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New evidences of Roundup® (glyphosate formulation) impact on the periphyton community and the water quality of freshwater ecosystems

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Abstract Argentina is the second largest world producer of soybeans (after the USA) and along with the increase in planted surface and production in the country, glyphosate consumption has grown in the same way. We investigated the effects of Roundup® (glyphosate formulation) on the periphyton colonization. The experiment was carried out over 42 days in ten outdoor mesocosms of different typology: "clear" waters with aquatic macrophytes and/or metaphyton and "turbid" waters with great occurrence of phytoplankton or suspended inorganic matter. The herbicide was added at 8 mg L^{-1} of the active ingredient (glyphosate) in five mesocosms while five were left as controls (without Roundup® addition). The estimate of the dissipation rate (k) of glyphosate showed a half-life value of 4.2 days. Total phosphorus significantly increased in treated mesocosms due to Roundup® degradation what favored eutrophication process. Roundup® produced a clear delay in periphytic colonization in treated mesocosms and values of the periphytic mass variables (dry weight, ash-free dry weight and chlorophyll *a*) were always higher in control mesocosms. Despite the mortality of algae, mainly diatoms, cyanobacteria was favored in treated mesocosms. It was observed that glyphosate produced a long term shift in the typology of mesocosms, "clear" turning to "turbid", which is consistent with the regional trend in shallow lakes in the Pampa plain of Argentina. Based on our findings it is clear that agricultural practices that involve the use of herbicides such as Roundup® affect non-target organisms and the water quality, modifying the structure and functionality of freshwater ecosystems.

Keywords Roundup® · Glyphosate · Periphyton · Water quality · Mesocosms · Clear and turbid shallow lakes

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Introduction

In Argentina, the area planted with soybean has increased from 370,000 to 17 million hectares (ha) since 1996. Almost 50% of the total planted area in the country (30 million ha in 2004/2005, Trigo and Cap 2006) was devoted to soybeans and more than 98% is glyphosate-tolerant. Nowadays, Argentina is the second largest world producer of soybeans (after the USA, James 2007) and along with the increase in planted surface and production in the country, glyphosate consumption has grown in the same way. Moreover, glyphosate is used not only for soybeans, but also for other crops like maize, cotton and canola, and for chemical fallow. This agricultural practice is a weed control mediated by the herbicide, for the

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preservation of soil water content to be used by different crops in rotation. These circumstances led Argentina to use 162 million kg of glyphosate in 2007 (CASAFE 2009). The speed at which the adoption of the new technologies evolved is an important fact but its consequences for the environment aren't yet fully understood.

Glyphosate [*N*-(phosphonomethyl)glycine] is a non-selective, broad spectrum, post-emergent agrochemical widely used in agriculture and silviculture in many countries for the control of grasses, sedges and broad-leaved weeds (Goldsborough and Brown 1988). Glyphosate's primary mode of action in plants and several microorganisms is the disruption of aromatic amino acid biosynthesis, through the inhibition of the enzyme 5-enolpyruvyl shikimic acid-3-phosphate synthase (EPSPS), which halts the production of chorismate (Amrhein et al. 1980). The process ultimately results in the cessation of aromatic amino acid synthesis, which in turn reduces protein synthesis and growth, and eventually causes cellular disruption and death (Salisbury and Ross 1994).

Nowadays, the products commonly used are formulations of glyphosate (e.g., Accord®, AquaMaster™, Rodeo®, Rondo®, Roundup®, Touchdown®), which in addition to the active ingredient include water and a surfactant system that enables the product to adhere to the surface of leaves so the active ingredient can penetrate them. Because of this, most of the studies carried out on the effects of glyphosate in aquatic environments have been performed using glyphosate formulations. Among these commercial formulations, one of the most used in the world is Roundup®, which contains 480 g L^{-1} of glyphosate, as the isopropylamine salt, and a surfactant, polyoxyethylene amine or POEA.

Glyphosate is usually assumed to be safe and non-toxic to the environment due to its fast biodegradation and/or adsorption by soil particulates. Nevertheless, off-target displacements from soils have already been reported (Peruzzo et al. 2008). Glyphosate may reach aquatic systems either by accidental or wind driven drift of the herbicide spray, or through transport in surface runoff (Edwards et al. 1980) and suspended particulate matter (Feng et al. 1990; Goldsborough and Beck 1989). It has been observed in Argentina that another way that glyphosate may reach water bodies is by direct human action, washing the tanks of the fumigation machines in streams and shallow water bodies near cultivation fields.

Most of the literature dealing with glyphosate impacts on aquatic organisms is based on laboratory bioassays (e.g., Relyea 2004; Schaffer and Sebetich 2004). Many toxicity studies are based on the effects on individuals, frequently only on a single species. Although this is a rapid way to identify the direct impacts of pesticides on organisms, it doesn't provide information about possible effects on organisms in their natural environments (Relyea 2005a). Field studies, on the other hand, have mostly focused on fish (Cavalcante et al. 2008; Langiano and Martinez 2008), invertebrates (Henry et al. 1994; Tsui et al. 2005), and amphibians (Costa et al. 2008; Relyea 2005b; Relyea et al. 2005). Monospecific tests may not be representative of what happens to populations present in natural waters and it is not possible to extrapolate the effects on the ecosystem from single species bioassays. That is the reason why studies on experimental mesocosms are so important, because they resemble the effect on natural communities and ecosystems as a whole.

Considering the amounts of glyphosate used in Argentina the gap in research on the impacts of this herbicide on the region is surprising. Most local research took laboratory bioassays into account, using freshwater algae (Asselborn and Zalocar de Domitrovic 1998; Sáenz et al. 1997), macrophytes and invertebrates (Achiorno et al. 2008). Others assessed glyphosate effects on *Lemna gibba* using field and laboratory studies (Sobrero et al. 2007). Scarce information is available on the effect of glyphosate based herbicides at community and/or ecosystem level (Pérez et al. 2007) despite the utility of this kind of studies.

