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# Differential toxicity and uptake of Diazinon on embryo-larval development of *Rhinella arenarum*

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## HIGHLIGHTS

- Significant lethal and sublethal effects of Diazinon on *Rhinella arenarum* embryos and larvae were reported.
- Stage-dependent toxicity of Diazinon was evaluated and analyzed.
- Remarkable teratogenic and neurotoxic effects of Diazinon were described.
- Concentration, time and stage-dependent uptake of Diazinon were reported and discussed.
- The study showed the threat of Diazinon for *R. arenarum* populations.

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## ABSTRACT

Diazinon, an anti-cholinesterase organophosphate, is an extensively used pesticide. The main objective of this work was to assess the lethal and sublethal effects of Diazinon and its comparison with the uptake by embryos and larvae of the common South American toad *Rhinella arenarum* by means of standardized bioassays during acute (96 h), short-term chronic (168 h) and chronic (504 h) exposures. Toxicity resulted time- and stage-dependent, thus the lethal concentration 50 for 96 h, 168 h and 504 h were 27.2; 20.1 and 6.8 mg Diazinon $L^{-1}$ for embryos and 8, 6.7 and 1.9 mg Diazinon $L^{-1}$ for larvae. It is noteworthy the remarkable differences found in the concentration which caused lethality with those causing adverse effects on development such as malformations (teratogenic effects). Therefore, the teratogenic index from 144 h was greater than two; the main adverse effects were axial flexures, irregular borders, wavy tail, microcephaly, malformed mouth and adhesive structures, gut miscoiling, underdeveloped gills, cloacal edema, desquamation and severe hydropsy. Moreover, the characteristic sublethal effect of Diazinon on larvae was abnormal behavior related to neurotoxicity with a NOEC-168 h of 4.5 mg Diazinon $L^{-1}$. Diazinon contents in *R. arenarum* were time-dependent and significantly related to exposure concentration for both embryos and larvae. Diazinon contents were also stage-dependent, as it was up to 27 times higher for organisms exposed from blastula stage onwards than early larvae. These facts and the Hazard Quotients, a numerical expression of ecological risk, of 2.73, which is above USEPA's Level of Concern, showed the threat that Diazinon represents for *R. arenarum* populations.



## 1. Introduction

Diazinon is an organophosphate pesticide; it has been extensively applied since the early 50's in agriculture to control insects in corn, fruit, citrus, bananas, vines, sugar cane, snuff, potatoes, coffee, cocoa, tea, horticultural crops, cotton and rice. It is also widely used to control ectoparasites in veterinary medicine and domestic aphids, beetles and mealybugs. After application, Diazinon is easily washed into surface waters and may reach ground water, polluting the whole aquatic environment; moreover it is one of the most stable water organophosphate (Kanazawa, 1975; Albanis et al., 1998; Hamm and Hinton, 2000).

The main mechanism of toxicity of this pesticide is based on its ability to inhibit acetylcholinesterase (AChE) (Fulton and Key,

*Abbreviations:* AS, AMPHITOX solution; BCF, bioconcentration factor; LC, lethal concentration; NOEC, no observable effect concentration.

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2001; Galloway and Handy, 2003), enzyme responsible for inactivating the neurotransmitter acetylcholine (ChE) (Beauvais et al., 2000). As a result of the neurotransmitter accumulation, cholinergic receptors suffer an over stimulation, taking place a nerve poisoning (Coppage and Braidech, 1976). These features express as hyperactivity, loss of coordination, tremors, muscle spasms, convulsions, abnormal swimming, and finally paralysis and death. Due to the physiological similarity between the nervous system of insects and non-target vertebrates, there is a possibility that the same mechanism can also affect the last ones (Chambers and Carr, 1995).

Although amphibian toxicity information of Diazinon is scarce, acute parameters as the LC50-96 h of 7.49 mg $L^{-1}$ and 9.84 mg $L^{-1}$ for *Rana booylii* larvae and *Xenopus laevis* embryos were informed (Sparling and Fellers, 2007; Modra et al., 2011). Moreover, chronic exposure of *Bufo melanostictus,* LC50–30 d of 6 and 7.5 mg $L^{-1}$ for embryos and larvae respectively were also reported (Sumanadasa et al., 2008). Among sublethal effects, Bridges (1997) found that frog larvae exposed to organophosphate pesticides suffered reduced activity, uncoordinated swimming, increased vulnerability to predators and depressed growth rates. Therefore, most toxicity studies explored adverse effects under acute exposure condition only in certain period of the life cycle; nevertheless it is of concern evaluating an eventual differential susceptibility to the pesticide among different developmental stages of a species with conservation purposes.

The decline of amphibians and the large number of malformations found in populations worldwide have caused increasing concern (Wake and Vredenburg, 2008). Some studies indicate that this fact could be related to their high susceptibility particularly during early life stages (van der Schalie et al., 1999). In addition, the risk for adverse effects might be enhanced by their preference to breed in shallow, lentic, or ephemeral water bodies in which pollutants might be concentrated. This is particularly relevant as morphological studies on amphibians from the middle region of Argentina, which is dominated mainly by agriculture, reported a high incidence of malformations, being *Rhinella arenarum* one of the species with the highest incidence of malformations (Peltzer et al., 2010). Moreover, as amphibian are considered keystone members of ecosystems and vital links in food chains, the contaminant could be magnified across trophic webs (Suter, 1993). As others organophosphates, Diazinon presents low water solubility and should be readily absorbed (Bowman and Sans, 1983). Bioaccumulation may be considered as an early exposure biomarker for adverse effect to toxic substances in ecosystems (Franke et al., 1994).

