The Effect of Cypermethrin, Chlorpyrifos, and Glyphosate Active Ingredients and Formulations on Daphnia magna *(Straus)*

Pablo M. Demetrio, Carlos Bonetto & Alicia E. Ronco

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The Effect of Cypermethrin, Chlorpyrifos, and Glyphosate Active Ingredients and Formulations on *Daphnia magna* (Straus)

Pablo M. Demetrio · Carlos Bonetto · Alicia E. Ronco

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Abstract Acute effects of active ingredients (a.i.) and formulations (F) of widely used pesticides were assessed by means of the *Daphnia magna* toxicity test. Studied pesticides were the insecticides, cypermethrin and chlorpyrifos, and the herbicide, glyphosate. Results were analyzed and compared according to statistical endpoints (LCx) and log-probit regressions of toxicity data. The potency of acute toxicity followed the order: chlorpyrifos F > chlorpyrifos a.i. > cypermethrin F > cypermethrin a.i. ≫ glyphosate F > glyphosate a.i. Three to five orders of magnitude differences between the toxicity (µg/L to mg/L) of insecticides and the herbicide were observed. A pairwise comparison between a.i. and F indicated that all formulations were more potent. Additionally, for the case of glyphosate, evidence suggests that the adjuvant contributes to formulation toxicity rather than to the enhancement of a.i. potency.

Keywords Glyphosate · Cypermethrin · Chlorpyrifos · Toxicity · *Daphnia*

The worldwide increased use of land with biotech crops has led to extensive research on the potential impact of this type of productive system on the environment. The genetically modified (GM) soybean cultivation has become a monoculture replacing conventional crops. The main producers are the USA, Brazil, Argentina, China, and India (Bindraban et al. 2009). Most often, the GM soybean that is resistant to the herbicide glyphosate, is grown using the no-tillage technique. Direct seeding requires the use of glyphosate, together with different insecticides, according to the types of pests in each region. The organophosphate, chlorpyrifos, and the pyrethroid, cypermethrin, are widely used insecticides in Argentina (CASAFE 2012). Studies have reported the presence of these compounds in surface waters in the region (Jergentz et al. 2005; Marino and Ronco 2005; Peruzzo et al. 2008; Ronco et al. 2008; Agostini et al. 2009; Mugni et al. 2011), pointing out the need for research on risk assessment to aquatic biota.

This study assessed the toxicity of these pesticides on the micro-crustacean, *Daphnia magna* (Crustacea: Branchiopoda: Cladocera), a widely employed reference organism in laboratory toxicity testing (Joncxyk and Gilron 2005). In our laboratory, the test with *Daphnia* was previously intercalibrated within the WaterTox program (Ronco et al. 2002), and is being routinely used in assessment of toxicants in environmental samples. The generalized used of this test in aquatic ecotoxicology is related to the easy maintenance of the organism under laboratory conditions, in addition to its short life cycle and high reproductive rate. Additionally, daphnids are asexually reproduced, hence reducing heterogeneity due to genetic variability when culturing. Organisms of the genus *Daphnia* belong to the plankton of continental waters (Ruppert and Barnes 1995). The genus is widely represented in Argentina with a dozen species (Adamowicz et al. 2004).

A relevant approach to the toxicity assessment of pesticide formulations (F) should include their ingredients (a.i. and adjuvants). Furthermore, it is worth comparing the data

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of the adverse effects of the a.i. with their formulations. Available information on the differential effects on non-target organisms has shown that the formulation of a pesticide is generally more toxic than its respective a.i. (Giesy et al. 2000; Tsui and Chu 2003; Martin and Ronco 2006; Sobrero et al. 2007; Pereira et al. 2009; Demetrio et al. 2012). The objectives of this study were to determine and compare the acute lethal effects on *D. magna* of three commercial formulations and their respective active ingredients of glyphosate, cypermethrin and chlorpyrifos, thereby contributing data that may result in more rational decisions about these pesticides in their environmental management.

Materials and Methods

Daphnia magna was obtained from the Watertox Bioassays Program of the International Development Research Center of Canada (Ronco et al. 2002). The organisms were maintained and fed in 2 L glass containers with a maximum density of 10 organisms/L, 21 ± 2°C, photoperiod of 16:8 L:D and a light intensity of approximately 800 lux. Culture water had a hardness of 160–180 mg $CaCO_3$/L, pH 7.8 ± 0.2 and dissolved oxygen >6 mg/L.

