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Mechanisms of toxic action of copper and copper nanoparticles in two Amazon fish species: Dwarf cichlid (*Apistogramma agassizii*) and cardinal tetra (*Paracheirodon axelrodi*)

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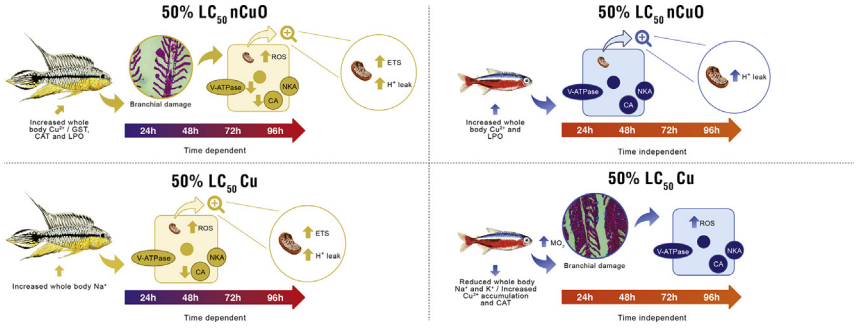
HIGHLIGHTS

- Copper nanoparticles were less toxic than copper to both fish species.
- Dwarf cichlid showed toxic effects time-dependent and Cardinal time-independent.
- nCuO promoted oxidative stress in dwarf cichlid, being the cardinal less affected.
- Cu affected metabolic dysfunctions in Cardinal but not in dwarf cichlid.
- Toxicity mechanisms are related to differential osmoregulatory strategies between the species.

GRAPHICAL ABSTRACT



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ABSTRACT

Copper oxide nanoparticles (nCuO) are widely used in boat antifouling paints and are released into the environment, potentially inducing toxicity to aquatic organisms. The present study aimed to understand the effects of nCuO and dissolved copper (Cu) on two ornamental Amazon fish species: dwarf cichlid (*Apistogramma agassizii*) and cardinal tetra (*Paracheirodon axelrodi*). Fish were exposed to 50% of the LC_{50} for nCuO (dwarf cichlid 58.31 μg L^{-1} and cardinal tetra 69.6 μg L^{-1}) and Cu (dwarf cichlid 20 μg L^{-1} and cardinal tetra 22.9 μg L^{-1}) for 24, 48, 72 and 96 h. Following exposure, aerobic metabolic rate ($\dot{M}O_2$), gill osmoregulatory physiology and mitochondrial function, oxidative stress markers, and morphological damage were evaluated. Our results revealed species specificity in metabolic stress responses. An increase of $\dot{M}O_2$ was noted in cardinal tetra exposed to Cu, but not nCuO, whereas $\dot{M}O_2$ in dwarf cichlid showed little change with either treatment. In contrast, mitochondria from dwarf cichlid exhibited increased proton leak and a resulting decrease in respiratory control ratios in response to nCuO and Cu exposure. This uncoupling was directly related to an increase in reactive oxygen species (ROS) levels. Our findings reveal different metabolic responses between these two species in response to nCuO and Cu, which are probably caused by the differences between species natural histories, indicating that different mechanisms of toxic action of the contaminants are associated to differential osmoregulatory strategies among species.

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1. Introduction

The progressive and substantial increase in the production and use of nanoparticles (NPs) in industrial and consumer products has promoted an increase in the input of NPs into aquatic systems (Moore, 2006; Service, 2004). Nanoparticles are materials with at least one dimension smaller than 100 nm (nm), and they exhibit unique properties which are desirable in some applications (EPA, 2007). Copper oxide nanoparticles (nCuO) have biocidal, antibacterial, antiviral and antifungal properties, and are commonly used in antimicrobial drugs and industrial applications like conductive films, lubricants, catalysts, and electronic devices (Chatterjee et al., 2012; Cheon et al., 2012; De Jong and Borm, 2008; Longano et al., 2012). They are also widely used in boat antifouling paints, and their release into the environment can induce toxicity to aquatic organisms.

The surface properties of metal oxide NPs are determined by their acidity constants and zero points of charge (Hristovski et al., 2007), which in turn influences their interactions with aquatic systems. Depending on the nature of the receiving waters, NPs may form stable colloidal suspensions of single particles or become agglomerated, aggregated, or fused (Nowack and Bucheli, 2007). These properties also affect their interactions with biological systems; NPs can cross cell membranes through diffusion or endocytosis, and they can accumulate in intracellular organelles, such as mitochondria (Lynch et al., 2006; Srikanth et al., 2016).

Previous studies have reported low toxicity of nCuO relative to Cu (Griffitt et al., 2007; Karlsson et al., 2013; Shaw et al., 2012; Srikanth et al., 2016). Dissolution of nCuO and the release of Cu ions likely contributes to Cu accumulation and bioactivity in zebrafish (Griffitt et al., 2009; Griffitt et al., 2008) and rainbow trout (Shaw et al., 2012). Even at low levels in the environment, waterborne copper ions (Cu^{2+}) are toxic to freshwater organisms and are widely recognized to induce histo-morphological alterations in target organs such as the gill (Griffitt et al., 2009). This damage can impair gas exchange, Na^+ and Cl^- homeostasis, and nitrogenous waste excretion, which may negatively affect fish performance and health (Grosell et al., 2012). These primary effects on respiration and ionoregulation have been suggested to imbalance the osmoregulatory functions of fish, increasing the energetic costs of ionoregulation to counter-act the increased Na^+ efflux and turnover rates induced by Cu exposure (Campbell et al., 2002; Grosell et al., 2002; Grosell et al., 2012). In this regard, it has been suggested that the main target-mechanisms for acute Cu toxicity in teleost fish are species-specific.

In rainbow trout, acute Cu exposure has negligible effects on whole animal oxygen consumption ($\dot{M}O_2$) (De Boeck et al., 2006), but consistently stimulates diffusive Na^+ losses and inhibits Na^+ uptake (Chowdhury et al., 2016). The latter responses were directly linked to the inhibition of gill Na^+/K^+-ATPase, V-type H^+-ATPase and carbonic anhydrase (CA) (Chowdhury et al., 2016). In contrast, common carp (*Cyprinus carpio*) and gibel carp (*Carassius auratus gibel*) exposed to Cu exhibited a decline in $\dot{M}O_2$, suggesting that the disruption of aerobic metabolism might be a key factor for acute toxicity in these species (De Boeck et al., 1995, 2006). Furthermore, in spite of the depletion of whole body Na^+ levels, acute Cu exposure does not inhibit Na^+ uptake in zebrafish (*Danio rerio*) but markedly reduces Ca^{2+} uptake (Alsop and Wood, 2011). To date, the effects of nCuO on the "osmoregulatory compromise" of freshwater teleost fish remains unclear.

In waterborne exposures, accumulation of Cu in gills and internal organs may have a substantial impact on cellular metabolism and whole animal respiration. Cu can promote mitochondrial dysfunction and exert deleterious effects on metabolic energy production of fish (Sappal et al., 2014, 2015). Intracellular Cu accumulation beyond toxicity thresholds can lead to excessive generation of reactive oxygen species (ROS), facilitated by the redox-active nature of Cu, which can cycle between Cu^+ and Cu^{2+} (Braz-Mota et al., 2017; Linder and Hazegh-Azam, 1996). Oxidative stress can alter electron transport capacity and/or disrupt mitochondrial membrane integrity, resulting in a decline in ATP production (Sappal et al., 2014, 2015). To avoid this, cells rely on enzymes such as glutathione S-transferases (GST), superoxide dismutase (SOD), and catalase (CAT) to neutralize ROS and prevent damage associated with DNA and lipid peroxidation (Sies, 1999).

Teleost fish that have evolved in acidic ion-poor environments, like "blackwaters" from the Amazon, display remarkable adjustments in their metabolism (Campos et al., 2017), ionoregulation (Duarte et al., 2013; Gonzalez et al., 2002; Matsuo and Val, 2007), and nitrogen waste excretion (Wood et al., 2014; Wood et al., 2017), to maintain their internal homeostasis in this challenging environment; for example, Amazonian Characidae species (such as *Paracheirodon axelrodi*, *P. innesi*, and *Hemigrammus* sp.) display a specialized acid-insensitive Na^+ transport mechanism in their gills (i.e. high affinity, low K_m) and a high capacity for Na^+ uptake (J_{max}). In contrast, Cichlidae species (e.g. *Pterophyllum scalare*, *Symphysodon discus* and *Apistogramma agassizii*) exhibit an acid-sensitive Na^+ transport system, with a low capacity and affinity for Na^+ uptake, but have gills will low intrinsic permeability (Duarte et al., 2013; Gonzalez and Wilson, 2001; Gonzalez et al., 2002; Wood et al., 2014). The two fish species cardinal tetra (*P. axelrodi*) and dwarf cichlid (*A. agassizii*) are very sensitive to Cu exposure (LC_{50}–96 h values of 40.1 and 45.9 μg Cu L^{-1}, respectively) (Duarte et al., 2009), which, in the case of cardinal tetra, seems to be strongly related to increased net Na^+ losses (Crémazy et al., 2016). The different ionoregulatory strategies exhibited by Amazon fish may lead to differences in the energetic costs of maintaining ionic and osmotic homeostasis when exposed to waterborne toxicants, as Cu and nCuO. This may be exacerbated by species-specific differences in habitat and lifestyle, with active swimmers like cardinal tetra potentially paying a more substantial energetic penalty for Cu exposure than the resident and sedentary dwarf cichlid. Detailing the mechanisms underlying acute Cu toxicity in these diverse species will, therefore, be valuable in understanding the broader impacts of Cu and nCuO toxicity in Amazonian fish.

With the growing threat of metal contamination in Amazonian waters (Silva et al., 1999; Sampaio, 2000), and the increased risk of nanomaterials toxicity to aquatic organisms (Moore, 2006), our goal is to understand the mechanisms of toxic action of Cu and nCuO in dwarf cichlid and cardinal tetra. We hypothesize that these two Amazon fish species will respond differently to exposure to these compounds because of their different metabolic and ionoregulatory strategies (Campos et al., 2017; Gonzalez and Wilson, 2001; Gonzalez et al., 2002).

