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Environmental Science and Pollution Research

ISSN 0944-1344
Volume 25
Number 24

Environ Sci Pollut Res (2018)
25:23857-23863
DOI 10.1007/s11356-018-2445-9

Springer

Environmental Science and Pollution Research (2018) 25:23857–23863
https://doi.org/10.1007/s11356-018-2445-9

RESEARCH ARTICLE

Differential sensitivity of developmental stages of the South American toad to a fungicide based on fludioxonil and metalaxyl-M

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Received: 30 October 2017 / Accepted: 29 May 2018 / Published online: 7 June 2018

Abstract

Agricultural fungicide application in Argentina has increased twice since 2008, with Maxim® XL (2.5% fludioxonil +1% metalaxyl-M) as one of the most used fungicide formulation. The toxicity of this pesticide on *Rhinella arenarum* was assessed by means of continuous (from embryo and larval development) and 24-h pulse exposure standardized bioassays. Lethality was concentration- and exposure time-dependent. Maxim® XL caused a progressive lethal effect along the bioassays with higher toxicity on embryos than larvae, obtaining 50% lethal concentrations at 96, 336, and 504 h of 10.85, 2.89, and 1.71 mg/L for embryos, and 43.94, 11.79, and 5.76 mg/L for larvae respectively. Lethal 504-h no observed effect concentration values for embryos and larvae were 1 and 2.5 mg/L respectively. A stage-dependent toxicity of Maxim® XL was also demonstrated within the embryo development, with early stages more sensitive than the later ones, and blastula as the most sensitive developmental stage. The risk quotients obtained for chronic risk assessment determined a potential threat for the survival and continuity of *R. arenarum* populations under these conditions. The results indicate that the levels of the fungicide reaching amphibian habitats could be risky for the early development of this amphibian species. This study also emphasizes the necessity to evaluate the chronic effects of fungicides in pesticide risk assessment.

Keywords Fungicide · Metalaxyl-M · Fludioxonil · Lethal effects · Amphibian · Standardized bioassays

Introduction

In the last few decades, pesticides have been used on an increasing scale throughout the world. Particularly, in Argentina, the use of pesticides raised more than 800% in the last two decades, and the crop areas increased nearly 50% (CASAFE 2017). Moreover, the application of fungicides has dramatically raised and they become the most important pesticides used in modern agriculture management programs (Reilly et al. 2012; Knäbel et al. 2014). Fungicide applications are frequent during the crop cycle (Knäbel et al. 2014), so chronic exposure scenarios become more probable, and environmental levels often exceed chronic toxicity threshold values (Reilly et al. 2012).

Maxim® XL, a fungicide formulation based on the active ingredients fludioxonil (2.5%) and metalaxyl-M (1%), is used to protect seed germination and the initial growing stages of crop plants (Syngenta 2017). Fludioxonil inhibits the transport-associated phosphorylation of glucose, whereas metalaxyl interferes with the development of the mycelium and the spores. This fungicide formulation is extensively used in Argentina with annual applications of approximately 450,000 kg (SENASA 2017). Metalaxyl levels were reported at high occurrence in water bodies at maximum concentrations of 8.015 μg/L while degradation products of metalaxyl were also found (Herrero-Hernández et al. 2013). Fludioxonil is considered persistent in aquatic environment according to the residues found 5 years after its application on grape crops (NRAAVC 2000). Despite the alarming ecotoxicological information provided by the company itself, that this commercial formulation is toxic to aquatic organisms (Syngenta 2017), there are scarce data about its toxicity, nor the toxicity of its active ingredients on nontarget organisms. However,