The tests followed the criteria used by Díaz-Báez et al. (2004). Neonates (<24 h) of *D. magna* were used for 48-h static toxicity tests. Final tests were performed in test tubes of 20 mL, in triplicate, with at least five concentrations of each toxicant in culture water as dilution media, with no feeding. Active ingredients of insecticides were added to the test medium mixed with ethanol (0.5 % v/v) due to the low solubility of these toxicants. Negative controls were done with and without solvent. The assessed endpoint was lethality (=immobilization). The acceptability criterion of the tests was <10 % lethality in negative controls. Routine sensitivity controls were performed using Cr(VI) as the reference toxicant (positive control) prepared from the salt $K_2Cr_2O_7$ (Sigma-Aldrich Analytical Reagent, Ciudad Autónoma de Buenos Aires, Argentina), with the computed LC_{50} of the positive control at the time of definitive tests as a criterion for acceptability (Díaz-Báez et al. 2004).

The insecticide formulations Glextrin® (250 mg/L of cypermethrin mix of isomers) and PirfosGlex® (480 mg/L of chlorpyrifos), the active ingredients, and the glyphosate (Technical Grade) were obtained from Gleba S.A. (BuenosAires, Argentina), and the herbicide Roundup® Max(74.4 % glyphosate) was obtained from Monsanto S.A. (St. Louis, Missouri, USA). Pesticide stock solutions (50 mg/L for the herbicide and 50 µg/L for the insecticides) were prepared in distilled water and used immediately to make test dilutions.

Concentrations in stocks and maximum exposure concentrations were measured by chromatographic methods (HPLC–UV for the herbicide, and GC–ECD for the insecticides). The analysis of glyphosate concentrations was performed following derivatization with 9-fluorenylmethylchloroformate (FMOC) by liquid chromatography (Peruzzo et al. 2008), using HPLC (Beckman, System Gold 126, San Diego, CA, USA) and a Supelco (Bellefonte, PA, USA) RP 18 column (4 mm × 250 mm) with 5 µm particle size and UV detection (206 nm). Analyses by GC–ECD (Carlo Erba, now Thermo Fisher Scientific, Milan, Italy) were carried out at: HP5 column Agilent (Santa Clara, CA, USA), 0.53 mm, 30 m, with a particle size of 1.5 µm, using 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. Solvents for pesticide analysis were from J. T. Baker (Xalostoc, México). Standards of glyphosate, cypermethrin, and chlorpyrifos used for chemical analysis were from SENASA (Argentine National Service for Food Sanitation Quality, Ciudad Autónoma de Buenos Aires, Argentina). 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. Recoveries of spiked samples were 92 %, 88 % and 95 % for cypermethrin, chlorpyrifos and glyphosate, respectively. The limits of detection were 0.025 and 0.01 µg/L for cypermethirn and chlorpyrifos, respectively; and 0.25 mg/L for glyphosate.

Calculations of $LC_{1/5/10/15/50/85}$ values were performed using a probit model (Finney 1971) with software for probit analysis (USEPA version 1.5). Computation of Chi square statistic against tabulated values ($\alpha = 0.05$) was the criterion used to test acceptance of the model. Slope (*b*) and elevation (*a*) for a given number of data (n) from each regression line pair (a.i. *vs* F) were compared by simple linear regression analysis (pairwise comparisons at a confidence of 99.95 %) to assess differences between them (Zar 1998).

Results and Discussion

Measured concentrations of stock solutions for each formulation or a.i. were the following, respectively: 49.2 ± 0.1 and 999.2 ± 0.1 mg/L of glyphosate; 51.0 ± 0.2 and 49.0 ± 1.1 µg/L of cypermethrin; 48.6 ± 0.8 and 51.2 ± 0.3 of chlorpyrifos. Definitive toxicity tests were carried out within the following ranges of measured concentrations: 5.1–15.2 and 49.8–981.8 mg/L of glyphosate, 1.1–8.8 and 0.9–11.5 µg/L of cypermethrin, 0.9–2.2 and

Table 1 48 h LCx values and 95 % confidence intervals for *Daphnia magna* for the studied formulations and a.i. (data obtained using log-probit model). Calculated and tabulated χ^2 values are given ($\alpha = 0.05$)

