; cyanotoxins; saxitoxins; DNA damages; Mitotic Index; Mode of action.

INTRODUCTION

Surface water bodies commonly end targets of industrial and urban wastes. As several chemicals present in wastewater induce adverse outcomes on living organisms, this is of increasing concern. Among the environmental contaminants, cyanobacteria toxins (*i.e.*, cyanotoxins) deserve attention because many regions worldwide have reported progressive eutrophication of water bodies, increasing the occurrence of cyanobacteria blooms (Heisler *et al.,* 2008). Among the cyanotoxins, microcystin is the most studied regarding the mechanisms of their toxicity, and thereby their capacity of acting as hepatotoxicants, genotoxicants, oxidative stress inducers, and tumor-promoting agents have already been described (Zegura *et al.,* 2011; Pamplona-Silva *et al.,* 2018; Díez-Quijada *et al.,* 2019). However, not all cyanotoxins have yet been sufficiently studied concerning their hazard, such as paralytic shellfish toxins (PSTs).

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PSTs belong to one of the four classes of cyanotoxins that mostly affect drinking water (He *et al.,* 2016). PSTs - generally known as saxitoxins - are well-described neurotoxins - a group of more than 50 analogs - which differ in molecular structure and toxicity, such as saxitoxins (STXs), neosaxitoxins (NeoSTXs), decarbamoylsaxitoxins (dcSTXs); gonyautoxins (GTXs), and C-toxins (Wiese *et al.,* 2010). However, to the best of our knowledge, no information regarding the toxicological effects of PSTs in plants, such as their capacity for inducing DNA damages (genotoxicity/mutagenicity) was described.

The species *Allium cepa* (higher plant, monocotyledon) is a standard test system used to evaluate genetic damages caused by environmental contaminants. This successful use of *A. cepa* in ecogenotoxicity testing is mainly due to its karyotype (large and few chromosomes), which favor this species in determining the genotoxic mode of action (MoA) (clastogenic and aneugenic) of a variety of chemicals. For this reason, the *Allium cepa* test is considered an efficient bioindicator in genotoxicity studies. (Leme & Marin-Morales, 2009, Hara & Marin-Morales, 2017; Pamplona-Silva *et al.,* 2018). Some studies suggest that there is a connection between the existence of cyanotoxins with chromosomal abnormalities and chromatid breaks (Zegura *et al.,* 2004; Zegura, 2011; Pamplona-Silva *et al.,* 2018) as a direct consequence of the increase of free radical production, the generation of DNA adducts, as well as cytoskeleton disorders and damages to DNA repair machinery. However, the potential toxicity of cyanotoxins and their mechanism of toxicity differs among them since cyanotoxins are structurally diverse chemicals (Zegura *et al.,* 2011; Laughinghouse IV *et al.,* 2012). Consequently, despite a positive correlation between some cyanotoxins (e.g., microcystins) and genotoxicity, these data cannot be extrapolated to other cyanotoxins, such as PSTs, for example.

Additionally, ecotoxicological investigations that employ plants are of great significance because they can demonstrate the toxicity of bioactive compounds on different levels of the food chain. Plants are considered the foundation of the food chain, providing food resources to other organisms (Ma *et al.,* 2010). Although direct natural contact of land plants with PSTs may be uncommon, irrigation with contaminated water increases the possibility of this occurring, something that has already been considered as a public health issue in Canada (Miller & Russell, 2017). Consequently, these assays are often used to monitor polluted environments (Leme & Marin-Morales, 2009; Pamplona-Silva *et al.,* 2018) and are well established for higher plants to make available accurate information about the toxicity of a significant amount of compounds.

In surface waters contaminated with low levels of cyanotoxins/cyanobacteria, after the plants are irrigated with it, cyanotoxins can be found mostly in plant roots, but in this case, at levels of concern to human health (>1 mg kg^{-1} – WHO, 1998). Besides, human dietary exposure to cyanotoxins may also occur, although information to date has been restricted to the intake of crustaceans, shellfish, and fish of marine origin (Ibelings & Chorus, 2007). The risk that freshwater cyanotoxins may pose to human health has yet to be better-evaluated (Ibelings & Chorus, 2007). As a result, identifying common pathways of human exposure to PSTs is necessary to determine the risk assessment to which humans are exposed.

The present study aimed to evaluate the occurrence and effects of cyanobacteria and their toxins in plants exposed to water samples from two different sites of Alagados Reservoir (Paraná-Brazil) during a bloom of cyanobacteria, using the *A. cepa* test. The choice of the Alagados Reservoir was made as a case study due to the records of cyanobacteria bloom dating back to the 1980s in this reservoir. Since 2002 (Yunes *et al.,* 2003), the intensity of potentially toxic cyanobacterial blooms has increased dramatically (Clemente *et al.,* 2010), and, as a result, PSTs have been the main cyanotoxin found in this reservoir (Yunes *et al.,* 2003; Clemente *et al.,* 2010; Calado *et al.,* 2017; Calado *et al.,* 2019).

