Co-exposure to titanium dioxide nanoparticles (NpTiO$_2$) and lead at environmentally relevant concentrations in the Neotropical fish species *Hoplias intermedius*

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ARTICLE INFO

ABSTRACT

Growing production and utilization of titanium dioxide nanoparticles (NpTiO$_2$) invariably lead to their accumulation in oceans, rivers and other water bodies, thus increasing the risk to the welfare of this ecosystem. The progressive launch of these nanoparticles in the environment has been accompanied by concern in understanding the dynamics and the toxic effect of these xenobiotic in different ecosystems, either on their own or in tandem with different contaminants (such as organic compounds and heavy metals), possibly altering their toxicity. Nevertheless, it remains unknown if these combined effects may induce damage in freshwater organisms. Therefore, this study aimed to analyze the consequences caused by NpTiO$_2$, after a waterborne exposure of 96 h to a Neotropical fish species *Hoplias intermedius*, as well as after a co-exposure with lead, whose effects for fish have already been well described in the literature. The characterization of NpTiO$_2$ stock suspension was carried out in order to provide additional information and revealed a stable colloidal suspension. As a result, NpTiO$_2$ showed some genotoxic effects which were observed by comet assay in gill, kidney and brain cells. Also, the activity of brain acetylcholinesterase (AChE) has not changed, but the activity of muscle AChE decreased in the group exposed only to PbII. Regarding the hepatic antioxidant system, catalase (CAT) did not show any change in its activity, whereas that of superoxide dismutase (SOD) intensified in the groups submitted only to PbII and NpTiO$_2$ alone. As for lipid peroxidation, there was a decrease in the group exposed to the NpTiO$_2$ alone and to the co-exposed group (NpTiO$_2$ + PbII). As far as metallothionein is concerned, its concentration rose for the co-exposed group (NpTiO$_2$ + PbII) and for the group exposed to PbII alone. Overall, we may conclude that NpTiO$_2$ alone caused DNA damage to vital tissues. Also, some impairment related to the antioxidant mechanism was described but it is probably not related to the DNA damage observed, suggesting that the genotoxic effect observed may be due to a different mechanism instead of ROS production.

1. Introduction

Due to the growing number of applications of nanomaterials, manufactured nanoparticles may enter the aquatic environment in large amounts [1]. The use of manufactured nanoparticles grows on a global scale every year, and an increasing in the nanotechnology business is expected at an annual ratio of around 17.5% from 2016 to 2022 (Global Nanotechnology Market [2]). Manufactured nanomaterials are described as nano-components having at least one of their dimensions

between 1 and 100 nm [3]. Titanium dioxide nanoparticles (NpTiO$_2$) are a group of manufactured nanomaterials with unique physiochemical properties on nanoscale, such as a high surface area, conductivity, chemical reactivity and penetration capacity [4–6] which differ from macroscopic bulk materials chemically similar in composition. NpTiO$_2$ are one of widely used nanoparticles at present, found in a considerable amount of consumer products, like cosmetics, sunscreens, catalysts, dyes and food colors [7] biomedical applications [8] as well as due to the emission of nanoparticles in the air by motorcycles, diesel powered

cars and metallurgical industries [9], which renders their presence in the aquatic environment inevitable [3].

Although studies about the toxicity of NpTiO$_2$ have been published, their results are uncertain and conflicting [10]. Also, making associations between different studies is a challenge within toxicology as there may be variations in: (a) sizes of nanoparticles; (b) size distribution; (c) purity of the nanomaterials utilized; (d) material surfaces with the same average size; (e) materials which may coat the nanoparticles; (f) crystalline structure; (g) sizes of aggregates formed in suspension/environment; (h) tests carried out, and, finally, (i) concentrations of nanomaterials utilized for each assay [11]. In addition, the extracellular microenvironment content may critically affect the behavior of NPs and their communication with cellular membranes. With regard to this, so far there is restricted data as to, for example, how various biomolecules mediate the interaction between these materials and the assembly of extra cellular matrix (ECM). ECM altered molecules, in turn, may affect cell differentiation, the immune cell response and cell longevity, as well as the inflammatory process [12].

Furthermore, as well as other metallic nanoparticles, NpTiO$_2$ are capable of adsorbing other chemical substances (which may be toxic) in their surface, and by doing so may interfere with the availability of both compounds in water [13]. Once they reach the aquatic environment, nanoparticles may interact with other components – not only those which are particulate or dissolved organic matter, but also those that are inorganic ions, such as metals, or hydrophobic organic contaminants [14,15]. Zhang et al. [16] and Sun et al. [17] confirmed that hypothesis by carrying out experiments with NpTiO$_2$ co-exposed with cadmium and arsenic, respectively, indicating an interaction between these substances and the nanoparticles, considering the increased absorption and accumulation of these substances in muscles of *Cyprinus carpio*.

On account of the lack of adequate protocols for NPs assessment in water as well as in any other matrix, only predictions and estimates were available for the presence of NPs in natural environments [18]. According to these predictions, NpTiO$_2$ may be found in freshwater bodies in concentrations varying from 3 ng L^{-1} through 1.6 mg L^{-1}.Similarly, temporal variation due to alterations in water levels showed variations up to a factor of 10 [1]. In addition, environmental studies with NPs are still at the beginning as it remains troublesome to monitor their presence and action in water bodies, where NPs are known to bind together into bigger agglomerates [19] and combine with living and non-living subjects. This binding property might lead to the alteration of NPs scale and precipitation characteristics [20,21]. In addition, despite a stimulus to the development of nanotechnologies in Latin America, no specific legislation was introduced to regulate the use of nanoparticles. Also, no specific regulations for the limits of detection in surface waters have been published by the Latin America government to date.

Despite the increased regulation over its use by many countries [22] lead is still employed on a large scale in the manufacturing process of the most diverse products [23]. The pollution caused by this metal is endless and on a large scale across the globe, affecting both human population and aquatic environment [23]. For fish species, adverse health effects (*e.g.* antioxidant imbalance and osmoregulatory dysfunctions) brought about by lead have been widely reported, but new findings on the carcinogenic, mutagenic and clastogenic characteristics of inorganic lead compounds are surfacing [24–26]. Hong et al. [27] found evidence of lead associating with DNA through a covalent bond between Pb^{2+} ion and DNA molecule.

Since fish depend exclusively on the aquatic environment to survive, any disturbance in this environment may be crucial for the survival of fish species [28]. In general, toxicity studies are conducted with a view to creating a cause/effect association between the contaminants. Thus, it is vital to conduct these tests with numerous species so we may reach a broad sensitivity spectrum, mainly when it comes to different fish communities in relation to specific contaminants [29]. Although

standard toxicity assays with water species may be useful for purposes of regulation, it is worthy of note that such usefulness depends on the choice of representative species from selected areas, with relevant ecological role [29].

Hoplias intermedius species [30], popularly known as *traíra*, is a freshwater carnivore, extensively dispersed throughout South America. That species, similarly to others from the same genus, is an appropriate test organism in assessing noxious effects of contaminants inasmuch as it is a predator, may be used in bioaccumulation studies, does not migrate and may be maintained in laboratory conditions [24,26,31–35].

Owing to the economic (commercial fishing) and social (subsistence fishing) relevance of *Hoplias intermedius*, as well as to the marked rise in production, consumption and discharge of NpTiO$_2$ in freshwater bodies, which had been previously exposed to the presence of other compounds, our project seeks to analyze the genotoxic and biochemical consequences of titanium dioxide nanoparticles and their associated waterborne exposure to inorganic lead in *Hoplias intermedius*.

