

Acute effects of cadmium on osmoregulation of the freshwater teleost *Prochilodus lineatus*: Enzymes activity and plasma ions

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ABSTRACT

Cadmium (Cd) is a trace element that is very toxic to fish. It is commonly found in surface waters contaminated with industrial effluents. When dissolved in water, Cd can rapidly cause physiological changes in the gills and kidneys of freshwater fish. The objective of this study was to evaluate the acute effects of Cd on the osmoregulation of the Neotropical fish *Prochilodus lineatus*. Juvenile fish were exposed to Cd at two concentrations [1 (Cd1) and 10 (Cd10) $\mu\text{g L}^{-1}$] for 24 and 96 h. The effects of Cd were evaluated through the analysis of ions (Na^+ , K^+ , Ca^{2+} , and Cl^-) and plasma osmolality, and by measuring the activities of enzymes involved in osmoregulation obtained from the gills and kidney. Fish exposed to Cd for 24 and 96 h showed a decrease in Na^+/K^+ -ATPase activity in the gills and kidney. The activity of carbonic anhydrase decreased in the gills after 24 h and in both tissues after 96 h of Cd exposure. The gill Ca^{2+} -ATPase activity also decreased with Cd exposure, with a concomitant drop in the plasma concentration of Ca^{2+} . Despite the hypocalcemia, there were no changes in the concentration of the ions Na^+ , K^+ , and Cl^- or in plasma osmolality. Among the enzymes involved in ion transport, H^+ -ATPase was the only enzyme that showed increased activity in gills, whereas its activity in kidney remained unchanged. The results of this study demonstrate that waterborne Cd at the maximum concentrations set by Brazilian guidelines for freshwater affects the gills and kidney functions of *P. lineatus*. Acute exposure to Cd resulted in the decrease of the activity of enzymes, which culminated with the loss of the fish's ability to regulate the levels of calcium in the blood, leading to hypocalcemia.

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1. Introduction

Metal contamination in freshwater ecosystems has increased in the past several decades, resulting in toxic effects to wildlife and damage to the environment. Cadmium (Cd) is a non-essential trace metal that is extremely toxic to aquatic biota. Surface waters normally contain relatively low concentrations of Cd; however, residues generated from industrial activities, mining, disposal of batteries, and use of fertilizers have contributed to the additional amounts of this contaminant in inland waters (Sorensen, 1991).

Fish are highly susceptible to Cd contamination (EPA, 2001). The toxic effects of Cd on fish are persistent and can be detected

within a few hours of exposure (De La Torre et al., 2000). Chronic and sub-chronic exposure models have recently been used in eco-toxicology studies (Kamunde and MacPhail, 2011; Maunder et al., 2011; Cao et al., 2012); however, since the toxic effects of Cd are quickly established in fish, it is important to identify the physiological and biochemical alterations caused by acute exposure to this metal. Freshwater fish mainly absorb waterborne Cd through their gill epithelia; hence, gills are the first target organs of xenobiotics (Verbost et al., 1988). Once inside the organism, Cd enters the blood circulation to reach other organs and accumulates most significantly in kidney, followed by liver and gills (Pretto et al., 2011).

In freshwater fish, the gills and kidneys represent the main organs responsible for osmotic control and acid-base regulation (Gilmour and Perry, 2009). In the presence of Cd, modifications in enzymatic activities and in membrane transport systems are among the first changes that can be detected (Viarengo, 1989). Immediately after exposure to Cd, ionic regulation is compromised (McGeer et al., 2000), leading mainly to hypocalcemia, which is

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caused by the inhibition of the basolateral Ca^{2+} -ATPase in gill cell membranes (Verbost et al., 1988). In addition to the role of basolateral Ca^{2+} -ATPase (CATPase) in calcium absorption, Na^+/K^+ -ATPase (NKA), H^+ -ATPase (HATPase), and carbonic anhydrase (CA) present in the gills and kidney are also involved in the uptake of electrolytes from water and glomerular filtrate, respectively, and in the control of systemic pH by regulating the elimination of H^+ or HCO_3^- (Perry et al., 2003). The enzyme CA catalyzes the hydration of CO_2 , generating HCO_3^- and H^+ ; these ions are involved in the transport of Na^+ and Cl^- (Boisen et al., 2003), which are also affected by the elimination of H^+ caused by the apical H^+ -ATPase (Lin and Randall, 1991; Perry and Fryer, 1997). The activity of NKA in the basolateral membrane results in an electrochemical gradient that causes calcium to enter the cells through the apical side. Thus, the activities of these enzymes are essential for the uptake of ions and maintenance of ionic balance in freshwater fish (Lin and Randall, 1991; Perry et al., 2003; Evans et al., 2005).

There are considerable differences in Cd sensitivity among fish species and it is not possible to make definitive statements in relation to exposure concentrations that induce acute toxicity (McGeer et al., 2012). In Brazil, the emission of toxic substances, such as metals, to aquatic ecosystems is regulated by the Brazilian National Environmental Council (CONAMA, 2005). But the maximum acceptable values in the present national regulation are mainly based on international criteria like those from the United States Environmental Protection Agency (EPA) and the European Union (E.U.) regulations. Consequently, they do not consider the potential deleterious effects of pollutants to Brazilian native species (Martins and Bianchini, 2011). *Prochilodus lineatus* is a Neotropical fish species of great ecological significance that inhabits the largest South American river basins and is sensitive to metals, such as aluminum (Camargo et al., 2009), lead (Monteiro et al., 2011), and copper (Nascimento et al., 2012), nonetheless there is no information available regarding Cd effects on this fish species.

