Bull Environ Contam Toxicol (2007) 79:537–543
DOI 10.1007/s00128-007-9277-5

Effects of the Glyphosate Active Ingredient and a Formulation on *Lemna gibba* L. at Different Exposure Levels and Assessment End-Points

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Received: 18 April 2007 / Accepted: 5 September 2007 / Published online: 17 October 2007

Abstract The use of formulations of the herbicide glyphosate in transgenic crops of the Pampa's plains of Argentina has extensively increased, though there is scarce information of its impact on non-target vascular plants from agro-ecosystem related surface waters. The sensitivity of a local clone of the macrophyte *Lemna gibba* L. to glyphosate active principle and Roundup Max formulation was studied in standardized laboratory conditions. Phytotoxic effects, considering the aquatic route, at a concentration range of glyphosate between 0.5 and 80 mg L^{-1} as active ingredient during 10 days of exposure were assessed on plant population growth, frond growth, shape and number, total chlorophyll content and colony architecture. Exposure to 1 mg L^{-1} of glyphosate (an expected environmental concentration) affects all the studied assessment endpoints, except for population growth and chlorophyll content. Equivalent concentrations of this herbicide as the active ingredient or RoundupMax indicate higher phytotocity of the formulation. Exposed plants at concentrations of herbicide between 1 and 7.5 mg L^{-1} exhibit after two days a recovery of the multiplication rate. Frond aggregation and longer stipe was detected between 1 and 15 mg L^{-1} of glyphosate, determining more open colony architecture. At higher concentrations of the herbicide fronds break-up. Comparisons with literature data indicate a higher sensitivity of the *L. gibba* local clone with respect to *L. minor* and algal species, and also a similar response to the herbicide in field experiments with the same species.

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Glyphosate has been introduced in the new technologies of agriculture, and is at present widely used around the world. In particular, the application of formulations to transgenic crops in the Pampás region of Argentina has increased 100-fold in the last decade to more than 12 million hectares (Satorre 2005). Glyphosate is a non-selective herbicide known to alter aromatic amino acid synthesis via the inhibition of the shikimate pathway, interfering in protein production and with other molecules that require these amino acids as precursors, such as auxins and polyphenols (Salisbury and Ross 1994; WSSA 1994; Blackburn and Boutin 2003). Recent reports assessing the impacts of herbicides on non-target primary producers show a wide range of responses for several aquatic and terrestrial species to glyphosate (Peterson et al. 1994; Ralph 2000; Blackburn and Boutin 2003; Martin et al. 2003; Michel et al. 2004; Martin and Ronco 2006). The comparisons of the toxicity of different herbicide families with a diverse mode of actions indicate a low effect for glyphosate on aquatic primary producers (Peterson et al. 1994; Ralph 2000; Michel et al. 2004). Exposures to formulations of glyphosate indicate that co-adjuvants enhance toxic effects (Tsui and Chu 2003). *Lemna gibba* L. has been recommended to be used as a reference organism for standardized methods of pesticide phytotoxicity assessment on non-target macrophytes (Boutin et al. 1993). This is a widely distributed and representative species of subtropical and temperate regions of lentic surface waters from Argentina. Particular attention was given to the study of the sensitivity

of a local clone in relation to collection clones of Lemnaceae (Sobrero et al. 2004) for future use in regional environmental assessments.

The large amounts of glyphosate used worldwide, combined with relevance at the regional level, sparked the interest for a controlled laboratory assessment of the impact of glyphosate on *L. gibba* using exposure concentrations that are representative of those found in the field. Considering the fact that this herbicide could interfere with plant metabolism, which has consequences at different levels of growth and development, and the possible differential effects of the active ingredient and formulation, the present study included a comparison of the impact on plant population growth, frond growth, shape and number, colony architecture, root growth and total chlorophyll content of *L. gibba* with respect to time. Comparisons between data collected in the present study, experimental data found in the open literature, and data collected in regional field studies are also included.

