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## Mutagenic Activity Assessment of Cristais River, São Paulo, Brazil, Using the Blue Rayon/*Salmonella* Microsome and the *Tradescantia pallida* Micronuclei Assays

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### ABSTRACT

Cristais River, located in the metropolitan region of São Paulo city, is used as source of drinking water and mutagenic activity was observed in the extracts obtained from its waters. This effect was associated to a discharge of a textile dye processing plant that contains, among other mutagenic compounds, the dyes C.I. Disperse Blue 373, C.I. Disperse Violet 93 and C.I. Disperse Orange 37. The objective of this work was to assess the mutagenic activity using the blue rayon hanging technique (BR)/*Salmonella* microsome assay and the *Tradescantia*-MCN mutagenicity test exposed in the field for 24 hours before and after the discharge of the cited industrial effluent. The BR/*Salmonella* microsome assay showed to be sensitive to detect the mutagenicity, after the textile dye processing plant discharge. The Trad-MCN assay, using plant cuttings exposed directly in the river showed an increased response after the industrial discharge. Due to its simplicity and low cost, the Trad-MCN assay seems to be a good alternative for screening surface waters for mutagenic activity. Both techniques have the advantage of better represent the environmental conditions because the extraction/exposure is performed in the field during 24 hours.

*Key words:* Azo dyes, blue rayon, *Salmonella*/microsome assay, textile effluent, *Tradescantia* micronuclei.

*Abbreviations:* BR – blue rayon; BDCP – Black Dye Commercial Product; DWTP – drinking water treatment plant; MCN – micronuclei; TLC – thin layer chromatography.

### RESUMO

#### Avaliação da atividade mutagênica do Ribeirão dos Cristais, São Paulo, Brazil, usando o blue rayon/*Salmonella* microssoma e o teste de micronúcleos com *Tradescantia pallida*

O ribeirão dos Cristais, localizado na região metropolitana de São Paulo, vem sendo utilizado para produção de água para abastecimento público após tratamento. Atividade mutagênica foi detectada em extratos orgânicos de águas coletadas após o lançamento de efluentes de uma indústria de tingimento de tecidos. Os corantes mutagênicos C.I. Disperse Blue 373, C.I. Disperse Violet 93 e C.I. Disperse Orange 37 foram detectados nesses extratos juntamente com outros compostos mutagênicos. O objetivo deste trabalho foi avaliar a atividade mutagênica dessas águas através da extração com blue rayon *in situ* (BR)/teste *Salmonella* microssoma e o teste Trad-MCN utilizando plantas expostas no corpo d'água antes e depois do lançamento. O teste BR/teste *Salmonella* microssoma se mostrou sensível na detecção de mutagenicidade após o lançamento do efluente industrial. A incidência de micronúcleos foi maior nas plantas expostas após o lançamento. Graças a sua simplicidade e baixo custo, o teste Trad-MCN com plantas expostas no corpo d'água parece ser uma alternativa

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viável para triagem de atividade mutagênica em águas superficiais. Ambas as técnicas têm como vantagem melhor representar as condições de campo, pois a extração/exposição é feita por períodos contínuos de 24 horas.

*Palavras-chave:* corantes azóicos, blue rayon, teste *Salmonella*/microsoma, efluentes têxteis, teste de micronúcleos *Tradescantia*.

## INTRODUCTION

Numerous chemicals derived from industrial, domestic and agricultural sources are released directly into surface waters. Surface runoff and atmospheric deposition also contribute to aquatic pollution. In many cases, these polluted waters are source for drinking water use or are employed directly for agricultural or industrial purposes. Among the organic contaminants present in the aquatic environment, mutagenic compounds have important consequences both for humans in terms of the safety of the drinking water supply and recreation activities as well for the ecological integrity of aquatic organisms (Ohe *et al.*, 1999).

