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Genotoxic effects of Roundup® on the fish *Prochilodus lineatus*

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ABSTRACT

Glyphosate-based herbicides, such as Roundup®, represent the most extensively used herbicides worldwide, including Brazil. Despite its extensive use, the genotoxic effects of this herbicide are not completely understood and studies with Roundup® show conflicting results with regard to the effects of this product on the genetic material. Thus, the aim of this study was to evaluate the genotoxic effects of acute exposures (6, 24 and 96 h) to 10 mg L^{-1} of Roundup® on the neotropical fish *Prochilodus lineatus*. Accordingly, fish erythrocytes were used in the comet assay, micronucleus test and for the analysis of the occurrence of nuclear abnormalities and the comet assay was adjusted for branchial cells. The results showed that Roundup® produces genotoxic damage in erythrocytes and gill cells of *P. lineatus*. The comet scores obtained for *P. lineatus* erythrocytes after 6 and 96 h of exposure to Roundup® were significantly higher than respective negative controls. For branchial cells comet scores were significantly higher than negative controls after 6 and 24 h exposures. The frequencies of micronucleus and other erythrocyte nuclear abnormalities (ENAs) were not significantly different between Roundup® exposed fish and their respective negative controls, for all exposure periods. In conclusion, the results of this work showed that Roundup® produced genotoxic effects on the fish species *P. lineatus*. The comet assay with gill cells showed to be an important complementary tool for detecting genotoxicity, given that it revealed DNA damage in periods of exposure that erythrocytes did not. ENAs frequency was not a good indicator of genotoxicity, but further studies are needed to better understand the origin of these abnormalities.

1. Introduction

Herbicides constitute a heterogeneous category of chemical products, specifically made for weed control [1], which can reach aquatic ecosystems intentionally or indirectly, through soil surface run-off, from areas where they are applied [2]. Experimental findings have revealed that various pesticides possess genotoxic or mutagenic properties which constitute initial risk factors in the generation of carcinogenic and reproductive effects in the long term [1].

Currently, among the various existing pesticides in the market, glyphosate is the most extensively used, and its use in agriculture is continuously expanding on farms that grow genetically modified crops because they can tolerate treatments with this herbicide [3]. Glyphosate is a broad-spectrum nonselective herbicide used for inhibition of unwanted weeds and grasses in agricultural, industrial, urban, forest and aquatic landscapes [4].

Roundup® is the commercial name of an herbicide product in which glyphosate is formulated as isopropylamine salt (IPA) and a surfactant, polyethoxylene amine (POEA), is added to enhance the efficacy of the herbicide [5,6]. Due to its high water solubility and its extensive use, the exposure of non-target aquatic organisms to this herbicide is a concern especially in systems of shallow waters [7].

The acute toxicity of glyphosate is considered to be low by the World Health Organization [8]. However, glyphosate-based commercial formulations are generally more toxic than pure glyphosate [9,10] mainly because surfactants, such as the POEA used in Roundup® formulation, are toxic to aquatic organisms [7]. Giesy et al. [11] observed that POEA was more toxic to fish than pure glyphosate. Tests for acute toxicity, carried out on carps (*Cyprinus carpio*), revealed that the median lethal concentration for 96 h (LC_{50}96 h) of glyphosate is very high, that is, 620 mg L^{-1} [12]. On the contrary, the LC_{50}96 h of the formulated product Roundup® was much lower, varying from 2 to 55 mg L^{-1}, depending on the species of fish, life stage and conditions of the test [13]. The LC_{50}96 h of Roundup® was determined as 13.7 mg L^{-1} to juveniles of the Neotropical fish *Prochilodus lineatus* [14], a detrivorous fish species commonly found in rivers of the south and southeast regions of Brazil and considered as a potential bioindicator species [15,16].

Although studies regarding the biologic effects of pesticides have increased over the last years, the results on the genotoxicity of these products are often incomplete, and sometimes contradictory.

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The genotoxic potential of Roundup® has been studied extensively by the use of various methods, but conflicting results have been encountered [17].

