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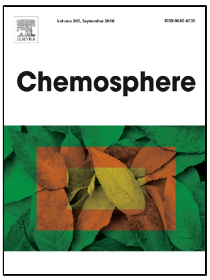

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Impact of interaction between *Limnoperna fortunei* and Roundup Max® on freshwater phytoplankton: an *in situ* approach in Salto Grande reservoir (Argentina)

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Highlights

- *Limnoperna fortunei* and Roundup Max® led to changes in phytoplankton and water quality.
- The interaction between stressors led to a decrease in algal biomass.

- Phytoplankton abundance showed a synergistic response in presence of both stressors.
- The interaction between *L. fortunei* and glyphosate led to a decrease in species diversity.
- Changes in biological variables involved the enhanced growth of a small opportunistic alga.

Abstract

The joint impact of the glyphosate-based commercial formulation Roundup Max® and the invasive mussel *Limnoperna fortunei* on phytoplankton and water quality was assessed in Salto Grande reservoir, a scenario were both stressors coexist. We performed an *in situ* mesocosm approach, through a 7-day experiment using 400-L enclosures. The following treatments were applied by triplicate: addition of 250 mussels (M); addition of 5 mg L^{-1} of active ingredient (a.i.) in Roundup Max® (R); addition of 250 mussels and 5 mg L^{-1} of a.i. in Roundup Max® (MR), and controls, without any addition (C). R showed higher total phosphorus (TP) and ammonium nitrogen ($N\text{-}NH_4^+$) concentrations due to the herbicide input, and a significant increase in algal abundance, biovolume and chlorophyll *a* levels (Chl-*a*). In M mussels grazed on phytoplankton, which resulted in subsequent phosphates (SRP) release. A decrease in species diversity was observed in R and M with respect to C. In MR, there were higher TP and $N\text{-}NH_4^+$ concentrations, a decrease in biovolume, an antagonistic effect on Chl-*a* and a synergistic effect on phytoplankton abundance. Species diversity and evenness showed a significant decrease due to the explosive growth of a small and opportunistic Chlorophyta, *Spermatozopsis exsultans*. The dominance of this species may be due to negative selectivity for *S. exsultans* and/or release of potential competitors by *L. fortunei*, and to the input of nutrients by Roundup Max® and/or removal of competitors by its toxicity.

Keywords

Glyphosate - *Limnoperna fortunei* - Roundup Max® - Invasive species - Mesocosms - Anthropogenic stressors

1. Introduction

Human population growth has led to agricultural intensification and the development of industries and urbanization-related technologies, resulting in a wide variety of environmental problems (Townsend, 2008). Overexploitation, habitat transformation, climate change, pollution and spread of alien species have been among the main direct anthropogenic drivers of ecosystem change over the last 50 years (Millenium Ecosystem Assessment, 2005). The effect of stressors may vary when applied individually or simultaneously. Indeed, the unpredictable impact of their joint action on the ecosystem has generated increasing interest in ecological research, particularly in the context of aquatic ecosystems (Crain et al., 2008; Townsend et al., 2008).

Argentina is among the countries producing the largest amount of genetically modified crops, which are currently cultivated on over 22 million hectares (Trigo, 2011). During 2013, about 240 million kg of herbicides (75% of which corresponded to glyphosate) were applied to control undesirable weeds mainly soybean, maize and cotton crops, and for chemical fallow (CASAFE, 2014). According to the National Service of Agri-Food Health and Quality Agency (SENASA, 2017) there are around 400 records of commercial formulations with glyphosate as active ingredient (a.i.) plus a mixture of substances (adjuvants) of unknown composition receiving "trade secret" protection. The adjuvants improve the penetration of the a.i. into the plants, thus increasing the efficiency of commercial formulations. Therefore, knowledge of the effects of commercial formulations is of paramount importance due to their massive use of in agriculture.

Many studies addressing the impact of glyphosate (a.i. and commercial formulations) on freshwater ecosystems have suggested toxic effects on non-target aquatic organisms and deterioration of water quality (Relyea et al., 2009; Pérez et al., 2007; Lipok et al., 2010; Vera et al., 2010; Avigliano et al., 2014; Pizarro et al., 2016a). Input of herbicides to aquatic systems may occur indirectly by wind-drift after spraying or by surface runoff (Feng et al., 1990; Peruzzo et al., 2008), or directly to control aquatic plants (Solomon and Thompson, 2003) or when washing tanks of fumigation equipment (Vera et al., 2010).

On the other hand, the golden mussel *Limnoperna fortunei,* native to Asian rivers (Morton, 1977), was transported into the Río de la Plata basin through ship ballast waters, the first record being in 1990 (Pastorino et al., 1993). More than 20 years later, this invasive bivalve dominates the benthic fauna of almost the whole Paraná-Plata watershed, reaching densities over 200000 individuals m^{-2} (Boltovskoy et al., 2006). The golden mussel has been characterized as an efficient "ecosystem engineer" due to its high filtering activity (Sylvester et al., 2005) and significant role in the recycling of nutrients (Cataldo et al., 2012a, Darrigran and Damborenea, 2011).

The joint study of anthropogenic stressors in freshwater ecosystems has acquired a general relevance in the last decade (Ormerod et al., 2010), and in regard to the combined effect of glyphosate and *L. fortunei* in particular, there is an increasing line of work motivated by the high resistance of this species to pollutants (Karatayev et al., 2007) and also to the potential ability of *L. fortunei* to modify their dynamics in water. Studies involving these two anthropogenic stressors have used both micro and mesocosm approaches under indoor and outdoor conditions. Di Fiori et al. (2012) demonstrated experimentally that *L. fortunei* participates in glyphosate dissipation in water. Outdoor mesocosm studies concerning the interaction between *L. fortunei* and glyphosate (a.i./commercial formulation) revealed synergistic and/or antagonistic effects on some variables of microbial communities (Pizarro et al., 2016b; Gattás et al., 2016). Moreover, De Stefano et al.

(2018) compared the interaction between *L. fortunei* and two glyphosate-based formulations using microcosms under laboratory conditions. To our knowledge, there are no field studies on the impact of the interaction between both stressors on freshwater microbial communities. *In situ* approaches provide realistic exposure scenarios which are difficult to replicate under laboratory conditions. They better represent situations that actually occur in the environment such as stressor interactions, and have the potential to incorporate indirect effects, mechanisms and system dynamics (Crane et al., 2007).

Salto Grande reservoir, located on the Uruguay River, Argentina, provides an interesting scenario for studying the *L. fortunei*-glyphosate interaction. In fact, the two stressors may coexist in this reservoir: *L. fortunei* was first recorded in Salto Grande in 2001 (Boltovskoy et al., 2006), and it is surrounded by crop fields where glyphosate-based herbicides are intensively used since the late 90s (Trigo, 2011). On this basis, the objective of the present study was to evaluate the joint effect of *L. fortunei* and the glyphosate-formulation Roundup Max® on water quality parameters and phytoplankton from an *in situ* mesocosm approach.

