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Bioaccumulation of nickel and its biochemical and genotoxic effects on juveniles of the neotropical fish *Prochilodus lineatus*

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ABSTRACT

Juveniles of the freshwater fish *Prochilodus lineatus* were exposed to three concentrations of nickel (Ni): 25, 250 and 2500 μg L^{-1} or water only for periods of 24 and 96 h to test for Ni bioaccumulation, its effects on antioxidant defenses and metallothioneins, and the occurrence of DNA damage. After exposure, the fish were sampled and tissue removed from the gills, liver, kidney and muscle to test for Ni accumulation and conduct biochemical (gills and liver) and genotoxic (blood cells and gills) analyses. The results showed that Ni accumulates in the organs in different proportions (kidney > liver > gills > muscle) and accumulation varied according to exposure time. Metallothionein (MT) levels increased in the liver and gills after exposure to Ni, implying that the presence of Ni in these tissues could induce MT synthesis. We also observed that Ni exposure affected antioxidant defenses, increasing lipid peroxidation in the liver of fish exposed to Ni for 96 h at the highest concentration tested. DNA damage increased in both blood cells and gills of fish exposed to all Ni concentrations, indicating the genotoxic potential of Ni on fish. We therefore concluded that Ni accumulates in various tissues and results in oxidative and DNA damage in *P. lineatus,* and that the maximum permitted Ni concentration set in Brazilian legislation (25 μg L^{-1}) for freshwaters is not safe for this species.

1. Introduction

Nickel (Ni) is a transition metal relatively abundant in the Earth's crust. It is of significant economic importance and is widely mined (Eisler, 1998). Brazil has the third largest nickel reserves in the world (IBRAM, 2012). Various anthropogenic processes, including mining, smelting, refining, and the manufacture of stainless steel and Ni–Cd batteries have resulted in Ni contamination in many aquatic areas (Bielmyer et al., 2013; Jayaseelan et al., 2014). Brazil's regulatory guidelines (CONAMA, 2005) stipulate 25 μg L^{-1} as the maximum permitted Ni concentration in inland waters. However, Ni concentrations in superficial natural freshwaters can reach 100 μg L^{-1} (CETESB, 2014) and levels of up to 90 mg kg^{-1} have been detected in suspended particles from watercourses of industrialized areas in southeastern Brazil (Jordão et al., 2005). High Ni levels (up to 13 5 mg kg^{-1}) have also been detected in the muscle tissue of various fish species collected from a Brazilian river basin with high levels of many heavy metals (Meche et al., 2010).

Nickel is well established as an essential nutrient for plants and terrestrial animals and evidence is mounting to suggest that Ni is probably essential to fish (Pyle and Couture, 2012). However, at elevated concentrations, it can be harmful (Bielmyer et al., 2013). It can significantly affect the physiology of aquatic organisms (Pane et al., 2003; Alsop et al., 2014) and oxidative stress is thought to be one possible mechanism of Ni-induced toxicity (Parthiban and Muniyan, 2011; Kubrak et al., 2013; Zheng et al., 2014). Although the precise mechanism of Ni toxicity is not clear, there is evidence that it can trigger the generation of reactive oxygen species (ROS) and inhibit antioxidant enzyme activity in fish tissue (Zheng et al., 2014). ROS readily interacts with DNA, proteins, and other important biomolecules, inflicting cellular and molecular damage and physiological dysfunction (Kubrak et al., 2012). One of the first enzymes involved in defense against ROS is superoxide dismutase (SOD), which catalyzes the conversion of reactive superoxide anions to hydrogen peroxide (H_2O_2) for subsequent detoxification by catalase (CAT) and glutathione-dependent peroxidase (GPx). GPx catalyzes the metabolic conversion of H_2O_2 and other peroxides to water, with concomitant oxidation of glutathione (GSH), one of the most important non-enzymatic antioxidants in the cell (Van Der Oost et al., 2003). Other important enzymes in antioxidant defense are glutathione reductase (GR), responsible for reducing glutathione (GSSG) and maintaining normal levels of GSH and glutathione-S-transferase (GST), an enzyme that catalyzes the conjugation of a variety of metabolites, including lipid peroxidation products with GSH (Modesto and Martinez, 2010).

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In view of the rising global demand for Ni and the corresponding potential for increased anthropogenic inputs, it is important to develop a deeper understanding of its effects on fish. However, little work has been done on Ni toxicity in fish compared to other more toxic metals, such as copper (Cu) or cadmium (Cd) (Pyle and Couture, 2012), and studies on the way Ni affects Neotropical fish need to be conducted.

Therefore, the aim of this study was to characterize Ni bioaccumulation in different tissues and its acute effects on a Neotropical fish, focusing on antioxidant defenses, lipid peroxidation, metallothionein levels and DNA damage. The freshwater fish *Prochilodus lineatus* (Order Characiformes), also known as curimba or curimbatá, was chosen because it is common in the rivers of the south and southeastern regions of Brazil and sensitive to a variety of metals, including aluminum (Camargo et al., 2009), cadmium (Silva and Martinez, 2014), copper (Nascimento et al., 2012) and lead (Monteiro et al., 2011; Ribeiro et al., 2014).

2. Material and methods

2.1. Animals

Juveniles of *P. lineatus* (Valenciennes, 1836), 8–10 months old, weighing 10.16 ± 0.36 g and measuring 8.95 ± 0.87 cm in total length (mean ± SE, $n=279$) were supplied by the fish hatchery at the State University of Londrina, Paraná, Brazil. The fish were acclimated in the laboratory for a period of five to seven days, in a 300-l tank containing dechlorinated freshwater, with constant aeration and a 12/12 photoperiod. The chemical and physical characteristics of the water were monitored continuously (*T*: 20.4 ± 1.1 °C; pH: 7.1 ± 0.3; DO: 7.7 ± 0.7 mg O_2 L^{-1}; conductivity: 82 ± 0.1 μS cm^{-1}; water hardness: 35 mg L^{-1} $CaCO_3$). During acclimation, the fish were fed every 48 h with commercial fish food containing 36% protein (Guabi®, BR). Feeding was suspended 24 h before commencing the toxicity tests.

