Interference of goethite in the effects of glyphosate and Roundup® on ZFL cell line

Natara D. G. da Silva [a], Cristiane E. A. Carneiro [b], Estefânia V. R. Campos [c], Jhones L. de Oliveira [c], Wagner E. Risso [d], Leonardo F. Fraceto [c], Dimas A. M. Zaia [b], Cláudia B. R. Martinez [a,d,*]

[a] *Programa de Pós-Graduação em Ciências Biológicas, Universidade Estadual de Londrina – UEL, Londrina, Paraná, Brasil.*

[b] *Departamento de Química, Universidade Estadual de Londrina – UEL, Londrina, Paraná, Brasil.*

[c] *Departamento de Engenharia Ambiental, Universidade Estadual Paulista – UNESP, Sorocaba, São Paulo, Brasil.*

[d] *Departamento de Ciências Fisiológicas, Universidade Estadual de Londrina - UEL, Londrina, Paraná, Brasil.*

*Corresponding author at: Departamento de Ciências Fisiológicas, Universidade Estadual de Londrina, Londrina, Paraná 86057-970, Brasil.

E-mail address: cbueno@uel.br (C.B.R. Martinez).

Abstract

Goethite (α-FeOOH) brings important perspectives in environmental remediation, as, due to its physicochemical properties, this iron oxide can adsorb a wide variety of compounds, including glyphosate. This study aimed to evaluate the effects of goethite nanoparticles (NPs), glyphosate (Gly), Roundup® (Rd), and co-exposures (Gly+NPs and Rd+NPs) on zebrafish liver cell line (ZFL). ZFL cells were exposed to NPs (1, 10, and 100 mg L^{-1}), Gly (3.6 mg L^{-1}), Rd (10 mg L^{-1}), and co-exposures (Gly+NPs and Rd+NPs), or only to saline for 1, 6, and 12 h. Cell viability was assessed by Trypan blue, MTT, and neutral red assays. The generation of reactive oxygen species and total antioxidant capacity were also determined, while genotoxicity was quantified by the comet assay. Both NPs and Rd in isolation produced cytotoxic effects at 6 h and genotoxic effects at 1 and 6 h. Rd+NPs resulted in synergistic effects, intensifying the toxicity. Cells exposed to Gly did not present toxic effects and Gly+NPs resulted in the suppression of toxic effects observed for NPs. The presence of other components in Roundup® seems to favor its toxicity compared to the active ingredient. In conclusion, according to the *in vitro* model, the concentrations used were not safe for the ZFL lineage.

Keywords: iron oxide; nanotoxicology; *in vitro* assays; *Danio rerio*; cell viability; DNA damage.

1. Introduction

Glyphosate, N-(phosphonomethyl) glycine, is an active ingredient that occupies first place in the ranking of commercialized herbicides worldwide (Benbrook, 2016), and is widely used in the control of invasive plants, both in agriculture and domestic use (Benbrook, 2016; De Gerónimo et al., 2018; Hanke et al., 2010; Tang et al., 2015). For this reason, the presence of glyphosate is increasingly common in aquatic environments (Bruggen et al., 2018; Maqueda et al., 2017), leading to contamination of surface and groundwater, mainly through processes such as surface runoff and soil leaching (Borggaard and Gimsing, 2008; Bruggen et al., 2018; Marques et al., 2014).

According to the World Health Organization, glyphosate toxicity is low for non-target organisms (WHO, 2005). This is based on the mode of action of glyphosate, which consists of its ability to reduce aromatic amino acids synthesis through specific inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, present in a small range of organisms, primarily green plants (Lopes et al., 2014; Lushchak et al., 2009). However, several authors suggest higher toxicity of glyphosate formulated products for aquatic organisms, demonstrating alterations in the activity of antioxidant enzymes (Lushchak et al., 2009; Modesto and Martinez, 2010b), cytotoxic effects (Goulart et al., 2015; Koller et al., 2012; Peixoto, 2005; Pereira et al., 2018), genotoxic effects (Cavalcante et al., 2008; Marques et al., 2014; Moreno et al., 2014), altered acetylcholinesterase activity (Modesto and Martinez, 2010b), and reproductive and behavioral changes (Bridi et al., 2017; Vanlaeys et al., 2018).

Roundup® is the commercial name of a popular glyphosate-based herbicide extensively used around the world (Caballero-Gallardo et al., 2016). Its composition includes, besides glyphosate (360 g L^{-1}), a mixture of surfactants, containing 15% polyoxyethylene amine (POEA), which appears to be related to the increased toxicity of the product (Lushchak et al., 2009; Moreno et al., 2014; Peixoto, 2005; Tsui and Chu, 2003; Vanlaeys et al., 2018). In this context, Navarro and Martinez (2014) reported several damages related to imbalance in the redox state of the neotropical fish *Prochilodus lineatus*, such as hemolysis, DNA damage, and lipid peroxidation after exposure to surfactant POEA. In addition, glyphosate-based herbicides with POEA were more toxic to

the microcrustacean *Artemia salina* and the zebrafish *Danio rerio* than formulations without POEA, both with 360 g of glyphosate L^{-1} (Rodrigues et al., 2017).

Considering the potential for contamination of water bodies by glyphosate, iron oxide nanoparticles present important perspectives in the remediation of contaminated aquatic environments (Liu et al., 2014). Due to their special physicochemical properties (large surface area and high surface reactivity), iron oxide nanoparticles can adsorb a variety of compounds such as anions, organic compounds/organic acids, and cations (Kharisov et al., 2012; Liu et al., 2014; O'Carroll et al., 2013; Tosco et al., 2014).

Among the most commonly used iron oxides, goethite (α-FeOOH) is highlighted as the most thermodynamically stable (Cornell and Schwertmann, 2003), as an abundant and cheap natural material (Liu et al., 2014), as well as for presenting great adsorption capacity for glyphosate (Jonsson et al., 2008; Liu et al., 2014; Sheals et al., 2002; Yang et al., 2018). Goethite may form monodentate complexes with glyphosate by binding to the phosphonate group or form bidentate bridges on the iron oxide surface in an inner sphere mode, while the carboxylate and amino group are noncoordinated to the surface (Barja and dos Santos Afonso, 2005).

Although several studies have evaluated the structure and characterization of goethite (Cornell and Schwertmann, 2003; Dultz et al., 2018; Liu et al., 2014), its adsorption capacity for glyphosate (Barja and dos Santos Afonso, 2005; Jonsson et al., 2008; Pessagno et al., 2008; Sheals et al., 2002; Waiman et al., 2012), and its potential for application in environmental protection (Liu et al., 2014; Orcelli et al., 2018), there is a lack of studies evaluating biological responses to exposure to goethite and, therefore, its toxicological impact, mainly for aquatic organisms, remains unknown.

In this context, studies at the cellular level are extremely important, since they provide information on the mechanisms of action of the agents tested and/or the cellular response and, thus, can be used preventively (Bols et al., 2005; Fent, 2007). The development of *in vitro* models has increased significantly in recent years and these play a key role in toxicological research, offering ethical, scientific, and economic advantages (Jennings, 2015; Singh et al., 2018; Srivastava et al., 2018). Fish cell lines are of growing importance in

eco/genotoxicology as they represent standardized experimental systems that are carried out in a controlled environment, giving fast, affordable and ethically eligible results (Žegura and Filipič, 2019).

In the present study, we used the hepatocyte cell line from *Danio rerio* (zebrafish), initially obtained by Ghosh et al. (1994), which presents characteristics of hepatic parenchyma cells. As the liver is the main organ of metabolism, detoxification, and homeostasis, the applicability of this lineage becomes relevant in *in vitro* toxicological studies. In addition, several studies have demonstrated the sensitivity of this lineage to metal toxicity (Chen and Chan, 2018; Sandrini et al., 2009; Tang et al., 2013), nanomaterials (Azevedo Costa et al., 2012; Morozesk et al., 2018; Thit et al., 2017), pharmaceutical products (Gajski et al., 2016; Novak et al., 2017), biodiesel (Cavalcante et al., 2014), gasoline (Lachner et al., 2015), and herbicides (Goulart et al., 2015; Lopes et al., 2018).

Thus, considering the potential risk of contamination of aquatic organisms by glyphosate and the limited studies evaluating biological responses to goethite, this work aimed to evaluate the possible cytotoxic, biochemical, and genotoxic effects of goethite nanoparticles (NPs), glyphosate, and the formulated product Roundup® on the hepatocyte cell line of *D. rerio* (ZFL), as well as to evaluate the interference of the nanomaterial in the effects of these herbicides.

2. Material and Methods

2.1. Preparation of goethite nanoparticles (NPs)

The goethite NPs were produced according to the protocol described by Schwertmann and Cornell (2000). A solution containing goethite NPs was dispersed in ultra-pure water and sonicated. The concentration of NPs in the stock solution was estimated by dry weight and the corresponding iron concentration. This solution was autoclaved and used for the preparation of exposure solutions at final concentrations of 1, 10, and 100 mg L^{-1} of NPs diluted in Dulbecco´s phosphate buffered saline with calcium, magnesium and glucose (Dulbecco's PBS: 136.9 mM NaCl; 2.68 mM KCl; 0.90 mM $CaCl_2$; 0.49 mM $MgCl_2.6H_2O$; 7.58 mM Na_2HPO_4; 1.47 mM KH_2PO_4; 5.55 mM glucose; pH

7.4). Considering the lack of data reporting the effects of goethite NPs at cellular level, the concentrations used in the present work were based on cytotoxicity results found in the literature for different iron oxide nanoparticles (Ankamwar et al., 2010; Bhattacharya et al., 2012; Karlsson et al., 2008; Singh et al., 2010). Furthermore, previous tests (MTT and NR) were performed in a range of 1 – 200 mg L^{-1} (data not shown) to select appropriate concentrations. Based on these results, the concentrations of 1, 10, and 100 mg L^{-1} were chosen to further investigate the toxic effects of goethite NPs in ZFL cells.

2.2. Characterization of goethite NPs

The goethite NPs exposure solutions (1, 10, and 100 mg L^{-1}) were characterized for both the hydrodynamic diameter and the polydispersity index using the dynamic light scattering technique (DLS) and zeta potential (ZP) through the electrophoresis light scattering technique. For this, a Malvern ZS90 particle analyzer (Malvern®) was used at a fixed angle of 90° to 25°C. The characterization tests were conducted in triplicate at 0, 24, and 48 h in the absence of stirring, in order to approximate the exposure performed in the toxicity tests.