Responsible editor: Philippe Garrigues

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USEPA (2011) reported that fludioxonil is highly toxic to aquatic organisms for both acute and chronic exposures. Thus, the 96-h 50% lethal concentration (LC 50) for fishes ranged from 0.23 to 1.5 mg/L (USEPA 2011). Among amphibians, Li et al. (2016) reported a 48-h LC50 of 1.57 mg fludioxonil/L and teratogenic effects for *Xenopus tropicalis* embryos. *Rana pipiens* larvae exposed to metalaxyl showed lethal and sublethal effects, with a very low no observed effect concentration (NOEC) value of 0.16 μg/L (Hutchinson and Czyrska 1975). Hayes et al. (2006) reported 35% mortality of leopard frogs (*Rana pipiens*) metamorphic larvae exposed to 0.1 μg/L metalaxyl. In mammals, metalaxyl induced bradycardia (Naidu and Radhakrishnamurty 1988) and affected the activity of monoamine oxidase in the heart (Naidu 1989). Moreover, metalaxyl could also act as a cotoxin or cocarcinogen (Paolini et al. 1996) and may induce chromosomal alterations (Hrelia et al. 1996). It was also reported that fludioxonil could induce breast cancer (Go et al. 2017).

Considering the growing worldwide use of Maxim® XL particularly in Argentinean crops, the high environmental persistence, and the high frequency of application, there is a clear need to assess the toxicity of this fungicide in the agroecosystem biota as amphibians. Specifically, the embryo–larval period of amphibians could occur in water reservoirs adjacent to agricultural fields where pesticides are applied (Hayes et al. 2006). Furthermore, amphibians are extremely sensitive because of their permeable skin, their aquatic and terrestrial life stages, and a rudimentary immune system (Wake and Vredenburg 2008). *Rhinella arenarum*, an amphibian species with an extensive dispersion in Argentina, is placed in the "least concern" topic according to the International Union for Conservation of Nature (IUCN 2017) so it is a species that it is allowed to use in laboratory. Moreover, previous researches on this species have regarded it as a valid and effective in vivo model to study toxic effects induced by different xenobiotics (Brodeur et al. 2009; Svartz et al. 2015; Perez Coll et al. 2017).

Laboratory tests are very valuable tools to evaluate the ecotoxicological hazard potential of sensitive organisms exposed to different xenobiotics as pesticides. In this regard, AMPHITOX is a set of standardized tests, among them, two of continuous exposures one from the start of embryo development and the other from early larval development with *Rhinella arenarum* as test organisms (Herkovits and Perez Coll 2003; Perez Coll et al. 2017). The bioassay employing larvae evaluates the toxic effects of xenobiotics, with an easy and immediate endpoint as lethality (Herkovits and Perez Coll 2003). The bioassay that uses embryos is an early life stage (ELS) toxicity test that includes a meticulous assessment of developing organisms that needs more expertise. Although the 24-h pulse exposure bioassay uses high concentrations of chemicals, it also provides important information about the toxic effects on biota in cases of environmental emergency. Also, this bioassay design offers additional information on the mechanisms of action of the pollutant and allows identifying the most sensitive stage within the life cycle of a species. All these bioassays allow disposing a more toxicity profile of the xenobiotic of the more sensitive life cycle period and are focused on the conservation purposes of species.

Previously, we emphasized the study of sublethal effects on metamorphosis and gonadogenesis of Maxim® XL on *R. arenarum* (Svartz et al. 2016). So, the objectives of the present study were to find the most sensitive developmental period by evaluating the lethal effects of Maxim® XL on the embryo and larval development of the South American toad *R. arenarum* and the most sensitive embryo stage by means of 24-h pulse exposure bioassays by using AMPHITOX. In addition, acute and chronic ecotoxicological risk assessments of Maxim® XL supported on the risk quotient approach (previously called hazard quotient) (USEPA 2017) on this amphibian species were also performed. These aims are in line with the need to dispose of conservation strategies for amphibian populations around the world.

