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Manuscript Draft

Manuscript Number: STOTEN-D-18-13607R1

Title: Can atrazine loaded nanocapsules reduce the toxic effects of this herbicide on the fish Prochilodus lineatus? A multibiomarker approach

Article Type: Research Paper

Keywords: Nanotechnology, oxidative stress, genotoxicity, osmoregulation, nanopesticide.

Corresponding Author: Dr. Claudia B R Martinez, Ph.D.

Corresponding Author's Institution: Universidade Estadual de Londrina

First Author: Laura L Andrade, Master

Order of Authors: Laura L Andrade, Master; Anderson E Pereira, PhD; Leonardo F Fraceto, PhD; Claudia B R Martinez, Ph.D.

Abstract: Atrazine (ATZ) is a widely used herbicide that has the potential to contaminate the environment and cause deleterious effects on non-target organisms. Release systems for ATZ have been developed to minimize this contamination, such as nanocapsules prepared with poly (ε-caprolactone) (PCL). The objective of this work was to investigate the effects of nanoencapsulated ATZ compared to ATZ on biomarkers of the freshwater teleost Prochilodus lineatus. The fish were exposed for 24 and 96 h to nanoencapsulated ATZ (nATZ) and atrazine (ATZ) at concentrations of 2 and 20 µg L-1, just to the PCL nanocapsules without the herbicide (NANO) in the corresponding amounts or only to dechlorinated water (CTR). The results showed that nATZ was less toxic compared to ATZ, as it did not promote an increase in glycemia, alterations in antioxidants, nor in carbonic anhydrase enzyme activity, and no increase in the frequency of micronuclei and other nuclear erythrocyte abnormalities either. However, exposure to nATZ, as well as to ATZ and PCL nanocapsules, resulted in a reduction in hemoglobin content, increase in erythrocyte DNA damage, as well as changes in Ca2+-ATPase activity, leading to a decrease in plasma Ca+2. The Integrated Biomarker Response Index (IBR) depicted that exposure to ATZ promoted changes in a greater number of biomarkers compared to nATZ, indicating that the nanoencapsulation of the herbicide protected the animal from the effects of ATZ.

Response to Reviewers: We have made specific answers to each the reviewers' questions. We believe the revised manuscript is a better description of our work and conclusions and we are grateful for the careful revision made by the reviewers.

Reviewer #1
1. In the Highlights, the authors should highlight your findings and lists them as 3 or 4 items.
Answer: As suggested we changed the highlights as it follows:
- The effects of nanoencapsulated (nATZ) and atrazine (ATZ) on fish were compared

- Fish were exposed to ATZ, nATZ or ony to the nanocapsules (NANO) for 24 and 96 h
- Genotoxic, biochemical and physiological biomarkers were measured
- ATZ promoted changes in a greater number of biomarkers compared to nATZ
- Nanoencapsulation of the herbicide protected the animal from the effects of ATZ

2. There are too many abbreviations in the manuscript, and I recommend the authors list an abbreviations in the manuscript.
Answer: All the abbreviations are clearly described along the text, mainly in the Material and Methods section. We did not to include a list of abbreviations, as requested, considering that we did not observe list of abbreviation in other articles published in Stoten.
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Answer: As suggested we reduced the description of the methods as much we could. It was not possible to describe some of the physiological and biochemical methods as brief as Zheng et al, because they have used detection kits (purchased from Jiancheng Bioengineering) following the manufacturer's protocol and we did not.
4. The Statistical method used is wrongly described. It must have been a two-factor analysis of variance with between animal factor and time factor.
Answer: As we wrote in the Material and Methods section (item 2.3), for each concentration (2 and 20 µg L^{-1}) at each experimental period (24 and 96 h), independent experiments were performed and the four treatments (CTR, NANO, ATZ, and nATZ) ran simultaneously. Considering this we made statistical comparisons only among the treatments performed simultaneously (CTR x Nano x Atz x nATZ) and that's why we used single factor analysis of variance (ANOVA or Kruskal-Wallis).
5. In the Results, all the P value should be marked in the significant different results.
Answer: As requested, besides F or H values we included P values.
6. In the Figures: the figure legends all abbreviations must be explained. Furthermore, the Y axis should be explained detailly. In addition, there are two many figures, in the manuscript, I recommend the authors combine some figures, such as Fig. 3 and Fig. 4; Fig. 5 and Fig. 6.
Answer: As requested, we merged Figs 5 and 6 into one. We did not merge Figs 3 and 4 because it would become a Figure with too many graphics which were not closely related.
7. The Discussion need to be improved and some recent research about ATZ should be noticed. Such as, a study suggested atrazine hinders PMA-induced neutrophil extracellular traps in carp via inhibition of ROS burst, autophagy and glycolysis Environmental Pollution 243 (2018) 282-291 (https://doi.org/10.1016/j.envpol.2018.08.070). How does the authors explain these different results? And current study hinted nATZ is less toxic than the conventional formulation. Is it due to the dosage form or the nanomaterials protection itself? Please also add discussion on the limitations of the study and future perspectives.
Answer: We thank the reviewer for this suggestion. We read this paper (Wang et al. 2018) and another recent paper by Wang et al (2019), and both of them are now cited in our work. Considering the possible protection effect of ATZ nanoencapsulation we wrote in the discussion that the lower toxicity of nanoencapsulated atrazine (in comparison to

free atrazine) could be due to the slower release of ATZ and consequently the lower amounts of the herbicide available to the organism.

Reviewer #2

1. Some information on environment levels of atrazine would help in understanding the potential risks associated. Add environmental levels of atrazine in the aquatic ecosystem.
Answer: In order to attend this request we included in the Introduction (at the end of the first paragraph) the following sentences: Field surveys have shown that this herbicide is commonly detected in surface waters in levels above the limits determined by the guidelines of the US Environmental Protection Agency (3 µg L-1; USEPA, 2014). In Brazil, ATZ concentrations between 0.3 and 5.4 µg L-1 were registered in surface freshwater (Loro et al., 2015, Vieira et al., 2017). In streams of North America this herbicide has been repeatedly detected at concentrations above 100 µg L-1 (Ehrsam et al., 2016).
2. The last paragraph in introduction should give the reader more insight into your study. Based on previous studies, what are your hypotheses/expectations for each of the things you are examining?
Answer: We presented information concerning atrazine effects on aquatic organisms along the second paragraph of the Introduction, as it follows: Several studies have shown that aquatic organisms such as bivalves (Santos and Martinez, 2014) and fish are sensitive to the ATZ (Blahova et al., 2013; Mela et al., 2013; Nwani et al., 2011; Zadeh et al., 2016). The freshwater fish Prochilodus lineatus exposed to ATZ showed reduced activity of antioxidant and biotransformation enzymes in the liver, genotoxic damage in different cells, alterations in plasma osmolality, and a decrease in the gill activity of carbonic anhydrase, as well as morphological alterations in the gills (Paulino et al., 2012; Santos and Martinez, 2012). Anyway, we changed the last paragraph of the Introduction in order to attend this request, as it follows: "In this context, this work aimed to evaluate the effects of nanoencapsulated ATZ compared with its conventional formulation on genotoxic, biochemical, and physiological biomarkers of the fish P. lineatus. This biological model was chosen as this is a neotropical freshwater fish widely used in human food and sensitive to various xenobiotics, among them ATZ (Paulino et al., 2012; Santos and Martinez, 2012). The results of this study will provide new information concerning the effects of nanopesticides on aquatic organisms which is essential for the safe use of nanocarrier systems in agriculture."
3. Line no. 143 - Add purity of used Atrazine.
Answer: The purity of atrazine (purity ≥ 98.0%) was added.
4. In general the discussion is so long for significant results presented, authors need improve the discussion section focusing the importance of study considering environmental relevant concentrations used and the relevance of this study. The discussion section is repetitive, when compared to the results section. This duplication should be eliminated. Please shorten the discussion.
Answer: We shortened the discussion a little and tried to reduce the duplication (results and discussion). However, we have to add some other comments, as suggested by the other reviewers. We believe the discussion is better now.
5. In the conclusion, no exact suggestion was proposed, because the authors did not thoroughly discuss their findings and did not conclude the real necessities for future study.

Answer: In order to attend this request, our conclusion was revised as as it follows: "The development of controlled release systems could mitigate the negative impacts of pesticides on the environment, as well as increase food safety. However, studies concerning the effects of these release systems are necessary for their regulation and use in the agricultural market. Thus, the present work is important to understand the toxicity of nanoencapsulated ATZ in a non-target organism by assessing its ecological risk. The results of the integrated analysis of the biomarkers showed that the ATZ presented a higher toxicity to P. lineatus compared to its nanoencapsulated formulation. This demonstrates that nanoencapsulated ATZ was able to protect the animal from the effects of the herbicide in a general way, indicating that nanoencapsulated ATZ is less toxic to P. lineatus compared to its conventional formulation. it is likely that the lower toxicity of nanoencapsulated atrazine compared to free atrazine is related to the slower release of ATZ and consequently the lower amounts of the herbicide available to the organism. However, more studies are needed to clarify the mechanisms underlying the different effects produced by the herbicide in the free and nanoencapsulated form, as well as the effects of PCL nanocapsules.

Reviewer #3
1. The graphical abstract looks too complicated, please make it simple and clear.
Answer: Graphical abstract was revised. We believe it is clearer now.
2. line 43, "This herbicide is widely used, for the control of weeds" , the comma should be eliminated.
Answer: Ok
3. In the "Fish handling, experimental design, and sampling", you should indicate the purity of atrazine. And you should correct the bold words into normal style words.
Answer: As requested we indicated the purity of atrazine (purity ≥ 98.0%) and corrected the bold words into normal style.
4. line 169, this unit of Celsius is not standard.
Answer: The unit of Celsius was corrected.
5. Please handle the title of each experimental program in method, italics or bold?
Answer: Titles of the experiments were standardized.
6. The introduction of computational software is lacking in statistical analysis.
Answer: As requested we included the following sentence in the end of the item 2.8 Statistical analyzes: All analyzes were carried out using the software SigmaPlot 11.0.
7. Figure 1 is not clear enough, and the picture Numbers A and B are inconsistent with those of other figures.
Answer: We removed Figure 1. Data concerning nanoparticles characterization are now presented in Table 1.
8. Each result section should have a general title. I think you can combine eight figures of the results properly and keep them within 6 in total
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9. Have you read the articles "Atrazine exposure triggers common carp neutrophil apoptosis via the CYP450s/ROS pathway" and "Atrazine hinders PMA-induced neutrophil extracellular traps in carp via the promotion of apoptosis and inhibition of ROS burst, autophagy and glycolysis"? The two articles are the new researches about atrazine, which are closely related to your research results. You can quote them.

Answer: We thank the reviewer for this suggestion. We read these two recent papers by Wang et al (2018 and 2018) and both of them are now cited in our work.
10. line 377, Brassica sp.,?
Answer: Brassica sp. is the target plant used in the work by Pereira et al 2014. We checked and it is corrected.

Cover Letter

Universidade Estadual de Londrina

Centro de Ciências Biológicas
Departamento de Ciências Fisiológicas
Laboratório de Ecofisiologia Animal
Dra. Cláudia Bueno dos Reis Martinez

Londrina, January 22, 2019

Daniel A. Wunderlin, Ph.D.
Associate Editor
Science of the Total Environment

Dear Editor,

Please find enclosed the revised version of the manuscript “Can atrazine loaded nanocapsules reduce the toxic effects of this herbicide on the fish *Prochilodus lineatus*? A multibiomarker approach” (STOTEN-D-18-13607) to be submitted to Science of the Total Environment.

We have now made all the changes suggested by the three reviewers. We believe this revised version of the manuscript is a better description of our work and we are grateful for the careful revision made by all the reviewers and the editor.

Looking forward hearing from you soon.

Yours sincerely.

Claudia Bueno dos Reis Martinez

Campus Universitário – Caixa Postal 10011 – CEP: 86057-970 – Londrina – Paraná
Fone: 43-33715146 E-mail: cbueno@uel.br; claudiabrmartinez@gmail.com

Can atrazine loaded nanocapsules reduce the toxic effects of this herbicide on the fish *Prochilodus lineatus*? A multibiomarker approach

Laura Lui de Andrade[1], Anderson do Espirito Santo Pereira[2], Leonardo Fernandes Fraceto[2], Claudia Bueno dos Reis Martinez*[1]

[1]*Department of Physiological Sciences, State University of Londrina (UEL), Londrina, Paraná, Brazil;* [2] *Institute of Science and Technology, São Paulo State University (UNESP), Sorocaba, Brazil.*

*Corresponding author

Claudia Bueno dos Reis Martinez - Laboratório de Ecofisiologia Animal, Departamento de Ciências Fisiológicas, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, Km 380, s/n - Campus Universitário, Londrina - PR, Brasil, CP 86057-970. Tel.: +55(43) 3371.5146. E-mail: claudiabrmartinez@gmail.com; cbueno@uel.br

Response to Reviewers

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Journal: Science of the Total Environment

Corresponding author: Claudia B R Martinez

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Answer: *Ok*

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Answer: *The unit of Celsius was corrected.*

5. Please handle the title of each experimental program in method, italics or bold?

Answer: *Titles of the experiments were standardized.*

6. The introduction of computational software is lacking in statistical analysis.

Answer: *As requested we included the following sentence in the end of the item 2.8 Statistical analyzes: All analyzes were carried out using the software SigmaPlot 11.0.*

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Abstract

Atrazine (ATZ) is a widely used herbicide that has the potential to contaminate the environment and cause deleterious effects on non-target organisms. Release systems for ATZ have been developed to minimize this contamination, such as nanocapsules prepared with poly (ε-caprolactone) (PCL). The objective of this work was to investigate the effects of nanoencapsulated ATZ compared to ATZ on biomarkers of the freshwater teleost *Prochilodus lineatus*. The fish were exposed for 24 and 96 h to nanoencapsulated ATZ (nATZ) and atrazine (ATZ) at concentrations of 2 and 20 μg L^{-1}, just to the PCL nanocapsules without the herbicide (NANO) in the corresponding amounts or only to dechlorinated water (CTR). The results showed that nATZ was less toxic compared to ATZ, as it did not promote an increase in glycemia, alterations in antioxidants, nor in carbonic anhydrase enzyme activity, and no increase in the frequency of micronuclei and other nuclear erythrocyte abnormalities either. However, exposure to nATZ, as well as to ATZ and PCL nanocapsules, resulted in a reduction in hemoglobin content, increase in erythrocyte DNA damage, as well as changes in Ca^{2+}-ATPase activity, leading to a decrease in plasma Ca^{+2}. The Integrated Biomarker Response Index (IBR) depicted that exposure to ATZ promoted changes in a greater number of biomarkers compared to nATZ, indicating that the nanoencapsulation of the herbicide protected the animal from the effects of ATZ.

Keywords: Nanotechnology, oxidative stress, genotoxicity, osmoregulation, nanopesticides.

1. INTRODUCTION

Atrazine (ATZ) is a triazinic herbicide used in the control of weeds, whose mechanism of action is the inhibition of the photosynthetic system, that result in the plant death (Nakka et al., 2017). This herbicide is widely used~~,~~ for the control of weeds in many crops as example, sugarcane, wheat, sorghum, nuts, and corn (Singh et al., 2018). Even though its use has been banned in European Union, ATZ is the second most consumed pesticide in the world, with annual consumption of 70,000 to 90,000 tons (Ehrsam et al., 2016; Singh et al., 2018). ATZ has high potential to contaminate soil, surface water, and groundwater due its high persistence and mobility in the environment (Cerejeira et al., 2003; Kumar et al., 2013; Schwab et al., 2006). Field surveys have shown that this herbicide is commonly detected in surface waters in levels above the limits determined by the guidelines of the US Environmental Protection Agency (3 µg L^{-1}; USEPA, 2014). In Brazil, ATZ concentrations between 0.31 and 5.4 µg L^{-1} were registered in surface freshwater (Loro et al., 2015, Vieira et al., 2017). In streams of North America this herbicide has been repeatedly detected at concentrations above 100 µg L^{-1} (Ehrsam et al., 2016).