Cristais River, located in the metropolitan region of São Paulo city, Brazil, is used as source of drinking water for ~ 60,000 inhabitants, and mutagenic activity was observed in the extracts obtained from its waters (Umbuzeiro *et al.*, 2004a). Kummrow *et al.* (2003) using the *Salmonella*/microsome assay and two different extraction techniques (XAD4 and blue rayon in columns), detected mutagenic activity in raw and treated water collected from the Cristais River. Umbuzeiro *et al.* (2004a) observed elevated mutagenic responses observed with the *Salmonella* strains YG1041 and YG1042 in relation to TA98 and TA100 respectively. Those results suggested that polycyclic aromatic compounds from the nitroaromatics class and/or aromatic amines were responsible for the observed effect. Using thin layer chromatography, Umbuzeiro *et al.* (2005a) detected three prevalent mutagenic dyes in the raw water samples: C.I. Disperse Blue 373, C.I. Disperse Violet 93 and C.I. Disperse Orange 37. They also found that a textile dye processing plant effluent was responsible for the mutagenic activity as well as for the presence of the cited dyes in the Cristais River waters. This effluent showed positive responses when tested in laboratory conditions using the *Tradescantia*-micronucleus assay (Trad-MCN) (Lobo *et al.*, 2004).\* Recently this effluent was chemically characterized by Oliveira *et al.* (2007) and the contribution of the azo dyes in the mutagenic activity was determined.

Testing water samples in mutagenicity tests usually require the collection of several liters of water and their transportation to the laboratory, which can lead to the alteration of the physical and chemical properties of the sample. Another difficulty is that the sample is usually collected at once; therefore variations of the quality of the water that occur during the day cannot be well represented. This can be avoided using techniques, which better represents the field conditions, especially if performed over a 24 hour-period.

The blue rayon hanging technique (Sakamoto & Hayatsu, 1990) and the blue rayon anchored technique (Kummrow *et al.*, 2006) are performed in the field for 24h-periods and when coupled with the *Salmonella*/microsome assay are useful for the assessment of mutagenic activity related to polycyclic compounds (Ohe *et al.*, 2004; Kummrow & Umbuzeiro, 2006) although with relative high cost.

The micronucleus assay is a widely used cytogenetic assay for detection of chromosomal damage (Ohe *et al.*, 2004). Plant bioassays are sensitive, simple, have low cost, and have been used worldwide. The *Tradescantia*/micronuclei assay is sensitive to several environmental contaminants (Trad-MCN) (Ma *et al.*, 1984; Rodrigues *et al.*, 1997; Guimarães *et al.*, 2000). Ohe *et al.* (2004), in a review about mutagens in surface waters, found that 37% of the mutagenicity/genotoxicity studies published from 1990 to 2004 were performed using the *Salmonella*/microsome assay. Although 17% used a micronucleus assay (*in vivo* and *in vitro*), only four were performed using the Trad-MCN. This assay was used in China and Italy to verify genotoxicity in water sources that were used for drinking water production and recreation activities (Duan *et al.*, 1999; Monarca *et al.*, 1998; Jiang *et al.*, 1999; Yang, 1999). In these studies the plant cuttings were exposed to the water samples in the laboratory.

The objective of this study was to assess the mutagenic activity using the blue rayon hanging technique coupled with the *Salmonella*/microsome assay and the *Tradescantia*-MCN with the plant cuttings exposed directly in the Cristais river, before and after the mutagenic textile dye processing plant effluent discharge.

## MATERIAL AND METHODS

### *Sampling sites*

Three different sites were chosen: Site 1, Cedro Creek, a tributary of the Cristais River, located in a protected area and used in this work as a reference site; Site 2, Cristais River, immediately downstream of the textile dye processing plant discharge and Site 3, Cristais River, at the DWTP intake (see Figure 1 for details). The exposures were performed in the river during 24 hours in July of 2003.

### *Blue rayon hanging technique and elution procedures*

The samplings were performed using the blue rayon hanging technique. The blue rayon (Sigma-Aldrich) was placed in three nylon nets (5 g of blue rayon each) and attached to

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a floating device (Sakamoto & Hayatsu, 1990) (Figure 2). This apparatus was immersed in the water for 24 hours. Blue rayon fibers were previously washed, tested for mutagenic activity with the strains TA98, TA100, YG1041 and YG1042 and only when the extracts presented negative results they were used in the field (Kummrow *et al.*, 2003). After the 24 hour period, the blue rayon fibers were taken out, transferred to beakers and transported to the laboratory in boxes protected from the light. In the laboratory, blue rayon fibers were washed with ultra-pure water in order to remove the non-adsorbed compounds and solids. The blue rayon fibers were dried with clean paper towels and eluted with 200 mL of methanol/ammonia (50:1 v/v) for each 1 g of blue rayon (Kummrow *et al.*, 2003). The eluates were reduced to 2-3 mL using a rotary evaporator, transferred to vials, evaporated to dryness with a gentle stream of nitrogen and resuspended in dimethylsulfoxide (DMSO) just before testing in the *Salmonella*/microsome assay.

