ToxEHS

B-esterases and Behavioral Biomarkers in Tadpoles Exposed to Pesticide Pyrethroid-TRISADA®

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Abstract

Objective: The ecotoxic effects of pesticide used for mosquito's control TRISADA® (TRI) [deltamethrin (D) 1% + tetramethrin (T) 0.33%, and piperonyl butoxide (PB) 0.29%] on amphibian larvae were investigated.
Methods: In the laboratory, *Rhinella arenarum* tadpoles were exposed to nominal concentrations of 0.0000 (control; CO), 0.0003125% (C1); 0.000625% (C2); 0.00125% (C3); 0.0025% (C4); 0.005% (C5) (v/v) of formulated TRI. Median lethal concentration (LC_{50}) (%) and 95% confidence limits (CL), the no-observed-effect concentration (NOEC), and the lowest-observed-effect concentration (LOEC) were quantified. The possible effects of TRI on B-esterases, evaluated through acetylcholinesterase (AChE) and carboxylesterase (CbE) activities, in addition to swimming performance (distance moved, mean speed, maximum speed, global activity, and resting time or immobility) were measured in tadpoles whose concentrations displayed survival rates higher than 50%.
Results: The 48 h LC_{50} of TRI was 0.00125% (v/v) [12.5 (D) + 4.1 (T) + 3.6 (PB); μg L^{-1}] (CL: 0.000811-0.001926%). The 48 h NOEC and LOEC values were 0.0003125% (v/v) [3.1 (D) + 1 (T) + 0.9 (PB); μg L^{-1}] and 0.000625% (v/v) [6.2 (D) + 2 (T) + 1.8 (PB); μg L^{-1}], respectively. At 48 h of exposure to upper sublethal TRI concentration assay (C3), AChE and CbE activities were significantly inhibited (68 and 84%, respectively) with respect to controls. Also, all the sublethal TRI concentrations caused significantly alterations of all swimming endpoints evaluated.
Conclusion: The current study established that pesticide TRI is toxic to *R. arenarum* tadpoles and had detrimental effects on the B-sterases activities and swimming activity at TRI sublethal concentrations.

Keywords: Amphibian, *Rhinella arenarum*, Pyrethroids mixture, B-esterases activity, Behaviour

Introduction

Greater than 70% of the worldwide amphibian populations are in decline[1]. Much of the interest on these declines is currently focused on the role of pesticides, which is shown in several recent studies that highlight the importance of research on the effects of amphibian exposure to novel stressors. Generally, pesticide application coincides with the amphibian aquatic larval phase, indeed tadpoles is exposed through lixiviation or runoff of field crop contaminated or pesticide spray drift into the breeding ponds. Thus, exposure studies during the aquatic phase are a highly relevant approach. The impact of pesticides on amphibian larvae includes death and many sublethal effects at different biological levels (metabolic changes, delay or increase metamorphosis, malformations, abnormal behavioural, endocrine disruption, and others)[2]. For the above mentioned, anuran amphibians have been considered as good bioindicators of aquatic ecosystems and its used in many laboratory and field studies to model organisms.

Pyrethroids (PY) are pesticides characterized by high insecticidal properties which produce alterations in the ion conductance of nerve cell plasmatic membranes. While PY have been used around the world, few severe problems have been reported, particularly because of its low mammalian toxicities and high biodegradability. For this reason, PY are often widely used in insecticide-based vector control in gardens, homes, and agriculture, and its take a main role in the last decades to prevent the spread of mosquito-borne diseases[3]. Resistance in the target organisms was an unexpected conse-

quence due to wide synthetic PY application and its uncontrolled use[4]. There are two types of synthetic PY type I (produce repetitive nerve discharges but not neurotransmitter release) (e.g. tetramethrin, permethrin, bifenthrin) and II (sodium channels is open for a longer period, do not induce repetitive discharges) (e.g. deltamethrin, cypermethrin, cyfluthrin, cycloprothrin, fenvalerate)[5]. Both PY types are mixed for control long-lasting insecticidal mosquito nets, indeed mixtures with piperonyl butoxide were also supplied to combat PY insect resistance[6]. Although low occurrence of acute toxicity was report for humans ecological effects on aquatic organisms were well documented[7]. In nature, amphibian tadpoles are likely to be sensitive to low-level of PY and generally their biological responses are synergizing with environmental factors[8].

