Chemosphere 100 (2014) 50–56

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

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

Carolina Mariel Aronzon [a], Damián J.G. Marino [b], Alicia E. Ronco [b], Cristina Silvia Pérez Coll [a,*]

[a] Instituto de Investigación e Ingeniería Ambiental (3iA), Universidad Nacional de San Martín, Campus Miguelete, 25 de Mayo y Francia, C.P. 1650 San Martín, Provincia de Buenos Aires, Argentina
[b] Centro de Investigaciones del Medio Ambiente, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calle 47 y 115, C.P. 1900 La Plata, Provincia de Buenos Aires, Argentina

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.

ARTICLE INFO

Article history:
Received 12 July 2013
Received in revised form 17 December 2013
Accepted 22 December 2013
Available online 31 January 2014

Keywords:
Amphibian embryo-larval development
Differential toxicity
Teratogenesis
Neurotoxicity
Uptake
Diazinon

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.

* Corresponding author. Address: Campus Miguelete, 25 de Mayo y Francia, C.P. 1650 San Martín, Provincia de Buenos Aires, Argentina. Tel.: +54 11 4006 1500x6023.
E-mail address: perezcoll@unsam.edu.ar (C.S. Pérez Coll).

0045-6535/$ - see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.chemosphere.2013.12.078

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-

pret the risk quotient and analyze the potential risk to non-target organisms and the need to consider regulatory action. The LOC value for risk is 1. If HQ > 1, harmful effects are likely due to the contaminant in question.

2.4. Uptake experimental protocols

For each experimental condition, triplicate batches of 60 embryos from early blastula stage (S.4) and larvae from complete operculum stage (S.25) were placed in covered 20-cm-diameter glass Petri dishes containing 150 mL of 1.5; 3 and 4 mg Diazinon L^{-1} and 0.5; 1.5 and 3 mg Diazinon L^{-1}, respectively. Exposure concentrations were selected to be subtoxic according to the effects obtained in preliminary bioassays. Embryos and larvae were removed at 96 h, 240 h and 504 h; then they were thoroughly washed with 200 ml of AS twice, dried, weighted and stored at −20 °C.

Biological samples were homogenized and extracted according to US EPA (1994). Extracts were analyzed in a gas chromatograph with a NPD at 250 °C, with a HP-5 of 30 m × 0.32 mm column. Hydrogen was used as carrier at 0.8 ml min^{-1}, split injection at 1:50 and 220 °C. Oven ramp from 160 °C to 250 °C, at 10°/min. Analyte identity was confirmed by mass spectrometry using a single quadrupole operating in SIM mode by monitoring ions m/z = 304, 179, 137, previously characterized by scanning (between 70 and 550 umas). Recovery was 97.8%.

2.5. Statistical analysis

Lethality data were statistically analyzed by the USEPA Probit Program (US EPA, 1988). Toxicity Profiles (TOPs), as isotoxicity curves (Herkovits and Helguero, 1998) were plotted based on LC10, 50 and 90 at different times. To assess statistical differences between LC50 values, a comparison was made, considering statistically significant differences when the higher LC50 and the lower LC50 ratio exceeded the critical value (95% confidence interval) according APHA (1980).

Bioconcentration Factors (BCF) were defined as the ratio of Diazinon concentrations in embryos or larvae and the Diazinon concentrations in the exposure media.

One-way ANOVA analysis was used to assess significant differences of Diazinon uptake among embryos and larvae exposed to different conditions. Multiple comparisons were performed using Tukey's test. Graph Pad Prism 3.0 was used.

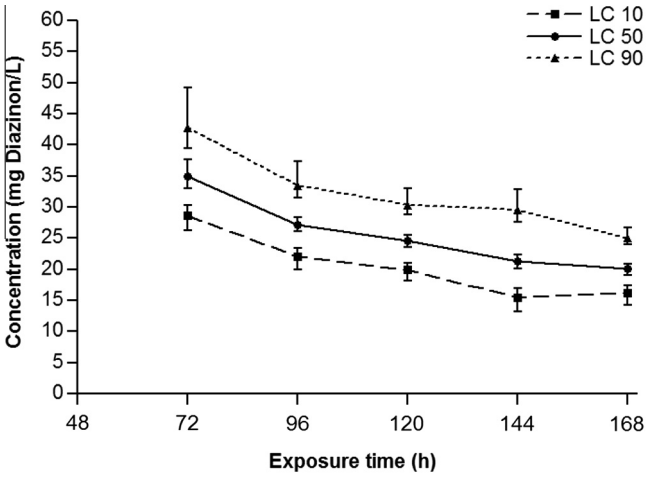

Fig. 1. Toxicity profile curves of Diazinon representing the lethal concentrations (LCs) 10%, 50%, and 90% in *Rhinella arenarum* embryos exposed from early blastula stage (S.4) onwards. Bars show 95% confidence intervals.

