

Biomarkers of waterborne copper exposure in the Neotropical fish *Prochilodus lineatus*



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ABSTRACT

The main goal of the present study was to investigate the effects of acute exposure to copper (Cu) using a Neotropical freshwater fish as sentinel species through multi biomarkers analysis at different biological levels. Juveniles of *Prochilodus lineatus* were kept under control condition (no Cu addition in the water) or exposed to environmentally relevant concentrations of waterborne Cu (5, 9 and 20 µg L⁻¹) for 96 h. These concentrations were selected to bracket the current Brazilian water quality criteria for Cu in fresh water (9 and 13 µg L⁻¹ dissolved copper). Endpoints analyzed included ethoxyresorufin-O-deethylase (EROD), glutathione-S-transferase (GST), catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) activity, reduced glutathione (GSH) and metallothionein-like protein (MT) concentration, lipid peroxidation (LPO) level, tissue damage index, and incidence of free melano-macrophages (FMM) and melano-macrophage centers (MMC) in the liver. They also included DNA damage (frequency of nucleoids per comet class, number of damaged nucleoids per fish and DNA damage score) in erythrocytes, as well as muscle and brain acetylcholinesterase (AChE) activity and behavioral parameters (swimming distance and velocity, time spent swimming and swimming activity in the upper and lower layers of the water column). Fish exposed to any of the Cu concentrations tested showed increased liver MT concentration and LPO level, higher number of damaged nucleoids in erythrocytes per fish, and inhibited muscle AChE activity. Also, increased liver SOD activity was observed in fish exposed to 9 and 20 µg L⁻¹ Cu. Fish exposed to 5 and 9 µg L⁻¹ Cu spent lower amount of time swimming. Fish exposed to 9 µg L⁻¹ Cu showed increased swimming distance and velocity while those exposed to 20 µg L⁻¹ Cu had lower swimming distance and velocity, as well as, spent less time swimming in the lower layer of the water column when compared to those kept under control condition. These findings indicate that Cu exposure at environmentally relevant concentrations (below or close to the current Brazilian water quality criteria) induced significant biological (histological, biochemical and genetic) and ecological (swimming and exploratory abilities) damages in the Neotropical fish *P. lineatus*. They also suggest that MT concentration, DNA damage (comet assay), LPO (TBARS method), SOD and AChE activity, together with swimming behavior analyses are potential biomarkers to assess and monitor areas impacted by Cu in fresh water.

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1. Introduction

In recent decades, urbanization and industrialization processes have resulted in alarming quantities of metals contaminating

aquatic systems, over and above the contribution made by naturally occurring geochemical processes (Ellingsen et al., 2007). Copper (Cu) mining and industries producing wire and cables, electronic devices, wood preservatives, fungicides, fertilizer additives, antifouling paints, among others, significantly contribute to the contamination of freshwater ecosystems. Indeed, naturally occurring Cu concentrations in these sites can raise up to 100 µg L⁻¹ or even above 100 mg L⁻¹ in mining areas (Grosell, 2012).

Cu is a vital component for key biological functions, acting as a cofactor of several enzymes such as copper/zinc superoxide

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dismutase (CuZn-SOD), involved in antioxidant defense, and mitochondrial cytochrome c oxidase involved in cellular respiration in aerobic organisms (Ellingsen et al., 2007). Additionally, Cu plays an essential role in connective tissue formation, neurotransmitter biosynthesis and iron homeostasis (Liu et al., 2008; Grosell, 2012). However, copper in excessive amounts in different tissues can be toxic to freshwater fishes, and its mechanisms of toxicity have been studied at concentrations beyond the 96-h LC₅₀ (lethal concentration to 50% of the tested organisms after 96 h of waterborne exposure). These mechanisms include absorption of Cu by the gill, which mimics the essential ion sodium. Higher Cu concentrations in gill tissue block ion uptake by inhibiting ATP-dependent basolateral enzymes. Plasma Na⁺ and Cl⁻ concentrations decrease by both reduced active uptake and increased diffusive loss, leading to ionoregulatory and osmoregulatory disturbances that eventually cause lethality (Grosell, 2012; Wood, 2012).

Under certain circumstances, transition from Cu(II) to Cu(I) can also result in the generation of reactive oxygen species (ROS), which if not detoxified efficiently can induce damage to cell components (Camakaris et al., 1999) through processes such as lipid peroxidation (Baker et al., 1998; Kim et al., 2014) and genotoxic effects (Vutukuru et al., 2006; Mustafa et al., 2012). Sensory organs that are in direct contact with water such as the olfactory epithelia and lateral line (hair cells in neuromasts) are also important targets for Cu toxicity, leading to impairment of behavioral effects mediated by these organs such as avoidance responses (DeForest et al., 2011) and swimming orientation (Johnson et al., 2007), respectively. Other ecologically relevant behavioral effects of Cu in fishes include decreases in swimming resistance (Beaumont et al., 1995) and foraging (Sandheinrich and Atchison, 1990).

In tropical freshwater fishes, Cu was shown to disrupt ion regulation in the Amazonian tambaqui *Colossoma macropomum* (Matsuo et al., 2005) and osmoregulation in the tilapia *Oreochromis niloticus* (Saglam et al., 2013). It was also reported to affect cellular morphology in gill tissues of the curimba *Prochilodus scrofa* (Mazon et al., 2002). Also, this metal can be accumulated in other target organs such as liver and kidney in the common carp *Cyprinus carpio* and the gibel carp *Carassius auratus gibelio* (Boeck et al., 2004). In the Neotropical catfish *Rhamdia quelen*, Cu exposure was shown to modify the cellular morphology and affect the biotransformation and antioxidant capacity in these tissues (Mela et al., 2013). Hematological parameters were also affected by Cu exposure in *P. scrofa* (Carvalho and Fernandes, 2006).

The Neotropical fish *Prochilodus lineatus* (Valenciennes, 1836), formerly described as *P. scrofa* (Steindachner, 1881) is an ecologically and economically important benthic iliophagus species (Revaldaves et al., 1997), thus being subjected to direct contact with chemical contaminants available in sediments as well as dissolved in water (Colombo et al., 2007). Freshwater fishes show a wide range of sensitivity to Cu, with *P. lineatus* showing a 96-h LC₅₀ corresponding to 29 µg L⁻¹ Cu (Mazon and Fernandes, 1999). Sub-lethal effects include increases in hematocrit (Hct) and decreases in red blood cells (RBC) counting and hemoglobin (Hb) concentration after waterborne exposure of *P. lineatus* to 16 µg L⁻¹ Cu (Cerdeira and Fernandes 2002), as well as severe gill damage after exposure to 25 and 29 µg L⁻¹ Cu (Mazon et al., 2002).

