Comparative toxicity of endosulfan and diazinon on the embryo-larval development of the South American toad, *Rhinella arenarum*

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Abstract: The toxicities of endosulfan and diazinon were comparatively analysed on the early development of the South American toad, *Rhinella arenarum*. Pesticides varied greatly in their effects on survival, with concentration-, time- and stage-dependent sensitivity. Endosulfan was 227 times more toxic than diazinon. The larval period was the most sensitive for both pesticides (LC-504 h: 0.01 mg L^{-1} and 1.92 mg L^{-1} for endosulfan and diazinon, respectively). Endosulfan toxicity increased 587 and 60 times from acute to chronic exposure in treatments with embryos and larvae, respectively, while diazinon toxicity increased about four and five times, respectively. Both pesticides caused important neurotoxic effects expressed as behavioural disturbances. Risk evaluation assessed by hazard quotient (HQ) was over 1, the level of concern (LOC), for both pesticides at chronic exposure periods and even at acute endosulfan exposure. Results obtained in the present study highlight that these pesticides should be considered potential threats for this species.

Keywords: endosulfan; diazinon; amphibians; embryo-larval toxicity; neurotoxic effects; organochlorine; organophosphate.

Reference to this paper should be made as follows: Aronzon, C., Svartz, G. and Pérez Coll, C. (2017) 'Comparative toxicity of endosulfan and diazinon on the embryo-larval development of the South American toad, *Rhinella arenarum*', *Int. J. Environment and Health*, Vol. 8, No. 3, pp.225–234.

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1 Introduction

Lately, agriculture development has been mainly characterised by the expansion of its boundary and the increased use of agrochemicals, which can control harmful pests with low cost and effort. However, the inappropriate use of pesticides may result in adverse effects on human health and non-target organisms, like amphibians (Mann et al., 2009). Moreover, the decline of amphibians and the large number of malformations found in populations worldwide have caused increasing concern (Mann et al., 2009).

The likelihood that exposure to pesticides may cause harmful ecological effects can be estimated numerically using the hazard quotient (HQ) approach (US EPA, 1998), which represents a useful diagnostic tool for water pollution control purposes. Most of the toxicity studies evaluate adverse effects only in acute exposure conditions, focusing on a certain period of the life cycle; however, assessing the toxic effects at different developmental stages would be necessary to find the most sensitive life period of a species for management and conservation decisions (Aronzon et al., 2011). Besides, extending the evaluation time, beyond the acute period to chronic exposure might show a more environmentally realistic approach.

Endosulfan is a synthetic organochlorine compound widely used in agriculture to control insects and acarus. It has been classified as ‘highly hazardous’ but is still largely used, particularly in some developing countries such as Argentina where it has been banned only recently (SENASA, 2013) and is still present in the environment (Etchegoyen et al., 2017). As is a persistent organic pollutant an almost worldwide distribution might be expected.

Diazinon is an organophosphate pesticide extensively applied and is one of the most stable organophosphorus pesticides, with a low degradation rate in water (IPCS, 1998), it has been classified as probably carcinogenic to humans (Group 2A) (International Agency for Research on Cancer, 2015).

In this context, the main aim of present study was to evaluate and compare the toxicity of endosulfan and diazinon on the embryo-larval development of the native toad, *Rhinella arenarum*, a keystone member of aquatic ecosystems of wide regions of South America by means of a standardised test (Herkovits and Pérez-Coll, 2003). Moreover, on the basis of the hazard quotient approach, the ecological risk assessments of both pesticides were performed.

2 Materials and methods

2.1 Acquisition of Rhinella arenarum embryos

Three pairs of *R. arenarum* adults, weighing ~200–250 g per animal were acquired in a non-impact site (Buenos Aires, Argentina: 35°11′S; 59°05′W). Embryos and larvae acquisition and husbandry were carried out according to Herkovits and Pérez-Coll (2003). Amphitox Solution (AS) composition is (in mg L^{-1}): Na^+ 14.75; Cl^- 22.71; K^+ 0.26; Ca^{2+} 0.36; HCO_3^- 1.45. The embryo-larval stages were identified according to Del Conte and Sirlin (1951). All experiments were conducted in accordance to international standards on animal welfare (Canadian Council on Animal Care in Science, 1993).

