

## Genetic and biochemical effects induced by iron ore, Fe and Mn exposure in tadpoles of the bullfrog *Lithobates catesbeianus*



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### ABSTRACT

For decades, the extraction of minerals has intensified in order to meet the demand of industry. Iron ore deposits are important sources of metals, such as iron (Fe) and manganese (Mn). The particulate ores can be dispersed during extraction, transport and storage, with potential to induce biological impacts. Amphibians are very sensitive to environmental stressors. Therefore, the present study aimed to assess the effects of iron ore, Fe and Mn exposure during the metamorphosis of *Lithobates catesbeianus*. Endpoints analyzed included morphological (biometrical and developmental analyses), whole body Fe and Mn concentration in, plasma ferritin concentration, erythrocyte DNA damage (measured through comet assay and micronucleus test) and liver activity of enzymes involved in oxidative status [glutathione S-transferase (GST) and catalase (CAT)]. Tadpoles were kept under control condition (no contaminant addition) or exposed to iron ore (3.79 mg/L as fine particulate matter); Fe (nominal concentration: 0.51 mg/L Fe as  $C_{10}H_{12}FeN_2NaO_8$ ; Fe-EDTA); and Mn (nominal concentration: 5.23 mg/L Mn as  $4H_2O.MnCl_2$ ) for 30 days. Virtually, no mortality was observed, except for one tadpole found dead in the iron ore treatment. However, tadpoles exposed to iron ore had longer tail than those kept under control conditions while tadpoles exposed to manganese chloride showed higher body length than control ones. Exposure to Fe and Mn induced a delay in tadpole metamorphosis, especially when these metals are presented not as a mixture (iron ore). Tadpoles exposed to iron ore had increased whole body Fe and Mn while those exposed to Fe and Mn accumulated each metal individually. Tadpoles exposed to any of the contaminants tested showed a significant increase in erythrocyte DNA damage and frequency of micronuclei. In addition, they showed higher liver GST activity respect with those kept under control conditions. Plasma ferritin concentration and liver CAT activity were higher only in tadpoles exposed to iron ore. These findings indicated that tadpoles accumulated Fe and Mn at the whole body level after exposure to the single metals or to their mixture as iron ore. In addition, they indicate that Fe and Mn accumulation can induce oxidative stress with consequent significant developmental, genotoxic and biochemical effects in *L. catesbeianus* tadpoles.

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### 1. Introduction

Iron ore deposits are associated with the environmental availability of metals such as copper (Cu), zinc (Zn) and magnesium (Mg). They are also important sources of iron (Fe) and manganese (Mn). Indeed, these are the main metals present in iron ore. Thus, iron ore mining and metal casting can influence the cycle of Fe and Mn (Lima and Pedrozo, 2001). In temperate environments, residues

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from iron ore mining have increased the levels of dissolved ions and particles in suspension, changing the water chemistry and metals bioavailability (Pereira et al., 2008).

For decades, the extraction of iron ore has been intensified in order to meet the industrial demand for steel production (Luz and Lins, 2004). Brazil is the second largest producer of iron ore in the world, while the Espírito Santo state (southeastern Brazil) is one of the largest iron ore exporters in the world (Lima and Pedrozo, 2001; Luz and Lins, 2004; IBRAM, 2012; Carvalho et al., 2014). Activities associated with this production involve iron ore beneficiation and transfer to ports. The iron ore is mined and carried out from the Minas Gerais state (southeastern Brazil), and further beneficiated in the Espírito Santo state. Therefore, the whole environment may be subjected to the impact of the dispersal of fine particulate iron ore resulting from this activity.

Iron is one of the most abundant elements on Earth, after oxygen, silicon and Mg (Cox, 1997). It is easily oxidized, being rarely found in its elemental form (Fe). It is primarily oxidized to the ferrous form ( $\text{Fe}^{2+}$ ) and thereafter to the ferric form ( $\text{Fe}^{3+}$ ) (Huebers, 1991; O'Neil, 1994; Bury et al., 2011).  $\text{Fe}^{3+}$  is prone to form iron hydroxides, which are insoluble (Lima and Pedrozo, 2001).

Iron speciation occurs by the oxidation of this element in the water column under the influence and in association with microorganisms and macroorganisms. In the aquatic environment,  $\text{Fe}^{2+}$  is oxidized to  $\text{Fe}^{3+}$ , with the resulting ferric hydroxide form being precipitated and deposited. However, there are benthic organisms favoring the conversion of  $\text{Fe}^{3+}$  into  $\text{Fe}^{2+}$  through redox reactions. Thus, Fe can diffuse back into the water column (Bury et al., 2011). The diffusion/return of  $\text{Fe}^{2+}$  in the water surface can occur by bioturbation, physical resuspension and upwelling (Lima and Pedrozo, 2001; Bury et al., 2011).

Iron is essential for life, being important for oxygen transfer, immune function, and DNA synthesis (Berg et al., 2008; Pan et al., 2009). However, the excess of Fe can be harmful, triggering a suite of free radical reactions, which can induce damage to proteins, lipids and nucleic acids (Berg et al., 2008). Animals have developed sophisticated systems for safely storing the excess of Fe. This metal is transported in the blood serum associated with transferrin and binds to specific receptors that are located on the cell membrane. Cellular uptake of Fe into the cell cytosol occurs when this metal is combined with apoferritin to form ferritin, which is a protein that acts as a cellular Fe store (Umbelino and Rossi, 2006). This protein is found mainly in the liver and kidneys (Berg et al., 2008).

