Glyphosate input modifies microbial community structure in clear and turbid freshwater systems

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RESEARCH ARTICLE

Glyphosate input modifies microbial community structure in clear and turbid freshwater systems

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Abstract Since it was commercially introduced in 1974, glyphosate has been one of the most commonly used herbicides in agriculture worldwide, and there is growing concern about its adverse effects on the environment. Assuming that glyphosate may increase the organic turbidity of water bodies, we evaluated the effect of a single application of 2.4± 0.1 mg l^{-1} of glyphosate (technical grade) on freshwater bacterioplankton and phytoplankton (pico, micro, and nanophytoplankton) and on the physical and chemical properties of the water. We used outdoor experimental mesocosms under clear and oligotrophic (phytoplanktonic chlorophyll *a*= 2.04 μg l^{-1}; turbidity=2.0 NTU) and organic turbid and eutrophic (phytoplanktonic chlorophyll *a*=50.3 μg l^{-1}; turbidity=16.0 NTU) scenarios. Samplings were conducted at the beginning of the experiment and at 1, 8, 19, and 33 days after glyphosate addition. For both typologies, the herbicide affected the abiotic water properties (with a marked increase in total phosphorus), but it did not affect the structure of micro and nanophytoplankton. In clear waters, glyphosate treatment induced a trend toward higher bacteria and picoeukaryotes abundances, while there was a 2 to 2.5-fold increase in picocyanobacteria number. In turbid waters, without picoeukaryotes at the beginning of the experiment, glyphosate decreased bacteria abundance but increased the number of picocyanobacteria, suggesting a direct favorable effect. Moreover, our results show that the impact of the herbicide was observed in microorganisms from both oligo and eutrophic conditions, indicating that the impact would be independent of the trophic status of the water body.

Keywords Herbicide · Organic turbidity · Microbial structure · Picocyanobacteria · Freshwater ecosystems · Trophic status · Mesocosms

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Introduction

In developed countries, agriculture has been implicated as the single largest cause of water pollution, mainly via non-point sources (Monaghan et al. 2007). Agriculture affects freshwater ecosystems, mostly due to the associated deforestation in the catchment area, fine sediment input, pesticide input, and nutrient enrichment from fertilizers. In particular, lakes located in lower terrain receive chemicals and other contaminants used or released along the basins. Since it was commercially introduced in 1974, glyphosate N-(phosphonomethyl) glycine has been one of the most commonly used herbicides in agriculture worldwide, thereby gaining the attention of the scientific community (Duke and Powels 2008). Glyphosate is a non-selective, broad-spectrum, postemergent agrochemical, mainly used for weed control (Goldsborough and Brown 1988). Glyphosate's primary mode of action in plants and

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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). In 2011, the worldwide application of glyphosate products was estimated to be about 650,000 tons (CCM International 2011). In South America, shallow water bodies receive a significant amount of this herbicide deriving principally from intensive cultivation of genetically modified soybean and no-till practice. Glyphosate may reach aquatic systems either by accidental or wind-driven drift of the herbicide spray, through transport in surface runoff, or as suspended particulate matter (Feng et al. 1990). Despite the fact that glyphosate is usually assumed to be safe and non-toxic to the environment because of its rapid biodegradation and/or adsorption by soil particles, it affects non-target organisms of varying complexity (e.g., Lajmanovich et al. 2011; Yadav et al. 2013). The impact of glyphosate on freshwater organisms at different levels of organization has been analyzed under various approaches, such as manipulative experiments involving laboratory and outdoor assays with micro and mesocosms (Relyea 2005; Vera et al. 2014). Moreover, important off-target displacements of glyphosate were described at the ecosystem level, causing structural and functional changes in freshwater consistent with a decrease in water quality due to the acceleration of eutrophication processes (Pérez et al. 2007; Vera et al. 2012). This is a matter of increasing concern because freshwater bodies provide important ecosystem goods and services to humanity.

In a short-term experimental study using 25-m^3outdoor mesocosms, Pérez et al. (2007) observed a decrease in the total abundances of micro and nanophytoplankton and a 40-fold increase in the abundance of picocyanobacteria, 11 days after a single addition of the glyphosate formulation Roundup®. After the experiment, these mesocosms received no further attention for over 1 year, providing Vera et al. (2010) with a unique opportunity to investigate the long-term effect of a glyphosate formulation on freshwater; unexpectedly, they found that the herbicide-treated mesocosms had shifted from a "clear" to a "turbid" state and showed a high abundance of picocyanobacteria, while the non-treated control mesocosms remained clear.

The Pampa plain of Argentina hosts shallow freshwater systems of contrasting typologies: clear water lakes with dense macrophyte stands and turbid ones dominated by dense phytoplankton assemblages (Allende et al. 2009); both types fit in the model of alternative stable states described by Scheffer et al. (1993) for shallow lakes. There are more than 10,000 water bodies in the Pampa plain (Dukatz et al. 2006), which is one of the regions of the world most affected by agricultural practices. The use of glyphosate in Argentina rocketed in the mid1990s, and its use reached 1.97×10^8 l during 2012 (CASAFE 2013). In this context, Vera et al. (2010) argued that although nutrient enrichment in water bodies of the region is mainly due to fertilizer input, organophosphorus-based pesticides such as glyphosate may contribute to increase nutrient loading, thus switching the system to a turbid state.

