1

Environmental Toxicology

Triple-Mixture of Zn, Mn, and Fe Increases Bioaccumulation and Causes Oxidative Stress in Freshwater Neotropical Fish

Luciana Fernandes de Oliveira,[a,b,]* Caroline Santos,[a] Wagner Ezequiel Risso,[a] and Claudia Bueno dos Reis Martinez[a]

[a]Laboratório de Ecofisiologia Animal, Departamento de Ciências Fisiológicas, Universidade Estadual de Londrina, Londrina, Paraná, Brasil
[b]Instituto Federal do Paraná, Campus Londrina, Londrina, Paraná, Brasil

Abstract: Metal bioaccumulation and oxidative stress biomarkers were determined in *Prochilodus lineatus* to understand the effects of short-term exposure to a triple-mixture of Zn, Mn, and Fe. Three independent tests were carried out, in which fish were exposed to 3 concentrations of Zn (0.18, 1.0, and 5.0 mg L^{-1}), Mn (0.1, 0.5, and 5.0 mg L^{-1}), and in the mix test to Fe (5.0 mg L^{-1}) and a mixture of Zn (1.0 mg L^{-1}) + Mn (0.5 mg L^{-1}), with and without Fe. After exposure for 96 h, tissues were removed for metal bioaccumulation analysis and oxidative stress biomarkers were determined in liver, along with DNA damage in blood cells. Our results revealed that Zn and Mn were bioaccumulated in fish tissues after exposure to 5.0 mg L^{-1}, whereas Fe only bioaccumulated in muscle and gills after mixture exposure. Results indicated that 1 metal interfered with the other's bioaccumulation. In *P. lineatus*, 5 mg L^{-1} of both Mn and Fe were toxic, because damage was observed (lipid peroxidation [LPO] in liver and DNA damage in blood cells), whereas Zn induced liver responses (metallothionein [MT] and reduced glutathione [GSH] increases) to prevent damage. In terms of bioaccumulation and alterations of oxidative stress biomarkers, we showed that Zn, Mn, and Fe triple-mixture enhances individual metal toxicity in Neotropical fish *P. lineatus*. *Environ Toxicol Chem* 2018;9999:1–8. © 2018 SETAC

Keywords: DNA damage; Metallothionein; Metal bioaccumulation; Metal mixture; Reduced glutathione; Lipid peroxidation

INTRODUCTION

Metals such as Zn, Mn, and Fe can occur simultaneously in the aquatic environment at high concentrations in consequence of the discharge of industrial or mining effluents (Jayaprakash et al. 2015; Oliveira et al. 2016). However, little is known about the metals' possible interactions and combined effects in aquatic animals. These metals are required for various functions in the organism but become toxic at levels exceeding a threshold above which the organism can no longer regulate them (Newman and Clements 2008).

Fish are widely used as biomonitors of aquatic environments and are sensitive to a variety of contaminants, including metals, which can accumulate in their tissues (Kennedy 2011). Zinc, Mn, and Fe play a part in regulating the production of antioxidants, such as metallothioneins (MTs), and can be components of the antioxidant metalloenzyme superoxide dismutase (SOD; Cu/Zn-SOD or Fe/Mn-SOD), acting as scavengers (Powell 2000; Aguirre and Culotta 2012). In spite of their antioxidant role, these ions can cause oxidative stress in fish (Gabriel et al. 2013; McRae et al.

2016) by increasing the production of reactive oxygen species (ROS) and impairing antioxidant defenses (Lushchak 2016).

The use of biomarkers associated with oxidative stress is therefore of interest for evaluating the toxicity of metals mixtures. Metals interact with other molecules and can affect the normal functioning of some cellular processes, such as cellular respiration, generating ROS. The cells mobilize antioxidant defenses that neutralize ROS, but in situations where ROS production exceeds antioxidant capacity, oxidative damage occurs in biomolecules, such as membrane lipids, proteins, and nucleic acids, characterizing oxidative stress. Antioxidant defenses involve some enzymes, such as SOD, catalase (CAT), and glutathione peroxidase (GPx), as well as other molecules, such as reduced glutathione (GSH) and MTs.

Our aim, in the present study, was to verify whether Zn, Mn, and Fe combinations at an environmentally relevant concentration increase bioaccumulation and toxicity in freshwater neotropical fish when considering oxidative stress biomarkers.

MATERIAL AND METHODS

Experimental design

Juveniles of *Prochilodus lineatus* ($n = 192$; total length: 11.7 ± 0.98 cm; weight: 13.8 ± 2.9 g) were subjected to acute

96-h semistatic tests (water renewal every 24 h). Three tests were conducted, exposing fish to Zn (Zn test), Mn (Mn test), or a Zn/Mn mixture, with and without Fe (mix test). In the Zn test, fish were exposed to the following nominal Zn concentrations: $0.18\,mg\,L^{-1}$, $1.0\,mg\,L^{-1}$, and $5.0\,mg\,L^{-1}$. In the Mn test, fish were exposed to Mn at nominal concentrations of $0.1\,mg\,L^{-1}$, $0.5\,mg\,L^{-1}$, and $5.0\,mg\,L^{-1}$. In the mix test, fish were exposed to intermediate nominal concentrations of Zn+Mn (Zn: $1.0\,mg\,L^{-1}$, Mn: $0.5\,mg\,L^{-1}$), Zn+Mn+Fe (Zn: $1.0\,mg\,L^{-1}$, Mn: $0.5\,mg\,L^{-1}$, Fe: $5\,mg\,L^{-1}$), and Fe alone ($5\,mg\,L^{-1}$). The tests were performed independently, each test with its own control (fish exposed to water only). Metals were added to the water as sulfate salts ($ZnSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, and $FeSO_4 \cdot 7H_2O$). Test concentrations of Zn, Mn, and Fe were chosen based on 2 criteria: 1) environmentally relevant concentrations observed in a stream near a coal mining area (Oliveira et al. 2016), especially the ones used in the mix test, and 2) the limit concentrations established in the Brazilian legislation (CONAMA 357/2005), either for class 1 and 2 (Zn: $0.18\,mg\,L^{-1}$, Mn: $0.1\,mg\,L^{-1}$), or for classes 3 and 4 (Zn: $5.0\,mg\,L^{-1}$, Mn: $0.5\,mg\,L^{-1}$, Fe: $5.0\,mg\,L^{-1}$).

