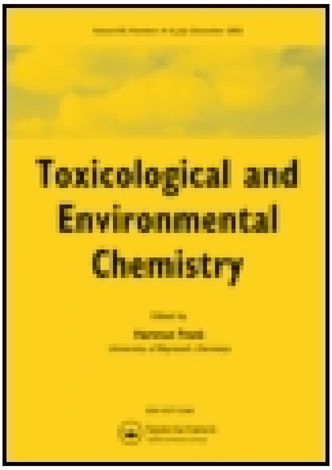

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Glyphosate is the most used pesticide in Argentina. *Hyalella curvispina* is a widely distributed and commonly abundant component of the invertebrate assemblages in shallow waters of southern South America. The aim of this study was to assess the acute toxicity of the increasingly common Roundup Full II®, commercial formulation of the herbicide glyphosate (66.2% active ingredient), to *H. curvispina* in laboratory and field assessments. The mean estimated 48-h LC_{50} of Roundup was 9.9 ± 1.7 mg L^{-1}. In a field experiment Roundup was applied to soybean plots. Simulated rain was generated the following day by means of irrigation sprinkler equipment. *H. curvispina* was exposed to runoff water and soy leaves. No mortality was observed. It is suggested that Roundup crop applications represent a low risk of acute toxicity to *H. curvispina* adults inhabiting water bodies adjacent to crop fields.

Keywords: acute toxicity; herbicide; Roundup; amphipod

1. Introduction

The Argentine Pampa is an extensive plain with a mild climate and fertile soils originally covered by grasslands. For a long time, farmers employed a mixed system of livestock and crop production, mainly wheat and corn. Soy was not a common crop until 1996, when the genetically modified soybean resistant to glyphosate was introduced into the Argentine market and fast adopted by farmers. Soy production has steadily increased since then to represent at present roughly one-half both of the total harvest and of the cultivated area in Argentina (53 million tons and 18 million ha, respectively; FAO 2012). Argentina is the third largest Roundup-resistant (RR) soybean producer after the USA and Brazil. In South America, soy is widespread in Brazil, Argentina, Uruguay, Paraguay and Bolivia (Bindraban et al. 2009). Glyphosate is the most used herbicide in Argentina (CASAFE 2012). Glyphosate consumption has expanded from 12 million liters in 1996 to 200 million liters at present (Aparicio et al. 2013). Glyphosate is a broad spectrum, nonselective herbicide extensively used for weed control. Glyphosate is not only used for soybean production. It is also used on other crops such as corn and cotton and for chemical fallow (Mugni 2009; Potter et al. 2011; Osterberg et al. 2012). Glyphosate is applied in Argentina at doses of 1.6–4 liters per hectare 2–3 times per growing season. Repeated herbicide applications in the field may represent a risk to adjacent surface waters. However, the environmental impact of such agricultural intensification remains largely unreported. Only two studies report glyphosate concentrations in Argentine surface water bodies. Peruzzo, Porta, and Ronco (2008) studied a first order stream surrounded by soy

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crops and four rivers draining intensively cultivated areas in the northeast of Buenos Aires province. Glyphosate was detected in 90% of the water samples. Aparicio et al. (2013) surveyed 44 streams in intensively cultivated areas in the southeast of Buenos Aires, assessing separately suspended and dissolved fractions. Glyphosate was detected in 15% of the water and 67% of the suspended matter samples.

Amphipods have often been used for testing insecticide toxicity to nontarget invertebrate fauna (Borgmann, Ralph, and Norwood 1989; Adam et al. 2009; Xuereb et al. 2009; Dutra et al. 2009; Wheelock et al. 2005). The freshwater amphipod *Hyalella curvispina* has a wide distribution and is often the dominant invertebrate in the benthic and epiphytic communities of shallow environments in southern South America (García, Rodrígues Capítulo, and Ferrari 2010).