MATERIAL AND METHODS

Collection sites and water sampling

The Alagados Reservoir (Fig. 1) is located in the basin of the lower Tibagi, among the cities of Ponta Grossa, Castro, and Carambeí, Paraná, Brazil (24º 52' to 25º 05' S and 49º 46' to 50º 06' W). This reservoir has an average depth of 8.1 meters, an average flow of 9.4 m^3/s, and accumulates about 27.7 million m^3 of water, whose residence time is approximately 40 days (IAP, 2009). Furthermore, it is currently responsible for water supplying the cities mentioned above and being used for power generation, recreation, fishing, and other human-related activities (Clemente *et al.,* 2010). The inhabitants that live in the settlements surrounding Brazilian reservoirs use fish as the primary source of protein (Calado *et al.,* 2019). Consequently, the Alagados Reservoir is monitored by the responsible environmental agency, as well as other reservoirs, since 1995. Also, the reservoir is classified as moderately degraded (Class III) (IAP, 2004; IAP, 2009) due to characteristics including a lengthened water residence time (from 41 to 120 days), eutrophication, a medium to a high diversity of phytoplankton, and cyanobacterial blooms (IAP, 2018).

Regarding its physical-chemical characteristics, the Alagados Reservoir has detailed information (IAP, 2004; IAP, 2009; IAP, 2018; Wojciechowski, 2013). The reservoir has an average annual temperature of 20°C, ranging from 12°C (winter) to 27°C (summer). The pH in this reservoir varies from 6.5 to 9.0, with an annual average of 7.8, with a trend described for an increase in pH in January and a decrease in pH in July. The concentration of total phosphorus to the sub-surface in the reservoir water varies between 0.01 and 0.17 mg L^{-1}, with an average of 0.04 mg L^{-1}. The reactive phosphorus in this same reservoir varies between 0.01 and 0.07 mg L^{-1}, with an average of 0.02 mg L^{-1} (Wojciechowski, 2013). Regarding rainfall, the reservoir is located in a region with a subtropical

climate, for which rainfall is regularly distributed so that the dry season and wet season are not limited, but periods of less or greater precipitation (Wojciechowski, 2013).

Water samples (3 water samples of 1 L each) were collected during the summer (period of highest luminosity indexes - March 2014 – Wojciechowski et al., 2016) for each one of the two sites in the Alagados Reservoir, as follows: site 1 (S1, 25°18′49″S, 50°3′34.11″W) located closer to the water supply catchment; site 2 (S2, 25°1′14.55″S, 50°2′17.81″W) located closer to small and private properties, which are mainly used for recreation. The sites differ, considering the adjacent land use to the reservoir margins, and may vary in their level of contamination (Calado *et al.,* 2017; Calado *et al.,* 2019). Also, the luminous intensity of the region in which the reservoir is located during the collection period (March 2014) must be taken into account, since this period is of greater luminous intensity, an important abiotic factor for the growth of cyanobacteria, as well as the emergence of blooms (Wojciechowski *et al.,* 2016).

Three samples per site were carried out using dark plastic containers at 10 cm below the water surface (euphotic zone). The samples were transported at 4°C and stored in the laboratory at -20°C and 4°C until the chemical analysis and bioassay were carried out.

Phytoplankton analysis

For the qualitative analysis of cyanobacteria, the water samples were collected using plankton nets (20 µM) and preserved in the Transeau solution. Cyanobacteria were identified according to Komárek & Anagnostidis (1986) and Anagnostidis & Komárek (1988). For quantitative analysis, water samples were collected with a bottle of Van Dorn and fixed with acetic-Lugol. The cell count was performed on an Olympus® IX-70 inverted microscope in sedimentation chambers to determine the concentration of cells present (cells/mL) (Utermöhl, 1958). A total of 400 cells were counted in randomized fields (Chorus & Bartram, 1999), reducing the count error to 10% (Venrick, 1978).

Chemical analysis of cyanotoxins

Water samples (total amount of 500 mL) were frozen in a freezer at -20°C and lyophilized to quantify cyanotoxins. The lyophilized extract was re-suspended in 0.5 M acetic acid and was placed in a shaker for 2 h for extraction. After that, the sample was filtered with cellulose acetate (0.45 µM) and analyzed using high-performance liquid chromatography (HPLC) (Oshima, 1995). The analyzed cyanotoxins were saxitoxin (STX), neosaxitoxin (neoSTX), and gonyautoxins (GTX1, GTX2, GTX3, GTX4, and GTX5). Cylindrospermopsin (CYN) and Microcystins were not detected in the water samples. The equipment was calibrated with PST standards obtained from the National Research Council, Canada, and standards were also run before and after sample analyses. The detection limits were determined as follows: STX, 5.90 ng mL^{-1}; NeoSTX, 6.14 ng mL^{-1}; GTX 1, 9.93 ng mL^{-1}; GTX 2, 9.03 ng mL^{-1}; GTX 3, 6.86 ng mL^{-1}; GTX 4, 3.24 ng mL^{-1}, and GTX 5, 9.78 ng mL^{-1}. All the samples were analyzed in triplicate. Detected concentrations were converted to STX equivalent (eq. STX) to compare the toxicity of each variant with that of STX (Hall *et al.,* 1990).

