

Investigation of the Genotoxic Potential of the Waters of a River Receiving Tannery Effluents by Means of the *in vitro* Comet Assay

Silvia Tamie Matsumoto¹, Mário Sérgio Mantovani², Mirtis I. Mallaguti³ and Maria Aparecida Marin-Morales^{3,*}

¹ Universidade Federal do Espírito Santo, Departamento de Ciências Biológicas, Av. Marechal Campus 1468, Maruípe, CEP: 29040–090, Vitória/ES., Brazil

² Universidade Estadual de Londrina, Departamento de Biologia Geral, Cx. Postal. 601, CEP: 86051–990, Londrina/PR., Brazil

³ Universidade Estadual Paulista-UNESP, IB-Campus de Rio Claro, Av.24-A, 1515, CEP: 13506–900, Rio Claro/SP., Brazil

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Summary The comet assay has been described as an efficient tool for the detection of changes in the DNA molecule of cells exposed to contaminating agents *in vivo* and *in vitro*. The possible environmental contamination due to the persistence of chromium residues from tannery effluents was determined in the waters of the “Córrego dos Bagres” stream, Municipal district of Franca/SP, by the comet assay on CHO-K1 cells. Water samples were collected during the four seasons of the year 2001 at three distinct stations along the river. The data suggest that the comet test showed good sensitivity for the environmental monitoring of these waters and indicated that this test can be efficient for the determination of the quality of waters contaminated with effluents containing heavy metal residues such as chromium.

Key words Comet assay, Chromium, Genotoxicity, CHO-K1.

Industrialization and urbanization processes have turned the question of environmental contamination increasingly critical. The contamination of aquatic ecosystems with substances originating from industrial wastes is a matter that is not always treated with the importance it deserves, despite the concern raised by the harmful effects of these substances, with a consequent alteration of continental water quality (White and Rasmussen 1998).

According to Mitchelmore and Chipman (1998), the comet assay is a methodology that can be potentially used to investigate breaks in the DNA strand, which can serve as a bioindicator of genotoxicity in fish and in other aquatic organisms. These biomarkers are important for the assessment of carcinogenic potential, of the effect on the reproductive system of organisms and of other adverse effects of pollution.

Recently, the comet assay has been used as an effective tool for environmental biomonitoring, permitting the detection of changes in the DNA molecule of cells exposed to chemical and physical agents *in vitro* or *in vivo* (Darroudi and Natarajan 1993, McNamee *et al.* 2000, Tice *et al.* 2000). It is a sensitive and rapid method for measuring DNA damage and repair in individual cells, with damage being detected by electrophoretic microgel migration (Olive and Banáth 1993, Klaude *et al.* 1996, Mitchelmore and Chipman 1998).

An important study conducted by Pandrangi *et al.* (1995) showed that the comet assay was efficient in assessing DNA damage provoked by heavy metal pollution in sediments of the Great Lakes (Canada). Nacci *et al.* (1992) reported a reduction in DNA strand breaks when organisms previously exposed to sediments contaminated with heavy metals were exposed to an environment

* Corresponding author, e-mail: mamm@rc.unesp.br

with controlled conditions (laboratory) without the presence of these pollutants. According to these investigators, this response reflects reversibility rather than persistence of damage after normalization of the environmental conditions.

Experimentally tested hexavalent chromium components proved to be potentially carcinogenic to man and animals since they induced cell transformations *in vitro*, causing various cytogenetic alterations in different biological systems such as point mutations, physicochemical changes in nucleic acids, chromosomal aberrations, and changes in the repair mechanism, among others (Levis *et al.* 1978).

The objective of the present study was to assess the possible environmental contamination derived from the persistence of chromium residues in tannery effluents dumped into the waters of the “Córrego dos Bagres” stream, Municipal district of Franca/SP, using the comet assay applied to CHO-K1 cells.

