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Atrazine promotes biochemical changes and DNA damage in a Neotropical fish species

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HIGHLIGHTS

- Atrazine effects were assessed in fish using biochemical and genetic biomarkers.
- Atrazine promoted a decreasing trend on biotransformation and antioxidant enzymes.
- DNA damage was observed in different cell types of fish exposed to atrazine.
- Atrazine did not increase ROS generation and LPO in the liver.
- Atrazine did not promote changes in muscle or brain AChE.

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ABSTRACT

The effects of Atrazine, an herbicide used worldwide and considered as a potential contaminant in aquatic environments, were assessed on the Neotropical fish *Prochilodus lineatus* acutely (24 and 48 h) exposed to 2 or 10 μg L^{-1} of atrazine by using a set of biochemical and genetic biomarkers. The following parameters were measured in the liver: activity of the biotransformation enzymes ethoxyresorufin-O-deethylase (EROD) and glutathione S transferase (GST), antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), content of reduced glutathione (GSH), generation of reactive oxygen species (ROS) and occurrence of lipid peroxidation (LPO); in brain and muscle the activity of acetylcholinesterase (AChE) and DNA damage (comet assay) on erythrocytes, gills and liver cells. A general decreasing trend on the biotransformation and antioxidant enzymes was observed in the liver of *P. lineatus* exposed to atrazine; except for GR, all the other antioxidant enzymes (SOD, CAT and GPx) and biotransformation enzymes (EROD and GST) showed inhibited activity. Changes in muscle or brain AChE were not detected. DNA damage was observed in the different cell types of fish exposed to the herbicide, and it was probably not from oxidative origin, since no increase in ROS generation and LPO was detected in the liver. These results show that atrazine behaves as enzyme inhibitor, impairing hepatic metabolism, and produces genotoxic damage to different cell types of *P. lineatus*.

1. Introduction

Atrazine is currently one of the most widely used herbicides and several studies have already detected its presence in water bodies at levels above the limits determined by local guidelines (Konstantinou et al., 2006; Davis et al., 2007). Despite being classified as moderately toxic, atrazine can promote toxic effects on aquatic animals (Solomon et al., 2008; Ventura et al., 2008). Some studies have been conducted on fish to evaluate the toxicity of atrazine (Yang et al., 2010) and focused on several aspects such as the biochemical (Dong et al., 2009), genetic (Ventura et al., 2008), histopathological (Paulino et al., 2012a) and physiological effects (Nieves-Puigdoller et al., 2007; Paulino et al., 2012b). However, the mechanisms of toxicity of this herbicide and its metabolites are not yet fully understood (Graymore et al., 2001).

The effects of atrazine can be evaluated by using biomarkers, that are biochemical or cellular changes in body fluids, cells, or tissues that indicate the presence of contaminants, and can detect more quickly the presence of toxic compounds, allowing earlier identification of change, before deleterious effects reach higher organization levels (Monserrat et al., 2007). The effectiveness of biomarkers has been demonstrated in several studies on the toxicity of pesticides to fish (Oruç et al., 2004; Dorval et al., 2005; Ramesh et al., 2009). However, the simultaneous use of several biomarkers is recommended because the evaluation of a single parameter can result in misinterpretation since not all of the damage that a contaminant or impacted environment can cause to an organism will necessarily be shown (Zorita et al., 2008).

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Biochemical biomarkers can provide information about the process of pesticide detoxification. When in contact with an organism, the toxic agent can be biotransformed by enzymes, which act to make the xenobiotic substance a less toxic compound and facilitate its excretion. In fish, the main enzymes that act on the biotransformation of xenobiotics are cytochrome P450 1A (CYP1A) and glutathione S-transferase (GST) (Van der Oost et al., 2003). However, these biotransformation processes can generate intermediates that are more toxic than the contaminant itself: reactive oxygen species (ROS), represented by the superoxide anion ($O_2^{\cdot-}$), the hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) (Valavanidis et al., 2006).

Organisms have both enzymatic and non-enzymatic antioxidant defences against ROS (Lushchak, 2011). One of the first enzymes that act in defence against ROS is superoxide dismutase (SOD), that catalyses the conversion of reactive superoxide anions ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2), which is subsequently detoxified by catalase (CAT) and glutathione dependent peroxidase (GPx). GPx catalyses the metabolism of H_2O_2 to water, involving a concomitant oxidation of reduced glutathione (GSH), one of the most important non-enzymatic antioxidants in the cell (Van der Oost et al., 2003). Another important enzyme in the antioxidant defence of organisms is glutathione reductase (GR), responsible for reducing oxidized glutathione (GSSG) and maintaining normal levels of GSH (Hermes-Lima, 2004).