Periphyton is a very important community in aquatic ecosystems and its role is very significant in shallow lakes where there is a great variety of habitats for its establishment considering the high proportion of littoral areas. Its importance in terms of production is evident in "clear" waters where its contribution to the total microbial production of the system is more than 77% greater than that of phytoplankton (Liboriussen and Jeppesen 2003). Periphyton possesses many attributes that makes it an ideal community to employ in water quality monitoring investigations. Because periphyton is a sessile community, it cannot avoid potential pollutants through migration or other means. Because periphyton integrates the influences of environmental conditions over long periods of time, they have been widely applied for monitoring purposes (Sabater and Admiral 2005). Some studies focused on the effect of Roundup® on structural and functional features of the periphytic algal fraction (Goldsborough and Brown 1988; Holtby and Baillie 1989) while Austin et al. (1991) used Vision®, another glyphosate-based herbicide, for a similar purpose.

The objective of the present study was to investigate the effect of the glyphosate formulation Roundup® on the periphyton accrual from water bodies with limnological properties similar to those on the Pampean plain of Argentina. We analysed the colonization of periphyton by means of structural and functional features of the community developed in artificial substrata placed in mesocosms that simulated different freshwater ecosystems. The experiment was carried out in ten shallow artificial lakes

(mesocosms) with the same morphometry but with different limnological characteristics, some with "clear" waters with aquatic macrophytes and/or metaphyton and others with "turbid" waters with a major presence of phytoplankton or suspended inorganic matter. The impact of the herbicide on the water quality of a heterogeneous group of shallow lakes was also discussed.

Materials and methods

Mesocosms' description

The experiment was carried out in 2006, between April and June, at the IIB-INTECH (National Institute of Biotechnological Investigations—National Technological Institute of Chascomús), Chascomús, Buenos Aires province, Argentina. The experiment employed artificial outdoor mesocosms which have a history of serving as useful experimental venues for ecotoxicological studies employing Roundup®. The ten mesocosms (depth: 1.2 m; area: 25 m^2), constructed in an area of approximately 1 ha, were built by accumulation and leveling of the land to form a hill where the excavations were made. Each excavation, which would be a mesocosm, was lined with black nylon for isolation to prevent percolation. The bottom of each excavation was covered with soil from places nearby to provide sediments to each environment (Fig. 1). Finally, they were filled with well water and were left to evolve. The first experiment, where the impact of Roundup® on microbial communities was tested, was developed during 2005. After the end of this experiment and in order to start the present study, the mesocosms were dried and afterwards refilled with new water. Before the refill, five mesocosms were randomly selected for Roundup® addition, with five to remain as controls. After the complete refill, the mesocosms were left to evolve naturally for about 1 year up to the beginning of the new experiment. At that moment, the ten mesocosms displayed different limnological characteristics, showing the typology representative of shallow lakes of the Argentine Pampean plain (Allende et al. 2009; Izaguirre and Vinocur 1994; Quirós and Drago 1999). The ten mesocosms were mainly eutrophic, with or without plants (rooted macrophytes and/or metaphyton), with clear to turbid waters. Turbidity was originated by organic (phytoplankton) or inorganic compounds (suspended matter).

Experimental design

At the beginning of the present experiment, five mesocosms were treated with Roundup® in order to attain an initial concentration of 8 mg glyphosate as active ingredient per L^{-1} in each treated mesocosm. The remaining five mesocosms were left as controls (without Roundup® addition). The nominal concentration of glyphosate was selected to be comparable and intermediate to the concentrations assayed in a previous experiment (Pérez et al. 2007). The tested concentration lies towards the higher edge of the range reviewed by Relyea (2006) as worst case scenarios, ranging between 1.4 (Canadian government) and 10.3 a.i. mg L^{-1} (Mann and Bidwell 1999).

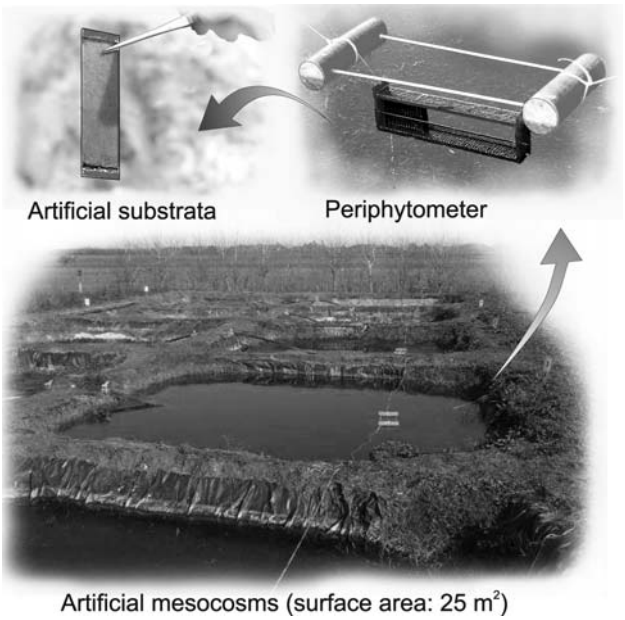

Fig. 1 Scheme with pictures of the outdoor mesocosms built for the experiment, a periphytometer and an artificial substratum for periphyton colonization

Water samples were collected from each mesocosm on six occasions using a Van Dorn-style bottle. The first samples were collected immediately before herbicide application ($t0$), except those for glyphosate determination that were collected immediately after application. The remaining samples were collected 3, 8, 14, 28 and 42 days after Roundup® application ($t1$–$t5$, respectively). The water samples were transported in 5-L plastic containers from which subsamples were taken for glyphosate determinations and analyses of physical, chemical and biological variables. The study of the periphyton assemblage was performed using artificial substrates which were placed in each mesocosm at the beginning of the experiment. Figure 1 shows a schedule of the mesocosms and the materials for periphyton analysis.

Statistical analyses

The Kruskal–Wallis non-parametric ANOVA by ranks test (KW) was used to compare the water chemistry variables

of "clear" and "turbid" mesocosms at *t*0 and between treatments over the course of the experiment. Simple linear regression analyses were performed, for each treatment, for log-transformed periphytic variables versus time. Prior to each regression analysis, Kolmogorov–Smirnov and Levene's tests were run in order to check data for normality and homoscedasticity, respectively. Regression analyses with auxiliary (dummy) variables were performed to test homogeneity between slopes, and differences between intercepts were assayed using analyses of variance procedures ($P < 0.05$).

