Bull Environ Contam Toxicol (2012) 88:15–19
DOI 10.1007/s00128-011-0463-0

Effects of Pesticide Formulations and Active Ingredients on the Coelenterate *Hydra attenuata* (Pallas, 1766)

Pablo M. Demetrio · Gustavo D. Bulus Rossini · Carlos A. Bonetto · Alicia E. Ronco

Received: 16 February 2011 / Accepted: 3 November 2011 / Published online: 18 November 2011

Abstract Lethal effects of active ingredients and formulations of widely used soybean pesticides were assessed with the *Hydra attenuata* toxicity test. Studied pesticides were insecticides chlorpyrifos and cypermethrin, and herbicide glyphosate. Results indicate the following toxicity trend: chlorpyrifos > cypermethrin > glyphosate. Tested active ingredients of insecticides and respective formulations did not significantly differ between them. Glyphosate formulation exhibited higher toxicity at low concentrations ($LC_{1–10}$) respect to active ingredient, reversing this behavior at higher concentrations ($LC_{50–90}$). Comparing *H. attenuata* sensitivity with existent toxicity data for aquatic organisms indicates that this species is poorly sensitive to tested insecticides and highly sensitive to the herbicide.

Keywords Herbicides · Insecticides · Toxicity · *Hydra*

Biotech crops have been increasing around the world, transgenic—soybean being one of the most extensively farmed. The United States, Brazil, Argentina, China, and India, in this order, are the five main producing countries in the world of the Roundup®-resistant (RR) variety of soybean. It is managed by a direct seeding technique using formulations of the obliged herbicide glyphosate as a weed killer (Bindraban et al. 2009) and several insecticides to control different insect pests. The principal insecticides used in Argentina are formulations of cypermethrin and chlorpyrifos, followed by endosulfan (CASAFE 2008). There are a few reports showing concentrations of concern in aquatic environments of agroecosystems in the region (Jergentz et al. 2005; Marino and Ronco 2005; Peruzzo et al. 2008; Ronco et al. 2008; Agostini et al. 2009).

In the present study we assessed the toxicity of the most widely used soy bean pesticides to the aquatic invertebrate *Hydra attenuatta*, a sensitive organism to different type of toxicants (Blaise and Kusui 1997; Trottier et al. 1997; Ronco et al. 2002). Reports on the biological effects of pesticides on hydra are scarce (USEPA 2007). This test has been intercalibrated within the WaterTox exercise (Forget et al. 2000; Ronco et al. 2002), with the participation of our laboratory among other six, being the test with hydra the one providing very good sensitivity and best certainty in toxicity assignment of samples within the battery of tests used in the program (Ronco et al. 2002). The simple primary structure of hydra comprising only an endoderm and ectoderm favor the exchange of substances with the media. Its small size, easiness of culture and high reproduction rate, supports the use of this organism in toxicity testing (Blaise and Kusui 1997). Additionally, the wide distribution of the genus *Hydra* in fresh water environments and the place in the food chain (secondary consumer), also are bioecological reasons sustaining the choice of this organism in the present study.

The investigation of the differential effects of active ingredients (a.i.) and formulations of pesticides in aquatic ecotoxicology in most cases indicates higher toxicity of formulations with respect to the active ingredient on non-target plants and animal species (Giesy et al. 2000; Tsui and

P. M. Demetrio · G. D. Bulus Rossini · A. E. Ronco (✉)
Centro de Investigaciones del Medio Ambiente, Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, CONICET. Calle 47 y 115, La Plata (1900), Buenos Aires, Argentina
e-mail: cima@quimica.unlp.edu.ar

C. A. Bonetto
Instituto de Limnología Dr. Raúl A Ringuelet, Universidad Nacional de La Plata, CONICET. Av. Calchaquí km 23.5, Florencio Varela (1888), Buenos Aires, Argentina

Chu 2003; Martin and Ronco 2006; Sobrero et al. 2007; Pereira et al. 2009). The objective of the present study is to assess and compare the acute effects on *H. attenuata* exposed to the active ingredients and commercial formulations of glyphosate, cypermethrin, and chlorpyrifos.

Materials and Methods

Hydra attenuata was obtained from the Watertox Bioassays Program of the International Development Research Center of Canada (Forget et al. 2000). Hydra was maintained and grown in glass bowls containing 0.5 L of Hydra medium at 20 ± 1°C with a 16 h light and 8 h dark photoperiod, following a procedure adapted from Trottier et al. (1997).

