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Micronucleus Test for Monitoring Genotoxicity of Polluted River Water in *Rana catesbeiana* Tadpoles

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Chromosomal aberrations have frequently been used to detect and quantify mutagenic agents. However, this method has the disadvantage of being time-consuming and requiring very accurate observation, according to Siboulet et al. (1984). Evaluation of micronucleus frequency *in vivo* is recommended by the regulatory agencies around the world to be conducted as part of product safety assessment (Krishna and Hayashi 2000). The *in vivo* micronucleus (MN) test, when performed appropriately, is useful for screening substances for their ability to cause both clastogenity (chromosome breakage) and aneugenicity (chromosome lagging due to disfunction of mitotic apparatus) (Krishna and Hayashi, 2000; see also Carrano and Heddle 1973; Schmid 1975). This test has been used successfully in many amphibian species to detect the presence of mutagens in fresh water (Fernandez et al. 1993).

Amphibian larvae (tadpoles) have been widely used as bioindicators to detect the presence of mutagenic agents in water, mainly because their high sensitivity makes them ideal for genotoxicity monitoring of aquatic environments (Jaylet et al. 1986, Fernandez et al. 1993, Ralph et al. 1996). In this study, we used *Rana catesbeiana* tadpoles to access genotoxicity of water samples obtained from the Paraíba do Sul River in the State of São Paulo, Brazil (23° to 24° lat S – 45° to 46° long W). The water in this river is collected to supply many cities in the region. The most important points for study are the Pindamonhangaba, Tremembé, and São José dos Campos sites. The river receives contaminants from many sources, including paper mill and industries (car, food, and chemical), accidental spills, landfill run-offs, pesticides, an oil refinery, and untreated human sewage (CETESB 2000).

MATERIALS AND METHODS

Rana catesbeiana tadpoles (n = 250) were obtained from a commercial supplier, and had morphological characteristics typical of stages 25 to 38, as defined by Gosner (1960). The tadpoles were maintained in cages (1 x 0.50 x 0.70 m) constructed of plastic mesh (2 x 2 mm holes) sowed with fishing line. Each cage had a metal framework (3/8 in. iron bars) to maintain its shape. The study was done at three sites along the Paraiba do Sul River. At site 1 (Pindamonhangaba), caged tadpoles were placed in the irrigation canals of a rice field. At sites 2 and 3 (Tremembé and São José dos Campos), the cages were placed in water at a station

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Table 1. Characteristics of the cities neighboring sites 1, 2 and 3.

Cities (sites)	*Urban population*	*Number of industries*	*Sewage**	
			collected	*treated*
Pindamonhangaba (1)	118,793	255	96	96
Tremembé (2)	29,850	8	90	0
Taubaté**	229,810	515	96	5
Caçapava**	66,418	80	89	89
S. J. dos Campos (3)	532,403	811	98	49

Source: The data were provided by the respective city halls.
* Percentages of the total sewage that are collected and treated by the city halls.
** Located between sites 2 and 3.

used to treat water for human consumption. Some characteristics of the cities neighboring the sites studied are shown in Table 1.

Two experiments were done, one in the summer (rainy season) and one in the winter (dry season). At each site, 45 caged tadpoles were submerged and the cage anchored underwater by its own weight. The tadpoles were not fed during the experiments. After 7-14 days of exposure (Ralph et al. 1996), 15 tadpoles from each site were collected (Jaylet et al. 1986) along with a sample of local water and transported in separate plastic bags to the laboratory.

Fifteen tadpoles were studied for each season as soon as they arrived from the suppliers, and frequency of micronuclei was determined. These 30 animals were designed as baseline. In order to test if tadpoles were responsive to a known mutagen, two additional groups were evaluated. One group. Composed by 25 animals (10 in the rainy and 15 in the dry season) were maintained during 14 days in cages at the site where they were collected and were provided with tap water and food daily. This group was designed as negative group. The group defined as positive (n=15) was kept in a solution of cyclophosphamide (Genuxal 1000 mg – ASTA medical) during 14 days at a concentration of 5 ppm (Rudek and Rozek 1992), in a small (1.5 l) aquarium containing 100 ml of solution/larva (Djomo et al. 1995). The solution was replaced every 96 h (APHA et al. 1998) and the tadpoles were fed every two days with commercial frog food. The aquarium solution was aerated and kept under natural conditions of lighting and temperature.

To determine micronuclei frequency, blood samples were collected from the ventral tail blood vessel (Forman and Just 1976) into heparinized (Liquemine – sodium heparin 25.000 I.U. /5 ml - Roche) micropipettes and two blood smears were prepared for each tadpole.

