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Toxicity and effects of a glyphosate-based herbicide on the Neotropical fish *Prochilodus lineatus*

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Abstract

The toxicity of Roundup, a glyphosate-based herbicide widely used in agriculture, was determined for the Neotropical fish *Prochilodus lineatus*. The 96 h-LC_{50} of Roundup was 13.69 mg L^{-1}, indicating that this fish is more sensitive to Roundup than rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). These differences should be considered when establishing criteria for water quality and animal well-being in the Neotropical region. Short-term (6, 24 and 96 h) toxicity tests were then performed to evaluate the effects of sub-lethal concentrations of the herbicide (7.5 and 10 mg L^{-1}) to *P. lineatus*. Roundup did not interfere with the maintenance of the ionic balance and there was no significant alteration in plasma cortisol levels in Roundup-exposed fish. However an increase in plasma glucose was noted in fish exposed to 10 mg L^{-1} of the herbicide, indicating a typical stress response. Catalase liver activity also showed an increase in fish exposed to 10 mg L^{-1} of the herbicide, suggesting the activation of antioxidant defenses after Roundup exposure. In addition, Roundup induced several liver histological alterations that might impair normal organ functioning. Therefore, short-term exposure to Roundup at subletal concentrations induced biochemical, physiological and histological alterations in *P. lineatus*.

Keywords: Acute toxicity; Biotransformation; LC_{50}; Liver histopathology; Osmoregulation; Oxidative stress; Roundup; Stress response

1. Introduction

Glyphosate is a broad-spectrum non-selective herbicide used for inhibition of unwanted weeds and grasses in agricultural, industrial, urban, forestry and aquatic landscapes (Çavas and Könen, 2007). It is perhaps the most important herbicide ever developed and its use continues to expand particularly in applications involving plant varieties that are genetically modified to tolerate glyphosate treatments (Williams et al., 2000). In Brazil, glyphosate is the most widely used herbicide and its consumption has increased 95% in the period from 2000 to 2004. In the state of Paraná (southern Brazil) 4562 tons of glyphosate were used in corn and soybean culture between 2000 and 2001 (Inoue et al., 2003). High concentrations of glyphosate were detected in water near to intense cultivation areas in southern Brazil (da Silva et al., 2003). The major formulation is Roundup, in which glyphosate is formulated as isopropylamine salt and a surfactant, polyethoxylene amine (POEA), is added to enhance the efficacy of the herbicide (Tsui and Chu, 2004; Releya, 2005). Due to its high water solubility and extensive usage (especially in shallow water systems), the exposure of non-target aquatic organisms to this herbicide is a concern (Tsui and Chu, 2003).

The acute toxicity of glyphosate is considered to be low by the World Health Organization (WHO, 1994). However, commercial glyphosate formulations are more acutely toxic than glyphosate (Amarante et al., 2002; Peixoto, 2005). Surfactants such as POEA in Roundup are the principal toxic component in the formulated products based on glyphosate to aquatic organism (Tsui and Chu, 2003). In a review of toxicological data, Giesy et al. (2000) found POEA to be more toxic to fish than glyphosate. Neskovic et al. (1996) carried out acute toxicity tests with carp (*Cyprinus carpio*) and found the median lethal concentration in 96 h (96 h-LC_{50}) of glyphosate to be fairly high, 620 mg L^{-1}. However, considering the formulated product Roundup, 96 h-LC_{50} varied from 2 to 55 mg L^{-1}, depending on the fish species, life stage and test conditions (Jiraungkoorskul et al., 2002).

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Nevertheless, glyphosate alone or with its formulation products was previously considered to be harmless in normal usage and at chronic exposure in previous testing approaches (Williams et al., 2000). However, toxic effects of Roundup at sub-lethal concentrations have now been demonstrated in fish (Marc et al., 2004). Sub-lethal concentrations of glyphosate, corresponding to less than 2% of the LC_{50}, caused ultrastructural damage in the liver of *C. carpio* (Szarek et al., 2000). Histological alterations were also observed in liver, gills and kidneys of Nile tilapia (*Oreochromis niloticus*) after acute and chronic exposure to sub-lethal concentrations of Roundup (Jiraungkoorskul et al., 2002, 2003).

Despite the fact that Roundup is widely used in Brazil, only a limited amount of information is available on its toxic effects to native freshwater fishes. Recently, acute effects of Roundup on metabolic and enzymatic parameters of *Leporinus obtusidens* and *Rhamdia quelen* were investigated by Glusczak et al. (2006, 2007). Apart from these studies, toxicological responses of Neotropical fishes to glyphosate-based herbicides remain poorly understood.

Prochilodus lineatus (Order Characiformes, Family Prochilodontidae) is native to the south and southeast regions of Brazil. This fish represents a well suited species to environmental monitoring as it is a bottom feeder animal which is in contact with xenobiotics in water and in sediment, and is sensitive to variations in water quality (Camargo and Martinez, 2006; Simonato et al., in press).

Thus, to obtain more information about the threat imposed by the use of glyphosate-based pesticides to Neotropical fish species this work was designed to determine the toxicity of Roundup to *P. lineatus* and to evaluate the responses of this fish at biochemical, physiological and histological levels, after acute exposure to sub-lethal concentrations of the herbicide.

2. Materials and methods

2.1. Toxicity tests

Juveniles of *P. lineatus*, weighing 16.32 g±8.35 (mean±S.E., $n=144$), were supplied by the Hatchery Station of Universidade Estadual de Londrina. Prior to the toxicity tests, fish were acclimated to laboratory conditions for a minimum of seven days in a 600-L tank with aerated dechlorinated water ($T \cong 22$ °C; pH ≅ 7.5; OD ≅ 8 mgO_2 L^{-1}; conductivity ≅ 90 μS cm^{-1}; $Na^+ \cong 0.086$ mM; $K^+ \cong 0.030$ mM; $Cl^- \cong 0.103$ mM; hardness ≅ 80 mg L^{-1} $CaCO_3$). During this period, fish were fed with commercial pellet food each 48 h. Animals were not fed during the toxicity tests.

Short-term (6, 24 and 96 h) static toxicity tests were performed to evaluate the toxicity of Roundup to *P. lineatus*. Preliminary tests were carried out to determine the appropriate concentration range for testing the chemical. The commercial formulation of glyphosate, Roundup (360 g glyphosate L^{-1} or 41% of glyphosate, Monsanto do Brasil LTDA), was used. Experiments were performed in 100 L glass aquaria containing 8 fish each, with continuously aerated dechlorinated water, with the same characteristics described for the acclimation period. The tests consisted of five groups of fish exposed to one of five Roundup concentrations (7.5, 10, 15, 20 and 30 mg L^{-1}) and a control group exposed only to water, without the herbicide. The number of dead fish was recorded every 6, 24 and 96 h, and the values of the median lethal concentration (LC_{50}) were estimated by the trimmed Sperman–Karber method (Hamilton et al., 1977).

