Water Air Soil Pollut (2013) 224:1404
DOI 10.1007/s11270-012-1404-1

Individual and Mixture Toxicity of Commercial Formulations Containing Glyphosate, Metsulfuron-Methyl, Bispyribac-Sodium, and Picloram on *Rhinella arenarum* Tadpoles

Rafael C. Lajmanovich · Celina M. Junges · Andrés M. Attademo · Paola M. Peltzer · Mariana C. Cabagna-Zenklusen · Agustín Basso

Received: 26 July 2012 / Accepted: 29 November 2012 / Published online: 17 February 2013

Abstract We investigated the effects of four commercial formulations of herbicides (glyphosate [GLY], metsulfuron-methyl [MET], bispyribac-sodium [BIS], and picloram [PIC]) individually, and in three 50:50 mixtures (GLY–MET, GLY–BIS, GLY–PIC) on the common toad *Rhinella arenarum* (Anura: Bufonidae) tadpoles. Enzymatic parameters such as, glutathione *S*-transferase (GST), butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) activities, as well as erythrocyte nuclear abnormalities (ENA) were studied. Interactions between herbicides in mixtures were evaluated and classified as additive, synergistic, or antagonistic. Toxicity results (48-h LC_{50}) showed that PIC was the most toxic herbicide, followed by BIS, GLY, and MET, while GLY–PIC was the most toxic mixture, followed by GLY–BIS, and GLY–MET. All commercial herbicide formulations and their mixtures significantly inhibited BChE activity in exposed tadpoles. The AChE activity was also inhibited by all herbicides and their mixtures, except by GLY–BIS. The inhibition of GST activity was only significant for GLY, MET, PIC, and GLY–MET. A significant increase in the frequency of ENA was found for tadpoles exposed either to commercial herbicide formulations or to mixtures, except for GLY. All the mixtures showed synergism for BChE activity while for AChE only the GLY–MET and GLY–PIC mixtures acted synergistically. GLY–MET showed synergism for GST, whereas for ENA, the mixture GLY–BIS was antagonistic. This study with *R. arenarum* tadpoles demonstrates that the interactions between three of the most intensively used herbicides in soybean crops results in synergistic effects on mortality and neurotoxicity and synergistic or additive effects in genotoxicity.

Keywords Amphibians · Herbicide mixtures · Cholinesterase · Erythrocyte nuclear abnormalities

R. C. Lajmanovich · C. M. Junges · A. M. Attademo · P. M. Peltzer
National Council for Scientific and Technical Research (CONICET), Buenos Aires, Argentina

R. C. Lajmanovich (✉) · C. M. Junges · A. M. Attademo · P. M. Peltzer · M. C. Cabagna-Zenklusen · A. Basso
Ecotoxicology Laboratory, Faculty of Biochemistry and Biological Sciences (FBCB-UNL), Ciudad Universitaria Paraje el Pozo s/n, 3000 Santa Fe, Argentina
e-mail: lajmanovich@hotmail.com

1 Introduction

In Argentina, like in many South American countries, the expansion of agricultural land has caused extensive habitat loss and degradation, which are among the greatest current and future threats to biodiversity (Dirzo and Raven 2003). In this context, a great

mixture of pesticides and their residues are present in a wide variety of aquatic habitats by agricultural lands (Wharfe 2004; Abrantes et al. 2010). To understand the effects of pesticides on amphibians, the natural first step is to understand the direct toxicity of pesticides. There are a growing number of excellent laboratory-based, single-species toxicology experiments that provide the LC_{50} values of different pesticides (Relyea et al. 2005). However, amphibians in nature live in much more complex environments that contain a great number of pesticide mixtures (Relyea 2009). Mixtures of pesticides may cause undesirable effects even if the individual substances are recorded as being below their no-observed effect concentration (NOEC) (Chèvre et al. 2008).

Glyphosate (GLY) [*N*-(phosphonomethyl) glycine]-resistant crops (GLY-RCs) are the transgenic crops most extensively grown worldwide, with soybean being the major GLY-RC (Gutterson and Zhang 2004). The Monsanto Company (2002) stated that "normal use" of GLY formulations (i.e., Roundup®) is not expected to cause unreasonable adverse effects to amphibians, including tadpoles (Giesy et al. 2000); however, according to our experience, the GLY "normal use" is not always strictly followed in field crops. On the other hand, numerous studies have reported deleterious effects of GLY formulations on larval amphibians (see the review of Govindarajulu 2008). However, a strong scientific debate about the impact of GLY on amphibians and human health remains (Carrasco 2011; Relyea 2006; Saltmiras et al. 2011; Thompson et al. 2006).

The intense use of GLY has increased the spread of weeds with tolerance to this herbicide (Vidal et al. 2010), and in consequence, GLY is usually mixed with a wide range of herbicides including metsulfuron-methyl (MET) (Semino 2008), bispyribac-sodium (BIS) (Valverde 2007), picloram (PIC) (O'Sullivan and Kossatz 1982), in order to improve the control of weed species. Furthermore, the mixtures can alter the toxicity of individual compounds, with effects on biological systems that can be antagonistic, additive or synergistic (Streibig and Jensen 2000).

MET is a sulfonylurea herbicide extensively used in agriculture, especially in rice and soybean fields (Pratap and Mashiat 2004). The half-life of MET in the soil is 30 days, and its water solubility is 9.5 mgl^{-1} (Barceló and Hennion 1997). Aquatic contamination by this product may occur in and around agricultural areas and may adversely affect aquatic fauna (Jonsson and Maia 1998). BIS is a pyrimidinyl carboxy herbicide very toxic to aquatic organisms, used to control grasses, sedges and broadleaved weeds, especially *Echinochloa* spp. (EFSA 2010). Water solubility of BIS is 73.3 gl^{-1} and its half-life in soil is 60 days (Rodrigues and Almeida 1998). PIC, in the pyridine family of compounds, is a systemic herbicide used for control of woody plants and a wide range of broadleaved weeds. It is very soluble in water (430 mgl^{-1}), but does not easily degrade in water; it is moderately toxic to birds, fishes, honeybees and earthworms (Britt et al. 2003). It has also been identified as an endocrine disruptor (Wylie 2006). A 50:50 mixture of PIC and 2,4-D known as Agent White, was used during the Vietnam War (Gupta 2009). PIC is listed in the soybean herbicide effectiveness ratings as one of the new options for weed management (Owen 2011).

Determinations of cholinesterases (ChEs) activity in amphibians are important tools for characterizing and monitoring exposure to pesticides (Sparling and Fellers 2007). The effects of commercial herbicides which affect ChEs (i.e., glyphosate, quinclorac, metsulfuron-methyl, imazethapyr, imazapic, clomazone) have been extensively studied in aquatic vertebrates such as fish (da Fonseca et al. 2008; dos Santos Miron et al. 2005, 2008; Glusczak et al. 2006, 2007; Modesto and Martinez 2010; Moraes et al. 2007, 2011; Pretto et al. 2011; Rossi et al. 2011; Salbego et al. 2010). Also, a recent study with glyphosate formulations (Lajmanovich et al. 2011) suggests that this herbicide inhibits ChEs in tadpoles.

Butyrylcholinesterase (BChE; EC 3.1.1.8) and acetylcholinesterase (AChE; EC 3.1.1.7) are two ChEs enzymes (Pezzementi et al. 2011). The main function of AChE is the rapid hydrolysis of acetyl esters such as acetylcholine, whereas BChE has no known specific natural substrate, although it is able to hydrolyze acetylcholine (Valbonesi et al. 2003). It has been reported that toxicity of anticholinesterase pesticides mixtures may deviate from concentration addition if the individual chemicals in a mixture interact via toxic processes to produce either antagonistic, synergistic or additive effects (Borgert et al. 2004). Moreover, glutathione *S*-transferase (GST; EC 2.5.1.18) are a multigene family of cytosolic enzymes involved in the conjugation of electrophilic metabolites with the tripeptide glutathione to yield a water-soluble conjugated metabolite. GST activity is used as a biomarker of

pesticide contaminated environment in ecological risk assessment (Otitoju and Onwurah 2007). In addition, some hematological parameters such as erythrocyte morphology can also be used as biomarkers and have potential applications in terrestrial and aquatic ecotoxicological studies (Cabagna et al. 2005; Attademo et al. 2011).

The objective of this study was to determine and compare the effects of the individual formulations of the herbicides: GLY, MET, BIS and PIC, and three binary combinations (GLY and MET, GLY and BIS, GLY and PIC) on *Rhinella arenarum* tadpoles. This information would help the assessment and characterization of potential amphibian's ecological risks after the application of those herbicide mixtures.

