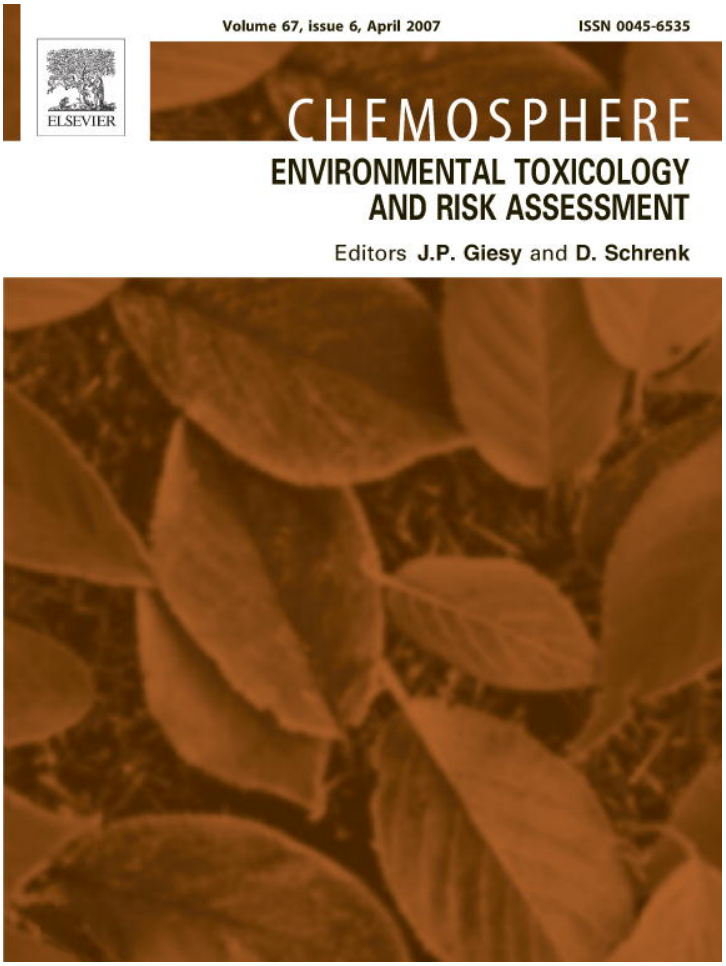

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Evaluation of genotoxicity and toxicity of water and sediment samples from a Brazilian stream influenced by tannery industries

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Abstract

This paper reports results of genotoxicity and toxicity studies of water and sediment samples collected from the Estância Velha stream of southern Brazil, a stream transporting both domestic sewage and effluents from regional factories working in the leather industry. Three sites were selected: in the stream headwaters (Site 1), located downstream of an urban area (Site 2), and near the basin outfall (Site 3). Results obtained with *Allium cepa* showed no evidence of chromosomal mutation, either in water or in sediment, during winter or summer seasons, but samples collected below Site 1 showed high toxicity. Physical and chemical analyses showed high concentrations of pollutants at these sites. Ecotoxicity tests with *Daphnia magna* and *Ceriodaphnia dubia* measured toxicity in water from Sites 2 and 3 in summer 2004. A toxic effect on *Hyalella azteca* was only found in sediment from Site 3 during winter 2003 and summer 2004. The results suggest that the synergy among different compounds in domestic and industrial sewage discharges can make it difficult to maintain system stability.

Keywords: Allium test; Toxicity; Genotoxicity; Environmental monitoring; Leather

1. Introduction

Waste from leather industries are of great concern to agencies responsible for environmental management. Some authorities consider it to be one of the ten most harmful to the environment, responsible for extreme pollution of water resources and generating substances leading to deterioration and death of a wide range of organisms (Aragon, 1990). International agencies have drawn attention to the lack of legislation specific to the leather industry, to the need for careful impact assessment studies when industrial projects are being planned, to the need for effluent dilution factors to be evaluated, to the adverse effects of chromium, and to the large amounts of organic matter associated with the leather industry effluents (Bosnic et al., 2000).