Bioassay with Allium cepa seeds

Seeds of *A. cepa*, from the same batch and variety ("Baia Periforme" onion; 2n = 16 chromosomes, TopSeed®—Agristar, Petrópolis, Rio de Janeiro, Brazil) were germinated in Petri dishes covered with filter paper (100 seeds/

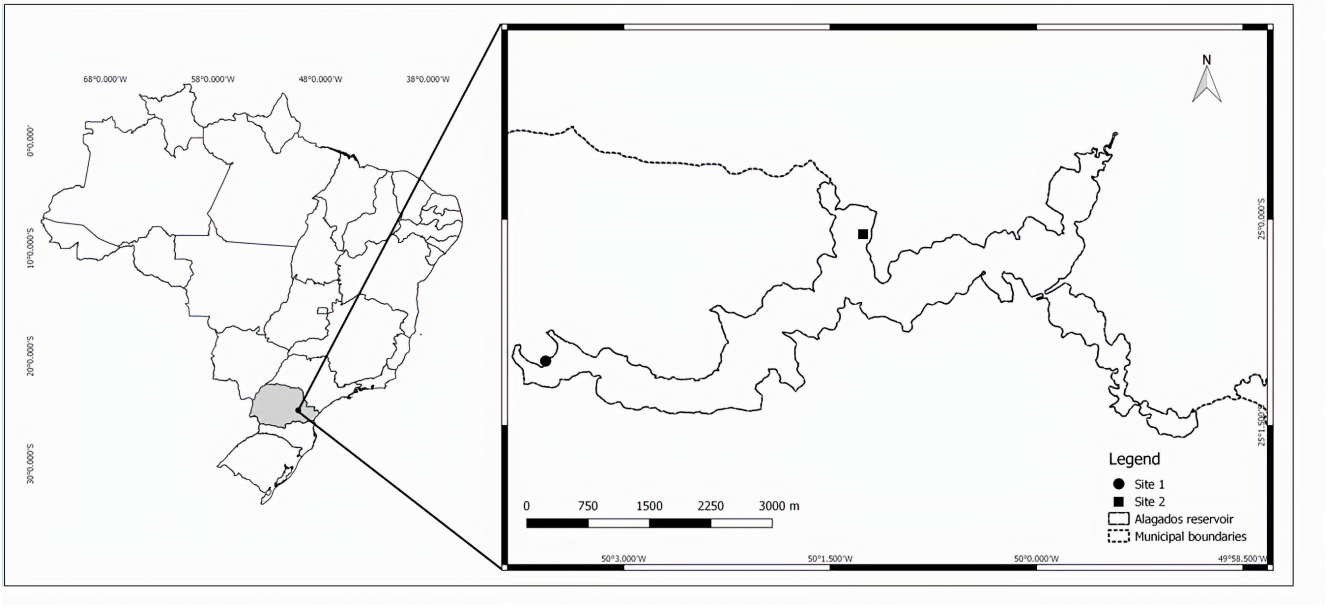

Figure 1. Alagados Reservoir and location of the sampling sites: site 1 (S1), water supply catchment; site 2 (S2), human settlements surroundings of the reservoir, and agriculture.

dish), containing raw water samples (S1 and S2). Water samples with high toxicity may inhibit *A. cepa* seeds from germinating, making the genotoxicity bioassay unfeasible. As a methodological approach, to permit the germination of the seeds and, consequently, the evaluation of the genotoxicity parameters (Fiskesjo, 1985), two dilutions of the raw samples were performed for each collection point, as follows: 75% dilution (75% raw sample + 25% ultrapure water) and 50% dilution (50% raw sample + 50% ultrapure water). The negative control (NC) was performed in ultrapure water and the positive control (PC) in methyl methanesulfonate (MMS, CAS 66-27-3, Sigma-Aldrich®) at 10 mg L^{-1} (Felisbino *et al.,* 2018; Santos Filho *et al.,* 2019). Methyl methanesulfonate is a compound with known genotoxic effects on A. *cepa* root cells (Leme *et al*., 2008). The experiment was carried out under controlled temperature (25°C) and in the absence of light, to prevent the photodegradation of the cyanotoxins in the samples.

After five days of exposure to the samples and their respective dilutions, the roots with ~ 2 cm in length were collected, fixed in Carnoy's fixative (ethanol-acetic acid - 3:1-v/v), and stored at 4°C until the cytological slides were prepared. The roots were submitted to the Feulgen reaction (Mello & Vidal, 1978), and cytological slides were mounted using the meristematic region of A. *cepa*, according to the protocol described by Leme & Marin-Morales (2009).

The meristematic cells (5000) per treatment (500 cells/slide, 10 slides/treatment) were evaluated under a light microscope (Olympus® BX 40; magnification, 400 x magnification). The dividing cells (Mitotic Index – MI, cytotoxicity), the cells bearing different types of chromosome aberrations (CA) and nuclear abnormalities (NA) (genotoxicity), and the micronucleated cells (mutagenicity) were counted and recorded (Montalvão *et al.,* 2019). The CA and NA were also categorized as aneugenic (*e.g.*, loss, delay, and adherence) and clastogenic (*e.g.*, chromosomal bridge and breaks) to determine the genotoxic mode of action (MoA).

Firstly, data were analyzed regarding the normality distribution using the Shapiro-Wilk test, as well as Levene´s homogeneity test. Since the data failed the assumptions of normality and homoscedasticity tests, statistical analyzes were performed using non-parametric tests. Statistical comparisons between treatments and NC were carried out using the non-parametric Kruskal-Wallis test, followed by post-hoc Student-Newman-Keuls tests. For comparisons between the collection sites, the Mann-Whitney U test was used, followed by Dunn's post-hoc test. The significance level was set at $p < 0.05$, and the analyses were run in R Project (2015).

RESULTS

Phytoplankton densities and cyanotoxins concentrations

Four species of cyanobacteria capable of producing cyanotoxins were detected in the water samples of the Alagados Reservoir. The densities of cyanobacteria in the tested water samples were, in general, higher for S1 than S2. The predominant cyanobacteria species was *Cylindrospermopsis raciborskii*, presenting a density of around 1 million cells mL^{-1} in the S1 sample and $4.9x10^5$ cells mL^{-1} in the S2 sample (Table 1). *C. raciborskii* was the dominant species found in this reservoir, and only PSTs have been found in concentrations higher than the permitted legislation limits (Clemente *et al.,* 2010; Calado *et al.,* 2017; Calado *et al.,* 2019).

