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Research Article

Cytotoxic and genotoxic potential of surface water from the Pitimbu river, northeastern/RN Brazil

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Abstract

In this study, the onion (*Allium cepa*) root test was used to evaluate the genotoxicity of the Pitimbu River (Natal city, Brazil) surface water. The water was collected at five sampling sites along the river and one sample was obtained after the treatment (flocculation, chlorination and pH correction) of the river water for human consumption. All raw river water samples increased the frequency of chromosomal abnormalities and/or micronuclei and two of the water samples produced alterations in the mitotic index of the root cells. Two of the water samples also altered root growth and two produced morphological modifications in the *A. cepa* roots. Water collected from a site near an industrial area was the most consistently toxic and genotoxic of the samples. Although the water chlorinated for human consumption was not genotoxic, the data indicate that surface water from the Pitimbu River contains toxic and genotoxic compounds that potentially may impact this aquatic ecosystem.

Key words: Allium cepa, micronucleus, cytotoxicity, genotoxicity, surface water.

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Introduction

Urban-industrial and agricultural wastes can add significant amounts of contaminants to surface water and sediments and, consequently, water pollution is a serious problem for the health of the biota and humans that interact with these aquatic ecosystems. The contamination of water resources by genotoxic compounds is a worldwide problem (Claxton *et al.*, 1998; Kong, 1998; Vargas *et al.*, 2001; Ohe *et al.*, 2003; Buschini *et al.*, 2004). Because of the importance of water quality to health, many toxicity and genotoxicity tests have been used in combination with physical and chemical analysis in order to evaluate both water and environmental quality (Smaka-Kincl *et al.*, 1996; Claxton *et al.*, 1998; Umbuzeiro *et al.*, 2001; Vargas *et al.*, 2001; Ohe *et al.*, 2003; Isidori *et al.*, 2004). Water quality is an important risk factor in cancer and relative risk has been estimated in areas supplied with mutagenic drinking water. The observed exposure-response relationship indicated a relative risk for lymphomas, pancreatic cancer and esophageal cancer compared with areas in which non-mutagenic drinking water was consumed (Koivusalo *et al.*, 1995; Tao *et al.*, 1999).

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Many types of genotoxicity and mutagenicity assays employing microorganisms and mammalian cells have used for monitoring of complex environmental samples such as river water (Verschaeve, 2002; Isidori *et al.*, 2004; Reinecke and Reinecke, 2004; Russo *et al.*, 2004). However, plant assays, such as the *Allium cepa* test, may have some advantages over microbial and mammalian cell tests for environmental monitoring. Plant assays are highly sensitive to many environmental pollutants, including heavy metals (Fiskesjo, 1988), and have been used for monitoring the potential synergistic effects of mixtures of pollutants, including hydrophilic and lipophilic chemicals (Grover and Kaur, 1999; Ateeq *et al.*, 2002; Rank *et al.*, 2002; De Campos and Viccini, 2003). Furthermore, the test plants can be directly exposed to complex mixtures or environmental samples either in the laboratory or *in situ* (Fiskesjo, 1985b; Grant *et al.*, 1992; Rank, 2003). Because of the large size of their chromosomes, higher plants are suitable for cytological analysis and the responses seen in plant tests are highly correlated with those seen in other biological systems, making plant tests good candidates for evaluating the genotoxicity of environmental samples (Grant, 1999; Sadowska *et al.*, 2001). Onion (*Allium cepa*) root-tip cells can be used to measure a variety of morphological and cytogenetic factors that can be used as toxicity indicators, such factors including root morphology and growth, mitotic index determination, and the in-

duction of micronuclei and aberrant metaphases and anaphases (Grant, 1994,1999; Evseeva *et al.*, 2003).

In this study, we used the *Allium cepa* test to evaluate the toxicity and genotoxicity of surface water collected at five sampling sites, and on the Pitimbu River, an important water source that supplies drinking water for the 170,000 habitants of the city of Natal in the northeastern Brazilian state of Rio Grande do Norte (www.serhid.rn.gov.br/igarn). Treated drinking water derived from one of the sites was also tested.

