Evaluation of Sinos River water genotoxicity using the comet assay in fish

Scalon, MCS.[a], *Rechenmacher, C.*[b], *Siebel, AM.*[b], *Kayser, ML.*[c], *Rodrigues, MT.*[c], *Maluf, SW.*[d], *Rodrigues, MAS.*[e] *and Silva, LB.*[f]*

[a]Programa de Pós-graduação em Qualidade Ambiental, Universidade Feevale, RS 239, 2755, CEP 93352-000, Novo Hamburgo, RS, Brazil

[b]Curso de Ciências Biológicas, Universidade Feevale, RS 239, 2755, CEP 93352-000, Novo Hamburgo, RS, Brazil

[c]Curso de Biomedicina, Universidade Feevale, RS 239, 2755, CEP 93352-000, Novo Hamburgo, RS, Brazil

[d] Laboratório de Citogenética, Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos, 2350, Rio Branco, CEP 90035-000, Porto Alegre, RS, Brazil

[e]Programa de Pós-graduação em Qualidade Ambiental, Grupo de Pesquisa em Tecnologias Ambientais, RS 239, 2755, CEP 93352-000, Novo Hamburgo, RS, Brazil

[f]Programa de Pós-graduação em Qualidade Ambiental, Grupo de Pesquisa em Indicadores de Qualidade Ambiental, Universidade Feevale, RS 239, 2755, CEP 93352-000, Novo Hamburgo, RS, Brazil

*e-mail: lucianosilva@feevale.br

Received February 21, 2010 – Accepted April 29, 2010 – Distributed December 31, 2010

Abstract

The Sinos River, in southern Brazil, is polluted by industrial discharges and untreated urban wastes. Fish genotoxicity biomarkers are valuable parameters for environmental risk assessment. In this study, we used the comet assay to detect genotoxicity due to multiple sources of pollution in the peripheral blood of a native fish species (*Hyphessobrycon luetkenii*). In addition, we analysed possible DNA damage from aluminum, lead, chromium, copper, nickel, iron and zinc contamination. Water samples were collected seasonally from three sampling sites and the fish were assessed under laboratory conditions. Water chemical analysis showed an increased level of aluminum and iron in most of the samples at sites 2 and 3, located in the middle and lower river course, respectively. The index of DNA damage assessed by the comet assay demonstrated no significant differences in different seasons or at the different sampling sites, while the frequency of cells with DNA damage was higher in water samples collected at sites 1 and 2 during the spring season. None of the metals studied seems to be associated with the increase in the frequency of cells with DNA damage observed during the spring season. The results of this study indicate that the Sinos River is contaminated with substances that are genotoxic to fish, including the waters near the river spring.

Keywords: genotoxicity, river pollution, biomarkers, metals.

Avaliação da genotoxicidade da água do rio dos Sinos utilizando o ensaio cometa em peixes

Resumo

O rio dos Sinos, no sul do Brasil, é poluído tanto por descargas industriais como por resíduos urbanos não tratados. Os biomarcadores de genotoxicidade em peixes são parâmetros valiosos para a determinação de risco ambiental. Neste estudo, utilizamos o ensaio cometa em sangue periférico de um peixe nativo (*Hyphessobrycon luetkenii*) para detectar a genotoxicidade devido a múltiplas fontes de poluição. Além disso, analisamos a possível influência da contaminação por alumínio, chumbo, cromo, cobre, níquel, ferro e zinco sobre o dano de DNA. Amostras de água foram coletadas sazonalmente em três pontos de coleta e os peixes foram expostos sob condições de laboratório. A análise química da água mostrou níveis elevados de alumínio e ferro na maioria das amostras dos pontos 2 e 3, localizados nos cursos médio e inferior do rio, respectivamente. O índice de dano de DNA estimado pelo ensaio cometa não demonstrou diferenças significativas tanto entre estações como entre pontos de coleta, enquanto que a frequência de células com dano de DNA apresentou valores superiores nas amostras de água coletadas durante a primavera nos pontos 1 e 2. Nenhum dos metais estudados parece estar associado com o aumento na frequência de células com dano de DNA observado durante a primavera. Os resultados deste estudo indicam que o rio dos Sinos está contaminado com substâncias genotóxicas para os peixes, incluindo as águas próximas à nascente do rio.