The slides were fixed for 5 min in methanol and air-dried before staining with the Feulgen method and Fast-green. The slides were covered with micro cover slips, fixed with Entellan-Merk (rapid mounting media for microscopy) and then coded, randomized and blindly scored by a single observer (Das and Nanda 1986). Between 1000 and 2000 red blood cells in each slide were scored (Djomo 1995; Fernandez 1993) in order to obtain the minimum number of cells required for statistical analysis (Djomo 1995).

The areas selected for scoring had optimal cell morphology, staining, and cell distribution (Krauter et al. 1987). The slides were examined using a Carl Zeiss microscope equipped with an oil immersion lens (X 1000).

The frequencies of MN in the control groups were compared using the Kruskal–Wallis test. One-way ANOVA was employed to evaluate the differences among the sites and the influence of time of exposure and season of the year. As dependent variable, we employed the frequency of MN, as well as its square root and rank transformations, in order to avoid the influence of heterocedasticity of the dependent variable. As predictive variables, we considered dummy indicators of site, period and season of exposure. The level of significance was set in 5%.

The water samples collected concomitantly with the tadpoles were analyzed by the Adolfo Lutz Institute in São Paulo, according to the guidelines established by the Brazilian Environmental Council (CONAMA 1986). Chemical analysis of the water samples showed that the values for most parameters were within acceptable limits, except for color, turbidity and iron content. The values for color, turbidity, bicarbonate alkalinity, non-carbonate hardness, carbonate hardness, total hardness, nitrite, nitrate, total iron and chlorides were higher in the rainy season than in the dry season. These values were also higher than the recommended values.

RESULTS AND DISCUSSION

The parameters analyzed are summarized in Tables 2 and 3. All parameters generally

Table 2. Physical and chemical parameters of the water samples analyzed (rainy season)

Parameters	Values*							
	Brazil**	Negative Control	Site 1 7 d	Site 1 14 d	Site 2 7 d	Site 2 14 d	Site 3 7 d	Site 3 14 d
Temperature (C°)	-	-	27	27	27.5	28	28	27
pH	6.0–9.0	-	6.7	6.5	6.0	6.6	6.0	6.5
Color (HU)	15.0	14	119	134	96	113	120	336
Turbidity (NTU)	5.0	0.64	17	23	18	18	15	54
Hydroxide alkalinity	0.0	0	0	0	0	0	0	0
Carbonate alkalinity	120	0	0	0	0	0	0	0
Bicarbonate alkalinity	250	18	24	26	24	26	24	24
Non-carbonate hardness	-	0	0	4	6	20	14	20
Carbonate hardness	-	10	20	26	24	26	24	24
Total hardness	500	10	20	30	30	46	38	44
Consumed oxygen	-	0.20	2.3	3.3	5.2	3.4	2.6	3.5
Albumin nitrogen	-	0	0.04	0	0.02	0	0.04	0
Ammonia nitrogen	0.50	0	0.02	0	0.01	0	0.02	0
Nitrite	1	0	0.12	0.05	0.13	0.06	0.14	0.05
Nitrate	10	0	0.4	2	0.5	1.6	0.3	1.6
Total iron	0.3	0.02	2	1.3	4.5	1.7	1.9	2.5
Chlorides	250	2.00	10	14	10	12	11	10
Total phosphorus	0.025	0	0	0	0	0	0	0

* d = days; chemical concentrations in mg/L; HU = Hazen Unit (mg PL-Co/L); NTU = Nephelometric Turbidity Unit (it increases when the total suspended solids in the water increases).
** Accepted values.

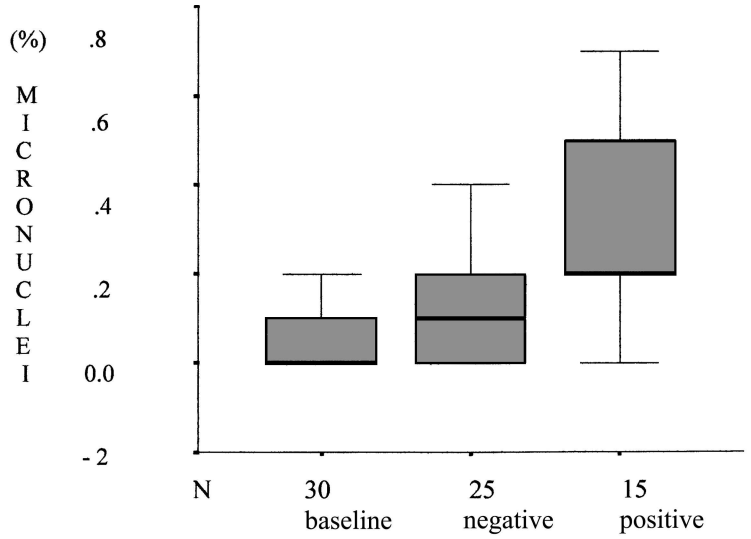

Figure 1. The frequencies of MN in erythrocytes of *Rana catesbeiana* tadpoles in the baseline and control groups. The results are shown as box plots.