Periphyton analysis

A special device (periphytometer), containing clear polycarbonate strips (1 mm thick) of known surface that served as artificial substrata, was suspended approximately 10 cm below the water surface in each mesocosm in special frames at the beginning of the experiment (Fig. 1). The substrata were allowed to be colonized by a periphytic community and samples were collected 8, 14, 28 and 42 days from the beginning of the experiment. On each sampling date, the periphyton on each substrate was removed by means of a fine brush and divided into aliquots for different analyses. Samples for qualitative algal determinations were fixed with 2% formalin and analyzed under an optical microscope at 1,000× magnification. Water samples for quantitative analysis were preserved with 1% acidified Lugol's iodine solution. Counts of periphyton algae were performed using the inverted microscope technique (Utermöhl 1958) at 400× magnification. The counting error (<15%) was estimated according to Venrick (1978).

The following variables were also considered: live and dead diatom abundance, algal classes' percentages, chlorophyll *a* concentration (P-Chl *a*), dry weight (DW), ash-free dry weight (AFDW) and primary production (PP). We considered as dead diatoms those individuals that presented a disorganized chloroplast at microscope level and/or broken frustules. All periphytic variables were expressed on an area basis. Periphyton chlorophyll *a* concentration was estimated from scraped material filtered through Whatman® GF/F filters. Filters were immediately wrapped in aluminum foil and stored at −80°C until processing. Pigments were extracted (overnight, at 4°C, in the dark in a nitrogen-saturated atmosphere) using 90% (by volume) aqueous acetone and the extracts were cleared by centrifugation at 3,000 rpm for 10 min. Pigment extracts were measured by ion pairing reverse-phase HPLC (modified from Mantoura and Llewellyn 1983) using an Aktabasic chromatograph (Amersham, Buckinghamshire, UK) controlled by the Unicorn program (Amersham, Buckinghamshire, UK). The method employed is described in Laurion et al. (2002). The HPLC system was calibrated with commercially available chlorophyll *a* standard from Sigma (Buchs, Switzerland).

DW was estimated from samples filtered through Whatman® GF/C filters pre-combusted to 440°C for 2 h prior to use and later weighting of the material dried at 60°C on a stove. AFDW was determined as the mass difference after 3 h′ calcination (440°C) of dry samples (APHA 2005).

PP was estimated by the ^{14}C-technique (Steeman-Nielsen 1952). One colonized substratum was incubated for 2 h in a 70 mL acrylic tube (clear cut-off at 400 nm) placed at the surface of an outdoor water bath. For each mesocosm, two tubes were incubated at a saturating, but not photoinhibiting irradiance level (ranging from 41.85 to 104.54 W m^{-2}), obtained by using a neutral density filter. The irradiance level was decided based on preliminary production *versus* irradiance curves. In addition, a single dark tube per mesocosm was used to estimate dark ^{14}C incorporation. Three μCi of ^{14}C labeled $NaHCO_3$ were added to each tube. After incubation, the material was scraped from the substratum side facing the light. This side was marked with an innocuous label prior to use. The scraped material was filtered through Whatman® GF/F filters, placed in a HCl saturated atmosphere and dried overnight. The activity of filters was measured in a scintillation counter with 2.5 mL of OptiPhase "HiSafe"3 scintillation solution. Dissolved inorganic carbon was determined from alkalinity by Gran titration, pH, and temperature (Stumm and Morgan 1996).

Physical and chemical variables of the mesocosms' water

Physical and chemical analyses were performed 3, 8, 14, 28 and 42 days after the Roundup® addition. Conductivity (Hach conductimeter), pH (Orion pH meter) and dissolved oxygen concentration (YSI 5000 meter) were measured in situ on each sampling date. Water temperature was recorded sub-superficially over the course of 1 day at *t*5 (after 42 days) in mesocosms E1 and E3 with a THERMOBUTTON Data Logger. At *t*0 water transparence was recorded in each mesocosm from vertical profiles of downward irradiance measurements (380–750 nm, every 1 nm), using a spectra-radiometer (USB2000, Ocean Optics). Profiles were obtained around 1 h from astronomic noon. Broadband (K_d PAR) vertical diffuse attenuation coefficients, for downwelling irradiance, were calculated by regressing log-transformed irradiance measurements against depth. Nephelometric turbidity values (Tn) were measured with an underwater turbidimeter (SCUFA, Turner®). Phytoplankton chlorophyll *a* was monitored daily using an underwater turbidimeter (SCUFA, Turner®).

Water samples for chemical analysis of major ions and nutrients were filtered immediately after sampling through

Whatman® GF/C filters. Soluble reactive phosphorus (SRP) was measured by the molybdate-ascorbic method, nitrate by the hydrazine reduction method followed by nitrite determination by diazotation and ammonium by the indophenol blue method, following the APHA (2005). Calcium and magnesium (atomic absorption spectrometry), sodium and potassium (flame photometry), bicarbonate (titration), sulphate (turbidimetry), and chloride ($AgNO_3$ titration) were determined following the APHA (2005). Total phosphorus (TP) were measured in the same way as SRP after acid digestion of unfiltered water samples.

Analyses of glyphosate were carried out before glyphosate addition in all mesocosms and on five sampling occasions (days 2, 8, 10, 11 and 14) at each treated mesocosm on water samples filtered through a 0.45 µm membrane filter. The analyses were performed by reversed-phase HPLC (high performance liquid chromatography) following derivatization with fluorenylmethyl chloroformate chloride (FMOC chloride), following Miles et al. (1986).

Results

Roundup® effect on periphyton community

Total periphyton mean algal abundance ranged from 9.6×10^4 to 88.2×10^4 indiv cm^{-2} and from 7.4×10^4 to 63.4×10^4 indiv cm^{-2} in control and treated mesocosms, respectively. This variable showed values always higher in control mesocosms, and even though it increased from the beginning of the experiment, an abundant decrease was seen on the last sampling date, for both control and treated mesocosms (Table 1).