Materials and methods

Sample collect

Water collects were performed during the four seasons of the year 2001 at three different stations along the “Córrego dos Bagres” stream, Municipal district of Franca/SP. The collect sites selected were: before the site of tannery effluent dumping (200 m upstream) – FA, dumping site – FB, and after the dumping site (200 m downstream) – FC (Fig. 1). At each collect, 2 liters of water were obtained at a depth of 30 cm according to routine criteria for chemical analyses. The water samples were placed in plastic bottles and carried in an insulated box with ice to Universidade Estadual Paulista – UNESP – Rio Claro/SP.

Physicochemical analysis of the water

The analyses were based on the Standard Methods for the Examination of Water and Wastewater (Franson 1995).

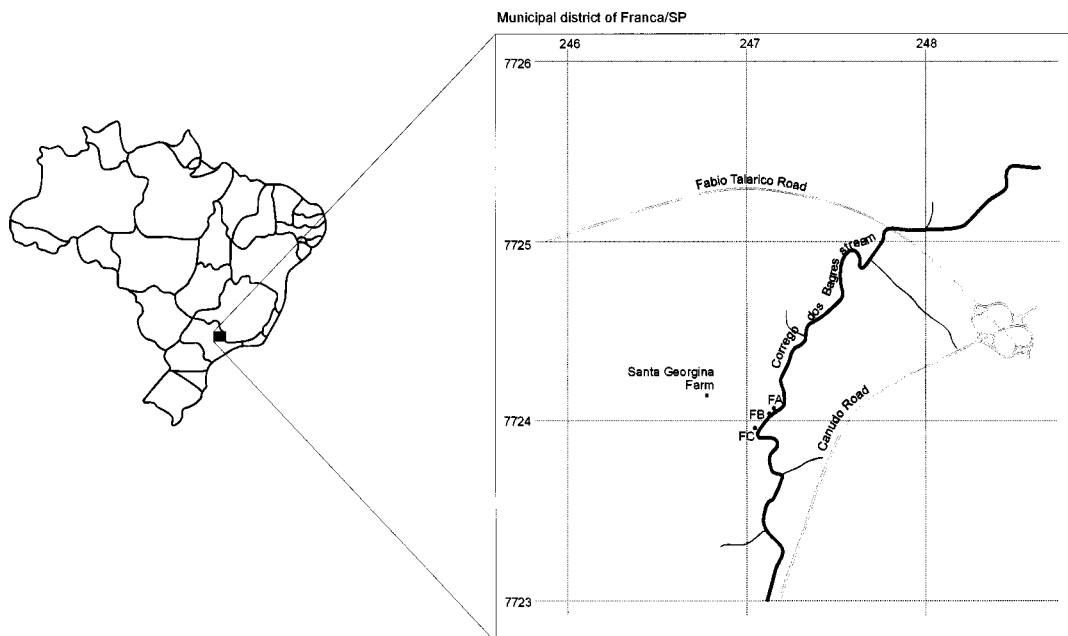


Fig. 1. Geographical localization of the collect site along the “Corrego dos Bagres” stream.

For cation determination, the samples were first acidified with HNO₃, pH 1, and the cations were analysed sequentially by inductively coupled plasma atomic emission spectrometry (ICP-AES) with ultrasonic nebulization. The following elements were determined: calcium, magnesium, strontium, silicon, iron, manganese, aluminium, zinc, chromium, cobalt, nickel, lead, cadmium, phosphorus, copper, and barium.

The standard solutions used to construct the calibration curves for the elements analyzed in 0.1% HNO₃ medium were obtained by appropriate dilutions of the standard solutions of 1000 ppm Titrisol (Merck).

Culture conditions and treatments

CHO-K1 cells were cultured in D-Mem+HAMF10 (1:1) medium supplemented with 10% Bovine Serum Albumin at 37°C. For the treatments a concentrated culture medium (stock) was prepared, to be diluted 3:1 with the water samples collected. The water samples collected at each station were added to the concentrated culture medium at the treatment time.

After 24 h of culture in normal medium, CHO-K1 cells were kept for 2 h (37°C) in the mixture of medium and water collected from the stream, whose pH was adjusted to 7.2 before treatment. The positive control was treated with methylmethanesulfonate (MMS) at the concentration of 10⁻⁴ M and the negative control was only kept in culture medium. Three independent experiments were performed.

