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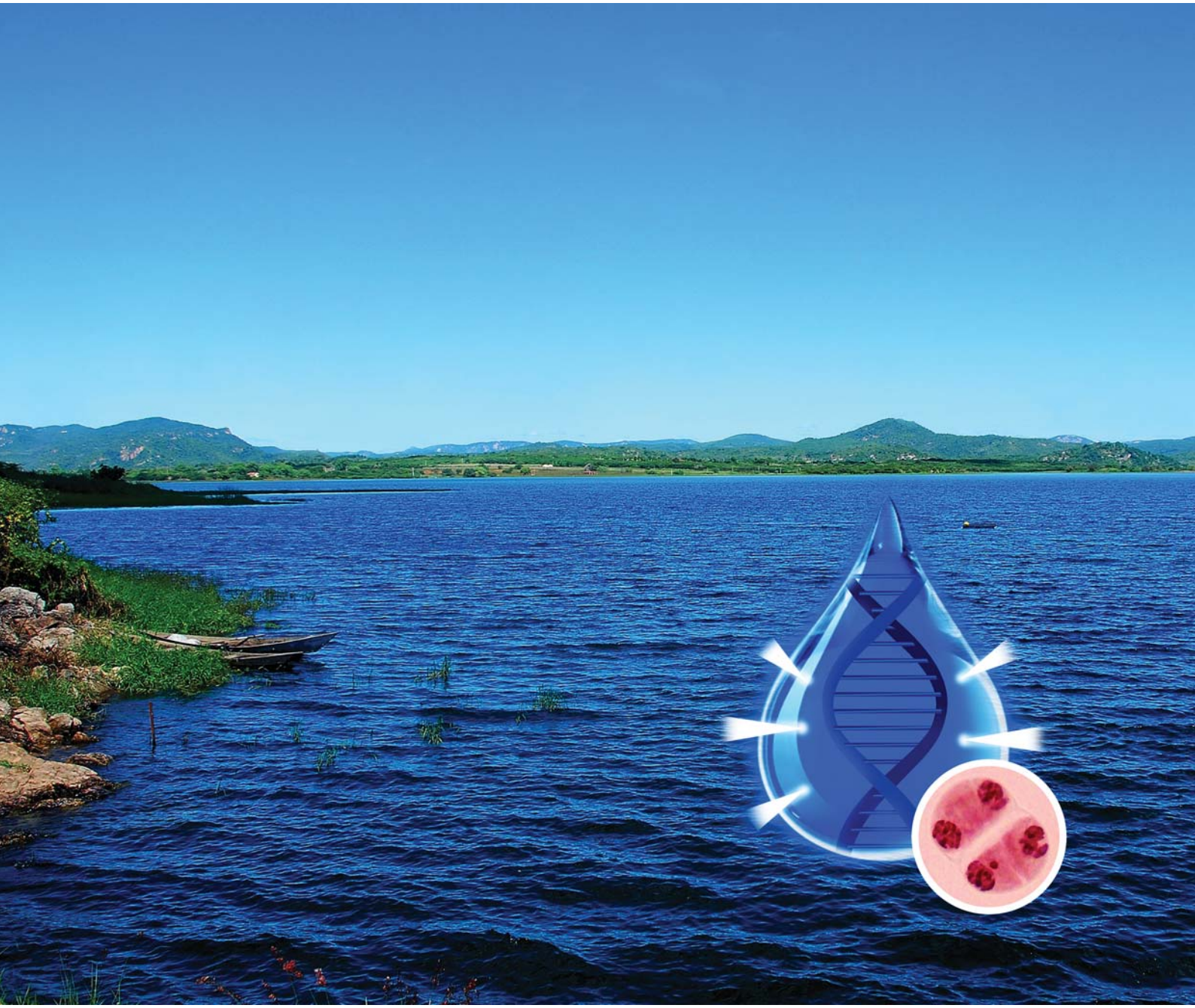

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PAPER

Micronucleus study of the quality and mutagenicity of surface water from a semi-arid region