LC_x	mg/L		µg/L			
	Glyphosate F	Glyphosate a.i.	Cypermethrin F	Cypermethrin a.i.	Chlorpyrifos F	Chlorpyrifos a.i.
1	4.88 (3.89–5.64)	31.3 (18.6–44.0)	0.73 (0.50–0.94)	0.78 (0.51–1.05)	0.02 (0.01–0.04)	0.09 (0.05–0.14)
5	5.90 (4.97–6.61)	53.7 (36.7–69.6)	1.08 (0.82–1.32)	1.24 (0.90–1.55)	0.05 (0.03–0.07)	0.19 (0.12–0.28)
10	6.53 (5.65–7.20)	71.8 (52.6–89.4)	1.34 (1.06–1.58)	1.57 (1.21–1.91)	0.07 (0.05–0.10)	0.29 (0.19–0.40)
15	6.99 (6.16–7.63)	87.3 (66.8–106.1)	1.54 (1.26–1.79)	1.86 (1.48–2.21)	0.10 (0.07–0.12)	0.38 (0.27–0.50)
50	9.34 (8.71–9.95)	199 (170–236)	2.81 (2.49–3.16)	3.73 (3.25–4.29)	0.30 (0.24–0.37)	1.22 (0.98–1.51)
85	12.5 (11.6–13.8)	456 (366–625)	5.12 (4.43–6.23)	7.48 (6.28–9.49)	0.93 (0.70–1.33)	3.86 (2.94–5.54)
90	13.4 (12.3–15.0)	555 (433–796)	5.90 (5.02–7.40)	8.83 (7.26–11.59)	1.21(0.89–1.83)	5.07 (3.74–7.67)
95	14.8 (13.4–17.0)	742 (554–1,143)	7.28 (6.02–9.56)	11.3 (8.97–15.6)	1.80 (1.26–2.95)	7.59 (5.32–12.5)
99	17.9 (15.7–21.7)	1,280 (875–22,700)	10.8 (8.42–15.6)	17.8 (13.24–27.5)	3.80 (2.40–7.28)	16.2 (10.2–31.5)
$\chi^2 calc$	5.76	5.89	6.51	5.74	3.69	5.96
$\chi^2_{0.05}$ *tab*	7.81	9.49	9.49	9.49	11.1	11.1

1.0–5.5 µg/L of chlorpyrifos for each formulation and a.i., respectively. Pesticide concentration did not decay below 25 % of the initial concentrations in any of the testing exposures.

The results of toxicity tests with *D. magna* (LC_1 through LC_{99}) for F (expressed as a.i.) and a.i. can be seen in Table 1. Regression analysis using the log-probit model for each compound is given in Table 2 and Fig. 1. Pairwise comparison between slopes and elevations of the obtained toxicity data are shown in Table 3.

The relative toxicant potency of the studied pesticides to *D. magna* was compared taking into account the concentration–response curve (C–R) and the interval of concentrations inducing a response (Eaton and Klaassen 2001). The following order of potency was detected: chlorpyrifos F > chlorpyrifos a.i. > cypermethrin F > cypermethrin a.i. $\gg$ glyphosate F > glyphosate a.i. Three to five orders of magnitude differences between the potencies (µg/L to mg/L) of insecticides and the herbicide for both F and a.i. were observed. The difference between insecticides and the herbicide can be explained by their distinct modes of action (Stenersen 2004) and the test organism. Only in the case of the herbicide can we reject the hypothesis of similar slopes in the log-probit lines of a.i. and F. The toxicity profile varies with the additional chemistry of the latter. In the insecticides, we cannot observe difference among slopes, but a significant difference between elevations, indicating an increment in the toxicity potency of the formulations.

There are several reports on the toxicity of the studied compounds to aquatic organisms. According to the USEPA database, the general trends are similar to the ones seen in our study. The highest numbers of studies on glyphosate are registered in the following order: fish, amphibians, and crustaceans. Our results of 48 h-LC_{50} with *D. magna* for glyphosate F are within the ranges observed by several authors. Al-Omar and Hassan (2000) obtained a 48 h-LC_{50} for a formulation containing 48 % a.i. of 9.6 and 10.5 mg/L a.i. obtained by the direct and probit methods, respectively. Hatman and Martin (1984) reported a 48 h-LC_{50} value of 7.9 mg/L a.i. Additionally, Alberdi et al. (1996) compared the sensitivity of two daphnid species to a formulation containing 48 % glyphosate; *D. magna* and the South American native species *D. spinulata* had LC_{50} values of 31.8 and 29.6 mg/L a.i., respectively. The authors did not find significant differences between the species or between 24 and 48 h of exposure time. Recently, Curha et al. (2013) reported small variability in the sensitivity, and mostly in the same order of magnitude, to the a.i. and formulations (acute, lethal) to different clones of *D. magna* when assessing different stages of development.