To evaluate Roundup effects, fish were exposed to two sub-lethal concentrations of the herbicide, corresponding to 55 and 75% of the 96 h-LC_{50}, or only to clean water (control groups), in 100 L glass aquaria containing 8 fish each. One experimental group for each Roundup concentration plus one control group were terminally sampled at: 6, 24 and 96 h. Replicates were carried out for each experimental time. The physical–chemical characteristics of the water during sub-lethal tests for both Roundup concentrations, in all the exposure periods, remained stable. The mean values (±SE) for control and experimental groups were, respectively, temperature: 21.7±0.3 °C and 21.7±0.3; pH: 7.5±0.1 and 7.4±0.1; dissolved oxygen: 7.2±0.1 and 7.2±0.1 mg O_2. L^{-1}; conductivity: 91.7±3.0 and 94.3±2.9 μS cm^{-1}.

Immediately after removal from the aquaria, the 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 livers were immediately removed and divided in two parts. One part was stored at −80 °C for enzymatic assays and the other was placed in Bouin's fixative for histopathological analysis.

2.2. Physiological and biochemical analysis

Blood samples were centrifuged (5 min, 5000 ×*g*) and plasma samples were stored frozen (−20 °C). Plasma sodium was measured by flame photometry. Plasma chloride concentration was determined by the thiocyanate method using a commercial kit (Analisa, Brazil). Plasma osmolarity was determined with a freezing point osmometer. Plasma glucose was analyzed using a colorimetric commercial kit (Glucox 500-Doles Reagentes, Brazil) based on the glucose–oxidase reaction. Cortisol was analyzed with a commercial immunoenzymatic kit (Diagnostic Systems, USA) and the reading carried out in a microplate reader at 450 nm.

Fish livers were homogenized in 10 volumes (w/v) of ice-cold 0.1 M K-phosphate buffer (pH 7.0) and centrifuged (14000 ×*g*) for 20 min at 4 °C, to obtain the supernatant for glutathione-*S*-transferase (GST) and catalase analyses. GST activity was determined as described by Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The change in absorbance was recorded at 340 nm and the enzyme activity was expressed in nmol min^{-1}.mg liver protein. Catalase activity was estimated from the rate of consumption of hydrogen peroxide (Beutler, 1975). Change in absorbance was recorded at 240 nm and enzyme activity was expressed in μmol min^{-1}.mg liver protein. Concentration of protein in the supernatant was measured by the method of Lowry et al. (1951). All samples were analyzed in duplicate.

2.3. Histopathological analysis

Liver samples were fixed in Bouin's fixative, embedded in paraffin and sectioned (5 μm). The slides were stained with

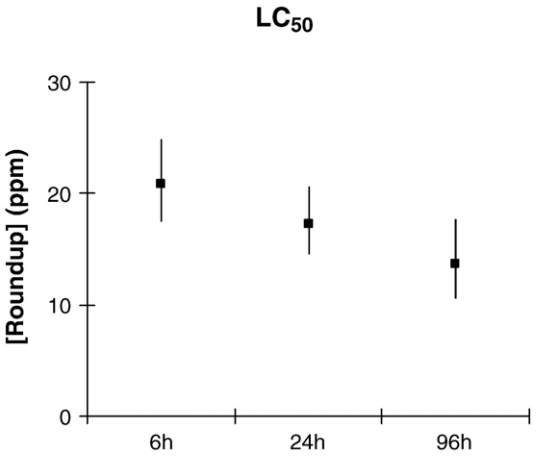

Fig. 1. LC_{50} values of Roundup estimated for *Prochilodus lineatus* for each exposure period (6, 24 and 96 h) and their respective confidence limits (vertical lines).

hematoxylin–eosin (HE), examined under an Olympus light microscope and photographed using a digital camera.

Liver tissue changes induced by Roundup were evaluated semi-quantitatively by the Degree of Tissue Change (DTC), which is based on the severity of the lesions and the possibility of recovery. For DTC calculation (modified from Poleksić and Mitrović-Tutundžić (1994)), liver changes were classified into three progressive stages of impairment of the hepatic function: stage I = changes that do not damage the liver tissue to such an extend that the organ cannot repair itself; stage II = repairable changes that are more severe and affect the associated tissue function; and stage III = changes that preclude the restoration of the structure of the liver, even with an improvement in water quality. The DTC was calculated by the sum of the number of lesions types within each of three stages multiplied by stage index, using the following mathematical equation proposed by Poleksić and Mitrović-Tutundžić (1994): $DTC = (1 \times \Sigma I) + (10 \times \Sigma II) + (100 \times \Sigma III)$; where I, II and III are the number of lesions of stage I, II and III respectively. The DTC value obtained for each fish was used to calculate the mean index for each Roundup-exposed group and their respective controls. The mean DTC was divided into 5 categories; 0–10: functionally normal liver, 11–20: slightly to moderately damaged liver, 21–50: moderately to heavily damaged liver, 51–100: severely damaged liver, and >100: irreparable damaged liver.

2.4. Statistical analysis

For the biochemical and physiological variables, differences between experimental and control groups, at each exposure time