3. Results

3.1. Embryo toxicity

3.1.1. Lethal effects

Diazinon LC10, 50 and 90 at different exposure times are represented in Fig. 1. NOEC-48 h for lethality was 30.0 mg Diazinon L^{-1}. Toxicity significantly increased from the acute (LC50–96 h: 27.2 mg Diazinon L^{-1}) to short-term chronic periods (LC 50–168 h: 20.1 mg Diazinon L^{-1}). By extending the exposure to 420 and 504 h, toxicity significantly increased reaching LC50 values of 12.1 mg Diazinon L^{-1} and 6.8 mg Diazinon L^{-1}, respectively. Although ecological risk represented as HQ values increased along time, in the case of exposure from early embryonic period, did not exceed 0.7. There were no significant differences in susceptibility between litters, and the coefficients of variation were always lower than 11.7%.

3.1.2. Sublethal effects

Embryos exposed to Diazinon from blastula stage showed different sublethal effects, being EC50–96 h 17.8 mg Diazinon L^{-1}. The main malformations were general underdevelopment, cellular dissociation, underdeveloped or agenesis of gills and tail flexures. Sublethal effects increased with exposure time reaching an EC50–144 h of 12.5 mg Diazinon L^{-1}. At short-term chronic exposure (168 h), controls reached the end of the embryonic development (S.25), while exposed embryos were still delayed. At this time, the main malformations observed were axial flexures, irregular borders, wavy tail, microcephaly, malformed mouth and adhesive structures, gut miscoiling, underdeveloped gills, cloacal edema, desquamation and severe hydropsy (Fig. 2). The TI at 96 h, 144 h and 168 h were 1.52, 1.71 and more than 2, respectively. The statistical analysis of lengths at 168 h of exposure showed that embryos exposed from 13.5 mg Diazinon L^{-1} were significantly shorter than controls (Fig. 3).

Treated embryos also exhibited neurotoxic effects from 96 h, after the beginning of neuromuscular activity, with a LOEC value of 6 mg Diazinon L^{-1}. Neurotoxic effects were exposure concentration and time-dependent. Thus, embryos showed gradual increases in spasmodic contractions and decreased response to stimulus. Finally, lack response to stimulation but even weak heartbeat was observed. Simultaneously to this lack of general response, a reduction in food intake was evident due to the persistence of complete pellets in the Petri dishes.

3.2. Larval toxicity

3.2.1. Lethal effects

Fig. 4 shows Diazinon LC10, 50 and 90 for early *R. arenarum* larvae at different exposure times. The toxicity profile showed a higher susceptibility of larvae, about 3 to 4.4 times, than embryos continuously treated from blastula stage. Larval susceptibility significantly increased from a LC50–24 h of 11.2 mg Diazinon L^{-1} to 6.7 mg Diazinon L^{-1} at 168 h (Fig. 4). Empirical NOEC values were 9 and 6 mg Diazinon L^{-1} at 48 and 168 h, respectively. Toxicity continued increasing to LC50 of 5.2 and 1.9 mg Diazinon L^{-1} when exposure was extended to 240 h and 504 h, respectively. HQ values also increased along time from acute to chronic exposure, to reach a maximal value of 2.7. There were no significant differences in susceptibility between litters, and the coefficients of variation were always lower than 10.5%.

3.2.2. Sublethal effects

The characteristic sublethal effect of Diazinon on larvae was mainly abnormal behavior associated with neurotoxicity and it