2.2. Experimental protocol

After acclimation, the fish were subjected to semi-static tests for 24 and 96 h in 100-L glass aquaria containing 80 L of freshwater. Eight fish were placed in each aquarium, maintaining a maximum density of 1 g fish per liter of water, and 80% of the water was renewed every 24 h. The fish were divided into four groups, one for each experimental treatment: the control group (Ni0) was exposed to dechlorinated water only; three experimental groups were exposed to 25 μg L^{-1} (Ni25), 250 μg L^{-1} (Ni250) and 2500 μg L^{-1} (Ni2500) Ni respectively, added to the water as $NiCl_2$ (Synth, Brazil) 24 h before beginning the tests. The lowest Ni concentration tested was based on the Brazilian guidelines (CONAMA, 2005) which set 25 μg L^{-1} as the maximum permitted Ni concentration in inland waters. The tests were performed for each experimental period (24 and 96 h) in independent experiments and for each exposure time, control (Ni0) and Ni groups (Ni25, Ni250 and Ni2500) were run simultaneously.

During the tests, water temperature, pH, dissolved oxygen and conductivity inside each aquarium were monitored using a multiparameter water quality meter (Hanna HI9828, USA) and kept stable (mean ± SE) at 21.4 ± 0.4 °C; 7.02 ± 0.03; 7.53 ± 0.36 mg O_2 L^{-1} and 78.8 ± 0.9 μS cm^{-1}, respectively. Water samples were collected every 24 h to determine Ni levels. Total Ni concentration was determined in non-filtered water samples and the concentration of dissolved Ni was determined in water samples filtered through a 0.45 μm syringe filter (Millipore Millex HV/PVDF). For both analyses, samples were acidified by adding HNO_3 and stored at 4 °C before Ni concentrations were determined by electrothermal atomic absorption spectrometry, using an atomic absorption spectrometer with graphite furnace atomizer (Perkin Elmer A700), and a Ni detection limit of 5 μg L^{-1}.

After the exposure periods (24 or 96 h), the fish were anesthetized using benzocaine diluted in water (0.1 g L^{-1}) and blood was taken from the caudal vessel using pre-heparinized syringes. The fish were then killed by medullar section and gill, liver, kidney and muscle tissue removed. Tissue samples were stored in an ultrafreezer at −80 °C prior to biochemical analysis. Blood samples and gill sub-samples were rapidly processed for the comet assay, as described below. These procedures were approved by the Animal Ethics Committee of the State University of Londrina.

2.3. Ni determination in the tissues

Ni content was quantified in gill, liver, kidney and muscle tissue, after drying at 60 °C. Dry tissue samples were fully digested (1:10, w/v) in 5 N HNO_3 (Suprapur, Merck) at 60 °C. Samples were then centrifuged (14,000 *g*, 20 min) and the supernatant used for Ni determination in an atomic absorption spectrometer with graphite furnace atomizer (Perkin Elmer A700).

2.4. Biochemical assays

Gills and liver samples were weighed and homogenized (gills 1:5 and liver 1:10, w/v) in K-phosphate buffer (0.1 M; pH 7.2), centrifuged (13,200 *g*; 4 °C; 20 min) and the supernatant separated for biochemical assays. The protein concentration in the liver homogenate was measured according to the method in Lowry et al. (1951), using bovine serum albumin as standard.

Superoxide dismutase (SOD) activity was determined by measuring inhibition of the reduction rate of cytochrome c by the superoxide radical at 550 nm and 25 °C, according to McCord and Fridovich (1969). Catalase (CAT) activity was determined by the H_2O_2 decomposition rate, based on the absorbance drop at 240 nm, according to Beutler (1975). Glutathione peroxidase (GPx) activity was estimated according to Hopkins and Tudhope (1973), by NADPH oxidation in the presence of H_2O_2 at 340 nm. Glutathione reductase (GR) activity was indirectly determined based on NADPH reduction in the presence of glutathione disulfide at 340 nm (Carlberg and Mannervik, 1975). The concentration of glutathione (GSH) was determined at 412 nm, according to Simonato et al. (2011). Glutathione S-transferase (GST) activity was determined after complexating glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm, according to Keen et al. (1976).

Lipid peroxidation (LPO) was determined based on the production of malondialdehyde (MDA), one of the end products of lipid peroxidation. MDA-content was determined by the TBARS assay, which measures thiobarbituric acid (TBA) reactive substances, by fluorescence readings (ex/em: 535/590 nm), according to Camejo et al. (1998). LPO was expressed in μ mol MDA mg $protein^{-1}$, based on an MDA standard curve.

Metallothionein (MT) content was determined based on the method described by Viarengo et al. (1997). For this assay, samples of gills and liver were homogenized (gills 1:5 and liver 1:10, w/v) in a buffer (Tris–HCl 20 mM, sucrose 0.5 mM and β-mercaptoethanol 0.01%, pH 8.6), centrifuged for 45 min (18650 *g*; 4 °C), and the supernatant was subjected to ethanol/acid chloroform fractionation to obtain a partially purified metalloprotein fraction. In this fraction, sulfhydryl groups (–SH) were quantified at 412 nm, using Ellman's reagent. Reduced glutathione (GSH) was used as standard and the metallothionein content was expressed in nmol GSH mg $protein^{-1}$.

2.5. DNA damage – comet assay

Freshly collected blood samples were diluted (1:1000, v/v) in phosphate buffered saline (PBS:NaCl 126.6 mM, KCL 4.8 mM, CaCl 1.5 mM, $NaHCO_3$ 3.7 mM, Na_2HPO_4 8.9 mM and NaH_2PO_4 2.9 mM) and kept on ice for the comet assay. Immediately after removal, the gills were cleaned with PBS, and gill filaments excised and transferred to microtubes containing PBS and kept on ice until cell dissociation, according to Cavalcante et al. (2008). Gill filaments were sectioned and pieces incubated in 0.05% trypsin (diluted in PBS Ca^{2+} and Mg^{2+} free) and homogenized by periodic manual inversion for tissue dissociation. The solution was then filtered (30 μm mesh) into a tube containing a 10% fetal bovine serum, centrifuged (10 min, 1000 *g*) and the pellet resuspended in PBS ready for the comet assay.

Before running the comet assay, erythrocyte and gill cell viability was determined using the Trypan blue exclusion method (Tice et al., 2000). For each fish, a total of 100 cells were scored per cell type, and viability expressed as the percentage of viable cells in the total number of cells counted. At least 80% of cells must be viable to run the comet assay.