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Several studies have shown that aquatic organisms such as bivalves (Santos and Martinez, 2014) and fish are sensitive to the ATZ (Blahova et al., 2013; Mela et al., 2013; Nwani et al., 2011; Zadeh et al., 2016). The freshwater fish *Prochilodus lineatus* exposed to ATZ showed reduced activity of antioxidant and biotransformation enzymes in the liver, genotoxic damage in different cells, alterations in plasma osmolality, and a decrease in the gill activity of carbonic anhydrase, as well as morphological alterations in the gills (Paulino et al., 2012; Santos and Martinez, 2012).

63 New technologies can minimize the damage of ATZ in the environment without
64 undermining the weeds control, such as nanotechnology (Fraceto et al., 2016; Mishra
65 et al., 2017). The development of nanoparticles as nanocarrier system can promote a
66 sustained release for pesticides and improve their efficacy and safety (Parisi et al.,
67 2015; Sekhon, 2014). Nanoparticles (NP) may allow more bioavailability and a more
68 controlled release specific to the target, as well as the use of optimized concentrations
69 (Wang et al., 2016). Because of this, NP may reduce the concentration of pesticides
70 applied in field as well as the frequency of their use, avoiding a temporal overdose,
71 reducing waste and the risks to non-target organisms and the environment (Kah and
72 Hofmann, 2014; Kah et al., 2013; Pascoli et al.,2018). Several studies have shown that
73 the use of NP as carrier systems for pesticides results in an increase in their
74 effectiveness in the target organism and a decrease in toxicity to non-target organisms
75 (Kumar et al., 2014; Oliveira et al., 2015a; Tong et al., 2017).

76 Release systems for ATZ based on polymer nanoparticles have been developed,
77 such as nanocapsules prepared with poly (ε-caprolactone) also known as PCL (Grillo et
78 al., 2012; Pereira et al., 2014). PCL is a polymer, soluble in several organic solvents and
79 commonly used for the preparation of NP as nanocarrier system for biologically active
80 compounds due to its biodegradability and biocompatibility characteristics (Pereira et
81 al., 2014; Sinha et al., 2004).

82 Nanoparticles of PCL containing ATZ have shown to be effective for the control
83 of target species (Pereira et al., 2014). This nanocarrier system did not cause damage
84 to corn (*Zea mays*), a non-target organism, but was more effective against the target
85 organism (*Brassica* sp.), indicating this system as a safe tool for the control of invasive
86 plants without affecting the growth of the crop (Oliveira et al., 2015a and 2015b).

Another work performed with two different target organisms (*Amaranthus viridis* and *Bidenspilosa*) showed that PCL nanocapsules loaded with ATZ were more effective in relation to ATZ (Souza et al., 2018) and ten-fold dilution of the ATZ-containing nanocapsules resulted in the same efficacy of the standard dose of its commercial formulation (Oliveira et al., 2015b; Souza et al., 2018).

Nevertheless, for their safe use, it is essential to investigate the toxicity of ATZ nanocapsules to non-target organisms. In the study carried out with the microcrustacean *Daphnia similis,* ATZ nanocapsules showed higher toxicity than the free herbicide (Clemente et al., 2013). On the other hand, in cytogenetic tests using human lymphocyte cultures, nanoencapsulation reduced the extent of damage to cells and in the test performed with the microalgae, *Pseudokirchneriella subcapitata* the use of the nanoencapsulated herbicide reduced the inhibition of its growth (Clemente et al., 2013). Genotoxicity tests using human lymphocytes and onion cells (*Allium cepa*) showed that nanoencapsulated ATZ was less toxic than the herbicide in its conventional formulation (Grillo et al., 2012).

In this context, this work aimed to evaluate the effects of nanoencapsulated ATZ~~,~~ compared with its conventional formulation on genotoxic, biochemical, and physiological biomarkers of the fish *P. lineatus.* This biological model was chosen as this is a neotropical freshwater fish widely used in human food and sensitive to various xenobiotics, among them ATZ (Paulino et al., 2012; Santos and Martinez, 2012). The results of this study will provide new information concerning the effects of nanopesticides on aquatic organisms~~,~~ which is essential for the safe use of ~~this~~ nanocarrier systems ~~use of these systems~~ in agriculture.

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~~In this context, the investigation of the effects of nanoparticles on non-target organisms is essential for their safe use in agriculture. Thus, this work aimed to evaluate the effects of ATZ nanoencapsulation, compared with its conventional formulation, at different concentrations and times of exposure, on genotoxic, biochemical, and physiological biomarkers of the fish *P. lineatus*. This biological model was chosen as this is a native freshwater fish widely used in human food and sensitive to various xenobiotics, among them ATZ (Paulino et al., 2012; Santos and Martinez, 2012).~~

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2. MATERIAL AND METHODS

2.1 Preparation of nanocapsules of PCL

PCL nanocapsules containing ATZ were prepared by interfacial deposition of preformed polymer (Grillo et al., 2012). Initially, two solutions were prepared, the organic and aqueous phase. The organic phase was composed by 100 mg of PCL, 200 mg of myritol® 380 oil, 40 mg of surfactant (sorbitan monostearate-SPAN® 60) and 10 mg of atrazine, for the dissolution of these compounds were added 30 mL of acetone, kept under magnetic stirring at 40°C. The aqueous phase was composed with 60 mg of the surfactant polysorbate 80-tween® 80 in 30 mL of deionized water. After the complete dissolution of these compounds, the organic phase (at room temperature) was inserted in the aqueous phase (under magnetic stirring), this final solution was kept under agitation for 10 minutes, and the volume was reduced to 10 mL by rotary evaporation. The final ATZ concentration was 1 mg mL^{-1}. As control we prepared nanocapsules without ATZ.

134

135 **2.2 Nanoparticles characterization**

136 Nanoparticles size (nm) and polydispersity index (PDI) were determined by
137 photon correlation spectroscopy (DLS), using a ZS90 analyzer (Malvern Instruments,
138 UK) at a fixed angle of 90°. Zeta potential values (mV) were also determined using the
139 ZS90 analyzer, by electrophoresis technic. Nano Tracking Analysis (NTA) was used to
140 obtain the size (nm) and the concentration of nanoparticles (nanoparticles mL^{-1}) using
141 a Model LM-10 instrument (Malvern Instruments, UK). Each sample was measured 5
142 times, with approximately 400 nanoparticles counted in each measurement. Samples
143 were not diluted and analyzed at 25°C.

144

145 **2.3 Fish handling, experimental design, and sampling**

146 Juveniles of *Prochilodus lineatus* (14.99 ± 0.42 cm and 24.40 ± 5.09 g, mean ±
147 SD, n = 160) provided by the Acqua Norte Fishery (Cambará, PR, Brazil) were
148 acclimated for a minimum of five days in 300 L tanks, containing dechlorinated water
149 and constant aeration, with a photoperiod of 12 h : 12 h. During acclimation, water
150 was partially renewed every 48 h and feeding occurred before the water renewal,
151 being suspended 24 h before the beginning of the experiments. The physical and
152 chemical parameters of the water were monitored (Horiba multi-parameter meter)
153 and remained stable (mean ± SD): temperature 23.22 ± 0.59 °C; pH 7.83 ± 0.16;
154 conductivity 260 ± 9 µS cm^{-1}; and dissolved oxygen 6.9 ± 1.06 mg L^{-1}

155 After acclimation, the fish were exposed, for 24 and 96h, to one of the
156 following treatments: Control (CTR), with fish exposed only to dechlorinated water.
157 Atrazine (ATZ) with fish exposed to free atrazine (Atrazine PESTANAL®, 45330 SIGMA,

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158 purity ≥ 98.0%) at concentrations of 2 µg L^{-1} or 20 µg L^{-1} (ATZ2 and ATZ20);
159 Nanoatrazine (nATZ), with fish exposed to PCL nanocapsules loaded with ATZ , at
160 concentrations of 2 µg L^{-1} or 20 µg L^{-1} (nATZ2 and nATZ20), prepared from a stock
161 solution of 1 mg nATZ mL^{-1}; Nanocapsules (NANO), with fish exposed to water
162 containing only PCL nanocapsules, without atrazine, in amounts corresponding to
163 those used in the treatments of 2 µg L^{-1} or 20 µg L^{-1} (NANO2 and NANO20), prepared
164 from a stock solution of 1 mg Nano mL^{-1}. The concentrations of ATZ tested were
165 defined considering that 2 µg L^{-1} corresponds to the maximum concentration of
166 atrazine allowed by the Brazilian legislation (CONAMA Resolution 357, 2005) for inland
167 waters and 20 µg L^{-1}corresponds to a concentration ten times higher than permitted
168 one.

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169 For each concentration (2 and 20 µg L^{-1}) at each experimental period (24 and 96
170 h), independent experiments were performed and the four treatments (CTR, NANO,
171 ATZ, and nATZ) ran simultaneously. In each experiment, ten fish were used per
172 treatment (n = 10), distributed in two boxes (50 L of water), with five fish in each,
173 totaling two replicates per treatment for each experiment. During the exposure
174 periods, the physical and chemical parameters of the water remained stable (mean ±
175 SD): temperature 24.28 ± 0.57 °C; pH 7.83 ± 0.15; conductivity: 117 ± 19 µS cm^{-1};
176 dissolved oxygen: 7.12 ± 0.57 mg O_2 L^{-1}. Water samples were collected for
177 characterization of the nanoparticles one hour after the addition of the nanoparticles
178 (NANO or nATZ) and at the end of the experiment (96h) as described above (item 2.2).

179 After exposure, the fish were anesthetized in benzocaine (0.1 g L^{-1}) and blood
180 was withdrawn from the caudal vein. Next, the animals were killed by medullary
181 section for removal of the gills and liver. An aliquot of whole blood was used for

hematological and genotoxic analyses. The remainder of the blood was centrifuged (1870 *g*, 15 min) and the plasma stored in frozen (-20 °C) for the determination of ions and glucose concentrations. Samples of the gills and liver were kept frozen (-80°C) for the biochemical analyses. This study was approved by the Ethics Committee on the Use of Animals of the State University of Londrina (Process CEUA nº 18819.2016.85).

2.4. Genotoxic biomarkers

The alkaline comet assay was performed with erythrocytes according to Singh et al. (1988), with modifications described by Vieira et al. (2016). Slides stained with GelRed were examined under a fluorescence microscope (Leica Microscope DM-2500, Germany) and genotoxic damage was quantified by the extent of DNA migration, determined visually on 100 nucleotides randomly selected from non-overlapping cells. Damage was classified into four comet classes: class 0 = no apparent damage; class 1 = tail length less than the nucleoid diameter; class 2 = tail length corresponding to one or two times the nucleoid diameter; class 3 = tail length greater than twice the nucleoid diameter. The DNA damage score was obtained by multiplying the number of cells in each class by the value of the comet class.

The frequency of micronuclei (MN) and other erythrocytic nuclear abnormalities (ENA) was determined according to Ueda et al. (1992). Slides stained with acridine orange in Sorenson's buffer (0.003%, pH 6.8) were analyzed under the fluorescence microscope at a magnification of 1,000x. For each fish, 3,000 cells were analyzed for the presence of micronuclei (MN), blebbed nuclei (BN), lobed nuclei (LN) and notched nuclei (NN), according to Carrasco et al. (1990). The mean frequency of

each ENA (MN, BN, LN, and NN), as well as the frequency of all ENAs added, for each group, was calculated and expressed per 1000 cells (‰).

2.5 Physiological biomarkers

Hematological analyses. Hematocrit (Hct) was determined by blood centrifugation (7 min, 1,200 *g*) in heparinized glass capillaries, using a microhematocrit centrifuge (Luguimac S.R.L., Model LC 5, Argentina).~~For hematocrit determination, blood was centrifuged (1,200 *g*, 7 min) in a micro capillary centrifuge (Luguimac SRL, Model LC 5, Argentina) in order to get the percentage of erythrocytes.~~ Hemoglobin was determined by the cyanometahemoglobin method in a spectrophotometer (Libra S32, Biochrom, UK) using a commercial kit (Labtest, Brazil). The number of erythrocytes per mm^3 of blood (RBC) was counted under microscope using a Neubauer chamber.

Plasma concentrations of ions and glucose. ~~The chloride concentration was determined by a commercial kit (Labtest, Brazil) using the mercury thiocyanate method.~~ Sodium and potassium concentrations were determined in a flame photometer (Digimed DM-62, Brazil). Concentrations of calcium and magnesium were determined in an atomic absorption spectrometer (Perkin Elmer Analyst 700, USA) ~~with a flame atomizer~~. Chloride and ~~Glucose~~ glucose concentrations ~~was~~ were determined using ~~a~~ commercial colorimetric kits (Labstest, Brazil), based on the mercury thiocyanate and the glucose oxidase methods, respectively ~~and was read~~ in a microplate spectrophotometer (Victor^3, PerkinElmer, USA) ~~at 550 nm.~~.

Branchial enzymes for ion transport. For the analyses of the Na^+/K^+- ATPase (NKA) and H^+-ATPase activities, gills filaments were homogenized (1:5, w:v) in SEID buffer (150 mM sucrose, 10 mM EDTA, imidazole, 2.4 mM sodium deoxycholate, pH 7.5) and

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centrifuged (Hettich®, Universal 320R, UK) (7500 *g*, 15 min, 4°C). The supernatant was incubated with ouabain (NKA inhibitor) or NEM (H^+-ATPase inhibitor) and the production of ADP was estimated during 30 min (Gibbs and Somero, 1989). The activity of Ca^{2+}-ATPase was measured according to Tellis et al. (2013) and Vijayavel et al. (2007) ~~with modifications~~. Briefly, ~~Samples~~ samples were homogenized (1:5, w:v) in SEID buffer, centrifuged (10000 *g*, 20 min, 4 °C), and the enzyme activity was determined by the quantification of inorganic phosphate released in the sample in a microplate reader (Bio-Tek Instruments, ELX 800, United States) at 620 nm. For carbonic anhydrase (CA) gills filaments were homogenized (1:10, w:v) in buffer (225 mM mannitol, 75 mM sucrose, 10 mMTris-base and 10 mM NaH_2PO_4, pH 7.4) and centrifuged (13600 *g*, 10 min, 4°C). The supernatant was added to a saturated solution of CO_2 and the reduction in pH resulting from the release of H^+ was measured (Quimis, pH meter - Q400AS, Brazil) for 20 seconds (Vitale et al., 1999).

2.6 Biochemical biomarkers

The liver was homogenized (1:10, w:v) in ~~potassium~~ K phosphate buffer (0.1 M, pH 7.0) and centrifuged (13000 *g*, 20 min, 4°C) for biochemical analyzes. The protein content of the supernatant ~~fraction~~ was determined based on a standard curve of bovine serum albumin (BSA) at 575 nm (Bradford, 1976).

Biotransformation enzymes. The CYP1A activity was determined by ~~the EROD (7-ethoxyurephrine-O-desethylase) assay,~~ measuring the increase in fluorescence given by the conversion of 7-ethoxyfurorufine (ETOX) to resorufin, every minute, during 10 min, in a microplate spectrophotometer at 590 nm (Eggens and Galgani, 1992). The glutathione-S-transferase (GST) activity was determined by the complexation of

reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) using a microplate spectrophotometer at 340 nm (Keen et al., 1976).

Antioxidants. The concentration of glutathione (GSH) was measured by the reaction of GSH with the 5,5-dithiobis-acid-nitrobenzoic substrate (DTNB), and thiolate was quantified using a microplate spectrophotometer at 412 nm (Beutler et al, 1963). Catalase activity (CAT) was measured by the decomposition of H_2O_2 following the decrease in the absorbance over time in a spectrophotometer (SpectraMax, Plus 384, USA) at 240 nm (Beutler, 1975). The superoxide dismutase (SOD) activity was determined by the inhibition of cytochrome c reduction quantified at 550nm (McCord and Fridovich, 1969). The glutathione peroxidase (GPx) activity was determined by the oxidation of NADPH in the presence of H_2O_2 at 340 nm (Hopkins and Tudhope, 1973).

Oxidative damage. Lipid peroxidation (LPO) was determined by measuring the reaction of ~~one of its products (~~malondialdehyde~~)~~ with thiobarbituric acid at 530 nm (Camejo et al., 1998). Protein oxidation was measured by the quantification of carbonylated proteins (PCO) from the reaction with 2,4-dinitrophenyldrazine (DNPH) to form hydrazones, detected at 360 nm (Levine et al., 1994).