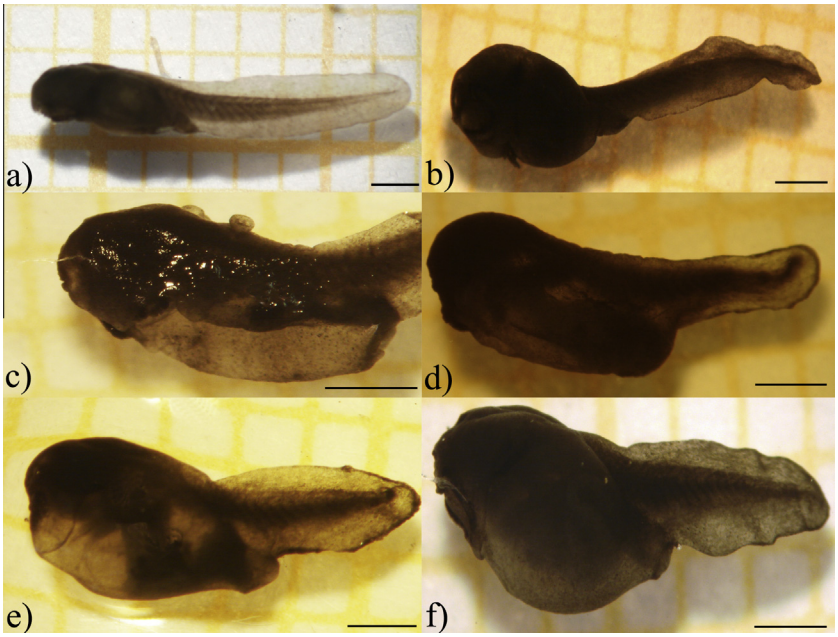

Fig. 2. Examples of optical microscopic views of malformations produced by Diazinon in *Rhinella arenarum* embryos continuously exposed for 168 h. (a) Control; (b) 9 mg Diazinon L^{-1}, note the axial flexures, irregular borders, wavy tail; (c), (d) 13 mg Diazinon L^{-1} and (e), (f) 18 mg Diazinon L^{-1}, observe the reduce body size, axial flexure, microcephaly, gut miscoiling, underdeveloped gills, cloacal edema, severe hydropsy and generalized edema. Scale: 1 mm.

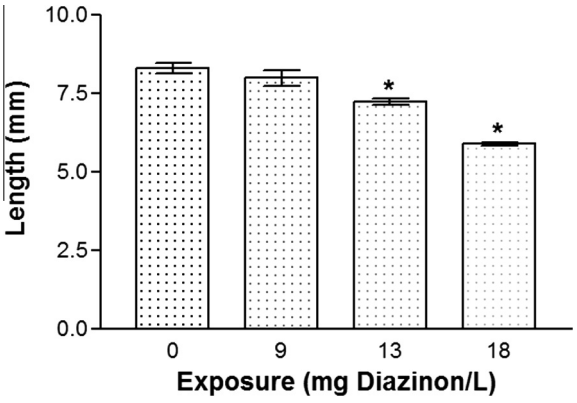

Fig. 3. Length of *Rhinella arenarum* embryos exposed from early blastula stage (S.4) onwards to different Diazinon concentrations for 168 h ($n = 30$). *Significant differences from control ($p \leqslant 0.05$).

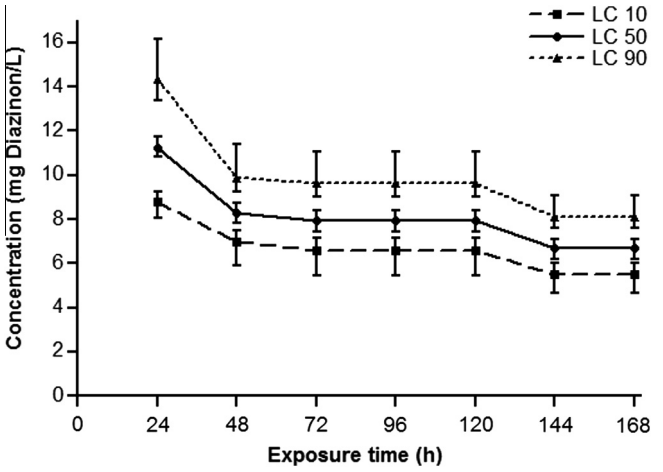

Fig. 4. Toxicity profile curves of Diazinon representing lethal concentrations (LCs) 10%, 50%, and 90% in *Rhinella arenarum* larvae exposed from complete operculum stage (S.25) onwards. Bars show 95% confidence intervals.

was registered within the first 24 h of exposure. Adverse effects were concentration and exposure time-dependent. Thus, larvae showed hyperkinesis with shorter trajectories and erratic swimming but then, they exhibited less frequent movements and non-feeding behavior. Finally, no movements and lack of response were registered. NOEC-168 h was 4.5 mg Diazinon L^{-1}. Observed edemas were also increasing with concentration and exposure time.

