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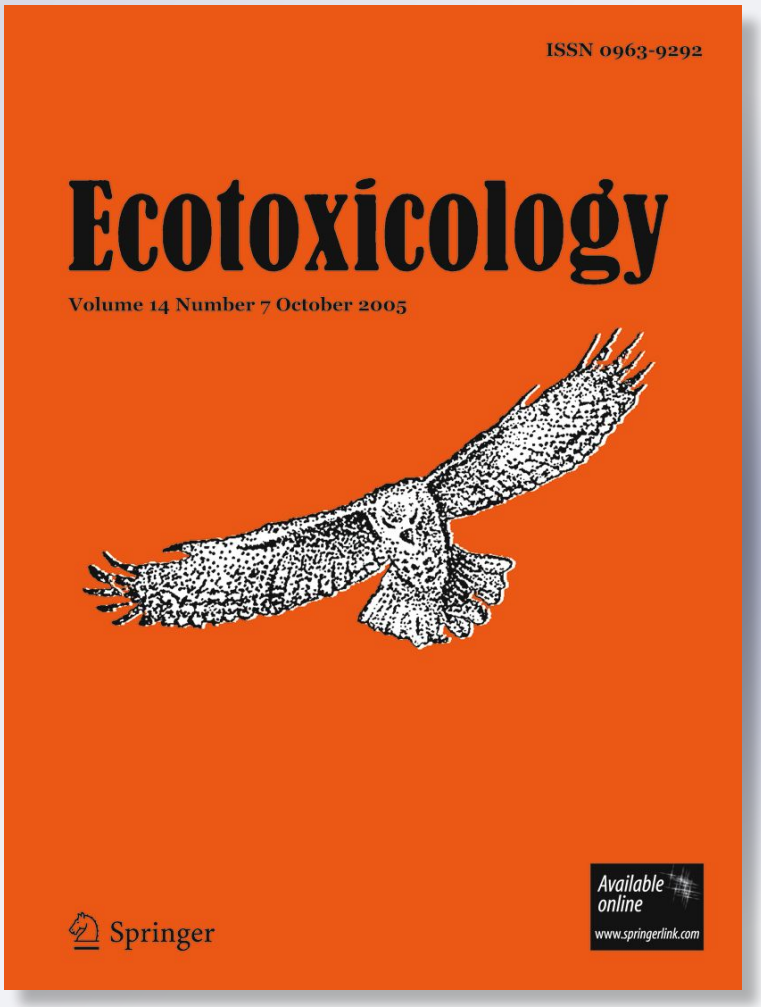

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Sublethal effects of atrazine on embryo-larval development of *Rhinella arenarum* (Anura: Bufonidae)

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Abstract Atrazine (ATR), one of the most widely used herbicides in the world, affects not only target organisms but also the biota in general. Here, the teratogenic and neurotoxic effects of ATR on *Rhinella arenarum* (South American toad) embryos, and larvae were evaluated by means of standardized bioassays during acute and chronic exposures. The herbicide had a significant incidence of malformations, with a Teratogenic Index (TI) of 3.28. The main effects were delayed development, reduced body size, microcephaly, axial flexures, wavy tail and edema. In addition, delayed development, reduced development of forelimbs, and edema were recorded at metamorphosis stages. Scanning electron microscopy allowed observing different degrees of cellular dissociation and persistent cilliar cells in specific regions like the adhesive structure and tail fin. Results obtained by ATR 24 h pulse exposures at six developmental stages pointed out blastula as the most susceptible developmental stage both for immediate and delayed adverse effects. A noteworthy recovery capacity from acute toxic effects was recorded from the neural plate stage onwards. Regarding neurotoxic effects, abnormal, and erratic swimming and spasmodic contractions were recorded. Both the teratogenic and neurotoxic effects reported in this study demonstrate the importance of evaluating sublethal effects in non-target organisms as they could imply reduced fitness of individuals and eventually a population decline. The Hazard Quotients (HQ) for ATR ranged from 0.14 to 10.80, and the fact that some of these values are above USEPA's level of concern indicate that ATR is likely a risk to *R. arenarum.*

Keywords Atrazine · *Rhinella arenarum* · Amphibian development · Sublethal effects · Teratogenesis · Neurotoxicity

Introduction

Among the growing impacts of agricultural activities is the generation of huge toxic residuals that affect the quality of soil and water. This can result in significant costs in the short, medium, and long term for both human and environmental health, reducing the biodiversity of native flora and fauna and encouraging the resistance and emergence of new pests and diseases (Herzog and Funderburk 1986). Atrazine (ATR) is a triazine compound (2-chloro-4-ethylamino-6-isopropylamino-s-triazine), used as a selective systemic herbicide to control the appearance of weeds in crops, pastures, and the growth of aquatic weeds in lakes and ponds. ATR is one of the most widely used herbicides in grain-producing countries such as the USA, Brazil, and Argentina, where 7.6 million L/year are used (CASAFE 2011). However, due to its toxicity, it has been banned in some regions, such as the European Union, since 2004 (Sass and Colangelo 2006). Among the most severe effects, ATR was associated with cancer, endocrine disruption, and teratogenesis in non-target vertebrates, including humans (Cooper et al. 2000; Pintér et al. 1990; Simic et al. 1994; Wetzel et al. 1994). Moreover, increased chromosomal aberrations were noted in lymphocyte cultures of farm workers exposed to ATR (Yoder et al. 1973).

