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Sublethal effects of waterborne copper and copper nanoparticles on the freshwater Neotropical teleost *Prochilodus lineatus*: a comparative approach

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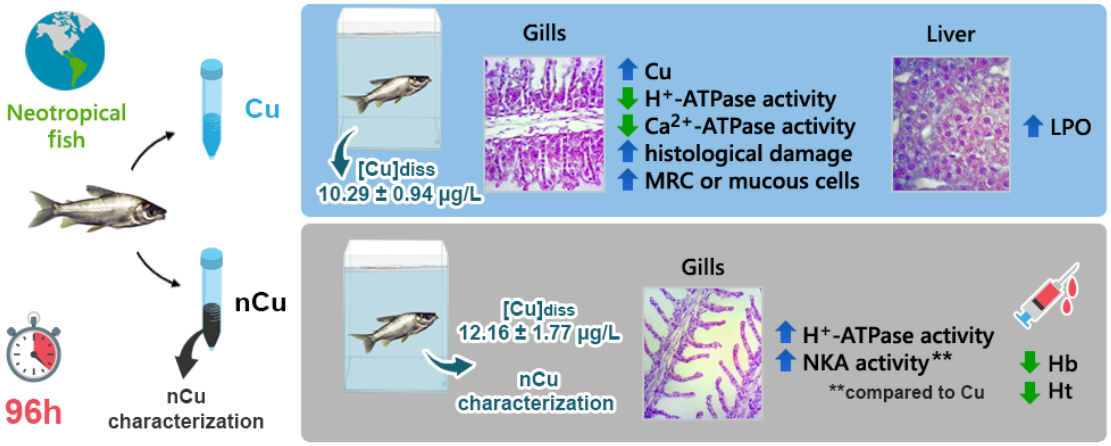
*Graphical Abstract



***Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

Highlights

- Copper nanoparticles (nCu) were less toxic than copper salt (Cu)
- The gills and liver of fish exposed to Cu were adversely affected
- Cu decreased but nCu increased H^+ pump activity in fish gills
- nCu increased Na^+/K^+ pump activity compared to Cu
- Only fish exposed to nCu became anemic

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Abstract

Copper nanoparticles can be a source of contamination in the aquatic environment, but their effects on fish and how these effects may differ from copper salts is not understood. Thus, this work aimed to compare the sublethal effects of copper nanoparticles (nCu) and copper chloride (Cu) on the Neotropical freshwater teleost *Prochilodus lineatus*, a species known for its sensitivity to copper. Juveniles (n = 8/group) were exposed to 20 μg L^{-1} of copper as $CuCl_2$ (Cu), 40 μg L^{-1} of copper nanoparticles (nCu), or only water (CTR), for 96 h. These concentrations were chosen to achieve similar dissolved copper concentration in both treatments (Cu: 10.29 ± 0.94 μg L^{-1}; nCu: 12.16 ± 1.77 μg L^{-1}). After the exposure, the following biological parameters were evaluated: copper accumulation in the gills, liver, gastrointestinal tract, kidney, and muscle; hematocrit (Ht) and hemoglobin content (Hb); branchial activity of Na^+-K^+-ATPase (NKA), H^+-ATPase (HATP), Ca^{2+}-ATPase (CaATP), and carbonic anhydrase (CA); glutathione content (GSH) and lipid peroxidation (LPO) in the liver; acetylcholinesterase activity (AChE) in the brain and muscle; and histopathology of the gills and liver. The gills of Cu-exposed fish were adversely affected, with increased copper content, inhibition of H^+-ATPase and Ca^{2+}-ATPase, and histological damage, including proliferation of mitochondria rich cells and/or mucous cells. In addition, LPO levels increased in the liver of Cu-exposed fish, indicating the occurrence of oxidative stress. Exposure to nCu promoted a decrease in Ht and Hb, indicating anemia, and an increase in branchial Na^+-K^+-ATPase and H^+-ATPase activities, which can be an adaptive response to metabolic acidosis. Overall, copper nanoparticles were less toxic than copper. However, the effects promoted by the nanoparticles were different from those promoted by copper. These results emphasize the need for a better understanding of copper nanoparticles

toxicity in order to establish safe concentrations and avoid environment impacts.

Keywords: Biomarkers. Nanotoxicology. Osmoregulation. Hematology. Fish. Oxidative stress.

1. Introduction

Although copper (Cu) is an essential element for organisms, it becomes toxic in excessive amounts. For freshwater teleosts, the gills are the primary target organ for copper toxicity. Cu ion mimics sodium ion, being absorbed by gills through the Na^+ pathway (Wood, 2012). Inside the cell, Cu inhibits Na^+-K^+-ATPase and promotes diffusive Na^+ and Cl^- loss via paracellular pathways. This leads to decreased plasma Na^+ and Cl^- concentrations and reduced plasma osmolality. As a result, fluid diffuses from plasma to red blood cells and tissues (Grosell, 2012). For the freshwater teleost *Prochilodus lineatus,* Cu can promote proliferation of pavement and mitochondria rich cells (MRC) in the gills (Mazon et al., 2002), as well as increases in hematocrit, red blood cell count, and hemoglobin concentration (Cerqueira and Fernandes 2002). Following waterborne exposure, Cu concentration initially increases in fish gills (Grosell, 2012), and then Cu is transported through the bloodstream to internal organs. Accumulation of Cu was observed in the gills, liver, intestine, and kidney of *P. lineatus*, with the highest concentration found in the liver (Mazon and Fernandes, 1999). Additionally, acute exposure of *P. lineatus* to environmentally relevant Cu concentrations promotes oxidative stress, with increased lipoperoxidation (LPO) in the liver and DNA damage in blood cells (Simonato et al., 2016).

Recently, the development of nanotechnology has brought attention to the benefits of using copper in the nanoparticle range. Copper nanoparticles (nCu) present catalytic, electric, optical, and biocidal properties (Huang et al., 1997; Kawamura, et al., 2015; Gawande et al., 2016; Perdikaki et al., 2016; Pantidos et al., 2018) applied in various materials, including printing inks (Rahman et al., 2018), sensors (Guo et al., 2016), as a contrast agent for imaging in diagnostics (Ding et al.,

2015), pesticides (Tegenaw et al., 2015), and antifouling paints (Adeleye et al., 2016). The substantial use of copper nanoparticles invariably results in their release into the aquatic environment (Keller et al., 2013; 2017), and the potential hazard to aquatic organisms must be evaluated.

For several freshwater teleosts, copper nanoparticles are usually less toxic than copper salts (Griffitt et al., 2007; Song et al., 2015; Braz-Mota et al., 2018). However, sublethal responses are not necessarily similar. Exposure of rainbow trout to 20 µg L^{-1} nCu for 96 h promoted a depletion in plasma Na^+ and K^+ in relation to fish exposed to $CuSO_4$, although copper was not accumulated in the gills of fish exposed to nCu, only in the gills of fish exposed to $CuSO_4$ (Shaw et al., 2012). In the same study, accumulation of Cu was observed in the intestine of fish exposed to nCu, but not in the intestine of fish exposed to $CuSO_4$. These results corroborate with the increased histological damage found in the gills of fish exposed to $CuSO_4$ in comparison to unexposed animals, in the same experimental conditions, and in the intestine of rainbow trout exposed to 100 µgL^{-1} nCu for 10 days (Al-Bairuty et al., 2013). In addition, dwarf cichlids (*Apistogramma agassizii*) exposed to 50% of LC_{50}-96 h of Cu displayed an increase in H^+-ATPase activity in the gills, whilst exposure to 50% of LC_{50}-96 h of nCuO decreased the activity of this enzyme in the same species (Braz-Mota et al., 2018). In the same study, whole body homogenates of dwarf cichlids and cardinal tetras (*Paracheirodon axelrodi)* exposed to nCuO exhibited an increase in LPO compared to fishes exposed to Cu after 96 h. Together, these findings suggest different bioavailability, uptake routes, and mechanisms of toxicity for ionic and nanoparticle forms of copper. In fact, uptake of nCu by fish probably occurs via the endocytosis pathway, rather than by ionic transporters as is the case for Cu ions (Shaw and Handy, 2011). However, there is a lack of studies concerning

nCu toxicity and its sublethal effects on Neotropical fish species, even though these species have been shown to be, in several cases, more sensitive to copper than fish from other geographic regions (Cerqueira and Fernandes, 2002; Duarte et al., 2009; Simonato et al., 2016). In this context, the Neotropical freshwater teleost *P. lineatus* is a widely distributed species in Brazil (Castro, 1990) with ecological relevance (Botta et al., 2000) and has presented sensitivity to metals, including copper (Mazon and Fernandes, 1999; Nascimento et al., 2012).

