Biochemical and Genotoxic Effects of Iron and Manganese in Oreochromis Niloticus (Teleostei: Cichlidae)

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Abstract

The Doce River, southeastern Brazil, in 2015 received iron mining tailings after the Fundão (MG) dam burst, which resulted in the leakage of about 50 million cubic meters of tailings mud, which have iron (Fe) and manganese (Mn) as main components, causing much damage to health to aquatic organisms, including death. Since exposure of aquatic organisms to metals can cause genotoxic damage and induce the generation of reactive oxygen species, causing oxidative damage to biomolecules, the present study aimed to evaluate the toxicity of the association between Fe and Mn in *Oreochromis niloticus* through genotoxic (micronucleus test and comet assay), and biochemical (CAT and GST enzymes) assays. The tested treatments were T1 = control group, T2 = 3.81 mg/L of Fe + 0.5 mg/L of Mn, and T3 = 7.62 mg/L of Fe + 5.23 mg/L of Mn, during 96-h bioassays. All animals exposed to the metals showed a significant increase in erythrocyte micronucleus frequency and DNA damage. The hepatic GST activity increased two times in animals exposed to T3 compared to control group. The results indicate that Fe + Mn caused genotoxic and biochemical changes in exposed fish. Therefore, excess metals in ecosystems, even those essential for organisms, can be dangerous for the local biota due to the risk associated with high concentrations of these metals.

1. Introduction

In November 2015, the Doce River (southeastern Brazil) suffered a large environmental impact. It received an influx of tailings from the ruptured Fundão dam in Mariana - Minas Gerais State, resulting in the leakage of approximately 50 million cubic meters of mud mining tailings. These tailings are mainly composed of hematite, goethite, kaolinite, quartz, and metals (Gomes et al. 2017; Queiroz et al. 2018). The major metals that compose iron ore are iron (Fe) and manganese (Mn) (Veronez et al. 2018). The increase in these metal concentrations in aquatic environments can impact the biota, causing imbalances in these ecosystems and associated organisms (Zhang et al. 2018).

Iron and Manganese are essential for living organisms and their metabolic functions. However, at high concentrations, they can become toxic and cause damage. Manganese is involved in several biological processes, such as the metabolism of carbohydrates, lipids, and proteins, and as an enzymatic cofactor (Keen 1984). However, Mn overload can cause damages, such as changes in the immune responses and deficiency in calcium absorption (Hernroth et al. 2004; Gunter et al. 2006). Iron participates in oxygen transport, DNA synthesis, and electron transport associated with cellular respiration (Crichton 1991). Iron overload can damage organs, tissues, and cells, cause histopathological changes and decrease the number of relevant cells to the immune system (Sousa et al. 2020).

The use of biomarkers has great relevance in environmental monitoring. These are molecular, cellular, or systemic markers of great importance to the evaluation of the organisms' response to the effects caused by a pollutant. Metals can also react with genetic material, producing genotoxic damage detected by the micronucleus test and the comet assay, respectively. Analyses like micronucleus test and comet assays are capable of detecting anomalies caused by contaminants in the animals' DNA and of measuring

physical and biochemical changes in the blood (Nussey et al. 1995). Among the various biochemical biomarkers studied, there are two very important ones, the enzymes glutathione-S-transferase (GST) and catalase (CAT), in the liver and gills. Glutathione-S-transferase is an enzyme of the phase II of biotransformation metabolism, conjugating xenobiotic to polar molecules (Gao et al. 2020). Catalase decomposes hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) (Aebi 1984), being part of the cells' antioxidant defense metabolism related to oxidative stress (Vasylkiv et al. 2011). The gills are extensively studied because are constantly exposed to environmental changes due to respiratory processes and the liver for being the main organ for detoxifying xenobiotic.

A good biological model is indicated to effectively evaluate an organism's response to the studied biomarkers. Bioindicator organisms are usually indicated because their condition reflects the environmental conditions. Fishes are good bioindicators, as they are constantly exposed to environmental variations and can metabolize, concentrate, and accumulate pollutants, in addition to being sensitive to biochemical and genotoxic analyses (Collins et al. 2004). *Oreochromis niloticus* (Linnaeus, 1758) is a fish species belonging to the Cichlidae family, native to the African continent, but widely distributed in reservoirs and rivers of tropical regions, including the Doce River. Furthermore, this species responds promptly to environmental changes caused by contaminants (Almeida et al. 2002).