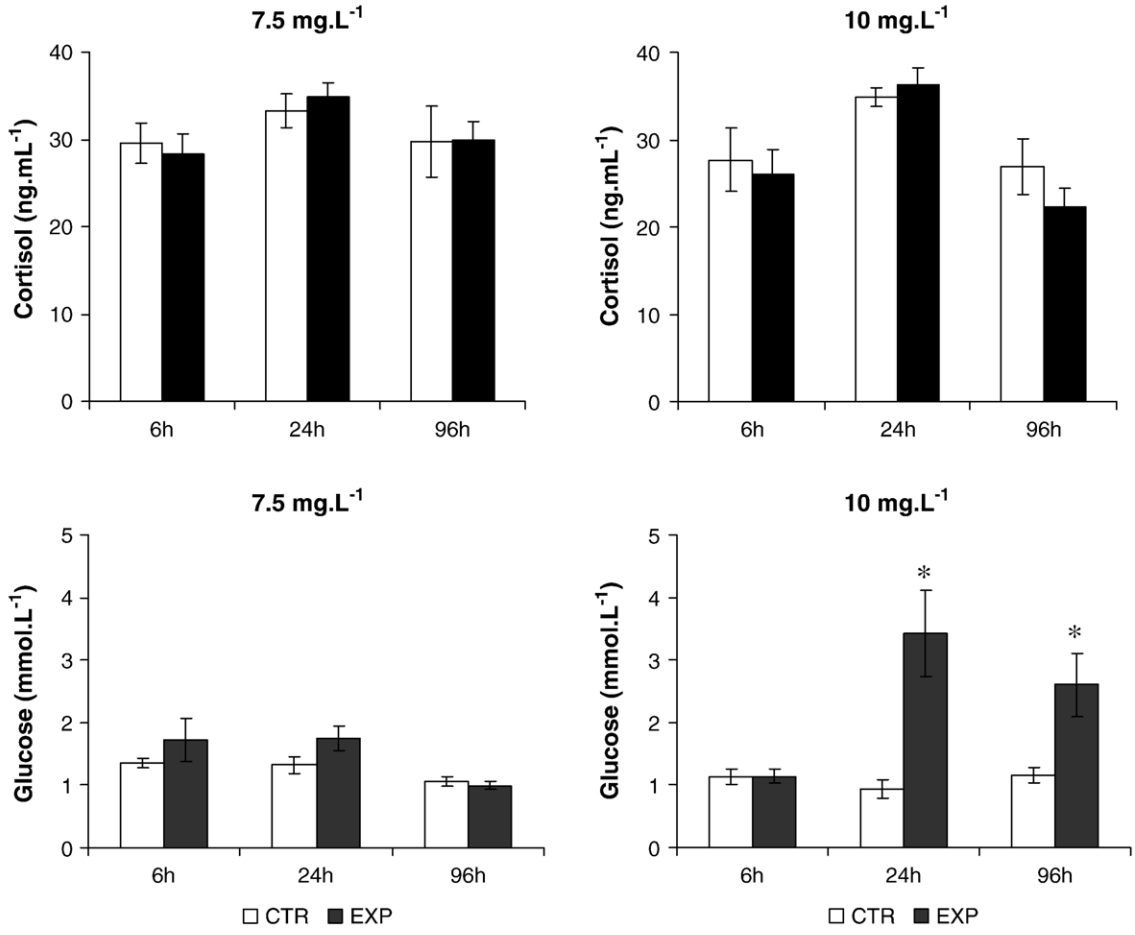

Fig. 2. Plasma cortisol and glucose concentrations of *Prochilodus lineatus* exposed to 7.5 or 10 mg L^{-1} of Roundup (EXP) or only water (CTR) for different experimental periods (6, 24 and 96 h). Bars represent means and vertical lines the SE (number of animals: 4–9). *Different from respective control ($P<0.05$).

(6, 24 and 96 h), were analyzed by Student's *t*-test. DTC values were log-transformed before statistical analysis, although non-transformed data are shown in Fig. 6. Differences between control groups and experimental groups, within the same Roundup concentration, were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $P \leq 0.05$ was taken as significant.

3. Results

3.1. LC_{50}

Roundup LC_{50} values for *P. lineatus* and their respective confident limits estimated for each exposure period are shown in Fig. 1. Roundup concentrations tested on the sub-lethal

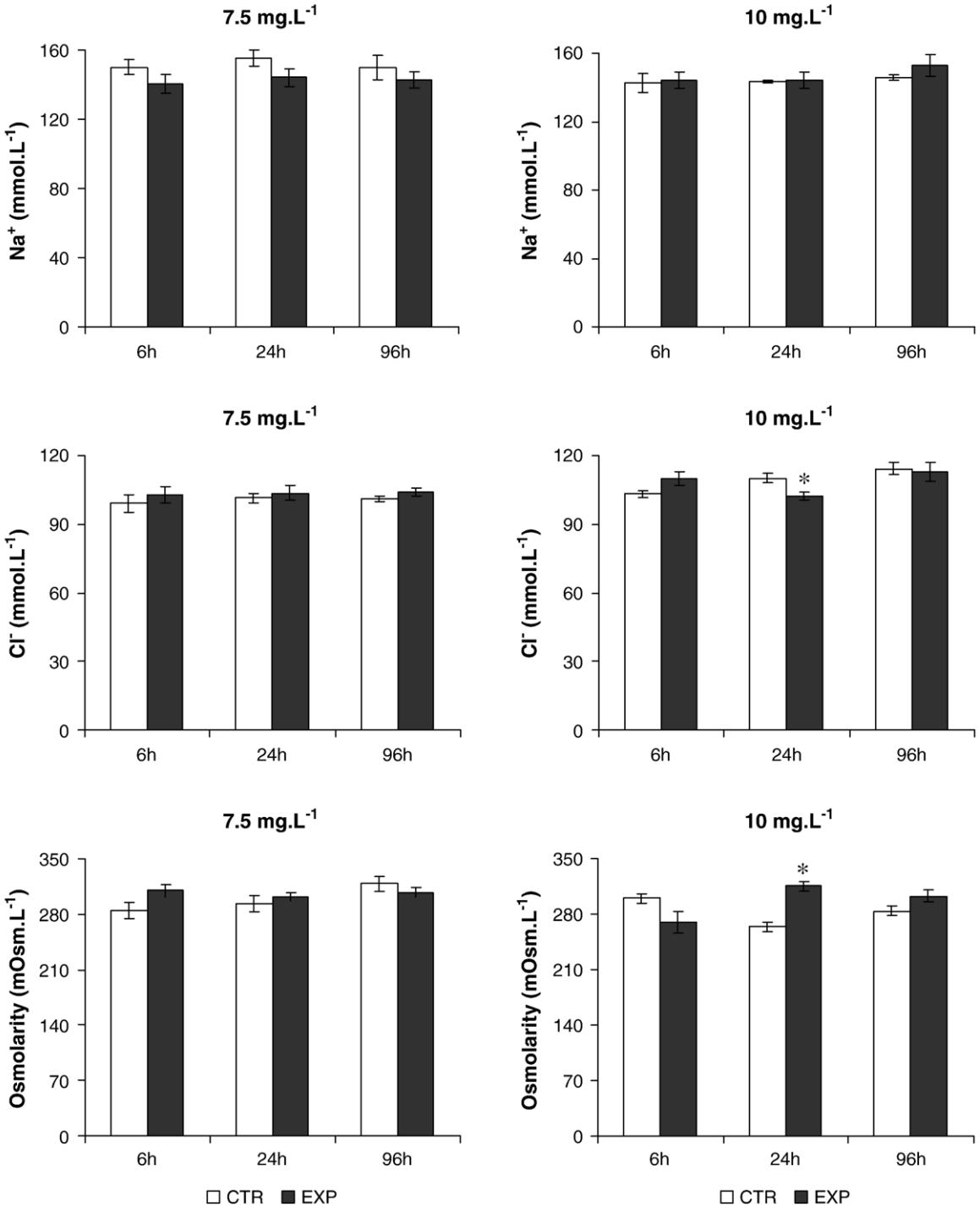

Fig. 3. Plasma concentrations of sodium and chloride and plasma osmolarity of *Prochilodus lineatus* exposed to 7.5 or 10 mg L^{-1} of Roundup (EXP) or only water (CTR) for different experimental periods (6, 24 and 96 h). Bars represent means and vertical lines the SE (number of animals: 6–12). *Different from respective control ($P<0.05$).

experiments were 7.5 and 10 mg L^{-1}, which correspond, respectively, to 25 and 55% of the 96 h-LC_{50}. All these values represent nominal concentrations of the formulated product.