#### ***Salmonella*/microsome microsuspension assay**

The blue rayon organic extracts were assayed for mutagenicity using the *Salmonella typhimurium* strains TA98 (*hisD3052*, *rfa*,  $\Delta$ *bio*,  $\Delta$ *uvrB*, pKM101) (Maron & Ames, 1983), YG1041 (a derivative of the TA98, able to produce high levels of nitroreductase and O-acetyltransferase) (Hagiwara *et al.*, 1993), TA100 (*hisG46*, *rfa*,  $\Delta$ *bio*,  $\Delta$ *uvrB*, pKM101) (Maron & Ames, 1983) and YG1042 (a derivative of the TA100, able to produce high levels of nitroreductase and O-acetyltransferase) (Hagiwara *et al.*, 1993) with and without S9 metabolic activation, in a microsuspension assay (Kado *et al.*, 1983). Overnight cultures of strains (around  $10^9$  cells mL<sup>-1</sup>) 5-fold concentrated by centrifugation (10,000 g at 4°C, for 10 min) were resuspended into 0.015 M sodium phosphate buffer. 50  $\mu$ L of cell suspension, 50  $\mu$ L of 0.015 M sodium phosphate buffer or S9 mix, and 5  $\mu$ L of the sample were added to a tube and incubated at 37°C for 90 min without shaking. After incubation, 2 mL of molten agar was added, and the mixture was poured onto a minimal agar plate. Colonies were counted after 66 h of incubation at 37°C using an automatic colony counter. The metabolic activation was provided by Aroclor 1254 induced Sprague Dawley rat liver S9 mix (MolTox, Boone, NC), which was prepared at a concentration of 4 % v/v. For TA98 and TA100, the positive controls were 0.125  $\mu$ g/plate of 4-nitroquinoline-oxide (4NQO) (Acros), and 0.625  $\mu$ g/plate of 2-aminoanthracene (2AA) (Sigma-Aldrich), both dissolved in dimethylsulfoxide (DMSO). For YG1041 the positive controls were 10  $\mu$ g/plate of 4-nitro-O-phenylenediamine (4NOP) (ICN Biomedicals Inc), and 0.0312  $\mu$ g/plate of 2AA. For YG1042 the positive controls were 5  $\mu$ g/plate of 2-Nitrofluorene (2NF) (Aldrich), and 0.625  $\mu$ g/plate of 2AA. All positive controls were dissolved in DMSO. The doses of the blue rayon extract tested were 25, 50, 100, 200 and 400 mg per plate and two plates of each dose were tested. For the negative control, five replicates were employed. The mutagenic ratio (MR) was calculated, dividing the mean of revertants per plate obtained for each dose by the mean of revertants obtained for the negative control.

#### ***Micronuclei in Tradescantia assay - Trad-MCN***

Cuttings of twenty plants of *Tradescantia pallida* (Rose) Hunt. cv. Purpurea Boom (Suyama *et al.*, 2002) that were cultivated in the garden of the University of São Paulo were exposed directly in the river for 24 hours (Figure 2). In this case, the experiment was performed only at the reference site, Cedro Creek (site 1), and at the intake of the Cristais River DWTP (site 3), ~ 6 km after the textile dye processing plant discharge. After exposure, the plant cuttings were taken to the laboratory and the young inflorescences were collected and immediately fixed in 1:3 acetic acid/ethanol solution for at least for 24 h. The inflorescences were dissected and early tetrads of the meiotic microspore mother cells were squashed in aceto-carmin stain on a microslide (Ma, 1981). Only preparations containing early tetrads were used. Scoring was performed by counting, under 400 x magnification, the number of micronuclei present in a random set of 300 early tetrads per inflorescence. Slides were coded before scoring.

