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The Micronucleus Assay in Fish Species as an Important Tool for Xenobiotic Exposure Risk Assessment—A Brief Review and an Example Using Neotropical Fish Exposed To Methylmercury

CARLOS ALBERTO MACHADO DA ROCHA,[1,2] RAQUEL ALVES DOS SANTOS,[3] MARCELO DE OLIVEIRA BAHIA,[2] LORENA ARAÚJO DA CUNHA,[1] HELEM FERREIRA RIBEIRO,[2] and ROMMEL MARIO RODRÍGUEZ BURBANO[2]

[1]Coordenação de Recursos Pesqueiros e Agronegócio, Insituto Federal de Educação, Ciência e Tecnologia do Pará, Brazil
[2]Laboratório de Citogenética Humana e Genética Toxicológica, Universidade Federal do Pará, Brazil
[3]Laboratório de Mutagênese, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Brazil

Micronucleus (MN) assay has been extensively used in the evaluation of DNA damage. Mutagenesis and genotoxicity studies employed this methodology to evaluate possible genotoxic risk due to exposition to hazardous xenobiotics in different organisms, including aquatic sentinel organisms. MN assay in such species is sensitive, fast, and an important biomarker of mutagenic exposure in the environment. The use of bioassays, considering the toxic effects of isolated or combined contaminants, is also important since the environmental variants are minimized. The aim of this study is to gather and evaluate published data on the use of fish MN assay in biomonitoring and genotoxicity assays. In addition, we show the results of both micronuclei and other nuclear abnormalities in erythrocytes from Colossoma macropomum*, exposed to methylmercury. Specimens ($n = 9$) were subjected to 2 mg/L of methylmercury, with an equal control group. Chi-square test was performed to compare the frequencies of nuclear abnormalities between control and treatment groups. The contingence table of χ^2 test showed a significant increase of altered cells in the exposed group. Our results support the importance of MN test as an effective indicator for genotoxicity in fishes, which can be used with exposition bioindicators of human populations exposed to chemical pollutants of consuming water.*

Keywords micronucleus assay, fishes, biomonitoring, genotoxicity

INTRODUCTION

Micronuclei (MN) were first described in the cytoplasm of erythrocytes more than a century ago and were called "fragment of nuclear material" by Howell or "intraglobularies corpuscules" in the terminology of Jolly in the late 18th century and early 1900. These structures are known by the hematologists as "Howell-Jolly bodies" (Kirsch-Volders et al., 2003).

Micronuclei (MN) are citoplasmatic chromatin masses that look like small nuclei as a result of lesions at the chromosomes or DNA strains, or at the level of proteins directly involved in chromosome segregation; formation of MN originating from chromosome fragments or chromosome loss events requires a mitotic or meiotic division (Heddle et al., 1983; Al-Sabti and Metcalfe, 1995). Scoring MN in interphase nuclei is technically

Address correspondence to Rommel Mario Rodríguez Burbano, Campus Universitário do Guamá, Av. Augusto Correa, 01, Universidade de São Paulo, Belém 66075-110, Brazil. E-mail: rommel@ufpa.br

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easier and faster than scoring of chromosomal aberration in metaphase nuclei (Al-Sabti and Metcalfe, 1995).

Salvadori and colleagues (2003) highlighted that, whatever the type of DNA damage, MN are originated during cell division. Then, DNA damage due to exposure to mutagenic agents is expressed in micronulceus form just after one cycle of cell division and is dependent on the proportion of cells under division.

The increased interest in environmental genotoxicity studies went ahead with the development of a great number of tests to evaluate genotoxic effects in aquatic environments. Considering this, micronucleus assay (MN), one of the most popular and promising tests on ecotoxicology, represents a cytogenetic indicator of DNA damage for over 30 years (Fenech et al., 2003).

The advantage in using fish models includes the facility by which teleostei, especially the small species, can be maintained and handled inside the laboratory under experimental conditions of toxic exposure. Besides, fishes frequently respond to chemical exposure as superior vertebrates, which validate this model to study potential teratogenic and carcinogenic compounds in humans (Al-Sabti and Metcalfe, 1995).