2. Materials and methods

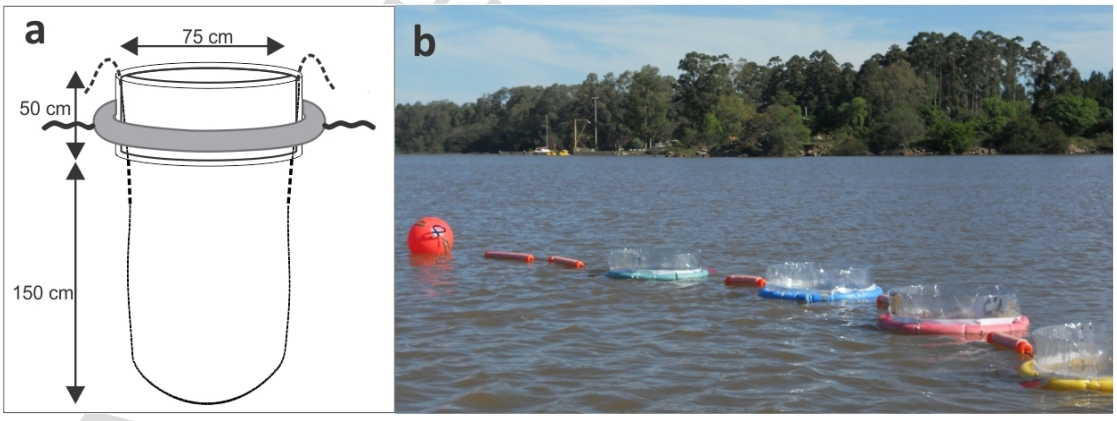
2.1. Study site

Salto Grande is a large reservoir (750 km^2) located on the Uruguay River (31.2° S, 57.9° W) (mean depth: 6.4 m; maximum depth: 35 m) (De León and Chalar, 2003). The construction of the reservoir was completed in 1979, commissioned by a binational Argentina and Uruguay organization named the "Joint Technical Commission for Salto Grande". It has a polymictic regime with short-lasting stratification during low flow period, and eutrophic characteristics (mean total phosphorus: 40 µg L^{-1}; mean chlorophyll *a*: 14.8 µg L^{-1}). There are recurrent cyanobacterial blooms

117 of *Microcystis* spp. and *Dolichospermum* spp. (O'Farrell et al., 2012). The reservoir provides a
118 variety of ecosystem goods and services at both local and regional scales, including hydropower
119 generation, fisheries, drinking water supply, navigation, irrigation and tourism.

120 *2.2. Field experiment*

121 Twelve 400-L mesocosms (experimental units) were placed in a bay (31.254681° S, 57.909536°
122 W) inside Salto Grande in October 2014. Each mesocosm consisted of a cylindrical polyethylene
123 bag (200 µm thick) suspended from a polyethylene terephthalate cylinder; a floating ring was
124 attached around the cylinder (**Fig. 1.a**). Three buoys were moored by one dead weight each, and a
125 rope attached to the top of each buoy linked four mesocosms to one another in a row, allowing
126 them to move along with the water flow (**Fig. 1.b**). The mesocosms were filled with reservoir
127 water, and treatments were applied 24 h after filling to stabilize the water system and minimize
128 the confinement effect. For an initial characterization of the mesocosms, measurements of water
129 pH, conductivity, temperature and dissolved oxygen were taken using field probes (HACH, Sension
130 156), and turbidity with a portable turbidimeter (2100P HACH).

131 

132 **Fig. 1.a.** Scheme of mesocosm, and **b.** Photograph of a set of mesocosms tied to a buoy in Salto Grande reservoir.

133

134 Each of the four mesocosms from each set was randomly assigned to one of the following
135 treatments: M, R, MR and C. Treatment M comprised 250 adult mussels placed in two submerged,

136 5-mm mesh plastic cages. Prior to the experiment, mussels had been collected manually from the
137 Delta of the Paraná River, kept in aerated aquaria with dechlorinated tap water and fed daily with
138 aquarium juvenile fish food. We used individuals 20 ± 1 mm in length that showed extended
139 siphons (as indicator of viability). Mussel density used lies within the range recorded for natural
140 water bodies (Boltovskoy et al., 2009) and is similar to those used in other field experiments
141 conducted in Salto Grande reservoir (Cataldo et al., 2012b). Treatment R consisted in the addition
142 of the commercial formulation Roundup Max® (74.7%: N-(phosphonomethyl) glycine
143 monoammonium salt, 25.3%: inert ingredients and adjuvants) at 5 mg L^{-1} of the a.i. This
144 concentration represents a "worst-case" scenario and is within the range recorded for natural
145 water bodies of the region after herbicide spraying (Ronco et al., 2008). In treatment MR mussels
146 enclosed as described above were exposed to Roundup Max® at 5 mg L^{-1} of the a.i. Treatment C
147 represented the controls (without mussels or commercial formulation). Water samples were
148 collected sub-superficially from each mesocosm after treatment application (day 0), on the next
149 day (day 1) and one week later (day 7), for the determination of glyphosate, total and dissolved
150 nutrients in water, and phytoplankton abundance, biovolume and chlorophyll *a.*

151 *2.3. Sample analyses*

152 The determination of glyphosate was performed by capillary electrophoresis, on a Beckman
153 P/ACE MDQ CE System (Beckman Coulter) with diode-array UV detection. Karat 8.0 Software
154 (Beckman Instruments) was used for data collection and processing. A fused-silica capillary
155 (diameter: 75 µm, total length: 57 cm, optical viewing window: 50 cm) was used at 25 ºC, and
156 conditioned with a running electrolyte (BGE) composed of 10 mM potassium biphthalate, 10 mM
157 TRIS, and 0.1 mM CTAB (pH 6.6) (adapted from Rojano-Delgado et al., 2010; Lanaro et al., 2015).
158 The stock standard solutions of glyphosate were prepared by dissolving 0.025 g of Glyphosate
159 Pestanal™ (CAS: 1071-83-6, Sigma-Aldrich, Buchs, Switzerland) in 50 mL of 18 milliohm Milli-Q

160 water and stored at -20 ºC. The standard working solutions were prepared by the dilution of the
161 stock solutions in Milli-Q water (after ruling out a possible effect of the sample matrix). Each
162 sample was filtered with a syringe through a 0.45-µm membrane into the injection vial, and then
163 transferred to the capillary by hydrodynamic injection (0.5 psi for 15 sec). The electrophoretic runs
164 were conducted under reverse polarity (-10 kV) and indirect detection (λ= 220 nm). Samples and
165 standard solutions were stored at 10 ºC in the sample tray during the electrophoretic runs. The
166 detection limit of the optimized procedure was 0.5 mg L^{-1}, and the limit of quantification was 1 mg
167 L^{-1}.