2.7 Integrated Biomarker Response Index (IBR)

An Integrated Biomarker Response Index (IBR) was calculated, as described by Beliaeff and Burgeot (2002) and modified by Sanchez et al. (2013). Only the biomarkers that showed significant and consistent changes were used for the calculation of the IBR and the calculations were performed as described by Vieira et al. (2016). Briefly, for each individual biomarker, the ratio between the mean value obtained in each treatment (NANO, ATZ, and nATZ) at each time and concentration, and the respective

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control value (CTR) was log10 transformed (Yi). In the next step, an overall mean (μ) and standard deviation (s) were calculated, considering all Yi values. Then, the Yi values were standardized using the formula: Zi = (Yi-μ)/s and the difference between Zi and Z0 (CTR) was used to define the deviation index of the biomarker (A). To obtain the integrated biomarker response index, the A value of each biomarker was calculated for the different treatments and the IBR was calculated by summing the absolute values of A.

2.8 Statistical analyzes

The results of each biomarker were compared between the different groups (CTR x NANO X ATZ X nATZ), for each concentration and experimental time, by single factor analysis of variance (ANOVA) or the Kruskal-Wallis test, according to the data distribution (normality and homogeneity of variance). When necessary, the differences were found by the Holm-Sidak or Dunn's method. Values of $p < 0.05$ were considered significant. All analyzes were carried out using the software SigmaPlot 11.0.

3. RESULTS

3.1 Nanoparticles characterization

The results of DLS ~~(Fig. 1a and 1b)~~ and NTA analyzes for NANO and nATZ samples showed a size variation and a reduction in nanoparticle concentrations along the experiment (~~Fig. 1~~Table 1). For NANO2, from time zero to the end of the experiment, we observed a decrease in size (from 254 nm to 107 nm) and in the concentrations of nanoparticles (from 1.45×10^8 to 8.03×10^7 nanoparticles.mL^{-1}). The same trend was observed for nATZ2, which showed a decrease in size (from 490 nm to

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331 nm) and in nanoparticles concentrations (from $3.05x10^8$ to $6.41x10^7$ nanoparticles~~.~~ mL^{-1}). For NANO20 and nATZ20, along the experimental period DLS analyzes showed a variation in the size of nanoparticles ~~(Fig. 1b)~~, whereas NTA indicated a variation the nanoparticles concentration. Samples of NANO20 showed a slight increase in size (from 277 nm to 323 nm) and a decrease in the concentrations of nanoparticles (from $1.67x10^8$ to $8.24x10^7$ nanoparticles~~.~~ mL^{-1}). While for nATZ20 we observed both a reduction of size (from 432 to 104 nm) and nanoparticles concentrations (from $1.58x10^8$ to $1.11x10^8$ nanoparticles~~.~~ mL^{-1}). The DLS methodology ~~(Fig. 1a and 1b)~~ indicates that there were aggregates, which could influence in the nanoparticles size. Due to the low concentration of nanoparticles in the samples, the size estimated by NTA can be more accurate and the NTA data ~~(Fig. 1 - Table)~~ showed that there were no relevant variations in the size during the assay.

3.2 Physiological biomarkers

Among the hematological parameters analyzed (Fig. ~~2~~1), the most consistent variations between treatments occurred in the hemoglobin content. At 96 h the fish of the NANO2, ATZ2, and nATZ2 groups demonstrated a significant decrease in the hemoglobin content in relation to their respective CTR ($F = 26.34$, $P < 0.001$), as well as the ATZ20 and nATZ20 groups in relation to their respective CTR ($F = 7.32$, $P = 0.001$) (Fig. ~~2A~~1A).

In relation to glucose (Fig. ~~3A~~2A), a point increase was observed only in fish exposed to ATZ20 in relation to their respective CTR at 24h ($F = 5.43$, $P = 0.004$). Among the plasma ions, point variations were observed in the concentrations of Na^+, K^+ and Cl^- (Figs. ~~3B~~2B, ~~3C~~2C, and ~~3D~~2D). The plasma concentration of Ca^{2+} varied more

consistently (Fig. ~~3E~~2E), with a significant reduction in the fish in the NANO2, ATZ2, and nATZ2 groups compared to their respective CTR, both after exposure for 24 (F = 17.07, P <0.001) and 96h (F = 57.77, P <0.001); and in the fish of the ATZ20 group in relation to the CTR at 24h (F = 4.95, P = 0.006). The concentration of plasma magnesium did not change (Fig. ~~3F~~2F).

Fish exposed to nATZ20 for 24 h showed significantly higher values in the branchial activity of Na^+/K^+-ATPase (F = 8.62, P <0.001) and H^+-ATPase (F = 4.75, P = 0.007) in relation to the respective CTR (Figures ~~4A~~ 3A and ~~4B~~3B). On the other hand, the activity of Ca^{2+}-ATPase (Fig. ~~4C~~3C) was significantly lower in the gills of the fish exposed to NANO2, ATZ2, and nATZ2 treatments, for 96h (F = 11.88, P <0.001) and in the fish of the NANO20 and ATZ20 groups at 24h (F = 6.06, P = 0.002) and the ATZ20 and nATZ20 groups at 96h (F = 5.79, P = 0.004). In relation to carbonic anhydrase (Fig. ~~4D~~3D), fish in the ATZ2 group presented significantly lower values of this enzyme activity at 96h (F = 8.44, P <0.001), while significantly higher values were observed in the ATZ20 group at 24 h (F = 4.24, P = 0.014).

3.3 Biochemical biomarkers

The hepatic activity of CYP1A did not vary significantly in the treatments and times tested (Fig. ~~5A~~4A). The fish exposed to ATZ2 for 24h presented significantly lower liver GST activity (Fig. ~~5B~~4B) than the respective CTR (F = 4.23, P = 0.013). In relation to the primary antioxidant enzymes (Figs. ~~5C~~4C, ~~5D~~ 4D and ~~5E~~4E) in the liver, significant variations in relation to the CTR were observed only in CAT and GPx activity. Fish exposed to ATZ20 demonstrated significantly higher CAT activity (Fig. 4D) at 24h (F = 4.88, P = 0.007), whereas at 96h CAT activity was significantly lower in the ATZ20 and

nATZ groups (F = 6.71, P = 0.002). The fish exposed to ATZ20 presented GPx activity (Fig. ~~5E~~4E) which was significantly higher at 24h (F = 7.06, P <0.001) and significantly lower at 96h (F = 23.33, P <0.001). On the other hand, significantly higher values of GSH (Fig. ~~5F~~4F) were observed in fish exposed for 96h to the treatments ATZ2 (F= 44.79, P = 0.009) and ATZ20 (F = 31.12, P = 0.003).

Oxidative damage was evaluated by the analysis of LPO (Fig. ~~6A~~54G) and PCO (Fig. ~~6B~~54H) in the fish liver. The results indicated significantly higher values of LPO only in fish exposed to ATZ2 for 24h (F = 4.61, P = 0.009). On the other hand, PCO did not demonstrate significant variation in the treatments and times tested.

3.4. Genotoxic biomarkers

In relation to DNA damage, fish exposed to ATZ2 and nATZ2 for 24 and 96 h (F = 30.34, P <0.001 and F = 136.74, P <0.001, respectively), as well as ATZ20 and nATZ20, for 24 and 96 h (H = 33.87, P <0.001 and F = 29.34, P <0.001, respectively), presented a DNA damage score significantly higher than those of the CTR group (Fig. ~~7A~~65A). Fish from the NANO2 (F = 136.74, P <0.001) and NANO20 (F = 29.34, P <0.001) groups also showed a significantly higher DNA damage score than the respective CTR at 96 h (Fig. ~~7A~~65A). ENA frequency, when considered together (MN + NN + LN + BN), showed a significant increase only in fish exposed to ATZ20 for 96h (F = 4.42, P = 0.015), in relation to the respective CTR (Fig. ~~7B~~65B). When analyzed individually, there were no significant variations in the frequency of each ENA between the different treatments. However, among the ENAs a higher occurrence of notched nuclei was observed (Fig. ~~7D~~65D), followed by blebbed nuclei (Fig. ~~7E~~65E), lobed nuclei (Fig. ~~7F~~65F), and MN (Fig. ~~7C~~65C), which were observed only in fish exposed to ATZ.

3.5 Integrated Biomarker Response Index (IBR)

IBR values were calculated considering the following biomarkers: hemoglobin, glucose, Ca^{2+}, Ca^{2+}-ATPase, NKA, H^{+}-ATPase, AC, GST, GSH, CAT, GPx, LPO, DNA damage, and ENA frequency. In the four experiments carried out, the fish of the groups exposed to ATZ presented the highest IBR values, while the fish exposed to the nanocapsules showed the lowest values. The IBR values presented higher values in fish exposed to the treatments with higher concentration and longer experimental time (Fig. ~~8A~~7~~6~~A). When the IBR values of the 4 experiments were considered together (Fig. ~~8B~~7~~6~~B), the highest mean value (IBR = 30.18) was observed for fish exposed to ATZ, followed by fish from the nATZ treatment (IBR = 23.41), and finally the nanocapsules (IBR = 18.90).

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4. DISCUSSION

The present work evaluated ~~alterations in~~ biomarkers of *P. lineatus* exposed to ATZ, as well as the effects caused by nanoencapsulation of ATZ. The results show that some of the effects observed in fish exposed to ATZ~~, such as the increase in lipoperoxidation and in ENAs frequency, the increase in glycemia and GSH content, and the alterations in carbonic anhydrase and GPx activities,~~ were not observed in fish exposed to the nanoencapsulated herbicide, or in fish exposed to nanocapsules alone. However, fish exposed to nATZ showed ~~the~~ some ~~same~~ alterations as the fish exposed to ATZ~~ in relation to hemoglobin content, increased DNA damage score, and the activity of Ca^{2+}-ATPase that resulted in a decrease in plasma Ca^{2+} concentration~~. The Integrated Biomarker Response Index (IBR) clearly showed a greater effect of ATZ in its

conventional formulation in relation to nATZ. The IBR also demonstrated that the nanocapsules without ATZ caused ~~alterations~~changes in a lower number of biomarkers, but the same changes as those observed in the fish exposed to the nATZ, suggesting that the effects of nATZ may have been caused by both the herbicide and the PCL nanocapsules.

The concentrations of nanocapsules containing ATZ, as well as those of nanocapsules without ATZ, decreased along the experiment, suggesting some absorption of these compounds by the organism. Nanocapsules can be inserted into cells due to their large surface area and small size and additional surface modifications may further enhance cell uptake (Hu and Gao, 2010; Yuan et al., 2016), whereby nanocapsules containing herbicides can penetrate the cells of animals (Clemente et al., 2013), which may explain the toxicity observed for the fish *P. lineatus*.

In the evaluation of NP toxicity, the morphology, surface area, coating, solubility, and the composition of the NP should be considered. Assays performed with PCL nanocapsules without ATZ showed their phytotoxicity for *Brassica* sp., which may be due to the substances present in their composition (Pereira et al., 2014). One of these components is polysorbate 80 surfactant (Tween 80). Yuan et al. (2016) showed that *Danio rerio* embryos exposed to chitosan nanocapsules modified with Tween 80 showed increase in the mortality rate, a decrease in the incubation rate, and an increase in malformations in a dose dependent manner, in addition to an increased level of intracellular ~~ROS~~ production of reactive oxygen species (ROS). Other compounds used in the organic phase of nanocapsules preparation are triglycerides of capric and caprylic acids. Capric acid may have been released during the metabolism of triglyceride, resulting in toxicity to the fish. The study by Yang et al. (2018) showed that

after being absorbed capric acid (or decanoic acid) induced oxidative stress by ROS generation, and induced LPO, causing apoptosis in human trophoblasts. The decanoic acid has a variety of biological activities, including antiproliferative and pro-apoptotic effects in human cells (Kim et al., 2014).

Regarding the effects of ATZ, fish exposed to the highest ATZ concentration for 24 h depicted an increase in hemoglobin concentration. This may have occurred in response to the stress caused by the herbicide and the need to increase the concentration of oxygen in the organism (George et al., 2017). This increase in hemoglobin coincides with increased glycemia, ~~also observed in fish exposed to the same concentration of ATZ for 24 h,~~ reinforcing the idea of a stress response. High concentrations of plasma glucose can be explained by the mobilization of glycogen reserves in response to the stress induced by the herbicide (Khan et al., 2016). The increase in glycemia after exposure to triazine herbicides has also been reported for the carp *Cyprinus carpio* (Blahova et al., 2014; Bhanu and Deepek, 2015; Khan et al., 2016). In fish exposed to nanoencapsulated ATZ these signs related to a stress response were not observed and could be due to the slower release of ATZ and consequently the lower amounts of the herbicide available. However, after 96 h of exposure to NANO2, ATZ (2 and 20), and nATZ (2 and 20) fish showed a decrease in hemoglobin content. It is known that free herbicide can cause inhibition of hemoglobin formation or premature mortality of red blood cells, affecting oxygen transport capacity (Akinrotimi et al., 2010). The same inhibition may have occurred in fish exposed to the encapsulated herbicide, as well as in fish exposed only to nanocapsules, which may also have interfered in the transport of oxygen.

Fish exposed to ~~The concentration of plasma Ca^{2+} was also very sensitive to the presence of~~ ATZ in both free and nanoencapsulated form, as well as to the nanocapsules, ~~since the fish exposed to NANO2, ATZ2, and nATZ2 for 24 and 96 h and to ATZ20 for 24h,~~ demonstrated a significant reduction in blood Ca^{2+}. These reductions largely coincide with decreases in activity of Ca^{2+}-ATPase. The maintenance of plasma calcium in freshwater teleost involves the uptake of this ion by the branchial epithelium, which occurs through the entrance of Ca^{2+} across the apical membrane, favored by the low concentration of intracellular Ca^{2+}, and its transport to plasma is directed by the Ca^{2+}-ATPase, located in the basolateral cell membrane (Marshall, 2002). Thus, ~~these results show that both the~~ free and nanoencapsulated herbicide, as well as ~~the~~ nanocapsules alone, negatively interfere with calcium homeostasis in a freshwater teleost species.

Gills play a vital role in the transport of ions (Na^+, K^+, Cl^-, Ca^{2+}, and Mg^{2+}) and thereby maintain the hydroelectrolytic and acid-basic balance in fish. Therefore, the quantification of ~~plasmatic~~ plasma ions is considered a sensitive biomarker to exposure to xenobiotics (Saravanan et al., 2011, 2015). In fish exposed to ATZ2 a significant reduction in Na^+ ~~(ATZ2)~~ concentration was detected at 24 h and a significant increase in potassium ~~(ATZ2)~~ at 96 h. Changes in the concentration of these ions may have been due to transient alterations in specific channels or exchangers, since exposure to free ATZ did not cause significant alterations in NKA activity. This lack of alterations in NKA agrees with the results of Paulino et al. (2012), who also did not verify changes in NKA activity after exposure of *P. lineatus* ~~at 2, 10, and 25 µg L^{-1} of~~to ATZ. On the other hand, after 24 h exposure to nATZ20 there was a significant increase in plasma Na^+, which coincides with the increase in the activity of enzymes NKA and H^+-ATPase. It is known

that NKA is important for the concentration of Na^+ as it contributes to Na^+ absorption by the gill epithelium (Aperia et al., 2016) leading to an increase in plasmatic levels. ~~This increase in NKA enzyme activity was a transient response since no increase was observed at 96 h.~~

In relation to the activity of the CA, nATZ did not alter its activity, unlike free ATZ, which promoted ~~an increase~~changes in CA activity ~~at 24 h, but at 96 h the herbicide led to a reduction in its activity.~~. ~~This~~ These ~~effect~~ changes may have been caused by a direct action of the ATZ on the enzyme, since a high species-specific affinity was also verified in the inhibition of CA by ~~agrochemicals~~ pesticides (Lionetto et al., 2012).