2.3. Quantification of iron (Fe)

Total and dissolved Fe concentrations were analyzed in the exposure solutions. For the analysis of total Fe, the solutions were fixed in nitric acid (HNO_3 0.5% - Fmaia, Brazil) and for dissolved Fe solutions, they were filtered (0.45 µm) and then fixed. The samples were analyzed using an atomic absorption spectrophotometer (EAA - Analyst 700, Perkin Elmer®, USA) through flame atomization.

2.4. ZFL cell line

The ZFL cell line was grown in 25 cm^2 flasks using medium containing 50% Leibovitz L-15 (Gibco®), 40% RPMI 1640 (Gibco®), and 10% fetal bovine serum (FBS) (Gibco®). The flasks were kept in a dry oven without addition of CO_2 at 28°C.

2.5. *In vitro* exposures

Concentrations of the selected herbicides, Roundup® (360 g glyphosate L^{-1}, Monsanto do Brasil LTDA) and glyphosate (CAS no. 1071-83-6, Milenia Agrociências S/A), were defined taking into account previous studies that our research group have already developed with the neotropical fish *P. lineatus* exposed to Roundup Transorb®, Roundup®, and glyphosate (Cavalcante et al., 2008; Modesto and Martinez, 2010a, b; Moreno et al., 2014).

Stock solutions of Roundup® and glyphosate were prepared at a concentration 100 times higher (100 and 36 mg L^{-1}, respectively) than the final exposure and diluted in Dulbecco's PBS before distribution to the plate wells to reached 10 and 3.6 mg L^{-1}, respectively. Exposure concentrations of Roundup® and glyphosate were based on the concentration of the active ingredient present in the formulation (360 g glyphosate L^{-1}).

All the experimental solutions were prepared in Dulbecco's PBS to avoid any interactions between the treatments and the compounds present in the culture medium, which could influence the results. In addition, preliminary tests demonstrated that Dulbecco's PBS was able to maintain ZFL cell viability (mitochondrial and lysosomal activity) for at least twelve hours (data not shown), which corresponds to the maximum exposure time used in this study.

ZFL cells were seeded at a density of 10^6 cells mL^{-1} on a transparent 96-well plate (TPP®) for the cytotoxic assays, on a black 96-well plate with a clear bottom (Perkin Elmer®) for the biochemical assays, and on a transparent 24-well plate (TPP®) for the genotoxic assay. After 24 h of cell attachment at 28°C, the cells were exposed for 1, 6, and 12 h to the following treatments: goethite NPs at 1 mg L^{-1} (N1), 10 mg L^{-1} (N2), and 100 mg L^{-1} (N3); glyphosate at 3.6 mg L^{-1} (Gly), and co-exposures to Gly plus goethite NPs (GlyN1, GlyN2, GlyN3); Roundup® at 10 mg L^{-1} (Rd); and Rd plus goethite NPs (RdN1, RdN2, RdN3). The cells of the control groups (CTR) were only exposed to Dulbecco's PBS. Three independent experiments were performed, and for each assay four plates were assembled per experimental time and, depending on the assay, the number of replicates per treatment was variable (cytotoxic and biochemical assays: eight replicates per treatment; genotoxic assay: two replicates per treatment).

2.6. Cytotoxic assays

Cytotoxicity was evaluated using different assays. To verify the integrity of the plasma membrane, the Trypan blue exclusion test (TB) was used prior to the comet assay. After exposure, the cells were detached from the wells, homogenized with 0.4% of the TB dye, and a total count of 100 cells was performed using a Neubauer chamber. Cell viability was expressed as the percentage of viable cells and the treatments with cell viability equal to or greater than 80% (Tice et al., 2000) were selected for ROS, ACAP, and comet assay.

Mitochondrial activity was assessed through the reduction in 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) salt according to the protocol of Mosmann (1983), with modifications. After exposure, the MTT salt was added to the wells for 4 h at a final concentration of 0.80 mM. Subsequently, the plate was centrifuged for 5 min at 200 g, dimethyl sulfoxide (DMSO, 99.5%) was added for solubilization of the formazan crystals, and the absorbance was determined on a microplate reader (Victor 3, Perkin Elmer®) at 540 nm.

Lysosomal integrity was also assessed by the neutral red (NR) retention assay. After exposure, the cells were incubated with NR dye (40 μg mL^{-1}) for 3 h. Next, the plate was centrifuged for 5 min at 200 g, fixed with formaldehyde (0.5%) in calcium chloride solution (1%) for 2 min, and exposed to an acid alcohol solution (1% acetic acid in 50 % ethyl alcohol) under constant stirring for 15 min. The absorbance was determined on a microplate reader (Victor 3, Perkin Elmer®) at 540 nm and, for MTT and NR assays, the results of the different treatments were given in relation to the CTR, which was considered as 100% cell viability.

2.7. Biochemical assays

The generation of reactive oxygen species (ROS) and the total antioxidant capacity against peroxyl radicals (ACAP) were determined according to the protocol of Amado et al. (2009) with modifications. After the exposures, the solutions were withdrawn and reaction medium (30 mM HEPES, 200 mM KCl, 1 mM $MgCl_2$, pH 7.2) was added to all wells. For each experimental treatment, eight replicates were performed per plate; four wells were treated with potassium phosphate buffer (0.1 M, pH 7.4) and the other four

received peroxyl, 2,2'-azobis radicals (2-methylpropinamide) dihydrochloride (10 mM ABAP, pH 7.2). The autofluorescence reading was performed on a microplate reader (Victor 3, Perkin Elmer®). This was carried out by adding the 2'-7'-dichlorofluorescein diacetate compound (1 mM H_2CDF-DA) and re-reading at 35°C for 70 min with readings every 5 min, excitation of 485 nm, and emission of 520 nm (Azevedo Costa et al., 2012). To quantify the ROS and ACAP, the fluorescence data were adjusted to a second-order polynomial function and the integral value was calculated. For ROS quantification, the values of the integrals of the samples treated with potassium phosphate buffer in isolation were analyzed and the results expressed as unit area of fluorescence over time (FU x min). To evaluate the ACAP, the difference in the area values of the samples treated and non-treated with ABAP were calculated. A greater difference between the areas indicated lower antioxidant capacity of the sample. To facilitate visualization of the results, ACAP data were inverted (1/relative area).

2.8. Genotoxic assay

To evaluate DNA damage, the comet alkaline test was performed according to Singh et al. (1988) with modifications. For this assay, a positive control (PC) was also performed using 0.5 mM methyl methanesulfonate (MMS). After exposure, aliquots of 20 µL of each sample were homogenized with low melting point agarose (5%) for the preparation of slides previously prepared with normal melting point agarose (1.5%), covered with coverslips, and placed in a refrigerator for 40 min. After this period, the coverslips were removed and the slides were immersed in lysis solution (2.5 mM NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO, pH 10) for 2 h. After the lysis, the slides were transferred to an electrophoresis cube, containing fresh and ice-cold alkaline buffer (1 mM EDTA and 300 mM NaOH, pH > 13) for 35 min. The electrophoresis was conducted at 25 V and 300 mA for 20 min. The slides were then neutralized (0.4 M Tris, pH 7.5) for 15 min and fixed with 100% ethanol for 10 min. The slides were stained with GelRed (Biotium®) and 100 nucleoids per slide were analyzed in a blinded test under a fluorescence microscope (Leica®, DM 2500) in a 40X objective. DNA damage was visually classified, according to the migration of the DNA fragments into four classes (Collins et al., 2008): class

0 (nucleoid without tail with few fragments around), class 1 (tail smaller than the nucleoid diameter), class 2 (tail with a length one to two times the diameter of the nucleoid), and class 3 (tail with a length greater than twice the diameter of the nucleoid). The damage score was obtained by multiplying the number of nucleoids observed in each class of damage analyzed by the value of the class.

2.9. Statistical analyzes

For each parameter evaluated, the results were compared between the different treatments CTR x N1 x N2 x N3, CTR x Gly x GlyN1 x GlyN2 x GlyN3, CTR x Rd x RdN1 x RdN2 x RdN3, and CTR x PC (where applicable) for each experimental time (1, 6, and 12 h) through parametric (ANOVA) and nonparametric analysis of variance (Kruskal-Wallis), according to distribution of data (normality and homogeneity of variance). When necessary, the differences were identified by the Student-Newman-Keuls (SNK) multiple comparisons test. Values of $P < 0.05$ were considered significant and the results are expressed as mean ± standard error (SE).

3. Results

3.1. Characterization of goethite NPs

The results indicate a significant increase in the mean diameter of goethite nanoparticles in N2 and N3 treatments at 0 h ($P < 0.001$) and 48 h ($P = 0.005$) (Figure 1A). For 24 h ($P = 0.080$), no difference in this parameter was verified. The DLS methodology indicates that there were aggregates, which could influence the size of the nanoparticles.

For the polydispersity index results, although significant differences were not observed for concentrations and times evaluated (0 h: $P = 0.975$; 24 h: $P = 0.132$; 48 h: $P = 0.071$) (Figure 1B), the values found were high (> 0.5), indicating a highly polydisperse system. In this case, the high polydispersity of the system also indicates the formation of aggregates in the solution.

Finally, the zeta potential results indicated some significant changes in the values as a function of time (0 h: $P < 0.001$; 24 h: $P = 0.003$; 48 h: $P < 0.001$) (Figure 1C); however, the values were lower than 30 mV, characterizing the solutions as unstable dispersions. The zeta potential infers on the surface

charge of the particles, an important parameter of stability. Thus, these results demonstrate system instability in solution, leading to aggregation and also increased polydispersity.

3.2. Quantification of iron

According to Table 1, the results showed an increase in total Fe concentrations as the NPs concentrations increased both in isolation and in co-exposure with glyphosate and Roundup® when compared to the respective CTR. Although these values were lower than the nominal values of the concentrations of 1, 10, and 100 mg L^{-1} of goethite, the intended gradient was produced. Similarly, the concentrations of dissolved Fe found in the different solutions containing NPs were lower when compared with the total Fe concentrations analyzed, and the highest concentrations of dissolved Fe were for solutions containing the highest concentration of NPs (N3, GlyN3, and RdN3) when compared to the respective CTR. In addition, the concentration of dissolved Fe found for N3 (8.36) was approximately 12 to 16 times higher, and for GlyN3 (7.10) and RdN3 (4.97), approximately 7 to 10 times higher when compared to the dissolved Fe values of the different treatments.