In aqueous solutions, copper nanoparticles undergo several transformations depending on water chemical and physical characteristics. Aggregation of nanoparticles can result in their sedimentation, influencing bioavailability in liquid phase, and dissolution of nanoparticles release free metal ions, which can be a source of toxicity (Griffitt et al., 2007; Handy et al., 2008; Shaw and Handy, 2011; Keller et al., 2017). For Cu toxicity, the dissolved fraction of aqueous media, traditionally defined as smaller than 0.45 µm, is more relevant than total copper due to its greater bioavailability to organisms. This classification of dissolved Cu encompasses dissolved ions, nanoparticles, and some bulk particles. Therefore, considering that aggregation of nanoparticles reduces bioavailability in the liquid phase, we decided to compare equal dissolved (<0.45 µm fraction) concentrations of copper and copper nanoparticles as a way of comparing equal bioavailable concentrations of salt and nano forms of copper in the water column. In this context, the aim of the present study was to evaluate the sublethal effects on different biological parameters of the Neotropical fish *Prochilodus lineatus* after acute exposure to similar dissolved concentrations of copper chloride and copper nanoparticles.

2. Material and methods

2.1 Cu and nCu stock solution and suspension

Copper nanoparticles (40-60 nm and ≥99.5% purity) were purchased from Merck (774111). Stock suspensions (200 mg L^{-1}) were prepared in ultrapure water and dispersed using an ultrasonic cleaner (Unique UltraCleaner 750) for 4h. A 5 g Cu L^{-1} stock solution was prepared from $CuCl_2.2H_2O$ in ultrapure water, stirred and acidified with HNO_3 1%. Both Cu and nCu solutions were prepared the day before the beginning of the experiment and used throughout the exposure period.

2.2 Fish handling and experimental design

Juveniles of *P. lineatus* (n=24; 14.74 ± 2.75 g; 11.68 ± 0.63 cm; mean ± SD) were purchased from Venites Aquaculture Station (Toledo, Paraná, Brazil) and acclimated for two months in a 500L tank containing dechlorinated tap water under continuous aeration, controlled temperature and a 12h:12h light/dark photoperiod. Throughout acclimation, the fish were fed every 48 h with commercial fish feed (Guabi, Brazil), which was interrupted 24 h prior to the beginning of the experiment and during the experiment.

After acclimation, fish were randomly divided into three groups (n = 8 fish/group) and kept individually in glass aquaria containing 10 L of water each. One group was exposed to 20 µg Cu L^{-1} (nominal) in the form of $CuCl_2$ salt (Cu treatment). This concentration was chosen as appropriate to observe toxic effects (Simonato et al., 2016) but no mortality of animals (LC_{50}-96 h for *P. lineatus* = 29 µg Cu L^{-1}, Mazon and Fernandes, 1999). Another group was exposed to 40 µg Cu L^{-1} (nominal) in the form of copper nanoparticles (nCu treatment). This concentration was defined based on previous tests with copper nanoparticles in order to match the concentration of

dissolved copper to the $CuCl_2$ exposure. A control group (CTR) was kept in water with no addition of Cu. The exposure occurred for 96 h in a semi-static system with renewal of 80% of the volume of water every 24 h.

2.3 Cu concentrations and water parameters

Water samples were collected daily to determine total (not filtered) and dissolved (filtered with 0.45 µm mesh filter, Millipore Millex HV/PVDF) concentrations of copper. Samples were acidified (1% HNO_3) and analyzed by graphite atomizer, atomic absorption spectroscopy (AAnalyst 700, Perkin Elmer, USA), with detection limit of 0.014 µg L^{-1}.

Water parameters remained constant throughout the acclimation and the experimental period. The following data correspond to the experimental period. Water temperature, dissolved oxygen, pH, total dissolved solids, conductivity, redox potential, and turbidity were monitored at initial (0 h) and final (96 h) times using a multi-parameter meter (HoribaU52) and corresponded to (mean ± SD, n=44) 22.18 ± 0.63 °C, 7.35 ± 0.32 mg O_2 L^{-1}, 7.60 ± 0.16, 0.05 g L^{-1}, 0.079 µS cm^{-1}, 358.48 ± 17.61 mV, and 15.71 ± 0.41 NTU. Hardness was (mean ± SD, n=12) 38.04 ± 6.88 mg $CaCO_3$ L^{-1}. Na^+ and K^+ concentrations were measured using a flame photometer (DM-62, Digmed, Brazil), and Ca^{2+} concentrations were measured using an AAnalyst 700 spectrophotometer (Perkin Elmer, USA). Electrolyte composition (n=18) corresponded to 0.09 mM Na^+, 0.01 mM K^+ and 0.09 mM Ca^{2+}.

2.4 Nanoparticles characterization

Copper nanoparticles images from a stock suspension were obtained by Easy Scan 2 Basic BT02217 AFM instrument (nanosurf, Switzerland). A TapAL-G

cantilever was used, and the equipment was operated in the noncontact mode with a scan mode of 100 Hz. AFM images were analyzed by ImageJ software (version 1.48V). A total of 100 nanoparticles were counted to obtain size and size distribution.

The water collected throughout the experiment (24, 48, 72, and 96 h) were characterized by nanoparticle tracking analysis using a Nanosight Model LM_{10} cell Instrument (laser with 532 nm) and a sCMOS camara, controlled by Nanosight v. 3.1 software (Malvern Instruments, UK). The analyses were performed five times at 25°C. In order to compare the water collected from aquaria (experimental conditions) with the original nanoparticles suspension, the stock suspension was diluted in ultrapure water in the same proportion done in aquaria during the experiment. This dilution is referred as time 0 h.

2.5 Fish handling after exposure and tissue sampling

Following exposure, the fish were anesthetized with benzocaine (0.1 g L^{-1}) and blood samples were collected from the caudal vein for hematological parameters and blood plasma analysis. After this, the fish were killed by medullar sectioning for the removal of organs. Individual samples of gills, liver, gastrointestinal tract, posterior kidney, muscle, and brain were stored in a freezer at -80°C for further biochemical, physiological, and bioaccumulation assays. Subsamples of gills and liver were fixed for histopathological analysis. Tissues were homogenized on ice for all biochemical and physiological assays.

2.6 Cu concentration in tissue samples

Individual samples of gills, liver, gastrointestinal tract (GI tract), posterior kidney, and muscle were completely dried at 60°C and subsequently

submitted to acidic digestion with suprapure nitric acid (5N) at 60 °C for 48 h, according to Alves and Wood (2006). Tissue digests were analyzed for Cu concentration through electrothermal atomic absorption spectrophotometry (AAnalyst 700, Perkin Elmer, USA) against reference standard solutions (Specsol, Brazil).

2.7 Hematological parameters and plasma ion content

For hematocrit (Hct) determination, heparinized microcapillaries were filled with blood, centrifuged (1200 × *g*, 7 min) and read using a standardized card. Hemoglobin (Hb) concentration was determined by a colorimetric method using cyanmethemoglobin (commercial kit, Labtest Diagnóstica, Brazil) and measured at 540 nm using a Libra S32 (Biochrom, UK) spectrophotometer. Mean Corpuscular Hemoglobin Concentration (CHCM) was calculated using Hb concentration and Hct percentage. Blood samples were centrifuged (1870 × *g*, 10 min, MCD2000, Hsiangtai, Taiwan) and plasma samples were stored at -20°C. Plasma samples were used for analysis of Na^+ and K^+ concentrations using a flame photometer (DM-62, Digmed, Brazil) and for determination of Ca^{2+} concentrations using atomic absorption spectroscopy (AAnalyst 700, PerkinElmer, USA) against standard curves (Specsol, Brazil).

2.8 Enzymatic activity in gills

Gill samples were stored in SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.5), homogenized (1:5 w/v) in SEI buffer with 2.4 mM sodium deoxycholate, centrifuged (10000 × *g*, 20 min, 4°C), and the supernatants were used for determination of Na^+-K^+-ATPase (NKA), H^+-ATPase (HATP), Ca^{2+}-ATPase (CaATP) and carbonic anhydrase (CA) activities.