Materials and methods

Obtaining *R. arenarum* embryos and larvae

Rhinella arenarum adults were obtained in a presumably non-polluted area of Lobos (Buenos Aires, Argentina). The methodology performed was based on the AMPHITOX protocols (Herkovits and Perez Coll 2003; Perez Coll et al. 2017). Oocytes were obtained by inducing ovulation with a subcutaneous injection of 5000 IU human chorionic gonadotropin (hCG) in the toad female. For embryo bioassays, the jelly coat was dissolved by immersing egg ribbons in a solution of 2% thioglycolic acid at pH 7.2 containing 1.35 mL saturated sodium hydroxide (NaOH) solution in 100 mL AMPHITOX solution (AS). This step was followed by a thorough wash of embryos. The composition of AS was NaCl 36 mg/L, KCl 0.5 mg/L, $CaCl_2$ 1 mg/L, and $NaHCO_3$ 2 mg/L prepared in distilled water. Embryos were staged according to Del Conte and Sirlin (1951) as follow: early blastula (S.4), neural plate (S.13), muscular activity (S.18), gill circulation (S.20), opercular folds (S.23), and complete operculum (S.25) stages. Embryos were kept in AS and maintained at 20 ± 2 °C. The AS was replaced entirely every 3 days and monitored weekly to ensure that the pH was at acceptable levels (7 ± 0.5).

Preparation of test solutions

Test solutions were prepared by diluting the commercial formulation Maxim® XL in AS to obtain the test solutions. MAXIM® XL is a broad-spectrum compound for the control of pathogens. This fungicide is commercialized in Argentina

by Syngenta and it is the result of the association of two active ingredients: 2.5 g/100 mL (2.5% *w*/*v*) of fludioxonil (CAS No. 131341-86-1) and 1 g/100 mL (1% *w*/*v*) of metalaxyl-M (CAS No. 70630-17-0).

Toxicity bioassays

For each bioassay, triplicates of ten organisms (embryos/larvae) were placed in covered 10-cm-diameter glass Petri dishes containing 40 mL of test solution. Table 1 specifies the conditions of toxicity bioassays.

Organisms were maintained at 20 ± 2 °C and a 12:12-h light:dark photoperiod. Larvae were fed with three granules (6 ± 0.5 mg) of balanced fish food TetraColor® per Petri-dish. For continuous treatments, test solutions were entirely replaced every 48 h. In the case of pulse treatments, after 24 h, embryos were thoroughly washed and kept in AS until 504 h post-exposure, replacing the medium every other day. Control groups were simultaneously maintained in AS without additions and were renewed every other day.

Survival was evaluated each 24 h by means of smooth movements of the Petri dishes, followed by stimulation with a light source. In case of no response, soft mechanic stimulation with a glass rod was made and finally heartbeat was checked.

Data analysis

Lethality data were statistically estimated by the USEPA Probit Program (USEPA 1988). Lethal concentrations 10, 50, and 90% (LC10, LC50, and LC90) at different times were represented by toxicity profile (TOP) curves. To establish statistical differences between LC values, a comparison was made considering the substantially difference when the higher/lower ratio exceeded the critical value (95% confidence interval) established by the American Public Health Association (2005). The effect of concentration and exposure time on survival was analyzed by generalized linear mixed models (GLMMs) assuming a binomial distribution of the error. To compare treatment means at a significance level of $p < 0.05$, Di Rienzo, Guzmán, and Casanoves (DGC) test (Di Rienzo et al. 2002) was performed with the aid of InfoStat software (Di Rienzo et al. 2015). Lethal NOEC (no observed effect concentration) value was the highest concentration without statistically significant differences compared with the control group.

Ecological risk evaluation

An ecological risk assessment of Maxim® XL for this native species was performed using the risk quotient approach (USEPA 2017). The risk quotient is calculated as the ratio of the expected environmental concentration (EEC) (Boutin et al. 1993; Boutin et al. 1995) and the level at which no adverse effects are expected (10% lethal concentration, LC10). The EEC for Maxim® XL was based on 10% of the maximum application rate given on manufacturers' labels. The maximum application concentration allowed for this commercial formulation is 175 g/L/ha. The EEC was calculated assuming a water depth of 15 cm and an area of 1 m^2 (Boutin et al. 1993, 1995). The risk quotient (RQ) was calculated as EEC/LC10 and compared with the USEPA level of concern (LOC) (USEPA 2017). After the risk quotient(s) is calculated, it is compared to the agency's level of concern (LOC). A LOC is a policy tool that the agency uses to interpret the risk quotient and to analyze potential risk to nontarget organisms and the need to consider regulatory action. The LOC value for risk is 1. If the risk quotient is greater than 1, harmful effects likely are because of the contaminant in question.

