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RESEARCH ARTICLE

Effects of the emulsifiable herbicide Dicamba on amphibian tadpoles: an underestimated toxicity risk?

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Abstract

The effects of exposure to the herbicide Dicamba (DIC) on tadpoles of two amphibian species, *Scinax nasicus* and *Elachistocleis bicolor*, were assessed. Mortality and biochemical sublethal effects were evaluated using acetylcholinesterase (AChE), glutathione *S*-transferase (GST), glutathione reductase (GR), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) activities and thyroid hormone (T4) levels. The LC_{50} value at 48h was 0.859 mg L^{-1} for *S. nasicus* and 0.221 mg L^{-1} for *E. bicolor* tadpoles. After exposure to sublethal DIC concentrations for 48 h, GST activity increased in *S. nasicus* but significantly decreased in *E. bicolor* with respect to controls. GR activity decreased only in *S. nasicus* at all the tested DIC concentrations. AChE activity was significantly inhibited in both *S. nasicus* and *E. bicolor* tadpoles at 48 h. DIC also caused significant changes in transamination, as evidenced by an increase in AST and ALT activities in both amphibian species. T4 levels were higher in DIC-treated tadpoles of both species than in controls. The DIC-induced biochemical alterations in glutathione system enzymes and transaminases indicate lesions in liver tissues and cellular function. Moreover, the observed AChE inhibition could lead to the accumulation of acetylcholine, excessively stimulating postsynaptic receptors, and the increase in T4 levels in both species may indicate an overactive thyroid. The commercial DIC formulation showed a high biotoxicity in the two amphibian native species after short-term exposure, controversially differing from the toxicity level indicated in the official fact sheet data. This fact highlights the need for an urgent re-categorization and reevaluation of DIC toxicity in native species.

Keywords Dicamba · Herbicide · Tadpoles · Biomarker · Antioxidant systems · Acetylcholinesterase · Transaminase · Thyroid hormone

Introduction

Pesticides damage the ecosystem and the health of organisms via the accumulation of risky substances (Geiger et al. 2011; Rasheed et al. 2019). Dicamba (DIC), a post-emergence broad-leaf benzoic acid (3,6-dichloro-2-methoxybenzoicacid) herbicide globally used, is the third most widely applied in Argentina (CASAFE 2019). This herbicide was developed because weeds acquired resistance to glyphosate and is widely used on lawns, grasslands, and several crops (maize, rice, cotton). DIC-resistant soybean and cotton seeds have been recently launched to the market. The commercialization of DIC-tolerant soybean seeds has been recently allowed by the Secretariat of Food and Bio-economy of the Ministry of Agro-industry (Resolution N° 30/2018) in

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Argentina. However, the toxic effects of DIC on wildlife have been scarcely explored (Ruiz de Arcaute et al. 2020).

Pesticides are among the several causes that contribute to the decrease in amphibian populations worldwide (Suarez et al. 2016). In addition, the biological traits of amphibians make them sensitive to low levels of pesticide pollution in water; therefore, they are among the vertebrates most threatened by anthropogenic activities (Chanson et al. 2008; Bishop et al. 2012). Since the application of pesticides to field crops usually coincides with the aquatic larval stage in spring and summer, pesticides cause deleterious effects on amphibian survival and metamorphosis (Peltzer et al. 2008). For this reason, several researchers have performed toxicological studies using amphibian as indicator vertebrates (Sánchez-Bayo and Goka 2012). Studies focusing on native freshwater organisms can be helpful to identify potential risks to sympatric species and may provide ecological knowledge about the ecosystem health (Ossana et al. 2013; Attademo et al. 2014). The biotransformation of non-polar xenobiotic agrochemicals in freshwater organisms generally involves phase-I and phase-II enzymatic reactions, which make compounds more water soluble for excretion (Brodeur et al. 2011; Colin et al. 2016). Phase-II reactions involve the addition of endogenous polar compounds (e.g., glucuronyl sugars or glutathione) to the original xenobiotic or its metabolites generated in phase-I reactions; thus, specific enzymes such as glutathione-*S*-transferase (GST, EC 2.5.1.18) are fundamental. The biotransformation of xenobiotics may also contribute to the generation of reactive oxygen species (ROS), i.e., molecules able to trigger oxidative stress. Many herbicides have also been shown to produce oxidative cellular damage by inducing redox cycling (Lajmanovich et al. 2012; Nikoloff et al. 2014; Pérez-Iglesias et al. 2017). Oxidative stress is caused by an imbalance between ROS production and the ability of an organism to detoxify them. This process may cause the formation of free radicals and the consequent DNA damage (Costa et al. 2008).

In organisms exposed to environmental contaminants, like amphibians, the antioxidant system, composed of low-molecular-weight enzymes and compounds such as glutathione reductase (GR, EC 1.8.1.7) (Livingstone 2001), is significantly altered. Therefore, these enzymes are commonly used as biomarkers, providing evidence of biotoxicity due to contaminant exposure in aquatic vertebrates (Van der Oost et al. 2003; Freitas et al. 2017; Gupta 2018).

The toxicity of xenobiotic compounds, such as herbicides, to amphibian tadpoles can also be studied by evaluating the activities of *B*-esterases (Lajmanovich et al. 2013, 2014), particularly acetylcholinesterase (AChE, Sánchez-Hernández 2007) which is considered a common biomarker to assess neurotoxic effects. AChE plays a key role in the neuromuscular system, preventing continuous nerve firings by hydrolyzation of acetylcholine at nerve endings (Jebali et al. 2013). In addition, the enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are involved in the metabolism of amino acids and, therefore, allow the strategic link of both protein and carbohydrate metabolisms; their activities are considered sensitive indicators of oxidative stress, since their increased levels may indicate tissue damage under toxic stress (Prashanth and Neelagund 2008). AST is a bilocular (cytoplasmic and mitochondrial) enzyme, whereas ALT is a unilocular (cytoplasmic) enzyme; the highest activity of both enzymes occurs in the hepatic tissue. Thus, their alteration under several physiological and pathological conditions (Li et al. 2012) indicates tissue damage in organs like the liver and kidney (Loteste et al. 2013).