2. Material and methods

2.1. Bioassays

Commonly known as "traíra", *Hoplias intermedius* (Characiformes, ERYTRINIDAE) [30] was chosen as a test organism for this study. The juvenile specimens were donated from the Hydrobiology and Fish Farming of Furnas (São José do Rio da Barra, state of Minas Gerais – Brazil), as this fish farming station has no sources of anthropogenic contamination.

Stock animals were acclimatized for approximately 40 days in tanks with 250 L capacity, filled with aerated and dechlorinated tap water, with precise photoperiod (12 h) and fed to satiation. In total, 73 juvenile specimens were utilized, of both sexes (43 females and 29 males), weight averaging 11.14 ± 1.361 g (mean ± SEM) and length of 18.62 ± 5.767 cm (mean ± SEM) on average. The animals were divided as follows: 18 fish for the negative control group (CN), 10 fish for the positive control group (CP) and 15 fish for each treatment (titanium dioxide nanoparticles - NpTiO$_2$, inorganic lead – Pb II, and a co-exposure group of those two contaminants - NpTiO$_2$ + PbII), adding up to 5 experimental groups.

After the acclimatization period, the fish were separated into five glass aquariums with a maximum capacity of 108 L, containing the appropriate number of specimens of H. *intermedius*. Having been acclimatized for a week in aquariums under the same conditions of aeration and photoperiod, the animals only received food in the first 3 days. Within 7 days of the acclimatization period, the experiment was started. During the bioassay, the fish did not receive any food so that we could prevent NpTiO$_2$ adsorption to the food or fecal material, also to prevent loss of water quality [36]. The average temperature of the aquarium was 23.09 ± 1.432 °C (mean ± SD) and the average concentration of dissolved oxygen was 8.332 ± 0.5626 mg.L^{-1} (mean ± SD) (Table 1).

The animals were divided as follows: 18 fish in the NC (negative control group) - no contaminant was added - 10 fish in the PC (positive control group) – in which 5 µg of methyl methanesulfonate (MMS)/g of animal weight was injected intraperitoneally 24 h before the end of the experiment, as seen in Piancini et al. [37] - and 15 fish for each treatment group (titanium dioxide nanoparticles - NpTiO$_2$, inorganic lead – Pb II, and a co-exposure group of those two contaminants – NpTiO$_2$ + PbII), adding up to 5 experimental groups. The fish were then submitted to waterborne exposure for a period of 96 h in semi-static condition, in which two thirds of filtered and dechlorinated tap water were renewed and re-dosed every 24 h. In the group exposed to NpTiO$_2$, its concentration amounted to 100 mg.L^{-1}, while in that submitted to lead, its concentration amounted to 0.033 mg.L^{-1}. For our co-exposure group, both contaminants, NpTiO$_2$ and lead, were utilized simultaneously with the same concentration above (see below for stock

Table 1

Average temperature and average rate of oxygen dissolved in the aquarium for each treatment (Negative Control - NC, NpTiO₂, NpTiO₂ + Pb II, Pb II, and Positive Control - PC) measured during waterborne experiment (96 h) (mean ± SD).

Temperature (˚C)					
NC	NpTiO$_2$	NpTiO$_2$ + PbII	Pb II	PC	Total
23.48 ± 1.95	22.60 ± 0.67	22.73 ± 1.02	23.46 ± 1.80	22.98 ± 1.55	23.09 ± 1.43

Dissolved oxygen (mg/L)					
NC	NpTiO$_2$	NpTiO$_2$ + PbII	Pb II	PC	Total
8.06 ± 0.73	8.40 ± 0.49	8.68 ± 0.15	8.40 ± 0.46	8.18 ± 0.78	8.33 ± 0.56

solutions). The experiment was carried out according to the Federal University of Paraná's Committee of Ethics for Animal Use, under certificate number 791. No fish mortality was reported during this experiment.

To sample, specimens of H. *intermedius* were anaesthetized with benzocaine (Sigma -Aldrich®, Ref: E1501) diluted in 10% ethanol, concentrated at $20 \, mg.L^{-1}$ [38]. Peripheral blood was sampled *via* caudal vein and then one blood smear slide was performed to each sample for Piscine Micronucleus Test analysis (PMT) and the remaining sampled blood was collected in microtubes containing 1.0 mL of fetal bovine serum (FBS), which were stored under refrigeration and in the dark, for the comet assay to be performed. After blood sampling, we euthanized the fish by means of spinal cord section, then weighed them and measured them lengthwise. After that, samples were taken from renal, liver, gill, brain and gonadal tissues and then placed in microtubes containing 500 μL of FBS and stored under refrigeration, in the dark, for the subsequent comet assay. A portion of the gonadal tissue was placed in another microtube containing 10% formaldehyde for subsequent sexing of each sample. Samples of the brain and liver, as well as a portion of the muscles were excised and stored in microtubes at −80 ˚C for biochemical assays.

2.2. Preparation of suspensions and dosing

NpTiO₂ (Sigma-Aldrich® - Ref: 637254 – 21 nm) were acquired as a white powder. We chose this specific product for we had employed the exact same particles in previous studies with fish [e.g. [6,39,40]]. According to the maximum concentration recommended by the OECD [41], we opted for a suspension of 100 mg of NpTiO₂/L, as concentrations above that would have no environmental applicability [39]. The suspension was sonicated during 30 min in ultrasonic bath (Schuster®, U-100) at a frequency of 42 kHz prior to each daily dosing.

In order to characterize the nanoparticles, we utilized TEM (transmission electron microscopy) images, as well as X-ray diffraction to verify the crystalline composition of the nanomaterial purchased from the supplier. For the calculation of the specific surface area, we utilized Quantachrome® porosimeter and carried out the method described by Brunauer-Emmett-Teller (Theory of multimolecular adsorption - BET method). Additionally, we conducted an analysis of porosity of the surface of the nanoparticles. The XPS technique (X-ray Photoelectron Spectroscopy) was also utilized for a more accurate investigation of the chemical structure from nanoparticles surface.

The characterization of the nanoparticle suspension was performed with Zetasizer® Nano Series ZS90 (manufactured by Malvern Instruments) which revealed the zeta potential (ζ), average hydrodynamic particle size as well as the index of polidispersity. The measurements were performed at 25 ˚C A polystyrene cuvette was employed for the analysis of the hydrodynamic size and polidispersity index. Each examination lasted on average 1 m 45 s and was conducted with a detection angle of 90˚ and red lasers of 633 nm. To determine the zeta potential (ζ), we placed the samples in the electrophoretic cell, in which we established a potential of ± 150 mV and averaged 12 runs per

sample. By means of the polydispersity index it was possible to calculate the percentage (%) of polydispersity [% Polydispersity (% Pd) = (PdI) ½×100], to verify the homogeneity of the particle sizes in colloidal suspension, classified as homogenous suspension when % Pd < 20 [42].

We utilized lead in its form Pb (NO₃)₂ (Vetec®, CAS number: 10099-74-8) at a concentration of $0.033 \, mg.L^{-1}$, according Brazilian law number 357/2005 [43] for waters class 3 (*i.e.* water utilized for human consumption, after undergoing conventional treatment), which establishes this concentration of lead as a maximum to be found in Brazilian waters.