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The present study evaluated the mutagenic potential of surface water from the Lucrecia dam. The Tradescantia-micronucleus (Trad-MCN) test and CBMN assay in human peripheral blood lymphocytes were applied, corresponding to an *in vivo* and *in vitro* system, respectively. Heavy metals and some physicochemical properties were also measured. Water samples were collected in November 2009 (dry season) and May 2010 (rainy season) at three different points. Results of both assays for raw water showed positive responses for the points analyzed when compared to the negative control. The CBMN assay showed that diluted water was still able to induce a significant increase in micronucleus frequency. For both assays, the highest mean MN was observed in the dry season. Chemical analyses detected an increase in heavy metal levels at the sampling points and in the different seasons. These findings indicate the presence of genotoxins, such as heavy metals, in the water, which may be affecting the entire ecosystem, as well as human health. More prolonged monitoring is recommended in order to better characterize this public water supply.

1. Introduction

The water quality of many reservoirs worldwide has been compromised by contaminant percolation into the water. This natural or anthropogenic phenomenon increases the level of genotoxic compounds in aquatic ecosystems, affecting environmental quality and the health of living beings inhabiting these ecosystems, including the human population.[1–3]

Against this backdrop of reduced water quality lies the Lucrecia dam (Fig. 1), a major surface water reservoir by volume in the semi-arid region of Rio Grande do Norte, Brazil. It has been contaminated by heavy metals and toxic cyanobacteria, as well as α and β radiation, resulting in potential fish genotoxicity.[4] The population using the water source studied has exhibited high cancer rates, generally associated with its consumption, displaying prevalence about three times higher than the entire state of Rio Grande do Norte.[5] Several studies have confirmed that a polluted water source is related to endemic cancers in human beings.[6,7]

In order to study environmental genotoxicity, it is important to consider that an environmental sample consists of a complex mixture of substances requiring the use of different genetic assessment methods. The micronucleus test is one of the most widely used, attracting increasing attention in laboratories that are active in the environmental mutagenesis field. Different cells from a number of organisms are being employed in these

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Environmental impact

Monitoring water contamination is extremely important, especially if it has an impact on public health. This is the case for Lucrecia dam, located in the semiarid region of Brazil, which is contaminated by heavy metals, cyanobacteria and radiation. The population that uses it for consumption has shown high rates of cancer. This study showed high frequencies of micronucleus in Trad-MCN and CBMN assays, which can be associated with chemical analyses. The CBMN assay validated Trad-MCN for mutagenicity of water analysis. These results demonstrate the mutagenic potential of this water, which may be affecting the entire ecosystem, as well as human health.