To obtain samples with the necessary volume to analyze all the proposed biomarkers, 2 semistatic tests (24-h renewal period) were run for each test (Zn, Mn, and mix tests). In total, 6 tests were performed (1 control group for each test), split into 2 blocks. In block 1, we collected samples for almost all biomarkers whereas block 2 provided samples for bioaccumulation analyses and MT content.

Fish were obtained from the fish farm facility at the State University of Londrina (Londrina, Brazil) and immediately transported to the laboratory. Prior to testing, the fish were acclimated for 5 d under a 12:12-h light:dark photoperiod in 300 L tanks containing carbon filtered soft water (hardness: 40.6 ± 11.3 ppm $CaCO_3$; dissolved organic carbon: 2.6 ± 0.9 mg L^{-1}) at constant temperature ($\sim$22.5 °C), pH ($\sim$7–8), oxygenation level (>85% saturation). During acclimation, the fish were fed twice (2-d intervals) on commercial fish food (Guabi®, 36% protein). Feeding was suspended before beginning the experiments and during the tests.

Quantification of total and dissolved metals at mean exposure

Every day, before and after water renewal, nonfiltered and filtered (Millipore Millex HV/PVDF 0.45-µm mesh filter) water samples were collected for analysis of total and dissolved Zn, Mn, and Fe. These samples were immediately fixed with nitric acid (1%) and stored in decontaminated (10% nitric acid for 24 h) polypropylene plastic tubes. Zinc was quantified by flame, and Mn and Fe were quantified by electrothermic ionization in a graphite furnace in an atomic absorption spectrophotometer (AAnalyst 700, Perkin Elmer, USA) against standard reference solutions (Specsol, Brazil).

Fish sampling

Fish tissues were sampled according to the protocol approved by the Animal Ethics Committee of the State University of Londrina (Process 20032.2013.65). Prior to sampling, the fish were anesthetized with benzocaine ($0.1\,g\,L^{-1}$) and blood samples collected from the caudal vein. One aliquot (10 µL) from each animal was preserved in fetal bovine serum (Gibco®) for subsequent use in the comet assay and another aliquot ($\sim$100 µL) was separated for bioaccumulation analyses. The fish were then killed by medullar section and samples were taken from the gills, liver, bile, kidney, head kidney, axial muscle, and brain. The sample tissues were washed (except blood) with physiological solution (Sigma PBS), immediately frozen, and stored at –72 °C.

Metal bioaccumulation

Metal concentrations were determined by flame (Zn and Fe) and electrothermic ionization in a graphite furnace (Mn) in an atomic absorption spectrophotometer (AAnalyst 700, Perkin Elmer, USA) against standard reference solutions (Specsol, Brazil) after acid digestion. Tissues (gills, liver, bile, kidney, head kidney, axial muscle, and brain) and blood cells ($n = 8$ per tissue) were completely dried at 60 °C and digested in suprapure 5 N nitric acid for 48 h at 60 °C, according to Alves and Wood (2006). Results are expressed in milligram per kilogram dry weight.

Biomarkers

Liver tissues ($n = 8$) were homogenized in potassium phosphate buffer (0.1 M, pH 7), centrifuged (14 000 g; 20 min, 4 °C), and the supernatants used to determine antioxidant and oxidative stress endpoints. The biomarker methods used in the present study were as defined previously (Oliveira et al. 2016, Vieira et al. 2016) with some minor modifications. Lipid peroxidation (LPO) was determined by the thiobarbituric acid substances fluorescence assay (ex/em: 535/590nm), according to Camejo et al. (1998), after incubating the supernatants for 1 h with thiobarbituric acid (1.3%) at 60 °C. To determine protein carbonylation levels, we used the method described by Levine et al. (1994), based on the reaction with 2,4-dinitrophenylhydrazine (10 mM, prepared in HCl 2 M) and consequent formation of dinitrophenyl hydrazones quantified at 360 nm. Nonprotein thiol levels were determined according to the method in Beutler et al. (1963), measured at 412 nm. Superoxide dismutase (Cu/Zn-SOD) activity was determined in microplates by the method that involves inhibiting the reduction rate of cytochrome *c* by the superoxide radical, at 550 nm and 25 °C, according to McCord and Fridovich (1969). Glutathione *S*-transferase (GST) activity was determined using the method described by Keen et al. (1976), in which GST conjugates reduced glutathione with 1-chloro-2,4-dinitrobenzene, monitored for 1 min in a spectrophotometer at 340 nm. Levels of MT-like proteins were determined using the method described by Viarengo et al. (1997) with modifications. Partially purified metalloprotein fractions were obtained from the supernatant after ethanol/acid chloroform fractionation, and sulfhydryl groups (–SH) were quantified at 412 nm. All biochemical biomarkers are expressed in relation to the total protein content, determined at 595 nm by the method in Bradford (1976).

Erythrocytes DNA damage was quantified by the length of DNA migration (comet tail length), visually determined in 100 randomly selected nonoverlapping cells from each fish ($n = 8$ for each experimental group), as previously described in Vieira et al. (2016). Deoxyribonucleic acid damage was expressed as a score calculated by the formula $(0 \times A) + (1 \times B) + (2 \times C) + (3 \times D)$, where A, B, C, and D correspond to the number of cells in each comet class: class $0 =$ absence of comet tail; class $1 =$ comet tail shorter than the diameter of the nucleoid; class $2 =$ comet tail longer than the diameter of the nucleoid; class $3 =$ comet tail more than twice the diameter of the nucleoid.

Statistical analysis

Data were first tested for normality and homogeneity of variance to check the statistical requirements. In some cases, data were log transformed to meet parametric demands. Parametric analysis of variance (ANOVA) was run for the majority of data, except for MT where nonparametric analysis (Kruskal-Wallis) was applied. Multiple comparison tests (Student–Newman–Keuls or Dunn's) were performed where recommended. Bioaccumulation and biomarker results for each tissue were compared separately for each test (Zn, Mn, and Mix tests). Pearson correlation coefficients were calculated for metal accumulation and biomarkers in the liver. Differences were considered significant for $p < 0.05$.

RESULTS

Mortality

Six independent semistatic tests were successfully performed, despite some mortality after 96 h. In the first block of tests, the highest Zn concentration (5.0 mg L^{-1}) caused the death of 3 fish (total $n = 8$). In the mix test control, only 1 fish died; in the Fe group, 2 fish died. In the second block, mortality occurred only in the mix test, with 2 fish dying after Zn+Mn exposure and 1 dying after Zn+Mn+Fe exposure. Mn concentrations were confirmed as sublethal to *P. lineatus*.