Materials and Methods

A local clone of *L. gibba* was isolated from plants collected in El Pescado stream, Buenos Aires Province, Argentina and used for testing. Stock cultures of *L. gibba* were maintained under standardized growth conditions using sterile nutrient solution: 250 μM NH_4NO_3, 220 μM $CaCl_2$, 406 μM $MgSO_4$, 30 μM K_2HPO_4, 500 μM $NaHCO_3$, 18 μM EDTA-$FeCl_3$, 17.8 μM H_3BO_3, 1.8 μM $MnCl_2$, 0.08 μM $CoCl_2$, 0.16 μM $ZnSO_4$, 0.08 μM $CuSO_4$, 1.4 μM Na_2MoO_4, pH 6.5, at 24 ± 2 °C, during a 16 h day with 80 μM m^{-2} s^{-1}, cool-white fluorescent light. Toxicity tests were performed in 500 mL jars, containing 300 mL of the sterile nutrient solution, starting with six fronds, under the same conditions of illumination and temperature as the ones used for culturing, using plants previously acclimated for one month (Sobrero et al. 2004). Assuming a single application of the herbicide in the field, a static laboratory test was chosen, with no renewal, during the 10 day exposure time. To ensure exponential population growth, 1 mL of a fivefold nutrient solution was added every 2 or 3 days during the exposure period. The pH of testing solutions was registered along with the experiments (Tsui and Chu 2003). The experimental design included at least four replications per concentration, with seven concentrations of herbicide (0.5–80 mg L^{-1}), testing both the active ingredient, a.i. (glyphosate acid, technical grade, 95%w/w) and the commercial formulation (Roundup®Max, 70.7%w/w a.i. as acid), including controls. Data in the tables and figures refer to nominal concentrations of the a.i. at the starting time. Verification of the concentrations of a.i. in formulation and in the toxicity test solutions, from the initial time through exposure, was done by HPLC–UV detection (206 nm) and previous derivatization with 9-fluoroenylmethyl chloroformate chloride (AOAC 1990; Peruzzo et al. 2003).

Herbicide phytotoxicity was assessed on growth rate (GR) measured at 2, 5, 7 and 10 days of exposure, and also on frond growth (FG), frond number per colony (FNC), total chlorophyll content (TCC) and root length measured at 7 and 10 days. The GR was calculated according to Environment Canada (1999). The FG was estimated from a longitudinal and transversal frond axis measurement, differentiating mother fronds from daughter fronds (comprising buds), where a mother frond refers to a completely developed frond with a bud or daughter frond attached, while a daughter frond refers to those already developed that are without buds but still attached to the mother frond. A description of the effects on colony architecture took into account FNC, frond shape, and length of stipe. The effects on TCC and root elongation were assessed according to Sobrero et al. (2004). The concentration range of the herbicide used in the experiments included exposure levels of the dissolved glyphosate to be expected in the aquatic environment. Data representing each assessment end-point were analyzed by factorial ANOVA. Significant differences between means were determined using Tukey multiple comparison test ($p \leq 0.05$) (Zar 1996). The statistical end-point estimates, IC_{10}, IC_{25} or IC_{50} (inhibition concentration producing 10, 25 and 50% effect, respectively), for GR, FG and TCC were calculated by non-parametric linear interpolation (Environment Canada 1999).

Results and Discussion

The results of the assessed effects of the a.i. and Roundup for all end-points analyzed by factorial ANOVA can be seen in Table 1. Significant differences of effects on growth rate ($p \leq 0.05$) were observed among the type of product, glyphosate concentration and exposure time. Interactions were observed between the exposure time and concentration, and between the product and concentration. No significant interaction was observed between the product and the exposure time.

