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### Short Communication

## Evaluation of the Genotoxicity of Petroleum Refinery Effluents Using the Comet Assay in *Oreochromis niloticus* (Nile tilapia)

M. M. HOSHINA & M. A. MARIN-MORALES\*

São Paulo State University – UNESP, IB, Campus de Rio Claro, Av. 24 A, 1515, CEP 13506-900, Rio Claro – SP, Brazil

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### ABSTRACT

In this study, the genotoxic effects of effluent and water samples from areas (Atibaia and Jaguari rivers) related to a petroleum refinery were assessed by comet assay carried out in erythrocytes of *Oreochromis niloticus* (Nile tilapia). It was registered, for the negative control test (pure ground water), the lowest levels of nucleus with damage, followed by the waters collected in the Jaguari river (environmental control). It was observed a higher level of nucleus with damage in the fishes exposed to the refinery effluents samples, which decrease with the increasing treatments levels. It was also observed, in relation to the negative control, a genotoxic effect in the Atibaia river sample collected after the effluents discharge. The results showed that, even after the treatments, the petroleum refinery effluents induced genotoxicity in the exposed fishes.

**Keywords:** water pollution, environmental impact, *Oreochromis niloticus*, comet assay, petroleum refinery effluents.

### RESUMO

#### **Avaliação da Genotoxicidade de Efluentes de Refinaria de Petróleo Utilizando Ensaio Cometa em *Oreochromis niloticus* (tilápia do Nilo)**

Neste estudo, os efeitos genotóxicos de amostras de efluente e água de áreas (rios Atibaia e Jaguari) relacionadas com uma refinaria de petróleo foram avaliados através do ensaio do cometa realizado com eritrócitos de *Oreochromis niloticus* (tilápia). Foi registrado, para o controle negativo (água de poço artesiano), os níveis mais baixos de núcleos com danos, seguido das amostras de água coletadas no rio Jaguari (controle ambiental). Foi observado um alto nível de núcleos com danos em peixes expostos aos efluentes da refinaria, os quais foram diminuindo conforme os efluentes foram sendo tratados. Também foi observado um efeito genotóxico, em relação ao controle negativo, na amostra coletada após a descarga dos efluentes no rio Atibaia. Os resultados mostram que, mesmo após passar por tratamentos, os efluentes da refinaria de petróleo induziram genotoxicidade nos peixes expostos.

**Palavras-chave:** poluição aquática, impacto ambiental, *Oreochromis niloticus*, ensaio do cometa, efluentes de refinaria de petróleo.

### INTRODUCTION

Contamination of aquatic resources is one of the most worrying subjects of humankind. Domestic and industrial effluents are the principal responsible for the contamination of aquatic environment (Claxton *et al.*, 1998; White & Rasmussen, 1998). Among those, petroleum refinery wastewaters may contain a wide range of organic and metallic pollutants, including oil and greases, phenols (creosols and xylenols),

sulphides, ammonia, suspended solids, nitrogen compounds, heavy metals among other substances (Brown & Donnelly, 1984). This industry uses and discharges large volumes of wastewaters to surface waters and, although most of the contaminants are treated and recovered in the refinery, a significant amount of toxic substances and compounds may still be present in its wastewater (Avci *et al.*, 2005).

\* Corresponding author: Maria Aparecida Marin-Morales, e-mail: mamm@rc.unesp.br

One of the ways to evaluate the genotoxic potential of physical, chemical and biological substances is the comet assay. This method is used to detect small DNA damages (single strand breaks, double strand breaks and alkali-labile sites of individual cells) (Singh *et al.*, 1988). Comet assay is considered sensitive, fast, cheap and requires small number of cells to be performed (Mitchelmore & Chipman, 1998; Sasaki *et al.*, 1997; Kosz-Vnenchak & Rokosz, 1997) and it has been applied, with great success, in erythrocytes of many species of fishes (Nacci *et al.*, 1996; Belpaeme *et al.*, 1996; Mitchelmore & Chipman, 1998) exposed to several genotoxic agents. This technique evaluates the potential of these substances to cause damages in DNA, which can be detected by the alkaline electrophoresis.

Thus, the aim of this work was to evaluate the possible genotoxic effect of effluents of a petroleum refinery located at Paulínia municipality (São Paulo State – Brazil) using the comet assay in erythrocytes of *Oreochromis niloticus*.

