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Mitochondria-rich cells adjustments and ionic balance in the Neotropical fish *Prochilodus lineatus* exposed to titanium dioxide nanoparticles

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ABSTRACT

Manufactured titanium dioxide nanoparticles (TiO_2-NP) have been intensely applied in numerous industrial products and may be a risk for aquatic systems as they are not completely removed from domestic and industrial wastes after water treatment. This study evaluated the osmo- and ionic balance, Na^+/K^+-ATPase, H^+-ATPase and carbonic anhydrase activities and the mitochondria-rich cells (MRC) in the gills and kidney of the Neotropical fish *Prochilodus lineatus* after 2 (acute) and 14 (subchronic) days of exposure to nominal 0, 1, 5, 10 and 50 mg L^{-1} TiO_2-NP. The nominal concentrations corresponded to 0.0, 0.6, 1.6, 2.7 and 18.1 mg L^{-1} suspended TiO_2-NP, respectively, in the water column one hour after NP introduction and were maintained for at least 24 h. Acute exposure to TiO_2-NP decreased plasma osmolality and Ca^{2+} levels. Na^+/K^+-ATPase, H^+-ATPase and carbonic anhydrase activities were inhibited in the gills, but not in the kidney. Total MRC density did not change in gills and kidneys. At gill surface, total MRC density decreased in fish exposed to 50 mg L^{-1} TiO_2-NP and the total MRC fractional surface area unchanged although, there were some changes in the fractional area of MRC with apical microvilli (MRCm) and MRC with apical sponge-like structure (MRCs). MRCm was more abundant than MRCs. After subchronic exposure, there was no change in plasma osmolality, ionic balance and enzyme activities. Total gill MRC density increased in the filament epithelium and renal tubules. In the gills, MRC contacting water exhibited some adjustments. Total MRC and fractional surface area unchanged, but there was an increase of MRCs contacting water at gill surface after exposure to10 and 50 mg L^{-1} TiO_2-NP. MRC proliferation in filament epithelium and in renal tubules as well as the increasing MRCs at gill surface may have contributed to avoid change in plasma osmolality, ionic balance and enzyme activities and suggested a cellular physiological and morphological response to restore and maintain osmotic and ionic homeostasis after subchronic exposure.

1. Introduction

The use of materials manufactured in the nanoscale (1–100 nm) dominates numerous industrial sectors due to their unique physical and chemical properties which provide reactivity differing from the same material at a larger scale. Manufactured titanium dioxide nanoparticles (TiO_2-NP), a metal oxide NP, have been intensely applied in numerous cosmetic and personal hygiene products, food, textile and electronics (Muller and Nowack, 2008) as well as water purification, due to their photocatalytic/photooxidation properties (Hosseini et al., 2015; Mobarakabad et al., 2015; Chang et al., 2017). This NP present in domestic and industrial wastes is not completely removed after water treatment and thus reaches aquatic ecosystems, where the NP are suspended in water and/or sedimented on the botton of the water body; these NP may threaten aquatic biota (Reijnders, 2012; Haynes et al., 2017).

TiO_2-NP are known to cause toxicity in many organisms or influence the effects of other water contaminants, thereby enhancing mortality (Chen et al., 2011; Czajka et al., 2015; Della Torre et al., 2015; Jovanovic et al., 2011, 2015; Tan and Wang, 2014; Zhang et al., 2007). In fish, TiO_2-NP suspended in the water may be absorbed through the lamellar gill epithelium during the respiratory process and, those in the sediment may be absorbed during food ingestion, depending on animal feeding habits. Thereafter, NP reaches other organs, via the

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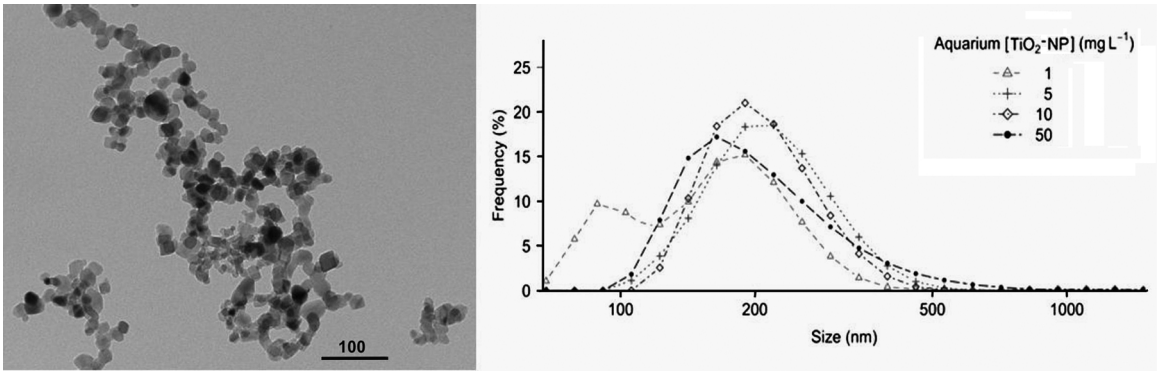

Fig. 1. TiO_2-NP agglomerate/aggregate size in aquarium water. (A) Transmission electron microscope image of the TiO_2-NP suspension. Scale bar in nm. (B) Frequency and distribution of TiO_2-NP agglomerate/aggregate size at different nominal TiO_2-NP concentrations.

Table 1
Mean (± SEM) of plasma ion (Na^+, K^+, Ca^{2+} and Cl^-) concentrations and osmolality of *Prochilodus lineatus* after acute (2 d) and subchronic (14 d) exposure to TiO_2-NP, except for Na^+ (acute exposure) which data show the median(first and third quartiles). *Indicates significant difference between each treatment and the control (0 mg L^{-1} TiO_2-NP) and letters indicate significant difference among groups (ANOVA, $p < 0.05$). Kruskal-Wallis was applied for Na^+ (acute exposure).