The genotoxic effects of pollutants can be monitored using *in vitro* and *in vivo* tests and micronucleus test and comet assay are the most widely utilized tests in genotoxic evaluations, mainly because of the sensitivity of both in detecting DNA damage and their rapid performance [4]. In fish, the micronucleus test involving peripheral blood erythrocytes is most commonly used [18]. As a complement to the micronucleus test, many authors examine the occurrence of morphologic alterations in the erythrocyte nucleus of fish, also called erythrocytic nuclear abnormalities (ENAs), as possible indicators of genotoxicity [19].

There are very few studies that examined the genotoxic effects of glyphosate-based herbicides on fish. In a study performed by Grisolia [20] it was reported that intra-abdominal injection of Roundup significantly increased the micronuclei frequencies on erythrocytes of fish *Tilapia rendalli*. In an other study [4] treatment with Roundup induced significant increases in frequencies of micronuclei as well as DNA damage, as revealed by comet assay, in peripheral erythrocytes of *Carassius auratus*.

In Brazil, glyphosate-based herbicides are most often utilized and their consumption increased 95% in the period of 2000–2004. Just in the state of Paraná (southern Brazil) alone, 4562 tons of glyphosate were used on soybean and corn crops, between 2000 and 2002 [21], and high concentrations of glyphosate have already been detected in water near to intense cultivation areas in southern Brazil [22]. Despite its extensive use, little is known about the genotoxic effects of this herbicide to Neotropical fish species.

In studies with fish, comet and micronucleus assays have been generally performed on peripheral blood erythrocytes due to their easy sampling and use [19,20,23–26]. Besides erythrocytes, other cell types such as gill cells have also been used, these cells have some advantages over erythrocytes because gill cells of fish exposed to a pollutant can demonstrate more frequent DNA damage than erythrocytes [23]. This can be explained by the fact that gill cells are continuously dividing and are also directly exposed to water contaminants [24].

The aim of this work was to evaluate the genotoxic effects of Roundup® in *P. lineatus* acutely exposed to the herbicide for different periods, using the comet assay, micronucleus test and the occurrence of erythrocytic nuclear abnormalities (ENAs).

2. Material and methods

2.1. Animals

Juveniles of *Prochilodus lineatus* (Valenciennes, 1847), with 9.6 ± 5.4 g and 9.7 ± 1.81 cm (mean ± S.D., $N = 50$), were supplied by the Hatchery Station of Londrina State University. Prior to the toxicity tests, fish were acclimated to laboratory conditions for a minimum of seven days in a 300-L tank with aerated dechlorinated water ($T \cong 25\,^{\circ}C$; pH ≅ 7.0) and a 14/10 h light/dark photoperiod. During this period, fish were fed every 48 h with commercial pellet food containing 36% of protein (Guabi®, BR). Animals were not fed during the toxicity tests.

2.2. Toxicity tests

Short-term (6, 24 and 96 h) static toxicity tests were performed to evaluate the genotoxic and mutagenic effects of 10 mg L^{-1} of Roundup® (360 g glyphosate L^{-1} or 41% of glyphosate, Monsanto Brazil LTDA) to *P. lineatus*. This Roundup® concentration corresponds to 75% of the LC_{50} of this herbicide to *P. lineatus* [14]. Experiments were performed in 100 L glass aquaria containing 6 fish each, with continuously aerated dechlorinated water. One negative control group (NC), exposed only to clean water was terminally sampled at each experimental interval along with the experimental groups exposed to Roundup®. Replicates were carried out for each acute experimental interval. During the tests water was continuously monitored for temperature, dissolved oxygen, pH and conductivity. The mean values (±S.D.) for NC and experimental groups were, respectively, temperature: 25.7 ± 0.5 and 25.0 ± 0.0 °C; pH: 7.4 ± 0.2 and 7.4 ± 0.1; dissolved oxygen: 6.9 ± 0.8 and 7.0 ± 0.8 mg O_2. L^{-1}; conductivity: 53.7 ± 10.4 and 62.8 ± 4.4 μS cm^{-1}. Positive control groups (PC), consisting of fish injected with the clastogenic agent cyclophosphamide (40 mg Kg^{-1}, Sigma–CAS no. 64-86-8) were terminally sampled 6, 24 and 96 h after treatment.

