



Mutagenic effects of tributyltin and inorganic lead (Pb II) on the fish *H. malabaricus* as evaluated using the comet assay and the piscine micronucleus and chromosome aberration tests

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Abstract

Genotoxicity studies on toxic metals and their organic compounds are very important, especially so in the investigation of the effects of these compounds on the aquatic environments where they tend to accumulate. The use of endemic aquatic organisms as biological sentinels has proved useful to environmental monitoring. We assessed the mutagenic potential of tributyltin (TBT) and inorganic lead (PbII) using samples of the fish *Hoplias malabaricus* (commonly called *traíra*) using the comet assay and the piscine micronucleus and chromosome aberration tests. Eighteen *H. malabaricus* were acclimatized in three individual aquariums, each containing six fish, six fish being exposed to 0.3 µg/g of body weight (bw) of TBT, six to 21 µg/gbw of PbII and six being used as controls. Exposure to TBT and PbII was achieved by feeding the fish every five days with *Astyanax* (a small fish that is part of the normal diet of *H. malabaricus*) which had been injected with solutions of TBT, PbII or with water (the control group). After two months the *H. malabaricus* were sacrificed and their peripheral blood collected and subjected to the comet and micronucleus assays, the chromosome aberration assay being conducted using kidney-tissue. Although the comet assay showed no mutagenic effects at the lead concentrations used but encountered results with TBT, the micronucleus and chromosome aberrations assays both indicated that TBT and PbII are potentially mutagenic ($p < 0.01$), the micronucleus assay showing morphological alterations of the nucleus.

Key words: Comet assay, micronucleus, chromosome aberrations, tributyltin, lead, fish.

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Introduction

The aquatic environment is the ultimate destination for almost all urban, industrial and agricultural wastes. Because of their real or perceived potential as mutagens or carcinogens, xenobiotic chemicals have often been the focus of attention, frequent pollutants of marine and/or fresh waters being inorganic lead (PbII) and organic tin compounds (Pain, 1995; Johnson, 1998; Alzieu, 2000; Godwin, 2001).

Organic tin compounds such as tributyltin (TBT) are used for many purposes, principally in antifouling paints and the plastics industry (Alzieu, 2000), several reports strongly suggesting that TBT can adversely affect the physiology of not only mammals (Mizunashi *et al.*, 2000) but

also marine organisms (Alzieu, 1991, 2000; Santos *et al.*, 2000). In spite of the fact that tin can bind to DNA no direct damage has yet been demonstrated, although some studies have shown apoptosis in mouse timocytes and hepatocytes exposed to different concentrations of TBT (Yamanoshita *et al.*, 2000).

Lead has been in use for thousand years, although the toxic effects of lead compounds were only recognized less than hundred years ago. Inorganic and organic lead enters the environment in several ways (Pain, 1995; Godwin, 2001) and can have diverse effects on organisms, including mimicking essential elements (*e.g.* calcium, magnesium, iron and zinc) with consequent effects on enzymes containing SH groups (Jennette, 1981), increased incorporation of erroneous nucleotides (Johnson, 1998) and the accumulation of free radicals due to alteration of the oxidative processes of cells and effects on repair mechanisms in which

lead has been implicated as a co-carcinogen (Fracasso *et al.*, 2002).

This paper reports the results a study of the neotropical fish *Hoplias malabaricus* in which this fish was exposed to TBT and PbII and the effects of the exposure assessed using the comet assay and the piscine micronucleus and chromosome aberration tests.

Material and Methods

Methodology

The fish we chose as a model was *Hoplias malabaricus* [Bloch, 1794] (Trahira, *traíra* in Portuguese), a neotropical piscivorous fish with a wide geographic distribution range, from Panamá to the rio Colorado in south of Argentina. This fish has the advantage that it is easily fed under laboratory conditions and, because it devours its prey whole, can be easily exposed to xenobiotics by feeding with a prey species previously injected with the material under investigation, a further advantage being that this species has 40 relatively large bi-armed chromosomes which makes studying the genetic effects of metals easier.