In this context, the objective of the present study was to evaluate the acute effects of waterborne Cd at the maximum concentrations set by Brazilian guidelines for freshwater on juveniles of *P. lineatus*. The parameters evaluated included the activity of the enzymes CATPase, HATPase, NKA, and CA in the gills and kidney, as well as the osmolality and plasma concentrations of Na^+ , K^+ , Ca^{2+} , and Cl^- , which would thus characterize the initial effects of Cd on the key components of osmoregulation in freshwater fish and the mechanism by which Cd affects plasma ion composition.

2. Materials and methods

2.1. Animals

Juvenile *P. lineatus* (12.4 ± 1.2 g; 11.2 ± 3 cm, $n = 251$) specimens were supplied by the Fish Culture Station of the State University of Londrina (EPUEL). Fish were acclimated for at least 5 days in tanks (300 L) containing non-chlorinated water, under constant aeration, and photoperiods of 12 h light and 12 h dark. During the acclimation period, fish were fed every 2 days with commercial fish feed (Guabi®, protein content of 36%). Feeding was suspended 24 h before and during the toxicity tests. The physical and chemical parameters of the water were monitored using a multi-parameter water quality meter (Horiba U-50).

2.2. Experimental protocol

Following acclimation, the animals were subjected to static acute toxicity tests for 24 and 96 h in 100-L glass tanks containing 80 L of water. Six to eight individuals were placed in each tank, keeping a maximum density of 1 g of fish per liter of water. Three

groups were formed for each experimental period: a control (CTR) group, where fish were only exposed to non-chlorinated water, and two experimental groups, where fish were exposed to $1 \mu\text{g L}^{-1}$ (Cd1) or $10 \mu\text{g L}^{-1}$ (Cd10) of Cd, added from a 1 mg L^{-1} cadmium chloride (CdCl_2) stock solution. All tests were carried out in duplicate. Cd concentrations were defined according to the maximal allowable concentrations of Cd in classes 1 and 2 ($1 \mu\text{g L}^{-1}$) and 3 and 4 ($10 \mu\text{g L}^{-1}$) freshwater, as defined by Brazilian legislation (CONAMA, 2005).

After the exposure period, the fish were anesthetized with benzocaine (0.12 g L^{-1}) and blood was drawn from the caudal vein into a pre-heparinized syringe. After blood collection, the animals were killed by medullar section. The gills and kidneys were removed using the procedure approved by the Animal Ethics Committee of the State University of Londrina (Process 35004.2011.18).

Blood samples were then transferred to 1.5-mL plastic tubes and kept on ice. Gill filaments were washed and separated from the gill arches, and together with kidney samples, were stored in plastic tubes containing the appropriate buffers for measurement of the activity of the enzymes NKA, HATPase, CATPase, and CA. All samples were kept frozen (-80°C) until analysis.

The water temperature, pH, dissolved oxygen, and conductivity were monitored during the entire experiment. Water samples were collected after each experimental time, fixed with HNO_3 ($\text{pH} \leq 2$), and analyzed by electrothermal atomic absorption spectrometry, using an atomic absorption spectrometer equipped with a graphite furnace atomizer (Perkin Elmer A700), to determine Cd concentrations. Total Cd concentration was measured in unfiltered water samples, whereas the concentration of dissolved Cd was determined in filtered water samples (0.45- μm filter).

2.3. Plasma analyses

Blood samples were centrifuged (10 min; $1870 \times g$), and plasma samples were frozen (-20°C). Osmolality was measured by determining the freezing point depression using an osmometer (Osmomat 030, Gonotec, Germany). A commercial test kit (Labtest Diagnóstica, Brazil) was used to determine the concentration of chloride by the mercury thiocyanate method using a microplate spectrophotometer (VictorTM, PerkinElmer) at 470 nm. The concentrations of sodium and potassium were determined in plasma samples diluted in deionized water (1:100) using a flame photometer (Analyser 900, Brazil). Calcium concentration was measured using the flame atomization method with an atomic absorption spectrometer (Perkin Elmer A700). Plasma samples were diluted to a ratio of 1:40 in 1% lanthanum chloride as a modifier.

2.4. Determination of NKA activity

NKA activity was determined in the homogenized fractions of gills and kidney according to the method described by Quabius et al. (1997). This method is based on the production of inorganic phosphorus in a medium after incubation in the presence and absence of ouabain. The excised organs were immersed in SEI buffer (0.3 M sucrose, 0.1 mM Na_2EDTA , 30 mM imidazole, 0.035% β -mercaptoethanol, pH 7.4) and stored in ultrafreezer at -80°C . Samples were homogenized in the SEI buffer with Triton X-100 (1:1000, w/v), centrifuged (13,600 $\times g$, 15 min, 4°C), and the supernatant was used for the enzyme assay and for the determination of total protein (Lowry et al., 1951). Each sample was incubated with a buffer solution (100 mM NaCl, 8 mM MgCl_2 , 30 mM imidazole, 0.1 mM EDTA, 3 mM ATP, pH 7.6) containing KCl (13 mM) or ouabain (2.5 mM). After a 30-min incubation in the dark, the reaction was stopped by adding a 1:1 mixture of 8.6% TCA and a color reagent (0.66 mM H_2SO_4 + 9.2 mM ammonium molybdate + 0.33 mM $\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$). A 650 mM phosphate solution

(Sigma) was used as standard. Readings (ELX Reader, BioTek) were performed at a wavelength of 620 nm and the enzyme activity was expressed in $\mu\text{M Pi mg proteins h}^{-1}$.

