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Genotoxicity evaluation of tilapia (*Oreochromis niloticus*) exposed to waters from two sites of Itajaí-Açu River (SC, Brazil)

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Abstract

The relationship between the genes and the effects attributed to them has been object of many studies, especially those seeking to establish the response of genes to environmental prod. The aim of this work was to establish a standard system to monitor effluents by using juvenile Nile tilapia (*Oreochromis niloticus*) as a bioindicator of genotoxicity, utilizing micronuclei test and comet assay. For this, the fish were exposed during 24, 48, 72 and 240 hours (10 days), to water samples collected at two sites of the Itajaí-Açú River: Ilhota and Blumenau, in the Santa Catarina State, Brazil. For positive control the herbicide 2,4-D (75 ppm) was utilized, and the negative control of each fish were the values obtained before the exposure to the river water, termed time zero (T_0). Water samples from both sites of the Itajaí-Açú River showed significant genotoxic effects in erythrocytes of the exposed fishes. The comet assay was a more sensitive test to detect genotoxic damage in shorter exposure times (24 and 48 hours) than the micronuclei test.

Key words: genotoxicity, comet assay, micronuclei test, *Oreochromis niloticus*, bioindicator

Avaliação da genotoxicidade em tilápias (*Oreochromis niloticus*) expostas às águas de dois locais do Rio Itajaí-Açu (SC, Brasil)

Resumo

A relação entre os genes e os efeitos a eles atribuídos tem sido objeto de muitos estudos, sobretudo aqueles que buscam estabelecer a resposta dos genes aos estímulos ambientais. Este estudo teve por objetivo padronizar um sistema de monitoramento de efluentes tendo juvenis de peixes exóticos da espécie tilápia (*Oreochromis niloticus*) como bioindicadores de genotoxicidade, utilizando as técnicas do teste do micronúcleo e ensaio cometa. Para isto, os peixes foram expostos no período de 24, 48, 72 e 240 horas (10 dias) às amostras de água coletadas em dois locais do Rio Itajaí-Açú: Ilhota e Blumenau, no Estado de Santa Catarina, Brasil. De controle positivo foi utilizado o herbicida 2,4-D (75ppm), e para o controle negativo foram atribuídos os valores obtidos de cada peixe antes da exposição às águas do rio, determinado como tempo zero (T_0). As amostras de água de ambos os pontos de coleta do Rio Itajaí-Açú mostraram efeitos genotóxicos significativos nos eritrócitos dos peixes expostos. O ensaio cometa mostrou ser mais sensível para detectar danos genotóxicos, nos menores tempos de exposição em relação ao teste do micronúcleo.

Palavras-chave: genotoxicidade, ensaio cometa, teste do micronúcleo, *Oreochromis niloticus,* bioindicador.

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INTRODUCTION

Pollution is spreading at unperceived speed, and there is a growing concern about water resources, in particular about conserving the environmental conditions necessary for survival for wild life aquatic organisms. For this, the knowledge of the quality of the natural water bodies, like lakes, streams, rivers, sees and others is essential, as are studies which evaluate the impact that pollution exerts on living beings, providing the necessary argument to enable adequate conservation, management and development programs (Barnhoorn & Van Vuren, 2004).

The sources of pollutants to water reservoirs are diverse, the most significant of them being city sewage, industrial waste and agricultural pesticides and fertilizers, which drain into rivers and may leach into the groundwater. Within the pollutants are many unlisted genotoxicants or products which originate them, whose consequences go beyond the killing of local organisms, affecting in unpredictable manner the generations to come, not only of aquatic organisms, but of all who depend on that water as drinking source (Claxton *et al*., 1998; Jha, 2004; Moron *et al*., 2006).

The Itajaí-Açú river, with an extension of 200 Km, is the main river of Santa Catarina state, draining a region of 15,500 Km^2, it crosses some of the most important cities of the state, like Brusque, Blumenau and Itajaí, receiving direct wastes from over 60 major polluting industries, mostly textile, metal-mechanic, fishery, soya oil, paper and starch-producing undertakings, so as spill waste from about 900,000 people, among other urban residues, most of them without any pre-treatment. The region is problematic, and the river, which often is colored in the dyeing tints of the textiles, is the source of the drinking water and for industrial use (Santos, 1996; Pereira-Filho *et al*., 2003).

