Stage-Dependent Toxicity of 2,4-Dichlorophenoxyacetic on the Embryonic Development of a South American Toad, *Rhinella arenarum*

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Received 1 July 2009; revised 3 December 2009; accepted 6 December 2009

ABSTRACT: The acute and short term chronic toxicity of both the herbicide butyl ester of 2,4-Dichlorophenoxyacetic acid (2,4-D) and a commercial formulation (CF) were evaluated on *Rhinella* (= *Bufo*) *arenarum* embryos at different developmental stages. Adverse effects were analyzed by means of the isotoxicity curves for lethality, malformations, stage-dependent susceptibility, and ultrastructural features. For all experimental conditions, the CF was more toxic, up to 10 times, than the active ingredient, being the open mouth stage (S.21) the most susceptible to the herbicide. For continuous treatment conditions, the early embryonic development was the most susceptible to 2,4-D and the LC50s for 96 and 168 h were 9.06 and 7.76 mg L^{-1} respectively. In addition, both the active ingredient and the CF were highly teratogenic, resulting in reduced body size, delayed development, microcephaly, agenesis of gills, and abnormal cellular proliferation processes as the main adverse effects. According to US EPA, 2,4-D in agricultural scenarios may be up to three times higher than the NOEC values for teratogenic effects reported in this study. Therefore, they might represent a risk for amphibians. This study also points out the relevance of reporting the susceptibility of embryos at different developmental stages to both the active ingredient and the CF of agrochemicals in order to protect nontarget organisms. © 2010 Wiley Periodicals, Inc. Environ Toxicol 26: 373–381, 2011.

Keywords: 2,4-dichlorophenoxyacetic acid; embryonic development; herbicide; amphibian embryo; teratogenesis; commercial agrochemical; stage-dependent toxicity

INTRODUCTION

There is an increasing concern due to the adverse effects of pesticides on nontarget organisms as they may be related to the loss of biodiversity. 2,4-Dichlorophenoxyacetic acid (2,4-D) was introduced in 1946 as an agricultural herbicide, and is one of the most widely used pesticides in the world

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Contract grant sponsor: Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)

Contract grant number: PIP2316, 5324

Contract grant sponsor: Universidad Nacional de San Martín

Contract grant number: SA-08-02

Published online 28 January 2010 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/tox.20564

(Wauchope et al., 1992). It is a systemic herbicide used in agriculture, pastures, forest management, home and gardens to control broadleaf weeds and aquatic vegetation.

2,4-D is a stable and persistent auxin-like substance that disturbs a large number of processes involved in plant growth and development, resulting in an overstimulation of growth and final death of plants (Hess, 1993; Zimdahi, 1993). Residues of the herbicide in aqueous systems can result from the deposition of spray drifts, the ''washout'' in the vapor or droplets from the atmosphere during rainfall, the run-off from treated fields, or from the herbicide application to control aquatic weeds. The butyl ester of 2,4-D acid is used in different commercial formulations (CF), which are mixtures of this active ingredient and other compounds like solvents and emulsifiers that increase the effectiveness, dispersion, persistence, and bio-availability of the active ingredient. These additives represent a major ecotoxicological concern because they can be more toxic than the active ingredient (Mann and Bidwell, 2001).

The toxicity of 2,4-D has been reported in several nontarget organisms, from bacteria to vertebrates (IPCS, 1989; Rodríguez and Amín, 1991; Charles et al., 2001). In animals, the mechanism of 2,4-D toxicity may range from oxidative stress (Oruc et al., 2004) to endocrine disruption (McKinlay et al., 2008). *In vitro* studies with animal cells have shown that the herbicide reduces the activity of mitochondrial enzymes involved in the production of ATP (Palmeira et al., 1994) and inhibits lipid metabolism and protein synthesis (Rivarola et al., 1992). In primary cultures of cerebellar granule cells it has been reported that 2,4-D produces a striking and dose-dependent inhibition of neurite extension, reduction in the cellular content of microtubules, disorganization of the Golgi apparatus, and inhibition in the synthesis of complex gangliosides (Rosso et al., 2000). Genotoxic effects produced by 2,4-D have been reported in yeast (Venkov et al., 2000) and in *Clarias batrachus* erythrocytes (Ateeq et al., 2002).