Therefore, due to the importance of understanding the synergistic effect of these metals, mostly due to the composition of iron ore, the objective of this study was to evaluate the toxicity of associated Fe + Mn using biochemical (CAT and GST enzymes) and genotoxic (micronucleus test and comet assay) biomarkers. Proposing the hypothesis that these metals in an association are toxic to *O. niloticus* even at low concentrations, causing enzymatic changes and damage to the genetic material.

2. Material And Methods

2.1 Acclimatization

Fifty juvenile individuals of *O. niloticus* (62.6 ± 4.9 g and 15.7 ± 1.33 cm) were acquired from the Aquamais fish farm located in Guarapari, Espírito Santo State, Brazil. They were rapidly transported to the Applied Ichthyology Laboratory at the University of Vila Velha (LabPeixe/UVV). There, they were maintained in 500-L polyethylene tanks with filtered water and constant aeration for four weeks for acclimatization. They were fed daily with a protein-rich (55%) feed.

We monitored the following water physicochemical parameters during the acclimatization period using an environmental multi-parameter YSI (EcoSense YSI DO 200 and EcoSense pH 100A): dissolved oxygen (8.16 ± 0.15 mg/L), temperature (24.60 ± 0.35°C), pH (6.97 ± 0.05), and electrical conductivity (148.65 ± 6.15 µS/cm). The contents of ammonia (0.92 ± 0.32 mg/L) and nitrite (0.007 ± 0.003 mg/L) were assessed colorimetrically. Hardness (5.95 ± 1.49 mg $CaCO_3$/L) and alkalinity (0.82 ± 0.14 mg $CaCO_3$/L) were determined by the titration method (APHA 1989). All analyses were performed every two days and the water in the tanks was replaced at the same frequency (70% of the water was exchanged). The tanks

were kept in a 12-h light/12-h dark photoperiod. The experiment was carried out with the approval of the Ethics, Bioethics, and Animal Welfare Commission (CEUA – UVV; 371–2016).

2.2 Experimental design

After the acclimatization period, 24 specimens of *O. niloticus* (54.16 ± 0.76 g and 15.45 ± 0.64 cm) were individually allocated to three treatments (n = 8 individuals per treatment). The following predetermined Fe (Iron EDTA; Sigma-Aldrich®) and Mn (Manganese Chloride - $MnCl_2.4H_2O$; Sigma-Aldrich®) concentrations were used in each treatment: T1 = control (without metal addition), T2 = 3.81 mg/L of Fe + 0.5 mg/L of Mn, and T3 = 7.62 mg/L of Fe + 5.23 mg/L of Mn. Each individual was placed in a 6-L glass aquarium with constant aeration. Feeding was suspended one day before the beginning of the bioassay (i.e., one day after the individuals' placement into the aquariums). The fishes remained 96 h at these conditions (experiment duration). The water physicochemical parameters (dissolved oxygen, temperature, pH, conductivity, hardness, alkalinity, nitrite, and ammonia) were determined at the beginning (time 0 h) and at the end of the experiment (time 96 h). No fish specimens died throughout the experimental period.

The Mn concentration of 0.5 mg/L was chosen because it is the maximum concentration allowed in freshwater bodies of class II by the CONAMA (National Environment Council) resolution 357/05 (Brazil 2005). The concentration of 5.23 mg/L was chosen for its previous usage in other assays on the same species (Coppo et al. 2018) and another animal species (*Lithobates catesbeianus*: Anura) (Veronez et al. 2018). The two Fe concentrations used (3.81 and 7.62 mg/L) were based on the maximum value (5.0 mg/L) allowed by CONAMA, with one lower and one higher than allowed (Veronez et al. 2018).

After the experimental period, the specimens were sedated with a Benzocaine solution (0.1 g/L), submitted to weight and dimension measurements, and had their blood collected by puncturing the caudal vein to perform the genotoxic assays. They were euthanized by cervical section and the tissues (liver and gills) were stored at -80°C (Ultra Freezer CL 120 – 80 V) until the biochemical analyses.

2.3 Genotoxic analyses

2.3.1 Micronucleus Test

The micronucleus test was performed according to Grisolia et al. (2005). After blood was drawn via a caudal puncture, a blood smear was made on microscopic slides. After drying, the slides were fixed with methanol and stained with 5% Giemsa for 40 min. The material was observed under a microscope to count the micronucleus in red blood cells (1000 cells per slide), with two slides per individual. The micronucleus identification was carried out according to the criteria proposed by Fenech et al. (2003) and the average micronucleus frequency (‰) in each treatment was calculated.