2.2 Test solutions

Twelve endosulfan (technical-grade PS81, purity of 99%, Supelco, CAS 115-29-7) exposure solutions ranged between 0.005 and 20 mg endosulfan L^{-1} were prepared by diluting the stock solution (1 g endosulfan L^{-1} in analytical grade acetone) with AS. The concentration of endosulfan in stock solution was analysed by HPLC-ESI-MS according to Svartz et al. (2014). Nine diazinon (purity 99%, CAS number: 333-41-5, Lot LB75417, Supelco Analytical) exposure solutions ranged between 0.5 and 45 mg diazinon L^{-1} were prepared by diluting the corresponding volume of the stock solution (3 g diazinon L^{-1} in analytical grade acetone) in AS. The concentrations of diazinon test solutions were analysed by HPLC-ESI-MS in SIM mode, positive detection according to Aronzon et al. (2014). The error between nominal and measured concentrations did not exceed 5%.

2.3 Toxicity experimental protocols

R. arenarum embryos at early blastula stage (S.4) and larvae at complete operculum stage (S.25) were continuously and separately exposed to endosulfan or diazinon for chronic (504 h) periods. Experimental condition was carried out according to Herkovits and Pérez-Coll (2003). In addition to AS control, a vehicle control was also done, with AS plus acetone at the highest concentrations used for endosulfan and diazinon test solutions, respectively; acetone concentration was always lower than 1.1%. Both controls were simultaneously maintained without differences, so the term ‘control’ in the rest of the manuscript stands for the means of both controls. Test solutions were renewed every other day and temperature was maintained at 20 ± 2°C. Lethal effects were evaluated with a Zeiss Stemi DV4 stereoscopic microscope (SM) and dead individuals were removed every 24 h. Larvae were fed with 6 ± 0.5 mg of balanced fish food TetraColor® for 24 h every other day. Toxicity experimental protocols were replicated three times using different clutches.

Behavioural alterations were evaluated (Denoël et al., 2013). Smooth movements of the Petri-dishes were done, and finally heartbeat was checked under the SM.

Ecological risk was numerically estimated using the hazard quotient (HQ) approach (US EPA, 1998), which is based on the comparison of the expected environmental concentration (EEC) (Boutin et al., 1995) with a standard toxicity end point (LC10). EEC for each compound was based on the maximum application rate given on manufacturer's labels, inferring an overspray exposure during aerial application, assuming a water depth of 15 cm and an area of 1 m^2. The maximum application rate allowed for an endosulfan and diazinon commercial formulation is 4.37 kg ha^{-1} active ingredient (Formulagro SRL) and 4.5 kg ha^{-1} active ingredient (Syngenta Crop Protection Inc.), respectively. The HQ calculated was compared with the USEPA level of concern (LOC). The LOC value for risk is 0.5 and 1 for acute and chronic exposure, respectively. If HQ > LOC value, harmful effects are likely due to the contaminant in question.

2.4 Statistical analysis

Lethal concentrations (LCs) were statistically estimated by the USEPA Probit Program (US EPA, 1988). Statistical differences between LC50 were carried according to APHA (2005) criteria. NOEC value of abnormal behaviour was calculated by chi square statistical analysis using GraphPad Prism 5.