At a concentration of 0.5 mg L^{-1} glyphosate a.i. or Roundup, no changes on the growth rate were detected during the exposure time. At 1 mg L^{-1}, a significant effect was observed during the first days, turning to a recovery of the growth rate (35 and 0% inhibition at second and fifth day, and 24 and 4% inhibition at the second and tenth day, with Roundup and a.i., respectively).

Statistical analysis of growth rate data indicated that Roundup was more toxic to *L. gibba* than was a.i.

Table 1 F-ratios and probability values from factorial ANOVA on the growth rate, frond growth and frond number per colony variables

Variable	Source of variation	df	*F*-ratio	*p*
Growth rate[a]	Glyphosate concentration	7	394.4	0.000*
	Product	1	37.2	0.000*
	Exposure time	3	8.6	0.000*
	Concentration × product	7	8.4	0.000*
	Concentration × exposure time	21	7.3	0.000*
	Product × exposure time	7	2.5	0.058
	Error	435	–	–
Frond Growth: Longitudinal Axis[a]	Glyphosate concentration	4	56.6	0.000*
	Product	1	8.0	0.005*
	Exposure time	1	17.6	0.000*
	Frond age	1	3632.0	0.000*
	Concentration × product	4	26.6	0.000*
	Concentration × exposure time	4	8.1	0.000*
	Concentration × age	4	3.8	0.004*
	Product × exposure time	1	5.0	0.026*
	Product × age	1	4.3	0.038*
	Exposure time × age	1	1.4	0.232
	Error	2261	–	–
Frond Growth: Transversal Axis[a]	Glyphosate concentration	4	155.5	0.000*
	Product	1	24.9	0.000*
	Exposure time	1	33.5	0.000*
	Frond age	1	1133.7	0.000*
	Concentration × product	4	37.9	0.000*
	Concentration × exposure time	4	6.8	0.000*
	Product × exposure time	1	0.3	0.579
	Product × age	1	15.4	0.000*
	Exposure time × age	1	45.8	0.000*
	Error	2257	–	–
Frond Number per Colony[a]	Glyphosate concentration	7	175.3	0.000*
	Product	1	1.1	0.297
	Exposure time	1	5.3	0.021*
	Concentration × product	7	3.2	0.004*
	Concentration × exposure time	7	12.3	0.000*
	Product × exposure time	1	0.0	1.000
	Error	714	–	–

[a] Interactions at higher levels were non-significant

* Significant differences

(Table 2). Although the IC_{10} values for the a.i. remained below 1 mg L^{-1} of glyphosate after 7 days of exposure, they increased to 4.6 mg L^{-1} on the tenth day. The IC_{25} or IC_{50} for the a.i. exhibits a slight increase in toxicity with time of exposure, and then remains constant after 7 days. Roundup, at the beginning of testing, is more toxic but then the effect decreases after 5 days of exposure until a higher effect is again observed. When measuring the difference between the growth rate statistical end-points as a quotient of the $IC_{a.i.}$ and $IC_{Roundup}$, it can be observed that in most cases Roundup is approximately twice as toxic as the a.i., although at 2 days of exposure the difference is much higher (from 3.6 up to 30 times).

Glyphosate effects on frond growth can be seen in Table 1 and Fig. 1. Factorial ANOVA of the response on longitudinal (LA) and transversal (TA) frond axes indicates differences due to products, product concentration, and time of exposure and age of fronds. Roundup exhibited high toxicity on frond growth at concentrations of 1 mg L^{-1} of herbicide (Fig. 1). On the other hand, this

Table 2 *Lemna gibba* statistical end-point values for growth rate and frond growth at different exposure times to glyphosate a.i. and Roundup formulation