If the generation of ROS exceeds the antioxidant capacity of the organism, oxidative stress occurs, which affects the normal functioning of the cell and may cause damage to proteins, enzyme inactivation, lipid peroxidation (LPO) and DNA strand breaks (Amado et al., 2009). Mechanism of pesticide toxicity has been usually associated with the increase of LPO (Singh et al., 2011) that is one of the major tools used to assess the effects of a pollutant on aquatic organisms (Valavanidis et al., 2006). On the other hand, genotoxic parameters, such as DNA strand breaks, are currently the most valuable biomarkers for environmental risk assessment and there are many reports linking the DNA damage to subsequent molecular, cellular and tissue level alteration of aquatic organisms (Ohe et al., 2004).

The activity of acetylcholinesterase (AChE) is another biochemical biomarker normally used to monitor aquatic environments mainly contaminated by pesticides. This enzyme can be inhibited by different types of agrochemicals, causing over-stimulation of muscle fibers and leading to paralysis, and even death, of animals (Ferrari et al., 2007).

The species *Prochilodus lineatus*, popularly known as curimba, is a Neotropical fish species of great economic importance and some studies have already shown the sensitivity of this fish to various types of pesticides (Cavalcante et al., 2008; Maduenho and Martinez, 2008; Modesto and Martinez, 2010a, 2010b). The objective of this work was to understand the effects of the herbicide atrazine on this fish species by using a set of biochemical and genetic biomarkers. To this end, it was measured the activity of hepatic biotransformation enzymes, enzymatic and non-enzymatic antioxidant defences in liver, AChE activity in the brain and muscle, the occurrence of LPO in liver and DNA damage in different tissues of *P. lineatus* after acute exposure to two environmentally relevant concentrations of atrazine.

2. Material and methods

2.1. Acclimation of animals and experimental design

Juveniles of *P. lineatus* (17.3 ± 7.9 g, 11.4 ± 1.8 cm, $N = 168$), supplied by the Fish Hatchery Station of Londrina State University, were acclimated for 7 d in 300 L tanks containing dechlorinated water and with constant aeration. During this time they were fed every 48 h with a commercial diet. They were not fed for 48 h before being exposed to the compound or during the toxicity tests. The physical and chemical parameters of the water were monitored throughout the acclimation period and remained constant (pH: 7.18 ± 0.5, conductivity: 118.25 ± 8.7 μS cm^{-1}, dissolved oxygen: 8.49 ± 0.9 mg O_2 L^{-1}, temperature: 21.96 ± 2.7 °C).

After this period, groups of eight fish were transferred to 100 L glass aquaria where they were subjected to static acute toxicity tests for 24 and 48 h. Experiments for each experimental period were run separately, and for each period (24 or 48 h) one group of fish was exposed to dechlorinated water only (control group) and two groups were exposed to water containing atrazine at concentrations of 2 μg L^{-1} and 10 μg L^{-1} (ATZ 2 and ATZ 10, respectively). The concentration of 2 μg L^{-1} corresponds to the maximum concentration of atrazine allowed by the Brazilian Guidelines (CONAMA Resolution 357, 2005) for continental waters and the concentration of 10 μg L^{-1} corresponds to 40% of the concentration of atrazine used on crops (Ventura et al., 2008).

2.2. Sampling

After the exposure periods the fish were removed from the aquarium and anesthetized in benzocaine (0.1 g L^{-1}) and blood samples were collected from the caudal vein. Following this the animals were killed by medullar section and the gills, liver, brain and muscle samples were removed. These procedures followed the standard protocols approved by the Committee for Animal Experimentation of Londrina State University. Immediately after excision the gills and liver were carefully washed with PBS (NaCl 126.6 mM, KCL 4.8 mM, $CaCl_2$ 1.5 mM, $NaHCO_3$ 3.7 mM, Na_2HPO_4 8.9 mM, NaH_2PO_4 2.9 mM) and gill filaments and part of the liver were transferred to microtubes for the cellular dissociation to be used in the comet assay. The remaining organs were stored in an ultrafreezer (−80 °C) for biochemical analysis.

2.3. Biochemical analysis in liver

Liver samples were homogenized (10× volume) in potassium phosphate buffer (0.1 M, pH 7.0) and centrifuged (20 min, 13 000 g, 4 °C) for biochemical analysis.

2.3.1. Biotransformation enzymes

The catalytic activity of CYP1A was determined by ethoxyresorufin-O-deethylase (EROD) activity through the conversion of 7-ethoxyresorufin, provided in the reaction medium (potassium phosphate buffer 0.1 M, pH 7.6, NADPH 2 mM, 7-ethoxyresorufin 0.1 mM), to resorufin, according to Eggens et al. (1992). The progressive increase in fluorescence resulting from the formation of resorufin was measured at 1-min intervals for 10 min (excitation: 530 nm; emission: 590 nm). The initial linear portion of the curve was used to evaluate the reaction rate and the EROD activity was expressed in pmol of resorufin min^{-1} mg of $protein^{-1}$, based on a resorufin standard curve.

The activity of GST was determined by the complexation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) in a spectrophotometer at 340 nm (Keen et al., 1976) and expressed as nmol of conjugated CDNB min^{-1} mg $protein^{-1}$.