NKA and HATP activities were determined according to the Gibbs and Somero (1989) protocol, adapted for a microplate reader. Supernatants were diluted to normalize the protein concentration of each sample to 1 mg mL^{-1}. Next, a reaction mixture (30 mM imidazole, 45 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 0.4 mM KCN, 1 mM ATP, 0.2 mM NADH, 3 $U.mL^{-1}$ pyruvate kinase, 2 $U.mL^{-1}$ lactate dehydrogenase, 0.1 mM fructose-1,6-diphosphate, 2mM phosphoenolpyruvate, pH 9.0) containing N-Ethyl-d_5-maleimide (NEM, 2 mM, inhibitor of H^+-ATPase), or ouabain (2 mM, inhibitor of NKA), or without inhibitors (total activity of the ATPases) was added to the supernatants. Absorbance of samples was measured in quadruplicate every minute for 15 min at 340 nm. NKA and HATP activities were calculated by the difference between media containing or not ouabain, and media containing or not NEM, respectively. Enzyme activities were expressed in µmol ADP. mg $protein^{-1}.h^{-1}$.

CaATP activity was measured according to the method described by Vijayavel et al. (2007) with modifications. The samples were incubated in a reactive solution (189 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris, 5 mM $CaCl_2$, 2 mM ouabain, pH 7.6) without ATP to determine the basal concentration of inorganic phosphate (Pi), and with ATP (3 mM) for the reaction to occur. Pi formation was measured using a staining solution (Ames, 1966) in a microplate reader (ELX 800, Bio-Tek Instruments) at 620 nm. Ca^{2+}-ATPase activity was quantified based on a phosphate standard curve (0.08-0.65 mM) and expressed in µmol Pi.mg $protein^{-1}$ min^{-1}.

CA activity was determined according to Vitale et al. (1999). The catalyzed reaction rate was quantified by adding water saturated with CO_2 to the supernatants and measuring pH decay during 20 seconds using a pH meter (Jenway 3510, EUA). The non-catalyzed reaction rate was quantified using buffer instead of

245 the supernatants. The activity of the enzyme was calculated as [catalyzed reaction
246 rate/ non-catalyzed reaction rate - 1].mg ptn^{-1}.

247 For all enzymatic assays, protein content in each supernatant was
248 determined using the Bradford method (Bradford, 1976) in a microplate reader at 595
249 nm.

250

251 *2.9 Acetylcholinesterase activity in muscle and brain*

252 Muscle and brain samples were homogenized in a phosphate buffer
253 solution (0.1 M, pH 7.5, 1:10 w/v) and centrifuged (16060 × *g*, 20 min, 4°C).
254 Supernatants were used for acetylcholinesterase (AChE) activity assay according to
255 the method described by Ellman et al. (1961) and adapted by Alves-Costa et al.
256 (2007). Nitrobenzoate formation was measured at 0, 3, and 6 minutes in a
257 spectrophotometer at 415 nm. Data were expressed in µmol min^{-1} mg protein^{-1}.
258 Protein content was determined according to Bradford (1976), in a microplate reader
259 at 595 nm.

260

261 *2.10 Antioxidant defenses and oxidative damage in liver*

262 Individual liver samples were homogenized in a phosphate buffer
263 solution (0.1M, pH = 7.0, 1:10 w/v), centrifuged (16060 × *g*, 20 min, 4°C), and the
264 supernatants were used for the assays described below.

265 Glutathione (GSH) content was determined according to the method
266 described by Beutler et al. (1963). Supernatants were acidified with trichloroacetic
267 acid (TCA 6%, 1:1 v/v) and centrifuged for protein decantation (1520 × *g*, 5 min, 4°C).
268 Dithionitrobenzoate (0.25 mM DTNB) was added to the resulting supernatants in a
269 phosphate buffer solution (0.1M; pH = 7.0). Thiolate formation absorbance was

measured at 412 nm and quantified based on a GSH standard curve (5-400 µM). The concentration of non-protein thiols was expressed in µg.mg ptn^{-1}.

Lipoperoxidation (LPO) was measured by the quantification of thiobarbituric acid reactive substances (TBARS assay), according to Camejo et al. (1998). Butylhydroxytoluene (BHT, 1mM), phosphate buffer solution (2.7mM KCl, 1.4 mM NaH_2PO_4, 137 mM NaCl, 10 mM Na_2HPO_4, pH 7.4), and trichloroacetic acid (TCA 50%) were added to the supernatants. A fluorescence reading (ex/em: 535/590nm) was performed for determination of the mixture autofluorescence. Thiobarbituric acid (TBA 1.3%) dissolved in 0.3% NaOH was added and the mixture was incubated at 60°C. After 1h, the fluorescence reading was repeated. TBARS concentration was determined based on a standard curve of malondialdehyde (MDA) and expressed in nmol.mg ptn^{-1}.

All biochemical biomarkers were expressed considering protein content in each liver supernatant determined by the Bradford method (Bradford, 1976) in a microplate reader at 595 nm.

2.11 Histological parameters in gills and liver

Gill and liver samples were fixed in Alfac solution (85 mL of ethanol 80%; 10 mL of formaldehyde 40% and 5 mL of glacial acetic acid) for 14 h, preserved in ethanol 70%, dehydrated in serial ethanol concentrations, diaphanized in xylol, and included in paraffin at 60 °C. Sections (5 µm) were cut using a semi-automated rotating microtome (Leica Biosystems, RM2245) and stained with hematoxylin and eosin for analysis under a light microscope (Zeiss, Primo Star, Germany). Damage to the tissue was evaluated semi-quantitatively by the Degree of Tissue Change (DTC), according to Poleksić and Mitrović-Tutundžić (1994). Tissue alterations were

295 classified as stage I alterations, which do not alter the normal functioning of the
296 tissue; stage II alterations, which impair the normal functioning of the tissue and are
297 reversible; and stage III alterations, which cause irreparable damage. DTC values
298 were calculated as follows: DTC = (1 × ∑I) + (10 × ∑II) + (100 × ∑III), where I, II, and
299 III correspond respectively to the number of alterations in stages I, II, and III. DTC
300 values between 0-10 indicate normal functioning of the organ; values between 11-20
301 indicate slight damage to the organ; values between 21-50 indicate moderate
302 changes in the organ; values between 50-100 indicate severe lesions; and values
303 above 100 indicate irreversible damage to the organ.
304
305 *2.12 Statistical analysis*
306 After verification of normality (Kolmogorov-Smirnov test) and
307 homoscedasticity (Levene Median test), data obtained for all treatments (CTR, Cu
308 and nCu) were compared using parametric (ANOVA) or non-parametric (Kruskal–
309 Wallis) analysis, followed by the Student–Newman–Keuls (SNK) or Dunn's test,
310 respectively. Values of $P < 0.05$ were considered significant.
311
312 **3. Results**
313 *3.1 Nanoparticles characterization*
314 AFM images obtained from the stock suspension revealed spherical
315 nanoparticles (Fig. 1A) with a mean size of 178 ± 90 nm (Fig. 1B), while nanoparticle
316 tracking analysis (NTA) of the same stock suspension showed an average
317 hydrodynamic size of 136 ± 28 nm (Fig. 2B). Particle size distribution obtained from
318 NTA indicates the presence of different populations in the stock suspension
319 (polydispersity of 0.5), with two main populations showing sizes around or higher

than 100 nm, as well as populations within the nanoparticle range, i.e., <100 nm (Fig. 2A). Water samples collected from aquaria also presented diverse particle size distribution, with multiple populations (Fig. 2A).

Copper nanoparticles initially had a size of 136 ± 28 nm, with an increase in the size (264 ± 21 nm) and a decrease in the concentration (from $7.35x10^{10}$ to $4.14x10^{7}$ particles/mL) after 24 h. This finding can represent an aggregation process in aquaria conditions. Furthermore, the size and the concentration remained constant throughout the experiment (24, 48, 72 and 96 h).

