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Genotoxic and biochemical effects of atrazine and Roundup®, alone and in combination, on the Asian clam *Corbicula fluminea*

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ABSTRACT

The present study aimed to evaluate biochemical and genotoxic effects of the herbicides atrazine (ATZ) and Roundup® (RD) separately, as well as their mixture, on the freshwater clam *Corbicula fluminea* after 96 h exposure. Animals were exposed to 2 and 10 ppb of ATZ (ATZ2 and ATZ10), 2 and 10 ppm of RD (RD2 and RD10) and the following mixtures: 2 ppb ATZ+2 ppm RD (AR2) and 10 ppb ATZ+10 ppm RD (AR10). Activities of ethoxyresorufin-O-deethylase (EROD), glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), as well as the multixenobiotic resistance mechanism (MXR), reduced glutathione concentrations (GSH) and lipid peroxidation (LPO) were measured in gills and digestive gland. DNA damage was determined in clams hemocytes through the comet assay. The gills were more susceptible to the action of the herbicides and the results showed that ATZ2 and ATZ10 caused a significant reduction in EROD and the mixture leads to a significant decrease in EROD and MXR. No significant change in the biotransformation parameters was observed in the digestive gland. Regarding the primary antioxidant defenses, SOD activity increased in the gills of clams exposed to ATZ10 and RD10 and in the digestive gland of animals exposed to RD2 and RD10, CAT activity was significantly reduced only in digestive gland of clams exposed RD10 while GPX increased in the gills after exposure to ATZ2 and RD10. The exposure to RD10 caused a significant increase in LPO in both gills and digestive gland. While the exposure to ATZ and RD separately did not increase DNA damage, the exposure to AR2 and AR10 caused a significant increase in the occurrence of DNA damage. In conclusion, this study showed that both herbicides applied alone caused effects on *C. fluminea*; ATZ interfered mostly in biotransformation while RD interfered mainly in antioxidant defenses leading to lipid peroxidation. The herbicides mixture showed antagonistic effects on the gills EROD and on lipid peroxidation in gills and digestive gland and synergistic effects on the gills MXR and on DNA damage in the hemocytes.

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1. Introduction

It is estimated that less than 0.1 percent of pesticides applied to crops reaches specific target (Pimentel and Levitan, 1986), leaving large amounts of toxic elements free to move into different environmental compartments and to contaminate soil and air, as well as surface and ground water (Belluck et al., 1991; Jablonowski et al., 2009). Brazil is the world's largest consumer of pesticides (ANDEF, 2012), and the class of herbicides is the most representative among the pesticides used in the country (IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis), 2010). In this scenario atrazine and glyphosate are worth noting, which represent the herbicides most used in Brazil and the world (Cox, 2001; IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis), 2010).

Atrazine (ATZ) is a broad-spectrum herbicide that does not strongly adsorb on sediments and its low rate of hydrolysis and photolysis can extend its presence in water (Graymore et al., 2001). This herbicide can be detected in freshwater environments (Gojmerac, 1996; Balinova and Mondesky, 1999) and also in estuarine and marine waters (Readman et al., 1993). In the United States, the detection of ATZ in groundwater was about 20 times more frequent than that of any other herbicide (Belluck et al., 1991) and ATZ concentrations of up to 108 $\mu g\ L^{-1}$ have been reported in the rivers of North America (USEPA (United States Environmental Protection Agency), 2002). In Brazil, ATZ was detected above the limit (2 $\mu g\ L^{-1}$) allowed by the Brazilian Guidelines (CONAMA, 2005) in samples from ground waters (Arraes et al., 2008) and from surface waters (Armas et al., 2007). Given its characteristics ATZ is considered a potential water contaminant (Eisler, 1989). Its toxicity is related to effects at different biological levels of organisms, such as genetic

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changes in bivalves (Bouilly et al., 2004), biochemical changes in gastropods (Russo et al., 2009) and teleosts (Santos and Martinez, 2012), reproductive changes in teleosts (Spanò et al., 2004) and amphibians (Hayes et al., 2002), and behavioral changes in bivalves (Flynn and Spellman, 2009), gastropods (Gerard and Poullain, 2005) and teleosts (Steinberg et al., 1995).

Glyphosate is a broad-spectrum herbicide most widely used around the world (Woodburn, 2000), primarily for agricultural applications, but it is also used in aquatic environments for the control of emergent and floating aquatic vegetation (Tsui and Chu, 2003). It is highly soluble in water and its half-life in water ranges from 7 to 70 days (Giesy et al., 2000), which makes glyphosate a potential contaminant of the aquatic environment. Glyphosate also has a high affinity for soil and may accumulate in the sediment (Giesy et al., 2000). Studies conducted in southern Brazil have already detected glyphosate in surface waters from different river basins located in agricultural areas at concentrations ranging from 20 to 30 ppb, up to 30 days after herbicide application (Da Silva et al., 2003), and at concentrations exceeding 100 ppb, up to 120 days after application of the herbicide in crops (Mattos et al., 2002), showing high persistence of glyphosate in the environment. An increasing number of studies report that glyphosate-based herbicides, such as Roundup® (RD), are potentially toxic to aquatic organisms, and may cause biochemical changes in crustaceans (Dutra et al., 2011), teleosts (Modesto and Martinez 2010) and amphibians (Costa et al., 2008), and also behavioral and histological changes in bivalves (El-Shenawy et al., 2003, 2009).

