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Roundup® exposure promotes gills and liver impairments, DNA damage and inhibition of brain cholinergic activity in the Amazon teleost fish *Colossoma macropomum*

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HIGHLIGHTS

- RD caused histopathology, increased hematological indexes and reduction of GST.
- RD increased hepatic levels of EROD and antioxidant defenses (GPx).
- RD caused DNA damage and inhibition of AChE activity.
- Alterations were dose-dependent.

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ABSTRACT

Roundup Original® (RD) is a glyphosate-based herbicide used to control weeds in agriculture. Contamination of Amazon waters has increased as a consequence of anthropogenic pressure, including the use of herbicides as RD. The central goal of this study was to evaluate the toxic effects of RD on juveniles of tambaqui (*Colossoma macropomum*). Our findings show that biomarkers in tambaqui are organ specific and dependent on RD concentration. Alterations in gills structural and respiratory epithelium were followed by changes in hematological parameters such as concentration of hemoglobin, particularly in fish exposed to the higher concentration tested (75% of RD LC_{50} 96 h). In addition, both RD concentrations affected the biotransformation process in gills of tambaqui negatively. Instead, liver responses suggest that a production of reactive oxygen species (ROS) occurred in fish exposed to RD, particularly in the animals exposed to 75% RD, as seen by imbalances in biotransformation and antioxidant systems. The increased DNA damage observed in red blood cells of tambaqui exposed to RD is in agreement with this hypothesis. Finally, both tested sub-lethal concentrations of RD markedly inhibited the cholinesterase activity in fish brain. Thus, we can suggest that RD is potentially toxic to tambaqui and possibly to other tropical fish species.

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1. Introduction

Roundup Original® (RD) is a non-selective glyphosate-based herbicide considered one of the most significant ever developed to control weeds in agriculture (Jiraungkoorskul et al., 2002). RD is used in several crops over the world, including Brazil, where it has been widely used in crops like rice, coffee, sugar cane, corn, soy, and wheat (Brazilian Ministry of Fisheries and Agriculture). Moreover, RD is also used to control weeds in Amazonian fish farms (Araújo et al., 2008).

Although RD is catalogued as a low toxic herbicide by Brazilian Health Surveillance Agency (ANVISA; IV degree), recent papers showed that it increases the toxicological risks to aquatic biota (Souza Filho et al., 2013; Sinhorin et al., 2014). According to Rodrigues and Almeida (2005), the high solubility of glyphosate (10,000–15,700 mg L^{-1} at 25 °C) facilitates its uptake by aquatic organisms. Furthermore, RD contains the surfactant POEA (polyoxyetilenoamina), which increases cell membrane permeability and thus increases the absorption of glyphosate salt by aquatic animals (Giesy et al., 2000).

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Biochemical, physiological, morphological, neurological, and genotoxic effects of RD have been previously reported for many teleost species (Neskovic et al., 1996; Jiraungkoorskul et al., 2002; Lushchak et al., 2009). Xenobiotic metabolism generates and accumulates reactive oxygen species (ROS) in fish tissues acutely exposed to RD, which in turn promotes the oxidation of lipids and proteins, and also results in oxidative damages in DNA. The biotransformation enzymes Ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferase (GST) are important to avoid ROS damages in the cells. EROD is responsible for introducing a functional group –OH in xenobiotic molecules, turning them more hydrophilic and thereby more easily excreted, while GST catalyzes the conjugation of reduced glutathione (GSH) with proper electrophilic substrates (Haluzová et al., 2011). The superoxide dismutase (SOD) is also one of the first enzymes of antioxidant defense system promoting the dismutation of O_2^- in hydrogen peroxide (H_2O_2), which is used as substrate by glutathione peroxidase (GPx) (Van der Oost et al., 2003). Genotoxic and neurotoxic effects in fish have been related to failures in these xenobiotic mechanisms (Singh et al. 1988). The enzyme Acetylcholinesterase (AChE) is essential in the control of many nervous functions in animals and usually shows strong inhibition by organophosphorated compounds, favouring the breakdown of acetylcholine followed by increased levels of AChE in synapses (Bretaud et al., 2000). In addition, genotoxic damage in fish erythrocytes has been extensively used as an important biomarker in toxicological studies because it detects single- and double-strand DNA breakdowns in animals acutely exposed to xenobiotics (Singh et al., 1988).

Recent studies have addressed the effects of RD exposure on Neotropical fish species. Studying *Piaractus mesopotamicus*, Shiogiri et al. (2012) showed no evident damage of gill morphology in animals exposed for 48 h to sub-lethal concentrations of RD, but did observed alterations in nuclear and cellular membranes, cytoplasmic vacuolization, lipid accumulation, and glycogen depression, suggesting a strong damage in liver of exposed animals. Short-term exposure to sub-lethal concentrations of RD promoted impairment of antioxidant and biochemical biotransformation system in *Prochilodus lineatus*, associated to lipid peroxidation of cell membrane in gills and liver, as well as genotoxic effects in both erythrocyte and gill cells (Cavalcante et al., 2008), and inhibition of brain acetilcholineterase (Modesto and Martinez, 2010). Altogether these data show that RD exposure may be potentially hazardous to Neotropical fish fauna, even at sub-lethal levels in aquatic environments.