The aim of this study was to assess the acute toxicity of the common glyphosate formulation Roundup Full II® to the nontarget organism *H. curvispina.* Fifty percent lethal concentration (48-h LC_{50}) was determined following laboratory standardized protocols. A complementary field experiment was performed to assess acute toxicity in runoff samples of soy cultivated plots after a Roundup application followed by a simulated rainfall produced by irrigation equipment.

2. Materials and methods

2.1. LC_{50} determination

The 48-h LC_{50} of glyphosate to *H. curvispina* was determined on six independent occasions during a nine-month period between May 2011 and February 2012. Specimens of *H. curvispina* were collected from an uncontaminated stream located 25 km south of La Plata city and transported to the laboratory, where they were reared for several weeks. They were kept in large plastic containers with stream water, which was gradually replaced with un-chlorinated tap water to compensate for evaporation losses. The locally abundant macrophyte *Lemna* sp. was placed on the surface of the water. *H. curvispina* specimens fed on the periphytic community of the *Lemna* rhizosphere and received a supplement of a mixture of fresh lettuce leaves and separate cultured algae twice a week.

Procedures for *H. curvispina* toxicity tests were adapted from standardized protocols for soil toxicity tests for *H. azteca* (US EPA 2000), as described by Mugni et al. (2013). Ten *H. curvispina* specimens, 5−10 mm in length, were exposed to different glyphosate concentrations in 100 mL of reconstituted, moderately hard synthetic water (APHA 1998), placed in 250-mL beakers. Three replicates of each concentration were tested. Tests were performed without feeding, at 22 ± 2 °C, and natural photoperiod. Dead individuals were removed immediately. Mortality was recorded at 48 h of exposure. As a validity criterion for the negative control, less than 10% was considered acceptable. Preliminary tests were conducted to choose an appropriate glyphosate concentration range within which to test lethal effects. As a standard laboratory quality control practice, a reference test with copper sulfate ($CuSO_4 \cdot 5\ H_2O$, 99.9%, Merck, Darmstadt, Germany) was performed. The 48-h LC_{50} positive control was 265 μg L^{-1} Cu(II). This value lies within the acceptable range in the control chart (225 ± 79 μg L^{-1} Cu(II)) conducted by Mugni (2009).

Toxicity tests were performed using Roundup Full II® formulation (66.2% active ingredient). A stock solution of Roundup (133 mg L^{-1}) was prepared with reconstituted moderately hard water (APHA 1998). Different exposure solutions were prepared by diluting the stock solution in reconstituted moderately hard water. Three replicates were performed. Nominal assayed glyphosate concentrations were 25, 20, 15, 10, 7, 5 and 2 mg L^{-1}. In the first LC_{50} determination glyphosate concentrations in replicates of the 2, 5 and 10 nominal doses were determined after a 2-h exposure; measured concentrations were 1.6, 3.6 and 8.7 mg L^{-1}, respectively. The LC_{50} concentrations were calculated taking into account recovery-corrected concentrations. Organisms were considered dead when no response was observed upon gentle prodding. Mortality data obtained from the 48-h exposures were used to estimate the LC_{50} and its 95% confidence limits by means of the Probit statistical analysis method.

2.2. *Glyphosate determination*

The glyphosate analytical determination was adapted from Peruzzo, Porta, and Ronco (2008). Samples were filtered through a Whatman 0.45 mm membrane (cellulose acetate). Glyphosate was derivatized by the addition of 0.25 mL of borate buffer 5% and 0.30 mL of FMOC-Cl (2 mmol L^{-1}) in $CHCl_3$ to 1 mL of water sample, at 40 °C, kept in the dark. The reaction was stopped after 24 h, by adding 0.30 mL H_3PO_4 (2%, Merck, Darmstadt, Germany), and kept refrigerated until analyzed. The derivatized product (Gly-FMOC) was analyzed by high performance liquid chromatography (HPLC) (CRB- 6A; detector FLD, RF-10 AXL, Shimadzu, Kyoto, Japan) using a Supelco/Ascentis RP 18 Column (3 μm particle size, length 100 mm and I.D: 3 mm). The mobile phase used was acetonitrile: 0.05 M phosphate buffer (pH 6), with a gradient elution starting at 10% acetonitrile and progressing linearly to 40% acetonitrile, flow: 0.5 mL min^{-1}; fluorescence detection conditions were: excitation, 266 nm, emission, 315 nm. The injected sample volume was 20 μL. The chromatographic measurements were done at 40 °C. The mean recovery of the complete analytical technique was 79% ± 5% of glyphosate. Solvents used for pesticide analysis were from J.T. Baker (Avantor Performance Materials S.A., State of Mexico, Mexico). The detection limit was 0.05 mg L^{-1}.