In the agricultural practices the application of more than one pesticide at the same time is common which means that mixtures of pesticides possibly will be present on the water surface (Cuppen et al., 2002; Van Den Brink et al., 2002). Thus, the organisms may possibly be simultaneously exposed to numerous contaminants, which can interact among themselves in different ways, causing different reactions in the organism, such as additive, synergistic or antagonistic effects (Eaton and Gilbert, 2008). ATZ and RD have overlapping periods of application in agriculture, i.e., they are likely to be simultaneously encountered in the environment, as observed by Armas et al. (2007). In this study, both herbicides were detected in samples of surface water, and the concentration of ATZ ranged from 0.6 to 2.7 $\mu g\,L^{-1}$, exceeding the maximum limit of 2 $\mu g\,L^{-1}$ allowed by the Brazilian Guidelines.

When in contact with an organism, contaminants such as herbicides can be biotransformed by mechanisms which act to make the xenobiotic substance a less toxic compound and facilitate its excretion (Santos and Martinez, 2012). In fish, phase I of the biotransformation pathway is mainly mediated by enzymes of the cytochrome P450 1A (CYP1A) subfamily (Van Der Oost et al., 2003), which is also present in mollusks, mainly in the digestive gland (Grosvick et al., 2006). Phase II comprises the xenobiotic conjugation reactions or its metabolites to reduced glutathione (GSH) by the action of glutathione S-transferase (GST) which have already been purified and characterized in mollusks (Fitzpatrick et al., 1995). Phase III, also known as the multixenobiotic resistance mechanism (MXR), is related to the presence of transport proteins (P-glycoprotein) associated with the plasma membranes, which accelerate the removal of the contaminant from the cell (Abou-Donia et al., 2002). These proteins have already been described in clams, such as *Corbicula fluminea* (Achard et al., 2004). However, these biotransformation processes can generate reactive oxygen species (ROS), represented by the superoxide anion radical, hydroxyl radical and hydrogen peroxide, which, if not neutralized by the antioxidant defenses, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione reductase (GR) and reduced glutathione (GSH), may promote oxidative damage to cell membranes (lipid peroxidation) and to the genetic material (Lushchak, 2011).

Bivalve mollusks are commonly used as sentinel organisms to monitor biological effects of environmental pollution because they are filter-feeding organisms which, consequently, may be exposed to large amounts of chemical pollutants (Sheehan and Power, 1999; Marigómez et al., 2013). Among them the freshwater clam *C. fluminea* (Müller, 1774) is known as one of the most important invasive species in aquatic environments (Sousa et al., 2008) and of particular relevance in biomonitoring studies (Shoults-Wilson et al., 2009). *C. fluminea* is originally from Asia and since the 1960s it has invaded South America (Belz et al., 2012). It is an opportunist species adaptable to a wide range of lentic and lotic habitats, which explains its considerable spread during the last decades (Vidal et al. 2002a). This species is known to accumulate various types of contaminants and several biochemical biomarkers have been studied in this clam after experimental exposure to different classes of contaminants (Vidal et al., 2001). Thus, *C. fluminea* can be considered as an interesting biological monitor (Vidal et al., 2002b; Rocha and Souza, 2012).

Thus, the present study aimed to evaluate the effects of ATZ and RD separately, as well as the mixture of these herbicides, on *C. fluminea* using biochemical and genotoxic biomarkers. For this purpose, parameters related to phase I (EROD), phase II (GST) and phase III (MXR) of biotransformation, as well as antioxidants enzymes (SOD, CAT, GPx and GR), the concentration of GSH, and the occurrence of lipid peroxidation (LPO) were measured in the gills and digestive gland, and DNA damage was determined in clams hemocytes.

2. Material and methods

2.1. Animals

Adult specimens of *C. fluminea* (Müller, 1774; Mollusca, Corbiculidae) measuring (mean ± SE, $n=90$) 6.40 ± 0.14 g with width of 3.10 ± 0.04 cm and height of 2.8 ± 0.02 cm, were collected in Lake Igapó (23°19′5.89″ S and 51°11′7.31″ W), located in Londrina, Paraná, Brazil. In the laboratory the clams were acclimated for 5 days in aquaria containing small stones (± 7 mm) and 40 L of dechlorinated tap water, constantly aerated. Water was renewed every 24 h until the beginning of the experiment.

2.2. Toxicity tests

Semi-static acute toxicity tests were performed in glass aquaria containing 10 L of dechlorinated tap water, constantly aerated, with 10 clams in each. Water was completely renewed every 24 h. Animals were exposed for 96 h to 2 and 10 ppb of ATZ (ATZ2 and ATZ10), 2 and 10 ppm of Roundup (RD2 and RD10) and the following mixtures of both herbicides: 2 ppb ATZ+2 ppm RD (AR2) and 10 ppb ATZ+10 ppm RD (AR10). Independent experiments were run to test each herbicide and the mixture, and control groups (CTR), with clams exposed only to dechlorinated water, were run simultaneously in each experiment. During the acclimation and in the course of the experiments a photoperiod of 12 h light/12 h dark was maintained and the water parameters remained as follows: pH 7.8, conductivity 80 $\mu S\,cm^{-1}$, turbidity 9.36 NTU, temperature 21 °C and dissolved oxygen 6.3 mg $O_2\,L^{-1}$.

ATZ concentrations were chosen considering that 2 and 10 ppb of ATZ (PESTANAL, analytical standard, Fluka) correspond, respectively, to the maximum concentration allowed for inland waters by the Brazilian Guidelines (CONAMA, 2005) and to 40 percent of the concentration normally used in crops (Ventura et al., 2008). For RD (Monsanto Brazil LTDA), the concentrations were defined based on the predicted concentration of RD following a single application of the herbicide in a water body with a depth of 0.15 m, with no foliar interception (which is 9 ppm) and half of the concentration when 50 percent of foliar interception was assumed (4.5 ppm; Giesy et al., 2000).