The genotoxic effect caused by some chemicals on fish genome has been the object of many studies, especially those aiming to assess the genetic responses to environmental stimuli (Bücker et al., 2006). Fishes can, in fact, be sentinel organisms that indicate the risk of human exposure to drinking water contaminated with genotoxic chemicals. Furthermore, fish and crustaceous species are among the most important vectors of human contamination once feeding is one of the major routes of exposure to toxicants in human populations (Al-Sabti and Metcalfe, 1995).

TECHNICAL ASPECTS AND ANALYSIS

A large number of studies using fish peripheral erythrocytes showed increase in MN frequency after laboratory exposure to different pollutants. The fish MN assay, as it has been actually called, is quick and simple: a drop of blood is smeared on a clean slide and dried at room temperature; slides are then fixed in absolute ethanol for 20 min and stained with Giemsa 10% (phosphate buffer pH 6.8); finally, the slides are analyzed with conventional microscopy to evaluate and quantify the presence of micronuclei (Benincá, 2006).

Two slides per sample are recommended for preparation, in which 4,000 erythrocytes are analyzed under 100× objective and scored for presence of both typical micronuclei and nuclear alterations manifested as changes in the normal elliptical shape of the main nuclei (Ayllon and Garcia-Vazquez, 2000). Therefore, the micronuclei test also accounts for nuclear abnormalities, as the occurrence of lobulated, segmented, and kidney aspect cells.

Although the significant differences between controls and exposed groups is usually reported using the Chi-square test (Ferraro et al., 2004; Lopez-Poleza, 2004; Cavas et al., 2005), other statistical analysis using non-parametric tests such as Mann-Whitney test (Lopes-Poleza, 2004; Grisolia et al., 2005; Bücker et al., 2006; Vanzella, 2006; Andreikënaitë et al., 2007), or Kruskal-Wallis test (Benincá, 2006; Matsumoto et al., 2006; Vanzella, 2006) are valid and recommended according to the data distribution.

Micronuclei are circa 1/20 to 1/10 smaller than the main nucleus (Ribeiro, 2003). However, in fish erythrocyte samples, they seem to vary from 1/30 to 1/10 since the chromosome size is shorter than in mammals (Al-Sabti and Metcalfe, 1995; Ayllon and Garcia-Vazquez, 2000).

ECOLOGICAL BIOMONITORING

The most accepted definition of biomonitoring is the use of systematic responses of a living organism to evaluate environmental changes, mostly trigged by human action. The biomonitoring programs are generally used to detect and control a problem and can be considered a promising tool for identification of pollutants that may affect human and environmental health; mostly with organisms (bioindicators) exposed to these pollutants, using biological system assays (biomarkers) (Da Silva et al., 2003). The use of biomarkers to measure biological responses in the affected organisms is very useful to simplify and lower costs of biological monitoring, especially in aquatic environments (Ramsdorf et al., 2008). These biomarkers consist of the formation of DNA adducts, chromosomal aberrations, DNA break and micronuclei frequency, and other nuclear abnormalities (Bombail et al., 2001).

The impact of toxic agents on the DNA integrity and function has been extensively investigated under environment conditions (Bombail et al., 2001). Considering endemic species, monitoring the presence of xenobiotic agents in aquatic environments can improve environmental quality and human health. Fishes are one of the most indicated organisms for the monitoring of aquatic environments (Van Der Oost et al., 2003). Localization and determination of pollutant concentration ensures the survival and health of these organisms in a way they can be used by human populations to supply their nutritional and leisure needs (Nicareta, 2004).

Micronucleus assay, originally developed in mammalian species, has been extensively used to evaluate the genotoxic risk of a large number of chemical agents (Heddle et al., 1983). Differing from other organisms belonging to the trophic chain, fishes are sensitive to relatively low concentrations of environmental pollutants (with possible mutagenic effects) and, therefore, are considered excellent bioindicators of environmental biomonitoring (Minissi et al., 1996).

In the last 10 years, the number of biomonitoring *in situ* studies using the MN assay in fish species has increased significantly. Some examples are presented in Table 1.