Material and Methods

Geography of the Pitimbu river

The Pitimbu river (5°50' S, 35°05' W to 6°00' S, 35°25' W), is located in the northeastern Brazilian state of Rio Grande do Norte (RN) between (Figure 1). The size of the hydrographic basin upstream of the Jiqui Pond totals 98 km^2. The river receives municipal wastewater from the cities of Macaiba, Parnamirim and Natal in RN state, as well as industrial and agricultural wastewater. This basin, also acts as the principal water source for the southern area of the city of Natal. The river empties into the Jiqui Pond, which provides 32% of the gross domestic water supply for the city of Natal, and is the principal source of water for the people in the southern part of the city. Pitimbu River water is treated for drinking using a conventional technique that includes flocculation, chlorination and pH correction. Due to its lack of turbidity and color, no coagulation is used in processing the water during the dry period (September to april) At Macaiba, the Pitimbu River passes through an agricultural area, while at Parnamirim, it passes through an important industrial area of RGN state.

Sampling

Surface water was collected from the Pitimbu river at five different sampling sites (sites 1 to 5) and a sample of treated drinking water was obtained at site 6 CAERN (Companhia de águas e esgotos do Rio Grande do Norte) water treatment station at site 6 (Figure.1), one set of samples being collected in January 2003 (Summer in the southern Hemisphere) and another during July (winter) of the same year. The sample sites were as follows: site 1 was at the headwaters of the Pitimbu River, located in the vicinity of the city of Macaiba; site 2 was located downstream of an agricultural area; site 3 was located close to the city of Parnamirim and an industrial area containing factories manufacturing textiles, soft drinks, candies, snacks and paper; site 4 was located downstream of the industrial area; site 5 was located at the Jiqui Pond; and site 6 was at a water treatment site run by the RGN Water and Sewage Company (Companhia de Águas e Esgotos do Rio Grande do Norte – CAERN) for the production of potable water using the water purification criteria of Statutory Instrument 518 (SI-518) of the Brazilian Health Ministry (Table 1), the purification process involving flocculation with aluminum sulfate, sedimentation, filtration, chlorination with sodium hypochlorite and pH correction.

The *Allium cepa* test

The procedure was based on previously described methods (Fiskesjo, 1985a; Rank and Nielsen, 1998). Bulbs of the common onion (*Allium cepa L.*) were purchased at a local supermarket and eight bulbs prepared for each sample. The bulbs were peeled and placed in distilled water for 48 h, after which some of the newly-formed root tips were