The stream flowing through the Estância Velha municipality of southern Brazil transports both domestic wastes and leather industry effluents. The stream forms part of the river system of the state of Rio Grande do Sul (RS), Brazil (Krebs and Reis, 1994), and contamination in one of the rivers downstream of Estância Velha was identified in 2001 (Vargas et al., 2001), by its mutagenic activity and the presence of metals in sediment, including iron, manganese, chromium, lead, copper, zinc and nickel.

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Hitherto, only physical and chemical tests have been used to evaluate the impact of industrial effluents on the stream. These tests satisfy criteria set by State agencies for environmental control, but several authors have recommended that physical and chemical analyses with tests using organisms be utilized to evaluate toxicity of compounds resulting from anthropogenic activity (Bertoletti, 1992; Smaka-Kincl et al., 1995). *Allium cepa* roots are regarded as one of the pioneering organisms for use in cytogenetic studies, and the *A. cepa* test has been used by the Environmental Protection Agency (US EPA) to evaluate toxicity and genotoxicity of industrial effluents (Fiskesjö, 1993; Grant, 1994). Ecotoxicological assays allow the extent of acute and chronic toxicity to be established through the use of *Daphnia similis*, *Daphnia magna*, *Ceriodaphnia dubia* and *Hyalella azteca* and other organisms, all of which are used by environmental protection agencies to evaluate industrial effluents by determining the toxicity of complex mixtures present in the environment (Ingersoll, 1995; Versteeg et al., 1997; Wang et al., 2004). Since the need has been identified to evaluate the impacts of leather-industry effluents on aquatic ecosystems (Class and Maia, 1994; Jordão et al., 1999), the aim of the present study was to assess the degree of toxicity present at three sites located along the Estância Velha stream, and to provide data for future projects on the management and recovery of areas affected by this industrial activity.

2. Materials and methods

2.1. Sampling

Samples were collected in the summer and winter of 2003 and the summer of 2004 at three different sites in the Estância Velha stream which flows from a lake fed by natural springs. Site 1 was located in the headwaters of the stream to the northeast of the urban area. Site 2 was located a further 4 km downstream, near to the urban area which produces not only domestic sewage but also the effluents from four sites for retanning and leather-finishing, a footwear factory and three chemical plants. Site 3, still further downstream and distant 8 km from the headwater Site 1, receives all industrial and domestic effluents discharged at upstream sites, together with effluent from eight tanneries, two of which process skins up to the finishing stage, and six tanneries specialized in retanning and leather-finishing. This site also receives effluents from a chemical plant and a foodstuff plant.

Water samples were collected midstream at each site and put in containers appropriate for each microbiological, physical and chemical analysis, with a volume of approximately seven liters for the ecotoxicological tests. Sediment samples of approximately 2 kg were collected at the same sites using a Correl-type collector (Mudroch and Macknicht, 1991); samples were placed in plastic bags and kept at low temperature in styrofoam boxes before transport to the laboratory according to Standard NBR 9898 (ABNT, 1987b).

2.2. Water and sediment analysis

For the microbiological, physical and chemical tests on water, the leather-industry standard NBR 9897 (ABNT, 1987a) was used, which in summer and winter 2003 gave the following measurements: chemical oxygen demand (COD), biological oxygen demand (BOD_5), pH, total suspended solids (TSS), total nitrogen, oil and grease, total chromium, temperature, phenol, dissolved oxygen (DO) and microbiological thermotolerant bacteria (MTB). Except for the phenols index determined by standard DIN 38406 H16 (2000), all other analyses followed the methodology described in Standard Methods, 20th edition (APHA-AWWA-WPCF, 1998). In the summer of 2004, total chromium, hexavalent chromium and mercury in water were determined at all sites.

In the winter of 2003, pH, total nitrogen and ammonia nitrogen were measured in the sediment. The tests followed standard procedures as described in the same reference. At all sites, indices of biodegradability were calculated in summer and winter of 2003. These were obtained from the relationship between BOD and COD as $f_b = BOD_5/0.65\ COD$ (Class and Maia, 1994; Emmanuel et al., 2005), varying from $f_b = 0.2$ for material that is practically non-biodegradable to $f_b = 0.9$ for completely biodegradable material.