Amphibians, which include a large number of endangered species, are also good indicators of environmental health (Simms, 1969; Herkovits et al., 1996; Houlahan and Findlay, 2000; Bueno-Guimarães et al., 2001; Wake and Vredenburg, 2008). Because of their high sensitivity to a wide diversity of environmental pollutants, mainly at embryonic and larval stages, they are widely used in ecotoxicological assessment studies (Herkovits and Pérez-Coll, 2003; Ferrari et al., 2005; Izaguirre et al., 2008).

Amphibians can be more vulnerable to the toxic effects of herbicides than other vertebrates due to their preference to breeding in shallow, lentic, or ephemeral water bodies (van der Schalie et al., 1999). The decline in amphibian populations and the large number of malformations found in many geographic regions contaminated with pesticides (Ouellet et al., 1997) seem to confirm their vulnerability to agrochemical pollutants. Early life stages involve very complex cellular differentiation and morphogenetic processes and are usually much more susceptible to noxious agents than adults. The toxicity studies of 2,4-D on amphibians have shown that the herbicide inhibits oocyte maturation (Stebbins-Boaz et al., 2004; LaChapelle et al., 2007) and arrests egg development (Losthe and Roth, 1946). The teratogenic effects of this herbicide has been reported in *Xenopus laevis* (Morgan et al., 1996), and metamorphosis impairment has been described in *Rana temporaria*, where it probably acts as an antagonist of thyroid hormones (Buslovich and Borushko, 1976). In a previous study in *Bufo arenarum* embryos continuously exposed to the same 2,4-D CF from the end of their embryonic development, the Lethal Concentrations (LCs) 50 for 24 and 168 h were 4 and 3 mg L^{-1} CF respectively. Toxicity Profiles (TOPs), isotoxicity curves based on the LCs, were also reported in that study (Pérez-Coll and Herkovits, 2006). Although those values may already imply a risk for amphibian embryos (EPA, 2004), based on previous stage-dependent susceptibility studies, it has been found that certain stages are highly susceptible to noxious agents (Herkovits et al., 1997; Castañaga et al., 2009). The main purposes of this study were to compare the toxicity of the active ingredient (2,4-D) and one of its commercial products on *Rhinella* (= *Bufo*) *arenarum* embryos and to compare the sensitivity between embryonic and early larval stages. The results are discussed focusing on changes in the susceptibility of the early life stages of *R. arenarum* to 2,4-D and its commercial product by analyzing the potential adverse effects on this widely distributed South American amphibian species.

MATERIALS AND METHODS

Rhinella arenarum Embryos

Rhinella arenarum adults, weighing ~200 to 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. Oocytes were fertilized *in vitro* with sperm suspensions in AS. 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 the stage required by each experimental protocol (Del Conte and Sirlin, 1951). Embryos used before hatching (muscular activity, S.18) 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.

Test Solutions

Stock solution of butyl ester of 2,4-D (purity: 99%; Lot VW169891, Merk) was prepared in acetone to a final

concentration of 2 g L^{-1}, while the commercial formulation (CF) was freshly prepared in deionized water to a final concentration of 4 g L^{-1}. The CF used, Esteron Ultra®, contains butyl ester of 2,4-D 100 g/100 cc (= 79.7 g acid equivalent to 2,4-D/100 cc), a tensioactive mixture (8 g/100 cc), and kerosene solvent (sufficient amount for 100 cc). Both substances were supplied by Dow AgroSciences Argentina S.A. Control embryos were simultaneously kept in AS and AS plus acetone at the highest concentration used for 2,4-D solution. Acetone concentrations were always lower than 1.1% (ASTM, 1993). 2,4-D concentrations at test solutions were verified by high-performance liquid chromatography UV. Analytical data oscillated around 17% of the nominal values.