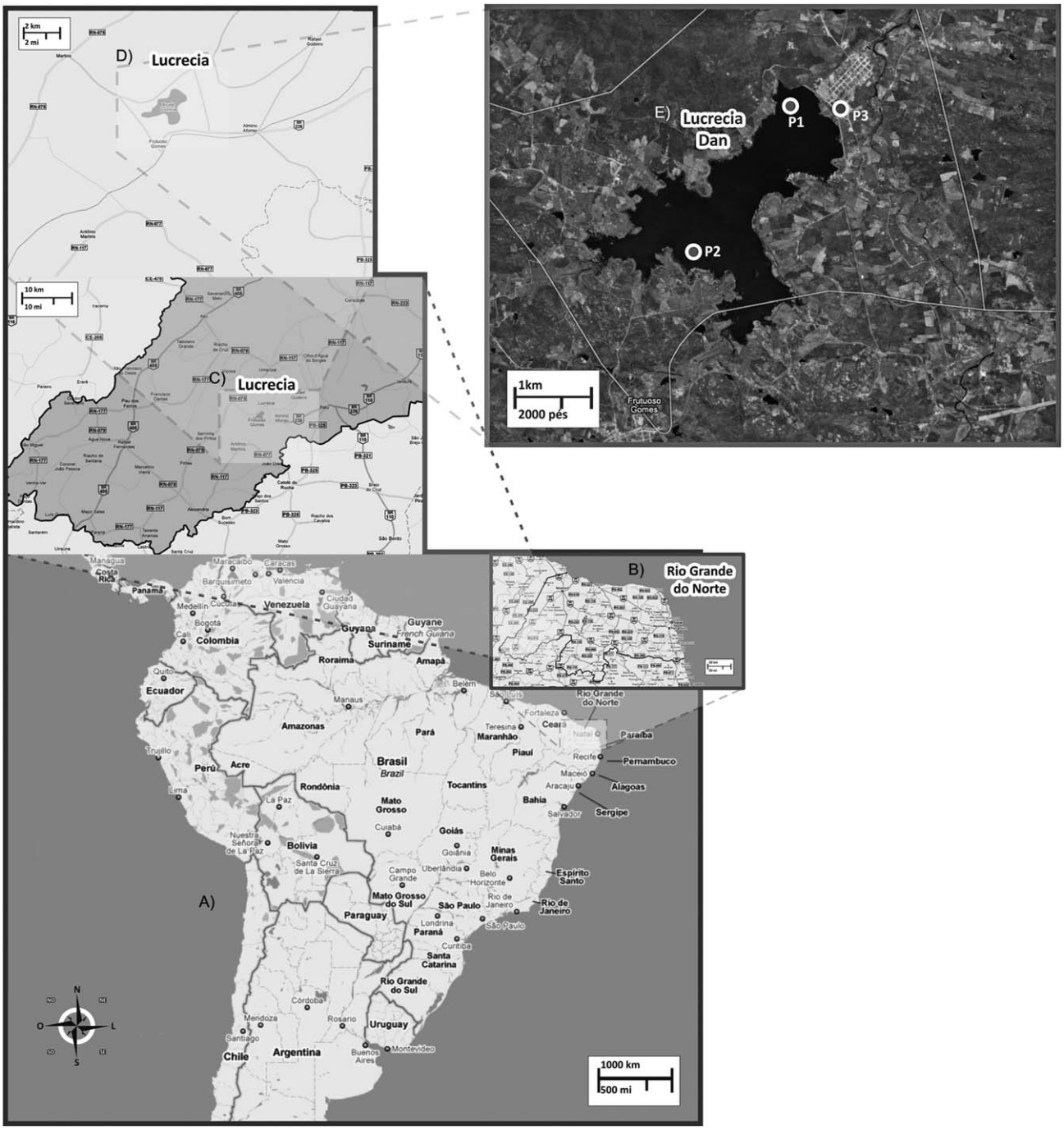

Fig. 1 Map representing the Lucrecia dam and its specific location in the semi-arid region of Rio Grande do Norte State in Northeast Brazil. (A) Brazil in South America and the location of Rio Grande do Norte State in the Northeast; (B) Lucrecia region in the southwest of Rio Grande do Norte; (C) Lucrecia dam in the Southwest of Rio Grande do Norte; and (D) Google Earth map depicting the Lucrecia dam and the city. P1, P2 and P3 are the water collection sites.

analyses, including plants,[8] mollusks,[9] fish,[10] human oral mucosa,[11] human cell lines[12] and human lymphocytes.[13]

The Tradescantia-micronucleus (Trad-MCN) bioassay is a very useful tool for screening mutagenic potential in the environment, due to its ability to detect low-level genotoxicity in either short-term *in situ* exposures or *in vivo* tests with unconcentrated water samples.[14–16] On the other hand, the micronucleus in peripheral blood lymphocytes is considered a predictor of increased cancer risk in humans.[17,18]

The aim of this study is to evaluate genotoxicity in the Lucrecia dam by applying the micronucleus test in two systems, one *in vivo* (Trad-MCN) and the other *in vitro* (CBMN), in both dry and rainy seasons. Cadmium, copper, zinc, lead, chromium, manganese and nickel levels were also measured.

2. Material and methods

2.1. Collection sites and sampling

Three monitoring sites were chosen to assess genotoxicity in the Lucrecia dam (Fig. 1): P1 (6° 33′ 7.62″S/93° 23′ 515″W), located near the water uptake for public distribution by CAERN (Rio Grande do Norte Water and Sewage Company), and most accessible to the population; P2 (6° 31′ 4.48″S/93° 23′ 8.46″W) on the opposite side of P1 at the confluence of tributary rivers; and P3 representing a household tap water.