The alkaline comet assay was performed according to Singh et al. (1988), with some modifications for blood cells (Vanzella et al., 2007) and gill cells (Cavalcante et al., 2008). The following basic assay steps were performed for both erythrocytes and gill cells: (a) lysis: one hour, at 4 °C, protected from light, in a lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1 mL Triton X-100, pH 10.0); (b) DNA unwinding: 30 min, in the dark, in an electrophoresis buffer (0.3 N NaOH, 1 mM EDTA, pH > 13); (c) electrophoresis: 20 min, 300 mA, 25 V, 1 V cm^{-1}; (d) neutralization: three 5-min washes in a buffer (0.4 M Tris, pH 7.5). Slides were then fixed with absolute ethanol for 10 min and kept under refrigeration pending cytological analyses. Two slides were prepared for each fish and each cell type.

The slides, stained with GelRed (15 μL of GelRed diluted in 45 mL distilled water and 5 mL NaCl 1 M), were analyzed under a Leica DM 2500 microscope with blue excitation filter (450–490 nm) and a 515-nm barrier filter 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 selected, non-overlapping cells per fish. DNA damage was classified as follows: class 0-no visible damage; class 1-short tail smaller than the diameter of the nucleoid; class 2-tail length up to twice the diameter of the nucleoid; class 3-tail length > twice the diameter of the nucleoid. The DNA damage score 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 DNA damage score for each treatment group and exposure period. Results for DNA damage in both cell types were also expressed as the mean number of damaged nucleoids (sum of classes 1, 2 and 3).

2.6. Statistical analysis

After checking for normality and homoscedasticity, the results obtained for each group (Ni0 × Ni25 × Ni250 × Ni2500) and each exposure period were cross-compared by parametric (ANOVA) or non-parametric analysis of variance (Kruskal–Wallis), and differences among treatment groups were identified by the Student–Newman–Keuls (SNK) or Dunn's test, respectively. Results are presented as mean ± SE. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Nickel concentration in the water and nickel accumulation in tissues

Total and dissolved nickel concentrations in the experimental aquaria during the 96-h exposure period were consistent with the nominal concentrations (Table 1). In the tissues, Ni concentrations indicated higher accumulation in the kidney, followed by the liver, gills and muscle (Fig. 1). Ni concentrations in the kidney varied from 2 to 16 μg Ni g^1, in the liver from 1.8 to 3.8 μg Ni g^{-1}, in the gills from 0.2 to 3.1 μg Ni g^{-1}, and in the muscle from 0.07 to 0.8 μg Ni g^{-1}. After 96 h exposure to 2500 μg Ni L^{-1}, the kidney (Fig. 1A) showed a significant increase (Kruskal–Wallis, $p < 0.001$) in Ni and contained 6 times more Ni than the liver (Fig. 1B) and gills (Fig. 1C). On the other hand, only the liver and gills showed a significant increase in Ni at the lowest exposure time (ANOVA, $p = 0.003$ and $p = 0.009$, respectively). After 96 h exposure, only fish exposed to the highest Ni concentration showed a significant increase (ANOVA, $p < 0.001$) in liver Ni concentration. The gills showed a significant increase (ANOVA, $p < 0.001$) in Ni content after 96 h of exposure to the two highest concentrations. Muscle (Fig. 1D) was the organ that least accumulated Ni and only after 96 h of exposure to the highest concentration there was a significant increase (ANOVA, $P < 0.001$) in tissue Ni concentration compared to the control (Ni0).

Table 1
Concentrations of total (T) and dissolved (D) nickel (μg L^{-1}) in the water from the experimental treatments (Ni0, Ni25, Ni250 and Ni2500) measured along the 96-h exposure period.

Experimental treatments	[Ni]	0 h	24 h	48 h	72 h	96 h
Ni0	T	ND	ND	ND	ND	ND
	D	ND	ND	ND	ND	ND
Ni25	T	24.1 ± 1.2	23 ± 0.9	22.8 ± 1.7	22.3 ± 1.9	21.8 ± 1.9
	D	23.7 ± 1.6	22 ± 1.5	21.8 ± 8.7	22.2 ± 2.1	19.6 ± 1.7
Ni250	T	252 ± 1.4	246 ± 1.6	235 ± 1.7	231 ± 2.1	227 ± 1.8
	D	233 ± 1.9	221 ± 3.5	224 ± 2.3	219 ± 1.8	211 ± 1.5
Ni2500	T	2480 ± 2.4	2420 ± 1.8	2370 ± 1.9	2290 ± 1.8	2210 ± 1.4
	D	2370 ± 2.1	2310 ± 14.9	2220 ± 2.9	2170 ± 2.3	2210 ± 2.8

ND: not detected. Values are mean ± SE, *n*: 8–16.