168 Filtered water samples (Whatman® GF/F filters) were used to determine the concentrations of
169 soluble reactive phosphorus (SRP) (ascorbic acid method) and ammonium nitrogen ($N\text{-}NH_4^+$)
170 (salicylate method) spectrophotometrically using reagent kits (HACH®), with a limit of detection of
171 0.001 mg L^{-1}, and a limit of quantification of 0.007 mg L^{-1} (SRP) and 0.02 mg L^{-1} ($N\text{-}NH_4^+$). Total
172 phosphorus (TP) was determined in unfiltered water samples after digestion with potassium
173 persulfate (Valderrama, 1981), and following the same methods as for SRP.

174 For phytoplankton quantification, water samples were preserved with 1% acidified Lugol's
175 solution. Counts were carried out following Utermöhl (1958), and the counting error (<20% for the
176 most abundant species) was estimated according to Venrick (1978). Biovolumes were estimated
177 following Rott (1981) and Hillebrand et al. (1999). To determine phytoplankton chlorophyll *a*
178 concentrations (Chl-*a*), water samples were filtered through Whatman® GF/F filters and stored at -
179 20 ºC. The pigment was extracted with hot ethanol (60-70 ºC) and spectrophotometrically
180 measured before and after acidification (HCl 1N). Final Chl-*a* was calculated according to Marker et
181 al. (1980).

182 Species diversity was calculated using Shannon-Wiener Index (Shannon and Weaver, 1963),
183 defined as $H' = -1\Sigma\ (p_i \ln p_i)$, where p_i is the relative abundance of each species or genera. Evenness

(E) was estimated as E=H´/ln R, where R is the number of species or genera. Grazing impact by *L. fortunei* on algae was calculated based on the formula modified from Cataldo et al. (2012a) as GI= B_i-B_f, where B_i and B_f are the percentages of the biovolume of each species or genus relative to the total biovolume on day 0 and 7, respectively. The mean GI was obtained from the three replicates of M and MR. Only taxa representing >0.5% of the total biovolume were included in the analysis.

2.4. Numerical and statistical analyses

Homoscedasticity and normality of the variables were tested with residual analysis. The differences between *treatments* (resulting from the crossing of the factors *mussels* and *Roundup Max®)* were analyzed with Mixed Linear Models for repeated measures in *time* (RM ANOVA), modeling the covariance matrix and heteroscedasticity (Di Rienzo et al., 2017). Akaike's criterion was used to select the most parsimonious models. Comparisons of the interaction between *time* and *treatments* and between *treatments* and *time* were performed with the DGC and Fisher's LSD tests.

Principal Component Analysis (PCA) was applied to the differences between final and initial biovolumes for the most representative species (>0.5% of the total biovolume), to assess the changes in species biovolume. We decided to employ this descriptive analysis considering the variability in some species biovolume registered, typical of outdoor experiments approach at community scale. The significance of the association between algal biovolume and GI by *L. fortunei* was evaluated by Pearson correlation coefficient for treatments M and MR.

The analysis of possible interactions between *mussels* and *Roundup Max®* were assessed by testing the null hypothesis of additivity of these stressors, incorporating the directions of their individual effects (Piggott et al., 2015). The significance of these interactions was performed using two-way ANOVA on the data of the response variables at day 7. For the interaction treatment, we calculated the expected values and their 95% confidence intervals (95 CI) for an additive effect to

208 compare them with the observed values and their 95 CI. These values and their respective CI were
209 relativized to -1 and 1 with respect to expected values (Lozano et al., 2018). When the interaction
210 is significant, it is synergistic if the observed joint effect is greater than that expected by additivity
211 and antagonistic if it is less than expected. All the statistical analyses were performed with
212 InfoStat® (2017), and its interface to R (R 3.3.1, R Core Team 2016).

213

214 **3. Results**

215 *3.1. Physical and chemical parameters of water*

216 The initial characteristics of the water in the mesocosms were: pH: 6.03 ± 0.35; conductivity:
217 48.34 ± 1.97 µS cm^{-1}; temperature: 23.40 ± 0.44 ºC; dissolved oxygen: 6.04 ± 0.63 mg L^{-1}; turbidity:
218 30.25 ± 0.75 NTU. Initial mean glyphosate concentration was 4.85 ± 0.11 mg L^{-1} in the treatments
219 with Roundup Max®, without significant variations throughout the experiment (R: 4.33 ± 0.44 mg
220 L^{-1}; MR: 4.21 ± 0.17 mg L^{-1}); no detectable glyphosate levels were detected in C and M.

221 At day 0, SRP concentration did not show significant differences between treatments (**Table**
222 **1**). No significant interaction was found between *time* and *treatments*, but the effect of *time* on
223 SRP concentration was significant (RM ANOVA, p= 0.004), with differences on days 1 and 7 with
224 respect to day 0 (DGC multiple comparisons, p< 0.05). In M, SRP concentration at day 7 increased
225 about 10 times compared to the initial SRP concentration.

226 For N-NH_4^+ concentration there was a significant interaction between *time* and *treatments*
227 (RM ANOVA, p< 0.0001). N-NH_4^+ concentration was significantly higher in R and MR than in M and
228 C at day 0 (**Table 1**). Although there was a decrease in N-NH_4^+ concentration in all treatments at
229 day 1, the difference observed at day 0 was also significant, and at day 7 values dropped to near
230 zero.

231 The concentrations of TP were 6 times higher in treatments with Roundup Max® than in those
232 without the formulation from day 0 to day 7 (**Table 1**). No significant interaction was found
233 between *time* and *treatments*, but the effect of *treatments* was significant (RM ANOVA, $p<$
234 0.0001).

235

Variables	Day	Treatments				Effect		
		C	M	R	MR	*Treatments*	*Time*	*Interaction*
SRP (mg L^{-1})	**0**	0.04 ± 0.01	0.04 ± 0.002	0.05 ± 0.002	0.06 ± 0.01	0.399 (3)	0.004 (2)	0.579 (6)
	1	0.11 ± 0.02	0.15 ± 0.05	0.13 ± 0.02	0.24 ± 0.07			
	7	0.20 ± 0.09	0.48 ± 0.24	0.10 ± 0.05	0.15 ± 0.01			
$N\text{-}NH_4^+$ (mg L^{-1})	**0**	0.12 ± 0.01	0.14 ± 0.01	0.47 ± 0.02	0.56 ± 0.05	<0.0001 (3)	<0.0001 (2)	<0.0001 (6)
	1	0.01 ± 0.01	b.d.l.	0.15 ± 0.003	0.15 ± 0.01			
	7	0.003 ± 0.003	b.d.l.	0.01 ± 0.01	0.003 ± 0.003			
TP (mg L^{-1})	**0**	0.23 ± 0.08	0.15 ± 0.04	1.63 ± 0.14	1.53 ± 0.07	<0.0001 (3)	0.468 (2)	0.487 (6)
	1	0.37 ± 0.02	0.29 ± 0.08	1.62 ± 0.05	1.50 ± 0.16			
	7	0.21 ± 0.05	0.19 ± 0.03	1.78 ± 0.13	1.65 ± 0.04			