2 Materials and Methods

2.1 Drugs

Acetylthiocholine iodide, butyrylthiocholine iodide, 5,50 dithiobis-2-nitrobenzoic acid salt were purchased from Sigma-Aldrich® (Germany). Reduced glutathione and 1-chloro-2,4-dinitrobenzene were supplied by Acros-Organics® (USA). All other chemicals for biomarker assessment were obtained from Biopack® (Argentina).

2.2 Experimental Design

Tadpoles of *Rinella arenarum* were selected as model test organisms. This common anuran has an extensive neotropical distribution (IUCN 2010), and it is frequently found in forest, wetlands, agricultural land, and urban territories (Peltzer et al. 2006). Its larvae exhibit aggregative behavior (Schmajuk and Segura 1982) and have been recently characterized by their sensitivity to GLY (Lajmanovich et al. 2011). Premetamorphic larvae were collected in January 2011 from temporary ponds in natural floodplains of the Paraná River (31°11′31″S, 60°9′29″W; Argentina). The average size (snout–tail tip) was 17±0.5 mm and weight was 0.055±0.009 g; Gosner stages (GS) 29–30 (Gosner 1960). The tadpoles were acclimated for 48 h to a 12-h light/dark cycle with dechlorinated tap water (DTW), pH 7.4±0.05; conductivity, 165±12.5 μmhos cm^{-1}; dissolved oxygen concentration, 6.5±1.5 mgl^{-1} hardness, 50.6 mgl^{-1} of $CaCO_3$ at 22±2°C, and fed on boiled lettuce (*Lactuca sativa*) at the beginning of the experiment.

For short-term (48-h) static toxicity tests, we used the commercial formulations of GLY (74.7 % active ingredient [a.i.], *N*-(phosphonomethyl) glycine; Ultra-Max®, Monsanto Co., Argentina; recommended application field rates of 1.1 to 2.3 kg a.i./ha; CASAFE 2007), MET (60 % a.i., methyl 2-(4-methoxy-6-methyl-1,3,5-triazin-2-ylcarbamoylsulfamoyl) benzoate; Metsulfuron 60®, Ciagro S.A., Argentina; recommended application field rates of 8–10 g a.i./ha; CASAFE 2007), BIS (40 % a.i., sodium 2,6-bis (4,6-dimethoxypyrimidin-2-yloxy) benzoate, Ectran®, Ipesa S.A., Argentina; recommended application field rates of 80 to 100 cm^3 a.i./ha; CASAFE 2007), and PIC (27.7 % a.i., 4-amino-3,5,6-trichloropicolinic acid, Tordon 24-K®, Dow AgroSciences, Argentina; recommended application field rates of 80 to 120 cm^3 a.i./ha; CASAFE 2007). The herbicides were tested as a complex commercial mixture because this is the form in which they are applied in cultivated fields and introduced into the environment.

Glass aquariums (12.5 cm diameter and 13.5 cm high) with 1 l of DTW and seven tadpoles per recipient with a loading ratio of approximately 0.38 (g/l) (N= 336 per treatment) were used in the experiments. Tests were conducted at 22±2 °C and at 12-h light/dark cycle. The nominal concentrations used to test single toxicities of four herbicides were: 0.0097, 0.0195, 0.039, 0.0781, 0.1562, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80, and 160 mg a.i. l^{-1}. The toxicity of the following mixtures GLY and MET, GLY and BIS, and GLY and PIC in 50:50 combinations were evaluated using the same nominal concentrations. Negative controls with DTW were used. Both control and test solutions were made in triplicate. Treatments were randomly assigned to the recipients, as was the order in which the recipients were sampled. For biomarkers assessment control and treated animals at all concentrations that had a survival rate >85 % at 48 h were killed according to ASIH (2004) criteria and with approval from the animal ethics committee of the Faculty of Biochemistry and Biological Sciences.

2.3 Enzymatic Determinations

Whole tadpoles were homogenized (on ice) in 0.1 %*t*-octylphenoxypolyethoxy ethanol (Triton X-100) in 25 mM tris (hydroxymethyl) aminomethane hydrochloride (pH 8.0) using a homogenizer.

The homogenates were centrifuged at 14,000 rpm for 15 min at 4 °C, and the supernatant was collected. Total protein concentrations in the supernatants were determined according to the Biuret method (Kingbley 1942). Enzyme kinetics assays were performed in triplicate. AChE and BChE activities were measured according to Ellman et al. (1961). The reaction mixture consisted of 0.01 ml extract, 2 mM dithio bis 2-nitrobenzoic acid, 20 mM acetylthiocholine, and butyrylthiocholine iodide (AcSCh and BuSCh, respectively); 25 mM Tris–HCl; and 1 mM $CaCl_2$ (pH=7.6). Assays were conducted at 25 °C. The variation in optical density was recorded at 410 nm for 1 min at 25 °C using a Jenway 6405 UV–VIS spectrophotometer. AChE and BChE activities were expressed as $nmol\,min^{-1}\,mg^{-1}$ protein using a molar extinction coefficient of 14.15×10^3 M^{-1} cm^{-1} for AChE, and 13.6×10^3 M^{-1} cm^{-1} for BChE. GST activity was determined spectrophotometrically by the method described by Habig et al. (1974) as adapted by Habdous et al. (2002) for mammal serum GST activity. The enzyme assay was performed at 340 nm in 100 mM Na phosphate buffer (pH 6.5), 2 mM CDNB, and 5 mM GSH. Enzyme kinetic assays were performed at 25 °C, and whole GST activity was expressed as $\mu mol\,min^{-1}\,mg^{-1}$ of protein using a molar extinction coefficient of 9.6×10^3 M^{-1} cm^{-1}. The data of enzymatic activity was expressed as the mean ± SEM.

2.4 Erythrocyte Nuclear Abnormalities

Blood was taken from each tadpole by cardiac puncture (Lajmanovich et al. 2005); blood smears were prepared on clean slides, fixed and stained by the May–Grunwald–Giemsa method (Dacie and Lewis 1995). Genotoxicity was tested using the presence of erythrocyte nuclear abnormalities (ENA) (Attademo et al. 2011), carried out in RBCs according to the procedures of Guilherme et al. (2008), by determination of the frequency of the following nuclear lesions: micronuclei (MN), lobed nuclei (L), binucleates or segmented nuclei (S), kidney shaped nuclei (K), and notched nuclei (N). The results were expressed as ENA frequency, the mean value (‰) of the sum (MN + L + S + K + N) for all the lesions observed.

2.5 Statistics and Data Analyses

Lethal concentration (LC_{50}) values and their respective 95 % confidence intervals (CI) were determined by the Trimmed Spearman–Karber method (Hamilton et al. 1977). The LC_{50} estimates were subjected to one-way analyses of variance (ANOVA) followed by post hoc contrast with Duncan's multiple range test. The mortality data were statistically evaluated by ANOVA using Dunnett's procedure for multiple comparisons in order to determine the NOEC and the lowest-observed effect concentration (LOEC).

NOEC values for all herbicide treatment were used for comparison of the ChEs and GST activities. The data were analyzed with one-way ANOVA and Dunnett's test for post hoc comparisons. In the same way, mutagenic activity data of ENA were analyzed using binomial proportion test (Margolin et al. 1983). These statistical analyses were performed using BioEstat software 5.0 (Ayres et al. 2008).

2.6 Toxicological Interactions Between Herbicides Based on LC_{50}

To determine whether the effects of the two herbicides in a 50:50 mixture were additive, synergistic, or antagonistic, the following equation from Marking (1985) was used:

$$(Am/Ai) + (Bm/Bi) = S$$

where A and B were the individual herbicides, i and m were the individual and mixture LC_{50}s, respectively, and S was the sum of activity. The toxicants are antagonistic if $S<1$, synergistic if $S>1$, or additive is $S=1$. Marking places an additive index value of 0 at $S=1$, a sum of activity at which the mixture components would be equitoxic (a demonstration of additive toxicity). If $S\leq1.0$, additive index (AI)$=1/S-1$. If for $S\geq1.0$, AI$=-S+1$. According to this scheme, when AI$=0$, components are simply additive; negative and positive numbers indicated less than additivity and more than additivity, respectively.

We used significant departures from additive toxicity as defined above to identify antagonistic and synergistic interactions between pesticides mixtures (Hertzberg and MacDonell 2002) or no interaction, where the predicted toxicity of the mixture is the sum of each chemical's predicted toxicity.