In Brazil, biomonitoring studies using aquatic species have also increased in the last years. Grisolia and Starling (2001)

Table 1 *In vivo* biomonitoring studies using the micronucleus assay in fish species

Organism	Cell type	Localization	Contaminant	Reference
Pholis gunnellus	Erythrocytes	Firth of Forth (Scotland)	Metals, hydrocarbons, and organochlorates	Bombail et al., 2001
Tilapia rendalli, *Oreochromis niloticus* and *Cyprinus carpio*	Erythrocytes	Lago Paranoá in Brasília (Brazil)	Domestic sewage	Grisolia and Starling, 2001
Anguilla Anguilla, Phoxinus phoxinus, and Salmo trutta	Erythrocytes	North Rivers of Spain	Metals, hydrocarbons, pesticides	Rodriguez-Cea et al., 2003
Mugil cephalus	Erythrocytes and gill cells	Mediterranean (Turkey)	Aromatic hydrocarbons	Cavas and Ergene-Gozukara, 2005
Geophagus brasiliensis	Erythrocytes	Santa Catarina (Brazil)	Domestic sewage	Benincá, 2006
Trematomus newnesi	Erythrocytes	Brazilian Antarctic Research Station "Comandante Ferraz"	Diesel oil and sewage	Campos, 2007
Astyanax sp. B and *A. Altiparanae*	Erythrocytes, hepatocytes, and kidney cells	Paraná (Brazil)	Agrotoxics	Ramsdorf, 2007
Clarias gariepinus, Oreochromis niloticus, *Oreochromis aureus,* and *Tilapia zilli*	Erythrocytes	River Nile, Abou Homos, Kafr Eldawar, and Lake Mariout (Egypt)	Heavy metals	Ali et al., 2008
Astyanax jacuhiensis	Erythrocytes	Rio Grande do Sul (Brazil)	Petrochemicals	Lemos et al., 2008

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evaluated the ability of wastewater from two municipal sewage treatment plants that debouch into Lake Paranoá to cause genetic damage using the MN test. They did not find significant differences between control and hypertrophic areas; however, cyclophosphamide and mitomycin C, used to test the sensitivity of the biological assay, significantly increased the MN frequency in *Tilapia rendalli*, *Oreochromis niloticus,* and *Cyprinus carpio*. In this study, *T. rendalli* was the most sensitive specie to both clastogens and *C. carpio* the most resistant. Lemos et al. (2008) performed a genotoxic evaluation in the Bom Jardim brook, a small stream that flows through an area under the influence of a petrochemical complex in Rio Grande do Sul, Brazil, using the MN assay in *Astyanax jacuhiensis*. The study found increased micronuclei frequencies and nuclear abnormalities in the exposed group when compared to the control; however, no differences were observed between samples collected in different sites from the brook (two ponds upstream from the industrial area). They showed that sites exposed to petrochemical influence were under higher genotoxic impact and that *A. jacuhiensis* was a sensible bioindicator.

GENOTOXICITY ASSAYS

The use of bioassays to evaluate the toxic effects of associated or isolated contaminants reduces significantly the influence of different environmental variables. Even though such results cannot be directly extrapolated to the environment, they are important to maintain of databases that can help to understand the interfering factors in the organism health and/or altering the environmental balance (Ramsdorf, 2007).

Genotoxicity assays using fish species can be performed *in vitro* and/or *in vivo*. In the *in vivo* assays, the tested agents are injected into the fishes or added to water or food (Cotelle and Ferard, 1999). Table 2 shows some examples of detection of genotoxic agents in an aquatic environment using the MN assay.

In fish species, the micronucleus assay is usually performed in erythrocytes since these cells contain a nucleus (Al-Sabti and Metcalfe, 1995). Erythrocyte samples are easily acquired and no cellular dissociation is required (Belpaeme et al., 1998).

The majority of genotoxicity studies with fish species are usually performed using micronucleus assay and/or the comet assay, which denotes the importance of discriminating what is genotoxic and what is mutagenic and also helps to evaluate low-dose effects (Bücker et al., 2006). Ferraro et al. (2004) evaluated the mutagenic potential of tributyltin (TBT) and inorganic lead (PbII) in *Hoplias malabaricus* using micronucleus assay, comet assay, and analysis of chromosomal aberrations. They observed that lead was highly mutagenic in all endpoints analyzed. However, genotoxicity of TBT was positive in the micronucleus assay and chromosomal aberrations but not in the comet assay, at least in the cellular type and concentrations tested.

Lopes-Poleza (2004) evaluated the genotoxic effects of methylmercury (CH_3Hg^+) in *Hoplias malabaricus*, using chromosomal aberrations (anterior kidney), micronuclei, and DNA damage by the comet assay in erythrocytes. The results showed that genotoxic effects of methylmercury are due to its accumulation in tissues and not in the circulating blood; thus, the erythrocytes were not a good bioindicator to demonstrate the hazardous effects of this compound in low concentrations.