2.3.2 Alkaline Comet Assay

The comet assay was performed under alkaline conditions and stained with silver nitrate according to Tice et al. (2000). The slides were previously coated with 1.5% agarose. The blood samples were diluted in an RPMI solution and mixed with low melting agarose. The slides passed through the electrophoresis

phase, in an electrophoretic buffer, followed by a 15-min electrophoretic run at 25 V and 300 mA. After the run, the slides were neutralized with TRIS buffer and placed in a fixative solution. Finally, they were hydrated and stained with silver nitrate. The comets' identification followed the criteria of Collins (2004) according to the shape and size of the tail (where the size of the tail is proportional to the number of DNA fragments). The cells were observed under an optical microscope at 40-fold magnification, and 100 cells per slide (two slides for each individual) were counted, classifying comets in classes from 0 (undamaged) to 4 (maximum damage).

2.4 Biochemical analyses

The branchial and hepatic tissues were homogenized with phosphate buffer (pH 7.0) and centrifuged (3030 g) for 30 min at 4°C to obtain the supernatants for the biochemical analyses.

Glutathione S-transferase activity (E.C. 2.5.1.18) was determined using phosphate buffer (pH 7.0), 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 1 mM glutathione (GSH) as substrate. Its activity was calculated by the absorbance reading obtained in a microplate reader at 340 nm. The absolute activity was estimated using the CDNB extinction coefficient (Habig and Jakoby 1981). The results obtained were expressed in g fresh tissue/min.

Catalase activity (E.C. 1.11.1.6) was assessed by observing the continuous decrease in hydrogen peroxide (H_2O_2) concentration according to Aebi (1984). Buffer was used as a reaction medium with 10 mM H_2O_2 and TE buffer (1 M Tris-HCl and 5 mM EDTA). The samples were read in a spectrophotometer at a wavelength of 240 nm. The results were expressed in μmol H_2O_2 metabolized/min/g of fresh tissue.

2.5 Concentrations of Iron and Manganese in the water

Water samples were collected in each experimental aquarium after contamination (0 h) and at the end of the experimental period (96 h). The samples were acidified with 10% of the total volume of the samples with nitric acid (65%) for dissolved Fe and Mn analyses. The samples were then filtered through pre-cleaned, non-sterile 13-mm filters, with 0.45-μm pores (Analytical). They were read on an Atomic Absorption Spectrophotometer (AAS) operating in flame mode (Thermo Fisher Scientific ICE3500, Waltham, MA, USA). For Fe, the limit of detection (LOD) was 3.12 μg/L, and the limit of quantification (LOQ) was 9.47 μg/L. The Mn presented LOD of 5.36 μg/L and LOQ of 16.25 μg/L.

2.6 Statistical analyses

Data normality was analyzed by a Shapiro-Wilk test. The catalase enzyme activity data were normalized by log10-transformation. The results for biochemical analyses, comet assay, and water physicochemical parameters were compared between treatments, and their differences detected by One-Way Analysis of Variance (ANOVA), followed by Tukey post-hoc test for multiple comparisons. The micronucleus test was analyzed by Dunnet's post-test. All statistical analyses were performed on the SigmaPlot 12.5 software and statistical significance was considered when $p < 0.05$.

3. Results

3.1 Metal concentration and water physicochemical parameters

At the beginning of the experiment, the actual dissolved Mn concentrations were 0.12 mg/L for T1 (control group), 0.22 mg/L for T2, and 3.49 mg/L for T3. For dissolved Fe, the concentrations were 0.45 mg/L for T1, 2.60 mg/L for T2, and 4.40 mg/L for T3. At the end of the experimental period, Mn concentrations were 0.03 mg/L for T1, 0.07 mg/L for T2, and 1.23 mg/L for T3. For Fe, concentrations were 0.54 mg/L for T1, 2.26 mg/L for T2, and 3.71 mg/L for T3.

Throughout the experimental period, dissolved oxygen, temperature, pH, hardness, alkalinity, nitrite, and ammonia remained constant, ensuring good water quality. Electrical conductivity presented a higher value in T3 when compared to T1 and T2 (Table 1).

Table 1
Physicochemical parameters and metal concentration of the experimental water carried out with specimens of *Oreochromis niloticus*, after the period of exposure to Fe + Mn for 96 h. Data are presented as mean ± standard deviation and different letters indicate statistically significant differences. T1 = control group (without metal addition); T2 = 3.81 mg/L of Fe + 0.5 mg/L of Mn; T3 = 7.62 mg/L of Fe + 5.23 mg/L of Mn.