The Amazon fish *Colossoma macropomum*, locally known as tambaqui, is economically important and is the most farmed native fish species in Brazilian Amazon (Santos et al., 2013). *C. macropomum* is also farmed in many tropical countries. Currently, the toxicological risk of RD to both natural fish stocks and farmed fish in the Amazon is increasing, as RD has been widely used in several agriculture crops in the region and applied as a non-selective herbicide around aquaculture fish tanks to control aquatic weed plants (Araújo et al., 2008). Although biochemical and physiological effects of RD on Neotropical species have been reported (Langiano and Martinez, 2008; Glusczak et al., 2011), there is a lack of information regarding toxicological risk of glyphosate-based herbicides to components of Amazon aquatic biota.

Few previous studies addressed the toxic effects of herbicides on tambaqui (Assis et al., 2010; Bravo et al., 2005). However, information of biomarkers of tambaqui exposed to sub-lethal concentrations of RD remains poorly understood. Thus, the central aim of this work was to investigate the effects of short-term exposure to two sub-lethal concentrations of RD on gill morphology and function, hematological parameters, biotransformation enzymes and antioxidant system in gills and liver of juveniles of *C. macropomum*. Additionally, we analyzed the effects of sub-lethal concentrations of RD on both neurological and erythrocytic DNA damage in tambaqui.

2. Material and methods

2.1. Experimental fish

Juveniles of *C. macropomum* (34.16 g ± 1.84; 10.98 cm ± 0.21) were obtained from a local fish farm (Fazenda Santo Antônio, Amazonas, Brasil). The animals were kept for two months in the Laboratory of Ecophysiology and Molecular Evolution (LEEM/INPA) in INPA's well water (in μmol L^{-1}; Na^+, 43; Cl^-, 31; K^+, 10; Ca^{2+}, 9; Mg^{2+}, 4; pH 6.28; 6.40 mg O_2 L^{-1} and 29 °C) in a 3000 L polyethylene tank with flow-thru water (1200 mL min^{-1}). They were fed dry food pellets, 26% protein content (Nutripeixe, Purina) twice a day. Feeding was suspended two days prior to the experiments. Experimental and holding procedures followed INPA's animal care guidelines and were previously approved by INPA's animal care committee (protocol number: 030/2012).

2.2. Toxicity tests

After acclimation period, 18 fish were individually transferred to 18 aerated glass aquarium (2 L), one fish in each aquarium, and allowed to recover overnight before start acute toxicity tests (96 h). The toxicity of RD (i.e. 360 g of glyphosate L^{-1}) was evaluated using two sub-lethal concentrations (nominal concentrations of 10 mg L^{-1} and 15 mg L^{-1} of glyphosate), in addition to a control group (n = 6 to each treatment). RD concentrations were chosen to represent 50% and 75% of LC_{50} 96 h to *C. macropomum*, already determined by Miyazaki et al. (2004). To start toxicity tests, RD solution was previously diluted 7.2 times (solution with nominal concentration of 50 g L^{-1} of glyphosate) and then added to the test aquaria to reach the nominal concentrations of 10 mg L^{-1} and 15 mg L^{-1} glyphosate. After the exposure, all fish were individually removed from aquaria and immediately anesthetized with buffered anesthetic (1 g L^{-1} MS-222 and 2 g L^{-1} $NaHCO_3$, Sigma Aldrich). Blood was sampled with a heparinized syringe from the caudal vein. Animals were euthanized by medullar section, measured and weighted, and samples of gills, liver and brain were removed by dissection. The samples were frozen in liquid nitrogen and stored at −80 °C freezer prior to analyze.

2.3. Analytical methods

2.3.1. Histopathology analysis of gills

Gills arches were fixed in 2.5% glutaraldehyde for 24 h and dehydrated in ethanol (70%, 80% and 96%). Samples were embedded in LEICA historesin, and serial sections of 5 μm thickness were prepared on glass slides, which were stained with toluidine blue. Samples were analyzed at 100× magnification in an optical microscope. Histopathological alterations were evaluated semi-quantitatively using the method described by Poleksic and Mitrovic-Tutundzic (1994). Indexes based on severity of lesions were used to assess gills tissue changes: $I = 1\sum I + 10\sum II + 100\sum III$, where stages I, II and III correspond to the degree of lesion: normal function of the organ (I = 0–10), mild to moderate damage (I = 11–20), moderate to severe (I = 21–50), severe (I = 51–100), and irreparable damage (I > 100).

2.3.2. Blood analysis

Hemoglobin concentration ([Hb]) was determined by cyanmethemoglobin method (Kampen and Zijlstra, 1964) in a spectrophotometer at 540 nm. Total erythrocyte counts (RBC) were read on a Neubauer chamber (Labomed microscope) using blood

diluted with formaldehyde citrate. Blood was centrifuged in microcapillary tubes and then hematocrit (Ht) was read using an appropriate card. Blood glucose content ($g\,dL^{-1}$) was determined electrochemically using a blood glucose portable reader (Accu-Check Advantage II/Roche). The [Hb], RBC and Ht values were used to calculate corpuscular parameters: medium corpuscular volume (MCV), medium corpuscular hemoglobin concentration (MCHC) and medium corpuscular hemoglobin (MCH).