2.3.2. Non-enzymatic antioxidant

Reduced GSH levels were estimated according to Beutler et al. (1963), using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) following Simonato et al. (2011). Supernatants of the acid extracts (1:1 *v*/*v* with 12%TCA) were added to 0.25 mM DTNB in 0.1 M potassium phosphate buffer, pH 8.0, and thiolate anion formation was determined at 412 nm against a GSH standard curve

(100–200 μmol of GSH). The GSH content was expressed in μg of GSH mg protein^{-1}.

2.3.3. Antioxidant enzymes

The activity of copper–zinc SOD was estimated by the inhibition of the reduction rate of cytochrome c by the superoxide radical, at 550 nm and 25 °C, and expressed as U SOD mg of protein^{-1}, where U represents the amount of SOD that promoted the inhibition of 50% of the reduction rate of cytochrome c (McCord and Fridovich, 1969).

The activity of CAT was determined by the rate of decomposition of H_2O_2 by the enzyme, which was evaluated by the decrease in absorbance at 240 nm (Beutler, 1975) and expressed in μmol H_2O_2 min^{-1} mg protein^{-1}. The determination of selenium-dependent GPx activity was basedon NADPH oxidation in the presence of GSH (0.95 mM) and H_2O_2 at 340 nm (Hopkins and Tudhope, 1973). GPx activity was expressed in μmol oxidized NADPH min^{-1} mg protein^{-1}. The activity of GR was determined indirectly based on the reduction of NADPH in the presence of oxidized GSH at 340 nm (Carlberg and Mannervik, 1975). GR activity was expressed in μmol min^{-1} mg protein^{-1}.

2.3.4. Generation of reactive oxygen species (ROS)

Measurements of ROS were conducted according to the method described by Ferreira-Cravo et al. (2007), which is based on the fact that non-fluorescent compound 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA) is oxidized by ROS present in the samples to the fluorescent compound dichloro-fluorescein (DCF). In order to run the assay samples of fresh liver were homogenized (1:10 – w/v) in a Tris–HCl buffer (10 mM, pH 7.75) plus EDTA (2 mM) and $MgCl_2$ (5 mM) and centrifuged (10000 g, 20 min, 4 °C). Supernatant was added to a reaction medium (HEPES 30 mM, KCl 200 mM, $MgCl_2$ 1 mM, H_2DCF-DA 40 μM, pH 7.2) in a microplate and the volume in each well was adjusted to 1 mg protein mL^{-1}. The fluorescence intensity was determined during 15 min, at 25 °C, using a fluorometer, with excitation and emission wavelength of 485 and 520 nm, respectively. Background fluorescence was determined previous to H_2DCF-DA addition. The results were expressed as fluorescence units (FUs) per mg of protein.

2.3.5. Lipid peroxidation

Lipid peroxidation was estimated from the production of malondialdehyde (MDA), which is one of the final products of lipid peroxidation. The MDA content was determined by the TBARS assay, which measures the thiobarbituric acid (TBA) reactive substances, at 530 nm, following the methodology described by Federici et al. (2007). Lipid peroxidation was expressed in equivalents of MDA as μmol MDA mg protein^{-1}.

2.4. Brain and muscle acetylcholinesterase (AChE)

Brain and muscle samples were homogenized (10× volume) in potassium phosphate buffer (0.1 M, pH 7.5), centrifuged (20 min, 13000 g, 4 °C), and the supernatant was removed for analysis of AChE. Enzyme activity was determined based on the colorimetric method of Ellman et al. (1961) adapted for reading on microplates, according to Alves Costa et al. (2007). The final concentration of the acetylthiocholine iodide substrate employed was 9 mM, while that of the DTNB color reagent was 0.5 mM for both tissues. Absorbance was determined in a microplate reader at 415 nm and the enzyme activity was expressed in nmol DTNB min^{-1} mg protein^{-1}.

2.5. Protein concentration

The total protein concentration in the samples used in the biochemical assays was measured using the method of Lowry et al. (1951), based on a standard curve of bovine serum albumin (BSA) at 700 nm. The method of Bradford (1976), which uses a BSA reference curve at 595 nm, was used to determine protein concentration in the samples used for estimating the amount of reactive oxygen species.

2.6. Genetic analysis – comet assay

The Comet assay was performed using the alkaline (pH > 13) version developed by Singh et al. (1988), with the modifications detailed by Cavalcante et al. (2008), using three cell types: erythrocytes, liver and gill cells. For tissue dissociation gill filaments and liver tissue were sectioned and the pieces were transferred to microtubes, incubated for 15 min at 30 °C in 0.05% trypsin (diluted in PBS Ca^{+2} and Mg^{+2} free) and homogenized by periodic manual inversion at room temperature. After that, the solution was filtered (30 μm mesh size) into a tube containing a fetal calf serum 10% to halt the enzymatic digestion. The resultant solution was centrifuged (10 min, 1000 g) and the pellet was resuspended in PBS to be used in the comet assay. Only blood, gill and liver samples with cellular viability greater than 80%, determined by the Trypan blue exclusion test, were used in the comet assay.