The fish exposed to nATZ did not present alterations in GST activity or GSH content, however fish exposed to their conventional formulation showed a decrease in GST activity ~~in the lowest concentration (ATZ2) and in the shortest time tested~~. GST is an enzyme mainly involved in phase 2 of biotransformation, a decrease in its activity was also seen in *P. lineatus* by Santos and Martinez (2012)~~, in *P. lineatus* exposed to 10 $\mu g.L^{-1}$ of ATZ~~. This decrease indicates that exposure to ATZ interferes with the detoxification system of the organism (Blahova et al., 2013). Regarding the hepatic content of GSH, fish exposed to ATZ ~~for 96 h~~ presented an increase in this parameter. A similar result was found in the work of Elia et al. (2012) in the teleost *Lepomis macrochirus* exposed to much higher concentrations of ATZ (6 and 9 $mg.L^{-1}$). The increase in GSH levels may be related to the ~~increase in~~higher demand for ~~their~~ conjugation ~~with the herbicide~~, in order to avoid bioaccumulation in the liver cells; but may also be ~~related to increased ROS production due to exposure to ATZ in order~~a response to prevent oxidative damage. It is known that GSH is efficient to avoid lipid

peroxidation, which agrees with the result found in this work, as there was no increase in lipid peroxidation in fish that presented an increase in GSH.

Primary antioxidant enzymes (SOD, CAT, and GPx) are the first line of defense against ROS. SOD represents a large family of enzymes that catalyze the dismutation of the superoxide (O_2^-) radical into hydrogen peroxide (H_2O_2). CAT and GPx are responsible for the detoxification of H_2O_2 (Nwani et al., 2011). In the present work, no consistent alterations in these antioxidant enzymes were detected in fish exposed to nATZ, however, some alterations occurred in fish exposed to ATZ. The results showed an increase in the activity of CAT and GPx in the first 24 h of exposure to ATZ20, which may be a response to the increase in ROS generated by exposure to the herbicide (John et al., 2001; Vasanth et al., 2013). Exposure to ATZ also led to an increase in antioxidant enzymes activity in *Poecilia sphenops* (Vasanth et al., 2013) and in CAT activity in female *Danio rerio* (Jin et al., 2010). On the other hand, after ~~96 h~~longer exposure to ATZ20, there was a reduction in CAT and GPx activity. ~~In animals exposed to nATZ20, only CAT activity showed a decrease.~~ Similar reductions in CAT and GPx have also been reported for the teleostei *Rhamdia quelen* (Mela et al., 2013), in *Labeo rohita* (Prabakaran et al., 2014), and in *D. rerio* embryos (Adeyemi et al., 2015) exposed to ATZ. In addition, Wang et al (2019) showed that ATZ exposure induced ROS accumulation by disrupting SOD, GSH and CAT functions in carp neutrophils after ATZ treatment. Thus, ATZ metabolism may lead to an excess generation of ROS while it interferes with the transduction of cellular antioxidant signaling pathway, impairing the balance of oxygen free radicals in cells and leading~~It is suggested that xenobiotics may inhibit the transcription of specific genes, which results in decreased mRNA levels, reflecting in lower activities (Silva et al., 2011). The low activity of these enzymes and~~

~~the production of excess ROS in animals exposed to ATZ could lead~~ to cellular damage (Wang et al., 2018~~Sun et al., 2006~~).

Lipid peroxidation (LPO) is among the primary harm caused by the excess of ROS, it is the initial step of damage in the cell membrane, which can lead ~~the~~ to cell ~~to~~ apoptosis~~; LPO can be caused by agrochemicals, metals, and other xenobiotics~~ (Livingstone, 2001). In the present study, an increase in LPO was observed only in the liver of the fish exposed to ATZ~~2, during the first 24 h~~, suggesting a pro-oxidant imbalance. The~~is~~ LPO increase ~~in LPO~~ in response to ATZ exposure was also reported for *C. punctatus* (Nwani et al., 2011), *L. rohita* (Prabakaran et al., 2014), and *P. sphenops* (Vasanth et al., 2013) ~~in response to exposure to ATZ.~~ ~~This increase was not observed in~~ Nevertheless, LPO increase was not observed in fish exposed to nAT~~, Z,~~ indicating that ATZ encapsulation was effective to avoid oxidative damage. ~~The increase in LPO was also reported for *C. punctatus* (Nwani et al., 2011), *L. rohita* (Prabakaran et al., 2014), and *P. sphenops* (Vasanth et al., 2013) in response to exposure to ATZ.~~ ~~Thus, it could be suggested that LPO may be associated with the excess of ROS resulting from ATZ metabolism, leading to membrane lipid peroxidation in liver cells.~~

In spite of being ~~effective~~ helpful in preventing ~~lipoperoxidation~~LPO, nanoencapsulated ATZ was not capable to avoid erythrocyte DNA damage, as an increase in damage score was observed in the erythrocytes of fish exposed to nATZ ~~at all times and concentrations tested~~, as was the case with ATZ, indicating the genotoxic potential of these compounds. S-triazine derivatives, which include atrazine, are capable of direct interaction with DNA, in a time-dependent manner (Oliveira-Brett and Silva, 2002). This interaction occurs by formation of adducts between the

herbicide and the purine bases of DNA, adenine and guanine. These results corroborate results ~~previously~~ found for the same fish species, which showed increased DNA damage in erythrocytes, liver and gill cells after exposure to ~~2 and 10 µg L^{-1} of~~ ATZ (Santos and Martinez, 2012). Grillo et al. (2012) performed genotoxic tests on human lymphocytes with the herbicide ATZ in conventional and nanoencapsulated formulation and their results also demonstrated that all treatments were significantly different from the CTR, but they were also different from each other, and the encapsulation of the herbicides decreased DNA damage by 50%.

Exposure to PCL nanocapsules alone for 96 h increased the DNA damage score in erythrocytes of *P. lineatus*. The hydrolysis of poly-epsilon-caprolactone results in the formation of 6-hydroxyhexanoic acid (Karande et al., 2017). In *Danio rerio* larvae, sublethal exposure to adipic acid esters, which is analogous to 6-hydroxyhexanoic acid, caused DNA damage in a dose-dependent manner, as well as the induction of genes related to stress (Boran and Terzi, 2017).

Regarding mutagenic effects, only fish exposed to ATZ20 ~~for 96 h~~ showed a significant increase in the frequency of ENAs. The causes to explain nuclear abnormalities are still uncertain, yet one theory attributes their appearance to alterations in cytoskeletal proteins, responsible for the maintenance of the nuclear shape (Ghisi et al., 2014). Among the ENAs, the lowest occurrence was MN, although it is worth mentioning that MNs were only detected in fish exposed to ATZ. It is known that the maximal induction of MN normally occurs one to five days after exposure (Nwani et al., 2011), which agrees with the formation of MN at 24 and 96 h of exposure as observed in this work. Exposure to xenobiotics may lead to alterations in mitotic spindle formation, causing damage or chromosomal losses, which may result in

MN formation (Viana et al., 2018). Previous studies showed that exposure to ATZ leads to the appearance of MN in a dose-dependent manner (Nwani et al., 2011; Piancini et al., 2015).

5. CONCLUSION

The development of controlled release systems could mitigate the negative impacts of pesticides on the environment, as well as increase food safety. However, studies concerning the effects of these release systems are necessary for their regulation and use in the agricultural market. Thus, the present work is important to understand the toxicity of nanoencapsulated ATZ in a non-target organism by assessing its ecological risk. The results of the integrated analysis of the biomarkers showed that the ATZ presented a higher toxicity to *P. lineatus* compared to its nanoencapsulated formulation. This demonstrates that nanoencapsulated ATZ was able to protect the animal from the effects of the herbicide in a general way, indicating that nanoencapsulated ATZ is less toxic to *P. lineatus* compared to its conventional formulation. it is likely that the lower toxicity of nanoencapsulated atrazine compared to free atrazine is related to ~~Considering the possible protection effect of ATZ nanoencapsulation we wrote in the discussion that the lower toxicity of nanoencapsulated atrazine (in comparison to free atrazine) could be due to~~ the slower release of ATZ and consequently the lower amounts of the herbicide available to the organism. However, more studies are needed to clarify the mechanisms underlying the different effects produced by the herbicide in the free and nanoencapsulated form, as well as the effects of PCL nanocapsules.

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842

Figure Captions

~~**Figure 1**. Characterization of the nanoparticles by DLS and NTA of NANO2 and NANO20, and nATZ2 and nATZ20 samples collected at time 0 and after 96 h. The images show the graphs obtained by DLS for the samples of NANO2 and nATZ2 (a), and NANO20 and nATZ20 (b). The table summarizes the size data obtained by DLS and NTA as well as the concentration of nanoparticles.~~

Figure ~~2~~1. Hemoglobin content (A), hematocrit (B) and number of erythrocytes per mm^3 (C) of *P. lineatus* exposed to 2 µg L^{-1} and 20 µg L^{-1} of PCL nanocapsules (NANO), atrazine (ATZ), and atrazine loaded nanocapsules (nATZ) or only to clean water (CTR), for 24 and 96 h. Results are mean ± SE (n= 6-10). Different letters indicate significant different between treatments ($p < 0.05$) for the same experimental tie and concentration.

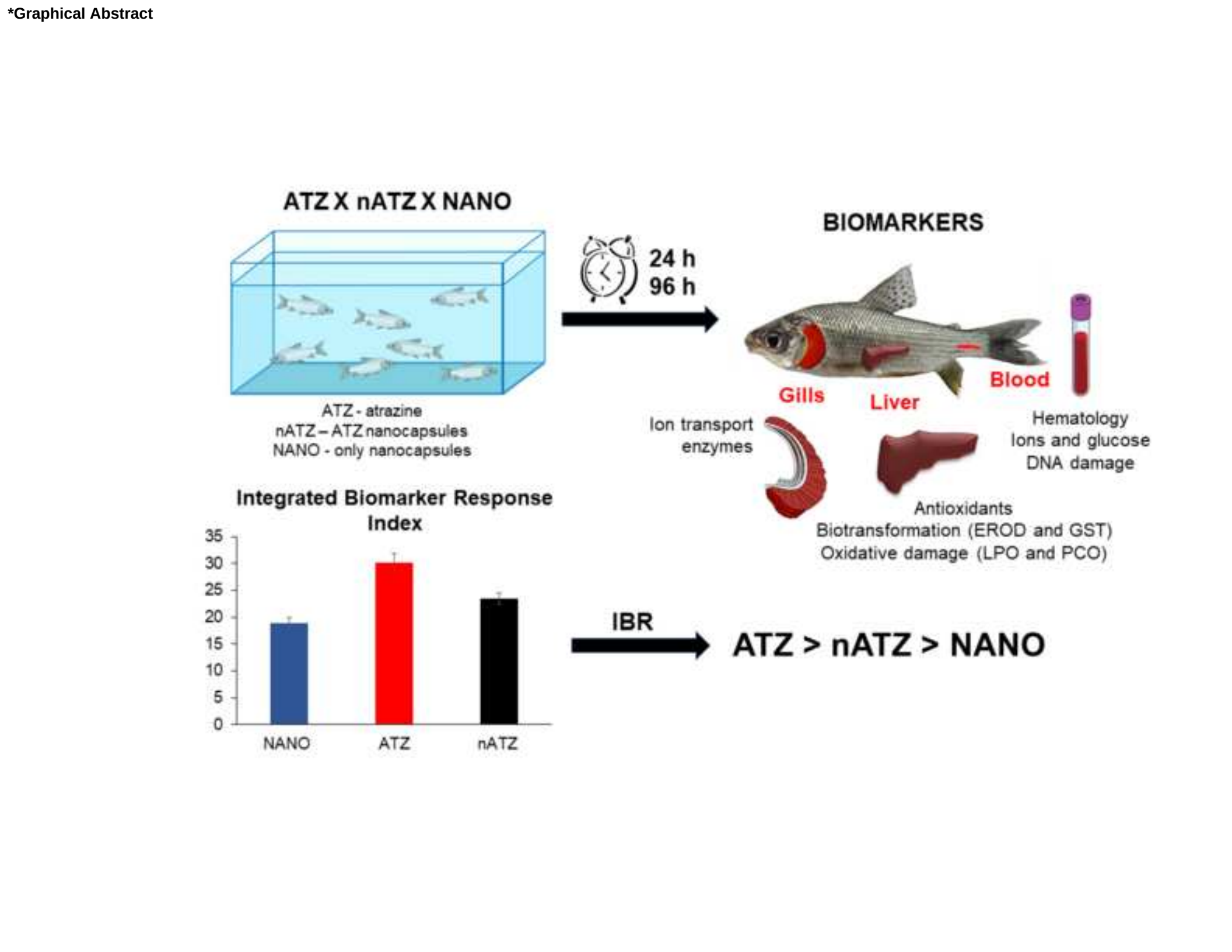
Figure ~~3~~2. Plasma concentrations of glucose (A), Na^+ (B), K^+ (C), Cl^- (D), Ca^{2+} (E) and Mg^{2+} (F) of *P. lineatus* exposed to 2 µg L^{-1} and 20 µg L^{-1} of PCL nanocapsules (NANO), atrazine (ATZ), and atrazine loaded nanocapsules (nATZ) or only to clean water (CTR), for 24 and 96 h. Results are mean ± SE (n= 6-10). Different letters indicate significant different between treatments ($p < 0.05$) for the same experimental tie and concentration.

Figure ~~4~~3. Branchial activity of Na^+/K^+-ATPase (A), H^+-ATPase (B), Ca^{2+}-ATPase (C) and carbonic anhydrase (D) of *P. lineatus* exposed to 2 µg L^{-1} and 20 µg L^{-1} of PCL

867 nanocapsules (NANO), atrazine (ATZ), and atrazine loaded nanocapsules (nATZ) or only
868 to clean water (CTR), for 24 and 96 h. Results are mean ± SE (n= 6-10). Different letters
869 indicate significant different between treatments ($p < 0.05$) for the same experimental
870 tie and concentration.
871
872 **Figure ~~5~~4.** Activity of EROD (A), glutathione S-transferase (B), superoxide dismutase
873 (C), catalase (D~~),~~) and glutathione peroxidase (E), ~~and~~ glutathione content (F), lipid
874 peroxidation (G) and carbonylated proteins (H) in the liver of *P. lineatus* exposed to 2
875 µg L^{-1} and 20 µg L^{-1} of PCL nanocapsules (NANO), atrazine (ATZ), and atrazine loaded
876 nanocapsules (nATZ) or only to clean water (CTR), for 24 and 96 h. Results are mean ±
877 SE (n= 6-10). Different letters indicate significant different between treatments ($p <$
878 0.05) for the same experimental tie and concentration.
879
880 ~~**Figure 65.** Lipid peroxidation (A) and carbonylated proteins (B) in the liver of *P. lineatus*~~
881 ~~exposed to 2 µg L^{-1} and 20 µg L^{-1} of PCL nanocapsules (NANO), atrazine (ATZ), and~~
882 ~~atrazine loaded nanocapsules (nATZ) or only to clean water (CTR), for 24 and 96 h.~~
883 ~~Results are mean ± SE (n= 6-10). Different letters indicate significant different between~~
884 ~~treatments ($p < 0.05$) for the same experimental tie and concentration.~~
885
886 **Figure ~~7~~65.** Score of DNA damage (A) and frequency (‰) of ENAs (B), micronucleus (C),
887 notched nucleus (D), lobed nucleus (E) and blebbed nucleus (F) of *P. lineatus* exposed
888 to 2 µg L^{-1} and 20 µg L^{-1} of PCL nanocapsules (NANO), atrazine (ATZ), and atrazine
889 loaded nanocapsules (nATZ) or only to clean water (CTR), for 24 and 96 h. Results are
890 mean ± SE (n= 6). Different letters indicate significant different between treatments (p

891 < 0.05) for the same experimental time and concentration. Inserts are
892 photomicrographs of the alterations represented in the graphs showing different
893 comet classes (stained with gelred) and ENAs (stained with acridine orange).
894
895 **Figure ~~8~~7~~7~~6.** Integrated Biomarker Response Index (IBR) calculated for *P. lineatus*
896 exposed to 2 µg L^{-1} and 20 µg L^{-1} of PCL nanocapsules (NANO), atrazine (ATZ), and
897 atrazine loaded nanocapsules (nATZ) or only to clean water (CTR), for 24 and 96 h (A),
898 and the mean IBR values considering together all exposure periods and concentrations
899 for each treatment (B).