Parameter	TiO_2-NP nominal concentration (mg L^{-1})				
	0	1	5	10	50
Acute exposure					
Na^+ (mEq L^{-1})	117 (115;121)	119 (107;121.5)	117.5 (115;119.5)	117.5 (115;119.8)	122 (119.8;123.2)
K^+ (mEq L^{-1})	3.2 ± 0.08[a]	3.7 ± 0.09*[b]	3.4 ± 0.07[ab]	3.2 ± 0.07[a]	3.5 ± 0.14 [ab]
Ca^{2+} (mEq L^{-1})	5.8 ± 0.25[a]	5.8 ± 0.13[a]	5.1 ± 0.14[ab]	4.7 ± 0.30*[b]	4.8 ± 0.2*[b]
Cl^- (mEq L^{-1})	96.7 ± 2.86	95.2 ± 1.84	97.9 ± 1.68	99.8 ± 2.12	93.1 ± 2.35
Osmolality (μOsmol kg^{-1})	234.4 ± 2.46[a]	222.2 ± 1.62*[b]	224.9 ± 1.17*[b]	222.2 ± 2.56*[b]	220.9 ± 1.6*[b]
Subchronic Exposure					
Na^+(mEq L^{-1})	139.1 ± 5.96	142.7 ± 8.27	140.6 ± 2.76	146.2 ± 4.46	139.9 ± 4.96
K^+ (mEq L^{-1})	4.1 ± 0.18	3.9 ± 0.11	4.3 ± 0.21	4.1 ± 0.12	4.2 ± 0.10
Ca^{2+} (mEq L^{-1})	4.9 ± 0.16	5.0 ± 0.16	5.4 ± 0.13	5.3 ± 0.09	5.1 ± 0.13
Cl^- (mEq L^{-1})	115.5 ± 2.97	111.9 ± 2.58	112.4 ± 4.06	111.2 ± 3.47	104.2 ± 3.02
Osmolalidade (μOsmol kg^{-1})	238.0 ± 5.43	246.6 ± 4.78	244.9 ± 5.02	243.7 ± 3.22	240.2 ± 4.49

bloodstream, where they may affect cellular biochemistry and physiology and, consequently, the morphology and function of a given tissue/organ. Gill morphology is altered after contact with irritant agents and it is characterized by increasing epithelial thickness and mucus secretion, which may prevent xenobiotic internalization (Ribeiro and Fernandes, 2013). Although the gills have a large surface area, such modifications affect gas exchange and the osmotic and ionic balance (Moron et al., 2003, 2009; Sakuragui et al., 2003).

TiO_2-NP induce gill epithelial thickening due cell hyperplasia and hypertrophy (Federici et al., 2007; Hao et al., 2009) and may affect the osmo-ionic homeostasis. TiO_2-NP also tend to accumulate in the kidney (Liu et al., 2009; Scown et al., 2009; Wang et al., 2007); such NPs may compromise renal function, which is the main organ for NP excretion (Iqbal et al., 2004; Xie et al., 2011). Mitochondria-rich cells (MRC) in the gills and kidney tubules are the main cells responsible for ionic transport. They contain large quantities of ATPases, which generate a driving force for ion uptake from the water by the gills and from the renal tubule lumen in the kidneys (Evans et al., 2005; Hirose et al., 2003; Tresguerres, 2016); cytosolic carbonic anhydrase provides the ions H^+ and HCO_3^- for Na^+/H^+ and Cl^-/HCO_3^- exchangers at cellular apical surface to maintain ionic homeostasis (Hwang and Lee, 2007; Lionetto et al., 2016). Changes in MRC by xenobiotics, such as NP, may alter cell function with consequent ionic-osmoregulatory disturbance (Camargo et al., 2009; Paulino et al., 2012).

The main goal of this study was to evaluate the action of TiO_2-NP on the ionic and osmoregulatory processes in the Neotropical fish, *Prochilodus lineatus* by testing the following hypotheses: 1) the osmo-ionic balance is affected by TiO_2-NP suspended in the water and 2) MRC and the enzymes related to ion transport (Na^+/K^+-ATPase, H^+-ATPase and carbonic anhydrase) are affected by TiO_2-NP.

2. Materials and methods

2.1. Preparation of TiO_2-NP suspension and characterization

TiO_2-NP powder (AEROXIDE® TiO_2 P25, 99.5% purity, 80% anatase and 20% rutile) was purchased from Evonik Degussa Industries, Brazil; the TiO_2-NP crystals had an average diameter of 21 nm and a surface area of 50 ± 15 m^2 g^{-1}. The TiO_2-NP stock suspension (10 g L^{-1}) was prepared in ultra-pure water (Milli-Q®) and dispersed for 30 min in a bath-type sonicator (frequency 40 KHz; Q335D, QUIMIS, Brazil) without solvents. The experimental nominal TiO_2-NP concentrations of 1, 5, 10 and 50 mg L^{-1} were obtained by stock solution dilution into aquarium water. Samples of aquarium water were taken in the middle of the water column at 0 and 24 h after TiO_2-NP dilution to determine the size, dispersion and sedimentation, zeta potential (ZP) and hydrodynamic diameter. The TiO_2-NP size and dispersion were determined using a transmission electron microscope (TEM, Philips CM-120, FEI Co., USA). ZP (surface charge) and hydrodynamic diameter in each suspension were assessed by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 spectrophotometer (Malvern Instruments, UK). Ti was measured by total-reflection X-ray fluorescence using an S2 PICOFOX spectrometer (Bruker, USA) after water acidification (2% nitric acid) and air dried.