2.3.3. Biotransformation enzymes

CYP1A levels were determined through the analysis of liver EROD activity. Liver samples were thawed on ice, homogenized in HEPES pH 7.5, centrifuged at 12,000 g for 20 min at 4 °C and the S9 post mitochondrial supernatant (PMS) collected for immediate use. EROD activity was measured using a modified method of Hodson et al. (1992). Reaction buffer was prepared with 0.1 mM HEPES pH 7.8, 0.12 mM $MgSO_4$, 40 mg L^{-1} bovine serum albumin (BSA), 0.5 mM NADPH (b-nicotinamide adenine dinucleotide phosphate, reduced form), and 50 μL PMS. Addition of 0.02 mM ethoxyresorufin initiated the reaction at room temperature for 2 min and addition of HPLC grade methanol terminated it. Resorufin standards (0.000–0.085 mM) and samples were centrifuged to precipitate proteins. The fluorescence of the supernatant was read on a SpectraMax M2 Spectrometer at excitation/emission wavelengths of 535/585 nm (slit 10 ex/10 em). EROD activity was expressed as picomoles of resorufin per mg of total protein per minute (pmol R. mg $protein^{-1}$. min^{-1}).

GST activities in gill and liver samples were determined according to the method described by Keen et al. (1976) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Change in absorbance was recorded at 340 nm, and the enzyme activity was calculated as nmol CDNB conjugate formed per min per mg protein using a molar extinction coefficient of 9.6 mM cm^{-1}.

2.3.4. Antioxidants enzymes

Copper–zinc SOD (CuZn-SOD) activities were determined in gill and liver samples according to the method of McCord and Fridovich (1969). Inhibition of reduction rate of cytochrome c by the superoxide radical at 550 nm and 25 °C is the basis of this method. SOD activity is expressed in U SOD per mg of protein, assuming one U of SOD as the quantity of enzyme that promotes the inhibition of 50% of reduction rate of cytochrome c. Activities of Selenium-dependent GPx (Se-GPx) were determined as described by Hopkins and Tudhope (1973), based on NADPH oxidation in the presence of GSH (0.95 mM) and H_2O_2 at 340 nm. GPx is expressed in μmol of NADPH oxidized per min per mg of protein using a molar extinction coefficient of 6.22 mM cm^{-1}.

2.3.5. Genotoxicity (comet assay in erythrocytes)

Damage of DNA was measured in erythrocytes using the comet assay method as described by Singh et al. (1988) and modified by Silva et al. (2000). Microscope slides were soaked briefly in standard melting agarose solution at 60 °C (1.5% normal melting agarose prepared in phosphate-buffered saline) and dried overnight. Blood was mixed with 0.75% low melting point agarose at 5% ratio (Gibco BRL) at 37 °C and immediately poured on pre-covered slides. A coverslip was used to spread the material and then the slides were immersed in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris; pH 10–10.5; 1% Triton X-100 and 10% DMSO). After two hours, the coverslip was removed. Slides were placed in an electrophoresis chamber filled with alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 12.6). Cells were exposed to alkali for 20 min. Subsequently; slides with DNA were electrophoresed for 20 min at 300 mA and 25 V at 4 °C. All steps were conducted under red light. Then, slides were washed three times (5 min each) with 0.4 M Tris buffer, pH 7.5, to buffer the excess alkali, and were submerged in silver solution (5% sodium carbonate, 0.1% ammonia nitrate, 0.1% silver nitrate 0.25% acid tungstosilicic and 0.15% formaldehyde) for 15 min at 37 °C to stain DNA. Randomly selected cells (100 cells from each of two replicate slides) were analyzed from each animal using an optical microscope (Leica DM205) at 100× magnification. Cells were visually scored according to tail size into five classes (from undamaged – 0 to maximally damaged – 4). An overall score was obtained by summation all cell' scores: from completely undamaged (sum 0) to maximum damage (sum 400) (Kobayashi et al., 1995).

2.3.6. Brain acetylcholinesterase (AChE)

AChE activity in brain was determined by the method of Ellman et al. (1961). Briefly, brain samples were homogenized in phosphate buffer 1:4 (0.1 M, glycerol 20%, pH 7.5). Homogenates were centrifuged for 20 min at 12,000 g (4 °C) and the supernatant used for measurement of AChE activity. Acetylthiocholine iodide (ATC) 9 mM was used as a substrate and 5,5′-dithio-bis (2-nitrobenzoic) acid (DTNB) as color reagent. Kinetic activity of AChE was measured using a Genesis Spectronic spectrophotometer at 415 nm.

2.3.7. Protein determination

Total protein was measured according to Bradford (1976) using a SpectraMax M2 and bovine serum albumin (BSA) as standard at 595 nm.

2.3.8. Statistical analysis

All data are presented as mean ± SEM ($n = 6$). Prior the comparative statistical tests, distribution and homogeneity of data were checked. One-way ANOVA followed by the Dunnet's test were used to determine differences in all analyzed parameters (Biomarkers responses) between fish exposed to the treatments (10 mg L^{-1} and 15 mg L^{-1} of RD) and the control groups. When data violated the premises of One-way ANOVA test, a non-parametric Kruskall–Wallis test was used. Statistical significance was accepted at the level of $P < 0.05$.