In the present paper, the micronucleus test (MNT) and the single cell gel electrophoresis (SCGE), were used to study the genotoxic potencial of Itajaí-Açú water by using erythrocytes of tilapia (*Oreochromis niloticus*)-as bioindicator. The MNT is a very sensitive and useful method that can detect both clastogenic and aneugenic activity (Al-Sabti & Matcalfe, 1995), and has been widely used as tool for the monitoring of water quality (Buschini *et al*., 2004; Maffei *et al*., 2009). Comet assay, first developed by Singh *et al*. (1988), is frequently used to evaluate the genotoxicity in environmental monitoring studies (Silva *et al*., 2000; Frenzilli *et al*., 2009). In literature, several *in vitro* and *in vivo* studies with the comet assay in fish are reported (Maffei *et al*., 2009; Hartmann *et al*., 2004) and the usefulness of this test in fishes as a model for monitoring genotoxicity of aquatic habitats seems widely accepted (Russo *et al.*, 2004; Frenzilli *et al*., 2009). The MNT is often applied in conjunction with the comet assay under alkaline conditions (Belpaeme *et al.*, 1998; Buschini *et al*., 2004; Bücker *et al*., 2006). Alkaline (pH > 13) version of the comet assay is capable of detecting DNA single-strand breaks, alkali-labile sites and DNA-DNA/DNAprotein cross-linking (Tice *et al*., 2000; Christofoletti *et al*., 2009).

The Nile tilapia *O. niloticus* is considered a good bioindicator for genotoxicants in water resources and for environmental monitoring (Andrade *et al.*, 2004a; Masutti *et al.*, 2006; Grisolia *et al.*, 2009). The choice of these species is because it is easily obtained and already well adapted to climactic conditions of Brazil. The Nile tilapia *O. niloticus* also have a commercial value added and it is cultivated in many regions of Brazil and according to FAO (1998) the tilapia is the most cultivated cichlid in the world.

The aim of this study was to develop a system using juvenile Nile tilapia as an *in vivo* diagnostic tool for the screening of environmental genotoxic effects of water obtained from the Itajaí-Açú River, Brazil.

MATERIAL AND METHODS

Samples

For the bioassay, juvenile specimens of the exotic species *Oreochromis niloticus* (Perciformes, Cichlidae), commonly known as Nile tilapia, were supplied by the Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina S.A. (EPAGRI), Hatchery Station from Camboriú city. Prior to the genotoxic tests individuals were acclimated to laboratory conditions for a week in tanks of 500 liters of tap water with aeration. After that a total of the 20 individuals (averaging 14 ±5 g in weight and 15 ±2 cm in length) 10 per each test, were used to test the waters of the Itajaí-Açú River at laboratory conditions. Fishes were exposed in 500 liter tanks filled with 200 liters of water from two locations. The sample point 1 (Ilhota) was located in Ilhota city (26º54'09.92"S, 48º49'54.18"W) and the sample point 2 (Blumenau) in Blumenau city (26º55'12.81"S, 49º03'31.90"W), both sample sites are in Santa Catarina State, Brazil (Fig.1). After exposition, blood was collected from the caudal vein of all fish at time zero (T_0) and after 24, 48, 72 and 240 hours of exposure and utilized to both micronucleus and comet assay tests. For each test, one slide was mounted per fish for each time of exposure. The fishes were not fed during the experiment and the temperature was ranging from 20 to 23 ºC under constant aeration.

The positive control group consisted of 12 individuals exposed to a sub-lethal concentration (75 ppm) of the herbicide 2,4-Dichlorophenoxyacetic acid (CAS No. 94-75-7, 98% purity) in buckets of 30 liters of capacity (Ateeq *et al*., 2002). The 2,4-D (DMA 806 BR, Dow Agrosciences Ind. Ltda) was supplied by the Experimental Station of EPAGRI from Itajaí city (Santa Catarina State, Brazil).

Micronucleus test (MNT)

The MNT first described by Schmidt (1975), was performed according to Grisolia and Cordeiro (2000) with minor modifications. Blood samples were obtained by caudal vein puncture using a heparinized syringe and smeared immediately onto clean glass slides; air dried overnight, and