Finally, it has been shown that thyroid hormones (THs), which are fundamental for metamorphosis in amphibians (Denver 2009), can be altered by a wide variety of chemicals (Miyata and Ose 2012). However, data on the chemical disruption of thyroid signaling in neotropical amphibians are scarce (Lajmanovich et al. 2019a). In tadpoles, alterations in the TH balance can lead to functional and structural changes in larval morphology, like heart defects and notochord malformations (Miyata and Ose 2012).

The response of enzymatic activities to DIC exposure in amphibian species is unknown; hence, the main objective of this study was to evaluate the acute and sublethal effects of DIC exposure on tadpoles of two anuran species, *Scinax nasicus* (Hylidae) and *Elachistocleis bicolor* (Leptodactylidae), commonly occurring in agricultural ponds of Argentina (Peltzer et al. 2006). To this end, the activities of AChE, GST, GR, AST, and ALT and the T4 levels were evaluated. This information may improve our knowledge about liver injuries and oxidative stress induced by a herbicide.

Materials and methods

Test organisms

S. nasicus and *E. bicolor* tadpoles were selected for this study because they are extensively distributed in forests, wetlands, agricultural lands, and riparian and urban areas of several neotropical countries, including Argentina, Brazil, Bolivia, Paraguay, and Uruguay. In the Red List of amphibians of Argentina, these species are listed as "not threatened" (Vaira et al. 2012). In agricultural lands, these anurans are likely exposed to contamination by pesticides during the breeding season and early developmental stages (Peltzer et al. 2006). Regarding their ecological habits, *S. nasicus* is nektonic and *E. bicolor* is benthonic-nektonic (Peltzer and Lajmanovich 2007).

We collected pro-metamorphic larvae (Gosner stages -GS-30-34; Gosner 1960) of both species from a non-agricultural site, located in a natural area of the city of Santa Fe (Santa Fe

Province, Argentina), with collection permission of the Ministry of Environment of Santa Fe (File N° 02101-0018518-1). Larvae were then transferred to aquaria of the Laboratory of Ecotoxicology of the School of Biochemistry and Biological Sciences of the Universidad Nacional del Litoral, Santa Fe, Argentina (FBCB-UNL), where they were acclimated to the following laboratory conditions: 12-h light/dark cycle with dechlorinated tap water, pH 7.4 ± 0.05, conductivity 162 ± 10.5 μmhos cm^{-1}, dissolved oxygen concentration 6.5 ± 1.5 mg L^{-1}, hardness 47.5 mg L^{-1} of $CaCO_3$, and temperature of 24 ± 2°C. The organisms used in the assays were treated in agreement with the standardized experimental laboratory protocols of the ASIH-American Society of Ichthyologists and Herpetologists (2004) and ASTM (2007), with minimal adjustments for local species, as previously described (Attademo et al. 2014). Specimens were euthanized according to the Animal Euthanasia Guide proposed by the Bioethics Committee and Institutional Animal Care and Use Committee of the FBCB-UNL (Res. CD N: 388/06) using a solution of 0.1% tricaine methanesulfonate (MS-222) buffered to pH 7.8 with $NaHCO_3$.

Dicamba determination

The commercial formulation used was the herbicide DIC Cowboy Elite SURCOS® (CAS 1918-00-9, 20% active ingredient). The safety criterion of DIC was class II (WHO (World Health Organization) 2019). In experimental assays, pesticides are usually assayed with the active ingredient; however, in the field, this herbicide is generally applied as a formulation, with active ingredients being usually combined with additives (Relyea 2009). These additives can increase the toxicity of the herbicide or improve its solubility (Eddleston et al. 2012). DIC is a post-emergent selective herbicide, with a recommended application field rate of 100–200 cm^3/ha. To confirm the concentration of the DIC commercial formulation and the nominal concentrations used in each treatment, we used an ultra-performance liquid chromatography (ACQUITY UPLC™, Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer (Micromass TQ Detector from Waters, Manchester, UK). We also evaluated features related to the chromatographic methodology, including the mobile phase composition, the ionization conditions, and the operating variable detection in multiple-reaction monitoring mode of the mass spectrometer. Separation was achieved in a rapid resolution column (C18, 2.1 × 100 mm, 1.7 μm) by means of a gradient elution, with a mix mobile phase of acetonitrile and water, both with 0.1% (v/v) formic acid. For mass detection, in addition to the retention time, two transitions from the DIC pseudomolecular ion ([M-H]-) for identification and the most abundant transition for quantification were used. The stability of the DIC test solutions was assessed at the start and end of the assay (Table 1 supplementary data). The resulting correlation coefficient (r) was 0.9934. The percentage of error between the DIC commercial formulation and the standard reference did not exceed 5%.

Experimental design

Tadpoles were exposed to DIC to calculate the median lethal concentration (LC_{50}), the no-observed-effect concentration (NOEC), and the lowest-observed-effect concentration (LOEC). Toxicity was tested using the following nominal concentrations: 0.01875, 0.0375, 0.075, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10, and 20 mg L^{-1}. A negative control with dechlorinated tap water was also used. Both the control and test concentrations were evaluated in triplicate, with 10 tadpoles per 1-L glass aquarium (n = 330 per species). Mortality of tadpoles and cumulative mortality after 48 h of exposure were recorded in each treatment. To prevent the aquarium water from being contaminated during the experiment, dead tadpoles were removed.