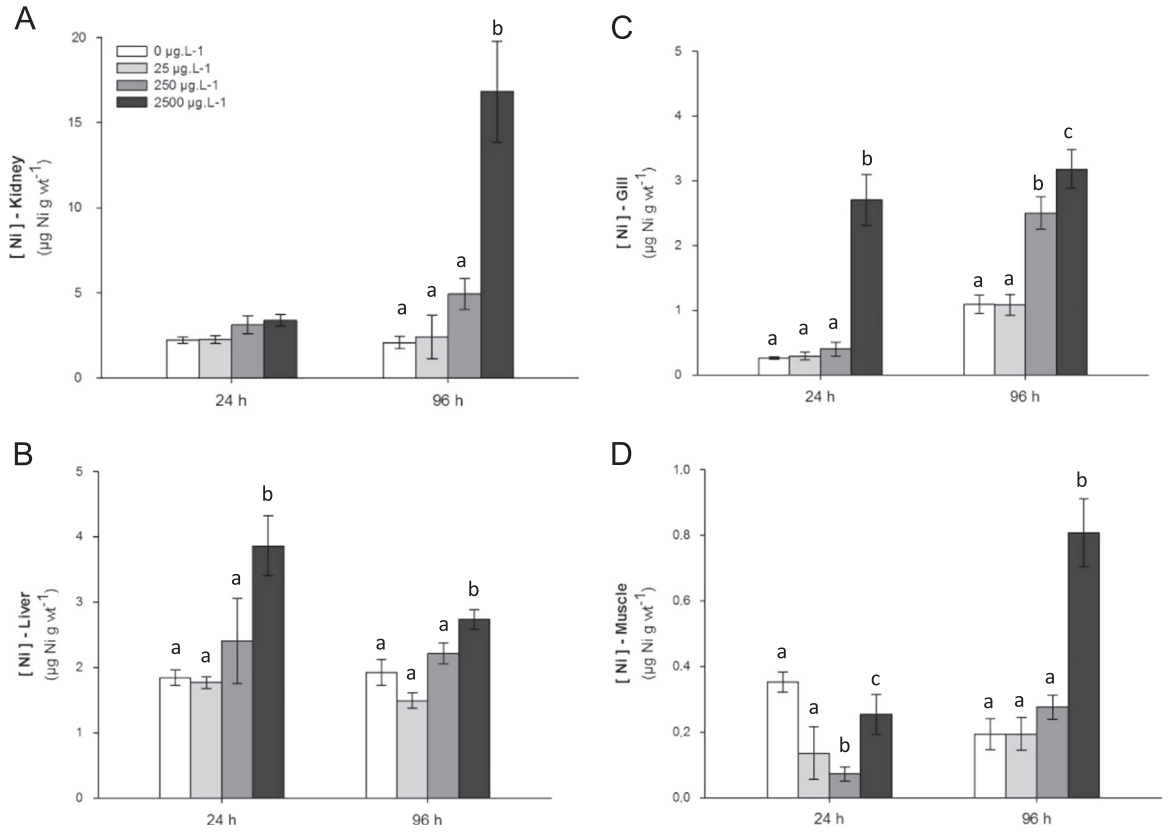

Fig. 1. Nickel concentrations (mean ± SE) in kidney (A), liver (B), gills (C) and muscle (D) of *P. lineatus* exposed to different Ni concentrations (25, 250 or 2500 µg L^{-1}) or only to water (0 µg L^{-1}), for 24 or 96 h. Note different scale used for different tissues. Different letters indicate significant difference between groups within the same exposure period ($p < 0.05$).

3.2. Metallothionein content – gills and liver

The liver (Fig. 2A) of fish exposed to Ni for 24 h showed a significant increase (ANOVA, $P < 0.001$) in MT concentration after exposure to all the Ni concentrations tested. Groups exposed to Ni for 96 h also showed a significant increase (ANOVA, $P < 0.001$) in liver MT, but only after exposure to the two highest Ni concentrations. The gills of fish exposed for 96 h to the two highest Ni concentrations also showed a significant increase (ANOVA, $P < 0.001$) in MT concentration (Fig. 2B).

3.3. Antioxidant defenses

Fish exposed to Ni for 96 h showed a significant decrease (ANOVA, $p < 0.001$) in hepatic SOD activity at all concentrations tested (Fig. 3A), and also in CAT activity (ANOVA, $p < 0.001$) at the two highest concentrations (Fig. 3B). These enzymes did not vary significantly (ANOVA, $p = 0.533$ and $p = 0.352$, respectively) between fish in the control group (Ni0) and groups exposed to Ni for 24 h (Fig. 3A and B). No significant variations (ANOVA, 24 h: $p = 0.241$ and 96 h: $p = 0.094$) among the different groups were found for the hepatic activity of the GPx enzyme (Fig. 3C). On the other hand, gill SOD activity increased significantly in fish exposed to 250 and 2500 µg Ni L^{-1} for 24 (ANOVA, $p = 0.005$) and 96 h (ANOVA, $p < 0.001$), compared to group Ni0 (Fig. 3D). However, CAT (ANOVA, 24 h: $p = 0.137$ and 96 h: $p = 0.182$) and GPx (ANOVA, 24 h: $p = 0.347$ and 96 h: $p = 0.403$) activities in the gills did not vary significantly between fish in the control group (Ni0) and those exposed to Ni (Fig. 3E and F).

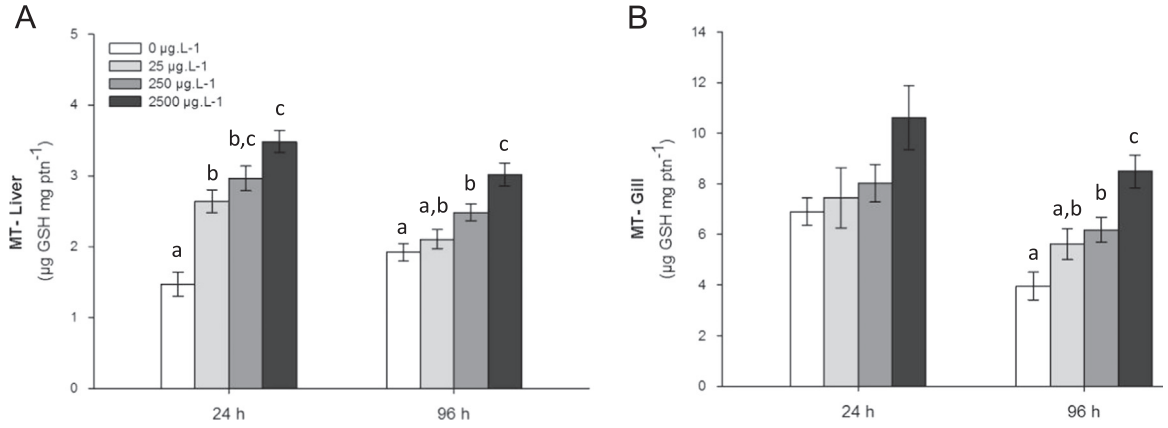

Fig. 2. Metallothionein (MT) content (mean ± SE) in liver (A) and gill (B) of *P. lineatus* exposed to different Ni concentrations (25, 250 or 2500 µg L^{-1}) or only to water (0 µg L^{-1}), for 24 or 96 h. Different letters indicate significant difference between groups within the same exposure period ($p < 0.05$).