Here, we report the results of an outdoor study using mesocosms, which was aimed to assess the effect of glyphosate on clear and organic turbid freshwater systems. Both typologies, also characteristics of different trophic status, are commonly found in typical shallow lakes in agricultural areas of Argentina. Considering that glyphosate promotes an increase in the organic turbidity of clear systems, we evaluated the effect of a single input of the herbicide glyphosate (technical grade) on microbial communities—bacterioplankton and phytoplankton—and on the physical and chemical properties of the water under two scenarios: (1) organic turbid type, eutrophic waters, with high phytoplankton chlorophyll *a* (chl *a*) concentration and water turbidity and (2) clear type, oligotrophic waters, with low chl *a* concentration and water turbidity. Considering that the impact of phosphorus addition to the water mediated by glyphosate could lead the evolution to eutrophication which will transform radically all the biotic components, we hypothesize that microbial communities from oligotrophic waters will be more sensitive to the herbicide addition than those from eutrophic waters.

Materials and methods

Experimental design

The experiment was carried out in March 2013 using 12 outdoor artificial mesocosms, half of which were under a clear water scenario (hereafter clear state) and the other half under an "organic turbid" water scenario (hereafter turbid state). The mesocosms consisted of ~70 l polythene bags which were installed in two large pools of 3000 l each. The pools were filled with tap water and then left to evolve naturally for about 6 months before the start of the experiment. At the beginning of this period, we put some individuals of submersed macrophytes (*Egeria* sp.) in one pool and samples of concentrated phytoplankton from a neighboring pool in the other. In this way, we obtained two shallow freshwater bodies with contrasting limnological characteristics, the former in a clear and oligotrophic state (chl a=2.04 μg l^{-1}; turbidity=2.0 NTU), and the latter in a turbid and eutrophic state (chl a= 50.3 μg l^{-1}; turbidity=1.0 NTU). Both systems had consolidated microbial communities at the beginning of the experiment (see t0 of Table 1).

One day before the start of the experiment, six mesocosms were filled with clear and six with turbid water. Three mesocosms from each pool were randomly selected for treatment with technical-grade glyphosate acid (≥95 %

Table 1 Mean values (±SD) of the physical, chemical, and biological variables registered at t0 (0 days) and t4 (33 days after glyphosate addition) for clear and turbid mesocosms for control and glyphosate treatments

		Clear state		Turbid state	
		Control	Glyphosate	Control	Glyphosate
pH	t0	7.3±0.03	7.2±0.01	7.3±0.1	7.4±0.1
	t4	7.8±0.1	7.7±0.02	7.8±0.1	7.9±0.02
Conductivity (μS cm^{-1})	t0	217.3±0.6	217.3±0.6	144.2±0.4	143.9±0.6
	t4	214.0±0.0	212.3±3.2	140.4±0.5	140.7±0.8
Turbidity (NTU)	t0	2.0±0.0	2.0±0.0	16.0±0.0	16.0±0.0
	t4	2.7±0.6	2.0±0.0	14.3±0.6	16.3±1.5
Dissolved oxygen (mg l^{-1})	t0	9.2±0.1	9.5±0.1	9.8±0.3	9.6±0.1
	t4	7.8±0.1	7.7±0.2	8.3±0.4	8.0±0.6
Temperature (°C)	t0	11.4±0.1	11.4±0.1	11.8±0.2	11.9±0.5
	t4	12.1±0.1	12.1±0.0	12.1±0.0	12.1±0.0
Suspended solids (mg l^{-1})	t0	5.0±0.0	3.3±1.5	30.3±2.5	26.3±8.1
	t4	5.0±5.3	6.0±2.6	24.7±8.5	21.0±1.0
P-PO_4 (mg l^{-1})	t0	0.05±0.02	0.03±0.01	0.03±0.01	0.04±0.02
	t4	0.02±0.01	0.02±0.01	0.01±0.00	0.02±0.01
TP (mg l^{-1})	t0	0.05±0.02	0.04±0.02	0.12±0.03	0.12±0.01
	t4	0.08±0.04**	0.70±0.02**	0.15±0.02**	0.73±0.04**
Chlorophyll *a* (μg l^{-1})	t0	2.1±0.7	2.0±0.3	43.4±6.0	57.3±6.4
	t4	2.7±0.8*	5.1±0.4*	59.0±6.0	61.8±9.3
Bacteria (ind ml^{-1})	t0	$1.19\times10^{6}\pm7.75\times10^{4}$	$1.19\times10^{6}\pm3.37\times10^{4}$	$2.77\times10^{7}\pm3.15\times10^{5}$	$2.75\times10^{7}\pm5.21\times10^{5}$
	t4	$1.30\times10^{6}\pm4.20\times10^{4}$**	2.15×106±3.47×10^{4}**	$6.12\times10^{6}\pm3.66\times10^{5}$**	$2.66\times10^{6}\pm7.02\times10^{4}$**
Picocyanobacteria (ind ml^{-1})	t0	$3.27\times10^{4}\pm5.16\times10^{3}$	$3.35\times10^{4}\pm4.01\times10^{2}$	$3.90\times10^{5}\pm3.91\times10^{4}$	$4.31\times10^{5}\pm9.62\times10^{4}$
	t4	$3.90\times10^{4}\pm1.57\times10^{3}$**	$7.95\times10^{4}\pm2.05\times10^{3}$**	$9.49\times10^{5}\pm1.50\times10^{4}$**	$2.33\times10^{6}\pm5.69\times10^{4}$**
Picoeukaryotes (ind ml^{-1})	t0	$2.23\times10^{4}\pm1.84\times10^{3}$	$2.29\times10^{4}\pm1.28\times10^{3}$	0.00±0.00	0.00±0.00
	t4	$2.17\times10^{4}\pm2.26\times10^{3}$**	$2.96\times10^{4}\pm7.23\times10^{2}$**	0.00±0.00	0.00±0.00
Micro+nanophytoplankton (ind ml^{-1})	t0	$1.93\times10^{5}\pm1.80\times10^{4}$	$2.44\times10^{5}\pm5.97\times10^{4}$	$6.43\times10^{4}\pm1.62\times10^{4}$	$5.06\times10^{4}\pm2.80\times10^{4}$
	t4	$8.92\times10^{4}\pm1.54\times10^{4}$	$1.39\times10^{5}\pm2.96\times10^{4}$	$4.46\times10^{4}\pm8.56\times10^{3}$	$4.87\times10^{4}\pm1.10\times10^{4}$
% Chlorophyceae	t0	99.0±0.7	97.6±0.3	80.1±3.1	79.0±4.1
	t4	96.8±0.9	90.4±1.7	86.3±3.6	89.0±2.2
% Desmidiaceae	t0	0.3±0.2	0.8±0.2	19.1±2.6	19.8±4.4
	t4	2.2±0.7	9.0±1.9	5.9±1.6	7.9±3.3
% Bacillariophyceae	t0	0.0±0.0	0.03±0.01	0.7±0.7	1.0±0.5
	t4	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
% Dinophyceae	t0	0.1±0.1	0.2±0.0	0.0±0.0	0.1±0.3
	t4	0.2±0.1	0.1±0.2	4.2±1.4	2.0±1.5
% Cryptophyceae	t0	0.6±0.5	1.3±0.2	0.0±0.0	0.0±0.0
	t4	0.6±0.2	0.4±0.3	0.4±0.4	0.4±0.4