3 Results

3.1 Embryo exposure

Acute endosulfan and diazinon NOEC values were 15 mg L^{-1} and 30 mg L^{-1}, respectively. From 96 h exposure, there was an important increase in toxicity for both pesticides, which was coincident with the beginning of the muscular response stage (S.18). Endosulfan toxicity increased 587 times from acute to chronic exposure (LC50-96 h = 17.62 mg L^{-1} to LC50-504 h = 0.03 mg L^{-1}) while diazinon toxicity increased four times during the same period (LC50-96 h = 27.2 mg L^{-1} to LC50-504 h = 6.8 mg L^{-1}). Differences in toxicity from acute to chronic periods were between 1.5 and 227 times, resulting endosulfan the most toxic pesticide at all exposure periods (Figure 1(a)). Embryos exposed to both pesticides exhibited neurotoxicity from 96 h, concurrently with the beginning of neuromuscular activity. Embryos showed spasmodic contractions, erratic swimming and loss of balance. These effects evolved over time into general weakness and reduction in food intake, up to total absence of spontaneous movements and even after light/mechanical stimulus. All these neurotoxic effects were concentration- and time-dependent. Thus, NOEC-168 h for endosulfan sublethal effects was as low as 0.01 mg L^{-1}, whereas this parameter for diazinon was 3 mg L^{-1}.

3.2 Larvae exposure

The LC50s of endosulfan and diazinon for larvae exposed from S.25 show a time-dependent toxicity of both pesticides. Although NOEC values at acute period were as high as 1 mg L^{-1} endosulfan and 6 mg L^{-1} diazinon, the chronic toxicity of endosulfan increased 63 times (LC50-96 h = 0.64 mg L^{-1}; LC50-504 h = 0.01 mg L^{-1}), whereas the toxicity of diazinon increased almost five times (LC50-96 h = 9.44 mg L^{-1}; LC50-504 h = 1.92 mg L^{-1}). The differences in pesticide toxicity at acute and chronic periods were between 15 and 192 times, resulting endosulfan the most toxic at all exposure periods (Figure 1(b)). Larvae, exposed to both pesticides, showed the same abnormal behaviour observed for exposed embryos when reached the larval stage; these abnormalities were associated with neurotoxicity. The main sublethal effects were erratic swimming, spasmodic contractions, reduction or lack of food intake and fewer movements up to narcosis. Adverse effects were concentration- and time-dependent. LOEC-168 h values for endosulfan and diazinon were 0.01 mg L^{-1} and 4.5 mg L^{-1}, respectively.

Figure 1 Lethal concentration 50 values of *Rhinella arenarum* continuously exposed to endosulfan and diazinon from blastula stage (S.4) (a) and complete operculum stage (S.25) (b) onwards. Error bars indicate 95% confidence interval. Light grey bars correspond to diazinon and dark grey bars correspond to endosulfan. Different letters indicate significant differences