The largest number of LC_{50} values in the literature for insecticides belong to invertebrates, followed by fishes and crustaceans (USEPA 2012). Present results here reported

Table 2 Regression parameters of log-probit lines for *Daphnia magna* 48 h toxicity data of formulations and a.i

	Glyphosate		Cypermethrin		Chlorpyrifos	
	F	i.a.	F	i.a.	F	i.a.
b	10.8	3.91	5.54	4.58	2.53	2.57
a	−5.39	−0.08	19.2	16.2	14.1	12.6
n	5	6	6	6	7	7
r	0.97	0.97	0.98	0.99	0.97	0.97
r^2	0.94	0.94	0.94	0.99	0.95	0.94

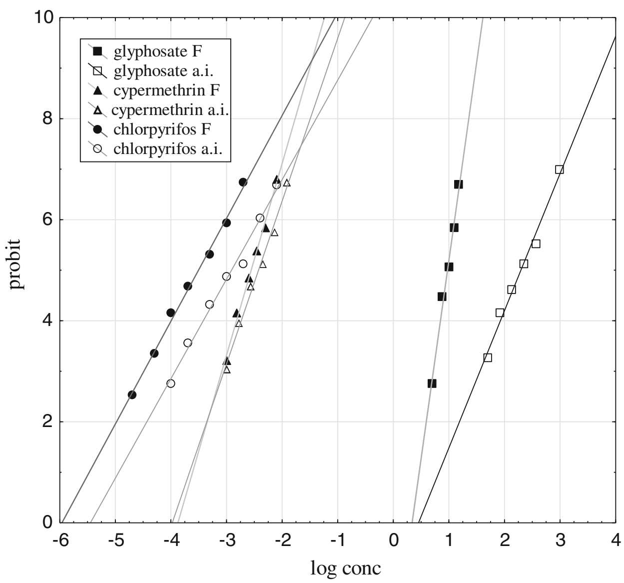

Fig. 1 Log-probit model regression lines for log concentration versus probit values for *Daphnia magna* following 48 h exposures to studied pesticides

Table 3 Results of statistical analysis for pair-wise comparisons of the log-probit regressions with 48 h *Daphnia magna* toxicity data from Table 2

Formulated versus a.i.	Glyphosate	Cypermethrin	Chlorpyrifos
Dif. among slopes	4.47	1.04	0.12
Critical value	2.36	2.31	2.23
Null hypothesis $b1 = b2$	R	A	A
Dif. among elevations	1.15	2.76	8.89
Critical value	2.31	2.26	2.2
Null hypothesis $a1 = a2$	A	R	R

A Accepted, *R* rejected

lie within reported ranges in the literature. Day and Kaushik (1987) informed a LC_{50} range of 0.12–5 µg/L in acute assays of synthetic pyrethroids to cladocerans, and emphasized the sensitivity of the group. Stephenson (1982) reported the LC_{50}-24 h of *D. magna* to cypermethrin a.i. of 2 µg/L, while USEPA (2012) reported values of 1–1.56 µg/L for formulated cypermethrin in acute tests.

Several determinations of chlorpyrifos a.i. LC_{50}-48 h to *D. magna* are available: 1.7 µg/L (USEPA 2012), 1.0 µg/L (Kersting and van Wijngaarden 1992), 0.6 µg/L (Moore et al.1998) and, the lowest, 0.19 µg/L (Kikuchi et al. 2000). Kikuchi et al. (2000) reported that the LC_{50} of chlorpyrifos to *D. magna* was the lowest of 11 assayed organochlorine insecticides, emphasizing its high sensitivity to chlorpyrifos. Several estimations of LC_{50}-48 h are also available for the formulated product: Foster et al. (1998) reported 0.25 µg/L and Diamantino et al. (1997) 0.32–0.34 µg/L. Van Wijngaarden et al. (1993) reported 0.8 µg/L for *D. longispina* and *Simocephalus vetulus.*

D. magna sensitivity was compared with reported values in ECOTOX (USEPA 2012). Only reports on aquatic organisms in acute exposures of $LC_{50} \leq 96$ h were considered. Records amounted to 4,753 for chlorpyrifos, 1,592 for cypermethrin, and 1,771 for glyphosate. Whenever more than one reference was available for the same species, the geometric mean was calculated. In this way available records for aquatic invertebrates were: 180 for chlorpyrifos, 83 for cypermethrin, and 49 for glyphosate. The sensitivity distribution was obtained for each pesticide. Glyphosate was located in the first half of percentiles. The LC_{50} of the formulated product was an order of magnitude lower than for the a.i., suggesting that only part of the toxicity could be attributed to the a.i. In relation with the insecticides, *D. magna* sensitivity to cypermethrin (a.i. and formulation) is in the third quartile, while chlorpyrifos is in the first. Particularly, sensitivity to the chlorpyrifos tested formulation is much higher (first decile) and close to others of this insecticide.

