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RESEARCH ARTICLE

Differential impact of *Limnoperna fortunei*-herbicide interaction between Roundup Max® and glyphosate on freshwater microscopic communities

F. Gattás[1] · A. Vinocur[1,2] · M. Graziano[1,3] · M. dos Santos Afonso[3] · H. Pizarro[1] · D. Cataldo[1]

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Abstract Multiple anthropogenic stressors act simultaneously on the environment, with consequences different from those caused by single-stressor exposure. We investigated how the combination of the invasive mussel *Limnoperna fortunei* and a widely applied herbicide, Roundup Max®, affected freshwater microscopic communities and water quality. Further, we compared these results with those induced by the combination of the mussel and technical-grade glyphosate. We carried out a 34-day experiment in outdoor mesocosms, applying the following six treatments: 6 mg L^{-1} of technical-grade glyphosate (G), the equivalent concentration of glyphosate in Roundup Max® (R), 100 mussels (M), the combination of mussels and herbicide either in the technical-grade or formulated form (MG and MR, respectively), and control (C). Herbicides significantly increased total phosphorus in water; R and MR showed greater initial total nitrogen and ammonium. R increased picoplankton abundance and caused an eightfold increase in phytoplankton, with high turbidity values; G had a lower effect on these variables. Herbicide-mussel combination induced an accelerated dissipation of glyphosate in water (MG 6.36 ± 0.83 mg G g DW^{-1} day^{-1} and MR 5.16 ± 1.26 mg G g DW^{-1} day^{-1}). A synergistic effect on ammonium was observed in MR but not in MG. MR and MG had an antagonistic effect on phytoplankton, which showed a drastic reduction due to grazing, as revealed by M. We provide evidence of differential effects of Roundup Max® and technical-grade glyphosate over water quality and microscopic communities, and in combination with mussels. However, in the combination of mussels and herbicides, mussels seem to play a leading role. In the presence of *L. fortunei*, the effects of higher nutrient availability provided by herbicides addition were counteracted by the filtration activity of mussels, which released nutrients, grazed on picoplankton and phytoplankton, and boosted the development of other primary producers, periphyton and metaphyton.

Keywords *Limnoperna fortunei* · Roundup Max® · Glyphosate · Invasive species · Multiple stressors · Microscopic communities · Outdoor mesocosms · Grazing rate

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✉ F. Gattás
florencia.gattas@ege.fcen.uba.ar

[1] Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, IEGEBA-CONICET, Universidad de Buenos Aires, Buenos Aires, Argentina

[2] Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

[3] Departamento de Química Inorgánica, INQUIMAE-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

Introduction

The aquatic environment can be exposed to multiple anthropogenic stressors, which may act additively, synergistically or antagonistically, making their combined effects unpredictable from their individual effects (Townsend et al. 2008). Improving our understanding of how these factors interact with each other is still a challenge to ecologists (Ormerod et al. 2010). Multiple-stressor effects are of great importance in assessing freshwater ecosystem condition (Christensen et al. 2006; Strayer 2010; Magbanua et al. 2015). Habitat transformation, climate change, overexploitation of species, spread of introduced species, and pollution have had an increasing impact on freshwater ecosystems over the past

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50 years (Millenium Ecosystem Assessment 2005). In particular, freshwater ecosystems are highly sensitive to herbicides used in agriculture and to invasive species (Tilman et al. 2001; Relyea 2005). In Argentina, about 240 million kilograms of herbicides (75 % of which contained glyphosate) were used in 2013, mainly for soybean and maize crops, and for chemical fallow (CASAFE 2014).

Although glyphosate is applied to control undesirable weeds, it may enter aquatic systems either indirectly through wind-driven drift of the herbicide spray or through transport in surface runoff (Edwards et al. 1980; Feng et al. 1990; Peruzzo et al. 2008), or directly, by washing the tanks of the fumigation machines (Vera et al. 2010) or to control aquatic plants (Solomon and Thompson 2003) even though in many countries, glyphosate formulations containing certain surfactants are prohibited for direct over-water use. Several studies have assessed the impact of glyphosate on freshwater ecosystems and non-target aquatic organisms (Relyea et al. 2005; Pérez et al. 2007; Lipok et al. 2010; Vera et al. 2010, 2012; Avigliano et al. 2014). Commercial formulations of glyphosate, which are actually applied in agricultural practice, contain a mixture of substances such as surfactants, which increase herbicide effectiveness by improving the penetration of the active ingredient into cell membranes. Nowadays, in Argentina, there are over 400 registered formulations of unknown composition. Roundup® (Monsanto, St. Louis, MO, USA), composed of the isopropylamine (IPA) salt of glyphosate, the surfactant polyethoxylated tallowamine (POEA), and water (Giesy et al. 2000), is one of the most used formulations worldwide. However, in general, the exact chemical composition of commercial formulations is proprietary and often confidential. Vera et al. (2012), who studied the effects of Glifosato Atanor®, suggested that it is composed of surfactants rich in phosphorus. Folmar et al. (1979) found that technical-grade glyphosate has a less toxic effect than Roundup® or the surfactant alone on aquatic invertebrates and vertebrates, suggesting that surfactants may be the primary toxic agent in the formulations. Likewise, freshwater green algae exposed the commercial formulations Ron-do® and Roundup® had lower growth rates than those exposed to glyphosate alone (Sáenz et al. 1997; Sáenz and Di Marzio 2009). Tsui and Chu (2003) reported that, in general, Roundup® and the surfactant had a higher acute toxicity than technical-grade glyphosate and IPA for the aquatic microorganisms tested (bacteria, microalgae, protozoa, and crustaceans), with the surfactant accounting for about 46 % of Roundup® toxicity. Studies using amphibians agree that the surfactant contributes to the majority of the toxicity of the commercial formulation (Relyea and Jones 2009; Fuentes et al. 2011; Moore et al. 2012).