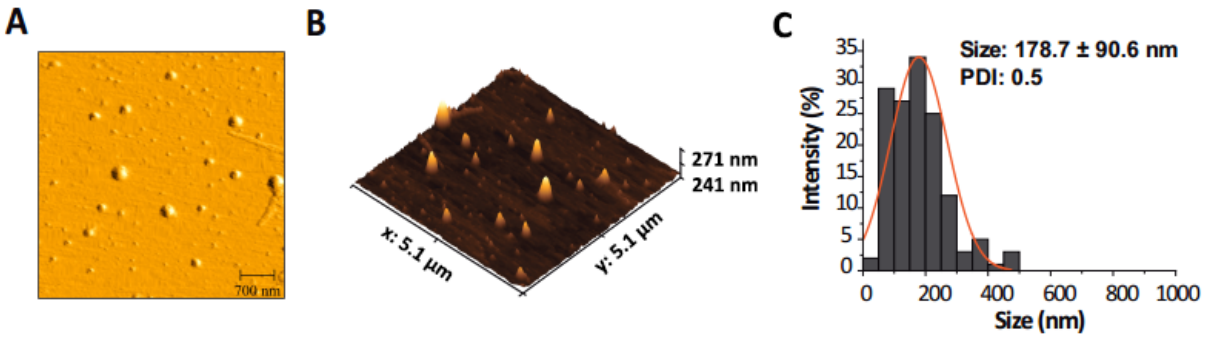
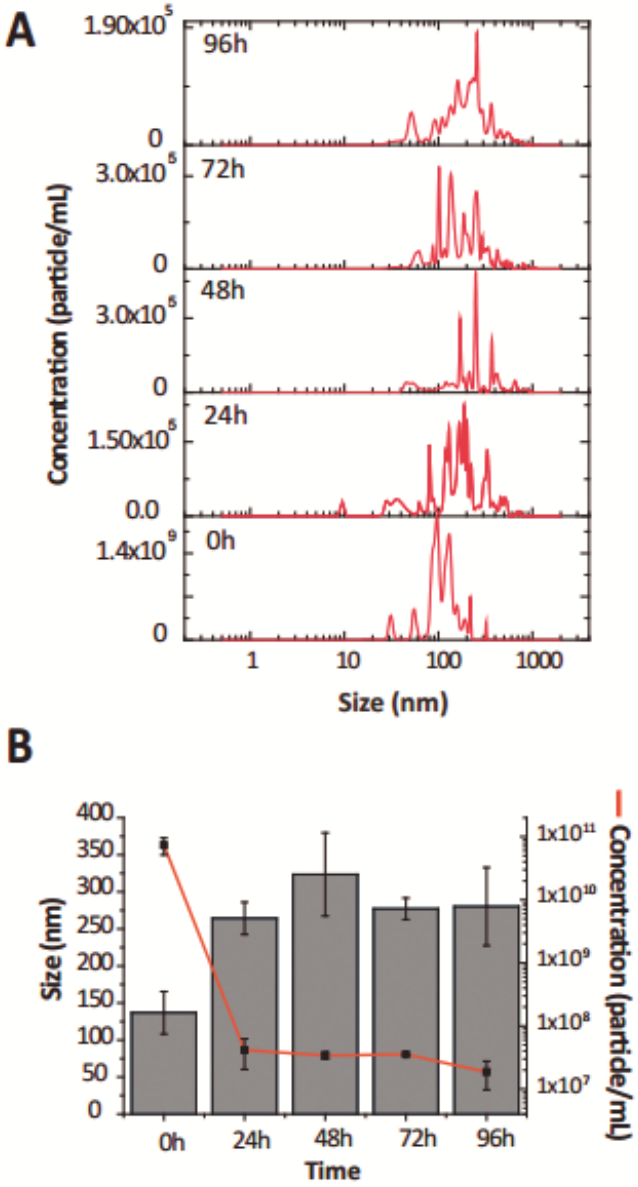


Figure 1 – Topographic image (A), 3D image (B) and particle size distribution (C) obtained from atomic force microscopy (AFM) of a stock suspension. PDI: polydispersity index.



335

336 **Figure 2 –** Particle size distribution (A) and size and concentration over time (B) obtained from
337 nanoparticle tracking analysis (NTA) of a dilution of the stock suspension (0 h) and of water samples
338 collected from aquaria (24 h, 48 h, 72 h, 96 h). In B, data are expressed as mean ± SD.

339

340 *3.2 Cu concentrations in the water*

341 Quantified Cu concentrations in the water of both treatments were
342 lower than the nominal concentrations. Despite this, Cu and nCu dissolved
343 concentrations were similar, as intended (Table 1).

344

Table 1 – Copper concentrations (µg L^{-1}) measured in the water of control (CTR), $CuCl_2$ treatment (Cu), and copper nanoparticle treatment (nCu). Data are expressed as mean ± SD (n=12-16). Water samples were collected every day before water renewal throughout the experimental period.

	CTR (µg L^{-1})	Cu (µg L^{-1})	nCu (µg L^{-1})
Cu (total)	2.19 ± 0.18	12.35 ± 0.69	15.28 ± 2.34
Cu (dissolved)	2.06 ± 0.24	10.29 ± 0.94	12.16 1.77

3.3 Cu concentration in tissues

Copper concentrations were significantly higher in the gills of fish exposed to Cu compared to control ($P < 0.001$; $H = 17.990$) (Fig. 3). No significant differences were found in the gills of fish exposed to nCu, and no significant differences were found in the liver, GI tract, posterior kidney, or muscle of fish exposed to Cu or nCu.

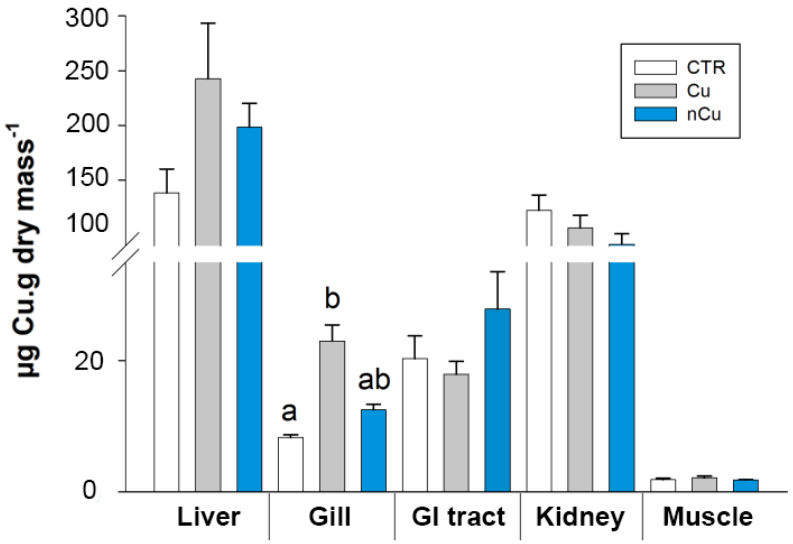


Figure 3 – Copper concentrations measured in the liver, gills, gastrointestinal tract (GI tract), posterior kidney, and muscle of *P. lineatus* from control (CTR), copper treatment (Cu), and copper nanoparticle treatment (nCu) after 96 h exposure. Data are expressed as mean ± SE (n=6-8). Different letters represent significant differences ($P < 0.05$) between treatments.

3.4 Hematological parameters and plasma ion content

Hemoglobin concentration decreased in fish exposed to nCu compared to control (P = 0.012) and Cu (P = 0.006) (F = 6.53). Hemoglobin concentration was not affected in fish exposed to Cu. Nanoparticle exposure also led to a decrease in hematocrit values compared to control (P = 0.044) and Cu (P = 0.023) (F = 4.571), whereas Cu exposure did not alter the hematocrit of the analyzed fish. There was no change in mean corpuscular hemoglobin concentration (MCHC) at any exposure (Fig. 4).