Experimental Protocols

The bioassays were conducted with *Rhinella arenarum* embryos, by following the AMPHITOX protocols (Herkovits et al., 2002; Herkovits and Pérez-Coll, 2003) for early life stages (AMPHIEMB) and seven-day exposure (AMPHISHORT), which allow the evaluation of teratogenesis and lethality, the main aims of this study. Duplicate batches of 10 embryos were placed in covered 10-cm-diameter glass Petri dishes containing 40 mL of AS with either the active ingredient (2,4-D) or the CF in different concentrations. Experiments were replicated four times. The CF concentrations were expressed as the concentration of 2,4-D in the solutions. The experimental conditions were as follows: (i) continuous exposure of embryos from the 2–4 blastomeres stage (S.3-S.4) up to the end of embryonic development (S.25) to the herbicides in seven concentrations ranging from 1 to 15 mg L^{-1} 2,4-D and from 0.25 to 3.85 mg L^{-1} CF; (ii) exposure of embryos in the following stages: blastula (S.3), gastrula (S.11), tail bud (S.17), muscular activity (S.18), gill circulation (S.20), open mouth (S.21), opercular folds-operculum closes on right side (S.23-24) and complete operculum (S.25) for 24 h to nine concentrations ranging from 1 to 26 mg L^{-1} 2,4-D and from 0.1 to 10 mg L^{-1} CF and subsequent change to AS; and (iii) continuous exposure of embryos from the end of their development (S.25) for 168 h to seven concentrations ranging from 8 to 20 mg L^{-1} 2,4-D and from 2 to 5 mg L^{-1} CF. Embryos were maintained at 20 ± 2°C and solutions were renewed every other day. Survival was evaluated every 24 h and dead individuals were removed. Larvae from S.25 were fed with Swiss chard *ad libitum* for 24 h, every other day. Teratogenic effects were studied with stereoscopic microscopy and photographs of experimental and AS/acetone control embryos were digitally recorded with a Sony DSC-S90 camera mounted on a Zeiss Stemi DV4 stereoscopic microscope. In addition, other embryos were prepared for Scanning Electron Microscopy (SEM) and observed in a scanning electron microscope JEOL 5800LV for ultrastructure evaluation.

Statistical Analyses

Lethality data were statistically analyzed by the US EPA Probit Program (EPA, 1988) and R. Cinto's Probit method modified by E.M. Rodríguez, with Abbot's correction and Lichfield's and Wilcoxon's alternative method; version April 1994 (Rodríguez and Amín, 1991). TOPs curves were plotted based on LC10, 50 and 90 from 24 to 168 h obtained by Probits. To establish statistical differences between the LC50 values obtained, a comparison was made, considering the difference statistically significant when the higher LC50/lower LC50 ratio exceeded the critical value (95% confidence interval) established by APHA (APHA, 1980).

RESULTS

Continuous Exposure from Blastula Stage Onwards

During the AMPHITOX test, both lethal and teratogenic effects of noxious agents during the embryonic development were evaluated by means of continuous treatment from blastula until complete operculum stage (Herkovits and Pérez-Coll, 2003).

Figure 1 compares the LC 10, 50, and 90 and their correspondent confidence limits (95%) for *Rhinella arenarum* embryos treated with 2,4-D and CF, at different exposure times, from early blastula stage up to 168 h. The figure shows that in all the cases the CF was significantly more toxic, 2.68 ± 0.05 times higher than its active ingredient ($P < 0.05$). The range of concentrations from LC10 to LC90 for 2,4-D was very broad (4.77 to 15.12 mg L^{-1}), whereas that for the CF was very narrow (1.8 to 4.65 mg L^{-1}). It is noteworthy that there was a multiple overlapping between the confidence limits for the LC10, 50, and 90 of CF within the acute period. The LC50 values for 2,4-D and CF at 24 h were 10.34 mg L^{-1} (9.52–11.24) and 3.89 mg L^{-1} (3.77–4.10) respectively. By extending the exposure of both the active ingredient and the commercial product to 168 h, the LC values decreased by about 25% to 7.76 mg L^{-1}(6.98–8.35) and 2.91 mg L^{-1} (2.64–3.14) respectively. This gradual increase in the toxicity was significant ($P < 0.05$) from 96 h LC50 onwards.

Twenty-Four Hour Exposure of Different Developmental Stages

To evaluate the stage-dependent susceptibility of the herbicide on *Rhinella arenarum* embryos, the adverse effects of the herbicide were studied by means of pulse treatments.