3.3. Cytotoxicity

The integrity of the plasma membrane (i.e. viability), assessed by TB, was greater than 90% for all times and experimental treatments tested. No statistical differences were found for NPs (1 h: $P = 0.220$; 6 h: $P = 0.622$; 12 h: $P = 0.153$), Gly and co-exposures (1 h: $P = 0.623$; 12 h: $P = 0.046$), and Rd and co-exposures (1 h: $P = 0.184$; 6 h: $P = 0.039$; 12 h: $P = 0.056$) in this parameter. Despite a significant decrease at 6 h ($P = 0.048$) for treatment GlyN3, the viability was still greater than 90% (Fig. 2A, B, and C).

When the MTT assay was employed, the results indicated that the N1 and N2 treatments were cytotoxic at 6 h ($P = 0.002$), resulting in a significant decrease in mitochondrial activity of the cells in relation to the CTR, with no change in this parameter at 1 and 12 h ($P = 0.655$ and $P = 0.466$, respectively) (Fig. 2D). For glyphosate alone (Gly) or in combination (GlyN1, GlyN2, and GlyN3), no significant alterations in viability were found (1 h: $P = 0.096$; 6 h: $P = 0.549$; 12 h: $P = 0.081$). The concentrations of NPs which demonstrated

cytotoxic effects when isolated (N1 and N2) showed that in association with glyphosate the effect was suppressed (Fig. 2E). For Roundup®, all treatments (Rd, RdN1, RdN2, and RdN3) at 6 h ($P < 0.001$) produced cytotoxic effects for ZFL cells, with a significant reduction in the viability of these organelles. In addition, the association of NPs with the herbicide did not reverse the cytotoxicity caused by the Roundup®. In contrast, the concentrations of NPs used seem to have negatively influenced this cytotoxicity, since N1 and N2 treatments also resulted in a decrease in viability, with a pronounced decrease in the co-exposure RdN2. No alteration in mitochondrial activity was observed for times of 1 and 12 h ($P = 0.279$ and $P = 0.652$, respectively) (Fig. 2F).

The results of lysosomal integrity through the NR retention test indicated that N1, N2, and N3 promoted a significant increase in viability of the cells at 1 h ($P = 0.003$) and this pattern of increase in viability was maintained in the N3 treatment at 6 h ($P < 0.001$). In contrast, N1 and N2 were cytotoxic at 6 h, and this cytotoxicity was more pronounced for N2. No alteration in this parameter was observed at 12 h ($P = 0.258$) (Fig. 2G). When glyphosate-containing treatments were evaluated, the same pattern for the MTT assay was repeated: glyphosate in isolation or in combination did not induce alterations in the viability of the ZFL line for any period tested (1 h: $P = 0.504$; 6 h: $P = 0.054$; 12 h: $P = 0.129$), and although the isolated NPs were cytotoxic, when associated with glyphosate this cytotoxic effect disappeared (Fig. 2H). In the treatments containing Roundup®, it was possible to verify that only the co-exposure RdN2 resulted in a significant reduction in the viability of these organelles at 6 h ($P < 0.001$). In this case, it is possible to suggest that N2, which was cytotoxic to ZFL cells at the same experimental time, appears to negatively influence the co-exposure cytotoxicity. For 1 and 12 h ($P = 0.629$ and $P = 0.103$, respectively), no alterations in this parameter were found (Fig. 2I).

3.4. ROS and ACAP

Regarding the generation of reactive oxygen species (ROS), no significant difference was found for NPs (1 h: $P = 0.134$; 6 h: $P = 0.174$; 12 h: $P = 0.637$) or Gly and co-exposures (1 h: $P = 0.045$; 6 h: $P = 0.806$; 12 h: $P = 0.023$) in all experimental times evaluated (Figs 3A and B). The results indicated that only the co-exposure RdN3 resulted in a significant increase in

ROS at 6 h (P = 0.034) and no significant alterations were observed in this parameter at 1 h and 12 h (P = 0.479 and P = 0.344, respectively) (Fig. 3C).

Concerning total antioxidant capacity against peroxyl radicals (ACAP), no significant difference between the treatments was found for NPs in the three different times of exposure (1 h: P = 0.028; 6 h: P = 0.241; 12 h: P = 0.334) (Fig. 4A). When glyphosate-containing treatments were evaluated, only treatment GlyN1 showed a significant increase in ACAP at 1 h (P = 0.028) and no significant alteration was observed for 6 and 12 h (P = 0.991 and P = 0.094, respectively) (Fig. 4B). Similarly, in the treatments containing Roundup®, only RdN1 and RdN3 produced a significant increase in this parameter at 1 h (P = 0.019) and no alterations were found for 6 and 12 h (P = 0.357 and P = 0.554, respectively) (Fig. 4C).

3.5. Genotoxicity

The DNA damage in ZFL cells exposed to the concentration of 0.5 mM MMS (PC) were significantly higher than their respective CTR at the three times tested (1 h: P < 0.001; 6 h: P = 0.029; 12 h: P = 0.029) (Fig. 5A), ensuring the efficiency of the procedure and validation of the methodology used.

The results for goethite NPs showed a significant increase in the DNA damage score, compared to the respective CTR, for the ZFL cells exposed to N1 and N2 after 1 h (P = 0.007), and the N3 cells after 6 h (P = 0.003). After 12 h (P = 0.157), no alteration in DNA score was observed (Fig. 5B). When the ZFL line was exposed to glyphosate in isolation (Gly) or in combination (GlyN1, GlyN2, and GlyN3), no significant alteration in the DNA damage score was identified (1 h: P = 0.056; 6 h: P = 0.463; 12 h: P = 0.327) (Fig. 5C). All the treatments containing Roundup® (Rd, RdN1, RdN2, and RdN3) resulted in a significant increase in the DNA damage score in relation to the CTR after 1 h (P = 0.008), with a more pronounced genotoxic effect for the co-exposures. After 6 h (P < 0.001), only Rd and RdN3 promoted a significant increase in DNA damage of the ZFL. On the other hand, after 12 h (P = 0.001) all co-exposures of Rd with NPs resulted in a significant increase in damage score, with RdN1 and RdN3 causing the greatest damage (Fig. 5D). Different comet classes observed in ZFL cells were shown in Fig. 5E.

4. Discussion

Considering the potential application of goethite NPs in environmental remediation, the present study evaluated the effects of this iron oxide and its interference in the effects of glyphosate and Roundup® on the hepatocyte cell line of *D. rerio* (ZFL), through different cellular assays for 1, 6, and 12 h. The results showed that the effects of isolated goethite NPs on ZFL cells were different from the effects produced in association with the selected herbicides: co-exposure with glyphosate suppressed the effects of goethite NPs; and co-exposure with the formulated product, intensified the effects of iron oxide NPs.

Regarding cell viability, the concentrations of 1 and 10 mg L^{-1} of goethite NPs were cytotoxic to the ZFL line after 6 h of exposure, interfering in mitochondrial metabolism and lysosomal integrity, without changes in the plasma membrane. Several *in vitro* studies have already demonstrated the ability of different iron oxide NPs to cause cytotoxic damage in lymphocyte cells (Sonmez et al., 2016), lung cell lines (Bhattacharya et al., 2012; Karlsson et al., 2008), neural cells (Costa et al., 2016), and keratinocytes and dermal microvascular endothelial human cells (Bayat et al., 2015) in different cellular targets (plasma membrane, mitochondria, and lysosomes).

Iron oxide NPs are more likely to cross biological membranes (Singh et al., 2010) due to their large surface area and high reactivity. Once internalized, these NPs can undergo the acid dissolution process within lysosomes (Xia et al., 2008), with subsequent liberation of free Fe ions (Fe^{2+}) (Laskar et al., 2012; Singh et al., 2010). As a result, the excess of these ions in cells can lead to iron imbalance and cause direct damage to the mitochondria, such as morphological alterations or decreases in mitochondrial membrane potential (Freyre-Fonseca et al., 2011; Levi and Rovida, 2009; Teodoro et al., 2011). In the latter case, the free iron can react with hydrogen peroxide and oxygen produced by the mitochondria to produce highly reactive hydroxyl radicals and ferric ions (Fe^{3+}) via the Fenton reaction (Singh et al., 2010). Another important factor to be considered is that depending on the surface charge of nanomaterials, it may interact with the inner surface of the lysosomal membrane, which could result in damage to this organelle (Asati et al., 2010; Cho et al., 2012).

From assessment of the concentrations of dissolved iron in the exposure solutions, it can be inferred that the positive cytotoxicity results are not related to the excess of free Fe ions. Although treatment N3 presented the highest values of dissolved iron, no cytotoxic damage was observed in ZFL cells exposed to 100 mg L^{-1} of goethite NPs. In contrast, the lowest dissolved iron values found in goethite NPs exposure solutions were at concentrations that resulted in damage to mitochondrial and lysosomal viability (N1 and N2).

To evaluate the possible DNA damage to ZFL cells, the comet assay alkaline version was used. This technique is widely used due to its high sensitivity to detect DNA molecule damage, such as single- and double-strand breaks, alkali-labile sites, incomplete excision repair sites, and cross-links (Singh et al., 1988; Tice et al., 2000). The results of this assay demonstrated the genotoxic potential of goethite NPs at concentrations of 1 and 10 mg L^{-1} after 1 h and the concentration of 100 mg L^{-1} after 6 h. DNA damage caused by iron oxide NPs has been reported in different cell lines and the concentrations of these NPs are quite varied (Auffann et al., 2006; Bhattacharya et al., 2012; Bayat et al., 2015; Karlsson et al., 2008; Sonmez et al., 2016). Moreover, the majority of studies report that DNA damage caused by exposure to nanoparticles is indirect, resulting from ROS formation by Fenton reaction (Rim et al., 2013; Singh et al., 2012). However, in the present study, the establishment of oxidative stress was not observed in any period of exposure and, in this particular case, it is suggested that dissolved iron values found for treatment N3 are related to the reported genotoxicity. In addition, it is possible that the DNA damage of the ZFL cells is due to the direct action of goethite NPs. According to Singh et al. (2010), super magnetic iron oxide nanoparticles can cause direct damage in the DNA by mechanisms still unknown.

Although the toxic potential glyphosate has been demonstrated for mussels *Perna perna*, and fishes *D. rerio* and *Jenynsia multidentata in vitro* (Sandrini et al., 2013), in the present work the herbicide was not cytotoxic and it did not promote oxidative stress or cause DNA damage to ZFL cells. In agreement, Lopes et al. (2018) reported the absence of cytotoxic effects on the same cell line exposed to glyphosate at concentrations of 0.65 and 3.25 mg L^{-1} for 24 and 48 h.