The current Brazilian environmental regulation (Resolution 357/2005 of the National Council for Environment —CONAMA) establishes the water quality criteria for Cu in freshwater as 9 and 13 µg L⁻¹ (on a dissolved Cu basis), depending on the use of the water (CONAMA, 2005). However, 28% of rivers that were monitored from 2009 to 2013 in the state of São Paulo (Southwestern Brazil) showed dissolved concentrations of Cu above these limits (CETESB, 2014). At this point, it is interesting to note that water quality criteria are generally derived based on lethality. However, the same Brazilian regulation also establishes that water chemical

contamination cannot cause any alteration in behavior, reproduction or physiology of life. In this context, the biomarker approach can thus be used as a potential tool to evaluate and monitor water quality in freshwater ecosystems. This approach is generally used to predict the alterations induced by chemical pollutants on biological processes which could lead to a pathological state (Travis, 1993). It has been successfully applied to assess the toxicity of chemical compounds in field studies in freshwater systems (Gillis, 2012; Gillis et al., 2014). However, examples where chemically induced biochemical, physiological, or morphological perturbations have been mechanistically linked to ecologically relevant behavioral responses in fish are limited (Bradbury et al., 2008).

In light of the background above, the aim of the present study was to analyze the effects of waterborne Cu exposure on genetic, biochemical, histological, and behavioral parameters in *P. lineatus* acutely exposed to environmentally relevant concentrations of Cu. This approach, using a suit of biomarkers, considering different biological levels, can provide an integrated picture of high ecological relevance concerning Cu effects in the organism as a whole. Additionally, data generated would allow us to test the adequacy of the current Brazilian water quality criteria for dissolved Cu in freshwater systems using a Neotropical fish as sentinel species.

2. Material and methods

2.1. Fish handling, experimental design, Cu exposure and tissue sampling

Juveniles of *P. lineatus* (10.1 ± 3.5 g wet body mass; 9.8 ± 1.2 cm total body length; mean \pm SD) were supplied by the Fish Hatchery Station of the State University of Londrina. Fish ($N=64$) were acclimated for five days in a 300-L tank containing dechlorinated tap water under constant aeration. Room photoperiod was fixed at a 12 h light:12 h dark cycle. The chemical and physical characteristics of the acclimation water were monitored continuously using a multiparameter water quality meter (Hanna HI9828, USA). The values (mean \pm SE) for temperature, pH, dissolved oxygen and conductivity of the water corresponded to 20.9 ± 0.8 °C, 7.1 ± 0.2 , 7.3 ± 0.4 mg O₂ L⁻¹, and 102.2 ± 6.8 µS cm⁻¹, respectively. At the second and fourth day of the acclimation period, fish were fed with commercial fish diet with 36% protein (Guabi, Brazil). Feeding was suspended 24 h prior to the beginning of the experiments and the animals were not fed during the experiments.

After acclimation, fish were randomly divided into four groups ($N=8$ fish per group). One group was kept under control condition (no Cu addition into the water) and the other three groups were exposed to different concentrations of waterborne Cu (5, 9 and 20 µg L⁻¹) for 96 h. Fish were exposed in glass aquaria containing 80 L of dechlorinated tap water. Water temperature, pH, dissolved oxygen, conductivity and hardness were monitored throughout the experiment. During the experiment, values (mean \pm SE) for water temperature, pH, dissolved oxygen, conductivity and hardness corresponded to 20.7 ± 0.5 °C, 7.0 ± 0.3 , 6.9 ± 0.4 mg O₂ L⁻¹, 94.5 ± 0.02 µS cm⁻¹, and 44.4 ± 2.6 mg CaCO₃ L⁻¹, respectively.

Every day, non-filtered and filtered (0.45-µm mesh filter, Millipore Millex HV/PVDF) water samples were collected from all aquaria for analysis of total and dissolved Cu concentration, respectively. All samples were acidified with HNO₃ and stored at 4 °C until Cu concentration measurements. Total and dissolved Cu concentrations were measured by graphite atomizer, atomic absorption spectroscopy (EAA ANALYST 700, PerkinElmer), with detection limit at 0.0014 µg L⁻¹.

After exposure, fish were anesthetized with benzocaine (0.1 g L⁻¹) and blood samples were collected by the caudal vein puncture and processed for the comet assay, as described below.

Fish were then killed by medullar sectioning, measured (total body length), weighed (wet body mass) and had the liver, brain and muscle dissected and stored at -80°C for biochemical analysis, as further described. Subsamples of the liver were fixed for histopathological analysis as described below.

2.2. Biochemical and histological parameters in liver

Individual liver samples were homogenized (10 mL g^{-1} tissue) in a phosphate buffer solution (0.1 M; pH 7.2). Samples were centrifuged ($15,000 \times g$, 30 min, 4°C) and the supernatants obtained were collected for analysis of the activity of biotransformation enzymes, antioxidant parameters and lipid peroxidation, as described below. For all biochemical biomarkers, the protein content of each liver supernatant fraction was determined using the Bradford's method (Bradford, 1976), using bovine serum albumin as standard.

2.2.1. Metallothionein-like proteins

The concentration of metallothionein-like proteins (MT) was determined following the methodology described by Viarengo et al. (1997) with modifications. Liver samples were homogenized (1:3 w/v) in buffer solution (0.5 M sucrose, 26 mM Tris, 0.5 mM phenylmethylsulfonyl fluoride, 1.3 mM β -mercaptoethanol) and centrifuged ($18,650 \times g$; 45 min, 4°C). The resulting supernatant was collected and subjected to ethanol/acid chloroform fractionation to obtain a partially purified metalloprotein fraction. Sulphydryl groups ($-\text{SH}$) were determined in this fraction with a spectrophotometer (412 nm), using the Ellman's reagent. GSH was used as standard and the MT concentration was expressed in nmol GSH mg protein $^{-1}$.

2.2.2. Antioxidants

Catalase (CAT) activity was determined according to the technique described by Beutler (1975), by monitoring the H_2O_2 decomposition from the decrease of absorbance at 240 nm. CAT activity was expressed in $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg protein}^{-1}$.

Selenium-dependent glutathione peroxidase (Se-GPx) activity was determined using the method described by Hopkins and Tudhope (1973), which is based on NADPH oxidation in the presence of reduced glutathione (GSH) at 0.95 mM and H_2O_2 at 340 nm. GPx activity was expressed in $\mu\text{mol oxidized NADPH min}^{-1} \text{ mg protein}^{-1}$, using a molar extinction coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$.

