Comparative Effects of Technical-Grade and a Commercial Formulation of Glyphosate on the Pigment Content of Periphytic Algae

María S. Vera, Ángela B. Juárez & Haydée N. Pizarro

Bulletin of Environmental Contamination and Toxicology

ISSN 0007-4861
Volume 93
Number 4

Bull Environ Contam Toxicol (2014)
93:399-404
DOI 10.1007/s00128-014-1355-x

Springer

Bull Environ Contam Toxicol (2014) 93:399–404
DOI 10.1007/s00128-014-1355-x

Comparative Effects of Technical-Grade and a Commercial Formulation of Glyphosate on the Pigment Content of Periphytic Algae

María S. Vera · Ángela B. Juárez · Haydée N. Pizarro

Received: 31 January 2014 / Accepted: 8 August 2014 / Published online: 17 August 2014

Abstract We investigated the potentially different effects of one of the most commonly used glyphosate formulations in Argentina, Glifosato Atanor®, and the technical-grade glyphosate on the pigment content, as biomass indicators of the algal fraction in a freshwater periphytic community. A laboratory bioassay was carried out in 250-ml beakers. Two treatments were used: technical-grade glyphosate acid and Glifosato Atanor® (isopropylamine salt of glyphosate 48 % w/v), which were at a concentration of 3 mg active ingredient per liter. Treatments and the control (without herbicide) were replicated in triplicate. The concentrations of chlorophyll *a* and *b* and carotenes were determined at 0, 2, 6, 10, 24, 48, 96 and 192 h after herbicide addition. A significant increase in pigment content was observed for both herbicides after a 2-day exposure. Moreover, the formulation had little or no effect compared to the active ingredient, suggesting that the additives of Glifosato Atanor® may not enhance glyphosate toxicity.

Keywords Glyphosate · Glifosato Atanor® · Periphyton · Laboratory bioassay · Photosynthetic pigments

M. S. Vera (✉) · H. N. Pizarro
Departamento de Ecología, Genética y Evolución, IEGEBA UBA-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires,
C1428EHA Ciudad de Buenos Aires, Argentina
e-mail: msolangevera@ege.fcen.uba.ar

Á. B. Juárez
Departamento de Biodiversidad y Biología Experimental, IBBEA UBA-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires,
C1428EHA Ciudad de Buenos Aires, Argentina

Glyphosate (N-(phosphonomethyl)glycine) is a non-selective, broad-spectrum and post-emergent herbicide known to disrupt aromatic amino acid biosynthesis in plants by inhibiting the shikimate pathway; this leads to a reduction in the synthesis of some proteins and growth and, eventually, cell disruption and death (Schönbrunn et al. 2001).

Glyphosate may reach natural water bodies near cultivation fields either indirectly through drift or surface runoff movements or directly by washing the tanks of the fumigation machines (Vera et al. 2010). In Argentina, almost 200 million kg of glyphosate were used during 2010 (CASAFE 2013); Glifosato Atanor®, which is one of the most commonly used formulations, is composed of 48 % w/v of glyphosate as isopropylamine salt, surfactants of unknown composition, and mostly water. A previous experiment (Vera et al. 2012) revealed that it also contains phosphorus additives which may be available for autotrophs. Berkovic et al. (2006) found glyphosate concentrations as high as 10.9 mg L^{-1} in freshwater from Argentina.

In freshwater bodies, glyphosate may adversely affect non-target primary producers such as plants and microalgae due to their physiological similarities. The effect of glyphosate or its formulations on algae has been studied chiefly in laboratory cultures of planktonic species (Lipok et al. 2010; Romero et al. 2011). Most toxicity bioassays involving sessile algae (Peterson et al. 1994) have been performed with unialgal cultures and only a few with entire periphytic communities (Holtby and Baillie 1989; Kish 2006). In addition, there is little information available comparing the effects of technical-grade glyphosate *versus* its commercial formulations on different algal species. The majority of these studies have shown that the formulation has a higher toxicity than the technical grade glyphosate (Powell et al. 1991; Sáenz and Di Marzio 2009; Sáenz et al.