Table 3. Physical and chemical parameters of the water samples analyzed (dry season).

Parameters	*Values**						
	Negative Control	Site 1 7 d	Site 1 14 d	Site 2 7 d	Site 2 14 d	Site 3 7 d	Site 3 7 d
Temperature (C°)	24	19.5	20	19.5	20	19.5	20
pH	-	6.8	6.6	6.7	6.6	6.7	6.6
Color (HU)	7	80	200	71	145	54	107
Turbidity (NTU)	0.9	11	30	10	20	6.7	15
Hydroxide alkalinity	0	0	0	0	0	0	0
Carbonate alkalinity	0	0	0	0	0	0	0
Bicarbonate alkalinity	20	20	20	16	14	20	16
Non carbonate hardness	0	8	0	10	2	10	0
Carbonate hardness	20	20	12	16	14	20	12
Total hardness	20	28	12	26	16	30	12
Consumed oxygen	0.2	1.7	1.9	1.3	1.3	1.2	1.3
Albumin nitrogen	0	0	0.14	0	0.11	0	0.06
Ammonia nitrogen	0	0	0.04	0	0.06	0	0.04
Nitrite	0	0.01	0.02	0.01	0.02	0.01	0.01
Nitrate	0.1	0.8	1.4	1.5	1	0.6	0.3
Total iron	0.06	0.4	0.7	0.3	0.5	0.3	0.3
Chlorides	8	7	12	14	11	9	9
Total phosphorus	0	0	0	0	0	0	0

* For the accepted levels in Brazil, see Table 2. d = days, HU = Hazen Unit (mg PL-Co/L), NTU = Nephelometric Turbidity Units, chemical concentrations in mg/L.

had higher values after 7 days than after 14 days, although bicarbonate alkalinity, carbonate hardness and total hardness were lower at 7 in comparison with 14 days.

The vast majority of micronucleated erythrocytes contained one MN, and only one cell had two MN.The shape of the MN varied from round (most common) to elongated and ring-shaped. Comparison of the frequency of MN in the erythrocytes of tadpoles exposed to cyclophosphamide (positive control) with the frequencies in the basal and negative control groups confirmed that the MN test was sensitive enough to detect the effects of a well-established mutagenic substance. A significant ($p < 0.001$) difference among groups was observed, positive group being different from baseline and negative groups (Figure 1).

Interestingly enough, the frequency of micronuclei in animals kept in aquaria receiving tap water (negative group), although non-statistically different from baseline, was somewhat high. It is possible that this event could be due to the presence of mutagenic substances generated by disinfection of surface drinking water, in particular water chlorination (Monarca et al., 2003).

The frequencies of MN observed at the three sites are shown in Figure 2. Significant effects were detected for season (dry > rainy, $p < 0.001$), period (7 > 14 days, $p = 0.038$) and site (site 1 < site 2 = site 3, $p = 0.012$).

In general, seven days of exposure resulted in the highest frequency of micronucleus in both seasons.

These results agreed with those by De Flora et al. (1993), who demonstrated the ability of organisms to eliminate damaged cells after a prolonged exposure. The genotoxicity results of our study also agreed with similar work done by Das and Nanda (1996) in this same area. These authors sought to explain the decreased frequency of MN in fish exposed to paper mill effluents over long periods of time, and suggested an erythropoiesis-inhibiting effect on cell division, with subsequent hindrance of the passage of affected cells into the blood stream.

In the case of *R. catesbeiana* tadpoles, the DNA repair mechanisms may be particularly effective, because the erythrocytes are nucleated cells, and there may be a decrease in chromosome damage during 14 days of exposure. Alternatively, the toxic substances may accumulate in the organism during the first 7 days and then be degraded over the following days. Similar results were reported by Lemos et al. (2001) for the fish *Pimephales promelas* exposed to hexavalent chromium for 7, 14 and 21 days.