2. Material and methods

2.1. Nanoparticle characterization

Copper oxide nanoparticles with an advertised diameter of <50 nm and a specific surface area of 29 $m^2\ g^{-1}$ were purchased from Sigma Aldrich (544868). Hydrodynamic diameter and zeta (ζ) potential were measured using Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) at 25 °C. Scanning electron microscopy (SEM) was used to confirm primary particle size as previously described (Callaghan et al., 2016). Concentrated exposure stock suspensions of 3 mg mL^{-1} were prepared immediately before use. The nCuO was dispersed by sonication (Sonics Vibra-Cell VCX 500) for 10 min, at 80% of the maximum capacity of the equipment.

2.2. Animals and housing

Dwarf cichlid (*Apistogramma agassizii*) and cardinal tetra (*Paracheirodon axelrodi*) were purchased from an ornamental fish shop in Manaus (Amazonas, Brazil), and transferred to the Laboratory of Ecophysiology and Molecular Evolution at the Brazilian National Institute for Research of the Amazon (INPA), where they were maintained

indoors for at least 1 month in 450 L fiberglass tanks, supplied with continuous aeration and flow through well water, vigorously aerated prior to use to reduce dissolve CO_2 (in μmol L^{-1}; Na^+, 43; Cl^-, 31; K^+, 10; Ca^{2+}, 9; Mg^{2+}, 4; pH 6.0, 6.40 mg O_2 L^{-1} and 29 °C). Throughout the acclimation period fish were fed ground dry commercial trout pellets once a day and held on a 12 h light/12 h dark photoperiod. Feeding was suspended 24 h before to the experiments. There was no mortality observed during the acclimation period.

2.3. Handling of fishes after exposure

Experimental and holding procedures followed the CONCEA (National Council of Animal use in Research and Education) animal care guidelines and were approved by INPA's animal care committee (protocol number: 026/2015). Before collection, the animals were euthanized by a blow to the head followed by severing the spinal cord with a scalpel. The fish were weighed and the gills were then removed for histological, mitochondrial and enzymatic analyses. Whole bodies were used for measurements of oxidative stress enzymes and ions. Tissues not used for histology or mitochondrial isolations were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. The gills and whole body were homogenized on ice for all enzymatic analyses.

2.4. Determination of acute nCuO toxicity

The nCuO toxicity to dwarf cichlid and cardinal tetra was evaluated through determination of the lethal concentration at 96 h of exposure (LC_{50}-96 h). Groups of 10 individuals of each species were transferred from the holding tanks to six 1.5 L glass aquariums and allowed to settle for at least 24 h before testing. Dwarf cichlid (0.89 ± 0.07 g) and cardinal tetra (0.67 ± 0.04 g) were subsequently exposed for 96 h to 6 nCuO concentrations (measured Cu concentrations ranging from 54 to 187 μg L^{-1} for dwarf cichlid and 59–91 μg L^{-1} for cardinal tetra), in addition to a control group.

All tests were performed in a semi-static system where 80% of water volume was replaced every 24 h, and each concentration of nCuO was tested in replicate twice. Water samples were collected every 12 h and stored at −20 °C for subsequent Cu analysis. Fish mortality levels at each concentration of nCuO tested were used to calculate the LC_{50}–96 h values using the trimmed Spearman Karber method.

2.5. Sub-lethal experimental series

After the acclimation period, groups of 21 fish of each species were transferred to 21 individual aerated glass tanks filled with 100 mL of well water, one fish per aquarium, and allowed to recover from handling overnight (dwarf cichlid, 1.0 ± 0.07 g and cardinal tetra, 0.78 ± 0.08 g). Fish were then exposed for 24, 48, 72 and 96 h to 50% nCuO LC_{50}-96 h (nCuO) (58.31 μg Cu L^{-1} to dwarf cichlid and 69.6 μg Cu L^{-1} to cardinal tetra) or to 50% Cu LC_{50}–96 h (Cu) (20 μg Cu L^{-1} to dwarf cichlid and 22.9 μg Cu L^{-1} to cardinal tetra), as reported by Duarte et al. (2009) and compared to a control group in clean water. The concentration of 50% of the LC_{50} was chosen to minimize lethality and ensure that the responses observed were the result of sub-lethal toxicity. The Cu concentrations were prepared from a stock solution of 1 M $CuCl_2 \cdot H_2O$ 24 h before the experiments. The experiments employed a semi-static system where 80% of the water volume was replaced every 24 h. During the nCuO exposures of 24, 48, 72 and 96 h, dwarf cichlid and cardinal tetra showed an average mortality of 1%, 4%, 3%, 0% and 1%, 3%, 1%, 0%, respectively. No mortality was observed in the control group. Identical experimental exposures were repeated three times to evaluate the effects of sub-lethal levels of Cu and nCuO. In the first experimental series we evaluated the osmoregulatory compromise, where we determined the routine metabolic rate, gill Na^+/K^+-ATPase (NKA), v-type H^+-ATPase and carbonic anhydrase (CA) activities, concentrations of major ions (Na^+, K^+, C a^{2+}, Mg^{2+} and Cl^-), Cu accumulation, activities of glutathione-S-transferase (GST), catalase (CAT), superoxide dismutase (SOD), and lipid peroxidation (LPO) in whole body of fish exposed to Cu and nCuO. In the second series of sub-lethal exposures we evaluated the mitochondrial physiology, and in this series, we measured gills Electron Transport System (ETS), leak state, Reactive Oxigen Species (ROS) in ETS and leak state, and Respiratory coupling ratio (RCR). The last experimental series we evaluated the morpho-histological alterations in the gills. In all the experiments the fishes were unfed, to avoid energy expenditure of digestion.

2.6. Osmoregulatory compromise

2.6.1. Metabolic rate

For each treatment and control group, individuals of both species ($n = 7$) were transferred to 70 mL glass chamber immersed in a water bath tank with aerated water. Routine $\dot{M}O_2$ was determined using an automated intermittent flow respirometry system (Oxy-4; Loligo Systems, Viborg, DEN). A pump interfaced to a DAQ-M (Loligo systems) data acquisition system controlled the measurement cycle, with a loop consisting of 3 phases: ambient flush (180 s), wait (120 s), and measurement (300 s). After 1 h of handling recovery in ambient flush conditions (duration established in preliminary tests), measurements were collected over 4 h. Metabolic rate was calculated as: $\dot{M}O_2 = \Delta O_2 * Vresp * B^{-1}$, where: ΔO_2 is the rate of change in oxygen concentration (mg O_2 h^{-1}), Vresp is the volume of the respirometry chamber, and B is the mass of the individual (kg).

2.6.2. Osmoregulatory enzymes

The activities of both NKA and v-type H^+-ATPase were determined by NADH oxidation in an enzymic reaction coupled to the hydrolysis of ATP (Kültz and Somero, 1995). The assay is based on the inhibition of NKA activity by oubain (2 mM), and v-type H^+-ATPase by N-ethylmaleimide (NEM, 2 mM). Gills were homogenized (1:10 *w*/*v*) in buffer (pH 7.5) containing (in mM): sucrose 150, imidazole 50, EDTA 10 and deoxycholic acid 2.5, and centrifuged at 2,000 ×*g* for 7 min at 4 °C. Supernatants were added to a reaction mixture containing (in mM): imidazole 30, NaCl 45, KCl 15, $MgCl_2$ 3.0, KCN 0.4, ATP 1.0, NADH 0.2, fructose-1,6-bisphosphate 0.1, PEP 2.0, with 3 U mL^{-1} pyruvate kinase and 2 U mL^{-1} lactate dehydrogenase. Samples were run with and without ouabain or NEM. Absorbance was followed over 10 min at 340 nm. NKA and H^+-ATPase activities were calculated by the differences between total activity and activities with ouabain and NEM inhibitors, respectively.

Gill CA activity was quantified according to the assay described by Vitale et al., 1999, based on Henry, 1991. Gills were homogenized (1:10 *w*/*v*) in phosphate buffer (10 mM, pH 7.4) and centrifuged at 2000*g* for 5 min at 4 °C. Supernatants (50 μL) were added in 7.5 mL of reaction buffer pH 7.4 containing (in mM): mannitol 225, sucrose 75, and tris-phosphate 10 and 1 ml of cold distilled water saturated with CO_2. Immediately after the addition of CO_2-saturated water, the reduction in pH was followed for 20 s, with pH readings every 4 s. Carbonic anhydrase specific activity (CAA) was calculated as: $CAA = [(CR/NCR)^{-1}]$ mg^{-1} total protein in the sample (CR: Catalyzed rate and NCR: non-catalyzed rate).

2.6.3. Ions content in water and whole body

In water and whole body samples, Cu and major cation (Na^+, K^+, Mg^{2+} and Ca^{2+}) concentrations were determined by plasma atomic emission spectrometry (Thermo Scientific iCAP 7600 ICP-OES), using an atomic spectroscopy standard Perkin Elmer Pure as reference. Total chloride was measured by the colorimetric assay described by Zall et al., 1956. Aqueous ammonia levels were measured using the colorimetric assay developed by Verdouw et al., 1978.

2.6.4. Antioxidant enzymes and lipid peroxidation

Whole body homogenates were prepared in buffer (pH 7.6) containing (in mM): Tris base 20, EDTA 1.0, dithiothreitol 1.0, sucrose 50, KCl 150. Homogenates were centrifuged at 15,000 *g* for 20 min at 4 °C, and used to determine GST, SOD and CAT activities, and LPO levels (1:10 *w*/*v* for GST and SOD and 1:4 w/v for CAT and LPO). GST activity was determined according to Keen et al., 1976 using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. Changes in absorbance were recorded at 340 nm, and activity was calculated as nmol CDNB conjugate min^{-1} mg $protein^{-1}$, using a molar extinction coefficient of 9.6 mM cm^{-1}. Superoxide dismutase (SOD) activity was quantified based on the inhibition of cytochrome *c* reduction rate by the superoxide radical at 550 nm and 25 °C (Turrens, 1997). Enzyme activity is expressed as U SOD mg $protein^{-1}$, where 1 U of SOD corresponds to the quantity of enzyme that promoted the inhibition of 50% of cytochrome *c*.