Days	Axis	Active ingredient			Roundup®		
		IC_{10}	IC_{25}	IC_{50}	IC_{10}	IC_{25}	IC_{50}
Growth rate inhibition (mg L^{-1})[a]							
2		$0.5 < IC < 1.0$[b]	15.1 (3.8–21.8)	33.1 (21.6–47.9)	$0.5 < IC < 0.9$[b]	$0.5 < IC < 0.9$[b]	9.2 (4.4–17.3)
5		$0.5 < IC < 1.0$[b]	11.4 (8.9–13.5)	22.6 (20.3–25.7)	2.1 (0.96–3.7)	6.5 (4.5–8.4)	15.9 (14.3–17.5)
10		4.6 (2.4–6.7)	10.7 (9.9–11.4)	20.5 (19.6–21.7)	2.5 (2.3–2.7)	5.0 (4.5–5.5)	11.6 (10.9–2.1)
Mother frond growth inhibition (mg L^{-1})[a]							
7	LA	14.1 (11.4–19.9)	>25	>25[b]	9.7 (6.1–16.3)	>25[b]	>25[b]
	TA	11.2 (10.1–12.4)	16.0 (13.2–23.3)	>25[b]	5.3 (3.9–6.2)	>25[b]	>25 [b]
10	LA	10.2 (8.1–11.9)	15.8 (12.8–23.2)	>25[b]	2.8 (1.4–4.3)	10.1 (7.8–12.2)	>25[b]
	TA	10.1 (8.9–10.9)	14.5 (13.2–17.9)	>25[b]	3.4 (3.1–3.8)	7.0 (6.3–8.1)	>25[b]
Daughter frond growth inhibition (mg L^{-1})[a]							
7	LA	$0.5 > CI < 1.0$[b]	16.2 (5.7–23.7)	>25[b]	2.7 (1.3–3.9)	6.2 (3.9–12.6)	>25[b]
	TA	$0.5 > CI < 1.0$[b]	7.3 (2.5–13.3)	>25[b]	2.8 (1.5–3.8)	6.0 (4.5–7.9)	13.6 (11.2–4.9)
10	LA	8.5 (3.98–10.19)	11.9 (9.2–14.5)	22.1 (17.1–24.8)	$0.5 < CI < 1.0$[b]	3.8 (1.1–9.1)	12.7 (6.8–14.9)
	TA	6.3 (1.22–9.30)	10.2 (7.9–12.3)	18.3 (14.2–22.9)	$0.5 < CI < 1.0$[b]	2.6 (1.1–4.5)	8.8 (6.5–10.9)

LA longitudinal axis, *TA* transversal axis

IC_{10}, IC_{25} and IC_{50}: inhibition concentration producing 10, 25 and 50% effect, respectively. Values in parentheses correspond to 95% confidence intervals

[a] Nominal glyphosate concentration as active ingredient or Roundup

[b] IC_{10} or IC_{25} estimation was not possible within tested concentration range

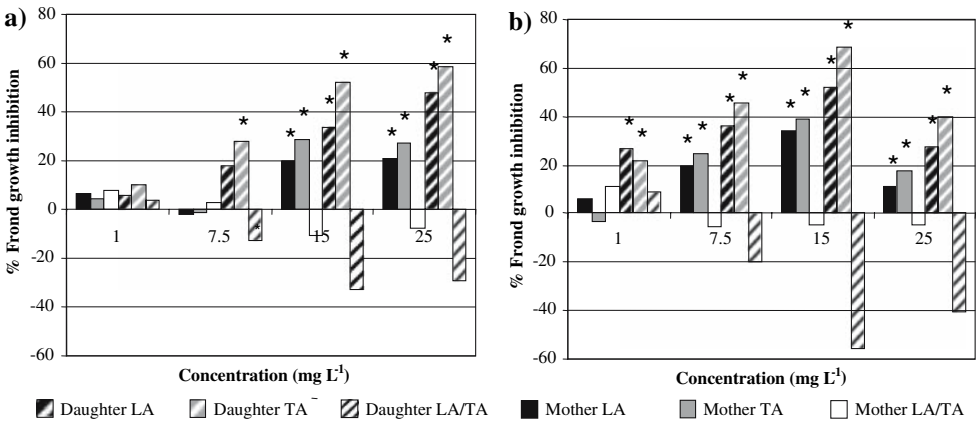

Fig. 1 *Lemna gibba* mother or daughter frond growth inhibition after 10 days exposure to glyphosate. **(a)** Plants exposed to glyphosate active ingredient; **(b)** Plants exposed to Roundup. *LA* longitudinal axis; *TA* transversal axis. *Significant differences with respect to the negative control

