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Roundup® causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish *Prochilodus lineatus*

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ABSTRACT

This work aimed to evaluate Roundup® effects on biochemical biomarkers of the neotropical fish *Prochilodus lineatus*. Fish were acutely exposed (6, 24 and 96 h) to 10 mg L^{-1} of Roundup® (RD) or only water (control) and samples of liver, for antioxidants analysis, and brain and muscle, for acetylcholinesterase (AChE) determination, were collected. Fish exposed to RD for 24 h showed reduction on superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, and increased glutathione (GSH) content. After 24 and 96 h, fish of RD group showed increased glutathione-S-transferase (GST) activity and lipid peroxidation. AChE activity was inhibited in brain after 96 h and in muscle after 24 and 96 h of exposure. Thus, acute exposure to RD stimulated the biotransformation pathway, with increased GST, but interfered on the antioxidant defenses, with reduction of SOD and GPx activity, leading to the occurrence of lipid peroxidation. Inhibition of AChE showed that RD acts as a contaminant with anti-AChE action.

1. Introduction

Roundup® (RD) is a glyphosate-based formulation that represents one of the most commonly applied herbicides in the world (Jiraungkoorskul et al., 2002), and the rapid increase of its use in agriculture is due to the cultivation of genetically modified crops (Roundup Ready®) to tolerate this herbicide (Giesy et al., 2000). Moreover, in Brazil the expansion of sugarcane cultivation has also contributed to increase the consumption of RD, since it is the main herbicide used in this crop. Glyphosate shows a high water solubility, varying from 10 000 to 15 700 mg L^{-1} at 25 °C (USEPA, 1993) and it has a low vapor pressure, which suggests that loss to the atmosphere from treated surfaces will be small (Battaglin et al., 2005). The half-life of glyphosate in aquatic environments is reported to range from 7 to 14 d (Giesy et al., 2000). Thus, glyphosate is frequently detected in many rivers in both agricultural and urban regions (Pesce et al., 2008) and this contamination represent significant toxicological risks to resident aquatic organisms (Çavas and Könen, 2007; Pesce et al., 2009). Glyphosate is moderately to very slightly toxic to aquatic animals (WHO, 1994), but the commercial formulation Roundup® (RD) is considered more toxic probably due to the addition of the surfactant polyoxyethylene amine (POEA) (Amarante et al., 2002). Recent studies (mainly from 2000 onward) are showing potentially adverse effects of Roundup, and its components glyphosate and POEA, on fish (Lushchak et al., 2009).

Several studies have demonstrated that RD is toxic to fish and can give rise to morphofunctional changes in these animals. Jiraungkoorskul et al. (2002) demonstrated that exposure to RD induces histological alterations in the gills, liver and kidney of the Nile tilapia *Oreochromis niloticus*. In the Neotropical fishes *Leporinus obtusidens* and *Rhamdia quelen* RD exposures inhibited the activity of brain acetylcholinesterase inducing metabolic alterations (Glusczak et al., 2006, 2007). Several authors have also shown that RD can be genotoxic to fish. Çavas and Könen (2007) found that *Carassius auratus* exposed to the herbicide presented dose-dependent increase of micronucleus frequencies, nuclear abnormalities and DNA breakage.

Many xenobiotics, such as pesticides, can produce reactive oxygen species (ROS) via several mechanisms, such as interference in electron transport in the mitochondrial membrane and subsequent accumulation of reactive intermediates, inactivation of antioxidant enzymes, depletion of non-enzymatic antioxidants and membrane lipid peroxidation (Winston and Di Giulio, 1991). ROS production associated with the presence of pollutants and the establishment of oxidative stress has been imputed as a possible mechanism of toxicity in aquatic organisms exposed to pesticides (Oropesa et al., 2008).