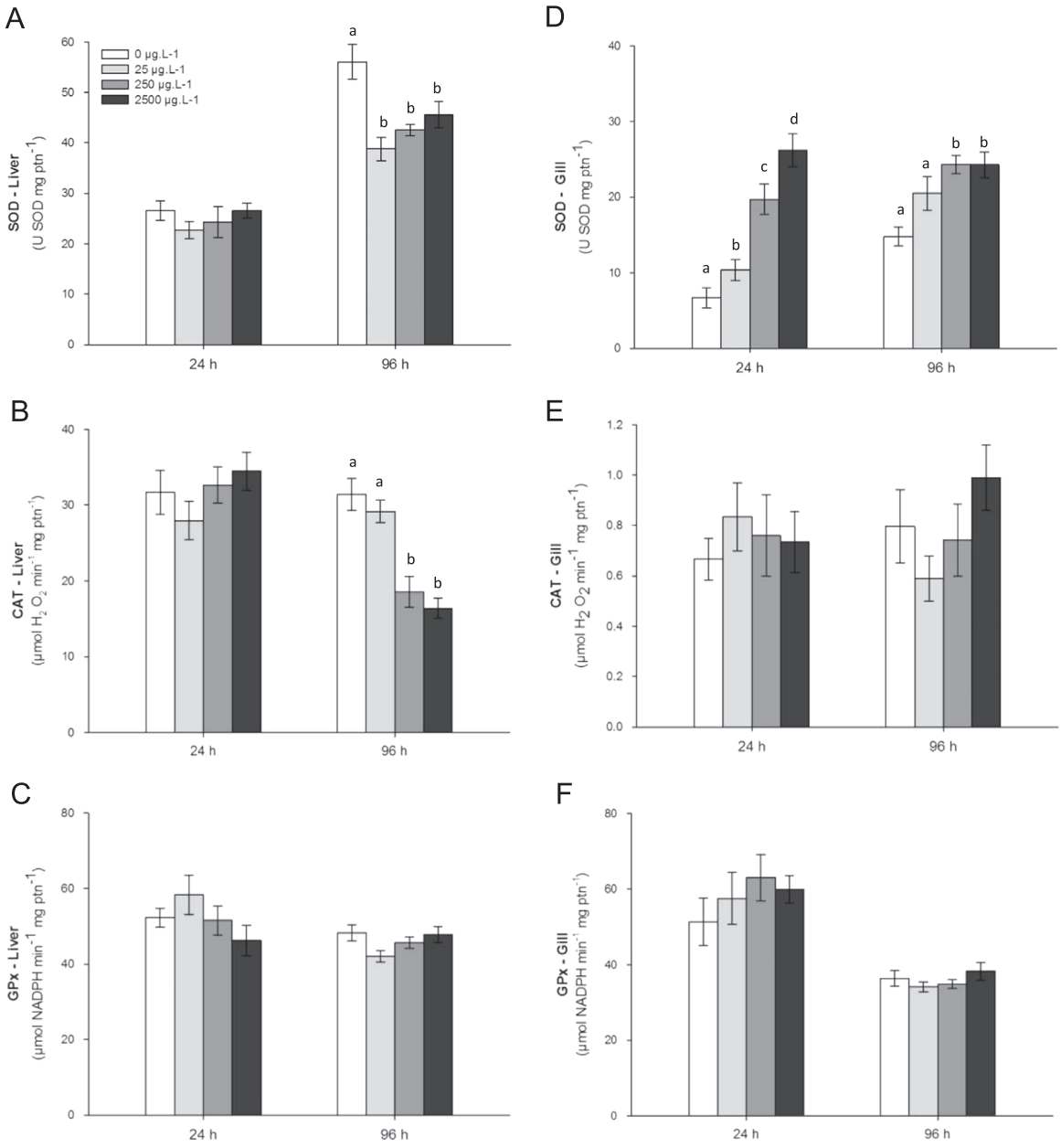

Fig. 3. Activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in the liver (A–C) and gills (D–F) of *P. lineatus* exposed to different Ni concentrations (25, 250 or 2500 µg L^{-1}) or only to water (0 µg L^{-1}), for 24 or 96 h. Values are presented as mean ± SE. Different letters indicate significant difference between groups within the same exposure period ($p < 0.05$).

There were no significant differences in the hepatic activities of GST (ANOVA, 24 h: $p=0.411$ and 96 h: $p=0.110$) and GR (ANOVA, 24 h: $p=0.383$ and 96 h: $p=0.060$) and in the liver GSH content (ANOVA, 24 h: $p=0.190$ and 96 h: $p=0.227$) between treatments, for any of the exposure periods (Fig. 4A–C). In the gills, GST activity (ANOVA, $p=0.414$) and GSH content (ANOVA, $p=0.316$) did not vary significantly between fish in the control group (Ni0) and groups exposed to Ni for 24 h (Fig. 4D and F). However, after 96 h there was a significant increase in gill GST (ANOVA, $p < 0.001$) in fish exposed to the highest Ni concentration and in GSH content (ANOVA, $p=0.009$) in fish exposed to 250 and 2500 µg Ni L^{-1}. In contrast, gill GR activity significantly increased (ANOVA, $p=0.024$) in fish exposed to all Ni concentrations for 24 h, compared to group Ni0, but no significative difference (ANOVA, $p=0.538$) was detected after 96 h exposure to any Ni concentration (Fig. 4E).

3.4. Lipid peroxidation

A significant increase (ANOVA, $p < 0.001$) in MDA was detected only in the liver of fish exposed to the highest Ni concentration (2500 µg L^{-1}) for 96 h (Fig. 5A). In gill tissue, there were no significant differences (ANOVA, 24 h: $p=0.065$ and 96 h: $p=0.289$) for any of the exposure times (Fig. 5B).

3.5. DNA damage

Different comet classes observed in *P. lineaus* erythrocytes are shown in Fig. 6A. There was a significant increase (ANOVA, $p < 0.001$) in the DNA damage score in erythrocytes (Fig. 6B) and gill cells (Fig. 6C) of *P. lineatus* after 24 and 96 h exposure to different Ni concentrations in comparison to fish from the Ni0 group.