On the other hand, the freshwater golden mussel *Limnoperna fortunei*, which is native to Asian rivers (Morton 1977), currently dominates the benthic fauna in most of the Río de la Plata basin, reaching densities of more than 200,000 individuals m^{-2} (Boltovskoy et al. 2006). After its accidental introduction in the ballast water of ships, it was first recorded along the banks of the Río de la Plata estuary in 1990 (Pastorino et al. 1993). *L. fortunei* is a filter-feeding bivalve that is considered an effective ecosystem engineer (Darrigran and Damborenea 2011) because of its conspicuous impacts on different communities and on water column, altering ecosystem structure and function. For example, it increases water transparency and decreases suspended matter (Boltovskoy et al. 2009; Cataldo et al. 2012a), enhances the abundance and richness of benthic invertebrates (Sylvester et al. 2007), and represents an important food supply for larval and adult fishes (Paolucci et al. 2007, 2010a, b; Cataldo 2015). Studies involving mesocosms revealed that, in the presence of *L. fortunei*, there was a decrease in particulate matter and algal density, an increase in ammonium, nitrates and phosphates in water, enhanced periphyton growth (Cataldo et al. 2012a, b) and frequent toxic cyanobacterial blooms (Cataldo et al. 2012b). Pizarro et al. (2015a), who studied the combined effect of technical-grade glyphosate and *L. fortunei* using outdoor mesocosms, observed that the mussel accelerated the dissipation of glyphosate promoting the availability of phosphorus for the microscopic communities. There was a decrease in phytoplankton abundance, an increase in periphytic chlorophyll *a*, and an enhanced development of mats of filamentous green algae (metaphyton). In addition, these authors found a synergistic effect on soluble reactive phosphorus concentrations, bacterioplankton, and water turbidity, among other variables.

Considering that what is currently used to control undesirable weeds are commercial formulations and not glyphosate alone, as a next step in this line of research, we focused on the possible joint impact of *L. fortunei* and a glyphosate-based commercial formulation on freshwater microscopic communities. The objective of the present study is to evaluate the combined effect of *L. fortunei* and Roundup Max® on freshwater picoplankton and phytoplankton, on periphyton colonization and on water quality parameters (i.e., nutrient concentrations and physical properties), using outdoor mesocosms. We hypothesize that Roundup Max® and technical-grade glyphosate may have different effects in the presence of *L. fortunei*, possibly due to the adjuvants and surfactants in the commercial formulation. In this regard, we also compared the effect of the active ingredient versus the formulation on mussels by exploring their filtration capacity and grazing activity.

Materials and methods

The experiment was carried out between March and April 2014, using three outdoor 3000-L aquaculture tanks

containing six 70-L polyethylene bags (mesocosms) each, located in the experimental field of Ciudad Universitaria, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (Buenos Aires, Argentina). To keep the experimental conditions as natural as possible, 4 months before the beginning of the experiment, the tanks were filled with groundwater and inoculated with water from neighboring artificial lakes. In this way, we obtained well-established microscopic communities that are representative of natural freshwater systems in the region. The mesocosms (experimental units) were filled with homogenized water from the three tanks. Each mesocosm was equipped with a device that provided continuous aeration, maintaining particles in suspension. To analyze periphyton colonization, four clean artificial substrates (high impact polystyrene strips of 35 × 5 × 0.2 cm) were attached vertically to one side of each mesocosm; they were submerged in the water, with one surface exposed to the water column, thus allowing colonization.

Adult mussels of *L. fortunei* were manually collected from the delta of the Paraná river and transported to the laboratory, rinsed with tap water, stored in aerated aquaria filled with dechlorinated tap water at 23–25 °C and fed with baby fish food daily. Before the beginning of the experiment, individual mussels (mean length 21.1 ± 2.9 mm) were placed on flat trays to check their viability based on the extension of siphons.

One mesocosm from each tank was randomly assigned to one of the six treatments (M, G, R, MG, MR, and C). Three mesocosms had two cages of plastic mesh with 5-mm pore size containing 100 mussels, which were suspended below the water surface (treatment M). Thus, mussel density lies within the range recorded for natural water-bodies (Boltovskoy et al. 2009) and is high enough to observe the effect of mussels, as previously assessed in experimental studies with mesocosms (Cataldo et al. 2012b; Pizarro et al. 2015b). Three mesocosms were treated with technical-grade glyphosate acid (gly) at 6 mg L^{-1} (≥95 % purity; CAS: 1071-83-6) (treatment G). Other three mesocosms were treated with Roundup Max® (74.7 % of N-(phosphonomethyl)glycine monoammonium salt and 25.3 % of inert ingredients and adjuvants; lot number az3.js06085) at 6 mg L^{-1} of the active ingredient (gly equivalent 67.9 % *w*/*w*) (treatment R). This concentration is within the range of glyphosate concentrations found in natural water bodies of the region after herbicide spray events (Ronco et al. 2008), thus representing a "worst-case" scenario. The combination of mussels and technical-grade glyphosate was assayed by exposing 100 specimens enclosed in two cages to 6 mg L^{-1} of technical-grade glyphosate (treatment MG). To test the combination of mussels and Roundup Max®, 100 mussels enclosed as described above were exposed to 6 mg L^{-1} of the active ingredient (treatment MR). The remaining three experimental units lacked mussels, technical-grade glyphosate or commercial formulation and served as controls (C).