The basic steps of the comet assay for the three cell types were executed as follows: (a) lysis: 1 h, at 4 °C, protected from light, in a lysis buffer (NaCl 2.5 M, EDTA 100 mM, Tris 10 mM, DMSO 10%, Triton X-100 1 mL, pH 10.0); (b) DNA unwinding: 30 min, in the dark, in an electrophoresis buffer (NaOH 0.3 N, EDTA 1 mM, pH > 13); (c) electrophoresis: 20 min, 300 mA, 25 V, 1 V cm^{-1}; (d) neutralization: three washes for 5 min each in buffer (Tris 0.4 M, pH 7.5). Slides were fixed with absolute ethanol for 10 min and kept under refrigeration until cytological analyses. The slides, stained with gelRed were analyzed with a Leica microscopy (DM 2500) adapted for fluorescence/epifluorescence, equipped with a blue excitation filter (450–490 nm) and a barrier filter of 515 nm at 1000× magnification. All slides were blind-reviewed. The extent of DNA damage was quantified by the length of DNA migration, which was visually determined in 100 randomly select and non-overlapping cells per fish. According to Kobayashi et al. (1995), DNA damage was classified in four classes 0: no visible damage; (1) a short tail smaller than the diameter of the nucleus; (2) a tail length 1–2 times the diameter of the nucleus; (3) a tail length > 2 times the diameter of the nucleus. The score of DNA damage for 100 comets was obtained by multiplying the number of cells in each class by the damage class, and ranged from 0 (all undamaged) to 300 (all maximally damaged). Results for DNA damage in each cell type were expressed as the mean score of DNA damage for each treatment group, for each exposure period.

2.7. Statistical analysis

The results of each parameter obtained at the same exposure period (24 or 48 h) were compared among treatment groups (CTR X ATZ2 X ATZ10) using the parametric one-way analysis of variance (ANOVA) or the non-parametric Kruskal–Wallis test, according to data distribution (normality and homogeneity of variance). Differences were analyzed by a post hoc Student–Newman-Keuls test (after ANOVA) or Dunn's test (after Kruskal–Wallis). Statistical significance was designated as $P < 0.05$.

3. Results

3.1. Hepatic activity of the biotransformation enzymes

In the fish exposed to 2 μg L^{-1} of herbicide for 24 h, the activity of EROD was significantly decreased when compared to the

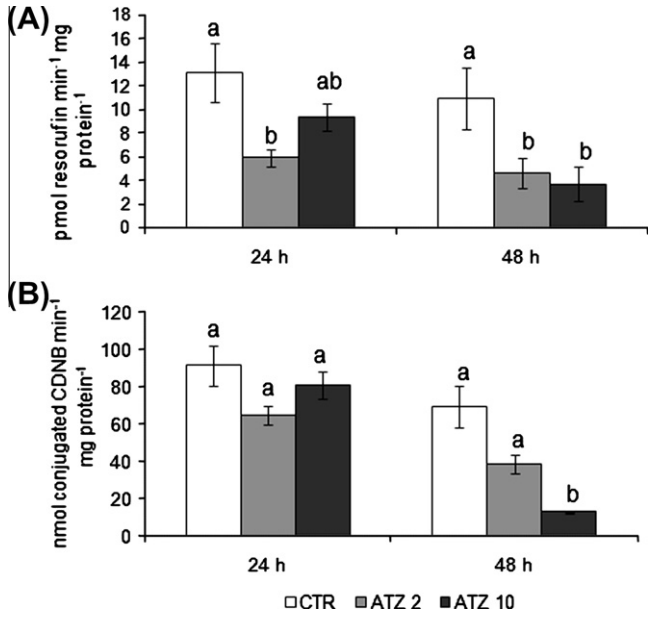

Fig. 1. Hepatic activity of (A) Ethoxyresorufin-O-deethylase (EROD) and (B) glutathione S-transferase (GST) in *P. lineatus* exposed to 2 and 10 μg L^{-1} of atrazine (ATZ 2 and ATZ 10) or only water (CTR) for 24 and 48 h. The columns represent the means and the vertical lines the standard errors. For each experimental period, the different letters indicate significant differences between the groups ($P \leqslant 0.05$).

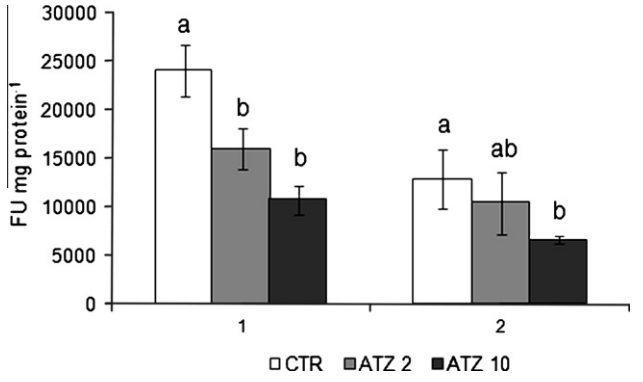

Fig. 2. Generation of reactive oxygen species (ROS) in the liver of *P. lineatus* exposed 2 and 10 μg L^{-1} of the herbicide atrazine (ATZ 2 and ATZ 10) or only water (CTR) for 24 and 48 h. The columns represent the means and the vertical lines the standard errors. For each experimental period, the different letters indicate significant differences between the groups ($P \leqslant 0.05$).

control. After 48 h of exposure to both concentrations of atrazine, the activity of EROD was significantly lower in comparison to the control (Fig. 1A). The fish exposed to the higher concentration of atrazine for 48 h also showed a significant reduction in GST activity (Fig. 1B).