B-esterases activities have been used to monitor vertebrate wildlife exposed to pesticides[9]. Particularly, acetylcholinesterase (AChE) and carboxylesterase (CbE) were recommended as useful biomarkers of amphibian tadpole exposure to anti-ChE chemicals[10]. PY pesticides usually inhibit some B-esterase enzymes production[11]. The modification in the B-esterases activity is generally related with motor activity in amphibian tadpoles[12,13] indicating several level of neurotoxicity[14]. In addition, locomotor activity biomarkers also provide integration of biochemical and physiological responses of aquatic vertebrates[15], in this regard, video-tracking software offers the possibility of precisely quantifying behavioural performance[16]. In addition, the holistic approach between two or more biomarkers and traditional toxicological endpoints (e.g., lethal concentration 50 (LC_{50}), no-observed-effect concentration (NOEC), and lowest-observed-effect concentrations (LOEC) take more relevance in evaluation of concentration-dependence curves[17]. Thus, in order to investigate the harmful effects of a pesticide PY formulations used for the control of mosquitoes, the objective of the current study was to assess the lethal effects on *Rhinella arenarum* tadpoles, and analyze responses at metabolic (AChE and CbE activities), and physiological (swimming behaviour) levels.

Results and Discussion

In the 48-h static bioassay no mortality was observed in the control group. LC_{50} nominal values and 95% confidence limits for 24 and 48 h of exposure to the insecticides TRI are summarized in Table 1, as well as the values for NOEC and LOEC. The mean value of the AChE activity in control (CO) tadpoles was 20.12 ± 4.85 nmol min^{-1} mg^{-1} protein at 48 h, whereas the mean value of the AChE activities at 48 h in exposed tadpoles were: C1 = 8.67 ± 3.47 nmol min^{-1} mg^{-1} protein; C2 = 12.65 ± 4.84 nmol min^{-1} mg^{-1} protein; C3 = 6.24 ± 0.12 nmol min^{-1} mg^{-1} protein. Tested concentrations of TRI affected significantly activity of AChE respect to the control AChE activity (KW = Dunnett's post hoc test $P < 0.05$; $P < 0.01$, Figure 1). The maximum percent-

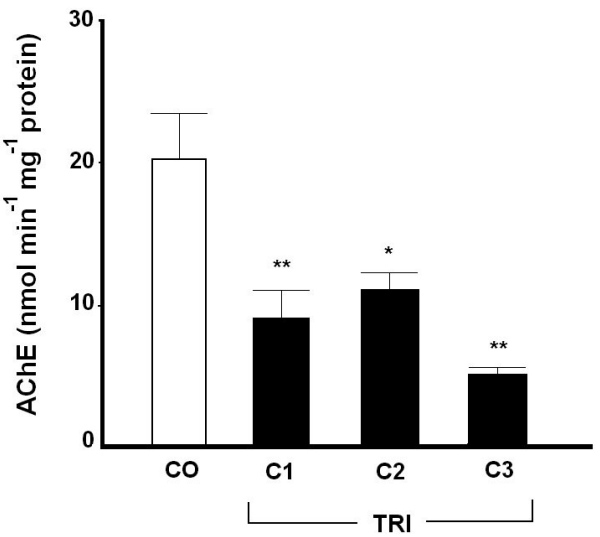

Figure 1. Acetylcholinesterase (AChE) activity in *Rhinella arenarum* tadpoles exposed (48 h) to sublethal percentage concentration of insecticide TRISADA® (TRI). CO = Control (dechlorinated tap water); (C1) 0.003125%; (C2) 0.000625%; (C3) 0.00125% (v/v). Data are expressed as mean ± SEM. Significant differences were $*P < 0.05$ and $**P < 0.01$ with respect to the control between different concentrations (Dunn's *post hoc* test). $n = 10$.

Table 1. Summary of medial lethal concentration percentage (LC_{50}), no-observed-effect concentrations (NOEC), and lowest-observed-effect concentrations (LOEC) (v/v %) of TRISADA® (TRI) on *Rhinella arenarum* tadpoles after 48 h exposure.