The cell density of other species of cyanobacteria found (*Aphanizomenon* sp., *Dolichospermum planctonicum*, *Oscillatoria* sp.) was almost 30 times lower than the cell density found for C. *raciborskii*. In addition, cylindrospermopsin (CYN) was not detected in this reservoir, as well as other types of cyanotoxins like microcystins, for example, as published by Calado *et al.* (2019).

The identified cyanobacteria are available for consultation in the herbarium (UPCB) located in the Department of Botany, Federal University of Paraná, Curitiba, PR, Brazil, under the numbers 91425 (S1) and 91426 (S2).

The cyanotoxins found in the water samples were STX, neoSTX, GTX2, and GTX3 (Table 2).

Bioassay with Allium cepa seeds

Cytogenotoxicity assays

Inhibition of cell division (reduced MI, cytotoxicity) was observed for both samples (S1 and S2) (Fig. 2). Comparing these effects between samples, the reduction of the MI was higher for S1 than S2 (50% dilution and raw sample) (Fig. 2 D-E). These water samples (S1 and S2) also significantly increased the levels of CA + NA (genotoxicity) (Fig. 3), and this level was even higher for S1 in comparison to S2 (raw sample) (Fig. 3 E). A significantly increased level of micronucleated cells was only observed to meristematic cells of *A. cepa* exposed to a raw sample of S1 (Fig. 4 A).

Concerning the genotoxic MoA, both samples induced different types of CA and NA (Table 3, Fig. 5). When clustering these abnormalities into the clastogenic and aneugenic categories, it was possible to analyze that these water samples induced both types of abnormalities (*i.e.*, clastogenic and aneugenic).

Table 1. Toxic cyanobacterial densities from the Alagados Reservoir (Paraná-Brazil) in March 2014.

Cyanobacteria species	Density (cells mL^{-1})	
	S1 water sample	S2 water sample
Aphanizomenon sp.	$5.8x10^4$	$7.3x10^4$
Cylindrospermopsis raciborskii	$1x10^6$	$3.9x10^5$
Dolichospermum planctonicum	$3.6x10^4$	$1.8x10^4$
Oscillatoria sp.	$3.4x10^3$	$2.7x10^3$
Total	**$1.1x10^6$**	**$4.8x10^5$**

Table 2. The concentration of PSTs and Eq. STXs concentrations found in water samples from the Alagados Reservoir (Paraná-Brazil, March 2014).

SITES	STX/ neoSTX (μg L^{-1})	GONYAUTOXINS (μg L^{-1})					TOTAL CONCENTRATION PSTs (μg L^{-1})	Eq. STXs (μg L^{-1})
		GTX1	GTX2	GTX3	GTX4	GTX5		
S1	5.34±0.08	<QL	12.06±0.13	6.82±0.47	<QL	<QL	24.21±0.34	13.18±0.25
S2	1.01±0.02	<QL	1.94±0.10	1.25±0.05	<QL	<QL	4.21±0.14	2.30±0.06

<QL: Below the quantification limit; Eq. STXs: equivalent amount in saxitoxins.

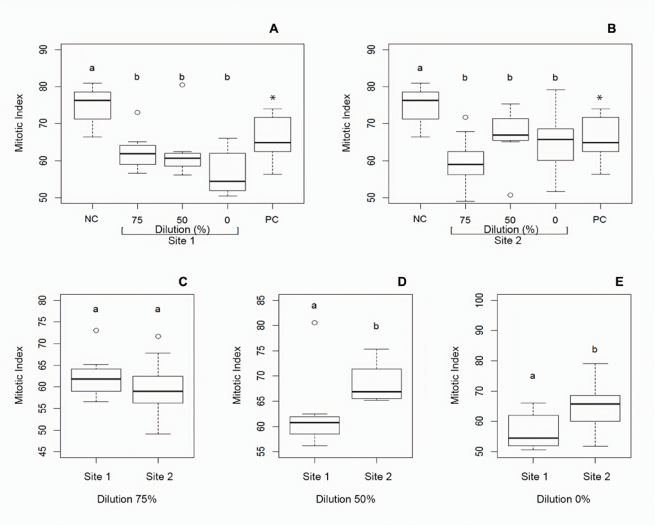

Figure 2. Cytotoxicity biomarker in *Allium cepa* meristematic cells exposed to water samples from the Alagados Reservoir (Paraná, Brazil). Mitotic index: comparison among treatments (**NC**: Negative Control; 75% dilution, 50% dilution; raw sample, **PC**: Positive Control) within S1 **(A)** and S2 **(B)**; Pairwise comparisons between the sites (S1x S2) treatments 75% **(C)**, 50% **(D)** e 0% - raw sample **(E)**. The results are expressed as median, 1st and 3rd quartile. Different lowercase letters indicate significant differences ($p < 0.05$) among treatments. The asterisk (*) indicates significant differences between NC and PC.