Results

Continuous exposure of embryos from early blastula stage (S.4) and larvae from complete operculum stage (S.25) onwards for 504 h

Mortality rates of embryos and larvae gradually increased with concentration and exposure time (Fig. 1). During the first hours, embryos exposed to Maxim® XL concentrations

Table 1 Conditions of the bioassays

Developmental stage	Treatment	Exposure concentrations (mg Maxim® XL/L)
Blastula (S.4)	Continuous exposure	0.25, 0.5, 3, 5, 10, 20
Complete operculum (S.25)		1, 5, 10, 30, 40, 50
Blastula (S.4) Gastrula (S.11) Neurula (S.13) Muscular activity (S.18) Gill circulation (S.20) Opercular folds (S.23) Complete operculum (S.25)	24-h pulse exposure	1, 5, 10, 15, 20, 30, 40, 50, 60

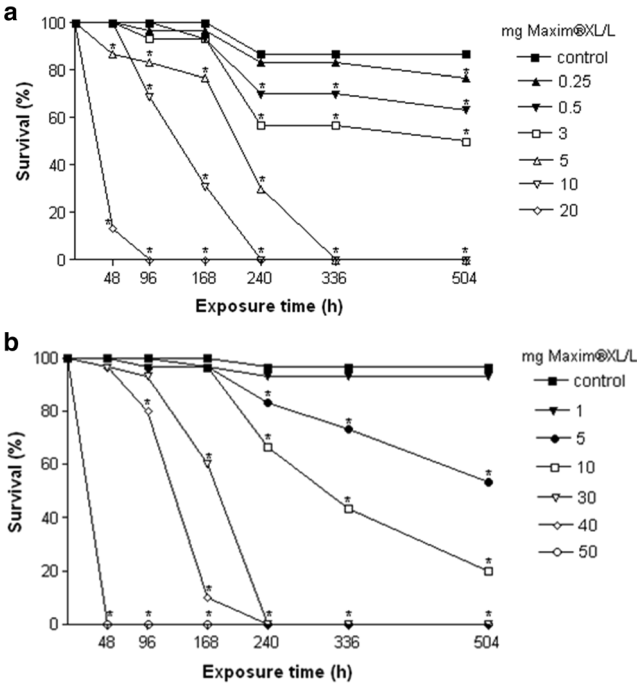

Fig. 1 Survival curves (%) of *R. arenarum* embryos (**a**) and larvae (**b**) continuously exposed to Maxim® XL. *Significantly different from controls ($p < 0.05$)

greater than 5 mg/L stopped their development during segmentation or initial gastrula (S.11) stages exhibiting cellular dissociation, irregular surface, and persistent yolk plugs (Fig. 2). As development advanced, reduced body size, underdeveloped tail and gills, microcephaly, axial curvatures, and wavy tails were also observed (Fig. 2). The toxicity of Maxim® XL was significantly higher in embryos than larvae at all exposure times. Mortality rates of embryos and larvae exposed to concentrations from 5 and 40 mg/L respectively were significantly higher than those in the controls during acute exposure (96 h). At chronic exposure (504 h), lethal NOEC values were lower than 0.25 mg/L (the lower concentration tested) and 1 mg/L for embryos and larvae respectively. Thereby, Maxim® XL toxicity in *R. arenarum* embryos and larvae increased considerably from acute to chronic exposure, with 96- and 240 h-LC50 of 9.83 and 3.85 mg/L and 43.94 and 11.79 mg/L for embryos and larvae respectively. However, from that time onwards, toxicity did not significantly increase (Fig. 3).

Twenty-four-hour pulse exposure at different embryonic stages

Figure 4 shows the pattern of decreasing sensitivity of embryos to the fungicide as development progresses. Blastula (S.4) and gastrula (S.8) stages were the most sensitive stages, and the end of embryonic development (S.25) was the most resistant, with 504-h LC50 of 10.08 and 52.69 mg/L for S.4 and S.25 respectively. In present study, no significant worsening of the lethal effect of Maxim® XL was detected by extending the evaluation time to 21 days.

