TOXICITY OF ENDOSULFAN ON EMBRYO-LARVAL DEVELOPMENT OF THE SOUTH AMERICAN TOAD RHINELLA ARENARUM

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Abstract: Endosulfan is a widely used pesticide despite its extreme toxicity to a variety of taxa and its worldwide ban. The aim of the present study was to evaluate the acute and chronic toxicity of endosulfan on the embryonic-larval development of the common South American toad Rhinella arenarum. The results showed that lethal and sublethal effects increased with concentration and exposure time. The sensitivity to endosulfan increased during the larval period, the complete operculum stage (S.25) being the most sensitive (504-h median lethal concentration [LC50] = 0.01 mg endosulfan/L; 10% lethal concentration [LC10] = 0.004 mg endosulfan/L). Endosulfan exposure caused morphological abnormalities such as general underdevelopment, edema, gill malformations, and cellular dissociation as well as neurotoxicity. Our results also showed that larvae exposed to concentrations of 0.005 mg endosulfan/L and 0.01 mg endosulfan/L completed metamorphosis earlier than controls, but with underdevelopment. The 240-h teratogenic index was 6.13, implying a high risk for embryos to be malformed in the absence of significant embryonic lethality. Because the hazard quotients for chronic exposure were over 1, the level of concern value and toxicity endpoints obtained in the present study for R. arenarum occurred at concentrations lower than the levels of endosulfan reported in the environment, this pesticide should be considered a potential risk for this species. Environ Toxicol Chem 2014;33:875–881. © 2013 SETAC

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INTRODUCTION

With the worldwide expansion of the agricultural frontier, large amounts of pesticides are increasingly polluting ecosystems. Endosulfan is a synthetic organochlorine compound used as an insecticide and acaricide. The routes of exposure in wildlife are through contact (skin, hair) and ingestion and, at high temperatures, by inhalation. Its mechanism of action is overstimulation of the central nervous system, inhibiting calcium and magnesium ATPase [1]. Technical-grade endosulfan is a mixture of 2 stereoisomers, alpha and beta, in a ratio of 7:3 [2]. The half-lives at 14 °C of alpha and beta stereoisomers in the Parana River, Santa Fe (an intensive agricultural area in Argentina), are 50 d and 33 d, respectively [3]. The US Environmental Protection Agency (USEPA) has classified endosulfan as category 1b, “highly hazardous,” and endosulfan is considered a persistent organic pollutant because of its high level of toxicity to living organisms, persistence in the environment, high potential for bioaccumulation, and long-distance migration capacity from its application sites. The use of endosulfan was recently banned by the United Nations Association, following the recommendation of the Scientific Committee, but with exemptions [4], so it is still largely used worldwide, particularly in some developing countries. The impact of this pesticide on wildlife in Argentina is thought to be very significant, considering that 5.5 million liters of endosulfan was applied in 2010 to control insects on soybean, alfalfa, cotton, sunflower, and corn crops [5].

Argentina has approximately 60 species of amphibians, and the toad we studied, Rhinella arenarum, occurs widely in the areas where endosulfan is used. Generally, amphibians are an important taxa affected by many pesticides that are sprayed on agricultural fields [6]. The pesticides accumulate in temporary ponds and result in high concentrations during spring and summer, coinciding with amphibians breeding and highly sensitive stages of development [6]. Moreover, their skin and egg membranes are highly permeable to pollutants. Many studies have examined the toxic effects of pesticides on the most sensitive life stages of amphibians, embryonic-larval development [7–9]. It was reported that endosulfan causes lethality in many species of amphibians, with 96-h median lethal concentration (LC50) values ranging from 1.3 μg/L to 120 μg/L [10], as well as sublethal effects, such as delay in the time to complete metamorphosis [11], malformations of gills [12], and neurotoxicity (hyperactivity, whip-like convulsions, narcosis, paralysis) [13,14]. Amphibian populations are declining in size and distribution worldwide as a result of agrochemical contamination as well as other anthropogenic and natural changes in their ecosystem [15]. Within this context, the importance of amphibians in both aquatic and terrestrial ecosystems can have large-scale consequences through alterations of food webs [16].