Palavras-chave: genotoxicidade, poluição de rios, biomarcadores, metais.

1. Introduction

The biodiversity of surface waters and freshwaters constitutes a valuable natural resource, in economic, cultural, aesthetic, scientific and educational terms. Their conservation and management are critical to the interests of all humans, nations and governments (Dudgeon et al., 2006). In densely populated countries, most or all rivers will receive huge amounts of waste derived directly from industry, agriculture and urban settlements or indirectly from the atmospheric deposition of airborne emissions (Frenzilli et al., 2009), and hence be contaminated with complex, ill-defined mixtures of chemicals. Most freshwater organisms will be exposed, to varying degrees, to this contamination and little is known about whether or not species are adversely affected by the chemicals present in their environment (Sumpter, 2009).

In order to assess exposure to or effects of environmental pollutants on aquatic ecosystems, there is a suite of fish biomarkers which may be examined. Genotoxic parameters are currently among the most valuable fish biomarkers for environmental risk assessment (Oost et al., 2003). The analysis of DNA alterations in aquatic organisms has been shown to be a highly suitable method for evaluating the genotoxic contamination of environments, being able to detect exposure to low concentrations of contaminants in a wide range of species. The comet assay or single cell gel electrophoresis has found wide application as a simple and sensitive method for evaluating DNA damage in fish exposed to various xenobiotics in the aquatic environment (Dhawan et al., 2009; Frenzilli et al., 2009).

Among the myriad of organic and inorganic substances released into aquatic ecosystems, heavy metals have received considerable attention due to their toxicity and potential to bioaccumulate at various trophic levels (Szefer et al., 1990). When assessing genotoxicity in fish, heavy metals have been considered as one of the major group of elements inducing DNA damage (Vargas et al., 2001; Valko et al., 2006; Barbosa et al., 2009).

In Rio Grande do Sul, Brazil's southernmost state, approximately 1.2 million inhabitants and a large number of different types of industry (including metallurgical companies and tanneries) depend on the Sinos River basin for water supply. The Sinos River is 190 km long and originates at an altitude of 740 m and flows into Guaíba Lake, near the city of Porto Alegre, the state capital. In the downriver direction, the river is divided into upper, middle and lower courses, following a gradient of pollution. The Sinos River in the upper course has low demographic density and suffers moderate impacts from small amounts of agricultural waste and relatively little influence from domestic sewage. The middle course has a major impact and has a larger number of inhabitants. In the lower course the demographic density is high and the river is heavily impacted by domestic and industrial sewage (FEPAM, 1999).

Studies investigating the genotoxicity of water samples collected from the Sinos River have yielded conflicting results. Moreover, genotoxicity assays in fish erythrocytes has not been used to evaluate the genotoxic impact of environmental pollutants from Sinos River water. The present work aimed to assess the genotoxic effects in fish exposed to samples from the Sinos River as well as to evaluate DNA damage from aluminum, lead, chromium, copper, nickel, iron and zinc contamination.

2. Materials and Methods

2.1. Sampling sites

Three sampling sites were selected in the Sinos River, with different levels of chemical impact. Sampling site 1, located near the river spring (29° 43' 26" S and 50° 16' 46'' W, town of Caraá), was expected to be an uncontaminated location and was used as the reference site. The two impacted sites were located in the town of Parobé (29° 41' 5" S and 50° 50' 52" W, sampling site 2) in the middle river course and in the town of Sapucaia do Sul (29° 47' 53" S and 51° 11' 24" W, sampling site 3) in the lower river course. Samples were collected four times at each site (seasonally) from November 2007 to July 2008, months which respectively correspond to spring and winter in the southern hemisphere.