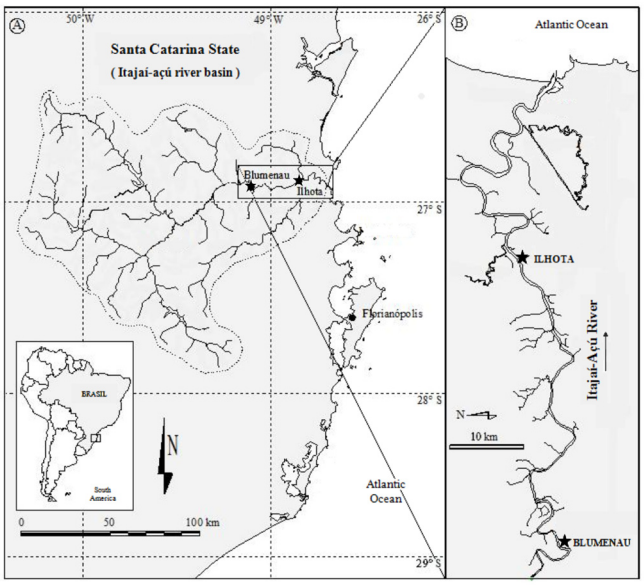

Figure 1. Map of the Brazilian state Santa Catarina (A), showing the Itajaí-Açú river basin (dashed line) and location of two sample sites (B) in Blumenau and Ilhota (closed stars).

then fixed in absolute methanol for 15 min and stained with 5% Giemsa solution for 15 min. At least 1,000 erythrocytes for each *O. niloticus* specimens were examined using a light microscope. Micronucleus (MN) were identified and scored microscopically under 100 x objective in an Olympus microscope. The main criteria for scoring the MN were the absence of connections with the main nucleus and a size smaller than 1/10 of the nucleus.

Alkaline Comet assay

The alkaline comet assay was performed basically as described by Hartmann *et al*. (2004). Fish blood, samples were collected from the caudal vein with a syringe. A 10 µL aliquot of fish blood was taken from each diluted sample and mixed with 75 µL low-melting-point (LMP) agarose (0.5%). The suspension was spread on slides previously coated with normal agarose, prepared in phosphate-buffered saline (PBS), which were then covered with a cover slip and conditioned in the refrigerator at 4ºC for 20 min, so that the agarose could solidify and the cover slip was carefully removed and a second layer of LMP agarose was added (75 µL). After agarose solidification, the cover slip was removed and slides were immersed into lysis solution (2.5M $NaCl_2$, 100mM Na2 EDTA, 10mM Tris, pH 10,1% sodium sarcosinate with 1% Triton X-100, and 10% DMSO added just before use) for at least 20 minutes, at 4ºC. Afterwards, slides were washed in ice-cold PBS in order to remove excess of salt and detergents, left in electrophoresis buffer (0.3 mM NaOH, 1 mM EDTA, pH > 13) during 20 min for DNA unwinding, and electrophoresed in the same buffer for 20 minutes (0.8 V cm^{-1}). Following electrophoresis, slides were neutralized in 400 mM Tris–HCl (pH 7.5), for 10 min. And fixed in absolute ethanol (for 5 min), let dry on air and stored in the dark until analysis. Observations were made on Olympus fluorescent microscope (1,000 x). The slides were stained at moment of analyses with DAPI (4,6-Diamidino-2-fenilindol) (3 µg mL^{-1}). A total of 100 comets were scored for each individual.

The samples were compared with the negative control group exposed to the hatchery water and the positive control 2,4-D (75 ppm) only at the 24 hours of exposure, which was the time proved to have been sufficient enough to show extensive DNA damage. All slides were independently coded and scored by a single observer. DNA migration was determined visually by the categorization of comets into different classes of migration. The damage classes were: I – II – III – IV – V – VI, and to the statistical analysis was attributed number values (Ranks) from 0 until 5 to each one of these classes. The parameters measured to analyze the electrophoretic patterns follow criteria proposed by Tice *et al*. (2000). Therefore, after get the frequencies for each damage class, it was calculated the sum of the ranks to each slide sample (individual), and this result was utilized to the comparison of the exposure times. Statistical analysis was performed using the non-parametric Mann–Whitney *U*-test, with the level of significance set at $p < 0.05$.

RESULTS AND DISCUSSION

The evaluation of wastes and effluents by genotoxicity assays provides useful data for hazard identification and comparative risk assessment (Claxton *et al.*, 1998). In our study we collected erythrocytes by caudal puncture of the *O. niloticus* without the need of sacrificing animals to conduct the study, and this was done after one week for acclimation (T_0), and 24 hours, 48 hours, 72 hours and 10 days of exposure. The fish demonstrated resistance to the stress of handling and the blood collection, corroborating data of Lemos *et al*. (2005) with other tilapia species, *Tilapia rendalli*, where the specimens could be returned to the environment after similar experimental procedures. That what was not the case when we used the electric fish *Eingenmannia virescens* (Bücker *et al*., 2006) or *Apteronotus bonapartii* (Carvalho *et al.*, 2006; Bücker *et al*., 2012), where a significant amount of fish died, probably because of the blood collection stress.