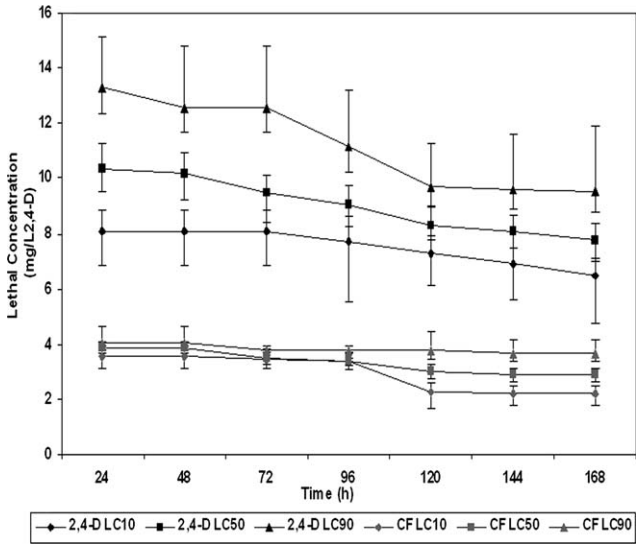

Fig. 1. Toxicity Profile curves of butyl ester of 2,4-Dichlorophenoxyacetic acid (2,4-D) and its Commercial Formulation (CF) on *Rhinella arenarum* embryonic development. The LCs 10, 50, and 90 and their corresponding confidence limits (95%) at different exposure times, from 24 h up to 168 h. In all cases the CF was significantly more toxic than the active ingredient ($P < 0.05$). By expanding the exposure to both the active ingredient and the commercial product to 168 h, there was a gradual increase in the toxicity from 96h onwards ($P < 0.05$).

Figure 2 shows the LC50 and its corresponding confidence limit (95%) for eight different developmental stages of *Rhinella arenarum* embryos exposed to 2,4-D and CF for 24 h. For all developmental stages evaluated, the LC values of the CF were several times higher than those of the active ingredient ($P < 0.05$), with a maximal difference of almost 10 times in S.21. In the case of 2,4-D, by comparing

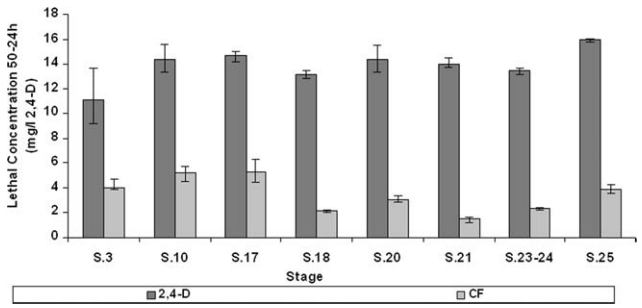

Fig. 2. LC50-24 h and its corresponding confidence limit (95%) of butyl ester of 2,4-Dichlorophenoxyacetic acid (2,4-D) and the Commercial Formulation (CF) on *Rhinella arenarum* embryos exposed for 24 h at eight different developmental stages. For all developmental stages evaluated, the toxicity of the CF was several times higher than the active ingredient ($P < 0.05$). The S. 21 (open mouth) was the most susceptible stage to the CF.

the eight stages evaluated, we were able to observed a limited stage-dependent susceptibility. In this context, although S.3 with an LC50 of 11.16 mg L^{-1} (13.34–14.58) seemed to be the most susceptible stage, there were no significant differences when compared with S.18 (LC50 = 13.14 mg L^{-1}, 12.77–13.47) and S.23-24 (LC50 = 13.42 mg L^{-1}, 13.20–13.64). However, there were significant differences between S.3 with all the other stages evaluated. Complete operculum was the least sensitive stage ($P < 0.05$), as its LC50 was 15.94 mg L^{-1} (15.78–16.07). Conversely, in the case of CF, a more conspicuous stage-dependent susceptibility was observed with S.21 as the most sensitive stage ($P < 0.05$) with an LC50 of 1.47 mg L^{-1} (1.16–1.68), followed by: S.18; S.23-24; S.20; S.3 and S.25, without significant differences between them. S.10 and S.17 were the least sensitive stages (5.17 mg L^{-1}, 4.56–5.78; 5.27mg L^{-1}, 4.48–6.31, respectively) without significant differences between them. It is noteworthy that when the adverse effects were recorded up to 168 h post exposure, neither the active ingredient nor the commercial product showed a significant increase in lethality.