Significant differences between treatments at t4 are indicated by asterisks: *$p<0.05$; **$p<0.01$

purity; CAS 1071-83-6) at a nominal glyphosate concentration of 2.5±0.1 mg active ingredient (a.i.) per liter. The remaining mesocosms without glyphosate served as controls. We chose this glyphosate concentration because it is comparable to those assayed in previous experiments (Vera et al. 2010, 2012). Moreover, the tested concentration is similar to the lower limit of the range of worst-case scenarios reviewed by Relyea (2005) (between 1.4 (Canadian government) and 10.3 a.i. mg l^{-1} (Mann and Bidwell 1999)). The first sample from each mesocosm was collected before the addition of herbicide (t0) and that for glyphosate determination immediately after its application; the following samples were collected 1, 8, 19, and 33 days after glyphosate addition.

Physical and chemical properties of the mesocosms' water

Water temperature, pH, dissolved oxygen, and conductivity were measured in situ on each sampling date with a Hach® portable multiparameter sensor and nephelometric turbidity with a 2100P Hach® portable turbidimeter. Total suspended solids (TSS) were estimated after filtration of a water sample through prerinsed and precombusted (530 °C, 2 h) GF/F filters (nominal pore size 0.7 μm), dried until constant weight at 103–105 °C. Water samples for chemical analysis were poured into polypropylene containers and immediately transported to the laboratory. Water was immediately filtered through Whatman® GF/F filters. Soluble reactive phosphorus (SRP) was determined by the molybdate-ascorbic method; total phosphorus (TP) from unfiltered water samples was converted to SRP after acid digestion with potassium persulfate; SRP and TP were analyzed following APHA (2005).

Glyphosate analysis

Glyphosate concentrations in the water samples were determined by ion chromatography (Zhu et al. 1999) using a DIONEX DX-100 chromatograph with a conductivity detector and a 25-μl sample loop. A DIONEX AS-4 was used as analytical chromatographic column. A mixture of $NaOH/Na_2CO_3$ 4 mM/9 mM was chosen as eluent with a flow rate of 1 ml min^{-1}. Data acquisition was performed using the Clarity Lite software (UK). The experimental error was below 5 %. The glyphosate dissipation rate (k) in water was estimated, assuming a first-order kinetics and using $C_t=C_0\ e^{(-kt)}$, where C_t is concentration at time t and C_0 is the initial concentration. The half-life ($t_{1/2}$) of glyphosate was calculated as $t_{1/2}=\ln 2/k$.

Phytoplankton and bacterioplankton

Water samples for the identification and quantification of the different size fractions of phytoplankton and bacterioplankton were obtained from each mesocosm on all sampling dates. Samples for micro (>20 μm) and nanophytoplankton (2–20 μm) identification were fixed with 2 % formalin and observed under a light microscope at ×1000 magnification. In general, Bourrelly's systematic scheme (1970, 1972, 1981) was followed, while Simonsen's (1979) system was followed for Bacillariophyceae. In the case of Cyanophyceae, we also introduced the taxonomical system of Komárek and Anagnostidis (1986) and Anagnostidis and Komárek (1988). For algae quantification, samples were fixed with 1 % acidified Lugol's iodine solution. Counts were performed using the inverted microscope technique (Utermöhl 1958) at ×400 magnification with a counting error of <15 %, estimated according to Venrick (1978).

Picophytoplankton (0.2–2 μm) and bacterioplankton samples were preserved with 2 % ice-cold glutaraldehyde and filtered through 0.2-μm pore-size black polycarbonate filters (Isopore GTPB 02500; Isopore, Billerica, Massachusetts, USA) stained with DAPI (Porter and Feig 1980). Then, each filter was mounted on a microscope slide with a drop of immersion oil (Immersol Zeiss 518 F; Zeiss, Jena Germany), and the autotrophic fraction (picocyanobacteria and picoeukaryote algae) and bacteria were quantified using an epifluorescence microscope (Olympus BX40). For abundance estimation, a minimum of 20 fields and 400 individuals were counted on each slide (error<15 %).