Bücker et al. (2006) exposed *Eingenmannia virescens* to benzene (50 ppm) during different periods. Although no significant results with the micronucleus were found, the comet assay suggested the genotoxicity of benzene once the authors observed a gradual increase in the number of damaged nucleoids in a dose-dependent response. These results suggest that the comet assay was more sensible than the micronucleus assay.

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Table 2 Genotoxicity studies using the micronucleus assay in fish species

Organism	Cell type	Contaminant	Reference
Oreochromis niloticus and *Tilapia rendalli*	Erythrocytes	Mitomycin C and cyclophosphamide	Palhares and Grisolia, 2002
Oreochromis niloticus	Erythrocytes and gill cells	Textile mill effluent	Cavas and Ergene-Gozukara, 2003
Hoplias malabaricus	Erythrocytes	TBT and lead	Ferraro et al., 2004
Hoplias malabaricus	Erythrocytes	Methylmercury	Lopes-Poleza, 2004
Cyprinus carpio, Carassius gibelio, and *Corydoras paleatus*	Erythrocytes, gill cells, and liver cells	Cadmium chloride and copper sulphate	Cavas et al., 2005
Oreochromis niloticus and *Tilapia rendalli*	Erythrocytes	Domestic sewage	Grisolia et al., 2005
Scophthalmus maximus	Erythrocytes	Dialkyl phthalate, bisphenol A and tetrabromodiphenyl ether	Bolognesi et al., 2006
Eigenmannia virescens	Erythrocytes	Benzene	Bücker et al., 2006
Prochilodus lineatus	Erythrocytes	Diesel oil	Vanzella, 2006
Oreochromis niloticus	Erythrocytes	Chromo	Matsumoto et al., 2006
Oncorhynchus mykiss	Erythrocytes	Mixture of heavy metals	Andreikënaitë et al., 2007
Trematomus newnesi	Erythrocytes	Diesel oil and sewage	Campos, 2007
Prochilodus lineatus	Erythrocytes	Aluminum	Galindo, 2007
Gambusia affinis	Erythrocytes	Residual hydrocarbons	Caliani et al., 2008
Carassius auratus auratus	Erythrocytes and epithelial of gill and fin	Mercury chloride and lead acetate	Cavas, 2008

Vanzella (2006) evaluated the genotoxicity of the soluble fraction of diesel (SFD) in *Prochilodus lineatus* using the comet and micronucleus assays. The animals were submitted to acute (6, 24, and 96 hr) and sub-acute (15 days) exposure to SFD 50% in water. The results demonstrated clastogenic and aneugenic effects of this tested fraction. Consequently, the combination of these two methodologies was adequate and advantageous.

Matsumoto et al. (2006) studied samples of water from Catfish Brook in Franca, a city in the Brazilian state of São Paulo. Erythrocytes from *Oreochromis niloticus* were submitted to comet and micronucleus assays. Samples from a tanning region with chrome effluent exhibited the highest level of DNA damage, supporting the hypothesis that this metal is genotoxic.

Galindo (2007) used RAPD, comet assay, and micronucleus assay to evaluate the genotoxic effect of aluminum in acid medium (pH 5.0) using the neotropical specie *Prochilodus lineatus*. The genotoxic effect was demonstrated with comet assay and RAPD. However, the level of micronuclei frequency was not increased and stayed near to control values. The authors concluded that, in the concentrations tested, aluminum was not mutagenic.

ASSESSMENT OF MUTAGENIC EFFECTS OBSERVED IN NEOTROPICAL FISH EXPOSED TO METHYLMERCURY

Fish Specimens

Colossoma macropomum Cuvier, 1818 (Characidae), well known as tambaqui, is an onivorous fish that belongs to the Amazon region in the Orinoco river and its effluents. This specie is abundant and has high aquaculture potential because they can be cultured and reproduced in captivity. It is the second biggest specie with squama and the biggest Characiforme in the Solimões/Amazonas Rivers, reaching 100 cm in native environment, weighting approximately 30 kg (Araújo-Lima and Goulding, 1998). With its tasty meat, it is widely appreciated in northern Brazil, with economic value in six of seven states in this region (Val et al., 2000).