Samples were collected from each experimental unit immediately after the application of the treatments—mussels and herbicides—(day 0), and at days 1, 6, 13, 20, and 34 after the beginning of the experiment. On each sampling date pH, conductivity, and dissolved oxygen were measured in the mesocosms with a field probe (HACH, Sension 156); turbidity was measured with a 2100P HACH portable turbidimeter. Temperature was monitored every 30 min using thermobuttons (Akribis Therm) located at the bottom of the bag, throughout the experiment.

The determination of glyphosate was performed by HPLC-UV chromatography after a derivatization step with 9-fluorenyl methoxycarbonyl chloride (FMOC-Cl) (Sancho et al. 1996; Stalikas and Konidari 2001). The analysis was carried out using a HPLC-UV system (Jasco Analytical Instruments, Easton, MD, USA) with a 5-μm Microsorb C18 column (250 × 4.6-mm inner diameter; Varian). The mobile phases were 5 mM ammonium acetate (A) and acetonitrile (B). The chromatographic separation of the products was done using a gradient program as follows: 0–3 min, 90:10 (mobile phase A/mobile phase B); 3–17 min, from 90:10 to 40:60; 17–24 min, 10:90; 24–35 min, 90:10 (adapted from Waiman et al. 2012; Meyer et al. 2009). The flow rate and the injection volume were 1 ml min^{-1} and 200 μl, respectively. Detection was at 265 nm. Calibration curves were constructed using the same matrix (mesocosm water) to prevent nonadditive matrix effects. The limit of quantification of the resulting procedure was 0.2 mg L^{-1}, and the percent variation coefficient was of 4.6 %.

The half-life of glyphosate in each herbicide treatment was calculated assuming first-order kinetics with data fitted to a negative exponential function, according to the following equation:

$$\left[Gly_f\right] = [Gly_i] \cdot e^{-k.t}$$

where $[Gly_f]$ and $[Gly_i]$ are final and initial glyphosate concentrations, respectively; k is a dissipation rate constant; and t is the time (days) from the beginning of the experiment. To calculate glyphosate dissipation per gram of dry weight of mussel per day, the soft tissue was removed from the valves, oven-dried at 60 °C to constant weight and weighed using a digital balance (precision of ±0.0001 g; KERN ARS 120-4).

Water samples were filtered through Whatman® GF/F filters, which were then stored at −20 °C until measurement of phytoplanktonic chlorophyll *a* (Chl-*a*). The pigment was extracted with hot ethanol (60–70 °C) and stored at 4 °C in darkness. After centrifugation (3000 rpm) for 10 min, the absorbance was determined at 665 and 750 nm in a spectrophotometer (HACH DR 2800) before and after acidification with HCl 1 N. Final Chl-*a* concentrations were calculated according to Marker et al. (1980). Unfiltered water samples were used to

determine total phosphorus (ascorbic acid method) and nitrogen (cadmium reduction method), after digestion with potassium persulfate (Valderrama 1981), and ammonium was determined from filtered water samples by the salicylate method. Measurements were made with a HACH DR 2800 spectrophotometer and the commercially available kits for the quantitative determinations (HACH Company, USA).

Phytoplankton abundance was estimated from unfiltered water samples fixed with 1 % acidified Lugol's solution, at days 0, 13, and 34. Cell counts were carried out following Utermöhl (1958); live or dead organisms (the latter with shrunken contents or empty) were identified to the class level. The counting error (<20 %) was estimated according to Venrick (1978).

For picoplankton analysis, water samples collected at days 0, 13, and 34 were fixed with 10 % ice-cold glutaraldehyde and filtered through 0.2-μm-pore-size black polycarbonate filters (MSI, Westboro, MA, USA) adding 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, 10 μg mL^{-1}) for staining of bacterial DNA (Porter and Feig 1980). After filtration, each filter was mounted between a microscope slide and cover glass, using a drop of immersion oil for fluorescence microscopy (Olympus), and stored at −20 °C until quantification. Bacterioplankton was counted under UV light, picoeukaryotes under blue light, and picocyanobacteria under green light in a minimum of 20 fields per slide (error < 15 %), using an epifluorescence microscope (Olympus BX40F4, Japan).

The artificial substrates for periphyton colonization were removed one at a time at days 6, 13, 20, and 34. The periphyton that developed on the strip below 1 cm from the water surface was scraped using a sharp blade (mean scraped area = 22.43 ± 3.81 cm^2). Periphytic Chl-*a* was determined as explained for phytoplanktonic Chl-*a*, and expressed as μg cm^2, based on the substrate scraped area.

On every sampling date, a digital photograph of each experimental unit was taken from above the water surface to assess metaphyton growth and estimate its percentage of coverage. The images were processed using the CobCal V 1.0 software (INTA, Argentina). At day 34, metaphyton was collected from each experimental unit with a strainer and its wet weight was measured with a digital balance. An aliquot was weighted and retained in Whatman® GF/F filters. Chl-*a* was determined as described for phytoplankton and periphyton and expressed in μg L^{-1}, based on the mesocosm volume.