To determine the CAT activity, the inhibition rate of H_2O_2 decomposition was monitored at 240 nm (Beutler, 1975), and expressed as μmol H_2O_2 min^{-1} mg $protein^{-1}$. The LPO levels were quantified in an assay based on the Fe^{+2} to Fe^{+3} oxidation by hydroperoxides in acid medium, in the presence of ferrous oxidation-xylenol orange, at 560 nm (Jiang et al., 1991). Total protein in gills and whole body samples were determined spectrophotometrically at 595 nm (SpectraMax M2, Molecular Devices Inc., Sunnyvale, CA, USA), according to the colorimetric assay (Bradford, 1976) using a bovine serum albumin (BSA) as standard.

2.7. Mitochondrial physiology

2.7.1. Electron transport system capacity and leak state

After dissection, gills were immersed in 2 mL relaxing BIOPS buffer (pH 7.1) containing (in mM): CaK_2EGTA 2.77, K_2EGTA 7.23, Na_2ATP 5.77, $MgCl_2 \cdot 6H_2O$ 6.56, taurine 20, imidazole 20, dithiothreitol 0.5, K-MES 50, sodium phosphocreatine 15 and sucrose 50. The gills were dried, weighed, and immerse in 0.5 mL of buffer MiR05 (pH 7.24) at 20 °C containing (in mM): EGTA 0.5, $MgCl_2 \cdot 6H_2O$ 3.0, potassium lactobionate 60, taurine 20, KH_2PO_4 10, Hepes 20, sucrose 160 and BSA 1.0, essentially fatty acid free, and homogenized by hand. The homogenate was diluted in 2.5 mL of Mir05 and oxygen consumption was analyzed in an Oxygraph 2k Oroboros- Innsbruck Austria (Gnaiger et al., 2000).

Oxygen was added into the gas phase above media prior to closing chambers to supersaturate gills using MiR05 Buffer. Complex I (CI) substrates (2 mM malate and 10 mM pyruvate) were added to measure state II respiration through CI in the absence of ADP (denoted 'Leak I'). Excess ADP (2.5 mM) stimulated oxidative phosphorylation (OXP-I, state III respiration), and glutamate (10 mM) was added to saturate CI. Cytochrome c (10 μM) was added to test outer membrane integrity. Phosphorylating respiration with CI and complex II (CII) substrates (OXP-I, II) was measured by the addition of succinate (10 mM). The respiratory electron transfer-pathway capacity (denoted "ETS"), repeated titrations of carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone (FCCP, 0.5 μM) were performed. The activity of CI, II and III complexes were then inhibited by the addition of rotenone (0.5 μM), malonate (15 mM), and antimycina (1 μM), respectively. RCR was calculated as ETS/leak state ratio.

2.7.2. ROS emission

ROS emission was measured in parallel with mitochondrial respiration. Superoxide dismutase (SOD; at 22.5 U mL^{-1}) was added to catalyze the reaction of the superoxide produced by the mitochondria and horseradish peroxidase (3 U mL^{-1}) was added to catalyze the reaction of hydrogen peroxide with Ampliflu Red (15 μM) and produce the fluorescent product resorufin (detected using an excitation wavelength of 525 nm and ampliometric filter set (AmR); Oroboros Instruments). The resorufin signal was calibrated with additions of exogenous hydrogen peroxide.

2.8. Gills histopathology

2.8.1. Light microscopy

Gill arches were fixed in 2.5% glutaraldehyde (GTA) for 24 h and subsequently dehydrated in ethanol (70%, 80% and 96%). Samples were embedded in Paraplast Plus (Sigma Aldrich), and serial sections of 3-μm thickness tissue were prepared on glass slides, which were stained with hematoxylin and eosin, and then contrasted with P*eriodic acid–Schiff* (*PAS*). Samples were analyzed at 100× magnification in a light microscope. The prevalence of histopathological lesions in branchial tissue, and gill lesion index were determined (Poleksic and Mitrovic-Tutundzic, 1994). To assess branchial tissue alterations, indices based on severity of lesions were calculated by: $I = 1 \sum I + 10 \sum II + 100 \sum III$ where stages I, II and III correspond to the degree of lesion, classified as: normal function of the organ ($I = 0$–10), mild to moderate damage ($I = 11$–20), moderate to severe ($I = 21$–50), severe ($I = 51$–100), and irreparable damage ($I > 100$).

2.8.2. Scanning electron microscopy

Gill arches were also fixed in 2.5% GTA and washed for 24 h with 16% glycerol for scanning electron microscopy (SEM) analyses. Samples were then dehydrated in stepwise ethanol solutions in water (30, 50, 70, 90, 95%) for 20 min at each step, followed by 2 × 20 min rinses in 100% ethanol. They were then critical point dried in liquid CO_2 (Bal-Tec CPD 030), mounted on aluminum specimen supports and sputter-coated with a gold layer in a metalizer (Bal-Ted SCD 050) and examined under an SEM (LEO 435VP).

2.9. Data analysis

The data are presented as mean ± SEM ($n = 7$ in series 1 and 2; $n = 6$ in series 3). Prior the comparative statistical tests, distribution and homogeneity of data were checked. Two-way ANOVA was used to determine differences in all analyzed parameters between fish exposed to the treatments (control, 50% LC_{50} Cu and 50% LC_{50} nCuO), and to evaluate the effect of exposure duration on the parameters, followed by Tukey post-hoc tests. When data violated the premises of ANOVA, a non-parametric Kruskall–Wallis test was used. A significance level of 5% ($p \leq 0.05$) was used in all test procedures. Histopathological alterations were evaluated semi-quantitatively using the method described by Poleksic and Mitrovic-Tutundzic (1994).

3. Results

3.1. Nanoparticle characterization

Characterization data for nCuO dispersed in filtered well water and a representative SEM image are shown in Supplemental Material (S1). The mean hydrodynamic diameter of nCuO was 185 nm, which suggests the occurrence of aggregation as well as a heterogeneous suspension formed by particles. The mean ζ potential was −18.2 mV, indicating a slightly stable colloid.

3.2. Acute toxicity of nCuO to dwarf cichlid and cardinal tetra

The Cichlid species was slightly more sensitive to nCuO, once the nCuOLC_{50}-96 h value for *A. agassizii* (dwarf cichlid) and *P. axelrodi* (cardinal tetra) were, respectivetly, 116.63 μg L^{-1} and 139.21 μg L^{-1} (Fig. 1). Cu LC_{50}-96 h to both fish species were previously determined Duarte et al. (2009) in similar well water conditions. nCuO was much less toxic to both dwarf cichlid and cardinal tetra compared to Cu (Cu LC_{50}-96 h for dwarf cichlid and cardinal tetra were, respectivetly, 40 μg L^{-1} and 45 μg L^{-1}), which is more toxic 2.9 times and 3.1 times, respectively (Fig. 1).

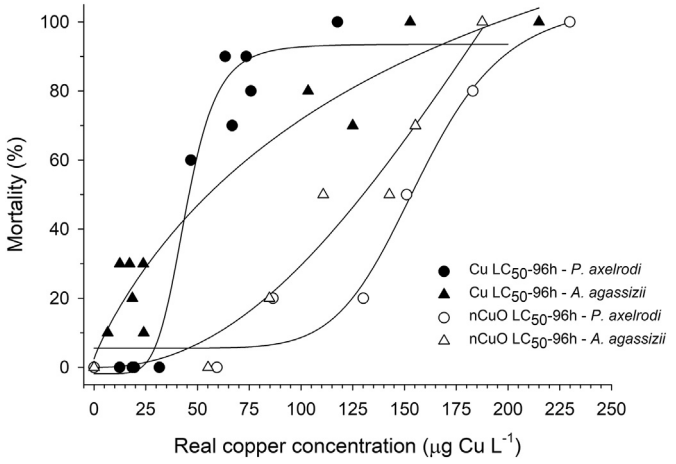

Fig. 1. Relationship between percentage of mortality and real Cu concentration in the acute toxicity tests to determine the lethal concentration (LC_{50}-96 h) of copper (Cu) and copper oxide nanoparticles (nCuO), to both *Apistogramma agassizii* (circles) and *Paracheirodon axelrodi* (triangles). Black symbols represent data from the present study with nCuO, while white symbols represent data of Cu toxicity from Duarte et al. (2009).

3.3. Effects of sub-lethal concentration of Cu and nCuO on the osmoregulatory compromise and Cu accumulation

Dwarf cichlid exposed to nCuO presented an increased $\dot{M}O_2$ over time, roughly doubling after 48, 72 and 96 h in comparison with fish exposed to nCuO for 24 h (Fig. 2A). There were no differences in routine $\dot{M}O_2$ in dwarf cichlid exposed to either Cu or nCuO compared with control (Fig. 2A). With regard to cardinal tetra, an increase in $\dot{M}O_2$ was observed in the 24, 48 and 72 h groups compared to control group exposed to Cu (Fig. 2B). Exposure to nCuO did not affect $\dot{M}O_2$ of cardinal tetra and, conversely, decreased respiration compared to the 72 h Cu group (Fig. 2B).

There were no exposure effects to either Cu or nCuO on branchial Na^+/K^+-ATPase activity in either dwarf cichlid or cardinal tetra throughout the entire experiment in comparison with control (Fig. 3A and D). In dwarf cichlid, gill v-type H^+-ATPase activity was inhibited compared to controls after 24 and 96 h of exposure to nCuO (Fig. 3B). In contrast, dwarf cichlid exposed to Cu for 96 h doubled H^+-ATPase activity in comparison with this same treatment in 24 h (Fig. 3B). There were no exposure effects to either Cu or nCuO in H^+-ATPase activity in cardinal tetra (Fig. 3E). Dwarf cichlid exposed to nCuO had its CA activity inhibited throughout the experiment, while Cu exposure inhibited activity of this enzyme only at 24 h (Fig. 3C). Conversely, CA activity in cardinal tetra showed no alterations, except in fish exposed to nCuO at 48 h (Fig. 3F).