3.2. Plasma cortisol and glucose

Plasma glucose of fish exposed to 10 mg L^{-1} of Roundup for 24 and 96 h was significantly higher than in the respective control. Animals submitted to the lower Roundup concentration did not show any variation in this parameter. Significant alterations in plasma cortisol were not observed between fish exposed to either herbicide concentrations, in any period of exposure, in relation to the respective controls (Fig. 2).

3.3. Osmo-ionic parameters

Fish exposed to 10 mg L^{-1} of Roundup during 24 h showed transitory alterations in plasma chloride concentrations and osmolarity, in comparison to the respective control groups. Significant variations in plasma sodium concentrations were not observed (Fig. 3).

3.4. GST and catalase activity

GST activity in liver did not vary between fish exposed to both herbicide concentrations, in any period of exposure, in relation to the respective controls. Hepatic catalase activity increased significantly in fish exposed to 10 mg L^{-1} of Roundup, during 24 h, in relation to respective control (Fig. 4).

3.5. Histopathological analysis

Fish exposed to both Roundup concentrations showed several pathological changes in the liver. The most frequent alterations were: cytoplasmic and nuclear degeneration (Fig. 5-D and -E); bile stagnation, which was identified as brownish-yellow granules in the cytoplasm (Fig. 5B); hyperaemia, that is, increased blood-flow in the liver (Fig. 5C); vacuoles in the

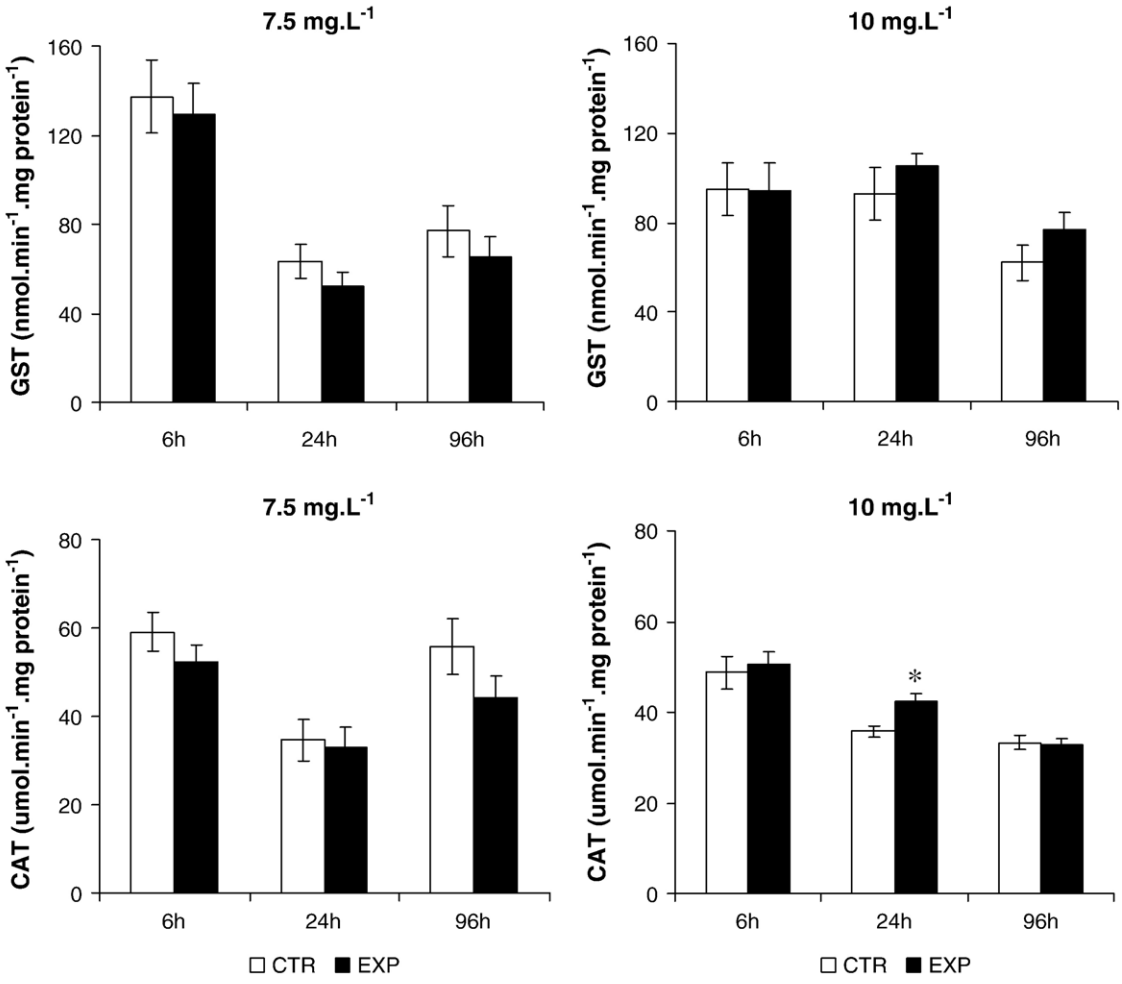

Fig. 4. Hepatic activity of glutathione-*S*-transferase (GST) and catalase of *Prochilodus lineatus* exposed to 7.5 or 10 mg L^{-1} of Roundup (EXP) or only water (CTR) for different experimental periods (6, 24 and 96 h). Bars represent means and vertical lines the SE (number of animals: 5–16). *Different from respective control ($P<0.05$).