3.3. Diazinon uptake

Uptake of Diazinon by embryos exposed from blastula stage was time-dependent, being significantly higher at 96 h of exposure with a maximal BCF of 107. However, BCFs felt to values between 1.2 and 2.8 when exposure time was extended to 504 h. Uptake was significantly related to exposure concentration up to 3 mg Diazinon L^{-1} at 96 h, and then it seems to reach a threshold with no significant differences between the highest concentrations (Fig. 5a). Uptake of Diazinon by larvae was not only concentration-dependent but also time-dependent, with a significant reduction when exposure time was extended to 504 h (Fig. 5b). The maximal BCF value of 4 was reached at 96 h. Thus, uptake was stage-dependent, as it was up to 27 times higher for organisms exposed from blastula stage onwards than early larvae.

4. Discussion

Present results show the high toxicity of Diazinon to embryos and larvae of *R. arenarum*, a widely distributed South American amphibian species. Toxicity of continuous exposure to the pesticide from the beginning of the embryonic and larval development were time-dependent, reaching LC50–504 h of 6.8 mg Diazinon L^{-1} and 1.9 mg Diazinon L^{-1}, respectively. Both values were between

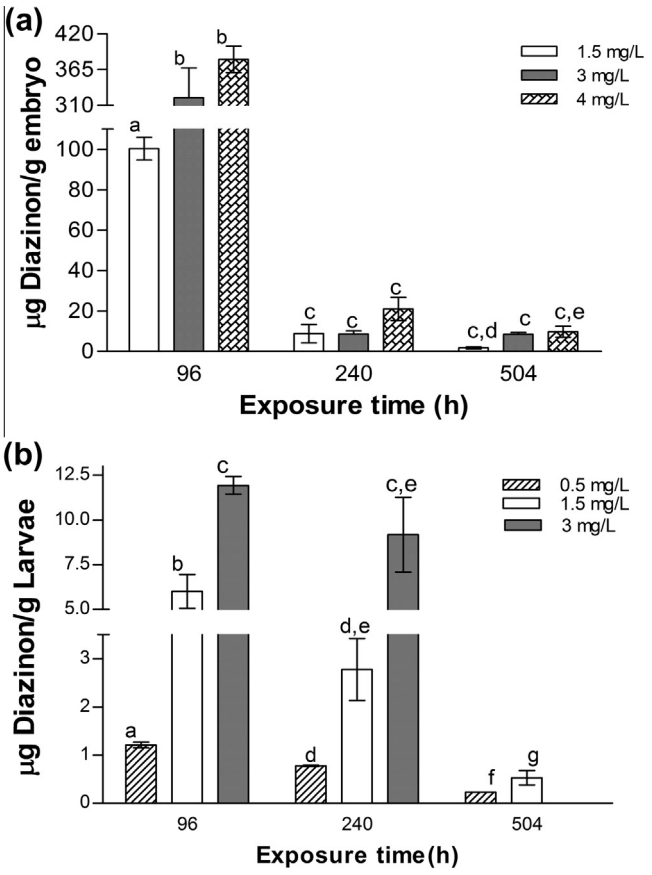

Fig. 5. Uptake of Diazinon by *Rhinella arenarum* embryos (a) exposed from early blastula stage (S.4) and larvae (b) exposed from complete operculum stage (S.25) up to 504 h (n = 30–40). Different letters indicate significant differences.

three and four times more toxic than the acute and short-term chronic periods.

Diazinon toxicity resulted higher to *Rana clamitans* and *Xenopus laevis* than to *R. arenarum* at the beginning of the development (Harris et al., 1998; Modra et al., 2011). Nevertheless, Diazinon toxicity on *R. arenarum* at larval stage was similar to *Rana boylii* (Sparling and Fellers, 2007) but resulted four times more sensitive than *Bufo melanostictus* (Sumanadasa et al., 2008). These stage and time-dependent toxicity highlight the importance of assess adverse effects during different developmental stages for amphibian species protection. Despite of the high Diazinon toxicity to *R. arenaum*, this organophosphate pesticide resulted less toxic than other organochlorine or pyrethroid pesticides such as endosulfan and cypermethrin, respectively (Svartz et al., 2013; Svartz and Pérez-Coll, 2013).