Time (h)	LC_{50} (%)	NOEC (%)	LOEC (%)
24	0.002806 (0.003149-0.003664) [28 (D) + 9.2 (T) + 8.1 (PB)]	0.00125 [12.5 (D) + 4.1 (T) + 3.6 (PB)]	0.0025 [25 (D) + 8.2 (T) + 7.2 (PB)]
48	0.00125 (0.000811-0.001926) [12.5 (D) + 4.1 (T) + 3.6 (PB)]	0.0003125 [3.1 (D) + 1 (T) + 0.9 (PB)]	0.000625 [6.2 (D) + 2 (T) + 1.8 (PB)]

Values in parenthesis correspond to the 95% confidence interval of each LC_{50} (%) estimate.
Values in square bracket are the nominal concentration (μg L^{-1}) of TRI compounds: (D) deltamethrin (T) tetramethrin (BP) piperonyl butoxide.

age of inhibition of AChE (68%) activity in TRI-treated tadpoles after 48 h was recorded in C3 (0.00125 v/v % TRI). The mean value of the CbE activity in control tadpoles was 12.54 ± 1.45 nmol min^{-1} mg^{-1} protein at 48 h, whereas the mean value of the AChE activities at 48 h in exposed tadpoles were: C1 = 3.12 ± 1.35 nmol min^{-1} mg^{-1} protein; C2 = 3.71 ± 2.05 nmol min^{-1} mg^{-1} protein; C3 = 1.99 ± 0.04 nmol min^{-1} mg^{-1} protein. TRI affected significantly activities of the CbE ($P < 0.01$; Figure 2) at all quantities tested, with a percentage of inhibition from 70% (C2) to 84% (C3). The sublethal exposure of tadpoles to TRI concentrations caused alterations of swimming endpoints (Figure 3), being

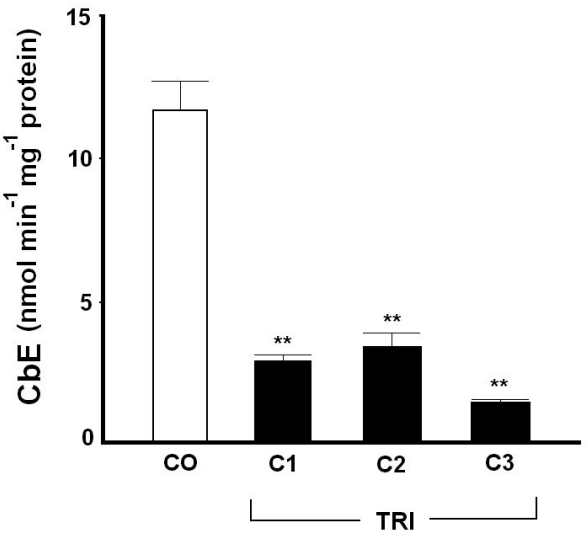

Figure 2. Carboxylesterase (CbE) activity in *Rhinella arenarum* tadpoles exposed (48 h) to sublethal percentage concentration of insecticide TRISADA® (TRI). CO = Control (dechlorinated tap water); (C1) 0.003125%; (C2) 0.000625%; (C3) 0.00125% (v/v). Data are expressed as mean ± SEM. Significant differences were $*P < 0.05$ and $**P < 0.01$ with respect to the control between different concentrations (Dunn's *post-hoc* test). $n = 10$.

distance moved ($F = 35.86$; $P < 0.0001$), mean speed ($F = 35.80$; $P < 0.0001$), maximum speed ($F = 45.61$; $P < 0.0001$), global activity ($F = 21.36$; $P < 0.0001$), significantly inhibited by TRI-exposure; while resting time in all treated tadpoles was greater respect to CO($F = 38.65$; $P < 0.0001$) (Table 2). Distance moved (r Spearman = 0.58), mean speed (r Spearman = 0.58), maximum speed (r Spearman = 0.57), and global activity (r Spearman = 0.59) showed a significantly positive correlation with AChE activity in the CO and exposed tadpoles ($P < 0.01$). On the other hand, resting time was negatively correlated with AChE activity (r Spearman = -0.54; $P < 0.01$).