Whole body levels of ions from dwarf cichlid and cardinal tetra exposed to Cu and nCuO are presented in Supplemental Material (S3). The particular increase of Na^+ levels in dwarf cichlid exposed for 72 h to nCuO contrasts with the low levels observed in the same treatment at 24 h. In contrast, cardinal tetra reduced Na^+ levels after 24 and 48 h of exposure to Cu-96 h, while nCuO had no effects whatsoever. No relevant changes were observed for Cl^-, Ca^{2+}, and Mg^{2+} during the experiment.

3.4. Antioxidant responses and cellular oxidative damage mediated by Cu and nCuO

The exposure of dwarf cichlid to nCuO stimulated whole body GST activity after 96 h, in comparison with animals exposed to nCuO over 24 to 72 h (Table 1). The GST activity in these fish was also increased relative to the animals exposed to Cu for 96 h. There were no effects on the GST activity in *P. axelrodi* exposed to Cu and nCuO (Table 1). Exposure of

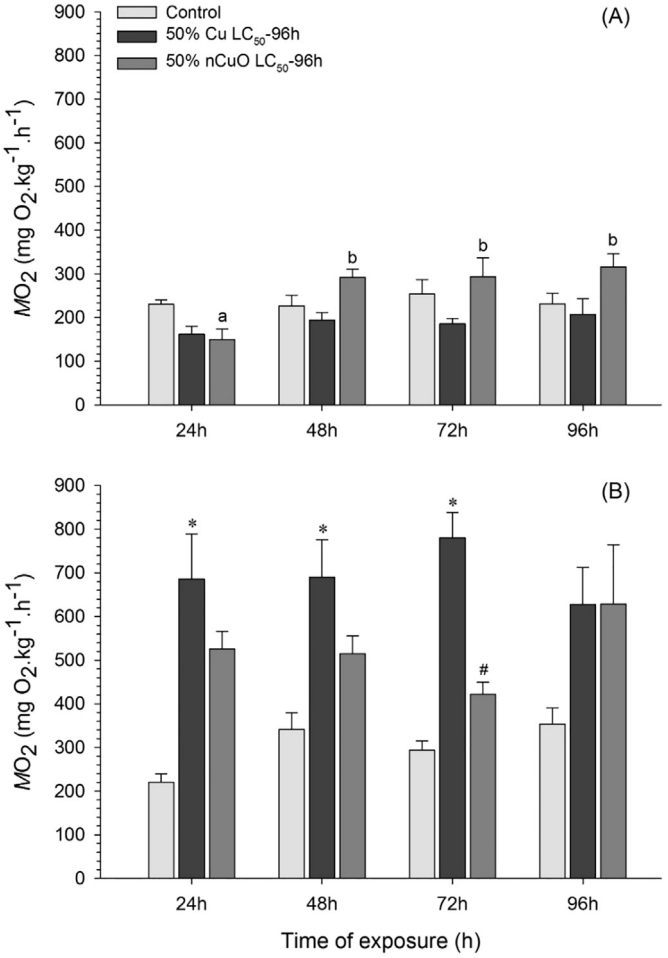

Fig. 2. Oxygen consumption ($\dot{M}O_2$) in (A) *Apistogramma agassizii* and (B) *Paracheirodon axelrodi* after 24, 48, 72, and 96 h exposure to either 50% Cu LC_{50}-96 h and 50% nCuO LC_{50}-96 h, and the control group. Data are presented as mean ± SEM ($n = 7$, at each treatment). Asterisks (*) represent significant differences between the group exposed to either Cu or nCuO and the control group, at each time of exposure ($p < 0.05$). Different letters represent statistical differences between the time of exposure within each treatment ($p < 0.05$). Symbol (#) represents significant differences between groups exposed to Cu and to nCuO at the same time of exposure ($p < 0.05$).

dwarf cichlid to nCuO induced whole body SOD activity after 96 h exposure, in comparison to both control fish to those exposed to Cu over 96 h. SOD activity was stimulated after 24 and 48 h of exposure to Cu, compared to both the control and nCuO groups in cardinal tetra, whereas in Cu exposure SOD activity was initially high but then decreased after 72 h.

CAT activity in whole body of dwarf cichlid increased in fish exposed to nCuO compared to the animals exposed to Cu. There were no effects on the CAT activity in *P. axelrodi* exposed to Cu and nCuO (Table 1). The levels of whole body LPO in dwarf cichlid increased after 48, 72 and 96 h in fish exposed to nCuO. Within the nCuO treatment group, LPO levels reached its highest value at 48 h and declined thereafter. At the 48 h of exposure, LPO levels were also higher than those in animals exposed to Cu at the same time. Differently, cardinal tetra LPO levels doubled only after 96 h exposure to nCuO relatively to the controls.

3.5. Effects of nCuO and Cu exposure to mitochondrial physiology

The exposure to nCuO increased the mitochondrial leak respiration in gills of dwarf cichlid after 24, 48 and 96 h in relation to control and, mitochondrial ROS production in leak state increased in 72 h to nCuO exposure (Fig. 4A). In fish exposed to Cu for 72 and 96 h leak respiration was increased when compared to control. Leak mitochondrial

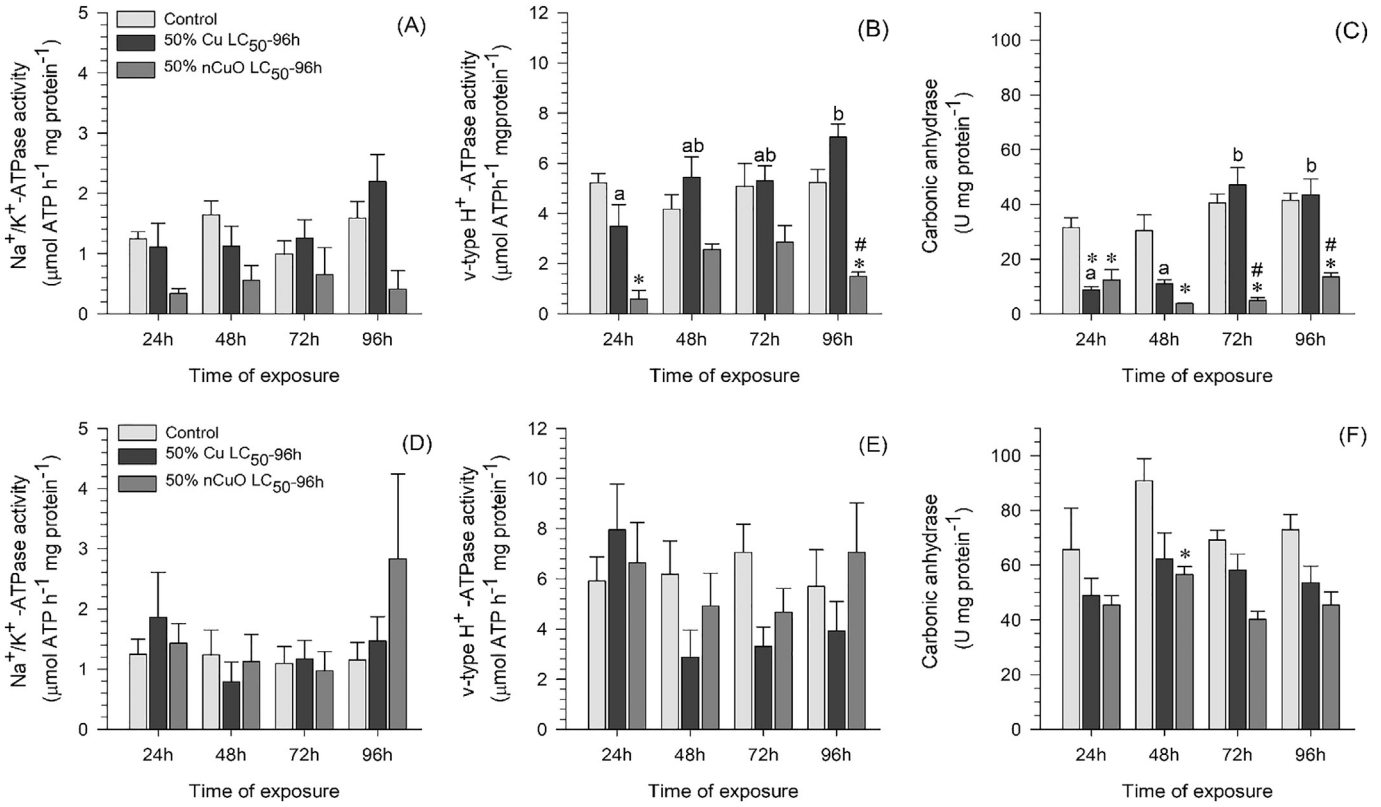

Fig. 3. Branchial activity of Na^+/K^+-ATPase, v-type H^+-ATPase, and carbonic anhydrase in *Apistogramma agassizii* (A, B and C, respectively) and *Paracheirodon axelrodi* (D, E and F, respectively) after 24, 48, 72 and 96 h exposure to either 50% of Cu LC_{50}-96 h and 50% of nCuO LC_{50}-96 h, and the control group. Data are presented as mean ± SEM ($n = 7$, at each treatment). Asterisks (*) represents significant differences between the group exposed to either Cu or nCuO and the control group at each time of exposure ($p < 0.05$). Different letters represent statistical differences between the time of exposure within each treatment ($p < 0.05$). Symbol (#) represents significant differences between group exposed to Cu and nCuO, at the same time of exposure ($p < 0.05$).