Water samplings were carried out in November 2009 and May 2010, corresponding to the dry and rainy seasons, respectively. About 3 L of surface water was collected at each point in pre-cleaned amber flasks at a depth of ~50 cm, stored at 4 °C for 4 days, then divided into aliquots and kept in a freezer at −20 °C, as described in Vargas *et al.*[19]

2.2. Water chemical analysis

The levels of cadmium, copper, zinc, lead, chromium, manganese and nickel were determined by Atomic Absorption Flame Spectrometry (Varian, model 50B). For this analysis, 1 L of water was obtained at each sample point (P1, P2 and P3), stored in amber glass at 4 °C and preserved at pH ≤ 2 with concentrated nitric acid, added immediately after collection. Samples were then taken to the laboratory and analyzed as soon as possible. In addition to heavy metal analysis, several physicochemical measures were also taken, such as pH, turbidity, total floating solids, ammonia nitrogen (NH_3), nitrite (NO_2^-) and nitrate (NO_3^-). One litre of water was obtained at each sampling point and stored in amber flasks at 4 °C until parameters were determined. All water chemical analysis followed APHA guidelines.[20]

2.3. Tradescantia-micronucleus assay

This assay was performed using *Tradescantia pallida* according to the protocol proposed by Ma.[21] Young stems with inflorescences of *T. pallida* were collected from plants cultivated in humus soil and fertilized with an N : P : K formulation (10 : 10 : 10). The plants were kept in full sunlight and watered daily with distilled water. Micronucleus tests employed raw water (undiluted) from each sampling point. The present study used formaldehyde (0.2%) as a positive control and distilled water as a negative control.

For the bioassay, about 15–30 cuttings of *T. pallida* were gathered at each point and maintained in a hydroponic system with Hoagland solution for 24 hours. Cuttings were then submitted to immersion assay in 560 mL of water. Twelve hours of exposure was followed by a 24 h recovery process in Hoagland solution. Inflorescences were then fixed in 1 : 3 aceto-ethanol solution (Carnoy's) for 24 hours and stored in 70% alcohol.

Slides were prepared from the buds, and micronucleus frequency in meiotic pollen mother cells was determined.[21] All the slides were analyzed in a blind and randomized manner. A total of 3000 tetrads were scored for each sample. Data were expressed as MCN/100 tetrads.

2.4. Lymphocyte culture and CBMN assay

A 5 mL peripheral blood sample was obtained from three healthy young male donors with normal karyotypes. These individuals were aged 20, 21 and 23 years, respectively, and all were non-smokers. In addition, none had any contact with the Lucrecia dam so as not to influence MN analysis. For each donor, one series of cultures was prepared with two parallel cultures for every sample tested. In order to evaluate dose response, both raw water and water diluted to 50% and 25% were used.

Whole blood cultures were then set up according to Huang *et al.*[22] Briefly, 0.3 mL of whole blood was added to 4.7 mL of RPMI 1640 culture medium containing 10% fetal bovine serum, 1% penicillin/streptomycin and 5% phytohaemaglutinin (PHA). Twenty-four hours after PHA stimulation, cultures were supplemented with 200 μL of water samples, previously sterilized in a Sartorius filter with a 0.22 μm pore membrane. The negative control was sterile distilled water and the positive control was 0.1 μg mL^{-1} of mitomycin-C. Forty-four hours after PHA stimulation, 4.5 μg mL^{-1} of cytochalasin B was added to the cultures to accumulate cells that had completed one nuclear division at the binucleated stage.[23] Blood cultures were incubated for 72 h at 37 °C. Lymphocytes were collected by centrifuge (1000 rpm × 5 min), treated with 0.075 M KCl and fixed 3 times in 5 mL of methanol/glacial acetic acid (3 : 1), which was freshly prepared. Cell suspension was dropped onto a slide and stained with Giemsa (1 : 40 phosphate buffer pH = 6.8). Micronucleus frequencies were then measured in 1000 binucleated cells for each donor, totaling 3000 cells per sample, in accordance with Fenech.[24] As in Trad-MCN, all slide analysis was carried out in a blind and randomized manner. Only after the analysis were slides identified.