3.2. Generation of ROS

The results showed that in animals exposed to 2 and 10 μg L^{-1} of atrazine for 24 h there was a significant decrease in ROS generation in comparison to the animals of the CTR group. In 48 h, only fish exposed to ATZ 10 showed a significant reduction in ROS generation in relation to the CTR group (Fig. 2).

3.3. Hepatic activity of the antioxidant enzymes

The hepatic activities of the antioxidant enzymes SOD, CAT and GPx were significantly reduced in fish exposed to both atrazine concentrations, for 24 and 48 h, in relation to their respective controls (Table 1). On the other hand, the liver activity of GR significantly increased in fish exposed to ATZ2 and ATZ10, in both experimental periods, when compared to respective controls (Table 1).

3.4. The hepatic content of GSH

Exposure to atrazine for 24 h did not lead to a significant alteration in the hepatic content of GSH. However, after 48 h exposure, atrazine promoted a reduction in the hepatic content of GSH that was significant only in fish exposed to the lower concentration of the herbicide (2 μg L^{-1}) in comparison to the respective control (Table 1).

3.5. Lipid peroxidation

Fish exposed to atrazine showed a reduction in the hepatic content of MDA compared to the respective control group. This reduction was significant in animals exposed to both concentrations of the herbicide for 24 h and also in those exposed to the lower concentration of atrazine for 48 h (Fig. 3).

3.6. DNA damage – the comet assay

Erythrocytes of fish exposed to atrazine for 24 and 48 h showed a concentration-dependent increase in the occurrence of DNA damage, this increase was significant only at the higher concentration of the herbicide (Table 2). For the liver cells a concentration-dependent increase in the occurrence of DNA damage was also observed after 48 h exposure, and this increase was significant only in fish exposed to ATZ10 (Table 2). In the case of gill cells, only fish exposed to the lower concentration of the herbicide for 48 h showed a damage score significantly higher than that of the control (Table 2).

Table 1
Hepatic activity of antioxidant enzymes superoxide dismutase (SOD, U mg $protein^{-1}$), catalase (CAT, μmol H_2O_2 min^{-1} mg $protein^{-1}$), glutathione peroxidase (GPx, μmol NADPH oxidized min^{-1} mg $protein^{-1}$), glutathione reductase (GR, nmol NADPH. min^{-1} mg $protein^{-1}$) and hepatic content of reduced glutathione (GSH, μg GSH mg $protein^{-1}$) in *P. lineatus* exposed to 2 and 10 μg L^{-1} of atrazine (ATZ 2 and ATZ 10, respectively) or only water (CTR), for 24 and 48 h.

Exposure time	Group	SOD	CAT	GPx	GR	GSH
24 h	CTR	36.6 ± 2.2^{a}	32.9 ± 3.6^{a}	153.6 ± 8.6^{a}	7.1 ± 1.1^{a}	7.7 ± 1.8^{a}
	ATZ 2	16.1 ± 1.1^{b}	10.6 ± 1.1^{b}	70.8 ± 2.8^{b}	25.3 ± 1.4^{b}	3.1 ± 0.9^{a}
	ATZ 10	19.2 ± 1.0^{b}	9.5 ± 3.8^{b}	91.3 ± 5.3^{b}	24.6 ± 2.5^{b}	3.4 ± 0.7^{a}
48 h	CTR	47.8 ± 7.4^{a}	40.6 ± 2.3^{a}	45.3 ± 6.6^{a}	7.1 ± 0.8^{a}	35.5 ± 6.5^{a}
	ATZ 2	13.7 ± 5.0^{b}	23.6 ± 1.9^{b}	21.5 ± 1.3^{b}	12.2 ± 1.4^{b}	14.3 ± 1.8^{b}
	ATZ 10	28.5 ± 3.7^{b}	20.1 ± 1.9^{b}	20.6 ± 0.6^{b}	16.0 ± 3.4^{b}	20.3 ± 1.6^{ab}

Data are means ± S.E., $n = 8$. Different letters indicate significant differences between the groups at the same exposure time ($P \leqslant 0.05$).