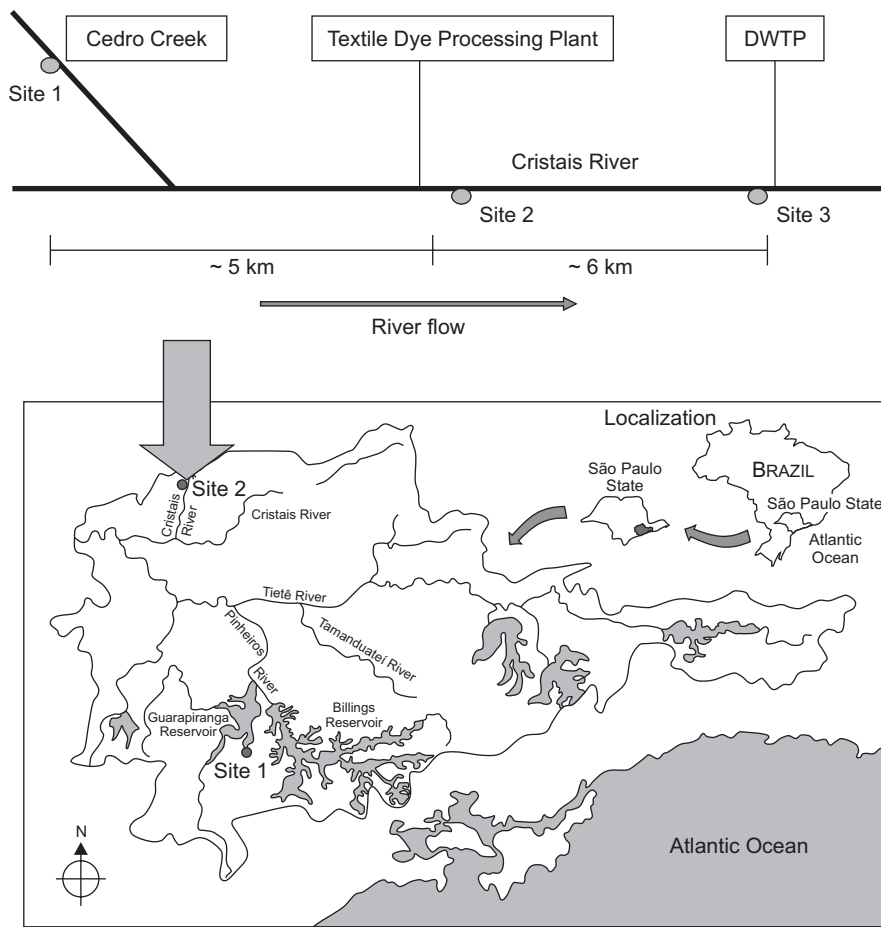
#### ***Statistical analysis***

For the *Salmonella*/microsome assay, the data were analyzed with the Salanal computer program using the Bernstein model (Bernstein *et al.*, 1982). Samples were considered positive when a significant difference among the tested doses and the negative control; a significant positive dose response were observed and additionally if at least in one dose the mutagenic ratio (MR) were greater than 2. If the mutagenic ratio was less than two the response was considered as marginal (Umbuzeiro *et al.*, 2004b).

For the Trad-MNC assay the data were analyzed using the non-parametric ANOVA Ranks-Kruskal-Wallis.

## **RESULTS AND DISCUSSION**

At Cedro Creek (site 1, Figure 1) no mutagenic activity was detected using the blue rayon hanging technique/*Salmonella* microsome assay for the strains TA98, TA100 and YG1042 (Table 1a and b). For the YG1041 a marginal (-S9) and positive (+S9) were observed with low mutagenic levels (Table 1a). After the discharge of the textile azo dye processing plant (site 2, Figure 1) positive results were detected with TA98 and YG1041 with and without S9, with higher mutagenic levels for YG1041 in the presence of S9 (49,000 rev./g BR) (Table 2a). This effect is probably related to the presence of polycyclic compounds because blue rayon fibers are selective to this type of molecules (Hayatsu, 1992) that are activated with S9. Because the mutagenic activity with the YG1041 increased 70 times and 15 times respectively with and without S9, in relation to the response with the TA98 strain, it is possible to suggest that the mutagens belong to the class of the nitroaromatics and/or the aromatic amines. The YG1041 strain produces much higher amounts of nitroreductase and O-acetyltransferase than TA98, which are very important enzymes in the activation of those types of compounds (Hagiwara *et al.*, 1993; Umbuzeiro *et al.*, 2005b). For TA100 and YG1042 no mutagenic activity was detected at site 2 (Table 2b).