Manganese can also be present in the aquatic environment under the oxidized state, thus forming  $\text{Mn}^{2+}$  and  $\text{Mn}^{4+}$  (Pereira et al., 2008). The latter is the predominant and more soluble form in water. The involvement of this metal in redox processes enables the release of the soluble part present in the sediment into the water column (Takeda, 2003).

As Fe, Mn is a trace metal essential for animals' life, acting primarily in the brain, where it is involved in the synaptic neurotransmission. This metal can be acquired through the diet or by absorption through the lungs. Its deficiency in the diet affects Mn homeostasis and neural activity. However, excess of Mn acts as a toxic agent to the brain, as this metal has pro-oxidant activity. Abnormal concentrations of Mn in the brain, especially in the basal ganglia, are associated with neural disturbances like the Parkinson's disease (Takeda, 2003; Vieira et al., 2012).

Factors influencing water quality can be harmful and affect aquatic animals' health under natural conditions. In aquaculture, including frog cultivation, changes in water quality can induce mortality of cultivated organisms and important economical losses for the producer. Frog production in captivity for commercial purposes has been a very common practice; the most used species is the bullfrog *Lithobates catesbeianus*. This species is found living in permanent water bodies, where it feeds and reproduces at a high rate.

In light of the above, the aim of the present study was to evaluate the effects of iron ore, Fe and Mn during the metamorphosis of the bullfrog *L. catesbeianus*. Endpoints analyzed included biometrical, developmental, genetic, and biochemical analyses. Whole body metal (Fe and Mn) concentrations were also considered.

## 2. Materials and methods

### 2.1. Tadpole acclimation

Tadpoles of *L. catesbeianus* were donated by a frog farmer (João Neiva, ES, southeastern Brazil). They were in stage 36 of development. This stage was selected because it is characterized as being the onset of hind limb externalization. It is also worth noting that the appearance of individual toes occurs in stage 37 of development (Gosner, 1960). Therefore, these stages comprise an important phase of transition from the aquatic to the terrestrial mode of life in anurans. Tadpoles were transferred from the frog farm to the Laboratory of Applied Ichthyology (FISHLAB, Vila Velha University, Vila Velha, Espírito Santo, southeastern Brazil) in plastic bags containing water bubbled with oxygen. Tadpoles were acclimated in 40-L tanks containing tap water for 10 days. Water was completely renewed every 3 days. Tadpoles were fed daily with a dry commercial feed containing 55% protein.

### 2.2. Experimental treatments

After acclimation, groups of six tadpoles were transferred to glass aquaria containing 10 L of tap water under constant aeration. They were kept under control conditions (no contaminant addition) or exposed to one of the following treatments for 30 days: iron ore treatment (3.79 mg/L as fine particulate matter); Fe treatment (0.5063 mg/L (1.379 nM) Fe as Fe-EDTA [ $\text{C}_{10}\text{H}_{12}\text{FeN}_2\text{NaO}_8$ ]); and Mn treatment (5.23 mg/L (26.426 nM) Mn as  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ). Concentrations of contaminants tested were selected based on the bioavailability of these elements in the Mäe-Bá pond (Anchieta, ES, southeastern Brazil) (Pereira et al., 2008). The iron ore (<75- $\mu\text{m}$  particle size) tested has the following metal composition: 264.0 mg/L Fe; 294.5 mg/L Mn; 3.5 mg/L Cu; and 3.4 mg/L Zn. Contaminants were added to the water 24 h prior the introduction of tadpoles. Each treatment was performed in triplicate ( $N=6$  per aquarium;  $N=18$  per treatment).

Tadpoles were fed daily with the commercial feed throughout the experiment. Experimental media were completely renewed every three days. Tadpoles were kept under the experimental conditions until reaching the stage 47 of development. This stage was selected to end the experiment because this is the phase where tadpoles are completing the whole metamorphosis, which is estimated to last approximately 30 days in *L. catesbeianus*. According to Gosner (1960), metamorphosis is essentially completed at the stage 47 of development, and the water dependence of tadpoles overcomes partial, with individuals becoming terrestrial.

The Committee of Ethics for Animal Use of the Vila Velha University (CEUA-UVV) approved all procedures performed in the present study (permit # 211/2011). All waste generated during the experiment and laboratory analyses was stored and further collected by a waste treatment company.

### 2.3. Water physicochemical parameters

Water physicochemical parameters were evaluated during the acclimation and experimental periods. Measurements were performed 24 h before and 24 h after the experimental medium renew. Dissolved oxygen, temperature, pH, and conductivity were measured using a multi-parameter analyzer (model 85, YSI, Yellow Springs, USA). Ammonia and nitrite concentrations were measured

**Table 1**

Physicochemical parameters of the acclimation and experimental media used to keep tadpoles of the bullfrog *Lithobates catesbeianus* under control condition or to expose them to iron ore, iron (Fe) and manganese (Mn). Data are expressed as median. Different letters in each row indicate significant difference among treatments ( $p < 0.05$ ). NM = not measured.