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An alarming amphibian population decline has been reported worldwide since 1969 (Alford and Richards 1999; Blaustein and Wake 1990; Houlahan et al. 2000; Kiesecker et al. 2001). There are several hypotheses on this phenomenon, including toxicity produced by chemical contaminants such as pesticides (Beebee and Griffiths 2005; Mann et al. 2009; Relyea 2009). These factors can interact synergistically to exacerbate the decline in population numbers. Amphibians can be exposed to pesticides in agroecosystems by many routes, but perhaps the most likely one is agricultural runoff in amphibian breeding sites (Hayes et al. 2003). Levels up to 12.7 mg/L ATR have been detected in natural waters (Bushway et al. 1991; Davies et al. 1994). Studies on the sublethal effects of ATR have reported the following: malformations: tail flexure, wavy tail, facial edema, and axial shortening with a no-observed-adverse-effect level (NOAEL) in *Bufo americanus* (American toad) embryos for deformity of 2.59 mg/L ATR (Allran and Karasov 2001); increase in intersex gonadal tissues in both *Rana pipiens* (northern leopard frog) and *Xenopus laevis* (African clawed frog) chronically exposed to 0.1 μg/L ATR (Carr et al. 2003; Hayes et al. 2002); and neurotoxic effects such as abnormal swimming and abnormal avoidance response in tadpoles of *Ptychadena bibroni* (broad-banded grass frog) chronically exposed to 0.2–0.6 μg/L (Ezemonye and Tongo 2009). The previous references correspond to studies with continuous exposure, but pulse-exposure experiments, by using high levels of contaminants, might bring valuable information about toxic effects on wildlife in cases of environmental emergencies. Also, these experimental designs might provide additional information to understand the mechanisms of action of a contaminant on a particular stage of the life cycle of a species.

Ecological risk can be estimated numerically using the Hazard Quotient (HQ) approach (USEPA 1998), based on the comparison of the expected environmental concentration (EEC) (Boutin et al. 1993, 1995) with standard toxicity end points (e.g., EC 10 values). Toxicity bioassays, e.g., FETAX (Morgan et al. 1996) and AMPHITOX (Herkovits and Pérez-Coll 2003) tests, represent useful tools to assess the risk of exposure of ecosystems to different physicochemical agents. Sublethal exposure of amphibians to pesticides may be valuable in the assessment of the sensitivity to contaminants that could produce detrimental effects, e.g., increased vulnerability to predation and a reduction in fitness, which could eventually affect amphibian populations (Little et al. 1990).

Despite the widespread use of ATR in the Argentine corn/soy belt, very few ecotoxicological studies have been conducted on native species. In a previous study from our laboratory, we found lethal effects of ATR on *Rhinella arenarum* embryos and larvae (Brodeur et al. 2009). In the present study, we attempted to test the hypothesis that sublethal effects of ATR on *Rhinella arenarum* are concentration- and developmental stage-dependent. To meet this objective, we evaluated the sublethal effects of ATR, mainly teratogenesis and behavioral disorders, in embryonic and larval stages by means of standardized bioassays using the common South American toad, *Rhinella arenarum*, which occurs in southern Brazil, Argentina (up to Chubut Province), Uruguay and Bolivia. Based on the HQ approach, an ecological risk assessment of ATR on this native species was performed.

Materials and methods

Acquisition of *Rhinella arenarum* embryos

To examine the potential for ATR to adversely affect the embryo-larval development of the common South American toad, *Rhinella arenarum,* three mating pairs of adults weighing approximately 200–250 g were acquired in Lobos (Buenos Aires province, Argentina: 35°11′S; 59°05′W). Frog care, breeding, embryo acquisition, and analysis were conducted according to the methods described in the AMPHITOX protocols (Herkovits et al. 2002; Herkovits and Pérez-Coll 2003). Briefly, ovulation of 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 preserved according to Pisanó (1956). Oocytes were fertilized in vitro using fresh sperm suspended in AS. The composition of AS was sodium chloride (NaCl) 36 mg/L, potassium chloride (KCl) 0.5 mg/L, calcium chloride ($CaCl_2$) 1 mg/L and sodium bicarbonate ($NaHCO_3$) 2 mg/L, prepared in distilled water. The jelly coat at early blastula stage was dissolved by immersing egg ribbons into a solution of 2 % thioglycolic acid at pH 7.2 with 1.35 mL of saturated sodium hydroxide (NaOH) solution every 100 mL in AS followed by a thorough wash of the embryos. Embryos were kept in AS and maintained at 20 ± 2 °C. The AS was replaced entirely every three days and monitored weekly to ensure that the pH was at acceptable levels (7 ± 0.5). Embryos were staged according to Del Conte and Sirlin (1951), and larvae were staged according to Echeverría and Fiorito de López (1981).

Toxicity bioassays

For treatments during embryonic stages (S4-S25), ten embryos were randomly placed in triplicate in 10 cm-diameter glass Petri dishes containing 40 mL of test solution. For treatments during prometamorphosis (S28), ten larvae were placed in triplicate in 20 cm-diameter glass

Petri dishes containing 100 mL of test solution. Test solutions were entirely replaced every 48 h. The toxicity bioassays were performed under the following conditions:

1) Continuous exposure from early blastula (S4) up to complete operculum stage (S25); 336 h (14 days). The ATR concentrations tested ranged between 5 and 30 mg/L.
2) Continuous exposure from early complete operculum stage (S25) up to late complete operculum stage (S25); 336 h (14 days). ATR concentrations tested ranged between 1 and 30 mg/L.
3) Continuous exposure from completed back legs (S28) until completion of metamorphosis; 1080 h (45 days). ATR concentrations tested ranged between 1 and 30 mg/L.
4) 24 h pulse-exposure with ATR in concentrations ranging between 1 and 40 mg/L in blastula (S4), neural plate (S13), muscular activity (S18), gill circulation (S20), opercular folds (S23), and complete operculum (S25). After the treatments, the embryos were thoroughly washed and kept in AS until 168 h post-exposure.