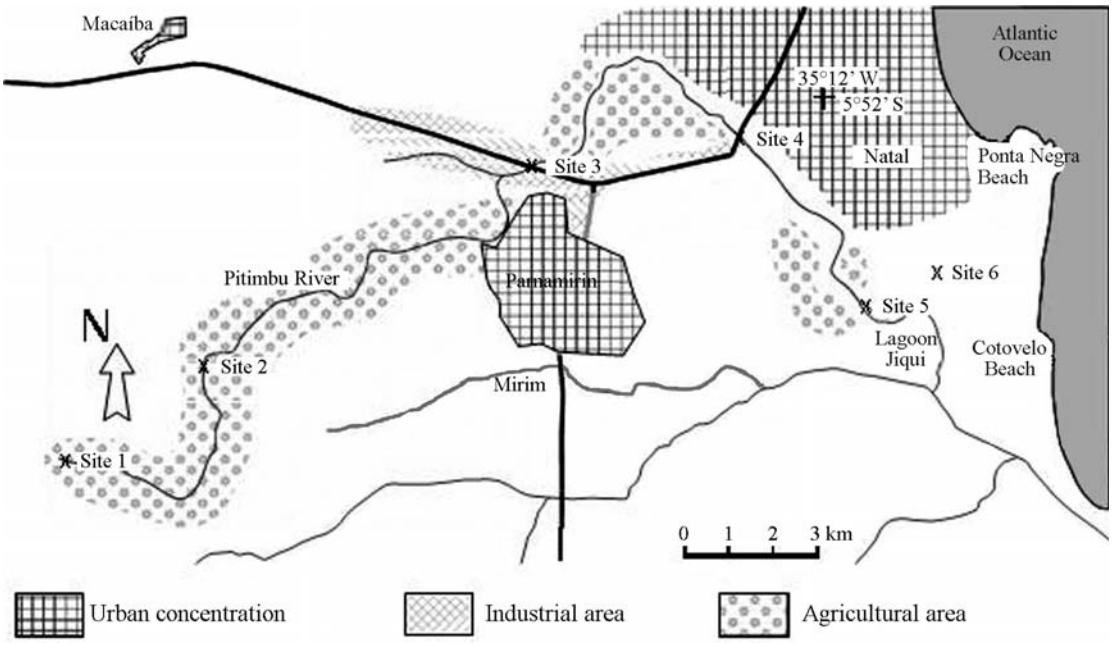

Figure 1 - Sampling sites in the Pitimbu River, Rio Grande do Norte, northeastern Brazil.

Table 1 - Chemical and bacteriological analysis performed on treated drinking water obtained from the Pitimbu river. The analysis was performed by the Rio Grande do Norte Water and Sewage Company (Companhia de Águas e Esgotos do Rio Grande do Norte) using the water purification criteria of the Brazilian Health Ministry Statutory Instrument 518 (SI-518).

Parameters	Free chlorine (mg L^{-1})	Total coliforms (colony forming units mL^{-1})	pH	Nitrates (mg L^{-1})	Turbidity (units)
Levels permitted by SI-518	0.2-5.0	Not found 95%	6.0-9.5	10.0	5.0
Assayed levels	0.5	100	6, 0	1.6	0.5

cut from each bulb and examined morphologically for any visible abnormalities. If the root-tip length was normal (2-3 cm) and cell organization apparently normal for all the root tips, individual sets of eight bulbs were placed in water from each sample site for 24 h at 25 °C ± 1 °C. Distilled water was used as a negative control and 300 mM aqueous hydrogen peroxide (Merck) as a positive control mutagen. After 24 h some root tips were removed from the bulbs, fixed 3:1 in ethanol:glacial acetic acid and kept overnight at 4 °C before being hydrolyzed in 1N HCl at 60 °C for 10 min, rinsed in tap water and stained in Schiff's reagent using Feulgen's method (Alexander *et al.*, 1950). Microscope slides were prepared by squashing the stained root tips in 45% (v/v) glacial acetic acid. One slide was prepared per bulb and all slides were coded and examined blind using bright-field microscopy at a total magnification of 40x. Genotoxicity was evaluated by counting the number of chromosomal breaks in 100 anaphase and metaphase cells per slide and also counting the number of micronuclei in 2000 interphase nuclei per bulb, micronuclei being recognized based on their being less than one-third the diameter of the nucleus, being separated from the main nucleus and having a staining intensity similar to that of the main nucleus. Cytotoxicity was assessed by recording the total mitotic index and the mitotic index for each stage of mitosis as calculated from a total of 2000 cells for each bulb. After 72 h treatment root length was measured with a electronic digital caliper (Starrett®727 series) and used as an index of toxicity, while macroscopically observable morphological modifications such as changes in root consistency and color and the presence of tumors, hook roots and twisted roots were also recorded. Since the water samples were collected during winter and summer, a total of 16 bulbs were analyzed for each sample.

Statistical analysis

The root was considered the experimental unit for statistical analysis. Mean values and standard deviations were calculated for each experimental group and evaluated using Student's *t*-test for independent samples.

Results

The t-test showed no statistically significant differences between the assay results obtained with water samples collected in the winter and summer of 2003 so the data were combined to generate Table 2. Roots exposed to distilled water for 72 h (negative control) had an average length of 4.4 cm and all showed normal morphology whereas roots exposed to water from site 3 showed a reduction in length while those exposed to treated water showed an increase in length. In addition, roots exposed to water from sites 1, 2 and 3 showed an increase in abnormal morphology, mainly in the form of twisted roots. There was an increase in the mitotic index of the root-tip cells from roots exposed to water from site 4 and site 6 (the treated drinking water), while water from the remaining four sites showed no significant cytotoxicity effects (Table 2). Root-tip cells from roots exposed to water from sites 1, 3 and 5 showed a significant increase in the frequency of chromosome damage. Although the sample from site 1, the most up-stream sampling site, and site 6 showed an increased frequency of chromosome damage, neither was statically significant. All the water samples and the positive, but not the negative, control produced mitotic damage in the root-tip cells, the most frequent abnormalities being multi-polar anaphases, c-mitosis and stickiness. Although the micronuclei frequency was higher in the root-tip cells exposed to water from all the sites and the positive control as compared to those exposed to distilled water, this increase was only statistically significant for the positive control and water from sites 1 and 3 (Table 2).