The equilibrium between antioxidant defenses and the generation of oxygen reactive species (ROS) is fundamental for the animal's homeostasis. When there is an imbalance between pro-oxidants and antioxidant defenses, the situation known as oxidative stress can be established, in which the excessive generation of ROS may lead to irreversible impairment of DNA and other macromolecules and even to death (Ahmad et al., 2000). Damage to

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membrane lipids – lipoperoxidation – is considered the greatest cause of cell injury and cell death and can be induced by pollutants such as herbicides (Glusczak et al., 2006). Superoxide dismutase (SOD), the first enzyme in the line of antioxidant defense, is responsible for catalyzing the conversion of the superoxide anion into hydrogen peroxide. Hydrogen peroxide then degrades into water and molecular oxygen via catalase (CAT), a family of enzymes which is present mainly in peroxisomes. Another very important peroxidase is glutathione peroxidase (GPx), which metabolizes a variety of peroxides, including hydrogen peroxide. Among the non-enzymatic antioxidants is the tripeptide glutathione in its reduced form (GSH), which acts as the main antioxidant in the cell and is a cofactor for the action of GST and GPx (Hermes Lima, 2004; Maran et al., 2009). Glutathione-S-transferase (GST) is an enzyme that acts in the process of biotransformation, catalyzing the conjugation of a variety of metabolites, including xenobiotic metabolites and lipoperoxidation products with GSH, transforming the toxic compound into more easily excretable one.

Inhibition of acetylcholinesterase (AChE) activity is a recognized effect of carbamate-based and organophosphorated insecticides in fish (Monserrat et al., 2002), but the determination of this enzyme in different fish tissues has also proved to be a sensitive method for detecting the presence of several herbicides (Sancho et al., 2000). Glusczak et al. (2007) reported that *R. quelen* showed significant reduction in AChE activity after exposure to RD. When the activity of this enzyme is reduced, acetylcholine is not broken down but remains accumulated in the synapses, altering the entire normal functioning of the nervous system (Dutta and Arends, 2003) and may affect the locomotion and equilibrium of exposed organisms (Bretaud et al., 2000).

In the Neotropical fish *Prochilodus lineatus*, acute exposure to sub-lethal concentrations of RD induced DNA impairment (Cavalcante et al., 2008), liver histological alterations and some biochemical variations that suggested the activation of antioxidant defenses (Langiano and Martinez, 2008). At present, more studies are necessary to evaluate oxidative stress parameters in *P. lineatus* exposed to Roundup to understand the mechanisms of toxicity of this herbicide. In this context, the goal of this work was to evaluate RD effects on the oxidative stress biomarkers and on AChE activity of *P. lineatus* after acute exposure to a sub-lethal concentration of the herbicide. This was done through the determination of the hepatic activity of the antioxidant enzymes (SOD, CAT, GPx and GST), the amount of GSH in the liver, the occurrence of lipoperoxidation (TBARS assay), and the AChE activity in the brain and muscle of *P. lineatus*.

2. Materials and methods

2.1. Animals

Juvenile specimens of *P. lineatus* (*n*: 72, weight: 10.9 ± 0.3 g), supplied by the hatchery station of State University of Londrina, were acclimated for a week in 300-L tanks containing dechlorinated and aerated water. During this period, they were fed every 48 h with commercial fish food containing 36% protein, but were not fed 48 h prior to and during the toxicity tests.

2.2. Toxicity tests

After acclimation, the fish were submitted to static acute toxicity tests (6, 24 and 96 h). The commercial formulation of Roundup® (360 g glyphosate L^{-1} or 41% of glyphosate, Monsanto do Brasil LTDA) was used at a nominal concentration of 10 mg L^{-1}. This concentration was chosen because it is sub-lethal to *P. lineatus* and previous studies have already shown that it induces liver histopathologies and genotoxic effects on this fish species (Langiano and Martinez, 2008). The tests were carried out in glass aquaria of 100 L, with six fish in each aquarium. A control group (CTR), with animals exposed only to water, without the contaminant, was sampled at each experimental time (6, 24 and 96 h), concomitantly with the groups exposed to Roundup (RD). All toxicity tests were carried out in duplicate. The following parameters were monitored during the experiments: temperature, dissolved oxygen, conductivity and pH of the water in the aquaria.

Immediately after their removal from the aquaria, the fish were anesthetized with benzocaine (0.1 g L^{-1}) and killed by medullary section. After the fish were weighed, the liver, brain and muscle were removed and frozen at −80 °C until the moment of the assays.