In our study, we used 18 adult *H. malabaricus* reared in captivity at a fish farm (Fazenda Canguiri, Universidade Federal do Paraná, Curitiba, Brazil) which were divided into three groups of six fish, one group being exposed to tributyltin (TBT), one group to lead (PbII) and one group kept unexposed as a control group. Each group was housed in a separate aquarium and acclimatized to a water temperature of 22 °C (\approx pH 7.6) and a 12 h light/dark photoperiod for 60 days before starting the experiments.

We exposed our *H. malabaricus* to TBT and PbII by feeding them with *Astyanax* (a small fish that forms part of the normal diet of *Hoplias*) which had been artificially contaminated by intraperitoneal injection of tri-n-butyltin or lead (Aldrich Chemical Co) in such a way as to produce a final concentration of 21 μ g PbII/g of body weight (bw) or 0.3 μ g TBT/g bw in the *H. malabaricus* after a suitable feeding schedule. The feeding schedule consisted of offering each *H. malabaricus* one *Astyanax* each day for four days over a period of 13 feed-cycles (about two months), *Astyanax* injected with TBT or PbII being fed to the appropriate experimental groups and *Astyanax* injected with water only being fed to the control group. Four days after the last feed-cycle the *H. malabaricus* were sacrificed after being anesthetized with 3-aminobenzoic acid ethyl ester (Sigma, São Paulo-SP, Brasil), blood being collected from the caudal vein for the comet assay and the piscine micronucleus test and kidney tissue for the chromosome aberration test.

Comet assay

For the comet assay, also known as the single-cell gel test (SCGE), we used the procedure of Singh *et al.* (1988), with a few modifications. Clean microscope slides were

prepared by covering them with a thin layer of 0.75% (w/v) NMP agarose (Gibco), two slides being prepared for each fish. For each slide, 10 μ L of whole fish blood was diluted in 1 mL of bovine calf serum and 10 μ L of this mixture placed in 120 μ L of LMP agarose gel (Gibco) which was then spread on the base layer of the slide and a cover slip added, the agarose gel being allowed to solidify by placing the slide in a refrigerator for 10 min. When the gel had solidified, the coverslips were gently removed and the slide placed in a lysis solution at 4 °C for 1 h to remove cellular proteins. After lysis, the slide was placed in an electrophoresis box containing an alkaline solution (pH > 13) for 25 min at 4 °C and then subjected to electrophoresis at 25 V, 300 mA, for 25 min, after which the slide was removed, neutralized, fixed in absolute ethanol and stained with 20 μ L of 10 μ L/mL ethidium bromide. Comets were scored using a Zeiss epifluorescence microscope. For each fish, 100 cells were visually analyzed according to the method of Kobayashi *et al.* (1995), the comets being classified as having: no detectable DNA damage and no tail (class 0); a tail with a length less than the diameter of the nucleus (class 1); a tail between one and two nuclear diameters long (class 2); a tail longer than twice the diameter of the nucleus (class 3). Apoptotic cells that presented totally fragmented nuclei were not scored (Speit and Hartmann, 1999). Differences between fish treated with TBT and PbII and untreated control fish were tested using the non-parametric Kruskal-Wallis test.

Piscine micronucleus test (PMT)

For each fish, two microscope slides were prepared by drawing a drop of fish blood over the slide to form a thin smear which was air-dried for 24 h, fixed with absolute methanol (Merck) for 1 h and stained for 20 min with 5% (w/v) Giemsa in pH 6.8 phosphate buffer (Merck). For each fish, 4000 erythrocytes were examined under 1000X magnification and scored for the presence of both typical micronuclei and nuclear alterations manifested as changes in the normal elliptic shape of the nuclei (Ayllon and Garcia-Vazquez, 2000). These two features were considered as nuclear abnormalities and scored together, frequencies being tested for significance using the χ^2 test.