2.3. Genotoxic analysis

2.3.1. Piscine micronucleus assay

According to the procedure employed by Vicari et al. [35], after collecting the blood sample, we smeared a single drop of blood on a slide. After drying overnight, these slides were placed in absolute Ethanol for 25 min, then colored with Giemsa 10% in phosphate buffer (pH 6.8) during 15 min. Analyzes were performed in light microscopy (1000× magnification - Olympus® CX 40), in which 2000 cells were evaluated per blood sample under blind analysis. Circulating erythrocytes were classified according to the descriptions made by Carrasco et al. [44], as described by Ayllon and Garcia-Vazquez [45], summed up then grouped as MN + ENA (micronucleus + erythrocytic nuclear alterations).

2.3.2. Comet assay (single cell gel electrophoresis)

We performed a comet assay with erythrocytes according to the protocol by Singh et al. [46], adapted by Ferraro et al. [24]. The comet assay with gill, kidney, liver, brain and gonadal tissue was executed as described by Ramsdorf et al. [26].

2.3.2.1. Comet assay in erythrocytes. Blood samples were collected and diluted in 1.0 mL FBS. From this dilution we collected 10 μL in cell suspension, which was added to agarose of low melting point (0.5% - Gibco®) at 37 ˚C. This mixture was put on a slide covered with agarose beforehand (NMP - 0.75% - Gibco®) then brought into the refrigerator for 10 min. After the cooling time, we removed the coverslips and placed the slides in a cold lysis working solution (Lysis stock solution: NaOH, 0.8%; NaCl, 2.5 M; Tris, 10 mM ; EDTA, 100 mM; *N*-lauryl-sarcocinate, 1%; Lysis working solution: 89 mL of lysis stock solution; 10 mL DMSO and 1 mL Triton X-100) at 4 ˚C for 24 h. The electrophoretic run was carried out at 1 V/cm within 25 min of adding an alkaline buffer (200 mM EDTA; 10 N NaOH; pH > 13). We then neutralized the samples with a TRIS buffer (0.4 M, pH 7.5), dried and put them in absolute Ethanol for 5 min.

2.3.2.2. Comet assay in fish tissue. Immediately after the removal of tissues (kidney, liver, gills, brain and gonads), we placed a small fraction of each sample tissue in a microtube containing 500 μL of FBS to be stored under refrigeration in the dark until disaggregation. The

fish tissues were then disaggregated with micro homogenizer (Tecnal®, model: TE-103) at 1500 rpm for about 30 s. 30 μL of the homogenate obtained were mixed with agarose LMP, to mount the slides. This procedure is like that employed for the erythrocytes, as described above.

For the visual analysis, we stained the slides with ethidium bromide (25 μL–10 μL/mL - Sigma-Aldrich®) and immediately analyzed in an epifluorescence microscope (400×magnification - Leica® - model: DFC300FX). We analyzed 100 nucleoids per fish. Damage was visually categorized [47] according to the relationship between the length of the 'tail' and the 'head' of the comet. In order to establish the scores, we multiplied the amount of nucleoids found in each class by its number.

2.4. Biochemical analysis

2.4.1. Sample preparation

Portions of the brain ($\pm$ 50 mg) and the axial muscle ($\pm$ 100 mg) were homogenized in a potassium phosphate buffer (0.1 M, pH 7.5) in a proportion of 1 (weight) to 10 (volume). This homogenized tissue was centrifuged for 20 min at 4 °C and 12.000×g. The supernatant was employed in the analysis of the activity of AChE in fish muscle and brain.

A portion ($\pm$ 100 mg) of the liver was homogenized in a potassium phosphate buffer (0.1 M, pH 7) in a proportion of 1 (weight) to 10 (volume). This homogenized tissue was centrifuged for 30 min at 4 °C and 15.000×g. The supernatant was employed to analyze the activity of superoxide dismutase (SOD), catalase (CAT) and to quantify lipid peroxidation (LPO).

Another portion ($\pm$ 80 mg) of the liver was homogenized in Tris-HCl/sucrose buffer (20 mM/500 mM, pH 8.6) in a ratio of 1 (weight) to 5 (volume). This homogenized tissue was centrifuged for 30 min at 4 °C and 15.000×g. The supernatant was employed in the quantification of the metallothioneins (MTs).

2.4.2. Acetylcholinesterase (AChE)

In order to measure AChE activity at 405 nm with 12 mM acetylthiocholine (ATC), we employed the procedure applied by Ellman et al. [48] and adapted for microplate by Silva de Assis [49]. The reading was performed each 15 s for 3 min and measured in $\mu mol.min^{-1}$. mg of $protein^{-1}$.

2.4.3. Catalase (CAT)

In order to measure CAT activity at 240 nm, we utilized 20 mM H_2O_2 as described in the Aebi protocol [50]. The reading of absorbance was performed every 15 s for 1 min and measured in $mmol.min^{-1}$. mg of $protein^{-1}$.

2.4.4. Superoxide dismutase (SOD)

We measured SOD activity at 440 nm by employing a method proposed by Gao et al. [51]. The quantity of SOD to inhibit the reaction of auto-oxidation of pyrogallol by 50% (IC 50) equals 1 unit (U). The results of SOD activity were presented as U of SOD. mg of $protein^{-1}$.

2.4.5. Lipid peroxidation level

We quantified the level of lipid peroxidation for each exposure group with the FOX assay [52] with readings realized at 570 nm. Lipid peroxides final concentration unit was nmol. mg of $protein^{-1}$.

2.4.6. Metallothioneins (MT)

We quantified the concentration of MTs at 412 nm through the method proposed by Viarengo et al. [53], with readings realized by means of standard curves of reduced glutathione (GSH) and 30% considering contents of metallothionein, whose unit of concentration was measured in μg. mg of $protein^{-1}$.

For the presentation of biochemical biomarkers results, the total concentration of proteins for each tissue was measured using the method published by Bradford [54].

2.5. Statistical analysis

We employed the Graph Pad Prism® software for statistical analyses. Data normality was verified with Kolmogorov–Smirnov's test, while Levene's was utilized to check how homogeneous the variances were. If the numbers showed a normal distribution, we utilized the ANOVA (1 criteria) test, followed by post-test T (LSD) for comet assay results and Student-Newman-Keuls post-test for biochemical assay results. If, however, the data did not show a normal distribution, or presented unequal variances, we applied the Kruskal-Wallis test for nonparametric data, then the Student-Newman-Keuls post-test, which establishes possible differences among the groups exposed, as well as between positive and negative control groups. The level of significance accepted was 0.05 ($p < 0.05$).

In order to observe which tissues showed higher DNA damage, we compared them with the test and post-test above (Kruskal-Wallis and Student-Newman-Keuls, respectively). We subtracted the score value of each individual from the average score in the control group, and then adjusted these amounts to make the score value positive. In this case, the gonad tissue was not included as it was not possible to pair these data. Variations between medians are deemed of statistical relevance when p < 0.05.

3. Results

3.1. Characterization of NpTiO$_2$

The crystalline structure which made up the NpTiO$_2$ was formed by 100% of anatase type, consisting of 28.42% titanium and 71.58% oxygen and with a hydrodynamic medium size of 45 nm and 107 nm for aggregate particles. The analysis by the BET method showed a specific surface area of nanoparticles of 83.47 m^2/g. Through transmission electron microscopy it was possible to check the morphological structure of NpTiO$_2$ powder (Fig. 1).