During 2003 the stream discharge Q was estimated as the product of mean water velocity and cross-section of wetted area A. This area was obtained from a channel survey, and mean water velocity was calculated from the time taken for a float to travel a distance of 2 m from five points of reference located at equal distances from the center of the stream to one of the banks (Pinto et al., 1976).

2.3. Ecotoxicological assays

2.3.1. Determination of toxicity and genotoxicity using *A. cepa*

Undiluted water and sediment samples collected in summer and winter of 2003 were tested using *A. cepa*. Copper sulphate was used as a positive control, at a concentration of $0.63\ mg\ l^{-1}$ and water from a natural source as a negative control (Fiskesjö, 1985). Vermiculite was used as a substrate to set up the positive and negative sediment controls.

Following Fiskesjö (1993), bulbs of healthy onions free from contact with agricultural pesticides were dried and placed in sets of 10 per sample collected, which were renewed daily and kept at a temperature of 20 °C. On the second day, roots approximately 2 mm long roots were collected for cytogenetic/genotoxicity analysis, and the material was fixed in Carnoy for at least 3 h and then in 70% alcohol under refrigeration. After fixing, the histological preparations were made using Feulgen stain for the meris-

tems, then "squashing" them with acetic acid and later fixing and staining with Fast-Green. Approximately four slides per bulb analyzed.

The genotoxicity evaluation consisted of the following assays: (a) mitotic index (MI), (count of 1000 cells per slide); (b) anomalies in the mitotic cycle such as the appearance of chromosomal bridges, and the presence of chromosomes or free fragments (count of 1000 cells per slide) and (c) presence of micronuclei (count of 3000 cells per slide). An optical microscope with 400 and 1000 magnifications was used for this purpose.

Toxicity was evaluated using by measuring root length and was determined by a 50% reduction in root length compared with the negative control (RC < 50%). After the roots were collected, bulbs were kept in illuminated solution t at a temperature of 20 °C for another four or five days. Data were analyzed by calculating means and standard errors of root growth rate and mitotic index; anomalies in the mitotic cycle were noted, together with the presence of micronucleus.

The correlation of significance between the sites sampled and the negative control was determined by analysis of variance (ANOVA) Tukey's test, program Graphpad Prism, version 3.0.

2.3.2. Determination of toxicity with D. similis, D. magna, C. dubia and H. azteca

Toxicity assays on water collected in winter 2003 used *D. similis*, following NBR 12713 (ABNT, 1993) and *D. magna* (US EPA, 1993). *H. azteca* (ASTM E1383-90, 1991) was used in assays on sediment samples. *D. magna* and *C. dubia* were used on water samples collected in summer 2004 following NBR 13373 (ABNT, 1995); *H. azteca* was used for sediment samples.

The Trimmed Spearman–Kärber (TSK), version 1.5 test was used to determine toxicity in the case of *D. similis*; for *H. azteca* and *D. magna*, Steel's Many-one rank test was used. The hypothesis tested was that no difference in death or immobility existed between the control group and the sample assayed. In the case of *C. dubia*, the test for presence of chronic and acute toxicity of a sample was by means of Student's *t*-test and Fisher's exact test.

3. Results

3.1. Results of physical and chemical analyses

The results of physical and chemical analyses in Table 1 show that of all the sites analyzed, the worst levels of physical and chemical parameters were found at Site 2 in summer 2003, and in winter, at Site 3. The analyses of summer 2004 did not detect either hexavalent chromium or mercury. Fig. 1 show that the biodegradability rate is lowest at Site 2 in winter ($f_b = 0.25$); high biodegradability ($f_b = 1.53$) was observed for Site 1 in the summer.

Average stream discharge in summer 2003 was 35 l s^{-1} at Site 1, 61.3 l s^{-1} at Site 2 and 144 l s^{-1} at Site 3. In winter, discharges were slightly higher: namely 38, 83 and 158 l s^{-1} at Sites 1, 2, 3, respectively. Fig. 2 show that concentrations of total nitrogen and ammoniacal nitrogen were higher in water at Site 3 than at the two sites further upstream. The pH values of the sediment did not vary during summer and winter in the samples collected at Site 2, where both had a pH of 7.5.

