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Sumário

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Carbofuran promotes biochemical changes in carp exposed to rice field and laboratory conditions

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ABSTRACT

Effects of carbofuran commercial formulation on oxidative stress parameters were studied in carps (*Cyprinus carpio*) exposed to 50 μg/L for 7 and 30 days under rice field and laboratory conditions. Thiobarbituric acid reactive substance (TBARS) levels were increased in the brain of fish after 7 and 30 days under rice field and laboratory conditions. In the liver and muscle, TBARS levels increased after 7 and 30 days under laboratory conditions, whereas in rice field the levels increased only after 30 days. Protein carbonyl content in the liver increased after 7 and 30 days under both experimental conditions. Acetylcholinesterase (AChE) activity was decreased in the brain and muscle after 7 and 30 days under both experimental conditions evaluated. The superoxide dismutase (SOD) activity increased in the liver after 7 and 30 days under rice field condition, whereas under laboratory condition this enzyme increased only after 30 days. The catalase (CAT) activity in the liver decreased after 30 days under rice field condition, whereas no changes were observed under laboratory conditions. In rice field, glutathione S-transferase (GST) decreased after 7 days but increased after 30 days, whereas no change was observed in fish exposed to carbofuran under laboratory conditions. These results suggest that environmental relevant carbofuran concentrations may cause oxidative stress, affecting biochemical and enzymatic parameters on carps. Some parameters could be used as biomarkers to carbofuran exposure.

1. Introduction

Dependence on pesticides has been increasing especially in tropical areas such as Brazil where agriculture has increased dramatically over the last decades (Sucahyo et al., 2008). Irrigated rice fields present enormous potential to expand the aquaculture production in rice-producing countries (Frei et al., 2007). In this system, seeding rice in water is recommended allowing fish to be cultivated in refuges located in the rice field. Concurrent rice–fish culture is an integrated system which allows the use of scarce resources such as water and land in a complementary way. Most pesticides used in rice fields may produce serious detrimental effects in the ecosystems, considering their toxic effects in non-target organisms including fish. In southern Brazil, most farmers use at least one pesticide in rice fields (Adhikari et al., 2004; Oruç and Usta, 2007; Clasen et al., 2012).

Carbofuran is a broad spectrum systemic carbamate insecticide, nematicide, and acaricide that banned in the United States and Europe because of unwanted toxic effects in birds, fish, mammals, insects and aquatic invertebrates (USEPA, 2006). It is also used to control coleopteran that damages irrigated rice crops in south-eastern Brazil (Plese, 2005; Pessoa et al., 2011). Contamination of water bodies adjacent to rice fields by carbofuran, mainly through runoff, is quite possible as a result of its widespread use in agriculture and relatively good solubility in water (320 mg/L at 20 °C). The concentration used in this study is based on the recommended dose of carbofuran commercial formulations to rice fields in Brazil, which is 4.0 kg/ha (Chelinho et al., 2012). The concentration was chosen to compare effects on carps exposed to different experimental conditions: rice field and laboratory. It has been shown that carbofuran concentration in irrigated rice fields in southeastern Brazil can reach maximum concentrations of 233 μg/L in lamina of water according to studies (Plese, 2005).

Several types of environmental pollutants may cause oxidative stress in fish. Studies have shown that carbofuran induces oxidative stress leading to the generation of free radicals with an increase of

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reactive oxygen species (ROS) and alteration in the antioxidant profile in different species of fish (Hernández-Moreno et al., 2010; Ensibi et al., 2012). ROS are involved in energy production, phagocytosis, cell growth regulation and cell signaling. The production of ROS associated with the presence of pollutants has been imputed as a possible mechanism of toxicity in aquatic organisms exposed to pesticides (Masella et al., 2005; Oropesa et al., 2009). Lipid peroxidation in fish has been suggested as one of the oxidative damage involved in pesticide-induced toxicity (Almroth et al., 2005). Parvez and Raisuddin (2005) suggested that protein carbonyl may serve as a general biomarker of oxidative stress. However, ROS also convert the amino groups of proteins and alter the structure or function of the proteins (Almroth et al., 2005). In line with oxidative damage caused by pesticide exposure the Acetylcholinesterase (AChE) is a key enzyme in the nervous system terminating nerve impulses by catalyzing hydrolysis of the neurotransmitter acetylcholine in acetate and choline. AChE is reported to be a specific biomarker of exposure to some pesticides including organophosphorus and carbamates, such as carbofuran (Lionetto et al., 2003). Representing antioxidant system the enzymes have a crucial role. Superoxide dismutase (SOD) is responsible for catalyzing the conversion of the superoxide anion into hydrogen peroxide. Hydrogen peroxide degrades into water and molecular oxygen via catalase (CAT), a family of enzymes which is present mainly in peroxisomes. Another important enzyme is glutathione S-transferase (GST), which acts in the process of biotransformation. It catalyzes the conjugation of a variety of metabolites, including pesticide metabolites and lipoperoxidation products, transforming the toxic compound into a more easily excretable metabolite (Parvez and Raisuddin, 2005; Clasen et al., 2012). Studies showed that several biochemical parameters are altered when exposed to carbofuran, such as metabolic parameters, AChE and TBARS indicating the toxicity of this pesticide for different fish species (Begum, 2004; Hernández-Moreno et al., 2010).

Considering the contamination potential of pesticides used in agriculture practices and possible contamination of fish, this study aimed at examining the effects of carbofuran at environmental relevant concentrations on the oxidative stress parameters in organs of *Cyprinus carpio* in rice fields and laboratory. Furthermore, we assessed the usefulness of these parameters as biomarkers of exposure to carbofuran due to the economic importance of the association rice–fish.

2. Materials and methods

2.1. Chemicals

A commercial formulation of the insecticide carbofuran (Furadan® 100 g) obtained from the FMC Química do Brasil Ltda (CAS 1563-66-2), containing ten percent carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl metylcarbamate) was used in the experiment. Bovine serum albumin, Triton X-100, hydrogen peroxide (H_2O_2), malondialdehyde (MDA), 2-thiobarbituric acid (TBA) and sodium dodecyl sulfate (SDS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals

Carps (weight, 15.0 ± 2.0 g; length, 10.0 ± 3.0 cm) were obtained from a commercial fish farm (RS, Brazil). Fish were acclimated in laboratory conditions for 10 days in tanks (250 L) containing clean water (free from insecticides) prior to experiments. They were kept in continuously aerated water with a static system and with a natural photoperiod (12 h light/12 h dark). After the acclimation period, 90 fish were transferred to points located in the rice field and other 30 fish were transferred to laboratory tanks. Both rice field and laboratory experiments were conducted for 7 and 30 days. In the period of acclimation, as in the period of exposure, the fish were fed twice a day with commercial fish pellets (42 percent crude protein, Supra, Brazil).

2.3. Experimental design

2.3.1. Rice field experiment

Fish were allocated to two groups, the control group (without insecticide) and exposure group (with insecticide). Each group was composed of 45 animals distributed in three tanks (triplicate) with 15 fish per tank. The fish were exposed to initial measured concentration of 50 μg/L of the insecticide for 7 and 30 days. The insecticide concentration used in this experiment corresponds to the concentration recommended in Brazil for use in rice culture. The control fish were placed in tanks with separate water supply from the exposure tanks, but conditions and placing of tanks were similar for both groups. During the experiment in the rice field, the fish were placed in submerged tanks, measuring 1.00 m (diameter) × 1.05 m (length). Fine-mesh plastic screens were used at the entrances and exits of water to avoid the presence of predators. Other conditions, such as climate changes, were not avoided in order to make the field experimental condition as real as possible. The following parameters were monitored during the experiments: temperature (24 ± 2.0 °C), pH (6.5 ± 0.2), dissolved oxygen (4.21 ± 2.0 mg/L), nonionized ammonia (0.8 ± 0.01 μg/L) and nitrite (0.06 ± 0.01 mg/L) of the water in the rice field. The insecticide concentration in water was monitored from the first day until it was not detected in either experimental condition (rice field and laboratory). The insecticide was analyzed by high-pressure liquid chromatography (HPLC) using the method described by Sabin et al. (2009). After each exposure period (7 and 30 days), the fish were killed by punching the spinal cord (behind the opercula), and a sample of five individuals was taken from the tanks and submitted to organs (brain, liver, and muscle) collected.

2.3.2. Laboratory experiment

Fish were distributed into 40 L tanks and allocated to two experimental groups as follows: the first was considered as a control group with 15 fish distributed into three tanks (5 fish per tank) containing insecticide-free water. The second with 15 fish distributed into three tanks (5 fish per tank) was exposed to initial measured concentration of 50 μg/L of the insecticide. Each group remained with the same experimental conditions for a period of 7 and 30 days. The insecticide concentration used in this experiment corresponds to the recommended concentration for growing rice. Moreover, these concentrations are likely to occur in the natural environment, close to agricultural areas. During the experimental period in the laboratory, the average water parameters were as follows: temperature 22.3 ± 2.0 °C, pH 6.6 ± 0.2 units, dissolved oxygen 6.4 ± 1.0 mg/L, nonionized ammonia 0.5 ± 0.01 μg/L, nitrite 0.06 ± 0.01 mg/L. After each exposure period (7 and 30 days) the fish were killed by punching the spinal cord (behind the opercula) and then the organs (brain, liver, and muscle) were collected.

2.4. Biochemical parameters

2.4.1. Tbars AsSAY

Lipid peroxidation was estimated by the TBARS assay, performed by a MDA reaction with TBA, which was spectrophotometrically measured according to Buege and Aust (1978). The liver, brain, and muscle were homogenized in 10 volumes (w/v) of potassium phosphate buffer (20 mM) and then TCA ten percent and TBA 0.67 percentwere added to adjust to a final volume of 1.0 mL. The reaction mixture was placed in a micro-centrifuge tube and incubated for 30 min at 95 °C. After cooling, it was centrifuged at 5000*g* for 15 min and optical density was measured by a spectrophotometer at 532 nm. The TBARS levels were expressed as nmol MDA/mg protein.