In turn, when associated with glyphosate, the observed toxicity effects of goethite NPs in ZFL cells disappeared. Glyphosate forms inner-sphere surface complexes on goethite by a ligand exchange mechanism, resulting in the formation of inner-sphere surface complexes, where the phosphonate group of Gly binds Fe^{+3} ions at the surface (Liu et al., 2014; Sheals et al., 2002). Considering the great adsorption capacity of goethite to glyphosate, it is possible that the formation of this complex resulted in a decrease in available Fe^{+3} ions and, consequently, the suppression of toxic effects on ZFL cells exposed only to goethite NPs. The type of contaminant and its interaction with iron-based NPs has been previously reported by Fajardo et al. (2015) who showed that different effects could be obtained depending on the chemical properties of the pollutant.

On the other hand, several authors have reported that glyphosate-based formulations may cause alterations in mitochondrial function, such as: inhibition of succinate dehydrogenase enzyme activity, transmembrane reduction capacity, and inhibition of ATP synthesis (Koller et al., 2012; Peixoto, 2005; Pereira et al., 2018; Ugarte, 2014; Vanlaeys et al., 2018). In the present study, the results demonstrated that Roundup® was cytotoxic, with a decrease in mitochondrial viability at 6 h and no effect on lysosomal and plasma membrane viability parameters. In agreement with the results found, Lopes et al. (2018), using the ZFL lineage, observed a significant reduction in mitochondrial metabolic activity after exposure to Roundup® (3.25 mg L^{-1}) at 24 and 48 h, whereas lysosomal integrity was reduced only after 24 h exposure, with no alteration in membrane viability at the two experimental times tested.

Among the effects described for fish, the genotoxicity of Roundup® has been pointed out as one of the most harmful (Cavalcante et al., 2008; Ghisi and Cestari, 2013; Guilherme et al., 2014; Marques et al., 2014; Moreno et al., 2014). Cavalcante et al. (2008), using the fish *P. lineatus*, exposed to 10 mg L^{-1} of Roundup®, showed a significant increase in erythrocytes DNA damage after 6 and 96 h of exposure and 6 and 24 h for gills, corroborating its genotoxic effect. In agreement with previous studies, the present work confirmed the genotoxic potential of Roundup® with a significant increase in DNA damage after 1 and 6 h of exposure. After 12 h, the DNA damage of the ZFL cells returned to CTR levels, which could indicate possible activation of the DNA

repair system, in order to restore the breaks caused by exposure to the formulated product. The DNA repair system is an important factor in the prevention of serious genetic damage, such as mutations, DNA breaks, and chromosomal aberrations (Marques et al., 2014), and although the comet assay does not make it possible to reliably infer the repair process, this test is used to detect genomic lesions that can be corrected.

According to the results found for isolated goethite NPs or isolated Roundup®, we found that both caused cytotoxic and genotoxic damage to the ZFL cells. When associated, the effects produced were more pronounced, even for the highest concentration of goethite NPs (100 mg L^{-1}), demonstrating cytotoxic characteristics for mitochondrial and lysosomal metabolism, ROS promotion, and ACAP activation, as well as genotoxic potential, even for the longest exposure time.

In relation to this intensified toxicity demonstrated by the co-exposure, it is possible to infer that both substances could act together, producing a synergistic effect and intensifying the toxicity for the ZFL cells. The entry of toxic molecules, such as Roundup®, into cells is facilitated due to the adsorbent capacity of nanomaterial. Thus, NPs can serve as carriers, even for contaminants, increasing the intracellular concentration of these compounds and, consequently, their potential toxicity (Azevedo Costa et al., 2012; Choi et al., 2007; Lei et al., 2018; Limbach et al., 2007). Moreover, the presence of the other substances in the formulated product, such as surfactants, could favor the entrance of NPs to the ZFL cells, causing the damage observed in this study. Pisanic et al. (2007) reported that coordination between the surfactant and nanostructures facilitates entry of both the nanostructures and surfactants into the cells or interaction with the cells.

Finally, considering the absence of toxic effects produced by glyphosate and the cytotoxic and genotoxic damage produced by Roundup® to ZFL cells in the present work, it is possible to suggest that the presence of other compounds in its composition may play an important role in the toxicity of this herbicide. The different compounds present in the Roundup® formulation have already been compared in several aquatic organisms and the order of toxicity of the chemical agents found was: POEA Roundup® glyphosate glyphosate isopropylamine salt (IPA), indicating that toxicity of the formulated product can

be attributed to the surfactant POEA (Tsui and Chu, 2003). In another study, it was pointed out that the genotoxic potential of Roundup® for the fish *Anguilla anguilla* is directly related to the genotoxicity of surfactant POEA (Guilherme et al., 2012). Likewise, Navarro and Martinez (2014) evidenced a significant increase in DNA damage of erythrocytes in the fish *P. lineatus* after exposure to POEA, supporting the genotoxic character of this surfactant. Although the toxicity of the surfactant POEA has been demonstrated in several studies, our data cannot support that the increase in Roundup® toxicity for ZFL cells is exclusively associated with POEA.

In summary, these data lead us to conclude that the concentrations of goethite NPs used were not safe for the ZFL lineage. In addition, it was shown that goethite NPs and Roundup®, both isolated, presented cytotoxic and genotoxic effects and, when co-exposed, produced a synergistic effect, intensifying the previously reported damage. On the other hand, glyphosate did not promote cytotoxic, biochemical, or genotoxic damage to ZFL cells and, in association, the toxic effects produced by isolated goethite NPs were suppressed. In comparison, given the lack of toxic effects of glyphosate, it is possible to suggest that the presence of other compounds in the formulated product favors the toxicity of this herbicide when compared to the active ingredient glyphosate.

Considering the lack of studies investigating the possible toxic effects of goethite NPs, especially for aquatic organisms, our study is extremely significant, as we evaluated the mechanism of action of nanomaterial at the cellular level, contributing to knowledge of the toxic effects generated by iron oxide NPs. In addition, the results may favor discussion and investigation of strategies to determine environmentally safe NPs concentrations that result in an effective tool for removing contaminants from polluted aquatic ecosystems.

Conflicts of interest

There are no conflicts of interest.

Acknowledgements

The authors would like to thank the Araucaria Foundation and CNPq (PRONEX: 24732/2012), the National Institute of Science and Technology in Aquatic

Toxicology (INCT-TA, CNPq: 573949 / 2008-5), and the São Paulo Research Foundation (#2017/21004-5) for financial support. To CAPES for granting a master's degree grant to N.D.G. da Silva and CNPq for the research grant to C.B.R. Martinez. This work is part of the Master dissertation of N.D.G. da Silva for the Post-graduate Program in Biological Sciences of the State University of Londrina.

References

Amado, L.L., Garcia, M.L., Ramos, P.B., Freitas, R.F., Zafalorf, B., Ferreira, L.R., Yunes, J.S., Monserrat, J.M., 2009. A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: Application to evaluate microcystins toxicity. Sci. Total Environ. 407, 2115-2123.

Amarante, O.P.J., Santos, T.C.R., Brito, N.M., Ribeiro, M.L., 2002. Glifosato: Propriedades, toxicidade, usos e legislação. Química Nova 25, 589-593.

Ankamwar, B., Lai, T.C., Huang, J.H., Liu, R.S., Hsiao, M., Chen, C.H., Hwu, Y.K., 2010. Biocompatibility of Fe_3O_4 nanoparticles evaluated by *in vitro* cytotoxicity assays using normal, glia and breast cancer cells. Nanotechnology 21, 75102.

Asati, A., Santra, S., Kaittanis, C., Perez, J.M., 2010. Surface-charge-dependent cell localization and cytotoxicity of cerium oxide nanoparticles. ACS Nano 4, 5321-5331.

Auffan, M., Decome, L., Rose, J., Orsiere, T., Meo, M., Briois, V., Chaneac, C., Olivi, L., Berge-Lefranc, J., Botta, A., Wiesner, M., Bottero, J., 2006. *In vitro* interactions between DMSA-coated maghemite nanoparticles and human fibroblasts: a physicochemical and cyto-genotoxical study. Environ. Sci. Technol. 20, 4367-4373.

Azevedo Costa, C.L., Chaves, I.S., Ventura-Lima, J., Ferreira, J.L.R., Ferraz, L., Carvalho, L.M., Monserrat, J.M., 2012. *In vitro* evaluation of co-exposure of arsenium and an organic nanomaterial (fullerene, C60) in Zebrafish hepatocytes. Comp. Biochem. Physiol. Parte C 155, 206-212.

Barja, B.C. and dos Santos Afonso, M., 2005. Aminomethylphosphonic acid and glyphosate onto goethite: a comparative study. Environ. Sci. Technol. 39, 585-592.

Bayat, N., Lopes, V.R., Sanchez-Dominguez, M., Lakshmanan, R., Rajarao, G.K., Cristobal, S., 2015. Assessment of functionalized iron oxide nanoparticles *in vitro*: introduction to integrated nanoimpact index. Environ. Sci. Nano 2, 380-394.

Benbrook, C.M., 2016. Trends in glyphosate herbicide use in the United States and globally. Environ. Sci. Eur. 28(3), 1-15.

Bhattacharya, K., Hoffmann, E., Schins, R.F.P., Boertz, J., Prantl, E., Alink, G.M., Byrne, H.J., Kuhlbusch, T.A.J., Rahman, Q., Wiggers, H., Schulz, C., Dopp, E., 2012. Comparison of micro- and nanoscale Fe^{+3}-containing (hematite) particles for their toxicological properties in human lung cells *in vitro.* Toxicol. Sci. 126, 173-182.

Bols, N.C., Dayeh, V.R., Lee, L.E.J., Schirmer, K., 2005. Use of fish cell lines in the toxicology and ecotoxicology of fish. Piscine cell lines in environmental toxicology. J. Biochem. Mol. Biol. 6, 43-84.

Borggaard, L.K. and Gimsing, A.L., 2008. Fate of glyphosate in soil and the possibility of leaching to ground and surface waters: a review. Pest Manag. Sci. 64, 441-456.

Bridi, D., Altenhofen, S., Gonzalez, J.B., Reolon, G.K., Bonan, C.D., 2017. Glyphosate and Roundup® alter morphology and behavior in zebrafish. Toxicology 392, 32-39.

Bruggen, A.H.C., He, M.M., Shin, K., Mai, V., Jeong, K.C. Finckh, M.R., Morris, J.G., 2018. Environmental and health effects of the herbicide glyphosate. Sci. Total Environ. 616-617, 255-268.