It is noteworthy the remarkable differences found in the concentration which caused lethality with those causing teratogenic effects. The pesticide induced important sublethal effects, the TI for embryos continuously exposed from the beginning of their development at 96 h, 144 h and 168 h were 1.52 and >2. These values highlight the teratogenicity of Diazinon on *R. arenarum* embryos, as values greater than 1.5 indicate a high risk for embryos to be malformed in absence of significant embryonic lethality (ASTM, 1993). Moreover, our results also highlight the marked sublethal potential of Diazinon on *R. arenarum*, as the informed TI value for *Xenopus laevis* was 1.3 (Modra et al., 2011). Thus, in the case of Diazinon, it is meaningful to consider teratogenesis as a relevant endpoint for risk assessment purposes.

The edemas observed under Diazinon exposure, have been also informed as an endothelium alteration, being the main characteristic effect also in exposed fish. This sublethal effect could be related to the local activation of the pesticide, leading to apoptosis and the lost of cellular integrity (Hamm and Hinton, 2000; Osterauer and Köhler, 2008).

Moreover, the present study showed the reduced body length caused by Diazinon on embryos continuously exposed to concentrations greater than 13.5 mg Diazinon L^{-1}. Despite this sublethal effect being non-specific, as it has been previously informed for other noxas (Sztrum et al., 2011; Aronzon et al., 2011a,b), the reduced body size observed might be consequence of the non-feeding behavior, which was strongly related to neurotoxicity. The reduced food intake has been previously informed for tadpoles exposed to endosulfan and showed that such feeding depression can be in part due to a reduction of movements (Denoël et al., 2013). Moreover, important neurotoxic effects of the pesticide were expressed as behavioral disturbances. These abnormalities were first recorded as hyperkinetic and erratic movements at low concentrations and shorter exposure periods, but at higher concentrations or prolonged exposure times, resulted in lack of movements and response to stimuli. This pattern of abnormal behavior is consistent with described neurotoxic effects, which would be caused by inhibition of the AChE activity, the main target of Diazinon (Fulton and Key, 2001). It has been also proved a correlation between the abnormal behavior, swimming, and changes in AChE activity (Beauvais et al., 2000). Present work highlights the importance to assess behavior as trait of the integrity of organisms, not only because it includes vital activities such as feeding but also because it implies, through locomotion, the use of environmental resources and the increase of the likelihood of predation (Bishop and Pettit, 1992; Denoël et al., 2013).

The marked decreased heartbeat was also shown in fishes exposed to Diazinon, and may also result from AChE inhibition, as ChE depresses frequency, power of contraction and excitation of the heart (Osterauer and Köhler, 2008).

The same neurotoxic pattern was observed in continuous treatment at the beginning of larval development but at lower concentrations and shorter exposure times. Thus, NOEC-24 h for abnormal behavior was 4.5 mg Diazinon L^{-1}. Besides, other sublethal effects as generalized edema were observed at the beginning of the short-term chronic period.

It is common to observe differences in susceptibility to physical and chemical agents among developmental stages, this should be taken into account in making decisions on acceptable levels for the species conservation as a whole (Hutler Wolkowicz et al., 2014; Sztrum et al., 2011; Aronzon et al., 2011a,b). This study highlights the increased susceptibility to Diazinon from embryo to larval amphibian development. Thus, larval period resulted between 3 and 5 times more sensitive than embryonic development. Nevertheless, in the case of organophosphates, there is a controversy: although a higher toxicity of the pesticide on early embryos of common carp and *Bufo melanostictus* were informed (Aydın and Köprücü, 2005; Sumanadasa et al., 2008), present results are in line with the increasing toxicity during the embryo-larval period informed in Medaka (Hamm et al., 2001), killfish (Takimoto et al., 1984) and even in *R. arenarum* exposed to parathion (Anguiano et al., 1994).This differential susceptibility to Diazinon exposure between embryonic and larval period might be related to the beginning of AChE activity in the development, which is well correlated to the muscular and nerve development (Gindi and Knowland, 1979).

Uptake of Diazinon by both embryos and larvae was concentration-dependent, mainly for the first 96 h of exposure. This concentration-dependence is coincident with that informed for fish (Keizer et al., 1991). The Diazinon uptake was also time-dependent with a decrease in the pesticide concentration as exposure time progress. One of the factors influencing this decline might be the

metabolic transformation of Diazinon to its metabolites and excretion, as it was showed for fishes (Kanazawa, 1975).

