Comparative toxicity of cypermethrin and a commercial formulation on *Rhinella arenarum* larval development (*Anura: Bufonidae*)

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Abstract: In this study we report the effects of cypermethrin (CY), a growing use insecticide, comparing the toxicity of the active ingredient (AI) and a commercial formulation (CF) on a non-target organism, *Rhinella arenarum*. The results showed a highly significant increase ($p < 0.05$) toxicity of both substances over time, obtaining a 96 h LC-50 of 28.07 mg CY/L and 11.20 mg CY/L, and a 336 h LC-50 of 0.0048 mg CY/L and 0.00065 mg CY/L, for AI and CF, respectively. Furthermore, the toxicity of CF was greater than AI at all exposure periods, reaching up to seven times. The larvae presented alterations in behaviour as hyperkinesia and spasmodic contractions up to total absence of movements. Considering these results and the risk assessment conducted, we conclude that CY represents a potential direct risk considering lethality, or indirect by sublethal effects for the survival of this amphibian in agroecosystems.

Keywords: *Rhinella arenarum*; larval development; amphibian; tadpoles; cypermethrin; commercial formulation; agrochemicals; lethal effects; sublethal effects; morphological alterations; toxicity bioassays.

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1 Introduction

Agroecosystems are exposed to different types of biocides affecting non-target organisms. Their presence in surface and groundwater is a result of drift and runoff during or after application. These pesticides produce toxic effects on human health and biota in general, so they are considered a problem of permanent analysis and review at global and regional levels.

Cypermethrin (CY), the alpha-cyano-3-phenoxybenzyl ester of 2,2-dimethyl-3-(2,2-dichlorovinyl)-cyclopropane-carboxylic acid, is a synthetic pyrethroid, similar to natural pyrethrins but more stable in the environment. Its structure is based on pyrethrum, an extract of dried flowers of chrysanthemums. It is used as non-systemic pesticide that acts by contact and ingestion to control a wide range of insects associated to cotton, cereal, vegetable and fruit crops. Owing to its low mammalian and bird toxicity, is also used to control pests affecting public health (flies, mosquitoes and cockroaches). Pyrethroids act primarily on the nervous system, by extending the open state of voltage-dependent sodium channels in nervous tissue. These altered sodium channels result in repetitive firing or depolarising block of the neuron, depending on how long the channel open state is prolonged (Soderlund et al., 2002; Narahashi, 2000).

CY is one of the most used insecticides in Argentina, where the application rates oscillate between 60 g and 280 g of active igredient per hectare, and more than 3,500,000 L per year are applied (CASAFE, 2011). Concentration range from 0.2 µg CY/L to 150 µg CY/L in streams near agroecosystems was reported (Garforth and Woodbridge, 1984; Jergentz et al., 2005; Marino and Ronco, 2005). In areas where the insecticide were applied in doses around 150 g CY/ha, a severe reduction of aquatic organisms was observed (Smies et al., 1980).

There are many studies about acute lethal and sublethal effects of this insecticide that have shown severe effects on different aquatic organisms (Tripathi and Singh, 2004; Shi et al., 2011; Wang et al., 2012). However, there is scarce information on effects produced by CY chronic exposure. Amphibian populations are actually on a global scale decline, and one of the causes could be the contamination of their habitat by agrochemicals (Beebee and Griffiths, 2005; Mann et al., 2009; Relyea, 2009). According to acute toxicity data, sublethal effects as axial flexure, eyes, head and intestines malformations, as well as alterations in behaviour were observed in *Hypsiboas pulchellus* (Agostini et al., 2010). In early stages of *Rana temporaria* exposed to concentrations as low as 1 µg CY/L, underdevelopment, reduced body size and tail flexure, were observed, while in latest stages, the inhibition of the metamorphosis process was informed (Paulov, 1990). At subcellular level, micronucleus induction in *Odontophrynus americanus* (Cabagna et al., 2006) and inhibition of the cholinesterase activity in *Rana tigrina* tadpoles (Khan et al., 2003) exposed to CY were reported.

Toxicity bioassays represent useful tools to assess the risk of exposure of ecosystems to different physicochemical agents. In this sense, the Amphitox test is a battery of bioassays that use embryo-larval stages of the common South American toad, *Rhinella arenarum* (Herkovits and Perez-Coll, 2003). Amphibians constitute key components on food webs, living near or in water reservoirs, so they are indirectly affected by pesticides. Moreover, the study on sublethal exposure to pesticides may be valuable in the assessment of the sensitivity to contaminants that could produce detrimental effects, e.g., increased vulnerability to predation and fitness reduction, which could eventually affect amphibian populations (Little et al., 1990).