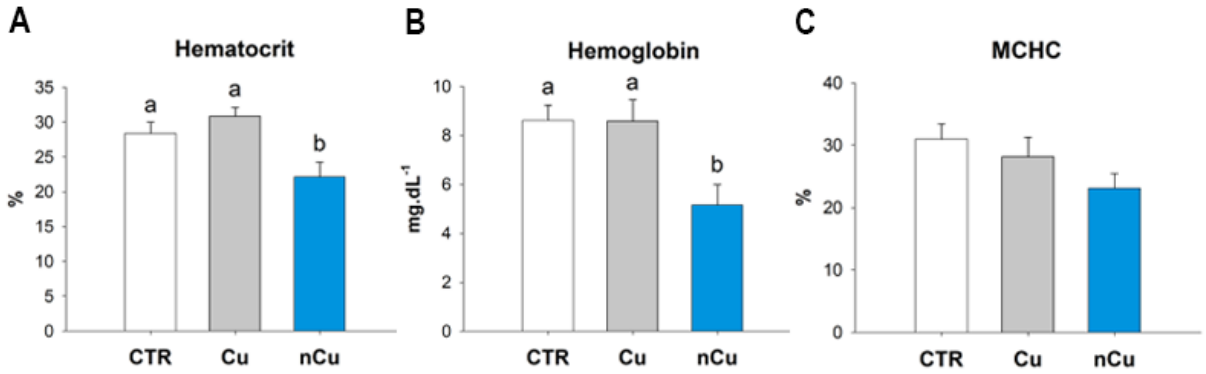


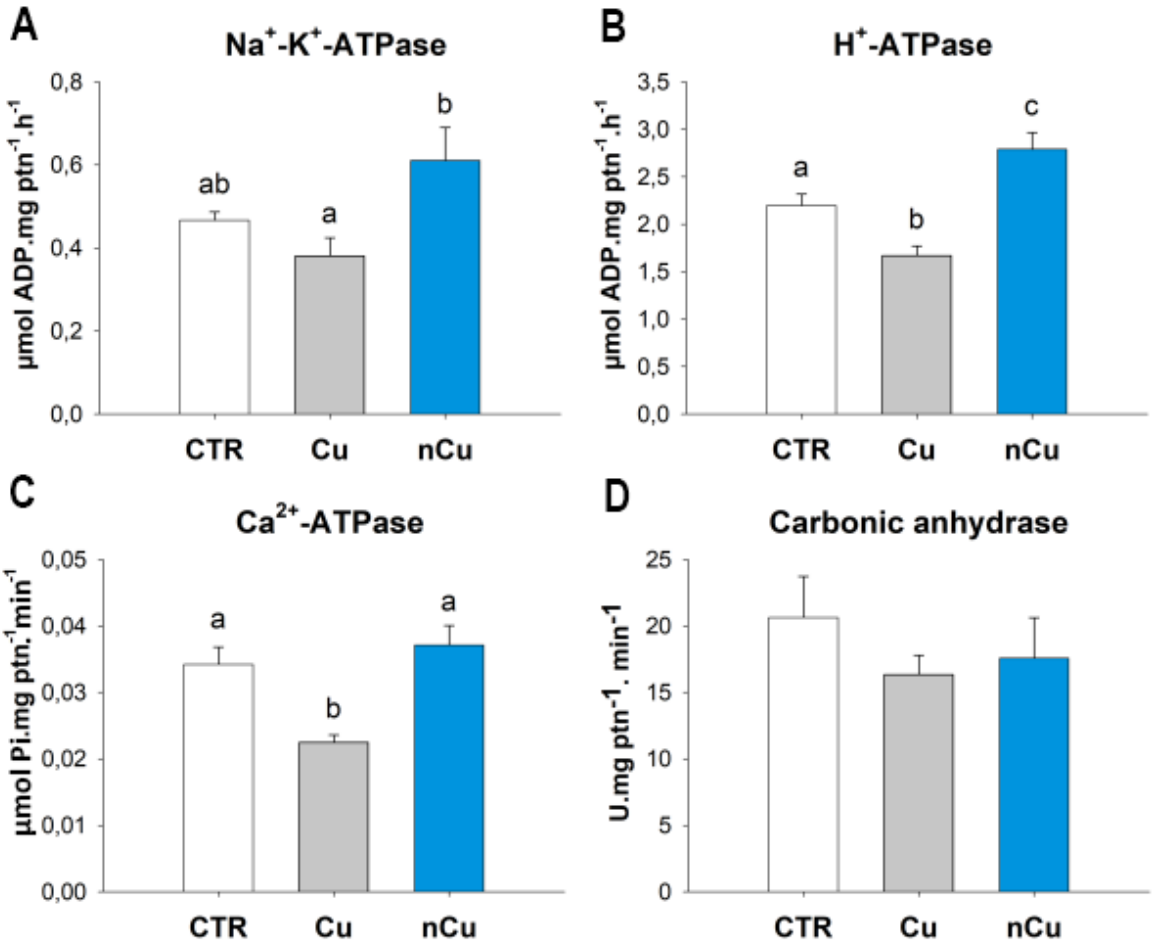
Figure 4 – Hematocrit (A), hemoglobin concentration (B), and mean corpuscular hemoglobin concentration (C) of *P. lineatus* from control (CTR), copper treatment (Cu), and copper nanoparticle treatment (nCu) after 96 h exposure. Data are expressed as mean ± SE (n=7-8). Different letters indicate significant differences between groups (P < 0.05).

There were no significant differences in Na^+, K^+, and Ca^{2+} plasma ions among treatments. Data from CTR were (mean ± SE, n=8): Na^+, 173.12 ± 4.49 mM; K^+, 6.55 ± 0.41 mM; Ca^{2+}, 0.62 ± 0.15 mM.

3.5 Enzymatic activity in gills

NKA activity increased in fish exposed to nCu compared to fish exposed to Cu (P = 0.015), but did not change compared to control fish (P = 0.059) (Fig. 5A). NKA activity was not affected by Cu exposure compared to control (P =

385 0.244) (F = 4.956). H^+-ATPase activity decreased in fish exposed to Cu compared to
386 control (P = 0.012) and to nCu (P < 0.001), whilst the activity of this enzyme
387 increased in fish exposed to nCu compared to control (P = 0.006) and to Cu (Fig. 5B)
388 (F = 16.475). Activity of Ca^{2+}-ATPase decreased in fish exposed to Cu compared to
389 control and to nCu (P < 0.05) (H = 13.299) (Fig. 5C). Carbonic anhydrase was not
390 affected by any exposure (Fig. 5D).
391



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393 **Figure 5 –** Activity of Na^+-K^+-ATPase (A), H^+-ATPase (B), Ca^{2+}-ATPase (C), and carbonic anhydrase
394 (D) in the gills of *P. lineatus* from control (CTR), copper treatment (Cu), and copper nanoparticle
395 treatment (nCu) after 96 h exposure. Data are expressed as mean ± SE (n=7-8). Different letters
396 indicate significant differences between groups (P < 0.05).
397

398 *3.6 Acetylcholinesterase activity in muscle and brain*

399 AChE activity in muscle and brain was not affected by any exposure

(Fig. 6). Enzymatic activity in muscle from CTR was 0.073 ± 0.002 µmol min^{-1} mg $protein^{-1}$ (mean ± SE, n=6), whereas activity in brain from CTR was 0.022 ± 0.002 µmol min^{-1} mg $protein^{-1}$ (mean ± SE, n=8).

3.7 Antioxidant defenses and oxidative damage in liver

GSH content in the liver of fish exposed to Cu or nCu was not affected (Fig. 7). However, lipoperoxidation increased in Cu-exposed animals compared to animals from control (P = 0.035) (F = 4.906). There was no significant difference in LPO after nCu exposure when compared to control (Fig. 3).

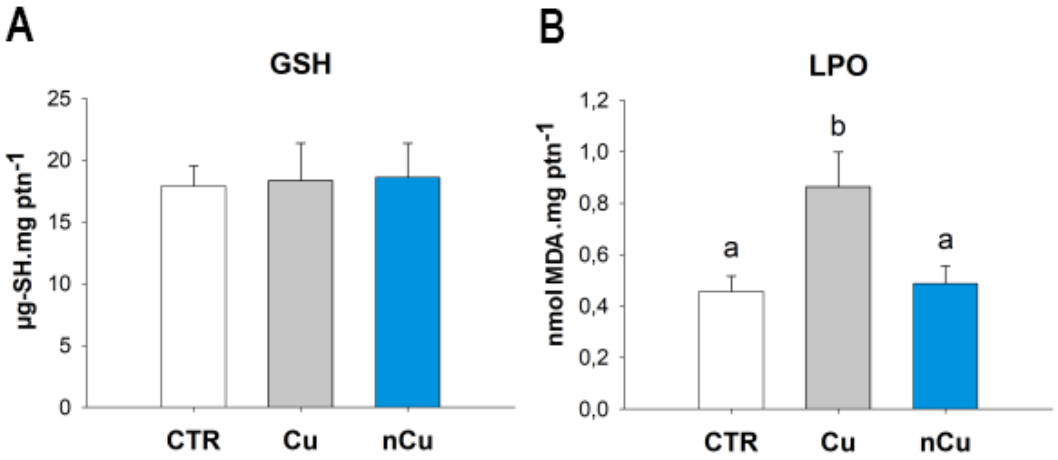

Figure 6 – Glutathione content (A) and lipoperoxidation (B) in the liver of *P. lineatus* from control (CTR), copper treatment (Cu), and copper nanoparticle treatment (nCu) after 96 h exposure. Data are expressed as mean ± SE (n=7-8). Different letters indicate significant differences between groups (P < 0.05).