end-point was not affected by the a.i., even at a concentration of 7.5 mg L^{-1}. The 7 and 10 day $IC_{10/25/50}$ values of the effect on the LA and TA of fronds were significantly lower for Roundup, which also indicated elevated effects of the formulation (Table 2). A differential effect on LA and TA elongation was also noticed as being more evident on the transversal axis, producing not only smaller, but also narrower fronds, with respect to the control. This observed response was higher in daughter fronds (Figs. 1, 2). Also, a differential sensitivity was detected in relation to the age of fronds (Figs. 1, 2). Mother fronds were always less inhibited than daughter fronds, and, as a result, it is more convenient to assess the effects on the latter. When considering all of the effects on frond growth in relation to exposure time, an incremental shift was detected, with the exception of the behavior of the a.i. at low concentration (Table 2). A significant enhancement of mother frond growth (LA) was detected at seven days of exposure at 1 and 7.5 mg L^{-1} of glyphosate for Roundup and a.i., respectively (results not shown). Effects on the frond growth could not be assessed at high concentrations of the herbicide due to high inhibition of the growth rate (i.e., at 25 mg L^{-1} of Roundup, see Fig. 1-, and 60 mg L^{-1} of a.i. -not plotted). Hence, inhibition on the frond growth is underestimated.

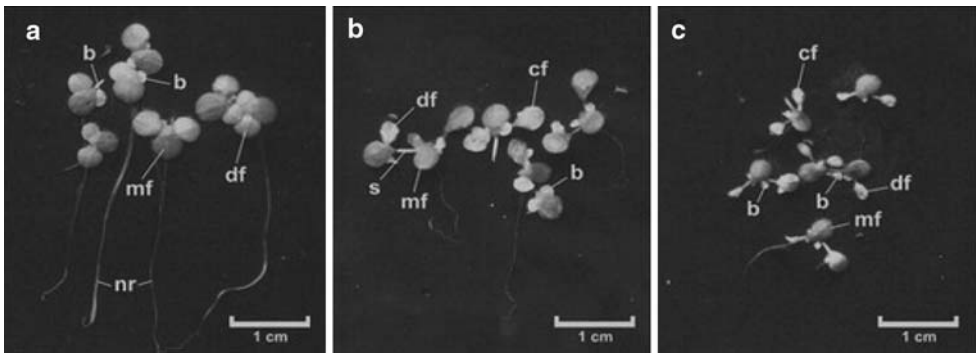

Fig. 2 Effect of Roundup on *Lemna gibba* frond growth, chlorophyll content and colony architecture after 10 day exposure. **(a)** Negative control; **(b)** 7.5 mg L^{-1}; **(c)** 15 mg L^{-1} of glyphosate. *mf* mother frond; *df* daughter frond; *b* bud; *s* stipe; *cf* chlorotic frond; *nr* normal root