Parameters	T1	T2	T3	*P*
Dissolved Oxygen (mg/L)	7.34 ± 0.50^{a}	7.22 ± 0.30^{a}	7.06 ± 0.37^{a}	0.37
Temperature (°C)	25.60 ± 0.18^{a}	25.40 ± 0.34^{a}	25.80 ± 0.40^{a}	0.11
pH (units)	7.20 ± 0.15^{a}	7.20 ± 0.21^{a}	7.20 ± 0.11^{a}	0.69
Conductivity (µS/cm)	172.60 ± 14.90^{a}	178.20 ± 16.40^{ab}	197.80 ± 22.10^{b}	0.02
Hardness (mg $CaCO_3$/L)	5.02 ± 0.70^{a}	4.62 ± 0.30^{a}	4.65 ± 0.45^{a}	0.22
Alkalinity (mg$CaCO_3$/L)	1.22 ± 0.90^{a}	1.66 ± 0.90^{a}	1.04 ± 0.70^{a}	0.33
Nitrite (mg/L)	0.009 ± 0.006^{a}	0.004 ± 0.002^{a}	0.006 ± 0.003^{a}	0.59
Ammonia (mg/L)	1.34 ± 0.33^{a}	1.67 ± 0.25^{a}	2.09 ± 0.93^{a}	0.12
Manganese (mg/L)	0.12 ± 0.02^{a}	0.22 ± 0.11^{ab}	3.49 ± 0.74^{b}	< 0.001
Iron (mg/L)	0.45 ± 0.03^{a}	2.60 ± 0.64^{b}	4.40 ± 0.47^{c}	< 0.001

3.2 Genotoxic analyses

Exposure to the association of Fe + Mn in *O. niloticus* individuals induced a significant increase in the frequency of micronucleated erythrocytes ($p \leq 0.001$). There was an increase in micronucleus incidence

by 11 times, for T2 compared to the control group, and 20 times for T3 compared to the control group (Fig. 1). The DNA damage index (DI) of the fishes' erythrocytes was high in the two treatments exposed to Fe + Mn. There was an increase of about 8 times for T2, and 22 times for T3, both concerning the control group ($p \leq 0.001$). T3 individuals were mainly classified as class 3 (severe damage) or 4 (very severe damage) in the comet assays (Fig. 2).

3.3 Biochemical analyses

The CAT activity had no significant differences in the gills ($p = 0.26$) (Fig. 3A) or the liver ($p = 0.75$) (Fig. 3B) of the fishes between treatments. On the other hand, the GST activity showed a significant increase in the liver samples ($p = 0.03$) in specimens exposed to the highest concentrations tested (T3) (Fig. 3D). The GST activity in the gills did not present significant differences ($p = 0.43$) (Fig. 3C).

4. Discussion

In the present study, there was an increase in the hepatic GST activity at the highest concentrations of metals. Furthermore, genotoxic damages were also detected in the two tested concentrations of Fe + Mn (T2 and T3). Similar results are reported by Coppo et al. (2018) and Passos et al. (2020) that found changes in biochemical, genetic, and physiological functions in *O. niloticus* exposed to high concentrations of Mn and in *Astyanax lacustris* (Characidae) exposed to the Doce River water. The exposure of aquatic organisms to metals, in addition to causing ionic and osmotic disturbances in some species, can alter aerobic and energetic metabolisms and induce the generation of reactive oxygen species (ROS), causing important oxidative damage to biomolecules, such as lipids, proteins, and DNA (Jijie et al. 2020). *O. niloticus* is a species of worldwide importance in aquaculture and, because of that, it is possible to understand not only the possible damage to the biota but possible risks to human health as well. Several authors have studied the effects caused by various contaminants in tilapias (Alkaladi et al. 2020; Ayyat et al. 2020; Chen et al. 2020; Lopes et al., 2020; Mahboob et al. 2020; Mohamed et al. 2020).

The exposure to Fe + Mn induced a significant increase in the frequency of micronucleated erythrocytes. The formation of micronuclei in the exposed tilapia reflects structural problems or chromosomal changes during mitosis; therefore, it is possible to identify the genotoxic potential of chemicals such as metals, even those essential to metabolism (Kample et al. 2018). The same result was found in acute exposure to Mn at concentrations of 3.88 and 7.52 mg/L in the fish species *C. auratus* (Valbona et al. 2018), as well as in a study with exposure to iron oxide (0.3 mg/L) in guppy fish (*Poecilia reticulate*) (Qualhato et al. 2017). Both studies detected the genotoxic potential of isolated metals, and our research, in this way, has been complementing the effects of these metals together.