## MATERIAL AND METHODS

### Samples collection

Water samples were collected in June 2004 at six different sites at the wastewater treatment and at rivers under the influence of a petroleum refinery (latitude 22° 48' 45" S, longitude 47° 11' 15" W) (Paulínia, São Paulo State – Southeastern

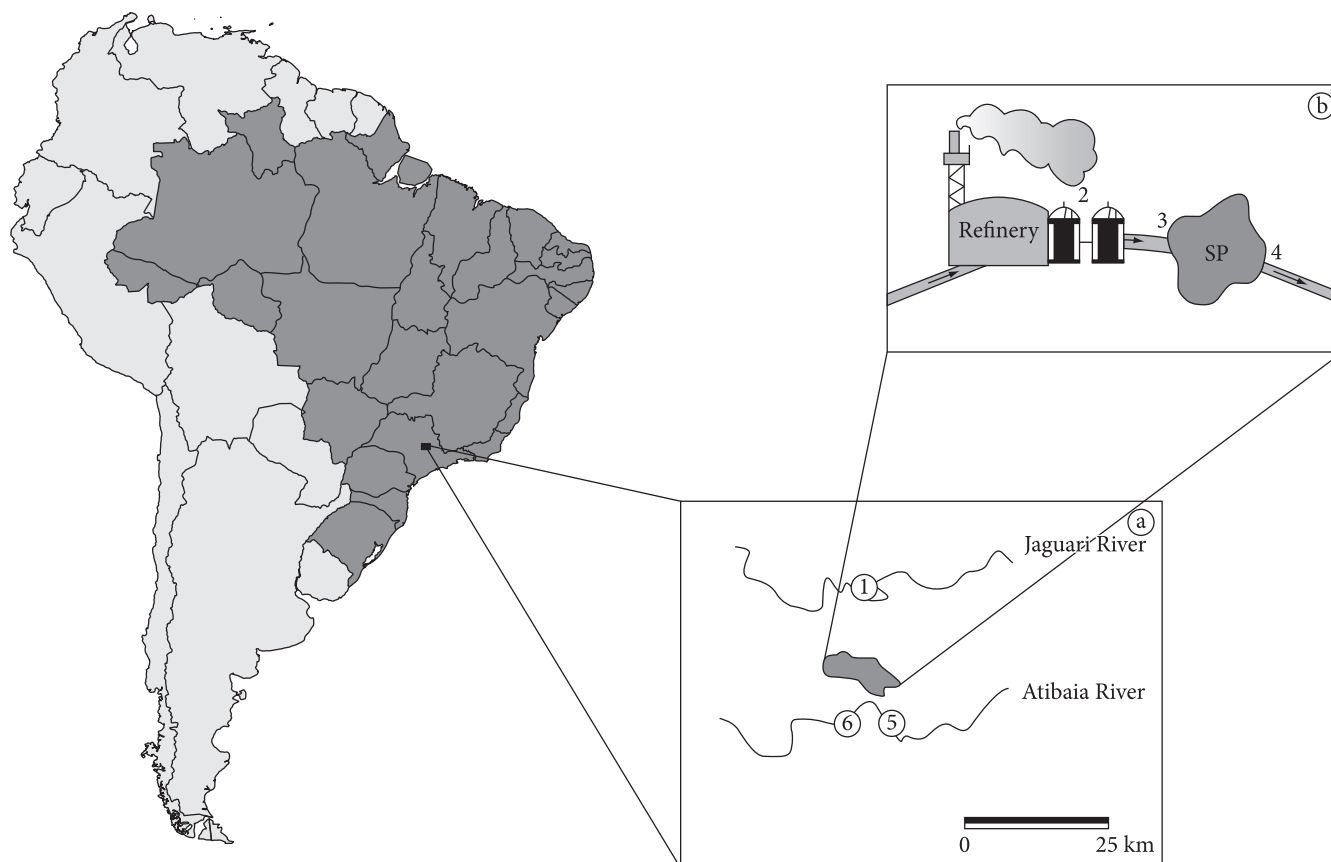
Brazil) as follows: - refinery water collection site at Jaguari river (Site 1); – wastewater treatment plant: after the physicochemical treatment and before the biological treatment (Site 2); entrance (Site 3) and output (Site 4) of the refinery stabilization pond; – Atibaia river: 1 km upstream (Site 5) and 1 km downstream (Site 6) the refinery effluent discharge (Figure 1).

### Test-organism

The test-organism used in the experiment was the Nile tilapia (*Oreochromis niloticus* - Perciformes, Cichlidae), which were raised on fish farms of UNESP (São José do Rio Preto Campus – SP). Before the experiments, they were acclimated in tanks with filtered water at ~ 23 °C and constant aeration for 1 week at the Laboratory of Water Toxicity (UNESP, Rio Claro Campus-SP). Individuals (mean size of 15 cm) were analyzed in order to avoid intra-specific differences related with size and age.