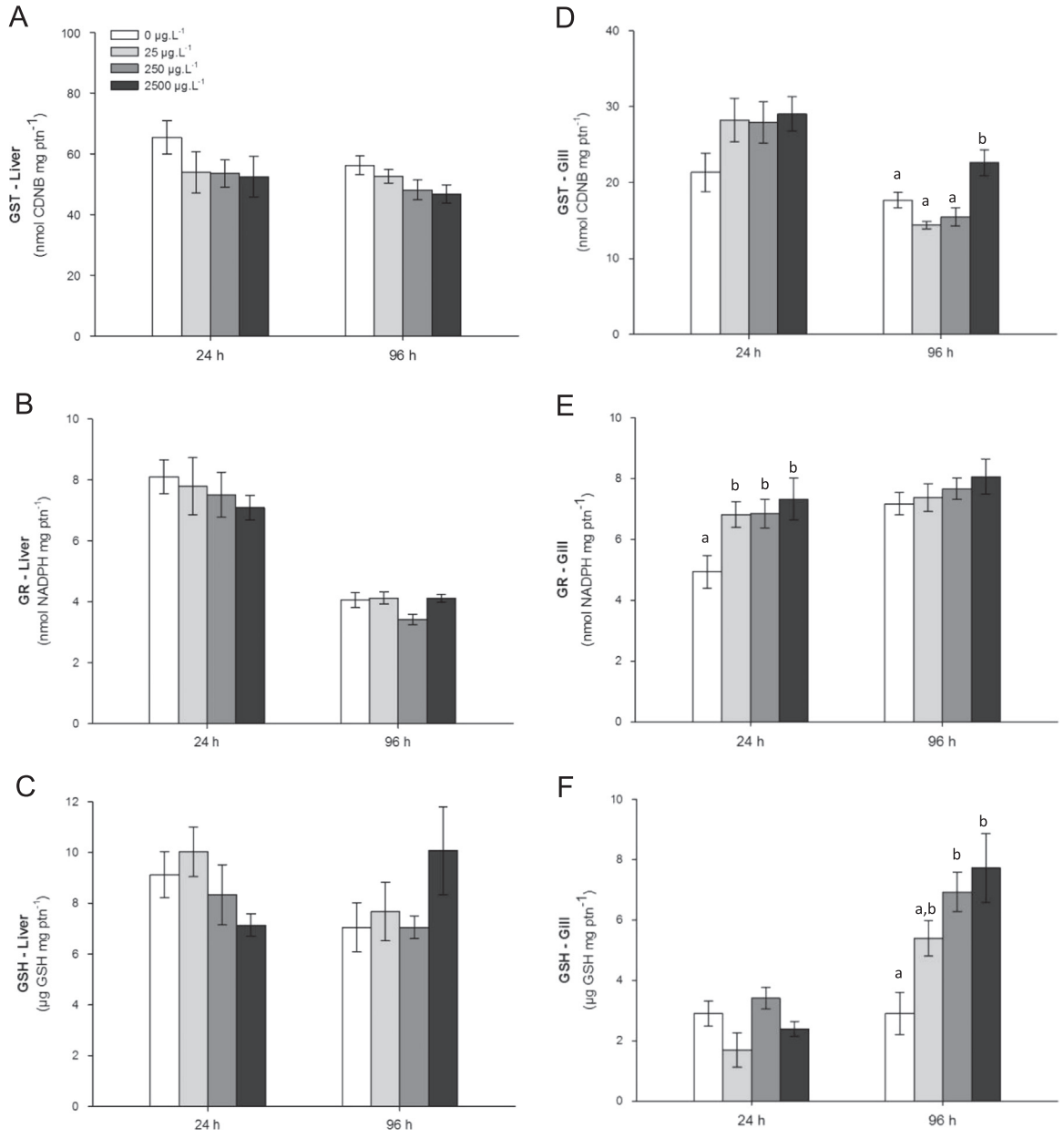

Fig. 4. Activity of glutathione-S-transferase (GST), glutathione reductase (CAT) and glutathione content (GSH) in the liver (A–C) and gills (D–F) of *P. lineatus* exposed to different Ni concentrations (25, 250 or 2500 µg L^{-1}) or only to water (0 µg L^{-1}), for 24 or 96 h. Values are presented as mean ± SE. Different letters indicate significant difference between groups within the same exposure period ($p < 0.05$).

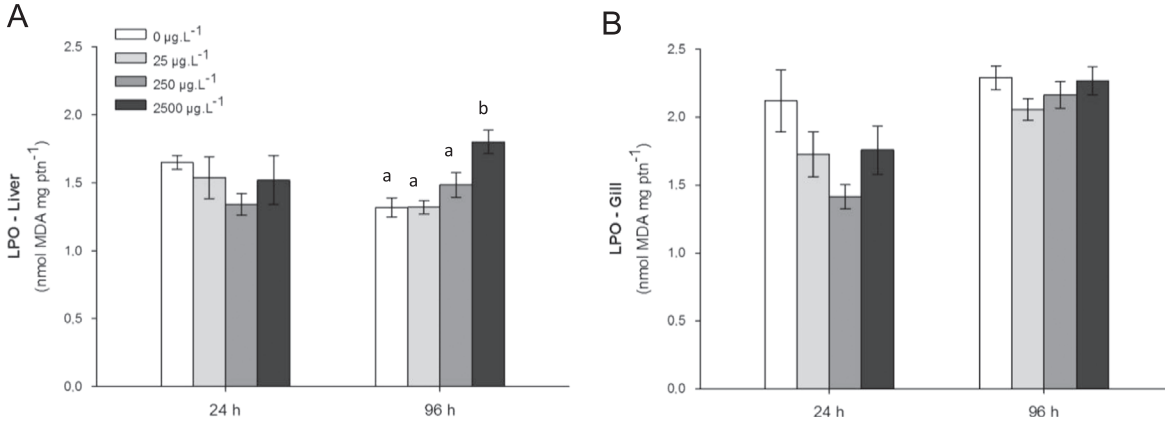

Fig. 5. Lipid peroxidation (LPO) in the liver (A) and gills (B) of *P. lineatus* exposed to different Ni concentrations (25, 250 or 2500 µg L^{-1}) or only to water (0 µg L^{-1}), for 24 or 96 h. Values are presented as mean ± SE. Different letters indicate significant difference between groups within the same exposure period ($p < 0.05$).

