Impact of endosulphan and cypermethrin mixture on amphibians under field use for biotech soya bean production

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Abstract: This study presents results at a local scale of the impact of pesticide aerial applications containing a mixture of endosulphan and cypermethrin on anurans assessed by means of field evaluations. Additionally, effects were compared with laboratory toxicity tests in standardised conditions. Field assessment with native population allowed the detection of dead anuran larvae of the species present in the stream of water after fumigation, in coincidence with the decrease in the frequency of live larvae ($p < 0.01$) from sampling nets. Cage experiments showed significant differences ($p < 0.01$) in anuran larvae survival before and after fumigations. Laboratory acute toxicity tests with endosulphan formulation demonstrated sublethal symptoms in *Hypsiboas pulchellus* larvae 24 h after initial exposure time. The 96 h LC-50 was 0.13 µg endosulphan/litre, which is over 1000 times lower than the cypermethrin LC-50 value. Endosulphan seems to be the insecticide responsible for the drastic effects on anuran larvae detected in the field during studied events.

Keywords: anuran; tadpoles; soya bean; endosulphan; cypermethrin; acute toxicity; *Hypsiboas pulchellus*; *Rhinella fernandezae*; *Leptodactylus latinasus*; field assessments; caged toxicity tests; laboratory toxicity tests.

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1 Introduction

The Argentine Pampa is an area of mild climate and fertile soils developed over deep loessic sediments. Originally it was covered by grasslands and for a long time the farmers employed a mixed system of livestock and crops, mainly wheat and corn. No tillage managerial practices were introduced until the soya bean resistant to glyphosate became available and the direct seeding was adopted by farmers. During the last few decades, the traditional production system has been replaced by intensive soya bean production. Land cultivated with soya beans increased from less than 40,000 ha in the 1970s to 8,300,000 ha at the end of the century (Schvarzer and Tavosnanska, 2007), reaching at present 18 million ha. Pesticide consumption increased from 6 million to 18 million kg only in the period from 1992 to 1997 (Bindraban et al., 2009), and has continued to increase more slowly since then. Formulations of the herbicide glyphosate and the insecticide cypermethrin are the most commonly used, followed by the organophosphate chlorpyrifos and the organochlorinated endosulphan.

Although some recent publications in the region deal with detailed aspects of pesticide effect on the biota (Jergentz et al., 2004a; Jergentz et al., 2004b; Martin and Ronco, 2006; Carriquiriborde et al., 2007; Ronco et al., 2008), the study of overall impact of such vast agricultural intensification is in progress.

Studies under laboratory conditions for the assessment of lethal and sublethal effects of the insecticides endosulphan (Brumhal and Shine, 2003; Goulet and Hontela, 2003; Brunelli et al., 2009) and cypermethrin (Campana et al., 2003; Cabagna et al., 2006) on different species of anuran larvae have shown severe effects associated with these compounds, although there is scarce information on the effects of pesticides on anuran populations from field studies (Relyea, 2004).

The present study presents results at a local scale of the impact on anurans of pesticide aerial applications containing a mixture of endosulphan and cypermethrin assessed by means of field studies (native populations and cage experiments). Additionally, the effects of insecticide formulations were compared with laboratory toxicity tests in standardised conditions.

2 Methods

2.1 Field studies

Studies were carried out at a local scale in a tributary of El Pescado stream (35°1′ S; 57°51′ W) of the Pampa's Plains, Province of Buenos Aires, Argentina. Three impact sites were situated along 1 km of the stream tributary in a sector traversing a cultivated field plot (Figure 1). Since there is no presence of undisturbed environments in the nearby area (Camilion et al., 2003; Ronco et al., 2007) that could be used as reference sites for comparisons, the strategy taken into account in the present study included comparisons between native anuran populations within the site before and after the fumigation of insecticides.

Two fumigation (aerial applications) events of a mixture of endosulphan (Thionex-L® formulation, 35% w/v) and cypermethrin (Sherpa® formulation, 25% w/v) at a proportion of 700 ml + 150 ml in 100 litre water per ha (total 64 ha) were studied (15 February 2005 and 15 March 2005). Events of potential contamination were taken at the time of pesticide application and the first rain after spray, no further than 15 days after treatment. Registration of pH, temperature and water depth in the stream of water was also done.