2.3. Antioxidant enzymes

The livers were weighed, homogenized (10× volume) in potassium phosphate buffer 0.1 M, centrifuged (20 min, 15 000*g*, 4 °C), and the supernatant removed for analysis of the biochemical parameters. The glutathione-S-transferase (GST) activity was determined according to the methodology proposed by Keen et al. (1976), following the complexation of the reduced glutathione (GST) with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm, and expressed as nmol of conjugated CDNB min^{-1} mg of $protein^{-1}$. The copper–zinc superoxide dismutase (CuZn-SOD) activity was determined by the method of Flohé and Otting (1984). This method is based on the measurement of the inhibition of the reduction rate of cytochrome *c* by the superoxide radical, at 550 nm. SOD activity was expressed in U SOD mg of $protein^{-1}$, with one U of SOD corresponding to the quantity of enzyme that promoted the inhibition of 50% of the reduction rate of cytochrome *c*. The catalase (CAT) activity was determined according to the technique described by Beutler (1975), by monitoring the H_2O_2 decomposition from the decrease of absorbance at 240 nm. CAT activity was expressed in μmol H_2O_2 min^{-1} mg of $protein^{-1}$. Selenium-dependent glutathione peroxidase (Se–GPx) activity was determined by the method of Hopkins and Tudhope (1973), based on NADPH oxidation in the presence of H_2O_2 at 340 nm. GPx activity was expressed in μmol oxidized NADPH min^{-1} mg of $protein^{-1}$.

2.4. Non-enzymatic antioxidant

Reduced glutathione (GSH) levels were measured according to Beutler et al. (1963), using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). Supernatants of the acid extracts (1:1 v/v with 12% TCA) were added to 0.25 mM DTNB in 0.1 M potassium phosphate buffer, pH 8.0, and thiolate anion formation was determined at 412 nm against a GSH standard curve. GSH content was expressed in μg of GSH mg of $protein^{-1}$.

2.5. Lipid peroxidation

Lipid peroxidation in the fish liver was estimated from the production of malondialdehyde (MDA), which is one of the final products of lipid peroxidation. The MDA content was determined by the TBARS assay, which measures the thiobarbituric acid (TBA) reactive substances, at 530 nm, following the methodology described by Satoh (1978). Lipoperoxidation was expressed as TBARS concentration in μmol mg of $protein^{-1}$, using a standard MDA curve.

2.6. Acetylcholinesterase

The brains and muscles were homogenized (10× volume) in potassium phosphate buffer (0.1 M, pH 7.5), centrifuged (20 min, 15 000*g*, 4 °C), and the supernatant was removed for analysis of

acetylcholinesterase. Enzyme activity was determined based on the colorimetric method of Ellman et al. (1961) adapted for reading on microplates, according to Alves Costa et al. (2007). The final concentration of the acetylcholine iodide substrate employed was 9 mM, while that of the DTNB color reagent was 0.5 mM for both tissues. Absorbance was determined in a microplate reader at 415 nm and the enzyme activity was expressed in nmol DTNB min^{-1} mg of $protein^{-1}$.

The protein concentration in all the samples of all the assays was determined by the method of Lowry et al. (1951), using bovine albumin as standard.

2.7. Statistical analysis

For each treatment group (CTR and RD), at each period of exposure (6, 24 and 96 h), twelve animals were sampled. However, the amount of liver tissue sampled from one fish was not enough for the determination of all the parameters and thus liver samples were distributed in order to get at least five or six fish samples for each biochemical analysis. For all the parameters analyzed, the differences between the control and experimental groups at each experimental time were analyzed by Student's *t*-test or the Mann–Whitney test, depending on the distribution of the data and the homogeneity of the variances. Values of $P \leqslant 0.05$ were considered significant.

3. Results

No fish mortality occurred during the toxicity tests. The physical and chemical parameters of the water were constant throughout the acclimatization period and the experiments (Table 1).