Biochemical biomarkers

The biochemical markers chosen, i.e., AChE, GST, GR, AST, and ALT activities and T4 levels, were determined in both control and DIC-treated tadpoles (n = 7–10, respectively) at the end of the experiments (survival rate > 85% at 48 h) (Lajmanovich et al. 2016). Relevant DIC concentrations recorded in the environment (ERC) were considered to determine the treatments concentrations (Zhu et al. 2014). When a chemical is found at these ERC, it may not necessarily lead to higher mortality but may significantly cause the disturbance of some biological functions (e.g., Saaristo et al. 2018); therefore, in the present study, the following concentrations, 0.01875, 0.0375, 0.075, 0.156, 0.312, and 0.625 mg L^{-1}, were considered to identify the sublethal effects of DIC on the larvae of the two anuran species studied. To determine AChE, GST, GR, AST, and ALT activities and T4 levels, samples of tadpoles at 48 h of treatments were weighed (g) and homogenized (1:10, w/v) in ice-cold 25 mM sucrose, 20 mM Tris-HCl buffer (pH = 7.4) with 1 mM EDTA, by using a polytron tissue grinder. The homogenates were then centrifuged at 10,000 rpm at 4 ± 1 °C for 15 min and stored at −80 °C until the analysis of biomarkers.

Acetylcholinesterase activity

AChE activity was determined colorimetrically following the procedure of Ellman et al. (1961). The reaction mixture (final volume = 930 μL) consisted of 25 mM Tris-HCl with1 mM CaCl2 (pH = 7.6), 10 μL 20 mM acetylthiocholine iodide, and 50 μL DTNB (3×10^{-4} M, final concentration). The variation in optical density was recorded at 410 nm at 25°C for 1 min by means of a JENWAY 6405 UV-VIS spectrophotometer. Total

protein concentration was determined using the Biuret method (Kingsley 1942), and AChE activity was expressed as nmol min^{-1} mg^{-1} protein using a molar extinction coefficient of 13.6×10^3 M^{-1} cm^{-1}.

Glutathione S-transferase activity

GST activity was determined by spectrophotometry at 340 nm in 100 mM Na-phosphate buffer (pH = 6.5), 20 μM 1-chloro-2,4-dinitrobenzene, and 50 μM reduced glutathione (GSH), following the method described by Habig et al. (1974) and adapted for mammal serum GST activity by Habdous et al. (2002). The assays were performed at 25 °C, and whole GST activity was expressed as nmol min^{-1} mg^{-1} protein using a molar extinction coefficient of 9.6×10^3 M^{-1} cm^{-1}.

Glutathione reductase

GR activity was assessed following the method of Ramos-Martinez et al. (1983), which measures the oxidation of NADPH at 340 nm in the presence of oxidized glutathione and 0.1 M Na-phosphate buffer (pH 7.0). GR activity was calculated by means of a millimolar extinction coefficient for the NADPH oxidation of 6.22 mM^{-1} cm^{-1}. Enzyme activity was expressed as nmol min^{-1} mg^{-1} protein. All kinetics assays were performed at 20–22°C, and non-enzymatic reaction was periodically confirmed in blanks (reaction mixture without the sample) so as to correct enzyme activity.

AST and ALT activities

The activities of both transaminases were determined using commercially available kits (Wiener Lab®), according to the manufacturer's instructions. The oxaloacetate and pyruvate formed were measured in their derivative form, 2,4-dinitrophenylhydrazone, at 505 nm at pH 7.5. All samples were analyzed in triplicate, at 37 °C, using a spectrophotometer. The enzymatic activity of the homogenates was expressed as units mg^{-1} protein.

T4 levels

The small size of the two tadpole species made it difficult to collect a sufficient amount of blood; therefore, the whole-body hormone content was measured (Lajmanovich et al. 2019a). The use of homogenates has been previously applied in different studies (e.g., Li et al. 2016). Total T4 levels were determined using enzyme-linked electrochemical luminescent immunoassay (ECLIA) kits (COBAS®, Roche Diagnostics, IN, USA), according to the manufacturer's instructions. The detection limit for T4 was 0.42 ng g^{-1}.

Statistical analysis

The mean lethal concentration (LC_{50}) values and their respective 95% confidence limits (95% CL) were calculated via the Trimmed Spearman-Karber method (Hamilton et al. 1977). Significant differences ($P \leq 0.05$) between LC_{50} values were determined by the criterion of non-overlapping 95% confidence intervals (APHA 1989). The data of biochemical parameters (enzymatic activity and hormone levels) were expressed as means ± standard error (SEM). The enzymatic activity of control and exposed animals was compared using Kruskal-Wallis and Dunn post hoc tests (Zar 1999). Kolmogorov-Smirnov test and the Levene test were used to confirm normality and homogeneity of variances, respectively. The results were statistically analyzed using GraphPad InStad®. Values were significant at $P < 0.05$.

Results

Acute assay (48 h)

The DIC LC_{50} values at 48 h were 0.221 mg L^{-1} (95 % CL 0.161–0.300) for *E. bicolor* and 0.859 mg L^{-1} (95 % CL 1.189–0.568) for *S. nasicus* tadpoles. No mortality was observed in controls. Stabilization of LC_{50} values was achieved after 24 h of exposure. The NOEC and LOEC values were 0.156 mg L^{-1} and 0.312 mg L^{-1}, respectively, for *E. bicolor* and 1.25 mg L^{-1} and 0.625 mg L^{-1}, respectively, for *S. nasicus.*

Acetylcholinesterase and stress oxidative response

E bicolor

In *E. bicolor* tadpoles, the DIC concentration of 0.155 mg L^{-1} significantly affected AChE activity (KW = 12.48; $P < 0.05$) with respect to the control (Dunn post hoc test $P>0.05$), whose mean value was 6.46 ± 0.47 nmol min^{-1} mg^{-1}protein at 48 h (Fig. 1a).