2.3. *Field experiment*

The field work was performed at the Experimental Field Station of the School of Agronomic Science at La Plata University, located 8 km southwest of La Plata City, Buenos Aires, Argentina (35 01° S, 57 59° W). Soy was grown in an experimental field divided into 8 × 30 m plots. Irrigation sprinkler equipment was installed. It consisted of a perimeter pipe, 3 cm in diameter, provided with nine sprinkler heads mounted at a distance of 15 m from one another. Each impact sprinkler head was a Senninger 7025 model, 9.5 mm in diameter, providing a simulated rain of 16 mm h^{-1} with drops of 0.7−1 mm in diameter. The whole system was fed with water from a well, pumped with a 60,000 L h^{-1} pump. The field has a slope of 1%. At the lower end of each plot, a small trench was dug into the soil in order to capture the runoff water. A 5-liter bucket was buried in the trench.

The soy was seeded on 28 December 2009, with 45 seeds/m^2, and at a spacing of 35 cm between furrows. A single glyphosate application was made using a tractor-mounted sprayer when the crop had grown enough to attain complete soil cover (2 February 2010). Three plots were treated with glyphosate at a dose of 4 L ha^{-1}, (2648 g active ingredient ha^{-1}). Four plots remained as controls without any application. The simulated rain episode was produced the day following the glyphosate application. It lasted until a surface runoff flux was observed, and stopped soon thereafter, in order to gather the whole runoff excess in the buried buckets. The runoff was transferred to dark bottles and immediately transported to the laboratory in coolers. The toxicity of the runoff water to the amphipod *H. curvispina* was assessed by means of laboratory toxicity tests. Three replicates from each plot were assessed. Procedures for toxicity tests to *H. curvispina* were the same as for the LC_{50} determinations. Soy leaves' toxicity to *H. curvispina* was also tested by adapted standardized protocols for soil toxicity test (USEPA 2000). Ten *H. curvispina* measuring 5−10 mm were exposed to a soy leaf in 150 mL of reconstituted moderately hard synthetic water (APHA 1998) kept in 250 mL beakers, in triplicate. Mortality was assessed after a 10-day exposure. The soy leaf was the only source of food for *H. curvispina*. Mortalities lower than 20% were considered as no effect.

3. Results and discussion

Table 1 summarizes the 48-h LC_{50} of glyphosate to *H. curvispina* determined on six independent occasions. The overall mean was 9.9 ± 1.7 mg L^{-1}. No mortality was observed in the controls. An important aspect in determining the suitability of a test for routine use is reproducibility (Sucahyo et al. 2008). The low variability observed among independent assays is indicative of the high reproducibility attained by *H. curvispina* toxicity testing with Roundup. Roundup appears to be moderately toxic to *H. curvispina* (>1, ≤10 mg L^{-1}; Giesy, Dobson, and Solomon 2000).

Table 2 compares glyphosate toxicity to *H. curvispina* with other nontarget invertebrates taken from the literature. Cladocera, amphipoda and copepoda are among the organisms most sensitive to Roundup exposure. However, a large variability in the LC_{50} reported by different authors for the same organism is observed, sometimes attaining a difference of one order of magnitude. It seems likely that differences in testing conditions and formulated products represent a source of variability. The apparently lower LC_{50} of *H. azteca* than of *H. curvispina* might be due to the fact that in the present work lethal concentrations were determined with adults while 7−12−day-old juveniles were utilized in the *H. azteca* determination (Tsui & Chu 2004). In the present study, we determined the 48-h LC_{50} while Tsui & Chu (2004) reported the 96-h LC_{50}. Different formulations were also used in these studies. Within this context, available information suggests that *H. curvispina* constitute a comparatively highly sensitive organism to glyphosate.