As a cytostasis parameter, the nuclear division index (NDI) was assessed according to the formula NDI = [$M1$ + 2($M2$) + 3($M3$) + 4($M4$)]/N, where $M1$–$M4$ indicates the number of cells with 1–4 main nuclei and for which 1000 cells per donor/sample (N) were scored, as recommended by Fenech.[25]

2.5. Statistical analysis

Statistical analyses were performed using R software 2.12.0.[26] Variables were evaluated for normality using the Kolmogorov–Smirnov test. For Trad-MN, different sample site groups were determined by ANOVA, followed by the Tukey post-hoc test. The Kruskal–Wallis test was applied for comparisons of MN means in human lymphocytes. Statistical differences were considered significant for $p \leq 0.05$.

3. Results

3.1. Physicochemical and heavy metal characterization

The physicochemical characterization of surface waters in the different seasons is summarized in Table 1. All properties analyzed, such as pH, turbidity, ammonium, nitrite and nitrate, were within the limits allowed by Brazilian law (Decree 518 MS, 2004).

Concentrations for the seven metals analyzed are shown in Table 2. During the dry season, the copper level at P1 was 38-fold higher than the Maximum Permitted Level (MPL) for class 2 and

Table 1 Physicochemical characterization of water samples collected from Lucrecia dam

Season	Parameter	Sampling points P1	P2	P3
Dry	pH	8.00	8.00	8.70
	Turbidity, NTU	3.33	3.33	0.10
	Total floating solids, mg L^{-1}	273.60	261.00	230.00
	NH_3, mg L^{-1}	0.24	0.04	0.03
	NO_2^-, mg L^{-1}	0.00	0.00	0.00
	NO_3^-, mg L^{-1}	0.00	0.00	0.00
Rainy	pH	7.07	7.46	7.77
	Turbidity, NTU	6.72	9.50	0.57
	Total floating solids, mg L^{-1}	207.80	208.30	207.70
	NH_3, mg L^{-1}	0.43	0.07	0.10
	NO_2^-, mg L^{-1}	0.00	0.00	0.00
	NO_3^-, mg L^{-1}	0.00	0.00	0.02

NTU = nephelometric turbidity unit.

3 waters, according to CONAMA,[27] and above the maximum concentration of chemical hazards in drinking water.[28] With regard to cadmium, all three collection points (P1, P2, P3) exhibited concentrations above the limits established by CONAMA[27] and P1 and P2 were above the maximum concentration of chemical hazards in drinking water.[28] Copper levels at P3 also exceeded legal limits. However, in the rainy season, cadmium (P2), lead (P1), manganese (P1 and P2) and nickel (P2) levels were above the limits permitted by both agencies.

3.2. Tradescantia-micronucleus assay

The frequency of micronuclei in the Trad-MCN observed at each sample site, for dry and rainy seasons, is summarized in Table 3. All dry season water samples demonstrated a significant increase in MN frequency compared to the negative control. In the rainy season, only P2 exhibited an increase in MN frequency.

A comparison of the collection points in the different seasons demonstrated a significant increase in MN frequency at P1 in the dry season compared to the rainy season ($p < 0.001$).

Table 2 Concentrations (mg L^{-1}) of the metals measured in the water samples from different points at the Lucrecia dam and maximum values allowed by CONAMA and WHO

Season	Metal	CONAMA[a]	WHO[b]	Sampling points/mg L^{-1} P1	P2	P3
Dry	Cd	0.001	0.003	0.004[c,d]	0.004[c,d]	0.003[c]
	Pb	0.010	0.010	0.007	0005	0.003
	Zn	0.180	3.000	0.006	0.020	0.008
	Cr	0.005	0.050	0.004	0.004	0.005
	Cu	0.009	1.000	0.349[c]	0.005	0.031[c]
	Ni	0.025	0.020	0.003	0.005	0.003
	Mn	0.100	0.100	0.079	0.067	0.007
Rainy	Cd	0.001	0.003	0.000	0.005[c,d]	0.000
	Pb	0.010	0.010	0.050[c,d]	0.000	0.000
	Zn	0.180	3.000	0.015	0.000	0.010
	Cr	0.005	0.050	0.000	0.000	0.000
	Cu	0.009	1.000	0.000	0.000	0.000
	Ni	0.025	0.020	0.000	0.050[c,d]	0.000
	Mn	0.100	0.100	0.195[c,d]	0.105[c,d]	0.000

[a] Maximum values allowed—resolution 357/2005 of the National Environment Council (CONAMA). [b] Maximum values allowed according to chemical hazards in drinking water of the World Health Organization (WHO, 2003). [c] Value above the limit allowed by CONAMA, 2005. [d] Value above the limit allowed by WHO, 2003.