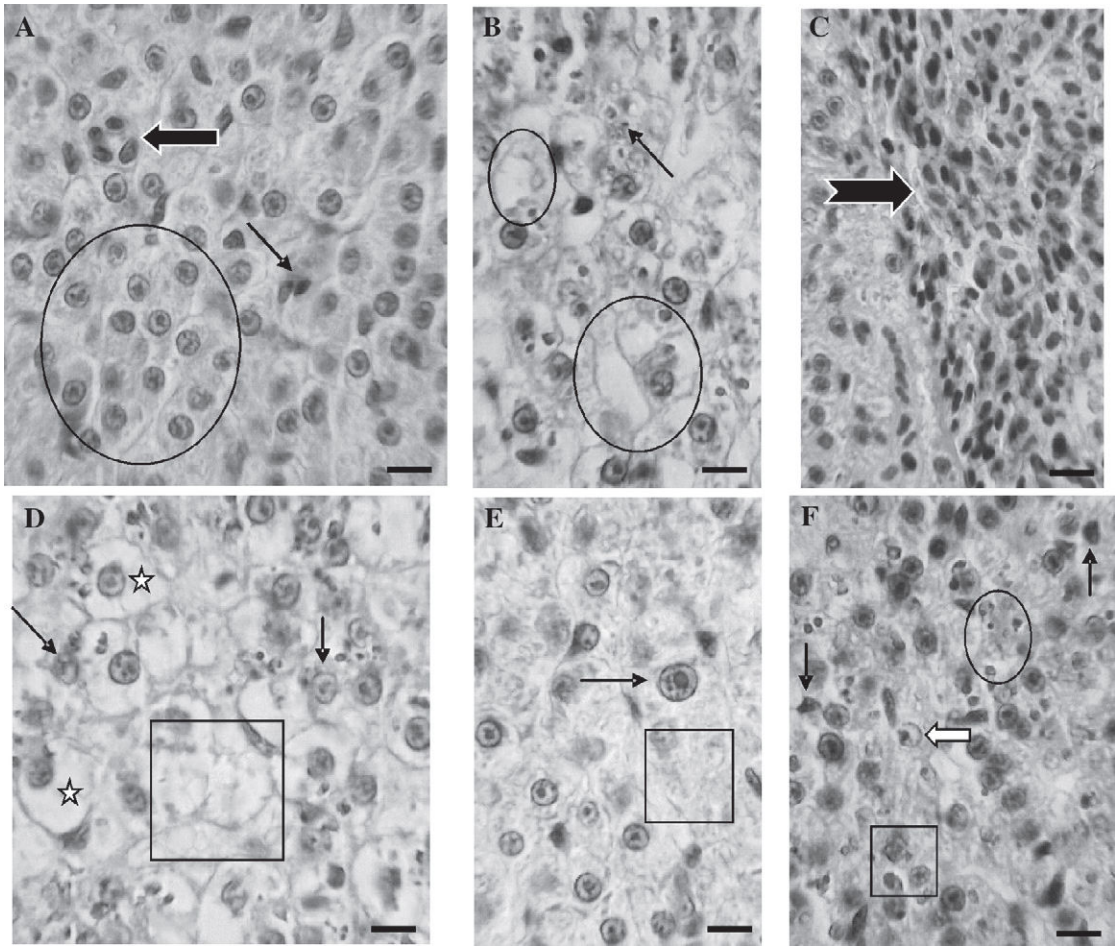

Fig. 5. Photomicrograph of the hepatic tissue of *Prochilodus lineatus* exposed to Roundup. (A) Normal hepatic tissue from control fish, showing hepatocytes (circle), sinusoid (arrow) and bile canaliculum (large arrow). (B) Bile stagnation (arrow) and vacuoles in the cytoplasm (circles) in fish exposed to 10 mg L^{-1} of Roundup for 24 h. (C) Hyperemia (large arrow) in fish exposed to 7.5 mg L^{-1} of Roundup for 24 h. (D) Cytoplasmic degeneration (square), nuclear degeneration (arrows), cellular hypertrophy (white stars) in fish exposed to 10 mg L^{-1} of Roundup for 24 h. (E) Nuclear hypertrophy (arrow) and cytoplasmic degeneration (square) in fish exposed to 7.5 mg L^{-1} of Roundup for 24 h. (F) Vacuoles in the nucleus (white arrow), bile stagnation (circle), pyknotic nuclei (arrows) and nuclear degeneration (square) in fish exposed to 7.5 mg L^{-1} of Roundup for 96 h. Scale bar corresponds to 100 μm.

cytoplasm (Fig. 5-B) and nucleus (Fig. 5-F). The frequency of occurrence of hepatic changes is presented in Table 1. DTC values determined for the liver of *P. lineatus* after 24 h of exposure to 7.5 mg L^{-1} of Roundup, and in all exposure periods to 10 mg L^{-1} of Roundup were significantly greater than respective controls (Fig. 6).

4. Discussion

Acute toxicity tests with glyphosate and Roundup have been done with different fish species, at different life stages and under different environmental conditions (Neskovic et al., 1996). In the present work, acute toxicity tests with the herbicide Roundup were developed with juveniles of the fish species *P. lineatus*, to determine LC_{50}. The LC_{50} of Roundup obtained for *P. lineatus* was 20.84 mg L^{-1} at 6 h, 17.32 mg L^{-1} at 24 h and 13.69 mg L^{-1} at 96 h (Fig. 1). These LC_{50} values were close to those found for *O. niloticus* by Jiraungkoorskul et al. (2002): 24 h-LC_{50} of 17.5 mg L^{-1} and 96 h-LC_{50} of 16.8 mg L^{-1}; although *P. lineatus* showed slightly greater sensitivity as exposure time increased (96 h). Servizi et al. (1987) found 96 h-LC_{50} values of Roundup for rainbow trout (*Oncorhynchus mykiss*) of 28 mg L^{-1} and 42 mg L^{-1} for Atlantic salmon (*Salmo salar*), which indicates a greater sensitivity for *P. lineatus* in relation to these coldwater species. It should be highlighted that safe concentrations of Roundup for temperate climate species, like salmon and rainbow trout, could be lethal for a Neotropical species.

Comparing the toxicity of Roundup with that of Trifluralin, another synthetic herbicide amply used in southern Brazil, it can be stated that Roundup is less toxic considering the 24 h-LC_{50} of Trifluralin for *P. lineatus* as 0.25 mg L^{-1} (Martinez and Cólus, 2002), much lower than the 24 h-LC50 for Roundup.

Roundup concentrations tested on the sub-lethal experiments (7.5 and 10 mg L^{-1}) might be considered environmentally realistic considering that at current application rates, a water body with no intercepting vegetation can have a maximum concentration of 3.7 mg glyphosate L^{-1} (Giesy et al., 2000), which corresponds to 9 mg of Roundup L^{-1}. The half-lives of glyphosate and POEA are 7 to 70 days and 21 to 28 days,

Table 1
Histological alterations found in the liver of *P. lineatus* following acute exposures (6, 24 or 96 h) to 7.5 or 10 mg L^{-1} of Roundup (EXP) or only to water (CTR), their respective stages of damage to the tissue and frequency of occurrence

Alterations	Stage	7.5 mg L^{-1}						10 mg L^{-1}					
		6 h		24 h		96 h		6 h		24 h		96 h	
		CTR	EXP	CTR	EXP	CTR	EXP	CTR	EXP	CTR	EXP	CTR	EXP
Melanomacrophage aggregates	I	+	+	++	++	+	+	0	+	++	+++	++	++
Cellular hypertrophy	I	+	++	0	++	+	++	0	+	++	++	+	+++
Nuclear hypertrophy	I	0	+++	0	+++	0	+++	0	++	0	+++	+	++++
Irregular shaped cells	I	0	+	+	+	0	+	0	+	++	+++	+	++
Irregular shaped nucleus	I	0	+++	0	+	0	++	0	+	+	++	0	++
Nuclei in the periphery	I	0	+	0	+	0	+	0	+	+	++	+	++
Vacuoles in the cytoplasm	I	+	+	+	++	0	+	0	+	+++	++++	++	+++
Vacuoles in the nucleus	II	0	++++	0	++++	+	+++	+	++++	0	++++	0	++++
Cytoplasmic degeneration	II	++	++++	+++	++++	+++	++++	+++	++++	++	++++	+++	++++
Nuclear degeneration	II	++	++++	++	++++	++	++++	++	++++	+++	++++	+	++++
Bile stagnation	II	+++	++++	+++	++++	+++	++++	+++	++++	+++	++++	+++	++++
Hyperemia	II	0	0	0	+++	0	+++	0	0	0	0	0	0
Pyknotic nuclei	II	0	+++	0	++++	+	++++	0	++++	0	++++	0	++++

Note: 0 = absent; + = fairly frequent; ++ = frequent; +++ = very frequent; ++++ = extremely frequent.

respectively, depending on site conditions (Giesy et al., 2000). Therefore, toxicity studies at relatively high glyphosate concentrations are environmentally relevant, particularly when fish are acutely exposed immediately after Roundup application (Çavas and Könen, 2007).