2.5. Determination of CATPase activity

To analyze CATPase activity, the gills and kidneys were stored in SEEI buffer (0.3 M sucrose, 0.1 mM Na_2EDTA , 0.1 mM EGTA, 30 mM imidazole, 0.035% β -mercaptoethanol, pH 7.6). The tissues were homogenized in 1:10 (w/v) SEEI buffer with Triton X-100 (1:1000) and centrifuged ($13,600 \times g$, 15 min, 4 °C). Aliquots of the supernatant were used for the determination of total protein (Lowry et al., 1951) and incubated in a buffer (4 mM MgCl_2 , 100 mM NaCl, 30 mM imidazole, 0.1 mM EDTA, and 3 mM ATP, pH 7.6) supplemented with 4 mM CaCl_2 , 1 mM EGTA and 20 mM KCl to measure total ATPase activity, and with 20 mM KCl and 1 mM EGTA to measure CATPase activity. After a 30-min incubation, the reaction was stopped by adding a 1:1 mixture of 8.6% TCA and a color reagent (0.66 mM H_2SO_4 + 9.2 mM ammonium molybdate + 0.33 mM $\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$). A 650 mM phosphate solution (Sigma) was used as standard. Readings (ELX Reader, BioTek) were performed at a wavelength of 620 nm and the enzyme activity was expressed in $\mu\text{M Pi mg proteins h}^{-1}$.

2.6. Determination of HATPase activity

A coupled pyruvate kinase/lactate dehydrogenase (PK/LDH) assay was used to measure HATPase activity, as described by Gibbs and Somero (1989). Gill filaments and kidneys were homogenized (1:10, w/v) in SEID buffer (150 mM sucrose, 50 mM imidazole, 10 mM EDTA, 2.4 mM sodium deoxycholate, pH 7.5) and centrifuged (7 min, 1000 × g at 4 °C). The supernatants were used to measure the concentration of total protein (Lowry et al., 1951) and diluted in SEID buffer to obtain a concentration of 1 mg mL⁻¹ for all samples. The pure reaction solution (30 mM imidazole, 45 mM NaCl, 15 mM KCl, 3 mM MgCl_2 , 0.4 mM KCN, ATP 1 mM, 0.2 mM NADH, PK at 3 U mL⁻¹, LDH at 2 U mL⁻¹, 0.1 mM fructose 1,6-bisphosphate, 2 mM phosphoenolpyruvate, pH 9.0) was used to measure total ATPase activity in the samples. The activity of HATPase was measured after the addition of 2 mM N-ethylmaleimide (NEM), an inhibitor of this enzyme, to the reaction solution. Samples were added to the different reaction mixtures in triplicate and readings were performed after a 30-min incubation. Absorbance readings were obtained every minute for 15 min at a wavelength of 340 nm (VictorTM, PerkinElmer). The HATPase activity was calculated using an extinction coefficient of 56.6 M⁻¹ cm⁻¹ and expressed in $\mu\text{mol ADP mg protein}^{-1} \text{ h}^{-1}$.

2.7. Determination of CA activity

CA activity was determined by using an electrometric method according to Vitale et al. (1999). The gills and kidneys were homogenized in buffer (225 mM mannitol, 75 mM sucrose, 10 mM Tris-base, and 10 mM Na_2PO_4 , pH 7.4), centrifuged at $13,600 \times g$ for 10 min (4 °C), and the supernatant was used for the assay. The catalytic activity of the enzyme was quantified by acidification of the saturated medium with CO_2 for 20 s using a pH meter (Jenway 3510). The slope of the curve generated by a decrease in pH as a function of time provided the rate of catalyzed (RC) reaction, and the rate of non-catalyzed reaction (RNC) was obtained by conducting pH readings in the absence of the sample (the test sample was replaced with buffer). On the basis of these two measurements, the activity of CA was calculated using the formula: $\text{CA} = [\text{RC}/\text{RNC} - 1] \text{ mg}^{-1} \text{ protein}$.

2.8. Statistical analysis

Results were expressed as the mean ± SE. After verifying the normality and homogeneity of variance, the results of the different parameters at each time (24 and 96 h) were compared among the different groups (CTR × Cd1 × Cd10) using one-way ANOVA, and differences were identified using the Student-Newman-Keuls (SNK) test of multiple comparisons when necessary. *p* values <0.05 were considered significant.

3. Results

No fish deaths were observed during the entire experimental period. The physical and chemical parameters of the water are presented in Table 1. There were no significant variations in the measurements of temperature, dissolved oxygen, pH, conductivity, and hardness among the experimental groups.