Reported glyphosate concentrations in streams and rivers of intensively cultivated areas in Argentina lie quite below the acutely toxic concentrations determined in laboratory exposures. Peruzzo, Porta, and Ronco (2008) reported glyphosate concentrations measured in four samplings of a first order stream and four rivers of NW Buenos Aires. Maximum measured concentration was 0.7 mg L^{-1}. Aparicio et al. (2013) reported glyphosate concentrations in three samplings of 44 streams of SW Buenos Aires. Maximum soluble and suspended concentrations were 4 μg L^{-1} and 298 μg kg^{-1}, respectively.

The resident invertebrate fauna of streams is subjected to ephemeral toxicity pulses produced by the rain events occurring soon after pesticide application in the surrounding crops (Jergentz et al. 2004; Mugni, Ronco, and Bonetto 2011). If a few samplings per year are made, peak concentrations will be missed. Glyphosate concentrations in runoff water will provide a more realistic approximation to pulse exposures.

Warnemuende et al. (2007) measured glyphosate runoff concentrations in experimental corn plots submitted to simulated rains. Two long rain episodes were produced, the following day after application and a week later. Several samples were taken during each rain event. Maximum glyphosate concentration (233 μg L^{-1}) was measured at the beginning of the first rain, decreasing progressively after that. The second rain showed the

Table 1. Acute toxicity of the Roundup Full II glyphosate formulation to the amphipod *Hyalella curvispina*.

	May 2011	August 2011	November 2011	December 2011	January 2012	February 2012
Point	Conc.(mg L^{-1}) 95% Conf. Lim	Conc.(mg L^{-1}) 95% Conf. Lim	Conc.(mg L^{-1}) 95% Conf. Lim	Conc.(mg L^{-1}) 95% Conf. Lim	Conc.(mg L^{-1}) 95% Conf. Lim	Conc.(mg L^{-1}) 95% Conf. Lim
LC 1.0	4.2 (3.2−5.0)	5.1 (2.6−6.6)	3.8 (2−5.1)	3.8 (2.6−4.8)	4.0 (2.5−5.1)	5.4 (3.0-6.6)
LC 5.0	5.4 (4.3−6.1)	6.4 (3.8−7.9)	5.3 (3.4−6.8)	5.0 (3.8−6.2)	5.0 (3.4−5.9)	6.2(4−7.2)
LC 10.0	6.0 (5.0−6.8)	7.3 (4.8−8.6)	6.3 (4.4−7.8)	5.9 (4.6−6.9)	5.5 (4.1−6.5)	6.6 (4.6−7.6)
LC 15.0	6.5 (5.6−7.3)	7.8 (5.5−9.2)	7.2 (5.2−8.7)	6.6 (5.3−7.6)	6.0 (4.6−6.9)	7.0 (5.0−7.8)
LC 50.0	9.1(8.3−10)	11.1 (9.6−12.5)	12.2 (10.4−14)	10.2 (8.9−11.6)	8.2 (7.2−9.2)	8.6 (7.5−9.3)
LC 85.0	12.8 (11.6-14.8)	15.8 (13.8−20.6)	20.6 (17.4−27.2)	15.8 (13.7−19.5)	11.3 (10.1−13.8)	10.6 (9.8−12.5)
LC 90.0	13.9 (12.4−16.3)	17.1 (14.7−23.8)	23.3 (19.3−32.2)	17.6 (15−22.3)	12.2 (10.7-15.4)	11.2 (10.2−13.6)
LC 95.0	15.7(13.8−18.9)	19.3 (16.2−29.3)	28.0 (22.4-41.9)	20.6 (17.2−27.3)	13.7 (11.8−18.3)	12.1 (10.9−15.7)
LC 99.0	19.6 (16.6−25.1)	24.2 (19.0−43.8)	39.6 (29.4−68.9)	27.5 (21.8−40.1)	16.9 (13.8−25.4)	13.8 (12−20.5)
Slope ± SE	−1.9 ± 0.72	−2.29 ± 1.44	−0.29 ± 0.72	−0.79 ± 0.66	−2.06 ± 1.2	−5.4 ± 2.5
Intercept ± SE	5.6 ± 0.68	5.5 ± 1.28	3.6 ± 0.59	4.32 ± 0.58	5.98 ± 1.1	9.04 ± 2.5

Table 2. Lethal concentrations of several Glyphosate formulations to different nontarget invertebrate species.