2.4.2. Protein carbonyl assay

The liver was homogenized in 10 volumes (w/v) of 10 mM Tris–HCl buffer pH 7.4 using a glass homogenizer. Protein carbonyl content was assayed by the method described by Yan et al. (1995), with some modifications. Soluble protein (1.0 mL) was reacted with 10 mM DNPH in 2 N hydrochloric acid. After incubation at room temperature for 1 h in the dark, 0.5 mL of denaturing buffer (150 mM sodium phosphate, pH 6.8, containing SDS 3.0 percent), 2.0 mL of heptane (99.5 percent) and 2.0 mL of ethanol (99.8 percent) were added sequentially, vortexed for 40 s and centrifuged at 10,000*g* for 15 min. The protein extracted from the interface was washed twice by resuspension in ethanol/ethyl acetate (1:1), and suspended in 1 mL of denaturing buffer. The carbonyl content was then measured spectrophotometrically at 370 nm. The total carbonylation was calculated using a molar extinction coefficient of 22,000 M/cm. The protein carbonyl content was expressed as nmol carbonyl/mg protein.

2.4.3. Acetylcholinesterase assay

AChE activity was measured as described by Ellman et al. (1961). Brain and muscle were weighed and homogenized in a Potter-Elvejhem glass/Teflon homogenizer with 50 mM sodium phosphate buffer, pH 7.2 (with one percent Triton X-100). The homogenates were centrifuged for 15 min at 3000*g* and 5 °C, and the supernatant was used as the enzyme source. Aliquots of the supernatant (50–100 μL) (brain and muscle, respectively) were incubated at 30 °C for 2 min with 0.1 M phosphate buffer, pH 7.5, 10 mM DTNB as chromogen. After 2 min, the reaction was initiated by the addition of acetylthiocholine (AcSCh; 0.5 mM) as the

substrate for the reaction mixture. The final volume was 2.0 ml. Absorbances were determined at 412 nm during 2 min. Enzyme activity was expressed as micromoles of AcSCh hydrolyzed/min/mg protein.

2.4.4. Superoxide dismutase assay

SOD activity was performed in the liver based on the inhibition of the radical superoxide reaction with adrenalin as described by Misra and Fridovich (1972). In this method, SOD present in the sample competes with the detection system for radical superoxide. A unit of SOD is defined as the amount of enzyme that inhibits the rate of oxidation of adrenalin by 50 percent. The oxidation of adrenalin leads to the formation of the colored product, adrenochrome. SOD activity was determined by measuring the rate of adrenochrome formation, measured at 480 nm, in a reaction medium containing glycine–NaOH (50 mM, pH 10) and epinephrine (1 mM). SOD activity was expressed in UI/mg protein

2.4.5. Catalase assay

CAT activity was assayed spectrophotometrically (Nelson and Kiesow, 1972). Liver was homogenized in 10 volumes (w/v) of 20 mM potassium phosphate buffer, pH 7.5, and centrifuged at 10,000*g* for 10 min at 4 °C. The assay mixture consisted of 2.0 mL potassium phosphate buffer (50 mM, pH 7.0), 50 μL H_2O_2 (0.3 M) and 50 μL homogenate. Change of H_2O_2 absorbance in 60 s was measured at 240 nm. CAT activity was calculated and expressed in μmol/min/mg protein.

2.4.6. Glutathione S-transferase assay

GST activity was measured in the liver using a procedure described by Habig et al. (1974) that involved CDNB as substrate. The assay mixture contained 1 mM CDNB (in ethanol), 10 mM GSH, 20 mM potassium phosphate buffer (pH 6.5), and 50 μL of the tissue homogenates. Enzyme activity was calculated from the changes in absorbance at 340 nm using a molar extinction coefficient of 9.6 mM/cm. The activity was expressed as μmol GS-DNB min/mg protein.

2.4.7. Protein determination assay

Protein was determined by the Comassie blue method using bovine serum albumin as standard. Absorbance of samples was measured at 595 nm (Bradford, 1976).

2.5. Statistical analysis

A comparison between two groups was made by the Student *t*-test. The results obtained were expressed as mean ± standard deviation (SD). The value of $p \leq 0.05$ was considered statistically significant for all analyses ($n=15$).

3. Results

Carbofuran residues were monitored in the water of the laboratory and rice field system to verify the presence of the active insecticide ingredients in the water. The concentration of carbofuran in the water in the rice field after 7 and 30 days reduced 30.96 percent and 92.32 percent, respectively. On the other hand, under laboratory conditions, the concentration of carbofuran in water reduced to 25.20 percent and 54 percent after 7 and 30 days of exposure, respectively (Fig. 1).

TBARS levels in the different organs of carps are shown in Fig. 2. In the brain, TBARS levels increased compared to the control group for both experimental conditions and periods tested (Fig. 2A and B). In the rice field, TBARS levels in the liver and muscle increased only after 30 days of exposure (Fig. 2A). However, under laboratory conditions, a significant increase in the liver and muscle TBARS was observed after both periods of 7 and 30 days (Fig. 2B). In addition, the protein carbonyl content increased in both experimental periods and under both rice field and laboratory conditions (Table 1). The AChE activity was inhibited in the brain and muscle after 7 and 30 days of exposure to carbofuran under both rice field and laboratory conditions (Fig. 3).

Carbofuran increased liver SOD activity after 7 and 30 days in the rice field, whereas the SOD activity increased in the laboratory only after 30 days. In the rice field, the CAT activity in the liver decreased after 30 days of exposure. However, under laboratory conditions, CAT activity did not show significant alterations in any experimental period (Table 1). In rice field, liver GST activity showed a reduction after 7 days of exposure. However, after 30 days of exposure in rice field, an increase was found in the activity of this enzyme. On the other hand, in laboratory, GST activity in liver did not show significant change after 7 or 30 days (Table 1).

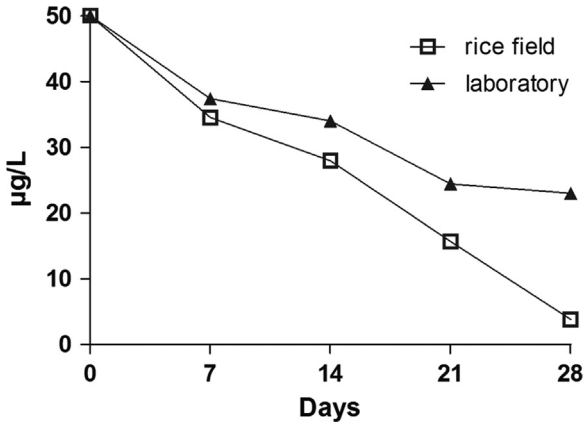

Fig. 1. The measured concentrations of carbofuran concentration (μg/L) in the water of rice field and laboratory conditions experiments.

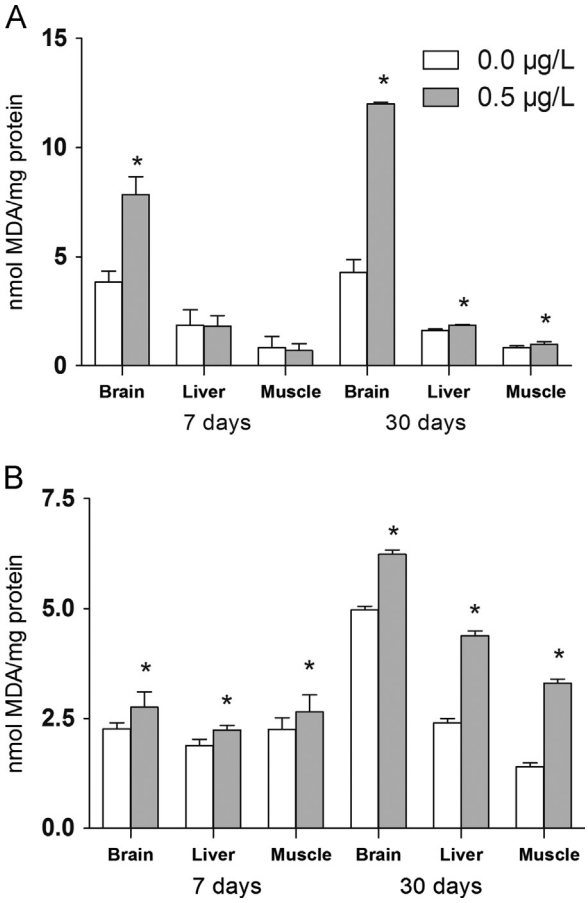

Fig. 2. TBARS levels in the brain, liver, and muscle of carps exposed to a carbofuran under rice field (A) and laboratory conditions (B) after 7 and 30 days. Data represent the mean ± SD ($n=15$). * indicates a significant difference from the control group at $p \leq 0.05$.

4. Discussion

In the present study, a set of oxidative stress parameters in carps was determined to evaluate possible effects of carbofuran considering two different experimental conditions: rice field and

Table 1
Effects of carbofuran on protein carbonyl, SOD, CAT and GST activities in the liver of carps after 7 and 30 days of exposure in field and laboratory conditions.

Rice field	7 days		30 days	
	0.0 μg/L	50.0 μg/L	0.0 μg/L	50.0 μg/L
Carbonyl	8.24 ± 0.94	9.22 ± 0.50*	9.37 ± 0.60	10.60 ± 0.15*
SOD	4.938 ± 0.38	7.587 ± 1.63*	4.774 ± 0.32	8.407 ± 0.73*
CAT	0.51 ± 0.08	0.59 ± 0.07	0.57 ± 0.03	0.43 ± 0.005*
GST	0.38 ± 0.09	0.27 ± 0.08*	0.31 ± 0.05	0.56 ± 0.07*
Laboratory				
Carbonyl	4.44 ± 0.43	8.92 ± 1.42*	6.67 ± 0.99	12.5 ± 1.07*
SOD	10.85 ± 2.5	10.34 ± 2.9	11.47 ± 1.3	15.61 ± 1.9*
CAT	0.304 ± 0.08	0.310 ± 0.05	0.530 ± 0.07	0.518 ± 0.06
GST	0.21 ± 0.01	0.25 ± 0.03	0.404 ± 0.051	0.443 ± 0.038

Protein carbonyl was expressed as nmol carbonyl/mg protein, SOD as UI SOD/mg protein, CAT as μmol/min/mg protein and GST as μmol GS-DNB/min/mg protein. Data are reported as mean ± standard deviation ($n=15$). *Significantly different from control group at $p \leq 0.05$.