In mosquito control programmes, the widespread use of synthetic pesticides such as organophosphates and PY had several downside effects such as the development of insect resistances or negative influence on environment and human health[18]. TRI resulted to be neurotoxic to *R. arenarum* tadpoles producing inhibition of AChE and detoxification by CbE[19]. These results were reinforced with the low swimming performance (low speed movements and global activities, and long period of inactivity) recorded for *R. arenarum* at all TRI concentration assayed.

In this context, amphibian species breed in a variety of freshwater ponds and often coexist with mosquito larvae in the summer months. In this season several pesticides including those to control mosquito larvae are massively applied[17]. In this regards, the present study provides relevant information on the hazardous potential of a widely used insecticide PY formulation for mosquito control on anuran tadpoles.

Considering the nominal concentration of the TRI formulations the 48 h LC_{50} values obtained would be composed of deltamethrin (12.5 μg L^{-1}), tetramethrin (4.1 μg L^{-1}) and piperonyl butoxide (3.6 μg L^{-1}). The low acute toxicity values for TRI in the present study are consistent with other researchers. The 48 h LC_{50} value of deltamethrin was 11.93 μg L^{-1} in *R. arenarum* tadpoles[20] and 4.4 μg L^{-1} for *Bufo bufo* tadpoles[21] More-

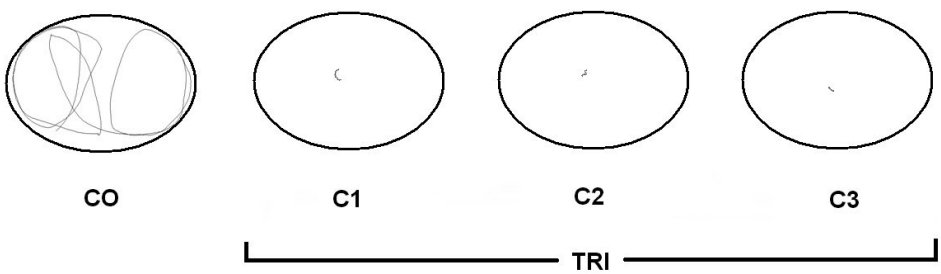

Figure 3. Representative video tracks of *Rhinella arenarum* tadpoles after exposition (48 h) to sublethal percentage concentration of insecticide TRISADA® (TRI). CO = Control (dechlorinated tap water); (C1) 0.0003125%; (C2) 0.000625%; (C3) 0.00125% (v/v).

Table 2. Summary of swimming parameters (mean ± SEM) evaluated in *Rhinella arenarum* larvae at 48 h exposed to sublethal percentage concentration of insecticide TRISADA® (TRI). CO = Control (dechlorinated tap water); (C1) 0.0003125%; (C2) 0.000625%; (C3) 0.00125% (v/v).

Behavioral parameters	Treatments of TRI exposure (%)			
	Control (CO)	C1	C2	C3
Distance moved (cm)	133.42 ± 19.36	6.01 ± 0.8**	6.92 ± 1.10**	2.68 ± 0.65**
Mean speed (cm/s)	2.22 ± 0.32	0.10 ± 0.01**	0.12 ± 0.02**	0.04 ± 0.01**
Maximum speed (cm/s)	5.72 ± 0.51	1.42 ± 0.14 **	1.68 ± 0.29**	1.10 ± 0.14**
Global activity (cm^2)	84.31 ± 13.07**	17.81 ± 1.19**	18.71 ± 2.33**	16.07 ± 0.84**
Resting time (s)	29.56 ± 4.71	59.99 ± 0.18**	59.97 ± 0.03**	59.99 ± 0.18**

Asterisks denoted significant differences with the CO (Dunnett's *post-hoc* test): $*P<0.05$; $**P<0.01$. $n=10$

over, Aydin-Sinan *et al*. (2012)[22] reported a LC_{50} value for deltamethrin after a 168 h of 6.26 μg L^{-1} in *Xenopus laevis* tadpoles; in contrast, Macagnan *et al*. (2017)[23] registered a 96 h LC_{50} value more high of 3.04 mg L^{-1} for *Physalaemus gracilis* embryos. Despite these data, there is no still information about the toxicity of the tetramethrin toxicity in anuran tadpoles. However, in zebrafish (*Danio rerio*) 48-h LC_{50} value of tetramethrin ranged between 51.7-87.9 μg L^{-1}(24). In addition, as to the piperonyl butoxide is primarily used as a synergist product for the control of insect pests[6] because it inhibits the cellular detoxification mechanisms[25], it is usually used in combination with some PY (i.e., tetramethrin and deltamethrin) providing high killing activity against target species[26]. The synergist piperonyl butoxide with PY is quite toxic to amphibians, suggesting that mechanisms of detoxification in amphibians are susceptible to this organic compound[27].