Acute toxicity tests with individual toxicants (active ingredients and formulations) were performed with *H. attenuata* according to Trottier et al. (1997), in quadruplicate using a minimum of five concentrations of each toxicant in culture water as dilution media. Tests were done in multiplates of 3 mL wells, exposing three organisms per well during 96 h with no renewal of test solutions. Temperature and light conditions were 21 ± 2°C and 16:8 L:D, respectively. Assessed end points were lethality (irreversible malformation like the tulip stage and the disintegration of organisms). The acceptability criterion of the tests was normal morphology appearance in all organisms of negative controls. Routine sensitivity controls were performed using Cr(VI) as the reference toxicant prepared from the salt $K_2Cr_2O_7$ (Sigma-Aldrich Analytical Reagent). Tests with insecticides as the active ingredient were done by adding ethanol (0.5%v/v) to the test medium due to the low solubility of these toxicants. Negative controls were done with and without solvent.

The insecticide formulations Glextrin® (250 mg/L of cypermethrin isomers mix) and PirfosGlex® (480 mg/L of chlorpyrifos), the active ingredients, and the glyphosate (Technical Grade) were obtained from Gleba S.A. (Buenos Aires, Argentina), and the herbicide Roundup® Max (74.4% glyphosate) was obtained from Monsanto S.A. (St. Louis, Missouri, USA).

Pesticide stocks solutions (100 and 25 mg/L, for the herbicide and insecticides, respectively) were done in distilled water and used immediately to prepare test dilutions in testing water. Concentrations in stocks were measured by chromatographic methods (HPLC–MS for the herbicide, and GC-ECD for the insecticides). HPLC–MS was performed in an Agilent Technologies Inc., USA, equipped with a binary pump and a diode array detector, and coupled with an MSD VL quadrupole with an electrospray ionization interface. The chromatographic separations (mobile phases, acetonitrile/water with 20 mm ammonium acetate, 50:50) were performed on a C-18 reverse-phase column (4 mm × 150 mm; 3 μm pore size). Analysis by GC-ECD were carried in a Carlo Erba Company; 15 m HP5 column (Agilent), 0.53 mm × 30 m, with a particle size of 1.5 μm, using with N_2 as a carrier, according to Marino and Ronco (2005). Verification of the maximum and minimum concentrations in testing dilutions (n = 4 per treatment) of each pesticide was also performed at the beginning and end of exposure. The method used in measuring the insecticides in the test dilutions were the same as the one for analyzing stock solutions. The analysis of glyphosate concentrations in test dilutions was done previous derivatization with 9-fluoroenylmethyl chloroformate chloride (FMOC) by liquid chromatography (Peruzzo et al. 2008) using HPLC (Beckman, System Gold 126) and a Supelco RP 18 column (4 mm × 250 mm) with 5 μm particle size and UV detection (206 nm). J. T. Baker solvents for pesticide analysis were used. Standards of glyphosate, cypermethrin, and chlorpyrifos used for chemical analysis were from SENASA (Argentine National Service for Food Sanitation Quality). Methods were subject to strict quality assurance and control procedures. For each set of samples a procedural blank and a matrix sample spiked with standards were used to determine the accuracy. The whole treatment recoveries were over 75% for all the tested compounds.

Calculation of $LC_{1;5;10;15;50;85}$ values was done using probit model (Finney 1971) with a specific software (Probit USEPA version 1.5). Slope and elevation comparisons were performed to assess differences in toxicity between the active compounds and the formulations of each pesticide (Zar 1998).

Results and Discussion

Measured concentrations (mg/L) in stock solutions of each formulation and a.i were, respectively: 98.3 ± 1.0 and 97.8 ± 1.1 of glyphosate; 24.0 ± 1.0 and 22.7 ± 1.4 of cypermethrin; 25.6 ± 0.8 and 25.3 ± 0.9 of chlorpyrifos. Tests were carried within the following measured concentration ranges: 13.7–36.1 and 15.8–22.2 for glyphosate; 7.4–14.2 and 7.5–19.7 for cypermethrin; 0.3–1.8 and 0.6–5.5 for chlorpyrifos, for each formulation or active ingredient, respectively. A maximum of 20% decay in concentration of each tested pesticide during 96 h testing period was detected.