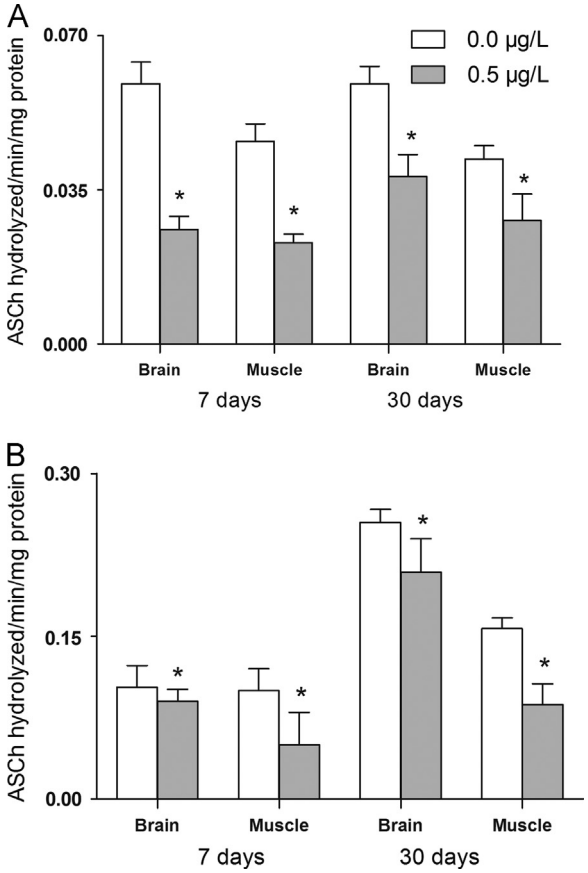

Fig. 3. AChE activity in brain and muscle of carps exposed to a carbofuran under rice field (A) and laboratory conditions (B) after 7 and 30 days. Data represent the mean ± SD ($n=15$). * indicates a significant difference from the control group at $p \leq 0.05$.

laboratory. There was a greater loss of pesticide in rice field conditions than under laboratory conditions, and residual carbofuran was found up to 28 days after first application. Carbofuran may be biotransformed or fish tissues may bioaccumulate this pesticide. Carbofuran is highly soluble in water therefore it is susceptible to leaching through the soil profile. Volatilization, photolysis, and oxidation are also important factors in the process of carbofuran dissipation and transformation into metabolites (Katsumata et al., 2005; Plese, 2005).

Despite the decrease of carbofuran in water, the lipid peroxidation phenomenon was evidenced due to the elevation in the TBARS levels in the brain, liver, and muscle in both experimental conditions. Pesticide-induced lipid peroxidation is a common response of fish, and some authors have also observed elevated levels of lipid peroxidation induced by deltamethrin (Sayeed et al., 2003) and 3,4-Dichloroaniline (Li et al., 2003). In a similar study, under rice field conditions, Clasen et al. (2012) observed TBARS levels increased for the same organs tested in the present study in carps exposed to a sublethal concentration of fipronil insecticide for 7, 30 and 90 days.

Another parameter to verify oxidative damage is the amount of protein carbonyl, which is the evidence of protein damage. Results observed for both experimental conditions indicated carbofuran-induced protein damage as well as the TBARS levels indicated the occurrence of lipid peroxidation in *C. carpio*. This elevation in protein carbonyl was reported by Parvez and Raisuddin (2005), to exposure *Channa punctata* by different experimental periods. The increase in TBARS levels could be associated with an increase in protein carbonyl content as a result of the formation of cytotoxic products. This toxic compounds generated by lipid peroxidation probably affect protein oxidation, inducing carbonyl formation. This increase in protein carbonyl causes changes in the activity of biological enzymes.

An important parameter used for toxicological evaluation is the measurement of AChE activity. The major effect of pesticides on AChE activity is the reduction of the activity after exposure (Miron et al., 2008; Modesto and Martinez, 2010). Results of this study are in agreement with those found by Golombieski et al. (2008), who showed an inhibition of AChE activity in the brain and muscle of carps exposed for 96 h to diafuran carbamate insecticide. Brain of goldfish exposed to carbofuran (50 or 500 μg/L) showed high levels of inhibition (Bretaud et al., 2000). Some authors suggest that the increase in TBARS levels could be associated with AChE inhibition (Sevgiler et al., 2004; Üner et al., 2006; Oruç and Usta, 2007). Our results suggest the same hypothesis because of the increase of TBARS levels in the brain and muscle, concomitant to AChE inhibition in the same organs after exposure to both conditions. Another hypothesis is the involvement of protein carbonyl to disrupt AChE activity. However, in the present study, only carbonyl in the liver was verified. In this sense, further studies are needed on AChE activity and its relationship with pro-oxidants, such as TBARS and protein carbonyl. Our results clearly indicated oxidative damage and the involvement of the insecticide tested causing lipid peroxidation and protein carbonylation. Some effects were observed on oxidative profile, indicating that even low concentrations of these insecticides affect fish organs and disrupt normal metabolism. In accordance with objectives of the present study, the TBARS, protein carbonyl measurements at different tissues could be used as a biomarker to carbofuran exposure, as well AChE activity in brain and muscle. The major toxic effect documented for carbofuran is AChE inhibition that represents disruption in cholinergic functions, other effects could be related to oxidative damage as showed at present study.

Considering the key antioxidant enzymes for the neutralization of ROS in all organisms, it is possible to detach SOD and CAT activities. These antioxidant enzymes are essential for the conversion of ROS in harmless metabolites, and they may be increased or inhibited under chemical stress. Clasen et al. (2012) have recently shown similar results in carps exposed to 0.65 μg/L of fipronil insecticide for 7, 30 and 90 days under rice field conditions. Changes in CAT and SOD activities are a typical response against pesticide toxicity. In the context of the present study, its effect showed to be directly associated with carbofuran residues found in

the water during the experiments. The decrease of CAT activity could be due to the flux of superoxide radicals, which have been reported to inhibit CAT activity as occurred in this study at 30 days in the rice field. Ensibi et al. (2012) found the same response in *C. carpio* after 4 and 30 days of exposure to carbofuran. CAT responses seem to be fish-specific and the variations observed were also obtained considering different agrochemical classes. Therefore, the use of CAT activity as an exclusive biomarker of toxicity is not recommended and it is necessary to verify the activity of different enzymes in order to understand antioxidant responses of fish (van der Oost et al., 2003).

GST activity in fish in polluted locations may be an important biomarker of pesticide toxicity. The most common response of GST against a toxic situation is the induction of activity that is considered beneficial to handle a stress condition. The increase observed in the GST activity is probably due to the increase in the biotransformation process of the xenobiotic by the fish exposed to carbofuran. The metabolism of the reactive species could be a cause of the activation of the defense mechanisms, as mentioned by Sayeed et al. (2003) when they exposed *C. punctata* to deltamethrin for short-term. The decreased enzyme activity may have occurred because liver is one of the first organs exposed to pesticides or other pollutants. However, this decrease may indicate a different response where the antioxidant defense is disrupted by carbofuran or its derived products, indicating a toxic situation caused by insecticide exposure. The inhibition of GST has also been reported in gills of mosquitofish exposed to carbofuran and liver of *Ancistrus multispinis* exposed to deltamethrin (Pimpão et al., 2007).

Results clearly demonstrated that carbofuran concentrations used in agriculture may cause changes in the biochemical parameters of carps under rice field and laboratory conditions. The measurement of brain TBARS and AChE activity could be taken into consideration to monitor insecticide toxicity to fish in contaminated water, as well the measurement of protein carbonyl in brain and muscle. Thus, some external stressors, such as carbofuran, may have a toxic effect on the *C. carpio* even when used at non-lethal concentration in different experimental conditions. The increased TBARS levels and protein carbonyl may have been caused due to the increase of free radicals as a result of fish stress condition after carbofuran intoxication.

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Imazapyr+imazapic herbicide determines acute toxicity in silver catfish *Rhamdia quelen*

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ABSTRACT

Imazapyr (IMY) and imazapic (IMI) are imidazolinone herbicides which have been associated in a commercial formulation (Kifix®). To date, there are no studies on the toxicity of an IMY+IMI herbicide in fish. This work aimed to assess the acute toxicity (24 and 96 h) of IMY+IMI (0, 0.488 and 4.88 μg/L) towards *Rhamdia quelen* through hematological, biochemical, immunological, ionoregulatory and enzymatic indexes. Red blood cell count was lower at 4.88 than at 0.488 μg/L (24 and 96 h); mean corpuscular volume was lower than control at both concentrations (24 h) and at 0.488 μg/L (96 h); lymphocytes declined at 4.88 μg/L comparing to control (96 h); and monocytes increased at 4.88 μg/L (96 h) in comparison with the respective control and with 4.88 μg/L at 24 h. Aspartate aminotransferase was higher at 0.488 μg/L (96 h) than the respective control and the respective concentration at 24 h; uric acid reduced at 4.88 μg/L comparing with 0.488 μg/L (96 h); and cortisol was lower at 4.88 μg/L compared to 0.488 μg/L and control (96 h). Herbicide exposure lowered plasma bactericidal activity at both concentrations (24 h) and at 0.488 μg/L (96 h); and plasma complement activity declined at 4.88 μg/L comparing with 0.488 μg/L and control (96 h), and was lower at all concentrations at 96 h than at 24 h. Plasma K^+ levels were higher at 4.88 μg/L than in the remaining groups (24 and 96 h); and Na^+ levels decreased at 4.88 μg/L compared to control (96 h). Na^+/K^+-ATPase and H^+-ATPase activities in gills were lower at 4.88 μg/L comparing with control (24 h) and with the respective concentration at 96 h; and AChE activity in brain was higher at 0.488 and 4.88 μg/L than control (24 h) and the respective concentrations at 96 h, while in muscle it was higher at 0.488 and 4.88 μg/L than control (96 h) and the respective concentrations at 24 h. The present findings demonstrate that, despite IMY+IMI targets the animal-absent AHAS enzyme, such formulation displayed an acute toxic effect upon *R. quelen* homeostasis by impacting on vital functions such as immune defense, metabolism, ionoregulation and neurotransmission.