Immediately after removal from the aquaria fish were anesthetized with benzocaine (0.1 g L^{-1}), and blood samples were taken from the caudal vein into heparinized plastic syringes. Subsequently animals were killed by cervical section and the gills were immediately removed. A small amount of each blood sample (10 μL) was diluted in 700 μL of phosphate-buffered saline (PBS: 126.6 mM NaCl, 4.8 mM KCL, 1.5 mM $CaCl_2$; 3.7 mM $NaHCO_3$; 8.9 mM Na_2HPO_4; 2.9 mM NaH_2PO_4) and kept in ice until the start of the comet assay.

Upon dissection, gills were immediately washed with PBS and filaments were gently cleaned using tiny brushes and then cut in small pieces. Gill filaments were stored in 700 μL of PBS and kept in ice until the moment of cell suspension preparation. All handling during gill dissection, dissociation, and preparations were performed on ice. The method for gill cellular suspensions preparation was based on Kilemade et al. [27]. Briefly, gill filaments were gently sectioned using disposable blades and sections were transferred to small plastic tubes, incubated for 15 min in 200 μL 0.25% trypsin – EDTA and homogenized by periodic manual inversion at room temperature for tissue dissociation. To halt the enzymatic digestion 200 μL of fetal calf serum was added to each tube. After 15 min the solution was filtered, leaving the larger undigested tissue pieces behind, the resulting cell suspensions were used in the comet assay.

2.3. Cell viability assay

Before running the comet assay, cell viability for erythrocytes and gill cells was determined using the trypan blue exclusion method. For each animal a total of 100 cells were scored per cell type, and the viability was expressed as the percentage of viable cells in the total number of cells counted. At least 80% of cells should be viable to run the comet assay [28].

2.4. Comet assay

Alkaline comet assay was performed according to Singh et al. [29] and Speit and Hartmann [30] with some modifications as described by Vanzella et al. [25]. Basic steps of the assay for both erythrocytes and gill cells were executed as follows: (a) lysis: one hour, at 4 °C, protected from light, in a lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1 mL Triton X-100, pH 10.0); (b) DNA unwinding: 30 min, in the dark, in an electrophoresis buffer (0.3 N NaOH, 1 mM EDTA, pH > 13); (c) electrophoresis: 20 min, 300 mA, 25 V, 1 V cm^{-1}; d) neutralization: three washes for 5 min each in buffer (0.4 M Tris, pH 7.5). Slides were then fixed with absolute ethanol for 10 min and kept under refrigeration until cytological analyses.

Slides stained with ethidium bromide (20 μg mL^{-1}) were analyzed under a Nikon fluorescence microscope (1000× magnification). All slides were independently coded and scored without knowledge of the code [28]. The extent of DNA damage was quantified by the length of DNA migration which was visually determined in 100 randomly select and non-overlapping cells per fish. DNA damage was classified in four classes (0: undamaged; 1: minimum damage; 2: medium damage; 3: maximum damage) and each comet assigned a value of 0–3 according to its class, the total score will be between 0 and 300 "arbitrary units" [31]. Results for DNA damage in erythrocytes and gill cells were expressed as the mean number of damaged nucleoids (sum of classes 1, 2 and 3) and the mean comet score for each treatment group (CN, Roundup® and CP), for each exposure period.

2.5. Micronucleus test and the occurrence of erythrocytic nuclear abnormalities (ENAs)

The micronucleus test was performed with fish erythrocytes according to the methodology of Hooftman and Raat [32] and the analysis of erythrocytic nuclear abnormalities according to Carrasco et al. [33]. Immediately after sampling blood was smeared on clean glass slides, dried overnight, fixed with methanol for 10 min and stained with Giemsa (5%). A total of 3000 erythrocytes per fish were examined under an Olympus optical microscope (1000× magnification). The mean frequencies of micronucleus (MN) and erythrocytic nuclear abnormalities (ENA) found in each experimental group were calculated and expressed per 1000 cells (‰). ENAs were classified, following Pacheco and Santos [34], into three categories: segmented nuclei (SN), lobed nuclei (LN) and kidney-shaped nuclei (KSN).