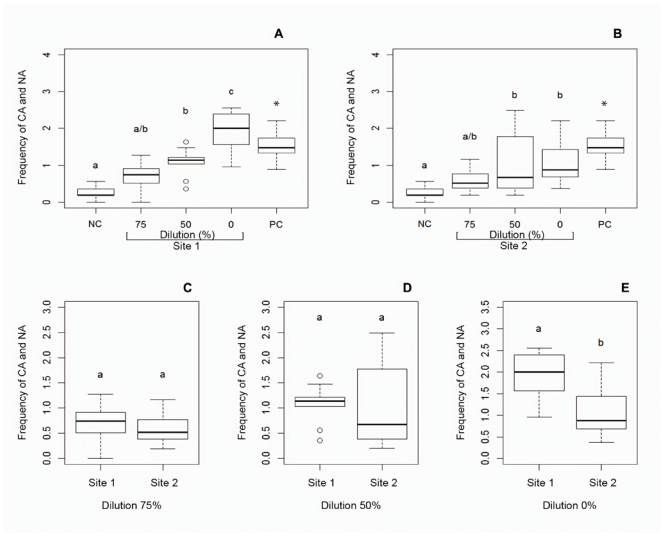

Figure 3. Genotoxicity biomarkers in *Allium cepa* meristematic cells exposed to water samples from the Alagados Reservoir (Paraná, Brazil). Frequency of chromosomal aberrations (CA) and nuclear abnormalities (NA): comparison among treatments (**NC**: Negative Control; 75% dilution, 50% dilution; raw sample, **PC**: Positive Control) within S1 **(A)** and S2 **(B)**; Pairwise comparisons between the sites (S1 x S2) treatments 75% **(C)** 50% **(D)**, and raw sample **(E)**. The results are expressed as median, 1st, and 3rd quartile. Different lowercase letters indicate significant differences ($p < 0.05$) among treatments. The asterisk (*) indicates significant differences between NC and PC.

Clastogenic damage was more significant than aneugenic damage at 75% dilution for both sites. For 50% dilution, clastogenicity was greater than aneugenicity only for S1, where higher concentrations of PSTs were found. In addition, considering the raw sample (0% dilution) for S2, it has been observed a more significant clastogenic damage than aneugenic damage. However, for S1, with the highest analyzed PSTs concentrations, clastogenic damage was similar to aneugenic damage (Fig. 6).

DISCUSSION

The Alagados Reservoir is susceptible to cyanobacterial blooms, previously reported in other studies (Yunes *et al.,* 2003; Clemente *et al.,* 2010; Calado *et al.,* 2017). A high number of cyanobacteria cells and the presence of PSTs were responsible for sporadic interruptions of supplying drinking water in the region (Wojciechowski *et al*., 2016). Our results demonstrated that the water samples collected during cyanobacteria bloom in this reservoir were able to induce cytotoxic, genotoxic, and mutagenic effects in the meristematic cells of *A. cepa*.

These effects are probably related to the presence of PSTs (STXs, neoSTXs, and GTXs 1, 2, and 3) detected in the water samples (S1 and S2) of Alagados Reservoir. The concentrations of PSTs, in terms of Eq. STXs, were above the limits for drinking water (3 μg L^{-1} Eq.STXs) established by the Brazilian law nº 2914 (Ministry of Health, 2011, Brazil) and proposed internationally as the freshwater concentration at which a health alert must be instituted (Batoréu *et al.,* 2005). It is known that PSTs can be produced by the cyanobacteria *Cylindrospermopsis raciborskii* (Lagos *et al.,* 1999), which were also detected in the water samples at densities higher than the maximum density acceptable by international water legislation standards (>20.000 cells mL^{-1}) (WHO, 2003; Ministry of Health Law nº 2914/2011, Brazil).

Cylindrospermopsis is one of the genera responsible for the production of PSTs in freshwater ecosystems and has spread to lakes in North America and Europe in the last 10–15 years as well as to water bodies in South America. Other cyanobacteria isolated in different parts of the world produce cylindrospermopsin (CYN) while the strains isolated in Brazil – in general – as well as specific strains from Alagados

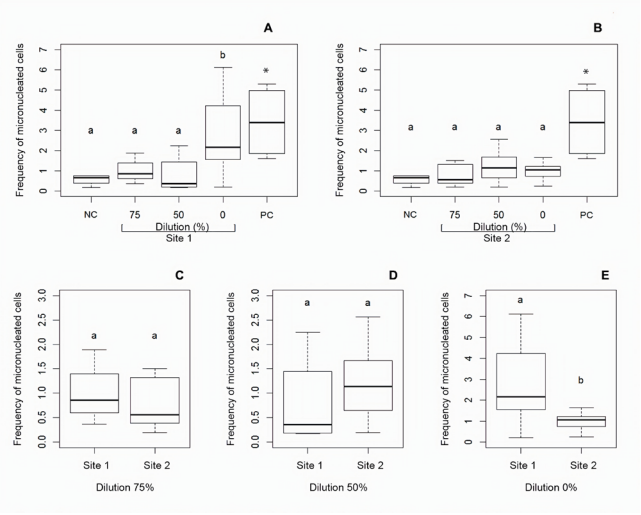

Figure 4. Mutagenicity biomarkers in *Allium cepa* meristematic cells exposed to water samples from the Alagados Reservoir (Paraná, Brazil). Frequency of micronucleated (MN) cells: comparison among treatments (**NC**: Negative Control; 75% dilution, 50% dilution; raw sample, **PC**: Positive Control) within S1 **(A)** and S2 **(B)**; Pairwise comparisons between the sites (S1 x S2) treatments 75% **(C)** 50% **(D)**, and raw sample **(E)**. The results are expressed as median, 1st and 3rd quartile. Different lowercase letters indicate significant differences ($p < 0.05$) among treatments. The asterisk (*) indicates significant differences between NC and PC.