We detected high levels of DNA damage, with the formation of class 3 and 4 comets in treatments T2 and T3, which are the highest DNA damage levels that can be found. The T3 fishes were the most affected, differing significantly from the other groups. Coppo et al. (2018) found that isolated Mn harms the replication of genetic material in *O. niloticus*. A study on the exposure of a guppy fish to iron oxide identified comet formation from short experimental exposures (3 and 7 days) to longer ones (14 and 21

days) (Qualhato et al. 2017). With these extents of damage, consequently, there is interference in the accuracy of the genetic material replication in the fish organism. Therefore, the comet assay is an important biomarker for checking acute DNA changes in the presence of Fe and Mn (Hariri et al. 2020). Both analyzes, micronucleus test and comet assay, proved to be efficient to evaluate the effects of these two metals together in *O. niloticus*, showing good biomarkers for this purpose.

GST is an enzyme of fundamental importance in protecting organisms from environmental stressors. Its activity may increase or decrease when exposed to metals, depending on the concentration and the period of exposure (Guilherme et al. 2008). The increase in the hepatic GST activity may indicate the onset of the organism's detoxification process against the metal, since, this enzyme participates in the biotransformation and conjugation of xenobiotic, and the liver plays an important role in metabolizing contaminants (Landi 2000; Moniruzzaman et al. 2020). Studies corroborate our results, through the detection of changes in aquatic organisms exposed to metals (i.e., Fe and Mn). Valbona et al. (2018), found a significant increase in GST activity in specimens of *Carassius auratus*, as well as Veronez et al. (2018) reported an increase in GST in liver tissues of tadpoles exposed to Fe, Mn, and iron ore. Thus, GST is a good biomarker to assess the degree of impact and the effects caused by Fe + Mn in fish and may contribute to the understanding of the mechanisms of action of these compounds in the face of environmental variation. On the other hand, CAT activity did not change in any treatment for both organs and hence cannot be considered a contamination biomarker for these associated metals in the gills and liver of *O. niloticus*. Other metabolic routes may have been activated (Pandey et al. 2003).

Even at low concentrations, associated Fe + Mn was potentially dangerous to fish specimens in the present study. Despite the importance of studying the effect of Fe and Mn together, especially due to the composition of iron ore, few studies portray their synergistic effects on fish. The rupture of the iron mining tailings dam in Mariana, Brazil released several toxic elements in the environment, such as Fe and Mn. According to Queiroz et al. (2018), seven days after the Fundão dam burst (in 2015), the concentration of Fe and Mn found in the sediment of the Doce River estuary were 34,900 and 586 mg/kg, respectively. In 2018, three years after the disaster, metal concentrations remained high, with 26,450 mg of Fe/kg and 1075 mg of Mn/kg (Passos et al. 2020). Thus, with resuspension events in the sediment, which are frequent in rivers, the metals associated with the sediment particles can become bioavailable again in the water column, contaminating the biota present in the river (Queiroz et al. 2018). Hence, understanding how these metals work together is extremely necessary. In general, mining activities are very damaging to ecosystems and the biota present, and in an accident, there is a very high risk of altering the food chain, with persistent damage to local biodiversity in the long term (Espindola et al. 2016).

5. Conclusion

In conclusion, associated Fe + Mn was toxic to the analyzed fish (*O. niloticus*) even at low concentrations. There was micronucleus formation in erythrocytes and damage in the genetic material, in addition to an increase in hepatic GST activity. These metals constitute the iron ores and remain present at high

concentrations in the Doce River after the disaster that occurred with the rupture of the tailings mud dam. Therefore, organisms that are present in ecosystems contaminated by these metals can suffer deleterious damage to their genetic material, cells, and systems.

Declarations

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Availability of data and material

The data analyzed during the current study are available from the corresponding author.

Credit authorship contribution statement

Larissa Souza Passos: Conceptualization, Investigation, Formal analysis, Data curation, Writing - original draft. Gabriel Carvalho Coppo: Investigation, Formal analysis, Data curation. Tatiana Miura Pereira: Investigation, Formal analysis. Barbara Teixeira Chisté: Investigation, Formal analysis. Julia Merçon: Investigation, Formal analysis. Taciana Onesorge Miranda Lopes: Investigation, Formal analysis. Adriana Regina Chippari Gomes: Conceptualization, Methodology, Writing - review & editing, Funding acquisition.