Phytoplanktonic chlorophyll *a* concentration (chl *a*) was estimated from triplicate samples (70–200 ml) collected onto Whatman® GF/F filters. These were immediately wrapped in aluminum foil and stored at −80 °C until processing. Chl *a* was extracted (overnight at 4 °C, in the dark and in a nitrogen-saturated atmosphere) using 90 % (by volume) aqueous acetone, and the extracts were cleared by centrifugation at 3000 rpm (~4710×*g*) for 10 min. Chl *a* extracts were measured by ion pairing reverse-phase HPLC (modified from Mantoura and Llewellyn 1983 and Hurley 1988), using a ÄKTA basic chromatograph (Amersham, Buckinghamshire, UK) with a Phenomenex® Gemini C18 column (250× 4.6 mm, 5 μm), controlled by the program Unicorn (Amersham, Buckinghamshire, UK). The method applied is described in detail in Laurion et al. (2002). Briefly, we used a gradient program that ramped from 85 % mobile-phase A (80:20 by vol. methanol/aqueous solution of 0.001 M ion-pairing and 0.001 M propionic acid) to 100 % mobile-phase B (60:40 by vol. acetone/methanol) in 30 min with a hold for 20 min. The HPLC system was calibrated with commercially available primary standards provided by the International Agency for ^{14}C Determination and from Sigma (Buchs, Switzerland).

Statistical analyses

For variables other than glyphosate, chl *a* and percentage of algal group, differences between treatments were tested using repeated-measures analysis of variance (RM ANOVA), considering as factors, the glyphosate concentration and time (five sampling times: 0, 1, 8, 19, and 33 days) for each water type separately. RM ANOVA was followed by the post hoc Tukey's test for multiple comparisons ($p<0.05$). For each water type, RM ANOVA was applied to detect statistical differences in glyphosate concentration among sampling times (0, 1, 8, 19, and 33 days). For chl *a* and percentage of algal group, differences between treatments were tested using one-way ANOVA at 33 days after glyphosate addition for each water scenario. Prior to every analysis, the Kolmogorov–Smirnov (with Lilliefors' correction), Levene median, and Mauchley

Sphericity tests were run to test for normality, homoscedasticity, and sphericity, respectively.

Results

Mean values (±SD) of the physical, chemical, and biological variables at day 0 and at day 33, for clear and turbid, states are shown in Table 1. There were significant differences in the abundance of microbial communities between water states, with picoplankton being more abundant in turbid systems and micro and nanophytoplankton more abundant in clear systems. Chlorophyceae is the best represented algal class for both systems. Chl *a* concentration also showed differences between water types. Below, we detail the results obtained during the study period.

Glyphosate evolution

After the herbicide application, mean glyphosate concentration (±SD) was 2.4±0.1 mg l^{-1} for both clear and turbid water. For clear water, treated mesocosms presented an estimate glyphosate dissipation rate (k) of 0.022 $week^{-1}$ with a half-life of 31.5 weeks, while for turbid waters, k value was 0.021 $week^{-1}$ with a half-life of 33.5 weeks. No significant differences in glyphosate concentration were observed for clear and turbid mesocosms along time ($p>0.05$) (Fig. 1).

Physical and chemical response parameters

The physical and chemical parameters did not show statistical differences between the treated and control mesocosms throughout the study period ($p>0.05$), with the exception of total phosphorus (Table 1). Both water states had circumneutral pH values, between minimum of 7.2 and maximum of 7.9. Conductivity was statistically higher for clear than for turbid waters at day 0 ($p<0.05$) (Table 1) and throughout the study period, with values remaining almost constant; maximum values of 222.0 and 147.2 μS cm^{-1} and minimum values of 203.3 and 133.5 μS cm^{-1} were recorded for clear and turbid waters, respectively. Nephelometric turbidity was always lower for clear waters, remaining almost invariable (2.0 NTU for clear and 16.0 NTU for turbid waters) throughout the study period. Dissolved oxygen values ranged between 7.3 and 9.5 mg l^{-1}, and the water of both systems was well-oxygenated throughout the study. Water temperature values were similar for both states during the experiment, ranging between 10.7 and 14.2 °C. Suspended solids varied between 2.6–9.6 and 13.6–40.0 mg l^{-1} for clear and turbid waters, respectively, with non-significant differences for factor treatment and time.

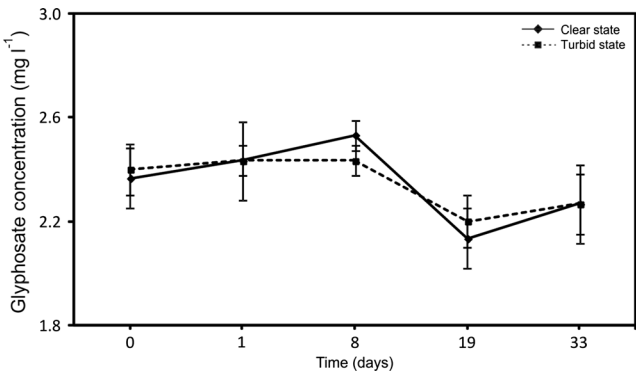

Fig. 1 Averages ±1SD of glyphosate in clear and turbid mesoscosms throughout the study period

At the beginning of the experiment (day 0), the concentration of TP for turbid waters was almost twice as much as in clear waters (Fig. 2, Table 1). Both water types showed a significant increase after herbicide addition; in comparison with controls, TP concentration values were ≈9 times higher for clear (Fig. 2a) and ≈5 times higher for turbid waters (Fig. 2b), remaining similar toward the end of the experiment. Significant differences in TP concentration were observed for treatment (RM ANOVA, $p<0.01$ (clear); $p<0.01$ (turbid)) and time (RM ANOVA, $p<0.05$ (clear); $p<0.01$ (turbid)).

Biological response parameters

Bacterioplankton followed a different pattern for each system after glyphosate addition. In clear water, treated mesocosms showed an increase in bacterial abundance from day 1 onward; at day 33, mean value of bacterial abundance was almost 2-times higher in treated mesocosms than in control mesocosms (Fig. 3a, Table 1). Clear mesocosms showed statistically significant differences for treatment (RM ANOVA, $p<0.01$), time (RM ANOVA, $p<0.01$), and time–treatment interaction (RM ANOVA, $p<0.01$). In contrast, the addition of herbicide to turbid water decreased bacterial abundance from 1 day onward. Although bacterioplankton abundance decreased significantly in both treated and control mesocosms (probably due to manipulative impact), it was significantly higher in the former. At day 33, mean bacterial abundance was almost an order of magnitude lower in treated mesocosms than in control mesocosms (Fig. 3b, Table 1). Turbid water showed statistically significant differences for treatment (RM ANOVA, $p<0.01$), time (RM ANOVA, $p<0.01$), and time–treatment interaction (RM ANOVA, $p<0.01$).