Chromosome aberration test (CAT)

Chromosome preparations were obtained by the short term culture of kidney cells (Fenocchio *et al.*, 1991), 30 metaphase plates from each individual being scored for alterations such as the number of breaks or gaps and the level of de-condensation. Observed frequencies for treated and controls were tested using the χ^2 test.

Results and Discussion

The comet assay showed a significant increase of tailed nucleoids in the erythrocytes of fishes treated with

PbII, while fish treated with TBT showed a lower frequency of comets than untreated controls (Figure 1).

The spontaneous (or basal) frequency of micronuclei (MN) in fish is normally very low and in our study no significant differences were observed in the PMT between unexposed control fish and fish exposed to TBT and PbII. However, statistically significant differences were seen between the frequency of erythrocytes with altered nuclear morphology as compared to conserved nuclear morphology (Table 1, Figure 2 a,b,c).

The number of micronuclei in fish cells is a highly variable feature and different authors report different numbers of spontaneous micronuclei for the same species (Gustavino *et al.*, 2001), although various authors (Hooftman and de Raat, 1982; Hose *et al.*, 1987; Ayllon and Garcia-Vazquez, 2000) have suggested that variations in the shape of nucleus could represent an alternative approach for detecting genotoxicity. In spite of the fact that a correlation between nuclear abnormalities and genotoxic effects has not yet been established, preliminary observations strongly suggest that such morphological alterations could be a manifestation of the effects of xenobiotics. It has been suggested that in genotoxicity studies with fish models both, alterations in nuclear morphology and micronuclei number, should be considered when using the PMT (Ayllon and Garcia-Vazquez, 2000; Hose *et al.*, 1987).

Analysis of chromosome aberrations using the CAT showed that TBT and PbII promote structural alterations

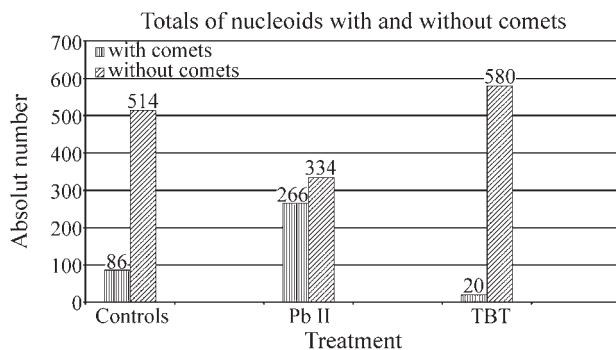


Figure 1 - Absolute number of comets found in each treatment.

Table 1 - Number of cells with micronuclei and morphological alterations in their nuclei.

Treatments	Number of cells with			
	Micronuclei		Nuclear alterations	
	Total (%)	Mean ± SD	Total (%)	Mean ± SD
Untreated control	2 (0.008)	0.33 ± 0.52	64 (0.27)	10.67 ± 3.61
* Tributyltin (TBT)	2 (0.008)	0.33 ± 0.52	102 (0.42)	17.0 ± 7.67
** Lead (PbII)	3 (0.012)	0.50 ± 0.55	176 (0.73)	29.33 ± 10.86

*Significant by the χ^2 test at $p < 0.01$.

**Significant by the χ^2 test at $p < 0.01$.

SD = Standard Deviation.

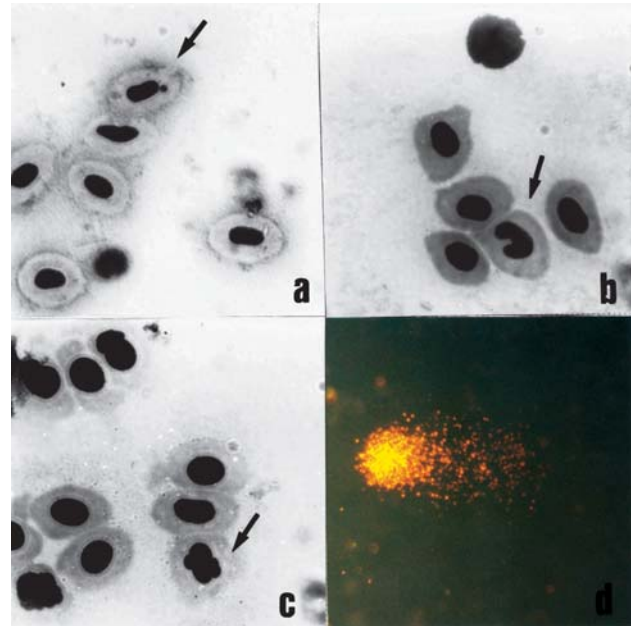


Figure 2 - a) Typical micronucleus, b) and c) morphological nuclear alterations, d) comet class 3.