Root-tip cells treated with the river water samples also showed alterations regarding the frequency of cells in different stages of mitosis (Table 3), with relatively small, but statistically significant increases (as compared to the negative control) in the frequency of metaphase cells occurring in roots exposed to water from sites 1, 2, 5 and 6, while larger increases in metaphase cell frequencies were produced by exposure to water from sites 3 and 4. The frequency of anaphase cells was significantly increased by exposure to water from sites 1 and 6, while water from site 4 produced a significant decrease in the frequency of anaphase cells (Table 3).

Discussion

Among the assays that have been used for evaluating water quality, plant systems are recognized as sensitive biomonitors of the cytotoxic and genotoxic effects of environmental chemicals and can be used for the detection of environmental mutagens both *in situ* and in the laboratory

Table 2 - Microscopic and macroscopic alterations in *Allium cepa* root tip cells treated with water from the Pitimbu river.

Sample	Genotoxic effects (mean ± standard deviation)		Cytotoxic effects (mean ± standard deviation)							Morphological root alterations	
	Chromosome breaks	Micronuclei	Mitotic index (%)	Fragments	Bridges	Laggards	Multipolar anaphases	c-mitosis	Stickiness	Length (cm)	Morphology (% normal)
Site 1	0	3.75 ± 0.56*	11.9 ± 1.36	0	5.0 ± 0.53	0	7.75 ± 1.63*	4.25 ± 0.47	0	3.9 ± 0.4	40 ± 3.86 *
Site 2	0	0.62 ± 0.34	13.9 ± 2.43	1.75 ± 0.1	4.25 ± 0.45	2.69 ± 1.35	23.79 ± 2.54*	8.52 ± 0.89*	4.25 ± 1.9*	4.2 ± 1.7	30 ± 5.43 *
Site 3	2.15 ± 1.7	1.5 ± 0.2*	8.0 ± 1.6	1.7 ± 1.2	10.82 ± 1.7*	0	24.75 ± 2.56*	7.58 ± 0.79*	0	2.3 ± 0.9 *	60 ± 3.24 *
Site 4	2.0 ± 1.35	0.87 ± 0.12	17.5 ± 2.56 *	2.45 ± 0.75	8.35 ± 0.56*	0	19.25 ± 1.32*	13.95 ± 2.65*	6.54 ± 0.7*	5.2 ± 0.2	90 ± 5.2
Site 5	5.25 ± 0.42*	0.75 ± 0.4	8.5 ± 3.12	4.62 ± 0.75*	15.73 ± 2.37*	6.7 ± 0.7	30.47 ± 1.56*	8.23 ± 1.5*	13 ± 2.43*	4.6 ± 0.6	90 ± 1.36
Site 6 (treated water)	0	0.75 ± 0.37	16.7 ± 1.34 *	0	5.7 ± 0.8	0	11.79 ± 1.56*	1.51 ± 0.45	2.6 ± 0.1	5.6 ± 1.2 *	90 ± 3.57
Negative control	0	0.37 ± 0.25	11.3 ± 2.35	0	2.35 ± 0.75	2.1 ± 0.5	3.45 ± 1.2	3.1 ± 0.5	0	4.4 ± 0.8	100
Positive control	1.2 ± 0.45	10.62 ± 1.4 *	5.1 ± 1.21 *	3.75 ± 0.25*	6.9 ± 1.45*	5.49 ± 1.15	18.75 ± 1.79*	8.91 ± 0.1*	16. 4 ± 1.75*	0.5 ± 1.3*	0

Notes: For each test we used one slide per bulb and 8 bulbs per water sample and season (winter and summer) to give a total of 16 bulbs for each sample site. The Mitotic index was calculated from a total of 2,000 cells. For chromosome aberration analysis we scored 100 anaphases and 100 metaphases. For the micronucleus test we scored 2,000 interphasic nuclei per slide.
*Values statistically significant when compared to the untreated control by Student's *t*-test at $p < 0.05$.