Furthermore, the uptake of Diazinon was markedly stage-dependent; it was from 1 to 27 times higher for exposures from the beginning of the embryonic development than the larval one. It is interesting to note that *R. arenarum* exhibited a high tendency to bioconcentrate Diazinon, thus in the case of embryos the BCF 96 h was 107. The average value informed for fish was 80.3 with a minimal and maximal value of 16 and 210 for *Tanichthys albonubes* and *Pseudorasbora parva*, respectively (ECOTOX Release 4.0 (http://cfpub.epa.gov/ecotox/)) (Kanazawa, 1978; Tsuda et al., 1997). On the other hand, the highest BCF 96 h for *R. arenarum* larval period was 4. This marked difference in the pesticide uptake between embryos and larvae might be due to the high lipid content of the yolk of embryos. Furthermore, a linear relationship between the bioconcentration ratio of Diazinon and fat content was observed in fishes (Kanazawa, 1975, 1978; Hamm et al., 2001). Bioconcentration has serious ecological implications because pesticides are retained in amphibian body which when fed on by predator could lead to biomagnification (ASTM, 1993; Suter, 1993; Ezemonye and Tongo, 2010).

Most toxicity assays focus on acute effects of a xenobiotic at a specific period of the life cycle; however, our results highlight the importance of evaluating toxicity during different amphibian embryo-larval stages for conservation purposes. Despite *R. arenarum* embryos were more resistant during the acute period than *Xenopus laevis* and *Rana booylii* (Sparling and Fellers, 2007; Modra et al., 2011), larvae resulted significantly more sensitive than other anurans such as *Bufo melanostictus* (Sumanadasa et al., 2008).

Moreover, the HQ approach provides a possibility to assessing the risk for adverse effects of Diazinon at different developmental stages of *R. arenarum*. It is noteworthy that for larval development, HQ resulted in values higher than 1 which represents the USEPA LOC. This fact highlights the threat that Diazinon represents for *R. arenarum* populations, mainly during the larval stages.

5. Conclusion

Diazinon resulted highly toxic for *R. arenarum*, increasing susceptibility from embryo to larval amphibian development. Uptake of Diazinon resulted exposure concentration, time and stage-dependent.

The wide range found in the concentrations which caused lethality with those causing teratogenic and neurotoxic effects, highlights them as relevant endpoints for risk assessment purposes. Moreover, these facts and the HQ obtained showed the threat that Diazinon represents for *R. arenarum* populations, mainly during the larval stages.

Acknowledgments

Cristina S. Pérez-Coll is member of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina, and Universidad Nacional de San Martín (UNSAM), Argentina. Carolina Aronzon has a CONICET-UNSAM fellowship. This study was supported by a research Grant PICT-2010-0891 from Agencia de Promoción Científica y Tecnológica, Argentina. We wish to thank Instituto Massone S.A. for providing the human chorionic gonadotropin.

References

Albanis, T.A., Hela, D.G., Sakellarides, T.M., Konstantinou, I.K., 1998. Monitoring of pesticide residues and their metabolites in surface and underground waters of Imathia (*N. Greece*) by means of solidphase extraction disks and gas chromatography. J. Chromatogr. A 823, 59–71.

American Public Health Association, 1980. Standard methods for the examination of water and wastewater., Washington, DC.

Anguiano, O.L., Montagna, C.M., Chifflet de Llamas, M., Guana, L., Pechen de D'Angelo, A.M., 1994. Comparative toxicity of parathion in early embryos and larvae of the toad, *Bufo arenarum* Hensel. Bull. Environ. Contam. Toxicol. 52, 649–655.

Aronzon, C., Sandoval, M., Herkovits, J., Pérez-Coll, C., 2011a. Stage-dependent toxicity of 2,4-dichlorophenoxyacetic on the embryonic development of a South American toad, *Rhinella arenarum*. Environ. Toxicol. 26, 373–381.

Aronzon, C.M., Sandoval, M.T., Herkovits, J., Pérez-Coll, C.S., 2011b. Stage-dependent susceptibility to copper in *Rhinella arenarum* embryos and larvae. Environ. Toxicol. Chem. 30, 2771–2777.

ASTM, 1993. Standard guide for conducting the Frog Embryo Teratogenesis Assay-Xenopus (FETAX). American Society for Testing and Materials. Standards on aquatic toxicology and hazard evaluation., Philadelphia, PA, pp. 1199–1209.

Aydın, R., Köprücü, K., 2005. Acute toxicity of diazinon on the common carp (*Cyprinus carpio L.*) embryos and larvae. Pestic. Biochem. Physiol. 82, 220–225.

Bantle, J., Dumont, J., Finch, R., Linder, G., Fort, D., 1998. Atlas of abnormalities. A guide for the performance of FETAX, Stillwater.