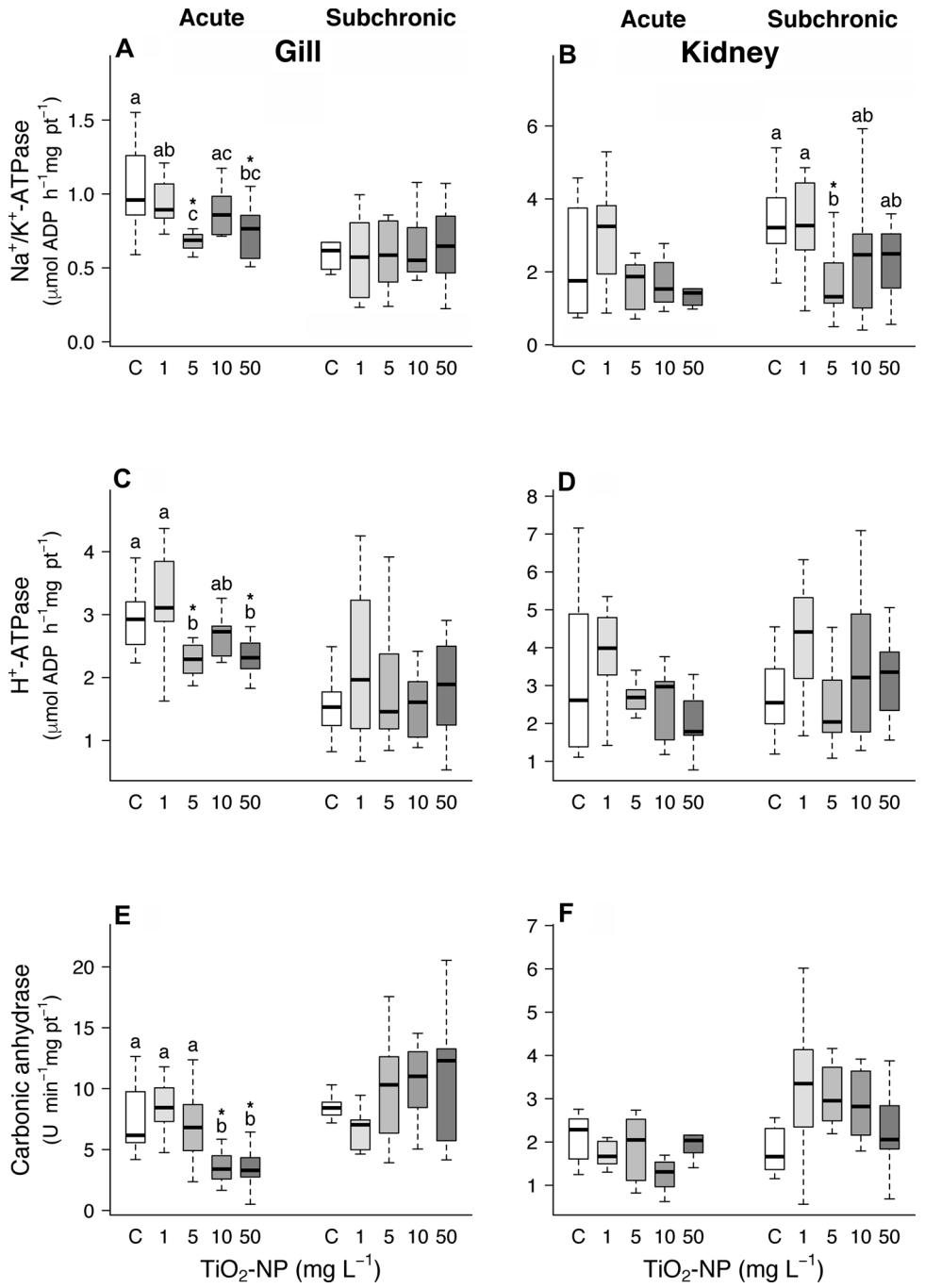

Fig. 2. Na^+/K^+-ATPase (A, B), H^+-ATPase (C, D) and carbonic anhydrase (E, F) activities in the gills (A, C, E) and kidneys (B, D, F) of *Prochilodus lineatus* from the control (C, 0 mg L^{-1} TiO_2-NP) and exposed groups (nominal: 1, 5, 10 and 50 mg L^{-1} TiO_2-NP) after acute and subchronic exposure. The box plots indicate the median, interquartile range (box) and extreme values (T bars). * indicates significant difference from the respective control and different letters indicate significantly difference among groups ($p < 0.05$).

2.2. Animals

Juvenile *Prochilodus lineatus* (body mass: 31.1 ± 0.39 g, total length: 14.4 ± 0.06 cm, i.e. less than 1-year-old, immature fish) were obtained from the Aquaculture Station at Furnas hydroelectric power-plant reservoir, São José da Barra, MG, Brazil. Fish were acclimated for two months in 1000 L tanks with dechlorinated flowing water, constant aeration and the following water physical and chemical characteristics: dissolved O_2 6.19–7.82 mg L^{-1}; pH 6.31–7.73; conductivity 40-58 µS cm^{-3}; alkalinity 35–43 mg L^{-1} as $CaCO_3$, total hardness 39–50 mg L^{-1} as $CaCO_3$ and temperature 22–26 °C. The photoperiod was 12 h light: 12 h dark. Fish were fed ad libitum with fish food (FRI-ACQUA 40, Fri-Ribe Rações, 40% protein).

2.3. Acute and subchronic exposure to NP-TiO_2

P. lineatus were exposed to nominal concentrations of 0, 1, 5, 10 e 50 mg L^{-1} TiO_2-NP for 2 d (acute exposure) or 14 d (subchronic exposure). These concentrations were used to assess sublethal effects during the exposure period; the 50% lethal concentration (LC_{50}) for TiO_2-NP was higher than 100 mg L^{-1} (nominal concentration) (Carmo, 2015). The fish (n = 10/200 L aquarium) were randomly distributed into five aquaria (in duplicate) having 200 L capacity and 55-cm water column. A semi–static system was used for experiments in which 80%