1997; Tsui and Chu 2003). Moreover, to our knowledge, no comparison has been reported between their effects on freshwater autotrophic communities (i.e. periphyton or phytoplankton).

The objective of the present study was to explore possible differential effects of the formulation Glifosato Atanor® and the technical-grade glyphosate acid (active ingredient, a.i.) on the algal fraction of a freshwater periphytic community, using a short-term laboratory bioassay. Our hypothesis was that the formulation additives and the a.i. alone may affect differently the pigment content of periphyton. We conducted a temporal analysis of the concentrations of chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) and total carotenes as biomass estimators for periphyton developed on artificial substrates.

Materials and Methods

Periphyton

Prior to the laboratory bioassay, aquatic plants (*Ceratophyllum* sp.) were placed in a 3,000-L pool filled with underground water to reproduce the limnological characteristics of a water body in a "clear water state" (Chl *a* 5.44 μg L^{-1} and nephelometric turbidity 1 NTU). After the pool developed into a true shallow pond with a well-established community of microorganisms in the water column, artificial substrata were submerged just below the surface. These consisted of clear 10-cm^2 polycarbonate strips. Substantial colonization was achieved after 21 days (i.e. maximum biomass peak).

Laboratory Bioassay

Bold's Basal Medium (BBM) for the laboratory testing was prepared with pool water filtered through GF/F Whatman® filters. Subsequently, the culture medium was filtered under sterile conditions through 0.22-μm white nitrocellulose filters (GAMAFIL S.A.), to obtain the sterile modified BBM used in the experiment.

Artificial substrata (i.e. polycarbonate strips) colonized by periphyton were removed from the pool and transported in cold and darkness to the laboratory. On that same day, sixty-three 250-mL autoclaved beakers (three replicates for each treatment and sampling–time combination) were filled with 100 mL of sterile modified BBM in a laminar-flow chamber. Three factors were randomly assigned to the beakers: control (C; no herbicide added), technical-grade glyphosate acid (G; ≥95 % purity; CAS: 1071-83-6), and Glifosato Atanor® (GA; isopropylamine salt of glyphosate 48 % w/v; surfactants of unknown composition and mostly water), with both herbicides at a nominal glyphosate concentration of 3 mg a.i. per liter, which is similar to that recommended for aquatic weed control (3.7 mg a.i. L^{-1}, Giesy et al. 2000) and it falls in the range of concentrations found in Argentine freshwater bodies (Berkovic et al. 2006). Initial glyphosate concentration in culture medium was analytically determined by ion chromatography (Zhu et al. 1999) using a DIONEX DX-100 instrument with a conductivity detector, a sample injection valve, and a 25-μL sample loop. Two plastic anion columns were coupled in series to serve both as pre-column (DIONEX AG-4) and analytical chromatographic column (DIONEX AS-4). The suppression was made using a micromembrane ASRS-ULTRA II. A mixture of $NaOH/Na_2CO_3$ 4 mM/9 mM was chosen as eluent with a flow rate of 2 mL min^{-1}. The actual values were 2.7 ± 0.081 mg L^{-1} for GA treatment and 2.9 ± 0.249 mg L^{-1} for G.

One colonized substratum was positioned on one side, on the bottom of each beaker.

The first sample (t0) was taken in triplicate before herbicide application. Then, each experimental unit was sealed with transparent film and randomly distributed in a culture chamber under controlled temperature (24 ± 1°C) and a 16:8 h light:dark cycle with cool fluorescent white light (50 μmol photon m^{-2} s^{-1}). The periphyton from three beakers (replicates) per treatment was collected on seven occasions: 2, 6, 10, 24, 48, 96, and 192 h following t0 (t1–t7).

Pigment content of periphyton was determined for every sampling time. Therefore, the community was harvested separately by scraping, and then subjected to centrifugation (10 min at 7,000×*g*). Chl *a*, Chl *b* and carotene concentrations were spectrophotometrically determined according to Lichtenthaler (1987). Samples were thoroughly ground in 80 % acetone; after 1-h incubation in the dark at 4°C, the extracts were clarified by centrifugation (10 min at 7,000×*g*) and their absorbances were read at 663.2, 646.8 and 470.0 nm using a UV/visible Shimadzu spectrophotometer. All variables were expressed on an area basis.