Fig. 1 depicts the primary antioxidant enzymes results. In comparison to respective control, fish exposed to the herbicide for 24 h showed a transient significant reduction of the hepatic SOD activity (Fig. 1A). The exposure to the herbicide for 6 and 24 h also promoted a significant decrease on GPx activity (Fig. 1C). CAT activity did not vary between control fish and RD exposed fish during any exposure times (Fig. 1B).

After 24 and 96 h of exposure to RD, the fish showed a significant increase in liver GST activity in relation to the controls (Fig. 2A). Animals submitted to the herbicide also showed a significant increase in GSH liver content after 6 and 24 h of exposure in comparison to respective controls (Fig. 2B).

Table 2 presents the results of the TBARS assay to estimate the lipid peroxidation in the liver of fish subjected to the toxicity tests. TBARS concentration was found to increase in all the experimental times; however, due to the high variability of the data, the differences were not statistically confirmed. Even so, it should be noted that the *P* values obtained for the analyses of 24 and 96 h, 0.068 and 0.057, respectively, are very close to the established level of significance ($P < 0.05$), indicating a strong tendency for increased TBARS concentration.

The acetylcholinesterase enzyme activity was significantly diminished in the fish brain after 96 h of exposure to RD, in comparison with its respective control (Fig. 3A). The enzyme activity was also inhibited in the muscle after 24 and 96 h of exposure to the herbicide compared with the respective controls (Fig. 3B).

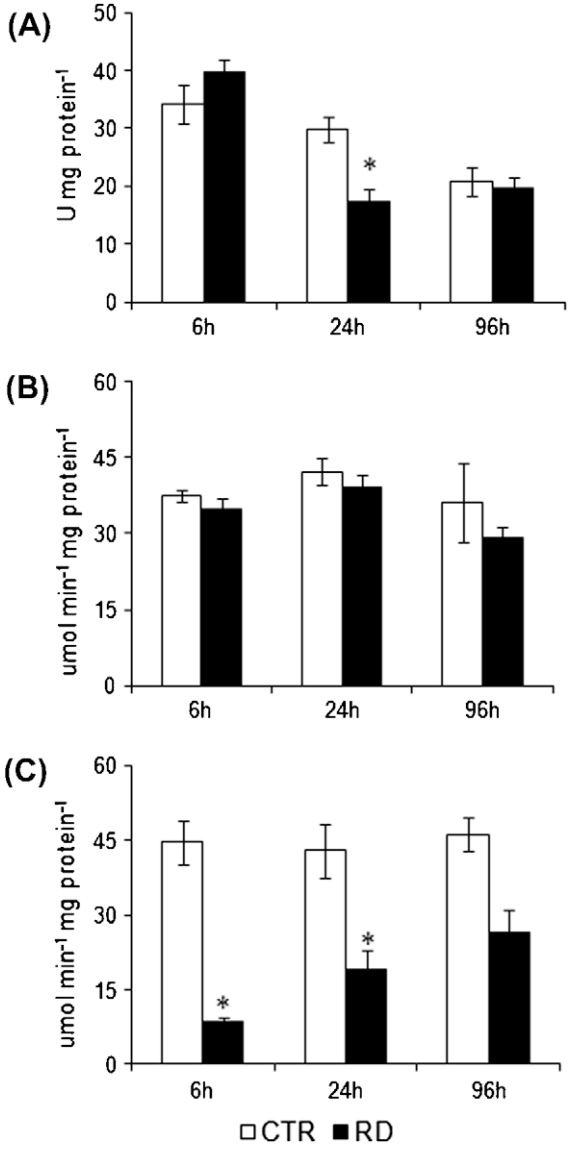

Fig. 1. Hepatic activity of superoxide dismutase (A), catalase (B) and glutathione peroxidase (C) of *Prochilodus lineatus* exposed to 10 mg L^{-1} of Roundup® (RD) or only water (CTR), for different experimental periods (6, 24 and 96 h). Data are means ± SE ($n = 5–6$). *Different from respective control, $P \leqslant 0.05$.

4. Discussion

In this work, a set of oxidative stress parameters of a Neotropical fish species was determined aiming the understanding of the possible mechanisms of toxicity of RD herbicide. Previous studies

Table 1
Physical and chemical parameters of the water samples collected during the acclimation period and during the tests (6, 24 and 96 h), for the control groups and Roundup exposed groups.