Preparation of test solutions

Toxicity tests were performed using technical-grade ATR (CAS No. 1912-24-9) with a purity of 98 %, purchased from Chem Service (West Chester, PA, USA). Preliminary tests allowed us to find the sublethal concentrations of ATR for *Rhinella arenarum* embryos and larvae. Test solutions between 10 and 30 mg/L ATR were prepared by directly weighing and dissolving the corresponding mass of ATR into one liter of AS. Test solutions under 10 mg/L ATR were prepared by dissolving a solution of 15 mg/L ATR with AS. Because some degree of precipitation was observed at the highest concentrations of ATR tested, 0.2 % acetone was added to the test solutions to insure homogenous dissolution. Controls both with AS and AS plus 0.2 % acetone were simultaneously maintained. The ATR concentrations in test solutions were verified by high-performance liquid chromatography/mass spectrometry (Brodeur et al. 2009). The error between nominal and measured concentrations did not exceed 5 %.

Data analysis

The teratogenic and neurotoxic effects of ATR were evaluated every 24 h. Abnormalities were observed under a binocular stereoscopic microscope (Zeiss Stemi DV4), photographed with a Sony DSC-S90 digital camera, and identified according to the "Atlas of Abnormalities" (Bantle et al. 1998). Embryos with typical adverse effects plus controls were fixed in formol 4 %, dehydrated in a gradient of ethanol, prepared for scanning electron microscopy by means of the critical point technique and observed in a Philips XL-30 operated at 10 Kw for ultra-structure evaluation.

Lethal and effective concentrations (LC and EC) were statistically estimated by the USEPA Probit Program (USEPA 1988). The results were considered statistically significant ($p < 0.05$) when the higher EC/lower EC ratio exceeded the critical value (95 % confidence interval) established by APHA (APHA 1980). The Teratogenic Index (TI) was calculated as LC 50/EC 50.

EEC for ATR was calculated as a percentage of the maximum application rate proposed, 2.7 kg/ha active ingredient, (Syngenta Crop Protection Inc. 2011). This percentage depends on exposure via spray drift, runoff, and washoff (10 %) or overspray exposure during aerial application (100 %). The EEC was calculated assuming a water depth of 15 cm and an area of 1 m^2 (Boutin et al. 1993, 1995). HQ is the ratio of the potential exposure to the substance and the level at which no adverse effects are expected (USEPA 1998), in this study was calculated as EEC/EC 10. The EC 10 was used instead of the no observable effect concentration (NOEC) to provide a more meaningful, yet conservative, estimate of the effect (Van der Hoeven 1997; Van der Hoeven et al. 1997). In the present work, we estimated two HQ approaches: HQ 1, based on 10 % of the maximum application rate proposed, and HQ 2, based on 100 % of that value.

After the risk quotient was calculated, it was compared to the USEPA level of concern (LOC). The LOC is a policy tool that the Agency uses to interpret 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 the HQ > 1, harmful effects are likely due to the contaminant in question.

Results

Because the two controls (AS and acetone solvent) did not differ statistically from one another, the term "control" in the rest of the manuscript stands for the means of both controls.

Continuous exposure from blastula stage

ATR caused highly relevant sublethal effects over 5 mg/L (NOEC value). Malformed embryos were observed from 48 h onwards and the percentage of affected embryos was proportional to ATR concentration with a maximal of 67 % with 30 mg/L ATR (Fig. 1). The main malformations were: delayed development, reduced body size, tail and

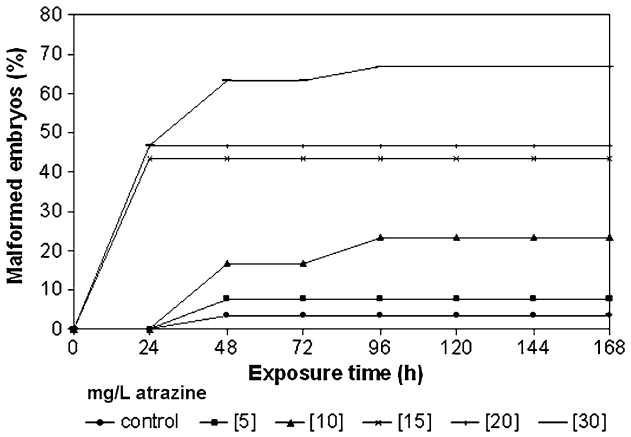

Fig. 1 Percentages of malformed *Rhinella arenarum* embryos continuously exposed to ATR from blastula

axial flexures, irregular border, different types of edema, wavy tail, microcephaly, malformed mouth and adhesive structures, gut miscoiling, underdeveloped gills, cloacal protrusion, and even cellular dissociation (Fig. 2). Treated embryos also exhibited neurotoxic effects such as abnormal and erratic swimming, spasmodic contractions and general weakness. A reduction in food intake was evident although not quantified. The EC50s for sublethal effects was about 8 mg/L and remained constant from 168 to 336 h of exposure. TI for ATR at 240 h was 3.28. Lethality was recorded only at concentrations higher than 20 mg/L ATR after 168 h of exposure, which is the time by which the embryos reached the completed operculum stage (S25). The HQ values remained constant along time from acute to chronic exposure, 0.54 (HQ 1) and 5.40 (HQ 2) (Table 1).