*Graphical Abstract



***Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

Highlights

The effects of atrazine (ATZ) and nanoencapsulated atrazine (nATZ) were compared

Fish were exposed to ATZ, nATZ or ony to the nanocapsules (NANO) for 24 and 96 h

Genotoxic, biochemical and physiological biomarkers were measured

ATZ promoted changes in a greater number of biomarkers compared to nATZ

Nanoencapsulation of the herbicide protected the animal from the effects of ATZ

Can atrazine loaded nanocapsules reduce the toxic effects of this herbicide on the fish *Prochilodus lineatus*? A multibiomarker approach

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Abstract

Atrazine (ATZ) is a widely used herbicide that has the potential to contaminate the environment and cause deleterious effects on non-target organisms. Release systems for ATZ have been developed to minimize this contamination, such as nanocapsules prepared with poly (ε-caprolactone) (PCL). The objective of this work was to investigate the effects of nanoencapsulated ATZ compared to ATZ on biomarkers of the freshwater teleost *Prochilodus lineatus*. The fish were exposed for 24 and 96 h to nanoencapsulated ATZ (nATZ) and atrazine (ATZ) at concentrations of 2 and 20 μg L^{-1}, just to the PCL nanocapsules without the herbicide (NANO) in the corresponding amounts or only to dechlorinated water (CTR). The results showed that nATZ was less toxic compared to ATZ, as it did not promote an increase in glycemia, alterations in antioxidants, nor in carbonic anhydrase enzyme activity, and no increase in the frequency of micronuclei and other nuclear erythrocyte abnormalities either. However, exposure to nATZ, as well as to ATZ and PCL nanocapsules, resulted in a reduction in hemoglobin content, increase in erythrocyte DNA damage, as well as changes in Ca^{2+}-ATPase activity, leading to a decrease in plasma Ca^{+2}. The Integrated Biomarker Response Index (IBR) depicted that exposure to ATZ promoted changes in a greater number of biomarkers compared to nATZ, indicating that the nanoencapsulation of the herbicide protected the animal from the effects of ATZ.

Keywords: Nanotechnology, oxidative stress, genotoxicity, osmoregulation, nanopesticides.

1. INTRODUCTION

Atrazine (ATZ) is a triazinic herbicide used in the control of weeds, whose mechanism of action is the inhibition of the photosynthetic system, that result in the plant death (Nakka et al., 2017). This herbicide is widely used for the control of weeds in many crops as example, sugarcane, wheat, sorghum, nuts, and corn (Singh et al., 2018). Even though its use has been banned in European Union, ATZ is the second most consumed pesticide in the world, with annual consumption of 70,000 to 90,000 tons (Ehrsam et al., 2016; Singh et al., 2018). ATZ has high potential to contaminate soil, surface water, and groundwater due its high persistence and mobility in the environment (Cerejeira et al., 2003; Kumar et al., 2013; Schwab et al., 2006). Field surveys have shown that this herbicide is commonly detected in surface waters in levels above the limits determined by the guidelines of the US Environmental Protection Agency (3 $\mu g\ L^{-1}$; USEPA, 2014). In Brazil, ATZ concentrations between 0.31 and 5.4 $\mu g\ L^{-1}$ were registered in surface freshwater (Loro et al., 2015, Vieira et al., 2017). In streams of North America this herbicide has been repeatedly detected at concentrations above 100 $\mu g\ L^{-1}$ (Ehrsam et al., 2016).

Several studies have shown that aquatic organisms such as bivalves (Santos and Martinez, 2014) and fish are sensitive to the ATZ (Blahova et al., 2013; Mela et al., 2013; Nwani et al., 2011; Zadeh et al., 2016). The freshwater fish *Prochilodus lineatus* exposed to ATZ showed reduced activity of antioxidant and biotransformation enzymes in the liver, genotoxic damage in different cells, alterations in plasma osmolality, and a decrease in the gill activity of carbonic anhydrase, as well as morphological alterations in the gills (Paulino et al., 2012; Santos and Martinez, 2012).

63 New technologies can minimize the damage of ATZ in the environment without
64 undermining the weeds control, such as nanotechnology (Fraceto et al., 2016; Mishra
65 et al., 2017). The development of nanoparticles as nanocarrier system can promote a
66 sustained release for pesticides and improve their efficacy and safety (Parisi et al.,
67 2015; Sekhon, 2014). Nanoparticles (NP) may allow more bioavailability and a more
68 controlled release specific to the target, as well as the use of optimized concentrations
69 (Wang et al., 2016). Because of this, NP may reduce the concentration of pesticides
70 applied in field as well as the frequency of their use, avoiding a temporal overdose,
71 reducing waste and the risks to non-target organisms and the environment (Kah and
72 Hofmann, 2014; Kah et al., 2013; Pascoli et al.,2018). Several studies have shown that
73 the use of NP as carrier systems for pesticides results in an increase in their
74 effectiveness in the target organism and a decrease in toxicity to non-target organisms
75 (Kumar et al., 2014; Oliveira et al., 2015a; Tong et al., 2017).

76 Release systems for ATZ based on polymer nanoparticles have been developed,
77 such as nanocapsules prepared with poly (ε-caprolactone) also known as PCL (Grillo et
78 al., 2012; Pereira et al., 2014). PCL is a polymer, soluble in several organic solvents and
79 commonly used for the preparation of NP as nanocarrier system for biologically active
80 compounds due to its biodegradability and biocompatibility characteristics (Pereira et
81 al., 2014; Sinha et al., 2004).

82 Nanoparticles of PCL containing ATZ have shown to be effective for the control
83 of target species (Pereira et al., 2014). This nanocarrier system did not cause damage
84 to corn (*Zea mays*), a non-target organism, but was more effective against the target
85 organism (*Brassica* sp.), indicating this system as a safe tool for the control of invasive
86 plants without affecting the growth of the crop (Oliveira et al., 2015a and 2015b).

Another work performed with two different target organisms (*Amaranthus viridis* and *Bidenspilosa*) showed that PCL nanocapsules loaded with ATZ were more effective in relation to ATZ (Souza et al., 2018) and ten-fold dilution of the ATZ-containing nanocapsules resulted in the same efficacy of the standard dose of its commercial formulation (Oliveira et al., 2015b; Souza et al., 2018).

Nevertheless, for their safe use, it is essential to investigate the toxicity of ATZ nanocapsules to non-target organisms. In the study carried out with the microcrustacean *Daphnia similis,* ATZ nanocapsules showed higher toxicity than the free herbicide (Clemente et al., 2013). On the other hand, in cytogenetic tests using human lymphocyte cultures, nanoencapsulation reduced the extent of damage to cells and in the test performed with the microalgae, *Pseudokirchneriella subcapitata* the use of the nanoencapsulated herbicide reduced the inhibition of its growth (Clemente et al., 2013). Genotoxicity tests using human lymphocytes and onion cells (*Allium cepa*) showed that nanoencapsulated ATZ was less toxic than the herbicide in its conventional formulation (Grillo et al., 2012).

In this context, this work aimed to evaluate the effects of nanoencapsulated ATZ compared with its conventional formulation on genotoxic, biochemical, and physiological biomarkers of the fish *P. lineatus.* This biological model was chosen as this is a neotropical freshwater fish widely used in human food and sensitive to various xenobiotics, among them ATZ (Paulino et al., 2012; Santos and Martinez, 2012). The results of this study will provide new information concerning the effects of nanopesticides on aquatic organisms which is essential for the safe use of nanocarrier systems in agriculture.

2. MATERIAL AND METHODS

2.1 Preparation of nanocapsules of PCL

PCL nanocapsules containing ATZ were prepared by interfacial deposition of preformed polymer (Grillo et al., 2012). Initially, two solutions were prepared, the organic and aqueous phase. The organic phase was composed by 100 mg of PCL, 200 mg of myritol® 380 oil, 40 mg of surfactant (sorbitan monostearate-SPAN® 60) and 10 mg of atrazine, for the dissolution of these compounds were added 30 mL of acetone, kept under magnetic stirring at 40°C. The aqueous phase was composed with 60 mg of the surfactant polysorbate 80-tween® 80 in 30 mL of deionized water. After the complete dissolution of these compounds, the organic phase (at room temperature) was inserted in the aqueous phase (under magnetic stirring), this final solution was kept under agitation for 10 minutes, and the volume was reduced to 10 mL by rotary evaporation. The final ATZ concentration was 1 mg mL^{-1}. As control we prepared nanocapsules without ATZ.

2.2 Nanoparticles characterization

Nanoparticles size (nm) and polydispersity index (PDI) were determined by photon correlation spectroscopy (DLS), using a ZS90 analyzer (Malvern Instruments, UK) at a fixed angle of 90°. Zeta potential values (mV) were also determined using the ZS90 analyzer, by electrophoresis technic. Nano Tracking Analysis (NTA) was used to obtain the size (nm) and the concentration of nanoparticles (nanoparticles mL^{-1}) using a Model LM-10 instrument (Malvern Instruments, UK). Each sample was measured 5

134 times, with approximately 400 nanoparticles counted in each measurement. Samples
135 were not diluted and analyzed at 25°C.
136
137 **2.3 Fish handling, experimental design, and sampling**
138 Juveniles of *Prochilodus lineatus* (14.99 ± 0.42 cm and 24.40 ± 5.09 g, mean ±
139 SD, n = 160) provided by the Acqua Norte Fishery (Cambará, PR, Brazil) were
140 acclimated for a minimum of five days in 300 L tanks, containing dechlorinated water
141 and constant aeration, with a photoperiod of 12 h : 12 h. During acclimation, water
142 was partially renewed every 48 h and feeding occurred before the water renewal,
143 being suspended 24 h before the beginning of the experiments. The physical and
144 chemical parameters of the water were monitored (Horiba multi-parameter meter)
145 and remained stable (mean ± SD): temperature 23.22 ± 0.59 °C; pH 7.83 ± 0.16;
146 conductivity 260 ± 9 µS cm^{-1}; and dissolved oxygen 6.9 ± 1.06 mg L^{-1}
147 After acclimation, the fish were exposed, for 24 and 96h, to one of the
148 following treatments: Control (CTR), with fish exposed only to dechlorinated water.
149 Atrazine (ATZ) with fish exposed to free atrazine (Atrazine PESTANAL®, 45330 SIGMA,
150 purity ≥ 98.0%) at concentrations of 2 µg L^{-1} or 20 µg L^{-1} (ATZ2 and ATZ20);
151 Nanoatrazine (nATZ), with fish exposed to PCL nanocapsules loaded with ATZ , at
152 concentrations of 2 µg L^{-1} or 20 µg L^{-1} (nATZ2 and nATZ20), prepared from a stock
153 solution of 1 mg nATZ mL^{-1}; Nanocapsules (NANO), with fish exposed to water
154 containing only PCL nanocapsules, without atrazine, in amounts corresponding to
155 those used in the treatments of 2 µg L^{-1} or 20 µg L^{-1} (NANO2 and NANO20), prepared
156 from a stock solution of 1 mg Nano mL^{-1}. The concentrations of ATZ tested were
157 defined considering that 2 µg L^{-1} corresponds to the maximum concentration of

158 atrazine allowed by the Brazilian legislation (CONAMA Resolution 357, 2005) for inland
159 waters and 20 μg L^{-1}corresponds to a concentration ten times higher than permitted
160 one.

161 For each concentration (2 and 20 μg L^{-1}) at each experimental period (24 and 96
162 h), independent experiments were performed and the four treatments (CTR, NANO,
163 ATZ, and nATZ) ran simultaneously. In each experiment, ten fish were used per
164 treatment (n = 10), distributed in two boxes (50 L of water), with five fish in each,
165 totaling two replicates per treatment for each experiment. During the exposure
166 periods, the physical and chemical parameters of the water remained stable (mean ±
167 SD): temperature 24.28 ± 0.57 °C; pH 7.83 ± 0.15; conductivity: 117 ± 19 μS cm^{-1};
168 dissolved oxygen: 7.12 ± 0.57 mg O_2 L^{-1}. Water samples were collected for
169 characterization of the nanoparticles one hour after the addition of the nanoparticles
170 (NANO or nATZ) and at the end of the experiment (96h) as described above (item 2.2).

171 After exposure, the fish were anesthetized in benzocaine (0.1 g L^{-1}) and blood
172 was withdrawn from the caudal vein. Next, the animals were killed by medullary
173 section for removal of the gills and liver. An aliquot of whole blood was used for
174 hematological and genotoxic analyses. The remainder of the blood was centrifuged
175 (1870 *g*, 15 min) and the plasma stored in frozen (-20^{o}C) for the determination of ions
176 and glucose concentrations. Samples of the gills and liver were kept frozen (-80^{o}C) for
177 the biochemical analyses. This study was approved by the Ethics Committee on the Use
178 of Animals of the State University of Londrina (Process CEUA nº 18819.2016.85).

179

180 **2.4. Genotoxic biomarkers**

The alkaline comet assay was performed with erythrocytes according to Singh et al. (1988), with modifications described by Vieira et al. (2016). Slides stained with GelRed were examined under a fluorescence microscope (Leica Microscope DM-2500, Germany) and genotoxic damage was quantified by the extent of DNA migration, determined visually on 100 nucleotides randomly selected from non-overlapping cells. Damage was classified into four comet classes: class 0 = no apparent damage; class 1 = tail length less than the nucleoid diameter; class 2 = tail length corresponding to one or two times the nucleoid diameter; class 3 = tail length greater than twice the nucleoid diameter. The DNA damage score was obtained by multiplying the number of cells in each class by the value of the comet class.

The frequency of micronuclei (MN) and other erythrocytic nuclear abnormalities (ENA) was determined according to Ueda et al. (1992). Slides stained with acridine orange in Sorenson's buffer (0.003%, pH 6.8) were analyzed under the fluorescence microscope at a magnification of 1,000x. For each fish, 3,000 cells were analyzed for the presence of micronuclei (MN), blebbed nuclei (BN), lobed nuclei (LN) and notched nuclei (NN), according to Carrasco et al. (1990). The mean frequency of each ENA (MN, BN, LN, and NN), as well as the frequency of all ENAs added, for each group, was calculated and expressed per 1000 cells (‰).

2.5 Physiological biomarkers

Hematological analyses. Hematocrit (Hct) was determined by blood centrifugation (7 min, 1,200 *g*) in heparinized glass capillaries, using a microhematocrit centrifuge (Luguimac S.R.L., Model LC 5, Argentina). Hemoglobin was determined by the cyanometahemoglobin method in a spectrophotometer (Libra S32, Biochrom, UK)

using a commercial kit (Labtest, Brazil). The number of erythrocytes per mm^3 of blood (RBC) was counted under microscope using a Neubauer chamber.

*Plasma concentrations of ions and glucose***.** Sodium and potassium concentrations were determined in a flame photometer (Digimed DM-62, Brazil). Concentrations of calcium and magnesium were determined in an atomic absorption spectrometer (Perkin Elmer Analyst 700, USA). Chloride and glucose concentrations were determined using commercial colorimetric kits (Labstest, Brazil), based on the mercury thiocyanate and the glucose oxidase methods, respectively in a microplate spectrophotometer (Victor^3, PerkinElmer, USA).

Branchial enzymes for ion transport. For the analyses of the Na^+/K^+- ATPase (NKA) and H^+-ATPase activities, gills filaments were homogenized (1:5, w:v) in SEID buffer (150 mM sucrose, 10 mM EDTA, imidazole, 2.4 mM sodium deoxycholate, pH 7.5) and centrifuged (Hettich®, Universal 320R, UK) (7500 *g*, 15 min, 4°C). The supernatant was incubated with ouabain (NKA inhibitor) or NEM (H^+-ATPase inhibitor) and the production of ADP was estimated during 30 min (Gibbs and Somero, 1989). The activity of Ca^{2+}-ATPase was measured according to Tellis et al. (2013) and Vijayavel et al. (2007). Briefly, samples were homogenized (1:5, w:v) in SEID buffer, centrifuged (10000 *g*, 20 min, 4 °C), and the enzyme activity was determined by the quantification of inorganic phosphate released in the sample in a microplate reader (Bio-Tek Instruments, ELX 800, United States) at 620 nm. For carbonic anhydrase (CA) gills filaments were homogenized (1:10, w:v) in buffer (225 mM mannitol, 75 mM sucrose, 10 mMTris-base and 10 mM NaH_2PO_4, pH 7.4) and centrifuged (13600 *g*, 10 min, 4°C). The supernatant was added to a saturated solution of CO_2 and the reduction in pH resulting from the release of H^+ was measured (Quimis, pH meter - Q400AS, Brazil) for

20 seconds (Vitale et al., 1999).