3. Results

Although there was no fish mortality during 96 h exposure to RD or in the control group, biomarkers responses in both gills and liver were markedly altered by exposure to RD sub-lethal concentrations. Fish exposed to both concentrations showed several pathological changes in gills. The most common histopathological changes and their respective frequencies are shown in Table 1. Filament epithelium hyperplasia and hypertrophy, epithelium lifting, aneurysm and rupture of lamellar epithelium and proliferation of mitochondria-rich cells are the changes observed in fish gills (Fig. 1), which were more frequent in fish exposed to RD 75% (Table 1).

Hematological parameters were altered only in fish exposed to 75% RD: [Hb] increased 1.3 times ($P < 0.05$); MCH increased 1.4 times ($P = 0.017$), and MCHC 1.2 times ($P < 0.05$) compared to control group (Table 2). There was no significant difference in RBC, Ht, MCV and blood glucose contents among all groups (Table 2).

Juveniles of *C. macropomum* exposed to RD 50% and 75% showed a decrease of 1.4 ($P = 0.017$) and 2.4 times ($P = 0.025$) in gills GST activity, respectively (Fig. 2A). However, no significant changes in GPx and SOD activity were observed (Fig. 2B and C).

GST activity was decreased by 1.7 times ($P = 0.017$) in liver of fish exposed to 75% RD (Fig. 2A) while GPx increased 2.5 times ($P = 0.017$) (Fig. 2B). There was no significant difference in SOD activity in fish liver exposed to RD (Fig. 2C). Interestingly, there was an increase of 1.8 times in liver EROD activity in fish exposed

Table 1
Histopathology and Indexes of Tissue Damage in gills of *C. macropomum* after 96 h exposure to 50% and 75% of RD LC_{50} 96 h. Values indicate the stages of damage as modified by Poleksic and Mitrovic-Tutundzic (1994). Kruskall–Wallis test was used. Data are means ± SEM, $n = 6$.

Lesion types	Stage	Control	Treatments	
			50% LC_{50} 96 h	75% LC_{50} 96 h
Filament hyperplasia and hypertrophy	I	0	++	++
Lamellar hyperplasia and hypertrophy	I	0	+++	+++
Lamellar fusion	I	0	0	0
Lamellar congestion	I	0	+	+
Lamellar epithelium lifting	I	0	++	+++
Mitochondria rich cells proliferation	I	0	+++	+++
Mucous cells proliferation	I	0	++	+++
Edema	I	0	+	0
Filament epithelium lifting	II	0	+	0
Lamellar fusion	II	0	+++	+++
Aneurism and rupture	II	0	++	+++
Necrosis	III	0	0	0
Fibrosis	III	0	0	0
Average histopathogical index		2.85	8.75	11.28[a]
Effects		Normal gill function	Normal gill function	Moderate to heavy damage

[a] Indicates significant difference from control group ($P < 0.05$).

to 75% RD concentration ($P = 0.017$) and no increase in fish exposed to 50% RD (Fig. 3).

Exposure to RD promoted significant genotoxic effect in tambaqui, evidenced by increases of 5.8 times ($P = 0.025$) and 6.4 times ($P = 0.017$) in erythrocytes DNA damage of fish exposed to 50% and 75% RD, respectively (Fig. 4). In addition to the genotoxic effect, short-term exposure to RD caused a neurological disturbance in fish, as seen by brain AChE inhibition of 1.8 times ($P = 0.017$) and 1.4 times ($P = 0.025$) in fish exposed to 50% and 75% RD, respectively (Fig. 5).

4. Discussion

Several studies have described the RD effects on biochemical and hematological parameters, DNA damage, and cholinergic activity of different freshwater fish species (Cavalcante et al., 2008; Glusczak et al., 2011; Modesto and Martinez, 2010a, 2010b). However, only sporadic studies have focused on RD effects in fish of the Amazon.

Exposure of freshwater fish to sub-lethal concentration of pesticides resulted in histological damage of several tissues (Hued et al., 2012), often associated with disruptions of morpho-functional properties. In the present work, the gills of *C. macropomum* exposed to RD showed hypertrophy and hyperplasia in filament and lamellar epithelium, epithelial lifting, aneurysm, rupture of lamellae epithelium, and proliferation of chloride and mucous cells, that were much more severe in fish exposed to RD 75%.

Similar type of histopathological lesions, in minor frequency, has been reported to *P. mesopotamicus* (Shiogiri et al., 2012) and *Cyprinus carpio* L. (Neskovic et al., 1996) after short-term exposure to 3.0; 3.5 and 4.0 mg L^{-1} of RD-Ready, and 5.0 and 10.0 mg L^{-1} of N-/phosphonomethyl/glycine, respectively. These non-specific responses of gill morphology, as epithelium lifting, hypertrophy, and hyperplasia are understood as compensatory responses of brachial tissue to reduce the toxic effects of direct and indirect contact with xenobiotic (Takashima and Hibiya, 1995). However, in an attempt to reduce the xenobiotic absorption in gills, these compensatory responses could result in increased water/blood barrier in branchial lamellae of fish, which had been directly related to disturbances in respiratory and ionic status of freshwater fish (Hughes et al., 1979).