The characterization of the NpTiO$_2$ suspension at 100 mg.L^{-1} revealed that nanoparticles were aggregated with 93.2% of the particles measuring 256 nm in size and 6.8% of them measuring 4216 nm, averaging 191.4 nm in size (Fig. 2). The percentage of polydispersity (Pd%) calculated was 58.22%, which reveals that the suspension is not homogeneous in relation to the size of the particles. The zeta-potential (ζ) calculated for this suspension was +37.4 mV, and thus it may be considered a stable colloidal suspension.

3.2. Genotoxic analysis

3.2.1. Micronucleus assay

Erythrocytic Nuclear Alterations (ENA) and the Piscine Micronucleus test (PMT) did not present alterations in the frequency of
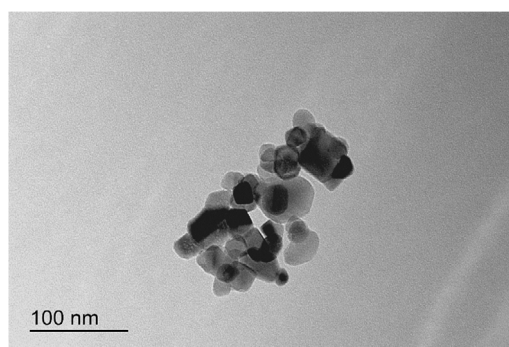

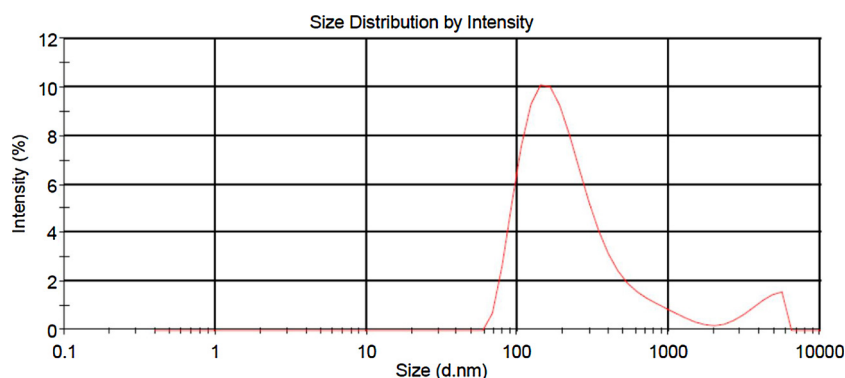**Fig. 1.** Electron micrograph of titanium dioxide nanoparticle (NpTiO$_2$) in stock suspension.

Fig. 2. Distribution of intensity of the particle size in NpTiO$_2$ stock suspension (10 mg/L), measured at 25 °C, showing the average particle size in nanometers. Graphic generated by Zeta Sizer® Nano ZS90 instrument (Malvern Intruments, UK).

micronuclei or in the nuclei of erythrocytes, neither in the contaminated groups nor in the negative control group (Fig. 3a).

There were no differences in the presence of the micronucleus alteration type nor in the other types of nuclear morphological alterations analyzed between the groups (Table 2).

3.2.2. Comet assay

The NC group differed significantly from the group co-exposed to NpTiO$_2$ and lead in the comet assay performed in erythrocytes. The group exposed only to lead, in turn, differed statistically from the group exposed only to NpTiO$_2$ (Fig. 3b).

Through the comet assay in gill tissue, it may be seen that the groups exposed to NpTiO$_2$ (p < 0.001) and the co-exposure group to contaminants NpTiO$_2$+Pb II showed a higher DNA damage in comparison with the NC group (p < 0.0001). Both the group submitted to lead and the NC showed lower levels of DNA damage and no significant differences between them were observed (Fig. 3c).

The comet assay performed in liver tissue revealed no difference between exposed groups in this experiment (Fig. 3d).

For kidney cells, Pb II treated groups (p = 0.0477), NpTiO$_2$+Pb II (p = 0.0405) and NpTiO$_2$ (p = 0.0114), although not differing significantly from one another, showed more marked damage in the DNA than the NC (Fig. 3e).

When analyzing the comet assay in brain tissue, animals co-exposed to NpTiO$_2$+Pb II (p < 0.0001) and those exposed only to NpTiO$_2$ (p = 0.0002) showed higher DNA damage in brain cells, but they did not differ statistically from each other. The group exposed only to lead did not differ from the negative control group (Fig. 3f).

Tissues with the highest DNA damage after waterborne exposure to Pb II were gills, liver, kidney and brain, showing statistically significant differences compared to erythrocytes (p = 0.0007; p < 0.0001; p < 0.0001 and p < 0.0001, respectively). Moreover, the kidney also showed similar DNA damage to the gills (p = 0.0291) (Fig. 4a).

After 96 h of waterborne exposure to NpTiO$_2$, gills showed higher DNA damage in comparison with erythrocytes (p = 0.0003) and liver (p = 0.038). Also, further brain damage could be observed in relation to blood (p < 0.0001), liver (p = 0.0009) and kidney (p = 0.0027) (Fig. 4b).

In the co-exposure group of NpTiO$_2$ and Pb II, we identified more severe brain damage than the gills (p = 0.009), erythrocytes (p < 0.0001), liver (p < 0.0001) and kidney (p = 0.0003). Furthermore, gills and kidney damages (respectively p = 0.0005 and p = 0.0128) were higher than that of erythrocytes (Fig. 4c).

As far as gonadal tissue is concerned, the exposure to NpTiO$_2$ did not lead to an increase in DNA damage in comparison with the NC group. The group co-exposed to contaminants (NpTiO$_2$+Pb II) had an intermediate position with regards to the group exposed to NpTiO$_2$ and that submitted to lead in terms of DNA damage effect, not differing from the negative control (Fig. 5a–c). DNA damage in gonadal tissue

was observed after exposure to Pb II, mainly in female gonads (Fig. 5b).

3.3. Biochemical analysis

Regarding the activity of brain AChE, we did not observe changes in any of the exposure groups (Fig. 6a). However, in the group exposed to Pb II, we observed a decrease in muscle AChE activity in comparison with the groups NC, NpTiO$_2$ and NpTiO$_2$+Pb II (Fig. 6b).

After analyzing the biomarkers of antioxidant mechanism in the liver, we identified a rise in SOD activity within the groups exposed to Pb II and to NpTiO$_2$ alone, in comparison with the NC group and with the one co-exposed to the contaminants (NpTiO$_2$ +Pb II) (Fig. 6c). However, the activity of catalase (CAT) was not altered in either exposure group (Fig. 6d).

The level of LPO increased in the group submitted to Pb II in comparison with those exposed to NpTiO$_2$ and to NpTiO$_2$+Pb II, although no differences were observed in comparison with the negative control (Fig. 6e).

As for the group submitted uniquely to Pb II and the co-exposure group (NpTiO$_2$ +Pb II), there was a rise in MT concentration in comparison with the group submitted to NpTiO$_2$ isolatedly and with the NC group (Fig. 6f).

4. Discussion

Studies describing the outcomes of nanoparticles in living aquatic beings are quite recent. One of the first publications showed significant oxidative imbalance in the brains of *Micropterus salmoides* following exposure to fullerene C$_{60}$ [55]. After this study, many have been the experiments with a variety of manufactured nanoparticles given their increasing application in human products and, consequently, their ubiquity in the aquatic environment in large scales with other contaminants [1,56].