3.2. Results of toxicity and genotoxicity tests

The evaluation of genotoxic effects, as determined by the mitotic index, number of micronuclei and chromosomal anomalies, showed no significant results, either in the negative control or among the different sites analyzed for water and sediment (Tables 2 and 3). In summer, significant

Table 1
Result of physical and chemical tests of water from different sites of Estância Velha stream in summer and winter of 2003

Physical–chemical analyses		Summer			Winter			Reference (maximum limit)
		Site 1	Site 2	Site 3	Site 1	Site 2	Site 3	
1	COD (mgO_2 l^{-1})	8	3610	350	11	172	301	405[a]
2	BOD_5 (mgO_2 l^{-1})	8	600	170	4	85	160	5[b]
3	pH	6.8	7.1	7.8	7.0	7.7	7.6	6.0–9.0[b]
4	TSS (mg l^{-1})	6	2909	137	17	49	128	135[a]
5	Total nitrogen (mg l^{-1})	2[c]	151[d]	102[d]	1[c]	35[d]	78[d]	10[a]
6	Oil and grease (mg l^{-1})	3	124	25	9	16	23	0[b]
7	Total chromium (mg l^{-1})	n.d	13.0	1.5	n.d	0.69	1.4	0.05[b]
8	Temperature (°C)	20	22	22	13	17	14	40[a]
9	Phenol (mg l^{-1})	0.02	0.10	0.08	n.d	n.d	0.02	0.003
10	D.O (mg l^{-1})	6.0	8.8	9.6	6.1	4.3	8.1	>5[b]
11	M.T.B (NPP 100 ml^{-1})	2.4×10^{2}	$>2.4 \times 10^{6}$	4.6×10^{6}	1.5×10^{2}	4.3×10^{5}	4.3×10^{5}	1.0×10^{3}[b]

n.d = Not detected; M.T.B = Microbiological thermotolerant bacteria; TSS = Total suspended solids.
Analyses performed had the following detection limits (minimum): Total chromium (0.01 mg l^{-1}), and Phenol (0.01 mg l^{-1}). Analyses performed in the summer of 2004: hexavalent chromium n.d (0.05 mg l^{-1} detection limit), mercury n.d (0.0001 mg l^{-1} detection limit).

[a] According to the operations license for tanneries in the region (according to SSMA 05, 1989).
[b] According to CONAMA 357 (2005) – class 2.
[c] Using the Kjedahl method.
[d] Using the Nessler method.

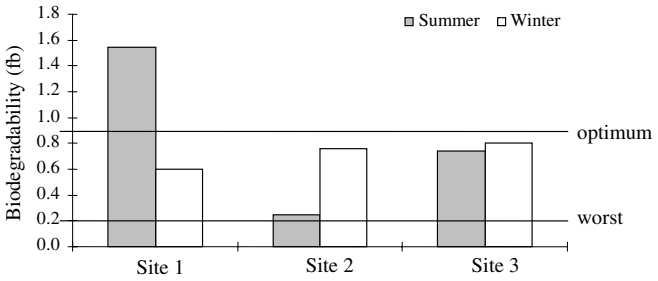

Fig. 1. Biodegradability rate at sampling sites of the Estância Velha stream in different seasons of 2003.

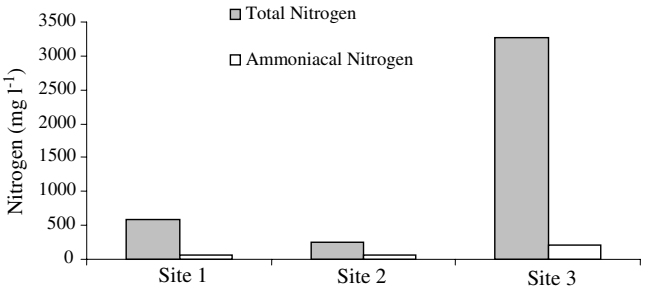

Fig. 2. Concentration of total nitrogen and ammoniacal nitrogen in sediment at sampling sites on the Estância Velha stream in winter 2003.

inhibition of *A. cepa* roots growth (RC < 50%) was observed at all sites ($P < 0.001$). During this season, Site 2 is outstanding with only 7% growth and a significant reduction of roots compared with the headwater Site 1, both for water ($P < 0.001$) and sediment ($P < 0.05$). In winter, water and sediments from Sites 2 and 3 were toxic as shown by inhibition of *A. cepa* roots. Water and sediment samples demonstrated a significant reduction in relation to negative control, except for water from Site 1. Site 2 (sediment) and Site 3 (water and sediment) also presented a reduction in relation to the headwater.