3.8 Histological parameters in liver and gills

At tissue level, gills of *P. lineatus* exhibit filaments comprising cubic epithelium, mitochondria rich cells, mucous cells, and blood vessels. Filaments divide into gill lamellae, which are formed by pavement epithelium and pillar cells (Fig. 6A). DTC values found for all groups indicate slight damage to the organ (CTR = 12.3 ±

3.1; Cu = 20.9 ± 3.2; nCu = 19.3 ± 3.8; mean ± SE), but DTC values were significantly higher in Cu exposure (P = 0.03, F = 3.796) (Fig. 8). Fish exposed to Cu exhibited frequent MRC or mucous cell hyperplasia, whilst this feature was not found in any fish from the control. Additionally, hyperplasia of these cells led to increased lamellar fusion. Frequency and severity of histological changes are shown in Table 3.

The liver of *P. lineatus* displays the typical pattern found in teleost fish, consisting of polyhedral hepatocytes with a large spherical nuclei and prominent nucleolus, arranged around sinusoids in a cord-like structure (Fig. 8C). Fish from all treatments exhibited some degree of damage, showing rare or fairly frequent alterations. Some exceptions were bile stagnation, frequent in all groups, and glycogen-type vacuoles in the cytoplasm, fairly frequent in CTR but very frequent in Cu and nCu groups (Table 4., Fig. 8D). DTC values ranged from 17 to 24, indicating slight to moderate damage to the organ in all groups. No significant differences were found among treatments (Fig. 9)

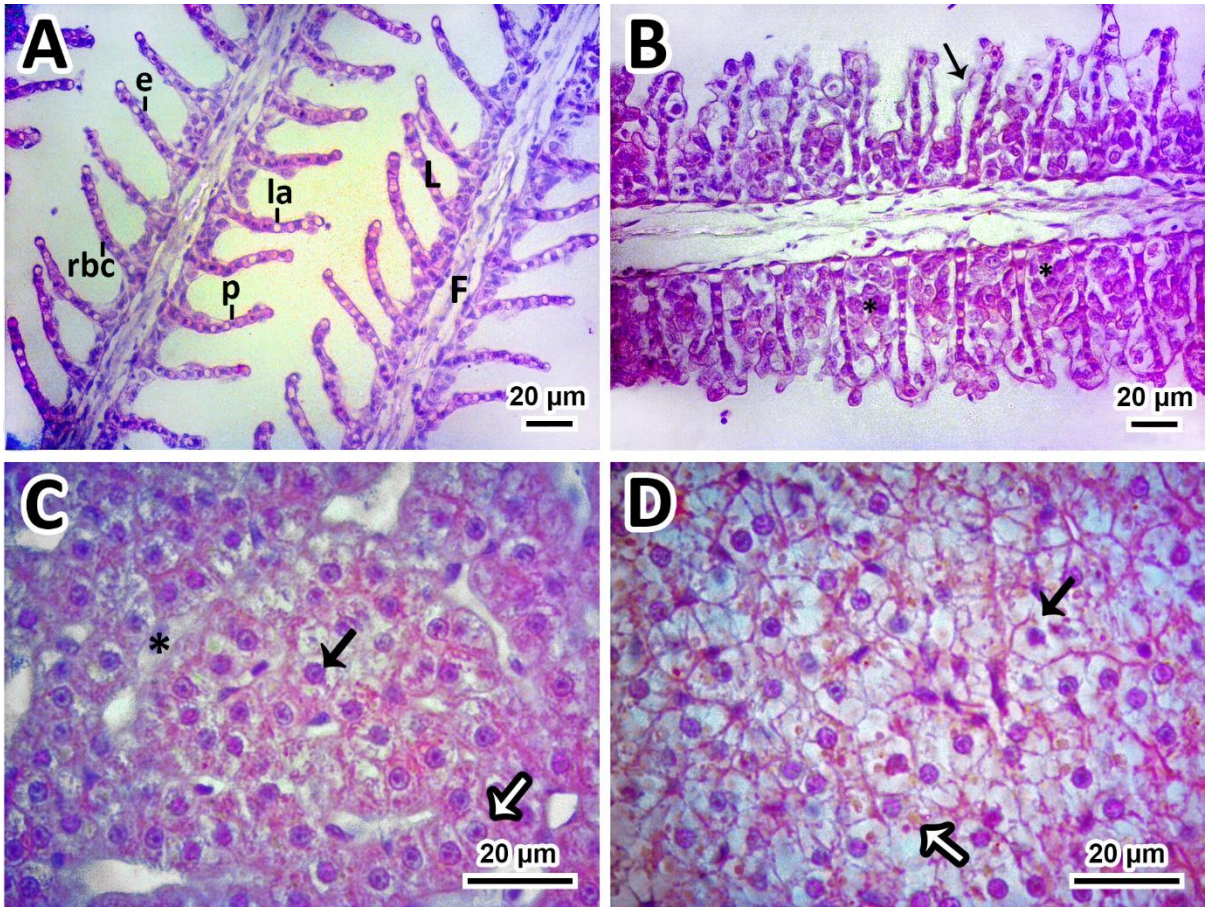

Figure 7 – Histological sections of gills and liver of *P. lineatus* under normal and pathological conditions. **A –** Gill section of a fish from control, showing normal architecture comprising of gill filament (F), gill lamellae (L), epithelial cells (e), red blood cells (rbc); lacuna (la), and pillar cells (p). **B –** Gill section of a fish exposed to Cu, showing epithelial lifting (black arrow) and MRC or mucous cell hyperplasia (*), which led to complete lamellar fusion. Hyperplasia of mitochondria rich cells or mucous cells was not separated considering that these cells can be mistaken in the histological processing used. **C –** Hepatic tissue of a fish from control, exhibiting polygonal shaped hepatocytes (white arrow), spherical nucleus with prominent nucleolus (black arrow), and sinusoids (*). **D –** Hepatic tissue of a fish exposed to Cu, showing cytoplasmic vacuolation (all cells), bile stagnation (white arrow), and rupture of cells (black arrow). Scale: 20 µm. H&E.

Table 2 – Histological changes and Degree of Tissue Change (DTC) in the gills and liver of *P. lineatus* from control (CTR), copper treatment (Cu), and copper nanoparticle treatment (nCu) after 96 h exposure (n=6-8). Different letters indicate significant differences between groups ($P < 0.05$). Statistics were only applied to DTC values.

Gills				
Changes	**Stage**	**CTR**	**Cu**	**nCu**
Lamellar/filament epithelial cell hypertrophy	**I**	0+	++	+
Lamellar epithelial lifting	**I**	++	++	++
Lamellar/filament epithelial cell hyperplasia	**I**	+	+	+
Half lamellar fusion	**I**	+	++	0+
Complete lamellar fusion	**I**	0+	++	+
Lamellar disarray	**I**	+	0+	+
Mitochondria rich cells or mucous cells hyperplasia	**I**	0	++	0+
Marginal channel expansion	**I**	0	+	0
Vascular congestion	**I**	+	+	+
Aneurysm	**II**	+	+	+
Presence of parasites	**I**	0	0+	0+
DTC		**12,29 ± 3,14[a]**	**20,88 ± 3,22[b]**	**19,29 ± 3,85[ab]**
Liver				
Changes	**Stage**	**CTR**	**Cu**	**nCu**
Disarrangement of hepatic cords	**I**	+	0+	+
Irregular shaped cells	**I**	0	0+	+
Irregular shaped nucleus	**I**	+	+	+
Cytoplasmic vacuolation	**I**	+	+++	+++
Cell hypertrophy	**I**	+	++	+
Nucleus hypertrophy	**I**	+	0+	+
Cell rupture	**II**	0+	+	+
Increased vessel frequency	**I**	+	0	0
Hyperemia	**II**	+	0+	0+
Dilated sinusoids	**I**	++	0+	+
Bile stagnation	**I**	++	++	++
DTC		**24,25 ± 4,42**	**17,5 ± 2,63**	**19,33 ± 3,95**

Note: 0 = absent; 0+ = rare; + = fairly frequent; ++ = frequent; +++ = very frequent; ++++ = extremely frequent.