2.2. Test organisms

The indigenous fish species *Hyphessobrycon luetkenii* (Boulenger, 1887) (Characidae) was selected for the exposure tests and the specimens were obtained from a local fish farm. Healthy fish were randomly divided into three groups of about 12 specimens and placed in a 9-L aquarium, with well-oxygenated, dechlorinated tap water at room temperature, for a 48 hours acclimatisation period; they were then released into aquariums with water from each sampling site within 4 hours after sampling. The exposure period was 48 hours with no food supply.

2.3. The comet assay

After the exposure period, the comet assay was performed on peripheral erythrocytes, according to Tice et al. (2000). Slides were pre-coated with normal melting point agarose. A mixture of 5 µL of blood sample collected from caudal veins of fish with 95 µL low melting point agarose (0.7%) was added to the slide and immediately covered with a coverslip and then kept for 10 minutes in a refrigerator to solidify. After solidification of the gel, coverslips were gently removed and the slides were immersed in cold, freshly made lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.2, to which 1% Triton X-100 and 10% DMSO had been added) and refrigerated at 4 °C for 1-24 hours. After the lysis, the slides were placed in a horizontal electrophoresis box side by side. The tank was filled with fresh electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) at 4 °C. The liquid covered the slides, which were then left in the solution for 20 minutes before the power was turned on. Electrophoresis was performed at 25 V and 300 mA (~0.95 V/cm) for 20 minutes. The steps above were carried out under red light to avoid induction

of DNA damage. After electrophoresis, the slides were gently removed from the tank, and neutralising buffer (0.4M Tris, pH 7.5) was added to the slides dropwise three times, letting it sit for 5 minutes each time. The slides were rinsed three times with distilled water, air dried for at least 24 hours, and then fixed and stained with silver stain according to Nadin et al. (2001).

For evaluation of DNA damage, 100 cells per individual were analysed under an optical microscope at 400x magnification. The cells were scored visually according to tail length into five categories, from undamaged (Type 0), to complete damage (Type IV) (Anderson et al., 1994). The extent of DNA damage was expressed as the mean percentage of cells with medium, high and complete damaged DNA, which was calculated as the sum of cells with damage types II, III and IV (Palus et al., 1999). From the arbitrary values assigned to the different categories (from Type 0 = 0 to Type IV = 4), a genetic damage index was calculated for each fish (Pitarque et al., 1999). Therefore, the total score per individual ranged from 0 (all undamaged) to 400 (all maximally damaged).

2.4. Water chemical analysis

Immediately after collection, water samples were analysed to determine the levels of aluminum, lead, chromium, copper, nickel, iron and zinc according to APHA (1998).

2.5. Statistical analysis

Statistical analysis was performed using ANOVA, followed by Duncan's multiple comparison test when appropriate. All analyses were carried out using the Statistical Package for the Social Sciences (SPSS) 15.0 for Windows, considering a significance level of $p \leq 0.05$.

3. Results

The results of water analyses for the three sampling sites are shown in Table 1. The heavy metals Al, Fe and Zn were detected in most samples at the three sampling sites. The occurrence of Pb, Cr, Cu and Ni was less frequent. Cu was not detected at site 1, but it was present in the four samples from site 3 and in two samples from site 2. Cr was found in only one sample (site 3 in the winter). At the three sampling sites Pb, Cr, Cu, Ni and Zn showed lower concentrations than the accepted levels established by the Brazilian environmental legislation for Class 1 water, while Al and Fe contents frequently exceeded the limits allowed at sites 2 and 3.

The DNA damage index estimated by the comet assay carried out on *H. luetkenii* erythrocytes showed no significant differences between sampling sites and sampling periods (Table 2). Table 3 shows that the percentage of cells with damaged DNA was not different between sampling sites in the four sampling periods. However, there were significant differences between sampling periods at sampling sites 1 ($p = 0.04$) and 2 ($p = 0.0001$). Spring showed higher values than autumn at site 1, while at site 2, Spring showed higher values than all the other three sampling periods.

4. Discussion

With increased concerns regarding the genotoxicity of pollutants, the use of sensitive bioassays may be a valuable tool to screen the genotoxicity of contaminated rivers. Studies investigating Sinos River water genotoxicity have yielded both positive and negative results. Vargas et al. (2001) found absence of mutagenic activity in the *Salmonella*/microsome test and presence of genotoxic activity in

Table 1. Levels of heavy metal present in the water samples collected from the Sinos River in different sampling periods.