Reservoir; thus far, produce PSTs (Lagos *et al.,* 1999, Calado *et al.,* 2017; Calado et al., 2019). In addition, for this species - *Cylindrospermopsis raciborskii* - and other cyanobacteria, light is one of the significant abiotic factors in regulating growth and the appearance of blooms. However, the species *C. raciborskii* is considered a species tolerant to various light conditions, which allowed the expansion of this species to regions of temperate climate. This means that in periods of higher light intensity, growth and blooms of this species are favored. In contrast, in periods of lower light intensity, the species is also adapted for development, achieving high growth rates under a relatively wide range of light intensities. This physiological adaptation can potentially be a competitive advantage in the phytoplankton community.

The risk of exposure to PSTs and their effects on some species are well documented in the literature (Landsberg, 2002), however primarily as a neurotoxicant. Few studies have demonstrated the capacity of PSTs in inducing cytotoxicity and causing primary DNA lesions (Clemente *et al.,* 2010; Da Silva *et al.,* 2014; Calado *et al.,* 2017) as shown by our study. Comparisons of the biomarkers of toxicity between the tested samples (S1 and S2) systematically point to a more significant harmful effect, even with a 25% sample dilution in water (75% of the raw sample) of the S1 sample, which is located near the public water supply catchment and had an equivalent concentration of PSTs 6 times higher than those found in S2 sample. The results demonstrate that the concentration of PSTs remains very high even after diluting the sample in 25% of water, evidencing its high toxicity. These results are significant because, even after water sample dilution, its toxicity remains high. This information is of fundamental importance because, in freshwater Class III (CONAMA resolution 357, 2005), used for public supply, animal watering, plants/crops irrigation, fishing, and recreation, the dilution of the contaminated water will not decrease the toxicity effects caused by the presence of PSTs in the concentrations found in this study. Besides, the mutagenicity observed only at S1 sample reinforces the dangerousness of high levels of PSTs contamination in waters, since the presence of micronucleated cells indicates irreversible genetic damage because MN contains acentric fragments or whole chromosomes from unrepaired damages of parental cells (Fenech, 2000; Kirsch-Volders *et al.,* 2011).

Table 3. Types of chromosomal aberrations **(CA)** and nuclear abnormalities **(NA)** observed in meristematic cells of *Allium cepa* exposed to dilutions and raw samples from the Alagados Reservoir (Paraná-Brazil, March 2014).

CA and NA	Dilutions							
			Site 1			Site 2		
	NC	75%	50%	RS	75%	50%	RS	PC
Clastogenic								
Chromosome bridge	0.038	0.389*	0.499*	0.714*	0.405*	0.626*	0.633*	0.412*
Chromosome break	0.057	0.093	0.036	0.184	0.037	0.096	0.061	0.674*
Total	0.095	0.482*	0.535*	0.898*	0.442*	0.722*	0.694*	1.086*
Aneugenic								
C-metaphase	0.038	0.056	0.160*	0.224*	0.037	0.048	0.000	0.056
Chromossome loss	0.057	0.056	0.036	0.224	0.037	0.144	0.102	0.094
Chromossome adherence	0.000	0.018	0.018	0.122	0.000	0.024	0.020	0.056
Chromossome delay	0.000	0.000	0.000	0.000	0.018	0.000	0.020	0.000
Nuclear bud	0.038	0.093	0.285*	0.612*	0.074	0.072	0.123	0.094
Total	0.227	0.686*	0.944*	1.998*	0.607	1.011*	0.960*	1.385*

NC= negative control (ultrapure water); 75% dilution of raw sample; 50% dilution of raw sample; RS= raw sample; PC= positive control (MMS to 10 mg L^{-1}); Data expressed in frequency (%). 5.000 cells were analyzed per treatment. The asterisk (*) indicates significant differences in comparison with NC ($p<0.05$), according to the Kruskal-Wallis test.