Caballero-Gallardo, K., Olivero-Verbel, J. and Freeman, J.L., 2016. Toxicogenomics to evaluate endocrine disrupting effects of environmental chemicals using the zebrafish model. Curr. Genomics 17(6), 515-527.

Cavalcante, D.G.S.M., da Silva, N.D.G., Marcarini, J.C., Mantovani, M.S., Marin-Morales, M.A., Martinez, C.B.R., 2014. Cytotoxic, biochemical and genotoxic effects of biodiesel produced by different routes on ZFL cell line. Toxicol. In Vitro 28, 1117-1125.

Cavalcante, D.G.S.M., Martinez, C.B.R., Sofia, S.H., 2008. Genotoxic effects of Roundup® on the fish *Prochilodus lineatus*. Mutat. Res. 655, 41-46.

Chen, Y.Y and Chan, K.M., 2018. Transcriptional inhibition of TCDD-mediated induction of cytochrome P450 1A1 and alteration of protein expression in a

zebrafish hepatic cell line following the administration of TCDD and Cd^{2+}. Toxicol. Lett. 282, 121-135.

Cho, W.S., Duffin, R., Thielbeer, F., Bradley, M., Megson, I.L., MacNee, W., Poland, C.A., Tran, C.L., Donaldson, K., 2012. Zeta potential and solubility to toxic ions as mechanisms of lung inflammation caused by metal/metal-oxide nanoparticles. Toxicol. Sci. 12, 1-31.

Choi, M.R., Stanton-Maxey, K.J., Stanley, J.K., Levin, C.S., Bardhan, R., Akin, D., Badve, S., Sturgis, J., Robinson, J.P., Bashir, R., Halas, N.J., Clare, S., 2007. A cellular Trojan horse for delivery of therapeutic nanoparticles into tumors. Nano Letters 7, 3759-3765.

Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivão, I., Giovannelli, L., Kruszewski, M., Smith, C.C., Štětina, R., 2008. The comet assay: topical issues. Mutagenesis 23, 143-151.

Cornell, R.M. and Schwertmann, U., 2003. The iron oxides: structure, properties, reactions, occurrences and uses. Secondedition: Wiley.

Costa, C., Brandão, F., Bessa, M.J., Costa, S., Valdiglesias, V., Kiliç, G., Fernández-Bertólez, N., Quaresma, P., Pereira, E., Pásaro, E., Laffon, B., Teixeira, J.P., 2016. *In vitro* cytotoxicity of superparamagnetic iron oxide nanoparticles on neuronal and glial cells. Evaluation of nanoparticle interference with viability tests. J. Appl. Toxicol. 36, 361-372.

De Gerónimo, E., Aparicio, V.C., Costa, J.L., 2018. Glyphosate sorption to soils of Argentina. Estimation of affinity coefficient by pedotransfer function. Geoderma 322, 140-148.

Dultz, S., Steinke, H., Mikutta, R., Woche, S.K., Guggenberger, G., 2018. Impact of organic matter types on surface charge and aggregation of goethite. Colloids Surf. A 554, 156-168.

Fajardo, C., Gil-Díaz, M., Costa, G., Alonso, J., Guerrero, A.M., Nande, M., Lobo, M.C., Martín, M., 2015. Residual impact of aged nZVI on heavy metal-polluted soils. Sci. Total Environ. 535, 79-84.

Fent, K., 2007. Permanent fish cell cultures as important tools in ecotoxicology. ALTEX 24, 26-28.

Freyre-Fonseca, V., Delgado-Buenrostro, N.L., Gutiérrez-Cirlos, E.B., Calderón-Torres, C.M., Cabellos-Avelar, T., Sánchez-Pérez, Y., Pinzón, E., Torres, I., Molina-Jijón, E., Zazueta, C., Pedraza-Chaverri, J., García-Cuélla, C.M.,

Chirino, Y.I., 2011. Titanium dioxide nanoparticles impair lung mitochondrial function. Toxicol. Lett. 202, 111-119

Gajski, G., Gerić M., Žegura, B., Novak, M., Nunić, J., Bajrektarević, D., Garaj-Vrhovac, V., Filipič, M., 2016. Genotoxic potential of selected cytostatic drugs in human and zebrafish cells. Environ. Sci. Pollut. Res. 23, 14739-14750.

Ghisi, N.C. and Cestari, M.M., 2013. Genotoxic effects of the herbicide Roundup® in the fish *Corydoraspaleatus* (Jenyns 1842) after short-term, environmentally low concentration exposure. Environ. Monit. Assess. 185, 3201-3207.

Ghosh, C., Zhou, Y.L., Collodi, P., 1994. Derivation and characterization of a zebrafish liver cell line. Cell Biol. Toxicol. 10, 167-176.

Goulart, T.L.S., Boyle, R.T., Souza, M.M., 2015. Cytotoxicity of the association of pesticides Roundup Transorb® and Furadan 350SC® on the zebrafish cell line, ZF-L. Toxicol. In Vitro 29, 1377-1384.

Guilherme, S., Santos, M.A., Gaivão, I., Barroso, C., Pacheco, M., 2012. Differential genotoxicity of Roundup formulation and its constituents in blood cells of fish (*Anguilla anguilla*): considerations on chemical interactions and DNA damaging mechanisms. Ecotoxicology21, 1381-1390.

Guilherme, S., Santos, M.A., Gaivão, I., Pacheco, M., 2014. Are DNA-damaging effects induced by herbicide formulations (Roundup® and Garlon®) in fish transient and reversible upon cessation of exposure? Aquat. Toxicol. 155, 213-221.

Hanke, I., Wittmer, I., Bischofberger, S., Stamm, C., Singer, H., 2010. Relevance of urban glyphosate use for surface water quality. Chemosphere 81, 422-429.

IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis), 2017. Relatórios de Comercialização de Agrotóxicos – Boletim de Produção, Importação, Exportação e Vendas de Agrotóxicos no Brasil. Available at: <https://www.ibama.gov.br/agrotoxicos/relatorios-de-comercializacao-de-agrotoxicos#boletinsanuais>.

Jennings, P., 2015. “The future of *in vitro* toxicology”. Toxicol. In Vitro 29, 1217-1221.

Jonsson, C.M., Persson, P., Sjöberg, S., Loring, J.S., 2008. Adsorption of glyphosate on goethite (α-FeOOH): Surface complexation modeling

combining spectroscopic and adsorption data. Environ. Sci. Technol. 42, 2464-2469.

Karlsson, H.L., Cronholm, P.,Gustafsson, J., Möller, L., 2008. Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes. Chem. Res. Toxicol. 21, 1726-1732.

Kharisov, B.I., Dias, H.V.R., Kharissova, O.V., Jiménez-Pérez, V.M., Pérez, B.O., Flores, B.M., 2012. Iron-containing nanomaterials: synthesis, properties and environmental applications.RSC Adv.2(25), 9325-9358.

Koller, V.J., Fürhacker, M., Nersesyan, A., Mišík, M., Eisenbauer, M., Knasmueller, S. 2012. Cytotoxic and DNA-damaging properties of glyphosate and Roundup in human-derived buccal epithelial cells. Arch. Toxicol. 86, 805-813.

Lachner, D., Oliveira L.F., Martinez, C.B.R., 2015. Effects of the water soluble fraction of gasoline on ZFL cell line: cytotoxicity, genotoxicity and oxidative stress. Toxicol. In Vitro 30, 225-230.

Laskar, A., Ghosh, M., Khattak, S.I., Li, W., Yuan, X.M., 2012. Degradation of superparamagnetic iron oxide nanoparticle-induced ferritin by lysosomal cathepsins and related immune response. Nanomedicine 7, 705-717.

Lei, C., Sun, Y., Tsang, D.C.W., Lin, D., 2018. Environmental transformations and ecological effects of iron-based nanoparticles. Environ. Pollut. 232, 10-30.

Levi, S. and Rovida, E., 2009. The role of iron in mitochondrial function. Biochim. Biophys. Acta 1790, 629-636.

Limbach, L.K., Wick, P., Manser, P., Grass, R.N., Bruinink, A., Stark, W.J., 2007. Exposure of engineered nanoparticles to human lung epithelial cells: influence of chemical composition and catalytic activity on oxidative stress. Environ. Sci. Technol. 41, 4158-4163.

Liu, H., Chen, T., Frost, R.L., 2014. An overview of the role of goethite surfaces in the environment. Chemosphere 103, 1-11.

Lopes, F.M., Varela Junior, A.S., Corcini, C.D., da Silva, A.C., Guazzelli, V.G., Tavares, G., da Rosa, C.E., 2014. Effect of glyphosate on the sperm quality of zebrafish *Danio rerio*. Aquat. Toxicol. 155, 322-326.

Lopes, F.M., Sandrini, J.Z., Souza, M.M., 2018. Toxicity induced by glyphosate and glyphosate-based herbicides in the zebrafish hepatocyte cell line (ZF-L).

Ecotoxicol. Environ. Saf. 162, 201-207.

Lushchak, O.V., Kubrak, O.I., Storey, J.M., Storey, K.B., Lushchak, V.I., 2009. Low toxic herbicide Roundup induces mild oxidative stress in goldfish tissues. Chemosphere 76, 932-937.

Maqueda, C., Undabeytia, T., Villaverde, J., Morillo, E., 2017. Behaviour of glyphosate in a reservoir and the surrounding agricultural soils. Sci. Total Environ. 593-594, 787-795.

Marques, A., Guilherme, S., Gaivão, I., Santos, M.A., Pacheco, M., 2014. Progression of DNA damage induced by a glyphosate-based herbicide in fish (*Anguilla Anguilla*) upon exposure and post-exposure periods – Insights into the mechanisms of genotoxicity and DNA repair. Comp. Biochem. Physiol. Part C166, 126-133.

Modesto, K.A. and Martinez, C.B.R., 2010a. Effects of Roundup Transorb on fish: hematology, antioxidant defenses and acetylcholinesterase activity. Chemosphere 81, 781-787.

Modesto, K.A. and Martinez, C.B.R., 2010b. Roundup causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brainof the fish *Prochilodus lineatus*. Chemosphere 78, 294-299.

Moreno, N.C., Sofia, S.H., Martinez, C.B.R., 2014. Genotoxic effects of the herbicide Roundup Transorb® and its active ingredient glyphosate on the fish *Prochilodus lineatus*. Environ. Toxicol. Pharm. 37, 448-454.