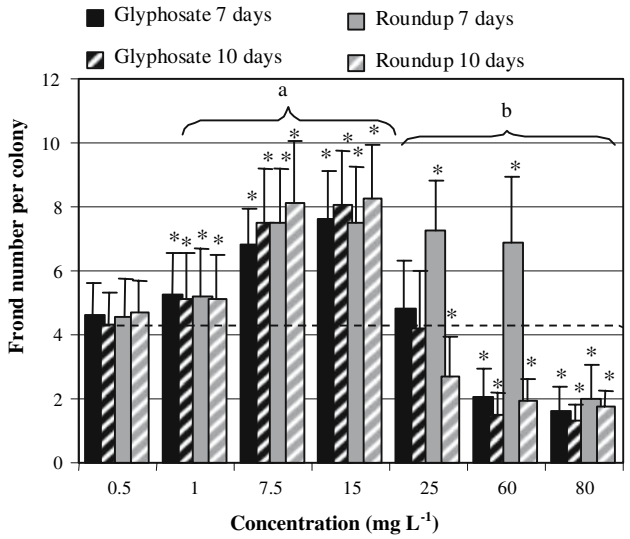

Fig. 3 Effect of glyphosate on *Lemna gibba* frond number per colony (FNC) at 7 and 10 day exposure. *Dotted line* indicates mean FNC for the negative control. **a**, **b** herbicide concentrations inducing aggregation and colony break up, respectively. *Error bars* correspond to standard deviation; * significant differences with respect to the negative control

The assessment of frond number per colony after 7 and 10 days of exposure is shown in Figs. 2 and 3. Results of the factorial ANOVA indicate a significant effect associated with the studied factors and their interactions, except for the type of product (Table 1). The response pattern shows a significant increment of the FNC (reduction of the abscission capacity), within the concentration interval of 1–15 mg L^{-1}, for both products and exposure times, which reach maximum values of 12 fronds per colony. At higher concentrations of glyphosate, a reduction of the FNC due to higher colony break-up was seen (Fig. 3).

Effects of exposure to the herbicide in relation to changes of architecture can also be detected through changes in frond shape and stipe length. Figure 2 shows that plants exposed to herbicide concentrations of 7.5 and 15 mg L^{-1} of Roundup for 10 days exhibit smaller fronds with a different shape with respect to the controls, as previously explained for frond growth. In addition, the stipe connecting the mother frond to the daughter frond and buds can be seen to be longer in the exposed plants. This abnormal stipe elongation was also observed on plants exposed to the a.i. Therefore, although there is a higher FNC, there is also a lower proportion of covered area per colony, yielding to a more open structure.

Regarding effects on chlorophyll content, chlorotic fronds were observed after 3 days of exposure to 7.5 mg L^{-1} and higher concentrations of glyphosate. This effect was evident in daughter fronds and buds, and almost undetectable in mother fronds (Fig. 2). Only at high concentrations of Roundup (over 25 mg L^{-1}) was the chlorosis even evident on mother fronds. Considering the differential response in TCC, the estimation of IC_{50} values for the whole population was meaningless. Additionally, Fig. 2 shows the effects on root elongation. Significant inhibition was observed for a.i. and Roundup at 7.5 mg L^{-1} glyphosate after 3 days of exposure.

The pH measurement indicates an increment during the exposure, from an initial value of 6.5 to a maximum of 7.8 after 10 days. The variation of glyphosate concentration along the time of exposure is given in Table 3. Results indicate a decay of 12% (for 1 mg L^{-1}) and 33% (for 15 mg L^{-1}) after 5 days. Then the loss remains fairly constant up to 10 days.

In general, the effects on the growth rate, frond growth and frond number per colony, in addition to changes in colony architecture of *L. gibba* can be seen at a concentration of glyphosate of 1 mg L^{-1} and higher. Although GR is a good assessment end point at this low concentration, a decrement effect is seen after longer exposures to both forms of the herbicide. A recovery in growth rate was evident, though the effects on the other assessed end-points remained clear and even increased during the exposure time. Recovery should not be taken as an indication of the health of the organisms or the population, considering the fact that the plant metabolism is still affected at other levels and consequently on other assessment end-points. Another aspect to be considered is the enhancement of certain plant attributes, such as frond growth, at low herbicide concentrations. Lockhart et al. (1989) also reported a hormetic

Table 3 Measured glyphosate concentrations in toxicity test media during experimental procedure.