3.1. Osmolality and plasma ions

Fig. 1 shows the results of osmolality and the concentrations of the plasma ions. After 24 h of exposure, only animals in Cd10 group showed significantly lower plasma Ca^{2+} concentrations (*p* = 0.03). After 96 h of exposure, the plasma Ca^{2+} concentrations decreased in Cd1 and Cd10 groups (*p* = 0.003). Exposure to Cd resulted in no changes in the concentrations of the other ions (*p* ≥ 0.69). No alterations in plasma osmolality for any of the experimental groups were observed (*p* ≥ 0.97).

3.2. NKA activity

NKA activity in the gills and kidney of Cd10 group decreased in relation to those of the CTR and Cd1 after 24 h (*p* < 0.03) and 96 h (*p* < 0.001) of exposure (Fig. 2). No significant differences between groups Cd1 and CTR were observed.

3.3. CATPase activity

After 24 and 96 h of exposure, Cd1 and Cd10 groups showed a significant decrease (*p* < 0.001) in gill CATPase activity in comparison to respective CTR (Fig. 3). In terms of enzyme activity in the kidney, a significant increase in CATPase activity was observed after 96 h of exposure to Cd1 (*p* < 0.001).

3.4. HATPase activity

No significant changes in kidney HATPase activity were observed in any of the experimental conditions tested (24 h: *p* = 0.085; 96 h: *p* = 0.443) (Fig. 4). Similarly, no significant differences in gill HATPase activity were observed between the groups CTR and Cd1 after 24 h and 96 h of exposure. However, a significant increase in gill HATPase activity was observed in group Cd10 after both exposure periods (*p* < 0.001).

3.5. CA activity

CA activity (Fig. 5) in the gills was significantly reduced under all exposure conditions in relation to their respective controls (*p* < 0.002). Cd10 group showed a greater reduction in the activity of gill CA than Cd1 after 96 h of exposure (*p* = 0.024). A significant decrease in kidney CA activity was observed in Cd1 and Cd10 groups after 96 h of exposure (*p* < 0.001).

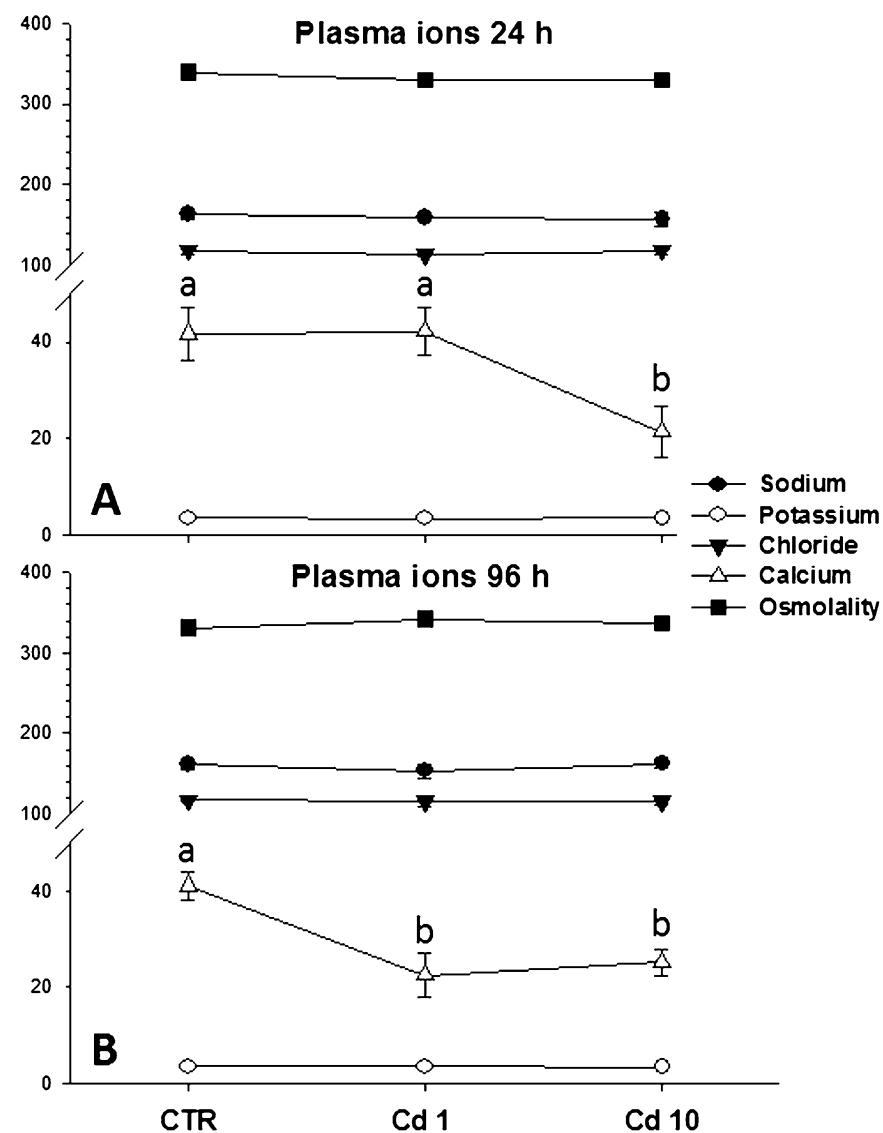


Fig. 1. Plasma concentrations (in mM) of sodium, potassium, chloride and calcium and plasma osmolality (in mOsm. Kg H₂O⁻¹) in juveniles of *P. lineatus* exposed for 24 h (A) and 96 h (B) only to water (CTR) or to Cd at concentrations of 1 µg L⁻¹ (Cd1) and 10 µg L⁻¹ (Cd10). Results are represented as means ± SE. Different letters indicate significant differences between the groups (CTR × Cd1 × Cd10) in the same period of exposure (*p* < 0.05; *n* = 6–8).