1. Introduction

The increasing demand for agriculture productivity has led to the indiscriminate application of agrichemicals worldwide (Narra, 2016). Only 0.1% of the used pesticides actually reaches the target organism, while the remaining results in contamination of the ecosystem (Carriger et al., 2006). Fish are likely to be targeted by such toxic compounds since water is a common element to most ecosystems and they can be found virtually everywhere in the

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aquatic environment, serving as sentinels for environmental biomonitoring (Weeks et al., 1992).

Imazapyr (IMY) and imazapic (IMI) are imidazolinone herbicides regularly used to limit weedy rice constraints in production systems, such as red rice (Rubin et al., 2014). Imidazolinone herbicides have been described as readily excreted before they can accumulate in animal blood or tissue (Gagne et al., 1991). Moreover, since the enzyme acetohydroxyacid synthase (AHAS), which is the target for these herbicides, is ubiquitously found in plants and microbes, they have been considered as *practically non-toxic* to some fish species (e.g., *Oncorhynchus mykiss* and *Lepomis macrochirus*) with a lethal concentration (LC_{50-96h}) > 100 mg/L (Gagne et al., 1991).

Nonetheless, a recent study assessing the toxicity of an imazethapyr+imazapic formulation (Only®) has reported enzymatic, metabolic and oxidative changes in *Cyprinus carpio* (Moraes et al., 2011). Then, although this class of herbicides is not subjected to bioaccumulation and is specifically toxic to plants, detrimental effects may arise from either acute or repeated exposure, since these synthetic compounds present physico-chemical features that permit them to be long-lived in the environment (Galon et al., 2014). In south Brazil, imazethapyr has been detected in surface/paddy water at a range of 0.05–0.113 μg/L, while imazapic was present at 0.007–0.085 μg/L (Silva et al., 2009). There is a paucity of information on the effects of rice herbicides of the imidazolinone class on fish, especially regarding an IMY+IMI formulation (Kifix®), which has not yet had its environmental occurrence assessed.

Hemato-biochemical variables are commonly used to investigate toxicity in fish exposed to anthropogenic toxic stressors like herbicides (van der Oost et al., 2003; Borges et al., 2007; Fonseca et al., 2008; Modesto and Martinez, 2010; Sharafeldin et al., 2015). Blood cells, hematocrit, proteins, glucose, steroidal hormones and enzymes such as transaminases are among the fish biomarkers which may be sensitive to water pollutants. Changes in some of these parameters may indicate endocrine disruption with hindering effects on reproduction, growth, behavior and disease resistance (van der Oost et al., 2003; Cericato et al., 2008,2009; Kreutz et al., 2011; Narra, 2016).

Because the immune system is quite complex and integrated with other organ systems and functions, it is particularly vulnerable to insult from xenobiotic stress (Weeks et al., 1992). An effective innate immune response is vital for the maintenance of the organisms' health upon exogenous burdens, hence the monitoring of its components in ecotoxicological investigations (Kreutz et al., 2011,2012; Danion et al., 2012).

The gills are the first organs in contact with the external milieu. Changes in the physico-chemical characteristics of water induce modifications in the gill epithelium cells, and important functions such as ionic transport are disturbed (Paulino et al., 2012). The gill enzymes Na^+/K^+-ATPase (NKA) and H^+-ATPase (HA), contained in the mitochondria-rich cells (MRC) and involved in K^+, Cl^- and Na^+ transport, play a major role in osmoregulation. The altered gill blood flow and paracellular permeability, which take place when fish experience environmental challenges, may influence the activity of these enzymes and thus disrupt ion balance and homeostasis (Vani et al., 2011; Paulino et al., 2012; Narra, 2016).

The activity of acetylcholinesterase (AChE) has been frequently studied as a biomarker in fish species exposed to herbicides (Golombieski et al., 2008,2009; Salbego et al., 2010; Moraes et al., 2011; Murussi et al., 2015; Narra, 2016). AChE is a versatile enzyme that rapidly hydrolyzes acetylcholine, a neurotransmitter with a key importance in the regulation of cognitive functions which is mainly found in the brain, cholinergic neurons and muscles (Pereira et al., 2013). Besides cholinergic neurotransmission, AChE is related to several non-cholinergic actions such as response to stressors and alteration in locomotion and orientation (Golombieski et al., 2008,2009; Salbego et al., 2010).

Silver catfish (*Rhamdia quelen*) is an omnivorous species which occurs from southern Mexico to central Argentina, and is widely cultivated in south Brazil where it is used in rice-fish culture (Marchezan et al., 2006). This species presents good growth, fertilization and hatching rates, and is well accepted by consumers, besides having a great ecological relevance and a key importance in the research field (Gomes et al., 2000; Baldisserotto, 2004). The present work aimed to assess, for the first time, the acute toxicity of an IMY+IMI herbicide in fish. The investigation was performed in laboratory-exposed *R. quelen* and analyzed parameters of hematology, biochemistry, immunology, ionoregulation and enzymology.

2. Materials and methods

2.1. Chemical

The commercial formulation of the herbicide (Kifix®-BASF, Brazil) used in the ecotoxicological trials is a mixture of IMY {(2-(4-isopropyl–4-methyl–5-oxo–2-imidazolin–2-yl)nicotinic acid)} and IMI {(2-(4-isopropyl–4-methyl–5-oxo–2-imidazolin–2-yl)–5-methylnicotinic acid} at 525 and 175 g/kg, respectively. Kifix® was added to the test water at 0.488 and 4.88 μg/L prior to the commencement of the tests. The lowest concentration was calculated based on the levels found for a similar herbicide (Only®) in surface waters of rice production areas in south Brazil (Silva et al., 2009). Then a tenfold higher concentration was also chosen to be used in the assays. The concentrations of each compound were determined in the water collected from the tanks at the beginning (0 h) and at the end of the experimental period (96 h) based on the methods proposed by Zanella et al. (2003) and Gonçalves et al. (2013). Compounds were determined by high-performance liquid chromatography with diode array detection (HPLC-DAD) after sample preparation via solid-phase extraction (SPE) using C18 (500 mg) and 100 mL of water sample with pH adjusted at 2.0. The analites were eluted with two aliquots of 1 mL of dichloromethane: methanol (65: 35, v/v), followed by solvent evaporation with a stream of nitrogen and dissolution in 500 μL of acetonitrile:water pH 3.0 (60:40, v/v). The HPLC-DAD analyzes were performed using column Synergi 4 μ Fusion RP−80 (250 × 4.6 mm, 4 μm), injection volume of 20 μL and detection at 254 nm. The absorption spectra were acquired from 200 to 350 nm for confirmation of the compounds (Table 1).

2.2. Fish maintenance and exposure method

Juvenile *R. quelen* (body net weight 165.3 ± 10.3 g and total length 25.7 ± 0.5 cm) were obtained from a fish culture near Santa

Table 1
Concentrations (μg/L) of imazapyr and imazapic in the water at 0 and 96 h after addition of an imazapyr+imazapic herbicide at 0.488 and 4.88 μg/L.

Samples	Imazapyr	Imazapic
0 h		
0.488	0.374 ± 0.06	0.102 ± 0.01
4.88	1.883 ± 1.15	0.739 ± 0.02
96 h		
0.488	0.264 ± 0.02	0.081 ± 0.01
4.88	1.912 ± 1.80	0.724 ± 0.03

Limit of detection: imazapyr – 0.015 μg/L; imazapic – 0.0075 μg/L. Limit of quantification: imazapyr – 0.05 μg/L; imazapic – 0.025 μg/L.

Maria (south Brazil) and transferred to the Fish Physiology Laboratory at the Federal University of Santa Maria (UFSM). Acclimation was performed in continuously aerated 250 L tanks for seven days under the following water quality parameters: 20.9 ± 0.03 °C, pH 6.7 ± 0.03 and dissolved oxygen 5.88 ± 0.42 mg/L. The fish were given commercial pellets to satiation once a day and feeding continued during the experimental period.

The animals were then allocated in 60 L tanks with permanent aeration and exposed for 96 h to one of the following three IMY+IMI herbicide concentrations (36 fish; 3 replicates/concentration; 4 fish/replicate): 0.0 (control), 0.488 and 4.88 μg/L. Water quality was monitored daily, and no changes were detected throughout the experimental period: 21.4 ± 0.05 °C, pH 7.5 ± 0.06, dissolved oxygen 6.5 ± 0.12 mg/L, total alkalinity 40 ± 1.2 mg/L $CaCO_3$, total hardness 20 ± 0.01 mg $CaCO_3$/L, total ammonia nitrogen 3.0 ± 1.0 mg/L, non-ionized ammonia 0.04 ± 0.02 mg/L and nitrite 0.18 ± 0.03 mg/L. Siphoning was performed 30 min after feeding with 30% of the water volume being replaced by herbicide solution. This protocol was approved by the Ethics Committee on Animal Experimentation of the UFSM (registration no. 067/2014).

After 24 h exposure and at the end of the experimental period (96 h), two fish from each tank (six fish per treatment at each given time, totaling 36 animals) were anesthetized with eugenol 50 mg/L (Cunha et al., 2010).

2.3. Hematological parameters

Blood was collected from the caudal vein using heparinized syringes. Whole blood parameters were evaluated using an automatic counter XS – 800i (Symex®): red blood cells (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), white blood cells (WBC) and thrombocytes (Trb). Blood smears were fixed in methanol and stained with Instant-Prov (NewProv®) for determination of the differential WBC count: lymphocytes, neutrophils, monocytes and eosinophils (at least 200 WBC were counted).

2.4. Biochemical parameters

Plasma was separated by centrifugation (3000 x g for 10 min at 4 °C) and biochemical parameters were evaluated in an automated Vitros 250 (Ortho-Clinical Diagnostics) using Johnson & Johnson kits (dry chemistry method). The analyzed variables were: total protein (TP), albumin (ALB), glucose (GLU), total bilirubin (TBI), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), high-density lipoprotein (HDL), total cholesterol (TC), urea (UR), uric acid (UA) and creatinine (CR). Plasma cortisol (COR) was determined in duplicate using an enzyme-linked immunosorbent assay (ELISA) kit (Diagnostics Biochem Canada Inc., Canada). Absorbance was estimated spectrophotometrically at 450 nm. The inter- and intra-assay variation coefficients were 5.15 ± 0.53% and 4.13 ± 0.67%, respectively. All tests were carried out in duplicate.