2.3. Sampling

Hemolymph was withdrawn from the sinus in the anterior adductor muscle and after that gills and digestive gland were immediately dissected. Hemolymph samples were stored on ice for the comet assay and the other tissues were kept frozen at −80 °C for the biochemical analysis.

2.4. Biochemical analysis

Gills and digestive gland were homogenized in potassium phosphate buffer (0.1 M, pH 7), centrifuged (16,000g, 4 °C, 20 min) and the supernatant was used for the measurement of all the biochemical parameters, except for MXR. Total protein determination was performed according to Lowry et al. (1951).

2.4.1. Biotransformation pathways

Etoxyresorufin-O-deethylase (EROD) activity was determined according to the method by Eggens et al. (1992), which determines the catalytic activity associated with CYP1A. The conversion of 7-etoxiresorufina (ethoxy) to resorufin was fluorimetrically monitored at 530 nm (excitation) and 590 nm (emission), every minute, for 40 min. EROD activity was expressed as pmol resorufin min^{-1} mg $protein^{-1}$. GST activity was determined according to the method by Keen et al. (1976), which measures the complexation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm for 1 min. Results were expressed as nmol CDNB conjugate min^{-1} mg $protein^{-1}$.The MXR activity was indirectly determined through the rhodamine B accumulation assay as proposed by Kurelec et al. (2000). In this assay, an increase in accumulated fluorescence represents a reduction in MXR. Fresh tissues were exposed to rhodamine B fluorescent dye (1 μM) for 2 h. Then, the tissues were weighed, frozen, homogenized in distilled water (1:7 w/v), centrifuged (1500g, 7 min, 4 °C) and the content of rhodamine B in the supernatant was determined by fluorescence measurement (excitation: 544 nm; emission: 590 nm). The concentrations of rhodamine B in tissue were determined against a standard concentration curve and the data were expressed as nmol rhodamine mg $tissue^{-1}$.

2.4.2. Antioxidant enzymes

SOD activity (CuZn-SOD) was determined according to the method of McCord and Fridovich (1969) which is based on the inhibition of the cytochrome c reduction rate promoted by the superoxide anion radical at 550 nm. SOD activity was expressed in U SOD mg $protein^{-1}$, where U is the amount of SOD that promotes 50 percent inhibition of the cytochrome c reduction rate. CAT activity was determined according to the method by Beutler (1975), which evaluates the decomposition rate of H_2O_2 by the decrease in the rate of absorbance at 240 nm and was expressed as μmol H_2O_2 decomposed min^{-1} mg $protein^{-1}$. GPx activity was determined based on the method proposed by Hopkins and Tudhope (1973). In this assay, the oxidation of NADPH in the presence of H_2O_2 is measured at 340 nm. GPx activity was expressed as μmol NADPH oxidized min^{-1} mg $protein^{-1}$. GR activity was determined according to Carlberg and Mannervik (1975) based on the reduction of NADPH in the presence of oxidized GSH at 340 nm. GR was expressed as μmol NADPH-oxidized min^{-1} mg $protein^{-1}$.

2.4.3. Non-enzymatic antioxidants

Reduced GSH levels were estimated according to Beutler et al. (1963), using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). Tissue samples were centrifuged (1500 g, 4 °C, 5 min) in trichloroacetic acid (TCA) 6 percent (1:1) and supernatants of the acid extracts 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. The GSH content was expressed in μmol GSH mg $protein^{-1}$.

2.4.4. Lipid peroxidation (LPO)

Lipid peroxidation was measured through the quantification of malondialdehyde (MDA), one of the LPO end-products present in the samples, according to the TBARS (thiobarbituric acid reactive substances) methodology, as proposed by Camejo et al. (1998) with some modifications for bivalve. Butylated hydroxytoluene (BHT 1 M), phosphate buffered saline (2 mM KCl; 1.4 mM NaH_2PO_4; 357 mM NaCl; 10 mM Na_2HPO_4; pH 7.4), trichloroacetic acid (TCA 50 percent) and thiobarbituric acid (TBA 1.3 percent) dissolved in 0.3 percent NaOH were added to the supernatant and the mixture was incubated at 60 °C for 1 h. A fluorescence reading was made (ex/em: 535/590 nm) and the TBARS concentration was determined from a malondialdehyde (MDA) standard curve. The TBARS concentration was expressed in μmol MDA mg $protein^{-1}$.

2.5. Genotoxic analysis

DNA damage in clams' hemocytes was quantified through the comet assay according to Fedato et al. (2010). The hemolymph was centrifuged (1160g for 20 min) and low melting point agarose was added to the remaining pellet which was distributed on slides previously covered with high melting point agarose (1.5 percent). The slides were then dried at 4 °C, coverslips were removed and the following steps were performed: I) lysis: 1 h, at 4 °C, protected from light, in a lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10 percent DMSO, 1 mL Triton X-100, pH 10.0); II) DNA unwinding: 30 min, in dark, in an electrophoresis buffer (0.3 N NaOH, 1 mM EDTA, pH > 13); III) electrophoresis: 30 min, 300 mA, 25 V; IV) neutralization: three washes for 5 min each in neutralization buffer (0.4 M Tris, pH 7.5); V) fixation with absolute ethanol for 10 min, followed by drying and storage of the slides under refrigeration (4 °C) until analysis. The slides were stained with GelRed and analyzed under a fluorescence light microscope adapted for fluorescence/epifluorescence, equipped with a blue excitation filter (450–490 nm) and a barrier filter of 515 nm at 1000× magnification. All slides were blind-reviewed and the extent of DNA damage was quantified by the length of DNA migration, which was visually determined in 100 randomly selected and non-overlapping cells per animal. DNA damage was classified in four classes – 0: no visible damage; 1: a short tail smaller than the diameter of the nucleoid; 2: a tail length 1 to 2 times the diameter of the nucleoid; 3: a tail length > 2 times the diameter of the nucleoid. The score of DNA damage for 100 comets was obtained by multiplying the number of cells in each class by the damage class, and ranged from 0 (all undamaged) to 300 (all maximally damaged).