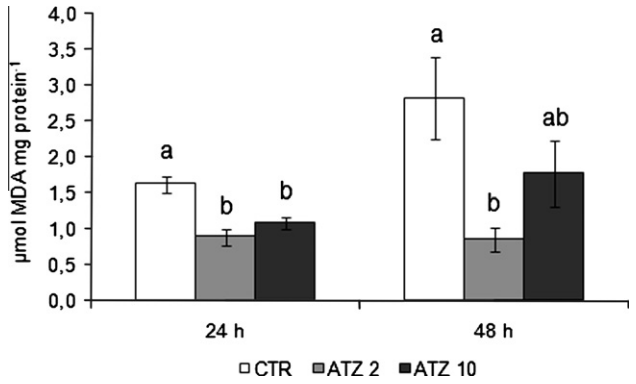

Fig. 3. Liver contents of malondialdehyde (MDA), a sub-product of lipid peroxidation, in *P. lineatus* exposed to 2 and 10 µg L^{-1} of atrazine (ATZ 2 and ATZ 10) or only water (CTR), for 24 and 48 h. The columns represent the means and the vertical lines the standard errors. For each experimental period, the different letters indicate significant differences between the groups ($P \leqslant 0.05$).

Table 2
DNA damage score in blood, liver and gill cells of *P. lineatus* exposed to 2 and 10 µg L^{-1} of atrazine (ATZ 2 and ATZ 10, respectively) or only water (CTR), for 24 and 48 h.

Exposure time	Group	Cell type		
		Blood	Liver	Gills
24 h	CTR	34.6 ± 8.1[a]	31.4 ± 6.5[a]	83.0 ± 11.0[a]
	ATZ 2	45.6 ± 3.7[ab]	40.3 ± 6.5[a]	105.2 ± 10.7[a]
	ATZ 10	83.9 ± 11.7[b]	42.1 ± 5.5[a]	112.2 ± 11.7[a]
48 h	CTR	51.4 ± 4.5[a]	81.7 ± 3.9[a]	64.3 ± 6.0[a]
	ATZ 2	123.0 ± 11.3[ab]	111.4 ± 14.1[ab]	117.2 ± 7.3[b]
	ATZ 10	140.7 ± 27.5[b]	127.0 ± 14.4[b]	72.0 ± 18.7[a]

Data are means ± S.E., $n = 8$. Different letters indicate significant differences between the groups at the same exposure time ($P \leqslant 0.05$).

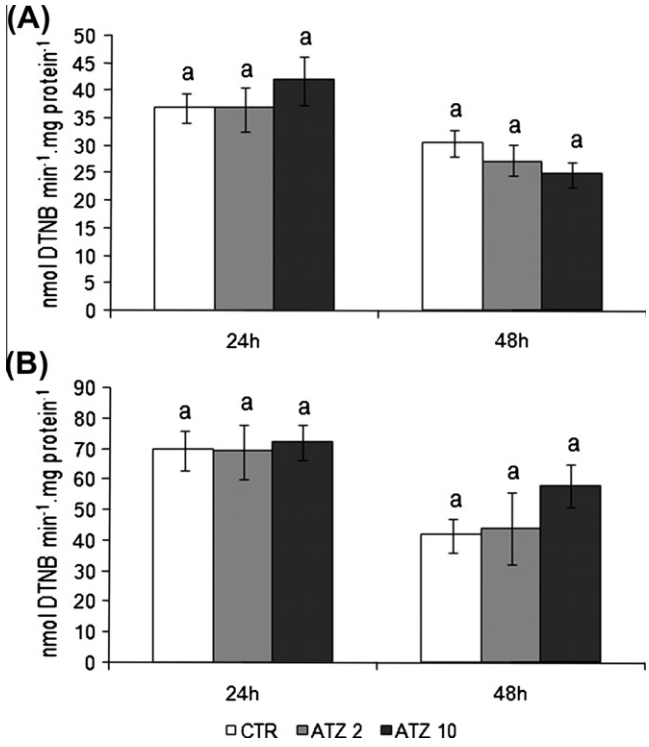

Fig. 4. Acetylcholinesterase activity in (A) brain and (B) muscles of *P. lineatus* exposed to 2 and 10 µg L^{-1} of atrazine (ATZ 2 and ATZ 10) or only water (CTR), for 24 and 48 h. The columns represent the means and the vertical lines the standard errors. For each experimental period, the different letters indicate significant differences between the groups ($P \leqslant 0.05$).

3.7. Acetylcholinesterase

The exposure to both atrazine concentrations, for 24 and 48 h, did not affect the brain or muscle activity of acetylcholinesterase in *P. lineatus* (Fig. 4A and B).

4. Discussion

In the current work, a general decreasing trend on the biotransformation and antioxidant enzymes was observed in the liver of *P. lineatus* exposed to atrazine; except for GR, all the other antioxidant enzymes (SOD, CAT and GPx) and biotransformation enzymes (EROD and GST) showed inhibited activity. These results suggest that atrazine, at the concentrations here applied behaves as enzyme inhibitor impairing hepatic metabolism.