The increased hemoglobin concentration, MCH, and MCHC reported here to tambaqui exposed to RD 75% are consistent with gill morpho-functional alterations that affect the respiratory compromise in fish. In general, exposure to aquatic toxic compounds impairs the respiratory function in freshwater fish through reduction of oxygen uptake and increase in oxygen demand to maintain vital cellular processes (Nowak, 1992).

These respiratory disturbances are associated with physiological adjustments although different responses were reported for several other freshwater fish species. Glusczak et al. (2006) observed a decrease in hematocrit (Ht), hemoglobin ([Hb]) and erythrocyte count (RBC) in *Leporinus obtusidens* after exposure to 3, 6, 10 and 20 mg L^{-1} of glyphosate herbicide. In contrast, Modesto and Martinez (2010a) reported a significant increase in hematocrit and number of erythrocytes in *P. lineatus* after 24 and 96 h of exposure to commercial formulation of Roundup-Transorb® indicating the release of new erythrocytes from hematopoietic tissues into blood stream. Our findings strongly suggest that increased hemoglobin level seen in tambaqui is an adaptive response to maintain tissue oxygenation. Tambaqui has been recognized as a hypoxia tolerant fish, presenting, among other specialized physiological traits, a high hemoglobin–oxygen affinity ($P_{50} = 2.4$ mm Hg) (Val and Almeida-Val, 1995). Thus, it is likely that the increased levels of blood hemoglobin parameters, associated with a very high oxygen carry capacity, would be an attempt to attenuate severe toxic effects of RD on their respiratory needs.

Mild changes in gill detoxification evidenced by GST activity, and no effects on antioxidant system (SOD and GPx) plus inhibition of GST in fish exposed to RD were observed in the present study. Glutathione-S-transferase, which catalyzes the conjugation of glutathione with xenobiotics in Phase II biotransformation system, has been widely recognized to play a significant role in the detoxification process in freshwater fish. Furthermore, several previous studies had reported increased GST activity in different tissues of fish exposed to different formulations of RD (Langiano and Martinez, 2008; Guilherme et al., 2010). However, some recent papers reported no effect of herbicide exposure on GST activity in gills of the freshwater mosquitofish *Gambusia yucatana* (Osten et al., 2005) and *Poecilia vivipara* (Harayashiki et al., 2013), suggesting that branchial GST is not directly involved in detoxification process in these fishes. The reduced biotransformation capacity seen in gills of tambaqui exposed to RD (GST) results from the direct toxic effect of RD on its respiratory functions. As mentioned the respiratory dysfunction is related to the histopathological damages of gills, rather than to oxidative stress generated by ROS in branchial tissue, and it is an indirect toxic effect of herbicide exposure.

Changes in biochemical biotransformation process and stimulation of antioxidant defenses occurred in tambaqui liver (75% RD) suggest that fish were under pro-oxidant stress. EROD is among the enzymes induced by CYP1A and catalyzes several xenobiotics during phase I biotransformation in liver (Van der Oost et al., 2003). EROD is an important biomarker for monitoring freshwater fish exposed to pollutants (Haluzová et al., 2011). At our best knowledge, this is the first report showing activation of hepatic EROD in a Neotropical fish species after RD exposure. Increased EROD activity has been reported in fish exposed to different herbicide formulations, as pyrethroid (Assis et al., 2009) and prochloraz (Haluzová et al., 2011). Although previous studies reported that RD exposure has no effect or causes a decrease of hepatic cytochrome