**Figure 1** – Map and diagram showing the location of the collection sites: site 1, Cedro Creek, reference site; site 2, after the textile azo dye processing plant discharge; and site 3, at the Drinking Water Treatment Plant in the Cristais River area.

**Table 1a** – Results of the *Salmonella*/microsome microsuspension assay for the blue rayon (BR) extract for site 1 – Cedro Creek (reference site) for TA98 and YG1041 in the presence and absence of S9.

Doses	Mean of Number of Revertants/plate, Standard Deviation (SD), and Mutagenic Ratio (MR)											
	TA98						YG1041					
	-S9			+S9			-S9			+S9		
mg blue rayon (BR)/plate	Mean	SD	MR	Mean	SD	MR	Mean	SD	MR	Mean	SD	MR
Negative control	26.2	4.4		25.3	4.7		83.0	3.8		80.2	8.7	
25	24.0	2.8	0.9	22.0	0.0	0.9	96.5	2.1*	1.2	83.0	2.8	1.0
50	23.5	5.0	0.9	30.5	6.4	1.2	109.0	9.9*	1.3	86.0	2.8	1.1
100	24.0	5.7	0.9	26.0	4.2	1.0	100.0	2.8*	1.2	95.0	9.9	1.2
200	18.0	4.2	0.7	24.0	7.1	0.9	118.0	2.8**	1.4	127.5	3.5	1.6
400	27.5	5.0	1.0	36.0	7.1	1.4	129.5	6.4**	1.6	175.0	5.7	2.2
Positive control <sup>a</sup>	102.5			275.0			830.0			820.0		
Result <sup>b</sup>	negative			negative			marginal			positive		
Potency (rev./g BR)	- <sup>c</sup>			-			***			470***		

\*: significant at 5% (ANOVA); \*\*: significant at 1% (ANOVA); \*\*\*: significant at 1% (positive dose response).

a: see material and methods for positive controls/doses. b: see material and methods for positive criteria. c: potency not calculated.

**Table 1b** – Results of the *Salmonella*/microsome microsuspension assay for the blue rayon (BR) extract for site 1 - Cedro Creek (reference site) for TA100 and YG1042 in the presence and absence of S9.

Doses	Mean of Number of Revertants/plate, Standard Deviation (SD), and Mutagenic Ratio (MR)											
	TA100						YG1042					
	-S9			+S9			-S9			+S9		
mg blue rayon (BR)/plate	Mean	SD	MR	Mean	SD	MR	Mean	SD	MR	Mean	SD	MR
Negative control	84.6	7.1		83.2	8.4		73.4	5.7		78.6	4.9	
25	74.5	2.1	0.9	86.5	9.2	1.0	69.5	2.1	0.9	51.0	0.0	0.6
50	79.5	7.8	0.9	80.5	9.2	1.0	72.5	6.4	1.0	57.0	7.1	0.7
100	74.0	1.4	0.9	79.5	0.7	1.0	74.5	13.44	1.0	89.0	0.0	1.1
200	74.5	5.0	0.9	84.5	5.0	1.0	65.5	6.4	0.9	72.0	12.7	0.9
400	88.0	0.0	1.0	84.5	9.2	1.0	69.0	2.8	0.9	66.0	8.5	0.8
Positive control <sup>a</sup>	570.0			770.0			221.0			820.0		
Result <sup>b</sup>	negative			negative			negative			negative		
Potency (rev./g BR)	- <sup>c</sup>			-			-			-		

a: see material and methods for positive controls/doses. b: see material and methods for positive criteria. c: potency not calculated.

Thin layer chromatography (TLC) was performed in the blue rayon extracts using the method described by Umbuzeiro *et al.* (2005a). At sites 2 and 3, the mutagenic dyes: Disperse Blue 373, Disperse Violet 93 and Disperse Orange 37 were detected among other unknown colored compounds (data not shown). The mutagenic profile of the blue rayon extract obtained from site 2 is similar to the one obtained for cited dyes (Umbuzeiro *et al.*, 2005a). It is possible that those dyes are contributing to the mutagenic activity detected at site 2. Compounds like the PBTAs, that are mutagenic benzotriazoles derived from the reduction/chlorination of the same type of the cited dyes, could also be contributing to the observed mutagenic activity. They are efficiently extracted by blue rayon and were found in several Japanese rivers (Ohe *et al.*, 2004; Watanabe *et al.*, 2005).