Continuous Exposure from the End of the Embryonic Development Onwards

Figure 3 compares the LC 10, 50, and 90 and their corresponding confidence limits (95%) at different exposure times for *Rhinella arenarum* embryos continuously treated

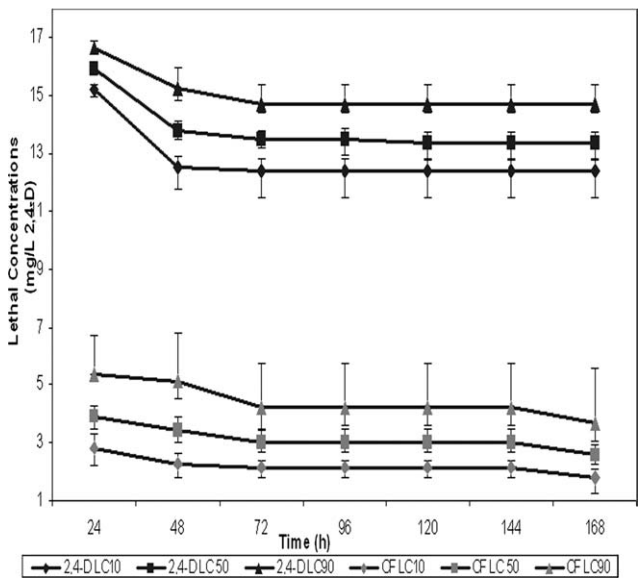

Fig. 3. Toxicity Profile curves of butyl ester of 2,4-Dichlorophenoxyacetic acid (2,4-D) and the Commercial Formulation (CF) on *Rhinella arenarum* embryos exposed from the end of the embryonic development onwards. The LCs10, 50, and 90 and their corresponding confidence limits (95%) from 24 h to 168 h. The CF was around four times more toxic than 2,4-D ($P < 0.05$).

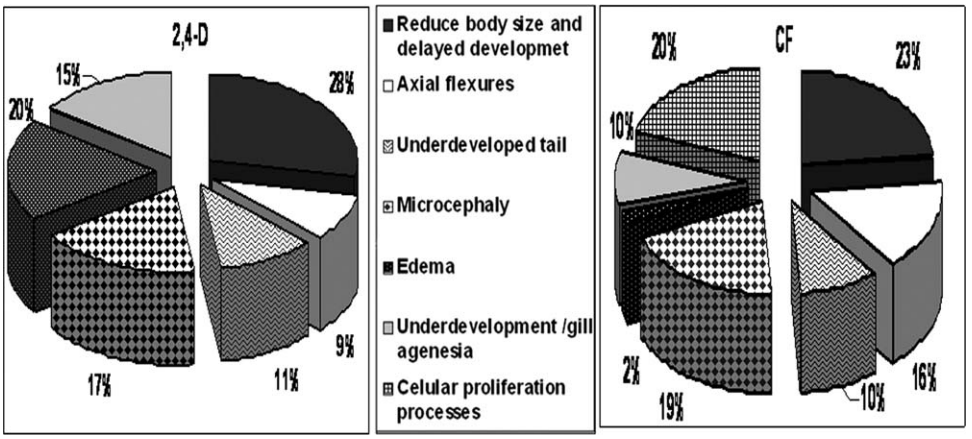

Fig. 4. Percentages of different abnormalities produced by continuous treatment of butyl ester of 2,4-Dichlorophenoxyacetic acid (2,4-D) and the Commercial Formulation (CF) on *Rhinella arenarum* embryos from blastula stage onwards up to 168 h.

with 2,4-D and CF from the complete operculum stage onwards. As that reported for exposures from blastula stage onwards, the CF was several times (4.45 ± 0.37) more toxic than 2,4-D ($P < 0.05$). The LCs50 for 2,4-D and CF were 15.92 mg L^{-1} (15.77–16.05) and 3.91 mg L^{-1} (3.51–4.31) at 24 h respectively, decreasing to 13.40 mg L^{-1} (12.78–13.74) and 2.58 mg L^{-1} (2.29–2.98) at 168 h. The toxicity of 2,4-D on the embryos exposed from S.25 onwards increased by almost 16% along the complete exposure. However, the toxicity significantly increased at 48 h, but did not change ($P > 0.05$) until the end of the exposure at 168 h. The toxicity of CF significantly increased at 72 h and then remained almost constant until 144 h. By the end of the exposure period (168 h), an additional increase was observed in the toxicity ($P < 0.05$), reaching a total increase of almost 34%, as compared with the 24 h LC50.

The 2,4-D LCs50 for embryos treated from blastula onwards and from the complete operculum stage onwards were significantly different ($P < 0.05$) from all the exposure times evaluated, while those for CF did not show significant differences ($P > 0.05$) in the LCs50 between both treatments.

The 2,4-D NOEC for lethality at 168 h for embryos exposed from blastula onwards and for larvae exposed from S.25 onwards were around 6.4 mg L^{-1} and 12.4 mg L^{-1} respectively. The CF NOEC for lethality was around 2 mg L^{-1} for both continuous treatments.