Morozesk, M., Franqui, L.S., Mansano, A.S., Martinez, D.S.T., Fernandes, M.N., 2018. Interactions of oxidized multiwalledcarbono nanotube with cadmium on zebrafish cell line: The influence of two co-exposure protocols on *in vitro* toxicity tests. Aquat. Toxicol. 200, 136-147.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival. J. Immunol. Methods 65, 55-63.

Navarro, C.D.C. and Martinez, C.B.R., 2014. Effects of the surfactant polyoxyethylene amine (POEA) on genotoxic, biochemical and physiological parameters of the freshwater teleost *Prochilodus lineatus*. Comp. Biochem. Phys. Part C 165, 83-90.

Novak, M., Žegura, B., Modic, B., Heath, E., Filipič, M., 2017. Cytotoxicity and genotoxicity of anticancer drug residues and their mixtures in experimental model with zebrafish liver cells. Sci. Total Environ. 601-602, 293-300.

O'Carroll, D., Sleep, B., Krol, M., Boparai, H., Kocur, C., 2013. Nanoscale zero valent iron and bimetallic particles for contaminated site remediation. Adv. WaterResour. 51, 104-122.

Orcelli, T., di Mauro, E., Urbano, A., Valezi, D.F., da Costa A.C.S., Zaia, C.T.B., Zaia, D.A.M., 2018. Study of interaction between glyphosate and goethite using several methodologies: an environmental perspective. Water Air Soil Pollut. 229, 1-18.

Park, J.H. and Park, E., 2011. Influence of iron-overload on DNA damage and its repair in human leukocytes *in vitro*. Mutat. Res-Gen Tox. En. 718, 56-61.

Peixoto, F., 2005. Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. Chemosphere 61, 1115-1122.

Pereira, A.G., Jaramillo, M.L., Remor, A.P., Latini, A., Davico, C.E., Silva, M.L., Müller, Y.M.R., Ammar, D., Nazari, E.M. 2018. Low-concentration exposure to glyphosate-based herbicide modulates the complexes of the mitochondrial respiratory chain and induces mitochondrial hyperpolarization in the brain *Danio rerio*. Chemosphere 209, 353-362.

Pessagno, P.C., Sánchez, R.M.T., dos Santos Afonso, M., 2008. Glyphosate behavior at soil and mineral – water interfaces. Environ. Pollut. 153, 53-59.

Pisanic, T.R., Blackwell, J.D., Shubayev, V.I., Fiñones, R.R., Jin, S., 2007. Nanotoxicity of iron oxide nanoparticle internalization in growing neurons. Biomaterials 28(16), 2572-2581.

Rim, K.T., Song, S.W., Kim, H.Y., 2013. Oxidative DNA damage from nanoparticle exposure and its application to workers' health: a literature review. Saf. Health Work 4, 177-186.

Rodrigues, L.B., Oliveira, R., Abe, F.R., Brito, L.B., Moura, D.S., Valadares, M.C., Grisolia, C.K., Oliveira, D.P., Oliveira, G.A.R., 2017. Ecotoxicological assessment of glyphosate-based herbicides: effects on different organisms. Environ. Toxicol. Chem. 36, 1755-1763.

Sandrini, J.Z., Bianchini, A., Trindade, G.S., Nery, L.E.M., Marins, L.F.F., 2009. Reactive oxygen species generation and expression of DNA repair-related genes after copper exposure in zebrafish (*Danio rerio*) ZFL cells. Aquat. Toxicol. 95, 285-291.

Sandrini, J.Z., Rola, R.C., Lopes, F.M., Buffon, H.F., Freitas, M.M., Martins, C.M.G., Rosa, C.E., 2013. Effects of glyphosate on cholinesterase activity of

the mussel *Perna perna* and the fish *Danio rerio* and *Jenynsia multidentata*: *in vitro* studies. Aquat. Toxicol. 130-131, 171-173.

Schwertmann, U. and Cornell, R.M., 2000. Iron oxides in the laboratory. Preparation and characterization. Second edition: Wiley.

Sheals, J., Sjöberg, S., Persson, P., 2002. Adsorption of glyphosate on goethite: molecular characterization of surface complexes. Environ. Sci. Technol. 36, 3090-3095.

Singh, N., Jenkins, G.J.S., Asadi, R., Doak, S.H., 2010. Potential toxicity of superparamagnetic iron oxide nanoparticles (SPION). Nano reviews 1, 5358-5373.

Singh, N., Jenkins, G.J.S., Nelson, B.C., Marquis, B.J., Maffeis, T.G.G., Brown, A.P., Williams, P.M., Wright, C.J., Doak, S.H., 2012. The role of iron redox state in the genotoxicity of ultrafine superparamagnetic iron oxide nanoparticles. Biomaterials 33, 163-170.

Singh, N.P., Mccoy, M.T., Tice, R.R., Schneider, E.L., 1988. A single technique for quantification of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184-191.

Singh, S., Khanna, V.K., Pant, A.B., 2018. Development of *in vitro* toxicology: a historic story. In: In Vitro Toxicology. Academic Press, Chapter 1, 1-19.

Sonmez, E., Aydin, E., Turkez, H., Özbek, E., Togar, B., Meral, K., Çetin, D., Cacciatore, I., Di Stefano, A., 2016. Cytotoxicity and genotoxicity of iron oxide nanoparticles: an *in vitro* biosafety study. Arch. Biol. Sci. 68, 41-50.

Srivastava, S., Mishra, S., Dewangan, J., Divakar, A., Pandey, P.K., Rath, S.K., 2018. Principles for *in vitro* toxicology. In: In Vitro Toxicology. Academic Press, Chapter 2, 21-43.

Tang, S., Allagadda, V., Chibli, H., Nadeau, J.L., Mayer, G.D., 2013. Comparison of cytotoxicity and expression of metal regulatory genes in zebrafish (*Danio rerio*) liver cells exposed to cadmium sulfate, zinc sulfate and quantum dots. Metallomics 5, 1411-1422.

Tang, T., Boënne, W., Desmet, N., Seuntjens, P., Bronders, J., van Griensven, A., 2015. Quantification and characterization of glyphosate use and loss in a residential area. Sci. Total Environ. 517, 207-214.

Teodoro, J.S., Simões, A.M., Duarte, F.V., Rolo, A.P., Murdoch, R.C., Hussain, S.M., Palmeira, C.M., 2011. Assessment of the toxicity of silver nanoparticles

in vitro: a mitochondrial perspective. Toxicol. In Vitro 25, 664-670.

Thit, A., Skjolding, L.M., Selck, H., Sturve, J., 2017. Effects of copper oxide nanoparticles and copper ions to zebrafish (*Danio rerio*) cells, embryos and fry. Toxicol. In Vitro 45, 89-100.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ. Mol. Mutagen. 35, 206-221.

Tosco, T., Papini, M.P., Vigghi, C.C., Sethi, R., 2014. Nanoscale zerovalent iron particles for groundwater remediation: a review. J. Clean. Prod. 77, 10-21.

Tsui, M.T.K. and Chu, L.M., 2003. Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere 52, 1189-1197.

Ugarte, R. 2014. Interaction between glyphosate and mitochondrial succinate dehydrogenase. Comput. Theor. Chem. 1043, 54-63.

Vanlaeys, A., Dubuisson, F., Seralini, G.E., Travert, C., 2018. Formulants of glyphosate-based herbicides have more deleterious impact than glyphosate on TM4 Sertoli cells. Toxicol. In Vitro 52, 14-22.

Waiman, C.V., Avena, M.J., Garrido, M., Band, B.F., Zanini, G.P., 2012. A simple and rapid spectrophotometric method to quantify the herbicide glyphosate in aqueous media. Application to adsorption isotherms on soils and goethite. Geoderma 170, 154-158.

WHO (World Health Organization), 2005. Water sanitation hygiene. Chemical hazards in drinking-water: Glyphosate and AMPA. Availableat: <http://www.who.int/water_sanitation_health/water-quality/guidelines/chemicals/glyphosateampa290605.pdf?ua=1>.

Xia, T., Kovochich, M., Liong, M., Mädler, L., Gilbert, B., Shi, H., Yeh, J.I., Zink, J.I., Nel, A.E., 2008. Comparison of the mechanisms of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. ACS Nano 2, 2121-2134.

Yang, Y., Deng, Q., Yan, W., Jing, C., Zhang, Y., 2018. Comparative study of glyphosate removal on goethite and magnetite: Adsorption and photo-degradation. Chem. Eng. J. 352, 581-589.

Žegura, Z. andFilipič, M., 2019. The application of the Comet assay in fish cell

lines. Mutat. Res. Gen.Tox.En.https://doi.org/10.1016/j.mrgentox.2019.01.011

Table 1. Mean concentrations of total and dissolved Fe (mg L^{-1}) in the exposure solutions of the different treatments.

Fe concentrations (mg L^{-1})								
	Total	Dissolved		Total	Dissolved		Total	Dissolved
CTR	0.76	0,67	**CTR**	0.76	0.67	**CTR**	0.76	0.67
N1	0.73	0.52	**Gly**	0.77	0.76	**Rd**	0.56	0.58
N2	5.23	0.69	**GlyN1**	1.48	0.97	**RdN1**	1.08	0.54
N3	60.0	8.36	**GlyN2**	6.23	0.95	**RdN2**	6.99	0.62
			GlyN3	62.2	7.10	**RdN3**	71.1	4.97

Figure captions

Fig. 1. Mean diameter (A), polydispersity (B), and zeta potential (C) of the goethite NPs present in the exposure solutions of 1, 10, and 100 mg L^{-1} (N1, N2, and N3) for 0, 24, and 48 h. Results are mean ± SE (n = 3). Different letters indicate significant differences between treatments for the same experimental time ($P < 0.05$).

Fig. 2. Cell viability (%) based on plasma membrane integrity (A-C), mitochondrial activity (D-F), and lysosomal integrity (G-I) for ZFL cells exposed to: 1, 10, and 100 mg L^{-1} of goethite nanoparticles (N1, N2, and N3); 3.6 mg L^{-1} of glyphosate (Gly) and the co-exposures (GlyN1, GlyN2, and GlyN3) and 10 mg L^{-1} of Roundup® (Rd) and the co-exposures (RdN1, RdN2, and RdN3) or only to the Dulbecco´s PBS (CTR) for 1, 6, and 12 h. Results are mean ± SE (n = 4). Different letters indicate significant differences between treatments for the same experimental time ($P < 0.05$).