Comet assay

The slides for microscope observation were mounted with 20 µl of the above cellular suspension and 120 µl of low melting point agarose at 37°C. The slides were bathed in a lysis solution (1 ml of Triton X-100, 10 ml of DMSO and 89 ml of lysis stock solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, ~68 g solid NaOH, and 10 g lauryl sarcosinate, pH 10]), in the dark, in a refrigerator at about 8°C for 1 h.

After lysis, the slides were placed in buffer containing 0.3 N NaOH and 1 mM EDTA, pH >13, for 20 min for DNA unwinding. Electrophoresis was performed for 20 min at 25 V, 300 mA (~1.0 V/cm) and the slides were then neutralized for 15 min in 0.4 M Tris, fixed in absolute ethanol for 10 min, and stained with ethidium bromide (0.02 µg/ml).

Comet assay analysis

The comet observations were performed at 400× using a Nikon epifluorescence microscope (filters B-3^A, excitation: λ=420–490 nm, barrier: λ=520 nm). One-hundred nuclei per sample were analyzed (treated and controls) and classified according to migration of fragments in classes 0, 1, 2 and 3 (Kobayashi *et al.* 1995): Class 0, cells without tail (not damaged) don't damaged that don't present tail; Class 1, cells with tail smaller than the nucleus' diameter; Class 2, cells with size of tail among 1–2 times the nucleus diameter; Class 3, cells with tail bigger than 2 times the nucleus' diameter.

Statistical analysis

Data were analyzed statistically by the χ^2 test (Pereira 1991) for comparison of the total number of comets of each treatment with the values observed in the negative and positive controls.

Results and discussion

All tests conducted on water samples from the three collect stations indicated the presence of more alterations than observed in the negative control tests, with these waters being considered more genotoxic than the negative control. The genotoxicity data obtained by the comet assay for

cells exposed to the water samples collected from the various stations showed lower values than those observed in the positive control (Table 1, Figs. 2, 3) and higher than those observed in the negative control. However, all of our results presented statistically significant values (0.01) regarding the genotoxic potential of the waters from the various collect sites. The comet assay on CHO-K1 cells appeared to be efficient in detecting the genotoxicity of waters contaminated with tannery effluents. Our data support those reported by Darroudi and Natarajan (1993) and McNamee *et al.* (2000), who stated that the comet assay is an efficient method for environmental biomonitoring.

The damage indices (cells with comets) observed for stations FB and FC were usually higher than those observed at FA, with FB (the site of effluent dumping) showing the highest impairment (Table 1, Fig. 2).

The FA site, corresponding to an upstream point in the river in relation to the dumping site and consequently a site not yet affected by the tannery effluent, did not show high indices of chromium contamination. Indeed the lowest values of total dissolved chromium (<0.01 to 0.05) were observed at this station (Table 2). Thus, the low indices of altered cells treated with the water from this site were correlated with the low chromium concentration existing there. The State Legislation of Sao Paulo-Brazil (CETESB 1995) establishes a maximum chromium level acceptable for class II and III waters (waters that receive effluents treated with substances considered not to be contaminants) of 0.05 mg/l. Our results agree with those reported by Pandrangi *et al.* (1995), who stated that the comet assay is an efficient method for the assessment of DNA damage induced by heavy metals.