Species	Glyphosate content (%)	Exposure time (h)	LC_{50} ($mg\ L^{-1}$)	Reference
Cladocera *Daphnia pulex*	48	96 h	0.7	Mensah, Palmer, and Muller 2013
Amphipoda *Hyalella azteca*	41	48 h	1.5	Tsui and Chu 2004
Nematomorpha *Chordodes nobilii*	35	96 h	1.7	Achiorno, Villalobos, and Ferrari 2008
Copepoda A*cartia tonsa*	41	48 h	1.8	Tsui and Chu 2003
Ephemeroptera *Baetis harrisoni*	48	96 h	2.7	Mensah, Palmer, and Muller 2013
Decapoda *Caridina nilotica*	48	96 h	2.8	Mensah, Muller, and Palmer 2011
Cladocera *Daphnia magna*	36	48 h	3.0	Folmar, Sanders, and Julin 1979
Cladocera *Daphnia magna*	41	48 h	3.0	Johnson and Finley 1980
Gastropoda *Burnupia stenochorias*	48	96 h	4.3	Mensah, Palmer, and Muller 2013
Cladocera *Ceriodaphnia dubia*	41	48 h	5.4	Tsui and Chu 2003
Cladocera *Ceriodaphnia dubia*	41	48 h	5.7	Tsui and Chu 2004
Cladocera *Daphnia magna.*	41	48 h	7.9	Hartman and Martin 1984
Cladocera *Daphnia magna*	36	48 h	9.7	Giesy, Dobson, and Solomon 2000
Amphipod *Hyalella curvispina*	66	48 h	9.9	This paper
Diptera *Tanytarsus flumineus*	48	96 h	12.2	Mensah, Palmer, and Muller 2013
Diptera *Chironomus plumosus*	36	48 h	18	Folmar, Sanders, and Julin 1979
Cladocera *Daphnia pulex*	36	48 h	19	Giesy, Dobson, and Solomon 2000
Cladocera *Daphnia magna*	48	48 h	20	Al-Omar and Hassan 2000
Coelenterate *Hydra attenuate*	74	96 h	22	Demetrio et al. 2012
Shrimp *Caridina nilotica* (40 days old)	48	96 h	25	Mensah, Muller, and Palmer 2011
Rotifer *Brachionus calyciflorus*	41	24 h	28	Xi and Feng 2004
Amphipod *Gammarus sp.*	36	48 h	43	Kreutzweiser, Kingsbury, and Feng 1989
Diptera *chironomus plumosus*	36	48 h	58	Giesy, Dobson, and Solomon 2000
Amphipod *Gammarus pseudolimnaeus*	36	48 h	62	Folmar, Sanders, and Julin 1979
Cladocera *Daphnia magna.*	48	48 h	62	Alberdi et al. 1996
Cladocera *Daphnia spinulata.*	48	48 h	63	Alberdi et al. 1996
Decapoda *Callinectes sapidus*	50	24 h	316	Osterberg et al. 2012

same pattern but attaining lower concentrations; maximum measured concentration was 25 μg L^{-1}.

Shipitalo, Malone, and Owens (2008) measured glyphosate losses in runoff from experimental soybean plots. Glyphosate concentrations decreased with time since application. Most of the glyphosate detections (90%) were the result of runoff events that occurred within 10 days since application. Maximum measured glyphosate concentration was 182 μg L^{-1} measured in a runoff event occurred the day following application. Screpanti et al. (2005) estimated field-scale runoff losses of glyphosate under natural rainfall conditions. Glyphosate was applied as pre-emergence herbicide on 350 m^2 (7×50 m) field plots cultivated with corn, throughout a three-year study. The maximum measured glyphosate concentration was 16 μg L^{-1}.