Sublethal Effects

The sublethal effects of 2,4-D and the CF resulted in a great number of different malformations. The NOEC value for malformations in embryos treated from blastula stage onwards with 2,4-D was 1.5 mg L^{-1}, while that for CF was 0.25 mg L^{-1}. The teratogenic potential of the herbicides, estimated as the ratio between the NOEC value for lethality and teratogenesis were around 4 and 8, respectively. No differences were observed between AS controls and acetone controls.

Figure 4 shows the percentages of different abnormalities observed in *Rhinella arenarum* embryos exposed to 2,4-D and CF from blastula stage onwards. The main adverse effects were: reduced body size and delayed development, edema, microcephaly, agenesis/underdeveloped/gills and tail/axial flexures.

Figure 5 shows examples of malformed *Rhinella arenarum* embryos exposed to 2,4-D and CF from blastula up to 168 h, when control embryos achieved the complete operculum stage. A SEM image of an experimental embryo (1b) shows reduced body size, delayed development, microcephaly, agenesis of gills, abnormal cellular proliferation processes with cellular dissociation and reduced tail. Stereoscopic microscope images of embryos with different degrees of malformations can be observed in d, e, and f. It is noteworthy that although, in general, a concentration-response relationship was observed for teratogenesis, in some cases very adverse effects were observed in embryos exposed to low concentrations and slight adverse effects were observed in embryos exposed to high concentrations, thus indicating the individual susceptibility to these noxious agents.

DISCUSSION

The results show the embryonic toxicity of the herbicide butyl ester of 2,4-D and its commercial formulation on *Rhinella arenarum*, a widely distributed South American amphibian species, in continuous and pulse exposures in eight developmental stages. The results highlight that the CF is significantly ($P < 0.05$) more toxic than the active