We estimated mussel grazing rates considering live phytoplankton and total picoplankton according to the equation of Quayle (1948):

$$G = (V \cdot f/N) \cdot \left\{ \left(C_f - C_i\right) / [(k-f) \cdot T] \right\}$$

$$f = (F \cdot N)/V$$

$$F = \frac{V \cdot \left[\ln\left(C_i/C_f\right) - \ln\left(C'_i/C'_f\right)\right]}{N \cdot T}$$

$$k = \left[\ln\left(C'_f/C'_i\right)\right]/T$$

where G is the grazing rate (cells ind^{-1} h^{-1}); V is the volume of the mesocosms (mL); f is the feeding coefficient; N is the total number of mussels per experimental unit; C_i and C_f are phytoplankton or picoplankton abundances (cells mL^{-1}) at days 0 and 34, respectively, in the mesocosms with mussels; k is the algal growth rate; T is the filtration time (duration of the experiment in hours); F is the filtration rate (mL ind^{-1} h^{-1}); and C'_i and C'_f are phytoplankton or picoplankton abundances days 0 and 34, respectively, in the control mesocosms.

Statistical analyses

Differences between treatments were analyzed by repeated-measures ANOVA (RM ANOVA), followed by Tukey's multiple comparisons tests, with previous evaluation of the model assumptions. It was considered as synergistic the significant interaction between factors resulting in a greater level than the expected by adding their individual effects, and as antagonistic when the combined effect of factors resulted in lower levels than the ones predicted by additivity. One-way ANOVA was used to test for differences between treatments in glyphosate dissipation rate, glyphosate half-life, phytoplankton composition, and grazing rate of mussel on picoplankton, followed by Tukey's test for multiple comparisons. Data were tested for normality and homoscedasticity before being analyzed. Statistical significance was set at a significance level of $p < 0.05$. The nonparametric Kruskal-Wallis test was used when data did not meet the assumption of normality or equality of variance after being transformed. RM ANOVA was performed with SPSS® software, while one-way ANOVA and the Kruskal-Wallis test, with InfoStat® software.

Results

Glyphosate concentration in water and dissipation

No significant differences in the initial concentration of glyphosate were observed among treatments with herbicides either alone or in combination (G 6.8 ± 0.1 mg L^{-1}; MG 6.9 ± 0.1 mg L^{-1}; R 6.7 ± 0.3 mg L^{-1}; MR 6.7 ± 0.3 mg L^{-1}) (RM ANOVA, $p = 1.00$ in all cases) (Fig. 1a). Glyphosate concentration showed a higher decreasing trend in treatments with both stressors combined (MG and MR) compared to treatments with the herbicide alone either in the technical-grade or formulated form (G

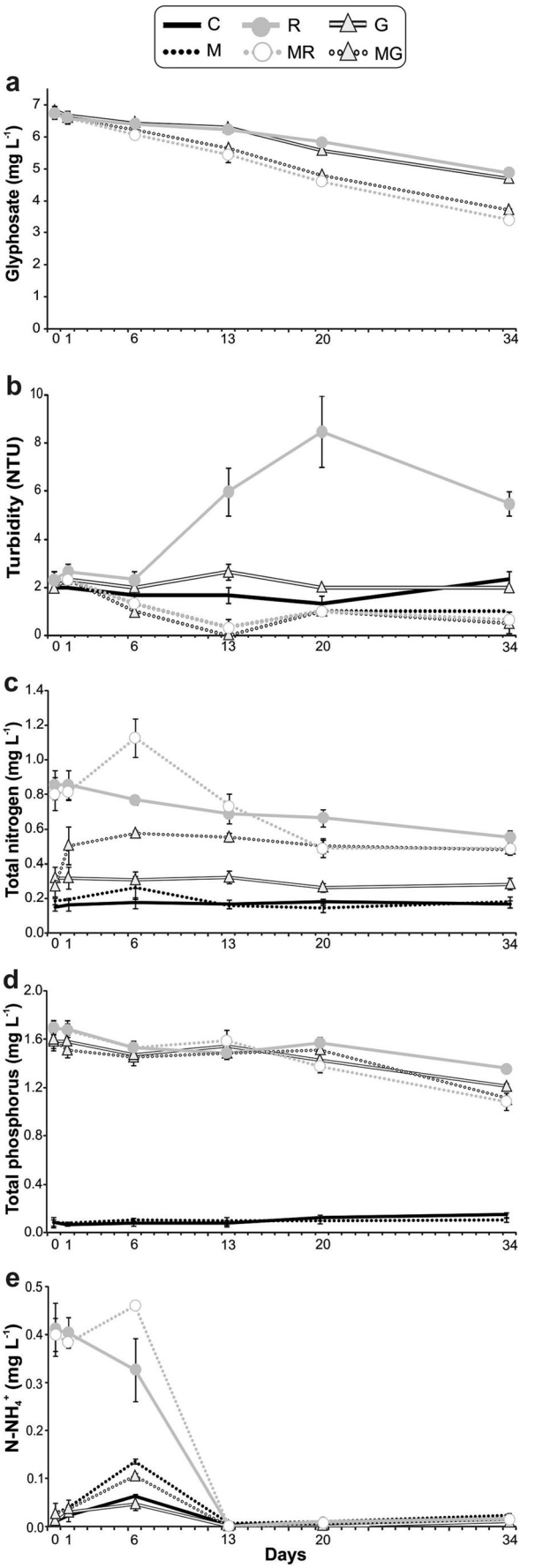

Fig. 1 Mean **a** glyphosate concentration (mg L^{-1}), **b** water turbidity (NTU), **c** total nitrogen (mg L^{-1}), **d** total phosphorus (mg L^{-1}), and **e** ammonium (mg L^{-1}) recorded in mesocosms at the beginning of the experiment (day 0) and at days 1, 6, 13, 20, and 34. *C* control, *G* glyphosate, *R* Roundup Max®, *M* mussels, *MG* mussels + glyphosate, *MR* mussels + Roundup Max®. *Bars* denote SE

and R), with significant differences from day 13 onward (RM ANOVA, $p < 0.05$ in all cases).