During the entire experiment both treatments were dominated by diatoms, ranging from 78.6 to 89.9% in control mesocosms and from 50.6 to 76% in treated mesocosms; an increase of cyanobacteria was observed from the first sampling onward in treated mesocosms (Table 1). The more representative species of diatoms that were registered were the ubiquitous *Gomphonema parvulum*, *Achnanthes minutissima* and *Amphora veneta*. Among the cyanobacteria, the more frequent species registered were *Chamaesiphon minutus*, *Chroococcus turgidus*, *Leptolyngbya faveolarum* and *Merismopedia hyaline*. A higher mortality of diatoms in the first stages of colonization was observed in treated mesocosms (Fig. 2a) but without significant differences between treatments. Diatom abundance (live + dead organisms) 8 days after the beginning of the experiment ranged from 3×10^2 to 1.9×10^5 indiv cm^{-2} and from 4.4×10^2 to 1.1×10^5 indiv cm^{-2} in control and treated mesocosms, respectively (Fig. 2b). From that day onward, diatom abundance increased until day 28 of the experiment and then a slightly decrease was observed. Treated mesocosms always showed significant lower values in relation to control ones at the same time level (slope $P = 0.815$; ordinate $P = 0.026$; Table 2).

Mean DW ranged from 174 to 893 µg cm^{-2} and from 75 to 387 µg cm^{-2}; mean AFDW ranged from 63 to 329 µg cm^{-2} and from 24 to 135 µg cm^{-2}, and mean P-Chl *a* concentration ranged from 0.4 to 4.9 µg cm^{-2} and from 0.2 to 1.7 µg cm^{-2}, in control and treated mesocosms, respectively, over the course of the experiment. Despite the major variation observed among mesocosms as regards periphyton variables (DW, AFDW, P-Chl *a*), values of the mass variables increased all during the experiment and they were always higher in control mesocosms (Fig. 3a–c). Comparing the regression lines obtained from DW, AFDW and P-Chl *a* temporal variations, we obtained significant differences between treated and control mesocosms. Treated mesocosms showed always lower values in relation to control ones at the same time level. The obtained linear regressions showed two lines with similar slope values (DW $P = 0.967$; AFDW $P = 0.967$; P-Chl *a* $P = 0.973$) and significant differences between intercepts (DW $P = 0.002$; AFDW $P = 0.015$; P-Chl *a* $P = 0.018$) for all the mass variables (Table 2) indicative of a delayed colonization of periphyton in treated mesocosms.

At the first periphyton sampling date, the PP ranged from 0.2 to 30.2 mg C m^{-2} h^{-1} and from 0.3 to 24.1 mg C m^{-2} h^{-1} in control and treated mesocosms, respectively; a slight decrease was observed until the end

Table 1 Mean total algal abundance, percentages of algal classes and mean primary production (PP) values of periphyton in control (*C*) and treated (*T*) mesocosms throughout the sampling period

	8 days		14 days		28 days		42 days	
	C	*T*	*C*	*T*	*C*	*T*	*C*	*T*
Algal abundance (10^4 ind cm^{-2})	9.6 (5.1)	7.4 (3.6)	55.5 (22.6)	38.0 (16.8)	88.2 (26.7)	63.4 (45.5)	64.3 (9.9)	48.9 (22.2)
Clorophyta (%)	12.8 (2.2)	21.7 (3.1)	6.9 (0.7)	18.2 (2.3)	5.1 (0.7)	15.3 (1.4)	8.4 (0.9)	6.9 (0.5)
Cyanobacteria (%)	8.5 (1.6)	24.3 (2.1)	12.2 (2.3)	31.3 (3.3)	4.9 (0.3)	20.8 (3.1)	11.0 (1.5)	15.4 (1.6)
Diatoms (%)	78.6 (3.8)	53.9 (3.7)	80.8 (2.8)	50.6 (4.1)	89.9 (0.7)	63.1 (3.5)	80.5 (2.3)	76.0 (2.4)
PP (mg C m^{-2} h^{-1})	14.9 (5.3)	6.7 (4.6)	9.8 (2.3)	5.5 (2.9)	10.6 (2.5)	4.0 (1.6)	8.3 (1.9)	6.1 (1.6)

Standard error in brackets

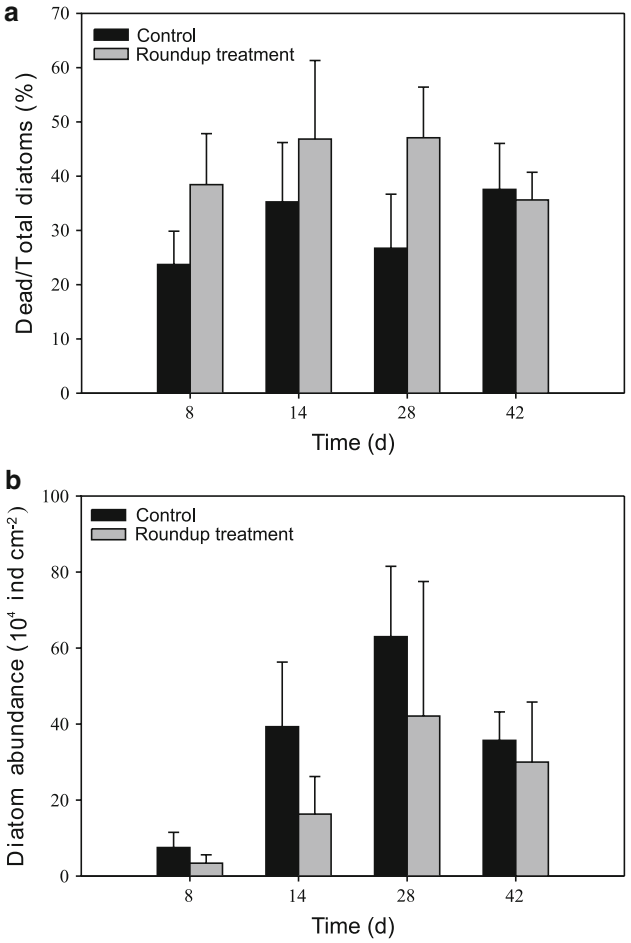

Fig. 2 **a** Mean ratio of dead/total diatoms and **b** diatom abundance in treated and control mesocosms throughout the sampling period. *Error bars* represent +1 SE

Table 2 Linear regression coefficients, origin ordinate and slope for control and treated mesocosms, for dry weight (DW), ash-free dry weight (AFDW), periphyton chlorophyll *a* concentration (P-Chl *a*) and diatom abundance (Diatoms)

	Ordinate (mean ± SE)		Slope (mean ± SE)	
	Control	Treated	Control	Treated
DW	2.03 ± 0.16	1.51 ± 0.29	0.02 ± 0.01	0.02 ± 0.01
	($P = 0.002$)		($P = 0.967$)	
AFDW	1.54 ± 0.21	1.10 ± 0.27	0.02 ± 0.01	0.02 ± 0.01
	($P = 0.015$)		($P = 0.967$)	
P-Chl *a*	−0.70 ± 0.29	−1.24 ± 0.32	0.03 ± 0.01	0.03 ± 0.01
	($P = 0.018$)		($P = 0.973$)	
Diatoms	4.37 ± 0.37	3.58 ± 0.46	0.03 ± 0.01	0.04 ± 0.02
	($P = 0.026$)		($P = 0.815$)	

SE represents the standard error. Significance levels between treatments in brackets

of the experiment (42 days). PP always showed higher mean values in control mesocosms, but without significant differences between treatments (Table 1).