Results of toxicity tests with *H. attenuata* for each formulation and active ingredient ($LC_{1;5;10;15;50;85}$ values) are shown in Table 1. Regression and correlation analyses for the log-probit model of formulation versus active ingredients are shown in Table 2. Only glyphosate and Roundup® Max showed significant differences among the slopes.

Table 1 Acute toxicity data for active ingredients and formulations

LC_x	Glyphosate formulation	Glyphosate a.i.	Cypermethrin formulation	Cypermethrin a.i.	Chlorpyrifos formulation	Chlorpyrifos a.i.
1	10.7 (7.1–13.3)	14.8 (12.6–15.9)	6.8 (4.8–7.8)	6.0 (3.7–7.6)	0.4 (0.2–0.5)	0.4 (0.2–0.6)
5	13.3 (9.7–15.6)	15.7 (13.9–16.6)	7.6 (5.8–8.5)	7.7 (5.3–9.2)	0.5 (0.3–0.6)	0.6 (0.3–0.8)
10	14.8 (11.4–17.0)	16.2 (14.7–17.0)	8.0 (6.5–8.9)	8.6 (6.5–10.2)	0.6 (0.4–0.7)	0.7 (0.4–0.9)
15	15.9 (12.8–18.1)	16.6 (15.2–17.3)	8.4 (7.0–9.2)	9.4 (7.3–10.9)	0.7 (0.5–0.8)	0.8 (0.5–1.1)
50	21.8 (19.5–24.2)	18.2 (17.4–18.9)	9.9 (9.0–10.7)	13.5 (11.9–15.5)	1.0 (0.8–1.2)	1.5 (1.2–1.8)
85	29.9 (26.7–36.3)	20.0 (19.5–22.2)	11.8 (10.9–13.5)	19.4 (16.7–25.5)	1.4 (1.2–2.0)	2.6 (2.1–3.7)

Results correspond to 96 h exposure and are expressed in mg/L of a.i. The 95% CI are given between parentheses

Table 2 Regression and correlation parameters of concentration response plots

Regression parameters	Glyphosate		Cypermethrin		Chlorpyrifos	
	Form	a.i.	Form	a.i.	Form	a.i.
b	7.58	25.86	13.96	6.63	6.35	4.32
a	−5.16	−27.59	−8.92	−2.51	5.05	8.60
n	7	5	5	6	5	7
r	0.89	0.95	0.97	0.93	0.96	0.94
r^2	0.80	0.91	0.94	0.87	0.92	0.88

form formulation, *a.i.* active ingredient

According to the results (LC_{50} values), the following order of toxicity for the tested pesticides is chlorpyrifos > cypermethrin > glyphosate. When each pesticide formulation is compared with its respective a.i., the ratios between observed effects for both forms in different points of the curves are not constant (Fig. 1). The three tested pesticides at low percentages of effect exhibit no differences (compared across the limits of confidence) between formulation and a.i.; but at high percentages of mortality (≥50%) differences are observed (Table 1). For the case of the cypermethrin and the chlorpyrifos the data do not present sufficient evidence to endorse the hypothesis of a differential toxicity between the formulation versus a.i. since the comparison of slopes and origins do not show significant differences ($\alpha = 0.05$). In the case of the glyphosate statistical significant differences ($\alpha = 0.05$) are observed for the slope, that is higher for the a.i. (Table 3).

The sensitivity of *H. attenuata* to the studied toxicants was compared with toxicity intervals of LC_{50} values for a set of selected freshwater species obtained from USEPA (2007). These data were organized by species giving 150 values for chlorpyrifos, 66 for cypermethrin, and 36 for glyphosate. Toxicity database intervals for the 10th and 90th percentiles of each compound were 0.35–853.87 μg/L for chlorpyrifos, 0.06–373.94 μg/L for cypermethrin, and 6,200–673,430 μg/L for glyphosate; indicating that sensitivity of *H. attenuata* to both insecticides is low (above the 90th percentile), and very high to the herbicide (below the 20th percentile).