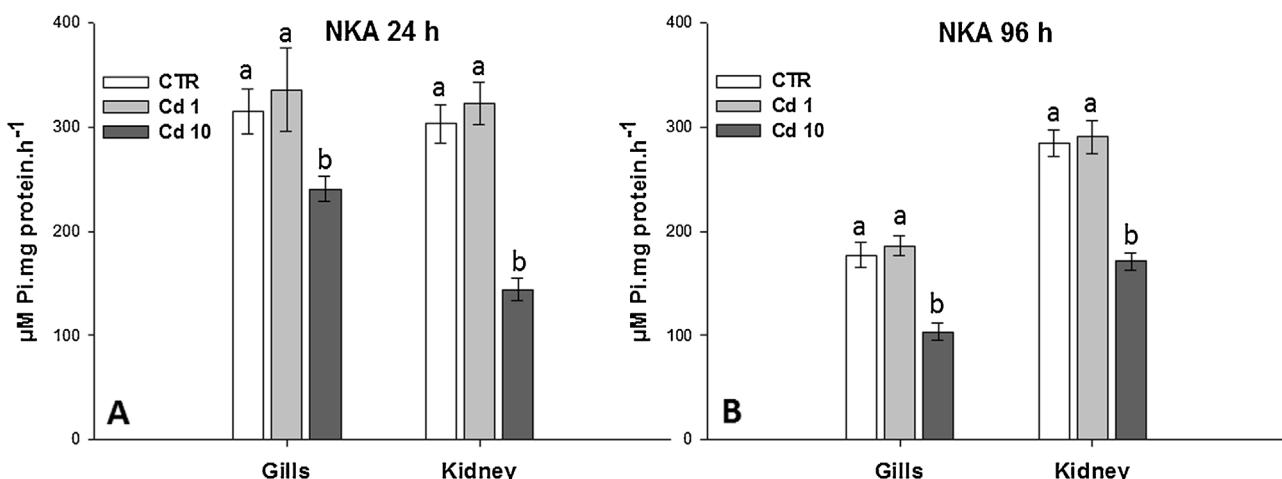


Fig. 2. Na⁺/K⁺-ATPase activity (mean ± SE) in gills and kidney of *P. lineatus* exposed for 24 h (A) and 96 h (B) only to water (CTR) or to Cd at concentrations of 1 µg L⁻¹ (Cd1) and 10 µg L⁻¹ (Cd10). Different letters indicate significant differences between the groups (CTR × Cd1 × Cd10) in the same period of exposure (*p* < 0.05; *n* = 10–14).

Table 1

Physical and chemical parameters and total and dissolved Cd concentrations in the water used in the experiments.

Water parameters	CTR	Cd1	Cd10
Temperature (°C)	19.55 ± 1.55	20.05 ± 1.10	19.80 ± 1.28
pH	6.73 ± 0.20	6.85 ± 0.35	6.93 ± 0.52
Dissolved oxygen (mg O ₂ L ⁻¹)	7.66 ± 0.98	8.1 ± 0.72	8.0 ± 0.87
Conductivity (μS cm ⁻¹)	86.0 ± 15.0	88.0 ± 12.0	92.0 ± 8.0
Hardness (mg CaCO ₃ L ⁻¹)	57.8 ± 3.2	55.1 ± 3.9	56.2 ± 4.1
Total Cd (μg L ⁻¹)	Nd	0.882 ± 0.079	7.91 ± 1.1
Dissolved Cd (μg L ⁻¹)	Nd	0.739 ± 0.066	6.81 ± 0.9

Results are mean ± SE ($n=8$). Nd: not detected.