2.6. Statistical analysis

The results of each parameter obtained for each experimental group (ATZ, RD and AR) were compared among treatment groups (CTR × 2 × 10) using either the parametric analysis of variance (ANOVA) or the non-parametric analysis (Kruskal–Wallis test) based on the data distribution (normality and homogeneity of variance). When an indication of a significant difference ($p < 0.05$) was observed, differences were analyzed by the post-hoc Dunn's test.

3. Results

As for the biotransformation mechanisms (Fig. 1), it was observed that among the organs analyzed, the gills appeared more susceptible to the action of the herbicides tested, since no significant change in the parameters analyzed in the digestive gland was observed. The exposure to ATZ caused a significant reduction in the EROD activity in gills, with a reduction of about 70 percent at 2 ppb and 79 percent at 10 ppb in relation to the respective control (Fig. 1A). Although no effect of RD on the EROD activity has been observed, the mixture of the two herbicides was able to promote a decrease in the activity of this enzyme in both AR2 and AR10, with a reduction of approximately 15 percent and 25 percent, respectively, compared to their respective control. Although these changes in the EROD have been observed, the GST activity remained unchanged during the experiments, despite the tendency for inhibition of this enzyme in the digestive gland after exposure to ATZ and to the mixture (Fig. 1B). As for the MXR effects, changes were observed only in the gills of the clams exposed to the higher concentration of the mixture, which showed a significant increase in the accumulation of rhodamine in comparison to the control, indicating the inhibition of the elimination mechanism via P-glycoprotein (Fig. 1C).

Regarding the primary antioxidant defenses (Fig. 2), an increase of SOD activity in the gills of clams exposed to the higher concentrations of ATZ and RD was observed (Fig. 2A), but the lowest concentrations of the herbicides and the mixture did not promote any changes in this enzyme in the gills. The SOD activity in the digestive gland increased after the exposure to both concentrations of ATZ and did not change after the exposure to RD and to the mixture. On the other hand, CAT activity of the digestive gland was significantly reduced by about 62 percent in clams exposed to RD10, but did not show any changes in the other testing situations (Fig. 2B). Conversely, GPx activity was altered only in the gills, where significant increases were observed after exposure to ATZ2 and RD10 (Fig. 2C). However, no significant changes were observed after exposure to the mixture.

The GR activity varied significantly only in the gills of the clams exposed to the lowest concentration of the mixture (AR2), where there was an increase of about 60 percent compared to its control (Fig. 3A). For the concentration of GSH (Fig. 3B), no significant change was observed in any of the organs analyzed after exposure to both herbicides, applied either separately or in a mixture.

Although some enzymatic antioxidant defenses, such as SOD and GPx, have been stimulated, the exposure to the higher RD concentration caused a significant increase in lipid peroxidation in both gills (370 percent) and digestive gland (350 percent; Fig. 4).