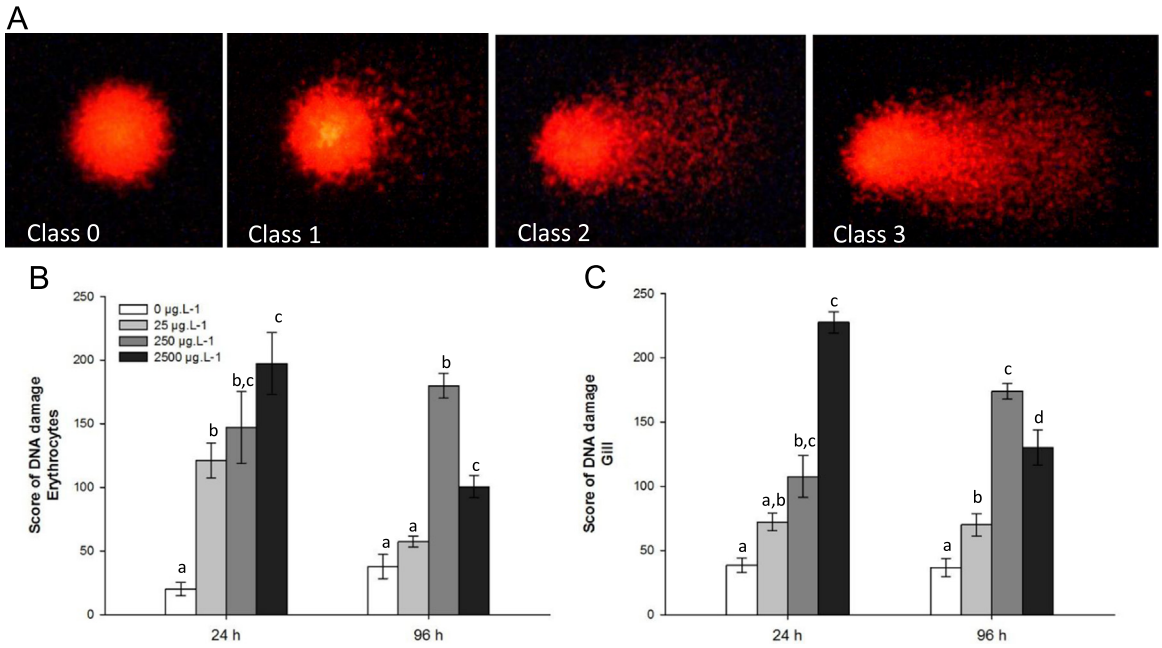

Fig. 6. (A) Photomicrographs of *P. lineatus* erythrocytes processed for the comet assay showing increasing degrees of DNA damage (original magnification: 1000 ×). Score of DNA damage (mean ± SE) in erythrocytes (B) and gill cells (C) of *P. lineatus* exposed to different Ni concentrations (25, 250 or 2500 µg L^{-1}) or only to water (0 µg L^{-1}), for 24 or 96 h. Different letters indicate significant difference between groups within the same exposure period ($p < 0.05$).

4. Discussion

The results of our experiments lead us to conclude that Ni causes significant alterations in *P. lineatus*, since it accumulates in various tissues, interferes with antioxidant defenses and is genotoxic. Studies on Ni toxicity to fish are rare and our study is significant in demonstrating the toxic effects of nickel on a neotropical fish. Studies like this can serve as references for the initial signs of Ni-induced damage, which could result in population level impacts after chronic exposure. The concentrations tested are of great ecological relevance and lend weight to the argument that the standards in the Brazilian legislation need to be based on studies of local species. Above all, the results of this study underline the importance of setting up assessment and monitoring programs in areas impacted by Ni, using native species. Furthermore, the results show how Ni can build up in the tissues of *P. lineatus*, and this could form the basis for a study to assess how bioaccumulation could adversely affect organisms that feed on this species.

Ni concentrations in the tissues showed that the highest accumulation is in the kidney, followed by the liver, gills and muscle. Accumulation of Ni in fish tissues is commonly rather tissue-specific and each tissue type has specific affinities for accumulation of different elements (Ptashynski et al., 2001; Ptashynski and Klaverkamp, 2002). However, the magnitude and the overall distribution patterns can vary according to the exposure source (i.e. through water, sediment or food), concentration of the metal species available in the environment, exposure time and the absorption capacity of each organism (Zhou et al., 2008; Leonard et al., 2014). In our study, the higher Ni accumulation in the kidneys and liver could be linked to the fact that after acute exposure, the organs with the fastest metabolic activity tend to accumulate more metals (Klavins et al., 2009). Moreover, these organs are important detoxification and excretion pathways (Malik et al., 2010). The kidney is the principal organ for excretion of metal species (Vinodhini and Narayanan, 2008) and the liver is considered a target organ, since it plays a part in eliminating and storing metals. The liver's high accumulation capacity results in the presence of proteins (MT) that bind to the metals and lower their toxicity, allowing the liver to accumulate metals in high concentrations. This was confirmed by the results for MT concentration, which showed that the liver exhibits a significant dose-dependent increase in MT concentration after 24 h and 96 h exposure to Ni. For this reason, it has been suggested that the liver is the best indicator of chronic exposure to metals (Jarić et al., 2011). Although the kidney accumulated 6 times more Ni than the liver and gills after 96 h, only the liver and gills showed an increase in Ni after just 24 h exposure. It is also important to point out that the gills were the only organ to show dose-dependent increased Ni levels after exposure to the two highest concentrations. This is probably because the gills are in direct contact with waterborne metals and are considered the main site for the uptake of these contaminants (Ptashynski et al., 2002). In contrast, muscle was the organ that least accumulated Ni, and this fact has already been noted by other authors (Vinodhini and Narayanan, 2008; Malik et al., 2010). In our study, muscle showed Ni accumulation only at the highest concentration and after 96 h exposure, but assessments of the effects of chronic exposure on muscle tissue are clearly important, since muscle is the main source of nutrition for other organisms that feed on fish, and Ni could therefore enter the food chain in this way.

Metallothioneins are proteins that act as chelators, protecting cells from damage caused by excess metals (Coyle et al., 2002; Nordberg et al., 2007). In addition to reducing the toxic potential of metals, these proteins store metals for synthesizing metalloenzymes and can also act as antioxidants (Paris-Palacios et al., 2003; Falfushynska and Stolyar, 2009). An increase in MT concentration is one of the most widely acknowledged responses to exposure to metals, however there are only few studies which show a correlation between Ni exposure and MT production in fish (Ptashynski et al., 2002; Giguère et al., 2006; Leonard et al., 2014). In our study, examinations of the liver and gills of *P. lineatus* showed that within 24 h, production of MT in the gills was on average twice that in the liver, and after 96 h it reached on average three times that recorded for the control group. Studies have already shown that the highest induction of MT is associated with

the organs involved in uptake, accumulation and excretion, such as the gills and liver (Filipovi and Raspor, 2003; Amiard et al., 2006). MT synthesis induced by metals is a direct response to an increase in intracellular concentration of the metal, mediated by regulatory factors. This being the case, it is thought that the presence of Ni stimulates MT synthesis when the amount exceeds that needed to satisfy the organisms requirements. A number of studies on different fish species and other metals have shown that MTs bind to these metal ions, and that the quantities of MT are correlated with levels of these metals in the tissue (Olsvik et al., 2000; Filipovi and Raspor, 2003; De Boeck et al., 2010).