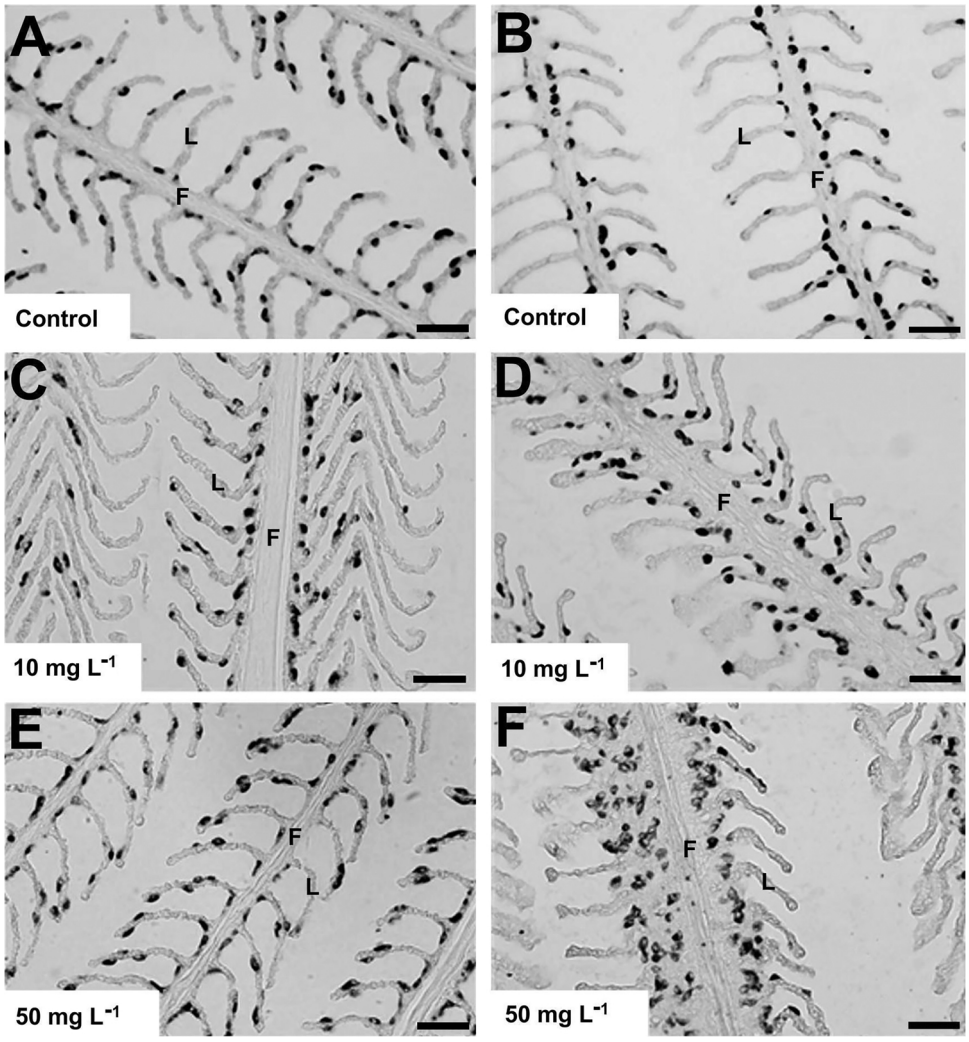

Fig. 3. Immunohistochemical staining for Na^+/K^+-ATPase in mitochondria-rich cells (MRC, arrow) in the gills of *Prochilodus lineatus* in the control group and groups exposed to TiO_2-NP, after acute (A, C, E) or subchronic (B, D, F) exposure. Note increasing MRC density in the filament epithelium after subchronic exposure to TiO_2-NP. F, filament; L, lamella. Scale bars = 50 µm.

water was renewed every 24 h. During subchronic exposure, fish were fed every 3 d immediately before water renewal.

At the end of 2 and 14 d, the fish were anaesthetized with benzocaine (0.1 g L^{-1}) and a blood sample was taken (~0.5 mL) via the caudal vein; thereafter the fish was killed by spinal section and the gills and kidneys were removed; samples from each organ were taken for physiological/biochemical (n = 12) and morphological (n = 8) analyses. Blood was centrifuged and the plasma was separated for ionic analyses; gill and kidney samples for biochemical analyses were immediately frozen and kept at −80 °C and samples for morphological analyses were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.3 or Bouin solution.

The Animal Experimentation Committee (Protocol no. 027/2011) and Environmental Ethics Committee (Protocol no. 002/2011) from Federal University of São Carlos, São Paulo, Brazil approved the experiments and procedures.

2.4. Plasma osmolality and ions

Plasma osmolality (mOsmol kg^{-1}) was assessed by determining the freezing point using a semi-µ-osmeter (µOSMETTE PRECISION SYSTEM, Natick, USA). Na^+ and K^+ concentrations (mEq L^{-1}) were measured using a flame photometer (DIGMED DM-61, DIGMED, Brazil). Calcium (Ca^{2+}) and chloride (Cl-) were measured using commercial kits (LABTEST kit 90 and 115, respectively) in a microplate reader (SpectraMax® M5 Multi-Mode, Molecular Devices, USA).

2.5. Enzyme activities in the gills and kidneys

The protein concentration (pt, mg mL^{-1}) of each sample from the gills and kidneys was determined using a commercial kit (Doles Micropote Pirogalol®, Doles Reagent, Goiânia, Brazil). The activity of Na^+/K^+-ATPase (µmol ADP mg pt^{-1} h^{-1}) and H^+-ATPase (µmol ADP mg pt^{-1} h^{-1}) was determined simultaneously according to the method described by Gibbs and Somero (1989) adapted for a microplate reader. ATPase activities were determined in the presence of ouabain (Na^+/K^+-ATPase inhibitor) and N-ethylmaleimide (H^+-ATPase inhibitor) and in the absence of enzyme inhibitors. Carbonic anhydrase activity (U mg pt^{-1} min) was determined by adding water saturated with CO_2 to the supernatant of the homogenate of each organ and determining the pH decay of the solution calculated as [(catalyzed reaction rate/non-catalyzed reaction rate) mg pt^{-1}] according to Vitale et al. (1999).

2.6. MRC identification and quantification in the gills and kidneys

Gills and kidney samples fixed in Bouin solution were processed for paraffin embedding and sectioned (8 µm in thickness) using a microtome (Microm HM 360, MICROM International GmbH, Germany).

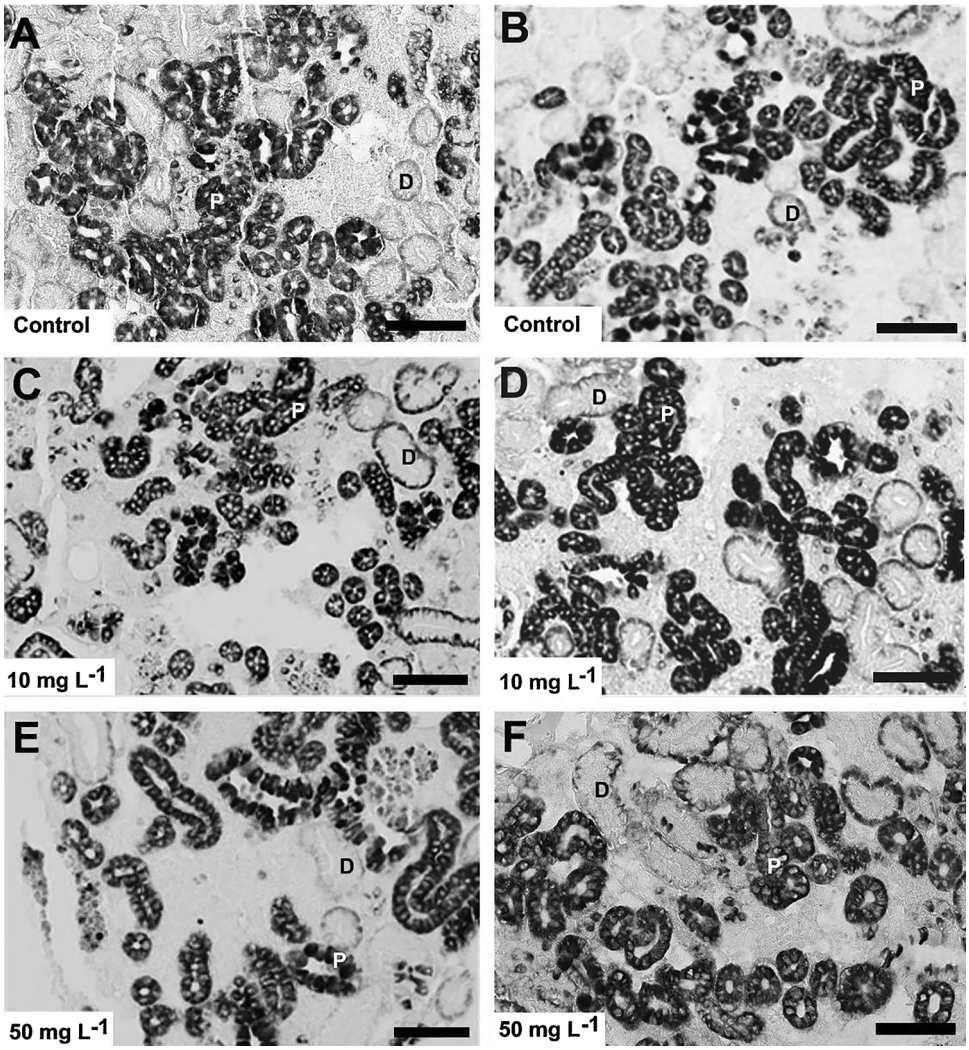

Fig. 4. Immunohistochemical staining for Na^+/K^+-ATPase in the mitochondria-rich cells (MRC) (arrow) in the kidneys of *Prochilodus lineatus* after acute (A, C, E) or subchronic (B, D, F) exposure to TiO_2-NP. P, proximal tubule; D, distal tubule. Scale bars = 50 μm.