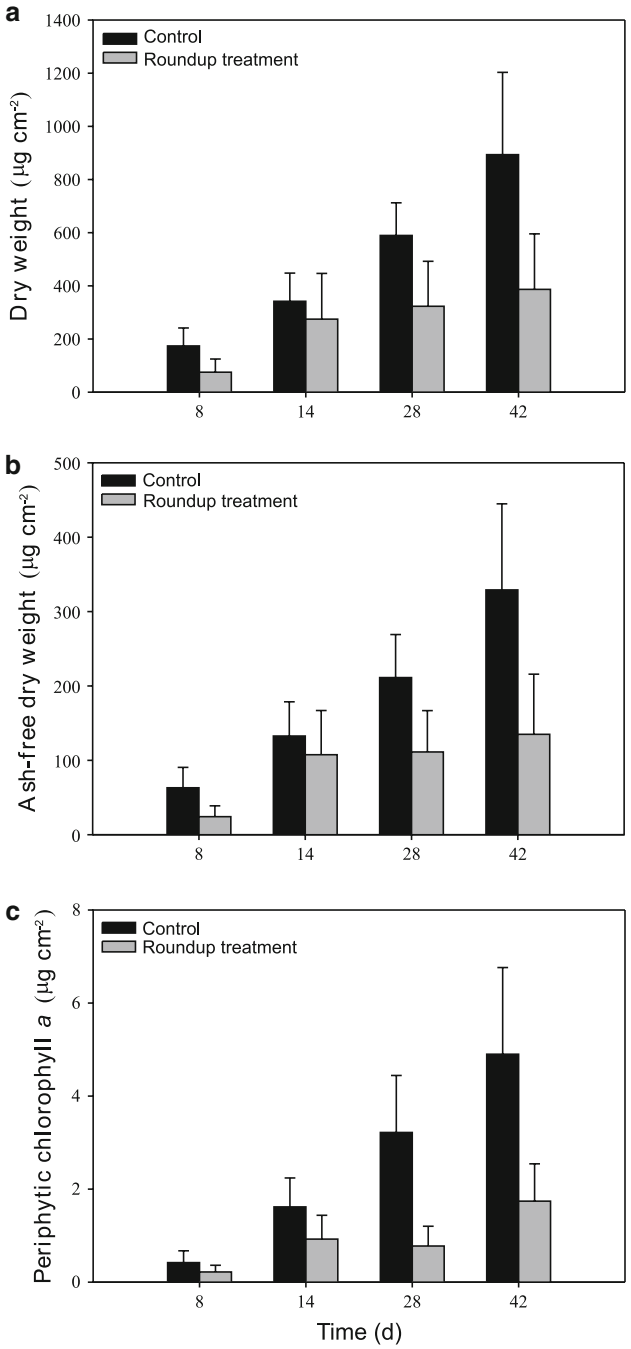

Fig. 3 Mean values of **a** DW, **b** AFDW and **c** chlorophyll *a* concentration in control and treated mesocosms throughout the sampling period. *Error bars* represent +1 SE

Mesocosms' water features

The ten mesocosms showed a great heterogeneity in limnological features at the beginning of the experiment. The physical and chemical characteristics before glyphosate addition, at *t*0, are shown in Table 3. Although the ten mesocosms showed different limnological properties at *t*0, we classified them considering the profiles of $K_d(\lambda)$ and

Table 3 Environmental variables recorded in each mesocosm (E) at *t*0

	E1	E2	E3[a]	E4	E5	E6[a]	E7[a]	E8[a]	E9[a]	E10
pH	8.55	9.02	9.39	8.52	8.5	8.38	8.93	8.52	8.89	8.85
Conductivity (mS cm^{-1})	2.57	2.72	2.78	2.73	2.79	2.77	2.73	2.61	2.75	2.77
Nephelometric turbidity (NTU)	0.80	9.10	1.90	1.80	16.40	3.50	3.50	0.70	1.60	1.60
K_dPAR (m^{-1})	[b]	1.77	4.26	0.89	4.11	0.70	[b]	1.66	2.26	6.37
Dissolved oxygen (mg L^{-1})	10.80	10.20	10.40	10.20	9.50	9.50	10.40	10.50	10.00	10.40
TP (μg L^{-1})	153.00	153.00	241.00	131.00	219.00	131.00	110.00	142.00	197.00	438.00
SRP (μg L^{-1})	6.40	7.60	3.80	7.60	15.20	9.00	7.60	6.40	4.60	3.80
N–NO_2 (μg L^{-1})	203.00	431.80	340.50	106.70	279.40	193.10	66.10	289.60	223.50	254.00
N–NO_3 (mg L^{-1})	11.29	2.33	1.82	9.83	2.33	8.37	11.65	12.38	6.92	2.98
N–NH_4 (μg L^{-1})	11.80	16.50	15.40	9.50	8.30	14.20	8.30	26.00	4.70	21.00
CO_3 (mg L^{-1})	62.15	95.62	152.99	43.03	52.59	47.81	95.62	47.81	100.40	95.62
HCO_3 (mg L^{-1})	738.79	738.79	646.44	738.79	787.40	743.65	675.61	753.37	690.19	729.07
SO_4 (mg L^{-1})	92.00	73.60	73.60	89.24	82.80	87.40	82.80	84.64	87.40	92.00
Cl (mg L^{-1})	394.79	394.79	404.81	324.65	394.79	424.85	404.81	384.77	414.83	354.71
Ca (mg L^{-1})	1.61	1.96	2.88	1.73	1.50	3.34	3.34	3.11	2.99	3.45
Mg (mg L^{-1})	23.45	28.50	23.35	20.65	14.75	21.95	20.30	21.50	20.45	21.50
Na (mg L^{-1})	520.00	615.00	545.00	515.00	440.00	630.00	620.00	590.00	630.00	615.00
K (mg L^{-1})	25.00	22.00	23.50	20.50	24.00	24.00	22.00	22.50	25.00	24.50
Phytoplanktonic chlorophyll *a* (μg L^{-1})	16.86	8.43	295.84	33.71	1.26	15.17	40.46	137.38	94.82	255.38
General aspect	Macrophytes	Inorganic	Organic (phytoplankton)	Macrophytes + metaphyton	Inorganic	Inorganic + organic (phytoplankton)	Macrophytes + metaphyton	Macrophytes	Organic (phytoplankton)	Organic (phytoplankton)
State	Clear	Turbid	Turbid	Clear	Turbid	Turbid	Clear	Clear	Turbid	Turbid