Statistical Analyses

For all variables, differences between treatments were assayed using two-factor analysis of variance (two-way ANOVA) for treatment (three levels: C, G, GA) and sampling time (eight levels: 0, 2, 6, 10, 24, 48, 96 and 192 h). When required, Tukey's post hoc tests were used for multiple comparisons. Prior to the analyses, data were tested for normality and homoscedasticity using normal probability plot of residuals and Bartlett's test, respectively. For all tests, the significance level was set at $p < 0.05$.

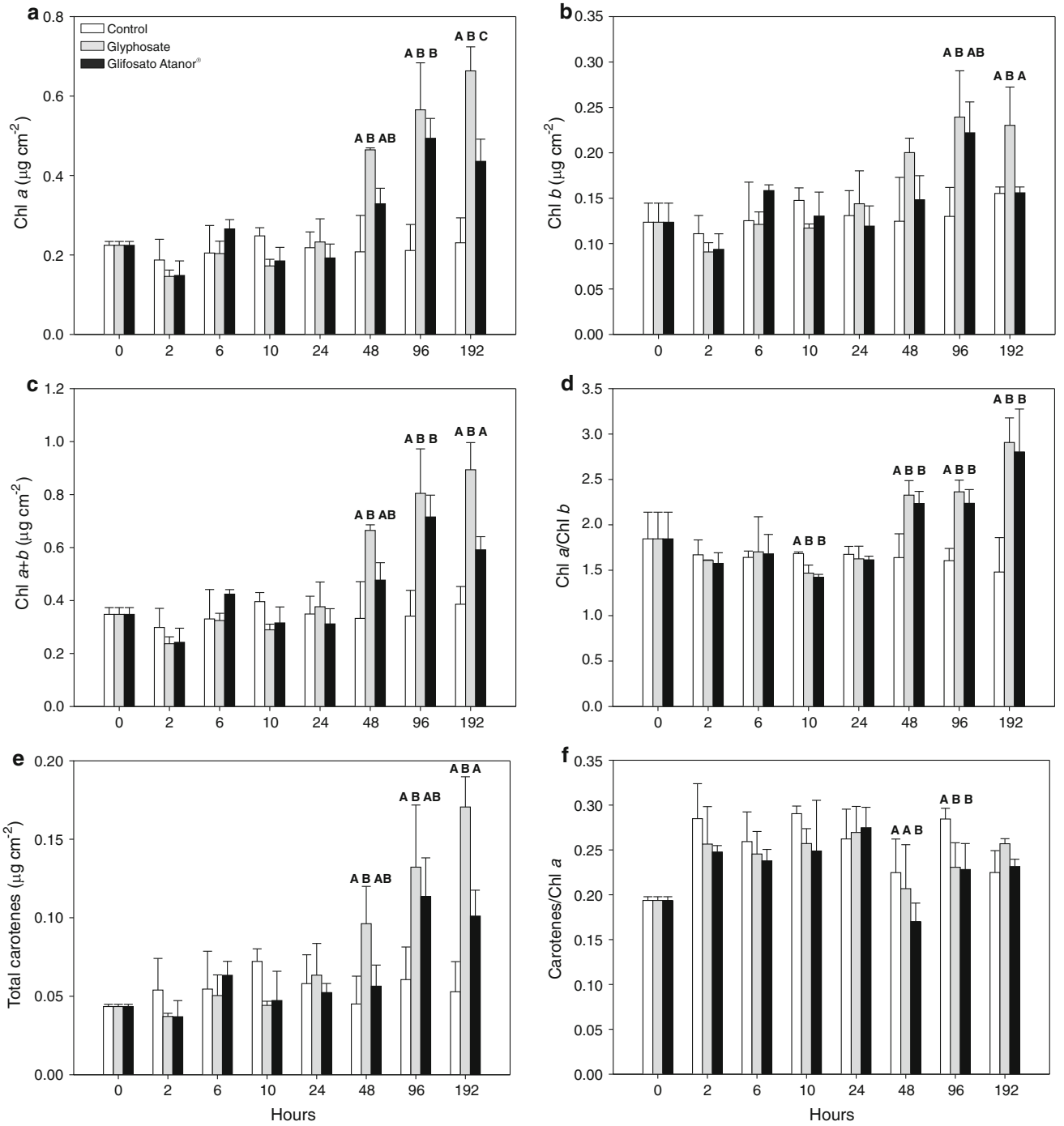

Fig. 1 Periphytic (**a**) chlorophyll *a*, (**b**) chlorophyll *b*, (**c**) total chlorophyll (Chl $a + b$), (**d**) chlorophyll *a*/*b* (Chl *a*/*b*), (**e**) total carotenes, and (**f**) total carotene/chlorophyll *a* mean values throughout the experiment for the different treatments. *Error bars* represent +1 SD. Significant differences between treatments ($p < 0.05$) on the same sampling date are indicated by *different letters*

Results

The mean Chl *a* content of periphytic algae ranged from 0.19 to 0.25 µg cm^{-2} for control microcosms, from 0.15 to 0.66 µg cm^{-2} for G-treated microcosms, and from 0.15 to 0.49 µg cm^{-2} for GA-treated microcosms. Two-way ANOVA showed significant differences in Chl *a* concentration for the time-by-treatment interaction ($p = 0.0001$). Mean comparisons revealed significant differences between treatments from t5 (48 h) to t7 (192 h), with G-treated samples showing a higher increase at 192 h (Fig. 1a). There were significant differences in Chl

a concentration between t6 (96 h) and t7 (192 h) compared with the previous sampling times for G- and GA-treated samples. At t7, mean Chl *a* concentration was 3 times higher for the G-treated samples than for the control and about twice that for the GA-treated samples (Fig. 1a). Chl *a* content in the control remained unchanged throughout the experiment.