There was a marked difference in the frequencies of MN between seasons. These findings may be explained by the dilution of toxic substances by precipitation during the rainy season, whereas in the dry season these substances were more concentrated. We correlated the mean frequency of micronuclei measured at the 3 experimental sites, after two periods and exposure and in the rainy and dry seasons – a total of 12 values – with the corresponding water quality parameters depicted in Tables 2 and 3. The most significant association (Spearman's non-parametric test) was negative and was obtained with pH ($r=0.696$, $p=0.012$). Although there are significant cross-correlation among the parameters of water quality determined in our study, and considering that the two aforementioned parameters may represent proxy variables of other non-measured components, it is tempting to speculate that

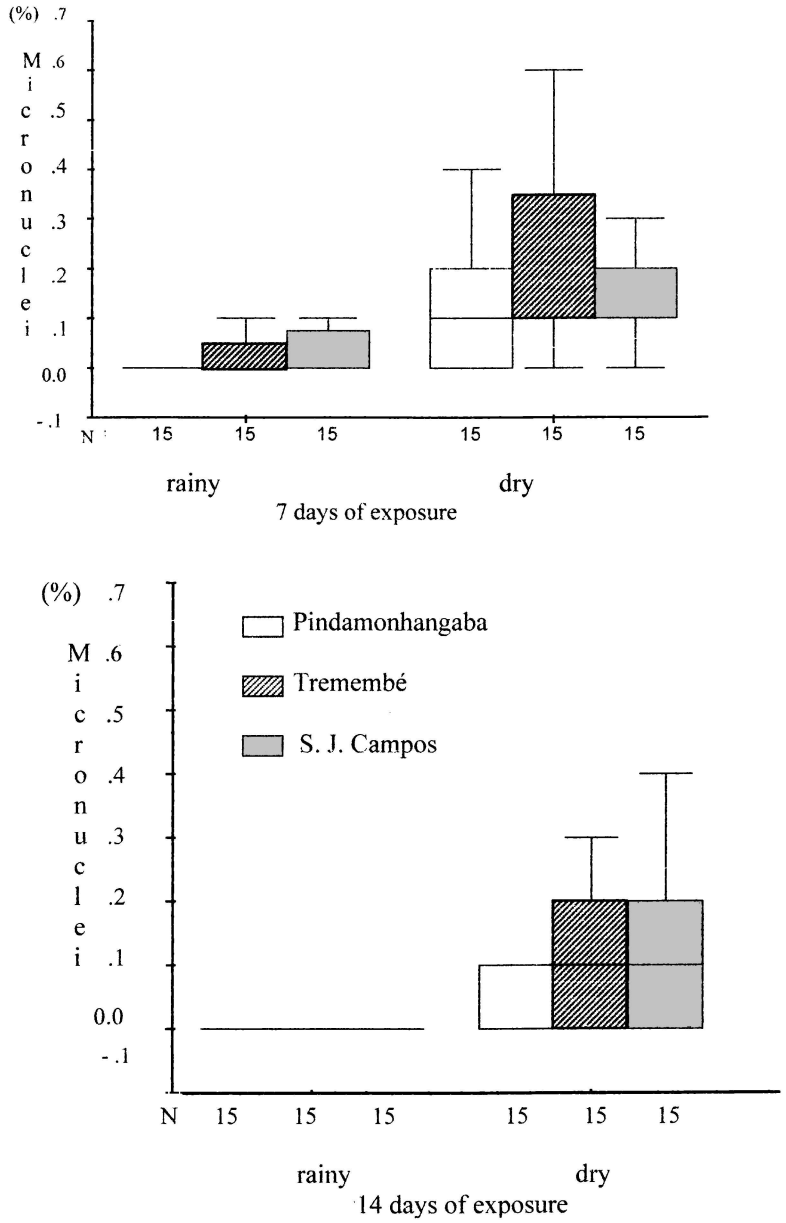

Figure 2. Box plots representing the values of micronuclei obtained in the three experimental sites for dry and rainy seasons and after exposure of 7 or 14 days. The horizontal bars represent the median values.

processes involving redox reactions participate of the process of micronuclei formation in the exposed animals.

As shown here, the MN test in *R. catesbeiana* tadpoles kept *in situ* in the areas of study was useful for assessing the genotoxicity of polluted water. In doing this test, a parallel negative control must be run to determine the species basal frequency of MN in order to provide a reliable database for comparison with altered frequencies. Determining the period of greatest genotoxicity is also important so as to obtain the highest sensitivity for the test. In the case of *R. catesbeiana* tadpoles, a 7 days exposure proved to be best.

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