According to Levis *et al.* (1978), chromium has a carcinogenic potential for man and animals

Table 1. Analysis of the changes observed in CHO-K1 cells treated with waters collected at distinct points along the stream which receives tannery effluents in the region of Franca/SP

	Cells		Class				Total of analysed cells	Score
	Normal	Altered	0	1	2	3		
Summer								
NC	296	4	296	4	0	0	300	4
PC	128	172	128	94	53	25	300	275
FA	272	28	272	24	4	0	300	32
FB	255	45	255	43	2	0	300	47
FC	222	78	222	49	27	2	300	109
Autumn								
NC	292	8	292	7	1	0	300	9
PC	240	60	240	45	11	4	300	79
FA	271	29	271	26	3	0	300	32
FB	246	54	246	48	4	2	300	62
FC	253	47	253	44	3	0	300	50
Winter								
NC	296	4	296	4	0	0	300	4
PC	204	96	204	65	24	7	300	134
FA	269	31	269	28	3	0	300	34
FB	253	47	253	38	6	3	300	59
FC	271	29	271	25	4	0	300	33
Spring								
NC	298	2	298	1	1	0	300	3
PC	191	109	191	75	26	8	300	151
FA	283	17	283	17	0	0	300	17
FB	276	24	276	21	3	0	300	27
FC	278	22	278	17	3	0	300	23

NC: Negative control, PC: Positive control, FA: Upstream from the site of effluent discharge, FB: Site of effluent discharge, and FC: Downstream from the site of effluent discharge.

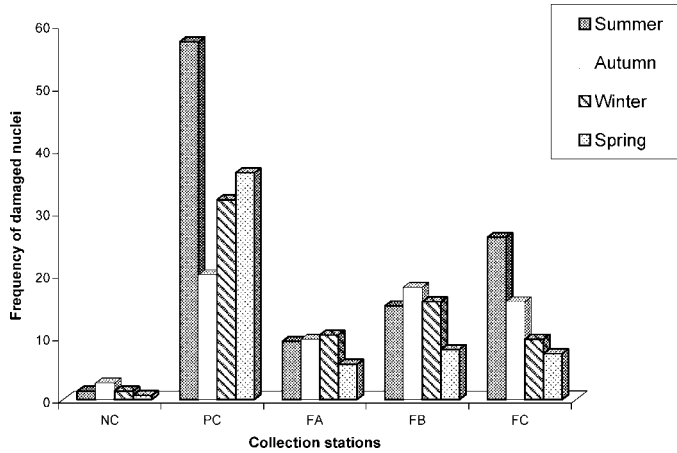


Fig. 2. Frequency of damage observed in CHO-K1 cells submitted to a culture medium containing water samples collected from the “Córrego dos Bagres” stream, for the 4 seasons of the year analyzed.

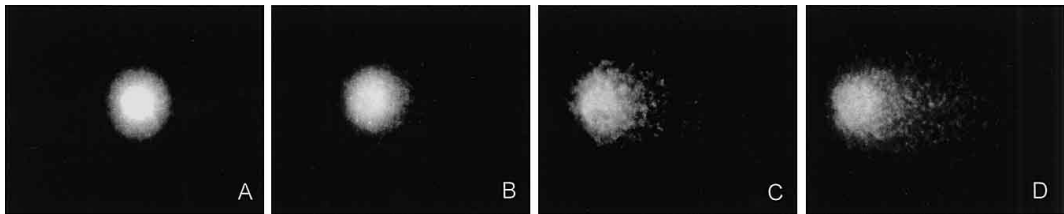


Fig. 3. Cell without DNA damage—comet class 0 (A) and cell with DNA damage: comet class 1 (B); comet class 2 (C); comet class 3 (D).

Table 2. Determination of elements (ppm) by ICP-AES in water samples collected at three different points in the four seasons of the year