2.5. Innate immunological parameters

Bactericidal activity (BA) in fish plasma was evaluated using two strains of *Aeromonas hydrophila*: a clinical strain isolated from infected fish and ATCC 7966. The inoculum was prepared in saline solution from cultures grown in Müller-Hinton agar (Himedia Laboratories) {(1×10^8 colony forming units (CFU)/mL; 0.15 optical density (OD) at 600 nm) (30 °C/24 h)}. The test was performed in 96-well plates where 100 μL of fish plasma was added to 100 μL PBS, and then 10 μL (1×10^6CFU) of inoculum was added to each well. The OD of the samples was measured at 600 nm for 8 h/30 °C, at 1 h intervals. The percentage of BA was calculated by comparison between negative control (samples without bacteria) and positive control (samples without plasma).

Total plasma complement hemolytic activity (CHA) was determined as in Morales-de la Nuez et al. (2009) using rabbit red blood cells as target. The natural agglutination activity (AA) of fish plasma was investigated using "U"-shaped 96-well plates as described by Sutili et al. (2014).

2.6. Ionoregulatory parameters

Concentrations of K^+ and Na^+ were determined in diluted plasma samples against known standards with a B262 Micronal flame photometer. Plasma levels of Cl^- were assessed via the colorimetric method of Zall et al. (1956).

2.7. Enzymatic parameters

Following blood collection, the fish were euthanized by sectioning the spinal cord and the gills, brain and a portion of muscle were carefully excised, placed on ice and maintained at −20 °C for one week pending enzymatic analysis. The activities of Na^+/K^+-ATPase (NKA) and H^+-ATPase (HA) were measured simultaneously using the method described by Gibbs and Somero (1989), with some modifications. The gills (100 mg) were homogenized in 1 mL homogenization buffer (150 mM sucrose, 50 mM imidazole and 10 mM EDTA pH 7.5), and 5 μL homogenate and 200 μL reaction solution (30 mM imidazole, 45 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 0.4 mM KCN 1 mM ATP, 0.2 mM NADH, 0.1 mM fructose 1,6 diphosphate, and 2 mM phosphoenolpyruvate) were added to each sample. Ouabain (2 mM) and NEM (N-ethylmaleimide, 2 mM) were used as inhibitors. The rate of NADH oxidation was monitored every 10 s over 10 min at 340 nm, at room temperature. The slope difference in the rate of NADH oxidation versus time between reactions with solutions that were inhibitor-free versus inhibitor-enriched (ouabain and N-ethylmaleimide) was used to determine NKA and HA activities, respectively. Both enzyme activities have been reported as nmol ADP/mg protein/h. All assays were run in quadruplicate in a microplate. Acetylcholinesterase (AChE; E.C. 3.1.1.7) activity was measured as described by Ellman et al. (1961), adapted for microplate. Aliquots of supernatant (10 μL each of brain and muscle) were preincubated at 30 °C for 2 min with 0.1 Mphosphate buffer, pH 7.5, and 1 mM DTNB as chromogen. After 2 min, the reaction was initiated by the addition of 1 mM acetylthiocholine (ASCh) as substrate for the reaction mixture. The final volume was 300 μL. Absorbance was determined at 412 nm for 2 min. Enzyme activity was expressed as μmol of ASCh hydrolyzed/mg protein/min. The protein concentration for all enzymes was determined by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as a standard.

2.8. Data analysis

The homogeneity of variances between groups was tested with the Levene's test. Comparisons between different groups were made using two-way ANOVA and Tukey's test (Statistica 7.0 Software). The significance threshold was set at 0.05. All values are presented as the mean ± standard error of the mean (mean ± SEM, $n=6$).

3. Results and discussion

3.1. Hematological parameters

RBC was greater at 0.488 μg/L compared to 4.88 μg/L at 24 h and 96 h. Values of MCV were lower in the presence of the

Table 2
Hematological parameters in *Rhamdia quelen* subjected to an imazapyr+imazapic herbicide at 0 (control), 0.488 and 4.88 μg/L and sampled at 24 and 96 h of exposure.

Variables	24 h			96 h		
	0	0.488	4.88	0	0.488	4.88
RBC (10^6/μL)	1.64 ± 0.10^{ab}	1.98 ± 0.05^{a}	1.44 ± 0.08^{b}	1.61 ± 0.10^{ab}	1.94 ± 0.10^{a}	1.52 ± 0.10^{b}
Hb (g/dL)	6.2 ± 0.4	6.9 ± 0.3	5.6 ± 0.2	6.0 ± 0.2	7.0 ± 0.4	5.5 ± 0.3
Ht (%)	22.4 ± 1.1	24.8 ± 2.1	22.6 ± 2.7	22.4 ± 1.3	24.1 ± 1.4	20.3 ± 1.0
MCV (fl)	141.0 ± 2.0^{a}	118.9 ± 2.3^{b}	128.0 ± 1.3^{b}	139.4 ± 2.2^{a}	124.0 ± 2.8^{b}	128.2 ± 1.5^{ab}
MCHC (g/dL)	26.2 ± 0.9	28.7 ± 0.3	27.9 ± 1.3	26.7 ± 0.7	29.3 ± 0.3	28.3 ± 1.0
WBC (10^3/μL)	78.4 ± 26.9	96.4 ± 5.8	92.2 ± 13.5	78.1 ± 26.2	97.3 ± 12.2	93.2 ± 13.5
Lϕ (10^3/μL)	84.0 ± 4.5	82.0 ± 3.2	78.3 ± 4.3	84.6 ± 6.3^{a}	74.3 ± 2.0^{ab}	59.0 ± 6.5^{b}
Nϕ (10^3/μL)	11.0 ± 5.0	6.0 ± 1.0	12.0 ± 4.4	5.6 ± 2.3	1.67 ± 1.2	$1.0 \pm 1.0^{*}$
Mϕ (10^3/μL)	4.3 ± 0.9	11.3 ± 2.2	8.33 ± 0.3	9.0 ± 4.5^{a}	24.0 ± 1.5^{ab}	$33.3 \pm 6.8^{b*}$
Eϕ (10^3/μL)	0.67 ± 0.3	0.67 ± 0.6	1.3 ± 0.3	0.67 ± 0.6	0.0 ± 0.0	6.6 ± 6.6
Trb (10^3/μL)	33.8 ± 8.5	47.0 ± 9.4	43.0 ± 14.0	35.6 ± 9.3	47.5 ± 15.3	50.6 ± 17.4

RBC=red blood cells; Hb=hemoglobin; Ht=hematocrit; MCV=mean corpuscular volume; MCHC=mean corpuscular hemoglobin concentration; WBC=white blood cells; Lϕ=lymphocytes; Nϕ=neutrophils; Mϕ=monocytes; Eϕ=eosinophils; Trb=thrombocytes. Different letters indicate significant difference between concentrations at a given time.

* Significant difference between times at a given concentration.

compound at 24 h and in the group subjected to 0.488 μg/L for 96 h, both comparing with the respective control. Lymphocytes decreased at 4.88 μg/L in relation to control at 96 h, whereas heterophils count at 4.88 μg/L was lower at 96 than at 24 h, and monocytes count was higher at 4.88 μg/L both comparing to control group at 96 h and to 4.88 μg/L group at 24 h. Hemoglobin, hematocrit, MCHC, total leukocytes, eosinophils and thrombocytes were not affected by the experimental conditions (Table 2).

Blood parameters are widely used as sensitive indicators of fish health status upon exposure to toxic stressors (van der Oost et al., 2003; Modesto and Martinez, 2010; Vani et al., 2011; Gholami-Seyedkolaei et al., 2013). According to Narra (2016), pesticides negatively influence O_2 blood carrying capacity, which leads to anemia. The latter author verified reduced RBC count, Hb and Ht in *Clarias batrachus* exposed to herbicides at day 7, but not at 24 h. On the other hand, similar results were observed at 24 h in *C. carpio* (Gholami-Seyedkolaei et al., 2013) and *Capoeta damascina* (Shahbazi et al., 2015), and at 96 h in *Leporinus obtusidens* (Glusczak et al., 2006). The current work did not find differences regarding the referred indexes within 24 or 96 h, but there was a significant decrease in RBC from 0.488 to 4.88 μg/L Kifix at both sampling times. Such depletion was not extensive enough to result in lower Hb concentration and Ht level. However, it may illustrate that the higher concentration displayed an initial inhibitory effect on erythropoiesis, disturbance of the osmotic regulation and/or destruction of erythrocytes in hematopoietic organs (Vani et al., 2011; Narra, 2016). Furthermore, water pollutants may cause aggregation of RBC in fish gills, thus reducing their number in the circulation (Narain and Srivastava, 1989). Contrastingly, Modesto and Martinez (2010) described increased RBC and Ht in *Prochilodus lineatus* exposed to a glyphosate-based herbicide (96 h), suggesting that it may have resulted from the release of RBC from blood deposits and/or from hemopoetic tissues as an adaptive response to the stressing agent. The reduced MCV values observed for the Kifix-exposed *R. quelen* at both concentrations (24 h) and at the lower concentration (96 h) may indicate that the RBC system reacted to the toxicant with anemia of the microcytic normochromic type, since MCHC remained within the reference interval (control) (Witeska, 2005).

Changes in the immune cellular composition of blood are usually the first physiological disturbances described in organisms exposed to environmental stressors (Weeks et al., 1992). Kifix, nonetheless, did not have an adverse effect on *R. quelen* WBC count. Differently, *R. quelen* (Kreutz et al., 2011), as well as other catfishes (Jeyapriya et al., 2013; Shahbazi et al., 2015), presented leukopenia following acute herbicide exposure.

In consistence with Kreutz et al. (2011) and Dal'Bó et al. (2015), lymphocytes were the most frequent WBC observed in control *R. quelen* in the IMY+IMI trials. Despite the unchanged WBC number, differential counts of organic defense blood cells revealed that exposure Kifix dwindled the number of lymphocytes in *R. quelen*. The same acute response was observed for glyphosate-exposed *R. quelen* (Kreutz et al., 2011) and *C. carpio* (Gholami-Seyedkolaei et al., 2013). Fish which are subjected to toxic stress are bound to present lymphopenia, which is the most consistent finding in a stress leukogram and may be due to cortisol-induced apoptosis, decreased efflux from lymph nodes and lymphotoxic effects (Witeska, 2005). However, lymphocytosis has also been described in fish subjected to herbicides (Modesto and Martinez, 2010; Jeyapriya et al., 2013; Narra, 2016). Toxic stress is said to boost lymphopoiesis and/or improve the release of lymphocytes from lymphomyeloid tissue, which is an adaptive response that makes the immune defense response more effective (Jeyapriya et al., 2013; Narra, 2016).