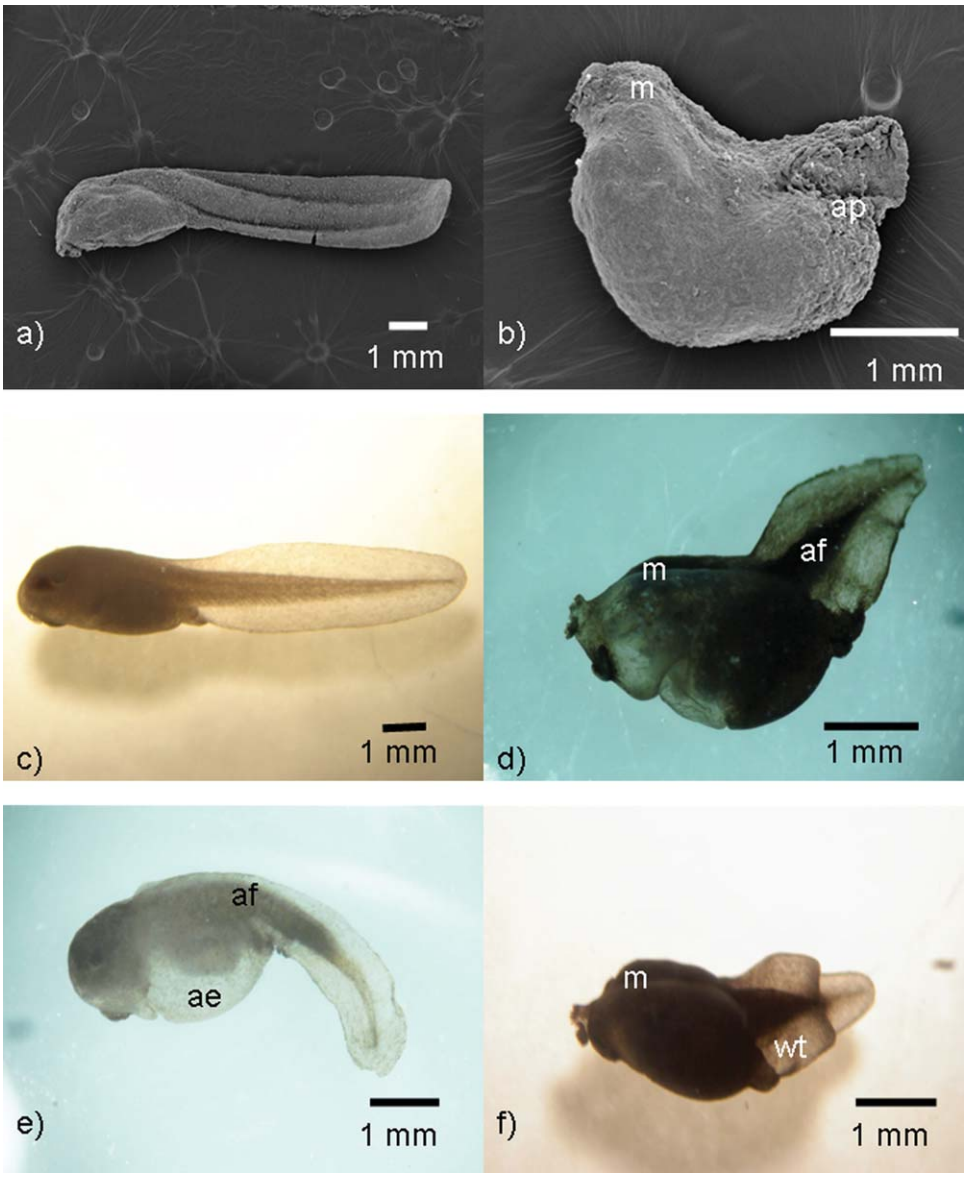

Fig. 5. *Rhinella arenarum* embryos exposed to butyl ester of 2,4-Dichlorophenoxyacetic acid (2,4-D) and the Commercial Formulation (CF) from blastula up to the end of the embryonic development. SEM images: (a) control embryo at S. 25, (b) embryo exposed to 2 mg L^{-1} CF with reduced body size, delayed development, microcephaly (m), agenesis of gills, abnormal cellular proliferation processes (ap) with cellular dissociation, and reduced tail. Stereoscopic microscopy images: (c) control embryo, (d) embryo exposed to 0.8 mg L^{-1} CF with reduced body size and delayed development, axial flexure (af) and cardiac edema (e) embryo exposed to 4.6 mg L^{-1} 2,4-D with underdeveloped gills, severe abdominal edema (ae), axial flexure; and (f) embryo exposed to 7.7 mg L^{-1} 2,4-D with general underdevelopment and reduced body size, microcephaly, and waving tail (wt). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

ingredient for all the experimental conditions evaluated. This higher toxicity may be related to the ability of additives to facilitate absorption of the herbicides into tissues (Green and Abdelghani, 2004), its intrinsic toxicity and eventually an additive or synergistic effect between the additives and the active ingredient. Considering that the toxicity reported for regulatory purposes is based on active ingredient data, our results point out that the toxicity due to the 2,4-D commercial products on nontarget organisms might be completely undervalued. Consequently, for risk assessment purposes on nontarget organisms it may be very relevant to take into account the concentration-response

relationship not only for the active ingredient but also for the commercial product. Moreover, the environmental concentrations of 2,4-D reported (Palma et al., 2004) do not explain the potential adverse effects in nontarget organisms, unless the toxicity of the commercial product applied in the field is incorporated in the risk assessment. There is very limited information on 2,4-D levels in surface waters in South America (Palma et al., 2004). However, models based on Estimated Exposure Concentrations (EEC) of the herbicide applied to different agricultural areas of the USA treated with 2,4-D predicted a peak surface water of 4000 μg L^{-1} 2,4-D, decreasing to around 2000 μg L^{-1} after 90 days (EPA, 2004). These herbicide concentrations are about from 3 to 1.5 times over the NOEC value for teratogenic effects on *R. arenarum* embryos, pointing out the risk of this herbicide for these amphibian species populations.

Although the most commonly used threshold in environmental toxicology is the LC50 48 h (Hoekstra, 1993), TOP curves might be very useful, as they show the concentrations exerting the same degree of adverse effects for different exposure periods. It is interesting to point out that the multiple overlappings between the confidence limits of those values for the CF from the 2–4 blastomeres stage onwards (Fig. 1) imply that the exposure to CF around LC10 may represent a risk for 50% of the population, while exposure to CF around LC50 might represent a risk for 90% of the population, in agreement with that previoulsy reported for other chemicals (Herkovits and Helguero, 1998; Herkovits and Pérez-Coll, 2000).