An increase in picocyanobacteria abundance after glyphosate addition was observed independently of the type of state. For clear water, mean picocyanobacteria abundance in treated mesocosms was 2-fold higher than in control mesocosms at day 33 (Fig. 4a, Table 1). For turbid water, mean picocyanobacteria abundance in treated mesocosms was almost 2.5-fold higher than in control mesocosms (Fig. 4b, Table 1). Both water types showed significant differences for

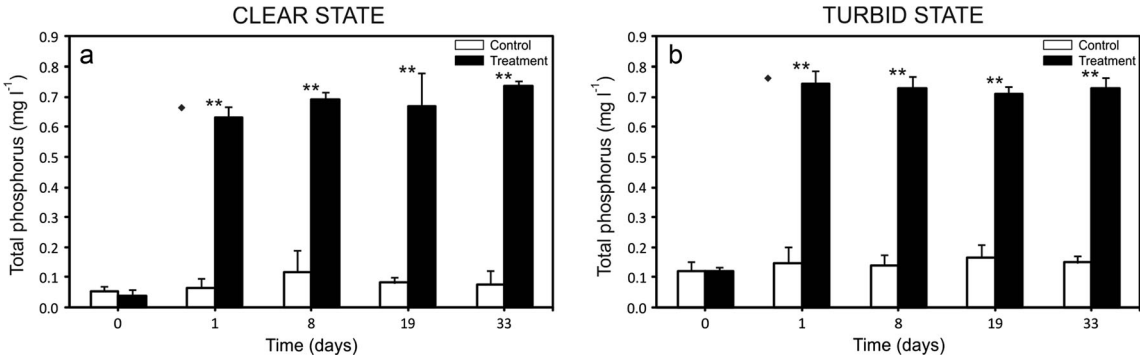

Fig. 2 **a**, **b** Mean values of TP concentration (mg L^{-1}) in control and treated mesocosms throughout the study period in **a** clear and **b** turbid freshwater systems. Error bars represent SD. Significant differences between treatments at the same sampling date are indicated by *asterisks* (**$p<0.01$). Significant differences between consecutive times within a treatment are indicated by *diamonds* ($\Diamond p<0.05$)

treatment (RM ANOVA, $p<0.01$ (clear); $p<0.01$ (turbid)), time (RM ANOVA, $p<0.01$ (clear); $p<0.01$ (turbid)), and time–treatment interaction (RM ANOVA, $p<0.01$ (clear); $p<0.01$ (turbid)). The picoeukaryotic fraction was absent in turbid waters from day 0 onward (Table 1). In clear water, picoeukaryote abundances ranged between $1.67\times10^4\pm1.84\times10^3$ and $2.17\times10^4\pm2.26\times10^3$ cells ml^{-1} in control mesocosms and between $1.82\times10^4\pm1.28\times10^3$ and $2.96\times10^4\pm7.23\times10^2$ cells ml^{-1} in treated mesocosms from day 0 onward (Fig. 5). Statistical differences were detected for treatment (treated higher than control mesocosms) (RM ANOVA; $p<0.01$), time (RM ANOVA; $p<0.01$), and time–treatment interaction (RM ANOVA; $p<0.05$).

Micro and nanophytoplankton abundance was higher in clear than in turbid mesocosms throughout the study period (Table 1); no significant differences were found between treated and control mesocosms and between times for both state types. For clear water, micro and nanophytoplankton abundance ranged between $8.92\times10^4\pm2.66\times10^4$ and $1.89\times10^5\pm2.94\times10^4$ ind ml^{-1} in control mesocosms and between $1.25\times10^5\pm1.02\times10^5$ and $2.15\times10^5\pm5.81\times10^4$ ind ml^{-1} in treated mesocosms from day 0 onward. For turbid water, their abundance ranged between $4.46\times10^4\pm1.48\times10^4$ and $7.64\times10^4\pm2.20\times10^4$ ind ml^{-1} in control mesocosms and between $4.87\times10^4\pm1.90\times10^4$ and $8.40\times10^4\pm2.29\times10^4$ ind ml^{-1} in treated mesocosms from day 1 onward. The percentages of algal groups were not significantly different between treated and control mesocosms during the study period for both water types (Table 1). Chlorophyceae was always the most represented algal class; percentages at day 33 were similar in control and treated mesocosms both for clear and turbid water (Table 1). In general, at day 33, turbid water showed a non-significant trend toward higher algal variety of classes.

Chl *a* concentration was lower in clear than in turbid water at day 0 (Table 1). For clear water, the effect of the glyphosate was statistically significant at day 33 (one-way ANOVA; $p<0.05$), with mean chl *a* value almost 2-fold higher in treated than in control mesocosms (Table 1). For turbid water, the effect of the herbicide was non-significant (one-way ANOVA; $p>0.05$), with mean chl *a* values similar for treated and control mesocosms at day 33 (Table 1).