The decrease in glyphosate concentration observed at day 34 with respect to the initial concentration was significantly higher in MG (46 %) and MR (49 %) than in treatments without mussels (G 31 % and R 28 %) (RM ANOVA, $p = 0.00$ in all cases). The presence of *L. fortunei* produced a glyphosate dissipation of about 6.36 ± 0.83 mg per gram of mussel dry weight per day in MG, and 5.16 ± 1.26 mg of glyphosate per gram of mussel dry weight per day in MR, with no significant differences between treatments. Glyphosate half-lives and dissipation rate constants differed significantly in treatments with mussels compared with treatments without mussels (one-way ANOVA for half-lives and dissipation rate constants, $p < 0.05$ in all cases). The half-life of glyphosate was longer in the treatments without mussels (65 ± 3 days for G and 79 ± 12 days for R) than in MG (39 ± 2 days) and MR (35 ± 1 days). The dissipation rate constant was lower in G and R (0.011 ± 0.001 and 0.009 ± 0.001 day^{-1}, respectively) than in MG (0.018 ± 0.001 day^{-1}) and MR (0.020 ± 0.001 day^{-1}).

Physicochemical parameters and nutrients of water

Initial mean daily water temperature was 18.4 ± 0.72 °C for all treatments. Except for a peak of 20.2 °C recorded at day 4, mean daily water temperature ranged between 16 and 18 °C during the first 24 days after the beginning of the experiment. Then, it dropped sharply to a mean of 12.0 ± 0.41 °C at day 34. Dissolved oxygen in water remained at saturation levels throughout the experiment (mean dissolved oxygen concentration 8.1 ± 0.45 mg L^{-1}). Initial mean conductivity was 154.3 ± 0.11 μS cm^{-1} for all treatments. In general, conductivity showed a decreasing trend over time, with significant differences among treatments at day 34 (RM ANOVA, $p = 0.016$); it was significantly lower in M (111.1 ± 1.14 μS cm^{-1}) compared with C (118.3 ± 0.95 μS cm^{-1}), G (118.7 ± 1.28 μS cm^{-1}), and R (119.0 ± 1.00 μS cm^{-1}) (RM ANOVA, $p < 0.05$). Initial mean pH was 7.2 ± 0.03 for all treatments, without any significant difference among treatments or any distinguishable trend over time. At day 34, the lower mean pH values were obtained for MG (6.9 ± 0.12) and MR (6.8 ± 0.13), with no significant differences with respect to C (7.2 ± 0.28), M (7.0 ± 0.31), R (7.2 ± 0.06), and G (7.5 ± 0.06).

Initial mean turbidity was 2.2 ± 0.18 NTU for all treatments (Fig. 1b). In R, water turbidity increased from day 6 onward

(RM ANOVA, $p < 0.05$ in all cases), with a maximum value of 8.5 ± 2.12 NTU at day 20. In treatments with mussels (M, MG, and MR), turbidity decreased over time, with values lower than 1 NTU at day 34. No significant differences were observed in mean turbidity between initial and final values in C and G.

The initial concentration of total nitrogen (TN) was significantly higher in treatments with Roundup Max® (0.8 ± 0.16 mg L^{-1} for MR and 0.9 ± 0.15 mg L^{-1} for R) than in the rest of the treatments (C 0.1 ± 0.03 mg L^{-1}; M 0.2 ± 0.18 mg L^{-1}; G 0.3 ± 0.11 mg L^{-1}; MG 0.3 ± 0.11 mg L^{-1}; RM ANOVA, $p = 0.00$ in all cases) (Fig. 1c). A decreasing trend in TN was observed throughout the experiment in R and MR. The latter treatment showed a significant peak (1.1 ± 0.11 mg L^{-1}; RM ANOVA, $p = 0.032$) at day 6, which decreased thereafter. MG also showed an increase in TN at day 6, and then remained steady until the end of the experiment (0.5 ± 0.05 mg L^{-1}). No changes in TN were detected for M, G, and C throughout the experiment. At day 34, R and MR had a reduction in TN of about 35 and 40 %, respectively (R 0.6 ± 0.07 mg L^{-1}, RM ANOVA, $p = 0.023$; MR 0.5 ± 0.06 mg L^{-1}, RM ANOVA, $p = 0.017$). Although no significant interaction was found between herbicides and mussels over time, Roundup Max® had a significant effect on TN from day 0 onward (RM ANOVA, $p = 0.000$ in all cases) and technical-grade glyphosate from day 1 ($p = 0.012$) onward ($p < 0.05$ in all cases). The mussels also had an effect on TN, but differences were statistically significant in day 6 (RM ANOVA, $p = 0.000$) and day 13 ($p = 0.024$).