Different commercial formulations are in use for each a.i. It is well known that availability and toxicity of the a.i. is modified by the adjuvant (Schmuck et al. 1994). Several studies have focused on the effects of adjuvants (Mayer et al. 1986; Schmuck et al. 1994; Cox and Surgan 2006). Mayer et al. (1986) compared the toxicity of 48 active ingredients and their commercial formulations by calculating the LC_{50} or EC_{50} quotients (i.e., LC_{50} a.i./LC_{50} F) for 161 pairs of data, assuming that values higher than one reflected greater toxicity for the formulated compound. With this assumption, increased toxicity was observed in 32 % of the studied formulations, while a decreased toxicity was observed in 11 %. Glyphosate was among those compounds evaluated, with its formulation resulting in an increase in toxicity by an order of magnitude. For the case of daphnids, Tsui and Chu (2003) reported two orders of magnitude between the LC_{50} of the a.i. and polyethoxylated tallow amine (POEA) formulated glyphosate, in accordance with our results. In particular, Servizi et al.

(1987) reported an LC_{50}-96 h of glyphosate to *D. pulex* of 25.5 mg/L, while the surfactant POEA yielded an LC_{50} of 3.8 mg/L, pointing to the likely contribution of POEA to the toxicity of glyphosate formulations in which is present.

Schmuck et al. (1994) obtained a data set under standardized laboratory conditions and compared the toxicities between the F and a.i. for several pesticides. There were 44 pairwise comparisons for algae, 61 for daphnids and 145 for fish. Assuming a natural variability in the quotient LC_{50} a.i./LC_{50} F between 0.5 and 2, they considered that the formulated toxicity was higher than that of the a.i. if the quotient was >2. Under this assumption the formulated toxicity was higher in 25 % of the tests with algae and fishes, and in 35 % of the tests with daphnids.

If the Mayer et al. (1986) criterion was adopted to analyze our data (Table 1), all the assayed formulated products induced higher toxicity. Although, according to the criteria of Schmuck et al. (1994), only glyphosate and chlorpyrifos formulations exhibited increased toxicity. Since with both criteria, one single point (LC_{50}) of each C–E (concentration–effect) function for F and a.i. is being considered, the comparison would be valid if the log-probit functions would have been parallel. In our pair comparisons (Tables 2, 3), we took into account the function, slope and y-intercept, and observed that each pair of the tested insecticides met the comparison according to Meyer et al. (1986) and Schmuck et al. (1994; were parallel), but not the herbicide. In the case of Roundup® (a glyphosate formulation), reports indicate that the adjuvant contributes to the formulated toxicity (Tsui and Chu 2003); although, since our log-probit regression lines differ, evidence suggests that different modes of action may be operative in the formulation containing glyphosate, as compared to the a.i alone.

Within the frame of the potential risk of the studied pesticides in the Argentine Pampa, the reported mean and maximum concentrations in regional environments amounted to 0.34–0.90 mg/L glyphosate (n = 15; Peruzzo et al. 2008), 17.26–194 μg/L cypermethrin (n = 12) and 4.05–10.80 μg/L chlorpyrifos (n = 10; Marino and Ronco 2005). A simple hazard quotient approach shows that according to the LC_{50} obtained in this research there is potential environmental risk associated with both tested insecticides (Hazard Quotient >1), but not with the herbicide (Hazard Quotient <1) within the interval of published concentrations.

In view of the reported differences in toxicity between a.i. and formulation for these pesticides, the importance of testing commercial formulations within ecotoxicological studies should be emphasized, since many regulatory thresholds are estimated from the LC_{50} of the a.i. in the formulation (Cox and Surgan 2006). Considering the results of this research, as well as the environmental relevance and actual patterns in the use of pesticides, there is a need for information on the differences in responses between a.i. and formulations in order to avoid underestimating their effect.

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