Continuous exposure from complete operculum stage

The NOEC value for sublethal effects during short-term chronic (168 h) exposure was 10 mg/L. The percentage of affected embryos was proportional to ATR concentration, with a maximal of 50 % with 30 mg/L. From 96 h onwards, malformations, mainly tail, axial flexures, different types of edema, malformed mouth and adhesive structures, and cellular dissociation in specific regions such as the stunted tail, were observed (Fig. 2). In acute exposure, treated larvae showed neurotoxic effects such as spasmodic contractions and general weakness at concentrations equal or higher than 15 mg/L ATR. A reduction in food intake was also observed. Lethality was recorded at concentrations higher than 20 mg/L ATR starting from 72 h of exposure onwards. Lethal and sublethal effects increased significantly over time ($p < 0.05$). HQ 1 and HQ 2 for acute exposure were about 0.18 and 1.8 respectively, whereas at 336 h their values were 1.08 and 10.8 respectively (Table 1).

Continuous exposure from prometamorphosis until completion of metamorphosis

Sublethal effects due to ATR exposure began at 168 h of exposure at concentrations higher than 15 mg/L (NOEC value). The most prominent morphological effects were malformations of the digestive tract, edema, wavy tail and developmental delay as reduced development of forelimbs (Fig. 2). The percentage of affected larvae was proportional to ATR concentration, with a maximal of 40 % with 20 mg/L. With regard to neurotoxicity, general weakness, lack of spontaneous mobility, loss of balance, and reduced food intake were observed. All these changes were concentration-dependent between 10 and 20 mg/L ATR. Lethal and sublethal effects increased significantly over time ($p < 0.05$). It is noteworthy that lethality was recorded with 20 mg/L ATR after 10 days of exposure and increased up to 100 % after 27 days of exposure. The HQ values increased over time. HQ 1 and HQ 2 for acute exposure were about 0.14 and 1.35 respectively, whereas at 336 h they reached values of 0.27 and 2.70 respectively (Table 1).

24 h Pulse-exposure at different developmental stages

Embryos treated with 40 mg/L ATR for 24 h did not result in lethality until 168 h post-exposure in the six developmental stages evaluated. Table 2 summarizes the LOEC values and the most conspicuous teratogenic and neurotoxic effects caused by ATR in embryos exposed at different developmental stages. It is noteworthy that the exposure to ATR during the early developmental stage (blastula) resulted in a wide range of teratogenic effects, both immediate (e.g., disruption of the gastrulation resulting in persistent yolk plug) and delayed to more advanced developmental stages (e.g., tail and axis flexures). Regarding neurotoxicity, all developmental stages evaluated showed abnormal and erratic swimming, spasmodic contractions, and general weakness (Table 2).

The NOEC values for the six embryonic stages evaluated at 24 and 168 h are plotted in Fig. 3. As an outstanding result, the NOEC values were at least two-fold lower at 24 than at 168 h post-exposure. This fact points out the recovery capacity from malformations as well as from neurotoxic effects caused by the lower exposure conditions. Based on the LOEC values, the gradient of susceptibility to ATR was: blastula > neural plate = complete operculum > opercular folds = gill circulation > muscular activity stage.

Discussion

Although most ecotoxicity studies focus on lethal effects, the results obtained by exposure to sublethal concentrations