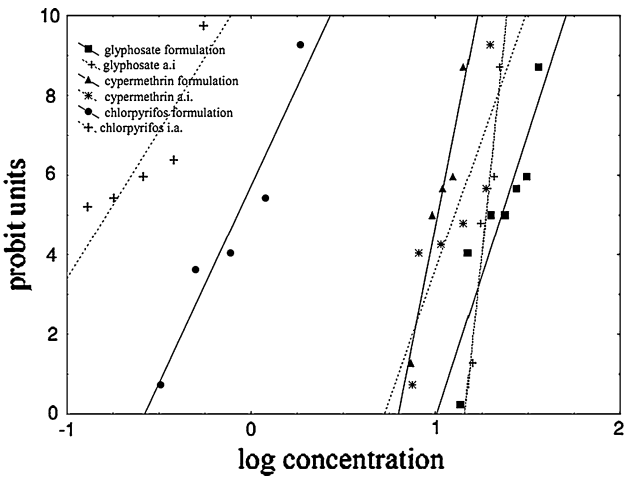

Fig. 1 Concentration response plots of active ingredients and formulations

Analysis of toxicity data for active ingredients and formulations of insecticides suggests no interactions between a.i. and their respective coadjuvants. Exposure to toxicants in hydra occurs without selective barriers like specialized epithelium tissues. These organisms have a simple anatomical and physiological structure (Berking 2003; Miljkovic-Licina et al. 2004). Hence, the presence of coadjuvants to assist toxicant uptake does not seem to be necessary to facilitate absorption. The actual toxicity of coadjuvants seems to be lower than the assessed toxicity for insecticide active ingredients, with no detectable

Table 3 Regression and correlation analysis for comparisons

Formulated versus a.i.	Glyphosate	Cypermethrin	Chlorpyrifos
Dif. among slopes	2.79	1.51	0.44
Critical value	2.31	2.36	2.31
Null hypothesis $b_1 = b_2$	R	A	A
Dif. among elevations	0.65	1.73	1.8
Critical value	2.26	2.31	2.26
Null hypothesis $a_1 = a_2$	A	A	A

Dif differences, *a.i.* active ingredient, *R* reject, *A* accept

additional contribution to formulation effect. Furthermore, the low sensitivity of *H. attenuata* to both of the tested insecticides, inhibiting acetylcholinesterase activity or interrupting impulse transmission along the axon (Stenersen 2004), could be due to the simple organization of these organisms, lacking a developed nervous system to centralize vital functions, consequently leading to a lower impact when compared with organisms having specialized or very specialized systems.

The toxicity of glyphosate has been reported for a few non-photosynthetic and photosynthetic organisms such as bacteria, protozoans, insects, fish, amphibians, algae and vascular plants (Tsui and Chu 2003; Martin and Ronco 2006; Ronco et al. 2007; Sobrero et al. 2007; USEPA 2007; Schneider et al. 2009). These reports indicate a toxicity pattern from higher toxicity in low complexity organisms (bacteria through hydra) and photosynthetic ones, and low toxicity in fish and amphibians. This fact contributes to the hypothesis that the effect of the herbicide active ingredient on the metabolic pathway of hydra would be different than that corresponding to other, more complex organisms. The shikimic acid metabolic pathway is assumed to be absent in animals. However (Starcevic et al. 2008) recently reported the presence of genes associated with the enzymes required in the shikimic pathway in marine cnidarians, suggesting an explanation to the observed toxicity of glyphosate to hydra.

We conclude that effects of formulation and active ingredients of cypermethrin and chlorpyrifos on *H. attenuata* do not differ. With glyphosate, higher and significant effects were detected for the formulation at lower concentrations, with a reversal of the behavior at higher concentrations. The general pattern of the biological effects of the studied compounds on the coelenterate is different from that observed for other animal species belonging to higher organization levels. Effects of the insecticides are low on *H. attenuata*, with this species being very sensitive to the herbicide.

Acknowledgments Funds for this study were from ANPCyT Project PICT-38350, Argentinean Ministry of Science and Technology. The authors wish to thank Dr. Damián Marino for chemical analysis of samples and Courtney Knipp for editorial suggestions.