Copper-zinc superoxide dismutase (CuZn-SOD) activity was determined according to McCord and Fridovich (1969). This method is based on the measurement of the inhibition of the reduction rate of cytochrome c by the superoxide radical at 550 nm and 25°C . SOD activity was expressed in SOD units mg protein $^{-1}$, with one unit of SOD corresponding to the quantity of enzyme that promoted the inhibition of 50% of the reduction rate of cytochrome c.

GSH concentration was determined according to the method described by Beutler et al. (1963), using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). Supernatants of acid extracts [1:1 v/v with 12% trichloroacetic acid (TCA)] were added to 0.25 mM DTNB in 0.1 M potassium phosphate buffer (pH 8.0). Thiolate anion formation was determined at 412 nm against a GSH standard curve. GSH concentration was expressed in $\mu\text{g GSH mg protein}^{-1}$, based on a standard curve built with GSH standard solutions (10–200 μmol).

2.2.3. Lipid peroxidation

The thiobarbituric acid reactive substances (TBARS) assay was performed according to Federici et al. (2007) as a measure of lipid peroxidation. Butylated hydroxytoluene (BHT; 1 M), phosphate buffer solution (2 mM KCl; 1.4 mM NaH_2PO_4 ; 357 mM NaCl;

10 mM Na 2HPO₄; pH 7.4), 50% TCA and 1.3% thiobarbituric acid dissolved in 0.3% NaOH were added to the liver supernatant and the mixture was incubated at 60°C for 1 h. Absorbance reading was performed in a spectrophotometer at 530 nm and the TBARS concentration was determined based on a standard curve built with malondialdehyde (MDA). TBARS concentration was expressed in $\mu\text{mol MDA mg protein}^{-1}$.

2.2.4. Biotransformation enzymes

EROD (7-ethoxyresorufin-O-deethylase) activity was measured using a protocol adapted for fluorescence microplate reader based on Burke and Mayer (1974). The assay was made of 50 μL of sample and 200 μL of reaction solution (0.1 M Tris; pH 7.5; 0.1 M NaCl; 2.6 μM 7-ethoxy-resorufin), which were incubated for 5 min in a microplate. Reaction was started by the addition of 10 μL of 2.6 mM NADPH. Resorufin formation was measured fluorometrically using the wavelength of 530 nm for excitation and 590 nm for emission. Measurements were performed at 27°C for 10 min. EROD activity was expressed in pmol resorufin $\text{min}^{-1} \text{ mg}^{-1}$ proteins.

Glutathione-S-transferase (GST) activity was determined as described by Keen et al. (1976) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Sample absorbance reading was performed at 340 nm. Enzyme activity was expressed as nmol CDNB conjugate formed $\text{min}^{-1} \text{ mg}^{-1}$ protein using a molar extinction coefficient of 9.6 mM cm^{-1} .

2.2.5. Histological parameters

Liver samples were preserved in Alfac fixative solution (ethanol 80%–85 mL; formaldehyde 40%–10 mL; glacial acetic acid–5 mL) for 16 h, dehydrated in a graded series of ethanol baths, and embedded in Paraplast Plus resin (Sigma). Sections (3–5 μm) were stained with hematoxinil/eosin and visualized in a Zeiss Axiophot photomicroscope. Free melano-macrophages (FMM) and melanomacrophages centers (MMC) were evaluated according to Rabitto et al. (2005). A liver lesion index was determined according to the method established by Bernet et al. (1999) and described in further details by Mela et al. (2013).

2.3. Genetic parameters in erythrocytes

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

The alkaline comet assay was performed in fish erythrocytes following procedures described by Singh et al. (1988). In studies with fishes comet assay is generally performed on peripheral blood erythrocytes due to their easy sampling with no need for a cell isolation step (Kilemade et al., 2004; Cavalcante et al., 2008). After sampling, an aliquot of blood was added to the low melting point agarose (0.5%). This mixture was placed on a glass slide previously covered with standard agarose, covered with coverslip, and remained in the refrigerator for 30 min. Slides were then subjected to the following steps: (a) lysis—1 h at 4°C , protected from light, in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1 mL Triton X-100, pH 10.0); (b) DNA denaturation—30 min in the dark in an electrophoresis buffer solution (0.3 N NaOH, 1 mM EDTA, pH 13); (c) electrophoresis—20 min, 300 mA, 25 V, 1 V cm^{-1} ; and (d) neutralization—three rinses for 5 min each with buffer solution (0.4 M Tris, pH 7.5). Slides were fixed with absolute ethanol for 10 min and kept under refrigeration until cytological analyses. Slides were stained with gelRed and analyzed under a Leica microscope (DM 2500) adapted for fluorescence/epifluorescence at 1000 \times 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 and non-overlapping nucleoids per fish. DNA damage was classified considering four classes, as following: 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 more than two times the diameter of the nucleus. Photomicrographs of *P. lineatus* erythrocytes showing nucleoids with different comet classes are shown in the study of Palermo et al. (2015). DNA damage score was obtained for 100 comets by multiplying the number of cells in each class by the damage class, and ranged from 0 (all undamaged) to 300 (all maximally damaged).

2.4. Acetylcholinesterase activity in muscle and brain

Muscle and brain samples were homogenized in phosphate buffer solution (0.1 M, pH 7.5) and centrifuged (10,000 × g, 20 min, 4 °C). Acetylcholinesterase (AChE) activity was measured spectrophotometrically at 405 nm, following the method described by Ellman et al. (1961). Results were expressed in nmol min⁻¹ mg protein⁻¹.

2.5. Behavioral parameters

To evaluate the effects of Cu on the spontaneous swimming activity, additional groups of juvenile *P. lineatus* (wet body weight = 12.2 ± 4.9 g, total body length = 10.1 ± 1.4 cm, n = 10 per treatment) were kept under control condition or exposed to Cu at 5, 9 and 20 µg L⁻¹ for 96 h, as previously described. After exposure, fish were individually transferred to a 30-L aquarium (40 × 50 × 15 cm water column height × length × width), installed inside an insulation box fitted with opaque glass on one side to allow back lighting. A video camera on the opposite side provided a lateral view of the fish, which was allowed to swim up and down for 40 cm (water column height) and swim towards or away for 15 cm (aquarium width) from the camera. Spontaneous swimming activity was analyzed by capturing videos using the SACAM software (Jorge et al., 2005), which calculated the following parameters: average swimming distance (cm) and velocity (cm s⁻¹), time spent swimming (s), and % of time spent in upper or lower layer of the water column. In last case, the water column was divided into two parts of equal heights. Each fish was tested individually and videos were recorded for 15 min, preceded by five min of fish adaptation to the test aquarium.

2.6. Statistical analysis

Data were expressed as mean ± SE. After checking data for normality and homoscedasticity, mean values for the different treatments were cross-compared using parametric (ANOVA) or non-parametric analysis of variance (Kruskal–Wallis), followed by the Student–Newman–Keuls (SNK) or Dunn's test, respectively. The significance level adopted was 95% (*p* < 0.05).