Thereafter, sections were deparaffinized, rehydrated and processed according to the avidin-biotin-peroxidase complex technique to identify Na^+/K^+-ATPase (Dang et al., 2000). Sections were incubated with a monoclonal Na^+/K^+-ATPase antibody (IgGα5, University of Iowa, USA) following by a secondary antibody (IgG goat anti-mouse peroxidase, Chemicon International, USA). Subsequently, 3-3-diaminobenzidine (DAB) + ammoniacal nickel sulfate in Tris-saline buffer containing H_2O_2 (0.03%) was applied to identify the enzyme-antibody complex. In each fish, MRC in the filament and lamella of gills were counted in 25 random fields and in the kidneys were counted 40 random fields; MRC density was expressed as number per mm^2.

Scanning electron microscopy was used to analyze the apical surface of MRC of the gills. Gill samples fixed with 2.5% glutaraldehyde in phosphate buffer pH 7.4 were dehydrated in an increasing ethanol series, followed by a 1,1,1,3,3,3-hexadethyldisilazane bath and air dried at room temperature. After coating with a gold layer (99%, Evonik Degussa, Brazil) by vacuum sputtering (FCD 004 BAUSER, Germany) digital images from five gill filaments in each sample were obtained using a scanning electron microscope (Philips- Inspect S50, FEI Company, USA) and analyzed using the software Motic Image plus 2.0 (Motic, China) according to Bindon et al. (1994) and Moron et al. (2003). The MRC density and fractional surface area of MRC (MRCFA) were calculated according to MRCFA = Σ surface of total MRC/photography area and the MRC density was calculated as MRC = MRCFA/MRC average surface area.

2.7. Statistical analyses

For data presenting normality, homogeneity of variance and linearity was applied one-way analysis of variance (ANOVA) followed by the Tukey's post-hoc test to identify where differences occurred. Dunnett's post-hoc test was used to compare differences between control and each of the treatments. For data that did not attended such assumptions was applied non-parametric analyses, Kruskal-Wallis and Dunn's post test were applied to plasma Na^+ content after acute exposure and H^+-ATPase activity in both gills and kidney after acute exposure. All statistical analyses were done using R 3.0.2 software (R Development Core Team, 2013) at a significance level of 0.05.

3. Results

No fish died during acute and subchronic exposure to TiO_2-NP; fish subjected to subchronic exposure (14 d) at 10 and 50 mg L^{-1} TiO_2-NP presented paler color compared to the controls.

3.1. TiO_2-NP characterization in water

The diameter of TiO_2-NP varied from 17 to 34 nm with a mean

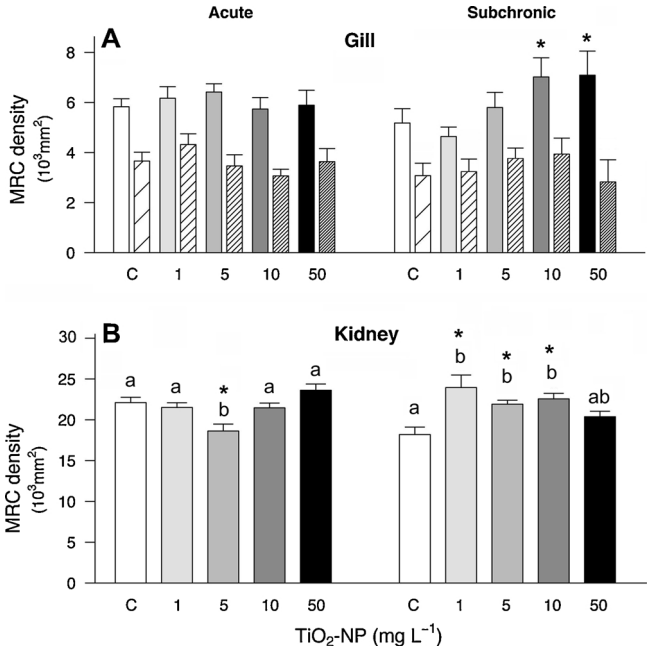

Fig. 5. Total mitochondria-rich cell (MRC) density in the filament (solid bars) and lamellae (striped bars) of the gills (A) and kidneys (B, solid bars) in *Prochilodus lineatus* from the control group and groups after acute and subchronic exposure to TiO_2-NP, identified by immunohistochemical for Na^+/K^+-ATPase. Values are means ± SEM. * indicates significant difference from the respective control and different letters indicate significant difference among groups ($p < 0.05$).

diameter of 24 ± 0.4 nm (mean ± SEM), similar to the size stated by the manufacturer Evonik Degussa (mean size = 21 nm diameter) (Fig. 1A). Zeta potential varied from −20.0 to −15.9 mV in aquarium water for TiO2-NP concentrations from 1 to 50 mg L^{-1} and the hydrodynamic diameter varied from 122 to 1990 nm. The aggregates/agglomerates formed by TiO_2-NPs ranged from 68 to 1718–2669 nm; most were around 180–230 nm (Fig. 1B).

At the nominal TiO_2-NP concentrations of 1, 5, 10 and 50 mg L^{-1} introduced into aquarium water, the concentrations of TiO_2-NPs suspended in water were, respectively, 0.6, 1.6, 2.7 and 18.1 mg L^{-1} after one hour (water taken from the middle of the water column) and were maintained for at least 24 h. However, the physiological, biochemical and morphological results were presented using the nominal concentration instead those in the water suspension. Ti^{+4} dissolved in water increased with the TiO_2-NP nominal concentration (1 to 50 mg L^{-1}) from 0.4 to 5.3 mg L^{-1} and slightly decreased after 24 h.