Initial concentrations of total phosphorus (TP) were significantly higher in treatments with herbicide alone or in combination (G 1.6 ± 0. 10 mg L^{-1}; MG 1.6 ± 0.15 mg L^{-1}; R 1.7 ± 0.11 mg L^{-1}; MR 1.7 ± 0.10 mg L^{-1}) than in C and M (C 0.1 ± 0.04 mg L^{-1} and M 0.1 ± 0.07 mg L^{-1}) (RM ANOVA, $p < 0.05$ in all cases) (Fig. 1d). TP concentrations decreased significantly throughout the experiment in treatments with herbicides, while no significant variations were detected for C and M. At day 34, TP was lower in the treatments with both stressors together (MG 1.1 ± 0.02 mg L^{-1} and MR 1.1 ± 0.12 mg L^{-1}) than in R (1.4 ± 0.04 mg L^{-1}) (RM ANOVA, $p = 0.012$ and $p = 0.003$, respectively), and G (1.2 ± 0.04 mg L^{-1}) had no significant differences with respect to MG and MR. The combination of mussels and both forms of the herbicide over time had no significant effect on TP. However, there was a significant effect of herbicides alone over time (RM ANOVA, $p < 0.05$ in all cases) and a significant effect of mussels alone, which was independent from time ($p = 0.014$).

The addition of Roundup Max® at day 0 led to a significant increase in ammonium concentration in R and MR (0.4 ± 0.08 and 0.4 ± 0.06 mg L^{-1}, respectively) with respect to the rest of the treatments (RM ANOVA, $p = 0.000$) (Fig. 1e). Ammonium concentration was higher at day 6 than at day 0 in all the treatments, except for R, especially in the treatments with mussels. This variable dropped sharply to values below 0.02 mg L^{-1} at day 13 in all the mesocosms. The combination of stressors had a significant effect on ammonium concentration over time, which was most clearly evidenced at day 6 (MG, RM ANOVA, $p = 0.002$, and MR, $p = 0.000$).

Biological response parameters

Phytoplankton

Initial phytoplankton abundances were similar in all the treatments ($44.99 \times 10^3 \pm 8.61 \times 10^3$ cells mL^{-1}) (Fig. 2a). The addition of Roundup Max® led to a sustained increase in phytoplankton abundance until day 13 (R: $236.77 \times 10^3 \pm 48.66 \times 10^3$ cells mL^{-1}; RM ANOVA, $p = 0.000$). There was an increasing but not significant trend with respect to day 0 in G (G $64.15 \times 10^3 \pm 24.35 \times 10^3$ cells mL^{-1}). In contrast, mussels produced a non-significant decrease with respect to day 0 in algal abundance (M 916 ± 140 cells mL^{-1}; MG $1.73 \times 10^3 \pm 400$ cells mL^{-1}; MR $2.54 \times 10^3 \pm 774$ cells mL^{-1}; RM ANOVA, $p > 0.050$ in all cases). In C, variations in phytoplankton abundance were not significant throughout the experiment. At day 34, phytoplankton abundances in R and G were 558 and 191 % higher than those in C, respectively (R $387.74 \times 10^3 \pm 18.75 \times 10^3$ cells mL^{-1}; G $171.44 \times 10^3 \pm 1.63 \times 10^3$ cells mL^{-1}; C $58.95 \times 10^3 \pm 9.80 \times 10^3$ cells mL^{-1}; RM ANOVA, $p = 0.000$ in both cases), while abundances in treatments with mussels were about four times lower than those in C (M $8.51 \times 10^3 \pm 2.83 \times 10^3$ cells mL^{-1}; MG $12.68 \times 10^3 \pm 2.44 \times 10^3$ cells mL^{-1}; MR $18.57 \times 10^3 \pm 9.58 \times 10^3$ cells mL^{-1}). In MG and MR, there was a significant reduction in phytoplankton abundance compared to C (RM ANOVA, $p = 0.001$ and $p = 0.003$, respectively) and a significant interaction between stressors ($p = 0.000$ in both cases).

Algal composition at day 0 was similar among treatments, with a dominance of genera of Desmidiaceae (48–53 % of total algae) and Chrysophyceae (31–39 % of total algae). Chlorophyceae were present in all treatments with abundances ranging between 5 and 13 %. Figure 2b shows the changes in the abundances of the different algal groups in the six treatments. When we calculated the difference in the abundances of the algal classes between final (at day 34) and initial (at day 0) values for each treatment, in G, R, and C, we observed an increase for Chlorophyceae (G $131.94 \times 10^3 \pm 3.90 \times 10^3$ cells mL^{-1}; R $290.25 \times 10^3 \pm 7.48 \times 10^3$ cells mL^{-1}; C $17.73 \times 10^3 \pm 4.43 \times 10^3$ cells mL^{-1}) and Desmidiaceae (G $16.15 \times 10^3 \pm 2.61 \times 10^3$ cells mL^{-1}; R $52.14 \times 10^3 \pm 4.66 \times 10^3$ cells mL^{-1}; C $7.97 \times 10^3 \pm 5.99 \times 10^3$ cells mL^{-1}). There was a decrease in the abundance of all algal