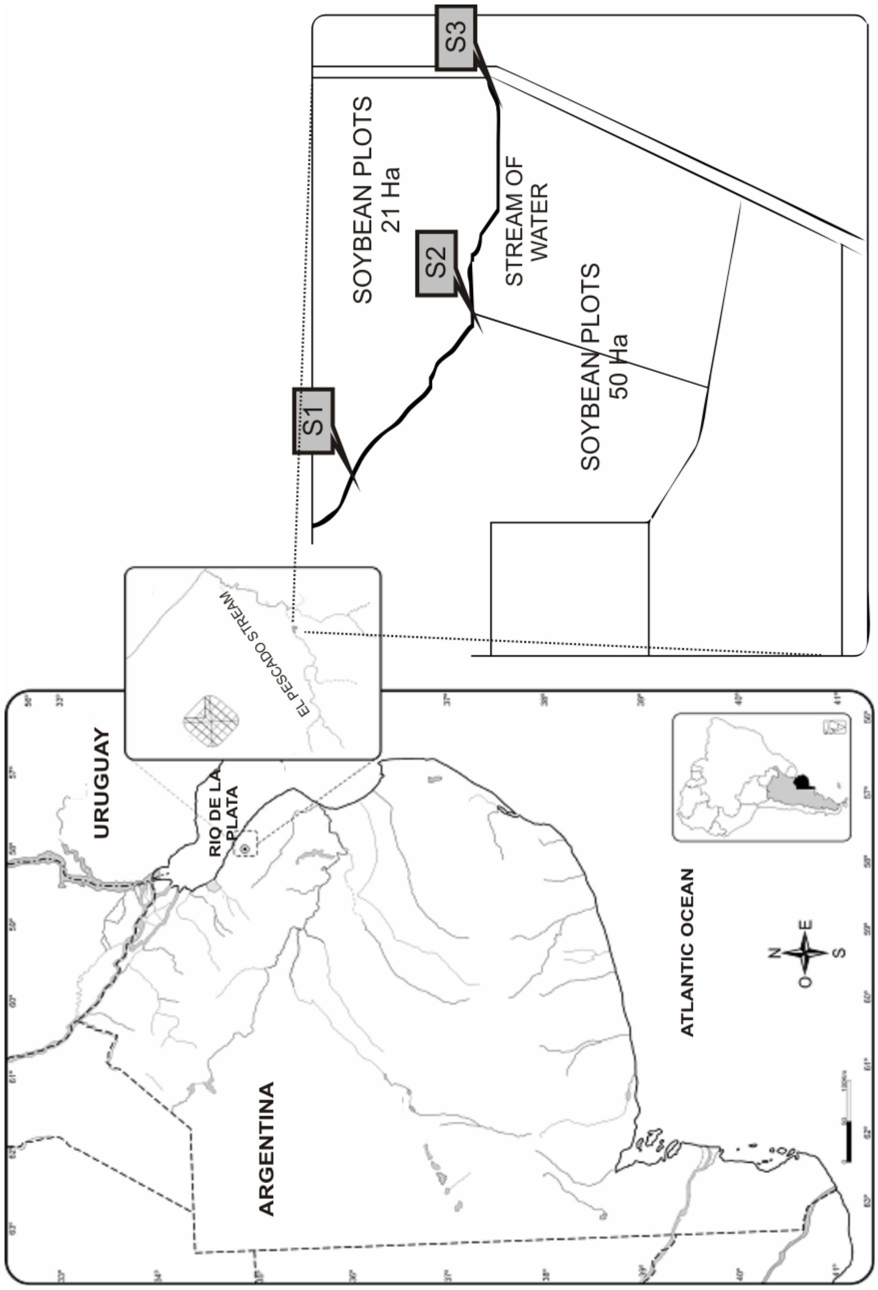
2.1.1 Assessment of effects on native populations

The study started during November 2004. Sampling of anuran larvae was done according to the quantitative sampling method (Heyer et al., 1994) for amphibian larvae, with ten replications per site. Sampling was performed using 20 cm diameter hand nets 1 mm mesh with 1 m passing rates, submerged between 10 cm and 30 cm. Net contents were placed on a dish for counting and species determination. Surveys were carried every 15 days during all the soya bean cultivation cycle, counting the number of larvae of each species per net (observed frequency). Particular attention was given to observations of dead individuals 24 h before and after each potential event of contamination, additionally a registration of larvae for counting mortalities or dying organisms along the water stream in the study sector was performed.