Table 4 shows toxicity to *D. similis*, *D. magna*, *C. dubia* and *H. azteca*. In winter 2003 no stream water showed toxic effects on *D. similis* and *D. magna*; regarding sediment toxicity, only sediment from Site 3 affected *H. azteca* significantly.

In summer 2004, acute toxicity in water was demonstrated by *D. magna* and *C. dubia* and in sediment by *H. azteca*, at Site 3 alone. Site 2 was the only site sampled that presented chronic toxicity to *C. dubia*.

4. Discussion

The present study clearly showed increased pollution, both in water and in sediment, from headwaters to lower

Table 2
Indices determined with *Allium cepa* in water from the Estância Velha stream in summer and winter 2003

Season	Samples	Mitotic index	Micronucleated cells	Microscopical effects*	Root length (cm)	Root length**
Summer	Site 1	1.8 ± 0.4	0.3 ± 0.2	0.8 ± 1.0	1.4 ± 0.4^{a}	47
	Site 2	1.3 ± 0.8	0.6 ± 0.2	0.8 ± 1.5	$0.2 \pm 0.1^{a,c}$	7[c]
	Site 3	1.8 ± 0.7	0.5 ± 0.5	0.8 ± 0.9	1.1 ± 0.4^{a}	38
	Negative control	1.5 ± 0.3	0.4 ± 0.1	1.0 ± 0.9	3.0 ± 0.3	100
	Positive control	0.9 ± 0.3^{b}	2.6 ± 1.0^{a}	3.1 ± 1.6^{a}	0.7 ± 0.5^{a}	23
Winter	Site 1	1.8 ± 0.6	0.5 ± 0.3	0.8 ± 1.1	2.6 ± 1.0	87
	Site 2	1.7 ± 0.4	0.6 ± 0.3	1.0 ± 1.1	1.3 ± 0.5^{a}	45
	Site 3	1.5 ± 0.7	0.4 ± 0.3	1.1 ± 0.9	$0.6 \pm 0.2^{a,c}$	19
	Negative control	1.3 ± 0.6	0.4 ± 0.1	0.9 ± 0.3	3.0 ± 1.0	100
	Positive control	0.4 ± 0.3^{a}	7.4 ± 3.5^{a}	4.0 ± 1.9^{a}	1.6 ± 0.4^{a}	55

Significant compared with negative control: [a]$P < 0.001$ and [b]$P < 0.05$; Significant compared with Site 1: [c]$P < 0.001$.
* Only the presence of chromosomal bridges has been identified.
** % of control.

Table 3
Indices determined with *Allium cepa* in sediment from Estância Velha stream in summer and winter 2003

Season	Samples	Mitotic index	Micronucleated cells	Microscopical effects*	Root length (cm)	Root length**
Summer	Site 1	2.0 ± 0.1	0.3 ± 0.5	0.7 ± 0.5	1.0 ± 0.3^{a}	29
	Site 2	1.9 ± 0.5	0.4 ± 0.4	1.0 ± 0.9	$0.5 \pm 0.4^{a,c}$	15[c]
	Site 3	1.8 ± 0.4	0.2 ± 0.1	1.2 ± 0.8	$0.5 \pm 0.4^{a,c}$	15
	Negative control	2.2 ± 1.0	0.3 ± 0.1	0.8 ± 0.6	3.4 ± 1.0	100
	Positive control	0.9 ± 0.7^{a}	4.3 ± 1.5^{a}	4.6 ± 1.9^{a}	1.1 ± 0.8^{a}	62
Winter	Site 1	2.1 ± 0.4	0.2 ± 0.1	0.9 ± 1.2	2.2 ± 0.7^{a}	63
	Site 2	2.1 ± 0.5	0.3 ± 0.1	1.1 ± 1.1	$1.1 \pm 0.4^{a,b}$	33
	Site 3	1.9 ± 0.1	0.4 ± 0.3	1.2 ± 0.8	$0.2 \pm 0.1^{a,b}$	6
	Negative control	2.1 ± 1.0	0.3 ± 0.0	1.0 ± 0.3	3.5 ± 1.0	100
	Positive control	1.0 ± 0.7^{a}	6.4 ± 2.8^{a}	3.2 ± 1.8^{a}	1.2 ± 0.3^{a}	33