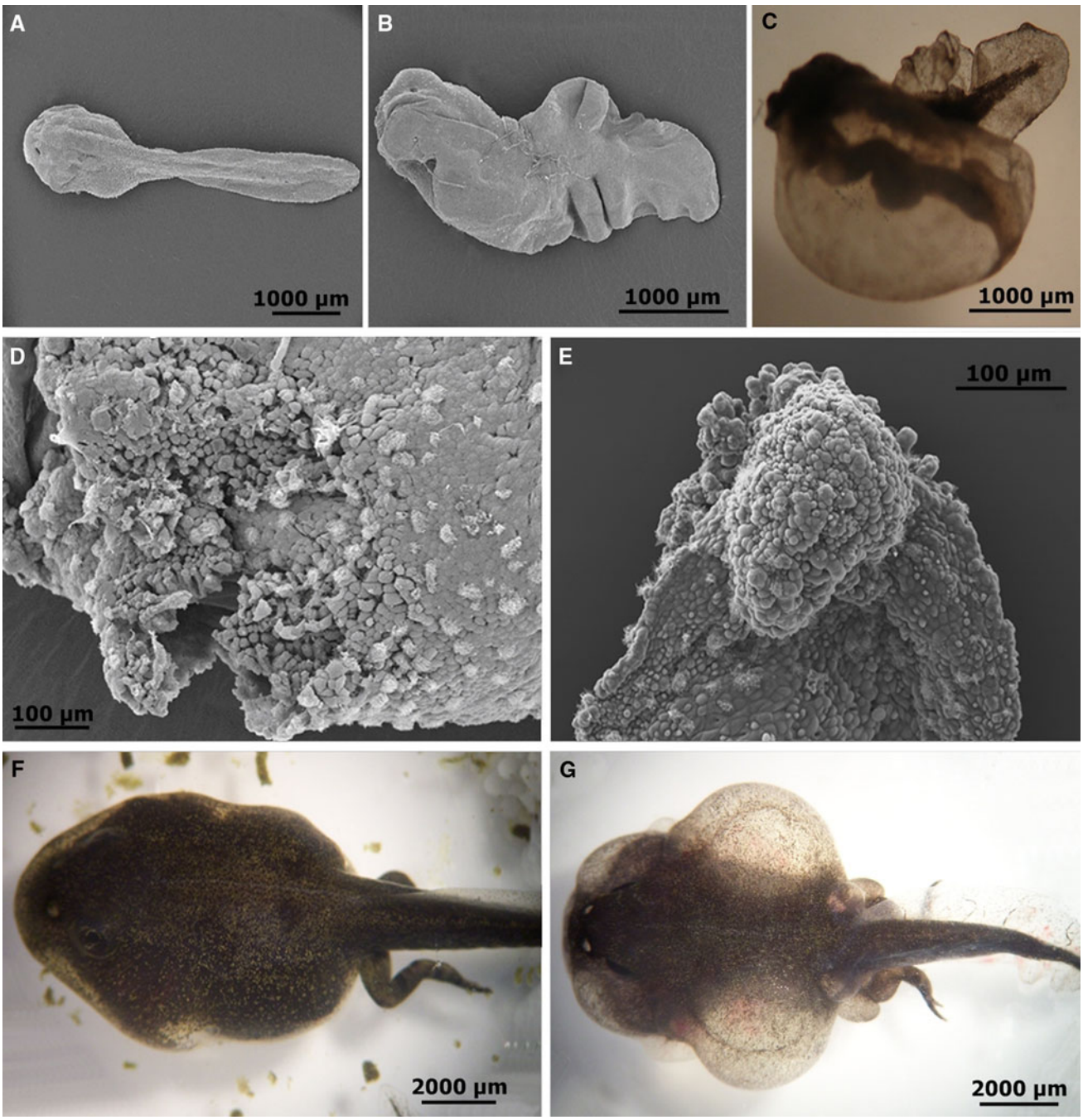

Fig. 2 Scanning electron and stereoscopic microscopy pictures of *Rhinella arenarum.* **a** Control larvae at S25; **b** and **c** Larvae continuously treated with 20 mg/L ATR from blastula (S4) onwards. The main anomalies were irregular border, flexures, pronounced edema, gut miscoiling, microcephaly, wavy and stunted tail. **d** and **e** Larvae continuously exposed to 15 mg/L ATR from S25. Details of malformed mouth and sucker; the stunted tail exhibits cellular dissociation. **f** Control prometamorphosis larvae (S28). **g** Larvae continuously exposed to 10 mg/L ATR from S28 onwards exhibit pronounced edema, tail flexures and reduced development of forelimbs

are also relevant from an ecological point of view. In the case of ATR, previous reports on sublethal effects on amphibians have focused on endocrine disruption during metamorphosis and gonadal development (Carr et al. 2003; Hayes et al. 2003). In this study, we addressed the sublethal toxicity of the herbicide ATR on the embryonic and larval development of a native amphibian from South America, *Rhinella arenarum*, and carried out a risk assessment for worst case scenarios. This contribution on teratogenic and neurotoxic effects of ATR on embryonic and larval stages complements previous studies conducted in our laboratory with *Rhinella arenarum* focusing on lethality at different

Table 1 Toxicity and hazard quotient (HQ) of ATR for *Rhinella arenarum* at embryonic and larval development

Stage	96 h			168 h			336 h	
	EC 10	HQ 1	HQ 2	EC 10	HQ 1	HQ 2	EC 10	HQ 1	HQ 2
S4	5	0.54	5.40*	5	0.54	5.40*	5	0.54	5.40*
S25	15	0.18	1.80*	10	0.27	2.70*	2.5	1.08*	10.80*
S28	20	0.14	1.35*	15	0.18	1.80*	10	0.27	2.70*

EC10 (mg/L) and HQ (EEC/EC10) values for acute (96 h) and chronic exposures (168, 336 h). HQ 1 is based on exposure via spray drift, runoff, and washoff (EEC = 2.7 mg/L) and HQ 2 is based on overspray exposure during aerial application (EEC = 27 mg/L). * HQ > 1 estimates harmful effects due to ATR exposure

ontogenic stages (Brodeur et al. 2009). The teratogenic effect with a NOEC of 5 mg/L, a EC50s of about 9 mg/L and the TI of 3.28 obtained in this study for *Rhinella arenarum* are in the range of adverse effects of ATR on *Xenopus laevis* embryos (Morgan et al. 1996) as well as on *Rana pipiens*, *Rana sylvatica* (Wood Frog) and *Bufo americanus* embryos (Allran and Karasov 2001). These values might be considered relevant, since a TI higher than 1.5 implies a high risk for embryos to be malformed in the absence of significant embryonic lethality (ASTM 1993). The wide range between lethal and effective concentrations of ATR points out that malformations are relevant end points to assess the population viability that might be affected by reduced fitness of individuals.

The delayed development and the reduced body size recorded in *Rhinella arenarum* embryos exposed to ATR are usual adverse effects exerted by environmental stressors in early life stages, e.g., cadmium (Herkovits et al. 1997), fungicides (Pérez-Coll et al. 2009), and 2,4-D (Aronzon et al. 2011). Microarray studies, carried out in *Xenopus laevis* larvae chronically exposed to 400 μg/L ATR, showed altered expression of key genes involved in growth, metabolism and the immune system (Langerveld et al. 2009). This might be related to the delayed development and reduced body size found as sublethal effects of ATR in this study. The reduced food intake, probably associated with a metabolic disruption, also contributed to the growth reduction in ATR-exposed embryos and larvae. Edema, also a noteworthy sublethal effect observed in amphibian embryos and larvae exposed to ATR, could be directly related to a disturbance in the ionic balance (Nieves-Puigdoller et al. 2007; Silvestre et al. 2002) and disruption of the endocrine system (Herkovits et al. 1980). It could also be a secondary consequence of heart malformations and urinary failure due to increased apoptosis in pronephric kidneys, as previously reported in ATR-treated tadpoles (Lenkowski et al. 2008). Abnormal behavior of tadpoles, such as erratic swimming, spasmodic contractions, and general weakness found in this study have also been reported for *Ptychadena bibroni* tadpoles (Ezemonye and Tongo 2009) and positively correlated with the concentration of ATR.