Evidently the combined effect of a PY type I (tetramethrin) and type II (deltamethrin) produces different interaction. For instance, toxicity of the permethrin (Type I) and cypermethrin (Type II) was significantly lower than the toxicity of cypermethrin alone. In accordance of Schleier & Peterson (2012)[28], the toxicity observed is most likely because of the competitive binding at the voltage-gated sodium channel, which is supported by physiological and biochemical studies of some PY[28].

Our results also reveal that sublethal concentrations of TRI significantly inhibited both AChE and CbE activities in tadpoles with respect to the control, showing a tendency that concentration-dependent-like inhibitory effect. The highest inhibition percentages of AChE (68%) and CbE (84%) were registered in tadpoles exposed to the highest TRI concentration tested (C3) (0.00125% v/v) and this may be explain by the role of AChE in cholinergic synapses of neurons in the central nervous system and skeletal muscle[29]. Moreover, the inhibition of AChE activity might be a consequence of the direct effect of deltamethrin + tetramethrin mixture on the active site of the enzyme or an indirect effect via the inhibition of enzyme synthesis[30]. In addition, in relationship with our results, several investigations have confirmed that deltamethrin exert secondary effects on the cholinergic system, particularly on AChE activity[31]. Besides Tu *et al*. (2012)[32] observed that AChE was strongly inhibited by deltamethrin exposition in the black tiger shrimp (*Penaeus monodon*). On the other hand, piperonyl butoxide possibly preventing the AChE inhibition in short term exposure[33] Thus, it has been suggested that inhibition of AChE activity at sublethal levels of a contaminant produce an adverse effect upon amphibian larvae health and ecological viability[12].

CbE activity exhibited a dose-response relationship, with activity decreasing with increasing TRI concentration in exposed *R. arenarum* tadpoles. CbE inhibition was also reported in some anuran tadpole species exposed to pesticides[13]. However, CbE are likely to be important for pesticide detoxification and it may be involved in the resistance mechanism of pesticide exposed organisms[11]. CbEs have an increased affinity over AChE for some PYs and it has been suggested that CbEs act as a "sink" for PYs, thus protecting the organism against PY toxicity[34]. In these sense, inhibition of CbE would dramatically increase the toxicity of TRI in exposed *R. arenarum*, rendering the enzyme unable to hydrolyze and thus detoxify PY[35].

Moreover, behavioral abnormalities recorded in TRI-treated *R. arenarum* tadpoles are clear indicators of toxicological effects. Tadpoles in the control group appeared active and healthy, whereas the TRI-treated tadpoles displayed typical signs of PY exposure, including uncoordinated swimming, spasmodic and erratic movement, and high period of immobility[36]. The results are similar to those observed with esfenvalerate[37], in which tadpoles exhibited convulsions and eventually died at sublethal levels. Indeed, it is expected that tadpoles deteriorate to death once the irreversible sublethal effects are induced, regardless of the persistence of PY exposure[38]. These behavioral effects are not surprising because PY pesticides induce a strong neurotoxic effect[39]. The signs

of toxicity effects characterized by motionlessness tadpoles were described in *R. arenarum* larvae after exposures to deltamethrin[20]. As mentioned above TRI is a potent inhibitor of AChE. The exposure of *R. arenarum* larvae may have resulted in an increased accumulation of acetylcholine (ACh) in the synaptic region; a causative factor for constant muscle contraction eventually leading to paralysis in the tail and body[40]. It can be interesting to consider the results of swimming behaviour and AChE activity, since both hypoactivity and hyperactivity can be associated with inhibition of AChE function[41]. In the same way, cypermethrin-induced tail/trunk deformities and asymmetrical movements in the exposed tadpoles[40].