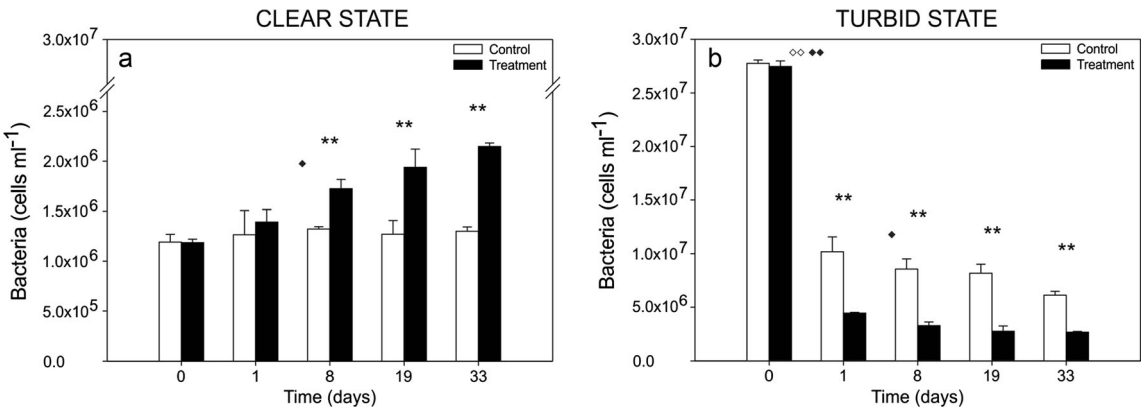

Fig. 3 **a**, **b** Mean abundance of bacteria in control and treated mesocosms throughout the study period in **a** clear and **b** turbid freshwater systems. Error bars represent SD. Significant differences between treatments at the same sampling date are indicated by *asterisks*: * $p<0.05$; ** $p<0.01$. Significant differences between consecutive times within a treatment are indicated by *diamonds*: $\Diamond p<0.05$; $\Diamond\Diamond p<0.01$, in *white* for control and *black* for treated mesocosms

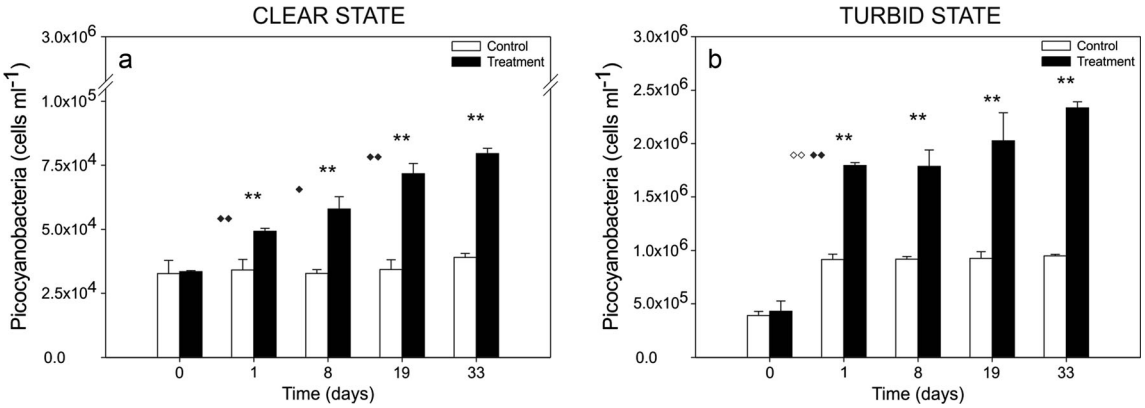

Fig. 4 **a**, **b** Mean abundance of picocyanobacteria during the experiment in control and treated mesocosms in **a** clear and **b** turbid waters. Error bars represent SD. Significant differences between treatments at the same sampling date are indicated by *asterisks*: *$p<0.05$; **$p<0.01$. Significant differences between consecutive times within a treatment are indicated by diamonds: ◊$p<0.05$; ◊◊$p<0.01$, in *white* for control and *black* for treated mesocosms

Discussion

Many studies have described the effect of glyphosate on freshwater microbial communities in terms of changes in their structure and function (e.g., Pesce et al. 2009, 2011; Pérez et al. 2011). Here, we evaluated the impact of a single input of glyphosate on different physical, chemical, and biological properties of the water under scenarios of different turbidity and trophic status. The most important result of our work is that, notwithstanding some differences between clear/oligotrophic and turbid/eutrophic scenarios, the microbial structure was markedly altered in both systems due to glyphosate addition. Moreover, as it has been already registered for freshwater (Pérez et al. 2007; Vera et al. 2010), the total phosphorus concentration is also affected by the herbicide addition in both turbidity states.

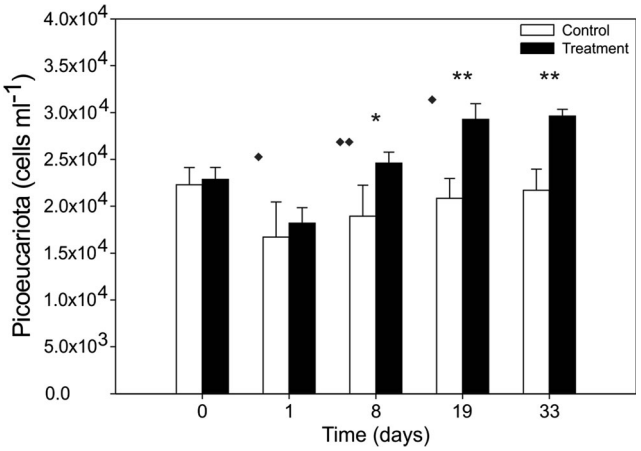

Fig. 5 Mean abundance of picoeukaryotes during the experiment in control and treated mesocosms in clear freshwater scenarios. Error bars represent SD. Significant differences between treatments at the same sampling date are indicated by *asterisks*: *$p<0.05$; **$p<0.01$. Significant differences between consecutive times within a treatment are indicated by diamonds: ◊$p<0.05$; ◊◊$p<0.01$, in *white* for control and *black* for treated mesocosms