236

237 **Table 1.** Mean values (± SE) of SRP, $N\text{-}NH_4^+$ and TP concentrations recorded at the beginning of the experiment (day 0) and at
238 days 1 and 7, and summary of *p*-values (RM ANOVA) for the effect of *treatments*, *time* and the interaction between *time* and
239 *treatments* (dF between brackets). C: Control, M: Mussels, R: Roundup Max®, MR: Mussels+Roundup Max®. b.d.l.: below
240 detection level,

241

242 *3.2. Phytoplankton*

243 Initial mean abundances of phytoplankton were 2119 ± 1080 cells mL^{-1} (**Fig. 2.a**). At day 1, both
244 M and MR showed a decrease in phytoplankton abundance with respect to initial values without
245 statistical significance (M: 512 ± 194 cells mL^{-1}; MR: 754 ± 134 cells mL^{-1}). There was a significant
246 interaction between *time* and *treatments* (RM ANOVA, $p<$ 0.0001). At day 7, abundance increased

247 significantly in C (9342 ± 4686 cells mL^{-1}), R (18829 ± 764 cells mL^{-1}) and MR (38661 ± 12484 cells
248 mL^{-1}) with respect to initial values (DGC comparisons, $p< 0.05$), while M did not show significant
249 changes (1995 ± 298 cells mL^{-1}).

250 Algal biovolume and Chl-*a* exhibited different patterns from those observed for total
251 abundances. At day 0 treatments showed similar biovolume (mean: $1.23 \times 10^6 \pm 7.48 \times 10^5$ μm^3 mL^{-1})
252 (**Fig. 2.b**). There was a significant interaction between *time* and *treatments* (RM ANOVA, $p= 0.011$).
253 At day 7, the biovolume in R was over 10 times higher than the initial value, showing
254 significant differences with the rest of the treatments (DGC comparisons, $p< 0.05$), while
255 biovolume values in M and MR were half or less than half those in C (DGC comparisons, $p< 0.05$).

256 Mean initial Chl-*a* concentration in all treatments was 3.02 ± 1.45 µg L^{-1}, with no statistical
257 differences between them (**Fig. 2.c**). There was a significant interaction between *time* and
258 *treatments* (RM ANOVA, $p= 0.001$). At day 7, Chl-*a* concentration in R was over 10 times higher
259 (42.09 ± 10.26 µg L^{-1}) than the initial value (DGC comparisons, $p< 0.05$). Final Chl-*a* concentration
260 in C, M and MR differed significantly from that in R (C: 7.98 ± 1.03 µg L^{-1}; M: 7.26 ± 1.45 µg L^{-1}; MR:
261 10.26 ± 1.45 µg L^{-1}).

262 **Figure 2.a.** Mean phytoplankton abundance (cells mL^{-1}), **b.** algal biovolume (μm^3 mL^{-1}), and **c.** Chl-*a* concentration (µg L^{-1})
263 recorded in mesocosms at the beginning of the experiment (day 0) and at days 1 and 7. Different lowercase letters
264 represent significant differences between *treatments* and over *time.* C: Control, M: Mussels, R: Roundup Max®, MR:
265 Mussels+Roundup Max®. Bars: ± 1SE.

266

267 The phytoplankton groups recorded were: Chlorophyta, Bacillariophyceae, Cyanobacteria,
268 Cryptophyceae, Chrysophyceae, Dinophyceae and Euglenophyceae. The differences between their
269 final and initial biovolumes and percentages based on total biovolume for each treatment are
270 shown in **Fig. 3**. The interaction of both stressors had no significant effect on the differences

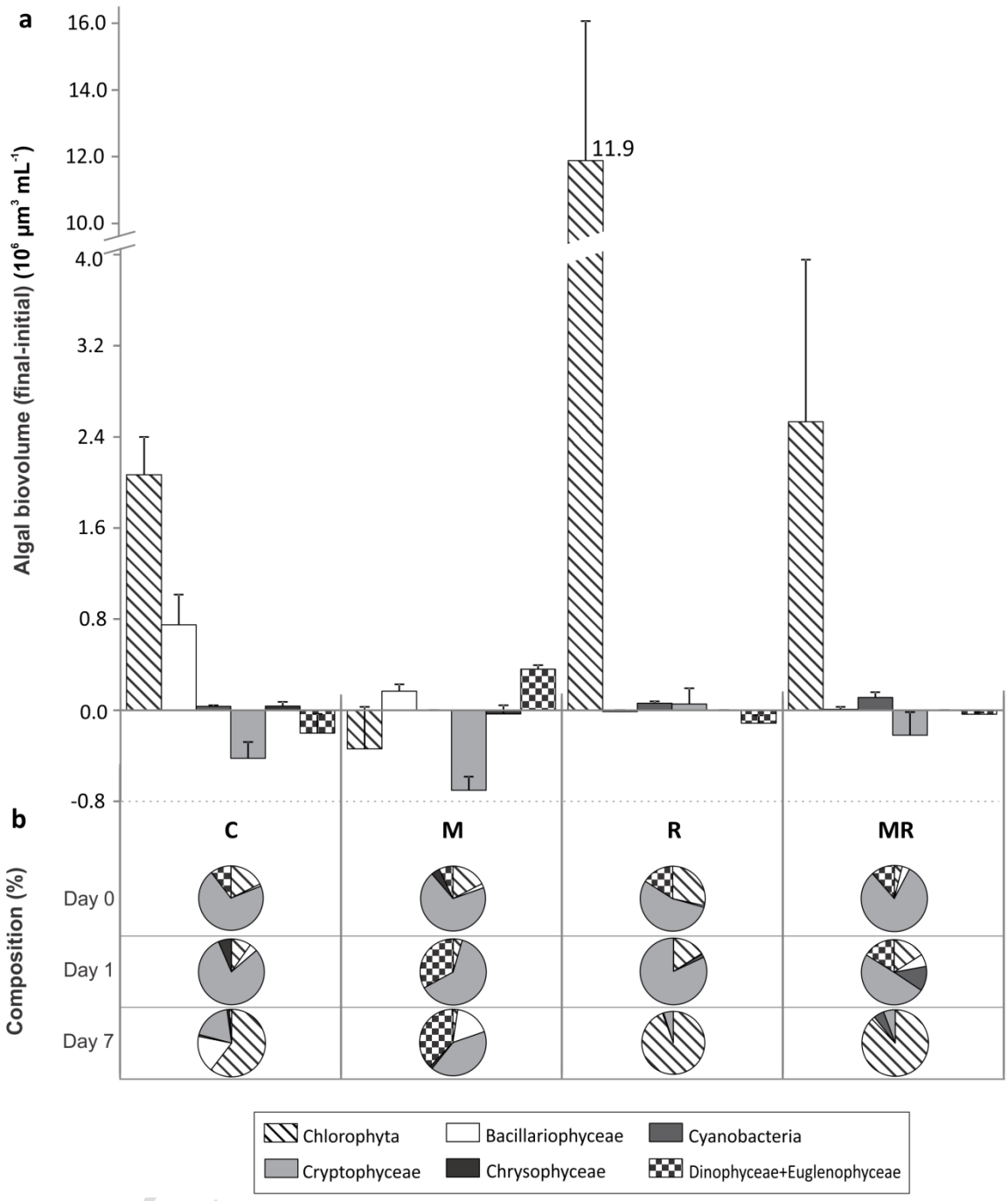
between final and initial biovolumes of each algal group. However, a significant effect of each individual stressor was recorded on Chlorophyta biovolume, which decreased in M (two-way ANOVA, p= 0.018) and increased in R (p= 0.013) (**Fig. 3.a**). *Roundup Max®* had a significant effect on Bacillariophyceae and Cryptophyceae (two-way ANOVA, p= 0.037 and p= 0.034, respectively), while *mussels* showed a significant effect on Dinophyceae+Euglenophyceae (two-way ANOVA, p= 0.030). Stressors exerted no significant effect on Cyanobacteria and Chrysophyceae.