3.2. Plasma osmolality and ionic concentration

The plasma osmolality and ions are shown in Table 1. After acute exposure, plasma osmolality decreased in all groups ($p < 0.05$). K^+ levels increased after exposure to 1 mg L^{-1} TiO_2-NP ($p < 0.05$) compared to control and 10 mg L^{-1} TiO_2-NP and Ca^{2+} levels decreased after exposure to 10 and 50 mg L^{-1} TiO_2-NP ($p < 0.05$) compared to control and 1 mg L^{-1} TiO_2-NP. No changes occurred in the plasma Na^+ and Cl- levels. After subchronic exposure, the osmolality and ion levels were unchanged compared to controls.

3.3. Na^+/K^+-ATPase, H^+-ATPase and carbonic anhydrase activity

In the gills, Na^+/K^+-ATPase activities decreased after acute exposure to 5 mg L^{-1} TiO_2-NP compared to the control and 1 mg L^{-1} TiO_2-NP ($p < 0.05$) and 50 mg L^{-1} TiO_2-NP compared to control (Fig. 2A). H^+-ATPase activities decreased after exposure to 5 and 50 mg L^{-1} TiO_2-NP compared to the control and 1 mg L^{-1} TiO_2-NP ($p < 0.05$) (Fig. 2C). Carbonic anhydrase activity decreased after exposure to 10 and 50 mg L^{-1} TiO_2-NP compared to the control, 1 and 5 mg L^{-1} TiO_2-NP ($p < 0.05$) (Fig. 2E). After subchronic exposure, no changed occurred in the enzyme activities (Fig. 2A, C, E).

In the kidneys, the enzymes were unchanged after acute exposure to TiO_2-NP, but after subchronic exposure Na^+/K^+-ATPase activity decreased in 5 mg L^{-1} TiO_2-NP compared to control and 1 mg L^{-1} TiO_2-NP ($p < 0.05$) (Fig. 2B, D, F).

3.4. MRC identification and quantification

Immunohistochemical staining for Na^+/K^+-ATPase was performed to identify MRC in the filament and lamellar epithelia of gills and renal tubules of the kidneys in the control group and after exposure to TiO_2-NP, as shown in Figs. 3 and 4. In the gills, total MRC density was unchanged after acute exposure and increased in the gill filament after subchronic exposure to 10 and 50 mg L^{-1} TiO_2-NP ($p < 0.05$), but did not change in the gill lamellae (Figs. 3 and 5). In renal tubules, strong stained MRC were localized in the proximal tubules, distal tubules were slightly stained in the basolateral cell membrane (Fig. 4). Strong stained kidney MRC decreased significantly after acute exposure to 5 mg L^{-1} TiO_2-NP, compared with all groups ($p < 0.05$), and increased after subchronic exposure to 1, 5 and 10 mg L^{-1} TiO_2-NP compared to the control ($p < 0.05$) (Fig. 5).

In the gills, the MRC contacting water were randomly distributed in the afferent and efferent side of the filament epithelium close to the lamellae and in the interlamellar region and, in the lamellar epithelium, among the pavement cells which presented convoluted microridges at the apical surface (Fig. 6). Two MRC types contacting water were identified in the gill epithelium according to their apical morphology; MRCm (type 1) with short microvilli on the apical surface and MRCs (type 2) with a concave apical surface and a sponge-like appearance (Fig. 6). After acute exposure, the MRCm density unchanged, but the density of MRCs decreased after exposure to 1 and 50 mg L^{-1} TiO_2-NP compared to the control and 10 mg L^{-1} TiO_2-NP ($p < 0.05$) resulting in significant decreasing of total MRC density at gill epithelial surface compared to the control ($p < 0.05$) (Fig. 7A). Total MRCFA unchanged, although MRCsFA contacting water increased in fish exposed to 10 mg L^{-1} TiO_2-NP compared to all groups ($p < 0.05$) (Fig. 7B). After subchronic exposure, at gill surface, the total MRC density did not change (Fig. 7A) however, the MRCs density increased at 10 and 50 mg L^{-1} TiO_2-NP compared to control and fish exposed to 1 and 5 mg L^{-1} TiO_2-NP ($p < 0.05$) (Fig. 7A). Total MRCFA did not change although MRCmFA decreased in fish exposed to 10 mg L^{-1} TiO_2-NP compared to control and 5 mg L^{-1} TiO_2; MRCsFA did not change significantly (Fig. 7B).

4. Discussion

TiO_2-NP is not soluble in water but remains suspended depending on the physicochemical properties of the water (Li et al., 2016; Ottofuelling et al., 2011). For example, in high salinity brackish waters and freshwater waters rich in nutrient, TiO_2-NP aggregate and precipitate rapidly; in humus-poor waters, TiO_2-NP are suspended in water for a longer period (Li et al., 2016). The zeta potential of TiO_2-NP in aquarium water indicates instability and a tendency to aggregate/agglomerate, favoring NP precipitation. This explains the sharp reduction in the nominal 1, 5, 10 and 50 mg L^{-1} TiO_2-NP concentrations compared to those suspended in the water column, i.e. 0.6, 1.6, 2.7 and 18.1 mg L^{-1} TiO_2-NP, respectively. Similarly, other studies reported a reduction in the TiO_2-NP concentration immediately after bring added into the water with up to 85% decrease in 24 h (Clemente et al., 2013; Hao et al., 2009; Ramsden et al., 2013).

Suspended TiO_2-NP into water accumulated in the gills of *P. lineatus* after subchronic (14 d) exposure (Carmo, 2015) indicated gill