Metals in water

In the water, metal concentrations were generally slightly lower than nominal, but the intended gradient was produced (Table 1).

Metals bioaccumulation

Metal concentrations in control fish (background concentrations) varied from 1 tissue to another (Figure 1), with Zn appearing at increasing levels as follows: muscle < blood cells < brain = gills < bile < liver < kidney < head kidney. For Mn, levels found in tissues were as follows: bile < blood cells < head kidney < brain < muscle < gills < kidney < liver; for Fe, levels were as follows: muscle < brain < bile < gills < kidney = head kidney < blood cells < liver.

In the Zn test, after exposure for 96 h, Zn levels increased in gills ($F = 10.6$), bile ($F = 3.9$), and kidney ($F = 6.3$) after the 5.0 mg L^{-1} Zn treatment compared with fish in other groups,

TABLE 1: Total (T) and dissolved (D) metal concentrations (mean ± standard deviation, $n = 4$) for all the treatments at the beginning (I) and end (F) over a 24 h-water renewal period[a]

		Zn test				Mn test					Mix test			
		Ctr	0.18	1.0	5.0	Ctr	0.1	0.5	5.0	Ctr	Fe	Zn+Mn	Zn+Mn+Fe
Zn (I)	T	<DL[b]	0.11±0.01	0.79±0.04	4.31±0.23	<DL	<DL	<DL	<DL	<0.05	<0.05	0.89±0.04	0.89±0.03
	D	<DL	0.11±0.02	0.77±0.02	4.22±0.17	<DL	<DL	<DL	<DL	<0.05	<0.05	0.89±0.05	0.75±0.07
Zn (F)	T	<DL	0.11±0.02	0.80±0.05	4.45±0.31	<DL	<DL	<DL	<DL	<0.05	<0.05	0.88±0.03	0.86±0.09
	D	<DL	0.11±0.02	0.77±0.04	4.26±0.21	<DL	<DL	<DL	<DL	<0.05	<0.05	0.86±0.03	0.71±0.06
Mn (I)	T	<0.1[c]	<0.1	<0.1	<0.1	0.15±0.05	0.38±0.03	4.46±0.60	<0.01	<0.02	0.38±0.08	0.34±0.07
	D	<0.1	<0.1	<0.1	<0.1	0.12±0.03	0.37±0.02	4.26±0.65	<0.01	<0.02	0.34±0.06	0.32±0.06
Mn (F)	T	<0.1	<0.1	<0.1	<0.1	0.08±0.02	0.32±0.07	4.51±0.34	<0.01	<0.02	0.29±0.12	0.32±0.04
	D	<0.1	<0.1	<0.1	<0.1	0.08±0.03	0.27±0.11	4.24±0.55	<0.01	<0.02	0.25±0.09	0.33±0.04
Fe (I)	T	<0.1	<0.1	<0.1	<0.1	<0.1[c]	<0.1	<0.1	<0.1	4.59±0.55	<0.1	4.86±0.60
	D	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	1.68±0.66	<0.1	0.82±0.20
Fe (F)	T	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	2.34±2.34	<0.1	3.49±1.15
	D	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.25±0.31	<0.1	0.74±0.63

[a]Metal concentrations are given in mg L^{-1}.
[b]DL = Detection limit for Mn: 1.5 μg L^{-1}; Fe: 5 μg L^{-1} and Zn: 1.5 μg L^{-1}.
[c]When values were low, the Less-than symbol (<) was used to simplify the reading of the table and avoid small numbers.
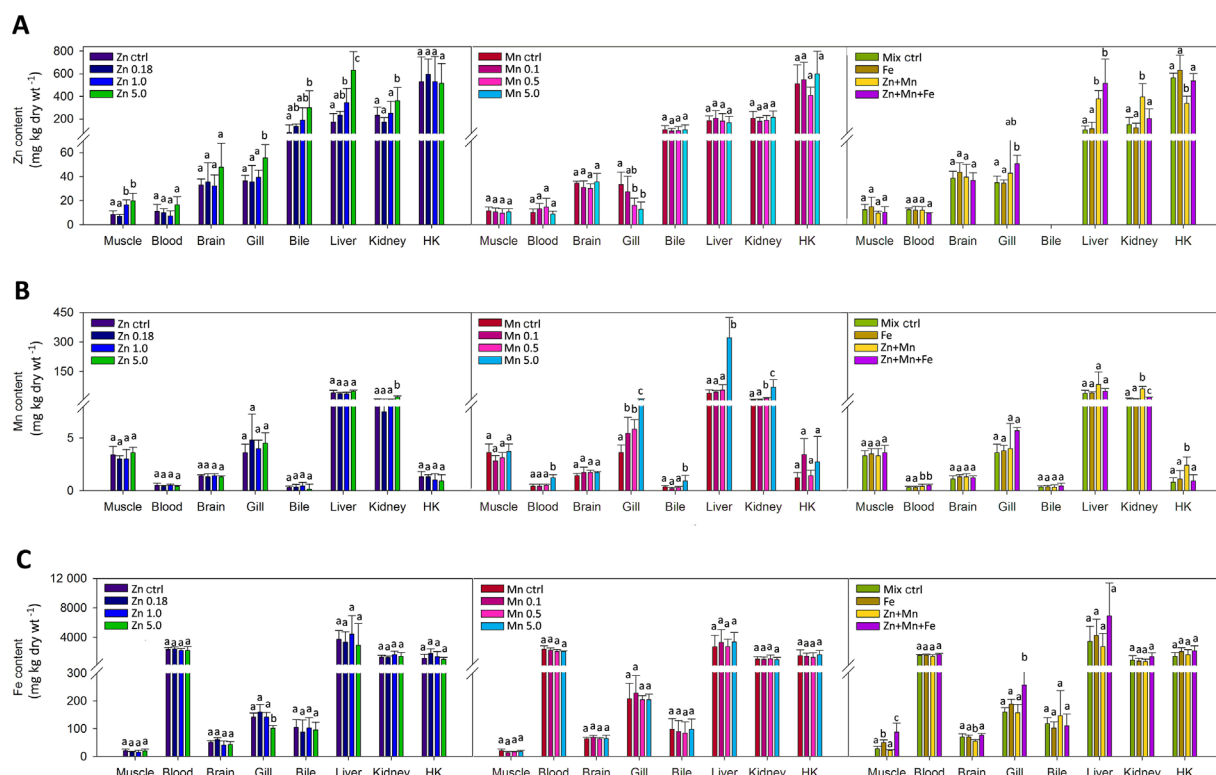
FIGURE 1: Concentrations of Zn (A), Mn (B), and Fe (C) in *Prochilodus lineatus* tissues (muscle, blood cells, brain, gill, bile, liver, kidney, and head kidney) after the Zn, Mn, and mix tests. Results are given as mean ± standard deviation, $n = 8$. For each tissue, different letters indicate significant differences among groups from the same test ($p < 0.05$). In the mix test, the amount of bile samples was insufficient to repeat the analysis. Ctrl = control; HK = head kidney.