At day 0, treatments presented a similar algal composition with over 55% of the total biovolume represented by Cryptophyceae, mainly species of the genera *Cryptomonas* (772709 ± 478726 $\mu m^3\ mL^{-1}$) and *Plagioselmis* (44770 ± 21172 $\mu m^3\ mL^{-1}$) (**Fig. 3.b**). Chlorophyta biovolume ranged between 4 and 28% of the total biovolume, mainly represented by *Pandorina morum* (148296 ± 314246 $\mu m^3\ mL^{-1}$), *Coenochloris* spp. (48532 ± 153473 $\mu m^3\ mL^{-1}$), *Micractinium pusillum* (25265 ± 60271 $\mu m^3\ mL^{-1}$), *Ankistrodesmus* spp. (13333 ± 20175 $\mu m^3\ mL^{-1}$) and *Chlamydomonas* spp. (10743 ± 16092 $\mu m^3\ mL^{-1}$). Dinophyceae (*Peridinium* spp.: 51351 ± 71828 $\mu m^3\ mL^{-1}$) and Euglenophyceae (*Euglena* spp.: 73513 ± 154980 $\mu m^3\ mL^{-1}$) corresponded to about 7 and 16% of the total biovolume. Chrysophyceae, Bacillariophyceae and Cyanobacteria represented in average less than 2% of the total biovolume.

At day 1, although Cryptophyceae persisted as the dominant group in all the treatments in terms of biovolume (more than 50% of the total biovolume), there were changes in M, with Dinophyceae and Euglenophyceae representing 33% of the biovolume.

At day 7, there were changes in the percentage of biovolume of the different phytoplanktonic groups in C, M, R and MR. In C, algal composition shifted toward the dominance of Chlorophyta species, representing about 60% of the total biovolume. The main species were *Micractinium pusillum* (mean biovolume: 840314 ± 506780 $\mu m^3\ mL^{-1}$), *Coenochloris* spp. (485324 ± 840606 $\mu m^3\ mL^{-1}$), *Ankistrodesmus gracilis* (312312 ± 90689 $\mu m^3\ mL^{-1}$), *A. fusiformis* (279279 ± 298931 $\mu m^3\ mL^{-}$

295 ^{1}), *Dictyosphaerium pulchellum* (172396 ± 127887 µm^3 mL^{-1}) and *Coelastrum microporum* (133841
296 ± 119405 µm^3 mL^{-1}). The percentage of Cryptophyceae decreased with respect to initial values,
297 representing 19% of the final biovolume (*Cryptomonas ovata:* 443243 ± 191930 µm^3 mL^{-1};
298 *Cryptomonas marssonii*: 334624 ± 368828 µm^3 mL^{-1}), while there was an increase in the
299 percentage of Bacillariophyceae, which constituted 18% of the final biovolume (*Fragilaria ulna*:
300 470828 ± 420046 µm^3 mL^{-1}; *Cyclotella meneghiniana*: 103087 ± 178552 µm^3 mL^{-1}). The percentage
301 of Chlorophyta increased at the end of the experiment in both R and MR, which represented more
302 than 85% of the total biovolume. In R, the species that contributed most to the total biovolume
303 were *Micractinium pusillum* (4737157 ± 430952 µm^3 mL^{-1}), *Coenochloris* spp. (3639929 ± 5147637
304 µm^3 mL^{-1}), *Ankistrodesmus gracilis* (1914413 ± 859994 µm^3 mL^{-1}), *Dictyosphaerium pulchellum*
305 (762522 ± 515742 µm^3 mL^{-1}), and *A. fusiformis* (720720 ± 318516 µm^3 mL^{-1}). In MR, *Micractinium*
306 *pusillum* (1714249 ± 2373660 µm^3 mL^{-1}) and *Spermatozopsis exsultans* (760439 ± 274834 µm^3 mL^{-}
307 ^{1}) contributed most to the total biovolume; however, *S. exsultans* was the most abundant species
308 (*S. exsultans* abundance: 31685 ± 11452 cells mL^{-1}; *M. pusillum* abundance: 2787 ± 3860 cells mL^{-}
309 ^{1}). In R and MR there was a decrease in the percentage of Cryptophyceae with respect to initial
310 values, representing 5 and 6% of the final biovolume respectively. In M, Cryptophyceae comprised
311 41% of the final biovolume (*Plagioselmis* spp. and *Cryptomonas* spp.), followed by 38% of
312 Dinophyceae and Euglenophyceae, and 17% of Bacillariophyceae. Species of Chlorophyta
313 comprised only about 2% of the total biovolume.



314

315 **Fig. 3.a.** Differences in the biovolume (µm^3 mL^{-1}) of the phytoplankton groups between the values obtained at the end
316 (day 7) and beginning (day 0) of the experiment for each treatment. Bars: ± 1SE. **b.** Average percentage of the biovolume
317 for each phytoplankton group with respect to the total biovolume of each treatment, on days 0, 1, and 7. C: Control, M:
318 Mussels, R: Roundup Max®, MR: Mussels+Roundup Max®.

319

Initially, all treatments had similar values of the Shannon-Wiener Index (H') (mean: 1.98 ± 0.08), with no significant differences among them. There was a significant interaction between *time* and *treatments* (RM ANOVA, p= 0.0002). At day 1, H' decreased (but not significantly) in M (1.52 ± 0.01), R (1.80 ± 0.04) and MR (1.47 ± 0.20). At day 7, H' increased significantly in C (2.38 ± 0.05) and decreased significantly in MR (0.62 ± 0.15); these values differed significantly from those in M (1.69 ± 0.32) and R (1.90 ± 0.07) (DGC comparisons, p< 0.05). Evenness showed a similar pattern as H', with a mean initial index of 0.72 ± 0.01; it decreased to 50% at day 7 in MR (0.35 ± 0.10), differing significantly from C (0.73 ± 0.02), M (0.69 ± 0.03) and R (0.69 ± 0.03) (DGC comparisons, p< 0.05).