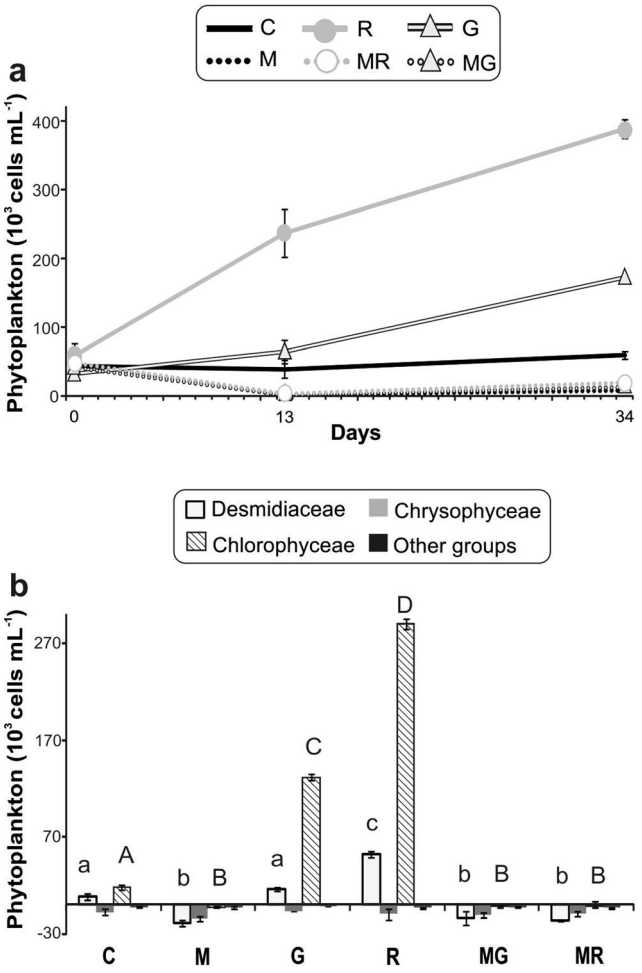

Fig. 2 Mean **a** phytoplankton abundance (cells mL^{-1}) recorded in mesocosms at the beginning of the experiment (day 0) and at days 13 and 34, and **b** differences in the abundance of algal classes between final (at day 34) and initial (at day 0) values for each treatment; *different lowercase letters* represent significant differences between treatments for Desmidiaceae (Tukey's test, $p \leq 0.05$); *different capital letters* represent significant differences between treatments for Chlorophyceae (Tukey's test, $p \leq 0.05$); *absence of letters* indicates non-significant differences between treatments (Chrysophyceae and "Other groups"). *C* control, *G* glyphosate, *R* roundup Max®, *M* mussels, *MG* mussels + glyphosate, *MR* mussels + Roundup Max®. *Bars* denote SE

groups in the treatments with mussels, either alone or in combination with herbicides, resulting in negative differences for Desmidiaceae (M $18.60 \times 10^3 \pm 6.20 \times 10^3$ cells mL^{-1}; MG $14.11 \times 10^3 \pm 12.32 \times 10^3$ cells mL^{-1}; MR $16.81 \times 10^3 \pm 1.12 \times 10^3$ cells mL^{-1}), Chrysophyceae (M $14.30 \times 10^3 \pm 3.94 \times 10^3$ cells mL^{-1}; MG $10.77 \times 10^3 \pm 4.16 \times 10^3$ cells mL^{-1}; MR $9.35 \times 10^3 \pm 4.15 \times 10^3$ cells mL^{-1}), and Chlorophyceae (M $2.55 \times 10^3 \pm 1.08 \times 10^3$ cells mL^{-1}; MG $1.67 \times 10^3 \pm 2.68 \times 10^3$ cells mL^{-1}; MR $236 \pm 5.39 \times 10^3$ cells mL^{-1}). Phytoplanktonic classes that represented less than 3 % of total initial and final abundances were classified as "Other groups" (i.e., Dinophyceae, filamentous Cyanobacteria, Euglenophyceae and Bacillariophyceae) and resulted in negative differences in all the treatments. Desmidiaceae exhibited a significantly higher growth in R than in C and G (one-way ANOVA, $p \leq 0.05$); Desmidiaceae abundance showed a negative trend at day 34 with respect to initial values in treatments with mussels (M, MG, and MR), which differed significantly from the other treatments but not from each other. The growth of Chlorophyceae was significantly greater in G and R with respect to C, and they also differed significantly between each other (one-way ANOVA, $p \leq 0.05$). As for Desmidiaceae, Chlorophyceae growth had a negative trend in the presence of mussels (M, MG, and MR), with values significantly different from their respective controls. The remaining groups (Chrysophyceae and Other groups) did not show significant differences in abundance between treatments (Fig. 2b).

The concentration of phytoplanktonic Chl-*a* followed an unclear trend over the study period in the different treatments, all of which showed mean values below 10 μg L^{-1}. Initial mean Chl-*a* was 3.8 ± 1.74 μg L^{-1} for all treatments, with values of about 2.8 ± 1.80 μg L^{-1} at day 34. Chl-*a* concentration ranged between these values in all the treatments during the study period, except for R which showed mean values higher than 5 μg L^{-1} and a peak of 29.0 ± 13.12 μg L^{-1} at day 20.

Picoplankton

At day 0 bacterioplankton abundance did not differ significantly among treatments (RM ANOVA, $p > 0.05$) (Fig. 3a). Except for R, all treatments showed a decrease in bacterioplankton abundance over time. In C, M, G, MG, and MR, we found significant differences between initial and final bacterial abundances (RM ANOVA, $p \leq 0.050$ in all cases). However, bacterioplankton abundance was lower in treatments with than without mussels; final bacterial abundance resulted significantly lower in treatments with mussels (M, MG, and MR) in comparison with R (RM ANOVA, $p = 0.003$ for R vs. M and MG, and $p = 0.011$ for R vs. MR). Although there was no significant interaction between stressors over time, the effect of mussels on bacterial abundances was evident throughout the experiment (RM ANOVA, $p = 0.000$).

Initial mean picocyanobacteria abundances were similar in all treatments ($26.36 \times 10^3 \pm 3.10 \times 10^3$ cells mL^{-1}) and remained steady for the rest of the study period, reaching final mean values of about $30.00 \times 10^3 \pm 8.90 \times 10^3$ cells mL^{-1}.