Fig. 3. Production of reactive oxygen species (ROS) in ZFL cells exposed to: A) 1, 10, and 100 mg L^{-1} of goethite nanoparticles (N1, N2, and N3); B) 3.6 mg L^{-1} of glyphosate (Gly) and the co-exposures (GlyN1, GlyN2, and GlyN3); and C) 10 mg L^{-1} of Roundup® (Rd) and the co-exposures (RdN1, RdN2, and RdN3) or only to the Dulbecco´s PBS (CTR) for 1, 6, and 12 h. Results are mean ± SE (n = 4). Different letters indicate significant differences between treatments for the same experimental time ($P < 0.05$).

Fig. 4. Antioxidant capacity against peroxyl radicals (ACAP) of ZFL cells exposed to: A) 1, 10, and 100 mg L^{-1} of goethite nanoparticles (N1, N2, and N3); B) 3.6 mg L^{-1} of glyphosate (Gly) and the co-exposures (GlyN1, GlyN2, and GlyN3); and C) 10 mg L^{-1} of Roundup® (Rd) and the co-exposures (RdN1, RdN2, and RdN3) or only to the Dulbecco´s PBS (CTR) for 1, 6, and 12 h. Results are mean ± SE (n = 4). Different letters indicate significant differences between treatments for the same experimental time ($P < 0.05$).

Fig. 5. A) Score of DNA damage in ZFL cells exposed to 0.5 mM of MMS (Positive Control or PC); B) 1, 10, and 100 mg L^{-1} of goethite nanoparticles (N1, N2, and N3); C) 3.6 mg L^{-1} of glyphosate (Gly) and the co-exposures (GlyN1, GlyN2, and GlyN3); and D) 10 mg L^{-1} of Roundup® (Rd) and the co-exposures (RdN1, RdN2, and RdN3) or only to the Dulbecco´s PBS (CTR) for 1, 6, and 12 h, quantified by the comet assay. Results are mean ± SE (n = 4). Different letters indicate significant differences between treatments for the same experimental time ($P < 0.05$). E) Photomicrographs of ZFL

cells processed for comet assay showing increasing degrees of DNA damage (original magnification: 1000x).

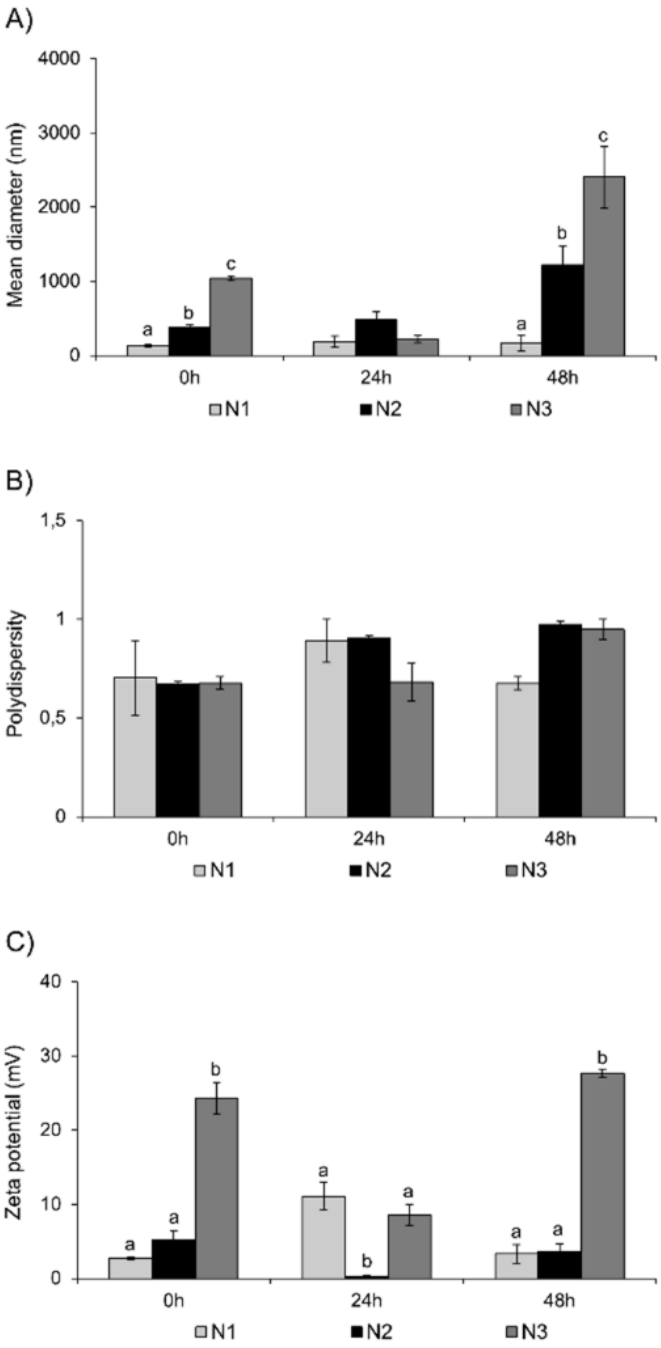
A)
Mean diameter (nm)
4000
3000
2000
1000
0
a
b
c
a
b
c
0h
24h
48h
N1
N2
N3
B)
Polydispersity
1,5
1
0,5
0
0h
24h
48h
N1
N2
N3
C)
Zeta potential (mV)
40
30
20
10
0
a
a
b
a
b
a
a
b
0h
24h
48h
N1
N2
N3

Fig. 1.

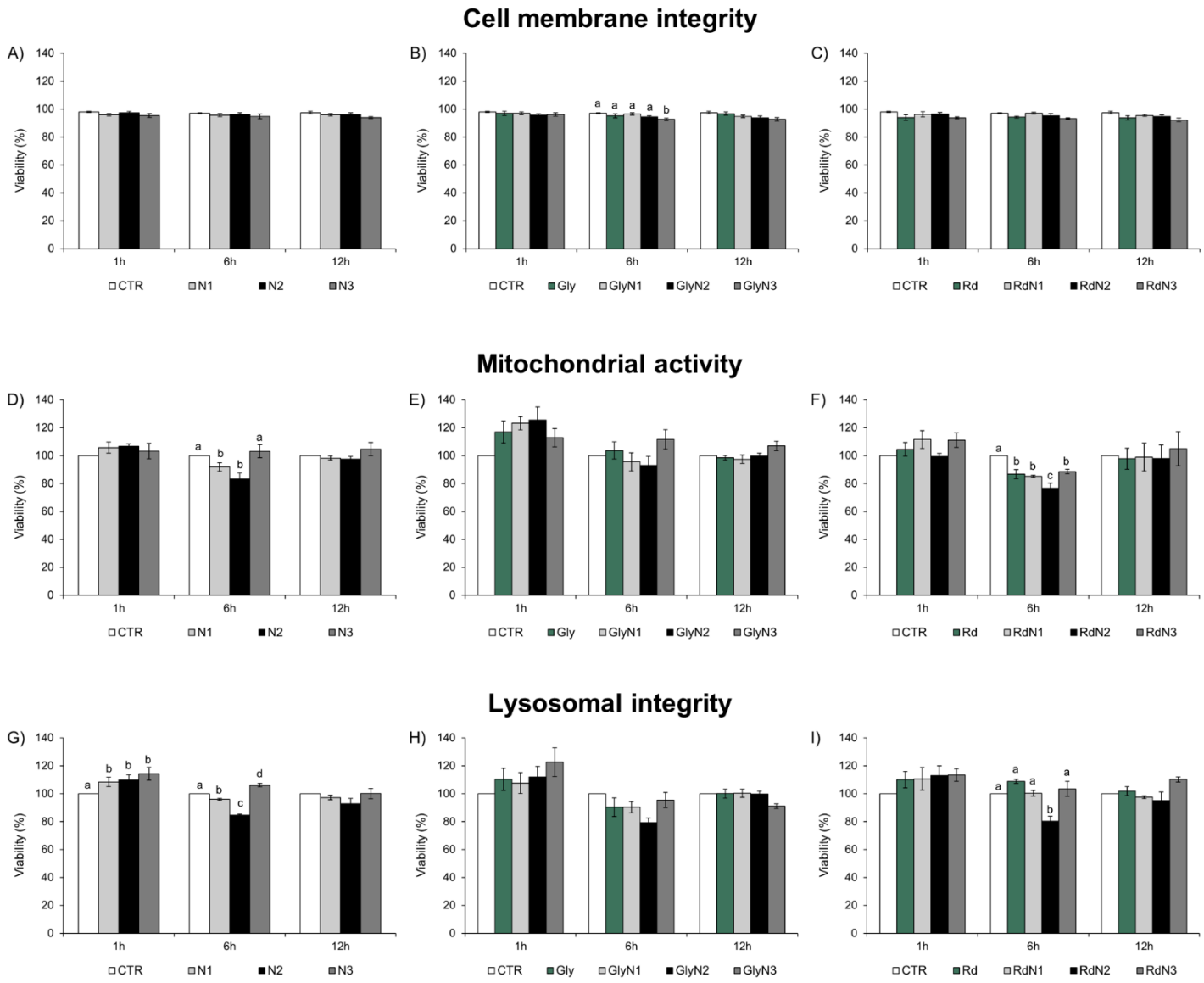
Cell membrane integrity
Mitochondrial activity
Lysosomal integrity
A)
B)
C)
D)
E)
F)
G)
H)
I)
Viability (%)
1h
6h
12h
CTR
N1
N2
N3
Gly
GlyN1
GlyN2
GlyN3
Rd
RdN1
RdN2
RdN3

Fig. 2.