(Grant, 1999). Toxic effects are evaluated in the *A. cepa* test by analyzing the macroscopic parameters of root growth and root morphology, while both genotoxicity and cytotoxicity can be monitored through cytological parameters such as the number of chromosome breaks and micronuclei, mitotic index and chromosome and mitotic damage. Positive results in higher plant systems indicate the presence of cytotoxic and/or genotoxic substances in the environment, and indicate the potential for direct or indirect risks for living organisms (Fiskesjo, 1993).

The Pitimbu river receives untreated wastes from agricultural operations (mainly at sites 1 and 2), industrial plants (mainly at sites 3 and 4) and domestic sewage (sites 1 to 4) and is the main water source for the Jiqui Pond (site 5) (Figure 1). In the present study, the treatment of *A. cepa* roots with surface water samples from the Pitimbu river caused alterations in mitotic index, chromosome and mitotic damage and/or changes in the frequency of micronuclei and also alterations in morphological parameters, mainly in the form of twisted roots. Although all the raw water samples showed some degree of genotoxicity compared to the distilled water control the water from site 3, located adjacent to an industrial area, appeared to be the most damaging as evidenced by its capacity to induced an increased micronuclei frequency and chromosome and mitotic damage as well as increasing the frequency of twisted roots and decreasing root length and (non significantly) mitotic index. The water sample obtained after treatment of the river water at the CAERN treatment plant produced a statistically significant increase of in mitotic index and multi-polar anaphase frequencies. However, overall it was less toxic and genotoxic than the raw river water samples. These data indicate the presence of cytotoxic and genotoxic substances in the raw river water that are reduced by the water treatment process.

The cytotoxic and/or genotoxic compounds present in the river water were not directly identified in this study but were assessed in the study by Santos *et al.* (2002), previously unpublished data from which is given in Tables 4 and 5. These tables show that there were high levels of potentially toxic metals such as aluminum, copper, iron, zinc and others in surface water and sediments from several Pitimbu river sites and that these levels were above those permitted by the Brazilian National Environment Council (Conselho Nacional do Meio Ambiente – CONAMA) Statutory Instrument 357/2005 (http://www.mma.gov.br/conama/res/res05/res35705.pdf). In particular, high concentrations of potentially toxic metals were detected by Santos *et al.* (2002) at a river site matching our sampling site 3, which displayed consistently high levels of genotoxicity.

Potentially toxic metals, resulting from some agricultural and industrial activities are one the most common environmental contaminants and several have been shown to be mutagenic and/or carcinogenic in both human and animal studies. Metals, such as arsenic (III), chromium (VI),

Table 3 - Distribution of mitotic phases in *Allium cepa* root tip cells treated with water samples from the Pitimbu river.

	Mitotic phase (%, mean ± standard deviation)			
Sample	Prophase	Metaphase	Anaphase	Telophase
Site 1	31.7 ± 12.0	25.6 ± 9.6*	24.5 ± 8.1*	18.2 ± 5.9*
Site 2	54.9 ± 5.7	24.7 ± 14.6*	12.2 ± 5.6	8.2 ± 4.7
Site 3	22.2 ± 9.0	54.1 ± 8.0*	14.5 ± 4.2	9.2 ± 9.1
Site 4	50.8 ± 3.3	33.6 ± 13.8*	7.4 ± 10.7*	8.2 ± 6.5
Site 5	52.0 ± 8.3	25.9 ± 4.6*	14.9 ± 6.5	7.2 ± 4.8
Site 6 (treated water)	45.1 ± 2.1	25.3 ± 13.8*	22.5 ± 16.7*	7.1 ± 5.6
Negative control	54.5 ± 3.6	18.9 ± 2.1	15.2 ± 4.8	11.4 ± 1.6
Positive control	55.5 ± 3.7	14.6 ± 7.3	6.7 ± 2.7*	23.2 ± 4.7*

*Values statistically significant when compared to the untreated control by Student's *t*-test at $p < 0.05$.