Among all the publications about titanium dioxide nanoparticles, there is a host of results about toxicity due to the different treatments and experimental models applied, as well as to the different physicochemical characteristics of NpTiO$_2$ - case study [57]. At the beginning of our experiments, the stock solution of NpTiO$_2$, as well as the NpTiO$_2$ powder (purchased from Sigma-Adrich®) were characterized to associate the physicochemical properties with the toxic capacity of nanoparticles [58], although there are still uncertainties regarding the relation between the physicochemical parameters of NPs and cellular intake [59]. Due to the need to perform the exposures in aqueous medium, some problems have been described in several ecotoxicological studies, such as the fast aggregation and precipitation of NpTiO$_2$ [60,61]. Aqueous solution pH plays an important role with regards to particle charge and size of aggregates of NpTiO$_2$. Also, in aqueous media, pH of the suspensions is one of the most critical factors affecting its zeta potential (ζ). By definition, zeta potential (ζ) measures
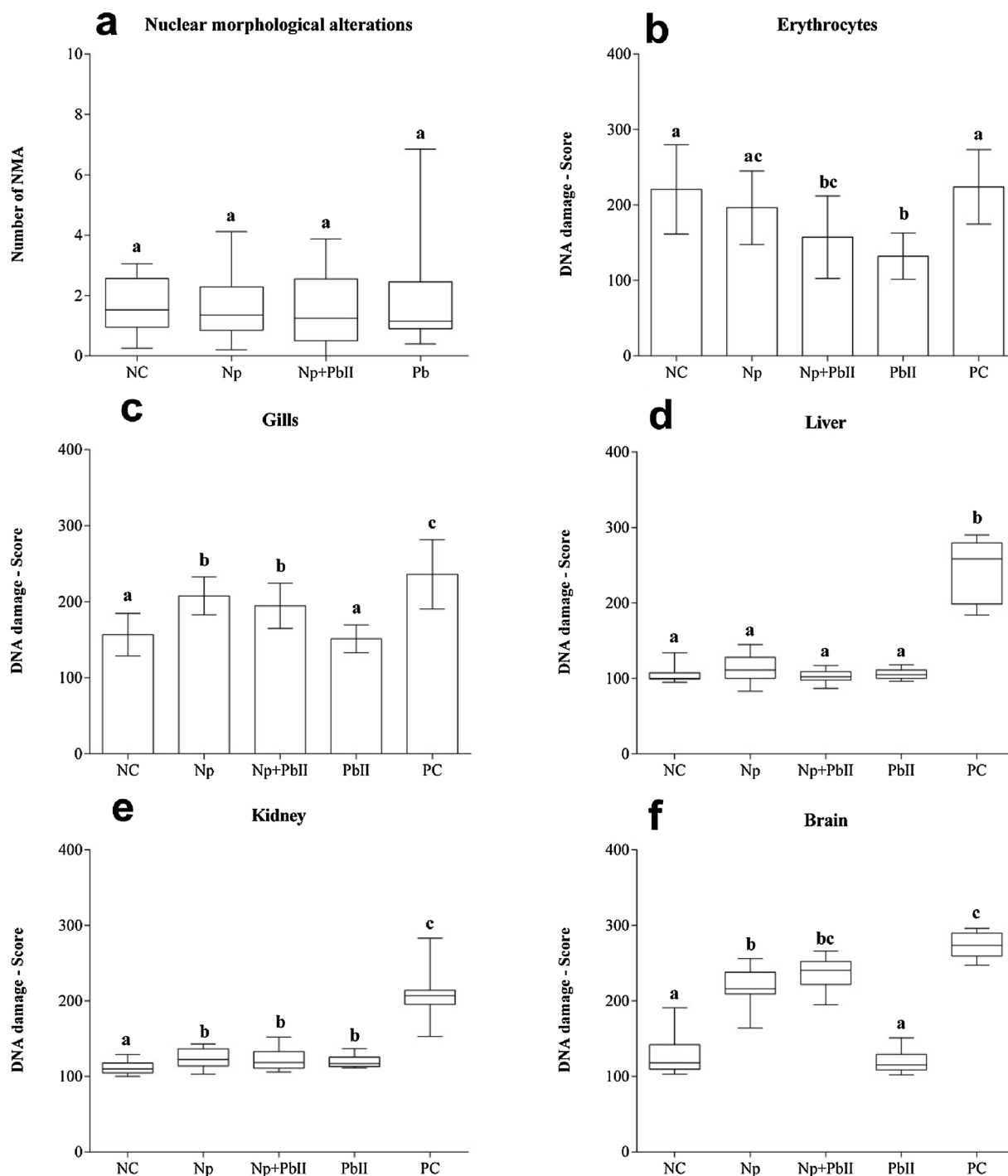
Fig. 3. Genetic biomarkers analyzed for *Hoplias intermedius* species after, after waterborne exposure to contaminants for 96 h in a semi-static regime. In (a) Number of nuclear morphological alterations (Micronucleus - MN + Erythrocytic Nuclear Alterations - ENA); (b) Scores of comet assay in erythrocytes; (c) Scores of comet assay in gill tissue; (d) the test scores comet in liver tissue; (e) score of the comet assay in kidney tissue; (f) score in the brain tissue comet assay. Different letters indicate statistically significant differences (p < 0.05). Data are expressed as median and quartiles.

the magnitude of the electrostatic charges between particles and is among the key parameters believed to affect stability of a colloidal system [58,42]. The pH_{pzc} (when the surface of an oxide possesses the same amount of negative and positive electrical charges) of anatase is 6.3 [62]. In theory, particles with a positive zeta potential (ζ) usually have a more acid pH, whereas pH tends to be basic when the value of this potential is negative [58]. In this study, the stock suspension of nanoparticles presented a zeta potential of +37.4 mV, which means that its stability is characteristic of the colloidal suspension. In addition, it is possible to infer that due to the pH being lower than pH_{pzc}, the

particles of these suspensions were mostly positively charged, thus allowing for a tough interaction with the biological membranes, mainly with the membranes of the gills cells, which in turn allows for the internalization of the particles, probably *via* endocytosis [58,63,3].

Test sample preparation required a sonication time of 30 min prior to the experiment to prevent NpTiO$_2$ from aggregating and precipitating in water [63]. On account of the well-known precipitation and aggregation of nano-TiO$_2$ in water, the authors judged it prudent to change the exposure media each 24 h while bioassays were conducted, in order to keep the exposure conditions constant. Semi-static exposure

Table 2

Frequencies of MN (micronuclei) and ENA (erythrocytic nuclear alterations) found in the experimental groups (Negative Control - NC, NpTiO$_2$, NpTiO$_2$ + Pb II, Pb II, and Positive Control - PC) after 96 h of waterborne exposure (mean ± SD).