Table 1
Activities of gluthatione-S-transferase (GST) and superoxide dismutase (SOD) (both enzymes expressed in U min^{-1} mg $protein^{-1}$), catalase (CAT) (µM H_2O_2 min^{-1} mg $protein^{-1}$) and lipid peroxidation (LPO) (µM cumene hydroperoxide mg $protein^{-1}$) in whole body of *Apistogramma agassizii* and *Paracheirodon axelrodi*, after 24, 48, 72, and 96 h exposure to either 50% of Cu LC_{50}-96 h and 50% of nCuO LC_{50}-96 h, and the control group ($n = 6$ at each treatment).

			GST	*SOD*	*CAT*	*LPO*
A. agassizii	Control	24 h	242.4 ± 19.7	18.1 ± 0.3	173.2 ± 17.2	30.1 ± 2.3
		48 h	265.1 ± 16.3	19.0 ± 0.5	175.1 ± 20.7	28.7 ± 1.1
		72 h	260.3 ± 16.2	18.7 ± 0.4	178.4 ± 16.0	25.7 ± 2.8
		96 h	218.7 ± 16.1	19.5 ± 1.0	181.1 ± 16.6	26.7 ± 2.4
	50% Cu LC_{50}–96 h	24 h	178.9 ± 20.9	18.6 ± 1.0	181.2 ± 12.6	31.7 ± 2.9
		48 h	239.8 ± 9.8	19.3 ± 1.1	287.2 ± 48.2	31.2 ± 3.2
		72 h	252.0 ± 29.6	19.9 ± 0.5	254.6 ± 45.9	40.5 ± 8.2
		96 h	163.1 ± 25.9	19.4 ± 0.6	204.9 ± 38.1	40.9 ± 6.9
	50% nCuO LC_{50}–96 h	24 h	191.4 ± 23.1a	23.7 ± 3.0	322.1 ± 36.3	53.0 ± 7.1 a
		48 h	177.4 ± 40.2a	23.1 ± 4.3	191.0 ± 33.0	90.1 ± 12.4b*#
		72 h	155.5 ± 16.3a	24.7 ± 0.6	357.2 ± 68.5	57.3 ± 6.2a*
		96 h	315.1 ± 27.6b#	31.7 ± 3.2*#	422.9 ± 109.7*	63.4 ± 7.6ab*
P. axelrodi	Control	24 h	444.0 ± 65.5	16.2 ± 0.9	97.5 ± 14.7	27.7 ± 2.5
		48 h	487.2 ± 34.8	15.3 ± 1.1	103.1 ± 15.2	24.9 ± 3.0
		72 h	457.1 ± 31.5	17.2 ± 0.6	105.2 ± 3.8	26.9 ± 3.0
		96 h	332.8 ± 42.2	15.6 ± 0.6	68.9 ± 13.4	22.7 ± 1.3
	50% Cu LC_{50}–96 h	24 h	425.9 ± 41.5	29.2 ± 2.4a*	115.1 ± 21.7	33.2 ± 4.0
		48 h	345.2 ± 36.2	23.7 ± 1.1ab*	130.5 ± 34.3	31.9 ± 2.5
		72 h	415.8 ± 45.9	20.3 ± 0.8b	101.7 ± 14.9	31.8 ± 2.4
		96 h	415.2 ± 47.0	20.9 ± 1.2b	80.3 ± 12.8	31.3 ± 3.9
	50% LC_{50} nCuO	24 h	366.6 ± 19.0	19.8 ± 1.8#	85.1 ± 25.1	27.9 ± 1.4
		48 h	349.3 ± 31.3	17.0 ± 0.9#	72.3 ± 17.5	38.7 ± 10.5
		72 h	287.8 ± 56.7	16.4 ± 1.3	60.5 ± 18.7	27.9 ± 4.5
		96 h	377.1 ± 29.4	21.7 ± 1.6	61.4 ± 14.3	47.2 ± 7.3*

Asterisks (*) represents significant differences between the group exposed to either Cu or nCuO and the control group at each time of exposure ($p < 0.05$).
Different letters represent statistical differences between the time of exposure within each treatment ($p < 0.05$).
(#) represents significant differences between group exposed to Cu and nCuO, at the same time of exposure ($p < 0.05$).

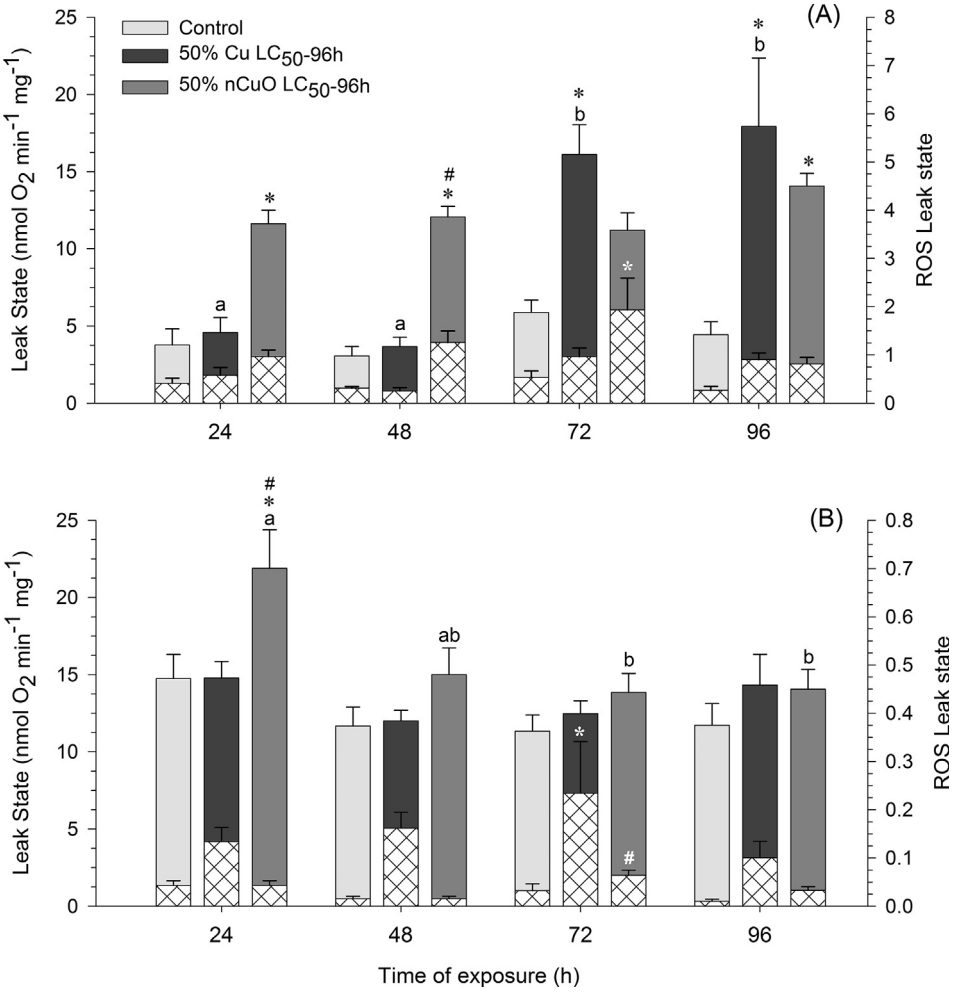

Fig. 4. Mitochondrial leak state respiration (solid bars) and ROS formation by mitochondrial respiration in leak state (cross-hatched bars) in gills of (A) *Apistogramma agassizii* and (B) *Paracheirodon axelrodi* after 24, 48, 72 and 96 h exposure to 50% of Cu LC_{50}-96 h and 50% of nCuO LC_{50}-96 h. Data are presented as Mean ± SEM ($n = 7$, at each treatment). Asterisks (*) represents significant differences between the group exposed to either Cu or nCuO and the control group at each time of exposure ($p < 0.05$). Different letters represent statistical differences between the time of exposure within each treatment. (#) represents significant differences between group exposed to Cu and nCuO at the same time of exposure ($p < 0.05$). Note that the scale of ROS production in Leak state of *P. axelrodi* (B) is ten times lower (one order of magnitude) than in *A. agassizii* (A).

respiration in cardinal tetra was stimulated only in 24 h of exposure to nCuO, which was different to fish at both control and exposed to Cu for 24 h, and the animals exposed to nCuO for 72 and 96 h (Fig. 4B). Mitochondrial ROS production in leak state of cardinal tetra increased after 72 h of Cu exposure in comparison with the control and to animals exposed to nCuO in 72 h (Fig. 4B).

The exposure to Cu for 72 and 96 h increased the ETS capacity in dwarf cichlid, whereas exposure for 96 h to nCuO improved ETS respiration, both in relation to control (Fig. 5A). ETS capacity in dwarf cichlid exposed for 72 and 96 h to Cu was also higher in comparison with fish exposed to the same treatment for 24 and 48 h, whereas the increased ETS respiration in fish exposed for 96 h to nCuO was also different from fish exposed for 24 and 48 h to the same treatment (Fig. 5A). ETS capacity in cardinal tetra was increased after 72 h of exposure to nCuO, in relation to control (Fig. 5B). After 24 and 72 h exposure to nCuO, mitochondrial ETS respiration of cardinal tetra were higher than those seen in fish exposed to Cu. In addition, ETS respiration after exposure to nCuO for 24 h was higher in relation to fish exposed to the same treatment for 96 h (Fig. 5B).

There were no effects of the exposure to either Cu or nCuO on mitochondrial ROS production at ETS respiration in gills of both fish species (Fig. 5). Respiratory coupling ratio (RCR), measured as ETS/leak state ratio, was decreased in dwarf cichlid after the exposure to nCuO for 24 and 48 h (S4). Cardinal tetra presented no RCR changes after exposure to either Cu or nCuO (S4).