Agrochemicals, in general, can be modified and become less toxic to organisms via biotransformation pathways. It is known that in several species of fish, the activity of the phase I biotransformation enzyme, CYP1A, may change in the presence of pesticides and other pollutants in the aquatic environment (Agradi et al., 2000; Van Der Oost et al., 2003). In present study, atrazine was responsible for the reduced hepatic activity of EROD (one of the ways of measuring CYP1A activity) in *P. lineatus* after 24 and 48 h of exposure. This result could be related both to a reduction in the amount of this enzyme in the liver. This idea is supported by Salaberria et al. (2009), who found that atrazine at doses of 2 and 200 µg kg^{-1} was able to decrease the gene expression of CYP1A in *Oncorhynchus mykiss*.

This inhibition of CYP1A by atrazine may affect the metabolism of endogenous compounds, such as sex hormones, thereby influencing the endocrine homeostasis of the organism, and so this herbicide may be considered an important endocrine disruptor (Spanò et al., 2004; Salaberria et al., 2009). Atrazine metabolism itself by cytochrome P-450-related enzymes may be of less importance in fish, considering that the chemical structure of atrazine allows direct conjugation to GSH, without activation by phase I enzymes (Wiegand et al., 2001). Glutathione-S-transferase, a major phase II biotransformation enzyme, in this case, would act on the metabolism of atrazine, compensating for the inactivity of EROD. However, the GST activity in *P. lineatus* exposed to atrazine was reduced, as well as the activity of EROD, after 48 h exposure to 10 µg L^{-1} of atrazine. This reduction in GST activity might be related with the low levels of GSH in the liver of the fish, since GSH is the main substrate for the action of GST in the metabolism of atrazine, and both were diminished, probably due to the large demand for herbicide conjugation with GSH.

The biotransformation of atrazine in hydrophilic compounds to facilitate its excretion is extremely important in order to restrict its accumulation; according to Solomon et al. (2008) this herbicide normally does not bioaccumulate since it is easily metabolized. However, for *P. lineatus*, atrazine at very low concentrations (2 and 10 µg L^{-1}) inhibited the major pathways for its own metabolism and then the herbicide could be accumulated within the hepatocyte. This idea was corroborated by the results of the comet assay, which showed an increase on DNA damage in the liver cells of fish exposed to 10 µg L^{-1} of atrazine for 48 h, the same group of fish that showed a significant decrease on GST activity. Atrazine has already been recognized in the literature as a potential genotoxic agent in various tissues of various organisms (Lin et al., 2005; Singh et al., 2008; Ventura et al., 2008) and the present work reinforces this idea, showing that atrazine caused DNA damage to the hepatocytes of *P. lineatus*.

Another result that should be highlighted in order to better understand the effects of atrazine on *P. lineatus* is the significant

decrease in ROS generation and MDA levels in liver tissues. The production of ROS occurs via the metabolism of endogenous and exogenous compounds, from cellular respiration and other processes, as a natural consequence of the cellular metabolism (Hermes-Lima, 2004). This decrease in ROS generation indicates that atrazine reduces the metabolism of liver cells, as clearly indicated by the reduced liver activity of the biotransformation and antioxidant enzymes in fish exposed to the herbicide. As it would be expected MDA levels in the liver of fish exposed to atrazine were also lower compared to MDA levels in the controls, thus there was no indication of lipid peroxidation in consequence of atrazine exposure. This hypothesis can be supported by Paulino et al. (2012a, 2012b), which reported that fish of the same species exposed to concentrations of 2, 10 and 25 μg L^{-1} of atrazine during 48 h and 14 d showed histological changes in the gills that would restrict oxygen uptake into the body causing hypoxia to various organs, including the liver.

In the liver of *P. lineatus* exposed to the two concentrations of atrazine for both experimental periods, except for GR, all the other antioxidant enzymes (SOD, CAT and GPx) showed reduced activity. These enzymes can be inhibited by different causes. For example, SOD can be inhibited by an excess of H_2O_2 in the cell. In turn, CAT activity can be inhibited when the levels of $O_2^{\cdot-}$ are high. In other words, the excess of substrate of one enzyme influences the activity of the other enzyme and vice versa. However, as in the present study no increase in ROS generation was observed it is very unlikely that the excess of H_2O_2 or $O_2^{\cdot-}$ had inhibited antioxidant enzymes activities. Another possibility is that atrazine would have interfered on the synthesis of these enzymes. However, studies conducted by Jin et al. (2010) showed that the fish species *Danio rerio*, also exposed to 10 μg L^{-1} of atrazine for 14 d, had increased mRNA levels of SOD and CAT in the liver. Thus, the most likely cause for the inhibition of these enzymes in *P. lineatus* exposed to atrazine would be a general reduction in the liver cell metabolism caused by the herbicide.