	Si	Ca	Sr	Mg	Pb	Fe	Mn	Cr	P	Al	Zn	Cu	Ba	Co	Ni	Cd
Summer																
FA	5.2	50.0	0.25	16.8	<0.05	0.21	0.07	0.05	1.5	0.33	0.11	0.01	0.04	<0.01	<0.01	<0.005
FB	6.0	20.7	0.12	5.5	<0.025	0.8	0.10	0.38	2.20	0.43	0.04	0.01	0.05	<0.01	<0.01	<0.005
FC	6.2	15.3	0.08	3.3	<0.025	0.3	0.04	0.23	0.11	0.14	0.05	<0.01	0.03	<0.01	<0.01	<0.005
Autumn																
FA	6.3	10.0	0.05	1.9	<0.025	0.3	0.01	<0.01	1.5	0.33	0.11	0.01	0.04	<0.01	<0.01	<0.005
FB	5.6	18.8	0.10	4.6	<0.025	0.5	0.04	0.11	1.2	0.12	0.05	<0.01	0.04	<0.01	<0.01	<0.005
FC	6.2	15.3	0.08	3.3	<0.025	0.3	0.04	0.05	1.1	0.07	0.01	<0.01	0.04	<0.01	<0.01	<0.005
Winter																
FA	6.4	9.2	0.05	2.1	<0.025	0.04	<0.01	<0.01	1.6	<0.05	0.01	<0.01	0.04	<0.01	0.01	<0.005
FB	7.1	24.3	0.10	5.6	<0.025	0.18	0.05	0.02	1.82	0.05	0.02	<0.01	0.04	<0.01	0.01	<0.005
FC	6.4	21.0	0.10	5.7	<0.025	0.15	0.08	0.02	1.22	<0.05	0.01	<0.01	0.05	<0.01	<0.01	<0.005
Spring																
FA	6.9	13.6	0.07	2.2	<0.025	0.16	0.03	<0.01	<0.1	0.10	0.01	<0.01	0.04	<0.01	<0.01	<0.005
FB	7.3	36.3	0.15	9.1	<0.025	0.28	0.05	0.07	0.40	0.06	0.02	<0.01	0.04	<0.01	0.01	<0.005
FC	7.2	25.5	0.12	6.3	<0.025	0.19	0.04	0.03	0.22	0.22	0.01	<0.01	0.06	<0.01	0.01	<0.005

by inducing cell transformations. Our results, suggest that the presence of higher chromium levels in the water can induce an increased percentage of damage to the DNA molecule (Fig. 3).

At the site located upstream from the dumping site (FA) a greater genotoxic effect was ob-

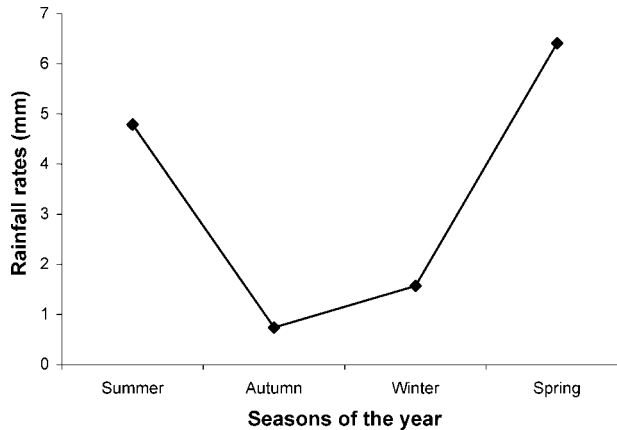


Fig. 4. Mean rainfall indices for the seasons of the year when water samples were collected from the “Córrego dos Bagres” stream.

served for the winter, fall and summer. According to the analyses performed on the samples from stations FB and FC (dumping site and downstream site, respectively), the same genotoxic effect of the waters was observed for all seasons of the year.

The present results indicate a greater water quality impairment by contaminants of tannery effluents, probably chromium, for the fall season, when the lowest rainfall rates were also observed (Fig. 4). Thus, a prolonged draught may compromise even more the water resources that receive effluents with a genotoxic potential.

The chemical analyses of water samples obtained at the 3 collect stations during the 4 seasons of the year showed that the FB site (dumping site) in general presented the highest rates of dissolved ions, as can be seen in Table 2.

Conclusion

The present data suggest that the comet test has good sensitivity for the environmental monitoring of waters, thus being efficient for the assessment of the quality of waters contaminated with effluents containing residues of heavy metals such as chromium.

On the basis of the chromium concentrations present in the waters sampled at the various collect stations (Table 2), we can see that the genotoxic effects of chromium detected by the comet assay were already observed at concentrations as low as 0.01 mg/l, contradicting the toxicity standards for chromium internationally established at levels starting from 0.05 mg/l.

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