DIC concentrations of 0.0187, 0.0375, and 0.155 mg L^{-1} also significantly affected GST activity (KW = 12.48, $P< 0.05$), with respect to the control, whose mean value was 60.63 ± 7.71 nmol min^{-1} mg^{-1} protein at 48 h (Fig. 2a).

The DIC concentrations tested did not affect GR activity significantly (KW = 5.96; $P > 0.05$), with respect to the control, whose mean value was 0.04 ± 0.01 nmol $min^{-1}mg^{-1}$ protein at 48 h (Fig 3a).

S. nasicus

In *S. nasicus* tadpoles, DIC concentrations of 0.0187, 0.312, and 0.625 mg L^{-1} significantly affected AChE activity (KW =

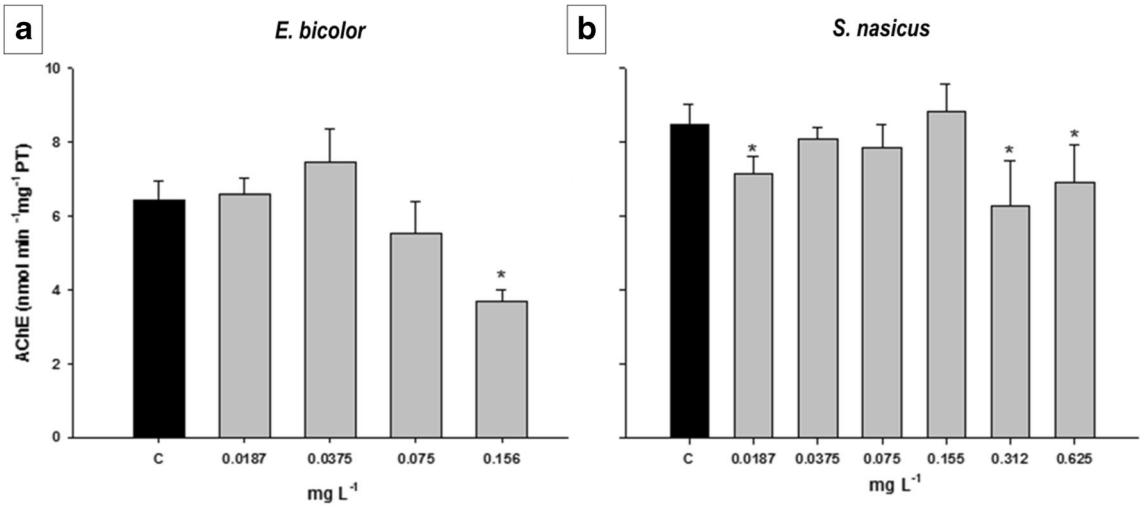

Fig. 1 Acetylcholinesterase (AChE) activity in *E. bicolor* (**a**) and *S. nasicus* (**b**) tadpoles exposed to the herbicide Dicamba for 48 h. Data are expressed as mean ± SEM, n = 7–10. Treatments are significantly different from control (C) at *$P < 0.05$; Dunnett's test

12.66; $P < 0.05$) with respect to control, whose mean value was 8.50 ± 0.52 nmol min^{-1} mg^{-1}protein at 48 h (Fig. 1b).

All DIC concentrations tested (except for 0.0187 mg L^{-1}) also significantly affected GST activity (KW = 6.24, $P< 0.05$), with respect to the control, whose mean value was 36.32 ± 1.95 nmol min^{-1} mg^{-1} protein at 48 h (Fig. 2b).

DIC also significantly affected GR activity (KW = 18.64, $P < 0.05$) with respect to the control, whose mean value was 0.06 ± 3.9^{-3} nmol min^{-1} mg^{-1} protein at 48 h (Fig. 3b).

AST and ALT activities

E. bicolor

In *E. bicolor* tadpoles, the DIC concentration of 0.075, 0.155, and 0.625 mg L^{-1} significantly inhibited AST activity (KW = 11.92; $P > 0.05$) with respect to the control, whose mean value was 7.87 ± 0.52 U mg^{-1} protein at 48 h (Fig. 4a).

The DIC concentration of 0.625 mg L^{-1} also significantly inhibited ALT activity (KW = 16.45, $P< 0.05$), with respect to the control, whose mean value was 7.23 ± 0.31 U mg^{-1} protein at 48 h (Fig. 5a).

S. nasicus

In *S. nasicus* tadpoles, all DIC concentrations (except at 0.0187 mg L^{-1}) significantly induced AST activity (KW = 12.76; $P< 0.05$) with respect to the control, whose mean value was 5.89 ± 0.15 U min^{-1} mg^{-1}protein at 48 h (Fig. 4b).

The DIC concentration of 0.625 mg L^{-1} also significantly induced ALT activity (KW = 11.49, $P < 0.05$), with respect to