but in muscle ($F = 15.3$) and liver ($F = 10.7$), increases were observed in both the 1.0 and 5.0 mg L^{-1} Zn treatments compared with the control. The lowest Zn concentration did not alter Zn levels in any tissue compared with the control. Manganese increased in the kidney ($F = 19.1$) after the 5.0 mg L^{-1} Zn treatment, and Fe levels dropped in gills ($F = 13.1$) after the 5.0 mg L^{-1} Zn treatment.

In the Mn test, fish blood ($F = 32.2$), gills ($F = 78.4$), bile ($F = 14.1$), liver ($F = 57.1$), and kidney ($F = 44.2$) from the 5.0 mg L^{-1} Mn group all accumulated Mn after 96-h exposure. Manganese levels in the gills increased for all 3 Mn concentrations tested compared with the control group ($F = 78.4$). A decrease in Zn concentrations was detected in the gills of fish from 0.5 and 5.0 mg L^{-1} Mn groups ($F = 5.3$).

For the Zn+Mn+Fe mixture, there was an increase in Fe levels in muscle ($F = 30.6$) and gill ($F = 8.5$) tissue, an increase in Zn in the gills ($F = 5.4$) and liver ($F = 23.0$), and an increase in Mn in blood cells ($F = 9.1$) and kidney tissue ($F = 52.9$). When Fe was not present in the mixture (Zn+Mn group), Fe levels dropped in the brain tissue ($F = 8.0$), but in the gills ($F = 5.4$) and liver ($F = 23.0$), Zn was still elevated, and Mn levels remained higher in blood ($F = 9.1$), kidney ($F = 52.9$), and head kidney ($F = 8.3$). A drop in Zn was observed in head kidney ($F = 11.8$) after exposure to Zn+Mn (Figure 2).

Biomarkers

Biomarkers were always compared among fish from the same test (Zn, Mn, or Mix tests) and not between different tests

because they were run independently, each having its own control group. Zinc and Mn induced significant changes in biomarkers only at the highest concentrations. Liver from the 5.0 mg L^{-1} Zn group showed higher nonprotein thiol ($p < 0.001$, $F = 9.42$) and MT ($p < 0.001$, $H = 24.63$) levels than the control and 0.18 mg L^{-1} Zn groups (Figure 3), together with a decrease in SOD activity ($p = 0.008$, $F = 4.90$; Figure 4). The 5.0 mg L^{-1} Mn group showed signs of oxidative stress, with higher LPO ($p = 0.036$, $H = 8.54$; Figure 5) and increased DNA damage ($p = 0.008$, $F = 5.11$; Figure 6). Although no differences were observed in biomarkers from fish exposed to intermediate concentrations of Zn and Mn, fish from the Zn+Mn+Fe group showed some significant alterations, including increases in liver nonprotein thiol ($p = 0.005$, $F = 5.45$) and LPO ($p = 0.043$, $F = 3.19$) compared with the control group (Figures 3 and 4) and reduced GST activity (Figure 4) compared with the Zn+Mn group ($p = 0.021$, $F = 3.89$). Finally, exposure to Fe alone induced DNA damage, with a damage score significantly higher than that of all other groups ($p = 0.004$, $F = 6.19$; Figure 6).

The Pearson's correlation matrix (Table 2) indicates that liver Zn levels showed a significant positive correlation with nonprotein thiol and MT, whereas liver Fe accumulation was correlated with nonprotein thiol and LPO levels.

DISCUSSION

In the present study, we showed that although intermediate concentrations of Zn (1.0 mg L^{-1}) and Mn (0.5 mg L^{-1})
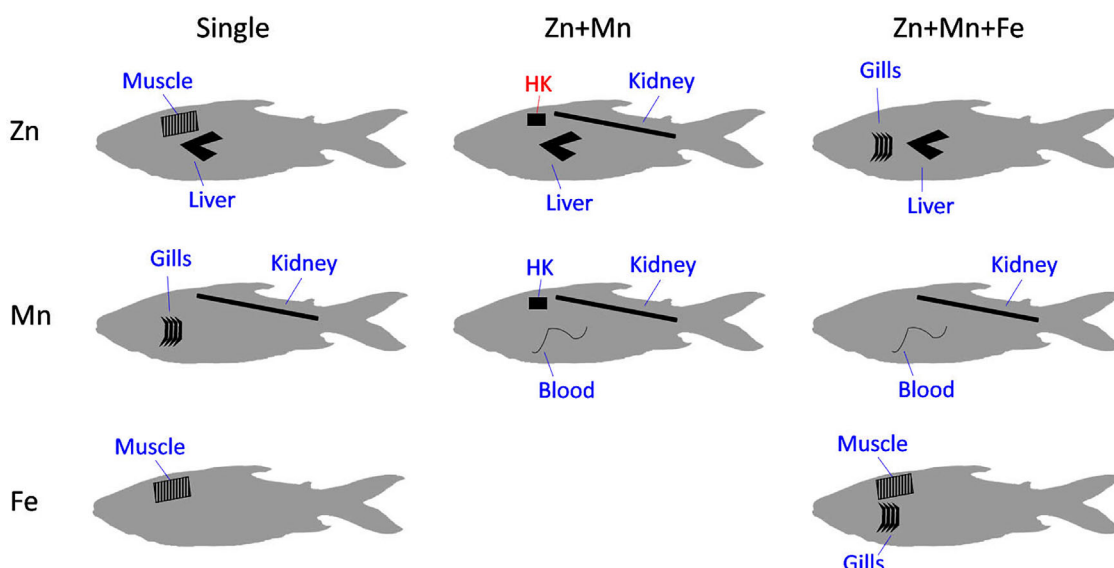
FIGURE 2: Organs in which metal bioaccumulation showed significant increases (blue) or decreases (red) after 96-h exposure to 1.0 mg L^{-1} Zn, 0.5 mg L^{-1} Mn, and 5.0 mg L^{-1} Fe (single metal exposures at the same concentrations as in the mixtures) compared with exposure to mixtures (Zn+Mn and Zn+Mn+Fe). HK = head kidney.