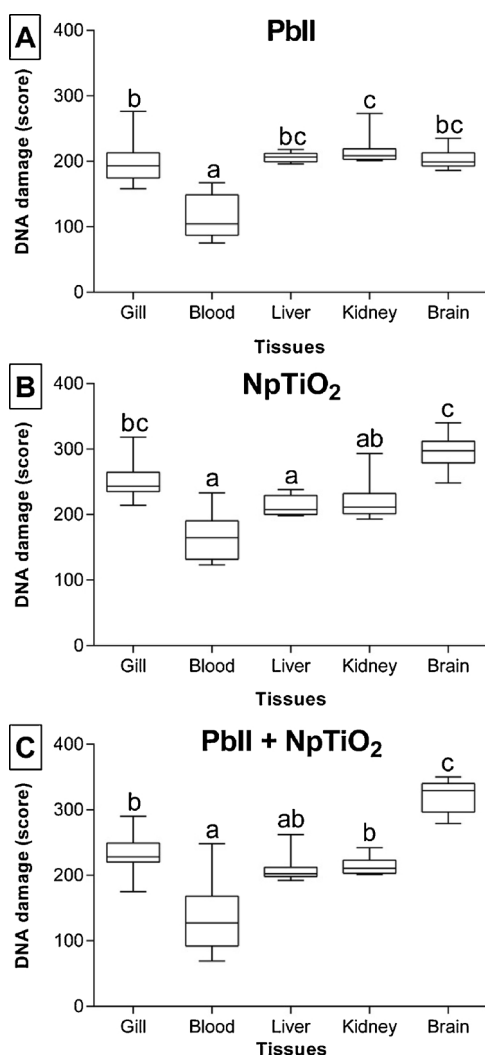|			Frequencies MN and ENA			
Treatments	Micronucleus	Blebbed	Lobed	Notched	Binucleated	Vacuolated
NC	2.00 ± 0.00	2.89 ± 0.03	2.87 ± 0.04	4.95 ± 0.35	2.36 ± 0.03	5.94 ± 0.64
NpTiO$_2$	2.10 ± 0.00	2.60 ± 0.02	3.00 ± 0.05	4.93 ± 0.39	2.43 ± 0.02	5.93 ± 0.77
NpTiO$_2$ + PbII	2.33 ± 0.01	2.33 ± 0.01	3.20 ± 0.05	4.67 ± 0.34	2.80 ± 0.02	5.66 ± 0.82
PbII	2.27 ± 0.02	2.53 ± 0.06	3.03 ± 0.07	5.07 ± 0.74	2.23 ± 0.03	5.86 ± 0.92
PC	2.25 ± 0.00	2.45 ± 0.02	2.85 ± 0.02	5.25 ± 0.40	2.45 ± 0.02	5.75 ± 1.17

Fig. 4. Differences in tissue response for *Hoplias intermedius* species when compared by DNA damage, after waterborne exposure to contaminants for 96 h in a semi-static regime. (A) Group exposed to Pb II; (B) Group exposed to NpTiO$_2$; and (C) Group exposed to NpTiO$_2$ + Pb II. Different lowercase letters indicate statistical difference between tissues, considering p < 0.05.
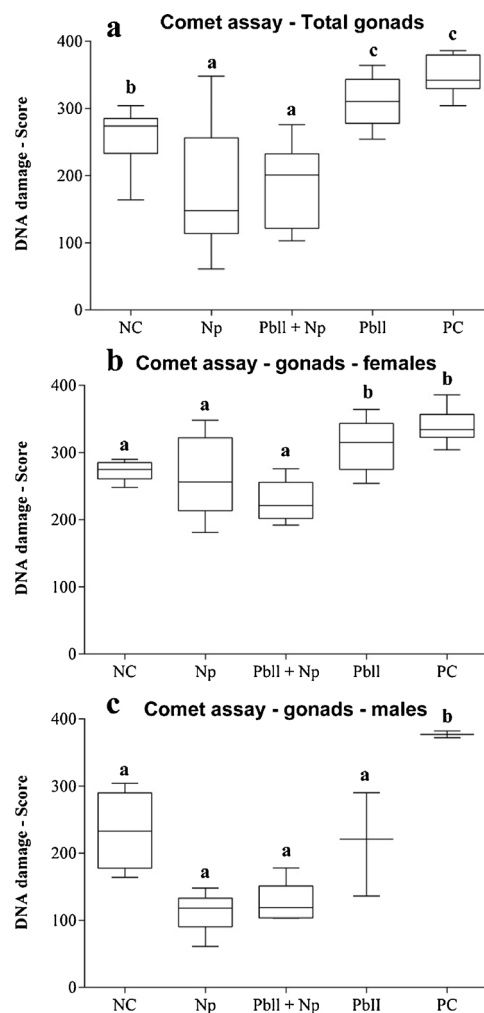

Fig. 5. Comet assay performed in gonadal tissue for *Hoplias intermedius* species after waterborne exposure to contaminants for 96 h in a semi-static regime. In (a) Scores of comet assay for male and female tissues together; (b) Scores of comet assay for gonadal tissue in females; (c) Scores of comet assay for gonadal tissue in males. Different letters indicate statistically significant differences (p < 0.05). Data are expressed as median and quartiles.

protocols were employed in a variety of similar studies including NpTiO$_2$ [64,65] and were recently recommended by Handy et al. [66].

Despite the publications on the effects of NpTiO$_2$, there are few reports in the literature about their synergy with other chemical compounds commonly seen in the aquatic ecosystem, such as metals [16,67,68]. It is known, however, that NpTiO$_2$ are capable of adsorbing to other metals, considered to be toxic (such as copper, for example) and may reduce the availability of metals co-existing in water [13].

In our research, we assessed the toxicity of NpTiO$_2$ (100 mg.L^{-1})

and PbII (0.033 mg.L^{-1}) in isolation and when these contaminants were co-exposed to a Neotropical fish species, *Hoplias intermedius*. The specimens were exposed in a semi-static waterborne exposure for 96 h to evaluate the genetic and biochemical effects caused by both contaminants. For piscine micronucleus test (PMT) and Erythrocytic Nuclear Alterations (ENA), there were no differences in the frequency of micronuclei nor ENA between exposure groups. Clemente et al. [6] exposed a fish species (*Piaractus mesopotamicus*) to NpTiO$_2$ at 0.1, 10 and 100 mg.L^{-1}, performing a waterborne exposure for 96 h. Following this exposure period, not only were micronuclei not found in
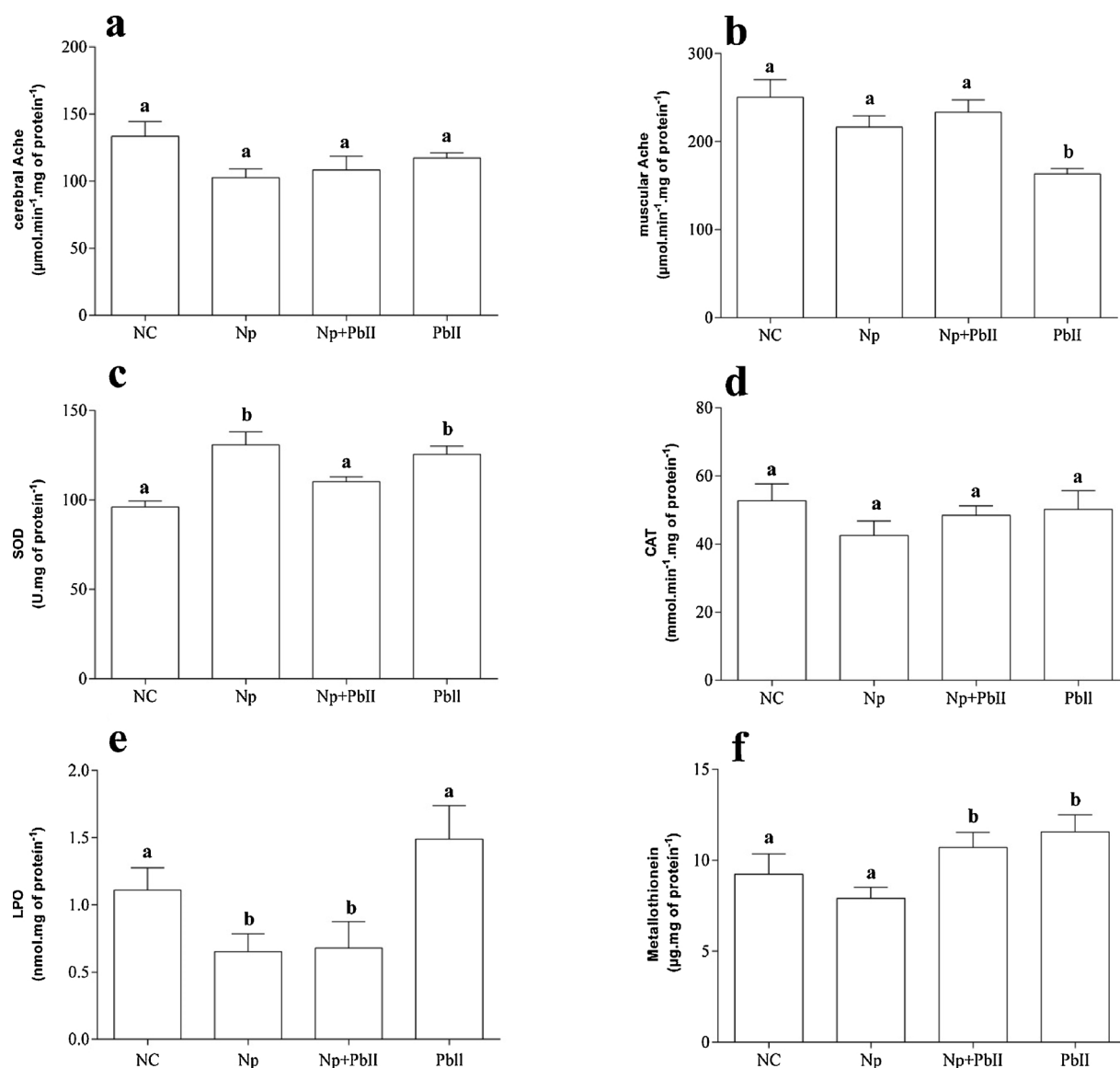
Fig. 6. Biochemical biomarkers analyzed for *Hoplias intermedius* species after waterborne exposure to contaminants for 96 h in a semi-static regime. In (a) Activity of brain acetylcholinesterase (AChE) and (b) of muscle acetylcholinesterase (AChE) activity. In (c), (d), (e) and (f) liver biochemical biomarkers, as (c) Catalase (CAT); (d) the activity of superoxide dismutase (SOD); (e) Lipid peroxidation (LPO); (f) metallothioneins (MTs). Different letters indicate statistically significant differences ($p < 0.05$). Data are expressed as mean ± standard error.