Figure 4 shows the biplot of the first two PCA axes where treatments C, M, R and MR were ordered according to changes in the biovolume of the different algal species. PC1 accounts for 93.0% of the total variance, with the greatest contribution made by changes in biovolume of *Micractinium pusillum* and *Coenochloris* spp., positively and strongly associated with the first axis (correlation= 0.98 for both species). PC2 accounts for 5.7% of the remaining variance, with a major contribution by changes in biovolume of *Spermatozopsis exsultans*, followed by *M. pusillum* and *Coenochloris* spp. *S. exsultans* shows the strongest correlation with PC2 (correlation= 0.99). PC1 clearly separates R from C, M and MR. In R, the mean biovolume difference of *M. pusillum* (4703914 $\mu m^3\ mL^{-1}$) and *Coenochloris* spp. (3397267 $\mu m^3\ mL^{-1}$) is higher than in C, M and MR. PC2 separates MR from the rest of the treatments because of a larger increase in the mean biovolume difference of *S. exsultans* (758969 $\mu m^3\ mL^{-1}$).

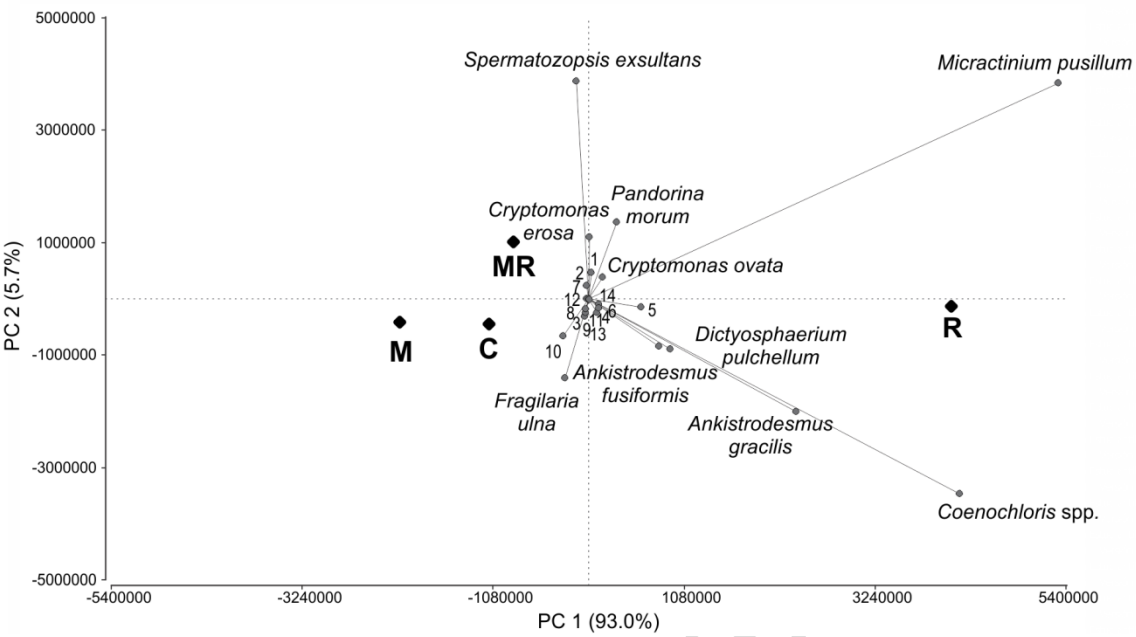


Fig. 4. Biplot of the Principal Component Analysis (PCA) of the difference between the final and initial algal biovolumes. Species were ordered decreasingly in terms of absolute values of changes in biovolume. The first ten species are identified by their names in the biplot and the others by the following numbers: 1: *Romeria* spp., 2: *Euglena* spp., 3: *Coelastrum microporum*, 4: *Scenedesmus opoliensis*, 5: *Cryptomonas marssonii*, 6: *Scenedesmus quadricauda*, 7: *Plagioselmis lacustris*, 8: *Fragilaria* spp., 9: *Cyclotella meneghiniana*, 10: *Peridinium* spp., 11: *Monoraphidium contortum*, 12: *Plagioselmis nannoplanctonica*, 13: *Chlamydomonas* spp., 14: *Mallomonas* spp. C: Control, M: Mussels, R: Roundup Max®, MR: Mussels+Roundup Max®.

No correlation was found between GI by *L. fortunei* and algal biovolume in M (r= 0.31, *p*= 0.18), while correlation was significant but weak in MR (r= 0.47, *p*= 0.04). Although algal biovolume decrease (by consumption, mainly) revealed no clear trend in any particular direction, some species showed extreme positive or negative GI values (**Table 2**).

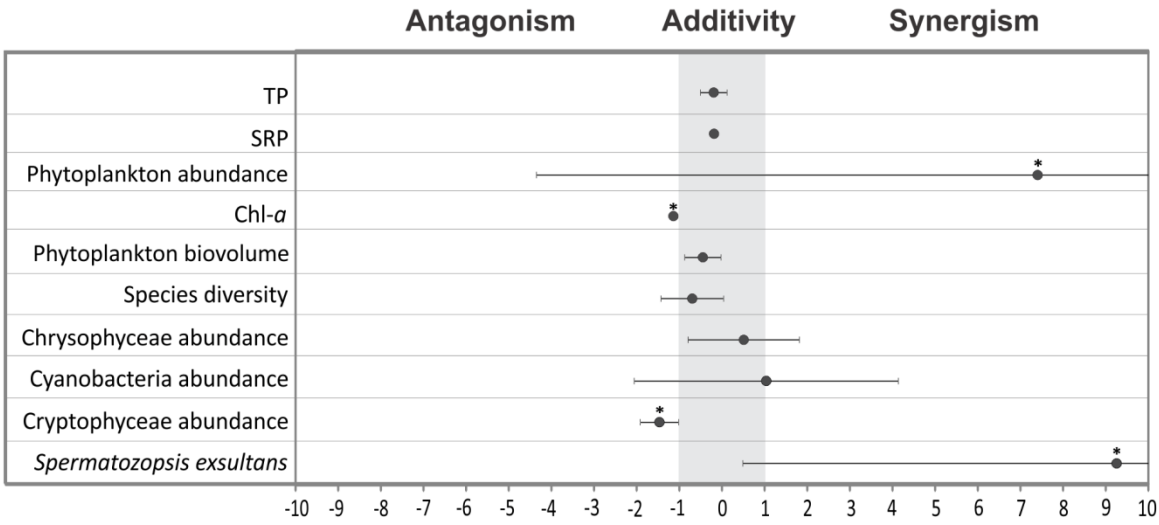
Species or genera	Biovolume (µm³)	Grazing Impact (%)		
		M	MR	C
Spermatozopsis exsultans	24	0.1	-41.6	-0.3
Micractinium pusillum	615	X	-43.2	-17.3
Cryptomonas erosa	819	-2.6	18.3	15.2
Cryptomonas marssonii	1013	5.0	18.2	16.5
Gomphonema spp.	1369	-5.9	X	-0.3
Cryptomonas ovata	3075	27.7	32.0	17.1
Peridinium spp.	5000	-18.4	17.1	-1.1
Fragilaria ulna	7466	-12.6	X	-10.4
Euglena spp.	17000	-24.5	X	10.4
Pandorina morum	37411	31.1	X	11.8

Table 2. Grazing impact (GI) of *L. fortunei* on phytoplankton for treatments M (Mussels) and MR (Mussels+Roundup Max®). The table shows the most extreme positive and negative values (GI >5% for at least one of the treatments). X: species or genera absent. The column of treatment C (Control) shows the mean percentage of the difference in biovolume of the species or genus between day 0 and 7 relative to the total biovolume in C.