Parameter	Acclimation	Control	Iron ore	Fe	Mn
Dissolved oxygen (mg/L)	7.5 <sup>a</sup>	6.2 <sup>a</sup>	6.3 <sup>a</sup>	6.3 <sup>a</sup>	6.3 <sup>a</sup>
Temperature (°C)	27.0 <sup>a</sup>	26.9 <sup>a</sup>	26.8 <sup>a</sup>	26.8 <sup>a</sup>	26.7 <sup>a</sup>
Conductivity (μS/cm)	99.0 <sup>a</sup>	88.9 <sup>a</sup>	86.8 <sup>a</sup>	87.6 <sup>a</sup>	97.4 <sup>a</sup>
Ammonia (mg/L)	0.13 <sup>a</sup>	0.64 <sup>b</sup>	0.53 <sup>b</sup>	0.54 <sup>b</sup>	0.58 <sup>b</sup>
Nitrite (mg/L)	0.001 <sup>a</sup>	0.01 <sup>a</sup>	0.01 <sup>a</sup>	0.01 <sup>a</sup>	0.01 <sup>a</sup>
pH (unit)	6.90 <sup>a</sup>	7.10 <sup>a</sup>	6.40 <sup>a</sup>	6.50 <sup>a</sup>	6.30 <sup>a</sup>
Dissolved Fe (mg/L)	11.01 <sup>a</sup>	11.01 <sup>a</sup>	16.32 <sup>b</sup>	24.98 <sup>b</sup>	NM
Dissolved Mn (mg/L)	0.019 <sup>a</sup>	0.019 <sup>a</sup>	0.220 <sup>a</sup>	NM	2.735 <sup>b</sup>

according to the methods described by APHA (2005). Water samples (10 mL) were filtered (0.45 μm-mesh filter), acidified with 150 μL of pure nitric acid, and analyzed for dissolved Fe and Mn concentrations. Analyses were performed by atomic absorption spectrophotometry (AAS 932 Plus, GBC, Hampshire, IL, USA).

#### 2.4. Morphological analyses

Morphological analyses were performed according to Rada et al. (2007), with some adaptations. Body, tail and total length were measured using a caliper. Tadpoles were weighed (wet body weight) on a precision scale. In addition, observations on the appearance of legs and the presence of anomalies were considered. Developmental stages of tadpoles were evaluated over the experiment based on a simplified table of Gosner (1960).

#### 2.5. Genetic and biochemical analyses

At the end of the experiment, tadpoles were anesthetized with 1 mL/L lidocaine. Blood samples were collected by puncturing the caudal vein with a heparinized syringe and used for the comet assay, micronucleus test and ferritin concentration analysis. After blood collection, tadpoles were killed by cervical section and had the liver dissected for enzymatic analyses. Whole bodies were used for metal concentration analyses.

##### 2.5.1. Comet assay

Blood samples were diluted 1:120 (v/v) in RPMI 1640 medium (RPMI-Roswell Park Memorial Institute) and used immediately. The alkaline comet assay was performed as described by Tice et al. (2000) and Andrade et al. (2004), with some modifications. Briefly, 5 μL of each diluted blood sample was added to 95 μL of 0.75% (w/v) molten low melting point agarose, and an aliquot of the mixture was spread on a microscope slide that was pre-coated with 1.5% (w/v) normal melting point agarose and topped with a coverslip. After agarose solidification, coverslips were removed and the slides were immersed in a lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0–10.5) containing 1% Triton X-100 and 20% DMSO. Slides were maintained in this lysis solution (4 °C) and kept in the dark for 2–3 h. Slides were then incubated in a freshly prepared alkaline buffer solution 300 mM NaOH and 1 mM EDTA, pH ≥ 13 for 20 min for DNA unwinding. Electrophoresis (15 min at 300 mA and 25 V) was performed using the same buffer solution. Every step was performed under indirect yellow light. After electrophoresis, slides were neutralized in a Tris solution (400 mM; pH 7.5), rinsed three times with distilled water, and dried over night at room temperature. Slides were fixed for 10 min in trichloroacetic acid (15% w/v), zinc sulfate (5% w/v), and glycerol (5% v/v). They were rinsed three times with distilled water and dried at 37 °C for 2 h. Dry slides were re-hydrated for 5 min in distilled water. They were then stained under constant shaking for 35 min. The staining solution was freshly prepared in the

dark and contained sodium carbonate (5% w/v), ammonium nitrate (0.1% w/v), silver nitrate (0.1% w/v), tungstosilicic acid (0.25%) and formaldehyde (0.15% w/v). Stained slides were rinsed twice with distilled water, submerged in the stop solution (acetic acid 1%), rinsed again with distilled water, and immediately coded for analysis. A total of 100 cells from each replicate (50 from each duplicate slide) were randomly analyzed under an optical microscope (100× magnification) to measure the length of comet's tail. Analysis of slides was performed considering 100 cells/tadpole using a visual classification based on the degree of DNA fragments migration from the nucleus. Cells were classified into class 0 (no damage), class 1 (little damage—when the tail length was smaller than the nucleus), class 2 (medium damage—when the tail length was between 1 and 2 times the nucleus diameter), class 3 (extensive damage—when the tail length was over 2 times the nucleus diameter), and class 4 (presence of apoptosis) (Kobayashi et al., 1995; Speit and Hartmann, 2006). The DNA damage index was calculated for each tadpole as the sum of the number of nucleoids that were observed for each damage class multiplied by the value of its respective damage class (0–4). Results were expressed as the mean DNA damage index for each experimental group, where 0 represents the absence of damage and 400 indicates the highest damage score.