The mean Chl *b* concentration was 0.15 ± 0.05 μg cm^{-2} and ranged from 0.08 to 0.29 μg cm^{-2} during the experiment. Two-way ANOVA yielded significant differences in Chl *b* concentration for the time-by-treatment interaction ($p = 0.003$). Post-hoc testing revealed significant differences in the Chl *b* concentration between treatments at t6 and t7. Significant differences were found between t6 and t7 compared with the previous sampling times for G-treated samples, while GA-treated samples showed a significant increase at t6 (Fig. 1b). There were no significant differences in Chl *b* concentration for the control group over the whole study duration.

The mean total Chl ($a + b$) concentration ranged from 0.30 to 0.40 μg cm^{-2} for control microcosms, from 0.24 to 0.89 μg cm^{-2} for G-treated microcosms, and from 0.24 to 0.72 μg cm^{-2} for GA-treated microcosms. Two-way ANOVA showed significant differences for the time-by-treatment interaction ($p = 0.0001$), with significant differences between herbicide treatments from t5 to t7 (Fig. 1c). For both G- and GA-treated samples, t6 and t7 differed significantly from the other sampling times; at t6, total Chl ($a + b$) concentration was increased about twofold when compared with control (Fig. 1c). The latter showed no changes in mean total chlorophyll during the experiment.

The mean Chl *a/b* ratio varied between 1.48 and 1.85 for control microcosms, between 1.47 and 2.91 for G-treated microcosms, and between 1.42 and 2.80 for GA-treated microcosms. Two-way ANOVA showed significant differences for the time-by-treatment interaction ($p = 0.0001$). Mean comparisons revealed significant differences between herbicide treatments at t3 (10 h; with lower values for G- and GA-treated samples than for C) and at t5 (with higher values for G- and GA-treated samples than for the control group; Fig. 1d). The sampling times t6 and t7 differed significantly from the other sampling times for G- and GA-treated samples. Both treatments showed an increase in the Chl *a/b* ratio of 40 % at t5 compared to C, reaching a 100 % increase on the last sampling date (8 days, Fig. 1d). The Chl *a/b* rate in the control group remained constant throughout the study.