3.6. Morpho-histological alterations induced by Cu and nCuO

The histopathology analyses confirm that the gills of dwarf cichlid displayed several morphological alterations after 96 h exposure to both nCuO and Cu, but only in fish exposed to nCuO was the integrity of gill epithelium mild to moderate damage (S5). In this fish species, the more frequent alterations after exposure to both nCuO and Cu were: lamellar epithelium lifting; aneurism and rupture; and lamellar hyperplasia and hypertrophy (Fig. 6).

Similarly, gill epithelium of cardinal tetra displayed several morphological alterations after 96 h exposure to both nCuO and Cu in contrast to dwarf cichlid, which presented mild to moderate damage in gills function only in fish exposed to Cu (S5). Cardinal tetra displayed moderate to heavy damage after 96 h exposure to Cu and normal gill function following 96 h exposure to nCuO (S5). The main morphological alterations in gill epithelium displayed by cardinal tetra after 96 h exposure to both nCuO and Cu were lamellar hyperplasia and hypertrophy, lamellar fusion, mitochondria rich cells proliferation and mucous cells proliferation (Fig. 7).

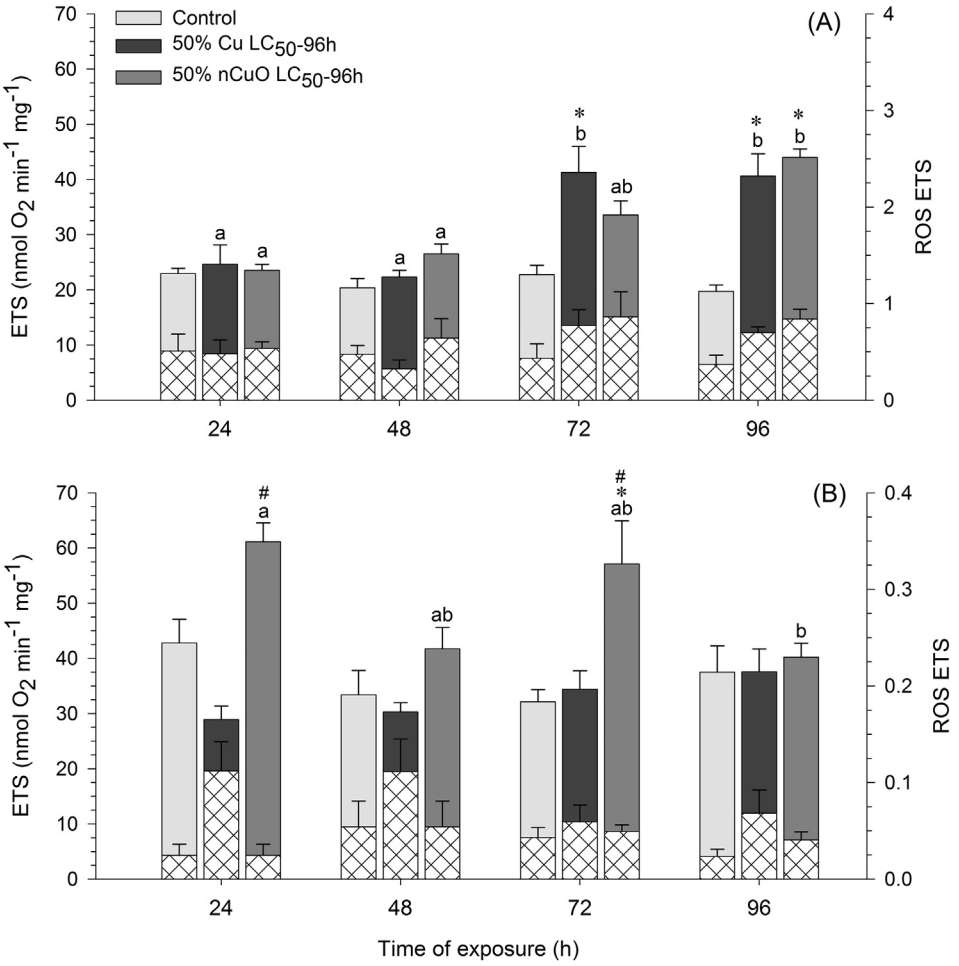

Fig. 5. Mitochondrial ETS (Electron Transport System) respiration (solid bars) and ROS formation by mitochondrial respiration in ETS (cross-hatched bars) in gills of (A) *Apistogramma agassizii* and (B) *Paracheirodon axelrodi* after 24, 48, 72 and 96 h exposure to 50% of Cu LC_{50}-96 h and 50% of nCuO LC_{50}-96 h. Data are presented as Mean ± SEM ($n = 7$, at each treatment). Asterisks (*) represents significant differences between the group exposed to either Cu or nCuO and the control group, at each time of exposure ($p < 0.05$). Different letters represent statistical differences between the time of exposure within each treatment ($p < 0.05$). (#) represents significant differences between group exposed to Cu or nCuO, at the same time of exposure ($p < 0.05$). Note that the scale of ROS production in leak state of *P. axelrodi* (B) is ten times (an order of magnitude) lower than in *A. agassizii* (A).

When we observed the surface of the gills in SEM analysis, the main alteration found in dwarf cichlid was the loss of microridges on the epithelium. In dwarf cichlid, microridges may provide some mechanical protection against trauma and aid in retaining mucous secretions on the epithelium, as this fish also showed some evidence of aneurisms and ruptures (Fig. 7). Cardinal tetra showed a similar loss of microridges in fish exposed to Cu in addition to some projections in the filaments in both Cu and nCuO exposed fish, suggesting hypertrophy in this region.

4. Discussion

In the present study we demonstrated that for dwarf cichlid (*A. agassizii*), nCuO negatively affected both mitochondrial physiology and gill osmoregulatory mechanisms and increased whole body Cu accumulation and gill lipid peroxidation, despite the activation of antioxidant enzymes (SOD and CAT). Interestingly, these effects were increased mainly after 72 h of exposure, indicating that toxic effects of nCuO to this species were time-dependent. In contrast, nCuO promoted only minor toxic effects in cardinal tetra (*P. axelrodi*), while dissolved Cu was quite harmful. Cardinal tetra exposed to Cu exhibited an increase in oxygen consumption, reductions in whole body Na^+ and K^+ levels, increased oxidative stress, and severe damage on the gill epithelium. The two species exhibited distinct responses to nCuO and Cu, suggesting that differences in their osmoregulatory strategies as pointed out by Gonzalez et al. (2002) may lead to different mechanisms of toxicity.

4.1. Acute toxicity of nCuO is lower than Cu for both dwarf cichlid and cardinal tetra

The physiological impacts of nCuO exposure in fish and the contribution of Cu dissolution to toxicity are unclear, so it is important to distinguish the effects of nanoparticles from dissolved metals. In the present work, dwarf cichlid was slightly more sensitive to nCuO compared to cardinal tetra, but Cu LC_{50}–96 h values previously obtained for these species (Duarte et al., 2009) were much more toxic than nCuO in overall (Fig. 1), this result is in accordance of previous studies (Griffitt et al., 2007) that also observed that *D. rerio* was more sensitive to dissolved Cu (LC_{50}-48 h of 0.25 mg Cu L^{-1}) than to nCuO (LC_{50}-48 h of 1.56 mg Cu L^{-1}). These authors pointed out that nanocopper has high capacity to aggregate into micron-sized particles and sediment rapidly, such that only 40–50% of the mass of added particles was present in suspension between 2 and 48 h after addition to their tanks. Copper bioavailability may still have been lower in the nCuO treatment, however, as SEM images and dynamic light scattering suggested that nCuO were aggregated in the waters of our treatments (S1). These results

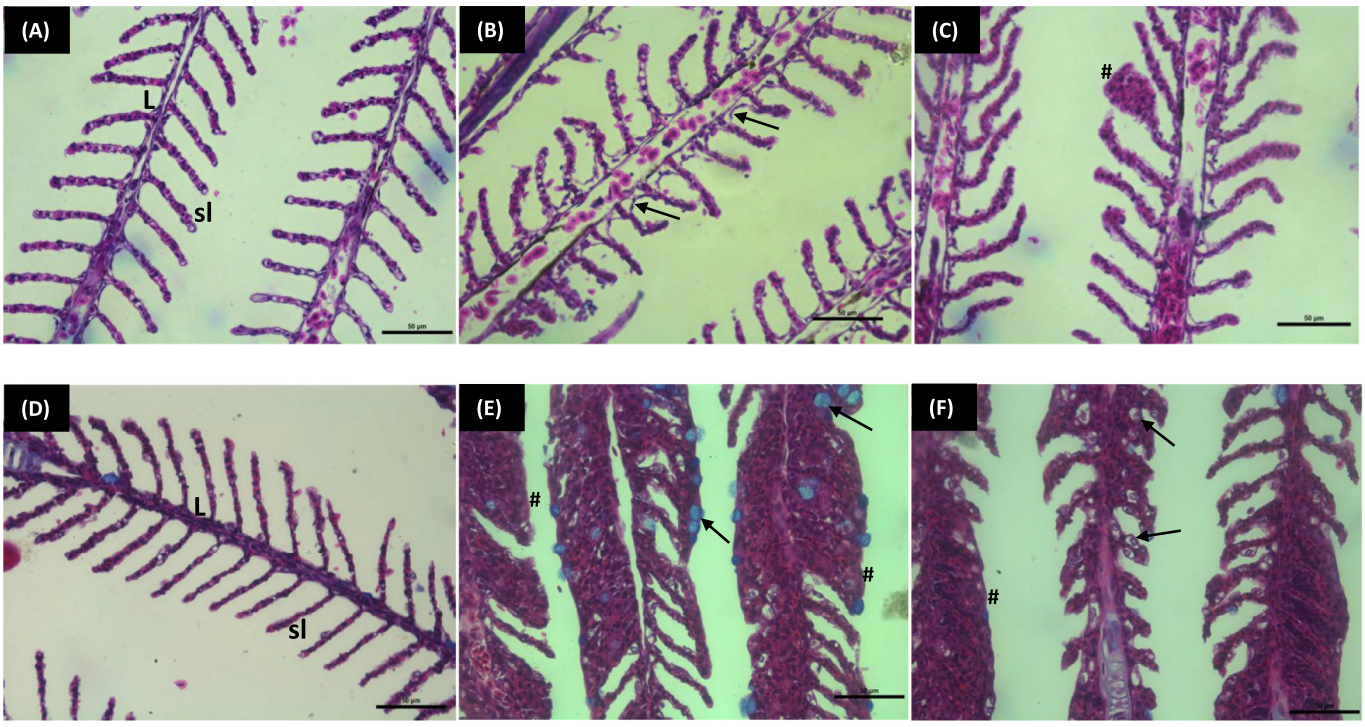

Fig. 6. Representative sections of gill of *Apistogramma agassizii* (A, B and C, respectively) and *Paracheirodon axelrodi* (D, E and F, respectively) after 96 h exposure. Animals exposed to 50% of Cu LC_{50}-96 h (B and E) and 50% of nCuO LC_{50}–96 h (C and F), $n = 6$. (A) control conditions; (B) filament epithelium lifting (←); (C) hyperplasia and hypertrophy of the filament epithelium (#); (D) control conditions; (E) Mucous cells proliferation (←) and filament fusion (#); (F) proliferation of mitochondria rich cells (←) and filament fusion (#). Note the normal structure of the fillament (F) and secondary lamellae (sl) in the control. Scale bar = 50 μm for all pictures.