3.3. Analysis of the interaction between stressors

The analysis of the impact of the interaction between *mussels* and *Roundup Max®* on biological, physical and chemical variables are shown in **Fig. 5**. Physical and chemical variables did not show significant interactions; in regard to phytoplankton variables, abundance showed a significant interaction between *mussels* and *Roundup Max®* (two-way ANOVA, p= 0.034), inducing a synergistic response in MR. In contrast, the interaction between *mussels* and *Roundup Max®* produced an antagonistic response in Chl-*a* (two-way ANOVA, p= 0.002). The analysis was performed for the abundance of the four most representative groups of phytoplankton at day 7. *Spermatozopsis exsultans* represents Chlorophyta, because it was the most abundant species and better reflected the interaction of stressors on green algae (which could not be included within the scale of **Fig. 5**). Both stressors acted synergistically on *S. exsultans* abundance (two-way ANOVA, p= 0.013), and also on total abundance of Chlorophyta (two-way ANOVA, p= 0.021). The

374 interaction between stressors had an antagonistic effect on Cryptophyceae (two-way ANOVA, *p*=
375 0.017).



376

377 **Fig. 5.** Values of each response variable recorded in the MR (Mussels+Roundup Max®), relative to those expected for
378 additive effects. The horizontal lines represent the 95 CI. Additivity values were relativized to -1 and 1. Circle with
379 asterisk indicates significant interaction. Circles located to the right of the gray zone (additivity) represent synergism and
380 those to the left represent antagonism.

381

382 **4. Discussion**

383 Studies on the joint effect of glyphosate and *Limnoperna fortunei* in water have been limited
384 to assays involving laboratory microcosms and outdoor mesocosms. This study is the first one to
385 assess the impact of both stressors *in situ* in Salto Grande reservoir. Interestingly, our results
386 differed from those obtained in other experimental approaches, reporting that the interaction
387 between Roundup Max® and *L. fortunei* decreases algal biomass (biovolume and Chl-*a*) and
388 species diversity, but exerts a synergistic effect on phytoplankton abundance. These results differ
389 with those obtained in laboratory studies using water from Salto Grande reservoir (De Stefano et
390 al., 2018), due to the growth of a small opportunistic species. Previous studies conducted in

391 mesocosms (Pizarro et al., 2016b; Gattás et al., 2016) and microcosms (Di Fiori et al., 2012)
392 reported notorious effects of both stressors already at day 1. In contrast, our study, involving
393 larger volumes of water, showed no acute effects, but there were relevant changes after seven
394 days. According to Peck (2011), changes in microbial communities are observed in a timescale from
395 minutes to days, due to rapid duplication times, as reported for many phytoplankton taxa, varying
396 between 0.19 and 3.95 divisions day $^{-1}$ (Tang et al., 1995; Reynolds, 1988).

397 Roundup Max® mainly affected the parameters of water quality, with higher TP concentration
398 in water from the beginning of the experience. Previous studies (Pérez et al., 2007; Vera et al.,
399 2010; Pizarro et al., 2016a,b) reported that the contribution of phosphorus to the water by the a.i.
400 in the commercial formulation is 14% of glyphosate molecular weight. There were higher N-NH_4^+
401 levels, as already recorded by Gattás et al. (2016) and De Stefano et al. (2018), supporting the
402 concept that the input of adjuvants and monoammonium salt of glyphosate from Roundup Max®
403 increases ammonium concentration. N-NH_4^+ concentration dropped sharply at day 7, which may
404 be attributed to phytoplankton uptake. Indeed, we observed a significant increase in algal
405 abundance and biovolume, with Chl-*a* exceeding 40 µg L^{-1} in R at the end of the experiment;
406 according to Wetzel (2001) these levels of Chl-*a* are characteristic of eutrophic conditions. In
407 addition, there were changes in algal composition and a lower specific diversity compared to C,
408 which may be associated with the input of nutrients from Roundup Max® and/or with the toxic
409 effect of the a.i. and adjuvants. Glyphosate may potentially have both positive and negative
410 influences on phytoplankton community structure because it acts either as a nutrient source for
411 tolerant or as a killer for non-tolerant microorganisms (Saxton et al., 2011). If certain species are
412 favored by increased nutrient input over others, the system would show lower evenness and/or
413 richness. Toxicity may cause the loss of species, resulting in lower species richness. In R, evenness
414 showed no significant differences but richness decreased 1.6 times compared to C. Although this

supports a possible toxic effect of Roundup Max® on some species, we cannot rule out a stimulating effect on others, as indicated by the increasing trend in the abundance of phytoplankton of larger biovolume in R (see below).

In M, heavy grazing of *L. fortunei* on phytoplankton and subsequent release of nutrients may explain the decrease in phytoplankton abundance and in Chl-*a* at day 1 and increased SRP concentration at day 7 (only significant for Chl-*a*). In addition, the decrease in specific diversity with respect to C was possibly related to lower richness (<50% that in C), considering that evenness remained unaffected. *In situ* studies using similar mesocosms (Cataldo et al., 2012a) showed that *L. fortunei* actively grazes on phytoplankton, leading to an abrupt decrease in algal numbers and to an increase in SRP concentration over a 24-h period. In agreement with our study, these authors found no association between algal species consumed by *L. fortunei* and cell biovolume in a range between 5 and 280.6 μm^3. Although mussels showed no directional grazing for any particular algal size, they significantly decreased the biovolume (and abundance) of Chlorophyta.

The joint effect of both anthropogenic stressors caused changes on chemical variables (TP and $N\text{-}NH_4^+$ concentrations) and on phytoplankton. Although *L. fortunei* is known to degrade glyphosate in water (Di Fiori et al., 2012), it did not decrease significantly at the end of our study. Taking into account the decrease in glyphosate observed in previous mesocosm experiments involving the simultaneous presence of Roundup Max® and *L. fortunei* (Gattás et al. 2016), and the volume and density of mussels used in our experiment, one might expect a reduction in glyphosate of about 0.007 mg L^{-1} after a 7-day period, which is far below the limit of detection of the method applied.