Downstream of the textile dye processing plant effluent discharge, at the DWTP intake (site 3, Figure 1), the blue rayon extracts also showed mutagenic activity with TA98 and YG1041 with and without S9, with an increased response for the YG1041 strain (30,000 rev./g of BR) (Table 3a). Comparing sites 2 and 3, waters from site 3 showed an increase in the mutagenic response without S9 in relation to site 2, for TA98 of 7.5 times and for YG1041, 3 times. In the presence of S9, the mutagenicity decreased, for TA98, in 11 times and for YG1041, in 1.6 times. It is possible to suggest that transformations occurred along the river, probably oxidation processes (e.g. photooxidation reactions) transforming indirect mutagens into other chemical species, such as hydroxyamines, that could be acetylated by the O-acetyltransferase of the *Salmonella* strains, causing the

observed increase in the mutagenicity without S9. Besides the Disperse Blue 373, Disperse Violet 93 and Disperse Orange 37, the PBTAs and other transformation products could be responsible for the mutagenic activity detected at site 3. At this site marginal responses were observed for TA100 and YG1042 at site 3 (Table 3b) indicating again a modification of the mutagenic activity along the river.

For the Trad-MNC assay, 12 plant cuttings exposed at site 1 – Cedro Creek and 9 from site 3 were scored for micronuclei. A number of 300 early tetrads were scored from each plant with some exceptions (Table 4) but a minimum of 5 plants and 1500 tetrads per site were analyzed, according to the recommendations of Rodrigues (1999). The micronuclei frequency obtained for each plant is presented in Table 4. Plant cuttings exposed at site 1 (reference site) presented a mean of micronuclei percentage of 1.87 (N = 12) with a standard deviation of 1.34. For site 3 (Cristais River at the intake of the DWTP), the mean of micronuclei percentage was 6.22 (N = 9), with a standard deviation of 2.89. The values were statistically different with a  $p < 0.05$  (Figure 3); therefore, water collected at site 3 induced more micronuclei in the plants exposed for 24 hours in comparison to the plants from site 1. We were not able to run a positive control in field conditions, but several other experiments have been performed exposing plant cuttings to formaldehyde at 0.1% in the laboratory and positive responses were obtained (data not shown). The fact that the DWTP source water (site 3) presented mutagenic response with a higher plant, besides bacteria, increases the level of concern related to its quality.

**Table 2a** – Results of the *Salmonella*/microsome microsuspension assay for the blue rayon (BR) extract for site 2 – Cristais River immediately downstream the textile dye processing plant discharge. For TA98 and YG1041 in the presence and absence of S9.

Doses	Mean of Number of Revertants/plate, Standard Deviation (SD), and Mutagenic Ratio (MR)											
	TA98						YG1041					
	–S9			+S9			–S9			+S9		
mg blue rayon (BR)/plate	Mean	SD	MR	Mean	SD	MR	Mean	SD	MR	Mean	SD	MR
Negative control	26.2	4.4		25.3	4.7		83.0	3.8		80.2	8.7	
25	25.0	2.8	1.0	74.5	3.5**	2.9	103.0	8.5*	1.2	1,100.0	70.7**	13.7
50	35.5	0.7*	1.4	96.0	26.9*	3.8	155.5	14.8**	1.9	1,370.0	17.9**	17.1
100	35.0	2.8	1.4	81.0	19.8*	3.2	260.0	17.0**	3.1	1,076.0	43.8**	13.4
200	35.5	6.4	1.4	58.0	1.4**	2.3	345.5	27.6**	4.1	1,085.0	77.9**	13.5
400	40.5	3.5*	1.5	52.0	1.4**	2.1	550.0	21.2**	6.6	903.0	51.6**	11.3
Positive control <sup>a</sup>	102.5			275.0			830.0			820.0		
Result <sup>b</sup>	marginal			positive			positive			positive		
Potency (rev./g BR)	38***			1,600***			2,900***			49,000***		

\*: significant at 5% (ANOVA); \*\*: significant at 1% (ANOVA); \*\*\*: significant at 1% (positive dose response).

a: see material and methods for positive controls/doses. b: see material and methods for positive criteria.

**Table 2b** – Results of the *Salmonella*/microsome microsuspension assay for the blue rayon (BR) extract for site 2 – Cristais River immediately downstream the textile dye processing plant discharge for TA100 and YG1042 in the presence and absence of S9.