The fish exposed to atrazine showed a reduced hepatic content of GSH and an increase in GR activity. GSH levels are mainly depend on the balance between GSH synthesis rate, conjugation rate (by GSTs), oxidation rate (non-enzymatically or by GPx), and oxidized glutathione (GSSG) reduction to GSH, by GR (Peña-Llopis et al., 2003). In the present study the increased activity of GR, in fish exposed to both concentrations of atrazine, indicates an attempt to maintain normal levels of GSH. The maintenance of GSH levels by GR, in this case, might contribute for the reduction of atrazine toxicity through its association with the herbicide molecule (Wiegand et al., 2001).

Damage to DNA can occur as a consequence of oxidative stress, xenobiotic metabolites or the xenobiotic itself. In the case of *P. lineatus*, atrazine itself was found to be genotoxic to the three tissues analyzed (blood, gill and liver), since no evidence was obtained to show the metabolism of this herbicide or the occurrence of oxidative stress. The results obtained from the genetic material of hepatocytes of fish exposed to 10 μg L^{-1} of herbicide for 48 h, as discussed above, may be a consequence of the accumulation of atrazine in the liver due to the reduction of GST activity. Assays to assess levels of damage in the genetic material of liver cells should be considered in toxicology studies, since the liver is the main organ responsible for xenobiotic metabolism and therefore is more susceptible to damage due to the presence of these toxic compounds in its cells (Rajaguru et al., 2003).

The blood cells of *P. lineatus* also demonstrated the genotoxicity of atrazine, since fish exposed to the higher concentration of atrazine for the two experimental periods showed increased levels of DNA damage in erythrocytes. Ventura et al. (2008) tested different concentrations of the same herbicide in *Oreochromis niloticus* and they also observed the genotoxic effects of atrazine on erythrocytes after 72 h of exposure. These data indicate that atrazine can affect the genetic material in erythrocytes of different fish species.

For the gill cells, a significant increase in DNA damage was found only in fish exposed to the lower concentration of atrazine for 48 h. The concentration of 10 μg L^{-1} of atrazine was genotoxic to the other tissues of *P. lineatus* and probably was also genotoxic to gill cells. However this higher atrazine concentration may have caused apoptosis of the gill cells and considering that apoptotic cells are not considered for the calculation of DNA damage score fish from ATZ 10 group showed a lower damage score less than the fish from ATZ 2.

The damage in DNA observed in different cells of *P. lineatus* exposed to the herbicide atrazine was probably not from oxidative origin, since no increase in ROS generation was detected in the liver and probably would not have been detected in the other organs tested either. According to Oliveira-Brett and Silva (2002), triazine herbicides, including atrazine, are able to directly bind to DNA through the mechanisms of intercalation and adduct formation between adenine and guanine. One of the main ways of preventing DNA damage is the conjugation of the xenobiotic with GSH, catalyzed by the action of GST (Chakraborty et al., 2009). Thus, as discussed above, atrazine needs GST to conjugate with GSH to become less toxic for the organism and its genotoxicity possibly gets worse as the result of a decrease in GSH content and GST activity.

Although atrazine exposure showed several effects on *P. lineatus*, the herbicide had no effect on brain or muscles acetylcholinesterase activity in this fish species. Data about the effect of atrazine on AChE activity in aquatic vertebrate species are scarce (Tyler Mehler et al., 2008). In a work conducted by Xing et al. (2010) juveniles of common carp (*Cyprinus carpio*) showed inhibited AChE activity in brain and muscle after 40 d exposure to different atrazine concentrations (4.28, 42.8 and 428 μg L^{-1}). This variation between the results obtained for *P. lineatus* and *C. carpio* may be produced by the differences in exposure periods, the size and the type of the fish. *P. lineatus* showed to be less sensitive to atrazine effects on AChE, even though its cholinergic activity was found to be influenced by other agrochemicals, for example, Diflubenzuron, Roundup and Roundup Transorb (Maduenho and Martinez, 2008; Modesto and Martinez, 2010a, 2010b).

In summary, the results obtained in this work show that atrazine was toxic to the Neotropical fish *P. lineatus*, even at low concentrations (2 and 10 μg L^{-1}), since it promoted significant alterations in the biochemical and genetic parameters evaluated. One effect of atrazine was a reduction in the hepatic activity of GST, which is primarily responsible for metabolism, and which caused the accumulation of this herbicide in the hepatocytes of the fish. The fish exposed to atrazine also showed inhibition of the biotransformation pathways of phase I, as shown by a reduction in the activity of EROD, which directly contributes to a reduction in the generation of reactive oxygen species in the liver and a consequent reduction in the activity of antioxidant liver enzymes. Moreover, atrazine stimulated the action of GR, which is responsible for the maintenance of normal GSH levels, which were found to be reduced in this work. Even with the reduction in antioxidant defence, atrazine did not cause lipid peroxidation in the liver of *P. lineatus*. In addition to the biochemical effects, atrazine caused DNA damage in the three types of tissue studied, blood, liver and gills, which did not have an oxidative origin since no increase in the generation of reactive oxygen species was detected. These results show that the limit of 2 μg L^{-1} of atrazine in fresh water, allowed by Brazilian legislation (CONAMA, 2005), is not a safe concentration for the fish *P. lineatus*.

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