erythrocytes, but also the frequency of ENA in the highest $NpTiO_2$ concentration tested ($100\,mg.L^{-1}$) was not significant, which corroborates the results observed in our study. Also, no significant results for ENA occurred in *Hoplias malabaricus* contaminated only with lead (Pb II) at doses of 7, 21, 63 and 100 mg/g, through intraperitoneal injections, demonstrating that this contaminant may not be efficient in causing DNA damage in these experiment [26].

In erythrocytes, through the analyses of the comet assay results, DNA damage was shown to decrease in the lead-only exposed group (Pb II) as well as in that submitted to nanoparticles associated with lead ($NpTiO_2 + Pb\ II$). In the group exposed only to $NpTiO_2$ this difference from the negative control group was not observed, which makes us believe that lead interacts with $NpTiO_2$ in a different manner, changing toxicological pattern presented as DNA damage. It is worth pointing out that the nanoparticle solution was stable. In these cases, stability would imply in solubility which would facilitate the interaction of these nanoparticles with the evaluated tissue [8].

Gills showed higher DNA damage when the fish were exposed to $NpTiO_2$ ($p < 0.001$) and to the association of contaminants

($NpTiO_2 + Pb\ II$) ($p < 0.001$). These organs were in immediate contact with water, which made them a target for toxic substances found in water bodies [17]. Also, during exposure to metals, the amount of mucus produced by the gills cells increases [69] which may have contributed to the adsorption of the titanium dioxide nanoparticles [17], resulting in greater DNA damage to gill cells when infected with $NpTiO_2$ alone or when in association with Pb II. In addition, it is important to relate the gill results with those found for erythrocytes, since both have a similar pattern of response. This similarity probably results from the gills being the most affected organs in a water route of exposure and, consequently, generate a large amount of reactive oxygen species (ROS). Also, similar results were found by Zhang et al. [16]. As gills are organs with a massive irrigation by blood, these reactive oxygen species go into the bloodstream, causing DNA damage to the erythrocytes [57].

This study revealed no changes to the comet assay performed in liver cells. Liver response to the contaminants is not considered fast because it seems to be related to the body and to the amount of time in which accumulation of a contaminant occurs [70]. As the total duration

of the experiment was 96 h, this period may not have been enough for genetic damage induction to liver cells or, if it occurred, it could have been reversed through the cell repair system [71]. In addition, liver and kidney organs are known to be related to the excretory function of exogenous substances in fish and the excretion capacity of nanomaterials for these organs still is a controversy [57].

Also concerning the liver, the evaluation of the antioxidant system comprises an important biochemical biomarker. Therefore, the activities of two important enzymes for the liver cells were evaluated: catalase (CAT) and superoxide dismutase (SOD). SOD is the metalloenzyme responsible for reducing oxygen free radicals, the anion superoxide (O^{2-}), acting on defense against reactive oxygen species (ROS) [72]. In the O^{2-} dismutation reaction, the SOD produces hydrogen peroxide (H_2O_2) and oxygen (O_2), and H_2O_2 is subsequently degraded, mainly by CAT. In this study, SOD activity revealed interaction between the effects of contaminants. Both the group exposed to NpTiO$_2$ and that exposed to lead showed increased activity of this enzyme in comparison with the NC group and to the one co-exposed to those compounds. However, when analyzing the increase in SOD observed in the co-exposure group (NpTiO$_2$ + Pb II), we observed such an increase was smaller than that shown by the groups separately. Although SOD acts by preventing the O^{2-} from causing any damage, the product of this reaction (H_2O_2) may also cause cell damage, since it is a reactive oxygen species (ROS) that may enter cell compartments and inactivate enzymes directly [73].

Furthermore, isolated exposure to Pb II and NpTiO$_2$ increased the activity of SOD, but even with this increase, the activity of CAT, which is the next enzyme in the antioxidant defense line, was not modified in none of the treatments. The results observed in CAT activity reflect no changes in LPO, indicating that even with the increase in SOD activity promoted by the treatment with Pb II or NpTiO$_2$, the antioxidant system of *Hoplias intermedius* was not impaired at the highest level to result in lipid damage. Also, the results observed in the SOD activity are consistent with several studies which report that exposure to metals in fish may promote oxidative stress as they reduce or increase enzyme activity of the antioxidant defense system [74-76]. Also, there are changes being reported in SOD activity in animals exposed to NpTiO$_2$ [77]. However, this result does not exclude the fact that other enzymes related to the same process as that in which SOD participates, such as GPx, suffered effects caused by the exposure to these contaminants as it was not possible to evaluate the activity of other enzymes functionally related to CAT and SOD. Unlike other peroxidases such as GPx, which convert the other peroxides, CAT only converts H_2O_2 [78].