Legislation[a]		Al	Pb	Cr	Cu	Ni	Fe	Zn
		0.1	**0.01**	**0.05**	**0.009**	**0.025**	**0.3**	**0.18**
Spring	Site 1	0.02	nd	nd	nd	0.006	0.05	0.01
	Site 2	0.56	nd	nd	nd	0.01	1.23	0.01
	Site 3	0.48	0.01	nd	0.01	0.02	1.74	0.04
Summer	Site 1	0.05	nd	nd	nd	nd	0.08	0.01
	Site 2	0.59	nd	nd	0.002	nd	1.93	0.01
	Site 3	0.05	nd	nd	0.004	nd	1.13	0.01
Autumn	Site 1	0.16	nd	nd	nd	nd	0.10	0.01
	Site 2	1.43	nd	nd	0.0025	nd	1.76	0.01
	Site 3	1.36	nd	nd	0.0042	nd	1.96	0.02
Winter	Site 1	nd	nd	nd	nd	nd	0.23	nd
	Site 2	0.18	0.0015	nd	nd	nd	0.66	nd
	Site 3	2.00	0.004	0.04	0.04	0.01	6.88	0.08

[a]Maximum values in mg/L allowed by Brazilian legislation – resolution 357/2005 of the National Environment Council (CONAMA) for Class 1 water. nd = Not detected by the method.

Table 2. Damage index estimated by the comet assay (mean ± standard deviation) in *H. luetkenii* erythrocytes exposed to water samples from the Sinos River.

Sampling site	Sampling period				p
	Spring	Summer	Autumn	Winter	
Site 1	54.3 ± 26.0 (10)	58.1 ± 14.5 (12)	64.6 ± 11.3 (9)	69.5 ± 14.2 (11)	0.20
Site 2	71.7 ± 34.7 (10)	64.6 ± 7.4 (12)	60.6 ± 9.8 (13)	65.3 ± 17.9 (8)	0.61
Site 3	50.8 ± 21.9 (10)	60.8 ± 16.0 (12)	66.4 ± 16.9 (12)	62.5 ± 15.0 (11)	0.22
p	0.22	0.49	0.54	0.57	

The number of fish analysed in each sample is presented between brackets. P values referring to ANOVA between sampling sites in the same season (last line) and between seasons at the same sampling site (last column).

Table 3. Percentage of cells of *H. luetkenii* with damaged DNA observed in the comet assay (%, mean ± standard deviation) after exposure to water samples from the Sinos River.

Sampling site	Sampling period				p
	Spring	Summer	Autumn	Winter	
Site 1	8.1 ± 8.1^{a} (10)	5.8 ± 4.5^{ab} (12)	1.8 ± 4.3^{b} (9)	2.7 ± 3.2^{ab} (11)	0.04
Site 2	14.3 ± 13.0^{a} (10)	2.8 ± 3.1^{b} (12)	0.4 ± 1.1^{b} (13)	2.4 ± 2.6^{b} (8)	0.0001
Site 3	6.4 ± 7.1 (10)	5.7 ± 7.9 (12)	1.1 ± 1.9 (12)	2.5 ± 4.2 (11)	0.10
p	0.18	0.35	0.46	0.97	

P values referring to ANOVA between sites in the same sampling period (last line) and between seasons at the same site (last column). Samples marked with different letters have significant difference between seasons at the same sampling site.

the microscreen phage-induction assay. Moreover, Terra et al. (2008) and Jacociunas et al. (2010) did not observe genotoxic effects using the micronuclei analysis in the V79 cell line (Chinese hamster lung) and the wing Somatic Mutation and Recombination Test (SMART) in *Drosophila melanogaster*, respectively.