Neutrophils and monocytes were the second and third most common leukocytes, respectively, in control *R. quelen* at 24 h, in agreement with Dal'Bó et al. (2015). Kreutz (et al., 2011), in turn, identified a slight predominance of monocytes over neutrophils. As well as in the current work, Modesto and Martinez (2010) similarly observed neutropenia in *P. lineatus* after 96 h herbicide-exposure (glyphosate), which is not a pattern observed in a stress leukogram (Witeska, 2005). On the other hand, the monocytosis reported in Kifix-exposed *R. quelen* is a typical effect of stress, and results from the migration of monocytes, which participate in phagocytosis, from the marginal compartment into circulation (Bush, 2004). Jeyapriya et al. (2013) observed increased neutrophils count in the catfish *Catla catla* at day 5 of exposure to monocrotophos, and no alteration in monocytes. Following acute exposure of *R. quelen* to glyphosate, neutrophils and monocytes counts remained unchanged (Kreutz et al., 2011).

Eosinophils were the least present WBC in *R. quelen* circulating blood, in accordance with Dal'Bó et al. (2015). These cells, which have a role in the inflammatory process, were not affected by the presence of IMY+IMI, in agreement with previous findings in *P. lineatus* after acute exposure to glyphosate (Modesto and Martinez, 2010). Eosinopenia, typically observed as a stress-induced response, was seen in *C. catla* after 5 days of exposure to monocrotophos (Jeyapriya et al., 2013).

Thrombocytes, which are required for hemostatic plug or fibrin clot, besides being important blood defense cells (Hill and Rowley,

Table 3
Biochemical parameters in *Rhamdia quelen* subjected to an imazapyr+imazapic herbicide at 0 (control), 0.488 and 4.88 μg/L and sampled at 24 and 96 h of exposure.

Variables	24 h			96 h		
	0	0.488	4.88	0	0.488	4.88
TP (g/dL)	4.3 ± 0.1	4.3 ± 0.2	4.0 ± 0.2	3.5 ± 0.1	3.2 ± 0.5	3.9 ± 0.2
ALB (g/dL)	1.5 ± 0.06	1.6 ± 0.11	1.4 ± 0.08	1.3 ± 0.06	1.3 ± 0.18	1.4 ± 0.09
GLU (mg/dL)	26.5 ± 6.5	14.6 ± 2.5	29.0 ± 5.5	18.0 ± 2.0	18.4 ± 3.9	20.6 ± 2.9
TBI (mg/dL)	0.33 ± 0.08	0.98 ± 0.21	0.41 ± 0.13	0.68 ± 0.14	0.88 ± 0.39	1.03 ± 0.36
ALT (U/L)	19.8 ± 3.7	29.8 ± 4.3	30.0 ± 1.9	24.0 ± 4.5	47.6 ± 2.6	27.0 ± 0.1
AST (U/L)	182.33 ± 4.7	261.66 ± 3.8	188.66 ± 3.0	189.0 ± 5.0[a]	501.5 ± 11.5[b*]	295.0 ± 26.0[ab]
ALP (IU/L)	16.5 ± 4.5	14.6 ± 4.6	20.7 ± 1.5	9.3 ± 0.2	9.0 ± 0.01	9.2 ± 0.01
TC (mg/dL)	209.0 ± 5.0	190.83 ± 5.0	167.0 ± 1.0	174.3 ± 1.2	186.6 ± 2.9	148.5 ± 2.5
HDL (mg/dL)	132.83 ± 1.2	124.83 ± 1.6	127.66 ± 1.1	120.0 ± 3.3	118.6 ± 1.6	97.6 ± 3.1
UR (mg/dL)	2.26 ± 0.1	3.25 ± 0.4	2.31 ± 0.2	2.2 ± 0.01	2.6 ± 0.3	2.1 ± 0.06
UA (mg/dL)	0.25 ± 0.02	0.25 ± 0.02	0.23 ± 0.02	0.26 ± 0.02[ab]	0.30 ± 0.03[a]	0.20 ± 0.01[b]
CR (mg/dL)	0.07 ± 0.01	0.10 ± 0.01	0.08 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
COR (ng/mL)	151.98 ± 7.2	150.28 ± 6.0	139.30 ± 9.0	175.40 ± 10.4[a]	163.20 ± 5.1[a]	130.90 ± 8.0[b]

TP=total protein; ALB=albumin; GLU=glucose; TBI=total bilirubin; ALT=alanine aminotransferase; AST= aspartate aminotransferase; ALP=alkaline phosphatase; TC=total cholesterol; HDL=high-density lipoprotein; UR=urea; UA=uric acid; CR=creatinine; COR=cortisol. Different letters indicate significant difference between concentrations at a given time.

[*] Significant difference between times at a given concentration.

1996), reduced in *R. quelen* acutely subjected to glyphosate (Kreutz et al., 2011). Blood clotting may be enhanced upon stress, but Trb number will not necessarily rise (Witeska, 2005).

3.2. Biochemical parameters

Values of AST increased at 0.488 μg/L after 96 h exposure both in relation to control and to 0.488 μg/L at 24 h. Uric acid was lower in the fish subjected to 4.88 μg/L than in those maintained at 0.488 μg/L for 96 h. Cortisol levels were lower at 4.88 μg/L than in the remaining groups exposed to the herbicide for 96 h. Glucose, albumin, total bilirubin, total protein, ALP, HDL, total cholesterol, creatinine, urea and ALT remained unchanged throughout the experimental period (Table 3).

The enzymes ALT and AST, usually considered together, are reliable biomarkers in short-term water pollution assessments, being sensitive indicators of tissue damage and cell rupture (van der Oost et al., 2003). Gholami-Seyedkolaei et al. (2013) have described the presence of these enzymes in liver, heart, skeletal muscle, kidney, pancreas, spleen, erythrocyte, brain and gills of *C. carpio*, and reported their elevation upon acute exposure to glyphosate. The authors explained that the herbicide may have accumulated in some of these sites and thus the enzymes were released into the blood stream. A similar event may have occurred with the IMY+IMI herbicide, evidenced by the rise in AST levels. *Rhamdia quelen* acutely subjected to cypermethrin and glyphosate also had increased AST plasma levels (Borges et al., 2007; Loro et al., 2015). AST and ALT increased after acute exposure of *Clarias garipinus* to dithiopyr (Attia and El-Badawi, 2015) and of *Oreochromis niloticus* to profenofos (Sharafeldin et al., 2015), while enhanced ALT has been described for *Pseudoplatystoma reticulatum* x *P. corruscans* exposed to glyphosate (Sinhorin et al., 2014). Pesticide stress has been accountable for inducing elevation of the transamination pathway, with amino acid input into the Krebs cycle to cope with the energy crisis (Philip and Rajasree, 1996).

The levels of UA in *Anguilla anguilla* presented a decline after 96 h-exposure to diazinon (Cerón et al., 1996), as it was seen in *R. quelen* under the effect of Kifix for the same period of time. In opposition, acute exposure of *C. garipinus* to dithiopyr (Attia and El-Badawi, 2015) and of *O. niloticus* to profenofos (Sharafeldin et al., 2015) resulted in increased UA levels. Waste proteins are broken down and excreted from the body in a variety of forms, such as UA (Phillips, 2004). When above normal values, this metabolite is considered an indicator of kidney dysfunction (Sharafeldin et al., 2015).

Cortisol is the most studied biomarker in order to verify primary stress response, being released as a result of the stimulatory effect of the adrenocorticotropic hormone (ACTH) in order to help fish maintain homeostasis (Barton, 2002). Cericato et al. (2008) observed that acute exposure to atrazine+simazine caused a rise in *R. quelen* COR, while methyl-parathion did not. However, both herbicides impaired *R. quelen* capacity of displaying a COR response to cope with an acute stress stimulus. A subsequent study confirmed that methyl-parathion-exposed *R. quelen* had a diminished ability to respond to ACTH, thus demonstrating endocrine disruption (Cericato et al., 2009). A similar outcome was seen in *Danio rerio* (Rosa et al., 2013). Fish that are environmentally contaminated with herbicides may present a reduced ability of mounting a normal COR response, thus becoming incapable of making suitable metabolic and ionic adjustments to cope with daily challenges (Rosa et al., 2013). There were also no differences in COR levels upon acute exposure of *P. lineatus* to glyphosate (Langiano and Martinez, 2008). Likewise, *R. quelen* in the Kifix-trials did not display elevated COR upon herbicide exposure. As suggested by Gesto et al. (2008), the herbicide may have promoted ACTH desensitization.

Increased TP after acute herbicide exposure has been reported in *L. obtusidens* (Fonseca et al., 2008), while decreased levels have been described in *R. quelen* (Borges et al., 2007) and *P. reticulatum* x *P. corruscans* (Sinhorin et al., 2014). Sharafeldin et al. (2015) also reported decreased TP as well as increased ALB in *O. niloticus*. Narra (2016) showed that herbicide-treated *C. batrachus* experienced a decrease in both TP and ALB, and explained that the outcome may represent a means of obtaining energy to surpass the stress, alteration in protein synthesis or increased denaturation, or an immunotoxic effect of the herbicides. The latter author also described hyperglycemia, suggesting enhanced glycogenolysis and reduction of glycolytic pathway. The typical stress-induced GLU elevation was also seen in other short-term toxicity tests (Borges et al., 2007; Langiano and Martinez, 2008; Attia and El-Badawi, 2015; Sharafeldin et al., 2015). Differently, Loro et al. (2015) found lower GLU in *R. quelen* and *L. obtusidens* after a 96h-exposure to glyphosate, and suggested that it may have resulted from the prompt use of this source of energy since the fish became hyperexcited under herbicide stress. A similar finding has been described for *L. obtusidens* exposed to 2,4-D for 96 h (Fonseca et al., 2008). The unchanged concentration of the waste product TBI in *R. quelen* indicates that Kifix did not cause hemolysis or liver

dysfunction (Mattsoff and Oikari, 1987). Elevation in ALP following acute exposure to profenofos has been reported in *O. niloticus* (Sharafeldin et al., 2015). Synthesis of ALP occurs in the intestinal epithelium, kidney, and liver, and is often increased in response to a biliary obstruction. In contrast, ALP decreased as exposure time increased (total 16 days) in glyphosate-treated *C. carpio* (Gholami-Seyedkolaei et al., 2013). The levels of TC decreased in *P. reticulatum* x *P. corruscans* (Sinhorin et al., 2014) and *O. niloticus* (Sharafeldin et al., 2015), but increased in *C. garipinus* (Attia and El-Badawi, 2015) and *R. quelen* (Borges et al., 2007) acutely exposed to pesticides. Such compounds may accumulate in liver and disrupt lipid metabolism, as well as affect permeability of hepatocytes, thus causing changes in TC and HDL (Yousef et al., 2003). One of the primary forms fish dispose of nitrogenous waste is UR (Phillips, 2004). Some works have shown an increase in this metabolite following acute exposure to pesticides (Borges et al., 2007; Sharafeldin et al., 2015). Another waste product is CR, which results specially from muscle breakdown. A rise in its levels indicates impairment of kidney function or structure, since its excretion is dependent on glomerular filtration (Sharafeldin et al., 2015). Such response was seen in some fish species upon acute herbicide exposure (Borges et al., 2007; Attia and El-Badawi, 2015; Narra, 2016).