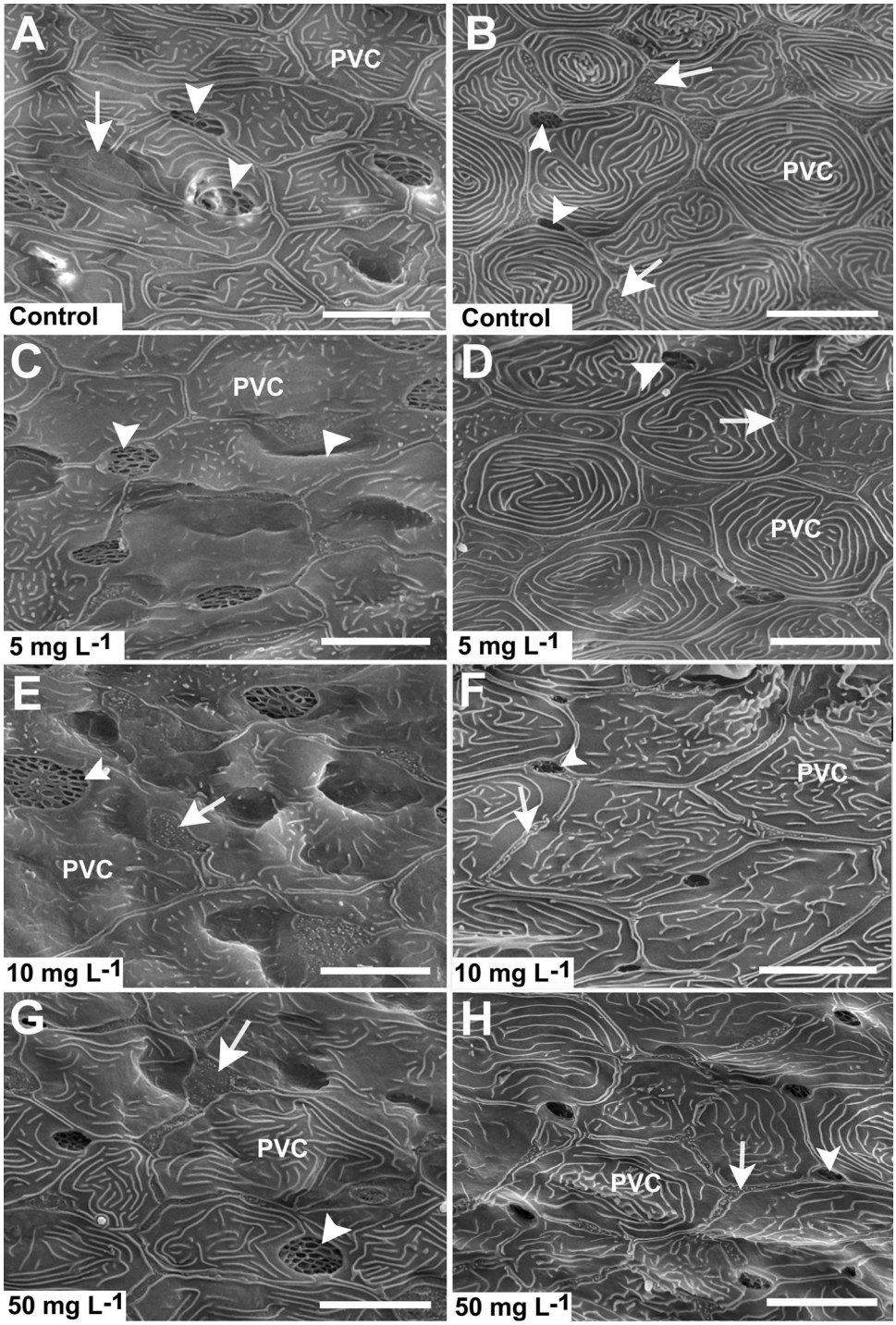

Fig. 6. SEM micrographs of the gill filament epithelium of *Prochilodus lineatus* from the control group (A, B) and groups exposed to TiO_2-NP (C, E, G) after acute (A, C, E, G) or subchronic (B, D, F, H) exposure. Note two mitochondria-rich cells (MRC) types contacting water: MRC with apical microvilli (MRCm, arrows) and MRC with apical sponge-like structure (MRCs, arrowheads). Pavement cell (PVC). Scale bars = 10 μm.

absorption probably during ventilation in the respiratory process. The kidney, liver, muscles and brain accumulation in the same fish species after subchronic exposure to TiO_2-NP also reported by Carmo (2015) suggested that the TiO_2-NP absorbed by the gill were, at least partially, transferred to the blood, reaching these organs. Previous studies in different freshwater fish species demonstrated Ti accumulation in several organs after TiO_2-NP exposure (Boyle et al., 2013; Hu et al., 2017; Ramsden et al., 2013; Zhang et al., 2007) emphasizing that the gills are the main organ for TiO_2 uptake (Zhang et al., 2007). Such TiO_2-NP accumulation may affect fish organ function.

In the present study, although exposure to different TiO_2-NP concentrations did not cause fish mortality, the decrease in plasma osmolality, as well as transitory increase in K^+ and decrease in Ca^{2+} levels shows that TiO_2-NP inside animal induced osmotic and ionic imbalance after acute exposure. This osmotic and ionic imbalance may be due to a reduction in gill Na^+/K^+-ATPase, H^+-ATPase and carbonic anhydrase activities, once these enzymes are responsible for creating a favorable gradient for ions' uptake (Hirose et al., 2003). Na^+/K^+-ATPase in the basolateral cell membrane and tubular system of MRC pump intracellular Na^+ into the interstitial space in exchange with K^+,

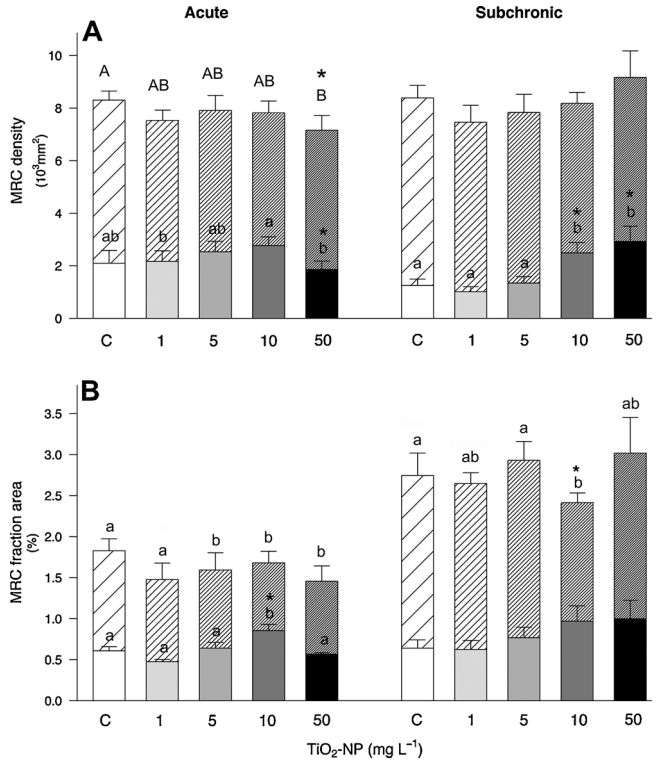

Fig. 7. Mitochondria-rich cells (MRC) density contacting water in the filament gill epithelium. MRC with apical microvilli (MRCm, striped bars) and MRC with an apical sponge-like appearance (MRCs, solid bars) densities in (A) and MRC fractional area in the (B) of *Prochilodus lineatus* from control group and groups exposed to TiO_2-NP after acute and subchronic exposure. Values are means ± SEM. * indicates significant difference from the respective control, different lower case letters indicate significantly MRCm and MRCs difference among groups ($p < 0.05$) and different upper case letters indicate significantly total MRC difference among groups ($p < 0.05$).