Ecological risk evaluation

A risk evaluation analysis for embryos and larvae continuously exposed to Maxim® XL starting at the blastula

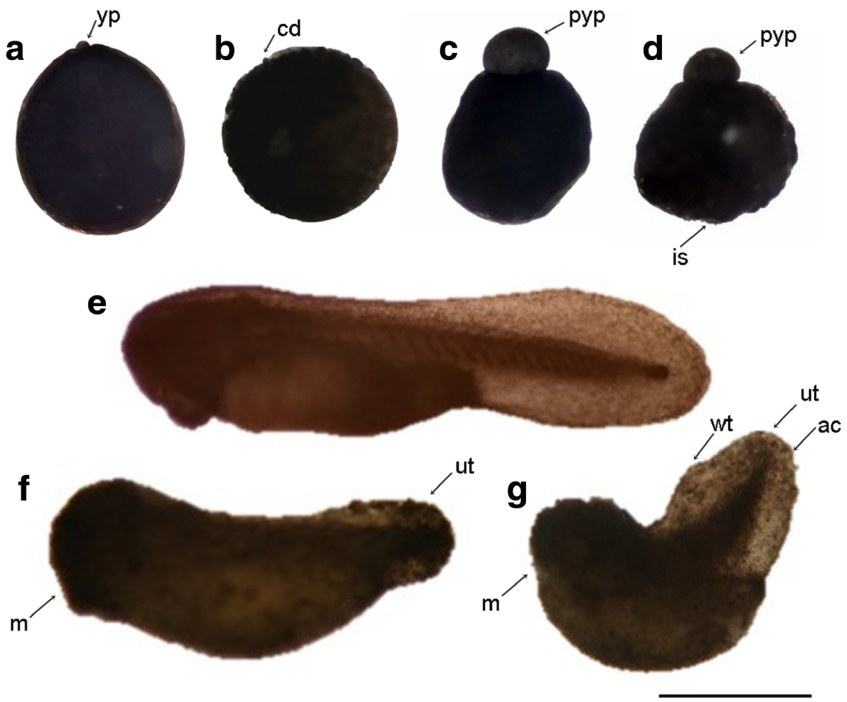

Fig. 2 Stereomicroscope photographs of *R. arenarum* embryos exposed to Maxim® XL from blastula stage (S.4). A–D Embryos observed at 24 h: control (S.12) (A); 10 mg/L Maxim® XL (B); 20 mg/L Maxim® XL (C, D). E–G Embryos observed at 72 h: control (S.20) (E); 5 mg/L Maxim® XL (F); 10 mg/L Maxim® XL (G). Abbreviation: ac, axial curvature; cd, cellular dissociation; is, irregular surface; m, microcephaly; pyp, persistent yolk plug; ut, underdeveloped tail; wt, wavy tail; yp, yolk plug. Scale bar, 2 mm