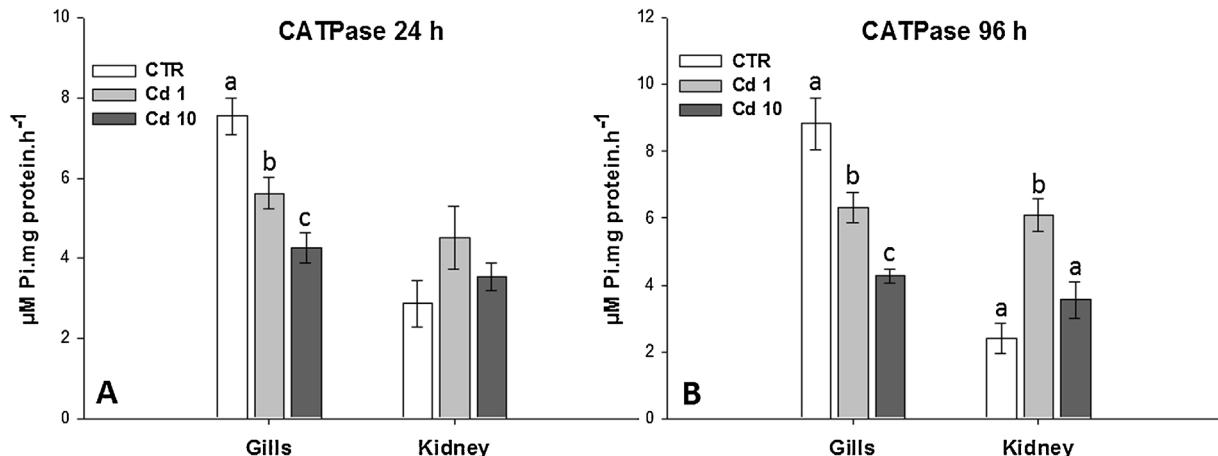


Fig. 3. Ca²⁺-ATPase activity (mean ± SE) in gills and kidney of *P. lineatus* exposed for 24 h (A) and 96 h (B) only to water (CTR) or to Cd at concentrations of 1 $\mu\text{g L}^{-1}$ (Cd1) and 10 $\mu\text{g L}^{-1}$ (Cd10). Different letters indicate significant differences between the groups (CTR × Cd1 × Cd10) in the same period of exposure ($p < 0.05$; $n = 6-8$).

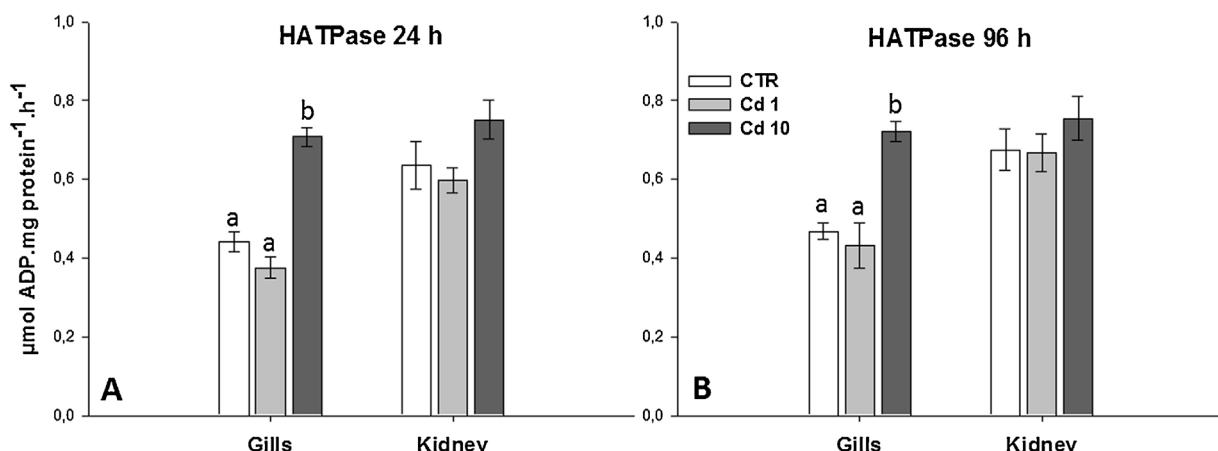


Fig. 4. H⁺-ATPase activity (mean ± SE) in gills and kidney of *P. lineatus* exposed for 24 h (A) and 96 h (B) only to water (CTR) or to Cd at concentrations of 1 $\mu\text{g L}^{-1}$ (Cd1) and 10 $\mu\text{g L}^{-1}$ (Cd10). Different letters indicate significant differences between the groups (CTR × Cd1 × Cd10) in the same period of exposure ($p < 0.05$; $n = 7-8$).

4. Discussion

Juveniles of *P. lineatus* subjected to acute Cd exposure exhibited alterations in the activity of the enzymes involved in acid-base and ionic regulation. This is the first study that has evaluated the effect of the maximum concentrations of Cd in freshwater, as allowed by Brazilian legislation, on a Neotropical fish species. The two concentrations of cadmium analyzed, 1 and 10 $\mu\text{g L}^{-1}$, were considered relatively low as most of the ecotoxicology studies use higher concentrations, ranging from 60 $\mu\text{g L}^{-1}$ to 1600 $\mu\text{g L}^{-1}$ (De La Torre et al., 2000; Garcia-Santos et al., 2006; Huang et al., 2009; Atli and Canli, 2013). Studies that use similar concentrations as those selected for the present investigation usually involve chronic and subchronic exposure (Franklin et al., 2005). Considering that not

all species of fish suffer the effects of Cd on the regulation of their osmotic systems even when subjected to more adverse testing conditions, *P. lineatus* is characterized as a species that is highly sensitive to Cd.