The aim of this study was to compare the toxicity of the active ingredient (AI) and a commercial formulation (CF) of CY, Glextrin 25®, on *Rhinella arenarum* larval development. To meet this objective, we evaluated the lethal and sublethal effects (morphological alterations and behavioural disorders mainly) of CY, by means of the standardised laboratory conditions of the Amphitox test. On the basis of the Hazard-Quotient approach (USEPA, 1998) an ecological risk assessment of CY on this native species was performed.

2 Methods

2.1 Acquisition of Rhinella arenarum tadpoles

Three adult mating pairs of the common South American toad, *Rhinella arenarum*, weighing ~200–250 g were acquired in Lobos (Buenos Aires province, Argentina: 35º11′S; 59º05′W). Toad care, breeding, embryo acquisition and analysis were conducted according to the methods described in the Amphitox protocols (Herkovits et al., 2002; Herkovits and Pérez-Coll, 2003). Briefly, ovulation of females was induced by means of an intraperitoneal injection of a suspension of one homologous hypophysis in 1 mL of Amphitox solution (AS) per female, preserved according to Pisanó (1956). Oocytes were fertilised *in vitro* using fresh sperm suspended in AS. The composition of AS was sodium chloride (NaCl) 36 mg/L, potassium chloride (KCl) 0.5 mg/L, calcium chloride ($CaCl_2$) 1 mg/L and sodium bicarbonate ($NaHCO_3$) 2 mg/L, prepared in distilled water. Embryos were kept in AS and maintained at 20 ± 2°C, until organisms reached the complete operculum stage, S.25 (Del Conte and Sirlin, 1951). The AS was replaced entirely every 48 h and monitored weekly to ensure that the pH was at acceptable levels (7 ± 0.5).

2.2 Test solutions

Toxicity tests were performed using technical grade CY (95.1% purity, CAS No. 52315-07-8), purchased from Sigma Aldrich SA, and the commercial formulation with 25% AI (Glextrin 25®, Gleba S.A.). The composition of the commercial formulation and the active ingredients is a mixture of isomers of cypermethrin: α cyano-3-phenoxybenzyl(±)-cis-trans-3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropanecarboxylate. Concentrations tested were 0.005; 0.025; 0.1; 0.5; 1; 2.5; 5; 10; 15; 20; 25 and 30 mg CY/L. Test dilutions were prepared from a stock solution containing 1000 mg CY/L. CY technical grade stock solution was dissolved previously in analytical grade acetone. The CY concentration in stock solution was verified by chromatographic methods

using a GC/MS (Agilent 5975C equipment) with a DB5-MS 30×0.25 mm column and 0.25 µm film thickness, he as a carrier, and a programme temperature 150°C – 8°C/min – 280°C. The error between nominal and measured concentrations did not exceed 5%.

2.3 Toxicity bioassays

For treatments, 10 tadpoles at early S.25 were randomly placed in 10 cm glass Petri dishes containing 40 mL of test solution. Tests were done using three replications per solution, and control groups in AS and acetone were simultaneously maintained. Test solutions were entirely replaced every 48 h. Tadpoles were fed with balanced fish food TetraColor® *ad libitum*. The toxicity bioassays were performed by continuous exposure of early tadpoles (S.25) up to late S.25, for acute (96 h), short-term chronic (168 h) and chronic (336 h) periods.

2.4 Data analysis

The lethal and sublethal effects were evaluated every 24 h. Abnormalities were observed under a binocular stereoscopic microscope (Zeiss Stemi DV4), photographed with a Sony DSC-S90 digital camera, and identified according to the 'Atlas of Abnormalities' (Bantle et al., 1998).

Lethal Concentrations (LC) and NOEC values were statistically estimated by the USEPA Probit Program (USEPA, 1988). The results were considered statistically significant ($p < 0.05$) when the higher LC/lower LC ratio exceeded the critical value (95% confidence interval) established by APHA (1980).

The Estimated Environmental Concentration (EEC) for CY was calculated as a percentage of the maximum application rate proposed, 400 g AI/L/ha (Gleba, 2012). This percentage depends on exposure via spray drift, runoff and washoff (10%) or overspray exposure during aerial application (100%). The EEC was calculated assuming a water depth of 15 cm and an area of 1 m^2 (Boutin et al., 1993, 1995). The Hazard Quotient (HQ) is the ratio of the potential exposure to the substance and the level at which no adverse effects are expected (USEPA, 1998), calculated in this study as EEC/NOEC. Two HQ approaches were estimated: HQ 1, based on 10% of the maximum application rate proposed, and HQ 2, based on 100% of that value.

After the risk quotient was calculated, it was compared with the USEPA Level of Concern (LOC). The LOC is a policy tool that the Agency uses to interpret the risk quotient and analyse the potential risk to non-target organisms and the need to consider regulatory action. The LOC value for risk is 1. If the HQ > 1, harmful effects are likely due to the contaminant in question.