2.6. Statistical analysis

Results are presented as the mean ± standard error. All the data were first tested for normality and homogeneity of variance to meet statistical demands. The results obtained for both controls (NC and PC) and for Roundup® group and negative controls, for each experimental period, were compared with each other using two-tailed Student t test. Differences between means were considered significant when $p < 0.05$. Erythrocytic nuclear abnormalities (ENA) other than micronuclei were considered together for statistical analysis and micronuclei were always considered separately from the other nuclear abnormalities.

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Table 1
Frequency of nucleoids observed in each comet class (0, 1, 2 and 3) and the number of damaged nucleoids (mean ± S.E.) in erythrocytes and branchial cells of *Prochilodus lineatus* exposed to Roundup (RDP) and the respective negative controls (NC) and positive controls (PC), taking into account the total number of fish (*N*) analyzed for each experimental period (6, 24 and 96 h)

	Time	Groups	*N*	Comet Classes				Damaged nucleoids (mean ± S.E.)
				0 (%)	1 (%)	2 (%)	3 (%)	
Erythrocytes	6 h	NC	6	70.5	23.0	3.2	3.3	29.5 ± 2.4
		RDP	6	54.5	33.3	8.8	3.3	45.5 ± 6.1*
		PC	6	25.7	63.1	10.2	1.0	74.3 ± 1.5*
	24 h	NC	5	77.8	17.6	2.8	1.7	22.2 ± 1.4
		RDP	6	78.5	18.7	2.2	0.7	21.5 ± 2.4
		PC	6	9.3	53.0	34.3	2.7	90.7 ± 1.6*
	96 h	NC	9	78.4	20.9	0.7	0.0	20.3 ± 1.6
		RDP	12	76.7	21.3	1.6	0.3	23.3 ± 1.1
		PC	8	25.1	69.0	4.5	0.3	74.9 ± 0.9*
Branchial cells	6 h	NC	6	71.5	24.5	2.2	1.8	28.5 ± 1.5
		RDP	5	66.6	28.0	3.2	2.6	33.8 ± 1.1*
		PC	7	63.9	24.7	7.3	4.1	36.1 ± 2.7*
	24 h	NC	6	71.2	23.4	3.0	2.4	28.8 ± 1.0
		RDP	6	61.2	29.2	4.8	4.5	38.5 ± 1.3*
		PC	4	65.0	23.3	6.8	5.0	35.0 ± 3.2
	96 h	NC	4	76.7	13.2	5.8	4.3	23.5 ± 2.3
		RDP	3	74.3	11.7	9.3	4.7	25.7 ± 1.7
		PC	4	65.0	23.3	6.8	5.0	35.0 ± 3.2*

One hundred nucleoids were analyzed per fish.
* Different from respective negative controls ($p < 0.05$).

3. Results

The cell viability assays which were run before the comet assays showed above 90% of viable erythrocytes and gill cells. The results obtained using the comet assay in erythrocytes of *P. lineatus* revealed that fish injected with cyclophosphamide (PC) showed a significant increase both in the number of damaged nucleoids and in the comet scores, in relation to their respective negative controls, in all experimental periods (Table 1 and Fig. 1). When branchial cells were used in the comet assay the results revealed that only after 6 h PC fish showed significant increase in the number of damaged cells and in the comet score, in relation to respective NC. After 96 h of cyclophosphamide injection a significant increase was observed in the number of damaged nucleoids, but the comet score remained similar to the one obtained with gill cells from NC (Table 1 and Fig. 1).