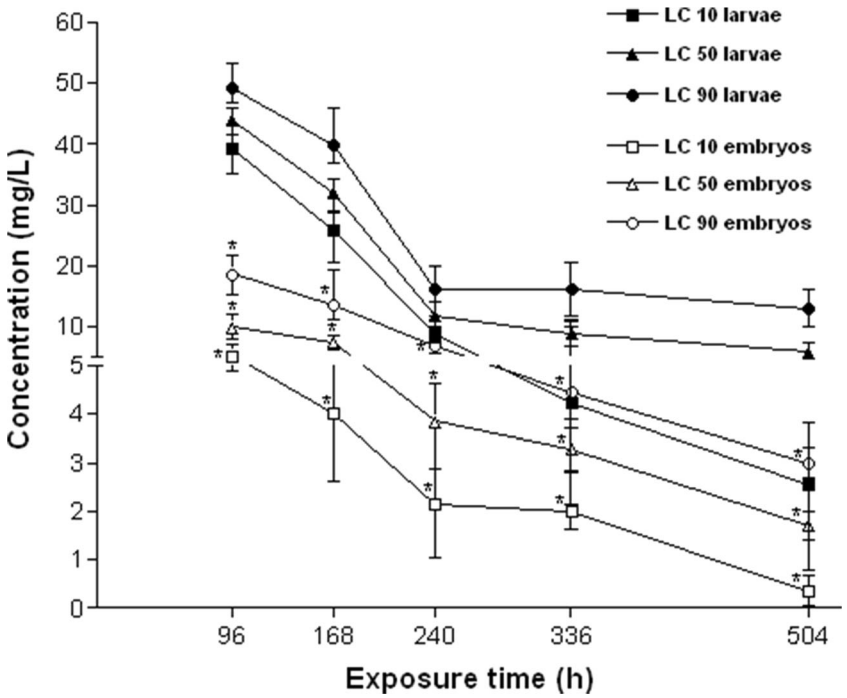

Fig. 3 Maxim® XL Toxicity Profile (TOP) curves of *R. arenarum* embryos and larvae continuously exposed for 504 h. Error bars indicate 95% confidence interval. Note that embryos were significantly more sensitive than larvae during the whole bioassay. *Statistically significantly different from the LCs obtained after the same exposure time from larvae ($p < 0.05$)

and complete operculum stages respectively was performed. The EEC for Maxim® XL was calculated as 10% of the maximum application rate allowed (175 g/L/ha), so the EEC was 1.75 mg/L/m^2. From this value, the risk quotients (RQ = EEC/LC10) for *R. arenarum* at the blastula and complete operculum stages at different exposure times were estimated (Table 2). The risk quotient values for both developmental periods at acute and short-term chronic exposures were below the LOC value, whereas for chronic exposure, the risk quotients were above the LOC value for larvae exposed from early embryo development.

Discussion

The results obtained in the present study highlight the relevant toxicity of the fungicide Maxim® XL on *R. arenarum* embryo–larval development. Moreover, it was shown that the sensitivity to the fungicide was higher in early embryos continuously exposed and then was decreasing, with 504-h LC50 of 1.71 and 5.76 mg/L for embryos and larvae respectively. Also, comparing lethal NOEC values at chronic exposure, embryo sensitivity was fourfold higher than the larval development. Furthermore, 24-h pulse exposure results confirmed this tendency, with fungicide toxicity fivefold higher in earlier

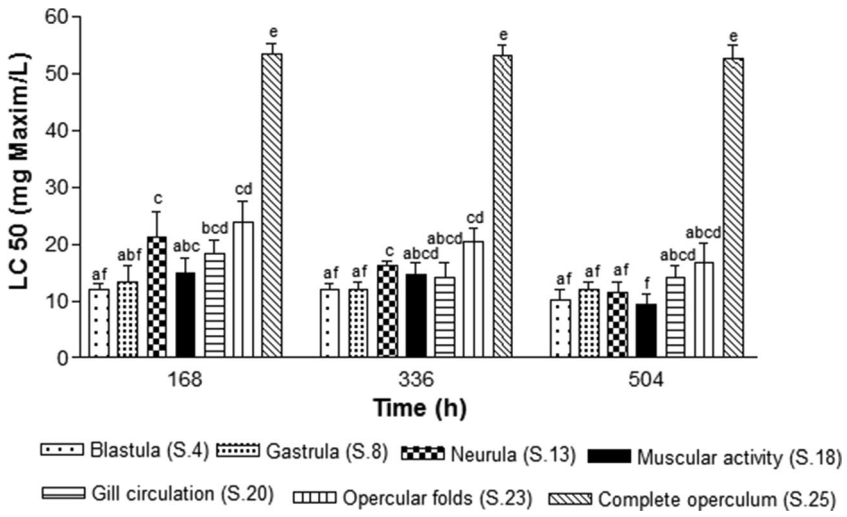

Fig. 4 Maxim® XL stage-dependent sensitivity of *Rhinella arenarum* embryonic stages assessed by 24-h pulse exposure experiments. Different letters indicate statistically significant differences ($p < 0.05$)