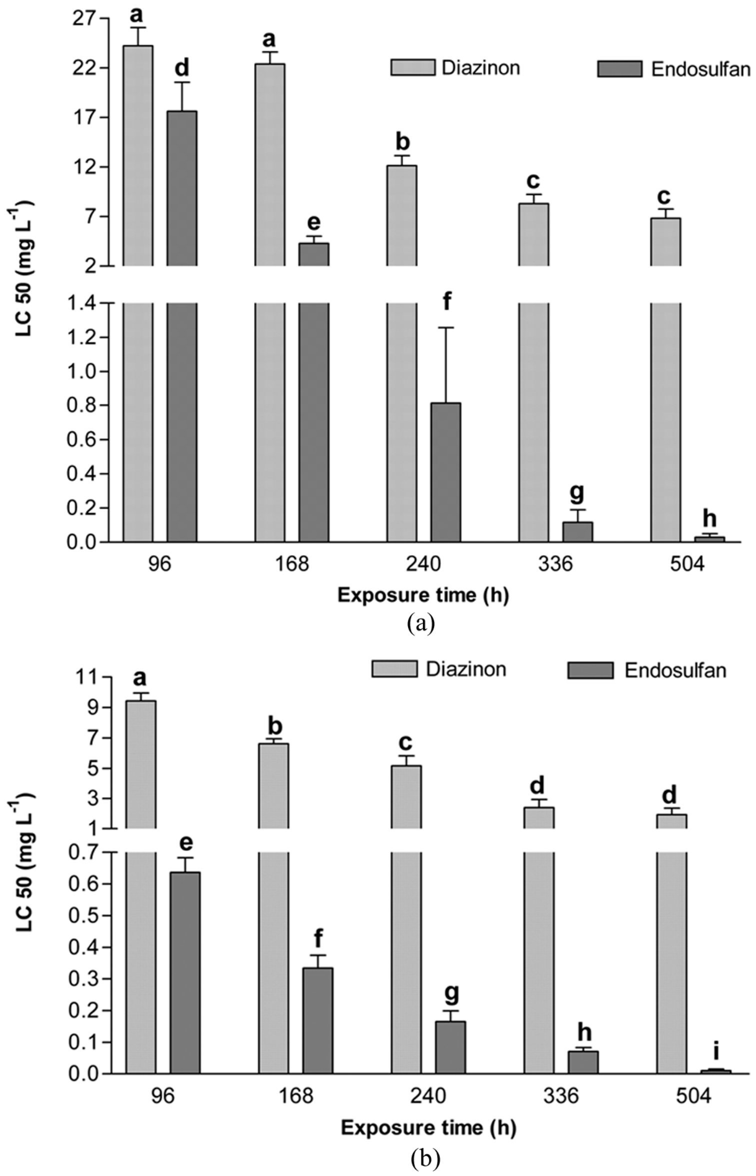

Table 1 Hazard quotient (HQ) values for acute (96 h), short-term chronic (168 h), and chronic (336 h, 504 h) periods to endosulfan and diazinon for *Rhinella arenarum* exposed from blastula (S.4) and complete operculum (S.25) stages onwards

	Exposure time (h)															
	96				*168*				*336*				*504*			
	Endosulfan		*Diazinon*		*Endosulfan*		*Diazinon*		*Endosulfan*		*Diazinon*		*Endosulfan*		*Diazinon*	
Developmental stages	LC10 (mg L^{-1})	HQ	LC10 (mg L^{-1})	HQ	LC10 (mg L^{-1})	HQ	LC10 (mg L^{-1})	HQ	LC10 (mg L^{-1})	HQ	LC10 (mg L^{-1})	HQ	LC10 (mg L^{-1})	HQ	LC10 (mg L^{-1})	HQ
Embryos (S.4)	11.55	0.04	20.80	0.14	0.62	0.7*	16.30	0.18	0.02	17.50*	5.83	0.51	0.007	62.40*	4.38	0.68
Larvae (S.25)	0.45	0.97*	7.80	0.38	0.22	2.00*	5.64	0.53*	0.04	10.90*	1.26	2.38*	0.004	109.30*	1.10	2.72*

*HQ > 0.5, and 1, at acute and chronic exposure, respectively. Estimates harmful effects due to the pesticide exposure.

3.3 Ecological risk evaluation

The results for embryos exposed to endosulfan highlighted that HQ values for 96 h were below the LOC value, whereas from 168 h onwards the HQ were above the LOC value. In the case of embryos exposed to diazinon, HQ values also increased along time but did not exceed 0.7 along the exposure period. In contrast, at larval exposure, the HQ values of endosulfan and diazinon also increased along time from acute to chronic period. HQ values for endosulfan were always over the LOC value, whereas HQ values for diazinon only exceed the LOC value from 168 h and reached a maximum value of 2.7 at chronic exposure (Table 1).

4 Discussion

This study shows the high toxicity of two widely used pesticides, endosulfan and diazinon, to embryos and larvae of the South American toad *Rhinella arenarum*. Although individual toxicities of both chemicals have been already assessed (Aronzon et al., 2014; Svartz et al., 2014) our comparative study also highlights the greatly different effects on amphibian survival, the relevance of performing toxicity bioassays during chronic periods, as toxicity of both pesticides was time-dependent, and the relevance of considering these results in an environmental scenario and in risk assessment.