[a] Roundup® added

[b] = no data

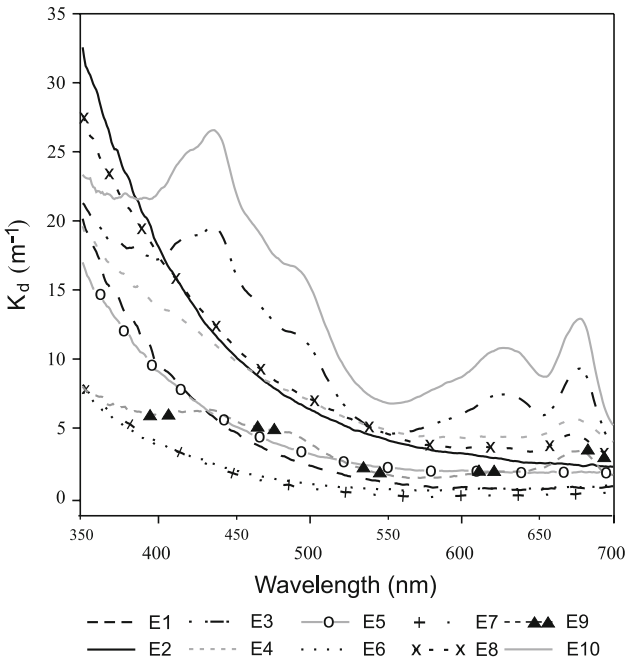

Fig. 4 Profiles of $K_d(\lambda)$ and K_d (PAR) against ultraviolet and photosynthetically active radiation (PAR, 400–700 nm) downward irradiance obtained for each mesocosm (E) at *t*0

K_d(PAR) against ultraviolet and photosynthetically active radiation (PAR, 400–700 nm) downward irradiance obtained for each mesocosms at *t*0 (Fig. 4), the values of nephelometric turbidity and phytoplanktonic chlorophyll *a* concentrations. We distinguished two groups of mesocosms: "turbid" (E2, E3, E5, E6, E9 and E10) and "clear" (E1, E4, E7 and E8) (Table 3). In the case of "turbid" mesocosms, some of them contained plenty of phytoplankton (e.g., E10) and others had a high amount of inorganic solids (e.g., E5).

The initial ionic concentrations in the different mesocosms were rather uniform (Table 3). The water was alkaline, attaining high pH (8.8 ± 0.3) and conductivity (2.7 ± 0.1 mS cm^{-1}), bicarbonate plus carbonate being the main anions and sodium the main cation. Dissolved oxygen averaged 10.2 ± 0.4 mg L^{-1}. Nitrate concentrations were high (mean 6.99 mg N L^{-1}), showing an extended variation range (1.8–12.3 mg N L^{-1}) and turned out to be significantly higher in the "clear" than in the "turbid" mesocosms (KW $P = 0.045$). Nitrite was also comparatively high (239 ± 107 μg L^{-1}), higher than the ammonium concentrations (3.6 ± 6.5 μg L^{-1}) but without significant differences. SRP concentrations were comparatively low (7.2 ± 3.3 μg L^{-1}) without significant differences between treatments; the lowest concentrations (3.3–4.6 μg L^{-1}) were measured in mesocosms attaining high phytoplankton development, and the highest (15 μg L^{-1}) in a mesocosm with high inorganic turbidity (Table 3). High inorganic nitrogen to SRP ratios, around 1,000, were recorded. TP concentrations ranged between 110 and 438 μg L^{-1}; the higher concentrations in the "turbid" mesocosms differed significantly from the "clear" ones from the second sampling date onward (KW $P = 0.0001$).

Initial values of phytoplankton chlorophyll *a* ranged from 11.9 to 280.0 μg L^{-1} in more contrasting mesocosms. The Chl *a* concentrations displayed a significant variation among mesocosms throughout the experiment, independently of glyphosate treatment addition. These variations were maintained during almost all the experiment without significant differences between treatments.

The ionic composition did not show significant differences between treatments throughout the experiment (Table 4). Sodium fluctuated between 475 and 635 mg L^{-1} while bicarbonate fluctuated between 510.3 and 956.5 mg L^{-1} without significant differences between treatments. Water pH ranged from 8.18 to 9.64 and dissolved oxygen from 5.7 to 11.5 mg L^{-1}. Mean water temperature at *t*5 was 9.33°C in E1 and 9.77°C in E3. Total P ranged between 88 and 460 μg L^{-1} in control mesocosms and between 131 and 1,110 μg L^{-1} in Roundup® inoculated mesocosms attaining significant differences between them (KW $P = 0.00003$). TP significantly increased after Roundup® addition in the glyphosate enriched treatments, a subsequent trend to decrease taking place (Fig. 5a). TP dissipation showed a significant lineal trend (ln TP = 6.9–0.04 day; $P = 0.0002$) as from the third day after the glyphosate application. After 42 days of glyphosate addition no significant differences were observed between mesocosms with and without Roundup®. The other measured nutrients, ammonium, nitrate, nitrite and SRP, varied during the experiment without showing any discernible pattern. Nitrate concentrations remained higher in the "clear" than in the "turbid" mesocosms. SRP increased from the first to the second sampling and remained high after that in both treatments. This behavior was probably due to phosphate release from sediments after the mixture of waters both in control and treated mesocosms at *t*0, because of glyphosate homogenization in treated mesocosms and in order to repeat the same mechanical action, in control ones. No significant differences were recorded in these nutrients between treatments.