2.6 Biochemical biomarkers

The liver was homogenized (1:10, w:v) in K phosphate buffer (0.1 M, pH 7.0) and centrifuged (13000 *g*, 20 min, 4°C) for biochemical analyzes. The protein content of the supernatant was determined based on a standard curve of bovine serum albumin (BSA) at 575 nm (Bradford, 1976).

Biotransformation enzymes. The CYP1A activity was determined by measuring the increase in fluorescence given by the conversion of 7-ethoxyfurorufine (ETOX) to resorufin, every minute, during 10 min, in a microplate spectrophotometer at 590 nm (Eggens and Galgani, 1992). The glutathione-S-transferase (GST) activity was determined by the complexation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) using a microplate spectrophotometer at 340 nm (Keen et al., 1976).

Antioxidants. The concentration of glutathione (GSH) was measured by the reaction of GSH with the 5,5-dithiobis-acid-nitrobenzoic substrate (DTNB), and thiolate was quantified using a microplate spectrophotometer at 412 nm (Beutler et al, 1963). Catalase activity (CAT) was measured by the decomposition of H_2O_2 following the decrease in the absorbance over time in a spectrophotometer (SpectraMax, Plus 384, USA) at 240 nm (Beutler, 1975). The superoxide dismutase (SOD) activity was determined by the inhibition of cytochrome c reduction quantified at 550nm (McCord and Fridovich, 1969). The glutathione peroxidase (GPx) activity was determined by the oxidation of NADPH in the presence of H_2O_2 at 340 nm (Hopkins and Tudhope, 1973).

Oxidative damage. Lipid peroxidation (LPO) was determined by measuring the reaction

of malondialdehyde with thiobarbituric acid at 530 nm (Camejo et al., 1998). Protein oxidation was measured by the quantification of carbonylated proteins (PCO) from the reaction with 2,4-dinitrophenyldrazine (DNPH) to form hydrazones, detected at 360 nm (Levine et al., 1994).

2.7 Integrated Biomarker Response Index (IBR)

An Integrated Biomarker Response Index (IBR) was calculated, as described by Beliaeff and Burgeot (2002) and modified by Sanchez et al. (2013). Only the biomarkers that showed significant and consistent changes were used for the calculation of the IBR and the calculations were performed as described by Vieira et al. (2016). Briefly, for each individual biomarker, the ratio between the mean value obtained in each treatment (NANO, ATZ, and nATZ) at each time and concentration, and the respective control value (CTR) was log10 transformed (Yi). In the next step, an overall mean (μ) and standard deviation (s) were calculated, considering all Yi values. Then, the Yi values were standardized using the formula: $Zi = (Yi-\mu)/s$ and the difference between Zi and Z0 (CTR) was used to define the deviation index of the biomarker (A). To obtain the integrated biomarker response index, the A value of each biomarker was calculated for the different treatments and the IBR was calculated by summing the absolute values of A.

2.8 Statistical analyzes

The results of each biomarker were compared between the different groups (CTR x NANO X ATZ X nATZ), for each concentration and experimental time, by single factor analysis of variance (ANOVA) or the Kruskal-Wallis test, according to the data distribution

277 (normality and homogeneity of variance). When necessary, the differences were found by
278 the Holm-Sidak or Dunn's method. Values of $p < 0.05$ were considered significant. All
279 analyzes were carried out using the software SigmaPlot 11.0.
280
281 **3. RESULTS**
282 **3.1 Nanoparticles characterization**
283 The results of DLS and NTA analyzes for NANO and nATZ samples showed a size
284 variation and a reduction in nanoparticle concentrations along the experiment (Table
285 1). For NANO2, from time zero to the end of the experiment, we observed a decrease
286 in size (from 254 nm to 107 nm) and in the concentrations of nanoparticles (from
287 $1.45x10^8$ to $8.03x10^7$ nanoparticles.mL^{-1}). The same trend was observed for nATZ2,
288 which showed a decrease in size (from 490 nm to 331 nm) and in nanoparticles
289 concentrations (from $3.05x10^8$ to $6.41x10^7$ nanoparticles mL^{-1}). For NANO20 and
290 nATZ20, along the experimental period DLS analyzes showed a variation in the size of
291 nanoparticles, whereas NTA indicated a variation the nanoparticles concentration.
292 Samples of NANO20 showed a slight increase in size (from 277 nm to 323 nm) and a
293 decrease in the concentrations of nanoparticles (from $1.67x10^8$ to $8.24x10^7$
294 nanoparticles mL^{-1}). While for nATZ20 we observed both a reduction of size (from 432
295 to 104 nm) and nanoparticles concentrations (from $1.58x10^8$ to $1.11x10^8$ nanoparticles
296 mL^{-1}). The DLS methodology indicates that there were aggregates, which could
297 influence in the nanoparticles size. Due to the low concentration of nanoparticles in
298 the samples, the size estimated by NTA can be more accurate and the NTA data
299 showed that there were no relevant variations in the size during the assay.
300

3.2 Physiological biomarkers

Among the hematological parameters analyzed (Fig. 1), the most consistent variations between treatments occurred in the hemoglobin content. At 96 h the fish of the NANO2, ATZ2, and nATZ2 groups demonstrated a significant decrease in the hemoglobin content in relation to their respective CTR ($F = 26.34$, $P < 0.001$), as well as the ATZ20 and nATZ20 groups in relation to their respective CTR ($F = 7.32$, $P = 0.001$) (Fig. 1A).

In relation to glucose (Fig. 2A), a point increase was observed only in fish exposed to ATZ20 in relation to their respective CTR at 24h ($F = 5.43$, $P = 0.004$). Among the plasma ions, point variations were observed in the concentrations of Na^+, K^+ and Cl^- (Figs. 2B, 2C, and 2D). The plasma concentration of Ca^{2+} varied more consistently (Fig. 2E), with a significant reduction in the fish in the NANO2, ATZ2, and nATZ2 groups compared to their respective CTR, both after exposure for 24 ($F = 17.07$, $P < 0.001$) and 96h ($F = 57.77$, $P < 0.001$); and in the fish of the ATZ20 group in relation to the CTR at 24h ($F = 4.95$, $P = 0.006$). The concentration of plasma magnesium did not change (Fig. 2F).

Fish exposed to nATZ20 for 24 h showed significantly higher values in the branchial activity of Na^+/K^+-ATPase ($F = 8.62$, $P < 0.001$) and H^+-ATPase ($F = 4.75$, $P = 0.007$) in relation to the respective CTR (Figures 3A and 3B). On the other hand, the activity of Ca^{2+}-ATPase (Fig. 3C) was significantly lower in the gills of the fish exposed to NANO2, ATZ2, and nATZ2 treatments, for 96h ($F = 11.88$, $P < 0.001$) and in the fish of the NANO20 and ATZ20 groups at 24h ($F = 6.06$, $P = 0.002$) and the ATZ20 and nATZ20 groups at 96h ($F = 5.79$, $P = 0.004$). In relation to carbonic anhydrase (Fig. 3D), fish in the ATZ2 group presented significantly lower values of this enzyme activity at 96h ($F =$

8.44, P <0.001), while significantly higher values were observed in the ATZ20 group at 24 h (F = 4.24, P = 0.014).

3.3 Biochemical biomarkers

The hepatic activity of CYP1A did not vary significantly in the treatments and times tested (Fig. 4A). The fish exposed to ATZ2 for 24h presented significantly lower liver GST activity (Fig. 4B) than the respective CTR (F = 4.23, P = 0.013). In relation to the primary antioxidant enzymes (Figs. 4C, 4D and 4E) in the liver, significant variations in relation to the CTR were observed only in CAT and GPx activity. Fish exposed to ATZ20 demonstrated significantly higher CAT activity (Fig. 4D) at 24h (F = 4.88, P = 0.007), whereas at 96h CAT activity was significantly lower in the ATZ20 and nATZ groups (F = 6.71, P = 0.002). The fish exposed to ATZ20 presented GPx activity (Fig. 4E) which was significantly higher at 24h (F = 7.06, P <0.001) and significantly lower at 96h (F = 23.33, P <0.001). On the other hand, significantly higher values of GSH (Fig. 4F) were observed in fish exposed for 96h to the treatments ATZ2 (F= 44.79, P = 0.009) and ATZ20 (F = 31.12, P = 0.003). Oxidative damage was evaluated by the analysis of LPO (Fig. 4G) and PCO (Fig. 4H) in the fish liver. The results indicated significantly higher values of LPO only in fish exposed to ATZ2 for 24h (F = 4.61, P = 0.009). On the other hand, PCO did not demonstrate significant variation in the treatments and times tested.

3.4. Genotoxic biomarkers

In relation to DNA damage, fish exposed to ATZ2 and nATZ2 for 24 and 96 h (F = 30.34, P <0.001 and F = 136.74, P <0.001, respectively), as well as ATZ20 and nATZ20, for

24 and 96 h ($H = 33.87$, $P < 0.001$ and $F = 29.34$, $P < 0.001$, respectively), presented a DNA damage score significantly higher than those of the CTR group (Fig. 5A). Fish from the NANO2 ($F = 136.74$, $P < 0.001$) and NANO20 ($F = 29.34$, $P < 0.001$) groups also showed a significantly higher DNA damage score than the respective CTR at 96 h (Fig. 5A). ENA frequency, when considered together (MN + NN + LN + BN), showed a significant increase only in fish exposed to ATZ20 for 96h ($F = 4.42$, $P = 0.015$), in relation to the respective CTR (Fig. 5B). When analyzed individually, there were no significant variations in the frequency of each ENA between the different treatments. However, among the ENAs a higher occurrence of notched nuclei was observed (Fig. 5D), followed by blebbed nuclei (Fig. 5E), lobed nuclei (Fig. 5F), and MN (Fig. 5C), which were observed only in fish exposed to ATZ.

3.5 Integrated Biomarker Response Index (IBR)

IBR values were calculated considering the following biomarkers: hemoglobin, glucose, Ca^{2+}, Ca^{2+}-ATPase, NKA, H^{+}-ATPase, AC, GST, GSH, CAT, GPx, LPO, DNA damage, and ENA frequency. In the four experiments carried out, the fish of the groups exposed to ATZ presented the highest IBR values, while the fish exposed to the nanocapsules showed the lowest values. The IBR values presented higher values in fish exposed to the treatments with higher concentration and longer experimental time (Fig. 6A). When the IBR values of the 4 experiments were considered together (Fig. 6B), the highest mean value (IBR = 30.18) was observed for fish exposed to ATZ, followed by fish from the nATZ treatment (IBR = 23.41), and finally the nanocapsules (IBR = 18.90).

4. DISCUSSION

The present work evaluated biomarkers of *P. lineatus* exposed to ATZ, as well as the effects caused by nanoencapsulation of ATZ. The results show that some of the effects observed in fish exposed to ATZ were not observed in fish exposed to the nanoencapsulated herbicide, or in fish exposed to nanocapsules alone. However, fish exposed to nATZ showed some alterations as the fish exposed to ATZ. The Integrated Biomarker Response Index (IBR) clearly showed a greater effect of ATZ in its conventional formulation in relation to nATZ. The IBR also demonstrated that the nanocapsules without ATZ caused changes in a lower number of biomarkers, but the same changes as those observed in the fish exposed to the nATZ, suggesting that the effects of nATZ may have been caused by both the herbicide and the PCL nanocapsules.

The concentrations of nanocapsules containing ATZ, as well as those of nanocapsules without ATZ, decreased along the experiment, suggesting some absorption of these compounds by the organism. Nanocapsules can be inserted into cells due to their large surface area and small size and additional surface modifications may further enhance cell uptake (Hu and Gao, 2010; Yuan et al., 2016), whereby nanocapsules containing herbicides can penetrate the cells of animals (Clemente et al., 2013), which may explain the toxicity observed for the fish *P. lineatus*.

In the evaluation of NP toxicity, the morphology, surface area, coating, solubility, and the composition of the NP should be considered. Assays performed with PCL nanocapsules without ATZ showed their phytotoxicity for *Brassica* sp., which may be due to the substances present in their composition (Pereira et al., 2014). One of these components is polysorbate 80 surfactant (Tween 80). Yuan et al. (2016) showed

397 that *Danio rerio* embryos exposed to chitosan nanocapsules modified with Tween 80
398 showed increase in the mortality rate, a decrease in the incubation rate, and an
399 increase in malformations in a dose dependent manner, in addition to an increased
400 level of intracellular production of reactive oxygen species (ROS). Other compounds
401 used in the organic phase of nanocapsules preparation are triglycerides of capric and
402 caprylic acids. Capric acid may have been released during the metabolism of
403 triglyceride, resulting in toxicity to the fish. The study by Yang et al. (2018) showed that
404 after being absorbed capric acid (or decanoic acid) induced oxidative stress by ROS
405 generation, and induced LPO, causing apoptosis in human trophoblasts. The decanoic
406 acid has a variety of biological activities, including antiproliferative and pro-apoptotic
407 effects in human cells (Kim et al., 2014).

408 Regarding the effects of ATZ, fish exposed to the highest ATZ concentration for
409 24 h depicted an increase in hemoglobin concentration. This may have occurred in
410 response to the stress caused by the herbicide and the need to increase the
411 concentration of oxygen in the organism (George et al., 2017). This increase in
412 hemoglobin coincides with increased glycemia, reinforcing the idea of a stress
413 response. High concentrations of plasma glucose can be explained by the mobilization
414 of glycogen reserves in response to the stress induced by the herbicide (Khan et al.,
415 2016). The increase in glycemia after exposure to triazine herbicides has also been
416 reported for the carp *Cyprinus carpio* (Blahova et al., 2014; Bhanu and Deepek, 2015;
417 Khan et al., 2016). In fish exposed to nanoencapsulated ATZ these signs related to a
418 stress response were not observed and could be due to the slower release of ATZ and
419 consequently the lower amounts of the herbicide available. However, after 96 h of
420 exposure to NANO2, ATZ (2 and 20), and nATZ (2 and 20) fish showed a decrease in

421 hemoglobin content. It is known that free herbicide can cause inhibition of hemoglobin
422 formation or premature mortality of red blood cells, affecting oxygen transport
423 capacity (Akinrotimi et al., 2010). The same inhibition may have occurred in fish
424 exposed to the encapsulated herbicide, as well as in fish exposed only to nanocapsules,
425 which may also have interfered in the transport of oxygen.

426 Fish exposed to ATZ in both free and nanoencapsulated form, as well as to the
427 nanocapsules, demonstrated a significant reduction in blood Ca^{2+}. These reductions
428 largely coincide with decreases in activity of Ca^{2+}-ATPase. The maintenance of plasma
429 calcium in freshwater teleost involves the uptake of this ion by the branchial
430 epithelium, which occurs through the entrance of Ca^{2+} across the apical membrane,
431 favored by the low concentration of intracellular Ca^{2+}, and its transport to plasma is
432 directed by the Ca^{2+}-ATPase, located in the basolateral cell membrane (Marshall,
433 2002). Thus, free and nanoencapsulated herbicide, as well as nanocapsules alone,
434 negatively interfere with calcium homeostasis in a freshwater teleost species.

435 Gills play a vital role in the transport of ions (Na^+, K^+, Cl^-, Ca^{2+}, and Mg^{2+}) and
436 thereby maintain the hydroelectrolytic and acid-basic balance in fish. Therefore, the
437 quantification of plasma ions is considered a sensitive biomarker to exposure to
438 xenobiotics (Saravanan et al., 2011, 2015). In fish exposed to ATZ2 a significant
439 reduction in Na^+ concentration was detected at 24 h and a significant increase in
440 potassium at 96 h. Changes in the concentration of these ions may have been due to
441 transient alterations in specific channels or exchangers, since exposure to free ATZ did
442 not cause significant alterations in NKA activity. This lack of alterations in NKA agrees
443 with the results of Paulino et al. (2012), who also did not verify changes in NKA activity
444 after exposure of *P. lineatus* to ATZ. On the other hand, after 24 h exposure to nATZ20

445 there was a significant increase in plasma Na^+, which coincides with the increase in the
446 activity of enzymes NKA and H^+-ATPase. It is known that NKA is important for the
447 concentration of Na^+ as it contributes to Na^+ absorption by the gill epithelium (Aperia
448 et al., 2016) leading to an increase in plasmatic levels.