individually did not induce any responses in or otherwise affect the liver and blood cells of *P. lineatus*, when mixed with Fe (5.0 mg L^{-1}) in environmentally relevant concentrations Zn and Mn caused oxidative stress. All metals individually tested caused hepatic and/or blood alterations after exposure to the concentration of 5.0 mg L^{-1}, but the same biomarker alterations were not necessarily observed. Manganese and Fe seem to be toxic because they caused oxidative damage (liver LPO) and DNA

damage in blood cells. In contrast, Zn at the same level stimulates liver responses to regulate and prevent metal toxicity (i.e., MT and GSH increase). On the other hand, when fish were exposed to the triple-mixture of Zn, Mn, and Fe, nonprotein thiol content also increased, but not enough to prevent LPO.

Although no biomarker alterations occurred, Zn accumulated in liver even after single exposure to 1.0 mg L^{-1} Zn (Figures 1 and 2). Liver as a target of Zn accumulation should be associated with
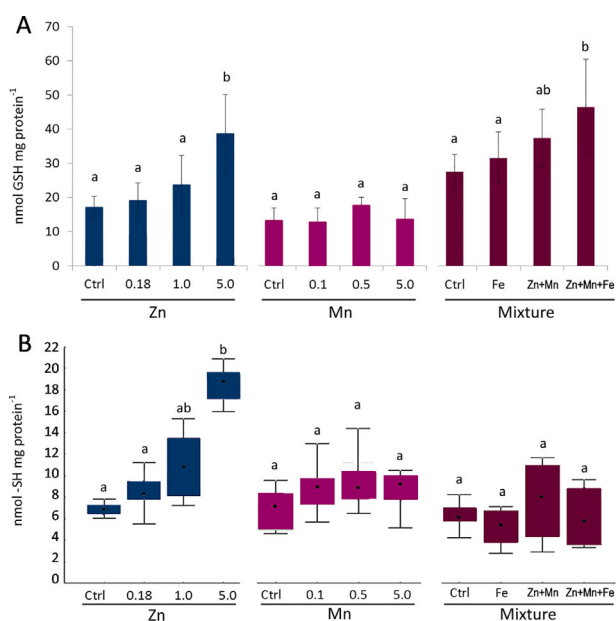
**FIGURE 3:** Nonprotein thiols (**A**) and metallothionein-like proteins (**B**; black dot: median; bars: 25–75%; lines: nonoutlier range; small dot: outlier) in liver from *Prochilodus lineatus* subjected to the Zn, Mn, and mix tests. Results given as mean ± standard deviation, $n = 8$. Different letters indicate significant differences among groups from the same test ($p < 0.05$). GSH = reduced glutathione; Ctrl = control; SH = sulfhydryl group.
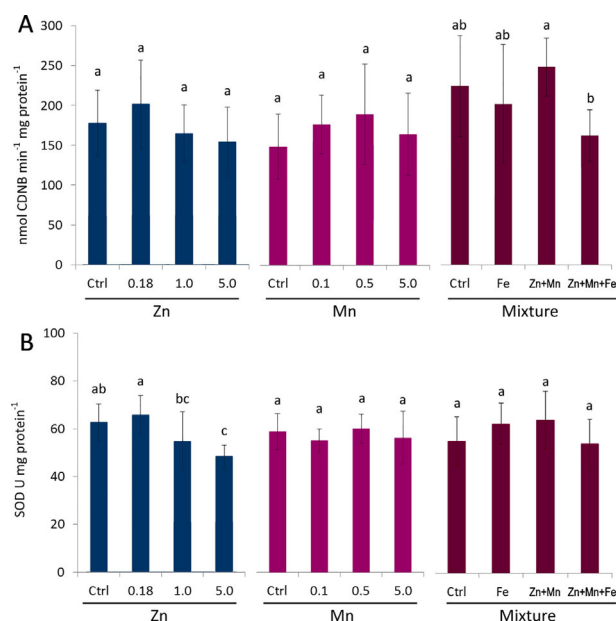

FIGURE 4: Activity of glutathione *S*-transferase (**A**) and superoxide dismutase (**B**) in the liver of *Prochilodus lineatus* subjected to the Zn, Mn, and mix tests. Results are given as mean ± standard deviation, $n = 8$. Different letters indicate significant differences among groups from the same test ($p < 0.05$). CDNB = 1-Chloro-2,4-dinitrobenzene; Ctrl = control; SOD = superoxide dismutase.
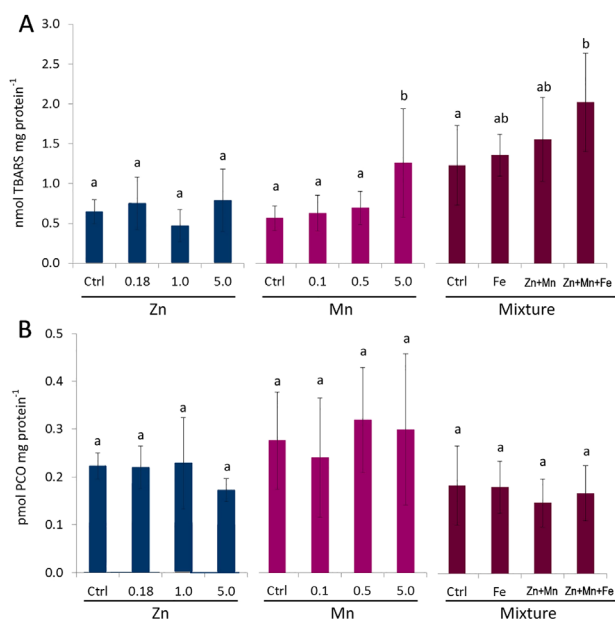
FIGURE 5: Lipid peroxidation (**A**) and protein carbonylation (**B**) in the liver of *Prochilodus lineatus* subjected to the Zn, Mn, and mix tests. Results are given as mean ± standard deviation, n = 8. Different letters indicate significant differences among groups from the same test ($p < 0.05$). TBARS = thiobarbituric acid substances; Ctrl = control; PCO = carbonylated proteins.
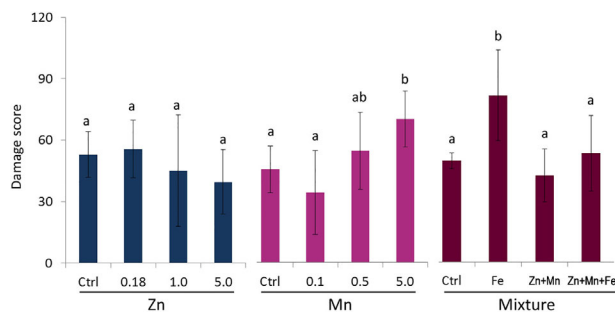
FIGURE 6: The DNA damage score in the erythrocytes of *Prochilodus lineatus* subjected to Zn, Mn, and mix tests. Results are given as mean ± standard deviation, n = 8. Different letters indicate significant differences among groups from the same test ($p < 0.05$).