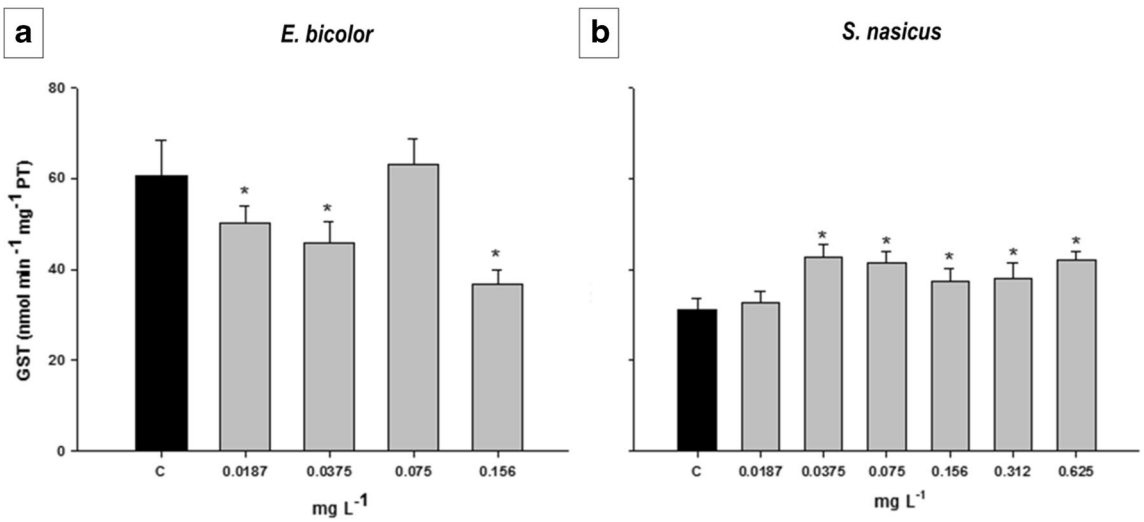

Fig. 2 Glutathione S-transferase (GST) activity in *E. bicolor* (**a**) and *S. nasicus* (**b**) tadpoles exposed to Dicamba for 48 h. Data are expressed as mean ± SEM, n = 7–10. Treatments are significantly different from control (C) at *$P < 0.05$; Dunnett's test

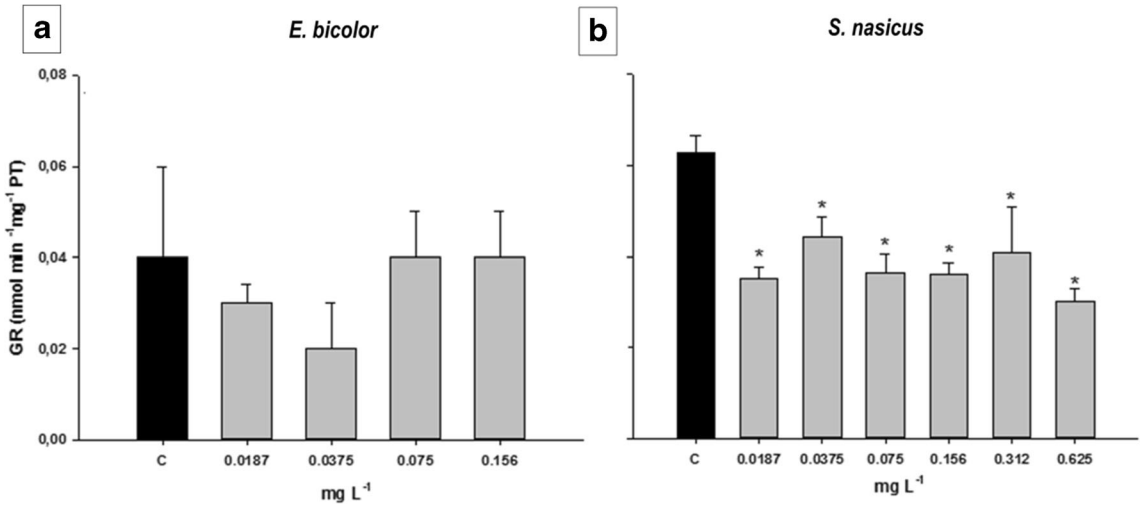

Fig. 3 Glutathione reductase (GR) activity in *E. bicolor* (**a**) and *S. nasicus* (**b**) tadpoles exposed to Dicamba for 48 h. Data are expressed as mean ± SEM, n = 7–10. Treatments are significantly different from control (C) at *$P < 0.05$; Dunnett's test

the control, whose mean value was 7.08 ± 0.22 U mg^{-1} protein at 48 h (Fig. 5b)

T4

DIC significantly affected T4 levels in both species (KW = 9.38, $P < 0.05$ for *E. bicolor* and KW = 11.69, $P < 0.05$ for *S. nasicus*), increasing them from the control at 0.156 mg L^{-1} DIC for *E. bicolor* (Fig. 6a) and 0.625 mg L^{-1} for *S. nasicus* (Fig. 6b). Mean T4 activity value in control tadpoles was 6.76 ± 0.28 ng g^{-1} for *E. bicolor* and 8.90 ± 0.64 ng g^{-1} for *S. nasicus* at 48 h.

Discussion

The massive pesticide use in modern agriculture has generated global concern due to the threat they pose to ecosystems and wildlife (Egea-Serrano et al. 2012). Knowledge about the toxicity and sublethal effects of herbicides is necessary to assess their environmental risks to wildlife and regulate their use (David and Kartheek 2016). In the present study, DIC LC_{50} values at 48h for *S. nasicus* and *E. bicolor* tadpoles were 0.859 mg L^{-1} and 0.221 mg L^{-1}, respectively, which reveal their sensitivity to this herbicide. Other studies have shown varied LC_{50} values for different non-target aquatic organisms exposed to DIC. For example, in *Rhinella arenarum* late-stage larvae, Soloneski et al. (2016) reported an LC_{50} for DIC (soluble formulation; Syngenta Agro S.A.) of 358.44 mg L^{-1} at 96 h. In rainbow trout and bluegill, Meister (1992) found that DIC LC_{50}values at 48 h were 35 mg L^{-1} and 40 mg L^{-1}, respectively. However, based on the acute toxicity here observed for DIC in the two evaluated species, the studied emulsifiable formulation seems to be more toxic than the soluble formulation, which reinforces the assumption that