The mean total carotene concentration ranged from 0.04 to 0.07 μg cm^{-2} for C microcosms, from 0.04 to 0.17 μg cm^{-2} for G-treated microcosms, and from 0.04 to 0.11 μg cm^{-2} for GA-treated microcosms. Two-way ANOVA indicated that there were significant differences for the time-by-treatment interaction ($p = 0.00001$), and mean comparisons revealed significant differences between herbicide treatments after 2 days of experiment (Fig. 1e). The sampling times t6 and t7 differed significantly from the other sampling times for G- and GA-treated samples. At t7, G-treatments showed a ~threefold increase in total carotene content as compared with C (Fig. 1e). Total carotene concentration in the control remained unchanged throughout the experiment.

The total carotene-to-Chl *a* ratio was used to analyze quantitative changes in carotene synthesis. The mean value of this ratio was 0.25 ± 0.04, and ranged between 0.16 and 0.32 during the experiment. Two-way ANOVA showed significant differences for the factors sampling time ($p = 0.0001$) and herbicide treatment ($p = 0.021$). A significant decline in the mean total carotene-to-Chl *a* ratio was found for the GA treatment at t5 compared with G and the control, while at t6 this ratio decreased significantly for the two treatments compared with the control (Fig. 1f).

Discussion

It is expected that glyphosate adversely affects the autotrophic fraction in the periphyton since it uses the shikimic acid pathway for the biosynthesis of aromatic amino acids (Schönbrunn et al. 2001). However, periphyton showed a significant increase in both chlorophyll and total carotenes after a 2-day exposure to both Glifosato Atanor® and the technical-grade glyphosate. This implies that the herbicides stimulated the development of organisms deriving from a mature periphyton community, which were later transferred to a culture medium supplemented with the appropriate nutrients and under unlimited light conditions. It is likely that the herbicides served as an extra source of nutrients for some fraction of the community, and/or that they triggered pathways for the synthesis of proteins and metabolites involved in stress response (Tukaj and Tujaj 2010), thus leading to increased growth.

In this experiment, the treatment with Glifosato Atanor® induced a twofold increase in Chl *a* content compared with the control and with test initiation. A significant increase in periphyton Chl *a* concentration induced by glyphosate-based herbicides has also been reported by other authors. This was observed by Holtby and Baillie (1989) in a natural water body after the application of Roundup® and by Vera et al. (2012) following a single application of 3.5 mg L^{-1} of Glifosato Atanor®. We also found an increase in the total chlorophyll (Chl $a + b$) concentration after 2 days of exposure to the herbicide treatments, changes similar to that reported by Kish (2006) for a periphytic community after a 7-day exposure to the lowest concentration (7.8 mg a.i. L^{-1}) of Roundup®. On the other

hand, a significant decrease in periphyton pigment concentration was observed following the addition of Roundup® (Vera et al. 2010). These contrasting results may reflect the differential responsiveness of periphyton to this herbicide, which could be due to many interacting factors such as differences in the formulation composition, the concentration of the a.i. and the water body morphometry, as well as indirect impacts resulting from changes in pressure of competition and herbivory.

Romero et al. (2011) observed a decreased synthesis of carotenes in *Chlorella kessleri* after exposure to Glifosato Atanor® at a concentration of 70 mg glyphosate L^{-1}. Such herbicide concentration in the water, which poses a threat to ecosystem integrity, is higher by an order of magnitude than that used by us. These results are, despite substantial deviation in the experiment approach, comparable to the present study covered for both Glifosato Atanor® as well as the technical-grade glyphosate.