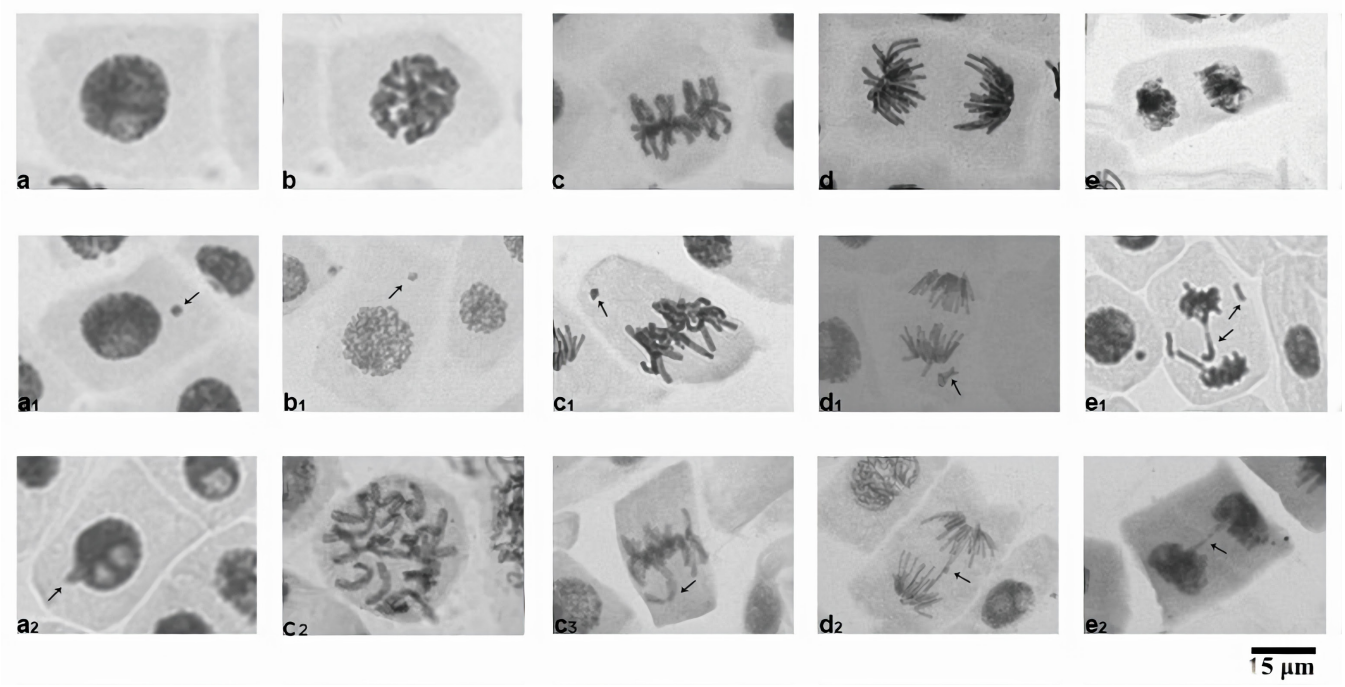

Figure 5. Chromosomal aberrations (CA) and nuclear abnormalities (NA) observed in meristematic cells of *Allium cepa* exposed to the water samples from the Alagados Reservoir. **(a)** Normal interphase nucleus; **(a_1)** Interphase with MN (arrow); **(a_2)** Interphase with nuclear bud (arrow); **(b)** Normal prophase; **(b_1)** Prophase with MN (arrow); **(c)** Normal metaphase; **(c_1)** Metaphase with MN (arrow); **(c_2)** C-metaphase; **(c_3)** Metaphase with chromosomal loss (arrow); **(d)** Normal anaphase; **(d_1)** Anaphase with chromosomal loss (arrow); **(d_2)** Anaphase with chromosomal bridge (arrow); **(e)** Normal telophase; **(e_1)** Telophase with chromosomal break, with chromosomal loss and delayed chromosome (arrows); **(e_2)** Telophase with chromosomal bridge (arrow).

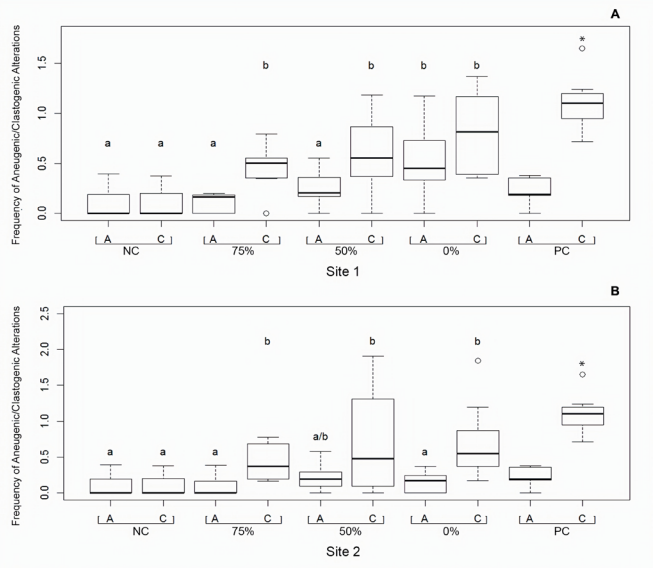

Figure 6. Genotoxic mode of action (MoA) observed in *Allium cepa* meristematic cells exposed to water samples from the Alagados Reservoir (Paraná, Brazil). Mechanisms of Action (MoA): Comparison of frequencies derived from clastogenic and aneugenic actions among treatments (**NC**: Negative Control; 75% dilution, 50% dilution; raw sample, **PC**: Positive Control) of S1 **(A)** and S2 **(B)**. A: Aneugenic; C: Clastogenic. The results are expressed as median, 1st and 3rd quartile. Different lowercase letters indicate significant differences ($p < 0.05$) between treatments and NC. The asterisk (*) indicates significant differences between NC and PC.

The data from our study showed that the types of CA observed indicates that the cyanotoxins presented in these water samples can act as clastogenic and aneugenic agents; however, the clastogenicity prevails as their main genotoxic MoA. Chromosomal bridge, a CA derived from clastogenic action, was the abnormality found most frequently, both at S1 and at S2 samples, and its level was also significant compared to NC. Bridges can result from dicentric chromosomes, formed from breaks in sequence, followed by chromosomal rearrangements. Thus, during anaphase, the two centromeres are pulled to opposite poles of the cell, forming an anaphase bridge, resulting in uneven chromosome breakage as the cell cycle progresses (Fenech 2000; Kirsch-Volders *et al.,* 2011).

Another type of CA found in our study was the C-metaphase; however, only at S1 (50% dilution and raw sample). The C-metaphase is originated by complete inactivation of the mitotic spindle of cells in the division, and their presence is, therefore, an evidence of aneugenic action (Kirsch-Volders *et al.,* 2011). CA as breakages, losses, and adhesions were also reported, always more frequently in the S1 than S2; however, they were not significant to NC. The nuclear bud (an NA) was observed in a significant frequency, but also only at S1 sample (50% dilution). Nuclear buds are originated attempting to eliminate exceeding genetic material, probably due to polyploidization events, through the formation of MN and latter minicells (Leme *et al.,* 2008).