Glyphosate dissipation in freshwater systems depends on many abiotic and biotic factors. Given the variety in the natural water body composition, a great range of half-life of glyphosate should be expected. The half-life of glyphosate determined did not differ significantly between turbid and clear waters, and values recorded (33.5 and 31.5 weeks, respectively) are among the highest reported for freshwater in the literature (Mercurio et al. 2014; Goldsborough and Brown 1988, and references cited therein). However, lower values were obtained by Pérez et al. (2007) (~6–7 days) and Vera et al. (2012) (~16 days) in similar experiments with outdoor mesocosms; the former two included sediments and all of them with water of different trophic status. Laboratory and field studies have demonstrated that the main mechanism of glyphosate removal from water is adsorption to suspended particulates followed by subsequent sedimentation and/or biodegradation (Zaranyika and Nyandoro 1993). The sediment composition is relevant for the adsorption of glyphosate, as clay minerals, which are rich in Fe^{3+} and Al^{3+}, have greater ability to retain the herbicide (Vereecken 2005; Khoury et al. 2010). Also, the adsorption of glyphosate decreases with the increase of pH values, inorganic phosphorus content, and cation concentration (Goldsborough and Beck 1989). The fact that the turbid and clear mesocosms used in our study shared a common water source may explain their similar glyphosate half-life. On the other hand, the differences found between our experiment and those abovementioned may be due to different water quality and presence/absence of sediments and the relative percentage composition of each mineral component (Pessagno et al. 2008).

The addition of glyphosate to the mesocosms caused direct and indirect changes in some physical and chemical variables. For example, it led to a significant increase in total phosphorus for both clear and turbid states. The significant rise of TP is in

agreement with previous studies (Pérez et al. 2007; Vera et al. 2010, 2012) reporting a marked increase in treated mesocosms; such result was fully explained by the phosphorus present in the glyphosate molecule. Phosphorus is one of the key factors influencing the entire metabolism of the water body, e.g., P could increase photosynthetic process and lead the evolution of an aquatic system to eutrophication. Therefore, the addition of glyphosate changes the system to a more eutrophic condition (Austin et al. 1991; Vera et al. 2012) which would be more evident in oligotrophic scenarios.

Saxton et al. (2011) stated that glyphosate in freshwater systems exerts positive and negative effects on distinct microbial communities, acting either as a nutrient source for herbicide-tolerant microorganisms or as a toxic to non-tolerant ones. In our experiment, the different microbial communities were favored, disfavored, or unaffected by glyphosate in relation to their relative abundances, depending on the water type. Algae possess the shikimate pathway which is involved in the biosynthesis of aromatic compounds (Duke et al. 2003), and its inhibition is the main mode of action of glyphosate. However, we found that the herbicide did not affect the abundance and class composition of the micro and nanophytoplankton for both clear and turbid waters. The effect of the herbicide on microalgae was analyzed mainly through laboratory assays, with varying results: low potential risk (Vendrell et al. 2009), negative effect (Sáenz et al. 1997), and positive effect (on certain cyanobacteria in the present study; Forlani et al. 2008; Arunakumara et al. 2013). Pérez et al. (2007), who used outdoor experiments (big pools of 25 m^2 surface) at the ecosystem scale, reported that the abundance of micro and nanophytoplankton was negatively affected by the glyphosate formulation Roundup®, while Vera et al. (2012) found that the glyphosate formulation Glifosato Atanor® had no significant effect on the structure of this community. Pesce et al. (2009) postulated that responses of natural microbial communities to glyphosate exposure can vary between experiments, especially when working at the ecosystem level where communities are composed of populations with different levels of sensitivity. Moreover, responses of some microbial communities could vary depending on the abiotic and biotic histories (Bonnineau et al. 2012; Pesce et al. 2011). Our results, like those before mentioned, show that the responses to the input of the herbicide are very complex, considering that living organisms have many responses to environmental change (Peck 2011).

The picoplankton fraction showed different trends in abundance and possibly in composition between clear and turbid waters. In clear waters, the increase in bacterial abundance after glyphosate addition was most likely due to the rise of populations using the herbicide as a nutrient source. An exhausted review about the utilization of glyphosate as phosphate source by bacteria has been recently published by Hove-Jensen et al. (2014). These authors have demonstrated that the soil bacteria *Sinorhizobium meliloti* of the family Rhizobiaceae is naturally able to utilize glyphosate as the sole P source. This ability to metabolize the herbicide is also present for other soil bacteria such as *Arthrobacter* sp. and *Pseudomonas* sp. (Schulz et al. 1985; Pipke et al. 1987). In a less important way, some soil bacteria have been described with the capability to metabolize glyphosate as C and N sources (McAuliffe et al. 1990; Obojska et al. 1999). There is much information about glyphosate degradation from aquatic habitats (e.g., Pérez et al. 2011; Mercurio et al. 2014). Forlani et al. (2008) described glyphosate metabolization in aquatic cyanobacteria (*Leptolyngbya boryana*, *Microcystis aeruginosa*, and *Spirulina platensis*), but very limited by low cell permeability to glyphosate and repressed when inorganic phosphorus is available. Likewise, in an outdoor experiment, Vera et al. (2012) observed the increase of bacterial abundance after the addition of the formulation Glifosato Atanor®. Moreover, some groups of bacteria can degrade glyphosate (Jacob et al. 1988), making phosphorus readily available to autotrophs. In our experiment, despite the slow dissipation of glyphosate in clear water, we found that the abundance of the picoeukaryotes fraction increased significantly from t1 onward, probably due to glyphosate biodegradation and the efficient phosphorus intake by this fraction. The small size of this fraction may account for its high uptake capability even under conditions of low nutrient concentration, providing it with a competitive advantage over other fractions (Callieri 2007). Glyphosate addition also favored the picocyanobacteria fraction, which showed a 2 to 2.5-fold abundance increase in treated mesocosms with respect to controls. This trend in the picocyanobacteria abundance was also described by Pérez et al. (2007), who observed a 40-fold abundance increase in mesocosms treated with Roundup® and by Vera et al. (2012), who reported an increase of about 1.5-fold in mesocosms—similar to those used by us—treated with Glifosato Atanor®. Cyanobacteria are known to be tolerant to glyphosate either by the overproduction of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPsynthase) or the production of a glyphosate-tolerant enzyme (Pérez et al. (op cit)). Ilikchyan et al. (2009, 2010) showed under laboratory conditions that picocyanobacteria of the genus *Synechococcus* and *Prochlorococcus*, which naturally use phosphonates (e.g., glyphosate) as P source, can hydrolyze them due to the expression of the phn genes; these encode functions for P uptake and C–P bond cleavage. In clear waters, the increase in abundance of the picoautotrophic fraction (picoeukariote+picocyanobacteria) was revealed by the significantly higher chl *a* concentration recorded in treated mesocosms.