3. Results

3.1. Cu concentrations

Total and dissolved Cu concentrations values for the treatments are shown in Table 1. Total and dissolved Cu concentrations were similar in the control treatment (no Cu addition into the water) and corresponded to 2.6 and 2.2 µg L⁻¹. In average, mean dissolved Cu concentrations corresponded to 78.4% of the total Cu concentration. In Cu treatments, dissolved Cu concentrations deviated only 15.3% of the expected nominal concentrations. Therefore, thereafter Cu

Table 1

Total and dissolved copper concentrations in the water used to expose the Neotropical fish *Prochilodus lineatus* to waterborne copper at different concentrations. Data are expressed as mean ± SE (*n* = 5–8).

| | Nominal copper concentration (µg L ⁻¹) | | | |
|----------------|--|-----------|------------|------------|
| | 0 | 5 | 9 | 20 |
| Total measured | 2.6 ± 0.2 | 8.9 ± 0.8 | 13.0 ± 0.7 | 23.8 ± 0.5 |
| Dissolved | 2.2 ± 0.1 | 6.2 ± 0.1 | 9.6 ± 0.1 | 20.3 ± 0.3 |

concentrations will be referred using the nominal concentrations for simplicity.

3.2. Biochemical parameters in liver

Fish exposed to Cu showed no significant difference in EROD, GST (Fig. 1B), CAT (Fig. 1C) and GPx (Fig. 1D) activity when compared to those kept under the control condition. However, SOD activity was 87.5 and 81.8% higher in liver of fish exposed to 9 and 20 µg L⁻¹ Cu than in liver of control fish (15.75 U SOD mg of protein⁻¹), respectively (Fig. 1E). GSH concentration was not affected by Cu exposure (Fig. 1F). MT concentration was 93.0, 112.5 and 110.5% increased in liver of fish exposed to 5, 9 and 20 µg L⁻¹ Cu when compared to the MT concentration observed in liver of control fish (4.87 nmol GSH mg protein⁻¹, respectively (Fig. 1G)). LPO concentration was 277.5, 374.7 and 234.6% increased in liver of fish exposed to 5, 9 and 20 µg L⁻¹ Cu when compared to the level found in liver of fish kept under control condition (0.157 µmol MDA mg protein⁻¹, respectively (Fig. 1H)). The lowest observed effect concentration (LOEC) for SOD, MT and LPO corresponded to 9, 5 and 5 µg L⁻¹, respectively.

3.3. Histological parameters in liver

Liver of both control and Cu-exposed fish exhibited a typical histology pattern, which was already described for most teleost fish, i.e., a very homogeneous tissue with sinusoids and polyhedral hepatocytes arranged in cords with spherical nuclei. FMM were observed only in Cu-exposed fish (Fig. 2A). MMCs showed up as granular or heterogeneous pigmented material (ranging from yellow to dark brown) and their presence was observed in both control and Cu-exposed fish (Fig. 2B). The Bernet's lesion index was not different among treatments (Fig. 2C).

3.4. Genetic parameters in erythrocytes

The number of damaged nucleoids per fish was higher in erythrocytes of Cu-exposed fish than in those of control fish (Table 2). Mean value of DNA damage score was 147% and 220% higher in erythrocytes of fish exposed to 5 and 20 µg L⁻¹ Cu when compared to the mean value of DNA damage score (65) observed in control fish, respectively. Erythrocytes of fish exposed to the highest Cu concentration showed mainly comets of class 3 (44%), followed by classes 2 and 1. The LOEC for the number of damaged nucleoids and DNA damage score was 5 µg L⁻¹ (Table 2).

3.5. Acetylcholinesterase activity in muscle and brain

Muscle AChE activity in control fish corresponded to 451.6 nmol min⁻¹ mg protein⁻¹. It was inhibited by 40.3, 42.7 and 41.8% in fish exposed to 5, 9 and 20 µg L⁻¹ Cu, respectively. The LOEC was 5 µg L⁻¹ (Fig. 3A). Brain AChE activity was not affected by Cu exposure (Fig. 3B).