Table 3 Frequency of micronuclei (MN% ± SD) obtained for *T. pallida* exposed to different raw water samples from the Lucrecia dam

Season	Sampling points P1	P2	P3
Dry	4.23 ± 1.11**/***	3.49 ± 0.59**	2.79 ± 0.75**
Rainy	2.43 ± 0.95	2.66 ± 0.80**	1.50 ± 0.36

Negative control: 1.46 ± 0.76; positive control: 4.25 ± 0.86***, /*** MN frequency for the P1 in the dry season compared to the rainy season, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Statistically significant compared to the negative control according to ANOVA.

3.3. Lymphocyte culture and CBMN assay

Table 4 shows CBMN assay results for human lymphocytes induced by water samples from the Lucrecia dam. In this assay, water was also diluted to 50% and 25% in order to observe the concentration response of micronucleus frequency, which was proportional to water concentration.

In the dry season, P1 was statistically significant for the three water concentrations compared to the control; P2 was also significant for raw water and water diluted to 50%. P3 showed no positive response for any sample. During the rainy season, P1 and P2 were only statistically significant for raw water. Raw water analyses were performed between the respective points, comparing dry and rainy seasons. No statistical difference was found between the seasons, although the mean MN frequency was higher in the dry season.

In addition to micronucleus frequencies, Nuclear Division Index (NDI) values are also shown in Table 4. This index exhibited no significant difference between the samples and the negative control, indicating the samples are not cytotoxic to human lymphocytes.

4. Discussion

Contamination of public water supplies is considered a major risk factor for human health. High concentrations of xenobiotics from urban, agricultural and industrial wastes have contributed to increased genotoxic activity in aquatic environments.[29,30] One of the most troublesome problems is the persistence of heavy metals, leading to ecosystem instability. They are highly toxic and, in contrast to organic compounds, are not biodegradable.[31] Water sample levels above those permitted by environmental agencies are strong indicators of anthropogenic activities.[32] In the present study, the levels of some metals, such as cadmium, copper, lead and manganese, were above maximum limits allowed by CONAMA[27] and WHO[28] at the different sampling points and in both seasons. Barbosa *et al.*[8] recorded similar results with water from Extremoz Lake, in the same state as our study, finding some metals above maximum allowable values. A study performed by Marcon *et al.*[4] at the Lucrecia dam from

Table 4 Frequency of micronuclei in human lymphocytes and N.D.I. for different dilutions of water samples from different points at the Lucrecia dam

		Sampling points					
		P1		P2		P3	
Season	Dilution	MN% ± SD	NDI	MN% ± SD	NDI	MN% ± SD	NDI
Dry	Raw water	15.00 ± 2.00**	1.961	11.00 ± 2.64**	2.004	6.67 ± 1.53	1.979
	50% diluted	8.33 ± 1.53*	1.972	8.00 ± 1.53*	2.022	5.67 ± 1.53	1.970
	25% diluted	7.33 ± 1.15*	1.922	5.67 ± 1.53	1.973	5.67 ± 0.58	2.001
Rainy	Raw water	12.67 ± 2.31**	1.989	8.67 ± 1.53**	1.930	7.00 ± 2.00	1.997
	50% diluted	6.67 ± 2.08	2.000	6.33 ± 2.31	1.959	5.33 ± 1.15	2.010
	25% diluted	6.33 ± 0.58	2.005	5.67 ± 1.15	1.960	6.00 ± 1.00	1.988

Negative control: 5.33 ± 0.57; NDI = 2.065 positive control: 41.33 ± 4.04***; NDI = 1.821*, NDI: Nuclear Division Index. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Statistically significant compared to the negative control according to the Kruskal–Wallis test.