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Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval

The experiment was carried out with the approval of the Ethics, Bioethics, and Animal Welfare Commission (CEUA – UVV; 371-2016).

Consent to participate

Written informed consent was obtained from individual participants.

Consent to Publish

Participants consent to the publication of the data.

Plant reproducibility

Not applicable.

Clinical Trials Registration

Not applicable.

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Figures

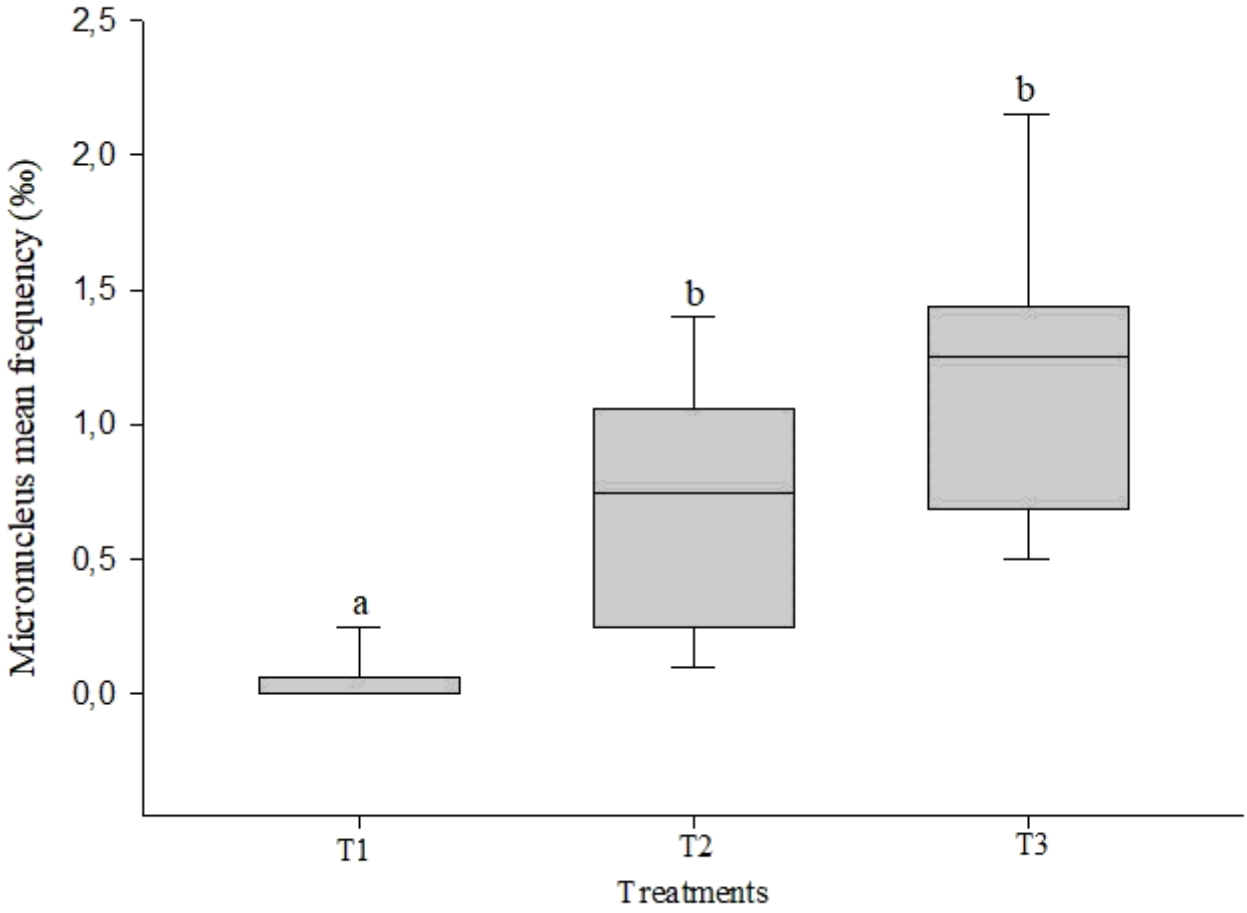

Figure 1

Micronucleus mean frequency (‰) in Oreochromis niloticus exposed to different concentrations of Fe and Mn during a 96-h exposure: T1 = without metal addition, T2 = 3.81 mg/L of Fe + 0.5 mg/L of Mn, and T3 = 7.62 mg/L of Fe + 5.23 mg/L of Mn. The results obtained were: T1: 0.06 ‰ ± 0.12; T2 0.69 ‰ ± 0.18; and T3 1.22 ‰ ± 0.20 of micronucleus. Different letters indicate significant differences ($p < 0.05$).