In the present work, we simulated the worst-case scenario by assessing the toxicity in runoff produced by a simulated rain event the day following a Roundup application in the soy plots. There was no *H. curvispina* mortality. Exposure of *H. curvispina* to soy leaves sampled immediately after the application did not produce mortality either. Our results are consistent with those reported by Screpanti et al. (2005), Warnemuende et al. (2007) and Shipitalo, Malone, and Owens (2008). These studies were performed within a similar experimental setup; glyphosate concentrations in runoff lie quite below the LC_1 (4.4 ± 0.7 mg L^{-1}, Table 1) assessed in the present study. Such concentrations should not produce any measurable *H. curvispina* mortality, as confirmed in the present study.

Ecological risk can be estimated by using the Hazard Quotient (HQ) approach (Giesy, Dobson, and Solomon 2000). The HQ is defined as the ratio between the maximum measured environmental concentration and the toxicity reference value (Giesy, Dobson, and Solomon 2000). If the HQ values exceed 1.0, harmful effects are expected. If the HQ is less than 1.0, harmful effects are not likely to occur. The reported maximum concentrations in regional environments amounted 0.7 mg L^{-1} glyphosate (Peruzzo, Porta, and Ronco 2008). The estimated HQ calculated with the reported environmental concentrations and the LC_{50} estimated in the present work resulted quite low (0.07), suggesting negligible risk of glyphosate to *H. curvispina* in the Argentine Pampasic streams. The estimated HQ remains low (0.16) even if the 1% lethal concentration is utilized for the calculation. Moreover, estimated HQ values for runoff water also attained quite low values (0.018−0.02) estimated using reported concentrations from Screpanti et al. (2005), Warnemuende et al. (2007) and Shipitalo, Malone, and Owens (2008) and the 1% lethal concentration.

Crustaceans have been widely used in aquatic toxicity testing (Graca et al. 2002; Sánchez-Bayo 2006; Barata et al. 2008; Dahl and Breitholtz 2008; Adam et al. 2010; Ding et al. 2011; Shen et al. 2012). Among aquatic crustaceans, *Daphnia* sp., *Ceriodaphnia* sp., *Gammarus* sp. and *Hyalella* sp. have often been used in aquatic toxicity testing for a variety of reasons, including their widespread distribution in aquatic environments and ease of culture under laboratory conditions. Familiarity with the organism and the availability of a large database may have contributed to their popularity (Hickey 1989). Because of its wide distribution in Mexico and the USA, *H. azteca* is routinely used as a test organism for toxicity assessment in aquatic environments of North America. *H. azteca* is not present in South America. *H. curvispina* is commonly the most abundant species in amphipod assemblages in a wide area of South America, extending from Rio de Janeiro, Brazil, on the Atlantic coast (22° S, 43° W), to Punta Arenas, Chile, on the Pacific (53° S, 70 W; Somma, Giusto, and Ferrari 2011). Such wide distribution overlaps with most of the agricultural areas in southern South America including the most important crop producer countries, Brazil, Argentina, Uruguay, Paraguay and Bolivia. Being sensitive to glyphosate and other pesticides (Mugni et al. 2013) and attaining a wide distribution area, *H. curvispina* represents a good model for environmental risk assessment.

4. Conclusions

Roundup acute toxicity to *H. curvispina*, assayed in laboratory experiments, *H. curvispina* exposures to soy runoff and measured glyphosate concentrations in streams and runoff waters suggest low risk of acute toxicity to *H. curvispina* derived from Roundup application in adjacent crops. Experiments reported in the present work refer to adults. Further studies are needed for juveniles, likely to be more sensitive.

Being widely distributed and often attaining high densities in shallow South America water bodies, *H. curvispina* seems suitable for use as a sentinel organism for environmental impact assessment.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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