The toxicity of endosulfan from acute to chronic exposure increased 587 times in treatments starting from S.4, and more than 60 times from S.25; while for diazinon, toxicity increased only about four and five times for embryo and larvae, respectively. Thus, considering survival, endosulfan was 227 times more toxic than diazinon.

It is very important to assess a possible time- and stage-dependent sensitivity to physicochemical agents (Aronzon et al., 2011; Greulich and Pflugmacher, 2003) for recommending more conservative and realistic tolerance thresholds for species conservation purposes. Results of this study show that the toxicity of both pesticides is concentration-and stage-dependent with a higher sensitivity at the larval period, highlighting the importance of performing toxicity bioassays at different developmental stages. Although the organophosphate stage-dependent pattern is not predictable (Sumanadasa et al., 2008), our results are in agreement with previous studies, where an increasing toxicity through the embryonic to larval period was reported (Hamm et al., 2001).

Toxicity of endosulfan for *R. arenarum* was in the range of those determined in studies with other amphibian species (Agostini et al., 2009). It is worth pointing out that, *R. arenarum* larvae exposed to diazinon were significantly more sensitive than other toad larvae (Sumanadasa et al., 2008).

It is highly remarkable that both pesticides caused important neurotoxic effects from the beginning of the exposure in line with Ezemonye and Tongo (2010). Furthermore, we have extended results of previous studies on xenobiotic neurotoxicity, as lack of correct equilibrium and posture lying on the lateral side, swirling, non-feeding behaviour and extensive paralysis caused by other chemicals, even endosulfan which was associated to movement reduction (Denoël et al., 2013). Neurotoxic effects might make organisms more vulnerable to predators and other environmental stressors such as infectious agents, influencing the physical condition of the animals or their reproductive successes.

The differential susceptibility to both pesticides with the larval period as the most sensitive might be related to the maturity of muscular and nervous systems, which is well correlated to the increase in the AChE activity (Gindi and Knowland, 1979). The present work highlights the importance of assessing behaviour as trait of the organism integrity, not only through vital activities such as feeding, but also by failed displacements that lead to misuse of environmental resources and increased likelihood of predation (Denoël et al., 2013).

Environmental levels of endosulfan were reported in the range of 0.1–100 µg L^{-1} (Dalvie et al., 2003), whereas for diazinon these parameters reached 3.1 mg L^{-1} (Shayeghi et al., 2006). The comparison between these environmental levels and the EEC obtained in this study of 43.7 µg endosulfan L^{-1} and 3.1 mg diazinon L^{-1} gives strength and realism to the prediction of ecological risk. Furthermore, the relevance of this work resides in the fact that the LC10-504 h of 4 µg L^{-1} for endosulfan and the LC10-504 h of 1.1 mg L^{-1} for diazinon show that the measured and predicted concentrations exceed the levels that allow the survival of *R. arenarum*. This information is particularly relevant considering both pesticide persistence and amphibian population health, which are likely to be compromised by the increasing use of pesticides and other agricultural chemicals (Mann et al., 2009). Indeed, *R. arenarum* is one of the species with the highest incidence of malformations and decreasing population size towards extinction in sites dominated by crops in Argentina (Bionda et al., 2013).

The different degree of toxicity of both pesticides on *R. arenarum* is reflected in the restrictions of their use. Thus, the extreme toxicity of endosulfan prompted its recent worldwide ban (SENASA, 2013), whereas only the home use of diazinon was prohibited at least in Argentina (Boletín oficial No. 31.404, 2008).

Taking into account the lethal and sublethal toxicity and the risk evaluation of endosulfan and diazinon performed in this study, these pesticides should be considered potential threats for non-target species, as in the case of the South American toad *R. arenarum*.

Acknowledgements

Carolina Aronzon, Gabriela Svartz and Cristina Pérez Coll are members of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina, and Universidad Nacional de San Martín (UNSAM), Argentina. This study was supported by a grant from CONICET (PIP No. 00140CO). We wish to thank Ferring Pharmaceuticals for providing the human chorionic gonadotropin.

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