2.7 Toxicological Interactions Between Herbicides Based on Enzymatic Activity and ENA

The experimental data for enzyme inhibition and ENA values were interpolated to estimate the median effective concentration (EC_{50}) and 95 % CI (estimated by a linear regression to produce a 50 % decrease in the biomarker measurements relative to controls; Laetz et al. 2009); (a) for single herbicide exposures (toxic potential [TP]) ($EC_{50\ TP}$) and (b) for binary mixture (BM) ($EC_{50\ BM}$). We used these endpoint comparisons to determine whether toxicological responses to BMs were additive, antagonistic, or synergistic. The criterion of non-overlapping 95 % CI was used to determine the significance of differences between EC_{50} values (Laetz et al. 2009).

3 Results

3.1 Toxicity Tests

Results of the acute toxicity bioassays are summarized in Table 1. Analyses of variance on LC_{50} values for *R. arenarum* tadpoles showed significant variations among four herbicides and their mixtures ($p<0.05$). PIC was the most toxic herbicide for assay tadpoles, followed by BIS, GLY, and MET. LC_{50} values at 48 h ranged from PIC=0.025 to LC_{50} MET=105.56 mg a.i. l^{-1}. GLY–PIC was the most toxic mixture, followed by GLY–BIS, and GLY–MET. LC_{50} values at 48 h ranged from GLY–PIC=0.051 to LC_{50} GLY–MET= 25.62 mg a.i. l^{-1}. According to Marking's additive index, all herbicide mixtures evaluated displayed synergistic toxicity, with a sum of activity of 2.04 to 2.18. Furthermore, the negative results of *AI* indicated low additivity.

Table 1 Summary of acute toxicity bioassay (48-h LC_{50}) results of different commercial herbicide formulations: glyphosate (GLY), metsulfuron-methyl (MET), bispyribac-sodium (BIS) and picloram (PIC) on *R. arenarum* tadpoles. GLY and MET, GLY and BIS, and GLY and PIC were tested in a 50:50 mixture

Herbicide treatment	48-h LC_{50} (mg a.i. l^{-1}) (95 % CI)	NOEC and LOEC (mg a.i. l^{-1})	S	AI
GLY	13.20[a] (11.57–15.05)	10 20	–	–
MET	105.56[b] (92.55–120.40)	80 160	–	–
BIS	0.20[c] (0.18–0.24)	0.1562 0.3125	–	–
PIC	0.025[d] (0.022–0.029)	0.0195 0.0390	–	–
GLY and MET	25.62[e] (18.89–34.56)	5 10	2.18	−1.18
GLY and BIS	0.4123[f] (0.3615–0.4703)	0.0781 0.1562	2.08	−1.08
GLY and PIC	0.051[g] (0.045–0.058)	0.0097 0.0195	2.04	−1.04

Values in columns followed by different letters are statistically different at the 5 % level of probability by Duncan's Multiple Range Test

S sum of biological activity, *AI* additive index, *NOEC* no observed effect concentration, *LOEC* lowest observed effect concentration

3.2 Enzymatic Activity

The mean value of AChE activity in the control tadpoles was 15.98±2.18 $nmol\,min^{-1}\,mg^{-1}$ protein at 48 h. AChE activity varied among groups exposed to different types of commercial herbicides and their mixtures, differing significantly in all cases respect to AChE activity of the control group ($p<0.01$) (except GLY–BIS). The percentage of AChE inhibition for the groups that were significantly different from controls ranged from PIC (24.73 %) to GLY–MET (52.07 %) (Fig. 1).

The BChE activity (mean ± SEM) in the control group was 4.15±1.04 $nmol\,min^{-1}\,mg^{-1}$ protein at 48 h. All commercial herbicide formulations and their mixtures inhibited BChE enzyme activity significantly ($p<0.01$) with respect to control. The percentage of inhibition ranged from 28.61 % (GLY–BIS) to 47.63 % (GLY–MET) (Fig. 2).

The mean value of GST activity in control tadpoles was 5.78±0.89 $\mu mol\,min^{-1}\,mg^{-1}$ proteins at 48 h. The inhibition of GST enzymatic activity with respect to the control group was significant ($p<0.01$) for GLY, MET, PIC, and GLY–MET. Indeed, an important inhibition of GST activity was observed in GLY and MET, with values that varied from 38.77 % in GLY to 48.06 % in MET (Fig. 3).

3.3 Blood Cell Morphology

Figure 4 shows the different types of red blood cells considered to quantify the ENA of circulating

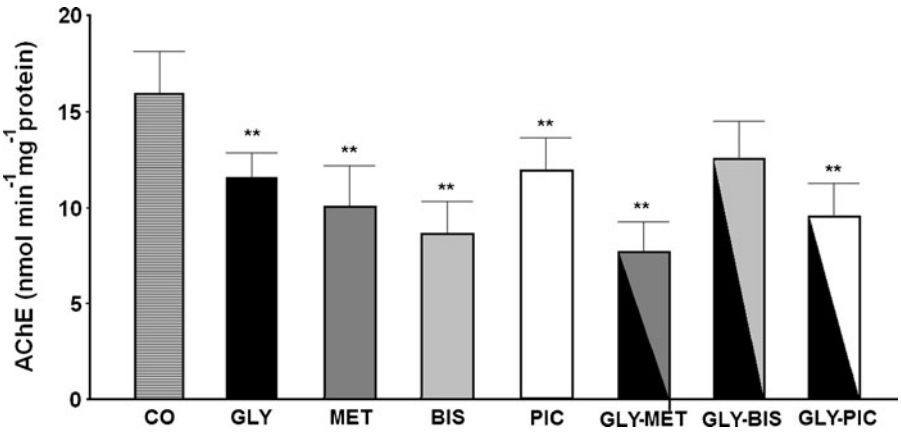

Fig. 1 Effects of commercial herbicide exposure (48-h) at no-observed effect concentration (*NOEC*) on the acetylcholinesterase (*AChE*) activity in *R. arenarum* tadpoles. *C0* control, *GLY* glyphosate, *MET* metsulfuron-methyl, *BIS* bispyribac-sodium, *PIC* picloram, and mixture of 50:50 GLY–MET, GLY–BIS, and GLY–PIC. Data are expressed as mean ± SEM, *N*=7–10. Significantly different from control (**$p<0.01$; Dunnett's test) (see Table 1 for NOEC values)

erythrocytes of *R. arenarum* tadpoles. Significant increase in the frequency of ENA ($p<0.01$) was found in those tadpoles exposed to NOEC of commercial herbicide formulations (except for GLY: 10 mg a.i. l^{-1}) and mixtures for assessed after 48 h (Table 2).

3.4 Toxicological Interactions Between Herbicides

Figure 5 shows the interaction between the EC_{50} values, which were calculated based on the results of the biomarkers studied to estimate the median effective concentration (EC_{50}) and 95 % CI for single herbicide exposures (toxic potential, TP) (TP EC_{50}) and for BM ($EC_{50\ BM}$). In the case of AChE, GLY–MET and GLY–PIC were synergistic; for BChE, all mixtures were synergistic; on the contrary, for GST only GLY–MET mixture was synergistic. For ENA, GLY–BIS was antagonistic; and in all other cases, the interactions were additive.

4 Discussions

Amphibians in the wild (especially in agricultural areas) are exposed to mixtures of pesticides. Furthermore, although brief episodes of high-concentration

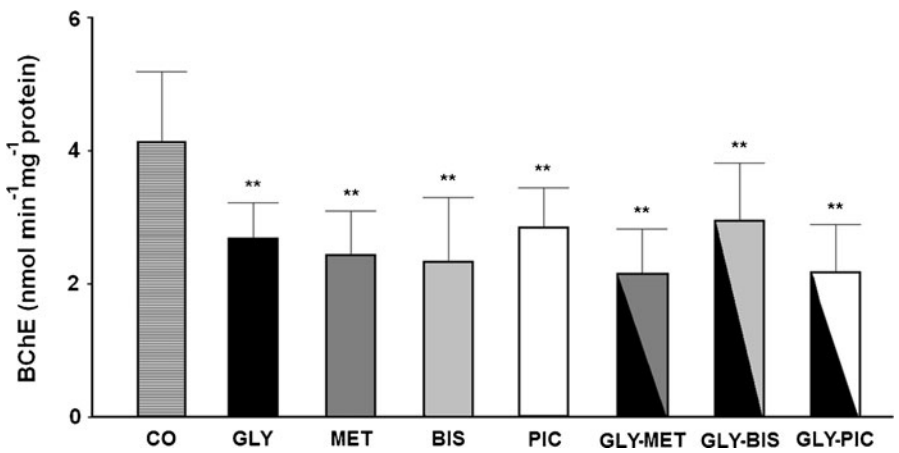

Fig. 2 Effects of commercial herbicide exposure (48-h) at no-observed effect concentration (*NOEC*) on the butyrylcholinesterase (*BChE*) activity in *R. arenarum* tadpoles. *C0* control, *GLY* glyphosate, *MET* metsulfuron-methyl, *BIS* bispyribac-sodium, *PIC* picloram, and mixture of 50:50 GLY–MET, GLY–BIS, and GLY–PIC. Data are expressed as mean ± SEM, *N*=7–10. Significantly different from control (**$p<0.01$; Dunnett's test) (see Table 1 for NOEC values)