In terms of MN induction erythrocytes from PC fish showed a significant increase in MN frequency after 24 and 96 h of the treatment with the clastogenic agent in relation to respective NC (Table 2). On the other hand, analysis of the frequency of other nuclear abnormalities (SN + LN + KSN) in erythrocytes of *P. lineatus* injected with cyclophosphamide did not show any significant increase with respect to NC in any experimental period (Table 2). The frequencies of ENAs verified for both negative and positive controls showed to be low, varying, respectively, from 2.11 to 4.11 and from 2.33 to 4.00 (‰). The type of nuclear abnormality more commonly detected was a kidney-shaped nucleus, which was observed more frequently after 96 h, both for NC and PC.

Table 2
Frequencies of micronuclei (MN) and other nuclear abnormalities (ENA) in erythrocytes of *Prochilodus lineatus* exposed to Roundup (RDP) and the respective negative controls (NC) and positive controls (PC), taking into account the total number of fish (*N*) analyzed for each experimental period (6, 24 and 96 h)

Time	Group	*N*	MN frequency (‰)	ENA frequency (‰)
6 h	NC	8	0	3.00 ± 0.34
	RDP	10	0	2.37 ± 0.16
	PC	7	0.05 ± 0.05	2.33 ± 0.38
24 h	NC	9	0.07 ± 0.05	2.11 ± 0.30
	RDP	12	0.05 ± 0.05	2.33 ± 0.46
	PC	8	0.71 ± 0.22*	2.33 ± 0.35
96 h	NC	9	0.18 ± 0.11	4.11 ± 0.38
	RDP	12	0.11 ± 0.08	3.67 ± 0.36
	PC	8	0.54 ± 0.09*	4.00 ± 0.30

Three thousand erythrocytes were analyzed per fish and results are shown as mean ± S.E.
* Different from respective negative controls ($p < 0.05$).

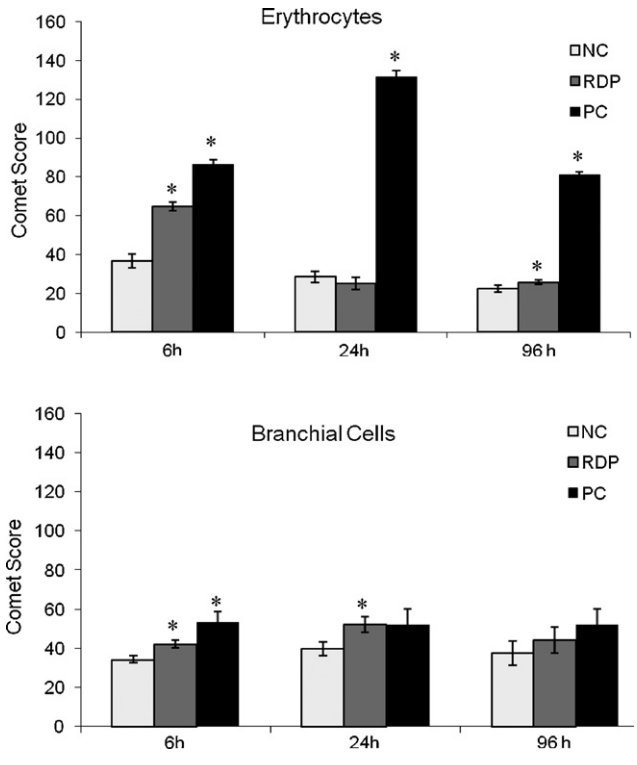

Fig. 1. Comet scores in erythrocytes and branchial cells of *Prochilodus lineatus* exposed to Roundup (RDP) and the respective negative (NC) and positive controls (PC) for each experimental period (6, 24 and 96 h). One hundred nucleoids were analyzed per fish. Bars represent means and vertical lines the S.E. *Significantly different from respective negative control ($p < 0.05$).

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Concerning Roundup® effects, fish erythrocytes exhibited significantly higher DNA damage after 6 and 96 h of herbicide exposure, as demonstrated by the significant increases in the comet scores in relation to respective NC (Fig. 1). The number of damaged nucleoids was significantly different from respective controls only in fish erythrocytes after 6 h exposure to Roundup® (Table 1). For branchial cells both the number of damaged nucleoids and the comet scores were significantly higher in fish exposed to the herbicide during 6 and 24 h in relation to respective NC (Table 1 and Fig. 1).