It is noteworthy the levels of endosulfan ranging from 0.1 μg/L to 100 μg/L have been reported in ground and surface water in intensive agricultural areas such as the Western Cape, Africa [17], with exceptional levels of up to 500 μg/L occurring after runoff events [18]. Moreover, there is abundant information confirming the presence of endosulfan at places far from the application site [19].

The aim of the present study was to evaluate the lethal and sublethal effects of endosulfan at embryonic and larval stages of R. arenarum, an amphibian species with a wide distribution throughout South America, using the standardized bioassay, AMPHITOX [20]. Stage-dependent sensitivity to endosulfan was also evaluated.

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An ecological risk assessment of endosulfan for this native species was performed using the hazard quotient approach [21]. The hazard quotient is the ratio of the expected environmental concentration (EEC) [22,23] and the level at which no adverse effects are expected (10% lethal concentration [LC10]). The EEC of a pesticide is a theoretical concentration based on a worst-case scenario for exposure of nontarget aquatic and terrestrial habitats interspersed within or adjacent to proposed use areas.

**MATERIALS AND METHODS**

**Acquisition of *R. arenarum* embryos**

To examine the potential effects of endosulfan on the embryonic-larval development of *R. arenarum*, 3 mating pairs of adults weighing approximately 200 g to 250 g per animal were acquired in a nonimpacted site, Lobos (Buenos Aires province, Argentina; 35°11’S, 59°05’W). Toad care, breeding, and embryo acquisition and analysis were conducted according to the methods described in the AMPHITOX protocols [20]. Briefly, AMPHITOX is a standardized test employing amphibian embryos that can be used to evaluate toxicity for acute, short-term chronic, chronic, and early-life-stage exposure to hazardous substances and samples. By plotting the LC10, LC50, and 90% lethal concentration, toxicity profile curves from 24 h to 504 h of exposure can be obtained, allowing the visualization of concentration and time exposure thresholds. By employing the early-life-stage test, it is also possible to evaluate malformations. Ovulation of females was induced by means of an intraperitoneal injection of a suspension of one homogenized toad pituitary containing 5000 IU human chorionic gonadotropin (hCG). The composition of AMPHITOX solution according to Pisanó [24], plus 5000 IU human chorionic gonadotropin (hCG). The composition of AMPHITOX solution was NaCl 36 mg/L, KCl 0.5 mg/L, CaCl2 1 mg/L, and NaHCO3 2 mg/L prepared in distilled water. Oocytes were fertilized and recorded with a Sony DSC-S90 digital camera, and neurotoxic effects were observed under a binocular stereoscopic microscope (Zeiss Stemi DV4), photographed and recorded with a Sony DSC-S90 digital camera, and identified according to Bantle et al. [26]. Embryos with significant adverse effects and controls were fixed in formalin 4%, dehydrated in a gradient of ethanol, prepared for scanning electron microscopy by means of the critical-point-drying technique [27], and observed in a Philips XL-30 operated at 10 kW for ultrastructural evaluation.

**Test solutions**

Test solutions were made using technical-grade endosulfan (PS81; Supelco) with a purity of 99%. A primarily stock solution containing 1000 mg endosulfan/L was made by dissolving endosulfan in analytical-grade acetone. The exposure concentrations were prepared by diluting the stock solution with AMPHITOX solution. Acetone concentration in test solutions was always lower than 1.1% [28]. Both AMPHITOX solution and acetone treatments were simultaneously maintained as controls. The concentration of endosulfan in stock solution was analyzed by high-performance liquid chromatography–electrospray ionization–mass spectrometry (negative mode), the identity of the compound was confirmed by scan detection, and the ion m/z 405 and m/z 407 were used for quantification [29]. The AMPHITOX solution was replaced entirely every 3 d and monitored weekly to ensure that the pH was at acceptable levels (7 ± 0.5).