##### 2.5.2. Micronucleus test

A drop of fresh blood sample from each tadpole collected as described above was smeared on a clean glass slide. Slides were left to dry overnight at room conditions. Blood smears were then fixed in methanol for 15 min, stained with a Giemsa solution (5% w/v) for 20 min, washed with distilled water, and left to dry at room conditions (Grisolia et al., 2005). After drying, 2,000 erythrocytes per tadpole (2 slides; 1,000 erythrocytes per slide) were analyzed for the presence of micronuclei under a light microscope (100× magnification). Blind analysis was performed with respect to the experimental condition. Micronuclei in erythrocytes were characterized as being small and non-refractive, with circular or ovoid chromatin bodies, displaying the same staining and focusing pattern as the main nucleus (Al-Sabti and Metcalfe, 1995).

##### 2.5.3. Ferritin concentration

Blood samples collected as described above were centrifuged (3000 rpm or 956 × g) for 10 min and serum was collected for ferritin concentration analysis using a commercial reagent kit (Ferritin, Bioclin). The analysis is based on the immune turbidimetric method used to determine the endpoint of the ferritin concentration by the photometric measurement of the antigen-antibody reaction between the latex particles that were stained with anti-ferritin antibody and ferritin present in the sample.

##### 2.5.4. Glutathione S-transferase and catalase activity

Liver samples were homogenized in a phosphate buffer solution (pH 7.0) and centrifuged (13,000 rpm or 17,949 × g) at 4 °C for

30 min. The supernatant obtained was used for enzyme activity and total protein concentration analyses.

Glutathione S-transferase (GST, EC 2.5.1.18) activity was determined using a phosphate buffer solution (pH 7.0) containing 1-chloro-2,4-dinitrobenzene (CDNB; 1 mM) and glutathione (GSH; 1 mM) as substrate. Enzyme activity was determined based on the extinction coefficient of CDNB (Habig et al., 1974; Habig and Jakoby, 1981). Catalase (CAT, EC 1.11.1.6) activity was determined by the continuous evaluation of the decrease in the hydrogen peroxide ( $H_2O_2$ ) concentration (Aebi, 1984). The reaction medium was prepared with a buffer solution (1 M Tris-HCl and 5 mM EDTA) containing hydrogen peroxide (10 mM). The total protein concentration in the liver homogenate was determined by the method of Lowry et al. (1951).

## 2.6. Whole body iron and manganese concentrations

Iron and manganese concentrations were measured in whole body of tadpoles after blood and liver samples collection. Tadpole bodies were dried to constant weight (dry weight), completely digested in 5 mL of  $HNO_3$  (Suprapur Merck), and diluted with 15 mL of MilliQ water. Fe and Mn concentrations were determined by atomic absorption spectrophotometry (AAS-932 Plus, GBC, New Hampshire, IL, USA) (Pedroso et al., 2007; Leonard et al., 2011; Carvalho et al., 2013). Data were expressed as mg metal/g dry weight.

## 2.7. Data presentation and statistical analysis

Data were previously checked for normality and homogeneity of variances. Water physicochemical and tadpole biometrical data showed normal distribution and homogeneity of variances. Therefore, they were expressed as mean  $\pm$  standard deviation and mean values for treatments were compared by one-way analysis of variance (ANOVA) followed by the Dunn's test. In turn, genetic (comet assay and micronucleus test), biochemical (plasma ferritin concentration and liver enzyme activity) and whole body metal (Fe and Mn) concentration data did not show a normal distribution. Therefore, data for these parameters were expressed as median and further analyzed by Kruskal-Wallis ANOVA, followed by the Dunn's test. In all cases, the significance level adopted was 95% ( $\alpha = 0.05$ ). Statistical analyses were performed using the Sigma Stat 12.5 software.

## 3. Results

### 3.1. Water physicochemical parameters

No significant changes in water physicochemical parameters (dissolved oxygen, temperature, conductivity, pH, nitrite and ammonia) were observed among treatments. For all treatments, mean ammonia concentration measured during the acclimation period was lower than that measured during the experimental period (Table 1).

Regarding metal (Fe and Mn) concentration in the water, there was a significant increase in Fe concentration after the addition of iron ore and Fe EDTA in the experimental medium when compared to that measured in the control group, as well as the water used for tadpoles acclimation (Table 1). Dissolved Mn concentration was significantly higher in the Mn exposure than in the other conditions (Table 1).

### 3.2. Morphological analyses

After the experimental period, only one tadpole was found dead in the iron ore treatment. Tadpoles kept under control con-

**Table 2**

Frequency (%) of developmental stages in *Lithobates catesbeianus* tadpoles exposed to iron ore, iron (Fe) and manganese (Mn) for 30 days.

Stage	Treatment			
	Control	Iron ore	Fe	Mn
36	0	0	5.5	0
37	0	0	0	11.1
38	0	11.7	22.2	22.2
39	16.6	0	16.6	5.5
40	5.5	17.6	0	5.5
41	0	5.8	5.5	5.5
42	16.6	11.7	11.1	16.6
44	27.7	0	16.6	0
47	33.3	52.9	22.2	33.3

**Table 3**

Biometrical data of *Lithobates catesbeianus* tadpoles exposed to iron ore, iron (Fe) and manganese (Mn) for 30 days. Data are expressed as median. Different letters in each column indicate significant differences among treatments ( $p < 0.05$ ).