2.1.2 Assessment of effects on caged organisms

Field experiments with caged anurans larvae of *Rhinella fernandezae* and *Hypsiboas pulchellus* obtained in the field were performed in 50 cm × 25 cm × 25 cm devices built with an iron frame and lined by a net 1 mm mesh, using 20 larvae per cage and three replications. Organisms were placed a week before each spray event and were checked daily. Mortalities were assessed every 24 h after insecticides application in the adjacent soya bean field during the three following days. Dead tadpoles were daily removed from cages during inspections. Devices were placed in the stream of water in vertical position fixed to an iron rod, with the bottom end on top of the sediment surface and top over the surface of the water level.

Figure 1 Study site area and stream of water location (S1: site 1, S2: site 2, S3: site 3)



2.2 Laboratory toxicity tests

Toxicity tests with anuran larvae were carried out using *H. pulchellus* larvae obtained from a pair in amplexus from a temporary unpolluted pond located in the flood plain of the El Pescado stream (35°01′ S, 57°59′ W) in a region free of agricultural activities (Natale, 2006). Eggs were acclimated in the laboratory at 25°C ± 1 and at a light: darkness ratio 16:8, until organisms reached stage 25 (Gosner, 1960), and then used for testing. Semi-static toxicity tests, with no feeding, were done exposing five organisms per replication, using eight concentrations of Thionex-L® formulation of endosulphan (35 g:100 cm^3) between 0.01 µg endosulphan/litre and 1000 µg endosulphan/litre, added of two negative controls and a positive control with 23 mg Cr (VI)/ litre according to Natale et al. (2006), with four replications. Tests were carried out in 1 litre glass jars using 500 ml of test solutions. Testing water was dechlorinated tap water (pH: 7.7–8.3; hardness: 180–250 mg $CaCO_3$/L). The tests were semi-static, with no feeding and with dilution refill every 24 h, time when the corresponding mortality readings were taken. Lethal and sublethal assessment end points (slight or profound narcosis) were measured.

2.3 Statistical analysis

Larvae frequency comparisons before and after insecticide application, for each sampling site, with data from registers of hand nets samplings of anuran larvae were assessed by means of the goodness fit test using Chi square (Zar, 1999). The observed value was taken from observed frequency (number of live individuals per net), after each application of insecticide event per study site, and the expected value from the observed frequency before each event. A logarithmic transformation was done for n = the number of individuals per net the variable V = Ln (n + 0.5).

Anuran larvae survival in field caged experiments were analysed by means of the goodness fit test using Chi square (Zar, 1999), considering as observed value the mean number of alive organisms in each cage after the event of potential contamination, and as expected value the number of alive organisms at initial time of experiment.

Laboratory toxicity tests were analysed by Probit analysis (Finney, 1971) and LC-50 values and the 95% confidence interval were calculated from estimated dose-response curves using the Probit Analysis Program, version 1.5 (USEPA, 1999).

3 Results

Mean water temperature, maximum and minimum were 25.5 (21.0–32.0)°C. Water levels ranged from 25.7 cm through 63.3 cm between sites. The pH remained between 6.5 and 7.0. No rain events occurred after 15 days of insecticides application in any of the studied events due to an unusual dry period in the region. Hence, assessment of the effects of the insecticides runoff could not be done.

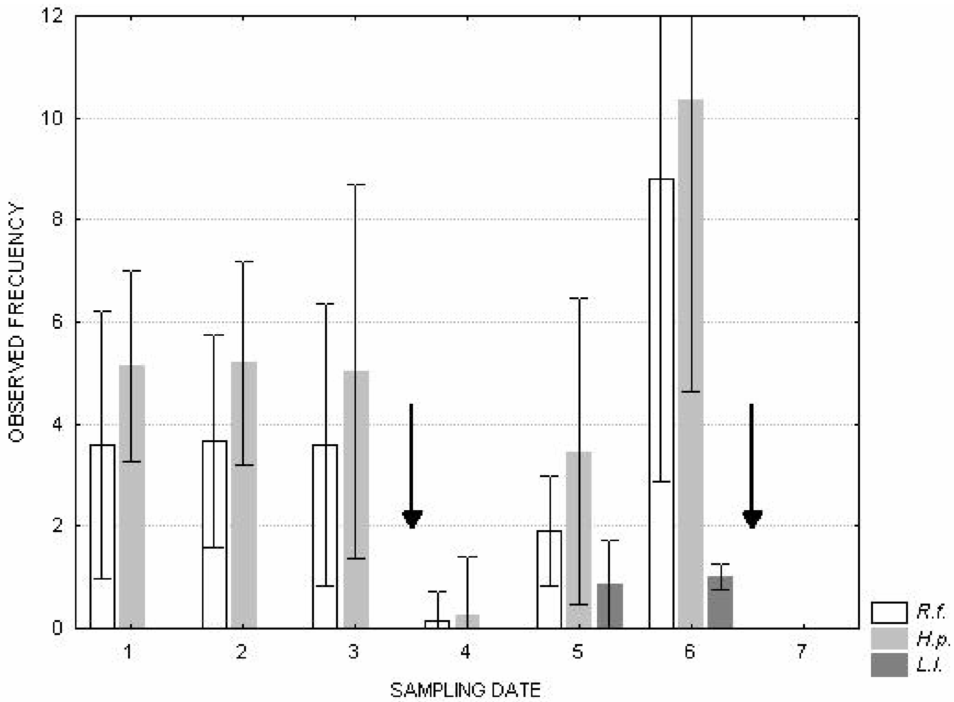
During the study three species of larvae were detected, listed as follows in order from the most to the least abundant: *Hypsiboas pulchellus*, *Rhinella fernandezae* and *Leptodactylus latinasus*.