By means of 24 h pulse exposures, the blastula stage was identified as the most susceptible to ATR affecting morphogenetic and cell differentiation processes. This result seems to point out that ATR uptake in blastula continued causing adverse effects as the development advanced, even until the last embryonic stages. The pulse exposure conditions provided the opportunity to record that most of the sublethal effects observed were, at least partially, reversed within 168 h post-treatment. These results could be related to the notorious shape regulation capacity of vertebrate organisms during their early life stages (Herkovits and Faber 1978), and also point out the magnitude of damage caused by ATR in the case of persistent malformations. Although concentrations used in 24 h pulse-exposures may be considered very high and not environmentally relevant, the results might anticipate the effects of the herbicide on amphibian development, as a result of spill situations. Furthermore, these results provide information that may help to elucidate the mechanisms of action of the herbicide on the processes of cell and organogenic differentiation that occur at different stages of embryo-larval development of amphibians.

The HQ approach provides a possibility to assess the risk for adverse effects of ATR at different developmental stages of *Rhinella arenarum*. It is noteworthy that for the embryo and larval stages evaluated, calculation of HQ 2 (based on direct applications) resulted in values higher than 1 even for acute exposure to ATR, which represents an USEPA LOC. The worst scenario was found for the embryonic stages, which showed a HQ 2 of 5.40. In the case of chronic exposure, the early larval development resulted in higher threat with HQ 2 of 10.80. Based on the results of HQ 1 (via spray drift, runoff, and washoff), except a value mildly higher than 1 in the case of 336 h exposure for early larval stages, ATR is not a potential risk for early life stages of this amphibian species.

Our results on teratogenesis and neurotoxicity are consistent with previous studies on ATR exposure in other amphibian species (Allran and Karasov 2001; Morgan et al. 1996; Rohr et al. 2003), confirming the relevance of reporting developmental disruptions that could increase susceptibility to predation (Brodie and Formanowicz 1983;

Table 2 Teratogenic and neurotoxic effects caused by ATR at different developmental stages

Stage	Time (h)	Teratogenic effects	Neurotoxic effects	LOEC (mg/L)
Blastula	24	Persistent yolk plug	N/A	15
		Cellular dissociation		
	96	Delayed development	Spasmodic contractions	20
		Reduced body size		
		Cellular dissociation		
		Microcephaly		
	168	Delayed development	Abnormal and erratic swimming	25
		Reduced body size	Spasmodic contractions	
		Edema	General weakness	
		Microcephaly		
		Axial flexures		
		Wavy tail		
		Malformed mouth and adhesive structures		
		Under developed gills		
		Cloacal protrusion		
Neural plate	24	Cellular dissociation	N/A	20
	96	Delayed development	N/A	25
		Reduced body size		
		Microcephaly		
		Cellular dissociation		
	168	Delayed development	Abnormal and erratic swimming	30
		Reduced body size	General weakness	
		Microcephaly		
		Under developed gills		
Muscular activity	24	Delayed development	General weakness	25
		Reduced body size		
	96	Delayed development	N/A	30
	168	N/A	N/A	>40
Gill circulation	24	Delayed development	Abnormal and erratic swimming	20
		Reduced body size	General weakness	
		Axial flexures		
		Under developed gills		
	96	Delayed development	General weakness	25
		Reduced body size		
	168	Delayed development	General weakness	35
		Reduced body size		
Opercular folds	24	Delayed development	Abnormal and erratic swimming	20
		Reduced body size	General weakness	
		Axial flexures		
		Edema		
		Wavy tail		
	96	Delayed development	General weakness	30
		Reduced body size		
	168	Delayed development	General weakness	35
		Reduced body size		

Table 2 continued

Stage	Time (h)	Teratogenic effects	Neurotoxic effects	LOEC (mg/L)
Complete operculum	24	Delayed development	Abnormal and erratic swimming	20
		Edema	General weakness	
	96	Delayed development	General weakness	25
		Edema		
	168	Delayed development	General weakness	30

N/A not available because there were no sublethal effects

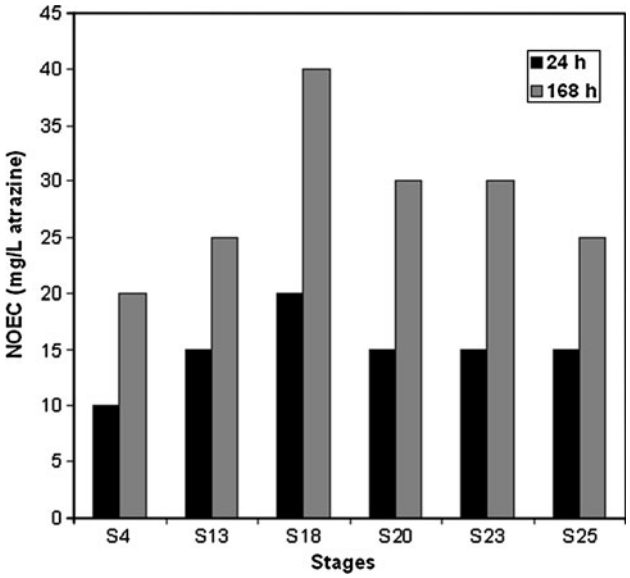

Fig. 3 NOEC values for teratogenic and neurotoxic effects at 24 and 168 h after pulse exposure of *Rhinella arenarum* embryos to ATR at different developmental stages

Cooke 1971) and thus influence later fitness (Semlitsch 1990). Furthermore, based on HQ assessment, ATR represents a threat for *Rhinella arenarum* during its embryo-larval period, especially in regions with intensive use of this herbicide, since it may potentially disrupt the population of this native amphibian.