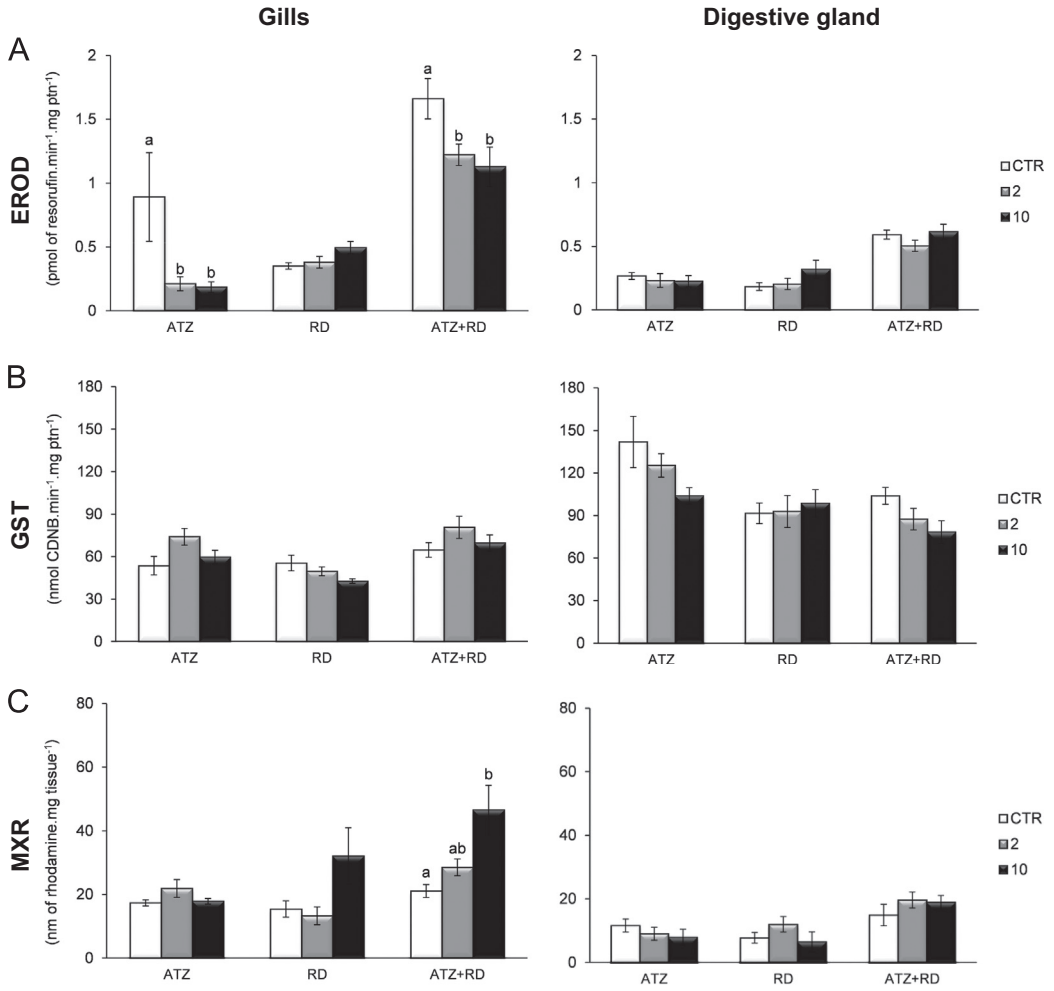

Fig. 1. Activity of (A) 7-ethoxiresorufina-O-deetilase (EROD), (B) glutathione-S-transferase (GST) and (C) accumulation of rhodamine (MXR) in the gills (I) and digestive gland (II) of *C. fluminea* exposed to 2 and 10 ppb of atrazine (ATZ2 and ATZ10), 2 and 10 ppm of Roundup® (RD2 and RD10), ATZ2 + RD2 mixture, ATZ10 + RD10 mixture or only to clean water (CTR). Values are presented as mean ± SEM ($n = 6–10$). Different letters indicate significant difference between the control group and the exposure groups of the same treatment ($p < 0.05$).

Moreover, in the digestive gland, both ATZ2 and AR10 caused a significant reduction in the occurrence of LPO (Fig. 4).

Different comet classes observed in *C. fluminea* hemocytes are shown in Fig. 5. Whilst the exposure to different herbicides separately has not been able to promote an increase in the occurrence of DNA damage in bivalve hemocytes, the exposure to the herbicides mixture caused a significant increase in the occurrence of such damage, both in AR2 and AR10, corresponding to approximately 114 percent and 124 percent, respectively, compared to their respective control group (Fig. 6).

4. Discussion

In the present work the gills and digestive gland of *C. fluminea* were chosen as target organs for the biochemical biomarkers considering that the digestive gland is a primary organ for bioaccumulation and is involved in pollutant detoxification and homeostasis maintenance (Cappello et al., 2013), while the gills are the main entrance of contaminants present in the environment (Rocha and Souza, 2012). The results showed that the gills exhibited a higher number of changes in the biotransformation mechanisms compared to the digestive gland, which did not present any significant alterations of EROD, GST and MXR. ATZ revealed to be a potential inhibitor of EROD activity, corroborating the data presented by Santos and Martinez (2012) in *Prochillodus lineatus* exposed to the same herbicide concentrations tested in this study. This inhibition may be the result of the ATZ action on the cytochrome P450 through the competition between ligands for the active site of the CYP1A enzyme (Kitteringham et al., 1998). Since metabolism tends to form less reactive and more readily excreted compounds than the parent compound (Van Der Oost et al., 2003), the inhibition of this mechanism would result in the persistence of the herbicide in the cell, which may facilitate its action as a toxic agent within the organism. RD did not appear as an interfering agent in the first phase of biotransformation. However, the mixture of the two herbicides caused inhibition of the gill EROD activity, probably by the ATZ action.

The GST has already been associated with the metabolism of contaminants, such as PCBs and PAHs in bivalves (Sheehan and Power, 1999) and identified as a metabolic pathway for ATZ metabolism in fish (Wiegand et al., 2001). Although GST is frequently used as an indicator of exposure to pesticides, sometimes appearing stimulated (Ezemonye and Tongo, 2010; Menezes et al., 2011) and sometimes inhibited (Santos and Martinez, 2012), in *C. fluminea*, this enzyme was not affected by ATZ or RD. Concerning the MXR mechanism, its reduction was observed only in the gills of bivalves simultaneously exposed to both herbicides. The decrease in this phase III mechanism appears to be the result