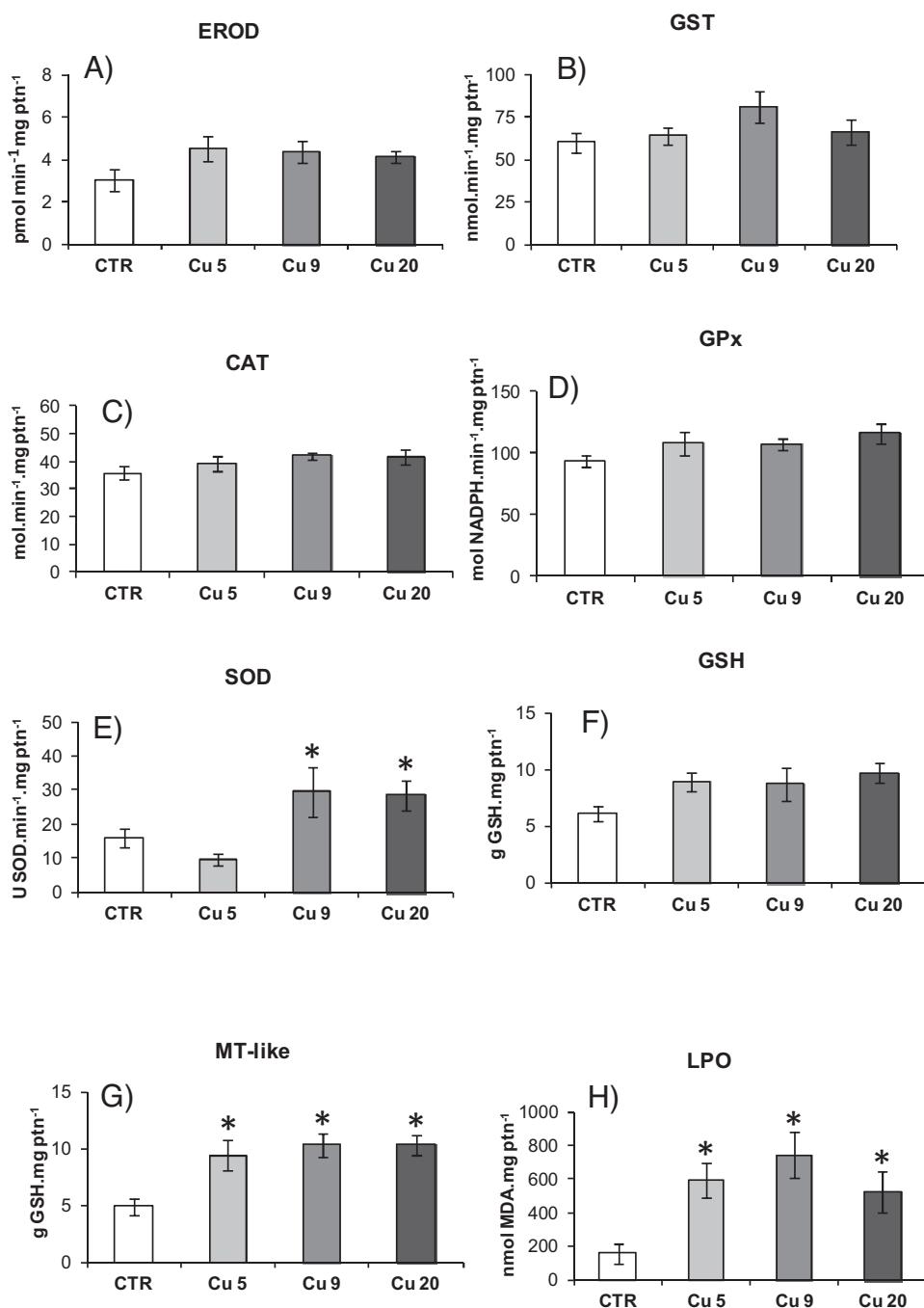


Fig. 1. 7-Ethoxresorufin O-deethylase (EROD) activity (A), glutathione-S-transferase (B), catalase (C), glutathione peroxidase (D) and superoxide dismutase (E) activity, GSH concentration (F), metallothionein-like proteins concentration (G), and lipid peroxidation (H) in liver of juvenile *Prochilodus lineatus* kept under control condition (CTR) or exposed to waterborne copper at 5, 9 and 20 $\mu\text{g L}^{-1}$ (nominal concentrations). Data are mean \pm SE, $n=6-8$. *Indicates different mean value from the control ($p < 0.05$).

Table 2

Mean frequency (%) of nucleoids per Comet class (0, 1, 2 and 3), number of damaged nucleoids per fish (mean \pm SE) and DNA damage score (mean \pm SE) in erythrocytes of the Neotropical fish *Prochilodus lineatus* exposed to different concentrations of waterborne copper (nominally: 0, 5, 9 and 20 $\mu\text{g L}^{-1}$). N: number of fish analyzed.

| Copper concentration ($\mu\text{g L}^{-1}$) | N | Comet class | | | | Damaged nucleoids | DNA damage |
|---|---|-------------|------|------|------|-------------------|-------------------|
| | | 0 | 1 | 2 | 3 | | |
| 0 | 5 | 31.8 | 64.6 | 0.2 | 0 | 64.8 \pm 6.8 | 65.0 \pm 6.7 |
| 5 | 5 | 7.5 | 37.2 | 43.0 | 12.6 | 92.8 \pm 5.7* | 161.0 \pm 28.6* |
| 9 | 5 | 15.6 | 71.9 | 12.0 | 0.5 | 84.4 \pm 2.7* | 115.0 \pm 10.6 |
| 20 | 7 | 6.1 | 23.7 | 25.7 | 44.4 | 93.8 \pm 2.4* | 208.4 \pm 28.9* |

* Indicates mean value significant different from the control condition (0 $\mu\text{g L}^{-1}$).

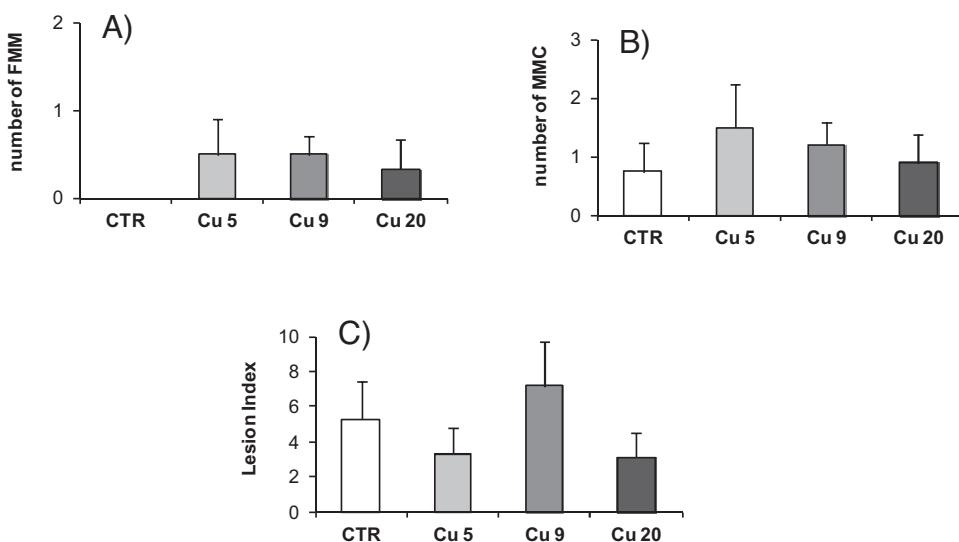


Fig. 2. Number of free melano-macrophages (FMM) (A), number of melano-macrophage centers (MMC) (B), and index of lesion injury (C) in liver of juvenile *Prochilodus lineatus* kept under control condition (CTR) or exposed to waterborne copper at 5, 9 and 20 $\mu\text{g L}^{-1}$ (nominal concentrations). Data are mean \pm SE, $n=6-8$. *Indicates different mean value from the control ($p < 0.05$).

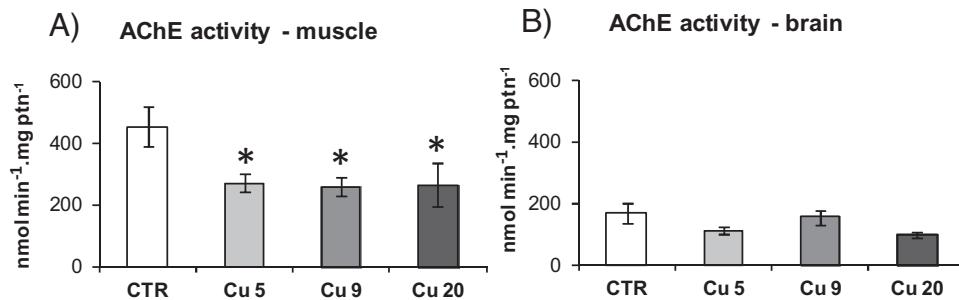


Fig. 3. Acetylcholinesterase activity in muscle (A) and brain (B) of juvenile *Prochilodus lineatus* kept under control condition (CTR) or exposed to waterborne copper at 5, 9 and 20 $\mu\text{g L}^{-1}$ (nominal concentrations). Data are mean \pm SE, $n=6-8$. *Indicates different mean value from the control ($p < 0.05$).