Table 4 - Analysis of Pitimbu river surface water. An asterisk indicates levels exceeding those permitted by current Brazilian legislation National Environment Council (Conselho Nacional do Meio Ambiente – CONAMA) Statutory Instrument 357/2005. The cytotoxic and/or genotoxic compounds present in the river water were not directly identified in this study but were assessed in the study by Santos *et al.* (2002), previously unpublished data from which is given in this table.

Analytical parameters	Mean values
Inorganic (mg L^{-1})	
HCO_3^-	32.4
Cl^-	29.5
Na^+	11.8
Ca^{2+}	6.2
Mg^{2+}	4.1
K^+	3.5
SO_4^{2-}	2.6
NO_3^-	1.6
NH_3	0.2
Fe^{3+}	0.8*
Al	0.1*
Ba	0.06
Cu	0.005
Cr	0.005
Cd	0.0005
Organic (mg L^{-1})	
Oil	10.8175*
Bacteriological (CFU 100 mL^{-1})	
Coliforms total	3.92×10^3*
Coliforms fecal	1.015×10^3*

Table 5 - Analysis of Pitimbu river sediments. An asterisk indicates levels exceeding those permitted by the Brazilian National Environment Council (see heading to Table 2). The cytotoxic and/or genotoxic compounds present in the river water were not directly identified in this study but were assessed in the study by Santos *et al.* (2002), previously unpublished data from which is given in this table.

Element	Mean value (mg kg^{-1})
Al	24,800
Fe	17,200
Mg	400
Ba	120
Zn	45*
Pb	25*
Cu	23*
La	22*
Ni	15
Te	6*
Mo,	2*
As	1.17
Hg	0.46*
Cd	0.224
Ag	0.185*

and nickel (II), increase the risk of cancer, especially lung and skin cancers (Harris and Shi, 2003; Leonard *et al.*, 2004). Plant assays have been considered useful tools for evaluating and ranking the toxicity and genotoxicity of metals (Fiskesjo, 1988; Steinkellner *et al.*, 1998; Evseeva *et al.*, 2003; Chandra *et al.*, 2005; Unyayar *et al.*, 2006). Various metals are known to induce chromosome breaks, fragments and micronucleus formation in plants and mammalian test systems (Knasmuller *et al.*, 1998). Among the effects induced are formation of DNA – DNA and DNA – protein cross-links, alterations of mitotic spindles formation producing aberrant mitotic stages, and induction of sister chromatid exchanges (SCE). The induction of micronuclei is usually caused by chromosome breaks or fragments or spindle poisoning, which is an anomalous disjunction of chromosomes during anaphase. Metals such as iron and copper can also cause indirect effects due to increased oxidative stress leading to cytotoxicity and DNA damage (Knasmuller *et al.*, 1998; Steinkellner *et al.*, 1998; Costa *et al.*, 2002; Harris and Shi, 2003; Waisberg *et al.*, 2003;

Leonard *et al.*, 2004; Chandra *et al.*, 2005; Unyayar *et al.*, 2006).

Although the treated water from site 6 in the Jiqui pond did not produce a significant genotoxic response, there was some indication of genotoxic potential. Chlorination is a common water disinfectant method which is able to reduce microbial water pollution, but which can also produce genotoxic and toxic compounds if precursors are present in the water to be treated and the level of chlorine is high (Komulainen, 2004). Surface water can contain variable levels of organic matter, including humic acids that are the main source of potentially toxic by-products of disinfection with chlorine, which can react with such compounds. The major chlorination by-products that have been the object of intensive evaluation are the trihalomethanes, halogenated acetic acids and chlorinated furanones, most of which are known carcinogens, although the cellular mechanisms of their carcinogenicity are poorly understood (Komulainen, 2004). Some authors have also used plant assay to assess the genotoxicity induced by the by-products of disinfection, with positive results having been obtained in *A. cepa* and *Tradescantia* assays with carboxylic acids and halogenated derivatives of peracetic acid, sodium hypochlorite and chlorine dioxide (Monarca *et al.*, 2002; Monarca *et al.*, 2003; Monarca *et al.*, 2005).

This report is the first to evaluate the genotoxicity of environmental samples from northeast Brazil, a region with deficiencies in basic sanitation and the treatment and disposal of urban-industrial and agriculture wastes. Our results indicate that Pitimbu river water contains significant levels of genotoxic activity.

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