Studies have been conducted that examine the relationship between PY insecticides and toxicity in tadpoles, but a clear relationship between ambient conditions and PY concentrations has not been identified. In this sense, their persistence in pond water is longer, and they may remain unchanged in sediments up to four months[42]. Therefore, examination of the effects of a short-term exposure to PY on biochemical markers in *R. arenarum* tadpoles could be informative for understanding the toxic-effect mechanism of these chemicals under real environmental conditions. Under the experimental conditions evaluated, B-esterases activity in *R. arenarum* tadpoles is equally sensitive endpoint than swimming activity. Thus, endpoints that integrate the behavioural and biochemical perspectives may provide a more sensitive indication of sublethal toxicity, and should hence be considered in the future.

Conclusion

In conclusion, the current study demonstrated that pesticide TRI is toxic to *R. arenarum* tadpoles and had a detrimental impact on the B-sterases activities and swimming activity at TRI sublethal concentrations. However, further studies are needed to elucidate the effects of toxicity on tadpoles exposed to TRI formulations, especially the role of surfactants and other hazardous components and the synergistic, additive or antagonistic combined effects of their active ingredients. Finally, we highlight the importance of using to use behavioral studies as a non-destructive biomarker that can be used as a tool in biomonitoring programs to assess the ecotoxicological risk in nontarget organisms.

Materials and Methods

Test Organisms and Experimental Design

Rhinella arenarum (Anura: Bufonidae) tadpoles were used as test organisms. This species has an extensive geographic distribution and abundance in the Neotropical region and is also listed as "not threatened" in the amphibian species categorization of Argentina. Its embryonic and larval stages are widely used in ecotoxicological tests due to its high sensitivity to water pollutions by pesticides. *R. arenarum* larvae were obtained from gelatinous egg strings collected from a site without agricultural activities located in natural area of Santa Fe city (Santa Fe province, Argentina). Eggs were transferred to tanks in to the Ecotoxicology Laboratory (Faculty of Biochemistry and Biological Sciences-FBCB-UNL), where they were acclimated under laboratory conditions at 12-h light/dark cycle with dechlorinated tap water (DTW), pH 7.2 ± 0.05; electrical conductivity, 164 ± 12.5 µS cm^{-1}; dissolved oxygen concentration, 6.5 ± 1.5 mg L^{-1} hardness, 49.5 mg L^{-1} of $CaCO_3$ at 22 ± 2°C, and feed on boiled lettuce (*Lactuca sativa*) until reached premetamorphic larvae at Gosner stages (GS) 26-30[43]. Larvae used in the experiments have been care according to the norms of ASIH (2004)[44] criteria and conforms to agreement from the animal ethics committee of FBCB (Res. CD N°: 388/06). Collection permit Nº: 02101-0018518-1 of Ministry of the Environment-Santa Fe.

Short-term static toxicity (48 h) was conducted using the insecticide TRISADA® (TRI), emulsifiable concentrates manufactured by Insumas S.R.L. This pesticide PY is compound by three ingredients: 1% (w/v) deltamethrin ([(S)-cyano-(3-phenoxyphenyl)methyl] (1R,3R)-3-(2,2-dibromoethenyl)-2,2-dimethylcyclopropane-1-carboxylate), 0.33% (w/v) tetramethrin (1,3-dioxo-4,5,6,7-tetrahydroisoindol-2-yl)methyl (1R,3R)-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropane-1-carboxylate), and 0.29% (w/v) piperonyl butoxide (5-[2-(2-butoxyethoxy) ethoxymethyl]-6-propyl-1,3-benzodioxole). The TRI is recommended to apply at concentrations of 10-20 cm^3 L^{-1} to control vectors of human's mosquitoes diseases. This commercial insecticide was applied as an insecticides mixture. Also, TRI is normally used for the control of flies, cockroaches, fleas, horsefly, lice and other insect pests in animals farming, factories and others agricultural exploitation. This product can be used in aqueous solution until 0.2%.