449 In relation to the activity of the CA, nATZ did not alter its activity, unlike free
450 ATZ, which promoted changes in CA activity.. These changes may have been caused by
451 a direct action of the ATZ on the enzyme, since a high species-specific affinity was also
452 verified in the inhibition of CA by pesticides (Lionetto et al., 2012).

453 The fish exposed to nATZ did not present alterations in GST activity or GSH
454 content, however fish exposed to their conventional formulation showed a decrease in
455 GST activity. GST is an enzyme mainly involved in phase 2 of biotransformation, a
456 decrease in its activity was also seen in *P. lineatus* by Santos and Martinez (2012). This
457 decrease indicates that exposure to ATZ interferes with the detoxification system of
458 the organism (Blahova et al., 2013). Regarding the hepatic content of GSH, fish
459 exposed to ATZ presented an increase in this parameter. A similar result was found in
460 the work of Elia et al. (2012) in the teleost *Lepomis macrochirus* exposed to much
461 higher concentrations of ATZ (6 and 9 $mg.L^{-1}$). The increase in GSH levels may be
462 related to the higher demand for conjugation, in order to avoid bioaccumulation in the
463 liver cells; but may also be a response to prevent oxidative damage. It is known that
464 GSH is efficient to avoid lipid peroxidation, which agrees with the result found in this
465 work, as there was no increase in lipid peroxidation in fish that presented an increase
466 in GSH.

467 Primary antioxidant enzymes (SOD, CAT, and GPx) are the first line of defense
468 against ROS. SOD represents a large family of enzymes that catalyze the dismutation of

469 the superoxide (O_2^-) radical into hydrogen peroxide (H_2O_2). CAT and GPx are
470 responsible for the detoxification of H_2O_2 (Nwani et al., 2011). In the present work, no
471 consistent alterations in these antioxidant enzymes were detected in fish exposed to
472 nATZ, however, some alterations occurred in fish exposed to ATZ. The results showed
473 an increase in the activity of CAT and GPx in the first 24 h of exposure to ATZ20, which
474 may be a response to the increase in ROS generated by exposure to the herbicide
475 (John et al., 2001; Vasanth et al., 2013). Exposure to ATZ also led to an increase in
476 antioxidant enzymes activity in *Poecilia sphenops* (Vasanth et al., 2013) and in CAT
477 activity in female *Danio rerio* (Jin et al., 2010). On the other hand, after longer
478 exposure to ATZ20, there was a reduction in CAT and GPx activity. Similar reductions in
479 CAT and GPx have also been reported for the teleostei *Rhamdia quelen* (Mela et al.,
480 2013), in *Labeo rohita* (Prabakaran et al., 2014), and in *D. rerio* embryos (Adeyemi et
481 al., 2015) exposed to ATZ. In addition, Wang et al (2019) showed that ATZ exposure
482 induced ROS accumulation by disrupting SOD, GSH and CAT functions in carp
483 neutrophils after ATZ treatment. Thus, ATZ metabolism may lead to an excess
484 generation of ROS while it interferes with the transduction of cellular antioxidant
485 signaling pathway, impairing the balance of oxygen free radicals in cells and leading to
486 cellular damage (Wang et al., 2018).

487 Lipid peroxidation (LPO) is among the primary harm caused by the excess of
488 ROS, it is the initial step of damage in the cell membrane, which can lead to cell
489 apoptosis (Livingstone, 2001). In the present study, an increase in LPO was observed
490 only in the liver of the fish exposed to ATZ, suggesting a pro-oxidant imbalance. This
491 LPO increase in response to ATZ exposure was also reported for *C. punctatus* (Nwani et
492 al., 2011), *L. rohita* (Prabakaran et al., 2014), and *P. sphenops* (Vasanth et al., 2013).

493 Nevertheless, LPO increase was not observed in fish exposed to nAT, indicating that
494 ATZ encapsulation was effective to avoid oxidative damage.

495 In spite of being helpful in preventing LPO, nanoencapsulated ATZ was not
496 capable to avoid erythrocyte DNA damage, as an increase in damage score was
497 observed in the erythrocytes of fish exposed to nATZ, as was the case with ATZ,
498 indicating the genotoxic potential of these compounds. S-triazine derivatives, which
499 include atrazine, are capable of direct interaction with DNA, in a time-dependent
500 manner (Oliveira-Brett and Silva, 2002). This interaction occurs by formation of
501 adducts between the herbicide and the purine bases of DNA, adenine and guanine.
502 These results corroborate results found for the same fish species, which showed
503 increased DNA damage in erythrocytes, liver and gill cells after exposure to ATZ (Santos
504 and Martinez, 2012). Grillo et al. (2012) performed genotoxic tests on human
505 lymphocytes with the herbicide ATZ in conventional and nanoencapsulated
506 formulation and their results also demonstrated that all treatments were significantly
507 different from the CTR, but they were also different from each other, and the
508 encapsulation of the herbicides decreased DNA damage by 50%.

509 Exposure to PCL nanocapsules alone for 96 h increased the DNA damage score
510 in erythrocytes of *P. lineatus*. The hydrolysis of poly-epsilon-caprolactone results in the
511 formation of 6-hydroxyhexanoic acid (Karande et al., 2017). In *Danio rerio* larvae,
512 sublethal exposure to adipic acid esters, which is analogous to 6-hydroxyhexanoic acid,
513 caused DNA damage in a dose-dependent manner, as well as the induction of genes
514 related to stress (Boran and Terzi, 2017).

515 Regarding mutagenic effects, only fish exposed to ATZ20 showed a significant
516 increase in the frequency of ENAs. The causes to explain nuclear abnormalities are still

517 uncertain, yet one theory attributes their appearance to alterations in cytoskeletal
518 proteins, responsible for the maintenance of the nuclear shape (Ghisi et al., 2014).
519 Among the ENAs, the lowest occurrence was MN, although it is worth mentioning that
520 MNs were only detected in fish exposed to ATZ. It is known that the maximal induction
521 of MN normally occurs one to five days after exposure (Nwani et al., 2011), which
522 agrees with the formation of MN at 24 and 96 h of exposure as observed in this work.
523 Exposure to xenobiotics may lead to alterations in mitotic spindle formation, causing
524 damage or chromosomal losses, which may result in MN formation (Viana et al., 2018).
525 Previous studies showed that exposure to ATZ leads to the appearance of MN in a
526 dose-dependent manner (Nwani et al., 2011; Piancini et al., 2015).
527
528 **5. CONCLUSION**
529 The development of controlled release systems could mitigate the negative
530 impacts of pesticides on the environment, as well as increase food safety. However,
531 studies concerning the effects of these release systems are necessary for their
532 regulation and use in the agricultural market. Thus, the present work is important to
533 understand the toxicity of nanoencapsulated ATZ in a non-target organism by
534 assessing its ecological risk. The results of the integrated analysis of the biomarkers
535 showed that the ATZ presented a higher toxicity to *P. lineatus* compared to its
536 nanoencapsulated formulation. This demonstrates that nanoencapsulated ATZ was
537 able to protect the animal from the effects of the herbicide in a general way, indicating
538 that nanoencapsulated ATZ is less toxic to *P. lineatus* compared to its conventional
539 formulation. it is likely that the lower toxicity of nanoencapsulated atrazine compared
540 to free atrazine is related to the slower release of ATZ and consequently the lower

amounts of the herbicide available to the organism. However, more studies are needed to clarify the mechanisms underlying the different effects produced by the herbicide in the free and nanoencapsulated form, as well as the effects of PCL nanocapsules.

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791

792 **Figure Captions**

793

794 **Figure 1**. Hemoglobin content (A), hematocrit (B) and number of erythrocytes per mm^3
795 (C) of *P. lineatus* exposed to 2 µg L^{-1} and 20 µg L^{-1} of PCL nanocapsules (NANO),
796 atrazine (ATZ), and atrazine loaded nanocapsules (nATZ) or only to clean water (CTR),
797 for 24 and 96 h. Results are mean ± SE (n= 6-10). Different letters indicate significant
798 different between treatments ($p < 0.05$) for the same experimental tie and
799 concentration.

800

801 **Figure 2.** Plasma concentrations of glucose (A), Na^+ (B), K^+ (C), Cl^- (D), Ca^{2+} (E) and Mg^{2+}
802 (F) of *P. lineatus* exposed to 2 µg L^{-1} and 20 µg L^{-1} of PCL nanocapsules (NANO),
803 atrazine (ATZ), and atrazine loaded nanocapsules (nATZ) or only to clean water (CTR),
804 for 24 and 96 h. Results are mean ± SE (n= 6-10). Different letters indicate significant
805 different between treatments ($p < 0.05$) for the same experimental tie and
806 concentration.

807

808 **Figure 3.** Branchial activity of Na^+/K^+-ATPase (A), H^+-ATPase (B), Ca^{2+}-ATPase (C) and
809 carbonic anhydrase (D) of *P. lineatus* exposed to 2 µg L^{-1} and 20 µg L^{-1} of PCL
810 nanocapsules (NANO), atrazine (ATZ), and atrazine loaded nanocapsules (nATZ) or only
811 to clean water (CTR), for 24 and 96 h. Results are mean ± SE (n= 6-10). Different letters
812 indicate significant different between treatments ($p < 0.05$) for the same experimental
813 tie and concentration.

814

Figure 4. Activity of EROD (A), glutathione S-transferase (B), superoxide dismutase (C), catalase (D) and glutathione peroxidase (E), glutathione content (F), lipid peroxidation (G) and carbonylated proteins (H) in the liver of *P. lineatus* exposed to 2 μg L^{-1} and 20 μg L^{-1} of PCL nanocapsules (NANO), atrazine (ATZ), and atrazine loaded nanocapsules (nATZ) or only to clean water (CTR), for 24 and 96 h. Results are mean ± SE (n= 6-10). Different letters indicate significant different between treatments ($p < 0.05$) for the same experimental tie and concentration.

Figure 5. Score of DNA damage (A) and frequency (‰) of ENAs (B), micronucleus (C), notched nucleus (D), lobed nucleus (E) and blebbed nucleus (F) of *P. lineatus* exposed to 2 μg L^{-1} and 20 μg L^{-1} of PCL nanocapsules (NANO), atrazine (ATZ), and atrazine loaded nanocapsules (nATZ) or only to clean water (CTR), for 24 and 96 h. Results are mean ± SE (n= 6). Different letters indicate significant different between treatments ($p < 0.05$) for the same experimental time and concentration. Inserts are photomicrographs of the alterations represented in the graphs showing different comet classes (stained with gelred) and ENAs (stained with acridine orange).

Figure 6. Integrated Biomarker Response Index (IBR) calculated for *P. lineatus* exposed to 2 μg L^{-1} and 20 μg L^{-1} of PCL nanocapsules (NANO), atrazine (ATZ), and atrazine loaded nanocapsules (nATZ) or only to clean water (CTR), for 24 and 96 h (A), and the mean IBR values considering together all exposure periods and concentrations for each treatment (B).

Table 1

Size and concentration of nanoparticles obtained by DLS and NTA in samples collected from the different experimental treatments (NANO2 and NANO20, and nATZ2 and nATZ20) at time 0 and after 96 h of fish exposure.

Treatment	Time (h)	Size (nm)		Nanoparticles mL^{-1}
		DLS	NTA	
NANO 2	0	254	184.2	1.45×10^8
	96	107.6	184.2	8.03×10^7
NANO 20	0	277.5	264	1.67×10^8
	96	323	274.3	8.24×10^7
nATZ 2	0	490	326.3	3.05×10^8
	96	331	188.5	6.41×10^7
nATZ 20	0	342.2	263.6	1.58×10^8
	96	104.8	268.3	1.11×10^8

Figures

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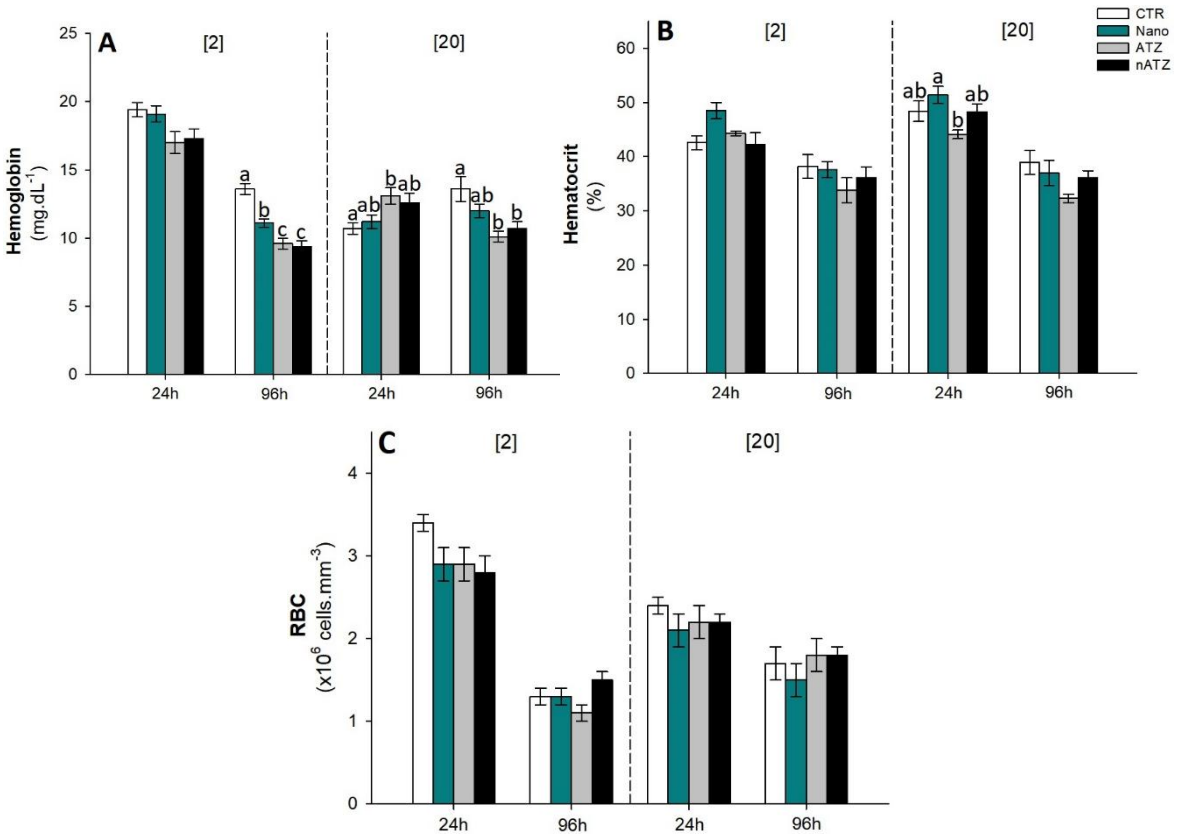