Nominal glyphosate concentration	Exposure time (days)					
	0		5		10	
	Mean measured concentration and SD	Percent decay	Mean measured concentration and SD	Percent decay	Mean measured concentration and SD	Percent decay
1	0.96 ± 0.042	4	0.88 ± 0.092	12	0.82 ± 0.122	18
2.5	2.40 ± 0.069	4	2.00 ± 0.095	20	1.95 ± 0.155	22
15	14.50 ± 0.048	3	10.05 ± 0.135	33	9.95 ± 0.259	34
25	24.50 ± 0.096	2	22.50 ± 0.322	10	21.30 ± 0.197	15

Concentrations are given in mg L^{-1} followed by standard deviation (SD). Measurements were done on two replicates

behavior on the growth of *L. minor* exposed from 2.3 to 22.8 mg a.i. L^{-1} of glyphosate. Also, Peterson et al. (1994) observed growth enhancement for several algae species near 3 mg L^{-1} of Roundup®. Therefore, apparent plant or colony recovery, together with inhibition of certain parameters and a hormetic type of response, should be carefully considered when setting non-effect guideline concentrations of glyphosate. The wide range of responses for the different end-points indicates the relevance of using a variety of parameters to assess the effects of the toxicant.

The response of the studied clone of *L. gibba* to glyphosate in comparison with different species indicates a higher sensitivity than other Lemnaceae, but within the range observed for algae and seeds (Lockhart et al. 1989; Peterson et al. 1994; Paradiso Giles 2000; Michel et al. 2004; Martin and Ronco 2006). At equivalent concentrations of the active ingredient, the Roundup®Max formulation shows a much higher toxicity on *L. gibba*. Higher effects of glyphosate formulations in comparison with a.i. have also been reported for another vascular plant (*Lactuca sativa*) (Martin and Ronco 2005), algae (*Selenastrum capricornutum* and *Skeletonema costatum*); bacteria (*Vibrio fischeri*), protozoans (*Tetrahymena pyriformis* and *Euplotes vannus*) and microcrustaceans (*Ceriodaphnia dubia*) (Tsui and Chu 2003). Reports indicate that surfactants account for up to 86% of the relative toxicity found in studies with algae and Roundup (Tsui and Chu 2003). The surfactants used in the Roundup formulation, polyoxyethylene amines (POEA) (Blackburn and Boutin 2003; Cox 2003; Tsui and Chu 2003), exhibit higher toxicity in an alkaline media (Krogh et al. 2003; Tsui and Chu 2003). Thus, the increment of toxicity with respect to time on *L. gibba*, even after an observed recovery, may be attributed to an additional activity of the mixture with increased pH.

Results from local field experiments using caged *L. gibba* and carried out in a surface water body adjacent to a soybean crop treated with Roundup®Max (using 1.5 kg ha^{-1}) indicate significant effects on biomass production (28% inhibition) and TCC (up to 75% inhibition) at five days after Roundup exposure (measured maximum concentration in water: 1.6 mg a.i. L^{-1}) (Martin et al. 2003; Peruzzo et al. 2003). There is agreement between this published data and the results reported here under laboratory exposure conditions, indicating that at similar low levels of herbicide, significant phytotoxic effects occur. Hence, according to the maximum recommended application dose of the Roundup®Max formulation for crops (3.2 kg ha^{-1}), corresponding to an expected environmental concentration (Peterson et al. 1994) of 1.6 mg a.i. L^{-1}, and adverse effects would be expected on aquatic plants associated with the use of the herbicide in crop management.

The local clone of *L. gibba* is a useful reference organism for the assessment of the impacts on non-target aquatic vascular plants, being that this species is affected by representative environmental concentrations of glyphosate found in water bodies of agroecosystems of the Pampa's plain.

Acknowledgments We thank P. Peruzzo for assistance in chemical analysis and C. Knipp for editorial suggestions. Financial support was from the National Agency for Promotion of Science and Technology ANPCyT (project PICT2000 8480) and the National University of La Plata, Argentina.

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