(e.g. gaps and breaks) but no numerical changes were observed (Table 2), although de-condensation of chromatin on chromatid arms (Figure 3) was observed.

Chromosome aberration test have not been widely used in fish due to the characteristics of their karyotypes, although *H. malabaricus* appears to be an appropriate biological model because this species has only about 40 medium sized bi-armed chromosomes. In our experiments, we frequently observed chromatin de-condensation which we scored as chromosome aberrations (Table 2, Figure 3).

All the end-points analyzed in this paper show that PbII significantly increases DNA damage, principally by inducing single strand breaks that could possibly initiate double strand breaks, as result of the inactivation or alteration of repair mechanisms (Obe *et al.*, 2002).

Fish treated with TBT scored statistically higher values in the piscine micronucleus and chromosome aberration tests but showed a decreased frequency of tailed

nucleoids in the comet assay. Although there is no completely satisfactory explanation for these observations TBT has also given ambivalent results in some other studies,

Table 2 - Absolute number of chromosomal alterations found in each treatment.

Treatments	Number of chromosome alterations				Total
	Breaks	Gaps	Numeric	De-condensation	
Untreated control	3	0	0	1	4
* Tributyltin (TBT)	11	0	0	8	19
** Lead (PbII)	23	1	0	9	33

*Significant by the χ^2 test at $p < 0.05$.

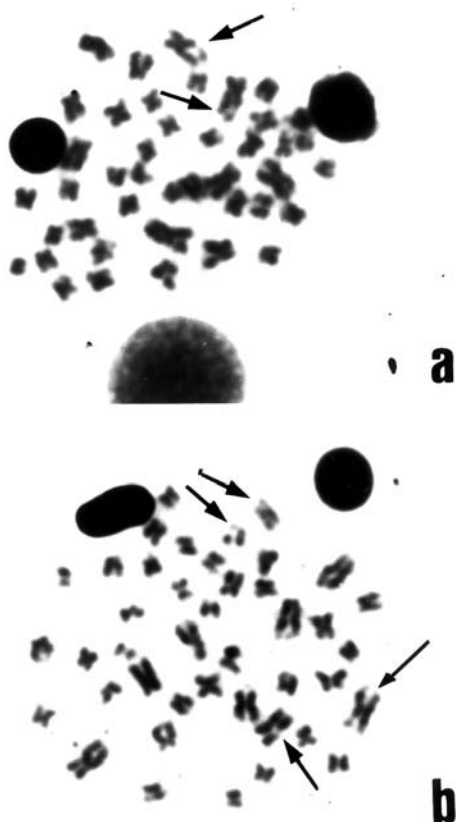


Figure 3 - Most frequent chromosome aberrations. Thin arrows indicate chromatid breaks and thick arrows chromatin de-condensation.

Table 3 - Number of cells in each comet assay class.

Treatments	Comet class			
	0	1	2	3
Untreated control	514	77	6	3
Tributyltin (TBT)	580	19	1	0
* Lead (PbII)	330	223	38	9

*Significant by the Kruskal-Wallis test at $p < 0.01$.

Yamanoshita *et al.* (2000) having reported that, depending on concentration, TBT can either cause apoptosis or protect against it. To resolve these questions new assays using different concentrations of TBT are needed.

This paper is the first to present results based on the use of three end-points to investigate the effects of toxic metals on an endemic neotropical fish, our data indicating that *H. malabaricus* represents a good experimental model for genotoxicity studies.

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