**Toxicity bioassays**

For treatments during embryonic stages, 10 embryos were randomly placed in triplicate 10-cm-diameter glass Petri dishes containing 40 mL of test preparation. For bioassays with larvae, 10 early larvae (S.25) were placed in triplicate 20-cm-diameter glass Petri dishes containing 150 mL of test preparation. The toxicity bioassays were performed under the conditions summarized in Table 1.

After 24-h-pulse treatments, embryos were thoroughly washed and kept in AMPHITOX solution until 504 h postexposure. Organisms were maintained at 20 ± 2 °C and on a 12:12-h light:dark photoperiod. Tadpoles were fed with 3 granules of balanced fish food TetraColor. Test solutions were entirely replaced every 48 h.

Lethal and sublethal effects were evaluated every 24 h, comparing the abnormal effects with the normal development and behavior of controls. The sublethal effects evaluated were developmental delay, cellular dissociation, irregular surface, persistent yolk plug, underdeveloped gills, microcephaly, wavy tail, and edemas. Neurotoxicity endpoints included spasmodic contractions, alterations in swimming, and narcosis. Feeding behavior also was qualitatively assessed. In addition, we evaluated endosulfan effect on metamorphosis. Abnormalities and neurotoxic effects were observed under a binocular stereoscopic microscope (Zeiss Stemi DV4), photographed and recorded with a Sony DSC-S90 digital camera, and identified according to Bantle et al. [26]. Embryos with significant adverse effects and controls were fixed in formalin 4%, dehydrated in a gradient of ethanol, prepared for scanning electron microscopy by means of the critical-point-drying technique [27], and observed in a Philips XL-30 operated at 10 kW for ultrastructural evaluation.

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| Developmental stage | Treatment | Exposure duration (h) | Bioassay duration (h) | Exposure concentrations (mg endosulfan/L) |
|---------------------|-----------|-----------------------|-----------------------|------------------------------------------|
| Blastula (S.4)      | Continuous| 504                   | 504                   | 0.01, 0.05, 0.1, 0.3, 0.5, 0.75, 1, 2.5, 5, 10, 15, 20 |
| Complete operculum  | Continuous| 2568 (107 d)          | 504                   | 0.005, 0.01, 0.05, 0.1, 0.5, 1           |
| (S.25)              | 24-h pulse| 24                    | 504                   | 0.1, 2.5, 5, 10, 15, 20                   |
| Blasula (S.4)       | 24-h pulse| 24                    | 504                   | 0.5, 1, 2.5, 5, 10, 15, 20                |
| Gastrula (S.11)     | 24-h pulse| 24                    | 504                   | 0.5, 1, 2.5, 5, 10, 15, 20                |
| Neurula (S.13)      | 24-h pulse| 24                    | 504                   | 0.5, 1, 2.5, 5, 10, 15, 20                |
| Muscular activity   | 24-h pulse| 24                    | 504                   | 0.5, 1, 2.5, 5, 10, 15, 20                |
| Gill circulation    | 24-h pulse| 24                    | 504                   | 0.5, 1, 2.5, 5, 10, 15, 20                |
| Opercular fold      | 24-h pulse| 24                    | 504                   | 0.5, 1, 2.5, 5, 10, 15, 20                |
| Complete operculum  | 24-h pulse| 24                    | 504                   | 0.5, 1, 2.5, 5, 10, 15, 20                |
solution was analyzed daily and was found to be stable over the exposure time. The error between nominal and measured concentration of the stock solution did not exceed 5%.