### Exposure experiment

Seven aquariums containing 20 L of water were used in the experiment, being one for negative control (pure ground water) and the others for each collection site. Five specimens of *Oreochromis niloticus* were randomly chosen and exposed for 72 hours in each aquarium (Grisolia & Cordeiro, 2000; Çavas & Ergene-Gözüraka, 2003) to the water samples in order to



**Figure 1.** Localization of the sampling sites at Paulínia municipality. A. #1, #5 and #6 at Jaguari and Atibaia rivers. B. #2, #3 and #4 at the refinery.

estimate the genotoxic effects. No food was supplied to the fish during the experiment.

No positive control was used since it was not found an adequate substance with the same exposition via. All the substances recommended as positive control (Ethyl methanesulphonate, mitomycin C, cyclophosphamide, colchicine, methylmethanesulphonate and others) are injected intraperitoneally in the fishes. Since the fishes were exposed directly to the waters, resulting in a different form of exposition, this could affect the comparison of the results.

### Comet assay

About 0.3 cc of whole blood was taken from each fish by heart puncture using heparinized syringes and 10  $\mu$ L aliquots were diluted in 1000  $\mu$ L of fetal bovine serum. Comet assay was conducted according to Singh *et al.* (1988) with some modifications. Microscope slides were pre-coated with normal melting point agarose 1.5% (v/v) and then were coated with 120  $\mu$ L of 0.5% (v/v) low melting point agarose at 37 °C, containing 10  $\mu$ L of the diluted blood. The slides were covered with coverslips and put in refrigerator for 20 minutes. After this period the coverslips were removed and the slides placed in lysis buffer (10 mM Tris, ~8 g of NaOH and 10 mL of 1% (v/v) sodium lauryl sarcosinate solution plus 1 mL of Triton X-100, 10 mL of DMSO and 89 mL of pH 10 lysis solution containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris and ~8 g of NaOH) in a refrigerator (4 °C) for at least 1 hour. After lysis, the slides were incubated in freshly prepared alkaline buffer solution (300 mM NaOH + 1 mM EDTA, pH > 13) for 20 minutes to denature the DNA and, then, submitted to electrophoresis at 25 V and 300 mA (1 V.cm<sup>-1</sup>) for 20 minutes. All these steps were carried out under indirect light. The slides were then neutralized with 0.4M Tris for 15 minutes, fixed in 100% ethanol for 10 minutes and left to dry at room temperature. The slides were stained with ethidium bromide (0.02 mg.mL<sup>-1</sup>) and analyzed under a fluorescence microscope (Leica DMLB) equipped with an excitation filter (420-490 nm) and a barrier filter ( $\lambda = 520$  nm), in 40 $\times$  objective lens. For each fish 100 nuclei were analyzed per blood sample.

On each slide, the cells were visually scored and allocated to one of four classes (0, 1, 2, 3) according to fragment migration as follows: class 0 - no tail; class 1 - a small tail less than the head (nucleus) diameter; class 2 - tail length equal to or up to twice the head diameter; and class 3 - tail more than twice the head diameter (Rigonato *et al.*, 2005).

The total score for 500 comets was obtained by multiplying the number of cells in each class by the damage class, according to the formula: Total score = (0  $\times$  n<sub>1</sub>) + (1  $\times$  n<sub>2</sub>) + (2  $\times$  n<sub>3</sub>) + (3  $\times$  n<sub>4</sub>), where n = number of cells in each class analyzed. Thus, the total score could range from 0 to 300. The results obtained were compared by the non parametric statistic Kruskal-Wallis test.

## RESULTS AND DISCUSSION

The results of the comet assay are shown in Table 1. Despite showing a high frequency of nucleoids of class 1 (small damage), the damage score for Site 1 was not significantly higher than the negative control. Site 1 (Jaguarí river) was considered as environmental control according to the results obtained in our analyses, and it is classified as Class 2 river according to the Resolution 357/2005 of the National Environment Council (CONAMA, 2005).

The highest score was observed for Site 2 (241.2  $\pm$  12.4), followed by Site 3 (186.4  $\pm$  18.8) and 4 (134.0  $\pm$  7). These values were significantly higher when compared to the negative and environmental controls. The high damage value at Site 2 was due to the presence of a great number of nucleoids of class 3. It was noticed that after the biological treatment (Site 3) the comet score decreased, since class 2 followed by 3 were the most frequent. The comet score also decreased at Site 4 (after the stabilization pond), where class 1 and 2 were the most frequent.

Although located in the rivers and free of the direct influence of the refinery output, Site 5 showed a slightly higher frequency of nucleoids of classes 2 and 3 in comparison to Site 1. Despite presenting a damage score about two times higher than Site 1, the damage score of Site 5 was not, however, significantly different from the negative and environmental control. On the other hand, there was an increase in the comet score in the

**Table 1.** Comet assay scores for erythrocytes of *Oreochromis niloticus* exposed to the water samples and negative control.