Treatment	Length (mm)			Wet body weight (g)
	Total	Body	Tail	
Control	85.3 <sup>a</sup>	37.4 <sup>a</sup>	53.5 <sup>a</sup>	5.9 <sup>a</sup>
Iron ore	91.7 <sup>a</sup>	37.7 <sup>a</sup>	73.3 <sup>b</sup>	6.5 <sup>a</sup>
Fe	101.7 <sup>a</sup>	38.6 <sup>a</sup>	55.5 <sup>a</sup>	7.6 <sup>a</sup>
Mn	103.6 <sup>a</sup>	40.6 <sup>b</sup>	63.8 <sup>a</sup>	7.9 <sup>a</sup>

**Table 4**

Classes of DNA damage in erythrocytes of *Lithobates catesbeianus* tadpoles kept under control condition or exposed to iron ore, iron (Fe) and manganese (Mn) for 30 days. Data are expressed as median. Different letters in each column indicate significant differences among treatments ( $p < 0.05$ ).

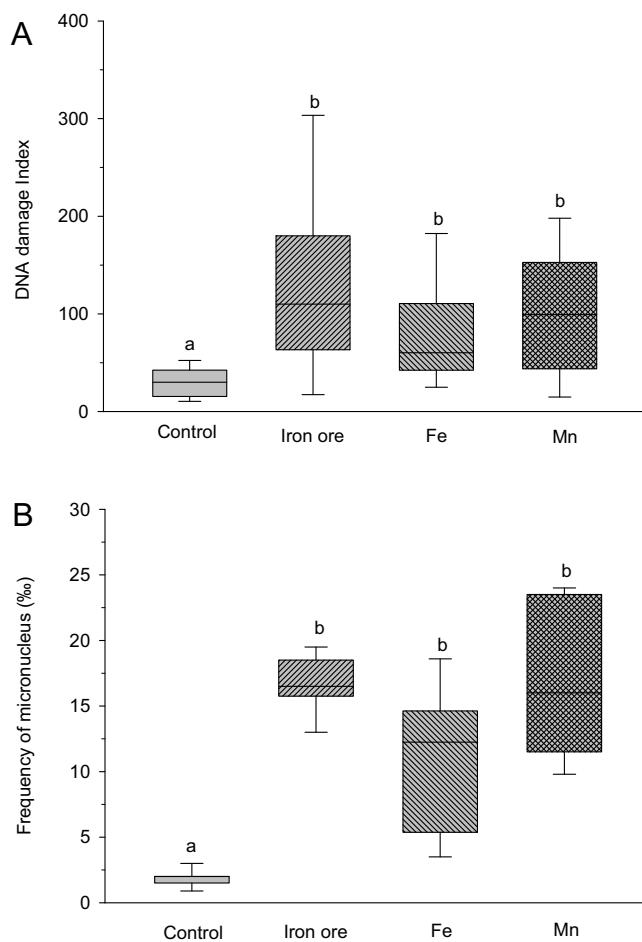
Treatment	DNA damage class				
	0	1	2	3	4
Control	81.8 <sup>a</sup>	18.2 <sup>b</sup>	4.0 <sup>a</sup>	0.0 <sup>a</sup>	0.0 <sup>a</sup>
Iron ore	14.0 <sup>b</sup>	21.0 <sup>a</sup>	12.5 <sup>b</sup>	19.0 <sup>b</sup>	1.5 <sup>b</sup>
Fe	10.5 <sup>b</sup>	9.0 <sup>b</sup>	8.5 <sup>b</sup>	6.8 <sup>b</sup>	1.0 <sup>b</sup>
Mn	22.0 <sup>b</sup>	19.5 <sup>b</sup>	23.5 <sup>b</sup>	6.5 <sup>b</sup>	0.5 <sup>b</sup>

ditions showed normal development, with 77.8% of them reaching the stages 42–7 of development. Approximately 50% of tadpoles exposed to the iron ore treatment reached the stage 47 of development with complete appearance of hind legs and tail absorption. However, the other 50% of tadpoles developed only until the stages 38–42. In tadpoles exposed to Fe and Mn treatments, there was a delay in development when compared to that observed in tadpoles kept under control conditions. In this case, 50% of the tested individuals developed only until the stages 36–41 (Table 2).

Regarding the biometrical analyses, no significant difference in tadpole total length and wet body weight was observed among treatments. However, tadpoles exposed to the iron ore treatment had significantly longer tail than those kept under control conditions. In addition, tadpoles exposed to the Mn treatment showed significantly higher body length than control tadpoles (Table 3).

### 3.3. Comet assay

Tadpoles exposed to one of the three treatments (iron ore; Fe; and Mn) showed a significantly increased DNA damage index (DI) in erythrocytes when compared to that observed in tadpoles kept under control conditions (Fig. 1A). Tadpoles from the three treatments showed more cellular fragments in class 2 (moderate damage), 3 (some severe damage) and 4 (severe damage) than those from the control group. Consequently they showed less cellular fragments belonging to class 0 (no damage) than the control tadpoles (Table 4).



**Fig. 1.** DNA damage index (range: 0–400) (A) and micronucleus frequency (B) in peripheral erythrocytes of *Lithobates catesbeianus* tadpoles kept under control conditions or exposed to one of the following treatments for 30 days: iron ore; iron (Fe) and manganese (Mn). The box represents the interquartile containing 50% of the observed values. The vertical line corresponds to the highest and lowest values observed and the horizontal line represents the median value for each experimental condition. Different letters indicate significant difference between control and treatment ( $p < 0.001$ ; Dunn's test).