Methylmercury

Mercury, like some other metals and several organomercurial compounds, has demonstrated mutagenic properties. This toxicity occurs on tubulin, the structural subunit of cellular microtubules, playing a role in cytoplasmic organization and formation of spindle fibers, interfering with the tubulin polymerization and causing contraction of the chromosomes in metaphase, delayed division of the centromere and reduced anaphasic movement (Cassidy and Furr, 1978; Thier et al., 2003).

Methylmercury (MeHg) is a compound classified as group 2B by IARC (International Agency for Research on Cancer), being indicated as a possible carcinogenic to humans (Hallenbeck, 1993) and, due to its property of integrating themselves in trophic chains, accumulates to a much greater extent when compared to other forms of mercury (Azevedo, 2003).

Neurotoxicity induced by MeHg increases the emergency of reactive radicals and accelerates free radical reactions. Oxidative stress on CNS can produce damage by several interacting mechanisms, including mitochondrial damage with increase in intracellular free Ca^{2+}, activation and inhibition of enzymes,

release of excitatory amino acids, metallothioneins expression, and microtubule disassembly (Nascimento et al., 2008).

METHODS

In the present study, we used young *C. macropomum* species obtained from Pisciculture Station of Federal Rural Amazon University in Castanhal City, Pará, Brazil (between 01°18′ 02″ S and 01°22′ 43″ S and 48°05′ 05″ W and 48°15′ 46″ W). Acclimatization to laboratory conditions for one month was done using dechlorinated tap water with the following physicochemical characteristics: temperature = 26 ± 1.3°C, pH = 6.5 ± 0.29, dissolved oxygen = 2.78 ± 0.55, total hardness = 6–49 mg/L (as $CaCO_3$), and conductivity = 12–97μ S/cm. Specimens were housed at a density of three specimens in a 30-L aquaria under constant aeration and 12 hr light/dark photoperiod. Preliminary experiments determined the concentration or maximum tolerated dose (MTD), at which the animals showed no reduction in survival and food uptake. A sublethal concentration of methylmercury (2 mg/L) was tested, and nine specimens were used as a test group with an equal control group. Blood samples were collected of each group after five days of treatment.

The blood was spread onto a microscope slide accord to Benincá (2006). Slides were observed for MN score in a transmission light microscope, using 1,000× magnification. Two thousand erythrocytes were analyzed per slide, and 4,000 per animal, including those cells with micronucleus and modifications in the nuclear shape. Only cells with well-preserved cytoplasm were considered. Coded and randomized slides were scored using a blind analysis by a single scorer. Statistical analysis considered the difference between groups that was <0.05 in the Chi-square test (BioEstat 5.0) (Ayres et al., 2007).

MNs were defined as round or oval intracytoplasmatic bodies not linked or connected to the main nucleus, with a diameter 1/30–1/10 of the major nucleus and on the same optical plane (Al-Sabti and Metcalfe, 1995; Ayllon and Garcia-Vazquez, 2000). Three other nuclear abnormalities were still considered: buds, lobes, and invaginations (Ayllon and Garcia-Vazquez, 2000; Bolognesi et al., 2006).

RESULTS AND DISCUSSION

In each animals kept in the fish tank with 2 mg/L of methylmercury, we observed 4,000 blood cells (Figure 1), with 1.02 ± 0.30% mean cells with alterations (Figure 2). Control animals presented a count of 0.85 ± 0.34% mean red blood cells with alterations. In animals subjected to methlymercury 2 mg/L, an increase in clastogenic or aneugenic accidents was observed, yielding the formation of micronuclei and other nuclear abnormalities.