Table 2 Toxicity and risk quotient of Maxim® XL for *Rhinella arenarum* at blastula and complete operculum stages for acute (96 h), short-term chronic (168 h), and chronic (336, 504 h) exposures

Stage	Exposure time (h)							
	96		168		336		504	
	LC10	RQ	LC10	RQ	LC10	RQ	LC10	RQ
Blastula (S.4)	5.18	0.34	4	0.44	2	0.875	0.36	4.86*
Complete operculum (S.25)	39.26	0.045	25.77	0.07	4.23	0.41	2.54	0.69

LC10. 10% lethal concentration (mg Maxim® XL/L); *RQ*, risk quotient values; *RQ > 1, estimates harmful effects from Maxim® XL exposure

stages (S.4–S.18) than complete operculum stage (S.25). It was observed that the fungicide did not continue producing a lethal effect in embryos exposed to pulse treatments after 24 h of exposure. So, the obtained toxicity parameters (LC50 values) remained constant up to 504 h. Previously, we have observed that embryos exposed to other xenobiotics such as endosulfan at different stages for 24 h showed effects in the further development in spite of stopping the exposure, as residual effects of the substance (Svartz et al. 2014). Different stage-dependent sensitivity studies assessing other pesticides such as cypermethrin reported higher sensitivity of larvae than embryos (Berrill et al. 1993; Biga and Blaustein 2013; Svartz et al. 2015). The differences in the toxicity patterns could be related to the action mode and the target site of the pesticides; for example, pyrethroid insecticides mainly act on sodium and calcium channels of nervous tissues, so the highest sensitivity of late stages could be related to the nervous system maturation (Svartz et al. 2015). In contrast, fungicides impact on basic cellular processes, inhibiting fungal biosynthesis of sterols and tubulin or cytochrome-c reductase activity (Casida 2009). As many biochemical pathways and processes are conserved across species, action modes of fungicides could predict analogous mechanisms of toxicity, target sites, and/or toxic effects for non-fungal species (Stenersen 2004). So, the higher toxicity observed in earlier stages could be related to the mechanisms of action of the active ingredients of Maxim® XL. Metalaxyl-M interacts with the RNA polymerase I template complex, thus inhibiting the incorporation of ribonucleotides in the nascent RNA (Marucchini and Zadra 2002). On the other hand, fludioxonil interferes with the formation of membranes (Yang et al. 2011). Both cellular functions are essential for segmentation and gastrulation processes and are characterized by intense mitotic and transcription activities necessary for subsequent embryo development. It is noteworthy that the embryo cell surface plays an important role in adhesiveness during morphogenesis and embryonic cell differentiation (Bellairs et al. 1978) that would be blocked by the fungicide leading to strong dissociation followed by arrested development of embryos.

Our earlier studies revealed that Maxim® XL in concentrations ranging from 0.25 to 2 mg/L caused effects on metamorphosis and gonadogenesis in *R. arenarum* after both acute and chronic exposures (Svartz et al. 2016). In that study, we also reported the high teratogenic effect expressed as a 96-h teratogenic index of 2.5 and behavioral alterations. Those sublethal effects are now complemented with lethality data obtained from different experimental approaches, thus completing the toxicological information of Maxim® XL during the development of *R. arenarum*.

The risk assessment highlights that RQ index could take values higher than the LOC threshold during the chronic exposure of *R. arenarum* embryos. These results emphasize the importance of chronifying the exposure since otherwise the risk would not have been detected. Maxim® XL represents a threat for *R. arenarum*, especially in regions with intensive use of this fungicide, thus may potentially disrupt the populations of this native amphibian.

Acknowledgements We wish to thank Ferring Pharmaceuticals for providing hCG hormones.

Funding information Cristina Perez Coll and Gabriela Svartz are scientists of CONICET. Mercedes Acquaroni has received a fellowship from the CONICET. This study was partially supported by the Universidad Nacional de San Martín.

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