GSH and MT content, because these are the primary cytosol ligands of Zn in cells (Colvin et al. 2008) due to the high affinity of this metal to cysteine. Metals are stored and/or detoxified in fish by molecules such as GSH and MT. Increases in these molecules

may cause the metals to accumulate and be retained in an inert form inside lysosomes and granules (Wood 2012). In *P. lineatus*, hepatic GSH and MT increases were positively correlated (Table 2) with Zn bioaccumulation in the liver after exposure to $5.0\,\text{mg}\,\text{L}^{-1}$, but not with Mn bioaccumulation.

There is a gap in our knowledge of metals excretion routes in fish (Wood 2012). Most Zn is thought to be excreted by the gills, but the intestine may also play an important role, as in mammals, whereas the kidney accounts for less than 1% of total Zn losses (Hardy et al. 1987). Metals can also be excreted in the bile in fish (Hauser-Davis et al. 2012), and our results lend weight to the idea that the bile should be considered a potential target for environmental metal contamination, because Zn and Mn were found to have bioaccumulated in the bile after exposure to the highest metal concentrations.

The levels and distribution of Zn, Mn, and Fe found in *P. lineatus* provide valuable data for future studies, mainly serving as basal values in field approaches. In general, Fe levels were the highest, followed by Zn, and then Mn. This seems to be a pattern in liver from freshwater fish (Supplemental Data). Iron plays an essential role as an oxygen carrier in vertebrates, forming the heme group of hemoglobin and acting as an electron acceptor or donor in cytochrome *c* oxidases in the respiratory chain (Bury et al. 2012). Elevated Fe levels were found in blood cells. In addition to the blood cells, liver, kidney, and head kidney, as well-perfused organs, exhibited the highest Fe background concentrations. In contrast, blood levels of Zn and Mn were lower, whereas liver and kidney tissues showed higher levels of Zn and Mn. These metals play a part in determining antioxidant status (Coassin et al. 1992; Powell 2000), and levels in fish liver may in part be related to the importance of this tissue in preserving the oxidative balance, because detoxification processes are usually triggered in the liver once multiple oxidative reactions and high free radical generation occur (Martínez-Álvarez et al. 2005). Although the gills were not among the organs with the highest background concentrations of the analyzed metals, an increase in gill metal bioaccumulation was observed after 96 h, even in fish exposed to intermediate concentrations. Because the gills are in intimate contact with the environment in situations of waterborne contamination, they are often shown to be bioaccumulation target tissues in fish (Wood 2012).

Our results show that although Zn was bioaccumulated in different tissues, the toxicity threshold for *P. lineatus* was not exceeded in the liver. Fish exposed in the $5.0\,\text{mg}\,\text{L}^{-1}$ Zn group did not exhibit any evidence of oxidative stress; on the contrary,

TABLE 2: Pearson's correlation coefficients for metal bioaccumulation in the liver and hepatic biomarkers

	Mn	Fe	NPSH	MT	LPO	PC	GST	SOD
Zn	−0.176	0.250	0.620*	0.713*	0.201	−0.470	−0.292	−0.521
	$p = 0.584$	$p = 0.433$	$p = 0.032$	$p = 0.009$	$p = 0.532$	$p = 0.123$	$p = 0.358$	$p = 0.082$
Mn		−0.149	−0.298	−0.020	0.195	0.461	−0.166	−0.114
		$p = 0.644$	$p = 0.347$	$p = 0.951$	$p = 0.545$	$p = 0.131$	$p = 0.606$	$p = 0.723$
Fe			0.680*	−0.265	0.701*	−0.451	−0.089	−0.111
			$p = 0.015$	$p = 0.406$	$p = 0.011$	$p = 0.141$	$p = 0.782$	$p = 0.731$

*Indicates significant differences ($p \leq 0.05$).
NPSH = nonprotein thiol; MT = metallothionein; LPO = lipid peroxidation; PC = protein carbonylation; GST = reduced glutathione; SOD = superoxide dismutase.

the increase in nonprotein thiol and MT-like protein levels in fish exposed to the highest Zn concentration showed that they were able to respond to the presence of Zn. Trace metals such as Zn and Cu are associated with MT regulation and can induce MT synthesis (Olsson 1993), making the liver cells more resistant to metal toxicity because this tripeptide, as well as GSH, bind to free Zn ions and remove them from the cytosol. Thus, in consequence of increases in nonprotein thiol and MT-like proteins, no other alterations were observed in *P. lineatus* biomarkers in the liver, nor in LPO or protein carbonylation concentrations. However, Cu/Zn-SOD activity decreased in the liver of these same fish, indicating that the antioxidant role of Zn is more related to nonenzymatic defenses than to antioxidant enzymes. A drop in SOD activity could be ascribed to Zn scavenging by GSH and MT, in turn reducing the availability of free metal ions to the enzyme.

Zn toxicity varies from 1 fish species to another and, based on oxidative stress biomarkers, there are no typical responses or effects. Several studies have shown that Zn causes oxidative damage to lipids and proteins (Loro et al. 2012; McRae et al. 2016) and can impair antioxidant defenses (Qu et al. 2014). Lipid peroxidation increased in the liver of *Galaxias maculatus* exposed to only $1.0 \, mg \, L^{-1}$ Zn for 96 h (McRae et al. 2016) and reductions in enzymatic and nonenzymatic antioxidants were observed in the liver of *Carassius auratus* exposed to 0.1 and $1.0 \, mg \, L^{-1}$ Zn for acute and subchronic periods (Qu et al. 2014). Variations in metal toxicity can be caused by differences in species sensitivity and experimental media properties, such as water hardness, temperature, and pH.