3.1 Field studies

3.1.1 Effects on native populations

Field assessment with native population allowed detection in both of the events of endosulphan-cypermethrin mixture applications dead anuran larvae of the species present in the stream of water, in coincidence with the decrease in the frequency of dead larvae detected in the samplings with nets (Figure 2). Also, dead invertebrates and fish were observed. Statistical analysis of results from larvae survival counting in each site by means of the Chi square test showed significant differences ($p < 0.01$) for sites 1 and 2 in both events. Site 3 only showed significant differences in the second spray event ($p < 0.01$).

Figure 2 Observed frequency of larvae (mean number of each species per net) found in the stream of water during samplings performed every 15 days. Arrows show application events of the cypermethrin-endosulphan mixture (R.f.: *R. fernandezae*, H.p.: *H. pulchellus*, L.l.: *L. latinasus*)



3.1.2 Effect on caged organisms

Results of the goodness fit test showed significant differences ($p < 0.01$) between the observed and expected frequencies of live organisms in both events. Percentages of mortalities before and after events of potential contamination could be seen in Table 1.

Table 1 Percentage of survival in caged experiments for each application event of an endosulphan-cypemethrin mixture, according to the test species and study site

Species	*First event*		*Second event*	
	S1	*S2*	*S1*	*S2*
Hypsiboas pulchellus	25%*	37%*	–	–
Rhinella fernandezae	27%*	57%*	66%*	40%*

Notes: *significant differences (p < 0.01). S1: site 1; S2: site 2.

3.2 Laboratory toxicity tests

3.2.1 Sublethal effects

Sublethal symptoms (slight and strong narcosis) could be detected 24 h after initial time of exposure (Table 2) with tested *H. pulchellus* larvae. Surprisingly, in all the dilutions sublethal effects were detected on the 100% of the 20 tested individuals of the corresponding dilution. The observed sublethal responses were none or all for each of the assessed concentrations.

Table 2 Letal and sublethal effects of endosulphan on *H. pulchellus* larvae at different exposure times in laboratory toxicity tests

Endosulphan (μg/L)	*Exposure time (h)*			
	24	*48*	*72*	*96*
0.01	SN	SN	NORM	NORM
0.1	SN	SN	NORM	NORM
1	SN	PN	MO	MO
5	SN	PN	MO	MO
10	SN	MO	MO	MO
50	SN	MO	MO	MO
100	MO	MO	MO	MO
1000	MO	MO	MO	MO
0 (Control group)	NORM	NORM	NORM	NORM

Notes: SN: slight narcosis; PN: profound narcosis; MO: mortality; NORM: normal.

3.2.2 Lethal effects

Table 2 also shows the concentrations at which mortalities were detected at different exposure times. A different behaviour was seen between the lethal and sublethal observations. An expected gradual increasing response (percentages of dead/alive individuals) was correlated with the increment of endosulphan concentrations allowing the application of Probit analysis for statistical end-point estimations (Table 3).

Table 3 Toxicity data for endosulphan given as LC-50 values with a 95% confidence interval (CI)

	24 h	*48 h*	*72 h*	*96 h*
LC-50	64.47	40.70	0.78	0.13
CI 95%	45.00–91.84	28.01–59.61	0.44–1.23	0.07–0.24
n	4	5	4	4
a	–0.6450	1.2266	5.2343	6.5310
b	3.1199	2.344	2.1404	1.7366

Notes: n = number of pairs of x, y values; a = y intercept (elevation), b = regression coefficient (slope).