3 Results

Because the two controls of active ingredient treatments (AS and acetone solvent) did not differ statistically from one another, the term 'control' in the rest of the manuscript stands for the mean of both controls.

3.1 Lethal effects

From the first hours, the increased lethal effects of CF were remarkable, for example at 96 h, the lethality of tadpoles exposed to AI was significant just from 20 mg CY/L, while for CF, the lethality began to be significant as low as 2.5 mg CY/L.

According to LC and NOEC values (Table 1), the CF was significantly more toxic to tadpoles than the AI at all exposure times, except at 240 h (no significant differences). In addition, the results showed a highly significant increase in toxicity by extending the exposure time for both the AI and the CF, obtaining 96 and 168 h LC-50 of 28.07 and 12.53 mg CY/L for AI, while the same parameters for the CF were 11.20 and 2.74 mg CY/L. The 336 h LC-50 for AI and CF were as low as 4.8 µg/L and 0.65 µg/L, respectively, indicating a linear increase in toxicity by extending the exposure time. For all times, the CF was more toxic than the AI, reaching up to seven times at 336 h.

Table 1 Toxicity data (LC-50 and NOEC values) of *Rhinella arenarum* tadpoles exposed to: (a) active ingredient (AI) and (b) commercial formulation (CF)

	96	*168*	*240*	*336*
(*a*) *Exposure time* (h)				
LC-50 (mg CY/L)	28.07	12.53	2.06	0.0048
NOEC (mg CY/L)	12.60	6.95	0.63	0.000061
(*b*) *Exposure time* (h)				
LC-50 (mg CY/L)	11.20	2.74	1.82	0.00065
NOEC (mg CY/L)	3.03	0.87	0.29	0.000036

3.2 Sublethal effects

The main sublethal effects caused by the AI and the CF on *Rhinella arenarum* were associated with behavioural alterations. From the first hours, this neurotoxicity was expressed as hyperkinesia, spasmodic contractions, twisting, erratic swimming and loss of balance. These effects over time evolved into general weakness up to total absence of spontaneous movement, or even after light/mechanical stimulus including at the lower concentrations. Furthermore, a reduction in food intake was observed. With regard to morphological alterations, tadpoles treated with CF and AI exhibited development delay, reduced body size, lateral curving of the tail, axial flexure and marked oedemas (Figure 1). All these abnormalities were concentration-dependent.

3.3 Ecological risk evaluation

The EEC for CY was calculated as a percentage of the maximum application rate proposed (400 g AI/L/ha; Boutin et al., 1993, 1995). This percentage could depend on:

a exposure via spray drift, runoff and washoff (10%), or

b overspray exposure during aerial application (100%).

So, the EEC calculated for CY taking account the first assumption (a) is 4 mg AI/L/m^2, and for (b) is 40 mg AI/L/m^2. Using these values, we estimated two hazard quotient approaches (HQ = EEC/NOEC) for each one of the toxics (CF and AI). Table 2

summarises the information of the HQ obtained at three different times. The obtained results highlight that HQ 2, for all exposure periods, were over the LOC value, both for AI and CF. The HQ 1 was over the LOC value for the AI from short-term chronic exposure, while for the CF, the HQ 1 was over the LOC value even from acute exposure onwards.

Figure 1 Stereoscopic microscopy pictures of *Rhinella arenarum* tadpoles exposed to different concentrations of the commercial formulation of CY (mg/L), at 192 h (top figures), and at 336 h (bottom figures) (see online version for colours)

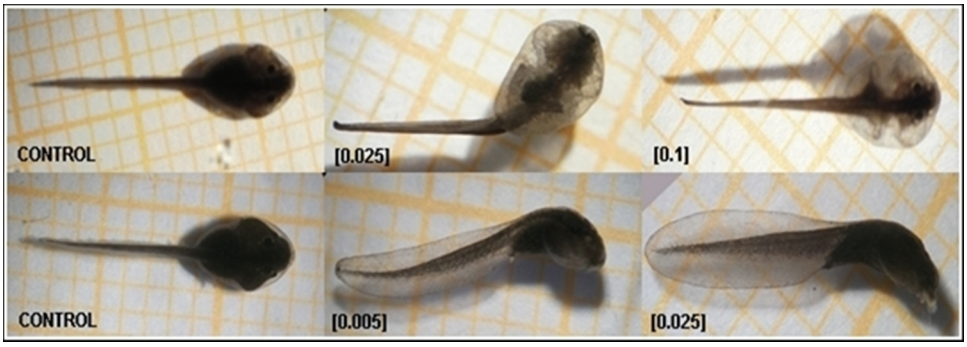

Table 2 NOEC (mg CY/L) and hazard quotient (HQ) values for *Rhinella arenarum* tadpoles at acute (96 h), short-term chronic (168 h), and chronic (336 h) exposures to active ingredient (AI) and commercial formulation (CF). *HQ > 1, estimates harmful effects due to CY exposure