	Temperature (°C)	pH	Dissolved O_2 (mgO_2 L^{-1})	Conductivity (μS cm^{-1})	Hardness (mg $CaCO_3$ L^{-1})
Acclimation	23.0 ± 2.3	6.84 ± 1.0	6.8 ± 0.2	65.2 ± 0.8	44
Control	25.7 ± 0.5	7.4 ± 0.2	6.9 ± 0.8	53.7 ± 10.4	44
Roundup	25.0 ± 0.0	7.4 ± 0.1	7.0 ± 0.8	62.8 ± 4.4	44

The values represent means ± SE ($n = 6$). Water hardness was determined only once for each experimental situation.

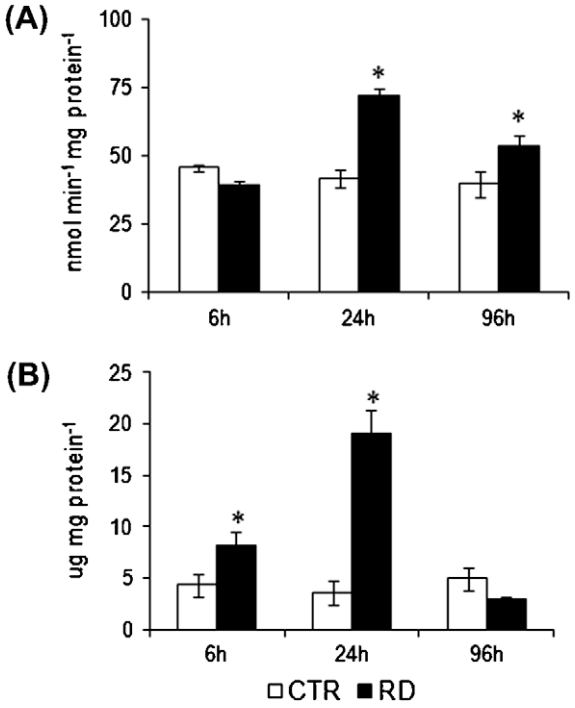

Fig. 2. Activity of glutathione-S-transferase (A) and concentration of reduced glutathione (B) in the liver of *Prochilodus lineatus* exposed to 10 mg L^{-1} of Roundup® (RD) or only water (CTR), for different experimental periods (6, 24 and 96 h). Data are means ± SE (n = 5–6). *Different from respective control, $P \leqslant 0.05$.

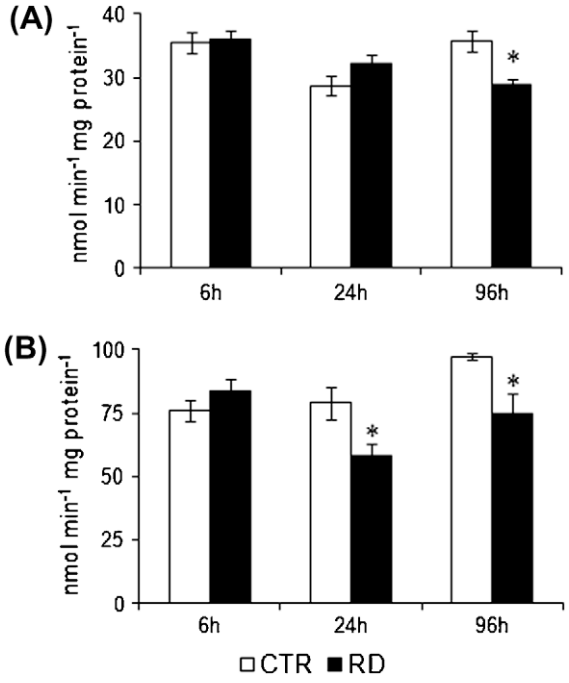

Fig. 3. Acetylcholinesterase activity in brain (A) and muscle (B) tissue of *Prochilodus lineatus* exposed to 10 mg L^{-1} of Roundup® (RD) or only water (CTR), for different experimental periods (6, 24 and 96 h). Data are means ± SE (n = 6). *Different from respective control, $P \leqslant 0.05$.