4 Discussion

The observed results have clearly shown drastic effects on two species of anuran larvae in relation to the mixture application of endosulphan-cypermethrin insecticides assessed by means of field assessments along a stream of water traversing a soya bean plot at a local scale. Having in mind that previous reports in the same place with equivalent methods have shown very low effects associated to cypermethrin applications (Ronco et al., 2008), the effects here reported could be mainly related to the toxicity of endosulphan by itself, or furthermore to additional toxicity due to its mixture with cypermethrin. Both of the species, *R. fernandezae* and *H. pulchellus*, used for testing in caged field experiments responded similarly in both application events. Detected mortalities in these experiments coincide with findings of dead anuran larvae in the stream of water, together with dead aquatic invertebrates and fish after fumigations. Additionally, the laboratory toxicity tests reported here with larvae of *H. pulchellus* have also demonstrated for the same formulation of endosulphan high levels of acute lethal and sublethal effects.

The relative acute toxicity (96 h exposure) of endosulphan and cypermethrin on *H. pulchellus* was compared by means of the Chemical Hazard Index (CHI) proposed by Birge et al. (2000) using *Oncorhynchus mykiss* LC-50 values for each toxicant as reference. Using the laboratory toxicity data of endosulphan obtained here and the LC-50 value for the reference species obtained by Capkin et al. (2006), a CHI (96 h LC-50): 0.13/1.75 = 0.074 was obtained. Also, taking into account the sensitivity of *H. pulchellus* to cypermethrin (given as the nominal values of the a.i. in Sherpa® formulation), with an LC-50 value and the 95% confidence interval of 241.9 [149.4–415.4] μg cypermethrin/L (unpublished data), and the LC-50 value for the reference species obtained by Stephenson (1982), the CHI (96 h LC-50): 241.9/2.8 = 86.393. This analysis shows that *H. pulchellus* is very sensitive to endosulphan and less sensitive to cypermethrin in comparison to the reference fish species. Also, it shows that endosulphan is 1860 fold more toxic to this anuran species than cypermethrin.

With the 96 h LC-50 data from the literature for amphibian larvae to endosulphan and cypermethrin from the ECOTOX Release 4.0 (http://cfpub.epa.gov/ecotox/) and the index proposed by Birge et al. (2000) we could compare the acute toxicity of endosulphan to *H. pulchellus* (CHI = 0.074) with published CHI values for other anuran species. It can be observed that this species is more sensitive than *Bufo bufo* 0.246 (Brunelli et al., 2009), *Rana tigrina* 1.028 (Gopal et al., 1981), *Rana clamitans* 8.571

(Harris et al., 1998) and *Bufo melanostictus* 70.286 (Vardia et al, 1984). Also, Lajmanovich et al. (2005) demonstrated micronucleous induction in erythrocytes of *H. pulchellus* at concentrations below 0.010 mg/L endosulphan in laboratory exposure tests.

Fort the case of cypermethrin, it was possible to estimate only one CHI value for the autochthonous species *Physalaemus biligonigerus* of 46.071, taking into account the 96 h LC-50 obtained by Izaguirre et al. (2000). These authors also demonstrated that a concentration of 12.9 µg/L induced apoptotic cell in prometamorphic larvae developing brain.

Additionally, the Predicted Environmental Concentration (PEC) of the insecticides taking into account the stream of water characteristics and the quantities of compounds used was estimated. If we consider that the area of the stream of water was 3,239.43 m^2 (estimated from a satellite image Digital Globe, ID 1010010002E3FD02, at the spray date, available from Google Earth, georeferenced, and using GIS Kosmo software to estimate the corresponding area), the mean water depth (40 cm), and the amount of products used (245 g endosulphan and 37.5 g cypermethrin per ha), the concentrations would be 61.2 µg/L of endosulphan and 9.4 µg/L of cypermethrin. If we take into account this information it can be seen the endosulphan PEC is much above the LC-50 value here reported. Furthermore, the cypermethrin PEC is below the corresponding LC-50 value. Hence, it could be attributed to endosulphan the majority of the toxic load. If we now consider the existent reports from the literature on studies of the toxicity of mixtures with both insecticides indicating no interactions (Price, 1981), we can conclude that information from the present study provides further elements for considering that endosulphan is the insecticide responsible for the drastic effects on anuran larvae detected in the field during the studied events. The present field surveys and laboratory assessments on the effects of endosulphan on anuran larvae, together with the existent information on the lethal and sublethal effects on other anuran species, provides further knowledge supporting the high risks associated to the use of the endosulphan in agroecosystems.

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