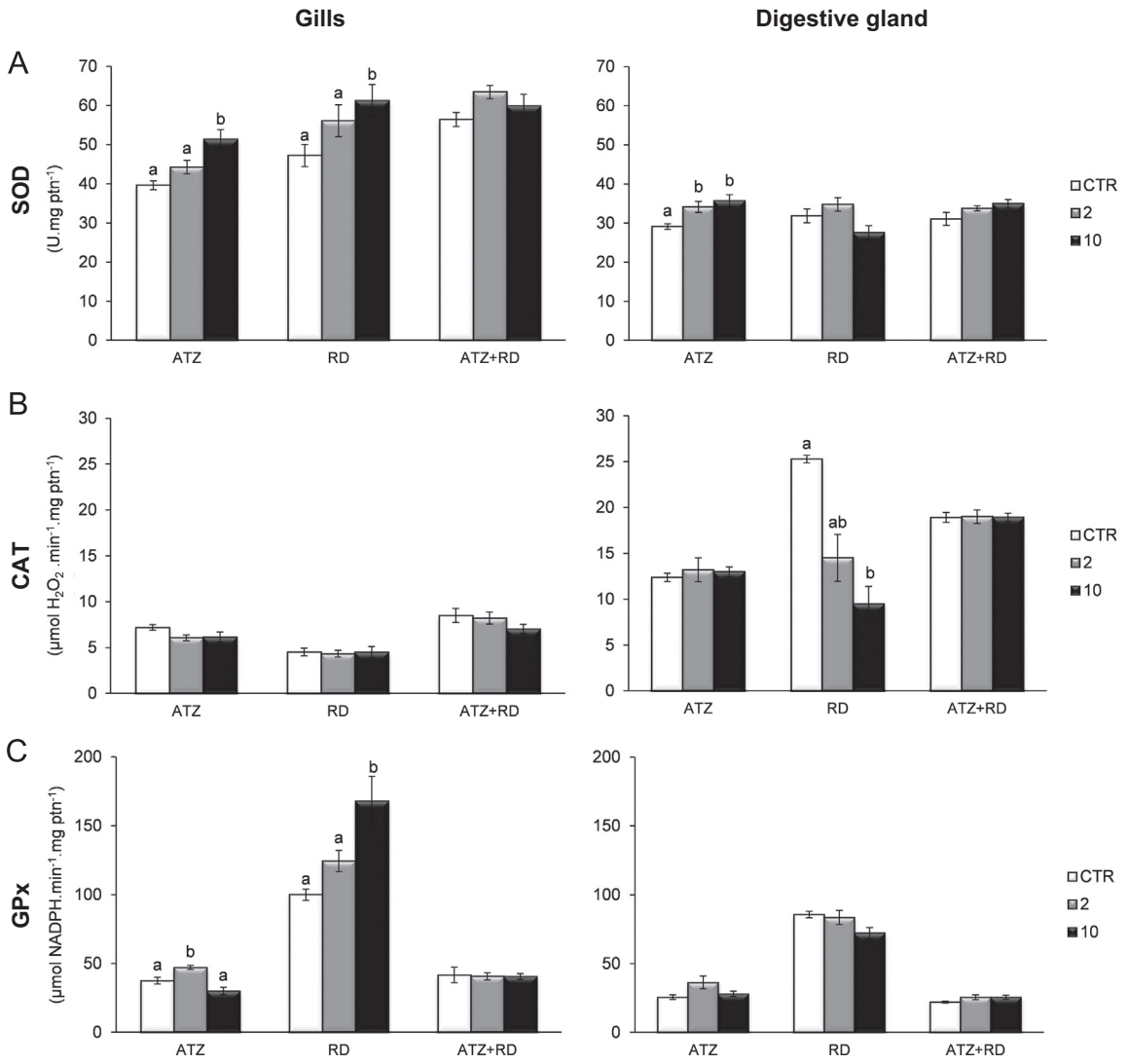

Fig. 2. Activity of (A) superoxide dismutase (SOD), (B) catalase (CAT) and (C) glutathione peroxidase (GPx) in the gills (I) and digestive gland (II) of *C. fluminea* exposed to 2 and 10 ppb of atrazine (ATZ2 and ATZ10), 2 and 10 ppm of Roundup® (RD2 and RD10), ATZ2+RD2 mixture, ATZ10+RD10 mixture or only to clean water (CTR). Values are presented as mean ± SEM (n=4–10). Different letters indicate significant difference between the control group and the exposure groups of the same treatment ($p < 0.05$).

of the potentiation of ATZ and RD effects, since the herbicides alone did not promote any effect on rhodamine B accumulation. The decrease of MXR associated with the decrease of EROD can hinder the elimination of the xenobiotic from the cell, inducing the accumulation of herbicides and their metabolites and thus making the organism more susceptible to their toxic effects.

The increase of SOD activity both in gills and digestive gland indicates that ATZ and RD are agents that stimulate the production of ROS, and ATZ shows higher oxidative capacity, as it interfered with the enzymatic activity of both organs. It is well known that the exposure of organisms to xenobiotics can increase the production of ROS (Lushchak, 2011), such as superoxide anion radical, which is directly neutralized by SOD and converted to hydrogen peroxide (H_2O_2) and oxygen (Aruoma, 1994). Such SOD increase results in an immediate increase in the concentration of H_2O_2 and both CAT and GPx may act in the neutralization of this oxygen species (Hermes-Lima, 2004). Since CAT remained unaltered in the gills during the exposure to ATZ and RD, while GPx was stimulated, the latter enzyme seems to play a central role in the gills, defending the organism against the excess of H_2O_2. In the digestive gland, CAT revealed to be more susceptible to RD as its activity was decreased, whereas SOD was more susceptible to ATZ that leads to an increase in SOD activity. This increase in SOD, not concurrent with an increase in GPx, as occurred in the gills, could be related to the fact that H_2O_2 can readily diffuse across the cell membrane (Aruoma, 1994). In this case, it may be suggested that H_2O_2 formed was possibly being released from the digestive gland by diffusion and not by enzymatic activity. GR activity as well as GSH concentrations appeared more resistant to herbicide exposure, since no changes were observed in these antioxidants by the action of the herbicides applied alone.