4. Discussion

The present study aimed to compare the effects of equal dissolved concentrations of copper chloride and copper nanoparticles in a Neotropical fish species known for its sensitivity to copper. Our approach consisted of analysis of biomarkers at different biological levels in order to understand the toxic responses as an integrated process. The gills of fish exposed to Cu were adversely affected, with increased copper content, iono- and osmoregulation disturbances and histological injury. In addition, oxidative damage was found in the liver of these fish. In contrast, nCu-exposed fish displayed different responses, with increased activity of branchial enzymes as well as the occurrence of an anemic condition.

Characterization of particles confirmed that nCu were aggregated and polydispersed, with particles showing sizes within and above the nanoparticle range. Thus, fish were exposed to copper nanoparticles. However, the biological effects found are not necessarily related to the nanoparticles, and even less related to the primary size of the nanoparticles used. In fact, the biological effects found are a result of a heterogenous suspension. This is particularly important as nanoparticles enter ecosystems, where phenomena such as aggregation can deviate particles from the nanoparticle range, which may change how they interact with biota.

Following waterborne exposure, Cu initially accumulates in the gills of freshwater fish, before being translocated to internal organs. With an increase in the metal concentration and/or time of exposure, accumulation takes place in the liver, the central compartment for metal homeostasis. In smaller quantities, other tissues also accumulate Cu (Grosell, 2012). In the present study, Cu concentrations were analyzed in the gills, liver, gastrointestinal tract, posterior kidney, and muscle of fish exposed to Cu and nCu. A higher concentration of Cu in relation to non-exposed

480 fish was found only in the gills of Cu-exposed fish. This is in accordance with the
481 already established knowledge that the gills are the main organ involved in Cu
482 uptake after waterborne exposure to this metal, and shows that acute exposure to
483 approximately 10 μg L^{-1} of dissolved Cu is capable of promoting Cu accumulation in a
484 tissue of *P. lineatus*. Moreover, in *P. lineatus* exposed to 25 and 29 μg Cu L^{-1} for 96 h
485 in similar water conditions as reported in the present study, i.e., soft water, similar
486 temperature and pH, Cu accumulated in the gills, liver, intestine, and kidney (Mazon
487 and Fernandes, 1999). This indicates a concentration dependence for Cu
488 accumulation in *P. lineatus*, as already reported (Mazon and Fernandes, 1999;
489 Mazon et al., 2002). In addition, Cu accumulation in *P. lineatus* seems to be time-
490 dependent (Takasusuki et al., 2004). Thus, in the concentration and time chosen in
491 the present study, it is possible that copper is under homeostatic control and does not
492 accumulate in internal organs.

493 Regarding nCu exposure, copper did not accumulate in any analyzed
494 tissue. Similar to our findings, exposure to 20 μg Cu L^{-1} for 96 h also promoted
495 accumulation of Cu only in the gills of rainbow trout, whilst the simultaneous
496 exposure to 20 μg nCu L^{-1} did not promote accumulation of Cu in any analyzed
497 tissues (Shaw et al., 2012). However, in the same study, accumulation of Cu in the
498 gills was noticed after 10 days of exposure to nCu. This suggests a time dependence
499 for Cu accumulation in the gills of fish after exposure to Cu nanoparticles. As the
500 authors pointed out, it seems unlikely that accumulation would be a result of Cu ions
501 released from nCu instead of internalized nanoparticles (Shaw et al., 2012). Indeed, it
502 has already been shown that Cu nanoparticles can be internalized in gill cells
503 (Mansano et al., 2018).

504 Furthermore, the similar Cu concentrations found in the

gastrointestinal tract (GI tract) of fish exposed to CTR, Cu, and nCu indicates that *P. lineatus* is not feeding from sedimented nanoparticles. However, a higher concentration of 100 µg nCu L^{-1} promoted accumulation of Cu in the intestine of rainbow trout after 96 h waterborne exposure with no feeding, while this feature was not found in Cu-exposed fish in the same time and concentration (Shaw et al., 2012). Additionally, in a higher time of exposure of 21 days with feeding, goldfish (*Carassius auratus*) exposed to 1 and 10 mg L^{-1} nCuO showed greater accumulation in the intestine, followed by the gills and the liver (Ates et al., 2015). Juvenile carp (C*yprinus carpio*) exposed to 100 mg L^{-1} nCuO for 30 days with feeding also presented higher accumulation in the intestine throughout the exposure period (after 1, 3, 5, 10, 15, 20, 25, and 30 days) (Zhao et al., 2011). Indeed, nCu can be internalized by intestinal cells, as evidenced by transmission electron microscopy images (Zhao et al., 2011). Therefore, understanding copper transport and distribution in the body of *P. lineatus* should be addressed by further studies with a higher concentration and/or longer time of exposure to nCu.

Hematocrit and hemoglobin concentration were not affected by Cu exposure, but decreased after nCu exposure. This indicates that nCu-exposed fish were anemic, with impairment in gas transport (Witeska, 2015). This finding suggests additional respiratory distress in nCu-exposed fish, compared to Cu-exposed fish. It is already known that Cu promotes respiratory injury, but lethality is often attributed to ionoregulatory disturbance (Grosell, 2012). For nCu, the events leading to lethality are not clear.

Additionally, Gupta et al. (2016) found that ferritin heavy chain (FHC) was down-regulated in the liver of juvenile carps (*C. carpio*) exposed to nCu. As ferritin plays an important role in iron metabolism (Percy et al., 1998), impairment in

the function of this enzyme could have led to the observed reduction in Hb concentration and Ht. In contrast, Shaw et al. (2012) and Al-Bairuty et al (2016) did not find any differences in Ht, Hb, or RBC of rainbow trout exposed to 20 µg nCu L^{-1} for 96 h. However, there is a lack of studies on hematological responses of fish after acute exposure to nCu. As rainbow trout and *P. lineatus* displayed different hematological responses after nCu exposure, further studies should evaluate these responses in other fish species.

The fish gill is the primary organ involved in osmotic and ionic regulation (Evans et al., 2005). Freshwater teleosts are hyperosmotic relative to the surrounding water, so the gill epithelia rely on active transporters for ion uptake, such as the membrane bound enzymes NKA, HATP, and CaATP. In the present study, Cu exposure promoted the inhibition of HATP and CaATP. Copper has been described as an ionoregulatory toxicant that inhibits enzymes through interaction with -SH residues (Viarengo et al., 1993). Besides, membrane enzymes can be inactivated as a result of ROS interaction and lipid peroxidation (Stark, 2005), a possibility in the present study, considering the evidence of oxidative stress found, that is, the increased LPO in the liver.

Apical HATP promotes the extrusion of protons to the water, increasing the electrical gradient that favors Na^+ entry into branchial cells through Na^+ channels. Once inside the cell, Na^+ is mainly pumped into the blood through the basolateral NKA, in exchange with K^+ (Grosell et al., 2002; Hwang et al., 2011). In the present study, inhibition of HATP after Cu exposure did not compromise Na^+ uptake, as plasma Na^+ levels were maintained. Possibly, NKA was able to generate the driving force for Na^+ absorption, since this enzyme was not affected by Cu exposure. For freshwater fish, inhibition of NKA is considered the mechanism of toxicity after

555 acute waterborne Cu exposure. It is possible that Cu accumulation in the gills of *P.*
556 *lineatus* exposed to Cu was below the threshold to promote NKA inhibition. However,
557 the present study shows that HATP and CaATP can be disturbed in concentrations
558 that do not affect NKA activity. In addition, maintenance of plasma Na^+
559 concentrations may be a result of the observed mitochondria rich cell (MRC)
560 hyperplasia, which is a common response in fish that encounter adversities in
561 maintaining the Na^+ and Ca^{2+} balance (McDonald and Wood, 1993), as will be
562 discussed below.