**Data analysis**

Lethal and effective concentrations were statistically estimated by the USEPA Probit program [30], effective concentrations being based on malformations. To examine statistical differences between the lethal concentration values obtained, a comparison was made, considering the difference statistically significant when the higher lethal concentration/lower lethal concentration ratio exceeded the critical value (95% confidence interval) established by the American Public Health Association [31]. The teratogenic index was calculated as the LC50 divided by the median effective concentration (EC50), establishing a teratogenic index >1.5 as a high risk for embryos to be malformed in the absence of significant embryonic lethality [28]. We conducted a two-way analysis of variance to evaluate the effect of endosulfan concentration and exposure time on lethality and metamorphosis. Tukey’s tests were used to compare treatment means where significant (p < 0.05). This analysis used GraphPad Prism version 6.03.

**Ecological risk evaluation**

The EEC for endosulfan was based on 10% of the maximum application rate given on manufacturers’ labels. The maximum application rate allowed for a commercial formulation (endosulfan 35%, 350 g/L active ingredient [32]) is 2.5 L of the product per hectare, resulting in a maximum application concentration of 4370 mg/L/ha active ingredient. The EEC was calculated assuming a water depth of 15 cm and an area of 1 m² [22,23]. The hazard quotient was calculated as EEC/LC10 and compared with the USEPA level of concern (LOC) [21]. The LOC is a policy tool that the USEPA uses to interpret the hazard quotient, analyze the potential risk to nontarget organisms, and evaluate the need to consider regulatory action. The LOC value for risk is 1. If the hazard quotient is greater than 1, harmful effects likely are because of the contaminant in question.

**RESULTS**

Because the 2 controls, AMPHITOX solution and acetone solvent, did not differ statistically, both treatments were combined and reported as the control in the rest of the present study.

**Continuous exposure from blastula stage (S.4)**

Exposure of embryos starting at the blastula stage for 96 h expressed acute lethal effects at concentrations greater than or equal to 15 mg endosulfan/L. After 96 h, there was an increase in toxicity of endosulfan increased that was coincident with the beginning of the larval stage (Figure 1). The toxicity profile curves based on lethal concentrations indicate a significant increase in toxicity with 96-h and 504-h LC10s of 11.55 mg endosulfan/L and 0.007 mg endosulfan/L, respectively. This was equivalent to a 587-fold increase in toxicity.

Sublethal effects in embryos noted during the initial 24 h of exposure included cellular dissociation, irregular surface, persistent yolk plug, and delayed development. As development advanced, underdeveloped gills, microcephaly, wavy tail, and marked edema were observed (Figure 2). Moreover, the animals exhibited neurotoxicity such as spasmodic contractions. As exposure increased, neurotoxic effects included 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 or mechanical stimulus. The no-observed-effect concentration 240 h for sublethal effects was 0.01 mg endosulfan/L, and the teratogenic index for endosulfan at 240 h was 6.13.

**Continuous exposure from complete operculum stage (S.25)**

Early *R. arenarum* larvae treated with concentrations more than 1 mg endosulfan/L showed lethal effects after 48 h of exposure, reaching 100% lethality at the end of the acute period. Toxicity profile curves show that this developmental stage was more sensitive to endosulfan than the blastula stage. Moreover,
whereas the 96-h LC10 was 0.45 mg endosulfan/L, it was only 0.004 mg endosulfan/L at 504 h, indicating a toxicity increase of more than 100-fold (Figure 3).

Sublethal effects were observed from 0.01 mg endosulfan/L onward and consisted of delayed development and wavy tail and also neurotoxicity such as erratic swimming, spasmodic contractions, and fewer movements up to narcosis. Furthermore, essential functions such as feeding were deeply affected by the pesticide; the 3 granules of food were intact after 24 h, whereas in plates of control larvae, the feces of the granules eaten were present. These effects were concentration dependent.