have already analyzed the effects of RD on some of these parameters separately (Glusczak et al., 2006, 2007; Langiano and Martinez, 2008). However, the present study involved an integrated examination of the possible alterations in the antioxidant defenses and the occurrence of oxidative lesions after the fish exposure to RD. The results indicated that this herbicide stimulates the biotransformation pathways but reduces the activity of some antioxidant enzymes and leads to the occurrence of lipid peroxidation. Moreover, the herbicide showed anticholinesterase activity in both brain and muscle tissues.

When one analyzes the activity of antioxidant enzymes, one must consider that these enzymes have a complex pathway of regulation and that their activities derive from two processes: production and inactivation. Protein carbonylation due to the direct action of reactive oxygen species (ROS) may cause loss of enzyme activity, and some lipoperoxidation products may react with amino acid residues, altering the function of the protein. Hence, many enzymes involved in the antioxidant defense process may also be inactivated by the excess of oxidants, and this oxidant may be its own substrate. Superoxide dismutase (SOD), for example, can be inactivated by hydrogen peroxide, catalase by the superoxide anion, and glutathione-S-transferase (GST) is easily inactivated by oxidants in general (Bagnyukova et al., 2006). In this study, SOD inhibition after 24 h of exposure to the herbicide may have been caused by the transitory accumulation of hydrogen peroxide in fish liver. Since CAT activity was not altered after exposure to RD, it is reasonable to assume that there was an accumulation of hydrogen peroxide in the cells. Hydrogen peroxide is a strong oxidant that can have various destinations inside the cell. H_2O_2 can be metabolized by CAT or GPx, generating H_2O and $O_2^{\cdot-}$ if it is not metabolized, hydrogen peroxide can generate hydroxyl radicals ($^{\cdot}OH$) through the Fenton reaction, which is the most reactive of the ROS and which reacts with lipids in the cell's plasma membranes (Hermes Lima, 2004). GPx, which could also metabolize this peroxide, also showed reduced activity after 6 and 24 h of exposure to the herbicide, contributing to the accumulation of H_2O_2, which may then have led to the reduction of SOD activity after 24 h or exposure.

The maintenance of catalase (CAT) activity observed in this study throughout all the experimental times is congruent with the findings reported by Glusczak et al. (2007), who also did not observe alterations in the activity of this enzyme in the liver of *R. quelen* exposed to 0.2 and 0.4 mg L^{-1} of RD for 96 h. CAT activity may be augmented or diminished in contaminated environments or after exposure to pollutants, depending on the chemical agent in question. Moraes et al. (2007) found increased CAT activity in the liver of *L. obtusidens* exposed to the herbicides clomazone and propanil. Pereira Maduenho and Martinez (2008) also found increased CAT in the liver of *P. lineatus* exposed to diflubenzuron. Conversely, Crestani et al. (2006) reported reduction of the hepatic activity of catalase in *R. quelen* exposed to clomazone.

The literature regarding the activity of catalase reports highly variable results. Therefore, the use of catalase activity as an exclusive biomarker of toxicity is not recommended and it is necessary to verify the activity of different enzymes in order to understand antioxidant responses of fish (Van der Oost et al., 2003). Thus, although CAT activity showed no alteration in this work, the hypothesis that exposure to RD generates ROS cannot be discarded,

Table 2
Malondialdehyde content (μmol MDA. mg protein^{-1}) in the liver of *Prochilodus lineatus* exposed to 10 mg L^{-1} of Roundup® or only water (Control), for different experimental periods (6, 24 and 96 h). The values of P obtained through the statistical comparison between control and Roundup groups, for each experimental period, are shown in the last column.

Period	Control	Roundup	P values
6 h	25.95 ± 5.65 (5)	31.81 ± 10.65 (5)	0.640
24 h	21.61 ± 5.02 (5)	43.55 ± 9.08 (5)	0.068
96 h	13.40 ± 0.97 (5)	38.27 ± 10.84 (6)	0.057

The values represent means ± SE (n).

since other enzymes of the antioxidant pathway exhibited alterations after exposure to the product.