#### 3.4. Micronucleus test

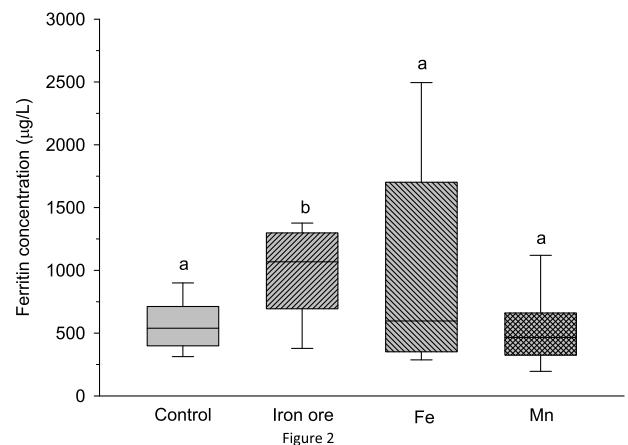
Erythrocytes of tadpoles exposed to iron ore, iron and manganese treatments showed a significantly increased (~15-fold) frequency of micronucleus than erythrocytes from control tadpoles (Fig. 1B).

#### 3.5. Ferritin concentration

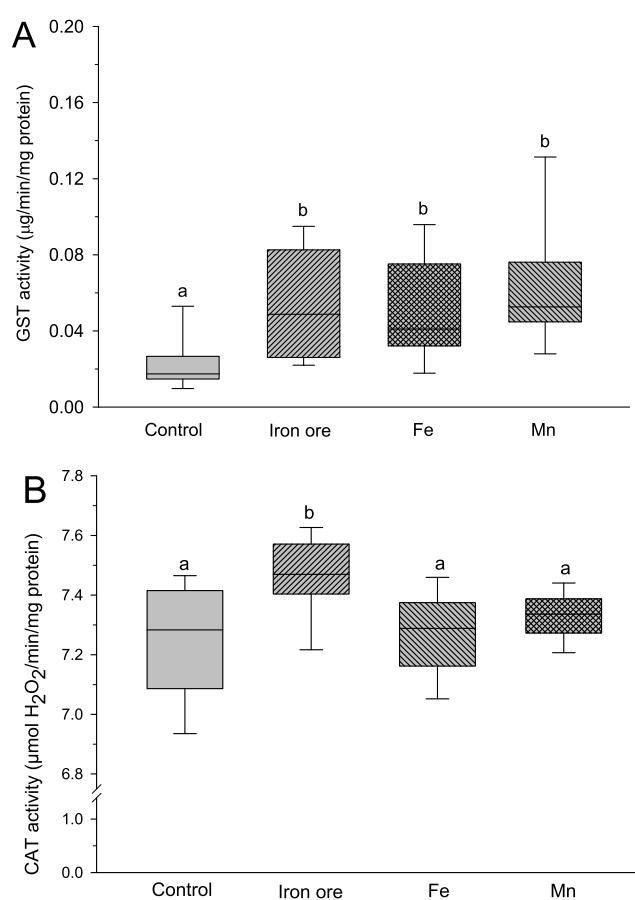
Blood ferritin concentration was higher in tadpoles exposed to iron ore treatment than in those kept under control conditions or exposed to the Fe and Mn treatments (Fig. 2).

#### 3.6. Enzyme activity

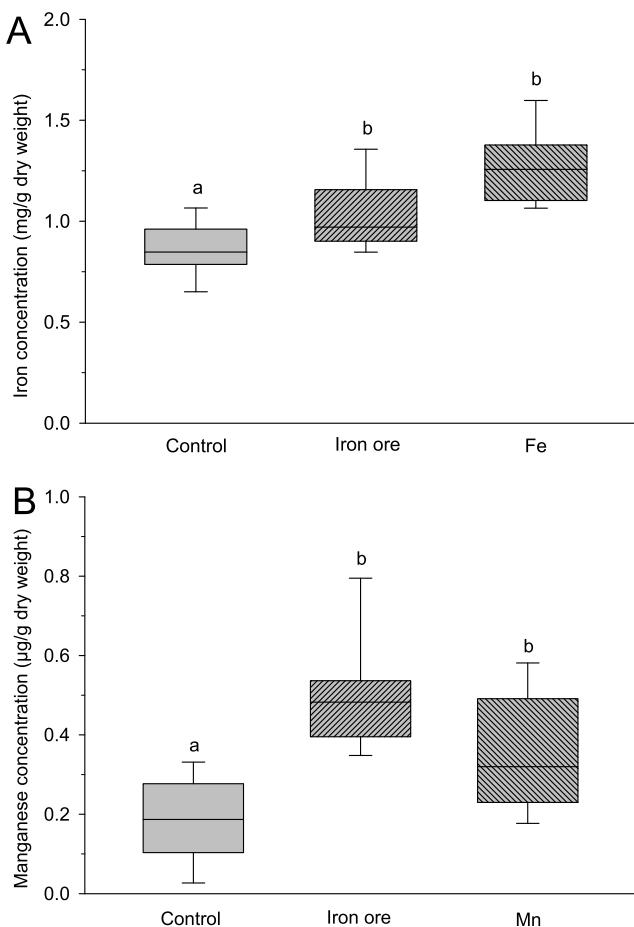
GST activity was increased in liver of tadpoles exposed to the iron ore, Fe and Mn treatments with respect to those of tadpoles kept under control conditions. However, no significant difference was observed among the treatments (Fig. 3A). In turn, catalase activity was increased only in liver of tadpoles exposed to the iron ore treatment when compared to the enzyme activity observed in liver of tadpoles kept under control conditions or exposed to the Fe and Mn treatments (Fig. 3B).