A change in the Chl *a*/*b* ratio can be used as an indicator of alterations in PSII photochemistry in Chlorophyta and higher plants (Pintilie et al. 2006; Samuel and Bose 1987). Physiological processes of senescence and exposure to contaminants may induce damage to the thylakoid membranes and affect the anchoring of chlorophyll molecules to the photosynthetic reaction center, thus leading to a lower Chl *a*/*b* ratio (Öncel et al. 2000; Samuel and Bose 1987). Romero et al. (2011) reported a decrease in Chl *a*/*b* ratio in *Chlorella kessleri* exposed to 70 mg a.i. L^{-1} of Glifosato Atanor®. We also observed a decrease in this ratio after 10 h of exposure to both herbicides, as well as a non-significant decrease in the content of total chlorophyll and carotenes. However, the Chl *a*/*b* ratio was found to increase from the second day to the end of the experiment for both glyphosate treatments. This result is in agreement with that of Vera et al. (2012), who obtained an increase in the ratio after a 21-days exposure to Glifosato Atanor® in an outdoor microcosms. The decline in the chlorophyll *a*/*b* ratio as early as 10 h after glyphosate addition could be attributed to potentially damaging effects of short-term exposure on the photosystems, before the occurrence of a change in the community composition. This may imply that the herbicides used here had adverse effects on the periphyton. On the other hand, variation in the taxonomic composition of the community may account for the increase in the Chl *a*/*b* ratio observed after a long-term exposure (2 days). A higher proportion of cyanobacteria and/or diatoms -which lack Chl *b*- relative to chlorophytes (Kirk 1994) may lead to a higher Chl *a*/*b* ratio. Although we did not analyze the taxonomic composition of periphyton, previous studies involving outdoor mesocosms and microcosms treated with Roundup® or Glifosato Atanor® (Pérez et al. 2007; Vera et al. 2010, 2012) revealed predominance of periphytic cyanobacteria over other algal groups, while diatoms were sensitive to the herbicides. These results suggest that there was a shift in the taxonomic composition of the periphytic algae toward a higher proportion of cyanobacteria. These are known to develop successfully under stress conditions, showing tolerance to glyphosate (Powell et al. 1991), or capacity for herbicide degradation (Forlani et al. 2008).

Glyphosate formulations are more toxic than the active ingredient to some non-target organisms. Sáenz et al. (1997), who performed laboratory bioassays using technical-grade glyphosate and a glyphosate formulation with an unknown surfactant (Rondo®), determined that additives increase the toxicity of glyphosate to freshwater green algae. Sáenz and Di Marzio (2009) reported that Roundup® was more toxic than technical-grade glyphosate for different species of green algae in culture. Using two species of cyanobacteria as test organisms, Powell et al. (1991) established the following toxicity ranking: Roundup® > glyphosate salt > glyphosate acid; in all cases cyanobacteria displayed some level of tolerance. Lipok et al. (2010) found that Roundup® 360 SL exerts a significantly greater toxicity than its main constituents – isopropylamine and glyphosate – to cyanobacteria and algae. Finally, according to Tsui and Chu (2003), the surfactant POEA is more harmful to two species of algae than Roundup® and glyphosate as acid and salt. Although no toxic effects were measured in our experiment, both herbicides produced an increase in algal biomass which persisted for up to 8 days of exposure. Moreover, the fact that the effect of Glifosato Atanor® was lower than or not significantly different from that of its a.i. suggests that the additives of this formulation do not increase glyphosate toxicity.

Our results showed that both glyphosate and one of its most commonly used commercial formulations in Argentina, Glifosato Atanor®, stimulate the growth of the autotrophic fraction in the periphyton, which plays an important role in the overall ecology of water bodies. Taking into account that we used herbicide concentrations similar to those measured in water bodies from Argentina, such findings pinpoint that freshwater microbial communities such as the periphyton may be at risk in the field.

Acknowledgments We thank Dr. Eugenia Di Fiori for field assistance and to the Associated Editor of the Journal for improving the manuscript. This work was supported by ANPCyT (PICT 2010-0908) and UBACyT O1/W550.

References

Berkovic A, Marino D, Lespade P, Ronco A (2006) Análisis de pesticidas asociados al cultivo de soja en aguas y sedimentos de un sector productivo de la pampa ondulada. Anales XXVI Congreso Argentino de Química, San Luis, 8–053

CASAFE (2013) Mercado Argentino de Productos Fitosanitarios 2013. Cámara de Sanidad Agropecuaria y Fertilizantes, Buenos Aires

Forlani G, Pavan M, Gramek M, Kafarski P, Lipok J (2008) Biochemical bases for a widespread tolerance of cyanobacteria to the phosphonate herbicide glyphosate. Plant Cell Physiol 49:443–456