suggest that nCuO have low bioavailability capacity compare to Cu^{2+}, and therefore could be considered less harmful to waterbodies. Nevertheless, it is essential to consider that NPs can release metal ions from their surface over time (Shaw and Handy, 2011; Siddiqui et al., 2015), which might contribute to the time-dependent toxicity observed in dwarf cichlid exposed to nCuO. In addition, the ingestion of nanoparticles can be an important route of exposure for aquatic organisms (Croteau et al., 2014). This may represent an alternate pathway for Cu uptake and for nano-specific effects, considering that ingested nCuO might act as a long-term source for exposure to Cu (Ates et al., 2014).

4.2. Dwarf cichlid and cardinal tetra exhibit different osmoregulatory compromise, Cu accumulation and oxidative stress responses to Cu and nCuO

Respiratory alterations have been pointed out as critical organismal responses to environmental challenges. Recent studies have suggested

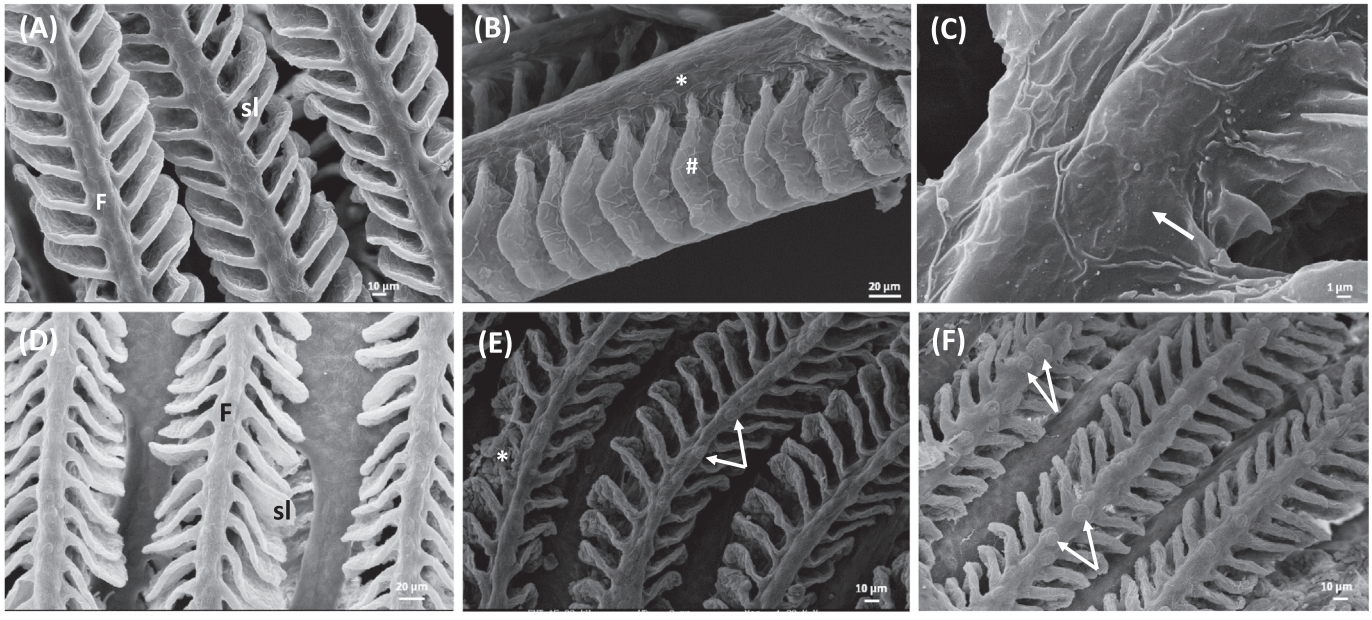

Fig. 7. Scanning electron micrographs of gills filaments of *Apistogramma agassizii* (A: control, B: Cu and C: nCuO) and *Paracheirodon xelrodi* (D: control, E: Cu and F: nCuO) after 96 h exposure to 50% of Cu LC_{50}-96 h and 50% of nCuO LC_{50}-96 h, $n = 3$. (A and D) regular spacing of secondary lamellae, sl, on the gill fillament, F, (B) epithelium with loss of microridges (*) and lamellar hyperplasia and hypertrophy (#); (C) epithelium with loss of microridges (arrow). (E) lamellar fusion (*) and gills projections evidentiated (arrow); (F) gills projections strongly evidentiated in nCuO exposure.

that different Amazon fish species can present distinct metabolic and osmoregulatory strategies facing environmental challenges (Campos et al., 2017; Duarte et al., 2013; Gonzalez and Wilson, 2001). One common respiratory response of fish exposed to Cu is the alteration of whole animal $\dot{M}O_2$ (Beaumont et al., 2000; De Boeck et al., 1995), which was found in the present work. $\dot{M}O_2$ increased in cardinal tetra following exposure to nCuO and Cu, respectively (Fig. 2).

Several studies have shown differences in the toxic responses to Cu and nCuO and, in general, these differences seem to be related to their different cellular targets (Karlsson et al., 2013; Siddiqui et al., 2015; Srikanth et al., 2016). During exposure to nCuO or Cu, dwarf cichlid showed no alterations in $\dot{M}O_2$ while cardinal tetra experienced a substantial increase in $\dot{M}O_2$ during the first 72 h of Cu exposure, which was on average 2.6 times higher than $\dot{M}O_2$ seen in control fish. This indicates that cardinal tetra may have less energy available to perform functions such as foraging, reproduction, or predator avoidance. Despite that, nCuO triggered no effects in cardinal tetra. These results show clear differences in the effects of Cu and nCuO on aerobic metabolism for some Amazon fish species, which are likely related to the differences in their metabolic and osmoregulatory strategies.

Aerobic ATP production is essential for driving the active transport of ions mediated by pumps and co-transporters in osmoregulatory tissues (Marshall, 2002). In freshwater fish, Cu can inhibit critical enzymes involved in Na^+ uptake, such as Na^+/K^+ATPase, v-type H^+-ATPase, Na^+/H^+ exchanger, and CA (Chowdhury et al., 2016; Grosell et al., 2012). Our findings revealed that, although both Cu and nCuO are ionoregulatory toxicants to dwarf cichlid, nCuO might be more effective in promoting ionoregulatory disturbances in this species. Gills of dwarf cichlid exposed to nCuO showed a marked and specific inhibition of v-type H^+-ATPase activity after 24 and 96 h, and inhibition of CA activity throughout all experimental exposure to nCuO. This is the first evidence that nCuO can have a similar inhibitory action as Cu on the activity of both v-type H^+-ATPase and CA in gills of freshwater fish. Under normal condition CA is responsible to promote the hydration of CO_2 with consequent liberation of H^+ and HCO_3^-, where H^+ can be used by the v-type H^+-ATPase to generate the electrochemical gradient to drive Na^+ uptake through Na^+ channels (ENaC) (Kumai and Perry, 2012), while supply HCO_3^- for exchange with Cl^-, by apical pendrin-like anion exchanger. Thus, the fact that both CA and v-type H^+-ATPase were simultaneously inhibited by nCuO (at 24 and 96 h of exposure) suggests that these enzymes might be an essential target for the ionoregulatory toxic action of nCuO in gills of dwarf cichlid.

Cardinal tetra, instead, was quite tolerant to both Cu and nCuO, displaying only a slight inhibition of CA in gills after 48 h of exposure to nCuO. Recently, a pharmacological study conducted with different Characidae fish species from the Rio Negro suggested that Na^+ uptake in cardinal tetra depends on both the ammonia excretion rate and the supply of H^+ mediated by CA activity (Wood et al., 2014). Exposure of cardinal tetra to similar levels of Cu used in this study (around 50 μg L^{-1}) resulted in the stimulation of Na^+, Cl^- and K^+ losses (Crémazy et al., 2016). Cu induced imbalances in Na^+ homeostasis of freshwater fishes is demonstrated to involve both the stimulation of Na^+ paracellular losses, by the displacement of Ca^{2+} ions from branchial tight junctions, and inhibition of Na^+ influx through the competition for Na^+ channels in apical membrane of ionocytes, and/or inhibition of different transporters, as CA, v-type H^+-ATPase and Na^+/K^+-ATPase (Alsop and Wood, 2011; Chowdhury et al., 2016; Crémazy et al., 2016; Grosell et al., 2002). In these regards, it has been proposed that the specialized high branchial Na^+ uptake system seen in cardinal tetra (Gonzalez and Wilson, 2001) may explain its tolerance to both high H^+ or Cu levels conditions (Crémazy et al., 2016). Thus, we presume that Cu accumulation in cardinal tetra exposed to both Cu and nCuO was below the threshold to promote inhibition of v-type H^+-ATPase and Na^+/K^+-ATPase, which are considered essential mechanisms for acute Cu toxicity in freshwater fishes. However, our data suggest that inhibition of CA plays a relevant role on nCuO acute toxicity in cardinal tetra, which might be mainly associated with impairments in ammonia excretion of fish, in accordance with recent findings that evidence that ammonia excretion rely on mechanisms independent of Na^+ uptake in cardinal tetra (Wood et al., 2014).