Although some antioxidant defenses, such as SOD and GPx, had been activated aiming to maintain the integrity of the organism, the 10 ppm concentration of RD promoted a significant increase in MDA concentration in both gills and digestive gland of clams, indicating lipid peroxidation. The augmented activity of SOD and GPx in the gills of clams exposed to RD10 suggests increased concentrations of the superoxide anion and hydrogen peroxide radicals (Simonato et al., 2011) that may have resulted in the generation of the hydroxyl radical (• OH) via the Fenton and the Haber–Weiss reactions (Hermes-Lima, 2004). This radical is the major cause of lipid peroxidation, since it attacks the polyunsaturated fatty acid chains of the membrane that can result in the conversion of hundreds of fatty acid side chains into lipid hydroperoxides (Halliwell and Chirico, 1993). The accumulation of these lipid hydroperoxides in the membrane disrupts its function

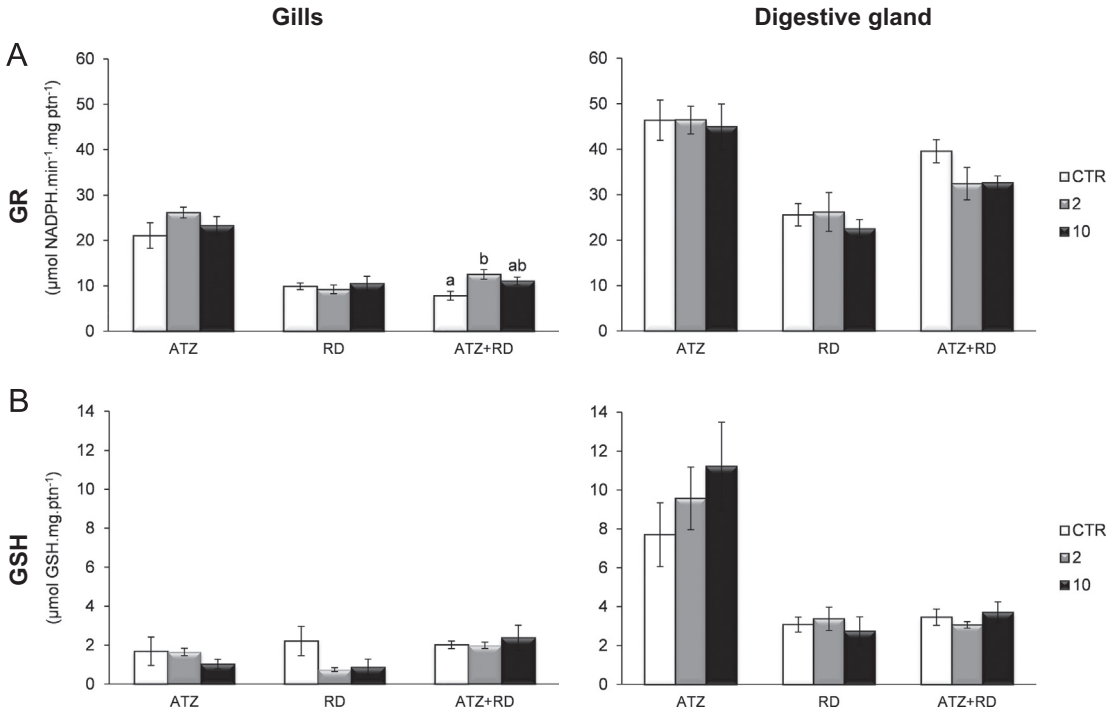

Fig. 3. (A) Activity of glutathione reductase (GR) and (B) concentration of reduced glutathione (GSH) in the gills (I) and digestive gland (II) of *C. fluminea* exposed to 2 and 10 ppb of atrazine (ATZ2 and ATZ10), 2 and 10 ppm of Roundup® (RD2 and RD10), ATZ2+RD2 mixture, ATZ10+RD10 mixture or only to clean water (CTR). Values are presented as mean ± SEM (n=4–10). Different letters indicate significant difference between the control group and the exposure groups of the same treatment ($p < 0.05$).

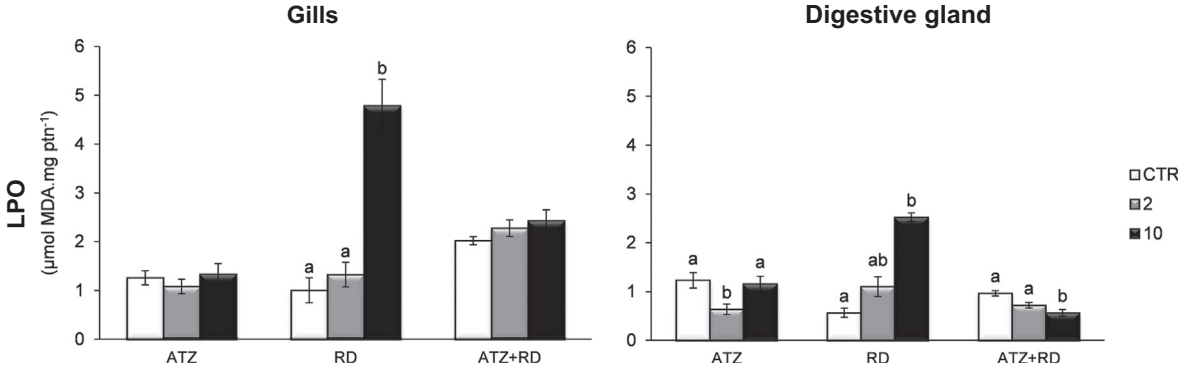

Fig. 4. Lipid peroxidation (LPO) in the gills and digestive gland of *C. fluminea* exposed to 2 and 10 ppb of atrazine (ATZ2 and ATZ10), 2 and 10 ppm of Roundup® (RD2 and RD10), ATZ2+RD2 mixture, ATZ10+RD10 mixture or only to clean water (CTR). Values are presented as mean ± SEM (n=4–10). Different letters indicate significant difference between the control group and the exposure groups of the same treatment ($p < 0.05$).