3.6. Behavioral parameters

Spontaneous swimming velocity was non-monotonically affected by Cu exposure. It was 337.9% increased in fish exposed to 9 $\mu\text{g L}^{-1}$ Cu and 67.4% reduced in fish exposed to 20 $\mu\text{g L}^{-1}$ Cu when compared to the mean value observed in control fish (2.25 cm s^{-1}). A similar pattern of response was observed for the swimming distance (Fig. 4A). Time spent swimming was also non-monotonically affected by Cu exposure. It was reduced by around 45% in fish exposed to 5 and 9 $\mu\text{g L}^{-1}$ Cu when compared to the mean value (625 s) observed in control fish (Fig. 4B). Control fish spent more than 80% of the swimming time in the lower layer of the water column. A similar pattern was found in fish exposed to 5 and 9 $\mu\text{g L}^{-1}$ Cu. However, a significant effect was observed in fish exposed to 20 $\mu\text{g L}^{-1}$ Cu, with fish spending more than 90% of the swimming time in the upper layer of the water column (Fig. 4C). The LOEC for swimming velocity, distance swam, time spent swimming and % of time spent in the different layers of water column corresponded to 9, 9, 5 and 20 $\mu\text{g L}^{-1}$, respectively.

4. Discussion

In the present study, Cu effects were observed in an ecologically and economically important Neotropical freshwater fish at environmentally relevant waterborne concentrations of the metal. Furthermore, the observed effects were seen in key parameters at different levels of biological organization. In addition, our data

indicate that the current Brazilian water quality criteria for Cu in fresh water guidelines do not protect juveniles of the Neotropical fish *P. lineatus* against several relevant biological effects of this metal. Acute exposure to dissolved concentrations of waterborne Cu at or below the current criteria (9 $\mu\text{g L}^{-1}$ dissolved Cu; CONAMA, 2005) induced the liver defense system against metal exposure by increasing metal and ROS scavenging capacity (increased MT concentration and SOD activity, respectively). However, the ability to deal with the metal exposure was overwhelmed in Cu-exposed fish, resulting in oxidative damage to lipids and DNA (genotoxic damage). In addition, Cu exposure also induced behavioral effects of ecological relevance (alteration in swimming pattern likely associated with muscle AChE inhibition). Many chemically induced biochemical, physiological, or morphological perturbations have been reported in cellular and organismal systems, but consequent behavioral effects on organism have not been established (Bradbury et al., 2008). Therefore our study showed that behavioral responses integrate genetic, biochemical and physiological processes. Thus, linking behavioral alterations to these types of observations we demonstrated the bridge between subcellular and cellular responses and ecological consequences (Bradbury et al., 2008).

The increase in liver MT levels is a well studied biochemical response and a useful biomarker for metal exposure in aquatic organisms (Amiard et al., 2006). MTs have been implicated in homeostasis and detoxification of trace metals in biological systems. The capabilities of a metal Cu to participate in cellular

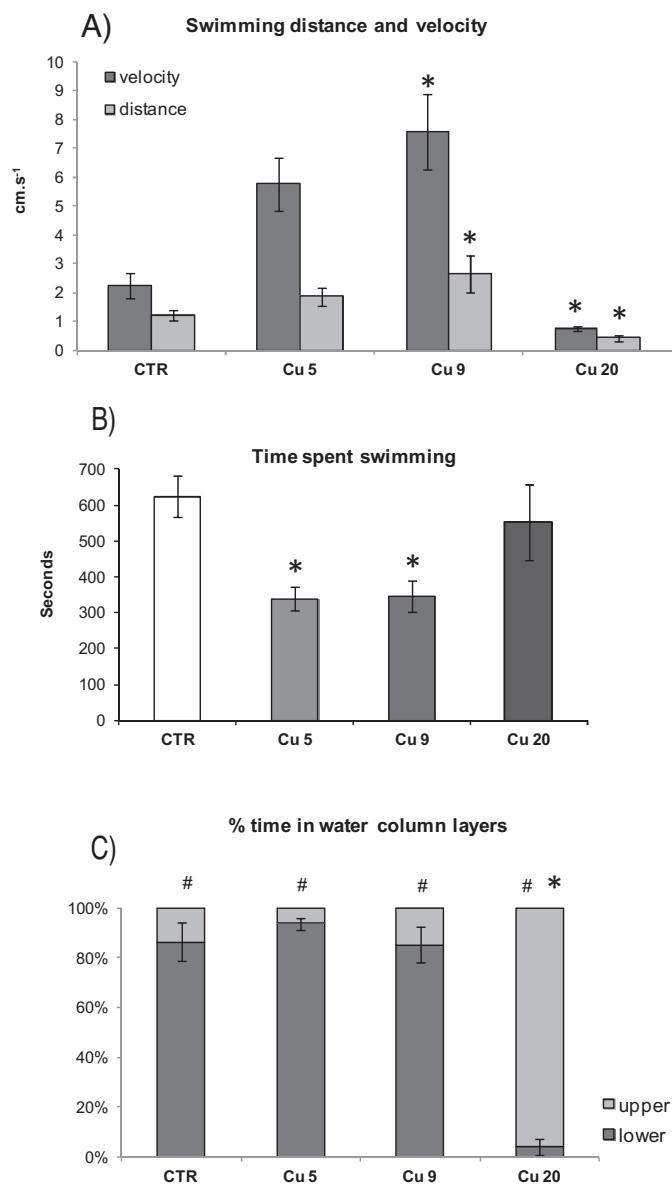


Fig. 4. Swimming distance ($\text{cm} \times 100$) and velocity (cm s^{-1}) (A), time spent swimming (s) (B) and % of time swimming spent in upper or lower layer of the water column (C) in juvenile *Prochilodus lineatus* kept under control condition (CTR) or exposed to waterborne copper at 5, 9 and 20 $\mu\text{g L}^{-1}$ (nominal concentrations). Data are mean \pm SE, $n=6-8$. *Indicates different mean value from the control. #Indicates mean values different for upper and lower layer of water column ($p < 0.05$).

generation of hydroxyl radicals ($\cdot\text{OH}$), in addition to its role as an essential nutrient, may underlie the existence of proteins such as MTs that tightly regulate the cellular transport and functions of these metals. In addition to reducing the toxic potential of metals, MTs can act as non-toxic metal reserves for metalloenzyme synthesis, protect against ionizing radiation and play a role as antioxidant (Di Giulio and Meyer, 2008). Liver is the main organ involved in Cu homeostasis (Mazon and Fernandes, 1999). The potency of the intracellular environment in chelating Cu is further boosted when Cu detoxification and storage systems, such as metal-binding proteins (MTs), are induced (Veld and Nacci, 2008).