3.3. Innate immunological parameters

At 24 h, Kifix-exposed *R. quelen* presented reduced BA against both strains of *A. hydrophila* at 0.488 μg/L, and against the clinical isolate at 4.88 μg/L, compared with control. At 96 h, control fish had greater BA against ATCC than those in 24 h-control, and at 0.488 μg/L there was a decline in the activity against ATCC in relation to the remaining ATCC groups at the same time point. Furthermore, *R. quelen* kept at 4.88 μg/L presented the lowest CHA among all groups at 96 h, and the groups assessed at the latter time point had lower CHA compared with each respective group at 24 h (Fig. 1). There was no AA of the bacterial cell and titer was not verified in the plasma agglutination assay.

When assessing the natural immune system of *R. quelen* treated with glyphosate for 24 h, Kreutz et al. (2011) did not verify any effect of the compound upon BA or CHA, but AA reduced in the exposed fish. A subsequent study on the same fish species also observed no effect of atrazine on CHA, but the chemical induced a decline in BA and AA after 24 h (Kreutz et al., 2012). A more prolonged assessment (28 days) identified a reduction in CHA in pendimethalin-exposed *O. mykiss* (Danion et al., 2012), in keeping with the present findings. The results described herein suggest that IMY+IMI, as well as glyphosate, atrazine and pendimethalin, may exert an immunosuppressive effect on *R. quelen* innate immune elements. Fish rely on a consistent non-specific humoral immune system to overcome infection by a wide range of microorganisms, and the immunodeficiency induced by the agrichemicals makes the fish prone to opportunistic pathogens (Kreutz et al., 2011, 2012).

3.4. Ionoregulatory parameters

Plasma K^+ levels were greater at 4.88 μg/L Kifix (24 and 96 h), while Cl^- remained unchanged and Na^+ reduced at the highest concentration of the herbicide (96 h) (Fig. 2). Borges et al. (2007) described elevated K^+ and Na^+ when the same species was acutely subjected to cypermethrin. In *P. lineatus*, acute exposure to atrazine did not affect Na^+ and Cl^- levels (Paulino et al., 2012), while glyphosate caused a slight reduction in Cl^- levels at 24 h, which was counterbalanced at 96 h (Langiano and Martinez, 2008). Changes in the gill epithelium following contact with pesticides may affect MRC density and/or function, and thus interfere

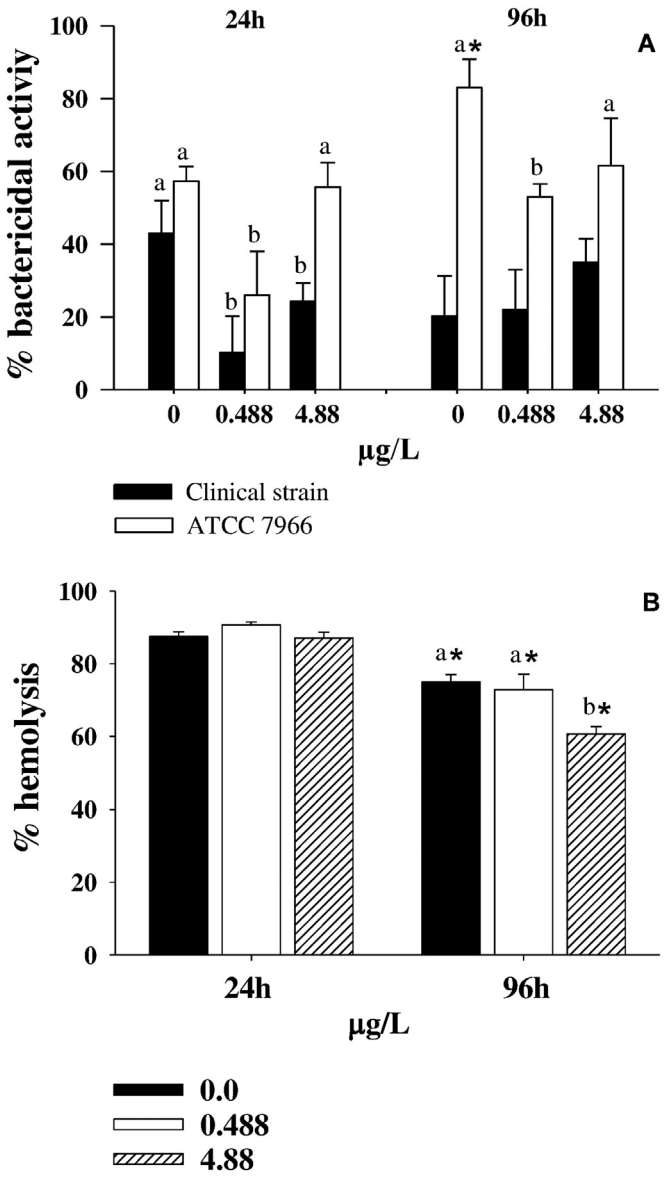

Fig. 1. Plasma bactericidal activity (A) against the fish pathogen *Aeromonas hydrophila* (clinical isolate and ATCC 7966), and total plasma complement hemolytic activity (B) in *Rhamdia quelen* subjected to an imazapyr+imazapic herbicide (24 and 96 h) at 0 (control), 0.488 and 4.88 μg/L. Different letters indicate significant difference between concentrations at a given time. (*) indicates significant difference between times at a given concentration.

with ion homeostasis (Paulino et al., 2012).

3.5. Enzymatic parameters

The fish maintained at 4.88 μg/L showed a significant inhibition in Na^+/K^+-ATPase activity comparing to the respective control at 24 h, and the enzyme activity was higher in both herbicide-exposed groups at 96 h than in the respective concentrations at 24 h. The activity of H^+-ATPase was also lower at 4.88 μg/L in relation to control at 24 h, and at 96 h the fish kept at 4.88 μg/L presented an induced activity of the enzyme compared to those exposed to the same concentration for 24 h (Fig. 3).

The enzymes NKA and HA are expressed in the MRC, where they play a crucial role in ion transport, and their activity level may indicate the pattern of ionoregulatory performance under certain environmental challenges (Paulino et al., 2012). No changes were verified in *P. lineatus* NKA activity following atrazine

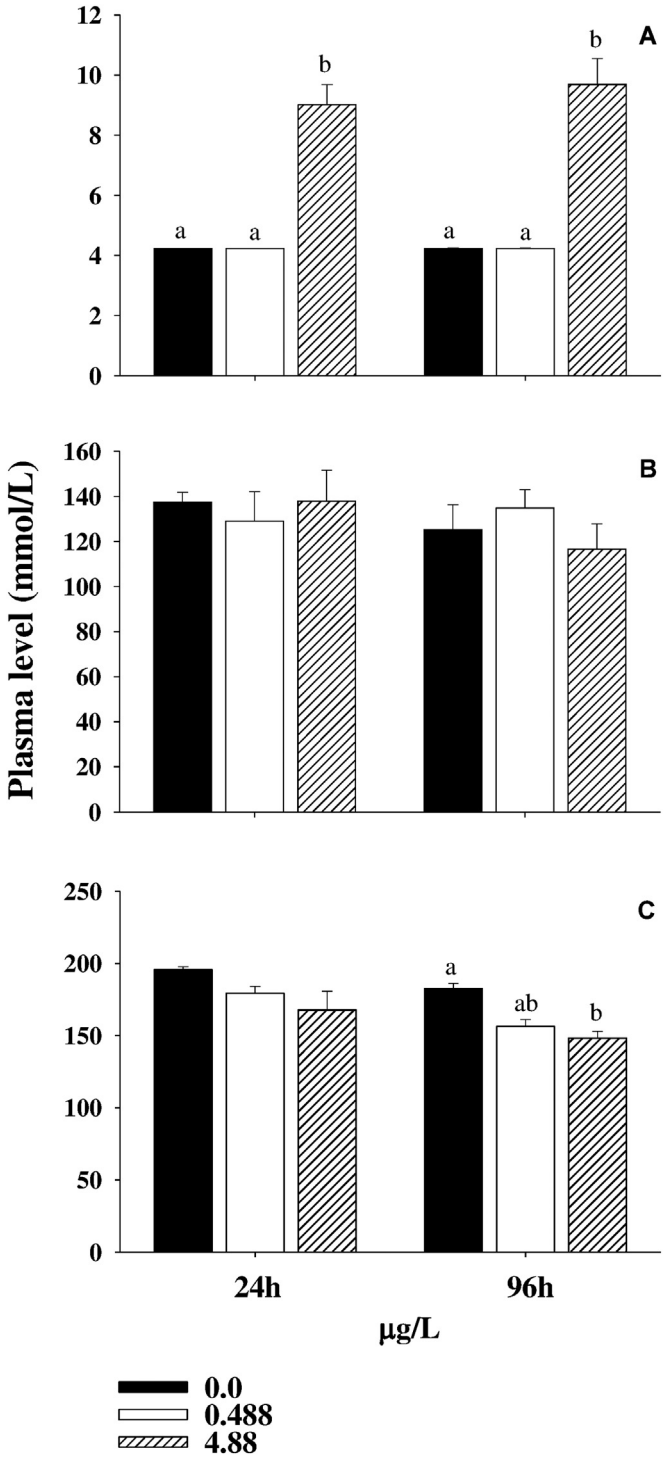

Fig. 2. Plasma levels of K^+ (A), Cl^- (B) and Na^+ (C) in *Rhqmdia quelen* subjected to an imazapyr+imazapic herbicide (24 and 96 h) at 0 (control), 0.488 and 4.88 µg/L. Different letters indicate significant difference between concentrations at a given time. (*) indicates significant difference between times at a given concentration.