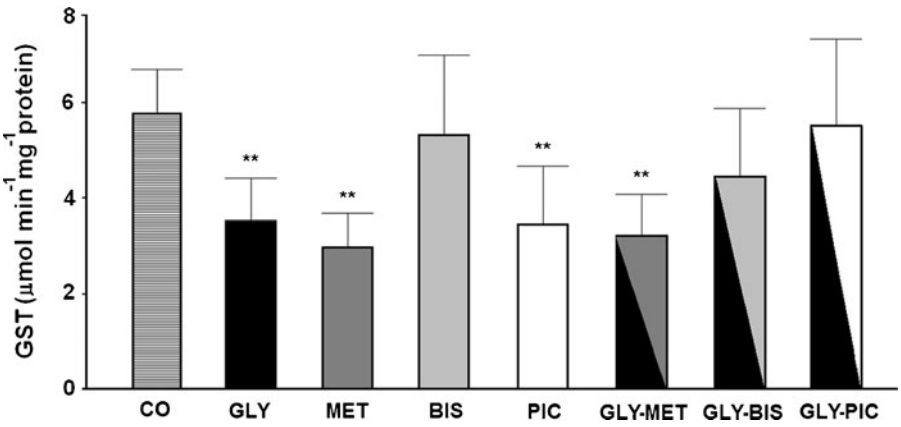

Fig. 3 Effects of commercial herbicide exposure (48-h) at no-observed effect concentration (*NOEC*) on the glutathione *S*-transferase (*GST*) activity in *R. arenarum* tadpoles. *C0* control, *GLY* glyphosate, *MET* metsulfuron-methyl, *BIS* bispyribac-sodium, *PIC* picloram, and mixture of 50:50 GLY–MET, GLY–BIS, and GLY–PIC. Data are expressed as mean ± SEM, N=7–10. Significantly different from control (**p<0.01; Dunnett's test) (see Table 1 for NOEC values)

exposure may occur, prolonged exposures to low concentrations of pesticide mixtures occur much more frequently (Hayes et al. 2006). In Argentina, 60 % of the cultivated surface is mainly used to grow genetically modified (GM) soybeans Roundup Ready (RR), and approximately 200 million l of GLY-based herbicides are used to produce 50 million tons of soybeans per year (Teubal 2009). In this area, the main application time of agrochemicals occurs from November to March, and it is coincident with the reproductive period of most local amphibian species (Peltzer and Lajmanovich 2007) as well as with the highest pluvial period, which causes intensive pesticide wash off (Peltzer et al. 2008), polluting aquatic ecosystems. In RR soybeans, optimal weed control often requires sequential applications of GLY, and the timing relative to weed emergence is important (Swanton et al. 2000). When GLY is sprayed two to three times annually at high rates, it imposes a high selection pressure on the weed flora. In 5–8 years, this may cause shifts in weed composition towards species that naturally tolerate GLY and other herbicides may be needed to control these weeds (Shaner 2000). Thus, herbicide combinations are considered potentially useful in controlling weeds resistant or tolerant to GLY (Vidal et al. 2010).

There is increasing discussion about the levels and potential toxicity of herbicides (i.e., glyphosate) in the field (Thompson et al. 2006). Some authors (e.g., Solomon and Thompson 2003; Thompson et al. 2006) claim that herbicide applications are not legal for small wetlands and that levels of these herbicides that are common in the field are either expressed in ppb or ppt level, but not in ppm levels as used in many laboratory studies. In contrast, other authors (i.e., Relyea 2006) argue that current application rates for these herbicide formulations are highly lethal to many amphibian tadpoles. In this context, by means of the recommended application field rates mentioned previously (i.e., 2.3 kg a.i./ha for GLY), considering a total of 150 l of water per hectares to apply herbicides (CASAFE 2007), the maximal initial concentrations in a pond embedded in the crops would be 11,454.6 mg a.i. l^{-1} for GLY, 40 mg a.i. l^{-1} for MET, 266.6 mg a.i. l^{-1} for BIS, and 221.6 mg a.i. l^{-1} for PIC. According to LC_{50} values calculated in our study, this

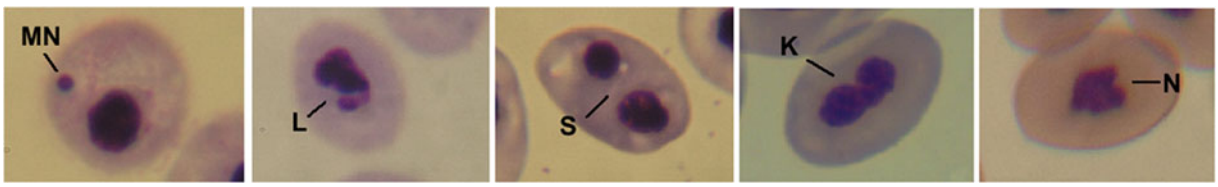

Fig. 4 Erythrocytes of *R. arenarum* tadpoles. Micronuclei (*MN*), lobed nuclei (*L*), binucleates or segmented nuclei (*S*), kidney-shaped nuclei (*K*), and notched nuclei (*N*). May–Grunwald–Giemsa stained blood smear (×1,000)

Table 2 Frequency of erythrocyte nuclear abnormalities (ENA) in *R. arenarum* tadpoles exposed to commercial herbicides (48-h) at no-observed effect concentration (NOEC)

Herbicide treatment	N	Total cell counted	Mean number of ENA (‰) (average ± SEM)
CO	9	9,000	0.11±0.11
GLY	9	9,000	0.22±0.14
MET	9	9,000	16±6.89***
BIS	8	8,000	26.5±11.33***
PIC	8	8,000	10±4.47***
GLY and MET	9	9,000	7.55±3.44***
GLY and BIS	11	11,000	0.72±0.72**
GLY and PIC	10	10,000	4.8±1.76***

Significantly different from control (**$p<0.01$, ***$p<0.001$; binomial proportion's test)

C0 control, *GLY* glyphosate, *MET* metsulfuron-methyl, *BIS* bispyribac-sodium, *PIC* picloram, and mixture of 50:50 GLY–MET, GLY–BIS, and GLY–PIC (see Table 1 for NOEC values)

would result in more than 50 % of the dead tadpoles for all mixes and individual herbicides except for MET alone.

GLY concentration is commonly expressed as mg a.i. l^{-1} or mg a.e. (acid equivalents) l^{-1}; i.e., 1 mg a.i. l^{-1} is equal to 0.75 mg a.e. l^{-1} (Relyea 2006). In the Asian common toad (*Bufo melanostictus*), the 48-h LC_{50} values calculated for GLY formulations was 49.44 mg a.i. l^{-1} (GS 25–26) (Jayawardena et al. 2011). In premetamorphic larvae (GS 29–30), the present investigation indicated a 48-h LC_{50} value of 13.20 a.i mgl^{-1} GLY (equivalent to 9.9 a.e. mgl^{-1}). However, in previous studies from our laboratory (Lajmanovich et al. 2011), the 48-h LC_{50} value was 2.42 a.e. mgl^{-1} for *R. arenarum* prometamorphic larvae (GS 36–38). In agreement with Lajmanovich et al. (2011), Vera Candioti et al. (2010) found that *R. arenarum* larvae in prometamorphosis (GS 37–39) are more sensitive than in premetamorphosis (GS 25) to carbamate pesticides. Also, Howe et al. (1998) noted an increase in sensitivity to atrazine as the amphibian larvae reached later stages.