Frequencies of MN and nuclear abnormalities in peripheral fish erythrocytes from groups of fish exposed to Roundup® and their respective negative controls groups are shown in Table 2. In contrast to comet results, both MN and ENAs frequencies registered in fish erythrocytes after herbicide exposure were not significantly different from the respective negative controls. As it was verified for negative control groups, the frequency of ENAs in erythrocytes of fish exposed to Roundup® showed to be low, varying from 2.33 to 3.67 (‰), and the type of nuclear abnormality more commonly detected was also a kidney-shaped nucleus, followed by segmented nuclei and lobed nuclei.

4. Discussion

Substantial progress has been made in the last decades to evaluate the impact of physical and chemical genotoxins in aquatic organisms [35]. The development of new methods and the application of assays that are more sensitive in the detection of genotoxicity for various xenobiotics in aquatic biota have been the main determinants for attaining these advances [36,37]. In the present work, the genotoxicity of the herbicide Roundup® was evaluated based on the comet assay applied to the analysis of peripheral blood erythrocytes and gill cells of *P. lineatus*, and based on the micronucleus test (in erythrocytes) and the test for erythrocytic nuclear abnormalities (ENAs).

Although the comet assay is suitable for genotoxicity studies in any nucleated eukaryotic cell [38], there may be various practical limitations to the application of this assay including the first stage of cell isolation [39]. For the comet assay to be applied in a reliable manner in cells from tissues such as gills and liver, it is necessary for the cells to be insolated using techniques that themselves do not cause DNA damage [28]. In fish, a tissue frequently chosen to perform the comet assay is blood because it is easy to collect and there is no need for a cell isolation step [27]. Besides erythrocytes, other cell types are used for monitoring the genotoxic effects of pollutants, thereby exploiting tissue-specific responses [38]. Thus, different tissues such as intestine, liver, gills, gonads, kidney, spleen and muscle are chosen for the determination of DNA damage by the comet assay [40]. However, regardless of the cell type to be studied, the results obtained in genotoxicity tests must be first checked in relation to the sensitivity of the test-organism and the overall credibility of the test system. In this context, the utilization of negative and positive control groups is part of the recommended guidelines [41].

In the present study, the results of the comet assay indicated that blood cells were more sensitive than the gill cells to DNA damage caused by cyclophosphamide. This stronger effect of cyclophosphamide in blood cells might be partially attributed to the route of administration of the genotoxic agent, which was by intraperitoneal injection, possibly resulting in a greater exposure of the erythrocytes than the gills cells [42].

The micronucleus test detects chromosomal fragments or acentric chromosomes that are not incorporated into the main nucleus after mitosis. Thus, for the detection of MN it is necessary that actively dividing cell populations undergo at least one cell cycle [18]. However, there is little information on the extent of the cell cycle in teleosts, considering that this cycle varies with temperature in poikilotherm animals, and the rate of erythropoiesis may vary in different fish species [43,44]. From the literature, it appears that a peak in micronucleated erythrocytes occurs 1–5 days after exposure, but in most fish species it takes place after 2 or 3 days [18]. Grisolia and Cordeiro [45] studied the effect of cyclophosphamide in peripheral blood erythrocytes of three fish species and observed an increase in MN frequency after 2–7 days of treatment. In the present study, cyclophosphamide induced an increased MN frequency in fish erythrocytes after 24 and 96 h of treatment. The absence of a significant MN increase after 6 h of cyclophosphamide injection is probably related to the short time interval of treatment, which was insufficient for the occurrence of a complete cell cycle and, consequently, for the detection of micronuclei in the erythrocytes examined.

In fish, besides the presence of micronuclei, there are various types of nuclear lesions in the erythrocytes, whose origin has not yet been very well elucidated [46]. Such abnormalities have been used by various authors as indicators of genotoxicity in fish [27,47–49]. Although the use of this method has indicated that cyclophosphamide induces a greater incidence of erythrocytic nuclear abnormalities (ENA) in other species of fish [19,23,50], this did not occur in the present work in relation to *P. lineatus* (Table 2). Pacheco and Santos [34] showed that at least 6 days exposure to cyclophosphamide was necessary to induce a significant increase in ENA frequency in *Anguilla anguilla*, and they suggested that a rapid catabolism of DNA-damaged erythrocytes and its slow replacement by the organism might be the cause of a delayed appearance.