Since of the lack of environmental concentration data deposited on ponds and uncertainties associated with the fate of this commercial products used for mosquito management[51], NOEC, LOEC, and LC_{50} values were calculated. Premetamorphic larvae at GS 26-30 ($n = 180$), with average size (snout-tail tip) 15 ± 0.25 mm and weight 0.042 ± 0.005 g were exposed to five nominal concentrations of TRI: C1 (0.0003125%), C2 (0.000625%), C3 (0.00125%), C3 (0.0025%), C5 (0.005%) (v/v), and a negative CO whit dechlorinated

tap water (DTW). Both control and test solutions were made in triplicate. In the bioassay were used the glass recipients with 1 L capacity and 10 tadpoles/recipients. Larval mortality was monitored and dead larvae were removed every 24 h, and the cumulative mortality in each treatment was calculated at 48 h of exposure. Therefore, a subsample of control and treated TRI tadpoles ($n = 10$; respectively) of each concentrations that had a survival rate $>50\%$ at 48 h were used to measured AChE and CbE activities, and behavioural alterations[12].

Enzymatic Assays

Each TRI-treated tadpole was homogenized (on ice) in 0.1% t-octylphenoxypolyethoxy-ethanol (triton X-100) in 25 mM tris (hydroxyl methyl) aminomethane hydrochloride (pH = 8.0) and using a polytron. Suspensions were centrifuged at 10,000 rpm for 15 min at 4 ± 1°C and the supernatant (crude extract) was extracted. The Biuret method was used to determine protein concentration in the supernatants[46]. When sample volume was enough, enzyme kinetic assays were carried out in triplicate or duplicate.

Acetylcholinesterase Activity

AChE activity was determined colorimetrically following Ellman *et al*. (1961)[47] procedure. The reaction mixture (final volume [Final Volume = 930 µL]) consisted of 25 mM Tris-HCl containing 1 mM $CaCl_2$ (pH = 7.6), 10 µL 20 mM acetylthiocholine iodide (AcSCh), and 50 µL DTNB (3×10^{-4} M, final concentration). The variation in optical density was recorded at 410 nm for 1 min at 25ºC using a JENWAY 6405 UV-VIS spectrophotometer. AChE activities were expressed as nmol min^{-1} mg^{-1} protein using a molar extinction coefficient of 13.6×103 M^{-1} cm^{-1}.

Carboxylesterase Activity

CbE activity was measured as described by Bunyan and Jennings (1968)[48]. The reaction medium (1940 µL) consisted of 25 mM Tris-HCl containing 1 mM $CaCl_2$ (pH = 7.6), and the supernatant. After a 5-min preincubation period, the reaction was initiated by adding 50 µL of 1-NA (46 µM, in acetone) and incubated at 25ºC for 10 min. The formation rate of naphthol was stopped by adding 500 µL of 2.5% (w/v) SDS and subsequently 0.1% (w/v) of Fast Red ITR dissolved in 2.5% (w/v) Triton X-100. The samples were left in the dark for 30 min for colour development. The absorbance of the naphthol-Fast Red ITR complex was read at 530 nm (using a molar extinction coefficient of 33.225×103 M^{-1} cm^{-1}).

Behavioral Activity

At the end of the exposure, one TRI-treated larvae or control was released in the center of a semi-circle recipient (27 × 49 cm) filled with 2 liters of DTW. After 30 s of acclimation, behavioral variables were recorded during five min. using a digital video camera (Motic®, 10.0 M pixel) placed just above the recipients. Ten replications were made for each treatment. The behavioral variables evaluated were: distance moved (cm), mean speed ($cm.s^{-1}$), maximum speed ($cm.s^{-1}$), global activity (cm^2), and resting time (s). Video data (.avi format) were automatically analyzed using video-tracking software (Smart 3.0.02, Panlab Harvard Apparatus®).

Statistical Analysis

LC_{50} values and their respective 95% confidence limits (CL) were calculated using the Trimmed Spearman-Karber method[49]. The mortality data were statistically evaluated by ANOVA using Dunnett's procedure for multiple comparisons in order to determine the NOEC and the LOEC. All biomarkers data were expressed as the mean ± SEM. AChE and CbE enzyme activities were analyzed with Kruskal-Wallis test and Dunn's test for post hoc comparisons. An analysis of variance (ANOVA) was applied to assess the effects of TRI treatments on behavioral endpoints followed by the Dunnett's test for pairwise comparisons[12]. Correlations between AChE activities and behavioral variables for each treatment and CO were analysed using a Spearman's correlation test. These statistical methods were performed using BioEstat software 5.0[50]. A value of $P < 0.05$ was considered significant.

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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