Despite its widespread use, no studies have been reported on the toxicity of MET and BIS in amphibian tadpoles. In this sense, *R. arenarum* tadpoles seem to be in the same sensitive range to MET as fish species, since its 48-h LC_{50} is 105.56 mg a.i. l^{-1}, while for rainbow trout (*Oncorhynchus mykiss*) 96-h LC_{50} values are 150 mg a.i. l^{-1} (Vencill 2002). In the peer review of pesticide risk assessment for BIS, the

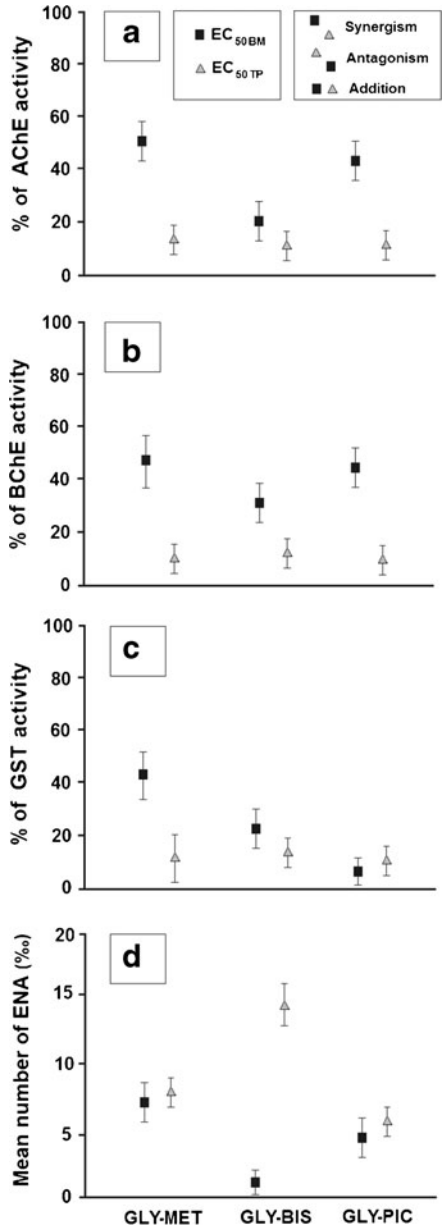

Fig. 5 Effects of commercial herbicide exposure (48-h) on the percentages of acetylcholinesterase (*AChE*) (**a**), butyrylcholinesterase (*BChE*) (**b**), glutathione *S*-transferase (*GST*) (**c**) inhibitions, and mean number of frequency of erythrocyte nuclear abnormalities (*ENA*) (**d**) in *R. arenarum* tadpoles. Median effective concentration calculated in binary mixture ($EC_{50\ BM}$) and median effective concentration (toxic potential) ($EC_{50\ TP}$). *GLY* glyphosate, *MET* metsulfuron-methyl, *BIS* bispyribac-sodium, *PIC* picloram in 50:50 mixture. Each point represents the mean (N=7–10), and error bars indicate the 95 % CIs of the mean

European Food Safety Authority (2010) indicated a low risk of MET to aquatic animals with values of 96-h LC_{50} for fish species between 10 and 95 mg a.i. l^{-1}. However, in our study the BIS at 48-h (LC_{50}=0.2 mg a.i. l^{-1}) indicates a greater toxicity of this herbicide for *R. arenarum* tadpoles. The only reference found in the literature about the toxicity of PIC in amphibian larvae indicates that it is moderately toxic to tadpoles with Median Tolerance Limit (48-h TL_{50}) values (defined as the pesticide concentration at which half of the test animals survived during the time interval tested) of 123 and 116 mgl^{-1} in *Adelotus brevis* (Tusked frog) and *Limnodynastes peroni* (Brown striped marsh frog), respectively (Johnson 1976). These values differ from those found for *R. arenarum* tadpoles (48-h LC_{50} =0.025 mg a.i. l^{-1}), because the first bioassay (referred to the toxicity of PIC) was performed with Tordon 50-D® (2,4-D-Picloram mixture). Based on our results, the herbicide Tordon 24-K® is very acutely toxic to amphibian tadpoles.

A discussion of the acute toxicity of GLY mixed with other herbicides requires knowledge of toxicities of the individual components of the mixture. In our experiments with *R. arenarum*, the single toxicity of commercial herbicide formulations had the following order: PIC > BIS > GLY > MET. In accordance with this trend, the toxicity of mixtures decreased as follows: GLY–PIC > GLY–BIS > GLY–MET. In this context, Deneer (2000) reported that the effects of most herbicide mixtures on aquatic organisms were additive in nature. According to Marking's equation additive index, the mixture of herbicides evaluated displays synergistic toxicity. The natural assumption when dealing with different chemicals mixed together would be that a mixture's toxicity equals the sum of the toxicities of its parts (Verbruggen and Van den Brink 2010). However, as this experiment has shown, mixture toxicity is far more complex than simple addition. Greater toxicity may be due not only to the toxic effects of the herbicides themselves but to supposedly inert additives such as a surfactant. For example, in commercial GLY formulations the polyethoxylated tallowamine surfactant (POEA) is cytotoxic and disrupts the membrane of sensitive respiratory surfaces (Partearroya et al. 1991). The synergistic relationship between the herbicides mixed together with surfactant was at least in part due to the surfactant's ability to facilitate absorption of the herbicides into tissues. Hence, this produces a greater toxicological impact of each herbicide (Green and Abdelghani 2004).

In pure chemical terms, GLY is an organophosphate that contains carbon and phosphorous (WHO/FAO 1996). In this sense, we observed that ChEs and GST activities in *R. arenarum* tadpoles exposed to GLY were inhibited compared to controls, confirming previous results with the same species (Lajmanovich et al. 2011). Moreover, inhibition of AChE activity in both brain and muscle tissue was observed in fish (*Rhamdia quelen* and *Leporinus obtusidens*) exposed to sublethal doses of commercial MET formulations (dos Santos Miron et al. 2005; Pretto et al. 2011). Also, another commercial formulation containing BIS caused changes in toxicology and oxidative stress parameters in tissues of *Cyprinus carpio*; thus, a decrease in AChE and GST activity may be the result of severe oxidative stress generated by exposure to this herbicide (Toni et al. 2010). On the other hand, there is limited data of PIC (technical grades or commercial formulations) about the effects as a ChEs inhibitor and other sublethal effects like on activities of antioxidant enzymes. We only found data for hexachlorobenzene, an impurity present in the technical grade of PIC (Cox 1998). This chemical induces oxidative damages and inhibits the activity of AChE and GST enzymes in juvenile common carps (*Cyprinus carpio*) (Song et al. 2006).

Usually, in the first contact with the toxic, the increase in GST activity is involved in metabolic detoxification. However, depletion of GST is associated with oxidative stress and cytotoxicity of pro-oxidant xenobiotics (Hazarika et al. 2003). Our results indicate that the activation of protective mechanisms is necessary for scavenging of produced reactive oxygen radicals. The present study also showed that commercial herbicides studied could cause neurotoxic effects on tadpoles. AChE is involved in the deactivation of acetylcholine at nerve endings, preventing continuous nerve firings, which is vital for normal functioning of neuromuscular systems, and is a common biomarker used in the assessment of neurotoxic effects. The AChE inactivation may have resulted from either amino acid residue and/or membrane lipid oxidation (Ballinger et al. 2005). Therefore, further investigations are needed to investigate the nature of these mechanisms in amphibians.

The blood of amphibians is a very plastic tissue (Barni et al. 2007), and variations of hematological

parameters in anurans have frequently been reported as a response to pesticide exposure (e.g., Attademo et al. 2011; Kundu and Roychoudhury 2009). In *Lithobates catesbeianus* tadpoles exposed to several Roundup® concentrations (6.75–27 mg GLY a.i. l^{-1}), a 24-h significant DNA damage was found in erythrocytes when tadpoles were compared with unexposed control animals (Clements et al. 1997). Furthermore, a recent study in Japanese medaka (*Oryzias latipes*) suggests that DNA damage is induced by acid alkanolamide a common surfactant in GLY formulations (i.e., Roundup®) (Uchida et al. 2012). Also, Bosch et al. (2011) reported that after 2 and 5 days of exposure to Roundup® (200–800 mg GLY a.i. l^{-1}), the micronucleated erythrocytes frequency in the adult toad *R. arenarum* was higher than the basal frequency. Additionally, Stone (2007) reported that chromosome aberrations increased in frequency in hamster ovary cells exposed to PIC. However, no genotoxicity data were found for MET and BIS. In our experiment, the GLY–NOEC (10 mg a.i. l^{-1}) mixture showed no difference in frequency of ENA with respect to the control group. However, the experimental data for the other three herbicides (MET, BIS and PIC) indicates some evidence of genotoxic potential. The results of genotoxicity of the herbicide combinations did not show a clear trend in synergy. However, in the case of GLY–BIS, the effect was antagonistic, which coincides with the low values of ENA obtained for this mixture. It is likely that the frequency of nuclear lesions may be altered by several factors, such as the erythropoiesis (Udroiu 2006). A decrease in erythropoiesis may be related to ENA decline (Marques et al. 2009); indeed, ENA frequency might have been falsely reduced.