Doses	Mean of Number of Revertants/plate, Standard Deviation (SD), and Mutagenic Ratio (MR)											
	TA100						YG1042					
	–S9			+S9			–S9			+S9		
mg blue rayon (BR)/plate	Mean	SD	MR	Mean	SD	MR	Mean	SD	MR	Mean	SD	MR
Negative control	84.6	7.1		83.2	8.4		73.4	5.7		78.6	4.9	
25	77.5	0.7	0.9	86.5	9.2	1.0	59.5	3.5	0.8	78.0	4.2	1.0
50	78.0	0.0	0.9	80.5	9.2	1.0	62.0	8.5	0.8	81.0	0.0	1.0
100	78.5	2.1	0.9	79.5	0.7	1.0	71.0	1.4	1.0	76.0	8.5	1.0
200	75.0	7.1	0.9	84.5	5.0	1.0	73.5	0.0	1.0	81.0	4.2	1.0
400	73.5	2.1	0.9	84.5	9.2	1.0	78.5	5.0	1.1	76.5	6.4	1.0
Positive control <sup>a</sup>	570.0			770.0			221.0			813.0		
Result <sup>b</sup>	negative			negative			negative			negative		
Potency (rev./g BR)	– <sup>c</sup>			–			–			–		

a: see *Material and Methods* for positive controls/doses. b: see material and methods for positive criteria. c: potency not calculated

The blue rayon/*Salmonella* microsome assay seems to be very sensitive to the mutagens present in the Cristais River waters and the results obtained have the advantage of indicating the type of mutagens present in the water, especially when different strains are used.

This is particularly important when multiple sources of mutagens are present and the genotoxic source must be identified. The results of the Trad-MCN assay were in agreement with the qualitative results observed for the BR hanging technique/*Salmonella* assay. Due to its simplicity and low cost, the Trad-MCN assay seems to be a good

alternative for screening river water for mutagenic activity, but because only one experiment was performed, more studies are needed to determine its efficiency.

Results of this study are in agreement with the data obtained for the same assays performed in the laboratory using the same water samples, with the advantage of not requiring the collection of several liters of water and their transportation to the laboratory. The 24-hour period field extraction/exposure procedures used in this work seem to be a good alternative for monitoring water for mutagenic activity because they can better represent the environmental conditions.

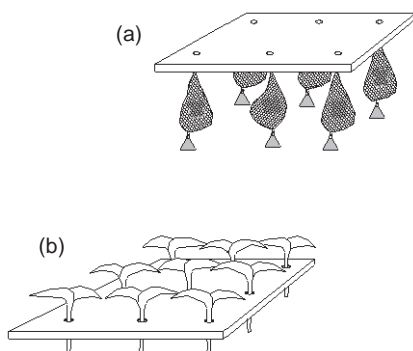


Figure 2 – Scheme of the nylon nets containing the blue rayon fibers (a) and the apparatus with *Tradescantia pallida* cuttings (b) exposed in the river water for 24 hours.

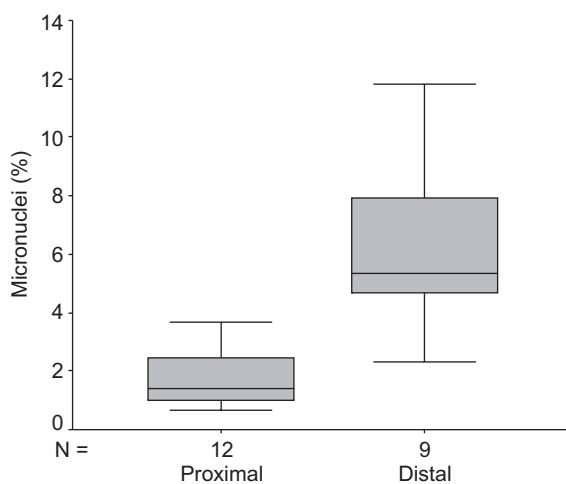


Figure 3 – Box plot graph of the incidence of micronuclei in plants exposed at site 1 – Cedro Creek and site 3 – Cristais River at the drinking water treatment plant intake.

Table 3a – Results of the *Salmonella*/microsome microsuspension assay for the blue rayon (BR) extract for site 3 – Cristais River at the drinking water treatment plant intake for TA98 and YG1041 in the presence and absence of S9.