Liver MT concentration was significantly increased when juvenile *P. lineatus* were acutely (96 h) exposed to Cu at any of the three concentrations tested (5, 9 and 20 $\mu\text{g L}^{-1}$). This finding is in agreement with a previous study performed with *P. lineatus* exposed to 16 $\mu\text{g Cu L}^{-1}$ (pH 8.0) and 98 $\mu\text{g Cu L}^{-1}$ (pH 4.5) (Carvalho et al.,

2004, 2015). In addition, previous studies have reported the effect of Cu on the increased MT expression in another freshwater fish, the common carp *C. carpio* exposed to 0.97 μM Cu (Boeck et al., 2003). These results indicate that liver MT concentration, which is already recognized as a biomarker in areas impacted with metals, proved to be a sensitive biomarker using this fish species.

In addition to MT, other defense systems are also playing an essential role in protecting aquatic organisms against the effects of metal exposure. In this context, antioxidant defense pathways comprise a range of potential and useful biomarkers for investigating ecotoxicological aspects. They provide sensitive and practical metrics for detection of environmental stress, reflecting the adverse biological responses towards anthropogenic environmental substances (Heath, 1995; Van Der Oost et al., 2003). However, their responses are variable, depending on the species and contaminant evaluated. They are not specific to particular pollutants and should

therefore be investigated in combination with other parameters rather than isolated. Redox active metals such as Cu can cause significant increases in ROS and may exert toxicity related to oxidative stress, which can lead to dysfunction in enzymes, lipids and DNA (Veld and Nacci, 2008).

Previous studies have shown that Cu exposure can either activate or inhibit antioxidant enzyme activities in fish, depending on the metal concentration, species and exposure route (Sanchez et al., 2005). In general, the response of the antioxidant defense system in fish results from a combination of biotic and abiotic factors (Martínez-Álvarez et al., 2005). In the present study, only SOD showed a significant response to Cu exposure, being increased in the liver of fish exposed to the two highest concentrations tested (9 and 20 $\mu\text{g L}^{-1}$ Cu). This effect may be related to the Cu ability in promoting the generation of ROS through the Fenton reaction (Hermes-Lima, 2004; Di Giulio and Meyer, 2008). In particular, Cu induces redox cycles through the Fenton reaction, which can accelerate the formation of hydroxyl radicals and cause severe oxidative damage to cell membranes (Eyckmans et al., 2011). The increased SOD activity combined with the lack of changes in the other antioxidant parameters analyzed (CAT, GPx, GST and GSH) led to an oxidative stress condition (increased LPO and DNA damage), indicating a significant Cu-induced disturbance in tissue/cell oxidative status (Kim et al., 2014).

Liver is the main organ involved in the detoxification and excretion of chemical pollutants. Indeed, it is highly specialized and uses various mechanisms to prevent cell damage (Mazon and Fernandes, 1999). However, liver tissue tends to exhibit higher metabolic activity than other tissues, suggesting that it could be more sensitive to toxic pollutants (Atli et al., 2006). In fact, increased liver LPO (TBARS method) and SOD activity were detected in the present study with LOECs corresponding to 5 and 9 $\mu\text{g L}^{-1}$ Cu, respectively. This finding suggests that liver LPO and SOD activity are sensitive to acute exposure to environmentally relevant Cu concentrations and can be potential biomarkers of Cu contamination in freshwater using the Neotropical characid *P. lineatus* as sentinel species, as previously reported for the cyprinid fish *Zacco platypus* exposed to 1.0, 4.5 and 15.0 $\mu\text{g L}^{-1}$ Cu for 4 days (Kim et al., 2014).

Although many studies have been conducted on Cu toxicity, there is paucity of information relating to Cu-induced DNA damage in aquatic organisms, especially fish (Mustafa et al., 2012). Cu is known to induce cellular and tissue damage, either through the generation of ROS or by direct interaction with biomolecules (Amado et al., 2006). However, fish DNA seems to be very sensitive to Cu exposure at environmentally relevant concentrations of the metal. In fact, a significant increase in the number of damaged nucleoids was observed in erythrocytes after acute exposure to dissolved Cu at 5 $\mu\text{g L}^{-1}$. Also, erythrocyte DNA damage based on the comet assay was also observed in the stickleback *Gasterosteus aculeatus* exposed to 3.2 $\mu\text{g L}^{-1}$ Cu (Santos et al., 2010). These findings suggest that DNA damage, measured through comet assay, is a potential biomarker of Cu exposure in freshwater fish, including the Neotropical curimbatá *P. lineatus*.

In addition to the biochemical and genetic effects discussed above, liver of Cu-exposed fish also showed the presence of FMM. Few studies have reported the presence of MMC in *P. lineatus* (Troncoso et al., 2012), and at the best of our knowledge the present study is the first to demonstrate the presence of FMM in this Neotropical fish species. According to Costa et al. (2009), teleost FMM are more nodular and more common in the spleen and kidney than in the liver. Indeed, they can be even absent in the liver of some species. Moreover, the number of FMM varies according to the fish species (Haaparanta et al., 1996). Some authors have suggested that histopathological analysis involving melano-macrophages could provide sensitive indicators of fish health or the occurrence of stressful environmental conditions (Rabitto et al., 2005). A higher

incidence of MMC is generally related to important hepatic lesion, such as degenerative and necrotic processes (Rios et al., 2007; Costa et al., 2011). However, the number of MMC was not affected by Cu exposure in *P. lineatus*. However, the presence of FMM was observed in all Cu-exposed fish and not in control fish, suggesting that groups of isolated FMM may function as MMC in *P. lineatus*, as reported for other species. However, the observed response seems to be complex and could not serve as useful biomarker under the experimental conditions tested in the present study.

Another morphological parameter analyzed in the liver of *P. lineatus* was the lesion index according to Bernet et al. (1999). As FMM and MMC, this parameter showed not to be a good biomarker of Cu exposure. The lack of morphological changes in the liver tissue of this species could be associated with the experimental conditions used in the present study (exposure time and Cu concentrations). In fact, significant morphological changes have been observed in the liver of the Neotropical catfish *Rhamdia quelen* after acute exposure to Cu (Mela et al., 2013). Indeed, it is also reported that longer exposure to Cu was responsible for an increased occurrence of morphological changes in the liver of the goby fish *Synechogobius hasta* (Liu et al., 2010). Another possibility to explain the lack of change in the liver of *P. lineatus* after acute Cu exposure is to consider that this species could more tolerant to copper than the other species of Neotropical fish tested (Mela et al., 2013). Thus, despite of the wide use of histological alterations as biomarker, in the present study the lack of morphological responses indicates that Cu did not cause significant histological changes in the liver of this fish.