	96 h			*168 h*			*336 h*		
	NOEC	*HQ1*	*HQ2*	*NOEC*	*HQ1*	*HQ2*	*NOEC*	*HQ1*	*HQ2*
AI	12.60	0.32	3.17*	6.95	0.58	5.76*	0.000061	65,573*	655,737*
CF	3.03	1.32*	13.20*	0.87	4.60*	45.98*	0.000036	111,111*	1,111,111*

4 Discussion

The results of this study highlight the high toxicity of CY, both for the active ingredient (AI) and the commercial formulation (CF) on *Rhinella arenarum* larval development. The CF exhibited higher toxicity than the AI, for lethal and sublethal effects, a very relevant fact considering that it is effectively applied in crops. This differential sensibility is showed in most of pesticides, and could be explained due to the effects of the coadjuvants (Mann and Bidwell, 1999; Relyea, 2009), producing additional toxicity and a magnification of the AI activity by synergic effects. At present time, CY is part of the 'new generation' pesticides, and is proposed to be an alternative to replace organochlorines such as endosulfan, the most used insecticide, which has been globally banished at 2011. So, the use of CY is likely to increase, and amphibians will be more likely to be exposed.

The acute toxicity values of CY presented rather differences from the results obtained for other amphibians. A 48 h LC-50 as low as 6.5 µg CY/L was reported for *Rana temporaria* tadpoles (Paulov, 1990), and for *Physalaemus biligonigerus* tadpoles

was determined that the 96 h LC-50 induced by CY was 129 µg CY/L (Izaguirre et al., 2000). In this study, although CY resulted lethal at concentrations exceeding the environmentally relevant range at acute exposure, the lethality at chronic exposure was extremely severe, highlighting the importance to extend the exposure time of the bioassays. It is also possible that if the exposure time of the pesticide had followed throughout *Rhinella arenarum* larval development, metamorphosis of tadpoles could have been delayed, as observed in *Rana arvalis* (Greulich and Pflugmacher, 2003). These metamorphosed tadpoles were distinguished by a much more compact physique (significant reduction in length, and significant increase in weight) so as to resist the adverse conditions. This fact was also observed by exposure to other pesticides (Brodeur et al., 2009; Svartz et al., 2012).

The morphological alterations observed in the present study were also reported in other researches. Tadpoles of *Hypsiboas pulchellus* exposed to CY showed lower body length, axial flexures, eye, head and intestine malformations (Agostini et al., 2010). The effects on behaviour reported such as hyperkinesia, spasmodic contractions, twisting, erratic swimming and loss of balance represent typical signs of cyano pyrethroid poisoning, and were also observed by Agostini et al. (2010). These alterations can be explained by the apoptosis that CY induces in the central nervous system (Izaguirre et al., 2000; Casco et al., 2006). In *Rhinella arenarum* pre-metamorphic tadpoles was observed that cellular apoptosis susceptibility (CAS) increases at the beginning of the exposure, suggesting a proliferative or regenerative effect, but decreases when the concentration and/or the biocide exposure time increases, suggesting compromise of the cellular cycle control and trigger of an apoptotic wave (Izaguirre et al., 2006).

In general, the amphibians must spend energy on pesticides detoxification to regain their physiological balance. This fact may increase the sublethal effects. Consequently, these alterations influence the fitness of the individuals (Semlitsch, 1990), making them more susceptible to predation, for example because they lose the ability to dart away (Ortiz-Santaliestra et al., 2010), or even making them more vulnerable to other environmental stressors, such as infectious agents, invasive species, changes in environmental physicochemical parameters, etc. The toxic effects are expressed at individual level, but in long-term would impact negatively on the viability of amphibian populations (Sparling et al., 2001), producing changes in abundance, age structure and dynamics, leading to the risk of population decline. Furthermore, these effects may influence other ecological levels and finally on ecosystems to which they belong.

The HQ approach provides a possibility to assess the risk for adverse effects of CY to *Rhinella arenarum* tadpoles. Taking into account the information resulted in this study, calculation of HQ 2 (based on direct applications, considering the worst scenario), both for IA and CF, resulted in values higher than 1 even for acute exposure, which represents the USEPA LOC. On the basis of the results of HQ 1 (via spray drift, runoff and washoff), CF had these values over the LOC just from acute exposure, while for IA these quotients were over the LOC only for chronic exposure. It is noteworthy that these results indicate that both AI and CF represent a potential risk at short-term chronic exposure, but CF means even more a risk at acute periods (at a normal scenario), at least for tadpoles of this amphibian species.

Considering all obtained results and contemplating the risk assessed based on the estimated HQ, contamination of water bodies by CY could result in severe adverse effects on the development of *Rhinella arenarum* tadpoles, disrupting the populations of this native amphibian.

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