When Roundup Max® was applied alone, the higher levels of nutrients led to an increase in algal abundance, biovolume and Chl-*a*. In MR, the two latter effects were counteracted by the

439 filtration activity of mussels while their grazing effect on algal abundance was diminished by other
440 processes (see below). The combination of both agents elicited an antagonistic effect on Chl-*a* and
441 an additive effect on biovolume, with a reduction of 97% and 78% in MR with respect to R,
442 respectively. The interaction of *L. fortunei* and Roundup Max® had a synergistic effect on
443 phytoplankton abundance different from that reported by De Stefano et al. (2018), in which
444 mussels played an important role in decreasing total phytoplankton abundance.

445 The differences observed between phytoplankton biomass and abundance in MR are most
446 likely due to the enhanced growth of *Spermatozopsis exsultans*, which is a small (length: 8-11 µm,
447 width: 1.5-3 µm) and quadriflagellate Chlorophyta (Melkonian et al., 1986). Its final abundance in
448 MR was more than ten times higher than that in R and accounted for the higher final total
449 abundance in MR than in the rest of the treatments, which is in contrast to what is expected for
450 algae exposed to glyphosate, since they possess the shikimic acid pathway (Schönbrunn et al.,
451 2001). Such increase in phytoplankton abundance has not been observed in previous mesocosm
452 and microcosm studies (Pizarro et al., 2016b; Gattás et al., 2016; De Stefano et al., 2018).
453 However, Cataldo et al. (2012b) reported an increase in the abundance of one species in
454 particular, *Microcystis* spp., in presence of *L. fortunei*. The PCA revealed a greater difference
455 between the final and initial biovolumes of *S. exsultans* in MR, and the interaction analysis of the
456 abundance of this species points to a synergistic effect. These results indicate that there was an
457 increase in the abundance of phytoplankton, which was mainly represented by tiny algae making
458 only a small contribution to the total biovolume.

459 Initial species diversity was within the range reported by De León and Chalar (2003) for the
460 reservoir (H': 0.5-4.1 bits ind^{-1}). At the end of the experiment, however, MR showed a significant
461 decrease in diversity, evenness and richness with respect to the rest of the treatments, suggesting

462 that the combination of both stressors homogenized the phytoplankton community (i.e., with one
463 dominant species), as expected for stressed ecosystems (Schindler, 1988).

464 Nutrient enrichment may favor large algae over small ones (Stockner, 1988; Masson et al.,
465 2000). Our results may be explained by two potential scenarios: 1) Roundup Max® alone induced
466 higher nutrient levels which enhanced algal biovolume; and 2) the interaction between this
467 herbicide and mussels favored small algae, with the cell biovolume of *S. exsultans* being the lowest
468 among the species recorded in this experiment (24 μm^3). According to Malone (1980) and
469 Cottingham (1999), smaller phytoplankters would outcompete larger ones under limiting nutrient
470 conditions, while larger organisms would outcompete smaller ones under heavy grazing
471 conditions. When stressors were applied individually, the results of algal biovolume were
472 consistent with those predicted by these authors, and when they were applied together this
473 variable followed the pattern observed in M.

474 The addition of Roundup Max® and the high filtering activity of *L. fortunei* may have favored
475 the dominance of *S. exsultans* through different processes. One possible mechanism could be a
476 negative selectivity of *L. fortunei* over *S. exsultans*. The GI analysis suggests that mussels may
477 consume phytoplankton regardless of size, but the extreme negative GI in MR may indicate that *L.*
478 *fortunei* did not consume *S. exsultans*. This possibility is reinforced by the positive but very low GI
479 values obtained in M for this species. Although these results may be explained by the small size of
480 *S. exsultans*, other studies indicate that *L. fortunei* can graze on picoplankton, comprising smaller
481 organisms than *S. exsultans* (Gattás et al., 2016). Interestingly, the invader *Dreissena polymorpha,*
482 which shares many ecological traits with *L. fortunei,* is capable of filtering 1-4 µm bacteria
483 (Silverman et al., 1995). On the other hand, *L. fortunei* has shown a negative selectivity for
484 flagellates like Cryptophyta (Rojas Molina et al., 2010), suggesting that the four flagella of *S.*
485 *exsultans* could be a hindrance to filtration. In our study, the most positive values of GI were

obtained for *Cryptomonas* spp. in MR and the abundance of Cryptophyceae exhibited an antagonistic response to both stressors in combination. The dominance of a *S. exsultans* could also be explained by its ability to better exploit nutrients. In line with this notion, studies carried out in the Uruguay River have reported *S. exsultans* as an opportunistic nanoflagellate abundant in autumn, winter and spring (O'Farrell and Izaguirre, 2014). Moreover, this species has been placed in codon X2 (Padisák et al., 2009) described as a functional group typical in meso-eutrophic environments (Reynolds et al., 2002), in agreement with the final trophic status in the mesocosms used for the MR treatment.

Other processes that may account for *S. exsultans* dominance are related to release of competition resulting from grazing activity of mussels or from Roundup Max® toxicity. On one hand, although mussels did not select any particular algal group, they exerted a decreasing effect on Chlorophyta biovolume and a positive grazing over certain Cryptophyceae, thus removing *S. exsultans* competitors. On the other hand, differential susceptibility of algal species to the adjuvants or the a.i. of Roundup Max® could have led to a shift in species composition (Brock et al., 2000). The application of Roundup Max® induced an increase in the biovolume of Chlorophyta and Cryptophyceae throughout the experiment, but the latter showed a decreasing trend in abundance, as this group was represented by species of larger biovolumes at the end of the experiment.

We performed a field manipulative experiment using 400-L mesocosms, which represent valuable tools for linking experimental reproducibility and ecological realism (Brock et al., 2000), and for addressing single and multiple-factor effects on microscopic communities and water quality (Caquet et al., 2000). The results of our study are consistent with previous results from outdoor mesocosms and indoor microcosms (Pizarro et al., 2016b; Gattás et al., 2016; De Stefano et al., 2018) concerning the effects of the interaction *L. fortunei* and glyphosate on water chemical

parameters and algal biomass. These are smaller-scale approximations used to assess the impacts of multiple stressors. However, the results from our field approach differ from those under laboratory conditions (De Stefano et al., 2018) in that the leading role of mussels in mitigating the impact of glyphosate on phytoplankton abundance was appeased. This highlights the importance of assessing numerous biological variables in these types of studies to gain a more complete understanding of the underlying processes.

We chose a commercial formulation which is widely used in agricultural fields of the region and a glyphosate concentration simulating a worst-case scenario. Nevertheless, caution should be taken when extrapolating results of experimental studies to complex ecosystems. The experiment was long enough to cause profound changes in phytoplankton and water quality. We assume that our results may reflect short-term effects of a punctual input of glyphosate into a water body after a fumigation event, and the joint presence of *L. fortunei*, an invader widely spread along the studied reservoir and the Río de la Plata basin.

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