Cortisol is the major corticosteroid hormone in fish and toxicants may have a significant effect on its dynamics (Mommsen et al., 1999). In the present work plasma cortisol values found for *P. lineatus* did not differ between control and Roundup-exposed group, varying from 22 to 36 ng mL^{-1} (Fig. 2). These values are quite low in comparison to the mean plasma cortisol concentration, 165 ng mL^{-1}, determined for *P. lineatus* submitted to confinement stress (Camargo and Martinez, 2006). The absence of a significant elevation in plasma cortisol might indicate a lack of cortisol response due to Roundup exposure or that the increase in cortisol had occurred before the first blood sampling, which was only 6 h after of the onset of the pollutant exposure. Most fish species tested show their highest plasma increase in cortisol within about 0.5–1 h after a stressful disturbance (Barton, 2002).

When fish were exposed to 10 mg L^{-1} of Roundup, for 24 and 96 h, a hyperglycemic response was observed (Fig. 2). This increase in plasma glucose is a very common response in fish under stress conditions and assists the animal by providing energy substrates to tissues such as brain, gills and muscles, in order to cope with the increased energy demand (Barton, 2002). The hyperglycemic response in many teleost species is mainly mediated by adrenaline and cortisol, and can occur by glycogenolysis and gluconeogenesis (Wendelaar Bonga, 1997). Although adrenaline is cleared rapidly from circulation after a stress (<30 min), plasma glucose remains elevated for longer periods suggesting that cortisol plays a role in the long-

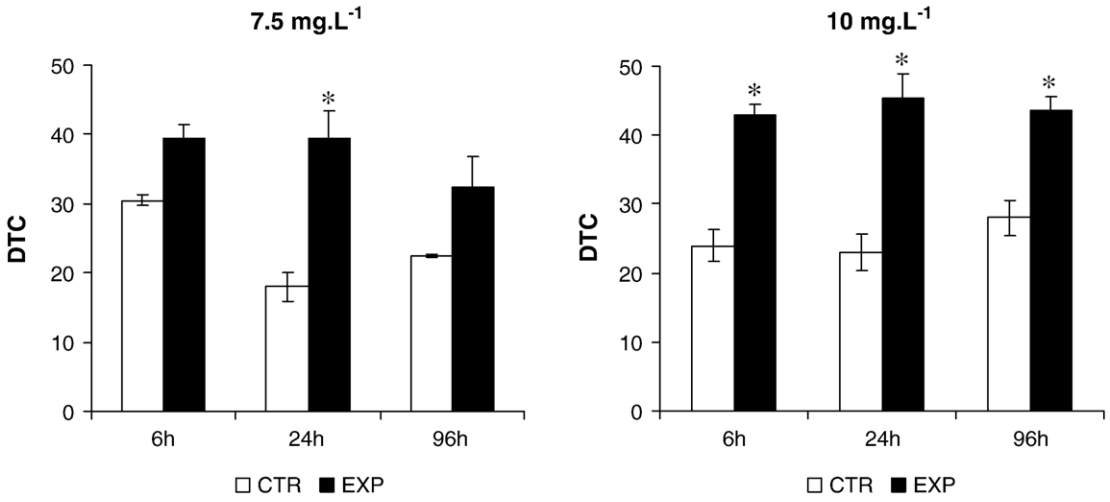

Fig. 6. Degree of tissue change (DTC) calculated for the hepatic tissue of *Prochilodus lineatus* exposed to 7.5 or 10 mg L^{-1} of Roundup (EXP) or only water (CTR) for different experimental periods (6, 24 and 96 h). Bars represent means and vertical lines the SE (number of animals: 6–8). *Different from respective control ($P<0.05$). DTC values were log-transformed before statistical analysis (ANOVA and Turkey's test), although non-transformed data are shown in the figure.

term maintenance of glucose levels (Barton, 2002). In the present study it was not possible to correlate the hyperglycemic response with cortisol circulating levels. However, plasma concentration of cortisol is determined to a large extend by the production and plasma clearance of the hormone, i.e. a sum of dynamic processes, all of which can regulate the physiological response to cortisol and cortisol-induced changes in tissue metabolism occur even in the absence of increased plasma cortisol levels (Mommsen et al., 1999).

In freshwater fish, the osmotic water influx and diffusive losses of ions, such as Na^+ and Cl^- are compensated by the excretion of large volumes of dilute urine and the active uptake to replace lost ions (Evans et al., 1999). This hydromineral balance can be affected by pollutants and concentrations of individual ions and total osmolarity in blood plasma are physiological variables that have been used as indicators of the effects of pollution on fish (Heath, 1995). In this study only minor transitory alterations were observed in plasma chloride and osmolarity of fish after 24 h exposure to 10 mg L^{-1} of Roundup (Fig. 3). The increased plasma osmolarity might be a consequence of the large increase (265%) in plasma glucose observed in those fish. These results indicated that the concentrations of Roundup used in this work did not interfere with the maintenance of the ionic balance of *P. lineatus*.

The liver is an organ performing various functions associated with the metabolism of xenobiotics in fish (Jimenez and Stegeman, 1990). Glutathione-*S*-transferases (GST) are a group of enzymes that catalyze the conjugation of reduced glutathione (GSH) with a variety of electrophilic metabolites, and are involved in the detoxification of both reactive intermediates and oxygen radicals (van Der Oost et al., 2003). It has been demonstrated that the activity of these enzymes may be enhanced in the liver of fish exposed to a variety of pollutants and even low level organic contamination can lead to increased hepatic GST activity in fish (Machala et al., 1997). However, in the present work *P. lineatus* exposed to the herbicide Roundup showed no variation in GST liver activity (Fig. 4), which might indicate that the metabolism of the compounds present in Roundup occurs by other biotransformation pathways.