563 Ca^{2+} uptake occurs mainly in MRC, through a trans-epithelial
564 pathway consisting of passive influx of ions through apical Ca^{2+} channels, followed by
565 active transport into the blood via CaATP and Na^+/Ca^{2+} exchanger (Perry, 1997). In
566 our study, although Cu inhibited the branchial CaATP, a change in plasma Ca^{2+}
567 concentrations was not observed. At the same time, we observed an increase in the
568 frequency of MRC and/or mucous cells in Cu-exposed fish. MRC proliferation could
569 act as a compensatory response to an ionoregulatory disturbance, maintaining the
570 homeostasis of the plasmatic ions analyzed. Indeed, Shephard and Simkiss (1978)
571 found that Cu ions inhibit CaATP in the gills of roach (*Rutilus rutilus*) *in vitro* and
572 promote proliferation of this enzyme *in vivo*. Acute exposure to copper also inhibited
573 branchial CaATP in tilapia (*Oreochromis niloticus*) (Atli and Canli, 2011), and
574 promoted MRC proliferation in this species (Pelgrom et al., 1995). MRC proliferation
575 was also found in the gills of *P. lineatus* exposed to 20, 25, and 29 µg Cu L^{-1} (Mazon
576 et al., 2002). Thus, MRC proliferation could be a useful biomarker of Cu
577 contamination in freshwater environments using the bioindicator *P. lineatus*, since
578 this fish species displays the same response to a wide range of Cu concentrations.

579 While fish exposed to Cu exhibited frequent MRC or mucous cell

hyperplasia, this was rare in fish exposed to nCu and absent in control fish. These histological changes were also found in the gills of the Amazon characid *P. axelrodi* exposed to Cu and, in contrast to our study, in fish exposed to nCu (Braz-Mota et al., 2018). Regarding mucous cells, secretion of mucous has several functions for fish, including a protective role against some metals (Reverter et al., 2018). However, hyperplasia of cells led to lamellar fusion, which can compromise gas exchange (Wilson and Taylor, 1993). Additionally, fish exposed to Cu exhibited frequent hypertrophy of pavement cells, whilst this change was fairly frequent in fish exposed to nCu and rare in control fish. Mazon et al. (2002) also reported hypertrophy of pavement cells in the gills of *P. lineatus* exposed to Cu. This feature results in increased water-blood diffusion distance, which, as well as being a barrier to toxicants, can also compromise gas exchange (Camargo and Martinez, 2007). Lastly, a fairly frequent marginal channel expansion was observed only in Cu-exposed fish. This can be related to an increase in blood flow, in order to increase diffusion of O_2 and compensate the trade-off of thickening of the lamellae. Cu is a well-known respiratory toxicant (Grosell, 2012; Mazon et al., 2002). Impairment of gas exchange can result in reduced partial pressure of oxygen (PO_2), affecting the supply of O_2 to tissues, and increased partial pressure of carbon dioxide (PCO_2), leading to respiratory and metabolic acidosis (Grosell, 2012). Finally, we found increased damage in the gills of fish exposed to Cu and nCu compared to control, with significantly greater injury in fish exposed to Cu. Al-Bairuty et al. (2013) also reported greater injury in the gills of fish exposed to $CuSO_4$ than to nCu.

With respect to nCu exposure, it is possible that NKA and HATP increased as a response to an acidosis condition. Acidosis occurs mainly due to lowered ventilation and/or due to anaerobic metabolism. Additionally, H^+ excretion

and Na^+ uptake are linked by the apical Na^+/H^+ exchanger (NHE), Na^+ channels (ENaC), and HATP, and the basolateral NKA (Perry and Gilmour, 2006). In the present study, Hb and Ht decreased, indicating impairment in oxygen uptake. Less oxygen in tissues could have increased anaerobic metabolism, leading to metabolic acidosis and subsequent increases in NKA and HATP as an adaptative response. Fish exposed to silver nanoparticles presented a decrease in blood PO_2 level without a change in basal metabolic rate, indicating impairment in gas exchange (Bilberg et al., 2010).

An increase in NKA activity was found in the gills of goldfish (*C. auratus*) exposed to suspensions of nCuO at concentrations of 40, 80, and 160 mg/L for 96 h (Xia et al., 2013). However, some studies reported a decrease in NKA (Griffitt et al., 2007; Shaw et al., 2012) and HATPase (Braz-Mota et al., 2018) activities in fish exposed to nCu. Braz-Mota et al. (2018) found that the different osmoregulatory strategies of two Amazon fish species resulted in different responses of each species to Cu and nCu. Thus, the ionoregulatory response we found for *P. lineatus* could be species-specific.

The liver has an essential role in Cu metabolism in fish (Grosell, 2012). This can be evidenced by the highest levels of Cu found in the liver compared to the other organs evaluated in this study. As Cu is required in small quantities for several cellular functions but can be toxic in higher amounts, the liver possess a fine regulation of Cu levels. Here we highlight the role of GSH as a Cu ligand (Grosell, 2012). In the present study, GSH concentration was not affected by Cu or nCu exposure, a result also found for this fish species at the same time of exposure to 5, 9, and 20 µg Cu L^{-1} (Simonato et al., 2016). This suggests that, in the experimental conditions presented, the liver is able to maintain normal GSH levels. However,

different times of exposure and antioxidant defenses must be addressed to better elucidate the role of this antioxidant in *P. lineatus* after waterborne Cu exposure. Furthermore, increased LPO was found in the liver of fish exposed to Cu. Lipoperoxidation results from the interaction of ROS with cellular constituents, and evidences a condition of oxidative stress. Cu is well known for the generation of ROS through the Fenton reaction (Grosell, 2012), and an alteration in the redox status with increased LPO has already been described for *P. lineatus* (Simonato et al., 2016).

Furthermore, the present study also evaluated histological changes in the liver of the fish. No significant differences were found for DTC values among exposures, however, frequency of irregular shaped cells increased in nCu exposure compared to CTR and Cu, frequency of cell hypertrophy was greater in Cu exposure compared to CTR and nCu, and rupture of cells was found in fish exposed to Cu and nCu, but were rare in the CTR group. Thus, in general, the frequency of cellular damage increased in Cu and nCu exposure compared to control. In addition, vacuoles in the cytoplasm ranged from fairly frequent in CTR to very frequent in Cu and nCu groups. Cytoplasmic vacuolization indicates the presence of poorly preserved or stained materials, such as fat or glycogen (Hibiya and Takashima, 1995). Toxic exposure can result in both accumulation or depletion of fat or glycogen in the liver of fish (Wolf and Wolfe, 2005). In the present study, vacuoles presented a glycogen-type morphology. In *P. lineatus*, Cu impairs the glycolytic capacity of the liver by reducing phosphofructokinase (PFK) and pyruvate kinase (PK) activities after 96 h exposure (Carvalho and Fernandes, 2008). Thus, additional impairment of glycogenolysis (leading to accumulation of glycogen in the liver) is possible and should be addressed in further studies. Finally, increased liver glycogen content can affect liver function, as demonstrated by Hilton and Dixon (1982).

Together, the histological findings suggest that the liver was adversely affected by Cu and nCu regarding hepatocyte morphology, and the DTC value for CTR was the highest among treatments due to impairment in blood flow, i.e., the observed hyperemia in these fish, which was rare in fish exposed to Cu and nCu. The liver is the site of several vital functions, such as nutrient storage, bile secretion, detoxification, and metabolism. These functions can be compromised due to altered cellular morphology (Hinton et al., 2001).

5. Conclusions

The current environmental guidelines in Brazil for protection of aquatic communities establishes that dissolved Cu in freshwater cannot exceed 13 μg L^{-1} (CONAMA, 2005). The present study shows that a concentration below 13 μg L^{-1} was able to promote adverse effects in the native species *P. lineatus.* Additionally, sublethal effects in *P. lineatus* were seen in concentrations as low as 5 μg L^{-1} (Simonato et al., 2016). As these authors pointed out, and we endorse, the current environmental regulation is not protecting a representative fish species, and therefore should be revised. Furthermore, although these guidelines include dissolved metals, proper regulation for metal nanoparticles is absent. In this regard, it is urgent to acknowledge the toxicity and effects of metals nanoparticles, and whether these are different from dissolved metals. In this way, it could be possible to determine if the already existing regulations for metals are suitable to protect aquatic life from nanoparticles. In the present study, a representative native fish species was exposed to similar dissolved concentrations of copper chloride and copper nanoparticles. Within the chosen biomarkers, exposure to copper nanoparticles was less toxic than exposure to the metal salt. However, copper nanoparticles promoted different

680 responses in comparison to those promoted by copper chloride, indicating that the
681 soluble and nano forms of the metal acted differently. To better elucidate the
682 questions raised by the present study, further investigations are needed.
683
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