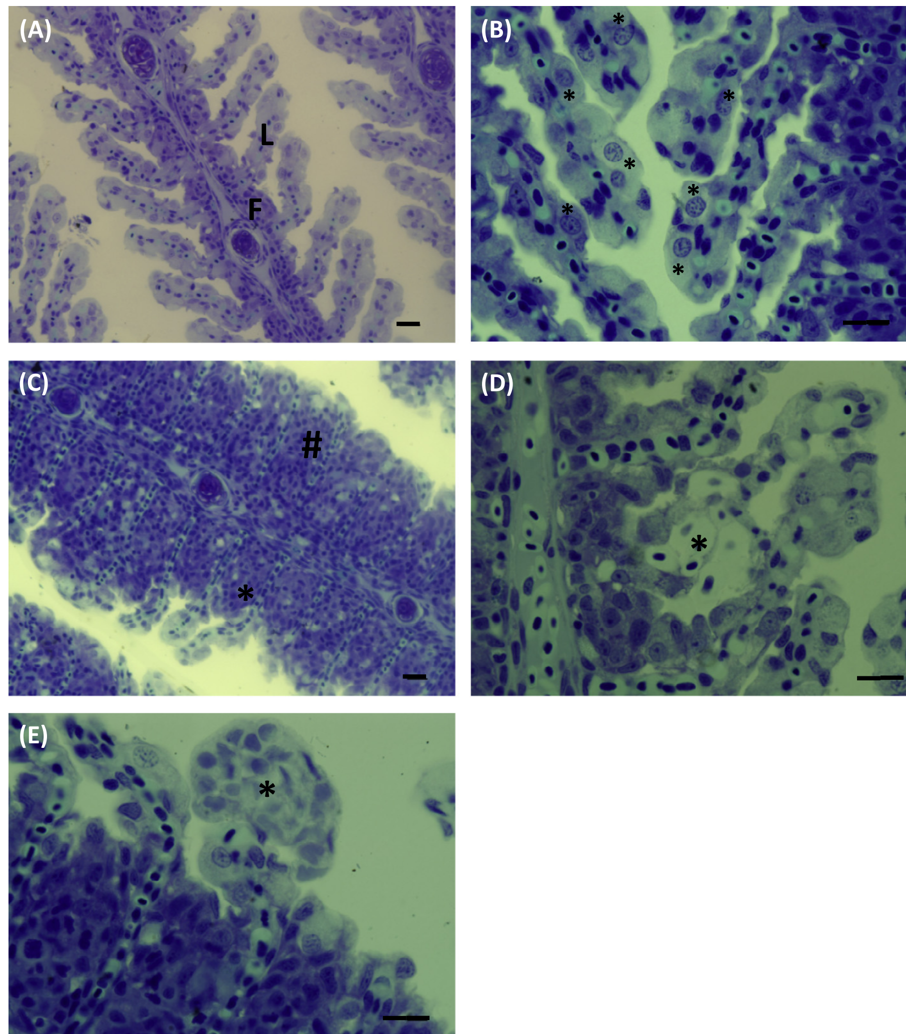

Fig. 1. Representative sagittal sections of gill of *Colossoma macropomum* after 96 h under control conditions and exposed to 50% and 75% of RD LC_{50} 96 h. (A) control conditions; (B) proliferation of mitochondria rich cells (*); (C) hyperplasia and hypertrophy of the filament epithelium (*) and filament fusion (#); (D) congestion (*); and (E) aneurism (*). Note the normal structure of the filament (F) and lamellae (L) in the control. Scale bar = 200 µm.

Table 2
Glucose levels, hematocrit (Ht), hemoglobin concentration ([Hb]), red blood cell count (RBC) and corpuscular constants: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) of *C. macropomum* after 96 h exposure to RD 50% and 75%. [Hb] and CHCM Kruskall–Wallis test was used. Data are means ± SEM, *n* = 6.

Hematological variables	Control	Treatments	
		50% LC_{50} 96 h	75% LC_{50} 96 h
Glucose	68.12 ± 5.8	58 ± 8.5	59.4 ± 8.5
Ht (%)	28.37 ± 0.8	27 ± 1.3	28.1 ± 1.2
Hb (g dl^{-1})	7.57 ± 0.2	7.483 ± 0.3	10.3 ± 0.9[a]
RBC count ($\times 10^6$ mm^{-3})	1.84 ± 0.1	1.74 ± 0.1	1.7 ± 0.1
MCV (mm^3)	167.72 ± 4.2	160.44 ± 4.6	177.4 ± 10.0
MCH (pg cel^{-1})	41.95 ± 3.0	42.88 ± 1.1	61.0 ± 5.5[a]
MCHC (%)	29.8 ± 2.9	27.8 ± 0.6	36.6 ± 2.9[a]

[a] Indicates significant difference in relation to control group ($P < 0.05$).

P450 by down-regulating genes of aryl hydrocarbon (ARNT) metabolism in freshwater organisms (Hoang-Le et al., 2011), EROD activity in liver was increased by 1.8 times in tambaqui exposed to RD 75%. According to Zanger and Schwab (2013), several xenobiotic as polycyclic aromatic hydrocarbons and β-naphthoflavone act as ligand to at least 13 Ah-receptor elements, which have been recognized to regulate the transcription of CYP1A genes in humans. Furthermore, evidences of AhR-independent elements of regulation of CYP1A genes transcription in human's hepatocytes has been reported (Yoshinari et al., 2008). Despite the mechanisms of CYP1A induction in the liver of tambaqui exposed to RD be unknown, such increased hepatic EROD activity would avoid the accumulation of toxic compounds, and reduce toxic effects of RD to the animals.

Phase II biotransformation system comprises the conjugation reactions of xenobiotics to reduced glutathione (GSH) throughout glutathione-S-transferase enzyme (GST). GST is used as a biomarker of fish exposure to aquatic pollutants; both induction and inhibition of GST in liver were previously reported (Guilherme et al., 2010; Menezes et al., 2011). GST activity is directly related to the type of glyphosate herbicide (Lushchak et al., 2009; Harayashiki et al., 2013). In contrast to the responses seen in gills, GST was inhibited in liver of tambaqui exposed to 75% RD, an opposite trend of EROD. According to Bernhardt (1995), activation of CYP1A through its catalytic cycle involves two-monoelectronic reduction of oxygen, and these reactions can generate ROS, such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). In addition, the interaction between superoxide and hydrogen peroxide may lead to formation of highly reactive hydroxyl radical ($HO^{\cdot}$)