In the co-exposure group to NpTiO$_2$ and Pb II (NpTiO$_2$ + Pb II), no changes were observed in the antioxidant system evaluated. Contrary to expectations, the combination of the two contaminants appears to prevent the effects caused by them isolately. In this context, several studies indicate that the potential of the nanoparticles adsorbing metal ions may change the Pb II input mechanisms and facilitate the removal of contaminants in the hepatic tissue cells [57,77,79]. This probably relates to the lack of effects observed and indicate that the chemical interaction between the contaminants may have reduced their availability and, as a consequence, their interaction with cellular components. Also, the adsorption of NpTiO$_2$ to Pb II may result in a stable molecule in physiological conditions. However, studies demonstrate an increase in metal internalization by liver cells, changes in induction of biochemical enzymes of the antioxidant system and even apoptosis, upon exposure to these two contaminants combined [16,80]. Hao et al. [64] conducted a study with the species *Cyprinus carpio*, in which the fish were exposed in water to 100 mg.L^{-1} and 200 mg.L^{-1} of NpTiO$_2$ for 20 days. Following this exposure, the liver showed a high frequency of necrotic and apoptotic cells, as well as a reduced quantity of lipid peroxidation and less intense activity of antioxidant enzymes.

The concentration of metallothioneins did not vary following exposure to NpTiO$_2$ and to Pb II. However, MTs are well known as capturers of metals. Also, their expression increases when exposed to a wide variety of metals, an increase which is also related to their protective function to the cell [81,82]. This increased concentration of MTs has been observed by Campana et al. [70] in *Halobatrachus didactylus* after being exposed to lead *via* intraperitoneal injection of 1 ng/g of Pb II.

The kidney cells showed higher DNA damage in the groups exposed to NpTiO$_2$ alone, in those co-exposed to contaminants (NpTiO$_2$ + Pb II) and in the group exposed to lead (Pb II) alone. The study by Patel et al. [83] showed accumulation of Pb II in a time-dependent manner by *Oncorhynchus mykiss* kidney after waterborne exposure with 0.5 mg.L^{-1} of Pb II, for 3, 8, 24 and 96 h. Through physiological analyses Patel et al. [83] suggest that kidneys perform an important role in lead metabolism and excretion routes, which could explain the larger damage caused by Pb II and its association with the titanium dioxide nanoparticles (NpTiO$_2$ + Pb II) in the *H. intermedius* kidney tissue. What is usually found in the literature is the fast response and sensitivity of the erythrocytes to the comet assay and a late response of the tissues [23], but in this study, despite being considered an acute experiment (96 h), we observe significant responses in the tissues chosen for certain analyses.

Among the most informative organ in this experiment, the brain showed significant results about titanium dioxide nanoparticles and its toxicity. The group exposed only to lead (Pb II) showed no differences in comparison with the negative control, but it did show differences in relation to the group co-exposed to the contaminants (NpTiO$_2$ + Pb II) and to the group exposed only to titanium dioxide nanoparticles (NpTiO$_2$). Recent studies suggest that, being a possible target organ, the brain is extremely relevant to the analysis of nanomaterials effects on fish [66]. Lesions found in this study may not be directly related to brain damage but are likely to be caused by increased blood pressure and respiratory disorders, which, in turn, may have been brought on by the accumulation of nanoparticles in the surface of the gills [waterborne exposure- 42]. Moreover, changes in brain enzymatic functions were found after contamination with metallic nanoparticles in *Oncorhynchus mykiss*, for variable exposure routes (*e.g.* waterborne and trophic exposure) [36,84,85]. The main problem, however, is related to the lack of studies confirming the existence of these nanoparticles in central nervous system, which is considered crucial to the analysis and further conclusions [66].

Acetylcholinesterase (AChE), which in fish is found in the brain, erythrocytes and muscle [71], is a widely used neurotoxicity biochemical biomarker [76] since this enzyme hydrolysizes neurotransmitter acetylcholine (ACh) in the synapse, thus facilitating the transmission of impulses from one neuron to another [86] and preventing the occurrence of continuous stimuli of the neuron, while maintaining normal operation of the motor and sensory system. For this research, we analyzed the AChE enzyme in the brain and in fish muscle. The activity of brain AChE did not vary significantly in the groups exposed to contaminants. Nevertheless, muscle AChE was less active in the lead-exposed group, which indicates a neurotoxic effect, since its activity did not suffer variations when it was associated with the nanoparticles. Regarding metals, this inhibition is commonly observed in the activity of this enzyme, which is related to high concentrations of contaminants [87,88]. Despite the concentration of lead in water not being very high, in accordance with the Brazilian law, we observed inhibition of AChE in fish muscle tissue submitted to lead. Evidence of neurotoxic effects caused by Pb II in fish AChE activity was identified by Lima et al. [89] and Richetti et al. [75] in zebrafish (*Danio rerio*), as well as in sea bream (*Sparus aurata*) by Souid et al. [76] among another species [74,90]

A few experiments have been performed to determine the reproductive effects of NpTiO$_2$ in fish species, although many studies published are with fish embryos (*e.g. zebrafish*) [68]. The normal development of these embryos is of fundamental importance to estimate survival of fish populations [66]. Thus, the comet assay in the gonads of male and female cells was performed to verify the occurrence of

damage to the genetic material of those cells. We hold the view that our findings generate data on this subject, but we consider the discussion on this topic to be particularly difficult, due to the absence of specific studies in gonadal fish tissue. Most studies were conducted in mice, and the results showed adverse effects on spermatogenesis, which led to histological changes, as well as on the viability of Leydig cells [reviewed in < **[91]]. In fish, studies with the cell line of rainbow trout gonad tissue (RTG-2) showed an increased cytotoxicity in some concentrations of *quantum dots* [92] and titanium dioxide nanoparticles, the exposure to which showed an increase in DNA strand breaks and cytotoxicity when combined with UVA radiation [93]. In our study, $NpTiO_2$ showed no genotoxicity but lead, in the concentration employed, was responsible for the rise in DNA strand breaks verified by single cell gel electrophoresis.

5. Conclusion

We observed a direct cause-effect relationship between inorganic lead and titanium dioxide nanoparticles ($NpTiO_2$) by analyzing the comet assay results. However, the group exposed only to $NpTiO_2$ showed genotoxicity in gills, kidneys and brain probably due to the mode of exposure employed in this study. Gills were particularly affected by $NpTiO_2$, whose adsorption into the epithelia may be at the root of the damage suffered by the organs above. Kidneys and brain, however, may also be target organs. With regards to the kidney, should it be confirmed that nanoparticles are excreted through it, we will be able to identify the damage caused to this organ. In relation to the brain, evidence suggests that $NpTiO_2$ may affect the central nervous system of some organisms. The liver did not seem to be affected by the $NpTiO_2$ at the concentrations utilized in this study. Notwithstanding the importance of our results, they ought to be dealt with cautiously. As the exposure lasted for only a few days, it may not be possible to verify an internal accumulation of these nanoparticles in the long term, which, in turn, could lead to a different toxic effect. Antioxidant mechanisms were also investigated in this work, as well as damage to macromolecules (LPO) and possible alterations in metallothionein concentrations. Thus, it was possible to observe altered results in biochemical biomarkers (SOD and LPO), which showed that the effects of nanoparticles are probably related to the chain reaction involving these enzymes. Despite these results, the genetic effect observed in some tissues may be due to mechanisms other than ROS production. Thus, further studies on the mechanism of $NpTiO_2$-induced cellular damage should be conducted in order to discover other ways in which $NpTiO_2$ may interact with the DNA molecule.

Disclosure statement

The authors confirm the non-existence of eventual interest conflicts. All of them signed this statement on the individual disclosure forms.

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