Among the larvae exposed to 0.005 mg endosulfan/L, 83% survived when they began the metamorphosis process (not significantly different from the control group, \( p > 0.05 \)), but only 46% completed the process (Figure 4). Before death, larvae exhibited general underdevelopment, lethargic behavior, and starvation. In the case of larvae exposed to 0.01 mg endosulfan/L, only 16% died before the metamorphosis and 30% completed it. Interestingly, larvae exposed to 0.005 mg endosulfan/L and 0.01 mg endosulfan/L began the metamorphosis earlier than the control group. However, these recently metamorphosed toads showed general underdevelopment compared with controls.

**24 h pulse exposure at different developmental stages**

Figure 5 shows the differential sensitivity of embryos and larvae to endosulfan. Sensitivity of embryos was lower in early embryonic stages but increased in late stages, particularly from the larval stage (i.e., 336-h LC10 value for S.4 and S.25 were 3.75 mg endosulfan/L and 0.3 mg endosulfan/L, respectively). Although no acute or short-term chronic lethality was observed during exposure at S.4, upon animals’ reaching larval stage it significantly increased, up to 5-fold in 10 d (240-h LC50 = 16.05 mg endosulfan/L, 504-h LC50 = 3.62 mg endosulfan/L). There was no significant lethality \(( p > 0.05)\) for embryos exposed from early stages (S.4–S.18), even at 20 mg endosulfan/L for acute and short-term chronic exposures, so LC50 could not be obtained. The most sensitive stage was S.25, with the sensitivity to endosulfan remaining relatively constant from 96 h until 504 h (96-h LC50 = 0.62 mg endosulfan/L; 504-h LC50 = 0.53 mg endosulfan/L).

The main sublethal effects observed at 336 h postexposure on larvae at all concentrations were edema, wavy tail, lateral and dorsal axis incurvation, underdeveloped opercular folds, underdeveloped gills, persistence of the oral adhesive apparatus, different degrees of cellular dissociation, persistence of ciliar cells, and neurotoxicity evidenced by spasmodic contractions and erratic swimming, up to absence of movements. Figure 6 shows the ultrastructure of malformed larvae caused by endosulfan 24 h-pulse exposure during muscular activity stage (S.18).

**Ecological risk evaluation**

A risk evaluation analysis for continuous exposures to endosulfan that were initiated at the blastula and complete operculum stages was performed. The EEC for endosulfan was calculated as 10% of the maximum application rate allowed (4370 mg active ingredient per L/ha), so the EEC was 0.0437 mg endosulfan/L/m². From this value, the hazard quotients (hazard quotient \( \frac{EEC}{LC10} \)) for both treatments were estimated (Table 2). The results for both stages highlight that hazard quotient values for acute and short-term chronic exposure periods were below the LOC value, whereas for chronic exposure the hazard quotient were above the LOC value.

**DISCUSSION**

The results obtained in the present study demonstrate lethal and sublethal effects produced by endosulfan on *R. arenarum* embryonic-larval development. The present study also reveals that larvae were almost 26 times and 3 times more sensitive than embryos at acute and short-term chronic exposures to endosulfan, respectively. Embryos and larvae exposed to endosulfan showed differences in their toxicity pattern in that the larvae were highly sensitive to the pesticide from the beginning of the exposure, whereas embryo sensitivity increased coinciding with
neuromuscular development, the main target organ of this pesticide. Moreover, the 24-h-pulse exposures confirmed this tendency in that S.25 was the most sensitive stage, and embryos from early stages (S.4–S.18) were more resistant even at higher endosulfan concentrations. Although the 24-h-pulse concentrations were high relative to those used in the continuous exposures, the pulsed experimental design may simulate environmental conditions such as overspray and accidental spills that result in high but transient concentrations. This information is useful for evaluating the differential sensitivity throughout the development of a species. The general pattern of the stage-dependent sensitivity obtained in the present study is in line with that reported by Harris et al. [33] in which Rana pipiens larvae at the metamorphic stage could not survive endosulfan concentrations that were sublethal for embryos.