The positive control, 2,4-D, caused a time-dependent increase of MN and extensive DNA damage at 24 h (Fig. 2 and 3), serving well as positive control for both assays, corroborating findings of Ateeq *et al*. (2002), were the herbicide 2,4-D was found to be genotoxic as well as cytotoxic in catfish *Clarias batrachus*, with a positive dose-response relationship.

MNT

Figure 2 shows the results for the MNT. Samples from Blumenau showed significant MN induction at 48, 72 and 240 h (10 days) of exposure. On the other hand, samples from Ilhota showed significant MN induction only at 72 h of exposure. The individuals exposed to the herbicide 2,4-D showed significant MN induction when compared with the negative control and also with individuals exposed to water samples from Ilhota and Blumenau.

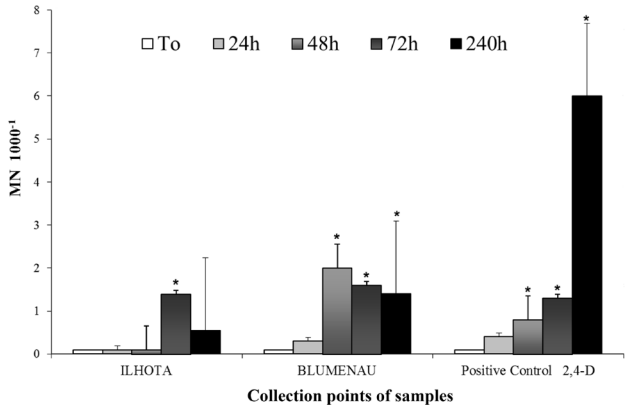

Figure 2. Mean number of micronuclei (MN 1000^{-1} erythrocytes) after exposure to water samples from Itajaí-Açú River in Ilhota and Blumenau cities. The bars represent the SE values. Positive control as 2,4-D (75 ppm). * $p < 0.05$ indicates difference from the T_0 using the Mann Whitney – *U* test.

Grisolia & Cordeiro (2000) utilized different substances known to be mutagenic (e.g. cyclophosphamide, mitomycin C) and compared the effects over three species, *Oreochromis niloticus; Cyprinus carpio* and *Tilapia rendalli*, the last one showed to be more sensitive when compared to the others using the MNT. Porto *et al*. (2005) utilizing MNT to evaluate the genotoxic effect of mercury pollution over Amazonian fish species concluded that MNT in fish erythrocytes may be useful for indicating genotoxity of mercury in Amazon Rivers.

In the MNT, the results for Ilhota showed lower rates of genotoxic effects in tilapia erythrocytes, than was found for Blumenau, having significant effects only at 72 h of exposure, despite being a downstream sample site from Blumenau city which is a large city. In the Blumenau, the results showed high-rates of genotoxic effects since 48 h of exposure up to the last time analyzed 240 h (10 days). This can be attributed to the large population of the Blumenau city, the activity of several industries, and therefore to its considerable proportion of contaminants discharged on the river (Resgalla-Jr *et al*., 2008). The results suggest that this pollution load may be diluted along the way down, since it receives the input of several minor water streams, or it may be that much of the genotoxicants sediment at the river bed, so that when the waters reach Ilhota they are already alleviated from much of the genotoxicants input of Blumenau. Work of White & Rasmussen (1998), suggest that household organic waste and a small amount of industrial waste are those most responsible for genotoxic effects in aquatic systems and associated biota.

Alkaline comet assay

The comet assay has been demonstrated to be a powerful tool for measuring the relationship between DNA damage and the exposure of aquatic organisms to genotoxic pollutants on environmental (Andrade *et al*., 2004b). This study provides data which will be useful for future work involving the biomonitoring of regions of the Itajaí-Açú River what it is important because the assessment of genotoxic effects is crucial to any comprehensive study of contaminants in aquatic environments. According to data in this study and the criteria established by Hartmann *et al*. (2004), the DNA is sensitive to pollutant exposure and effects (DNA breakage) serve as an early warning parameter. According by Lemos *et al*. (2005) to preserving the organism and ecosystem, the comet assay is a non-invasive and non-destructive methodology.