The results of present work indicate that *Rhinella arenarum* embryos are more susceptible to 2,4-D than other amphibians like *Xenopus laevis*. In this species, the LC50 for natural water samples or for artificial maintaining media, in laboratory conditions, were >270 mg L^{-1} and 254 mg L^{-1}, respectively (Morgan et al., 1996). These values were about 20 times higher than the results obtained in the present study. This difference may be related, at least partially, to the FETAX toxicity test conditions (ASTM, 1993), which use a higher salinity and temperature in the maintaining media (Herkovits and Pérez-Coll, 2003).

The stage-dependent susceptibility reported in this study strongly supports the relevance of evaluating adverse effects of noxious agents at different developmental stages. In the case of the CF for some developmental stages exposed for 24 h, e.g., muscular activity, open mouth and opercular folds-operculum closes on right side, embryos were more susceptible than in the case of continuous treatment from blastula exposed for 168 h. A possible explanation of this result might be an adaptation process in the continuously treated embryos, resulting in an enhanced resistance by the time they reach the most susceptible developmental stages. Pre-exposure to noxious agents might enhance the resistance of living organisms to challenges even against lethal concentrations (Herkovits and Pérez-Coll, 1995). In the case of 2,4-D, although no remarkable stage-dependent susceptibility was observed, the blastula stage was the most susceptible, whereas the complete operculum was the least sensitive. Therefore, the increasing lethality obtained in the continuous treatment condition seems to be directly related to the exposure time to this agrochemical. Consequently, stage-dependent susceptibility studies provide information that is not available by means of continuous treatment exposures and reveal which are the most susceptible stages for each environmental agent with teratogenic or lethal effect on early life stages.

Stage-dependent susceptibility has been reported for several environmental agents such as lead (Pérez-Coll and Herkovits, 1990), cadmium (Herkovits et al., 1997) atrazine (Brodeur et al., 2009) and UVB (Castañaga et al., 2009). As a general pattern, the results confirm that organogenic stages are very susceptible to environmental stressors. In addition, in the case of 2,4-D, embryos at blastula stage are also very susceptible (although not significantly different from S.18 and S.23-24). For the CF, the stage-dependent susceptibility is more pronounced: the most susceptible stage (open mouth) is about 3.6 times less resistant than the least sensitive stage (tail bud). The differential susceptibility for different noxious agents and for each developmental stage might be related to specific morphogenetic processes, cellular differentiation processes, and toxicity mechanisms of the environmental agent.

There is an increasing concern due to the large number of malformed amphibians found in many geographic regions (Schmidt, 1997), in many cases from agroecosystems (Ouellet et al., 1997). In this study, both 2,4-D and the CF caused various teratogenic effects. The genotoxic effects previously reported for 2,4-D (Venkov et al., 2000; Ateeq et al., 2002) may be the cause of the malformations reported in this study. Reduced body size and underdeveloped structures may be consequences of the interference of the herbicide with the activity of mitochondrial enzymes involved in the production of ATP (Palmeira et al., 1994), and the inhibition of lipid metabolism and protein synthesis (Rivarola et al., 1992). Although gross malformations resulted in most cases in lethality, some affected embryos survived until the end of the embryonic development. It is noteworthy that for most abnormalities the percentage of adverse effects in embryos treated with 2,4-D and CF were almost similar, except for edema, which was much higher in 2,4-D-treated embryos, and abnormal cellular proliferation processes, which were present only in CF-treated embryos. These results show the eventual interaction mechanisms between the ingredients in the CF with the active ingredient producing some different sublethal effects. The teratogenic potential of 2,4-D and the CF may be estimated as the ratio between the NOEC value for lethality and teratogenesis, which result in (values of) around 4 and 8, respectively. These values might be considered very high, since an agent with a teratogenic index higher than 1.5 (ASTM, 1993) implies a high risk for embryos to be

malformed in the absence of significant embryonic lethality. The large difference between the lethal and teratogenic concentrations contributes to consider malformations as a relevant end point for population viability by reducing fitness of individuals. In conclusion, the remarkable increase in the toxicity of the commercial formulation of 2,4-D is a fact that may be applied to other CFs. Therefore, for environmental and human health protection purposes, the toxicity of the commercial products seems to be relevant for risk assessments. Besides, further research is needed in order to attain a better understanding of the toxicity mechanisms of the compound and the possible implications of the malformations caused by agrochemicals on amphibian populations.

The authors wish to acknowledge Dow AgroSciences Argentina S.A for providing the herbicides.

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