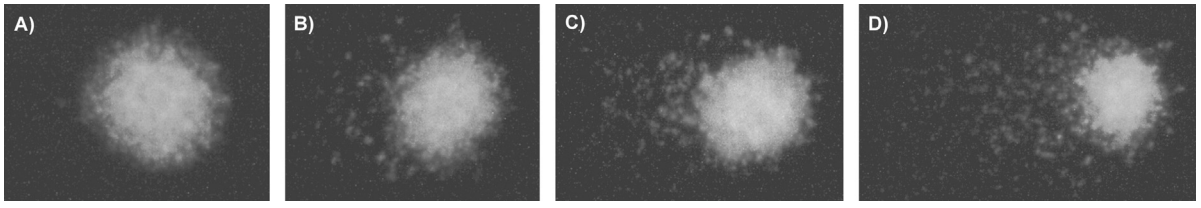

Fig. 5. Photomicrograph of *C. fluminea* hemocytes processed for the comet assay showing increasing degree of damaged DNA: A) comet class 0, B) comet class 1, C) comet class 2 and D) comet class 3. Magnification: 1000 ×.

and may cause its collapse. In addition, the membrane may decompose and produce a range of highly toxic products which can form adducts with DNA (Marnett, 1999). While RD applied alone revealed to be an inducer of lipid peroxidation both in gills and digestive gland of the clams, ATZ did not promote any enhancement on LPO; on the contrary, it leads to a reduction of LPO in the digestive gland of clams exposed to ATZ2.

The exposure of the clams to the herbicide mixture had an antagonist effect on the activity of the primary antioxidant enzymes, such as SOD in the gills and digestive gland, CAT in the digestive gland and GPx in the gills, considering that the effects observed after exposure to the herbicides alone were eliminated in the presence of ATZ plus RD. The mixture promoted a synergistic effect only on the gills GR activity and only with the smallest

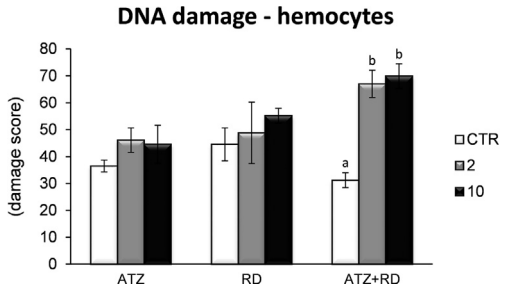

Fig. 6. DNA damage in hemocytes of *C. fluminea* exposed to 2 and 10 ppb of atrazine (ATZ2 and ATZ10), 2 and 10 ppm of Roundup® (RD2 and RD10), ATZ2+RD2 mixture, ATZ10+RD10 mixture or only to clean water (CTR). Values are presented as mean ± SEM (n=4–10). Different letters indicate significant difference between the control group and the exposure groups of the same treatment ($p < 0.05$).

concentration of herbicides. This may indicate that the animal undergoes a situation of excessive utilization of GSH, whose optimal levels within the cells were maintained via recycling of oxidized glutathione (GSSH).

The exposure to both herbicides simultaneously also showed an antagonist effect on the occurrence of lipid peroxidation in both gills and digestive gland. Besides the neutralization of the toxic effect on the digestive gland observed after RD exposure, a reduction in the oxidative damage to the membrane in organisms exposed to a higher concentration of herbicide mixtures was also observed when compared to their respective CTR group. This decrease in the MDA level may be the result of the protective effect of phospholipid hydroperoxide glutathione peroxidase (PHGPx), not analyzed in this study. This specific type of enzyme was found to perform a protective role against lipid peroxidation in the digestive gland of the mussel *Perna perna* (Almeida et al., 2004).

Furthermore, the mixture of ATZ and RD has been proven to have a genotoxic effect on *C. fluminea*, although there was no increase in DNA damage of the bivalve hemocytes exposed to the herbicides separately. Genotoxic effects of these two isolated herbicides have already been observed in the fish *P. lineatus* by Santos and Martinez (2012) and Modesto and Martinez (2010). ATZ, as well as other herbicides of the group of triazines, can bind directly to DNA through intercalation mechanisms and formation of adducts between the herbicide and the adenine and guanine bases (Oliveira-Brett and Silva, 2002). The formation of adducts with DNA is also responsible for the genotoxicity of RD (Peluso et al., 1998). Some studies have also shown that ATZ shows some synergistic interaction with some insecticides (Belden and Lydy, 2000; Lydy and Austin, 2004; Schuler et al., 2005), and can act by increasing the permeability to other pesticides. This could justify the genotoxic action of the mixture tested in this work.

In conclusion, this study showed that both herbicides applied alone revealed to be potentially toxic to *C. fluminea*, ATZ interfered mostly in biotransformation while RD interfered mainly in antioxidant defenses leading to lipid peroxidation. The herbicide mixture had diverse effects depending on the mechanism, revealing antagonistic effects on gills EROD and on lipid peroxidation in gills and digestive gland and synergistic effects on the gills MXR and on DNA damage in the hemocytes. Thus, the mixture effects are complex and variable, either intensifying or mitigating the adverse effects promoted by each compound alone and showed a greater effect on the gills, which is the organ with closer contact with the environment. Although a reasonable picture of the potential effects of both herbicides, including biotransformation mechanisms, antioxidant defenses, oxidative damages and DNA damages, was given in the present work, complementary analyses are necessary for a better understanding of the mechanisms of action of ATZ and RD, especially related to the mixtures. Although very important in situations of exposure to xenobiotics, the antioxidant defenses did not present any relevant changes so as to ensure their use as biomarkers. The freshwater bivalve *C. fluminea* revealed to be a good biomonitor of environments contaminated by the herbicides ATZ and RD; EROD and LPO appeared as potential biomarkers for the herbicides in question, respectively. As for exposure to the mixture, the comet assay and the multixenobiotic resistance mechanism (MXR) presented themselves as good biomarkers of this type of contamination.

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