Fig. 1

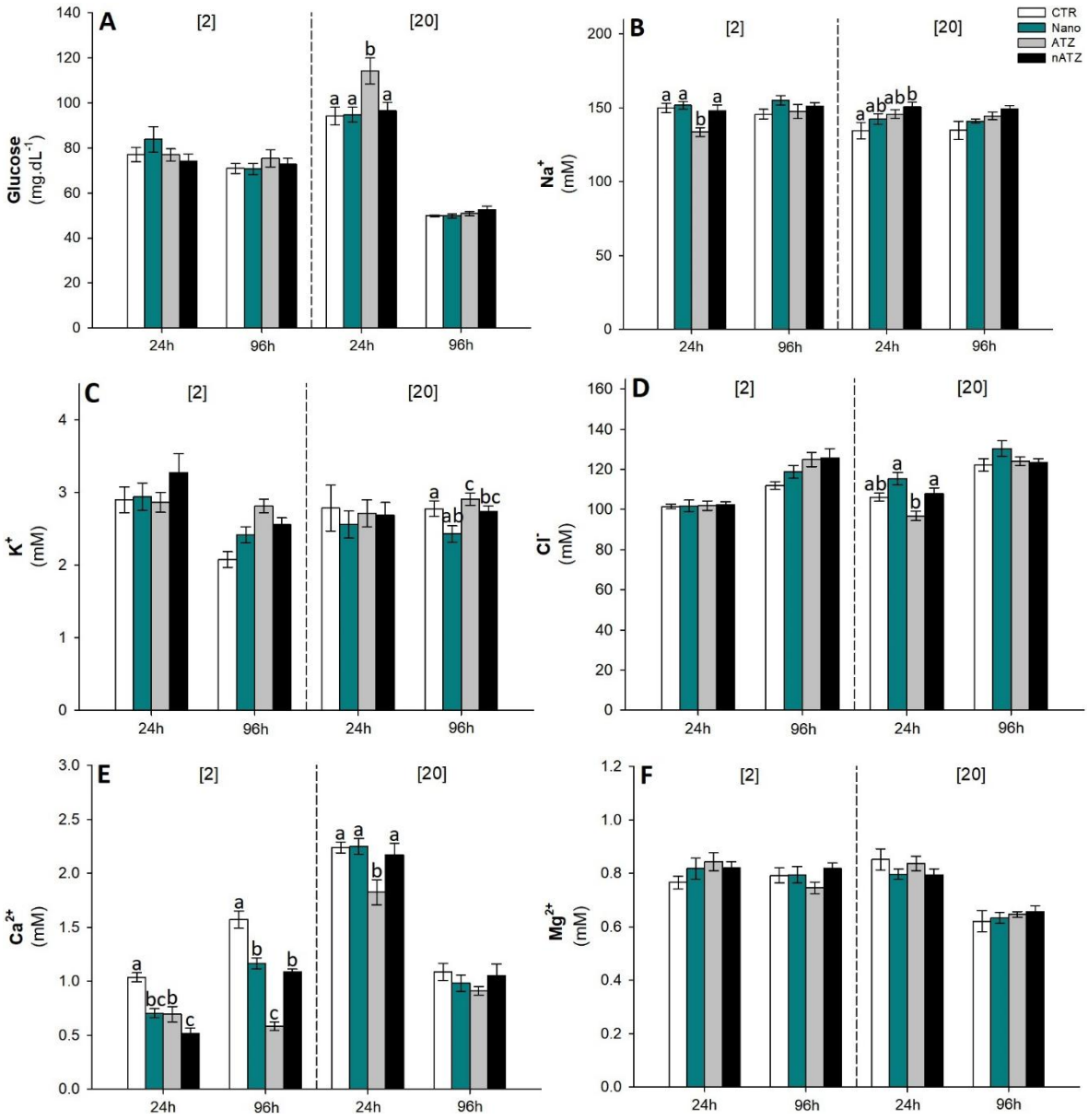
A
[2]
[20]
Glucose (mg.dL-1)
B
Na+ (mM)
CTR
Nano
ATZ
nATZ
C
K+ (mM)
D
Cl- (mM)
E
Ca2+ (mM)
F
Mg2+ (mM)
24h
96h

Fig. 2

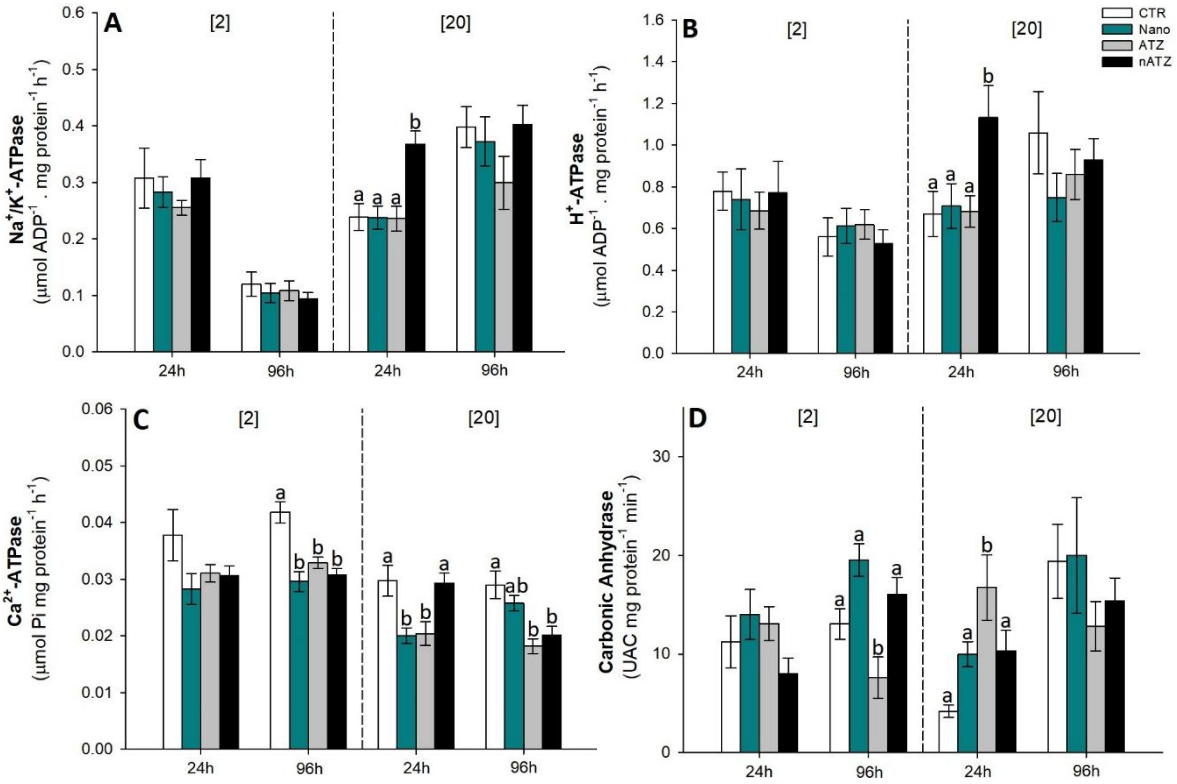
A
[2]
[20]
Na+/K+-ATPase
(µmol ADP-1 . mg protein-1 h-1)
B
H+-ATPase
(µmol ADP-1 . mg protein-1 h-1)
CTR
Nano
ATZ
nATZ
C
Ca2+-ATPase
(µmol Pi mg protein-1 h-1)
D
Carbonic Anhydrase
(UAC mg protein-1 min-1)
24h
96h

Fig. 3

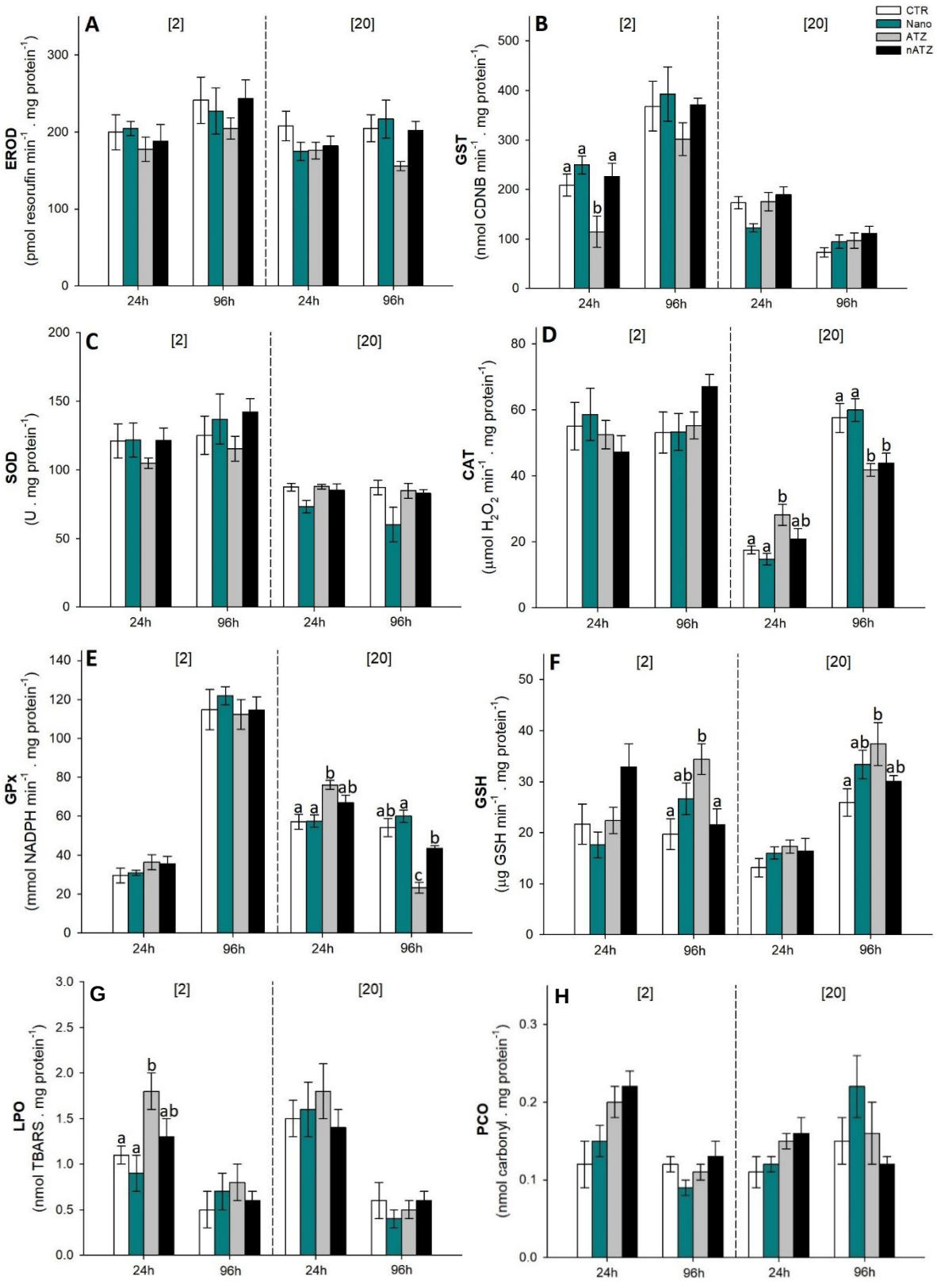
A
[2]
[20]
EROD
(pmol resorufin min-1 . mg protein-1)
B
GST
(nmol CDNB min-1 . mg protein-1)
CTR
Nano
ATZ
nATZ
C
SOD
(U . mg protein-1)
D
CAT
(µmol H2O2 min-1 . mg protein-1)
E
GPx
(mmol NADPH min-1 . mg protein-1)
F
GSH
(µg GSH min-1 . mg protein-1)
G
LPO
(nmol TBARS . mg protein-1)
H
PCO
(nmol carbonyl . mg protein-1)
24h
96h

Fig. 4

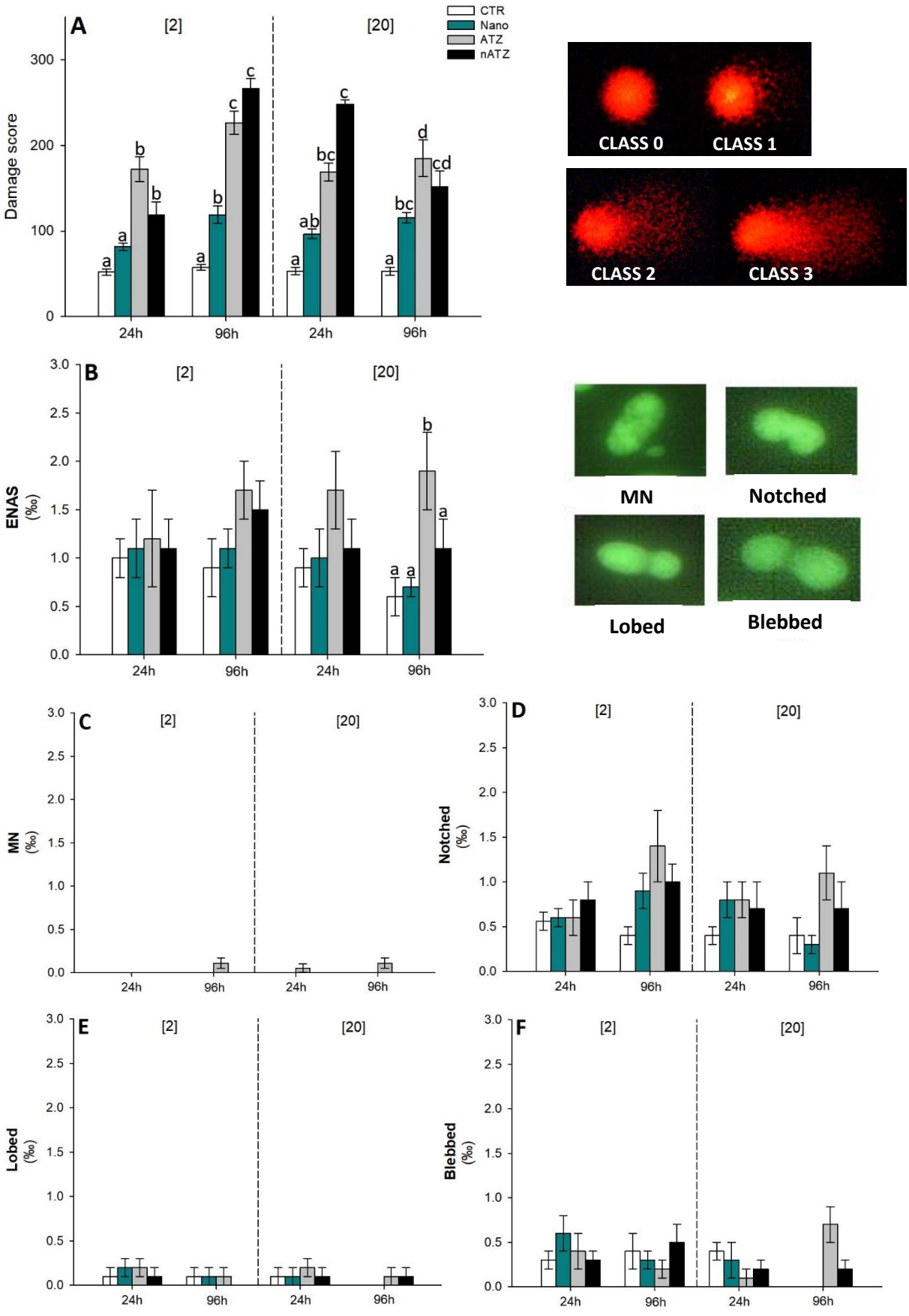
A
[2]
[20]
CTR
Nano
ATZ
nATZ
Damage score
300
200
100
0
24h
96h
24h
96h
CLASS 0
CLASS 1
CLASS 2
CLASS 3
B
[2]
[20]
ENAS (‰)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
24h
96h
24h
96h
MN
Notched
Lobed
Blebbed
C
[2]
[20]
MN (‰)
D
[2]
[20]
Notched (‰)
E
[2]
[20]
Lobed (‰)
F
[2]
[20]
Blebbed (‰)

Fig. 5

A

IBR					
	24h		96h		
	[2]	[20]	[2]	[20]	Mean
NANO	17,56	17,64	21,97	18,42	**18,90**
ATZ	25,59	31,89	29,82	33,43	**30,18**
nATZ	21,44	22,74	26,30	23,17	**23,41**

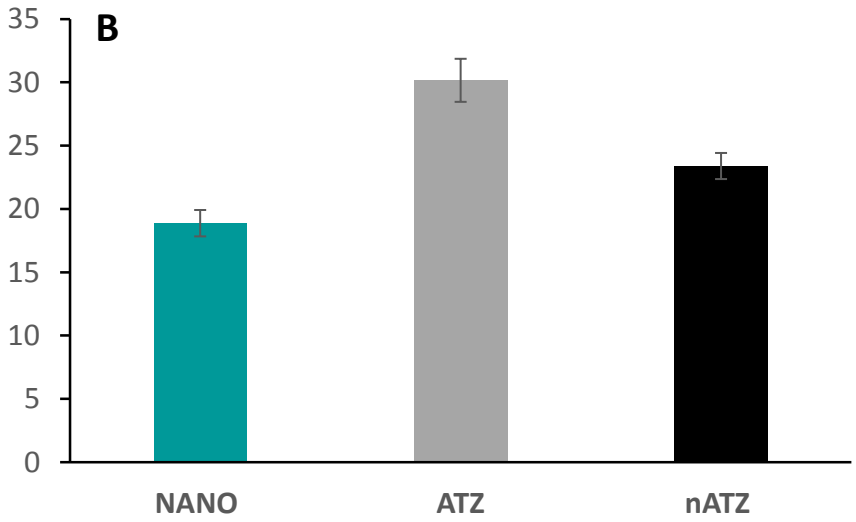

Fig. 6