The main aim of present study was to evaluate the toxic effects of Diazinon on the South American toad *R. arenarum* reporting lethal and sublethal effects. The differential susceptibility and uptake among embryos and larvae were also analyzed as well as their correlations. The results were discussed in relation to environmental concern and the toxicity mechanisms of the pesticide.

## 2. Materials and methods

### 2.1. R. arenarum embryos and larvae

Six couples of *R. arenarum* adults, weighing approximately 200–250 g were obtained in Lobos (Buenos Aires province, Argentina: 35° 11′ S; 59° 05′ W). Ovulation of *R. arenarum* 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, plus 500 IU of human chorionic gonadotropin (Mann and Bidwell, 2000). Oocytes were fertilized *in vitro* with sperm suspensions in AS. The AS composition is (in mg $L^{-1}$): $Na^+$ 14.75; $Cl^-$ 22.71; $K^+$ 0.26; $Ca^{2+}$ 0.36; $HCO_3^-$ 1.45. After fertilization, embryos were kept in AS at 20 ± 2 °C until reaching blastula (S.4) and larval stages (S.25).The stage of embryos and larvae were defined according to Del Conte and Sirlin (1951). Embryos were dejellied by means of a 2-min treatment with 2% thioglycolic acid solution, neutralized at pH 7.2–7.4 with 1.35 mL of saturated NaOH solution every 100 mL in AS, and then thoroughly washed.

### 2.2. Test solutions

A Diazinon (purity 99%, CAS number: 333-41-5, Lot LB75417, Supelco Analytical) stock solution of 3 g $L^{-1}$ was prepared by dissolving the corresponding volume, in acetone. Test solutions, ranging in concentrations between 1.5 and 45 mg Diazinon $L^{-1}$, were prepared by diluting the corresponding volume of the stock solution in AS. Diazinon test solutions were filtered by 0.45 nylon membrane and directly analyzed by HPLC–ESI–MS in SIM mode, positive detection. The ions $m/z = 305$, $m/z = 169$, and $m/z = 69$ were used to quantification and identification. The solutions were daily analyzed and maintained the stability. Recoveries assays were done and were of 97.8%.The error between nominal and measured concentrations did not exceed 5%.

### 2.3. Toxicity experimental protocols

*R. arenarum* embryos and larvae obtained from six different litters were continuously exposed to Diazinon from early blastula (S.4) and complete operculum (S.25) stages onwards for acute (96 h), short-term chronic (168 h) and chronic (504 h) periods.

For each experimental condition, triplicate batches of 10 embryos or larvae were placed in covered 10-cm-diameter glass Petri dishes containing 40 mL of test solutions. Simultaneously, control embryos or larvae were maintained in AS without additions. It was also tested another control of AS plus acetone at the highest concentration used for Diazinon test solutions. Test solutions were renewed every other day and temperature was maintained at 20 ± 2 °C. Lethal and sublethal effects were evaluated and dead individuals were removed every 24 h. Larvae were fed with balanced fish food TetraColor® *ad libitum* for 24 h every other day.

Sublethal effects were studied with Stereoscopic Microscopy (SM). Photographs of embryos and larvae were digitally recorded with a Sony DSC-S90 camera mounted on a Zeiss Stemi DV4 stereoscopic microscope. The teratogenic index (TI) was estimated as the ratio between the LC50 and the EC50. EC50 was based on the morphological abnormalities, and were identified according to the "Atlas of abnormalities" (Bantle et al., 1998). Behavioral alterations such as abnormal fast rotations which are a sign of neurotoxic stress; lying on the lateral or dorsal side, abnormal breathing, feeding and swimming patterns were evaluated (Denoël et al., 2012). Smooth movements of the Petri-dishes, followed by stimulation with a light source were done. In case of no response, soft mechanic stimulation with a glass rod was made and finally heartbeat was checked under Zeiss Stemi DV4 stereoscopic microscope.

Ecological risk can be numerically estimated using the Hazard Quotient (HQ) approach (US EPA, 1998) based on the comparison of the Expected Environmental Concentration (EEC) (Boutin et al., 1993, 1995) with a standard toxicity end point (e.g., EC10 values). EEC for Diazinon was calculated as a percentage of the maximum application rate proposed, 4.5 kg $ha^{-1}$ active ingredient (Syngenta Crop Protection Inc.). This percentage depends on overspray exposure during aerial application (100%). The EEC was calculated assuming a water depth of 15 cm and an area of 1 $m^2$. HQ in this study was calculated as EEC/LC10. In present study, we estimated HQ based on the maximum application rate proposed, to provide a more meaningful, yet conservative, estimation of the effect. After HQ was calculated, it was compared with the USEPA Level of Concern (LOC). The LOC is a policy tool that the Agency uses to inter-