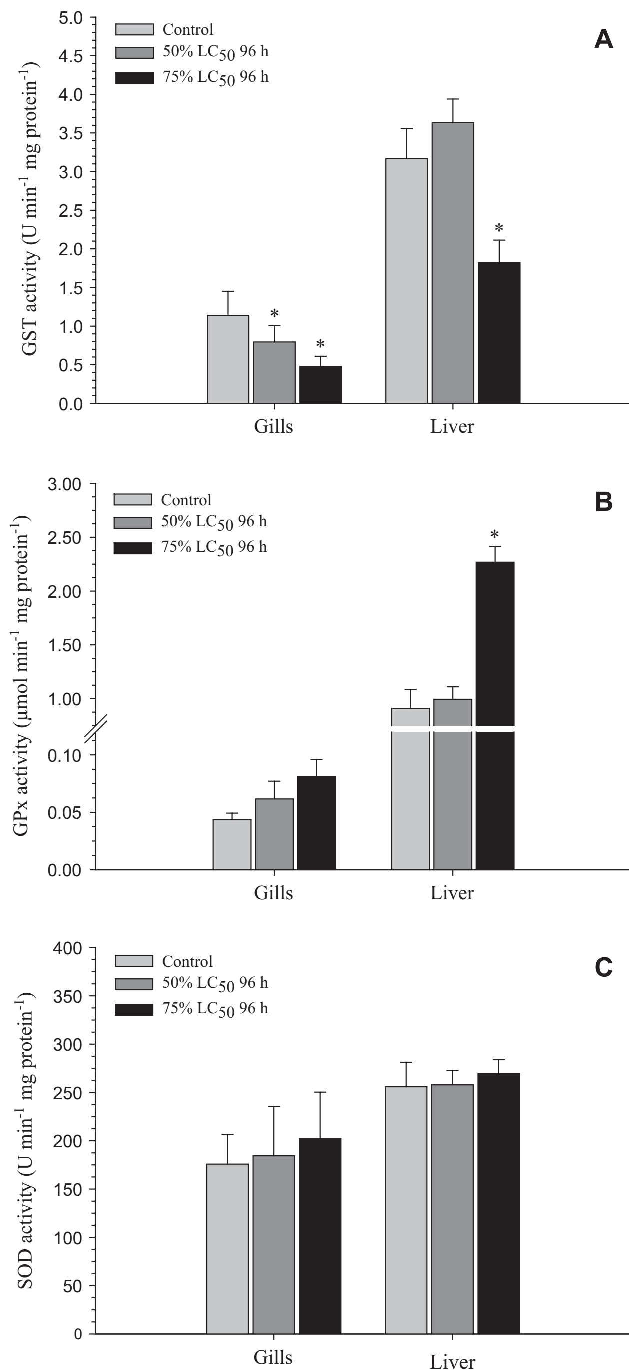

Fig. 2. Liver and gill glutathione-S-transferase (GST) (A), glutathione-peroxidase (GPx) (B), and superoxide dismutase (SOD) (C) of *C. macropomum* after 96 h exposure to RD 50% and 75%. Columns represent means and vertical lines represent SEM. *Indicates significant difference compared to control group ($P < 0.05$).

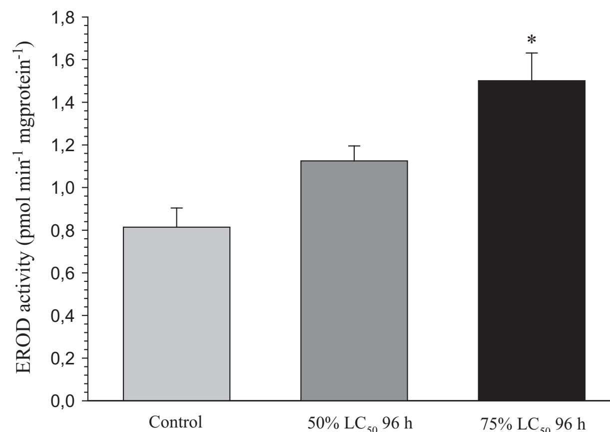

Fig. 3. Liver Ethoxyresorufin-O-deethylase (EROD) activity of *C. macropomum* after 96 h exposure to RD 50% and 75%. Columns represent means and vertical lines represent SEM. *Indicates significant difference compared to control group ($P < 0.05$).

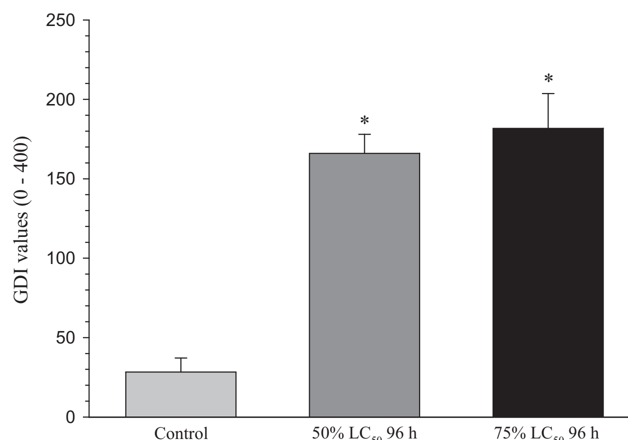

Fig. 4. Genetic Damage Indicator (GDI) in erythrocytes of *C. macropomum* after 96 h exposure to RD 50% and 75%. Columns represent means and vertical lines represent SEM. *Indicates significant difference compared to control group ($P < 0.05$).