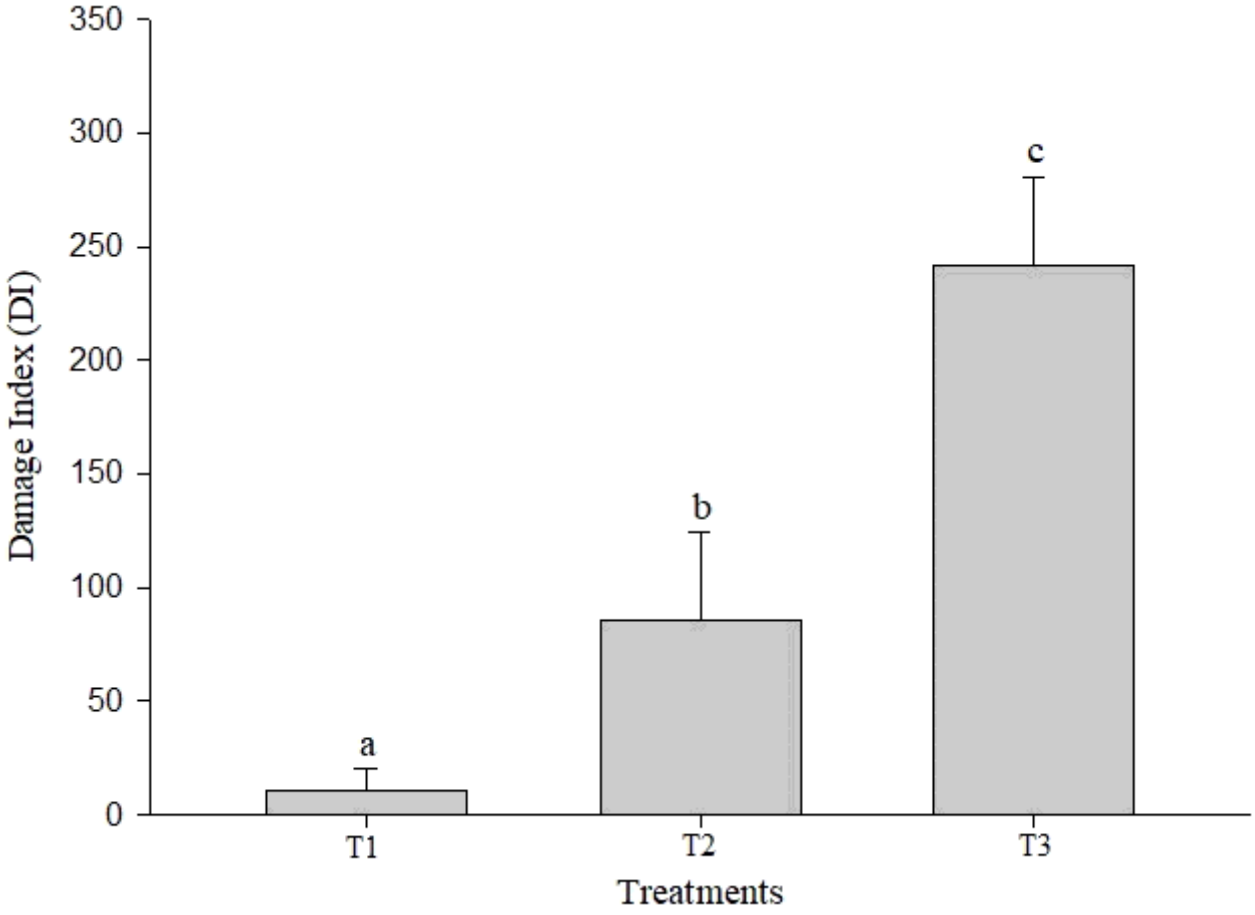

Figure 2

DNA damage index (DI) in Oreochromis niloticus exposed to different concentrations of Fe and Mn during a 96-h exposure: T1 = without metal addition, T2 = 3.81 mg/L of Fe + 0.5 mg/L of Mn, and T3 = 7.62 mg/L of Fe + 5.23 mg/L of Mn. The results obtained were: T1: 10.69 ± 9.76; T2: 85.94 ± 38.15; and T3: 241.81 ± 38.41. Data are presented as mean and standard deviation and different letters indicate significant differences ($p < 0.05$).