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

Holtby LB, Baillie SJ (1989) Effects of the herbicide Roundup (glyphosate) on periphyton in Carnation Creek, British Columbia. In: Reynolds PE (ed) Proceedings of the Carnation Creek Herbicide Workshop, Forestry Canada, Canada, pp 224–231

Kirk JTO (1994) Light and photosynthesis in aquatic ecosystems. Cambridge University Press, Cambridge

Kish PA (2006) Evaluation of herbicide impact on periphyton community structure using the Matlock periphytometer. J Freshw Ecol 21:341–348

Lichtenthaler HK (1987) Chlorophyll and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol 148:349–382

Lipok J, Studnik H, Gruyaert S (2010) The toxicity of Roundup® 360 SL formulation and its main constituents: glyphosate and isopropylamine towards non-target water photoautotrophs. Ecotoxicol Environ Saf 73:1681–1688

Öncel I, Keleş Y, Üstün AS (2000) Interactive effects of temperature and heavy metal stress on the growth and some biochemical compounds in wheat seedlings. Environ Pollut 107:315–320

Pérez GL, Torremorell A, Mugni H, Rodríguez P, Vera MS, do Nascimento M, Allende L, Bustingorry J, Escaray R, Ferraro M, Izaguirre I, Pizarro H, Bonetto C, Morris DP, Zagarese H (2007) Effects of the herbicide Roundup on freshwater microbial communities: a mesocosm study. Ecol Appl 17:2310–2322

Peterson HG, Boutin C, Martin PA, Freemark KE, Ruecker NJ, Moody MJ (1994) Aquatic phytotoxicity of 23 pesticides applied at expected environmental concentrations. Aquat Toxicol 28:275–292

Pintilie M, Oprica L, Surleac M, Dragut Ivan C, Creanga DE, Artenie V (2006) Enzyme activity in plants treated with magnetic liquid. Rom J Phys 51:239–244

Powell HA, Kerby NW, Rowell P (1991) Natural tolerance of cyanobacteria to the herbicide glyphosate. New Phytol 119:421–426

Romero DM, Ríos de Molina MC, Juárez AB (2011) Oxidative stress induced by a commercial glyphosate formulation in a tolerant strain of *Chlorella kessleri*. Ecotoxicol Environ Saf 74:741–747

Sáenz ME, Di Marzio WD (2009) Ecotoxicidad del herbicida glifosato sobre cuatro algas clorófitas dulceacuícolas. Limnetica 28:149–158 (in Spanish)

Sáenz ME, Di Marzio WD, Alberdi JL, Tortorelli MC (1997) Effects of technical grade and a commercial formulation of glyphosate on algal population growth. Bull Environ Contam Toxicol 59:638–644

Samuel K, Bose S (1987) Bleaching of photosyntethic pigments in *Chlorella protothecoides* grown in the presence of SANDOZ 9785 (4-chloro-5-dimethylamino-2 phenyl-3 (2H) pyridazinone). J Biosci 12:399–404

Schönbrunn E, Eschenburg S, Shuttleworth WA, Schloss JV, Amrhein N, Evans JNS, Kabsch W (2001) Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. PNAS 98:1376–1380

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

Tukaj S, Tujaj Z (2010) Distinct chemical contaminants induce the synthesis of Hsp70 proteins in green microalgae *Desmodesmus subspicatus*: heat pretreatment increases cadmium resistance. J Therm Biol 35:239–244

Vera MS, Lagomarsino L, Sylvester M, Pérez G, Rodríguez P, Mugni H, Sinistro R, Ferraro M, Bonetto C, Zagarese H, Pizarro H (2010) New evidences of Roundup® (glyphosate formulation) impact on the periphyton and the water quality of freshwater ecosystems. Ecotoxicology 19:710–721

Vera MS, Di Fiori E, Lagomarsino L, Sinistro R, Escaray R, Iummato MM, Juárez A, Ríos de Molina MC, Tell G, Pizarro H (2012) Direct and indirect effects of the glyphosate formulation Glifosato Atanor® on freshwater microbial communities. Ecotoxicology 21:1805–1816

Zhu Y, Zhang F, Tong C, Liu W (1999) Determination of glyphosate by ion chromatography. J Chromatogr A 850:297–301