The enzymatic activity assays have provided a better understanding of the physiological mechanisms involved in Cd toxicity. CA, NKA, HATPase, and CATPase are abundant in the kidney and branchial epithelia, which are the main organs involved in ion uptake and maintenance of ion homeostasis in freshwater teleost fish (Perry et al., 2003). The activity of kidney and gill NKA seems to be sensitive to the concentration of Cd, as animals in the group Cd10 showed inhibition within the first 24 h of exposure, whereas those of group Cd1 showed no alterations throughout the 96 h of exposure. When the gill tissues of *Anguilla anguilla* were incubated in the

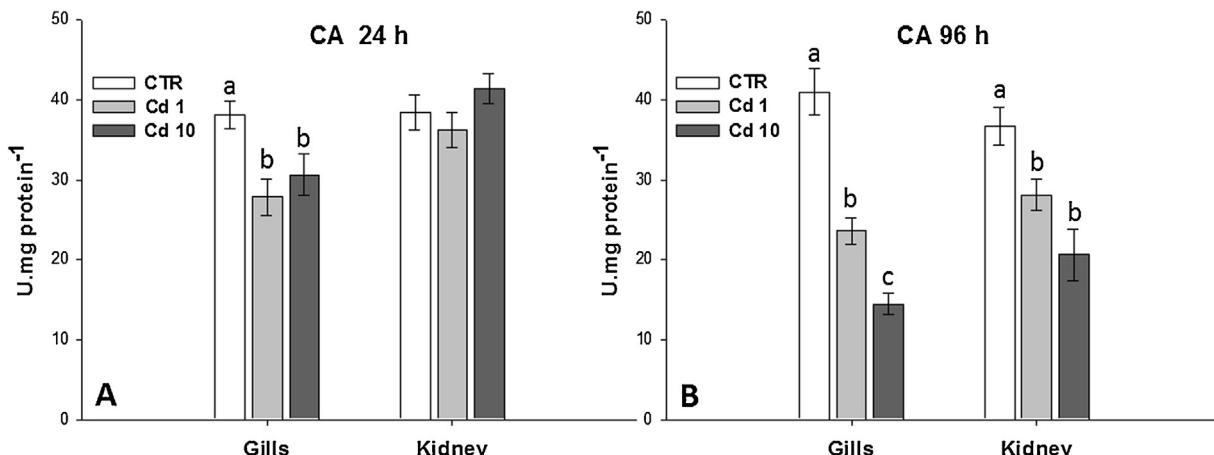


Fig. 5. Carbonic anhydrase (CA) activity (mean \pm SE) in gills and kidney of *P. lineatus* exposed for 24 h (A) and 96 h (B) only to water (CTR) or to Cd at concentrations of $1 \mu\text{g L}^{-1}$ (Cd1) and $10 \mu\text{g L}^{-1}$ (Cd10). Different letters indicate significant differences between the groups (CTR \times Cd1 \times Cd10) in the same period of exposure ($p < 0.05$; $n = 7-14$).

presence of Cd ($363 \mu\text{g L}^{-1}$), an inhibition of NKA was observed after 10 min of exposure and maximum effects were demonstrated after 1 h (Lionetto et al., 2000). In fact, in vitro studies have demonstrated that micromolar concentrations of Cd cause rapid inhibition of NKA activity in tissues of several fish species (Van Kerkhove et al., 2010). *Cyprinus carpio* showed an irreversible inhibition of NKA activity after a 14-day exposure to $1.6 \mu\text{g L}^{-1}$ Cd, which lasted for at least 19 days after recovery (De La Torre et al., 2000). Atli and Canli (2013) also observed a decrease in NKA activity in the liver and kidney of *Oreochromis niloticus*, following acute exposure to Cd.

The mitochondria-rich cells (MRCs) in the gills of *P. lineatus* are mainly found along the filaments and less frequently occur in the lamellae (Camargo et al., 2009). However, after exposure to aluminum, these authors demonstrated that the MRCs were no longer found in the lamellae and those in the filaments showed a significant reduction in quantity, thus resulting in a decrease in gill NKA activity. Despite the specific effect of each xenobiotic on a particular species, this change in the ultrastructure of the gills of *P. lineatus* might not be exclusive to aluminum exposure; it could also occur as a result of exposure to Cd. According to Kine-Saffran et al. (1993), NKA inhibition by Cd could occur through competition for binding sites of K^+ or Mg^{2+} ions, which probably involve the interaction of Cd with the $-\text{SH}$ group. Metals target the sulphydryl radicals of proteins, which could be oxidized by Cd, resulting in the breakage of hydrogen bonds and alteration in the three-dimensional structure of proteins (Dawson, 1982).

Gill HATPase activity is linked to the absorption of Na^+ , which in turn is favored by the electrochemical gradient. As Cd causes a drop in the activity of basolateral NKA, lower quantities of Na^+ are removed from the MRC. Thus, the intracellular milieu becomes less electronegative, with a higher concentration of Na^+ , which impairs the entry of this ion through its channel in the apical membrane (Lin and Randall, 1991; Hawkins et al., 2004). The increase in gill HATPase activity in *P. lineatus* enhances cellular electronegativity and favors absorption of Na^+ . This is likely an adaptive response that enables the fish to maintain stable plasma levels of Na^+ when faced with Cd stress.