Toxicity values of endosulfan for R. arenarum obtained in the present study are in the range of those determined in studies with other amphibian species. For acute lethality, the 96-h LC50 for Bufo bufo was 0.43 mg endosulfan/L [12], whereas it was 0.015 mg endosulfan/L and 0.13 mg endosulfan/L for Rana clamitans [33] and Hypsiboas pulchellus [13], respectively. In reference to sublethal effects, Broomhall and Shine [34] observed developmental delay in Litoria freycineti tadpoles exposed to 0.03 μg endosulfan/L or 1.3 μg endosulfan/L.

Figure 6. Scanning electron microscopy pictures of Rhinella arenarum larvae exposed to 24-h pulses to different endosulfan (ES) concentrations during the muscular activity stage (S.18) fixed at 96 h: lateral and dorsal axis incurvation from 10 mg ES/L (magnification ×21 [A]); underdeveloped opercular folds and underdeveloped gills from 5 mg ES/L; malformed, thicker, and shorter gill filaments than in the control group; increasing effect with concentration (×68 [B] and ×200 [C]); persistence of the oral adhesive apparatus at 5 mg ES/L and 10 mg ES/L (×200 [D]); axis incurvation and thicker fin at 15 mg ES/L and 20 mg endosulfan/L (×45 [E]); different degrees of cell surface dissociation, persistence of ciliated cells in specific regions of the epithelium and general disorganization of the epithelium (×375 [F]).
Exposure of *B. bufo* tadpoles to 0.05 mg endosulfan/L and 0.1 mg endosulfan/L resulted in increased incidences of mouth and skeletal malformations and reduced body weight [11], whereas alterations in the ultrastructure and cell composition of gills, indicating impaired gas exchange and osmoregulation in the gills, were observed in tadpoles exposed to 0.2 mg endosulfan/L [12]. The teratogenic potential of this pesticide represented by a 240-h teratogenic index of 6.13 is 4 times the threshold level to be considered a high risk for embryos to be malformed in the absence of significant lethality [28]. Furthermore, we confirm and extend results of previous studies on neurotoxicity, such as lack of correct equilibrium and lying on the lateral side, swirling, nonfeeding behavior, and extensive paralysis [14,35]. The neurotoxicity effect could be associated with the increased synaptic concentrations of several neurotransmitters, including the decreased acetylcholinesterase activity as observed in wild frogs exposed to endosulfan [36] as well as the neuronal degeneration in cerebral targets, such as the mesencephalon and hypothalamus reported for endosulfan-exposed fish [37]. Behavioral markers have relevant potential as early warning systems when other toxicity parameters such as mortality are absent.

It is well known that thyroid hormones regulate the metamorphosis process in amphibians [38]. Adverse scenarios such as pesticide exposures can induce the activity of these hormones [39], accelerating larval development and completing metamorphosis earlier, a phenomenon characteristic of amphibian larvae called phenotypic plasticity [40]. However, these larvae have reduced weight and length, decreasing their terrestrial fitness [41]. The results obtained in the present study on metamorphosis are consistent with this hypothesis, showing that exposed larvae at concentrations as low as 5 μg endosulfan/L completed the metamorphosis earlier with general underdevelopment. These developmental disorders can make them more vulnerable to predation or other environmental stressors such as infectious agents, invasive species, and changes in physical and chemical parameters of the environment, influencing the physical condition of the animals or their reproductive success [42].

Comparing the endosulfan environmental levels reported between 0.1 μg/L and 100 μg/L [17], reaching exceptional levels of 500 μg/L [18], with the EEC of 43.7 mg/L obtained in the present study, we can affirm the realistic prediction of this parameter. Also, given the 504-h LC10 as low as 4 μg endosulfan/L determined in the present study, the measured and predicted endosulfan concentrations exceed the levels that allow survival of *R. arenarum* larvae. Based on this risk assessment scenario, hazard quotients could be greater than 10 times the level of concern, highlighting that endosulfan represents a threat for *R. arenarum* embryonic-larval development.

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