Doses  mg blue rayon (BR)/plate	Mean of Number of Revertants/plate, Standard Deviation (SD), and Mutagenic Ratio (MR)											
	TA98						YG1041					
	-S9			+S9			-S9			+S9		
	Mean	SD	MR	Mean	SD	MR	Mean	SD	MR	Mean	SD	MR
Negative control	25.2	2.3		29.2	2.3		140.8	5.7		115.6	11.0	
25	30.5	3.5	1.2	37.0	1.4*	1.3	182.0	17.0*	1.3	716.0	5.7**	6.2
50	34.0	4.2	1.3	38.0	0.0	1.3	283.5	7.8**	2.0	905.0	91.9**	7.8
100	62.0	7.1**	2.5	40.5	5.0*	1.4	485.0	44.3**	3.4	935.0	35.4**	8.1
200	123.5	14.9**	4.9	63.0	4.2**	2.2	1,068.0	14.1**	7.6	1,163.0	65.0**	10.1
400	126.5	9.2**	5.0	83.5	2.1**	2.9	1,410.0	28.3**	10.0	1,307.5	2.1**	11.3
Positive control <sup>a</sup>	450.0			298.0			662.0			1,356.0		
Result <sup>b</sup>	positive			positive			positive			positive		
Potency (rev./g BR)	300***			140***			7,800***			30,000***		

\*: significant at 5% (ANOVA); \*\*: significant at 1% (ANOVA); \*\*\*: significant at 1% (positive dose response).

a: see material and methods for positive controls/doses. b: see material and methods for positive criteria.

**Table 3b** – Results of the *Salmonella*/microsome microsuspension assay for the blue rayon (BR) extract for site 3 – Cristais River at the drinking water treatment plant intake for TA100 and YG1042 in the presence and absence of S9.

Doses  mg blue rayon (BR)/plate	Mean of Number of Revertants/plate, Standard Deviation (SD), and Mutagenic Ratio (MR)											
	TA100						YG1042					
	-S9			+S9			-S9			+S9		
	Mean	SD	MR	Mean	SD	MR	Mean	SD	MR	Mean	SD	MR
Negative control	111.2	4.1		109.4	10.6		78.2	6.1		92.4	11.0	
25	104.5	13.4	0.9	157.5	21.9	1.4	81.0	8.5	1.0	96.0	2.8	1.0
50	106.0	1.4	0.9	143.0	1.4*	1.3	80.5	5.0	1.0	74.5	6.4	0.8
100	122.5	5.0	1.1	146.5	6.4*	1.3	80.0	0.0	1.0	100.0	2.8	1.1
200	130.0	22.6	1.2	156.5	23.3	1.4	101.5	5.0**	1.3	100.0	4.2	1.1
400	116.0	15.6	1.0	178.0	8.5**	1.6	103.0	4.2**	1.3	89.5	13.4	1.0
Positive control <sup>a</sup>	1,796.0			1,349.0			311.0			1,273.0		
Result <sup>b</sup>	negative			marginal			marginal			negative		
Potency (rev./g BR)	- <sup>c</sup>			410***			160***			-		

\*: significant at 5% (ANOVA); \*\*: significant at 1% (ANOVA). \*\*\*: significant at 2% (positive dose response); \*\*\*\*: significant at 1% (positive dose response).

a: see material and methods for positive controls/doses. b: see material and methods for positive criteria. c: potency not calculated.

**Table 4** – Number of plant cuttings analyzed and the frequency of micronuclei obtained for each site 1 and 3.

Cristais River site	Number of tetrads scored	Number of micronuclei	Percentage of micronuclei
1	303	3	0.99
	103	3	2.91
	101	1	0.99
	100	5	5.00
	300	2	0.67
	300	6	2.00
	300	4	1.33
	300	3	1.00
	300	11	3.67
	200	3	1.50
	300	2	0.67
	300	5	1.67
		<i>Sum = 2,907, N = 12</i>	<i>Sum = 48</i>
3	129	3	2.33
	203	24	11.8
	189	15	7.94
	134	12	8.96
	300	14	4.67
	200	8	4.00
	244	13	5.33
	106	6	5.66
	244	13	5.33
	<i>Sum = 1,749, N=9</i>	<i>Sum = 108</i>	<i>Average = 6.22%, SD = 2.89</i>

\*SD = standard deviation.

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