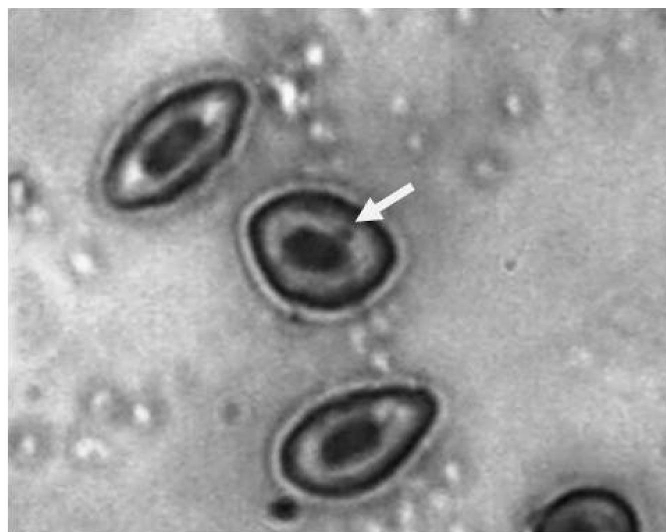

Figure 1 Photomicrograph (1,000×) of erythrocytes from *C. macropomum* treated with methylmercury 2 mg/L, showing a micronucleated erythrocyte.

In widespread informal gold mining in the Amazon Basin, mercury is used to capture the gold particles as amalgam. Mercury releases into the environment, resulting in contamination of freshwater fish with methylmercury (Grandjean et al., 1999). Methylmercury is an organic neurotoxic form of mercury, the one that is easier to bioaccumulate in organisms.

Spontaneous levels of micronuclei in fish species are relatively low (Ferraro et al., 2004). Our results demonstrated low frequencies of micronuclei in controls as in the exposed group. When we considered morfonuclear alterations and micronuclei altogether, exposed group and controls were significantly different.

MN assay in fish erythrocytes is a mutagenicity assay, being less sensitive than comet assay, which demonstrates genomic lesions that can be repaired (Russo et al., 2004; Bücker et al., 2006; Ramsdorf, 2007), reducing the number of stable lesions present in DNA molecule.

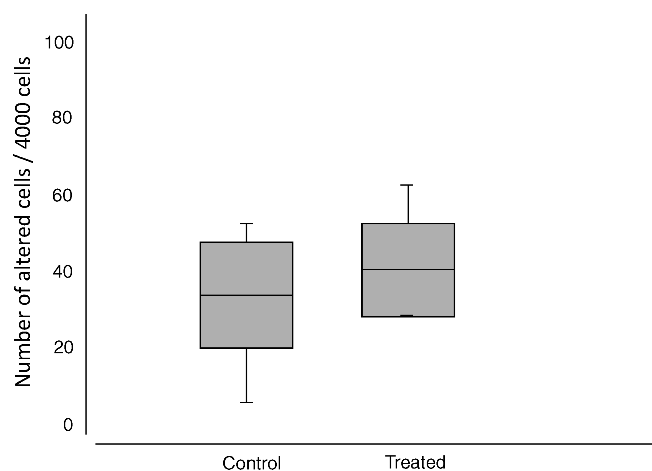

Figure 2 Number of cells with micronuclei and morphological alterations in their nuclei (per 4,000 cells). Significant difference was observed between the control and treated animals (χ^2 test; $p < 0.05$).

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On the other hand, the observation of nuclear abnormalities. besides the presence of MN, also can be considered a useful indicator of genotoxic and cytotoxic effects of contaminants in aquatic organisms (Cavas and Ergene-Gozukara, 2003; Dailianis et al., 2003; Ferraro et al., 2004, Cavas et al., 2005; Baršienë et al., 2006; Matsumoto et al., 2006).

Our results corroborate the importance of MN in mutagenic exposure assessment in fishes, which can be used as sentinel organisms for indicating the potential for human exposures to genotoxic chemicals in drinking water.

GENERAL CONCLUSION

When compared to other DNA damage detection techniques, micronucleus assay has some advantages: (1) it can be performed rapidly; (2) it is not complex; (3) it presents low costs; (4) its preparation and analysis are simpler and faster than chromosomal aberrations. Although the MN assay cannot give information about the type of chromosomal breakage, it is informative when the exposure causes aneugenic effects. Taken together, all the above-mentioned aspects render this methodology high applicability in the routine of mutagenesis studies. Research on environmental biomonitoring requires fast results and reproducibility. Exploration of the MN assay in fish species is welcome in order to standardize and improve the assessment of genotoxicity in target tissues. We assessed the mutagenic potential of methylmercury 2 mg/L using samples of the fish *Colossoma macropomum* (commonly called *tambaqui*) through piscine micronucleus test. Our results confirm the mutagenic effect of methylmercury. The sensibility to this compound and the economic relevance of *C. macropomum* show that such species can be used to monitor acute effects of metallic pollutant spilled in freshwater of Amazonic ecosystems.

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