**Fig. 2.** Plasma ferritin concentration in *Lithobates catesbeianus* tadpoles kept under control condition or exposed to one of the following treatments for 30 days: iron ore; iron (Fe) and manganese (Mn). The box represents the interquartile containing 50% of the observed values. The vertical line corresponds to the highest and lowest values observed and the horizontal line represents the median value for each experimental condition. Different letters indicate significant difference between control and treatment ( $p < 0.004$ ; Dunn's test).



**Fig. 3.** Glutathione S-transferase (GST) activity (A) and catalase (CAT) activity (B) in liver of *Lithobates catesbeianus* tadpoles kept under control conditions or exposed to one of the following treatments for 30 days: iron ore; iron (Fe) and manganese (Mn). The box represents the interquartile containing 50% of the observed values. The vertical line corresponds to the highest and lowest values observed and the horizontal line represents the median value for each experimental condition. Different letters indicate significant difference between control and treatment ( $p < 0.001$ ; Dunn's test).



**Fig. 4.** Iron (A) and manganese (B) concentration in *Lithobates catesbeianus* tadpoles kept under control conditions or exposed to one of the following treatments for 30 days: iron ore; iron (Fe) and manganese (Mn). The box represents the interquartile containing 50% of the observed values. The vertical line corresponds to the highest and lowest values observed and the horizontal line represents the median value for each experimental condition. Different letters indicate significant difference between control and treatment ( $p < 0.001$ ; Dunn's test).

### 3.7. Whole body iron and manganese concentration

Whole body iron concentration increased in tadpoles exposed to the iron ore and Fe treatments when compared with those kept under control conditions (Fig. 4A). In addition, whole body Mn concentration was higher in tadpoles exposed to the iron ore and Mn treatments compared with those kept under control conditions (Fig. 4B).

## 4. Discussion

The presence of metals can occur naturally in the aquatic environment but at small quantities. However, sewage discharge from industrial activities has increased the environmental concentration of these chemicals (Oakes et al., 2004). For example, Pereira et al. (2008) conducted a monitoring study in the M  e-B   Pond (Anchieta, Esp  rito Santo State, southeastern Brazil), which receives the effluent of an iron ore pelletizing company. These authors reported bioaccumulation of Fe, Pb, Ni and Hg in the gastropods *Melanoides tuberculata* and *Pornacea hastrum*, with Mn being the most bioaccumulated metal. Therefore, iron mining and processing can thus contribute as a relevant source of chemical pollutants, especially metals, in tropical aquatic environments (Pereira et al., 2008).

In present study, we observed high Fe levels in the control tap water used to maintain tadpoles in laboratory. The increased levels

of Fe observed are likely due to the addition of ferric chloride during the water treatment performed by the municipal water treatment plant (CESAN) (Vila Velha, Esp  rito Santo State, southeastern Brazil). Indeed, this chemical is used with the purpose of clotting water impurities, which are in suspension, into a fine colloidal state. Therefore, if tadpoles would have been exposed to water at low Fe levels than those presently tested, the resulting effects would be likely more significant than those actually reported in the present study. Anyway, the increased bioavailability of Fe and Mn in the iron ore treatment resulted in bioaccumulation of these metals in the whole body of *L. catesbeianus* tadpoles after 30 days of exposure. In agreement with this result, a significant bioaccumulation of Fe and Mn was also observed in *L. catesbeianus* tadpoles exposed to the Fe and Mg treatments, respectively.

Iron is an essential micronutrient for life, particularly in vertebrates. In this case, ferritin is used for cellular storage of iron (Umbelino and Rossi, 2006), thus exerting a cytoprotective effect. An increase in the concentration of this protein indicates that excess iron in the body is being mobilized, but the storage of this metal cannot be ensured, and it can thus remain free (Pan et al., 2009). The increase in plasma ferritin concentration observed in tadpoles exposed to iron ore treatment can be explained by the greater availability of Fe in the iron ore than in the Fe-EDTA treatment. Changes in liver Fe accumulation and ferritin concentration were shown to occur according to the developmental stage of metamorphosis in *Rana catesbeiana* tadpoles (Bury et al., 2011). According to these authors, changes observed can be associated with an increase in Fe absorption, thus explaining the appearance of immature blood cells. Although iron is an essential micronutrient, excessive accumulation of this metal induced morphological, genotoxic and biochemical effects in tadpoles of *L. catesbeianus* exposed to iron ore, Fe and Mn treatments. Regarding morphological disturbances, a delay in metamorphosis was observed in tadpoles of *L. catesbeianus* exposed to any of the treatments tested (iron ore, Fe and Mn). The longer delay was observed when Fe (Fe treatment) and Mn (Mn treatment) were added to the water not as a mixture (iron ore treatment). As observed in the present study with Fe and Mn, James and Little (2003) also observed a delay in metamorphosis of *Bufo americanus* tadpoles exposed to a high concentration (540 µg/L) of waterborne Cd. In addition, Lounbourdis et al. (1999) reported a tendency of growth delay in tadpoles of *Rana ridibunda* exposed to Cd for 15 and 30 days. Therefore, growth delay in metamorphosis of tadpoles induced by metals exposure could be suggested as a factor contributing to the decline of amphibians. A delay in metamorphosis would lead to tadpoles to spend more time in the most vulnerable stages due to their reduced size, thus making them more susceptible to predators. In addition to the observed delay in metamorphosis, significant changes in biometrical parameters were also found after exposure of *L. catesbeianus* tadpoles to iron ore and Mn treatments. Indeed, tadpoles exposed to the iron ore treatment showed longer tail than those kept under control conditions. Furthermore, tadpoles exposed to the Mn treatment had higher body length than those kept under control conditions. These findings are in agreement with the higher growth reported for tadpoles of *B. americanus* exposed to low Cd concentrations (5 and 54 µg/L) (James and Little, 2003).