maintaining low Na^+ inside the cell and favoring Na^+ uptake by the apical membrane contacting water (Bystriansky and Schulte, 2011). Na^+ and Cl^- uptake from water is linked to the excretion of H^+ and HCO_3^- via Na^+/H^+ or NH_4^+ and Cl^-/HCO_3^- exchangers. Cytosolic carbonic anhydrase catalyzes the reversible hydration of CO_2 generating H^+ and HCO_3^- for ion exchangers at the apical membrane of the cell (Hwang and Lee, 2007; Lionetto et al., 2016). Vacuolar proton ATPase (V-H^+-ATPase) on the apical membrane of MRC and pavement cells (PVC) also contribute to the intracellular electrochemical gradient for Na^+ uptake by pumping H^+ out of the cell; Na^+ from the water enters into the cell passively through a proposed epithelial Na^+ channel (Hirose et al., 2003; Evans et al., 2005; Tresguerres, 2016). In the kidneys, a similar process occurs in the renal tubule cells taking Na^+ and Cl^- from the tubular lumen and producing diluted urine (Perry et al., 2003; McDonald, 2007).

The decrease in plasma osmolality after acute exposure to TiO_2-NP may be partially explained by lower Ca^{2+} levels, suggesting possible inhibition of Ca^{2+}-ATPase activity as Ca^{2+} is actively absorbed by MRC. This enzyme generates a driving force for Ca^{2+} uptake through the gills and intestine (Flik et al., 1995; Wendelaar Bonga and Lock, 2008) and can be inhibited by transition metals such as Ti due to the affinity of the enzyme for metal ions (Atli and Canli, 2011; Wang et al., 2013). However, the inhibition of Na^+/K^+-ATPase, as occurred in the present study after acute exposure to TiO_2-NP, also contributed to reduce Ca^{2+} uptake by the gills due to the involvement of the Na^+/Ca^{2+} exchanger in the basolateral of MRC membrane for calcium extrusion into the interstitial medium (Hwang, 2009).

The inhibition of Na^+/K^+-ATPase, H^+-ATPase and carbonic anhydrase after acute exposure may have been mediated by Ti binding to the enzyme and/or TiO_2-NP interactions with these enzymes at high concentrations (above 5 mg L^{-1}). Some transition metals displace Zn^{2+} in the active site or bind to histidine and cysteine residues at sites other than the active one, which can inhibit carbonic anhydrase (Lionetto et al., 2016) as well as Na^+/K^+-ATPase and H^+-ATPase (Li et al. 1996; Atli and Canli, 2011, Hegazi et al., 2015). The absence of changes in plasma Na^+ and Cl- levels in *P. lineatus*, during acute exposure may be partially explained by unchanged renal enzyme activities or intestinal enzymes. In *Oncorhynchus mykiss*, a concentration-dependent tendency toward Na^+/K^+-ATPase inhibition after TiO_2-NP exposure without an effect on plasma Na^+ and Cl- concentrations was, in part, compensated for by unchanged intestinal Na^+/K^+-ATPase (Federici et al., 2007). After subchronic exposure to TiO_2-NP, the maintenance of osmo-ionic balance could be due to the changes in the density of MRCs contacting water, which are localized on gill filaments, but also due to a trend toward increasing apical surface area in the gills. MRC density increased in the renal proximal tubules probably also contributed to avoid osmotic unbalance caused by TiO_2-NP exposure.

The distribution of MRC in the filament and lamellar epithelia in the gill of *P. lineatus* is characteristic of Brazilian freshwater fish as the continental aquatic systems have soft and ion-poor water (Fernandes et al., 2007). Although, the total MRC density, identified by immunohistochemical staining for Na^+/K^+-ATPase, unchanged in the gill filament and lamellar epithelia after acute exposure, the alterations in total MRC contacting water and the MRCFA suggested some morphological adjustment. Such adjustments were clear after subchronic exposure to TiO_2-NP and suggested functional difference between the two MRC morphological types in the gill epithelium of *P. lineatus*. The MRC increasing only in the filament epithelium showed a redistribution of these cells after exposure to TiO_2-NP. Despite MRCm were more abundant than MRCs at gill surface, only MRCs density increased after subchronic exposure to high TiO_2-NP concentrations (10 and 50 mg L^{-1}) which suggested physiological and morphological adjustments to maintain ion uptake. MRC proliferation increases the ion transport capacity of the gills and the MRCFA represents the direct relationship with Na^+ and Cl- influx as was demonstrated in rainbow trout (*Oncorhynchus mykiss*), American eel (*Anguilla rostrata*), brown bullhead catfish (*Ictalurus nebulosus*) and tilapia (*Oreochromis mossabicus*) (Perry et al., 1992b). A similar relationship between MRCFA and Ca^{2+} uptake were reported in *O.mykiss*, *A. rostrata* and *I. nebulosus* (Perry et al., 1992a). Thereafter, inter-specific differences in the rates of ionic uptake between species have been attributed to MRC density at gill surface and MRCFA (Perry et al., 1992a,b); MRCFA is the consequence of MRC density and/or individual MRC apical surface area. In general, a change in MRCFA is the first event required to maintain ionic balance, followed by a change in MRC density, a response with greater energy demand (Moron et al., 2003). Tolerance to Ti accumulation in the gills and kidneys after subchronic exposure to TiO_2-NP, resulting in more MRC and unchanged osmotic and ionic balance, may be related to metallothionein (MT) expression in the new cells, thereby increasing fish tolerance to metals (Roesijadi, 1996; Wendelaar Bonga and Lock, 2008). Tilapia (*Oreochromis mossambicus*) exposed to copper exhibit higher MT levels in the gill cells, including MRC, after 2 d of exposure; this increased continuously until 14 d of exposure (Dang et al., 1999). However, in the liver of *P. lineatus*, Ti accumulation does not stimulate MT expression after TiO_2-NP exposure (Carmo, 2015).

5. Conclusions

In the Neotropical fish *P. lineatus*, acute exposure to high TiO_2-NP concentrations caused osmotic and ionic unbalance decreasing plasma osmolality and Ca^{2+} levels. The inhibition of $Na^+/K+$-ATPase, H^+-ATPase and carbonic anhydrase activities in the gills may, at least in part, explain osmotic and ionic unbalance. However, the maintenance of plasma Na^+ and Cl^- levels suggest partial compensation by the

kidney in which these enzymes were not inhibited. Despite acute effects, the changes in total MRC density in gill filaments and renal tubules as well as the changes in density of each MRC type suggested a cellular physiological and morphological response to restore/maintain osmotic and ionic homeostasis after subchronic exposure. Further studies are necessary to understand the functional differences between the two MRC types in gills and their molecular adjustment to maintain osmotic and ionic balance even after exposure to high concentrations of TiO_2-NP.

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