Biochemical and genetic changes induced by Cu exposure, as observed in the present study with the Neotropical fish *P. lineatus*, are of great concern and high toxicological relevance. However, in addition to these parameters we have analyzed the Cu effects on swimming behavior. The behavior of a fish reflects the integrated output of the nervous system at the organismal level in response to stimuli perceived in the environment. Chemically induced behavioral changes are ecologically relevant and must be considered in terms of adverse effects on an organism's ability to survive and reproduce (Bradbury et al., 2008). In fact, effects of chemical contaminants on swimming behavior have been little explored and however can be consistent indicators of mal-adaptive responses in fish exposed to these contaminants (Little and Finger, 1990).

In the present study, time spent swimming decreased by ~55% in juvenile *P. lineatus* exposed to 5 or 9 $\mu\text{g L}^{-1}$ Cu when compared to control fish. However, this response was paralleled by increased swimming velocity and distance in fish exposed to 9 $\mu\text{g L}^{-1}$ Cu. This can be explained by the darting swimming behavior that was observed, with bursts of high speed swimming. A similar behavior was reported in the zebrafish *Danio rerio* exposed to 16 and 40 $\mu\text{g L}^{-1}$ Cu (Tilton et al., 2011). On the other hand, *P. lineatus* exposed to 20 $\mu\text{g L}^{-1}$ Cu spent as much time as the control fish swimming, but exhibited a circling behavior and swam at a significantly lower speed, remaining in the upper layer of the water column. This pattern is in line with the findings reported by Schmidt et al. (2005) for the carp *C. carpio* exposed to tributyltin-chloride (TBT). These authors attributed this behavior to an increased metabolic rate with a consequent higher demand for oxygen during TBT exposure. Another possible explanation for this surface swimming behavior is that gas exchange is impaired due to gill lesions induced by Cu exposure. In fact, lamellar histological damage induced by Cu has been described in other fish species such as *C. carassius* (Schjolden et al., 2007), *Pelteobagrus fulvidraco* (Chen et al., 2012), *C. carpio* (Karan et al., 1998) and *S. hasta* (Liu et al., 2010).

The different pattern of effects in swimming behavior at the highest concentration of Cu tested (20 $\mu\text{g L}^{-1}$) may be due to damage that goes beyond the peripheral nervous system. Due the inhibition of AChE in brain and in muscle produces alterations in

behavior and leads to hyperstimulation of muscle fibers, the determination of AChE activity is usually evaluated in brain and muscle tissue because the neuromuscular system of fish is principally cholinergic and its activity is essential for normal muscle function (Payne et al., 1996). Although no change in brain AChE activity was observed in *P. lineatus*, the behavior showed by Cu-exposed fish suggests possible effects on brain areas related to balance and orientation. Erratic swimming, darting and loss of balance have been observed in *Labeo rohita* exposed to malathion (Patil and David, 2010). These effects were attributed to the hyperexcitation caused by inhibition of AChE activity, which was also observed in *P. lineatus* exposed to any of the concentrations of Cu tested (5, 9 and 20 µg L⁻¹). Although a mechanistic explanation for the AChE inhibition observed after exposure to metals has not been established, Frasco et al. (2005) reported that Cu inhibited AChE activity in vitro experiments. Also, Cu at high concentrations was shown to inhibit AChE activity in gastropods (Cunha et al., 2007).

The effect of Cu on swimming velocity in *P. lineatus* was non-monotonic, increasing at 9 µg L⁻¹ Cu and decreasing at 20 µg L⁻¹ Cu. A similar pattern was also reported for the guppy *Poecilia vivipara* exposed to 200 and 500 µg L⁻¹ phenanthrene, when a significant alteration in the pattern of habitat exploration and ability to capture prey were also evident relative to control fish (Torreiro-Melo et al., 2015). The alterations on swimming behavior observed in *P. lineatus* involving bursts of higher swimming velocities at 9 µg L⁻¹ Cu or slower velocity circle swimming in the surface at 20 µg L⁻¹ Cu could affect prey capture abilities of exposed fish, another ecologically relevant behavioral parameter.

Regarding environmental guidelines, it is important to note that Brazil has established many of its water quality criteria based on North American and European standards (Martins and Bianchini, 2011). However, climate and continental watersheds in Brazil are quite different from their American and European counterparts. Therefore, the assessment of the bioavailability and toxicity of metals like Cu using waters with typical characteristics of Brazilian freshwater systems and native species, as performed in the present study, should play an important role in refining the current water quality criteria. Indeed, the best strategy for correctly assessing the health of an ecosystem and predicting environmental risks is to integrate physicochemical measurements with ecotoxicological analysis in the scope of toxicity tests (Martins and Bianchini, 2011).

Findings from the present study expand our knowledge on the acute effects of Cu at environmentally relevant concentrations in fresh water, using water with typical pH and hardness of Brazilian rivers inhabited by the native fish *P. lineatus*. In this context, Brazilian water quality criteria for Cu in fresh water are 9 and 13 µg L⁻¹ in order to protect aquatic communities (CONAMA, 2005). However, our study indicated a LOEC as low as 5 µg L⁻¹ for liver MT, liver LPO, muscle AChE, damaged nucleoids and DNA damage score in erythrocytes. Also, it reports a LOEC of 9 µg L⁻¹ Cu for liver SOD activity, swimming velocity and time spent swimming. Similarly, toxic effects of Cu were also detected on multiple biochemical and histological parameters at concentrations below the mentioned guideline of 9 µg L⁻¹ Cu in the native Neotropical fish *R. queLEN* (Mela et al., 2013). Therefore, these LOEC indicate that the current quality criteria for Cu in fresh water should be further revised, if the studied species are to be protected. It is important to note that the 96-h LC₅₀ for Cu in *P. lineatus* is 29 µg L⁻¹ (Mazon and Fernandez, 1999), which is within the range of tolerance for the most sensitive freshwater fishes (USEPA, 2007). In fact, it ranges from 22.1 µg L⁻¹ in rainbow trout *Oncorhynchus mykiss* to 178.3 µg L⁻¹ in the darter *Etheostoma nigrum*, and up to 2231 µg L⁻¹ in the bluegill *Lepomis macrochirus*. Finally, it is important to emphasize that our study reinforces the sensitivity and potentiality of some parameters such as liver MT, SOD and LPO, DNA damage in erythrocytes, and muscle AChE, which could be used as biomarkers together with swimming

behavior analyses to evaluate and monitor areas impacted by Cu in Brazilian freshwater systems.

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