Besides the morphological and developmental disturbances discussed above, genotoxic effects were also observed after exposure of *L. catesbeianus* tadpoles to the iron ore, Fe and Mn treatments. According to Dhawan et al. (2009), metals can cause deleterious effects in somatic or germ cells. Considering that the comet assay was employed, results from the present study indicate that exposure to the iron ore, Fe and Mn treatments induced DNA damage in erythrocytes of *L. catesbeianus* tadpoles. A similar induction in DNA damage was observed in *Hypsiboas faber* tadpoles collected from a

coal mining area when compared to those collected in a reference area (Zocche et al., 2013).

In addition to DNA damage, exposure to iron ore, Fe and Mn treatments also induced the formation of micronuclei in erythrocytes of *L. catesbeianus* tadpoles. Micronuclei are structures similar to the nucleus, being formed by chromosome sequences that are separated from the main nucleus. They can occur naturally or due to the influence of contaminants (Cavalcante et al., 2010). As observed in the present study, a significant increase in the number of cells containing micronuclei was observed in *Xenopus laevis* tadpoles exposed to Cd (2, 10, or 30 mg/L) for 12 days (Mouchet et al., 2006). Taken together, these findings suggest that exposure of tadpoles to different metals can induce breaks in the chromosome or cause a dysfunction of the mitotic spindle apparatus during cell division (Winter et al., 2007). It is worth noting that no additive or synergistic effect was observed after exposure to metals as a mixture. The fact that similar genotoxic effects (DNA damage and micronuclei formation) were observed in *L. catesbeianus* tadpoles exposed to fine-particulate iron ore and in those exposed to the single metal (Fe and Mn) supports this idea.

Regarding biochemical effects, liver of *L. catesbeianus* tadpoles exposed to the iron ore, Fe and Mn treatments showed an increased GST activity. Similar results were found by Gabriel et al. (2013) in the fish *Colossoma macropomum* acutely exposed (96 h) to Mn (3.88 mg/L). It is important to note that GST plays a key role in the detoxification of various xenobiotic metabolites, neutralizing and bringing them soluble in water and consequently more easily excreted (Ezemonye and Tongo, 2010). The combined exposure to Fe and Mn in the iron ore treatment, along with the other iron ore components, also increased the CAT activity in the liver of *L. catesbeianus* tadpoles. However, exposure to Fe or Mn treatment alone did not alter the activity of this antioxidant enzyme. Paulino et al. (2012) suggested that the lack of CAT response could be explained by the increased activity of other antioxidant enzymes, such as glutathione peroxidase (GPx), or other forms of antioxidant defense. This situation may have occurred in the liver of *L. catesbeianus* tadpoles exposed to Fe or Mn treatment alone. As GST, CAT is also an enzyme involved in the antioxidant defense system. In this case, it acts by removing the hydrogen peroxide ( $H_2O_2$ ) produced during the biotransformation of xenobiotics, thus metabolizing it into  $O_2$  and water (Van der Oost et al., 2003).

Both Fe and Mn are known as redox-active metals involved in cellular oxidative status. Indeed, Fe plays a central role in oxidative stress by the formation of hydroxyl radicals through Fenton and Haber-Weiss reactions (Kanti Das et al., 2015). In turn, Mn plays a key role in cellular adaptation to oxidative stress. It is a cofactor for Mn superoxide dismutase (Mn-SOD) and part of non-proteinaceous Mn antioxidants. Therefore, this metal protects against the potential oxidative damage induced by reactive oxygen species (ROS), but without the deleterious side effects of Fenton chemistry (Aguirre and Culotta, 2012). However, as observed in the present study, it has been demonstrated that chronic exposure to Mn leads to an increase in markers of oxidative stress. In addition, a shift of  $Fe^{3+}$  towards  $Fe^{2+}$  in vertebrate brain tissue has been described under chronic exposure to Mn, thus enabling the action of the Fenton reaction (Fernsebner et al., 2014). Also, it is worth noting that the antioxidant properties of Mn are vulnerable to Fe. In fact, excessive amounts of cellular Fe can outcompete Mn for binding to Mn-SOD. In this case, Fe counteracts the benefits of non-proteinaceous Mn antioxidants, through Fenton chemistry (Aguirre and Culotta, 2012).

In light of the above, exposure to increased Fe and Mn concentrations alone (Fe and Mg treatments, respectively) or in combination (iron ore treatment) is inducing an oxidative stress condition in *L. catesbeianus* tadpoles. This condition is likely associated with a higher generation of ROS through Fenton chemistry after expo-

sure to Fe and Mn, either alone or in combination (iron ore). The increased response of the liver enzymatic antioxidant system (GSH and CAT activity), as an attempt to protect against a possible oxidative stress induced by metal exposure (Machado et al., 2013), as well as the genotoxic effects (DNA damage and increased frequency of micronuclei) observed in erythrocytes of tadpoles exposed to iron ore, Fe and Mn strongly support this idea.

In conclusion, chronic exposure to waterborne iron ore, Fe and Mn at environmentally relevant concentrations induced Fe and/or Mn accumulation in tissues of *L. catesbeianus* tadpoles with consequent morphological, developmental, genotoxic, and biochemical effects associated with a metal-induced oxidative stress condition.

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