Copper accumulation was observed in dwarf cichlid in nCuO exposures, while for cardinal tetra bioaccumulation was evident in both Cu and nCuO exposures. Our results indicate that nanoparticles may dissolve in solution with a consequent release of the metallic ion during the experiments, which contribute to the bioaccumulation of Cu seen here for cardinal tetra, despite having observed an aggregation of the particles in the water. Gradual dissolution of Cu from the Cu nanoparticles has been observed in other studies (Griffitt et al., 2007; Siddiqui et al., 2015). According to Wang et al. (2012), the primary uptake pathway of the nCuO is the endocytosis and, once inside the cell, the nanoparticle releases metal ions. This accumulation can promote damage to macromolecules and generate oxidative stress (Srikanth et al., 2016). Although in lower concentrations, Griffitt et al. (2007) also observed dissolved Cu in the nanocopper treatment, where Cu levels were similar following exposure to nanocopper and its soluble fraction. The authors suggested that the mechanisms responsible for the effects of nanocopper are unclear, and probably they do not appear to be mediated only by increased gill uptake. Additionally, cardinal tetra accumulated 2–3 times more Cu than dwarf cichlid after exposure to both Cu and nCuO. These differences can be explained by the specialized Na^+ transport system seen in cardinal tetra, with very high Na^+ affinities and high Na^+ uptake rates, which appears to be supported through unique and novel mechanisms (Gonzalez and Wilson, 2001; Gonzalez et al., 2002; Matsuo and Val, 2007; Wood et al., 2014). Thus, once Cu^{2+} may compete with Na^+ for the active transport sites in apical membrane of ionocytes, it would increase Cu uptake and accumulation by fish. It is in accordance of recent findings that demonstrated a higher Lethal Accumulation (LA50) of Cu in gills of cardinal tetra (Wood et al., 2014) in relation to those LA50 values typically reported for temperate fish species, such as rainbow trout and fathead minnow, following acute exposure to Cu (Niyogi and Wood, 2004).

As we have presented above, Cu induces distinct physiological toxicity and some studies have reported that nCuO induce oxidative stress through catalytic production of reactive oxygen species at the nanoparticles' surface (Siddiqui et al., 2015; Srikanth et al., 2016). This capacity to directly produce ROS has been described as an important mechanism of nCuO toxicity in fish (Srikanth et al., 2016). In dwarf cichlid, the increase in GST activity in nCuO compared to Cu exposed fish showed that the nanoparticle treatment required higher biotransformation activity and this was time-dependent. Furthermore, the activation of SOD, and CAT following exposure to nCuO suggests a coordinated response to an oxidative stress. The accompanying increase in LPO values indicates that oxidative lipid damage also occurred, so that the antioxidant responses were insufficient to avoid damage. These toxic effects of the oxidative stress resulting from ROS production can be assigned to the small size and large surface area of the nCuO, which may cause lipid damage, as was observed here for dwarf cichlid, in addition to oxidation of proteins and DNA, as found in previous studies (Siddiqui et al., 2015; Srikanth et al., 2016). SOD activity also increased in cardinal tetra after Cu exposure, but no lipid damage was observed in this treatment, indicating that SOD activity was sufficient to deal with increased ROS production in this species. There were no alterations in any oxidative stress enzymes after exposure to nCuO over the period studied for cardinal tetra. Despite that, a sharp increase in LPO levels occurred after 96 h of nCuO exposure, indicating that the antioxidant enzymes were not able to protect from the oxidative stress caused by the nCuO over this exposure duration.

4.3. The strategies of mitochondrial coupling respiration to cope with ROS production in gills of dwarf cichlids are different from cardinal tetra

Respiratory capacities for oxidative phosphorylation and electron transport were affected in different ways in cardinal tetra and dwarf

cichlid, indicating a species-specific metabolic stress response. In dwarf cichlid, increases in the maximum oxidative phosphorylation (ETS) were observed to nCuO or Cu, indicating an increase in energy demand. This species seems to have developed a distinct adjustment to sustain the supply of ATP to maintain ionoregulatory function of the gills. This may be in response to the cost of dealing with the observed gill tissue damage, which requires an activation of protein synthesis, and energetically-expensive process. Cardinal tetra likely increased ETS respiration to compensate for imbalances in Na^+ homeostasis, as discussed above. In this case, the increase in energy demand may be coming from the requirement for transporter synthesis, and/or from the differentiation of new ionocytes to deal with the loss of those damaged by Cu.

The integrity of the inner mitochondrial membrane is essential to the function of oxidative phosphorylation and electron transport, and when this integrity is compromised, uncoupling occurs (i.e., proton (H^+) leak across the mitochondrial inner membrane). There is a hypothesis that this mechanism can help prevent the production of ROS (Brookes, 2005). In dwarf cichlids an elevation in proton leak respiration was observed, this can contribute to oxidative protection and low oxidative damage. This could be considered as a strategy to survive exposure to toxins that trigger oxidative stress, such as nanoparticles and metals. It is hypothesized that increases in proton leak conductance avoid conditions that favour ROS generation by allowing mitochondria to operate at a lower membrane potential (Cunha et al., 2011). Therefore, decreases in mitochondrial membrane potential are expected to be associated with increases in leak respiration and/or decreases in oxidative phosphorylation respiration.

RCR values were lower in dwarf cichlids exposed to nCuO, indicating a higher mitochondria uncoupling ability in this species. This may reflect an early onset of mitochondrial deterioration, but it is also a strategy mechanism for this species, which increased leak state. In contrast, the mitochondria of cardinal tetra showed constantly uncoupled (even in the control), which can be understood as a strategy of the species to deal with oxidative stress once this specie showed lower ROS content compared to the dwarf cichlid, even under normal conditions. Furthermore, cardinal tetra showed high ETS efficiency after exposed to nCuO, this can be explained by the fact that the cardinal is more dependent on the Na^+ flux and, therefore, needs a higher efficiency in the production of ATP, being advantageous for this species the maintenance of a coupled mitochondria for a more significant energy generation. On the other hand, dwarf cichlid, which showed low ionic transport capacity, could maintain the metabolic rate and uncouple the mitochondrial membrane.

4.4. The morphological consequences from exposure of Cu and nCuO are different to gills in both fish species

Morphological alterations were observed in both LM and SEM images for both species. The main histopathological alterations in the gill tissue of the dwarf cichids were lamellar epithelium lifting, hyperplasia, and hypertrophy, which were more frequent in the nCuO treatment. Similar branchial damage has been observed in rainbow trout and zebrafish after exposure to nCuO (Al-Bairuty et al., 2013; Vicario-Parés et al., 2018). Additionally, SEM images revealed some particles in the surface of the gill epithelium only in the treatment with nCuO, which may be assumed to be nanoparticle aggregations (Fig. 7). The histopathological changes in dwarf cichlids' gills are likely a consequence of oxidative damage, as this species exhibited a clear oxidative stress response (S5). In contrast to the cichlids, cardinal tetra gill tissue damages were much more severe after exposure to Cu and included hypertrophy and hyperplasia in filaments and lamellar epithelium, epithelial lifting, aneurysm, lamellar rupture, and proliferation of chloride and mucous cells, this damage could compromise respiratory gas exchange and contribute to increased metabolic demand, as observed by the increase in $\dot{M}O_2$ in this species and as previously noted for *Oncorhynchus mykiss* (Al-Bairuty et al., 2013). Moreover, we observed that cardinal tetra exposed to Cu presented a MRC proliferation, which, although beneficial to ionic regulation, might impair gas transfer through thickening of the diffusion barrier elicited increase oxygen consumption. This process is energetically expensive and might be due to the building of those new ionocytes and to the synthesis of all proteins/transporters in them. The strong alteration exhibited in gill morphology suggests that the cardinal tetra is more sensitive to Cu than to nCuO.

5. Conclusions

The two fish species studied here showed distinct sensitivities to Cu and nCuO, suggesting that each toxicant may exert its effects through a unique mechanism of action. In general, the dwarf cichlid showed more alterations associated with oxidative stress, whereas cardinal tetra showed energy metabolic effects.

The dwarf cichlid presented mitochondrial adaptation mechanisms, as they could maintain their metabolic rate, even though uncoupling their mitochondria as a response to increased ETS. ROS generation promoted the activation of the anti-oxidant enzymes and the effects in gills lamellar epithelium were reduced. Additionally, all these effects in these cichlids occurred as a time-dependent response to nCuO exposure, where the effects were intensified after 72 h of exposure. These results can be explained by the low ionic transport capacity and permeability of their gills, as well as occurred in other species belonging to Cichlidae family (Gonzalez et al., 2002), which might directly influence $\dot{M}O_2$, and the capacity for uptake and metabolism of toxicants. In contrast, the cardinal tetra showed more metabolic dysfunctions under exposure to Cu, and the extent of these impairments was not time-dependent. This characid fish species showed an increase of energy demands, as observed by the increase in $\dot{M}O_2$ that can be explained by the severe compromising of the gills morphology and a higher Cu accumulation. Cardinal tetra presented a higher ion transport capacity in their gills, suggesting that the energy expenditure for their osmoregulatory mechanisms is higher. This may explain the increase in $\dot{M}O_2$ and consequent alterations in metabolic functions, as the higher requirement for ATP, and the maintenance of uncoupled mitochondria a compensatory mechanism for the maintenance of lower ROS content. Our findings reveal that different mechanisms of toxic action of contaminants are associated with differences in the osmoregulatory strategies among species here studied.

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