Beauvais, S.L., Jones, S.B., Brewer, S.K., Little, E.E., 2000. Physiological measures of neurotoxicity of diazinon and malathion to larval rainbow trout (*Oncorhynchus mykiss*) and their correlation with behavioral measures. Environ. Toxicol. Chem. 19, 1875–1880.

Bishop, C.A., Pettit, K.E., 1992. Declines in Canadian amphibian populations: designing a national monitoring strategy. In: Minister of supply and services Canada (Ed.), Ontario, pp. 243–250.

Boutin, C., Freemark, K.E., Keddy, C.J., 1993. Proposed guidelines for registration of chemical pesticides: nontarget plant testing and evaluation. Service (Headquarters), Environment Canada, Ottawa.

Boutin, C., Freemark, K.E., Keddy, C.J., 1995. Overview and rationale for developing regulatory guidelines for nontarget plant testing with chemical pesticides. Environ. Toxicol. Chem. 14, 1465–1475.

Bowman, B.T., Sans, W.W., 1983. Further water solubility determinations of insecticidal compounds. J. Environ. Sci. Health B 18, 221–227.

Bridges, C., 1997. Tadpole swimming performance and activity affected by acute exposure to sublethal levels of carbaryl. Arch. Environ. Contam. Toxicol. 16, 1935–1939.

Coppage, D.L., Braidech, T.E., 1976. River pollution by anticholinesterase agents. Water Res. 10, 19–24.

Chambers, J.E., Carr, R.L., 1995. Biochemical mechanisms contributing to species differences in insecticidal toxicity. Toxicology 105, 291–304.

Del Conte, E., Sirlin, L., 1951. The first stages of *Bufo arenarum* development. Acta Zool. Lilloana 12, 495–499.

Denoël, M., D'hooghe, B., Ficetola, G.F., Brasseur, C., De Pauw, E., Thomé, J.-P., Kestemont, P., 2012. Using sets of behavioral biomarkers to assess short-term eff ects of pesticide: a study case with endosulfan on frog tadpoles. Ecotoxicology 21, 1240–1250.

Denoël, M., Libon, S., Kestemont, P., Brasseur, C., Focant, J.F., De Pauw, E., 2013. Effects of a sublethal pesticide exposure on locomotor behavior: a video-tracking analysis in larval amphibians. Chemosphere 90, 945–951.

Ezemonye, L., Tongo, I., 2010. Acute toxic effects of endosulfan and diazinon pesticides on adult amphibians (*Bufo regularis*). J. Environ. Chem. Ecotoxicol. 2, 73–78.

Franke, C., Studinger, G., Berger, G., Bohling, S., Bruckmann, U., Cohors-Fresenborg, D., Johncke, U., 1994. The assessment of bioaccumulation. Chemosphere 29, 1501–1514.

Fulton, M.H., Key, P.B., 2001. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. Environ. Toxicol. Chem. 20, 37–45.

Galloway, T., Handy, R., 2003. Inmunotoxicity of organophosphorous pesticides. Ecotoxicology 12, 345–363.

Gindi, T., Knowland, J., 1979. The activity of cholinesterases during the development of *Xenopus laevis*. J. Embryo Exp. Morph. 51, 209–215.

Hamm, J.T., Hinton, D.E., 2000. The role of development and duration of exposure to the embryotoxicity of diazinon. Aquat. Toxicol. 48, 403–418.

Hamm, J.T., Wilson, B.W., Hinton, D.E., 2001. Increasing uptake and bioactivation with development positively modulate diazinon toxicity in early life stage medaka (*Oryzias latipes*). Toxicol. Sci. 61, 304–313.

Harris, M.L., Bishop, C.A., Struger, J., Ripley, B., Bogart, J.P., 1998. The functional integrity of northern leopard frog (*Rana pipiens*) and green frog (*Rana clamitans*) population in orchard wetlands. II Efects of pesticides and eutrophic conditions on early life stage development. Environ. Toxicol. Chem. 17, 1351–1363.

Herkovits, J., Helguero, L.A., 1998. Copper toxicity and copper-zinc interactions in amphibian embryos. Sci. Total Environ. 221, 1–10.

Hutler Wolkowicz, I.R., Herkovits, J., Pérez Coll, C.S., 2014. Stage-dependent toxicity of bisphenol-a on *Rhinella arenarum* (Anura, Bufonidae) embryos and larvae. Environ. Toxicol. 29, 146–154.

Kanazawa, J., 1975. Uptake and excretion of organophosphorus and carbamate insecticides by fresh water fish, motsugo, *Pseudorasbora parva*. Bull. Environ. Contam. Toxicol. 14, 346–352.