Hepatocytes like any other cells are dependent on antioxidant enzymes for protection against reactive oxygen species (ROS), produced during the biotransformation of xenobiotics (Landis and Yu, 1995). Among the enzymes that comprise this defense system is catalase, responsible for the removal of hydrogen peroxide (H_2O_2) which is metabolized to O_2 and water (van Der Oost et al., 2003). The hepatic activity of catalase in fish exposed to Roundup showed a significant increase in the group exposed to 10 mg L^{-1} for 24 h, in relation to is respective control (Fig. 4), which may be due to Roundup-mediated oxyradical production. This is corroborated by Pieniazek et al. (2004) who also verified an increase in catalase activity in human erythrocytes incubated in a solution containing 1500 mg L^{-1} of Roundup for 1 h. After 96 h of exposure to 10 mg L^{-1} of Roundup catalase liver activity returned to basal levels, apparently indicating that ROS production was already compensated. Catalase activity in the liver of other Neotropical fish species, *R. quelen*, also did not change after 96 h exposure to Roundup (Glusczak et al., 2007). However, even without catalase induction in the liver of *P. lineatus* after 96 h of Roundup exposure, the hypothesis of oxidative stress in the liver tissue should not be dismissed.

The liver of fish can be considered a target organ to pollutants since it participates in biotransformation and excretion of xenobiotics (Thophon et al., 2003). Alterations in its structure can be significant in the evaluation of fish health (Myers et al., 1998) and reflect the effects of a variety of environmental pollutants (Hinton et al., 1992). In the present research, fish exposed to Roundup showed several liver histological alterations (Fig. 5). Quantitative analysis of these hepatic changes showed that the liver of *P. lineatus* was affected after exposure to Roundup, with mean DTC values of 44 at 7.5 mg L^{-1} and 48 at 10 mg L^{-1}, indicating the occurrence of alterations that might impair normal organ function, although these lesions were reversible upon removal of the toxic agent from the water.

The histological alterations most frequently observed were cellular and nuclear degeneration, increase in nuclear volume, cytoplasmatic and nuclear vacuolization, the presence of pyknotic nuclei and bile stagnation (Table 1). These alterations appear to be commonly developed in fish exposed to Roundup considering that Jiraungkoorskul et al. (2002), in a study using *O. niloticus* exposed to 36 mg L^{-1} of Roundup, also observed the occurrence of the same hepatic lesions.

Increased cellular and nuclear volume of hepatocytes (stage I alterations) can be considered as responses to the stressor agent, since they indicate the activation of the liver functions and do not interfere with the hepatic performance, but rather indicate the intensification of hepatocytes metabolic activity under adverse conditions (Takashima and Hibyia, 1995). These alterations were frequently found in fish exposed to both Roundup concentrations, at all experimental times. In contrast, cytoplasmic and nuclear degeneration represent more serious lesions (stage II alterations) which, although reversible, can impede the functions performed by the liver, since the metabolically active tissue area is diminished. These alterations can correspond to direct effects caused by exposure to the xenobiotic and were found in all the fish exposed to Roundup and to a lesser degree in all the control fish (Table 1), probably as a normal process of cellular renovation in the tissue. Szarek et al. (2000) studied the hepatic ultrastructure in *C. carpio* exposed to Roundup and also verified the occurrence of different sized vacuoles in hepatocytes, as well as mitochondrial degeneration and an increase in the Golgi apparatus, showing that Roundup in water results in lesions to hepatocytes.

Nuclear vacuolization and pyknotic nuclei were also found in fish exposed to both concentrations of Roundup at all experimental times (Table 1). The vacuolization of the nucleus occurs in the liver of many aquatic vertebrates and invertebrates in the presence of pollutants (Oliveira Ribeiro et al., 2002) and can lead to the subsequent degeneration of the nucleus, indicated by the presence of pyknotic nucleus.

Bile stagnation was observed indistinguishably in the liver of control fish as well as of fish exposed to Roundup (Table 1). This alteration consists of the manifestation of a physiopathological condition caused by a lack of bile metabolism and excretion (Pacheco and Santos, 2002), in which bile secreted by hepatocytes remains inside cells and is not released into the digestive tract (Simonato et al., in press). In the present study, the occurrence of

this alteration was not associated with the presence of a xenobiotic, since it was also observed in the control group, and may reflect some nutritional problem resulting from the feeding of the fish in captivity.

In conclusion, the results of this work showed that Roundup could be less toxic to *P. lineatus* than other herbicides widely used in Brazil; however, this Neotropical fish species is more sensitive to this glyphosate-based herbicide than fish from temperate climates and these differences should be considered when establishing criteria for water quality and animal well-being in the Neotropical region. Exposure to sub-lethal concentrations of Roundup promoted an increase in plasma glucose, indicating a typical response to stress. The induction of liver catalase activity indicates the activation of antioxidant defenses, probably due to increased hydrogen peroxide generation. Roundup exposure also induced a variety of liver histological alterations that might impair normal organ functioning. At present it is clear that more studies related to stress response and oxidative stress in *P. lineatus* exposed to Roundup, are necessary to understand the mechanisms of toxicity of this herbicide.

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References

Amarante Jr., O.P., Santos, T.C.R., Brito, N.M., Ribeiro, M.L., 2002. Glifosato: Propriedades, toxicidade, uso e legislação. Quím. Nova 25, 589–593.

Barton, B.A., 2002. Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. Integ. Comp. Biol. 42, 517–525.

Beutler, E., 1975. Red Cell Metabolism: A Manual of Biochemical Methods. Grune and Straton, New York.

Camargo, M.P., Martinez, C.B.R., 2006. Biochemical and physiological biomarkers in *Prochilodus lineatus* submitted to in situ tests in an urban stream in southern Brazil. Environ. Toxicol. Pharmacol. 21, 61–69.

Çavas, T., Könen, S., 2007. Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and the comet assay. Mutagenesis 22, 263–268.

da Silva, M.D., Peralba, M.D.R., Mattos, M.L.T., 2003. Determinação de glifosato e ácido aminometilfosfônico em águas superficiais do arroio passo do pilão. Pesticidas: R. Ecotoxicol. Meio Ambient. 13, 19–28.

Evans, D.H., Piermarini, P.M., Potts, W.T.W., 1999. Ionic transport in the fish gill epithelium. J. Exp. Zool. 283, 641–652.

Giesy, J.P., Dobson, S., Solomon, K.R., 2000. Ecotoxicological risk assessment for Roundup herbicide. Rev. Environ. Contam. Toxicol. 167, 35–120.

Glusczak, L., Miron, D.S., Crestani, M., Fonseca, M.B., Pedron, F.A., Duarte, M.F., Vieira, V.L.P., 2006. Effect of glyphosate herbicide on acetylcholinesterase activity and metabolic and hematological parameters in piava (*Leporinus obtusidens*). Ecotoxicol. Environ. Saf. 65, 237–241.

Glusczak, L., Miron, D.S., Moraes, B.S., Simões, R.R., Schetinger, M.R.C., Morsch, V.M., Loro, V.L., 2007. Acute effects of glyphosate herbicide on metabolic and enzymatic parameters of silver catfish (*Rhamdia quelen*). Comp. Biochem. Physiol. C. 146, 519–524.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione-*S*-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Hamilton, M.A., Russo, R.C., Thurston, R.V., 1977. Trimmed Spearman–Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11, 714–719.