The idea, based on the liver biomarkers, that the fish were able to respond to the presence of Zn runs counter to the evidence: mortality of 40% of the fish in the $5.0 \, mg \, L^{-1}$ Zn group. These conflicting results may indicate that liver and blood were not the tissues most affected by Zn exposure. It is known that Ca homeostasis is affected by Zn, so other biomarker analyses targeted to Ca signaling and transport pathways should be more appropriate.

In contrast to the results obtained for Zn exposure, LPO and DNA damage were observed in the liver and blood cells of individuals exposed to Mn ($5.0 \, mg \, L^{-1}$). Manganese accumulates primarily in cell mitochondria and because of its high pro-oxidant capacity, it affects oxidative respiration, increasing ROS production and leading to mitochondrial dysfunction (Farina et al. 2013; Dolci et al. 2013). In the liver tissue of *P. lineatus*, neither nonprotein thiol and MT-like protein concentrations nor Cu/Zn-SOD activity responded to Mn exposure, leading to oxidative damage as indicated by the increase in hepatic LPO. In goldfish (*C. auratus*) exposed for 96 h to 0.1 mM ($\sim 5.5 \, mg \, L^{-1}$) Mn, LPO also increased in the liver, hepatic CAT dropped, and there was no impact on SOD activity. Only GPx activity increased under these conditions (Vieira 2012). The study from Vieira et al. (2012) corroborates our idea that concentrations of approximately $5 \, mg \, L^{-1}$ Mn cause oxidative damage in freshwater fish after exposure for 96 h, even though the Mn concentrations may be not sufficient to induce significant alterations in antioxidant mechanisms.

Like Mn, Fe is a pro-oxidant that easily changes its oxidation state, alternating between 2+ to 3+, endowing this metal with important functions in living organisms (Bury et al. 2012). Because of this characteristic, Fe is involved in the Fenton reaction that produces the hydroxyl radical ($HO^{\bullet}$), the most ROS radical, initiating a cycle of cellular damage, including DNA damage. In the present study, the erythrocytes of fish exposed to Fe suffered more DNA damage than control or mixture groups, in spite of the fact that the dissolved Fe concentration in the water was lower ($1.68 \pm 0.66 \, mg \, L^{-1}$) than the total concentration ($4.59 \pm 0.55 \, mg \, L^{-1}$). This effect was not found in fish from the Zn+Mn+Fe group, in which the level of dissolved Fe was even lower ($0.82 \pm 0.20 \, mg \, L^{-1}$) and may not have been sufficient to cause DNA damage.

Contaminant interactions could occur at a chemical level or affect toxicokinetic or toxicodynamic parameters (Sexton and Hattis 2007). Our results indicate that changes in toxicodynamics probably occurred as a consequence of alterations of metal toxicokinetics and biomarkers once internal Zn levels in the target organ (liver) overshot a threshold (Ashauer and Escher 2010). In fish exposed to the mixture Zn+Mn+Fe, Zn levels in the liver ($492.9 \pm 202.2 \, mg \, kg^{-1}$) were sufficient to boost nonprotein thiol, suggesting that this Zn level exceeds the threshold for this biomarker. On the other hand, the same Zn level did not significantly boost hepatic concentration of MT-like proteins. The initial Zn is thought to bind to nonprotein thiol (GSH, for instance), and when that becomes scarce, the Zn starts to bind to MTs (Hogstrand and Wood 1996). In the present study, however, only nonprotein thiol increased and liver integrity was not maintained, as indicated by increased LPO. Great care is required in interpreting and predicting the effects of metal mixtures, because they depend on a number of issues, such as the endpoint considered, exposure time, toxicant concentrations (Ashauer and Escher 2010), and especially the toxicodynamics.

CONCLUSIONS

Essential metals become toxic when they overshoot essential requirements and regulation thresholds, which vary from 1 fish species to another and from 1 metal to another. In *P. lineatus*, levels of $5 \, mg \, L^{-1}$ of Mn and Fe seem to be toxic, because at this concentration metals caused oxidative damage (liver LPO) and DNA damage in blood cells. In contrast, Zn at the same level stimulates liver responses to regulate and prevent metal toxicity (i.e., MT and GSH increase). Metal toxicity can affect different functional mechanisms (not only the induction of oxidative stress) and also target organs or tissues (especially the gills) other than liver and blood cells. Complementary approaches including other biomarkers are important for elucidating the toxicity of these metals. In regard to oxidative stress biomarkers, we have shown that when combined, Zn, Mn, and Fe enhance toxicity, because fish exposed to a mixture of these metals exhibited alterations that were not observed after exposure to only 1 of these metals at the same concentration.

REFERENCES

Aguirre JD, Culotta VC. 2012. Battles with iron: Manganese in oxidative stress protection. *J Biol Chem* 287:13541–13548.

Alves L, Wood CM. 2006. The chronic effects of dietary lead in freshwater juvenile rainbow trout (*Oncorhynchus mykiss*) fed elevated calcium diets. *Aquatic Toxicol* 78:217–232.

Ashauer R, Escher BI. 2010. Advantages of toxicokinetic and toxicodynamic modeling in aquatic ecotoxicology and risk assessment. *J Environ Monit* 12:2056–2061.

Beutler E, Duron O, Kelly BM. 1963. Improved method for the determination of blood glutathione. *J Lab Clin Med* 61:882–888.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.

Brasil. 2005. Resolução CONAMA n°357, de 17 de março de 2005. Classificação de águas, doces, salobras e salinas do Território Nacional.

Bury NR, Boyle D, Cooper CA. 2012. Iron. In Wood CM, Farrell AP, Brauner CJ, eds, *Fish Physiology: Homeostasis and Toxicology of Essential Metals*. Academic Press, Cambridge, MA, USA, pp 202–253.

Camejo G, Wallin B, Enojärvi M. 1998. Analyses of oxidation and antioxidants using microtiter plates. In Amstrong D, ed, *Free Radical and Antioxidants Protocols*. Humana Press, New York, NY, USA, pp 377–387.