K_d(PAR) did not vary significantly between treatments, averaging 4.28 m^{-1} (±3.36 SD), and ranging from 0.56 to 16 m^{-1}. Nephelometric turbidity (Tn) did not vary significantly between treatments and values averaged 5.59 NTU (±5.7 SD), ranging between 0.5 and 24.6 NTU.

Glyphosate evolution

Considering that glyphosate residue adjusted to a logarithmic function assuming a first-order kinetic (ln

Table 4 Mean ionic composition at the beginning of the experiment in all mesocosms and in the control and treated mesocosms throughout the experiment

	CO_3^{2-} meq L^{-1}	HCO_3^- meq L^{-1}	SO_4^{2-} meq L^{-1}	Cl^- meq L^{-1}	NO_3^- meq L^{-1}	Ca^{2+} meq L^{-1}	Mg^{2+} meq L^{-1}	Na^+ meq L^{-1}	K^+ meq L^{-1}	$\sum$ anions meq L^{-1}	$\sum$ cations meq L^{-1}
Initial (t_o)	2.40	11.60	1.80	11.00	0.11	0.13	1.80	25.20	0.60	26.90	27.70
Control (t_{1-5})	5.00	12.40	2.00	11.30	0.06	0.20	2.20	25.30	0.60	30.70	28.30
Treated (t_{1-5})	5.00	12.10	2.00	11.50	0.08	0.17	2.20	24.80	0.60	30.60	27.80

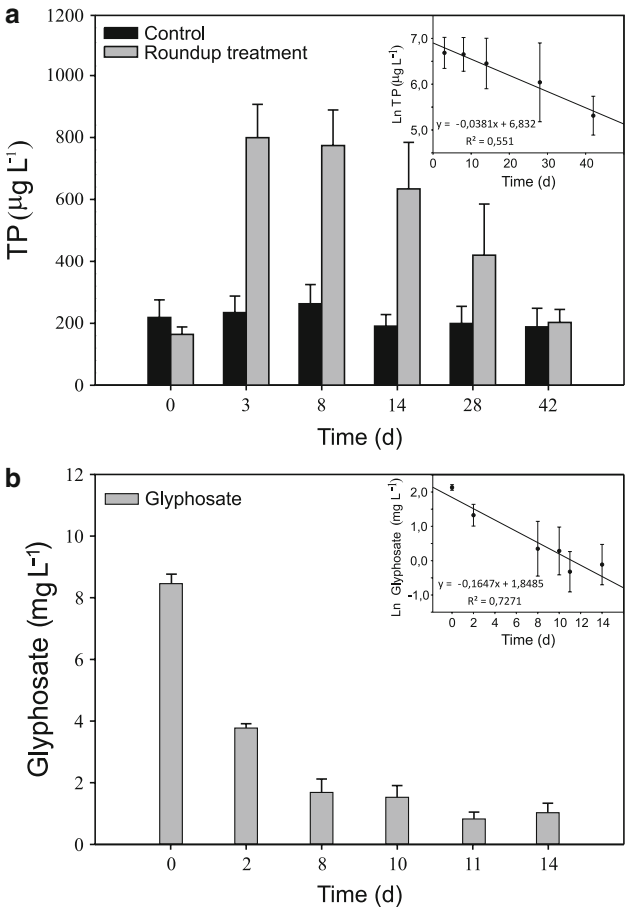

Fig. 5 **a** Total phosphorus concentration in glyphosate enriched treatments against controls throughout the experimental period; negative linear relationship obtained between ln TP in enriched mesocosms and time. **b** Glyphosate dissipation from mesocosms' water and glyphosate residue fitted to a logarithmic function assuming first-order kinetics. *Error bars* represent +1 SE in histograms and ± 1 SD in regressions

glypho = −0.165 day + 1.8; $P = 0.000001$) (Fig. 5b), glyphosate dissipation from treated mesocosms presented an estimate dissipation rate (k) of 0.165 day^{-1} (±0.022 SD) with a half-life of 4.2 day. The glyphosate concentrations shortly (1 h) after the herbicide application were similar among mesocosms, presenting a mean value of 8.456 mg L^{-1} (±0.686 SD). Higher differences in glyphosate concentration were observed among mesocosms at the end of the experiment, relative to initial values (Fig. 5b). One of the five treated mesocosms (E3) was excluded from dissipation analysis due to its erratic behavior in glyphosate concentrations throughout the experiment.

Discussion

The present study demonstrated that there was a clear delay in the periphytic colonization of new substrata in large treated outdoor mesocosms, with limnological characteristics similar to those of shallow lakes of the Pampa plain. This delay could be attributed to a direct toxicological effect of Roundup®. Considering that periphyton is one of the most significant microbial communities as a base of food webs in shallow lakes (Vadeboncoeur and Steinman 2002), the consequences on the ecology of the system would be important. Although both treatments, with and without herbicide, exhibited a biomass increase, in control mesocosms the magnitude of the biomass accrual was higher than in those receiving Roundup®, and this effect remained evident until the end of the lengthy experiment. Austin et al. (1991) showed an opposite behavior with an enhancement of AFDW and algal densities as a result of glyphosate (mediated by Vision® formulae) addition. These authors demonstrated the enhancement of soluble phosphorus concentration as a result of the degradation of glyphosate in oligotrophic streams. Thus, periphytic primary producers could develop higher biomass in such nutrient poor environments. Our mesocosms were always eutrophic and the differences in growth can be attributed to the toxicological effect of Roundup®.

Despite the lack of significant differences between treatments, the trend to a decrease in primary production in treated mesocosms was clear and was consistent with the results of Goldsborough and Brown (1988). They found that the photosynthetic activity of periphyton decreased with an increasing amount of herbicide, and that the limiting concentration for this effect depended on the physical and chemical properties of the water bodies and other factors, including transport limitation in thick periphyton films and degradation of the herbicide by periphytic organisms as a phosphorus source. Consistently, in our

experiment, the major effect can be seen upon 8 days of colonization, when the periphyton films were thin and the amount of non-algal material was the lowest. It is important to point out that the toxicity is produced by the joint effect of both glyphosate and POEA, which is the surfactant of the commercial formulation Roundup® whose toxicity was shown to be higher than glyphosate (Struger et al. 2008).