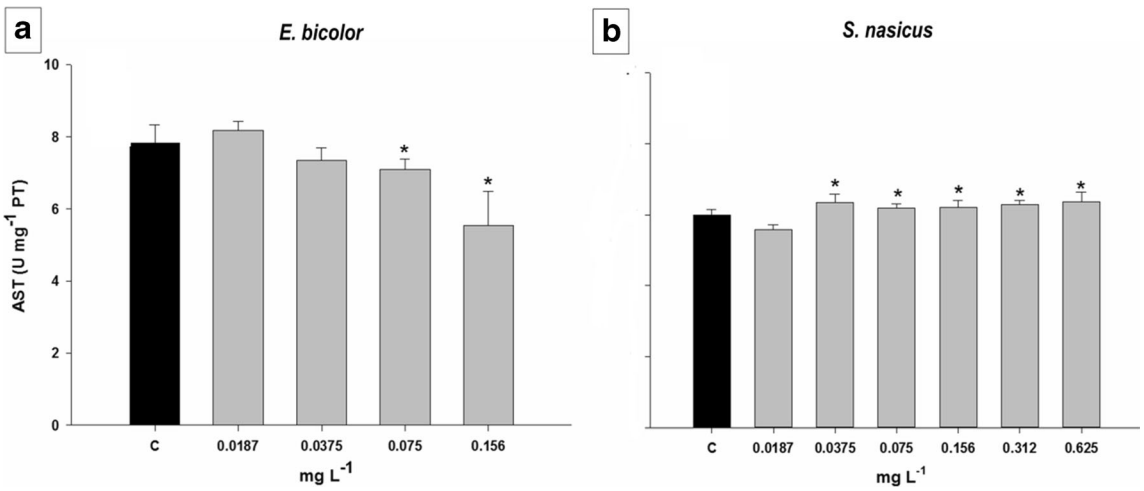

Fig. 4 Aspartate aminotransferase (AST) activity in *E. bicolor* (**a**) and *S. nasicus* (**b**) tadpoles exposed to Dicamba for 48 h. Data are expressed as mean ± SEM, n = 7–10. Treatments are significantly different from control (C) at *$P < 0.05$; Dunnett's test

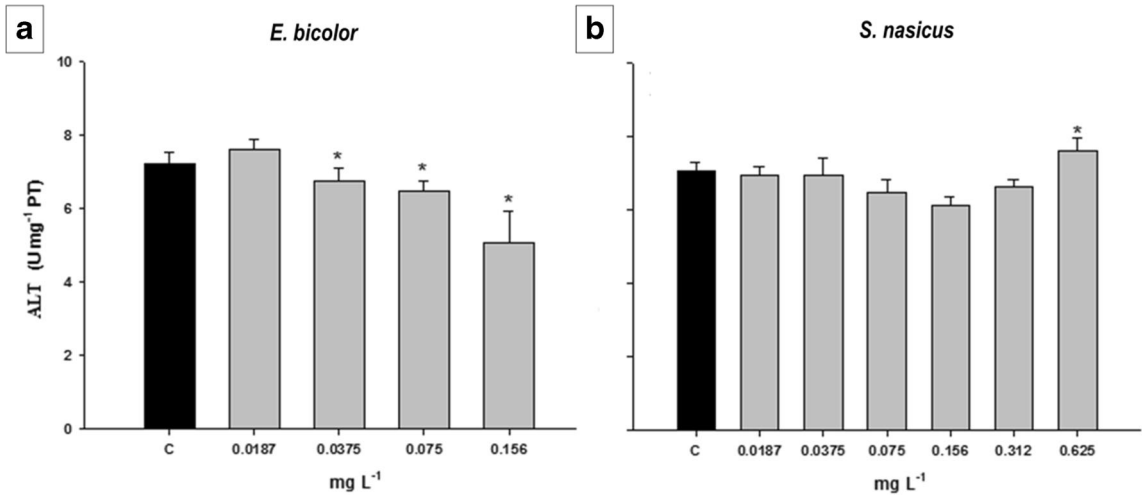

Fig. 5 Alanine aminotransferase (ALT) activity in *E. bicolor* (**a**) and *S. nasicus* (**b**) tadpoles exposed to Dicamba for 48 h. Data are expressed as mean ± SEM, n = 7–10. Treatments are significantly different from control (C) at $^*P < 0.05$; Dunnett's test

coformulants increase the toxicity of pesticide emulsifiable concentrates (Eddleston et al. 2012). The comparison of the effects of an organophosphosphate active ingredient (dimethoate) alone and in emulsifiable concentrate on Gottingen minipig suggested that solvents may play a crucial role in pesticide toxicity. Martinuzzi et al. (2019) observed similar results when studying the effects of a dimethoate emulsionable pesticide on *R. arenarum* tadpoles. Commercial formulations including not only the active ingredient but also coformulants should be included in environmental risk assessments to estimate the toxicity of the compounds introduced into different environments.

The herbicide DIC poses a potential threat to aquatic life due to its relatively high water solubility and its frequent presence in freshwater environments (Zhu et al. 2014). In 15 drinking water reservoirs of North America, DIC was detected at a maximum concentration of 1040 ng L^{-1} (Donald et al. 2007). Furthermore, in three sites of California (USA) considered free of agricultural inputs from 2008 to 2011, DIC was found to be one of the most frequent herbicides both in water and sediment samples (Ensminger et al. 2013). In Argentina, no data on DIC concentrations have been recorded to date. Furthermore, according to the data shown in Table 1, the evaluated DIC emulsifiable formulation (LC_{50}: 0.221–0.859 mg L^{-1}) would be one of the most toxic systemic herbicide described for tadpoles known to date, since values for other herbicides such as glyphosate range between 30 and 40 mg L^{-1} (Lajmanovich et al. 2015) and values for the herbicide 2,4 D LC_{50} are about 1040 mg L^{-1} (Curi et al. 2019). To the best of our knowledge, this study provides the first experimental evidence of the acute sublethal effects of DIC on tadpoles of *S. nasicus* or *E. bicolor*, two neotropical anuran species native to Argentina. The species most sensitive to DIC exposure under laboratory conditions was *E. bicolor*. This