The assumption of dose addition or concentration addition for mixtures of anticholinesterase pesticides has also been extended to aquatic life (Junghans et al. 2006). In biomarkers studied to identify the interactions between herbicides in 50:50 mixtures, it was first necessary to normalize each concentration–response curve using the calculated EC_{50} concentration for that individual chemical. In our study all herbicide mixtures showed synergism for ChEs activities, except for GLY–BIS, which was additive. In fact, certain pesticides combinations showed a clear pattern of synergism even at these relatively low levels. For example, diazinon and chlorpyrifos were synergistic when combined at 7.3 and 0.1 μg a.i. l^{-1}, respectively (Laetz et al. 2009).

5 Conclusions

Overall, our results indicate that PIC was the most toxic herbicide for *R. arenarum* tadpoles, followed by BIS, GLY, and MET, while GLY–PIC was the most toxic mixture, followed by GLY–BIS, and GLY–MET. Also, all commercial herbicides tested and their mixtures in sublethal concentrations (NOEC) inhibit ChEs activity and induce genotoxicity in tadpoles exposed.

Finally, *R. arenarum* tadpoles exposed to mixtures of the most intensively used herbicides in crops of soybean crops, showed mortality synergy, either concentration-synergistic or additive neurotoxicity and genotoxicity. This implies that single-chemical assessments will systematically underestimate the actual risks to amphibian species in waterbodies where mixtures of herbicides potentially occur.

Acknowledgments We thank Oscar Scremin for critical reading of the manuscript and for helping with the English language, Marcelo Peart, Rubén Pavot for providing the herbicides for trials, and Ing. Marta Anglada (Faculty of Agricultural Sciences [FCA], UNER) for help in calculating the initial concentration and formulation of herbicides applications. This study was supported in part by National Council for Scientific and Technical Research (CONICET), National Agency for Promotion of Science and Technology (ANCyT) and Course of Action for Research and Science Promotion (CAI+D-UNL).

References

Abrantes, N., Pereira, R., & Gonçalves, F. (2010). Occurrence of pesticides in water, sediments and fish tissues in a lake surrounded by agricultural lands: concerning risks to humans and ecological receptors. *Water, Air, and Soil Pollution, 212*, 77–88.

ASIH. (2004). *American Society of Ichthyologists and Herpetologists Guidelines for use of live amphibians and reptiles in field and laboratory research*. Washington, DC: Herpetological Animal Care and Use Committee of the ASIH.

Attademo, A. M., Cabagna Zenklusen, M., Lajmanovich, R. C., Peltzer, P. M., Junges, C., & Basso, A. (2011). B esterase activities and blood cell morphology in the frog *Leptodactylus chaquensis* (Amphibia: Leptodactylidae) on rice agroecosystems from Santa Fe Province (Argentina). *Ecotoxicology, 20*, 274–282.

Ayres, M., Jr., Ayres, D., & Santos, A. (2008). *BioEstat. Versão5.0*. Belém, Pará, Brazil: Sociedade Civil Mamirauá, MCT-CNPq.

Ballinger, C. A., Cueto, R., Squadrito, G., Coffin, J. F., Velsor, L. W., Pryor, W. A., & Postlethwait, E. M. (2005). Antioxidant-mediated augmentation of ozone-induced

membrane oxidation. *Free Radical Biology & Medicine, 38*, 515–526.

Barceló, D., & Hennion, M. C. (1997). *Techniques and instrumentation in analytical chemistry. Trace determinants of pesticides and their degradation products in water*, vol. 19. Elsevier.

Barni, S., Boncompagni, E., Grosso, A., Bertone, V., Freitas, I., Fasola, M., & Fenoglio, C. (2007). Evaluation of *Rana esculenta* blood cell response to chemical stressors in the environment during the larval and adult phases. *Aquatic Toxicology, 81*, 45–54.

Borgert, C., Quill, T., McCarty, L., & Mason, A. (2004). Can mode of action predict mixture toxicity for risk assessment? *Toxicology and Applied Pharmacology, 201*, 85–96.

Bosch, B., Mañas, F., Gorla, N., & Aiassa, D. (2011). Micronucleus test in post metamorphic *Odontophrynus cordobae* and *Rhinella arenarum* (Amphibia: Anura) for environmental monitoring. *Journal of Toxicology and Environmental Health Science, 3*, 155–163.

Britt, C., Mole, A., Kirharn, F., & Terry, A. (2003). *The herbicide handbook: guidance on the use of herbicides on nature conservation sites*. Peterborough: English Nature/ FACT.

Cabagna, M., Lajmanovich, R. C., Stringhini, G., & Peltzer, P. M. (2005). Hematological studies in the common toad (*Bufo arenarum*) in agrosystems of Argentina. *Applied Herpetology, 2*, 373–380.

Carrasco, A. E. (2011). Reply to the letter to the editor regarding our article (Paganelli, A., Gnazzo, V., Acosta, H., López, S. L. & Carrasco, A.E. (2010). *Chemistry and Research Toxicology, 24*, 610–613.

CASAFE. (2007). *Guía de productos fitosanitarios para la República Argentina* (13th ed.). Buenos Aires, Argentina: Tomo 1 Cámara de Sanidad Agropecuaria y Fertilizantes.

Chèvre, N., Maillard, E., Loepfe, C., & Becker-van Slooten, K. (2008). Determination of water quality standards for chemical mixtures: extension of a methodology developed for herbicides to a group of insecticides and a group of pharmaceuticals. *Ecotoxicology and Environmental Safety, 71*, 740–748.

Clements, C., Ralph, S., & Petras, M. (1997). Genotoxicity of select herbicides in *Rana catesbeiana* tadpoles using the alkaline single-cell gel DNA electrophoresis (comet) assay. *Environmental and Molecular Mutagenesis, 29*, 277–288.

Cox, C. (1998). Herbicide factsheet Picloram. *Journal of Pesticide Reform, 18*, 13–20.

da Fonseca, M. B., Glusczak, L., Moraes, B. S., de Menezes, C. C., Pretto, A., Tierno, M. A., Zanella, R., Gonçalves, F. F., & Loro, V. L. (2008). The 2,4-D herbicide effects on acetylcholinesterase activity and metabolic parameters of piava freshwater fish (*Leporinus obtusidens*). *Ecotoxicology and Environmental Safety, 69*, 416–420.

Dacie, S. J., & Lewis, S. M. (1995). *Practical haematology* (8th ed.). Livingstone, Edinburgh: Churchill.

Deneer, J. W. (2000). Toxicity of mixtures of pesticides in aquatic systems. *Pest Management Science, 56*, 516–520.

Dirzo, R., & Raven, P. H. (2003). Global state of biodiversity and loss. *Annual Review of the Environmental and Resources, 28*, 137–167.

dos Santos Miron, D., Crestani, M., Shettinger, M. R., Morsch, V. M., Baldisserotto, B., Tierno, M. A., Moraes, G., & Pimentel Vieira, V. L. (2005). Effects of the herbicides clomazone, quinclorac, and metsulfuron methyl on acetylcholinesterase activity in the silver catfish (*Rhamdia quelen*) (Heptapteridae). *Ecotoxicology and Environmental Safety, 61*, 398–403.

dos Santos Miron, D., Pretto, A., Crestani, M., Glusczak, L., Schetinger, M. R., Loro, V. L., & Morsch, V. M. (2008). Biochemical effects of clomazone herbicide on piava (*Leporinus obtusidens*). *Chemosphere, 74*, 1–5.

Ellman, L., Courtey, K. D., Andreas, V., Jr., & Featherstone, R. M. (1961). A new rapid colorimetric determination of cholinesterase activity. *Biochemical Pharmacology, 7*, 88–95.

European Food Safety Authority. (2010). Conclusion on the peer review of the pesticide risk assessment of the active substance bispyribac (unless otherwise stated all data evaluated refer to the variant bispyribac-sodium). *EFSA Journal, 8*, 1692.

Giesy, J. P., Dobson, S., & Solomon, K. R. (2000). Ecotoxicological risk assessment for Roundup® herbicide. *Reviews of Environmental Contamination and Toxicology, 167*, 35–120.