The reduction of GPx activity after 6 and 24 h of exposure would be explained by a decrease in the availability of the tripeptide GSH. GSH acts as the main non-enzymatic antioxidant of cells, in addition to being a substrate for GST and GPx activity; hence, depending on the situation, there may be a lack of this tripeptide for some of these processes (Monteiro et al., 2006). However, at the experimental times of 6 and 24 h GSH concentration has increased. Therefore, a shortage in GSH content does not explain the reduced GPx activity.

Both GST and GPx contribute to the detoxification of oxidative stress products, and the contribution of GST is more significant than that of GPx (Maran et al., 2009). It has been demonstrated that GST activity can be altered in polluted locations, and that the presence of organic contaminants may lead to the increased activity of this enzyme (Machala et al., 1997). In the present work, the increase observed in GST activity after 24 and 96 h of exposure is probably due to the increase in the biotransformation process of the xenobiotic by the animal exposed to RD, and metabolization of the lipoperoxides formed by the Fenton reaction may also be occurring, indicating the activation of the defense mechanisms. This increase of enzyme activity is consistent with the findings of Cavalcanti et al. (2008), who, by means of the Comet assay, observed increased DNA impairment of erythrocytes of *P. lineatus* exposed to 10 mg L^{-1} of RD for 96 h. In other words, even with the increase in GST activity, the defense pathway does not suffice to eliminate the products of the contaminant, which end up impairing the animal's DNA.

Another consequence of the insufficiency of antioxidant pathways is lipid peroxidation, which is one of the main indicators of oxidative stress (Falfushynska and Stolyar, 2009), and which can be generated in various fish tissues after exposure to pollutants such as herbicides (Miron et al., 2008). In the present study, the occurrence of lipid peroxidation was indicated by the increased concentration of TBARS in the liver of *P. lineatus*, after 24 and 96 h of exposure to RD. Elevation of TBARS levels has also been described in muscle of the fish *R. quelen* after 96 h of exposure to 0.2 and 0.4 mg L^{-1} of RD (Glusczak et al., 2007). RD also induced lipid peroxidation in the liver and skeletal muscle of bullfrog tadpoles (*Lithobates catesbeiana*) after 48 h of exposure to 1 mg L^{-1} of the herbicide (Costa et al., 2008). These authors suggest that the generation of oxygen reactive species and oxidative stress are involved in the toxicity induced by RD, and the findings of the present work reinforce this idea.

The reduction of acetylcholinesterase (AChE) activity observed in this study in brain and muscle after 96 h of exposure are in agreement with the result found in brain of *L. obtusidens* and *R. quelen* exposed to the same herbicide (Glusczak et al., 2006, 2007). However, this is the first report of AChE inhibition in muscle of fish exposed to RD. Several studies relate the activity of this enzyme to different species and contaminants (Miron et al., 2005; Üner et al., 2006; Rodríguez-Fuentes et al., 2008; Pereira Maduenho and Martinez, 2008; Falfushynska and Stolyar, 2009), since AChE sensitivity varies among species, and brain and muscle are the tissues most commonly used in assays (Sancho et al., 2000). The accumulation of acetylcholine due to reduction of enzyme activity may affect the fleeing and reproductive behavior of fish (Saglio and Trijasse, 1998; Bretaud et al., 2000), interfering directly in the survival of the species.

The results of the present study demonstrated that acute exposure to RD stimulated the biotransformation pathway, with augmentation of GST activity, and interfered in the antioxidant defenses of *P. lineatus*, with reduction of SOD and GPx activity. The animal's antioxidant defenses did not suffice to neutralize the ROS probably produced during the biotransformation process, leading to the occurrence of membrane lipid peroxidation. Thus, the exposure of *P. lineatus* to the herbicide leads to a situation of oxidative stress. Furthermore, the inhibition of brain and muscle AChE indicates that RD acts as a contaminant with anti-AChE action. However, more work is necessary to discriminate what component of the formulated product, glyphosate or POEA, could be responsible for ROS generation and AChE inhibition.

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