Figure 3 shows the mean of the sum of the ranks for the comet analysis. An increase in damaged nucleoids with increasing exposition time can be seen for both sampling points, revealing a time-dependent relationship between exposure and damage level in fish erythrocytes.

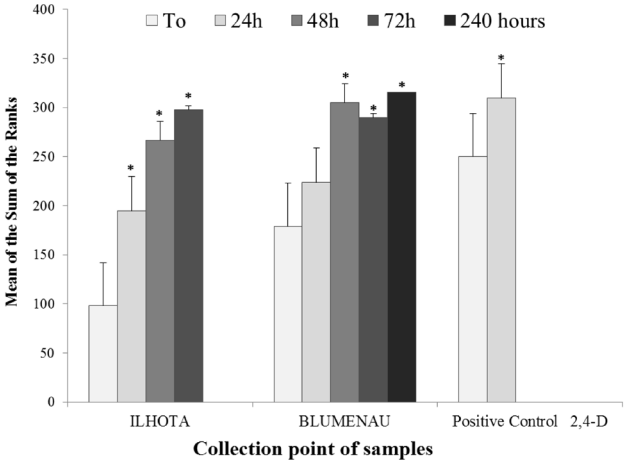

Figure 3. Mean sum of the ranks on different exposure times to water samples from Itajaí-Açú River in Ilhota and Blumenau cities. The bars represent the SE values. Positive control as 2,4-D (75 ppm). *$p < 0.05$ indicates difference from the T_0 using the Mann Whitney – *U* test.

Genotoxic effects were observed by comet assay on erythrocytes of fishes exposed to the waters of the Itajaí-Açú River in both sample sites (Ilhota and Blumenau). The level of damage found in both sample sites for higher exposure times is similar to the effects observed in the positive control group, where the samples showed a significant amount of damage. In the case of Ilhota, there was a significant increase in the mean of the sum of the ranks ($p<0.05$) already for 24 h of exposure. The effects were also time-dependent, since the values increased steadily with time up to 72 hs. In sample point of Blumenau, the comet assay showed no significant difference between T_0 and 24 h of exposure, however for the remaining times the damage showed a gradual increase from 48, 72 until 240 h (10 days) (Fig. 3). It was also noticed that there was an increase in the number of cells in the higher damage classes, when the exposure time increases (data not shown). On the other hand, the number of cells in the class I of damage was not showed to increase, suggesting a reduction of viable cells during exposure time. Bücker *et al.* (2006) in a study to evaluate the genotoxic action of benzene (50 ppm) in different specimens of electric fish (*Eingenmannia virescens*) also using MNT and Comet assay found similar results to those reported here.

It was unexpected the high level of DNA damage exhibited by the negative control of the herbicide 2,4-D exposure in the

comet assay. The most probable explanation for this are space and competition stress, since in this case 12 individuals were maintained in buckets of 30 liters of capacity, whereas for the river water testing 10 fish were exposed in 500 liter tanks filled with 200 liters of water. This points to unexpected effects of fish density and swimming space in generating an increase in DNA damage, possibly related to oxidative stress, which need further studies, but does not affect the results obtained for the river waters in the present work, since each experiment (actually each fish) has its own negative control.

The comet assay revealed significant DNA damage of the river water of both sites since the first analysis time up. When we compare the results between MNT and the comet assay for both sample sites it can be observed that for Ilhota only at the 72 h exposure time significant MN effects are present, whereas for Blumenau the MNT detected mutagenic effects already from 48 h up, what may be explained by the higher load of pollutants in this sample site. These results corroborate the sensitivity and speed of the comet assay in readily detecting DNA damaging agents in comparison to the MNT, because it does not depend on cell division. On the other hand, the MNT reveals that part of this DNA damage cannot be repaired, actually leading to irreversible mutations – highlighting the usefulness of utilizing both tests in combination.

The species *O. niloticus* demonstrated to be a sensitive organism to pollution and showed a pattern of DNA damage for environmental testing, thereby proving to be an adequate bioindicator of genotoxicity of river waters. A more realistic assessment could be provided by the analysis of native free-swimming fish caught from the polluted environment, and the comparison with fish of the same species caught from a reference area (Grisolia *et al*., 2009), which involves a more complicated setting. However, this study suggests that a monitoring program for the waters of this river using the exposure of the organisms *in situ* and continuously over time would be important. In conclusion, both techniques MNT and comet assay were effective and complementary to detect the presence of genotoxic effects in the tilapia erythrocytes, indicating that this fish can be used as a relevant parameter for biomonitoring the genotoxicity of aquatic environments.

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