Glusczak, L., dos Santos Miron, D., Crestani, M., Braga da Fonseca, M., de Araujo Pedron, F., Duarte, M. F., & Vieira, V. L. (2006). Effect of glyphosate herbicide on acetylcholinesterase activity and metabolic and hematological parameters in piava (*Leporinus obtusidens*). *Ecotoxicology and Environmental Safety, 65*, 237–241.

Glusczak, L., dos Santos Miron, D., Moraes, B. S., Simões, R. R., Schetinger, M. R., Morsch, V. M., & Loro, V. L. (2007). Acute effects of glyphosate herbicide on metabolic and enzymatic parameters of silver catfish (*Rhamdia quelen*). *Comparative Biochemistry and Physiology C: Toxicology Pharmacology, 146*, 519–524.

Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae, with notes on identification. *Herpetologica, 16*, 183–190.

Govindarajulu, P. P. (2008). *Literature review of impacts of glyphosate herbicide on amphibians: what risks can the silvicultural use of this herbicide pose for amphibians in B.C. (Wildlife report no. R-28) B.C*. Victoria, BC: Ministry of Environment.

Green, R. M., & Abdelghani, A. A. (2004). Toxicity of a mixture of 2,4-dichlorophenoxyacetic acid and monosoduim methanearsonate to the red swamp crawfish, *Procambarus clarkii*. *International Journal of Environmental Research and Public Health, 1*, 35–38.

Guilherme, S., Válega, M., Pereira, M. E., Santos, M. A., & Pacheco, M. (2008). Erythrocytic nuclear abnormalities in wild and caged fish (*Liza aurata*) along an environmental mercury contamination gradient. *Ecotoxicology and Environmental Safety, 70*, 411–421.

Gupta, C. R. (2009). *Handbook of toxicology of chemical warfare agents*. London: Academic Press.

Gutterson, N., & Zhang, J. Z. (2004). Genomics applications to biotech traits: a revolution in progress? *Current Opinion in Plant Biology, 7*, 226–230.

Habdous, M., Vincent-Viry, M., Visvikis, S., & Siest, G. (2002). Rapid spectrophotometric method for serum glutathione *S*-transferases activity. *Clinical Chemistry Acta, 326*, 131–142.

Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione *S*-transferases, the first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry, 249*, 7130–7139.

Hamilton, M. A., Russo, R. C., & Thurston, R. V. (1977). Trimmed Spearman–Karber method for estimating median lethal concentrations in toxicity bioassays. *Environmental Science and Technology, 11*, 714–719.

Hayes, T. B., Case, P., Chui, S., Chung, D., Haeffele, C., Haston, K., Lee, M., Mai, V. P., Marjuoa, Y., Parker, J., & Tsui, M. (2006). Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? *Environmental Health Perspectives, 114*, 40–50.

Hazarika, A., Sarkara, S. N., Hajare, S., Kataria, M., & Malik, J. K. (2003). Influence of malathion pretreatment on the toxicity of anilofos in male rats: a biochemical interaction study. *Toxicology, 185*, 1–8.

Hertzberg, R., & MacDonell, M. (2002). Synergy and other ineffective mixture risk definitions. *Science of the Total Environmental, 288*, 31–42.

Howe, G. E., Gillis, R., & Mowbray, R. C. (1998). Effect of chemical synergy and larval stage on the toxicity of atrazine and alachlor to amphibian larvae. *Environmental Toxicology and Chemistry, 17*, 519–525.

IUCN (2010). IUCN Red List of Threatened Species. Version 2010.4. Available at http://www.iucnredlist.orgUH. Accessed 27 Oct 2010.

Jayawardena, U. A., Navaratne, A. N., Amerasinghe, P. H., & Rajakaruna, R. S. (2011). Acute and chronic toxicity of four commonly used agricultural pesticides on the Asian common toad, *Bufo melanostictus* Schneider. *The Journal of National Science Foundation of Sri Lanka, 39*, 267–276.

Johnson, C. R. (1976). Herbicide toxicities in some Australian anurans and the effect of subacute dosages on temperature tolerance. *Zoology Journal of the Linnean Society, 59*, 79–83.

Jonsson, C. M., & Maia, A. H. N. (1998). Toxicidade aguda do herbicida clomazone no peixe *Hyphessobrycon scholzei*: avaliacao da concentracao letal mediana e de alteracoes no conteudo de nutrientes. *Pesticidas: Revista de Ecotoxicologia e Meio Ambiente, 8*, 101–110.

Junghans, M., Backhaus, T., Faust, M., Scholze, M., & Grimme, L. H. (2006). Application and validation of approaches for the predictive hazard assessment of realistic pesticide mixtures. *Aquatic Toxicology, 76*, 93–110.

Kingbley, G. R. (1942). The direct Biuret method for the determination of serum proteins as applied to photoelectric and visual calorimetry. *The Journal of Laboratory and Clinical Medicine, 27*, 840–845.

Kundu, C. R., & Roychoudhury, S. (2009). Malathion-induced sublethal toxicity on the hematology of cricket frog (*Fejervarya limnocharis*). *Journal of Environmental Science and Health. Part. B, 44*, 673–680.

Laetz, C. A., Baldwin, D. H., Collier, T. K., Hebert, V., Stark, J. D., & Scholz, N. L. (2009). The synergistic toxicity of pesticide mixtures: implications for risk assessment and the conservation of endangered Pacific salmon. *Environmental and Health Perspective, 117*, 348–353.

Lajmanovich, R. C., Cabagna, M., Peltzer, P. M., & Stringhini, G. (2005). Micronucleus induction in erythrocytes of the *Hyla pulchella* tadpoles (Amphibia: Hylidae) exposed to insecticide endosulfan. *Mutation Research, 587*, 67–72.

Lajmanovich, R. C., Attademo, A. M., Peltzer, P. M., Junges, C. M., & Cabagna, M. (2011). Toxicity of four herbicide formulations with glyphosate on *Rhinella arenarum* (Anura: Bufonidae) tadpoles: B-esterases and glutathione *S*-transferase inhibitors. *Archives of Environmental Contamination and Toxicology, 60*, 681–689.

Margolin, B. H., Collings, B. J., & Mason, J. M. (1983). Statistical analysis and sample-size determinations for mutagenicity experiments with binomial responses. *Environmental Mutagenesis, 5*, 705–716.

Marking, L. L. (1985). Toxicity of chemical mixtures. In G. M. Rand & S. R. Petrocelli (Eds.), *Fundamentals of Aquatic Toxicology* (pp. 164–176). New York: Hemisphere Publishing Corporation.

Marques, S. M., Antunes, S. C., Pissarra, H., Pereira, M. L., Gonçalves, F., & Pereira, R. (2009). Histopathological changes and erythrocytic nuclear abnormalities in Iberian green frogs (*Rana perezi* Seoane) from a uranium mine pond. *Aquatic Toxicology, 91*, 187–195.

Modesto, K. A., & Martinez, C. B. (2010). Effects of Roundup Transorb on fish: hematology, antioxidant defenses and acetylcholinesterase activity. *Chemosphere, 81*, 781–787.

Monsanto Company (2002). Monsanto, Glyphosate and wildlife, Backgrounder. URL<HUhttp://www.monsanto.com/monsanto/content/products/productivity/roundup/gly_wildlife_bkg.pdfUH> Accessed 30 Dec 2010.

Moraes, B. S., Loro, V. L., Glusczak, L., Pretto, A., Menezes, C., Marchezan, E., & de Oliveira Machado, S. (2007). Effects of four rice herbicides on some metabolic and toxicology parameters of teleost fish (*Leporinus obtusidens*). *Chemosphere, 68*, 1597–1601.

Moraes, B. S., Clasen, B., Loro, V. L., Pretto, A., Toni, C., de Avila, L. A., Marchesan, E., Machado, S. L., Zanella, R., & Reimche, G. B. (2011). Toxicological responses of *Cyprinus carpio* after exposure to a commercial herbicide containing imazethapyr and imazapic. *Ecotoxicology and Environmental Safety, 74*, 328–335.

O'Sullivan, P. A., & Kossatz, V. C. (1982). Influence of picloram on *Cirsium arvense* (L.) Scop, control with glyphosate. *Weed Research, 22*, 251–256.

Otitoju, O., & Onwurah, I. N. E. (2007). Glutathione *S*-transferase (GST) activity as a biomarker in ecological risk assessment of pesticide contaminated environment. *African Journal of Biotechnology, 6*, 1455–1459.