(Haber and Weiss, 1934), which is recognized as GST inhibitor (Kehrer, 2000). Thus, it is possible that the first biotransformation phase CYP1A metabolizes glyphosate-based herbicide in tambaqui liver generating ROS and inhibiting phase II GST.

GPx activity was significantly higher (2.5 times) in the liver of tambaqui exposed to 75% RD, whereas no effect was seen in SOD. SOD and GPx are recognized as free radicals scavengers, acting to neutralize superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) produced by different pathways that involve endogenous compounds and various xenobiotics (Kehrer, 2000). In fact, GPx and SOD varies according to RD exposure relatively to glyphosate formulation and fish species (Lushchak et al., 2009; Menezes et al., 2011).

GPx immediately produces H_2O_2 (Hermes-Lima, 2004). GPx increases in tambaqui liver support the hypothesis that the exposure to higher RD concentration stimulates production of ROS, despite no increase in liver SOD activity. According to Lushchak et al. (2009), many pathways induce oxidative stress in animals exposed to xenobiotic. However, it is accepted that herbicides decrease antioxidant potential by inhibition of antioxidant enzymes (Modesto and Martinez, 2010a). Thus, it is possible that tambaqui exposed to RD 75% increased GPx activity in liver as an attempt to eliminate excess of ROS generated by the first biotransformation phase.

Although the exposure to RD 75% promoted biotransformation and antioxidant defense in tambaqui, the exposure to other RD concentrations induced genotoxic effects as seen by the increase in DNA damage. Similar results were reported in other teleosts as *P. lineatus* (Cavalcante et al., 2008) and *Anguilla anguilla* (Guilherme et al., 2010) acutely exposed to RD sub-lethal concentrations. Damage to DNA occurs at pro-oxidant conditions generating oxidative stress (Santos and Martinez, 2012). To prevent DNA damage the xenobiotic conjugation with GSH must occur through GST, which makes xenobiotic excretion easier (Hermes-Lima,

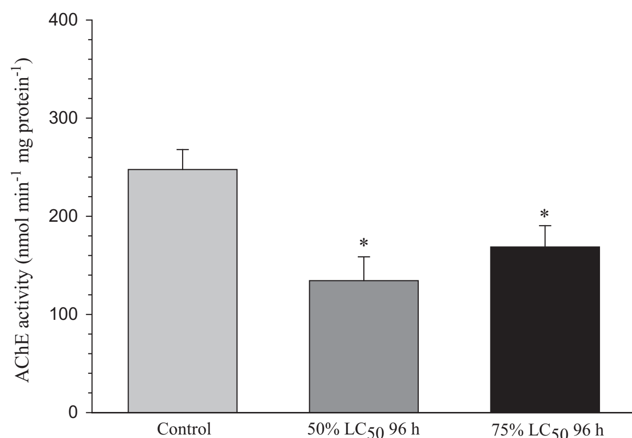

Fig. 5. Brain acetylcholinesterase (AChE) activity of *C. macropomum* after 96 h exposure to RD 50% and 75%. Columns represent means and vertical lines represent SEM. *Indicates significant difference compared to control group ($P < 0.05$).

2004). However, if the ability to generate antioxidant defense is not enough to neutralize ROS produced by herbicide exposure or EROD, cellular and DNA injuries might occur (Guilherme et al., 2010), as observed in the present analysis.

In addition to genotoxic effect, exposure for 96 h to RD promoted significant inhibition of brain AChE in tambaqui (1.8 and 1.4 times reduction in AChE activity in fish exposed to 50% and 75% RD, respectively). Several studies reported a relationship between AChE and contaminants in the aquatic environment (Glusczak et al., 2006; Modesto and Martinez, 2010b). The decrease of brain AChE activity observed for tambaqui in this study is in agreement with the findings for other Neotropical fishes, as *P. lineatus* (Modesto and Martinez, 2010b) and *L. obtusidens* (Glusczak et al., 2006) exposed to sub-lethal concentrations of this herbicide. The reduction in brain AChE activity generates the accumulation of acetylcholine at synapses, which, in turn, affects the normal functioning of nervous system and may affect locomotion and equilibrium (Bretaud et al., 2000). As brain AChE of tambaqui is sensitive to sub-lethal concentrations of RD, it may cause severe behavioral impairments of tambaqui.

RD concentrations tested in the present study were higher than the ones detected in natural freshwater bodies, which ranged between 0.01 and 1.7 mg L^{-1} (Guilherme et al., 2010). However, these herbicides are new synthetic agents to aquatic environments, and their effects on population are still poorly predictable (Lushchak et al., 2009). Our findings indicate that an acute exposure to RD sub-lethal concentrations promotes histopathological damage in gills, which are associated with alteration in hematological parameters. Moreover, activation of EROD and disturbances in liver antioxidant defense (GPx), suggest an increased ROS production in these animals, leading to an intense pro-oxidant condition, causing the genotoxic and neurological effects observed in this study. Thus, RD is potentially toxic to tambaqui and possibly to other tropical fish species.

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