Among the main algal groups, diatoms (Bacillariophyceae) appeared to be the most affected by the herbicide, with the lowest abundances in treated mesocosms. Despite differences among mesocosms and time, the more representative species of diatoms that were registered were the ubiquitous *Gomphonema parvulum*, *Achnanthes minutissima* and *Amphora veneta*. Taking into consideration that the dead diatoms immersed in the periphytic matrix presented higher numbers in Roundup® than in control mesocosms it is clear that the herbicide produced mortality and a decrease in the recruitment of new organisms. Cyanobacteria, on the other hand, emerged enhanced in number in treated mesocosms. These organisms, typical of extreme environments including herbicide stressed habitats, may resist glyphosate by different strategies. Besides the overproduction of EPSP synthase or the production of a glyphosate-tolerant enzyme (Powell et al. 1991) some cyanobacteria have the ability to degrade glyphosate and use it as a phosphorus source (Forlani et al. 2008; Lipok et al. 2007). Pérez et al. (2007) also observed higher proportion of periphytic cyanobacteria and registered a 40-fold increase in planktonic picocyanobacteria abundance as a result of Roundup® addition.

An important finding of this study is that, regardless the limnological type, the P content of the added glyphosate caused the increase of TP in all treated mesocosms. Pérez et al. (2007) also observed a TP increase after the addition of Roundup®, but in mesocosms with similar limnological properties. Considering that phosphorus represents 14% of glyphosate's molecular weight, the increased amount of TP in the first sampling, 3 days after glyphosate addition, accounted for 76% of the added P. Taking into account that glyphosate is fast dissipated from the water -we registered a half-life of 4.2 days similar to those reported in the literature-, three processes occur simultaneously: the incorporation of the herbicide in macrophytes and microorganisms such us phytoplankton and periphyton; glyphosate degradation by bacteria and fungi (Castro et al. 2007; Liu et al. 1991), and its immobilization upon contact with sediments, soils and clay minerals because of the formation of surface complexes with metal ions (Pessagno et al. 2005). In soils the most important metabolic pathway of glyphosate is the transformation into sarcosine and aminometilphosphonic acid (AMPA), which is further degraded to carbon dioxide (Giesy et al. 2000). Microbial degradation of AMPA has been reported to proceed at a slower rate than glyphosate breakdown, being detected in samples much more frequently compared to glyphosate (Kolpin et al. 2006). Since AMPA contains the P moiety of the glyphosate, and considering that we measured a glyphosate dissipation rate four times faster than the dissipation rate of TP, we suggest that the metabolic pathway of glyphosate degradation is quantitatively larger in our experiment. However, the glyphosate adsorbed to particles has a longer half-life and will return to the water as the equilibrium reaction is slowly modified (Barja and dos Santos Afonso 2005) eventually resulting in a long term effect.

The ten outdoor mesocosms used resembled the limnological physiognomy of the surrounding shallow natural lakes. Within the Pampa plain a host of shallow lakes shows two main contrasting typologies: "clear" water lakes, with dense macrophyte stands sustaining luxuriant periphyton growth, and "turbid" ones in which dense phytoplankton assemblages replace the macrophyte-periphytic dominance or which have a high amount of inorganic suspended solids (Allende et al. 2009). Since the glyphosate half-life was no longer than 1 week it was assumed that no long term effect could be attained, and that after a year of recovery it would be safe to start a new experiment in the same mesocosms. However, most of the "turbid" mesocosms in the present experiment were those treated with glyphosate in the previous experiment and the mesocosms used as controls in the first experiment remained "clear" at present. Unexpectedly, we detected that a single application of glyphosate in 2005 shifted the mesocosms from a "clear" to a "turbid" state which remained until the next year. As was discussed above, the glyphosate may be adsorbed to sediments and a slow later desorption might produce a long turn effect suppressing growth of the most sensitive groups and favoring the abilities to compete of the more resistant algae. This trend in long term effect was suggested by Holtby and Baillie (1989) who reported an enhancement of periphytic production as a response to increased levels of phosphorus produced by a unique application of Roundup® done 1 year before their experiment, carried out in natural streams.

Agriculture intensification occurred in the last decades within the Pampa plain (Mugni et al. 2005) and agrochemical consumption sharply increased. Quirós et al. (2002) suggested that most of the Pampean shallow lakes were in a "clear" water state at the beginning of this process and have now turned to a "turbid" water phase. Their work mainly discussed the effect of fertilizer applications, presenting evidence that nutrient loads into regional water bodies increased as a consequence of higher fertilizer applications, turning lakes from a "clear" to a "turbid" phase. Despite in Argentina the fertilizers are the main responsible to nutrient loading to water bodies due to the high amount used in agriculture (between 50 and

100 kg ha^{-1}), it has to be considered that the use of pesticides with phosphorus, such us glyphosate, increases even more the nutrient loading. The present study showed that a single glyphosate addition produced a long term shift in the water bodies' typology which is consistent with the regional trend suggested by Quirós et al. (2002).

The enrichment of the systems related to glyphosate addition was reported in the literature (Austin et al. 1991). The possibility of the acceleration of the eutrophication process and its consequences for natural environments are serious. In such situations, the whole ecology of the system turns to conditions where the physical and chemical properties of the water produce a decrease in biodiversity with the probable development of resistant species that might grow explosively. One of the most common and potentially toxic bloom forming cyanobacteria are *Microcystis aeruginosa,* usually detected in water bodies worldwide including in shallow lakes of the Pampean region (Izaguirre and Vinocur 1994). Forlani et al. (2008) have demonstrated that this species is capable of using glyphosate as a phosphorus source. Thus, these cyanobacteria are not only not affected adversely by glyphosate but their development is even enhanced by the herbicide, worsening the overall ecological condition of the shallow lakes near glyphosate-tolerant cultivation fields.

Aquatic ecosystems around the Pampean region of Argentina—more than 10,000 water bodies (Dukatz et al. 2006)—are at risk of being affected by the toxicological properties as well as the eutrophication potential of the glyphosate. In our study, the periphyton interacted with other communities and with the abiotic environment, enabling valid extrapolative inferences from our results to be made for natural aquatic systems. Based on the findings obtained in our work as well as those obtained in previous researches, it is clear that agricultural practices that involve the use of herbicides such as Roundup® affect non-target organisms and water quality, modifying the structure and functionality of freshwater ecosystems.

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