Coassin M, Ursini F, Bindoli A. 1992. Antioxidant effect of manganese. *Archi Biochem Biophys* 299:330–333.

Colvin RA, Bush AI, Volitakis I, Fontaine CP, Thomas D, Kikuchi K, Holmes WR. 2008. Insights into Zn^{2+} homeostasis in neurons from experimental and modeling studies. *Am J Physiol Cell Physiol* 294:726–742.

Dolci GS, Dias VT, Roversi K, Roversi Kr, Pase CS, Segat HJ, Teixeira AM, Benvegnú DM, Trevizol F, Barcelos RCS, Riffel APK, Nunes MAG, Dressler VL, Flores EMM, Baldisserotto B, Bürger ME. 2013. Moderate hypoxia is able to minimize the manganese-induced toxicity in tissues of silver catfish (*Rhamdia quelen*). *Ecotoxicol Environ Safety* 91:103–109.

Farina M, Avila DS, Rocha JBT, Aschner M. 2013. Metals, oxidative stress and neurodegeneration: A focus on iron, manganese and mercury. *Neurochem Int* 62:575–594.

Gabriel D, Riffel APK, Finamor IA, Saccol EMH, Ourique GM, Goulart LO, Kochhann D, Cunha MA, Garcia LO, Pavanato MA, Val AL, Baldisseroto B, Llesuy SF. 2013. Effects of subchronic manganese chloride exposure on tambaqui (*Colossoma macropomum*) tissues: Oxidative stress and antioxidant defenses. *Arch Environ Contam Toxicol* 64:659–667.

Hardy RW, Sullivan CV, Koziol AM. 1987. Absorption, body distribution and excretion of dietary zinc by rainbow trout (*Salmo gairdneri*). *Fish Physiol Biochem* 3:133–143.

Hauser-Davis RA, Bastos FF, Oliveira TF, Ziolli RL, Campos RC. 2012. Fish bile as a biomarker for metal exposure. *Mar Poll Bull* 64:1598–1595.

Hogstrand C, Wood CM. 1996. The physiology and toxicology of zinc in fish. In Taylor EW, ed, *Toxicology of Environmental Pollution: Physiological, Molecular, and Cellular Approaches*. Cambridge University Press, Cambridge, UK, pp 61–84.

Jayaprakash M, Kumar RS, Giridharan L, Sujitha SB, Sarkar SK, Jonathan MP. 2015. Bioaccumulation of metals in fish species from water and sediments in macrotidal Ennore creek, Chennai, SE coast of India: A metropolitan city effect. *Ecotoxicol Environ Safety* 120:143–255.

Keen JH, Habig WH, Jakoby WB. 1976. Mechanism for several activities of the glutathione S-transferase. *J Biol Chem* 20:6183–6188.

Kennedy CJ. 2011. The toxicology of metals in fishes. In Farrell AP, ed, *Encyclopedia of Fish Physiology*. Academic Press, Cambridge, MA, USA, pp 2061–2068.

Levine RL, Williams JA, Stadtman EP, Shacter E. 1994. Carbonyl assays for determination of oxidatively modified protein. *Met Enzymol* 233:346–357.

Loro VL, Jorge MB, Silva KR, Wood CM. 2012. Oxidative stress parameters and antioxidante response to sublethal waterborne zinc in a euryhaline teleost *Fundulus heteroclitus*: Protective effects of salinity. *Aquat Toxicol* 110–11:187–193.

Lushchak VI. 2016. Contaminant-induced oxidative stress in fish: A mechanistic approach. *Fish Physiol Biochem* 42:711–747.

Martínez-Álvarez RM, Morales AE, Sanz A. 2005. Antioxidant defenses in fish: Biotic and abiotic factors. *Rev Fish Biol Fisher* 15:75–88.

McCord JM, Fridovich I. 1969. Superoxide dismutase. An enzymatic function for erythrocuprein (hemocuprein). *J Biol Chem* 244:6049–6055.

McRae NK, Gaw S, Glover CN. 2016. Mechanisms of zinc toxicity in the galaxiid fish, *Galaxias maculatus*. *Comp Biochem Physiol C* 179:184–190.

Newman MC, Clements WH. 2008. Ecotoxicology: A comprehensive treatment. CRC Press, Boca Raton, FL, USA, p 852.

Oliveira LF, Cabral MT, Vieira CED, Antoniazzi MH, Risso WE, Martinez CBR. 2016. Metals bioaccumulation and biomarkers responses in the Neotropical freshwater clam *Anodontites trapesialis*: Implications for monitoring coal mining areas. *Sci Total Environ* 571:983–991.

Olsson PE. 1993. Metallothionein gene expression and regulation in fish. In Hochachka PW, Mommsen TP, eds, *Molecular Biology Frontiers*, Vol 2. Elsevier, Atlanta, GA, USA, pp 259–278.

Powell SR. 2000. The antioxidant properties of zinc. *J Nutr* 130: 1447S–14454S.

Qu R, Feng M, Wang X, Qin L, Wang C, Wang Z, Wang L. 2014. Metal accumulation and oxidative stress biomarkers in liver of freshwater fish *Carassius auratus* following in vivo exposure to waterborne zinc under different pH values. *Aquat Toxicol* 150:9–16.

Sexton K, Hattis D. 2007. Assessing cumulative health risks from exposure to environmental mixtures—Three fundamental questions. *Environ Health Perspec* 115:825–832.

Viarengo A, Ponzano E, Dondero F, Fabbri R. 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: An application to Mediterranean and Antarctic molluscs. *Mar Environ Res* 44:69–84.

Vieira CE, Costa PG, Lunardelli B, Oliveira LF, Cabrera LC, Risso WE, Primel EG, Meltti PC, Fillmann G, Martinez CB. 2016. Multiple biomarker responses in *Prochilodus lineatus* subjected to short-term in situ exposure to streams from agricultural areas in Southern Brazil. *Sci Total Environ* 542:44–56.

Vieira MC, Torronteras R, Córdoba F, Canalejo A. 2012. Acute toxicity of manganese in goldfish *Carassius auratus* is associated with oxidative stress and organ specific antioxidant responses. *Ecotoxicol Environ Safety* 78:212–217.

Wood CM. 2012. An introduction to metals in fish physiology and toxicology: Basic principles. In Wood C, Farrell AP, Brauner CJ, eds, *Fish physiology: Homeostasis and Toxicology of Essential Metals*. Academic Press, Cambridge, MA, USA, pp 1–53.