Significant compared with negative control: [a]$P < 0.001$; Significant compared with Site 1: [b]$P < 0.001$, [c]$P < 0.05$.
* Only the presence of chromosomal bridges has been identified.
** % of control.

Table 4
Results of acute and chronic toxicity assays performed using *Daphnia similis*, *Daphnia magna*, *Ceriodaphnia dubia* and *Hyalella azteca* in samples of surface water and in sediment at three sites of the Estância Velha stream

Water					Sediment	
Station	Winter 2003		Summer 2004		Winter 2003	Summer 2004
Sample/test	*D. similes* 48 h	*D. magna* 48 h	*D. magna* 48 h	*C. dubia* 7 days	*H. azteca* 10 days	*H. azteca* 10 days
Site 1	n.d	n.d	n.d	n.d	n.d	n.d
Site 2	n.d	n.d	n.d	Detected[b]	n.d	n.d
Site 3	n.d	n.d	100%[a]	100%[c]	100%[a]	75%[a]

n.d: No effect detected. Samples evaluated at a concentration of 100%.
[a] Mortality obtained in the test.
[b] Chronic toxicity determined by the statistical difference between the reproduction or survival of the control population.
[c] Acute toxicity determined by mortality that is statistically different from that which occurred in the control population during the first 48 h of exposure.

reaches of the Estância Velha stream. Although a treatment plant exists for the treatment of industrial wastewater, physical, chemical and microbiological tests identified contributions both from industrial wastes and untreated domestic sewage. The high concentration of chromium, organic compounds and thermotolerant bacteria from Site 1 onwards indicates the presence of wastes from the leather industry. Since factories for leather and other industrial products are sited in urban areas, the poor quality in samples collected at Sites 2 and 3 of the stream in summer, and at Site 3 in winter, is a matter for community concern.

Class and Maia (2003) state that physical and chemical indices of the magnitude observed in this study, which exceed limits established by government authorities, are a matter of concern for the leather industry. These authors found values in untreated tannery effluents similar to those found at Site 2 during the summer of 2003, where the physical and chemical conditions were poorest. Similarly, Aragon (1990) reported high concentration of organic matter in tannery effluents, probably originating from processes for the extraction of proteins and skin greases, the use of detergents and high sulphide concentrations, together accounting for more than 70% of the BOD and COD of leather-industry effluent.

In this study, no indication of chromosomal mutation was observed for *A. cepa* either in water or sediment samples from the Estância Velha stream. Studies with the TA98 and TA100 strains are in progress, and no positive mutagenic response has been found either in the presence or absence of metabolic activation.

Toxic effects on *A. cepa* were observed for nearly all samples in both summer and winter, the exception being Site 1 in winter. Samples taken in winter from Sites 2 and 3 showed particularly marked toxicity, corroborating results of physical and chemical tests on the water, where the BOD, oils and greases, total chromium and tolerant bacteria are beyond legal limits. Furthermore, cytotoxic effects have also been observed for *Salmonella thyphimurium* (Mitteregger et al., in preparation). Similar results were found by Lopez et al. (1997) in evaluating the tannery effluent where the presence of trivalent chromium(III) associated with organic matter had a cytotoxic effect on *S. thyphimurium*, although no mutagenic compounds were identified after primary and secondary effluent treatment.