References

Agostini MG, Natale GS, Ronco AE (2009) Impact of endosulfan and cypermethrin mixture on amphibians under field use for biotech soya bean production. Int J Environ Health 3:379–389

Berking S (2003) A model for budding in hydra: pattern formation in concentric rings. J Theor Biol 222:37–52

Bindraban PS, Franke AC, Ferraro DO, Ghersa CM, Lot LAP, Nepomuceno A, Smulders MJM, van de Wiel CCM (2009) GM related sustainability: agroecological impacts, risks and opportunities of soy production in Argentina and Brazil. Plant Research International, Wageningen UR

Blaise C, Kusui T (1997) Acute toxicity assessment of industrial effluents with a microplate-based Hydra attenuata assay. Environ Toxicol Water Qual 12:53–60

CASAFE (2008) Cámara de Sanidad Agropecuaria y Fertilizantes, Buenos Aires, Argentina. Available from http://www.casafe.org. Accessed Dec 2010

Finney DJ (1971) Probit analysis. Cambridge University Press, London

Forget G, Gagnon P, Sanchez WA, Dutka BJ (2000) Overview of methods and results of the eight international development research centre (IDRC) WaterTox project. Environ Toxicol 15: 264–276

Giesy JP, Dobson S, Solomon KR (2000) Ecotoxicological risk assessment for Roundup herbicide. Rev Environ Contam Toxicol 167:35–120

Jergentz S, Mugni H, Bonetto C, Schulz R (2005) Assessment of insecticide contamination in runoff and stream water of small agricultural streams in the main soybean area of Argentina. Chemosphere 61:817–826

Marino D, Ronco A (2005) Cypermethrin and chlorpyrifos concentration levels in surface water bodies of the Pampa Ondulada, Argentina. Bull Environ Contam Toxicol 75:820–826

Martin ML, Ronco A (2006) Effects of mixtures of pesticides used in the direct seeding technique on non-target plant seeds. Bull Environ Contam Toxicol 77:228–236

Miljkovic-Licina M, Gauchat D, Galliot B (2004) Neuronal evolution: analysis of regulatory genes in a first-evolved nervous system, the hydra nervous system. BioSystems 76:75–87

Pereira JL, Antunes SC, Castro BB, Marques CR, Gonçalves AM, Gonçalves F, Pereira R (2009) Toxicity evaluation of three pesticides on non-target aquatic and soil organisms: commercial formulation versus active ingredient. Ecotoxicology 18:455–463

Peruzzo P, Porta A, Ronco A (2008) Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. Environ Pollut 156:61–66

Ronco A, Gagnon P, Diaz-Baez MC, Arkhipchuk V, Castillo G, Castillo LE, Dutka BJ, Pica-Granados Y, Ridal J, Srivastava RC, Sánchez A (2002) Overview of results from the watertox intercalibration and environmental testing phase II program: part

1, statistical analysis of blind sample testing. Environ Toxicol 17:232–240

Ronco A, Carriquiriborde P, Natale G, Martin M, Mugni H, Bonetto C (2008) Integrated approach for the assessment of biotech soybean pesticides impact on low order stream ecosystems of the Pampasic Region. In: Chen J, Guo C (eds) Ecosystem ecology research. Nova Science, Hauppauge, pp 209–239

Schneider MI, Sanchez N, Pineda S, Chi H, Ronco AE (2009) Impact of glyphosate on the development, fertility and demography of *Chrysoperla externa* (Neuroptera: Chrysopidae): ecological approach. Chemosphere 76:1451–1455

Sobrero MC, Rimoldi F, Ronco AE (2007) Effects of the glyphosate active ingredient and a formulation on *Lemna gibba* L. at different exposure levels and assessment end-points. Bull Environ Contam Toxicol 79:537–543

Starcevic A, Akthar S, Dunlap WC, Shick JM, Hranueli D, Cullum J Long PF (2008) Enzymes of the shikimic acid pathway encoded in the genome of a basal metazoan, *Nematostella vectensis*, have microbial origins. Proc Natl Acad Sci USA 105:2533–2537

Stenersen J (2004) Chemical pesticides: mode of action and toxicology. CRC Press, Florida

Trottier S, Blaise C, Kusui T, Johnson EM (1997) Acute toxicity assessment of aqueous samples using a microplate-based *Hydra attenuata* assay. Environ Toxicol Water Qual 12:265–271

Tsui MTK, Chu LM (2003) Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere 52:1189–1197

USEPA (2007). ECOTOX User Guide: ECOTOXicology Database System. Environmental Protection Agency. Version 4.0. Available: http://www.epa.gov/ecotox/

Zar JH (1998) Biostatistical analysis. Prentice Hall, New Jersey