Evaluation of the genotoxicity and mechanisms of action confirms that the data obtained for MN showed S1 samples to have higher genotoxicity, analyzing the raw sample from S1 and S2, probably due to an equivalent concentration of PSTs 6

times higher for S1 than for S2. Additionally, the frequency of aneugenic responses from S1 was more significant than those found for S2, for which there was no evidence of an aneugenic action, only a clastogenic one, in comparison with NC. Raw samples of S1 showed a higher degree of aneugenicity in comparison with S2, but there were no statistical differences between the two mechanisms in S1.

Studies with other biological indicators already showed both quantitative and qualitative effects of PSTs on genetic biomarkers. Melegari and colleagues (2012) reported an increase in the frequency of MN after exposure to PSTs in neuronal cells of mice, with evidence of an aneugenic mode of action. Costa *et al.* (2012) found an increase of nuclear morphological changes in fish erythrocytes exposed to PSTs. Additionally, *in vivo* tests showed evidence of genotoxicity in the comet assay, for the fish species *Hoplias malabaricus*, after trophic exposure to PSTs, as well as *in vitro* effects to the neural cells of the same species (Da Silva *et al.,* 2011; Da Silva *et al.,* 2014).

Studies in Alagados Reservoir are recurrent. More recent results with fish in this area show that fish accumulate PSTs in the Reservoir and the genotoxic biomarkers showed an increase in the cytogenotoxicity. Some toxins produced by C. *raciborskii* are known inducers of oxidative stress and can lead to DNA damage (Zegura *et al.,* 2011; Calado *et al.,* 2017). The possible mechanism to induce this damage is the DNA strand breakage that can promote the micronucleus formation and the loss of chromosomes (Kirsch-Volders *et al.,* 2011). Recently, Calado and colleagues (2019) demonstrated that concentrations of PSTs in fish muscles in a depuration experiment decreased when compared to the field samples. However, after 90 days, PSTs were still present in the fish muscles.

Oxidative stress caused by PSTs has already been reported by scientific studies, especially in fish (Clemente *et al.,* 2010; Da Silva *et al.,* 2014; Calado *et al.,* 2017). According to these studies, PSTs act as inductors of oxidative stress due to their action on the selective permeability of membranes, causing a reduction of the antioxidant enzymes activities in the inner of the cells (Da Silva *et al.,* 2011). The DNA damages observed in our study may be related to the unbalance between the generation of reactive oxygen species (ROS) and oxidant defenses that can be disrupted by these cyanotoxins (PSTs).

ROS can damage DNA by the oxidation of nucleobases (*e.g.*, 8-oxo-7, 8-dihydroguanine), and these lesions may turn in DNA strand breaks mainly through DNA repair mechanisms (Collins, 2014). In turn, breaks in DNA molecule, although may lead to loss of genetic material due to the formation of acentric fragments, they can also lead to specific types of CA, such as structural chromosomal rearrangements and chromosomal bridges (Leme & Marin-Morales, 2009) – the latter observed in our study. ROS can also induce chromosomal instability by their interaction with tubulin, which is the main constituent of microtubules (MTs) of the mitotic spindle. For example, in cases of unbalance of the redox system, ROS can interfere indirectly or directly on tubulin polymerization, disrupting the MTs and activating proteins that will bind to the MTs, leading to their cleavage (Livanos *et al.,* 2014). The absence of a functional mitotic spindle can result in chromosomal abnormalities, such as C-metaphases (Leme *et al.,* 2008), which were also observed in our study at significant levels.

Finally, the *A. cepa* test showed a sensitive tool for evaluating the toxic effects of water contaminated with cyanotoxins. The genetic damages rose as increases the concentration of PSTs in the samples of Alagados Reservoir. Although the cyanobacteria bloom is the main environmental impact observed in the Alagados Reservoir, mainly because of anthropic activities in the reservoir surroundings, a mixture of contaminants might be present in this water body, enhancing the toxic effects described in this study. However, because human activities such as overpopulation, urban sprawl, and agriculture occur mainly around S2, the largest number of genetic alterations evidenced at S1 may be due to PSTs. Laughinghouse IV *et al.* (2012) and Pamplona-Silva *et al.* (2018) also demonstrated that *A. cepa* is a good test model to predict the genetic damages caused by microcystins, another cyanotoxin commonly found in water bodies. Although caution should be taken in translating toxicity data among species due to differences among them (*e.g.*, physiological, complexity), recent studies have shown good data correlation between *A. cepa* and mammalian test systems (Palmieri *et al.,* 2016; Hara & Marin-Morales, 2017). So, these results may be used as an alert to other species, as well as to the human population.

CONCLUSION

In conclusion, the present finding showed that the water from the Alagados Reservoir (South Brazil), which has high levels of PSTs could induce cytotoxic effects and compromise the genetic material (genotoxicity/mutagenicity) of *A. cepa*. It is important to emphasize that in complex samples, the toxic impact of PSTs can be amplified by other substances present in the environment and therefore induce a more toxic effect. These data, together with those previously reported in the literature, suggest that the degradation of water quality in this reservoir may be endangering the health of the associated biota, having severe implications for the integrity of the ecosystem. The results suggest that proper management of water quality and control of cyanobacteria blooms may substantially reduce contamination in this area. Furthermore, *A. cepa* is a good test system for estimating the genetic damages caused by cyanotoxins.

DISCLOSURE STATEMENT

The authors declare that there are no conflicts of interest.

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