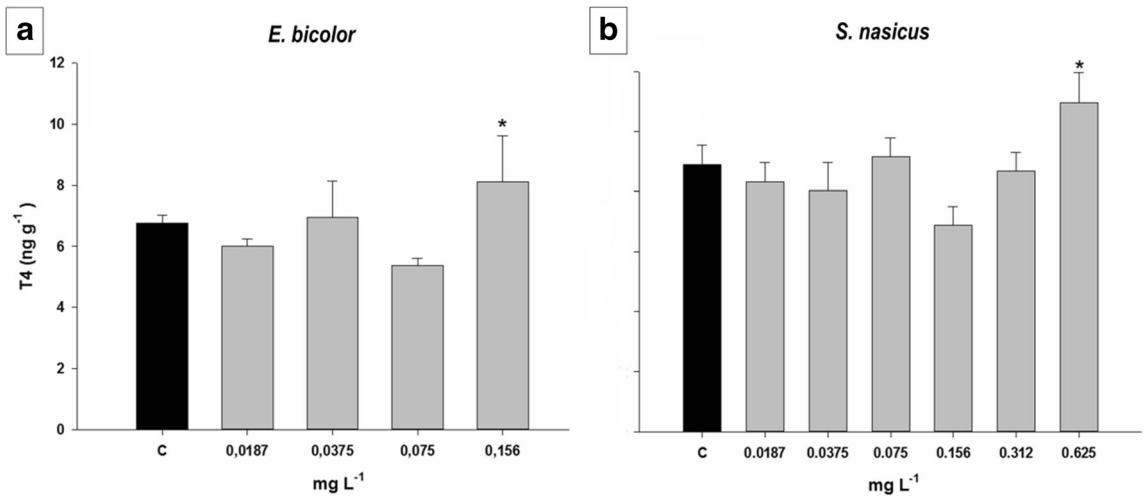

Fig. 6 Level of thyroid hormones (thyroxine; T4) in *E. bicolor* (**a**) and *S. nasicus* (**b**) tadpoles exposed to Dicamba for 48 h. Data are expressed as mean ± SEM, n = 7–10. Treatments are significantly different from control (C) at $^*P < 0.05$; Dunnett's test

Table 1 Comparison of LC_{50} (48 and 96 h) values for commonly used herbicides (commercial formulations) reported in different anuran species and the stage of development at which the determination was made (GS for most studies, except in *: NF refers to Nieuwkoop and Faber (1956) stages of development)

Herbicide (commercial formulation)	Species (stage of development)	LC 50 (mg L^{-1})		References
		48 h	96 h	
2,4-Dichlorophenoxyacetic acid				
Amina Zamba®	*Physalaemus albonotatus* (25–26)	1040.2	350	Curi et al. (2019)
Dicamba				
Banvel®	*R. arenarum* (35–37)	525.05	358.44	Soloneski et al. (2016)
Cowboy Elite	*Scinax nasicus* (30–34)	0.859		This study
SURCOS®	*E. bicolor* (30–34)	0.221		
Metsulfuron-Methyl3				
Metsulfuron 60®	*R. arenarum* (29–30)	105.56		Lajmanovich et al. (2013)
Glyphosate				
Credit®	*R. arenarum* (35–37)	85.96	78.18	Soloneski et al. (2016)
Roundup Ultra-Max®	*R. marina* (25)	3.7	3.5	Sookoo et al. (2017)
	R. arenarum (29–30)	13.20		Lajmanovich et al. (2013)
	R. arenarum (36-38)	2.4		Lajmanovich et al. (2011)
Atrazine				
Siptran 500sc®	*R. schneideri* (30)	31.1	22.2	Pérez-Iglesias et al. (2018)
	P. gracilis (25)		47.9	Flores Sturza (2017)
Paraquat				
Gramoxone Supert®	*R. marina* (25)	172	56	Sookoo et al. (2017)
	Engystomops pustulosus (25)	2.9	0.2	
	S. nasicus (25–26)	29.97	21.99	Lajmanovich et al. (1998)
Glufosinate ammonium				
Liberty®	*Hypsiboas pulchellus* (26–30)	21.47		Peltzer et al. (2013)
Propanil				
Propanil Trust®	*H. crepitan* (25)		16.54	Triana Velasquez et al. (2016)
	R. humboldti (25)		5.09	
Butachlor				
Not specified	*P. megacephalus* (25–26)	2.62	1.52	Geng et al. (2005)
	M. ornata (25–26)	0.85	0.53	
Diuron				
Karmex ®	*R. marina* (25)	3		Sookoo et al. (2017)
	E. pustulosus(25)	1.1		
Flurochloridone				
Twin Pack Golds®	*R. arenarum* (33)		2.96	Nikoloff et al. (2014)
Rainbows ®	*R. arenarum* (33)		2.85	
Trifluralin				
Treflan 4D	*Leptodactylus clamitans* (25)		2.81	Weir et al. (2012)
Pendimethalin				
Prowl 400EC	*L. clamitans* (25)		2.47	Weir et al. (2012)
Imazethapyr				
Pivot H®	*Hypsiboas pulchellus* (36)	1.55		Pérez-Iglesias et al. (2015)
Cycloxydim				
Focus® Ultra	*Xenopus laevis* (NF stage 48)		0.9	Wagner et al. (2015)
Bispyribac-Sodium				
Ectran®	*R. arenarum* (29–30)	0.20		Lajmanovich et al. (2013)
Picloram				
Tordon 24-K®	*R. arenarum* (29–30)	0.025		Lajmanovich et al. (2013)

species has benthonic habits and rest in the bottom of ponds to forage and escape from predators (Peltzer and Lajmanovich 2007). These ecological traits might increase the potential uptake of contaminants and, therefore, be associated with the interspecific variation to pollutants sensitivity. In this sense, toxicity studies involving multiple species of amphibians are important because species within a community may differ markedly in their sensitivity (Relyea 2009). The herbicide significantly inhibited AChE activity in *S. nasicus* and *E. bicolor* tadpoles after 48 h of exposure. Thus, AChE is a common biomarker used to assess neurotoxic effects. In previous studies, we found that disruption of AChE activity in tadpoles increases mortality, reduces activity, and increases the vulnerability to predators (e.g. Attademo et al. 2015,

2016). Similarly, Ruiz de Arcaute et al. (2019) also found inhibition of AChE activity in the freshwater fish *Cnesterodon decemmaculatus* treated with sublethal DIC concentrations in chronic tests. Therefore, further research is required to elucidate the mechanisms of DIC neurotoxicity in amphibians.