Ni interacts with the tissues and eventually inhibits the activity of antioxidant enzymes, resulting in oxidative stress (Kubrak et al., 2013). In the present study, in terms of hepatic antioxidant defenses, exposure to Ni for 96 h resulted in a reduction in SOD activity. This has already been observed in studies on other fish species (Parthiban and Muniyan, 2011; Zheng et al., 2014), and could indicate that the metal is affecting SOD. After 96 h, hepatic CAT also decreased at the two highest Ni exposure concentrations, and this could trigger an increase in the concentration of H_2O_2 which inhibits the activity of SOD (Sampson and Beckman, 2001). On the other hand, the drop in CAT activity could be associated with the binding of metal to –SH groups on the enzyme molecule, altering enzyme activity (Atli and Canli, 2007). Removing H_2O_2 is an important strategy used by aquatic organisms to counter oxidative stress. Consequently, the observed drop in liver CAT activity and the stability of GPx, which remains unchanged, may result in a failure to protect the liver cells against H_2O_2 production (Karadag et al., 2014). Hepatic GSH levels also remained unchanged and no significant variation was observed in GST and GR activities. GSH is one of the most important non-enzymatic antioxidants in the cell and GR is an enzyme responsible for catalyzing the reduction of GSSG (oxidized form) to GSH (reduced form) (Maran et al., 2009; Modesto and Martinez, 2010). Therefore, the lack of response in levels of GSH and in GR activity might have contributed for the occurrence of lipid peroxidation in the liver, after 96 h exposure to Ni 2500.

In gill tissue, exposure to nickel increased SOD activity at both exposure times indicating an increase in the antioxidant response in the gills after exposure to this metal. Furthermore, GSH levels significantly increased in the two higher Ni concentrations after 96 h exposure. It is important to point out that GSH plays a part in non-enzymatic antioxidant defense and is considered the primary line of defense against ROS, directly neutralizing the pro-oxidants or as a substrate for catalyzing enzymatic reactions by GST and GPx (Halliwell and Gutteridge, 2005). The increase in GSH levels is considered indicative of environmental stress (Ballesteros et al., 2009). Gill GST increased significantly over a period of 96 h at the highest Ni concentration, and this could be linked to cell protection and elimination of toxic compounds, since the gills are a major point of environmental contact for the organism and are responsible for various regulatory mechanisms, such as gaseous exchange, excretion, acid-base regulation and ion transport (Fernandes et al., 2007). These results show how Ni interferes in gill antioxidant defense, especially after 96 h exposure to the highest Ni concentration, implying that prolonged exposure at environmentally relevant concentrations could result in gill function impairment.

When the antioxidant defense system is insufficient or inactive, oxidative damage such as lipid peroxidation can occur. We know that oxidative stress occurs when the quantity of ROS increases significantly or the antioxidant defense system becomes inactive (Manoj and Padhy, 2013). In our study, we observed that the activation of antioxidant defenses was not sufficient to prevent the damage to the liver cells, given the significant increase in liver MDA levels after 96 h exposure to the highest Ni concentration. Metal-induced oxidative stress could give rise to structural changes in the enzymes and render them inactive (Che et al., 2007), as was observed in our study, where exposure to Ni resulted in a drop in hepatic SOD and CAT activity, causing oxidative damage. Excess Ni could have interacted with the H_2O_2 resulting in an increase in hydroxyl radical concentration via the Fenton reaction, this radical being the main cause of lipid peroxidation (Florence et al., 2002). The drop in CAT affects the Fenton reaction and increases the probability of lipid peroxidation (Bagnyukova et al., 2005). Nickel's capacity to induce LPO has already been described in various fish species, such as *Salvelinus namaycush* (Ptashynski et al., 2001), *Coregonus clupeaformis* (Ptashynski et al., 2002), *Cirrhinus mrigala* (Parthiban and Muniyan, 2011) and *Carassius auratus* (Zheng et al., 2014). The inhibition of the hepatic activity of antioxidant enzymes observed in our study suggests that the defense mechanisms are compromised in response to excessive generation of ROS causing the damage observed after 96 h. However, in the gills, the increase in SOD and GSH and the absence of any change in other antioxidant parameters could have helped in the organism's defense, since there was no increase in levels of MDA.

Our results indicated that Ni toxicity increases significantly in proportion to exposure time and that the antioxidant defense system was sufficient to prevent lipid oxidative damage only in the gill cells, since LPO was found in the liver. The gills are the first organs to be exposed to contaminants in the water, given their high contact surface area and permeability (Fernandes et al., 2007). Therefore, gill antioxidant defenses were activated on initial exposure to Ni, and were able to prevent LPO over the experimental periods. In other words, the organs examined vary in their sensitivity to Ni, and the liver was more sensitive to this pollutant than the gills.

Ni also proved to be genotoxic to *P. lineatus*, causing damage to the DNA in erythrocytes and gill cells. DNA integrity has been proposed as a sensitive and effective genotoxicity indicator for environmental monitoring (Frenzili et al., 2004) and we used the comet test to measure this kind of damage. It is one of the most widely used tests for assessing breaks in the DNA strands of aquatic animals (Ohe et al., 2004). We found that at almost all the Ni concentrations tested and for both exposure periods, there was a significant increase in the damage scores for blood and gill cells. The antioxidant defense pathways of these fish were probably insufficient to protect it against the possible damaging effects that Ni is capable of triggering in the DNA. However, we did detect a lower number of damaged nucleoids after 96 h exposure, both for erythrocytes and gill cells, compared to exposure for 24 h. This reduction could be due to the activity of the DNA repair system in fixing the breaks caused by exposure to Ni. On the other hand, it could also indicate that the cells had died, since if the repair system is inefficient or is not activated, cell death by necrosis or apoptosis can occur (Sandrini et al., 2009; Zheng et al., 2014). However, studies on the genotoxic effect on Ni on fish are still very rare.

The results of our study showed that Ni changes the antioxidant defenses of *P. lineatus*, and causes lipid peroxidation in the liver and DNA damage in gill cells and erythrocytes. Ni reached various tissues through circulation in the blood, accumulating in higher proportions in the kidneys and liver, organs whose function is to remove toxic substances from circulation to protect the organism. MT synthesis was also induced, probably to minimize the effects of the Ni. The various Ni treatments had significant effects on biochemical variables, and these parameters could be used as Ni exposure biomarkers in order to elucidate its toxicity mechanisms, in conjunction with the comet assay. It is also evident that the maximum permitted Ni concentration in Brazilian natural fresh water, set by CONAMA (2005), is unsafe for *P. lineatus*.

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