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References

Syngenta Crop Protection Inc. (2011) Gesaprim 90 WDG® product manual. http://www.syngenta.com.ar/(S(cuciqh55tkot4eby2fwumdqn))/Cultivos/viewimage.aspx?id=746. Accessed 17 November 2011

Alford RA, Richards SJ (1999) Global amphibian declines: a problem in applied ecology. Ann Rev Ecol Syst 30:133–165

Allran JW, Karasov WH (2001) Effects of atrazine on embryos, larvae, and adults of anuran amphibians. Environ Toxicol Chem 20(4):769–775

Aronzon CM, Sandoval MT, Herkovits J, Pérez-Coll CS (2011) Stage-dependent toxicity of 2,4-dichlorophenoxyacetic on the embryonic development of a South American toad, Rhinella arenarum. Environ Toxicol 26(4):373–381

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

Bantle JA, Dumont JN, Finch RA, Linder G, Fort DJ (1998) Atlas of abnormalities. A guide for the performance of FETAX, 2nd ed.Oklahoma State University, Stillwater

Beebee TJC, Griffiths RA (2005) The amphibian decline crisis: a watershed for conservation biology? Biol Conserv 125:271–285

Blaustein AR, Wake DB (1990) Declining amphibian populations: a global phenomenon? Trends Ecol Evol 5:203–204

Boutin C, Freemark KE, Keddy CJ (1993) Proposed Guidelines for Registration of Chemical Pesticides: nontarget plant Testing and Evaluation. Technical Report Series 145. Canadian Wildlife Service (Headquarters), Environment Canada, Ottawa, 91p

Boutin C, Freemark KE, Keddy CJ (1995) Overview and rationale for developing regulatory guidelines for nontarget plant testing with chemical pesticides. Environ Toxicol Chem 14(9):1465–1475

Brodeur JC, Svartz G, Perez-Coll CS, Marino DJG, Herkovits J (2009) Comparative susceptibility to atrazine of three developmental stages of *Rhinella arenarum* and influence on metamorphosis: non-monotonous acceleration of the time to climax and delayed tail resorption. Aquat Toxicol 91(2):161–170

Brodie ED, Formanowicz DR (1983) Prey size preference of predators: differential vulnerability of larval anurans. Hepetologica 39(1):67–75

Bushway RJ, Perkins LB, Fukal L, Harrison RO, Ferguson BS (1991) Comparison of enzyme-linked immunosorbent assay and high-performance liquid chromatography for the analysis of atrazine in water from Czechoslovakia. Arch Environ Contam Toxicol 21(3):365–370

Carr JA, Gentles A, Smith EE, Goleman WL, Urquidi LJ, Thuett K, Kendall RJ, Giesy JP, Gross TS, Solomon KR, Van Der Kraak G (2003) Response of larval *Xenopus laevis* to atrazine: assessment of growth, metamorphosis, and gonadal and laryngeal morphology. Environ Toxicol Chem 22(2):396–405

CASAFE (2011) Cámara Argentina de Sanidad Agropecuaria y Fertilizantes. http://200.69.30.152/~admin33/estad/Mercado2010.htm Accessed 17 November 2011

Cooke AS (1971) Selective predation by newts on frog tadpoles treated with DDT. Nature 229:275–276

Cooper RL, Stoker TE, Tyrey L, Goldman JM, McElroy WK (2000) Atrazine disrupts the hypothalamic control of pituitary-ovarian function. Toxicol Sci 53(2):297–307

Davies PE, Cook LSJ, Barton JL (1994) Triazine herbicide contamination of Tasmanian streams: sources, concentrations and effects on biota. Aust J Mar Freshw Res 45(2):209–226

Del Conte E, Sirlin L (1951) The first stages of *Bufo arenarum* development. Act Zoo Lilloana 12:495–499

Echeverría DD, Fiorito De López LE (1981) Estadios de la metamorfosis en *Bufo arenarum* (Anura). Physis Secc. B. Bs. As 40(98):15–23

APHA, editor (1980) American Public Health Association. Standard Methods for the Examination of Water and Wastewater, 15th edn. APHA-AWWA-WPCF, Washington DC, 1000 p

Ezemonye L, Tongo I (2009) Lethal and sublethal effects of atrazine to amphibian larvae. Jordan J Biol Sci 2(1):29–36

Hayes T, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA, Vonk A (2002) Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proc Natl Acad Sci U S A 99(8):5476–5480

Hayes T, Haston K, Tsui M, Hoang A, Haeffele C, Vonk A (2003) Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. Environ Health Perspect 111(4):568–575

Herkovits J, Faber J (1978) Shape: its development and regulation capacity during embryogenesis. Acta Biotheor 27(3–4):185–200

Herkovits J, Pérez-Coll CS (2003) AMPHITOX: A customized set of toxicity tests employing amphibian embryos. Symposium on multiple stressor effects in relation to declining amphibian populations. In: Linder GL, Krest S, Sparling D, Little EE (ed) Multiple Stressor Effects in Relation to Declining Amphibian Populations. ASTM International STP 1443, USA, pp 46–60