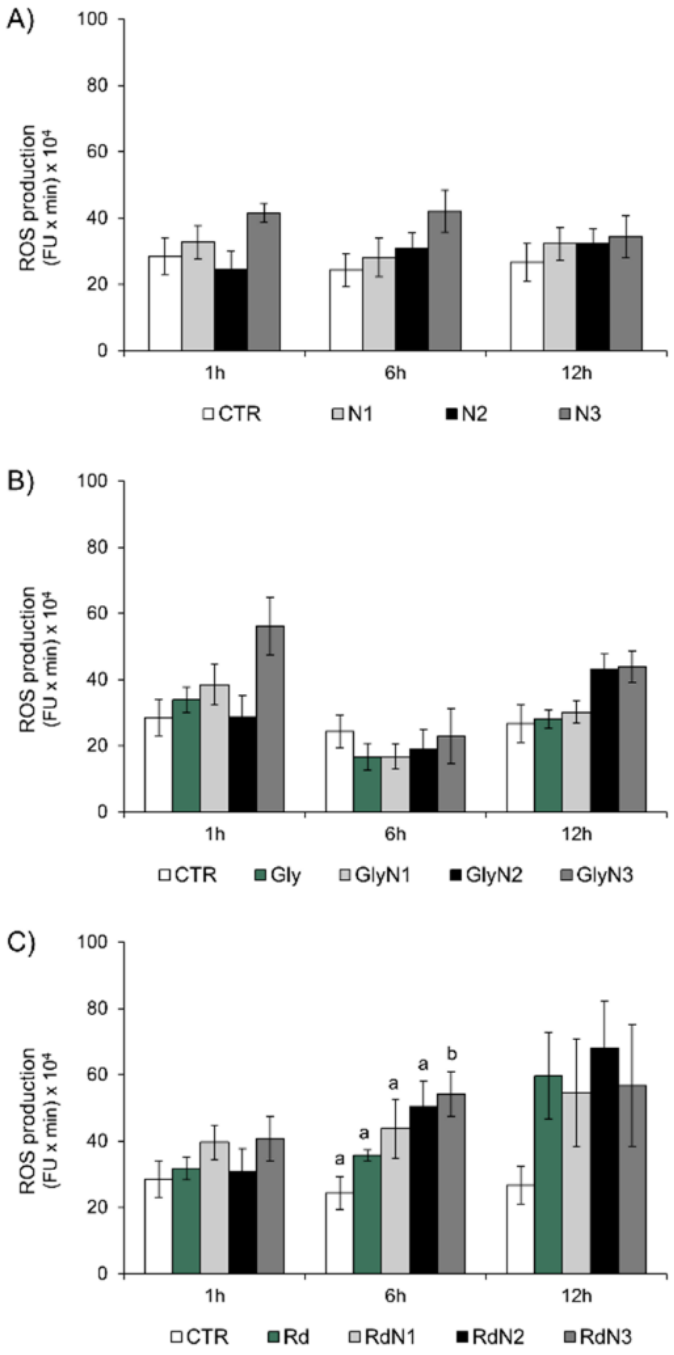
A)
ROS production (FU x min) x 10⁴
100
80
60
40
20
0
1h
6h
12h
CTR
N1
N2
N3
B)
ROS production (FU x min) x 10⁴
100
80
60
40
20
0
1h
6h
12h
CTR
Gly
GlyN1
GlyN2
GlyN3
C)
ROS production (FU x min) x 10⁴
100
80
60
40
20
0
a
a
a
a
b
1h
6h
12h
CTR
Rd
RdN1
RdN2
RdN3

Fig. 3.

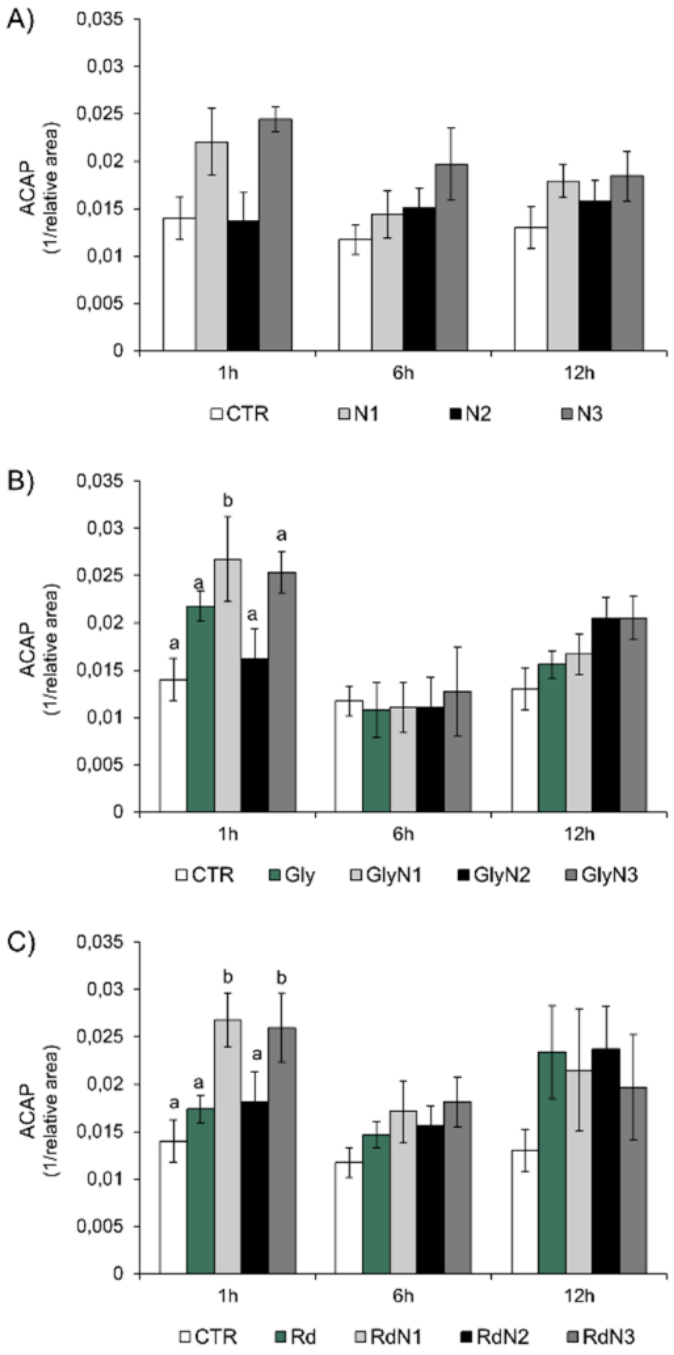
A)
ACAP (1/relative area)
0,035
0,03
0,025
0,02
0,015
0,01
0,005
0
1h
6h
12h
CTR
N1
N2
N3
B)
ACAP (1/relative area)
a
a
b
a
a
1h
6h
12h
CTR
Gly
GlyN1
GlyN2
GlyN3
C)
ACAP (1/relative area)
a
a
b
a
b
1h
6h
12h
CTR
Rd
RdN1
RdN2
RdN3

Fig. 4.

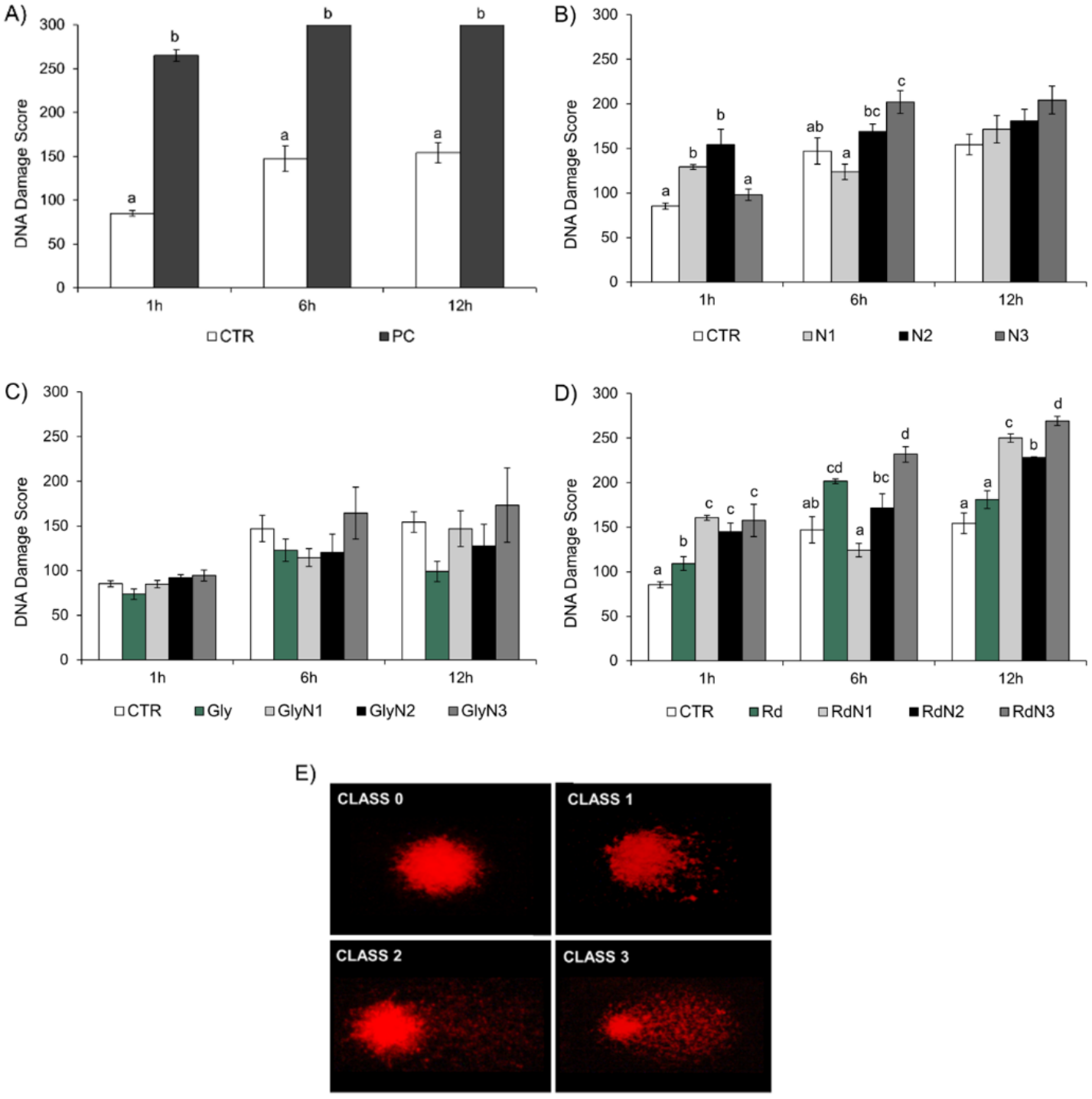
A)
DNA Damage Score
300
250
200
150
100
50
0
a
b
a
b
a
b
1h
6h
12h
CTR
PC
B)
DNA Damage Score
a
b
b
a
ab
a
bc
c
1h
6h
12h
CTR
N1
N2
N3
C)
DNA Damage Score
1h
6h
12h
CTR
Gly
GlyN1
GlyN2
GlyN3
D)
DNA Damage Score
a
b
c
c
c
ab
cd
a
bc
d
a
a
c
b
d
1h
6h
12h
CTR
Rd
RdN1
RdN2
RdN3
E)
CLASS 0
CLASS 1
CLASS 2
CLASS 3

Fig. 5.

Declaration of interests

 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: