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Comparative impact of two glyphosate-based formulations in interaction with *Limnoperna fortunei* on freshwater phytoplankton

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ABSTRACT

Although contamination and invasive species are two of the most relevant anthropogenic drivers affecting ecosystems, their joint impact on the environment has been poorly investigated. Glyphosate, directly or indirectly, contaminates freshwater systems which in turn may be invaded by the golden mussel *Limnoperna fortunei*. Under laboratory conditions, we studied the combined effect of technical-grade glyphosate, Roundup Max® and Glifosato Atanor®, in scenarios with and without *L. fortunei,* on phytoplankton from Salto Grande Reservoir (Uruguay River, Argentina). We expected that the effects of the interaction on phytoplankton and water quality would vary with the form of herbicide applied. The assay was conducted for 14 days (Tf) using 3-L bottles as experimental units. Eight treatments were performed in triplicate: C: Control; M: mussel; G: technical-grade glyphosate acid; R: Roundup Max®; A: Glifosato Atanor®; MG: mussel + technical-grade glyphosate acid, MA: mussel + Glifosato Atanor® and MR: mussel + Roundup Max®. The active ingredient was applied at 6 ppm. The dissipation of glyphosate in water was 1.5–2.6 times higher in presence of mussels. Treatments G and A showed an increase in phytoplankton abundance, mainly the cyanobacteria *Microcystis* spp. wich rised to 289% and 639% at Tf, respectively, relative to their values at Ti. Roundup Max® limited the growth of *Microcystis* spp., as its abundance decreased 59% relative to Ti. *L. fortunei* reduced phytoplankton abundances at Tf. Evenness increased significantly in M, MG, MR and MA, while it decreased in G, R and A relative to C. The interaction of factors produced a significant synergistic increase in periphyton; periphytic chlorophyll *a* concentration was $0.81 \pm 0.02\ \mu g\ cm^{-2}$ for MR; $0.09 \pm 0.02\ \mu g\ cm^{-2}$ for MA and $0.02 \pm 0.01\ \mu g\ cm^{-2}$ for MG. *Limnoperna fortunei* appeared as the driving force in the interaction. The assay described here allows for the rapid assessment of the impact of these types of agents on freshwater.

1. Introduction

Some human activities are direct drivers of ecosystem change, such as habitat fragmentation, climate change, species overexploitation, pollution and the introduction of invasive species (Millennium Ecosystem Assessment, 2005). In turn, direct drivers are influenced by indirect ones (e.g. technological development and the phenomenon of economic globalization), which also affect the structure and functioning of ecosystems. Most of these drivers are studied separately, but in fact they act simultaneously and the results of the interactions may be synergistic, antagonistic or additive (Townsend et al., 2008).

Over the past 50 years, the world population has doubled (World Population 2017) leading to an increase in human demands (Ojima et al., 1993). This resulted in greater land use and other practices that degrade soil fertility and water quality (Clark et al., 1986; Turner et al., 1993). Further intensification of human activities has caused the removal of native species, introduction of invasive species, changes in hydrological flows, and pollution of land, air and water (IGBP, 1990; WCED, 1987). The expansion of agricultural land has intensified the application of fertilizers and herbicides (Foley et al., 2005). An excessive input of nutrients to water bodies by the use of fertilizers is one of the main sources of pollution, reducing the capacity of these environments to assimilate and process wastes in the water. In consequence, numerous inland water bodies are deteriorated due to eutrophication. On the other hand, cyanobacterial blooms have emerged as an increasingly common event in lakes and coastal waters, and related eutrophication has become an issue of global concern (Bell and Codd, 1996; Carmichael, 1994; Conroy et al., 2005; Kalff, 2002).

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The herbicide glyphosate is the active ingredient of different commercial formulations applied worldwide; over 826 million kg of glyphosate were globally used for agricultural and non-agricultural practices in 2014 (Benbrook, 2016). In Argentina, where glyphosate is the main agrochemical used, over 320 million kg were sold in 2013 (CASAFE, 2013). Although glyphosate is applied to control undesirable weeds, it can reach water bodies by direct application or by indirect transport through wind and runoff after intense rainfall. Commercial formulations contain the active ingredient (glyphosate), inert elements and water. The active ingredient corresponds to a glyphosate salt, usually the isopropylamine salt (IPA), but there are also formulations with potassium or ammonium salt. The inert elements are solvents, surfactants and humectants of unknown composition which increase the permeability of the plant cuticle acting as a barrier to herbicide uptake (Lanctôt et al., 2014). The formulations vary depending on the type and concentration of the active component and on the type and concentration of surfactant or adjuvant aggregate (Giesy et al., 2000). Studies using algae and other organisms such as amphibians agree that the components of glyphosate-based formulations (i.e. surfactants and additives) contribute to the majority of the toxicity of the commercial formulations (Relyea and Jones, 2009; Fuentes et al., 2011; Lipok et al., 2010).

The golden mussel *Limnoperna fortunei* is an invasive freshwater species native to Southeast Asia, which was introduced to the coasts of La Plata River in the 1990s (Pastorino et al., 1993) through the ballast water of transoceanic ships. Adults are sessile and feed by filtering plankton and organic matter from water; they are involved in transferring part of the organic matter from the water column to the benthos, which may lead to the alteration of nutrient dynamics (Karatayev et al., 1997). The mussel-induced impacts are manifold and include increased water transparency due to its high filtration rates of up to 350 mL h^{-1} $individual^{-1}$ (Sylvester et al., 2005), as well as decreased phytoplankton abundance (Cataldo et al., 2012b) by inducing changes in the structure and function of freshwater ecosystems (Boltovskoy et al., 2009). In addition, the golden mussel exerts a grazing control on seston, modifying the proportion and availability of nutrients which may generate serious imbalances such as those favoring blooms of *Microcystis* spp. (Cataldo et al., 2012a).

Previous studies have shown that *L. fortunei* can reduce the half-life of glyphosate by more than 4-fold at a rate of 50.2 ± 3.4 mg per gram of mussel dry weight per day (Di Fiori et al., 2012).

Pizarro et al. (2015a) evaluated the impact of the combined effect of technical-grade glyphosate and *L. fortunei* on freshwater microbial communities using three concentrations of acid glyphosate (1, 3 and 6 ppm) in outdoor mesocosms. These authors found that the joint effect depended on the concentration of herbicide used and that the higher the herbicide dose, the higher the total phosphorus concentration and the larger the availability of phosphates. In addition, they reported that metaphyton was abundant in the treatments with glyphosate and mussels due to increased availability of nutrients and that both stressors acted synergistically on phosphate concentration, bacterioplankton abundance and water turbidity. Gattás et al. (2016), who investigated the combined effect of *L. fortunei* plus acid glyphosate with that of *L. fortunei* plus Roundup Max® on microbial communities in outdoor mesocosms, obtained different results according to the type of herbicide.

Taking into account that herbicides are currently applied as commercial formulations, the present work is focused on the effect of *Limnoperna fortunei* in combination with glyphosate and two widely used commercial formulations (Roundup Max® and Glifosato Atanor®) on water quality and phytoplankton using microcosms under controlled laboratory conditions. We analyzed physical and chemical variables of the water and the phytoplankton specific composition and diversity in an experiment of 14-days long. We used natural water from the Salto Grande Reservoir, which is a sink for the agrochemicals used in the region and harbors the mussel since the 2000s. We hypothesized that the effect of the herbicides and the golden mussel on phytoplankton and water quality would vary depending on whether they are used alone or in combination. We expected that the type of herbicide used in combination with mussels would influence the results.

2. Materials and methods

2.1. Experimental design

We conducted a manipulative laboratory experiment during April 2015 using water from the Salto Grande Reservoir (31°15′38, 16″S; 57°57′11, 84″W). It is a large (780 km^2), subtropical reservoir built in 1979 by damming the Uruguay River, where cyanobacterial blooms are increasingly common in mid-summer (Berón, 1990; Quiros and Luchini, 1983; De León and Chalar, 2003; Chalar, 2006). Boltovskoy et al. (2013) have reported a significant increase in the abundance of *L. fortunei* larvae in Salto Grande Reservoir since 2000. This, together with an extensive agrochemical use due to the dramatic expansion of the industrial agriculture in the region, makes the Salto Grande Reservoir a suitable model for studying the interaction between both anthropogenic stressors.

The assay was performed in an incubation chamber for 14 days under controlled light (1250 ± 180 Lux, LD 12:12 photoperiod), aeration and temperature (24 ± 1 °C) conditions. We used 24 microcosms (experimental units) consisting of 3-L plastic (PET, polyethylene terephthalate) bottles (Fig. 1) filled with water from the Salto Grande Reservoir, which was collected on the day of the beginning of the experiment. Air was supplied to each experimental unit via a centrally located aeration hose (10 mm in diameter) to allow recirculation of water and avoid sedimentation. Eight treatments were performed in triplicate: C: Control; M: mussel; G: technical-grade glyphosate acid (95% purity, CAS: 1071-83-6); R: Roundup Max® (74.7% of *N*-(phosphonomethyl) glycine monoammonium salt (CAS: 40465-66-5) and 25.3% of inert ingredients and adjuvants); A: Glifosato Atanor® (43.8% of *N*-(phosphonomethyl) glycine monopotassium salt (CAS: 39600-42-5) and 56.2% of inert ingredients and adjuvants); and treatments with mussels and herbicide, namely MG: mussel + technical-grade glyphosate acid, MA: mussel + Glifosato Atanor® and MR: mussel + Roundup Max®. Treatment C only included water from Salto Grande. Both Roundup Max® and Glifosato Atanor® are among the most used herbicides in Argentina. The active ingredient was applied at a concentration of 6 ppm in treatments G, R, A, MG, MR and MA. We chose this

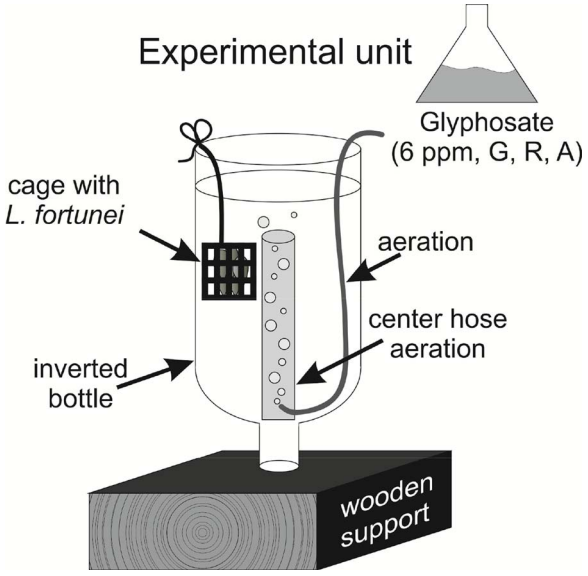

Fig. 1. Scheme of an experimental unit (microcosms) showing the cage with individuals of *L. fortunei* for treatments with mussels. G: technical-grade glyphosate acid; R: Roundup Max®; A: Glifosato Atanor®.

concentration because it is close to the highest value recorded in naturals systems (Mann and Bidwell, 1999).

Adult mussels of *Limnoperna fortunei* were collected from the delta of the Paraná River and transported in containers with water to the laboratory. Upon arrival, they were placed in tanks with dechlorinated water at 23–25 °C under continuous aeration and fed daily with baby fish food, during one week for acclimatization. Before the start of the experiment, we selected actively filtering individuals (with siphons extended) with a mean shell length of 20 ± 5 mm and mussels of similar size were separated in groups of 5 in plastic mesh cages (5-mm pore size). One cage was introduced into each of the microcosms corresponding to the treatments with *L. fortunei* (M, MG, MR and MG); cages were suspended below the water surface (Fig. 1).

2.2. Glyphosate analysis

Water samples from all treatments were collected immediately after the application of herbicides (initial time, Ti) and 14 days later (final time, Tf) and stored at −20 °C until glyphosate residue analysis. Samples were thawed, homogenized and a chloromethane extraction protocol was performed with 3 mL to 10 mL of water. In a 15 mL Falcon tube, 10 mL extracted water samples were added and centrifuged to avoid solid residues (17,000*g* × 10 min). The liquid phase was then transferred to a UPLC polypropylene holder. Glyphosate concentrations of water samples where determined through UPLC Waters Acquity (Ultra Performance Liquid Chromatography) with SQD detector (single quadrupole mass detector) using ESI negative mode. Chromatographic separation was set with 1% acetic acid in water: MeOH, at the following gradient; (95:5)-(95:5) 0–2 min, (95:5)-(0:100) 2–5 min, (100:0)-(95:5) 5–6 min, (95:5) 6–10 min, as the mobile phase. Hypercarb 2.1 × 100 mm 5 µm column was used. Selected ion monitoring mode was used in the quantification analysis (ion 168 *m/z* and ion 150 *m/z*). Calibrations curves were constructed in water covering the range from 5.00 $\mu g L^{-1}$ to 15.00 $mg L^{-1}$. The limit of detection was 1.00 $\mu g L^{-1}$.

2.3. Physical and chemical variables

Water, pH and conductivity were measured with a portable multimeter (Hach® sension 156 m) and turbidity with a Hach® 2100P turbidimeter. Water samples for chemical analysis were taken immediately after adding herbicides and mussels at Ti and at Tf. For chemical determination of dissolved nutrient concentrations (ammonium, nitrate + nitrite and soluble reactive phosphorus), samples of 200 mL were taken from each microcosm and filtered through 0.7 µm-pore fiberglass filters (Whatman® GF/F) (APHA, 2005). The soluble reactive phosphorus ($P\text{-}PO_4^{-3}$) was determined using the ascorbic acid method, nitrite + nitrate ($N\text{-}NO_3^- + N\text{-}NO_2^-$) by cadmium reduction to nitrite (Mackereth et al., 1978) and ammonium ($N\text{-}NH_4^+$) by the salicylate method (APHA, 2005). The detection limit for nutrients was 0.001 $mg L^{-1}$ and all the determinations were made spectrophotometrically (spectrophotometer Hach® DR 2800).

2.4. Biological variables

At the beginning and the end of the experiment, quantitative determinations of phytoplankton were carried out from unfiltered water samples fixed with 1% acidified Lugoĺs solution. Total abundance (live and dead organisms) was measured following Utermöhl (1958) method. Individuals with organized cell structure including chloroplasts and cell wall (e.g. frustules of diatoms) were considered alive. Abundance of *Microcystis* spp. was also calculated as the number of cells mL^{-1} considering the total number of cells by colony following Chorus and Bartram (1999) through the use of Analyze® software. The counting error (< 20%) was estimated according to Venrick (1978). Species diversity was estimated using the Shannon-Wiener index (Shannon and Weaver, 1963) calculated as $\acute{H} = -1\Sigma$ (pi ln pi), where pi is the relative abundance of each species. Evenness was calculated as $E = \acute{H}/\ln R$, where R corresponds to the species richness found in the sample.

Periphyton chlorophyll *a* (pChl *a*) was obtained from the periphyton grown on the aeration tubes of the microcosms, which acted as artificial substrata for the development of the attached community. The periphyton was removed by scraping a known area of the substrate surface and brought to a known volume with distilled water. Then, samples were filtered through Whatman® GF/F filters and frozen at −20 °C until quantification. Pigment extraction was performed with hot (60–70 °C) ethanol, and stored in darkness overnight at 4 °C. Subsequently, the samples were centrifuged for 10 min at 3000 rpm. For spectrophotometric analysis, absorbances were recorded at 665 and 750 nm before and after acidification with HCl 1N. The final concentration was estimated following Marker (1980), related to the surface of the scraped substrate and expressed per unit area.

For the duration of the experiment, mussels were checked daily for survival as described above.

2.5. Statistical analyses

Differences in the concentration of glyphosate and soluble nutrients between treatments were analyzed using a two-factor repeated measures ANOVA (DMR ANOVA). When interactions were significant ($P < 0.05$) mean comparisons were made using the Bonferroni test. The assumptions of the models were validated. When the interaction between factors (mussels and herbicides) was significant, we tested if the combined effect was synergistic or antagonistic (joint effect much larger or smaller than the sum of the separate effects, respectively). Statistical analyses were performed with SPSS® software.

Differences in phytoplankton and *Microcystis* spp. abundances and evenness between Tf and Ti were studied using general linear models as well for pChl *a* (only at Tf). The assumptions of homogeneity of variance and normality were tested with Shapiro-Wilks and Levene tests, respectively. The Akaike information criterion was used to select the most appropriate variance structure. Treatments were compared with the LSD Fisher test.

Cluster analysis was performed to group treatments according to phytoplankton species abundances. Unweighted pair-group moving average (UPGMA) clustering, using a Bray Curtis similarity matrix (Romesburg, 1984) was applied using Infostat® software.

3. Results

3.1. Survival of Limnoperna fortunei

Survival was 100% in all the experimental units throughout the study period, with all mussels being healthy and showing active filtration.

Mean values (± SE) of the physical, chemical, and biological variables at Ti and Tf for all treatments are shown in Table 1.

3.2. Glyphosate analysis

The actual mean value of glyphosate added to microcosms was 5.96 ± 0.26 $mg L^{-1}$. At Ti and Tf, undetectable values were registered for C and M treatments which mean that the water from Salto Grande reservoir, at the site and date of sampling, did not have detectable glyphosate concentration. At Tf, the concentration of glyphosate decreased in all herbicide treatments either with or without mussels (DMR ANOVA $P < 0.05$) (Fig. 2). In treatments without mussels, the final mean concentrations were 4.74 ± 0.77 $mg L^{-1}$ for G; 3.46 ± 0.38 $mg L^{-1}$ for R and 4.26 ± 0.21 $mg L^{-1}$ for A treatment, representing an herbicide dissipation of 20%, 41% and 28% respectively. At Tf G treatment was significantly different than the rest of treatments (DMR ANOVA $P < 0.05$) with the exception of A treatment (DMR ANOVA $P > 0.05$). In treatments with mussels, the final

Table 1
Mean values (± SE) of the physical, chemical and biological variables recorded at inicial time (Ti) and at final time (Tf) by treatments. C: Control, M: Mussel, R: Roundup Max®, G: glyphosate, A: GlifosatoAtanor®, MR: Mussel + Roundup Max®, MG: Mussel + glyphosate acid and MA: Mussel + GlifosatoAtanor®. ud: undetectable. The symbol ▲ indicates significant differences between control and each treatment (DMR ANOVA $P < 0.05$), and the symbol* indicates significant differences between Ti and Tf for each treatment (DMR ANOVA $P < 0.05$).

Variable	Treatments							
	C	G	R	A	M	MG	MR	MA
pH								
Ti	6.89 ± 0.05	7.36 ± 0.15	7.05 ± 0.04	7.13 ± 0.01	7.20 ± 0.17	7.11 ± 0.06	6.94 ± 0.04	7.05 ± 0.04
Tf	7.68 ± 0.20	6.89 ± 0.04	6.92 ± 0.01	6.95 ± 0.03	7.04 ± 0.08	6.88 ± 0.02	6.92 ± 0.02	7.02 ± 0.06
Conductivity (µS cm^{-1})								
Ti	53.00 ± 0.15	55.10 ± 2.75	56.13 ± 0.07	56.07 ± 0.07	54.33 ± 1.48	59.83 ± 7.48	56.87 ± 1.07	56.23 ± 0.03
Tf	43.30 ± 0.40	56.13 ± 3.07	54.93 ± 0.68	55.00 ± 0.38	51.83 ± 3.54	59.03 ± 9.09	53.40 ± 1.10	49.4 ± 4.86
Turbidity (NTU)								
Ti	17.33 ± 0.33	17.00 ± 0	19.00 ± 1.00	16.33 ± 1.20	17.33 ± 0.33	17.33 ± 0.33	19.33 ± 1.86	16.67 ± 0.88
Tf	6.00 ± 0	6.00 ± 0	6.00 ± 0	6.33 ± 0.33	6.00 ± 0	6.00 ± 0	6.00 ± 0	6.00 ± 0
Ammonia N-NH_3 (mg L^{-1})								
Ti	0.007 ± 0.007	ud	0.313 ± 0.003▲	0.020 ± 0.012	ud	ud	0.307 ± 0.007▲	0.023 ± 0.013
Tf	ud	ud	ud	0.003 ± 0.003	ud	ud	0.003 ± 0.003	ud
Nitrite + Nitrate N-NO_3^- + N-NO_2^- (mg L^{-1})								
Ti	0.667 ± 0.088	0.600 ± 0.058	0.700 ± 0.058	0.700 ± 0.100	0.700 ± 0	0 0.633 ± 0.033	0.600 ± 0.100	0.667 ± 0.033
Tf	0.467 ± 0.033	0.500 ± 0	0.367 ± 0.033	0.4 ± 0.058	0.467 ± 0.033	0.533 ± 0.033	0.433 ± 0.033	0.467 ± 0.033
Phosphates P-PO_4 (mgL^{-1})								
Ti	0.069 ± 0.010	0.039 ± 0.015	0.024 ± 0.009	0.021 ± 0.005	0.027 ± 0.014	0.027 ± 0.016	0.022 ± 0.008	0.022 ± 0.004
Tf	0.038 ± 0.014	0.040 ± 0.014	0.030 ± 0.013	0.038 ± 0.013	0.040 ± 0.010	0.091 ± 0.016	0.264 ± 0.007▲*	0.176 ± 0.003▲*
Phytoplankton abundance (cells mL^{-1})								
Ti	18176 ± 3377	23319 ± 7011	19082 ± 4516	7938 ± 2212▲	24505 ± 4412	20282 ± 4307	28553 ± 3881	16336 ± 2428
Tf	14434 ± 1551	63144 ± 13955▲*	11130 ± 9068	49033 ± 15745▲*	186 ± 151▲*	5478 ± 2904▲*	6030 ± 3499▲*	3238 ± 1842▲*
***Microcystis* spp. abundance (cellsmL^{-1})**								
Ti	16199 ± 4067	21483 ± 5480	18116 ± 4526	7354 ± 2171	23085 ± 4321▲	19154 ± 3846	27968 ± 3706▲	15594 ± 2465
Tf	11193 ± 1397	62415 ± 13819▲*	10763 ± 9110	47664 ± 15698▲*	43 ± 5▲*	4063 ± 2743▲*	5013 ± 3676▲*	691 ± 566▲*
Periphyton Chlorophyll *a* (µg cm^{-2})								
Tf	0.011 ± 0.004	0.002 ± 0.007	0.009 ± 0.003	0.0148 ± 0.003	0.004 ± 0.001	0.021 ± 0.016▲	0.810 ± 0.020▲	0.093 ± 0.023▲

concentrations were 2.83 ± 0.58 mg L^{-1} for MG; 2.15 ± 0.13 mg L^{-1} for MR and 1.69 ± 0.24 mg L^{-1} for MA treatment, with an herbicide dissipation of 52%, 64% and 72% respectively. No significant differences were registered for the final concentrations among treatments with *L. fortunei* (Fig. 2). Mussels reduced glyphosate 2.6 times for technical-grade glyphosate acid and Glifosato Atanor® and 1.5 times for Roundup Max®.

3.3. Physical and chemical variables

There were no significant differences in pH and conductivity values either among treatments or between time (DMR ANOVA $P > 0.05$) (Table 1). The pH values were circumneutral, ranging from 6.89 ± 0.02 (MG, Tf) to 7.7 ± 0.2 (C, Tf), while the conductivity ranged from 59.8 ± 7.5 µS cm^{-1} (MG, Ti) to 43.3 ± 0.4 µS cm^{-1} (C, Tf). At Ti, mean turbidity of treatments was 17.5 ± 1.1 NTU (DMR ANOVA $P > 0.05$), and at Tf values were very similar among treatments (≈6 NTU). Treatments had no effect on this variable; in the control, an experimental artifact may account for the decrease in turbidity observed at Ti as compared to Tf.

The concentration of ammonium at Ti was significantly higher in treatments with Roundup Max® than in the control (R: 0.31 ± 0.003 mg N-NH_4^+ L^{-1} and MR: 0.31 ± 0.007 mg N-NH_4^+ L^{-1}, DMR ANOVA $P < 0.05$, Table 1). There was a non-significant trend ($P > 0.05$) toward increased ammonium concentration in treatments with Glifosato Atanor® (A: 0.02 ± 0.012 mg N-NH_4^+ L^{-1} and MA: 0.023 ± 0.013 mg N-NH_4^+ L^{-1}). Ammonium concentrations at Ti were not detectable in treatments M, G and MG, while at Tf these were very low or undetectable in all treatments. A significant decrease in ammonium concentration at Ti in comparison to Tf was observed in treatments with commercial formulations (R, MR, A and MA, DMR ANOVA $P < 0.05$). There were no significant differences in nitrate + nitrite concentration among treatments either at the beginning or at the end of the experiment, ranging from 0.4 ± 0.058 mg (N-

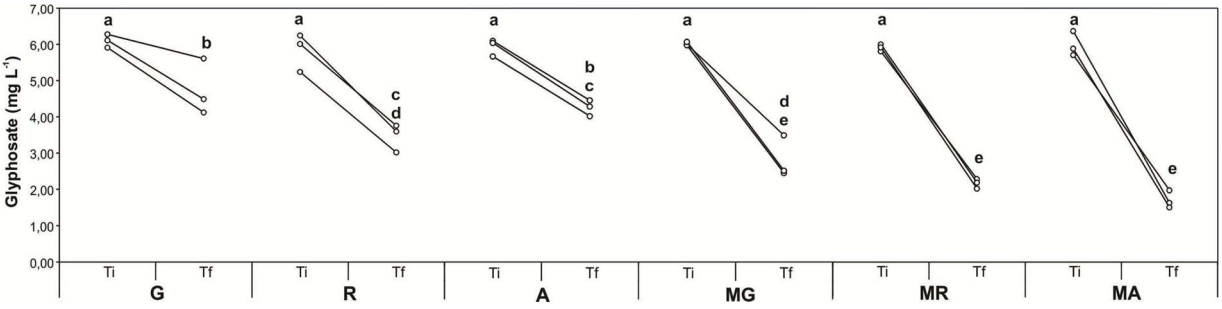

Fig. 2. Glyphosate concentration of each replicate by treatment, at initial time (Ti) and final time (Tf). G: glyphosate, R: Roundup Max®, A: Glifosato Atanor®, MG: Mussel + glyphosate; MR: Mussel + Roundup Max® and MA: Mussel + Glifosato Atanor®. Different letters mean statistically significant differences (DMR ANOVA $P < 0.05$, N = 3).

NO_2^- + N-NO_3^-) L^{-1} (A; Tf) to 0.7 ± 0.06 mg (N-NO_2^- + N-NO_3^-) L^{-1}(R; Ti) (Table 1).

No significant differences were found in the concentration of P-PO_4^{-3} among treatments and the control at Ti (mean value 0.031 ± 0.005 mg P-PO_4^{-3} L^{-1}) (DMR ANOVA $P > 0.05$, Table 1). The interaction mussel*glyphosate*time was significant (DMR ANOVA $P < 0.05$) and simple effects showed that the joint presence of mussels and herbicides increased the concentration of P-PO_4^{-3} at Tf in relation to the control in treatments with formulations (DMR ANOVA $P < 0.05$), these being 0.26 ± 0.01 mg P-PO_4^{-3} L^{-1} for MR and 0.18 ± 0.003 mg P-PO_4 L^{-1} for MA. The concentration of P-PO_4^{-3} in MR was significantly higher than that in MA, which in turn, was significantly higher than that in the control and in the rest of the treatments (DMR ANOVA $P < 0.05$). In MG, a clear trend toward an increased concentration of phosphates was observed, although it was not significant compared to the control (DMR ANOVA $P > 0.05$).

3.4. Biological variables

The presence of *L. fortunei* reduced phytoplankton abundances, with differences between Tf and Ti yielding negative values (M: −24319 ± 2514; MR: −22523 ± 4144; MG: −14804 ± 2802; MA: −13098 ± 387 cells mL^{-1}) (Fig. 3, Table 1). On the other hand, herbicide treatments produced varying responses: significantly positive values were observed for G and A, (G: 39825 ± 4016; A: 41095 ± 9378 cells mL^{-1}, DMR ANOVA $P < 0.05$), while R did not differ significantly from C (C: −3741 ± 2354; R: −7952 ± 3808 cells mL^{-1}, DMR ANOVA $P > 0.05$). The interaction glyphosate*-mussel was significant (DMR ANOVA $P < 0.05$).

During the study, we identified 32 algal species distributed in 7 groups (Table 2).

Chlorophyceae and Bacillariophyceae presented the highest values of species richness, while Cyanobacteria was the most abundant group, largely represented by *Microcystis* spp., as it accounted for more than 80% of the total abundance of this group; *Microcystis* spp. were always registered either as solitary cells or as small colonies (size < 60 µm). At Ti, the relative abundance of phytoplankton groups was similar in all treatments including the control, with 96.1 ± 0.8% of Cyanobacteria and 3.2 ± 0.7% of Cryptophyceae while the remaining 0.7% was distributed among the other Classes (Fig. 4). At Tf, the relative abundance of phytoplankton groups did not differ significantly from that at Ti in the control treatment. Bacillariophyceae increased significantly ($P < 0.05$) in treatments with mussels (M, MR, MG and MA). In M there was a significant increase in the relative abundance of Bacillariophyceae (20.2 ± 6.3%) and Cryptophyceae (24.2 ± 8.7%) ($P < 0.05$ for both cases). In MA Bacillariophyceae was the dominant group (68.9 ± 6.8%) followed by Cyanobacteria (26.6 ± 6.2%). With regard to the abundance of *Microcystis* spp., there was a significant decrease at Tf in treatments M, MR, MG y MA with respect to the initial values; contrarily, in A and G treatments the abundance of these species increased significantly at Tf, while treatments C and R remained without changes (DMR ANOVA $P < 0.05$) (Table 1).

Evenness is another component of diversity, besides species richness and composition. Evenness increased significantly in treatments with mussels (M, MG, MR and MA), while it decreased significantly in treatments without mussels (G, R and A) when compared to the control ($P < 0.05$ for both cases) (Fig. 5). Accordingly, the Shannon index increased significantly at Tf in all treatments with mussels (M, MG, MR and MA; DMR ANOVA $P < 0.05$), with a mean value (± SE) of 0.49 ± 0.06 (Fig. 6).

Table 2
List of species registered in the phytoplankton in all treatments. u.i. unidentified.

Phytoplankton groups	Species
Chlorophyceae	*Monoraphidium contortum*
	Monoraphidium arcuatum
	Monoraphidium minutum
	Monoraphidium sp.
	Scenedesmus sp.
	Spermatozopsis exsultans
	Chlamydomonas sp.
	Merismopedia sp.
	Desmidiaceae u.i.
Bacillariophyceae	*Aulacoseira granulata*
	Fragilaria ulna
	Fragilaria sp.
	Nitzschia acicularis
	Nitzschia sp.
	Navicula sp.
	Skeletonema sp.
Cyanobacteria	*Merismopedia* sp.
	Microcystis spp.
	Pseudanabaena mucicola
	Phormidium sp.
	Dolichospermum sp.
	Aphanothece sp.
	Planktolyngbya sp.
Cryptophyceae	*Cryptomonas marssonii*
	Cryptomonas ovata
	Plagioselmis lacustris
	Plagioselmis nannoplanctonica
Chrysophyceae	*Ochromonas* sp.
	Salpingoeca sp.
Dinophyceae	*Ceratium furcoides*
	Peridinium sp.
Euglenophyceae	*Euglena* sp.

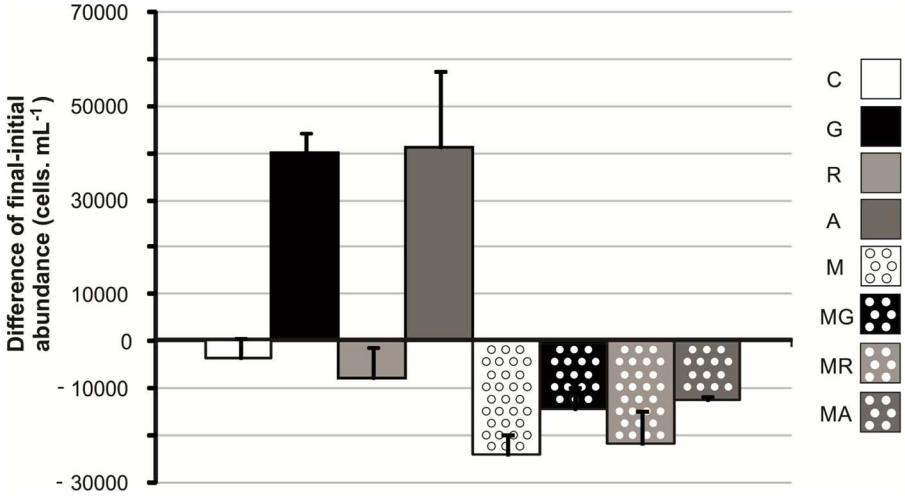

Fig. 3. Differences between final and initial abundances of phytoplankton by treatment. C: Control, M: Mussel, R: Roundup Max®, G: glyphosate, A: Glifosato Atanor®, MR: Mussel + Roundup Max®, MG: Mussel + glyphosate and MA: Mussel + Glifosato Atanor®. Bars = +1 SE; N = 3.

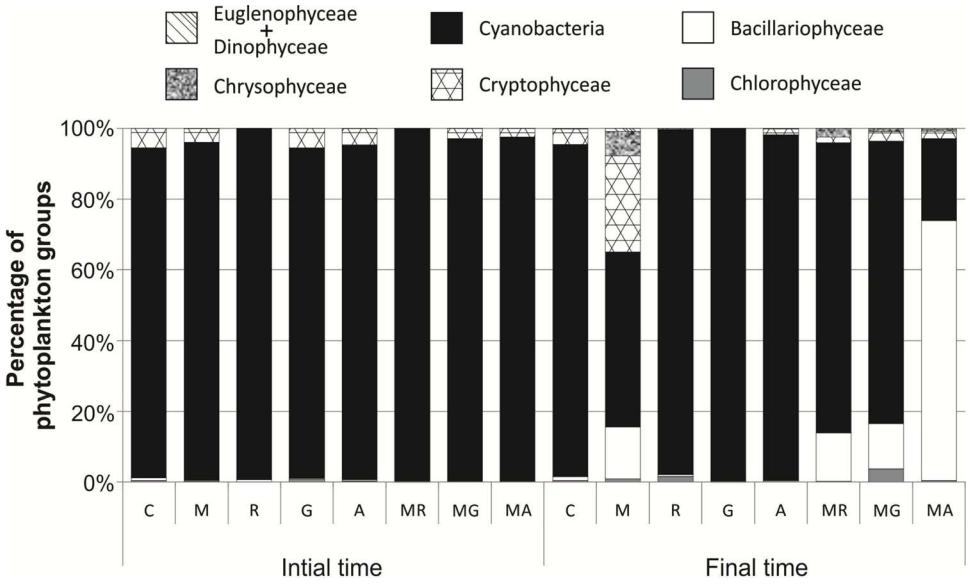

Fig. 4. Mean percentage of phytoplankton groups by treatment at initial and final time. C: Control, M: Mussel; R: Roundup Max®; G: glyphosate; A: Glifosato Atanor®; MR: Mussel + Roundup Max®; MG: Mussel + glyphosate and MA: Mussel + Glifosato Atanor®. N = 3.

The dendrogram obtained from the abundance of the phytoplankton groups using the Bray-Curtis index on transformed data (Fig. 7) had a cophenetic coefficient of 0.944. Two groups were evident at first sight: one comprising the treatments with the stressors alone and the other the interaction treatments. The control treatment is separated from the two groups as an outgroup.

Fig. 8 shows the mean values of pChl *a* from the periphyton on the plastic tubes of the aeration system, for each treatment at Tf. Treatments C, M, R, G and A showed very low, almost undetectable pChl *a* values. The interaction glyphosate*mussel was significant (DMR ANOVA $P < 0.05$); MR had the highest pChl *a* concentration ($0.81 \pm 0.02\ \mu g\ cm^{-2}$) of all groups −including the control-, followed by that in MA ($0.09 \pm 0.02\ \mu g\ cm^{-2}$) and MG ($0.02 \pm 0.01\ \mu g\ cm^{-2}$); pChl a concentration in MG was higher than that in the remaining treatments and control (M, R, A and G, Fisher LSD contrasts $P < 0.05$).

4. Discussion

It has been demonstrated that the joint effect of *Limnoperna fortunei* and glyphosate on the structure of freshwater microbial communities and water quality is different from the effect of each factor considered separately (Pizarro et al., 2015a). Gattás et al. (2016) reported that *L. fortunei* seemed to be the most powerful driver of the interaction, which elicited different responses depending on whether it involved glyphosate or a commercial formulation (Roundup Max®). In this study another widely used glyphosate-based formulation, Glifosato Atanor®, was included in the analyses. We also observed differences in the responses of phytoplankton from the Salto Grande reservoir to various types of herbicides, and although all of them promoted the diversity of phytoplankton, the presence of mussels modulated their effects yielding dissimilar results. Once again, *L. fortunei* appeared as the leading anthropogenic driver of the interaction because of its important role in mitigating the impact of glyphosate and commercial formulations on phytoplankton. In addition, mussels in combination with herbicides produced a synergistic effect on periphyton development.

Considering that the dissipation of glyphosate in water is mediated by different processes, such as adsorption to suspended particulates followed by subsequent sedimentation and/or biodegradation (Zaranyika and Nyandoro, 1993) in the present work we observed the decrease of glyphosate concentration in all treatments with different patterns. For those treatments without mussels, the dissipation of the herbicide showed some differences among them, being Roundup Max® the formulation with the highest rate. This dissimilarity was probably

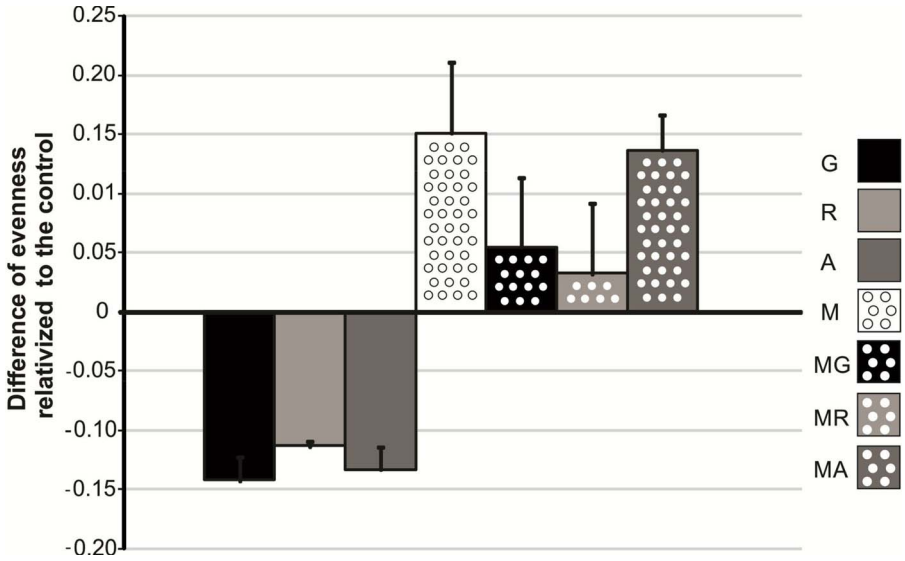

Fig. 5. Phytoplanktonic eveness of each treatment related to the control at final time. M: Mussel; R: Roundup Max®; G: glyphosate; A: Glifosato Atanor®; MR: Mussel + Roundup Max®; MG: Mussel + glyphosate and MA: Mussel + Glifosato Atanor®. Bar: +1SE. N = 3.

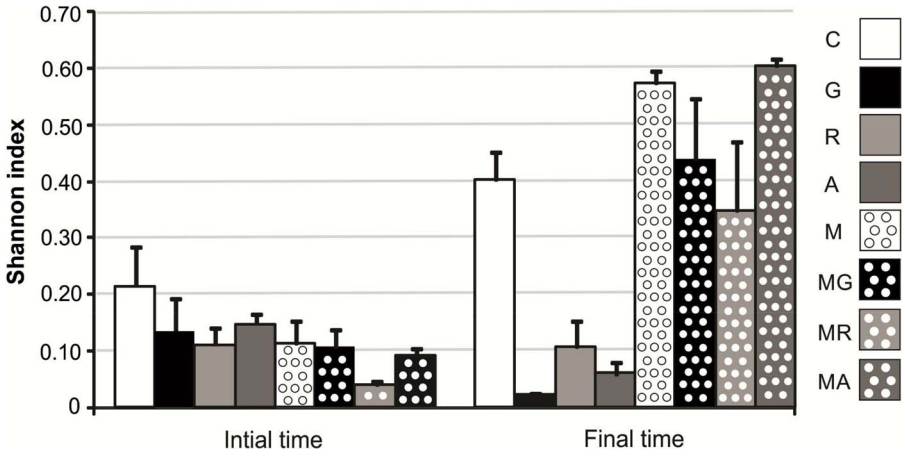

Fig. 6. Mean values of Shannon index of phytoplankton by treatment at initial and final time. C: control; M: Mussel; R: Roundup Max®; G: glyphosate; A: Glifosato Atanor®; MR: Mussel + Roundup Max®; MG: Mussel + glyphosate and MA: Mussel + Glifosato Atanor®. Bar: +1SE. N = 3.

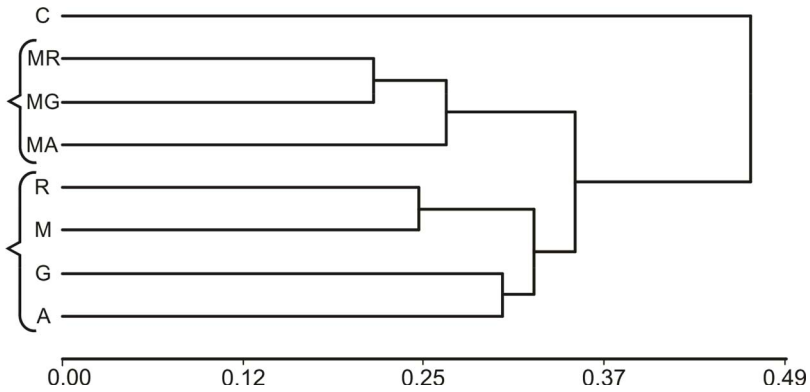

Fig. 7. Cluster of phytoplankton composition by treatments at Tf using UPGM linkage from Bray Curtis index. Curly braces separate groups of treatments. C: Control, M: Mussel, R: Roundup Max®, G: glyphosate, A: Glifosato Atanor®, MR: Mussel + Roundup Max®, MG: Mussel + glyphosate and MA: Mussel + Glifosato Atanor®.

due to the different presentation of glyphosate used in the treatments (i.e. technical-grade glyphosate acid for G treatment, glyphosate monopotassium salt for A treatment and glyphosate monoammonium salt for R) which could cause differences in the dissipation rates. The presence of *Limnoperna fortunei* accelerated the decrease of the glyphosate between 1.5 to 2.6 times depending of the type of herbicide. Similar result were observed by Pizarro et al. (2015b) where *L. fortunei* reduced 4-times the half-life of glyphosate, in an outdoor mesocosms experiment. The strong degradation power of the golden mussel was first described by Di Fiori et al. (2012) who mentioned that this ability is possibly mediated by bacteria associated to their metabolism or by microbial biofilm communities developed in their shells.

In our study, the addition of Roundup Max® and Glifosato Atanor® caused rapid changes in water chemistry as indicated by the significant increase in ammonium concentration at Ti. In the case of Roundup Max®, this may have been due to its active ingredient, which is a monoammonium salt of *N*-phosphonomethylglycine that dissociates in aqueous solution releasing ammonium (Gattás et al., 2016). The ammonium increase resulting from the addition of Glifosato Atanor® would be explained by the formulation adjuvants of unknown specific composition because its active ingredient is a monopotassium salt of glyphosate (Giesy et al., 2000). Ammonium concentration was reduced to almost null at Tf, probably because it was used as a nutrient for the metabolic processes of phytoplankton, periphyton, and/or other

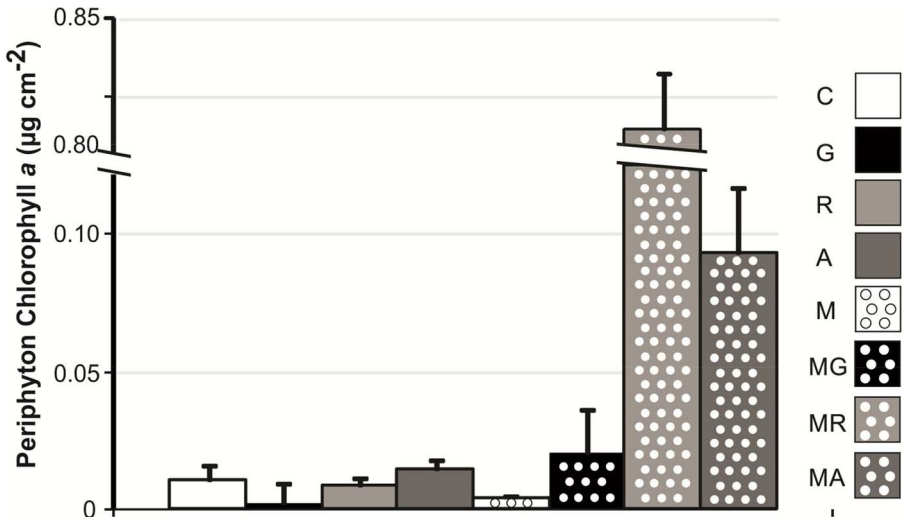

Fig. 8. Mean values of periphytic chlorophyll *a* concentration at final time. C: Control, M: Mussel, R: Roundup Max®, G: glyphosate, A: Glifosato Atanor®, MR: Mussel + Roundup Max®, MG: Mussel + glyphosate and MA: Mussel + Glifosato Atanor®. Bar = + 1SE. N = 3.

communities which were not considered in the present work (e.g. bacterioplankton).

We also found that neither the formulations assayed nor the acid glyphosate initially contributed with soluble reactive phosphorus to the water. A similar result has been reported in field studies using acid glyphosate (Pizarro et al., 2015a), Glifosato Atanor® (Vera et al., 2012) and Roundup® (Pérez et al., 2007; Vera et al., 2010). The increase of this nutrient at Tf in MR, MA and MG treatments suggests a joint effect of glyphosate and the golden mussel. Previous studies indicate that *L. fortunei* is capable of increasing nutrient concentrations, mainly phosphates, by two different mechanisms: i) the mussel shows a high predation rate on planktonic algae and bacteria, which are subsequently digested and released as large amounts of nutrients to the medium (Cataldo et al., 2012a,b); and ii) the mussel is able to reduce glyphosate concentration in water (Pizarro et al., 2015a), as it was here demonstrated considering the dissipation values of the herbicide in treatments with mussels. Also, differences in the concentration of soluble reactive phosphorus between the MR and MA treatments at Tf may result from the dissimilar chemical composition of the commercial formulations. Although initially they do not supply P-PO_4 to the water, it is possible that they have additives with phosphorus which may be degraded by mussel activity, with the nutrient being eventually released to the medium. This may diminish the negative effect on organisms considering that the substances present in the formulations may be even more harmful than glyphosate (Tsui and Chu, 2003). Interestingly, Vera et al. (2012) determined that the adjuvants of Glifosato Atanor® introduce much more phosphorus into the water than the active ingredient alone. Such load and quick release of nutrients mediated by mussels would lead to water enrichment, which in turn promotes eutrophication processes, as stated by Pizarro et al. (2015a).

In regard to the impact of herbicides on phytoplankton structure, the differences observed at the end of the experiment were most likely due to the composition of the herbicide assayed. Our results suggest that glyphosate and Glifosato Atanor® would stimulate the growth of phytoplankton, mainly Cyanobacteria, as its final abundances rised 289% and 639% with respect to their initial values in treatments G and A, respectively. Final Cyanobacteria abundance in treatment A triplicated the values registered in the control, while in G treatment the abundance was four-fold higher than the control. *Microcystis* spp. was the dominant component of Cyanobacteria, representing more than 98% of the final cyanobacteria abundances in treatments A and G. Forlani et al. (2008) have demonstrated that some strains of *Microcystis* sp. are capable to metabolize glyphosate in culture assays and that the herbicide is not only harmless but even beneficial to this organism in terms of growth. As mentioned above, Salto Grande Reservoir is currently experiencing strong blooms of *Microcystis* spp. (Chalar, 2006) with serious environmental and health consequences. Based on our results, glyphosate, the more used agrochemical in the region, is probably one of the major factors responsible for exacerbating this problem in Salto Grande reservoir. However, the fact that the stimulus for the development of *Microcystis* spp. appears to be related to the type of formulation makes one cautious about generalization. In this line of reasoning, the abundance of *Microcystis* spp. exposed to Roundup Max® decreased 59% with respect to their initial value, suggesting that the adjuvants in this formulation would have a negative impact on its growth.

The significant decrease in phytoplankton abundance in the treatment with *L. fortunei* alone with respect to C would have resulted from its high filtration rates. Considering an average filtration rate of 100 mL ind^{-1} h^{-1} (Cataldo et al., 2012b), mussels would filter the entire microcosm volume in ~5 h, thus explaining the decrease in phytoplankton abundance of 99.2 ± 0.3% observed after 14 days. These results are in agreement with previous studies carried out under different experimental conditions reporting a decrease in phytoplankton biomass in the presence of *L. fortunei* (Sylvester et al., 2005; Cataldo et al., 2012a,b). In the interaction treatments (i.e. MR, MA and MG), agents acted synergistically producing large amount of nutrients derived from herbicides and their recycling by the action of mussels. The intense grazing pressure exerted by the mussel would have counteracted the increase in phytoplankton abundance produced mainly by glyphosate and Glifosato Atanor®. However, increased nutrient availability favored organisms such as periphyton, which cannot be predated because they are attached to a substrate and out of reach of mussels (Cataldo et al., 2012a; Lowe and Pillsbury, 1995). In our study, this group showed higher development in interaction treatments than in those with agents treated separately. Moreover, periphyton development was possibly related to a decrease in phytoplankton abundance which reduced competition for nutrients.

Although the three treatments with herbicides showed a significant growth of periphyton, it was highest in MR. Interestingly, Roundup Max® seemed to work in a different way from Glifosato Atanor® and technical-grade glyphosate: i) alone, it reduced the development of phytoplankton, mainly represented by *Microcystis* spp.; and ii) in interaction with *L. fortunei*, it stimulated the growth of periphyton to a higher extent than did the other two herbicides. These differences could be attributed to their different chemical compositions. A similar behavior was reported by Pizarro et al. (2015a), who found a profuse growth of filamentous algae forming metaphyton when *L. fortunei* interacted with glyphosate in outdoor mesocosms. Comparing their experiment with ours, the development of periphyton or metaphyton would be explained by differences in the characteristics of the experimental units (microcosms under controlled indoor *vs.* mesocosms under outdoor conditions) and in the source of the water used. Moreover, a possible effect of herbicide type on mussel filtration rate deserves further investigation.

Mussels not only affected the abundance but also the diversity of phytoplankton, which showed a trend toward higher evenness. As it was discussed for the zebra mussel *Dreissena polymorpha* another freshwater invader ecologically similar to *Limnoperna fortunei*, the phytoplankton composition may be altered both indirectly and directly by the activity of mussels (Bastviken et al., 1998). In our experiment, *L. fortunei* altered nutrient content in the water column that could favor the development of certain groups instead others or could remove phytoplankton at a rate that faster growing organisms become more abundant. Moreover, the golden mussel could directly remove phytoplankton selectively, including size preferences, as it was stated by Boltovskoy et al. (2015). Direct and indirect actions could interact, causing increased phytoplankton-specific diversity in treatments in which mussels were present. In addition, the MA treatment induced a great development of Bacillariophyceae probably due to mussel-herbicide interaction and to the chemical composition of Glifosato Atanor®. Nonetheless, phytoplankton diversity was similar in all interaction treatments revealing, once again, that the different agents of change induce complex responses in the aquatic communities.

The effects on water observed in the present work would clearly modify the trophic state of the system and since phytoplankton and periphyton form the basis of aquatic trophic networks, any change affecting these communities will be transferred to higher trophic levels with potential impacts on the structure and functioning of the entire ecosystem (Woodward, 2009; Woodward et al., 2010). From our results it is clear that the availability of nutrients in the medium is affected by the agents (*L. fortunei* and glyphosate: Roundup Max®, Glifosato Atanor®, glyphosate acid) either separately or in interaction with one another. *L. fortunei* appeared as the driving force in the interactions, thus confirming its extraordinary capacity as an ecosystem engineer (Karatayev et al., 2015).

We analyzed the impact of the joint effect of two anthropogenic stressors, glyphosate in different forms and *L. fortunei*, on the phytoplankton from Salto Grande Reservoir. In this sense, it should be highlighted that our results were obtained from microcosms under controlled laboratory conditions and may differ from those obtained under natural conditions due to different reasons. The variables related

to the communities in the experimental system were most likely affected by confinement, since in small water bodies there is more availability of nutrients (Pesce et al., 2009) and herbicides and mussels exert a higher pressure. Although herbicide concentrations and mussel density were selected and scaled proportionally to the volume of the microcosms to represent a realistic scenario, the experimental conditions were far from those of the Salto Grande Reservoir. Nevertheless, our results are of great value and worthy of being explored in more realistic situations. Certainly, manipulative studies using community assemblages allow us to approach reality in a more holistic way. There is a wide range of effects exerted directly on some populations and indirectly through changes in their interactions, producing profound alterations in the biological communities that may go undetected in monospecific trials (Relyea et al., 2005; Schäfer et al., 2011). In this regard, our experiment can be considered as a very rapid test for assessing the impact of this type of agents on freshwater at the ecosystem level.

Anthropogenic factors affecting the environment do not operate in isolation from each other, and their interactions tend to be highly complex. Understanding the joint effects of multiple change drivers is a major challenge to deploy political strategies to mitigate their harmful environmental effects. Salto Grande provides many important ecological services, including hydroelectric energy and recreational opportunities for local people. Predicting how the combination of the most widely used herbicide in the region and the successful invader will affect this reservoir may serve as a useful decision-making tool for its sustainable management.

5. Conclusions

The interaction of glyphosate, either as technical grade or as commercial formulations (Roundup Max® and Glifosato Atanor®) with *Limnoperna fortunei* produced different impacts on phytoplankton from Salto Grande Reservoir according to the type of herbicide. The growth of *Microcystis* spp., a bloom-forming cyanobacteria, was increased by technical-grade glyphosate and Glifosato Atanor®, and decreased by Roundup Max®. The invasive mussel emerged as the driving force in the interaction. *L. fortunei* not only affected the abundance but also the diversity of phytoplankton which showed a trend toward higher evenness.

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