Our results suggest that DIC exposure increases the response of glutathione to counteract the formation of oxidative stress. In *E. bicolor* tadpoles exposed to DIC for 48 h, GST levels showed a rapid and significant decrease compared to controls, whereas in *S. nasicus* tadpoles, GST showed a significant increase. The variation in the GST response can be due to the target tissue or organ, species, or diet considered for evaluation (Rudneva et al. 2010). Studies analyzing GST in several aquatic species exposed to different herbicides have shown enzymatic induction or inhibition (e.g., Samanta et al. 2014). These results are in agreement with findings of Ruiz de Arcaute et al. (2019), who demonstrated that DIC significantly induced GST in the fish *C. decemmaculatus*.

In the present study, GR decreased in *S. nasicus* at 48 h of exposure at all DIC concentrations tested. Results of GST and GR indicate that DIC affected the enzyme performance related to antioxidant response; therefore, these enzymes seem to be suitable biomarkers to evaluate chemical exposure in contaminated aquatic ecosystems.

Here, DIC also caused statistically significant changes in transamination, as evidenced by the increase or decrease in AST and ALT activities. Interestingly, these changes occurred at very low DIC concentrations, indicating the high toxicity of this chemical in *S. nasicus* and *E. bicolor* tadpoles. Increased ALT and AST activities are usually indicative of liver disease because of their biological location. The increase in both AST and ALT may also be a sign of inflammatory disease or liver injury (Ayalogu et al. 2001). In their experiment, Gabriel et al. (2011) observed that by exposing the fish *Heterobranchus bidorsalis* to graded concentrations of the insecticide cypermethrin, the animal needed more energy for the detoxification, biotransformation, and excretion of the toxicant to minimize its effects. This fish species was able to do this by using carbohydrates, i.e., the main source of energy used under chronic stress. In fish, the reduction of the protein fraction can be due to carbohydrate degradation. Thus, the interaction here observed between carbohydrate and protein synthesis (transamination) may be due to the degradation and probable use of carbohydrates and proteins for metabolic processes.

DIC-exposed tadpoles showed both an increase and a decrease in transaminase activities. An increment in AST and ALT activities depicts the effective use of amino acids for metabolism (Tiwari and Singh 2004), whereas a decrease protects the structural membrane integrity of the hepatic cell (Pari and Amali 2005). Similar findings were reported by Güngördü et al. (2016), who evaluated the toxic effects of a glyphosate-based herbicide and a methidathion-based insecticide, both individually and in combination, on pre-metamorphic tadpoles of three anuran species (Denver et al. 2002).

Finally, our results also showed higher T4 levels in DIC-treated tadpoles of both amphibian species than in controls. Since thyroid hormones play a key role in development, DIC might disrupt the normal development of the exposed tadpoles; further studies are necessary to test this hypothesis. In previous studies, we also found an increase in T3 and T4 in *R. arenarum* tadpoles exposed to a glyphosate-based herbicide (Lajmanovich et al. 2019b). Similarly, Cao et al. (2016) found increased T4 levels in zebrafish larvae after exposure to a herbicide applied to control weeds in rice fields (Chang et al. 2013). Accordingly, more studies are necessary to better understand the potential molecular mechanisms associated with changes in T4 levels (Lajmanovich et al. 2019b). DIC was classified as category 4 in the ecotoxicology profile (LC_{50} for aquatic vertebrates > 180 mg L^{-1}, Bunch et al. 2012) due to its low acute toxicity (practically non-toxic). According to our results, the ecotoxicological classification of DIC for emulsifiable formulations needs urgent revision because it is highly toxic to amphibians.

Conclusions

In the last years, an increasing number of reports have shown that substances present in the environment are endocrine disruptors in amphibian species (Bókony et al. 2018). Indeed, these substances are underestimated as potential toxic pollutants in water bodies and have thus become a potential risk to aquatic organisms. The results of the present study showed that a DIC commercial formulation has high biotoxicity in *S. nasicus* and *E. bicolor* tadpoles, evidenced in terms of biochemical impairments such as inhibition of AChE activities and variation in oxidative stress enzymes (GST and GR), transaminase (AST and ALT) activities, and hormone (T4) levels. These results highlight the need for an urgent revision of the environmental regulations of these commercial herbicide formulations. Finally, these results provide evidences that can be used to prevent risks using the precautionary approach.

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Availability of data and materials Supporting data of the study are available in this published article (supplementary information). Besides, datasets generated and analyzed during the study are available from the corresponding author on reasonable request.

Author contribution Andres M. Attademo Conception: Design, execution, interpretation, and writing.

Rafael C. Lajmanovich: Conception, design, execution, and interpretation.

Paola Peltzer: Design and interpretation.

Ana Paula Cuzziol Boccioni: Execution and interpretation.

Candela Martinuzzi: Execution and analyses.

Fernanda Simonielo: Execution and interpretation

Maria Rosa Repetti: Execution and analyses.

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Declarations

Ethics approval and consent to participate The animals used in this research have been treated according to the criteria of the ASIH (2004) and with approval bythe Animal Ethics Committee of the Facultad de Bioquímica y Ciencias Biológicas (FBCB), Universidad Nacional del Litoral (UNL), Santa Fe, Argentina (http://www.fbcb.unl.edu.ar/pages/investigacion/comite-deetica.php).

Consent to publish Not applicable.

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