Owen, M. D. K. (2011). Herbicide Guide for Iowa Corn and Soybean Production. Iowa State University Extension Weed Science-94, 2010. Available at: www.weeds.iastate.edu.

Partearroya, M. A., Pilling, S. J., & Jones, M. N. (1991). The lysis of isolated fish (*Oncorhynchus mykiss*) gill epithelial-cells by surfactants. *Comparative Biochemistry and Physiology. C, 100*, 381–388.

Peltzer, P. M., & Lajmanovich, R. C. (2007). Amphibians. In M. H. Iriondo, J. C. Paggi, & M. J. Parma (Eds.), *The Middle Paraná River: limnology of a subtropical wetland* (pp. 327–340). New York, USA: Springer Publishing.

Peltzer, P. M., Lajmanovich, R. C., Attademo, A. M., & Beltzer, A. H. (2006). Diversity of anurans across agricultural ponds in Argentina. *Biodiversity and Conservation, 15*, 3499–3513.

Peltzer, P. M., Lajmanovich, R. C., Sánchez-Hernández, J. C., Cabagna, M., Attademo, A. M., & Bassó, A. (2008). Effects of agricultural pond eutrophication on survival and health status of *Scinax nasicus* tadpoles. *Ecotoxicology and Environmental Safety, 70*, 185–197.

Pezzementi, L., Nachon, F., & Chatonnet, A. (2011). Evolution of acetylcholinesterase and butyrylcholinesterase in the

vertebrates: an atypical butyrylcholinesterase from the Medaka *Oryzias latipes*. *PLoS One, 6*, e17396.

Pratap, S., & Mashiat, A. (2004). Efficacy of metsulfuron-methyl on weeds in wheat and its residual effects on succeeding soybean crop grown on vertisols of Rajasthan. *Indian Journal of Weed Science, 36*, 34–37.

Pretto, A., Loro, V. L., Menezes, C., Moraes, B. S., Reimche, G. B., Zanella, R., & de Ávila, L. A. (2011). Commercial formulation containing quinclorac and metsulfuron-methyl herbicides inhibit acetylcholinesterase and induce biochemical alterations in tissues of *Leporinus obtusidens*. *Ecotoxicology and Environmental Safety, 74*, 336–341.

Relyea, R. A. (2006). The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities: response. *Ecological Applications, 16*, 2027–2034.

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

Relyea, R. A., Schoeppner, N. M., & Hoverman, J. T. (2005). Pesticides and amphibians: the importance of community context. *Ecological Applications, 15*, 1125–1134.

Rodrigues, B. N., & Almeida, F. S. (1998). *Guia de herbicidas*. Paraná, Brazil: Londrina.

Rossi, S. C., Dreyer da Silva, M., Piancini, L. D., Oliveira Ribeiro, C. A., Cestari, M. M., & Silva de Assis, H. C. (2011). Sublethal effects of waterborne herbicides in tropical freshwater fish. *Bulletin of Environmental Contamination and Toxicology, 87*, 603–607.

Salbego, J., Pretto, A., Gioda, C. R., de Menezes, C. C., Lazzari, R., Radünz Neto, J., Baldisserotto, B., & Loro, V. L. (2010). Herbicide formulation with glyphosate affects growth, acetylcholinesterase activity, and metabolic and hematological parameters in piava (*Leporinus obtusidens*). *Archives of Environmental Contamination and Toxicology, 58*, 740–745.

Saltmiras, D., Bus, J. S., Spanogle, T., Hauswirth, J., Tobia, A., & Hill, S. (2011). Letter to the editor regarding the article by Paganelli, A., Gnazzo, V., Acosta, H., López, S. L., & Carrasco, A. E., 2010. *Chemical Research in Toxicology, 24*, 607–608.

Schmajuk, N. A., & Segura, E. T. (1982). Behavioral regulation of water balance in the toad. *Herpetologica, 38*, 296–301.

Semino, S. (2008). Can certification stop high soy pesticide use? *Pesticides News, 82*, 9–11.

Shaner, D. L. (2000). The impact of glyphosate-tolerant crops on the use of other herbicides and on resistance management. *Pest Management Science, 56*, 320–326.

Solomon, K. R., & Thompson, D. G. (2003). Ecological risk assessment for aquatic organisms from over-water uses of glyphosate. *Journal of Toxicology and Environmental Health, Part B, 6*, 289–324.

Song, S. B., Xu, Y., & Zhou, B. S. (2006). Effects of hexachlorobenzene on antioxidant status of liver and brain of common carp (*Cyprinus carpio)*. *Chemosphere, 65*, 699–706.

Sparling, D. W., & Fellers, G. (2007). Comparative toxicity of chlorpyrifos, diazinon, malathion and their oxon derivatives to larval *Rana boylii*. *Environmental Pollution, 147*, 535–539.

Stone, R. (2007). Epidemiology. Agent Orange's bitter harvest. *Science, 315*, 176–179.

Streibig, J. C., & Jensen, J. E. (2000). Action of herbicides in mixtures. In A. H. Cobb & R. C. Kirkwood (Eds.), *Herbicides and their mechanisms of action* (pp. 153–180). Sheffield, UK: Sheffield Academic Press Ltd.

Swanton, C. J., Shrestha, A., Chandler, K., & Deen, W. (2000). An economic assessment of weed control strategies in no-till glyphosate-resistant soybean (*Glycine max*). *Weed Technology, 14*, 755–763.

Teubal, M. (2009). Expansión del modelo sojero en la Argentina. De la producción de alimentos a los commodities. In P. Lizarraga & C. Vacaflores (Eds.), *La persistencia del campesinado en América Latina* (pp. 161–197). Tarija, Bolivia: Comunidad de Estudios JAINA.

Thompson, D. G., Solomon, K. R., & Wojtaszek, B. F. (2006). The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. *Ecological Applications, 16*, 2022–2027.

Toni, C., de Menezes, C. C., Loro, V. L., Clasen, B. E., Cattaneo, R., Santi, A., Pretto, A., Zanella, R., & Leitemperger, J. (2010). Oxidative stress biomarkers in *Cyprinus carpio* exposed to commercial herbicide bispyribac-sodium. *Journal of Applied Toxicology, 30*, 590–595.

Uchida, M., Takumi, S., Tachikawa, K., Yamauchi, R., Goto, Y., Matsusaki, H., Nakamura, H., Kagami, Y., Kusano, T., & Arizono, K. (2012). Toxicity evaluation of glyphosate agrochemical components using Japanese medaka (*Oryzias latipes*) and DNA microarray gene expression analysis. *The Journal of Toxicology Science, 37*, 245–254.

Udroiu, I. (2006). The micronucleus test in piscine erythrocytes. *Aquatic Toxicology, 79*, 201–204.

Valbonesi, P., Sartor, G., & Fabbri, E. (2003). Characterization of cholinesterase activity in three bivalves inhabiting the North Adriatic Sea and their possible use as sentinel organisms for biosurveillance programmes. *Science of the Total Environmental, 312*, 79–88.

Valverde, B. E. (2007). Status and management of grass-weed herbicide resistance in Latin America. *Weed Technology, 21*, 310–323.

Vencill, W. K. (2002). *Herbicide handbook* (8th ed.). Lawrence, KS: Weed Science Society of America.

Vera Candioti, J., Natale, G. S., Soloneski, S., Ronco, A. E., & Larramendy, M. L. (2010). Sublethal and lethal effects on *Rhinella arenarum* (Anura, Bufonidae) tadpoles exerted by the pirimicarb-containing technical formulation insecticide Aficida. *Chemosphere, 78*, 249–255.

Verbruggen, E. M. J., & Van den Brink, P. J. (2010). *Review of recent literature concerning mixture toxicity of pesticides to aquatic organisms*. Bilthoven, Netherlands: RIVM report/National Institute for Public Health and the Environment (601400001/2010).

Vidal, R. A., Rainero, H. P., Kalsing, A., & Trezzi, M. M. (2010). Prospección de las combinaciones de herbicidas para prevenir malezas tolerantes y resistentes al glifosato. *Planta Daninha, 28*, 159–165.

Wharfe, J. (2004). Hazardous chemicals in complex mixtures — A role for direct toxicity assessment. *Ecotoxicology, 13*, 413–421.

WHO/FAO (1996). *Data sheets on pesticides*. World Health Organization/Food and Agriculture Organization. No 91, Glyphosate, WHO/PCS/DS/96.91.

Wylie, P. L. (2006). Screening for 926 pesticides and endocrine disruptors by GC/MS with deconvolution reporting software and a new pesticide library. Agilent Application note 5989–5076.