Fishes are excellent specimens for the study of the mutagenic potential of contaminants present in water samples since they can metabolise, concentrate and store waterborne pollutants. They can serve as useful genetic models for the evaluation of pollution in aquatic ecosystems (Elliott et al., 1988). The comet assay has been used successfully to investigate the effects of genotoxic pollutants on the integrity of DNA (Dhawan et al., 2009; Frenzilli et al., 2009). Evaluation of Sinos River water genotoxicity using the comet assay in fish was performed for the first time in the present study.

As a consequence of industrial production, a large quantity of toxic material is released into surface waters. Due to the elevated concentrations of metals present in many types of wastewaters, metals are ubiquitous contaminants in aquatic ecosystems (Roche and Boge, 1996); therefore, they are among the most intensely studied contaminants. They do not only deteriorate the physicochemical equilibrium of the aquatic body, but they also disrupt the food web and bring about morphological, physiological and cytogenetic changes in aquatic inhabitants. Genotoxic studies on aquatic organisms exposed to polluted waters containing heavy metals have implicated DNA damage (Vargas et al., 2001; Matsumoto et al., 2006; Yadav and Trivedi, 2009; Barbosa et al., 2009).

In the present study the chemical analysis of the water showed presence of metals in the examined Sinos River water samples. The data obtained were compared with the values established by the Brazilian Environmental Standards. The results of this work indicate that the river receives the discharge of heavy metals rich in industrial effluents from the industries of the Sinos River basin. However, the levels of lead, chromium, copper, nickel and zinc were lower than the legally established concentrations for Class 1 water. Al and Fe exceeded the accepted limits in most of the water samples from sites 2 and 3. Aluminum, although it is not a heavy metal, was included in this work due to its toxicity to fish. Aluminum can lead to heavy loss of electrolytes, extreme gill damage and circulating collapse in fish (Savory and Wills, 1991)

From the two parameters of genotoxicity estimated by the comet assay in *H. luetkenii* erythrocytes, only the frequency of cells with higher level of DNA damage showed significant difference in relation to the sampling period. That result suggests that in the spring season, the potency of genotoxicans in the Sinos River was higher than in other sampling periods, both near the river spring (site 1) and in the middle river course (site 2). However, the increase in that parameter of genotoxicity does not seem to be related to differences between sampling periods regarding the presence or concentration of the heavy metals analysed. The methods used to evaluate genotoxicity in the present study, as well as in other studies, have the advantage of detecting and quantifying the genotoxic impact without requiring a detailed knowledge of the identity and of the

physical/chemical properties of the contaminants present (Frenzilli et al., 2009).

Leather processing is an important economic activity in the Sinos River basin. Among tannery effluent constituents, chromium has been accounted for the genotoxicity observed in streams affected by tanneries (Lopez et al., 1998; Chandra et al., 2004; Çavaş and Ergene-Gözükara, 2005; Matsumoto et al., 2003, 2006; Mitteregger et al., 2007). Matsumoto et al. (2006) found genotoxic effects on erythrocytes from fish exposed to water taken from sites where the total chromium concentration was 0.01 mg/L. In our study, the levels of chromium were below the detection limit, except for the winter water sample from site 3, which presented a chromium concentration of 0.04 mg/L. Despite the presence of chromium, no increase in genotoxicity was observed. To explain differences among studies of chromium-contaminated rivers, in addition to differences in exposure time, biomarkers and test organisms, additive, synergistic, or antagonistic interactions among the chemicals should be considered (Lambolez et al., 1994).

The findings highlighted the value of genotoxicity biomarkers as sensitive parameters of environmental pollutant contamination and their importance in the biomonitoring of aquatic ecosystems. It is possible that the changes registered by the comet assay parameter may be attributed to the exposure of fish to substances other than those metals measured in water samples. Because the contaminants in aquatic environments seldom occur as single chemicals, both the metals detected in our study and other classes of environmental contaminants are likely to induce genotoxic damage in the study area. White and Rasmussen (1998) present evidence to support the contention that despite the noteworthy genotoxicity of some industrial wastewaters, domestic wastewaters constitute a greater genotoxic hazard to aquatic systems and their associated biota. If we consider that most domestic wastewaters in the Sinos River basin receive no treatment, it is the domestic sources of contamination that should be investigated and controlled.