2006 to 2009 showed the presence of heavy metals. This demonstrates that they have not decreased over the years and suggests that their persistent presence is a serious problem.

Given the economic and social importance of the Lucrecia dam for the population, different organisms and experimental models must be used to obtain a clearer picture of mutagenic activity in surface water in order to determine the real risks this water poses. In the present study, an *in vivo* and *in vitro* system was used. The *in vivo* assay, using the Trad-MCN, revealed a significant increase in mean micronucleus frequency for the collection points analyzed in the different seasons. Similar results were recorded for the *in vivo* genotoxicity assay conducted by Marcon *et al.*,[4] with micronucleus and nuclear abnormality levels of *Oreocchromis niloticus* collected at the Lucrecia dam. These findings in both *in vivo* studies suggest that all organisms living in this ecosystem are likely affected.

The Trad-MCN assay proved to be effective and sensitive for analyzing the genotoxin effects of the water studied. Research performed on rivers in China[33] and Austria,[34] using Trad-MCN, showed that it was also sensitive in detecting the genotoxicity of these rivers. Although the Trad-MCN is used worldwide to monitor air quality,[35,36] urban sludge,[37] and soil,[38] there are no recent reports that used this test to evaluate water pollution. It is important to point out that clone 4430 is the most frequently used in the Trad-MCN bioassay; however, the plant has not adapted to hot climates, such as that of Northeast Brazil. In accordance with authors who showed similar sensitivity in both plant species[37,39] and the use of *Tradescantia pallida* as a bio-indicator,[40–42] we also suggest that *T. pallida* can replace clone 4430 in the Trad-MCN assay, at least in tropical regions. Furthermore, this is the first study in Brazil to use the micronucleus test in *Tradescantia pallida* to assess water resources. Thus, due to its versatility, practicality and reliability, the *T. pallida* micronucleus test may provide an early assessment of water quality under real conditions and can be used, along with other bioassays and physicochemical analyses, to monitor threatened ecosystems.

In addition to using the Trad-MCN assay to perform preliminary screening of mutagenic pollution in this area, we conducted the CBMN assay in human lymphocytes to investigate the response in an *in vitro* system using human cells. Micronucleus frequency was found to decay proportionally with increased dilution for all points analyzed, demonstrating a high concentration of genotoxins in undiluted water. A similar study carried out by Amaral *et al.*[43] applied SMART in *Drosophila melanogaster* to evaluate the genotoxicity of the Caí River, employing crude samples as well as 50% and 25% concentrations. El Asslouj *et al.*[44] performed the CBMN assay to assess the genotoxicity of the Bounoumossa River, adding 50 μL, 100 μL, 200 μL and 400 μL of wastewater to the lymphocyte cultures. The authors recorded an increase in micronucleus frequency proportional to increasing doses. This assay is important since micronucleus frequency in peripheral blood lymphocytes is a predictor of cancer risk, due to its association with early carcinogenesis events.[17] Thus, CBMN in human lymphocytes has been extensively used in molecular epidemiology and cytogenetics for biomonitoring human populations exposed to genotoxic agents or bearing a susceptible genetic profile.[45,46]

The CBMN assay also revealed genotoxicity in raw water from point P1 as well as for water diluted by 50% and 25% analyzed in the dry season, while point P2 showed positive responses for raw water and water diluted by 50%. The use of diluted water clearly shows its toxic impacts, such as the cytotoxic and/or genotoxic effects of the different concentrations. This study established that genotoxin concentrations in the dry season were so high that even when water was diluted, significant mutagenic responses were still observed.

P1 exhibited more copper (Cu) than cadmium (Cd) in the dry season and more manganese (Mn) than lead (Pb) in the rainy season. Only Cd was observed in the dry season at P2, while in the rainy season Mn > Ni > Cd. At P3, Cu was greater than Cd in the dry season. Metals form a particularly complex class of mutagens, since they can interact in different ways with cellular machinery.[47] The adverse effects of some heavy metals on plant, animal and human DNA are well documented, possibly leading to decreased fertility and cancer induction.[8,48–50] This is due to two main mechanisms: oxidative damage and interference with DNA replication and repair.[51,52]