Studies on the genotoxic potential of glyphosate and formulations based on this product, such as Roundup®, exhibit great variation due to the different formulations tested, doses applied, methods employed and organisms studied [4]. Such facts could explain, in part, the conflicting results that have been published with regard to the effects of these products. According to some of these studies, glyphosate and glyphosate-based herbicides can result in both the absence [51–54] and the incidence [53–59] of DNA damage.

In the present study, the comet assay revealed a significant increase in DNA damage in erythrocytes and gill cells in animals exposed to Roundup® for 6 h. However, after 24 h exposure, the erythrocytes and gill cells exhibited different behaviors (Table 1). At this time, the DNA damage in erythrocytes of *P. lineatus* exposed to Roundup® diminished returning to the mean score found in the respective control group (Fig. 1). It is possible that the repair system of fish had acted on the DNA of the erythrocytes or that the damaged cells had been removed by the spleen [18]. However, in the gill cells, DNA damage in fish exposed to Roundup® for 24 h remained increased, in relation to the respective negative control (Fig. 1). A possible explanation for this difference between erythrocytes and gill cells would be that the repair system in gill cells is slower and consequently damaged cells could have remained longer in the gill tissue, resulting in an increased comet score after 24 h.

The biotransformation of xenobiotics often results in the production of reactive intermediates such as reactive oxygen species (ROS), which are highly toxic and can cause oxidative damage to DNA. Although organisms are equipped with an antioxidant defense system to protect tissues against oxidative lesions, if the rate of ROS production exceeds the capacity of defense mechanisms, cellular and DNA lesions can occur [44,60]. Thus, it is possible that the increased DNA damage in erythrocytes of *P. lineatus* after 96 h of exposure to Roundup® could be due to ROS generated by the metabolism of the herbicide, which could have interacted with DNA of exposed fish, resulting in the lesions detected by the comet assay. In fact, *P. lineatus* exposed to 10 mg L^{-1} of Roundup® for up

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to 96 h showed a significant increase in hepatic catalase activity, indicating the activation of antioxidant defenses, probably due to the increased production of ROS [14].

While the comet assay showed a positive response following Roundup exposure, the MN test using *P. lineatus* erythrocytes did not indicate any genotoxic effect of the sub-lethal concentration of Roundup® (10 mg L^{-1}) here employed, which corresponds to 4.1 mg L^{-1} of glyphosate. This result agrees with Çavas and Konen [4] who investigated the effects of glyphosate in *Carassius auratus* and observed that the lowest glyphosate concentration capable of inducing a significant increase in the number of micronucleated erythrocytes was 5 mg L^{-1}, after 96 h exposure. The sensitivity of the MN assay in fish erythrocytes has been always debatable due to its low level induction and it is not surprising that a correlation between MN induction and comet response under *in vivo* conditions in *P. lineatus* is not apparent [44].

Among the three methods employed in this study, the frequency of ENAs was the least efficacious in the identification of damage to the genetic material caused by the herbicide Roundup®. Considering that not even cyclophosphamide was capable of inducing an increase in ENAs frequency, it is recommended that for *P. lineatus* the comet assay and MN test be adopted as tools in studies of genotoxicity.

In conclusion, the results of this work showed that Roundup® produced genotoxic effects on the fish species *P. lineatus*. The comet assay with gill cells showed to be an important complementary tool for detecting genotocixity, given that it revealed DNA damage in periods of exposure that erythrocytes did not. ENAs frequency was not a good indicator of genotoxicity, but further studies are needed to better understand the origin of these abnormalities. Finally, the use of the comet assay represents an efficient tool for monitoring genotoxic agents in aquatic ecosystem.

Conflict of interest statement

None.

Acknowledgments

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