Acknowledgements – This work was partially supported by a research grant from Conselho Nacional de Desenvolvimento Cientıfico e Tecnológico (CNPq – number 477192/2007-6) and by a Student Fellowship from Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) to an undergraduate student (CR).

References

American Public Health Association - APHA, 1998. *Standard Methods for the Examination of Water and Wastewater*. 20th ed. Washington, D.C.: APHA/AWWA/WPCF.

ANDERSON, D., YU, TW., PHILLIPS, BJ. and SCHEZER, P., 1994. The effect of various antioxidants and other modifying agents on oxygen-radical generated DNA damage in human lymphocytes in the comet assay. *Mutation Research,* vol. 307, p. 261-271.

BARBOSA, JS., CABRAL, TM., FERREIRA, DN., AGNEZ-LIMA, LF. and BATISTUZZO DE MEDEIROS, SR., 2009. Genotoxicity assessment in aquatic environment impacted by the presence of heavy metals. *Ecotoxicology and Environmental Safety*. doi:10.1016/j.ecoenv.2009.10.008.

ÇAVAŞ, T. and ERGENE-GÖZÜKARA, S., 2005. Induction of micronuclei and nuclear abnormalities in *Oreochromis niloticus* following exposure to petroleum refinery and chromium processing plant effluents. *Aquatic Toxicology*, vol. 74, p. 264-271.

CHANDRA, S., CHAUHAN, LKS., PANDE, PN., GUPTA, SK., 2004. Cytogenetic effects of leachates from tannery solid waste on the somatic cells of *Vicia faba*. *Environmental Toxicology*, vol. 9, p. 129-133.

DHAWAN, A., BAJPAYEE, M. and PARMAR, D., 2009. Comet assay: a reliable tool for the assessment of DNA damage in different models. *Cell Biology and Toxicology*, vol. 25, p. 5-32.

DUDGEON, D., ARTHINGTON, AH., GESSNER, MO., KAWABATA, ZI., KNOWLER, DJ., LEVEQUE, C., NAIMAN, RJ., PRIEUR-RICHARD, AH., SOTO, D., STIASSNY, MLJ. and SULLIVAN, CA., 2006. Freshwater biodiversity: importance, threats, status and conservation challenges. *Biological Reviews*, vol. 81, p. 163-182.

ELLIOTT, M., GRIFFITHS, AH. and TAYLOR, CJL., 1988. The role of fish studies in estuarine pollution assessment. *Journal of Fish Biology,* vol. 33, p. 51-61.

FRENZILLI, G., NIGRO, M. and LYONS, BP., 2009. The Comet assay for the evaluation of genotoxic impact in aquatic environments. *Mutation Research*, vol. 681, p. 80-92.

Fundação Estadual de Proteção Ambiental Henrique Luiz Roessler – FEPAM, 1999. *Pró-Guaiba, Rede de Monitoramento Ambiental*: Monitoramento das águas superficiais do Rio dos Sinos. FEPAM/CORSAN/DMAE.

JACOCIUNAS, LV., DIHL, RR., LEHMANN, M., REGULY, ML. and de ANDRADE, HH. 2010. Recombinagenic activity of water and sediment from Sinos River and Araçá and Garças Streams (Canoas, Brazil), in the Drosophila wing spot test. *Science of the Total Environment*, vol. 408, p. 571-577.

LAMBOLEZ, L., VASSEUR, P., FERRAD, JF. and GISBERT, T., 1994. The environmental risks of industrial waste disposal: an experimental approach including acute and chronic toxicity studies. *Ecotoxicology and Environmental Safety*, vol. 28, p. 317-328.

LOPEZ, LC., BASSI, M., TZICAS, E., MORETTON, J., 1998. Genotoxicity study of a leather tannery's waste waters. *Journal of the Society of Leather Technologists and Chemists*, vol. 82, p. 159-161.

MATSUMOTO, ST., MANTOVANI, MS., MALAGUTTII, MIA., DIAS, AU., FONSECA, IC. and MARIN-MORALES, MA., 2006 Genotoxicity and mutagenicity of water contaminated with tannery effluents, as evaluated by the micronucleus test and comet assay using the fish *Oreochromis niloticus* and chromosome aberrations in onion root-tips. *Genetics and Molecular Biology*, vol. 29, p. 148-158.