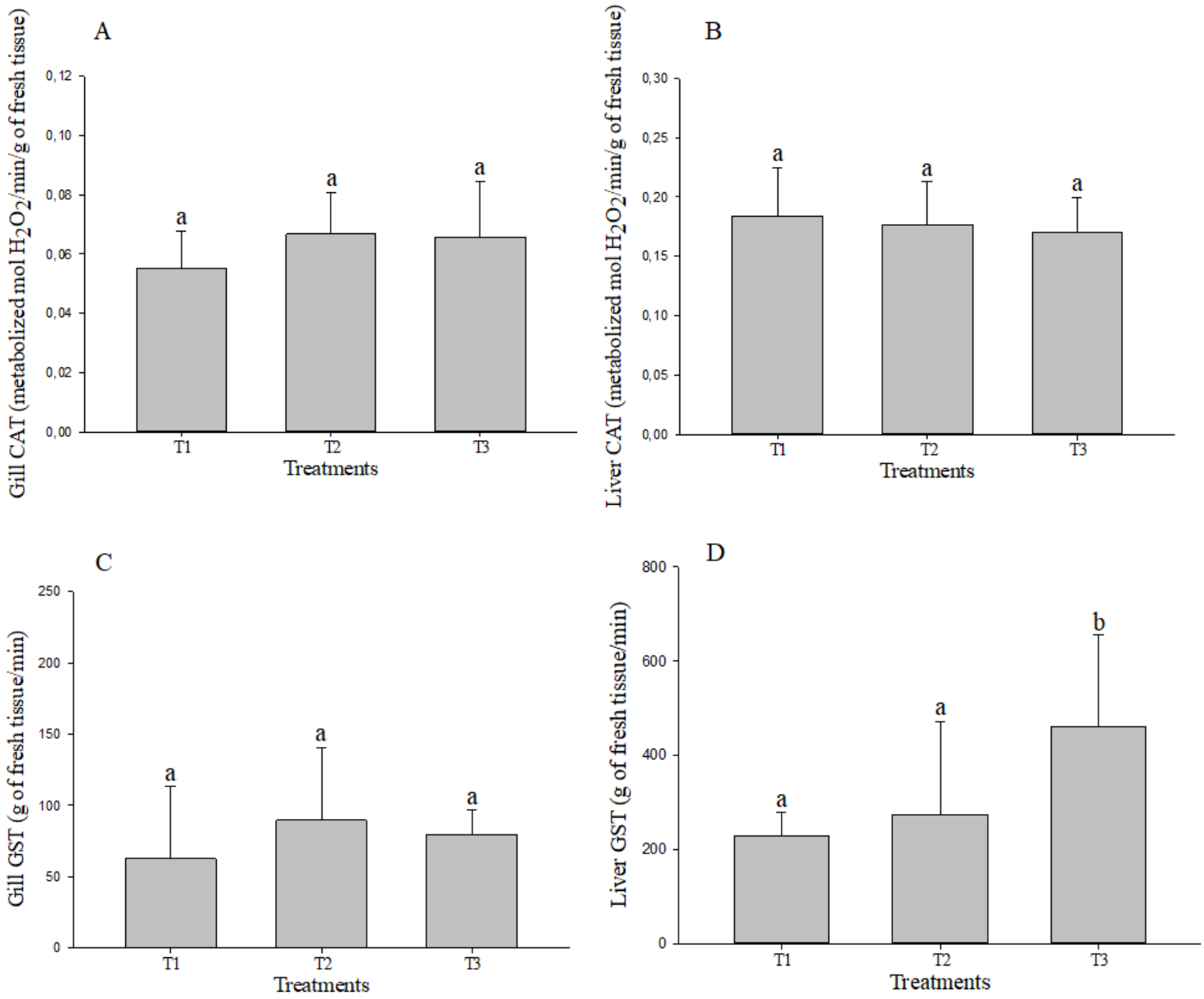

Figure 3

CAT enzyme activity in the gill (A) and the liver (B), and GST enzyme activity in the gill (C) and the liver (D) of Oreochromis niloticus exposed to different concentrations of Fe and Mn: T1 = without metal addition, T2 = 3.81 mg/L of Fe + 0.5 mg/L of Mn, and T3 = 7.62 mg/L of Fe + 5.23 mg/L of Mn, for a 96-h period. The results are expressed as mean and standard deviation. Different letters indicate significant differences ($p < 0.005$).