Kanazawa, J., 1978. Bioconcentration ratio of diazinon by freshwater fish and snail. Bull. Environ. Contam. Toxicol. 20, 613–617.

Keizer, J., D'Agostino, G., Vittozzi, L., 1991. The importance of biotransformation in the toxicity of xenobiotics to fish. I. Toxicity and bioaccumulation of diazinon in guppy (*Poecilia reticulata*) and zebra fish (*Brachydanio rerio*). Aquat. Toxicol. 21, 239–254.

Mann, R., Bidwell, J., 2000. Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. Aquat. Toxicol. 51, 19–29.

Modra, H., Vrskova, D., Macova, S., Kohoutkova, J., Hajslova, J., Haluzova, I., Svobodova, Z., 2011. Comparison of diazinon toxicity to embryos of *Xenopus laevis* and *Danio rerio*; degradation of diazinon in water. Bull. Environ. Contam. Toxicol. 86, 601–604.

Osterauer, R., Köhler, H.-R., 2008. Temperature-dependent effects of the pesticides thiacloprid and diazinon on the embryonic development of zebrafish (*Danio rerio*). Aquat. Toxicol. 86, 485–494.

Peltzer, P.M., Lajmanovich, R.C., Sanchez, L.C., Attademo, A.M., Junges, C.M., Bionda, C.L., Martino, A.L., Bassó, A., 2010. Morphological abnormalities in amphibian populations from the mid-eastern region of Argentina. Herpetol. Conserv. Biol. 6, 432–442.

Sparling, D.W., Fellers, G., 2007. Comparative toxicity of chlorpyrifos, diazinon, malathion and their oxon derivatives to larval *Rana boylii*. Environ. Pollut. 147, 535–539.

Sumanadasa, D.M., Wijesinghe, M.R., Ratnasooriya, W.D., 2008. Effects of diazinon on larvae of the Asian common toad (*Bufo melanostictus*, Schneider 1799). Environ. Toxicol. Chem. 27, 2320–2325.

Suter, G., 1993. Ecological risk assessment. Lewis p., Boca Raron FL.

Svartz, G.V., Hutler Wolkowicz, I.R., Pérez Coll, C.S., 2013. Toxicity of endosulfan on embryo-larval development of the South American toad, *Rhinella arenarum*, Environ. Toxicol. Chem. http://dx.doi.org/10.1002/etc.2506.

Svartz, G.V., Pérez-Coll, C.S., 2013. Comparative toxicity of cypermethrin and a commercial formulation on *Rhinella arenarum* larval development (ANURA: BUFONIDAE). Int. J. Environ. Health 6, 320–329.

Sztrum, A.A., D'Eramo, J.L., Herkovits, J., 2011. Nickel toxicity in embryos and larvae of the South American toad: effects on cell differentiation, morphogenesis, and oxygen consumption. Environ. Toxicol. Chem. 30, 1146–1152.

Takimoto, Y., Hagino, S., Yamada, H., Miyamoto, J., 1984. The acute toxicity of fenitrothion to killifish (*Oryzias latipes*) at twelve different stages of its life history. J. Pesticide Sci., 463–470.

Tsuda, T., Kojima, M., Harada, H., Nakajima, A., Aoki, S., 1997. Relationships of bioconcentration factors of organophosphate pesticides among species of fish. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 116, 213–218.

US EPA, 1988. Users guide for a computer program for PROBIT analysis of data from acute and short-term chronic toxicity test with aquatic organisms. Biological methods, Environmental monitoring and support Lab. United States Environmental Protection Agency.

US EPA, 1994. Test methods for evaluating solid waste, Physical/Chemical Methods. SW 846.

US EPA, 1998. Guidelines for ecological risk assessment. Ecological risk assessment Step 2. United States Environmental Protection Agency. Washington, DC. <http://www.epa.gov/R5Super/ecology/html/erasteps/erastep2.html>.

van der Schalie, W.H., Gardner Jr., H.S., Bantle, J.A., De Rosa, C.T., Finch, R.A., Reif, J.S., Reuter, R.H., Backer, L.C., Burger, J., Folmar, L.C., Stokes, W.S., 1999. Animals as sentinels of human health hazards of environmental chemicals. Environ. Health Perspect. 107, 309–315.

Wake, D.B., Vredenburg, V.T., 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proc. Nat. Acad. Sci. 105, 11466–11473.