Herkovits J, Ponce B, Olabe J (1980) Efecto de la prostaglandina F2 alfa sobre la proliferación celular y el transporte de Na, K, Ca y Mg en células epiteliales embrionarias. Medicina 40:858–859

Herkovits J, Cardellini P, Pavanati C, Pérez-Coll CS (1997) Susceptibility of early life stages of *Xenopus laevis* to cadmium. Environ Toxicol Chem 16(2):312–316

Herkovits J, Pérez-Coll C, Herkovits FD (2002) Ecotoxicological studies of environmental samples from Buenos Aires area using a standardized amphibian embryo toxicity test (AMPHITOX). Environ Pollut 116(1):177–183

Herzog DC, Funderburk JE (1986) Ecological bases for habitat management and pest cultural control. In: Kogan M (ed) Ecological theory and integrated pest management practice. Wiley, New York, pp 217–250

Houlahan JE, Findlay CS, Schmidt BR, Meyer AH, Kuzmin SL (2000) Quantitative evidence for global amphibian population declines. Nature 404(6779):752–755

Kiesecker JM, Blaustein AR, Belden LK (2001) Complex causes of amphibian population declines. Nature 410(6829):681–684

Langerveld AJ, Celestine R, Zaya R, Mihalko D, Ide CF (2009) Chronic exposure to high levels of atrazine alters expression of genes that regulate immune and growth-related functions in developing *Xenopus laevis* tadpoles. Environ Res 109(4):379–389

Lenkowski JR, Reed MJ, Deininger L, McLaughlin KA (2008) Perturbation of organogenesis by the herbicide atrazine in the amphibian *Xenopus laevis*. Environ Health Perspect 116(2):223–230

Little EE, Archeski RD, Flerov BA, Kozlovskaya VI (1990) Behavioral indicators of sublethal toxicity in rainbow trout. Arch Environ Contam Toxicol 19(3):380–385

Mann RM, Hyne RV, Choung CB, Wilson SP (2009) Amphibians and agricultural chemicals: review of the risks in a complex environment. Environ Pollut 157(11):2903–2927

Morgan MK, Scheuerman PR, Bishop CS, Pyles RA (1996) Teratogenic potential of atrazine and 2,4-D using FETAX. J Toxicol Environ Health 48(2):151–168

Nieves-Puigdoller K, Björnsson BT, McCormick SD (2007) Effects of hexazinone and atrazine on the physiology and endocrinology of smolt development in Atlantic salmon. Aquat Toxicol 84(1):27–37

Pérez-Coll CS, Sandoval MT, Herkovits J (2009) Efectos letales y teratogénicos del Funguicygon® en embriones de *Bufo arenarum*. In SETAC (ed) Química y Toxicología Ambiental en América latina. Desafíos, perspectivas y nuevos enfoques. ISBN: 978-987-25370-0-5, Argentina, pp 86–88

Pintér A, Török G, Börzsönyi M, Surján A, Csík M, Kelecsényi Z, Kocsis Z (1990) Long-term carcinogenicity bioassay of the herbicide atrazine in F344 rats. Neoplasma 37(5):533–544

Pisanó A (1956) Efficienza funzionale e structura dell′ ipofisi di anfibio. Arch Zool Ital 42:221–227

Relyea RA (2009) A cocktail of contaminants: how mixtures of pesticides at low concentrations affect aquatic communities. Oecologia 159(2):363–376

Rohr JR, Elskus AA, Shepherd BS, Crowley PH, McCarthy TM, Niedzwiecki JH, Sager T, Sih A, Palmer BD (2003) Lethal and sublethal effects of atrazine, carbaryl, endosulfan, and octylphenol on the streamside salamander (*Ambystoma barbouri*). Environ Toxicol Chem 22(10):2385–2392

Sass JB, Colangelo A (2006) European Union bans atrazine, while the United States negotiates continued use. Int J Occup Environ Health 12(3):260–267

Semlitsch RD (1990) Effects of body size, sibship, and tail injury on the susceptibility of tadpoles to dragonfly predation. Can J Zool 68(5):1027–1030

Silvestre F, Trausch G, Spanò L, Devos P (2002) Effects of atrazine on osmoregulation in the Chinese mitten crab, *Eriocheir sinensis*. Comp Biochem Physiol C: Toxicol Pharmacol 132(3):385–390

Simic B, Kniewald J, Kniewald Z (1994) Effects of atrazine on reproductive performance in the rat. J Appl Toxicol 14(6):401–404

USEPA (1988) EPA Probit analysis program for calculating EC/LC values. Version 1.5. United States Environmental Protection Agency, Cincinnati, OH

USEPA (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. Accessed 17 November 2011

Van der Hoeven N (1997) How to measure no effect. Part III. Statistical aspects of NOEC, ECx and NEC estimates. Environmetrics 8(3):255–261

Van der Hoeven N, Noppert F, Leopold A (1997) How to measure no effect. Part I. Towards a new measure of chronic toxicity in ecotoxicology, introduction and workshop results. Environmetrics 8(3):241–248

Wetzel LT, Luempert LG III, Breckenridge CB, Tisdel MO, Stevens JT, Thakur AK, Extrom PJ, Eldridge JC (1994) Chronic effects of atrazine on estrus and mammary tumor formation in female Sprague-Dawley and Fischer-344 rats. J Toxicol Environ Health 43(2):169–182

Yoder J, Watson M, Benson WW (1973) Lymphocyte chromosome analysis of agricultural workers during extensive occupational exposure to pesticides. Mutat Res 21(6):335–340