Collection sites	Number of cells with comet class (total of 500 cells per collection site)				Damage score (Mean $\pm$ S.D)
	Migration categories (Mean $\pm$ S.D)				
	0	1	2	3	
Negative control	94.4 $\pm$ 3.78	5.0 $\pm$ 4.00	0.4 $\pm$ 0.89	0.2 $\pm$ 0.45	6.4 $\pm$ 3.8
Site 1	68.0 $\pm$ 11.31	28.6 $\pm$ 14.72	2.2 $\pm$ 2.17	1.2 $\pm$ 1.79	36.6 $\pm$ 7.1
Site 2	1.2 $\pm$ 1.64	14.8 $\pm$ 6.10	25.6 $\pm$ 4.34	58.4 $\pm$ 5.32	241.2 $\pm$ 12.4**
Site 3	3.2 $\pm$ 3.11	24.2 $\pm$ 13.22	43.8 $\pm$ 14.65	28.8 $\pm$ 4.66	186.4 $\pm$ 18.8**
Site 4	15.0 $\pm$ 4.74	44.8 $\pm$ 5.40	31.4 $\pm$ 4.72	8.8 $\pm$ 3.7	134.0 $\pm$ 7**
Site 5	47.2 $\pm$ 3.27	29.8 $\pm$ 1.92	18.8 $\pm$ 2.77	4.2 $\pm$ 1.79	80.0 $\pm$ 8.4
Site 6	37.8 $\pm$ 5.12	35.2 $\pm$ 3.11	19.8 $\pm$ 3.27	7.2 $\pm$ 1.92	96.4 $\pm$ 10.6*

Damage Score: 0 = no damage; 300 = maximum damage; \* = significantly different in relation to the negative control (Kruskal-Wallis test at p = 0.05);

\*\* = significantly different in relation to Site 1 (Kruskal-Wallis test at p = 0.05).

erythrocytes of the fishes exposed to water collected after the discharge of the effluent at Atibaia river (Site 6), which was statistically different from the negative control.

The data obtained in this study showed that the waters from Site 2 presented a strong genotoxic effect, due to the dominance of class 3 nucleoids (high damage) and the highest comet score. Barros *et al.* (2006) assessed the toxicity of effluents from the same petroleum refinery herein studied using the freshwater planarian *Dugesia (Girardia) tigrina*, and observed toxic effects only in planarians exposed to water samples from Site 2. The waters of this site did not pass by the complete treatment performed by the petroleum refinery, thus it still contain a great variety of contaminants, such as polycyclic aromatic hydrocarbons (PAHs). According to Hamoutene *et al.* (2002), polycyclic aromatic hydrocarbons (PAHs) in the petroleum refinery effluents are the principal responsible for damages in DNA.

The petroleum refinery studied in this work perform physicochemical and biological treatments (flotation, aerated tanks, activated sludge, clarifier tanks and stabilization pond) in the effluents before the release in the Atibaia river. Although the damage values observed for Site 2, Site 3 and Site 4 did not differ statistically between themselves, there was a tendency of decrease in the values of the comet score towards increasing treatment levels. This might indicate that the treatments are removing some substances that are potentially genotoxic. In addition, although the refinery effluents present a genotoxic potential, this genotoxicity is significantly diminished when the effluent is mixed with the waters of the receptor river. The water sample from this receptor site (Site 6) presented damage score only significantly higher than the negative control, but neither in relation to waters from the environmental control (Site 1) nor from the same river before the discharge of the effluent (Site 5).

Souza & Fontanetti (2007), using the comet assay in *Oreochromis niloticus* to study the influence of petroleum refinery effluents in the Paraíba do Sul river, observed that was a significant increase in the comet score after the discharge of the effluents in the river, indicating that the studied effluents can interfere in the quality of the water of this river. However, depending on the effluent treatment processes adopted by the refineries, different impacts can be seen on the receiving rivers. In the case of the present study, the increase in the genotoxicity for the waters of the Atibaia river, after receiving the refinery effluent, was not very high and further studies are required to confirm if this effluent interfere in the quality of this river.

Hoshina *et al.* (2008) assessed the mutagenic effects of the same petroleum refinery effluents and waters of the Atibaia river herein studied. The authors observed an increase in the induction of micronucleus in erythrocytes of fishes exposed to the refinery effluents, indicating a change in the water quality of the Atibaia river after the discharge of the petroleum effluents. This is in disagreement with our results since the mutagenic effects observed for the waters of the Atibaia river do not seem to correspond to the genotoxic effects seen. These difference could be caused by other substances and/or compounds derived

from the effluents of other industries located upstream in the Atibaia River.

Despite all the treatments, the final effluent seems to still have genotoxic and mutagenic effects. Even reducing the effects, there is no guarantee that treatments performed by the refinery could prevent damages to exposed organisms. Thus, further studies must be done to fully assess the impact of this effluent to the Atibaia River.

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