The chromium found in water downstream from Site 1 is probably associated with tanning skins and retanning leather (Class and Maia, 1994). The values observed are of great concern to the sector since usually the concentrations of chromium salts used are about 2.0–3.0% of the mass of skins. The high concentration of total chromium found in the samples analyzed at Sites 2 and 3, and the absence of hexavalent chromium(VI), suggest the presence of only trivalent chromium(III), which is 1000 times less toxic than chromium VI (ASTDR, 2000) and this may explain the absence of positive results to *A. cepa* genotoxicity. In addition, Rutland (1991) emphasizes that the non-existence of chromium VI in tannery residues is due to their absence in skin tanning, for which chromium III is exclusively used, usually in the form of basic sulphide of trivalent chromium ($[Cr(OH)(H_2O)_5]SO_4$), preventing fiber putrefaction and improving its hydrothermal stability, effects not achieved when using chromium VI.

The presence of oils and greases and thermotolerant bacteria found at all sites analyzed suggests an accumulation of organic matter from domestic sewage produced in the neighboring community, and may be associated with the toxicity identified in *A. cepa*. The results described by Smaka-Kincl et al. (1995) and White and Rasmussen (1998) support the hypothesis that the discharge of untreated domestic sewage is the main cause of the toxic effect observed in *A. cepa*. This effect probably results from the action of domestic sewage components on the proteins that regulate the cell cycle, causing an acute effect by necrosis and cell death (Dartsch et al., 1998; Diana et al., 2000), possibly diminishing the genotoxic effect observed (Rank and Nielsen, 1998).

Ecotoxicological evaluation using *D. magna*, *Ceriodaphnia dubia* and *H. azteca* as test organisms was adequate to determine the presence of a toxic effect of the samples collected from the headwater and downstream sites.

Sediment analysis with *A. cepa* and *H. azteca* also identified the presence of toxicity in the sample collected at Site 3 during the winter of 2003. This toxicity is probably

associated with a high concentration of ammoniacal nitrogen found at this point. Other toxicity responses with ammoniacal nitrogen were observed with *D. magna* (Emmanuel et al., 2005) and *D. pulex* (Vidal et al., 2004). Their source may be the residues from the skin dehairing process, for which products such as sulphides and amines are used, together with lime to raise the pH (Hoinacki et al., 1994).

Assays using *D. magna*, *C. dubia* and *H. azteca* to evaluate water and sediment during the summer of 2004 indicate that the Estância Velha stream continues to receive a large volume of residues with high toxic content at Sites 2 and 3, clearly indicating the need for monitoring by ecotoxicity tests. The high toxicity in terms of the chronic effect with *C. dubia* the next year (summer 2004) in samples from Site 2, and in terms of the acute effect at Site 3, showed the higher sensitivity of this organism to the ecotoxicity test than *D. magna*. These results agree with those of Versteeg et al. (1997), suggesting that *C. dubia* is more sensitive to toxicity than other Daphnidea for complex mixtures, especially for industrial effluents containing inorganic compounds. Aragon (1990) also expresses concern about the presence of products used in finishing leathers, such as anilines, paints, lacquers, solvents and fixatives, which present risks to health and to the environment. This author also points out that products in skin and leather conservation, such as bactericides and fungicides, respectively, have been identified in tannery effluents with toxic effects on bioindicator organisms such as *D. magna* and *Danio rerio* (Tisler et al., 2004).

It is interesting to note that *A. cepa* proved more sensitive for detecting toxicity than the other ecotoxicity tests used, and that at Site 2 toxicity is higher in summer; the physical and chemical quality of samples, including total chromium and biodegradability rate, was then also at its poorest at this site, followed by Site 3 in winter. These results are supported by the fact that water flow is higher at Site 3 than at Site 2.

In conclusion, the two downstream sites receive extensive domestic residues and industrial effluents from the leather industry, causing severe degradation in water quality and showing the need for continuous monitoring to provide a basis for improvement. The discharge of wastes with different chemical characteristics leads to complex mixtures with time-varying composition which therefore subjects the existing biota to a continually-varying challenge. Hitherto, these risks have not been identified by the routine tests by industries in the region, so that the river system biota remains at high risk.

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