Heath, A.G., 1995. Water Pollution and Fish Physiology, 2nd ed. CRC Press, Florida.

Hinton, D.E., Baumann, P.C., Gardner, G.R., Hawkins, W.E., Hendricks, J.D., Murchelano, R.A., Okihiro, M.S., 1992. Histopathologic biomarkers. In: Huggett, R.J., Kimerle, R.A., Mehrle, J.R., Bergman, H.L. (Eds.), Biomarkers: Biochemical, Physiological and Histological Markers of Anthropogenic Stress. Lewis Publishers, Boca Raton, pp. 155–209.

Inoue, M.H., Oliveira Jr., R.S., Regitano, J.B., Tormena, C.A., Tornisielo, V.L., Constantin, J., 2003. Critérios para avaliação do potencial de lixiviação dos herbicidas comercializados no Estado do Paraná. Planta Daninha 21, 313–323.

Jimenez, B.D., Stegeman, J.J., 1990. Detoxification enzymes as indicators of environmental stress on fish. In: Adams, S.M. (Ed.), Biological Indicators of Stress in Fish. . American Fisheries Symposium, vol. 8. AFS, Bathesda, pp. 67–79.

Jiraungkoorskul, W., Upatham, E.S., Kruatrachue, M., Sahaphong, S., Vichasri-Grams, S., Pokethitiyook, P., 2002. Histopathological effects of Roundup, a glyphosate herbicide, on Nile tilapia (*Oreochromis niloticus*). Sci. Asia 28, 121–127.

Jiraungkoorskul, W., Upatham, E.S., Kruatrachue, M., Sahaphong, S., Vichasri-Grams, S., Pokethitiyook, P., 2003. Biochemical and histopathological effects of glyphosate herbicide on Nile tilapia (*Oreochromis niloticus*). Environ. Toxicol. 18, 260–267.

Landis, W.G., Yu, M.H., 1995. Introduction to Environmental Toxicology: Impacts of Chemicals upon Ecological System. Lewis publishers, Boca Raton.

Lowry, O.H., Rosenbrough, N.J., Faar, A.L., Randall, R.J., 1951. Protein measurements with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Machala, M., Petřivalský, M., Nezveda, K., Ulrico, R., Dušek, L., Piačka, V., Svobodová, Z., 1997. Responses of carp hepatopancreatic 7-ethoxyresorufin-*O*-deethylase and glutathione-dependent enzymes to organic pollutants — a field study. Environ. Toxicol. Chem. 16, 1410–1416.

Marc, J., Mulner-Lorillon, O., Bellé, R., 2004. Glyphosate-based pesticides affect cell cycle regulation. Biol. Cell 96, 245–249.

Martinez, C.B.R., Cólus, I.M.S., 2002. Biomarcadores em peixes neotropicais para o monitoramento da poluição aquática na bacia do rio Tibagi. In: Medri, M.E., Bianchini, E., Shibatta, O.A., Pimenta, J.A. (Eds.), A Bacia do Rio Tibagi, Londrina, pp. 551–577.

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fish. 9, 211–268.

Myers, M.S., Johnson, L.L., Olson, O.P., Sther, C.M., Horness, B.H., Collier, T.K., McCain, B.B., 1998. Toxicopathic hepatic lesions as biomarkers of chemical contaminant exposure and effects in marine bottomfish species from the northeastand Pacific Coast, USA. Mar. Pollut. Bull. 37, 92–113.

Neskovic, N.K., Poleksic, V., Elezovic, I., Karan, V., Budimir, M., 1996. Biochemical and histopathological effects of glyphosate on carp (*Cyprinus carpio*). Bull. Environ. Contam. Toxicol. 56, 295–302.

Oliveira Ribeiro, C.A., Schatzmann, M., Silva de Assis, H.C., Silva, P.H., Pelletier, E., Akaishi, F.M., 2002. Evaluation of tributyltin subchronic effects in tropical freshwater fish (*Astyanax bimaculatus*, Linnaeus, 1758). Ecotoxicol. Environ. Saf. 51, 161–167.

Pacheco, M., Santos, M.A., 2002. Biotransformation, genotoxic and histopathological effects of environmental contaminants in European eel (*Anguilla anguilla* L.). Ecotoxicol. Environ. Saf. 53, 331–347.

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

Pieniazek, D., Bukowska, B., Duda, W., 2004. Comparison of the effect of Roundup® Ultra 360 SL pesticide and its active compound glyphosate on human erythrocytes. Pestic. Biochem. Physiol. 79, 58–63.

Poleksić, V., Mitrović-Tutundžić, V., 1994. Fish gills as a monitor of sublethal and chronic effects of pollution. In: Müller, R., Lloyd, R. (Eds.), Sublethal

and Chronic Effects of Pollutants on Freshwater Fish. Fishing News Books, Oxford, pp. 339–352.

Releya, R.A., 2005. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecol. Appl. 15, 618–627.

Servizi, J.A., Gordon, R.W., Martens, D.W., 1987. Acute toxicity of Garlon 4 and Roundup herbicides to salmon and trout. Bull. Environ. Contam. Toxicol. 39, 15–22.

Simonato, J.D., Guedes, C.L.B., Martinez, C.B.R., in press. Biochemical, physiological, and histological changes in the Neotropical fish *Prochilodus lineatus* exposed to diesel oil. Ecotoxicol. Environ. Saf. doi: 10.1016/j.ecoenv.2007.01.012.

Szarek, J., Siwick, A., Andrzejewska, A., Terech-Majewska, E., Banaszkiewics, T., 2000. Effects of the herbicide RoundupTM on thse ultrastructural pattern of hepatocytes in carp (*Cyprinus carpio*). Mar. Environ. Res. 50, 236–266.

Takashima, F., Hibyia, T., 1995. An Atlas of Fish Histology Normal and Pathological Features, 2nd ed. Gustav Fisher Verlag, Kodansha.

Thophon, S., Kruatrachue, M., Upatham, E.S., Pokethitiyook, P., Sahaphong, S., Jaritkhuan, S., 2003. Histopathological alterations of white seabass, *Lates calcarifer*, in acute and subchronic cadmiun exposure. Environ. Pollut. 121, 307–320.

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

Tsui, M.T.K., Chu, L.M., 2004. Comparative toxicity of glyphosate-based herbicides: aqueous and sediment porewater exposures. Arch. Environ. Contam. Toxicol. 46, 316–323.

van Der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.

Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591–625.

WHO, World Health Organization, 1994. Glyphosate: Environ. Health Criteria 159. Genève.

Williams, G.M., Kroes, R., Munrot, I.C., 2000. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Regul. Toxicol. Pharmacol. 31, 117–165.