MATSUMOTO, ST., MANTOVANI, MS., MALLAGUTI, MI. and MARIN-MORALES, MA., 2003. Investigation of the genotoxic potential of the waters of a river receiving tannery effluents by means of the *in vitro* comet assay. *Cytologia*, vol. 68, p. 395-401.

MITTEREGGER, H., SILVA, J., ARENZON, A., PORTELA, CS., FERREIRA, ICFS. and HENRIQUES, JAP., 2007. Evaluation of genotoxicity and toxicity of water and sediment samples from a Brazilian stream influenced by tannery industries. *Chemosphere,* vol. 67, p. 1211-1217.

NADIN, SB., VARGAS-ROIG, LM. and CIOCCA, D., 2001. A silver staining method for single-cell gel assay. *Journal of the Histochemical Society*, vol. 49, p. 1183-1186.

OOST, R. van der, BEYER, J. and VERMEULEN, NPE., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology*, vol. 13, p. 57-149.

PALUS, J., DZIUBALTOWSKA, E. and RYDZYNSKI, K., 1999. DNA damage detected by the comet assay in the white blood cells of workers in a wooden furniture plant. *Mutation Research,* vol. 444, p. 61-74.

PITARQUE, M., CREUS, A., MARCOS, R., HUGHES, JA. and ANDERSON, D., 1999. Examination of various biomarkers measuring genotoxic endpoints from Barcelona airport personnel. *Mutation Research,* vol. 440, p. 195-204.

ROCHE, H. and BOGE, G., 1996. Fish blood parameters as a potential tool for identification of stress caused by environmental factors and chemical intoxication. *Marine Environmental Research*, vol. 41, p. 27-43.

SAVORY, J. and WILLS, MR., 1991. In MERIAN, E. (Ed.). *Metals and Their Compounds in the Environment*. Weinheim: VCH Verlagsgesellschaft. ch. II.1.

SUMPTER, JP., 2009. Protecting aquatic organisms from chemicals: the harsh realities. *Philosophical Transactions of The Royal Society A-Mathematical Physical And Engineering Sciences*, vol. 367, p. 3877-3894.

SZEFER, P., SZEFER, K. and SKWARZEC, B., 1990. Distribution of trace metals in some representative fauna of the Southern Baltic. *Marine Pollution Bulletin*, vol. 21, p. 60-62.

TERRA, NR., FEIDEN, IR., FACHEL, J., LEMOS, CT. and NUNES, EA. 2008. Ecotoxocological evaluation of sediment and water samples from Sinos River, Rio Grande do Sul, Brazil, using Daphnia magna and V79 cells. *Acta Limnologica Brasiliensia*, vol. 20, p. 65-74.

TICE, RR., AGURELL, E., ANDERSON, D., BURLINSON, B., HARTMANN, A., KOBAYASHI, H., MIYAMAE, Y., ROJAS, E., RYU, JC. and SASAKI, YF., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis*, vol. 35, p. 206-221.

VALKO, M., RHODES, CJ., MONCOL, J., IZAKOVIC, M. and MAZUR, M., 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions*, vol. 160, p. 1-40.

VARGAS, VMF., MIGLIAVACCA, SB., MELO, AC., HORN, RC., GUIDOBONO, RR., FERREIRA, ICFS. and PESTANA, MHD., 2001. Genotoxicity assessment in aquatic environments under the influence of heavy metals and organic contaminants. *Mutation Research,* vol. 490, p. 141-158.

YADAV, KK. and TRIVEDI, SP., 2009. Chromosomal aberrations in a fish, *Channa punctata* after in vivo exposure to three heavy metals, *Mutation Research,* vol. 678, p. 7-12.

WHITE, PA. and RASMUSSEN, JB. 1998. The genotoxic hazards of domestic wastes in surface waters. *Mutation Research*, vol. 410 p. 223-236.