Ca^{2+} is essential for the growth of young individuals, regardless of its levels in water, and Ca^{2+} needs to be absorbed by the gills in order to maintain plasma homeostasis (Perry and Flik, 1988). Cd inhibits the active uptake of Ca^{2+} through the gill epithelia and interferes with the metabolism of this ion (Verbost et al., 1988; Pratap and Wendelaar Bonga, 2007). In fact, waterborne Cd inhibited the activity of the CATPase in the gills, but not in the kidney of the fish. Under normal circumstances, CATPase that is

involved in the absorption of ions in the gills is not a limiting factor for Ca^{2+} intake (Perry, 1997) but its activity is essential for the maintenance of plasma concentrations of Ca^{2+} . In the gills of *P. lineatus*, inhibition of CATPase was accompanied by a drop in the plasma Ca^{2+} . In the kidney, the activity of CATPase was slightly affected by Cd; however, the increase in the activity of this enzyme after a 96-h exposure to Cd could be due to increased tubular reabsorption, since hypocalcemia was already present during this period. These results suggest that the kidneys cannot compensate for the main functions of the gills in ionic absorption. The increased activity of CATPase was not sufficient to revert the low plasma concentration of Ca^{2+} , which is one of the toxic effects of Cd. In *O. niloticus*, acute exposure to Cd ($100 \mu\text{g L}^{-1}$) inhibited the activity of CATPase in both gill and kidney tissues (Atli and Canli, 2013).

A small amount of Cd must enter the cell to reach a minimal concentration threshold to inhibit the activity of CATPase (Verbost et al., 1988; Franklin et al., 2005). This effect is commonly observed at Cd concentrations above $3 \mu\text{g L}^{-1}$ (Giles, 1988; Reid and McDonald, 1988). For example, Hollis et al. (2000) observed no effects in *Oncorhynchus mykiss* gills when these animals were exposed to $2 \mu\text{g L}^{-1}$ of Cd for 30 days. In another study involving trout, the plasma levels of Ca^{2+} did not decrease after chronic exposure to $3.6 \mu\text{g L}^{-1}$ of Cd; this effect was only demonstrated in fish exposed to $6.4 \mu\text{g L}^{-1}$ (Giles, 1988). On the basis of the results of the present study, the threshold of inhibition of CATPase in *P. lineatus* was reached at Cd concentrations of 1 and $10 \mu\text{g L}^{-1}$. However, inhibition of CATPase only occurred after prolonged exposure to the lower concentration ($1 \mu\text{g L}^{-1}$).

Gill CA activity decreased in all the experimental conditions, whereas in the kidney, a reduction in enzyme activity was only observed after a 96-h exposure to Cd. The isoforms of CA and NKA have been co-localized in the proximal tubular cells of fish (Georgalis et al., 2006). However, these enzymes are not uniformly affected by Cd. The continuous flow of water through the gills and the large surface area obtained from the gill lamellae in contact with the external environment favor exposure of the cells to this contaminant. Despite the absence of long-term accumulation of Cd in the gills, metals enter the organism through its thin epithelium and this probably explains the high sensitivity of the proteins obtained from this tissue. Gill CA showed the highest sensitivity to Cd compared to the other gill ATPases in *P. lineatus*. A possible explanation for the decreased activity of this enzyme could be the high similarity of Cd to Zn, which results in the competition for the binding site of CA, where Zn is displaced and replaced with Cd, a metal with higher affinity for the enzyme.

The gills of freshwater teleost fish are not only responsible for gaseous exchange and elimination of nitrogen, but also serve as the main organ controlling the coupled activities of osmoregulation and acid–base balance (Evans et al., 2005). Inhibition of ATPases and CA activity in the gills compromises both ion uptake and pH balance, and this is performed mainly by elimination or conservation of H⁺ and HCO₃[−]. In freshwater teleosts, acid–base regulation is performed by metabolic compensation, which is mostly executed by the gills, whereas kidneys contribute up to 30% of whole body H⁺ excretion during systemic pH changes (Georgalis et al., 2006; Gilmour and Perry, 2009). Exposure of *P. lineatus* to 10 µg L^{−1} Cd for 96 h thus causes CA inhibition in the gills and kidneys, compromising the main compensatory homeostatic mechanisms involved in the control of hydrogen ion concentration.

A comparison of the results obtained from different tissues has shown that the gill ATPases are more sensitive to waterborne Cd than kidney ATPases. The surface of the gills directly faces the xenobiotics present in the environment, thus causing stress and triggering physiological changes that can initiate an integrated compensatory response to handle the toxic effects of Cd. However, in the case of Ca²⁺, regulation at the kidney level did not efficiently prevent hypocalcemia. On the other hand, the concentration of Na⁺, K⁺, and Cl[−] and plasma osmolality did not significantly change, even after all the effects of Cd on gill ATPases. In this case the kidney function was shown to be essential for the maintenance of osmo-ionic conditions.

This is the first study that has analyzed the combined effects of acute Cd exposure on the ATPases HATPase, NKATPase, and CATPase and CA in kidney and gills of a Neotropical fish. In summary, the sub-lethal effects of Cd on *P. lineatus* were more rapidly detected in the ATPases and CA than in the concentrations of plasma ions, except for Ca²⁺. Transport enzymes in the gills were sensitive and rapidly responded to the toxic effects of Cd. These characteristics suggest that ATPase activity can be used as a biomarker for Cd contamination. Among the ATPases, the activity of CATPase, along with the analysis of the plasma levels of Ca²⁺, can be regarded as important tools in classical evaluations of Cd toxicity in freshwater fish.

In conclusion, these results demonstrate that the Neotropical fish *P. lineatus*, a species of great ecological significance to South American aquatic wildlife, is sensitive to low concentrations of water-dissolved Cd. Acute exposure to Cd led to a decrease in the activity of CA and NKA in the gills and kidneys, as well as in the activity of the gill CATPase, which culminated with the loss of the fish's ability to regulate the levels of calcium in the blood.

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