Glyphosate led to a decrease in bacterial abundance in turbid mesocosms, probably due to the presence of herbicide-sensitive species. Chan and Leung (1986) demonstrated that

glyphosate affects the growth, respiration, and enzyme activity of the freshwater bacteria *Pseudomonas chloraraphis* and *Aeromonas hydrophila*. Glyphosate inhibits not only the activity of EPSP synthase but also that of phospho-2-oxo-3-deoxyheptonate aldolase and 3-dehydroquinate synthase, as well as $Fe\ 2^{+}$ transport in several bacteria (Roisch and Lingens 1980). However, it should not be ruled out a possible indirect effect of glyphosate, through a top–down impact by protozooplankton who often presents differential predation on picoplankton. A possible decrease in density of predators due to the herbicide impact could lead, in our case, the increase in density of the picoplankton, through the process of trophic cascading (Bengtsson et al. 2004). In this sense, Jürgens et al. (1997) highlighted the importance of ciliates as bacterivorous organisms. A novel result of our study concerning the autotrophic fraction of the picoplankton is that glyphosate addition to turbid mesocosms increases the abundance of picocyanobacteria regardless of its initial high proportion. As mentioned above, picocyanobacteria may be directly favored by the herbicide, while potential competitive interactions between autotrophs in treated mesocosms should be ruled out because the abundance of the other autotrophic fraction, micro and nanophytoplankton, was unaffected. This unexpected trend in picocyanobacteria from eutrophic scenario suggests that glyphosate could produce another kind of impact, direct or indirect, in this autotrophic fraction that deserves further studies. Due to the small size, the increase of 1.5-fold rise in picocyanobacteria abundance may not have been enough to induce a significant increase in chl *a* concentration in treated mesocosms. There are many ecological consequences due to the increment of picocyanocteria abundance in water bodies because they comprise an extremely important element of aquatic ecosystem functionality (Callieri 2007). Picocyanobacteria constitute a relevant component of the microbiological loop, incorporate the dissolved organic matter into the food web, and comprise the main food source of the nanoplanktonic protozoans and larger zooplankton. Moreover, despite the knowledge of its toxicity still being scarce, some reports regarding the secretion of microcystins, neurotoxins, or LPS by picocyanobacteria have been published (Jakubowska and Szeląg-Wasielewska 2015). This aspect of picocyanobacteria would contribute to the impoverishment of water quality and hazard not only for the biota but also for people.

The fact that the final response of microbial community involves the interactions of populations and organisms with each other and with their abiotic environment adds further complexity to the analysis. In this sense, it is important to include protists to complete and enhance the understanding of the glyphosate impact on microorganisms as it was demonstrated by Mbanaso et al. (2013). Flagellates and ciliates may be directly affected by glyphosate, altering their predation pressure on picoplankton. Moreover, the responses of the biological communities could depend on their structure at the initial stage of the system, previous to the input of the glyphosate, as it has been observed in the bacterioplankton in our study, where the final response was related to the initial structure of the community. On the other hand, our findings are based on mesocosm experiments, used as a proxy of natural conditions, and on the active ingredient of commercial formulations used in agricultural activities that contain adjuvants and surfactants. Therefore, more research on freshwater is needed, based on different limnological scenarios and also testing diverse formulations, in order to make appropriate generalizations on the impact of herbicides, such as glyphosate, at ecosystem level of analysis.

Conclusions

The strong environmental impact caused by agricultural activities and agrochemicals has been extensively documented worldwide (Millenium Ecosystem Assessment 2000). In Argentina, the widespread use of glyphosate has brought about profound changes in the practice of agriculture, favoring economic growth at the expense of social and environmental losses (Pengue 2005). The shallow lakes present in the Pampa plain, the area of highest agricultural productivity in the country, are directly affected by glyphosate, and its consequences are being continuously investigated (Peruzzo et al. 2008; Vera et al. 2012). Quirós et al. (2002) proposed that most of the Pampean shallow lakes were in a clear water state until the beginning of the agricultural intensification in the 1980s, after which there was a sharp rise in the use of agrochemicals, mainly glyphosate. Vera et al. (2010) demonstrated that a single input of glyphosate has a long-term impact, producing a shift in the water of outdoor mesocosms from clear to organic turbid, probably due to increased growth of picocyanobacteria, as previously reported by Pérez et al. (2007). We believe that our results are ecologically relevant since they indicate that glyphosate induces an increase in picocyanobacteria abundance not only in clear but also in organic turbid waters, with functional consequences in the whole systems. Moreover, considering that glyphosate adds phosphorus to the water, it could be estimated that in less rich nutriment conditions, the aquatic communities could be more sensitive to changes than in eutrophic systems. However, our results show that the impact of glyphosate was observed in microorganisms from both oligo and eutrophic conditions, being therefore independent of the trophic status of the water body.

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