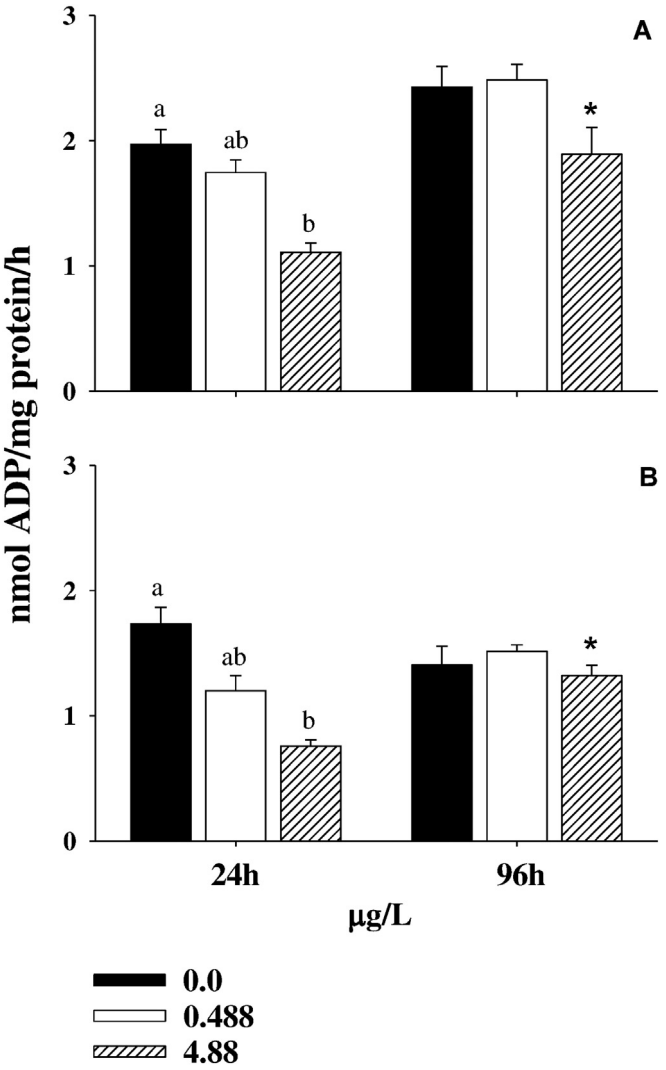

Fig. 3. Na^+/K^+-ATPase (A) and H^+-ATPase (B) in gills of *Rhamdia quelen* subjected to an imazapyr+imazapic herbicide (24 and 96 h) at 0 (control), 0.488 and 4.88 µg/L. Sampling was performed at 24 and 96 h of exposure. Different letters indicate significant difference between concentrations at a given time. (*) indicates significant difference between times at a given concentration.

exposure, although the herbicide induced MRC death at 48 h (Paulino et al., 2012). On the contrary, reduced NKA activity has been described in *C. catla* exposed to deltamethrin by Vani et al. (2011), as well as in *C. batrachus* exposed to single and cartel herbicides by Narra (2016), who stated that pesticides may directly interfere with NKA and its lipophilic metabolites. In agreement with the latter findings, 4.88 µg/L Kifix inhibited NKA activity at 24 h in *R. quelen*. Nonetheless, at 96 h the enzyme activity at the mentioned concentration was significantly higher than at the first time point, which may suggest an adaptation of the gill structure after a more prolonged herbicide exposure. The exact same response pattern was observed for HA (Fig. 3). Thus, the changes induced by Kifix upon NKA and HA activities at 24 h were not severe enough in order to promote reduction in the ions levels.

In silver catfish brain, AChE activity was higher in fish exposed to the herbicide than in control group at both sampling times, and the enzyme activity of the herbicide-exposed fish reduced from 24 to 96 h. In muscle, the activity of AChE was higher at both concentrations of the herbicide at 96 h compared to the respective control and to the respective concentrations at 24 h (Fig. 4).

The enzyme AChE accumulates in the synaptic cleft when pesticides interfere with its normal function, determining over stimulation and disruption of nerve impulses which culminates with ataxia, loss of equilibrium, aberrant swimming, central respiratory paralysis, seizures, coma and ultimately death. Herbicide-induced AChE inhibition has been extensively documented in gills, brain, liver and muscle of various fish species (Fonseca et al., 2008; Golombieski et al., 2008,2009; Modesto and Martinez, 2010; Salbego et al., 2010; Moraes et al., 2011; Vani et al., 2011; Gholami-Seyedkolaei et al., 2013; Pereira et al., 2013; Narra, 2016), including

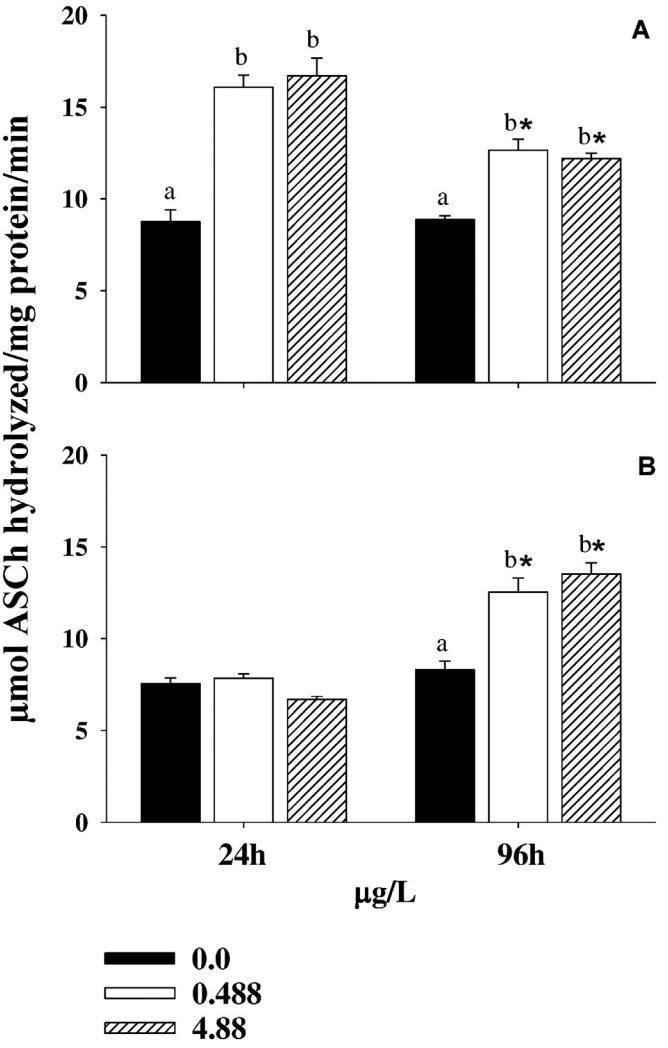

Fig. 4. Acetylcholinesterase activity in brain (A) and muscle (B) of *Rhamdia quelen* subjected to an imazapyr+imazapic herbicide (24 and 96 h) at 0 (control), 0.488 and 4.88 µg/L. Different letters indicate significant difference between concentrations at a given time. (*) indicates significant difference between times at a given concentration.

R. quelen (Miron et al., 2005; Glusczak et al., 2007; Murussi et al., 2015).

The activation of AChE upon herbicide contamination, as seen in the present work, is less well described than its inhibition. This response pattern was observed in acute-exposure trials in *R. quelen* brain (quinclorac and metsulfuron-methyl) (Miron et al., 2005), *Aristichthys nobilis* and *C. carpio* brain (metsulfuron-methyl) (Golombieski et al., 2009), and *C. carpio* and *R. quelen* muscle (clomazone) (Murussi et al., 2015). A long-term study, 30 days, also detected enhanced AChE activity in muscle, brain and spinal cord in *Anabas testudineus, Heteropneustes fossilis* and *O. niloticus* under the effect of the herbicide Almix (Samanta et al., 2014). AChE activity similarly increased in muscle of *L. obtusidens* subjected to a longer exposure time, 90 days, to glyphosate (Salbego et al., 2010). The only investigation assessing the effect of imidazolinone herbicides (imazethapyr+imazapic) upon AChE activity in fish (*C. carpio*) sampled the animals at day 7 (laboratory and rice field conditions) and at days 30 and 90 (only field), and found diverse results. At day 7, the activity of the enzyme was induced in brain (laboratory and field) but inhibited in muscle (laboratory). At day 30, it declined in brain and increased in muscle. At day 90, it was inhibited in muscle (Moraes et al., 2011). The induction of AChE activity in the Kifix-exposed *R. quelen* may suggest enhanced hydrolysis of the neurotransmitter acetylcholine, which consequently reduces activation of nicotinic and muscarinic receptors, as proposed by Golombieski et al. (2009). Nonetheless, more insights into the effects of AChE activation remain to be clarified (Salbego et al., 2010; Moraes et al., 2011; Murussi et al., 2015).

4. Conclusion

This study aimed to assess, for the first time, the acute toxicity of an IMY+IMI herbicide using a fish species as a bioindicator. Results indicated that the formulation had a potential toxic effect on parameters of hematology, biochemistry, immunology, ionoregulation and enzymology. Such response suggests that IMY+IMI posed a risk to *R. quelen* homeostasis, displaying immune depressant and neurotoxic effects, among others, despite targeting the animal-absent AHAS enzyme. Environmental herbicide contamination is an ongoing matter of concern, and novel evidence, as the one provided in the present investigation, highly contribute to future actions towards a tighter regulation of crop production methods.

Conflict of interest

The authors declare that there are no conflicts of interest.

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