Studies on copper (Cu) have shown it is required in trace amounts for metabolic pathways, but that it is toxic in excessive amounts, causing genotoxic endpoints such as sister chromatid exchanges and chromosomal aberrations.[53] Prá *et al.*[54] illustrated that copper induced mutagenicity, as evaluated by the MN test in mice. Cadmium (Cd) toxicity is well documented in a number of studies.[55–59] Cadmium may lead to carcinogenesis through various action mechanisms including inhibition of DNA repair,

induction of oxidative stress, aberrant gene expression and apoptosis resistance/inhibition.[52] Manganese (Mn) is reported to cause neurological disorders in excessive doses,[60] since it can cause oxidative stress, mitochondrial dysfunction and neuroinflammation.[61] Lead (Pb) toxicity has been studied for many years through several end-points; however, data related to the mutagenic, clastogenic and carcinogenic properties of lead and its compounds are still conflicting. Moreover, biochemical and molecular action mechanisms of lead remain unclear.[62] Nickel exposure is associated with some cancers and the carcinogenic actions of nickel compounds are thought to involve oxidative stress, genomic DNA damage, epigenetic effects, and regulation of gene expression by activation of certain transcription factors related to corresponding signal transduction pathways.[50]

Raw water data from Trad-MCN and CBMN assays demonstrate that P1 exhibited the highest mean MN in both assays. This may be related to the fact that the point receives wastewater from multiple sources. In addition, water has nowhere to drain, resulting in a large number of genotoxins. Genotoxicity findings for water samples from the dry season are higher than those from the rainy season in both assays. This is due to decreased contaminant concentrations in the rainy season caused by the dilution factor.[63] On the other hand, point P2 showed the strongest response to climate conditions for both experiments. This may be owing to the confluence area at this point, where converging tributaries carry large amounts of toxicants to the dam. Point P3 represented treated water distributed to the population. As expected, all mutagenicity findings for this point showed negative results, except for Trad-MCN in the dry season. This may be associated with the presence of excessive amounts of cadmium and copper.

Comparison of mutagenic responses in raw water found in the Trad-MCN and CBMN assays for the different seasons shows that both agree in 66.66% of responses. These data demonstrate that the CBMN assay, which is well validated worldwide, supports most responses recorded in the Trad-MCN, confirming its importance and sensibility in monitoring aquatic ecosystems. Differences between *in vitro* and *in vivo* bioassays may have arisen because culture cells were directly exposed to damage during the *in vitro* assay, while toxic compounds could have been metabolized during *in vivo* assays. Differences may also have been due to different DNA damage responses by different cell types used in each assay.[30,64] Additionally, for the assay using a plant, phytotoxicity of the absorbed metal is considered complex, depending on factors such as metal concentration and speciation, oxidation state, concentration, duration of exposure and complex hyperaccumulation mechanisms.[65,66]

The two types of micronuclei tests used in this study demonstrate the capacity of these Lucrecia dam water samples to induce chromosome breakage and/or dysfunction of the mitotic apparatus. It is important to underscore that the aquatic environment is often the ultimate recipient of an increasing range of contaminants, making it difficult to determine which class of compounds is responsible for genotoxic contamination. Among aspects to be considered are the effects of synergy and antagonisms that occur as a result of the mixture formed by chemical compounds, which can be influenced by local characteristics and transported, transformed and/or bioaccumulated.[38,67] Heavy metals are persistent at the Lucrecia dam. Their action mechanism may be compromising the entire ecosystem, but they are likely not the only genotoxic agents present in the water. There are a number of environmental factors, such as radioactivity and toxic cyanobacteria, as described by Marcon *et al.*,[4] as well as other possible organic compounds. Taken together, they may be responsible for the mutagenic effects of this water and could be endangering the health of all organisms, including the human population.

5. Conclusion

All results obtained in this study show the presence of genotoxic compounds, including heavy metals, in surface water at the Lucrecia dam. These may induce chromosomal mutations and could be correlated with the high cancer rate found in the local population. These findings demonstrate the importance of using different organisms in genotoxicity assays in conjunction with analytical methods for the characterization of complex environmental mixtures. In addition, more prolonged monitoring is recommended to better characterize this public water supply.

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