Initial mean abundances of picoeukaryotes were similar in all the treatments (Fig. 3b). Treatments with mussels either alone or in combination showed no significant variations in mean picoeukaryote abundances over time. In R, there was an increase in the abundance of this community during the first weeks after the beginning of the experiment, with significant differences at day 13 compared with C (RM ANOVA, $p = 0.008$), G ($p = 0.007$), and M, MR, and MG ($p = 0.000$

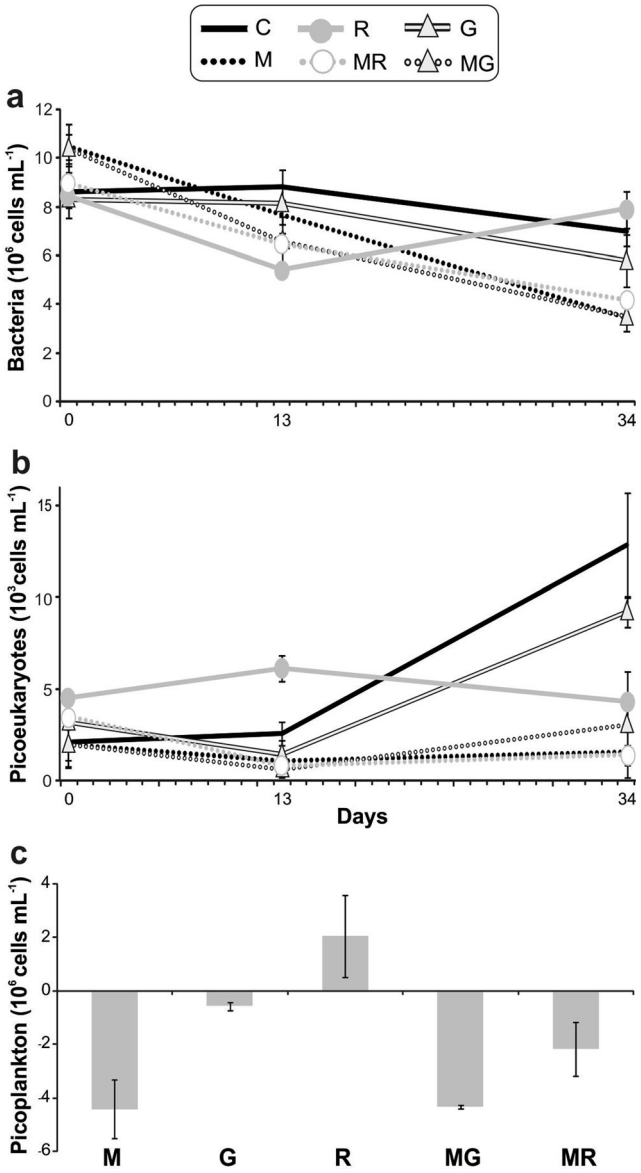

Fig. 3 Mean **a** abundance of bacterioplankton (cells mL^{-1}) and **b** abundance of picoeukaryotes (cells mL^{-1}) recorded in mesocosms at the beginning of the experiment (day 0) and at days 13 and 34, and **c** mean differences in total picoplankton abundances between final (at day 34) and initial (at day 0) values, with respect to the control, for each treatment. *C* control, *G* glyphosate, *R* Roundup Max®, *M* mussels, *MG* mussels + glyphosate, *MR* mussels + Roundup Max®. *Bars* denote SE

in all cases). At day 34, mean values increased significantly in C and G (RM ANOVA, $p = 0.000$ and $p = 0.010$, respectively).

Figure 3c shows the changes in the abundances of total picoplankton (i.e., bacteria, picocyanobacteria, and picoeukaryotes) for each treatment throughout the experiment, expressed as the differences between final (at day 34) and initial (at day 0) values with respect to C. The presence of mussels (M, MG, and MR) produced a strong decrease in picoplankton abundance (M $3.45 \times 10^6 \pm 2.02 \times 10^5$ cells mL^{-1}; MG $3.50 \times 10^6 \pm 1.01 \times 10^6$ cells mL^{-1}; MR $4.22 \times 10^6 \pm 4.99 \times 10^5$ cells mL^{-1}), as opposed to the treatment with Roundup Max® only (R $7.92 \times 10^6 \pm 1.34 \times 10^6$ cells mL^{-1}), in which picoplankton abundance increased about twice. Picoplankton abundance was not significantly affected by the addition of technical-grade glyphosate (G $5.83 \times 10^6 \pm 1.49 \times 10^6$ cells mL^{-1}).

Periphyton

The first samples, taken at day 6, contained a very low concentration of periphytic Chl-*a*, ranging between 0 and 0.01 μg cm^{-2}, with no significant differences between treatments (Fig. 4a). In all treatments with mussels, periphytic Chl-*a* concentration increased at day 13, reaching values significantly higher than those at day 0 (RM ANOVA, $p = 0.005$). In MR, there was a peak of periphytic Chl-*a* concentration at day 13 (0.11 ± 0.09 μg cm^{-2}), while in MG and M maximum values were recorded at day 20 (0.15 ± 0.05 and 0.17 ± 0.13 μg cm^{-2}, respectively). In C, periphytic Chl-*a* concentration was detected at day 20. Chl-*a* concentrations were higher in R and G than in C, but differences were not significant. The decrease in the concentrations of periphytic Chl-*a* recorded at day 34 in M, MG, and MR was not significant; however, these values were higher than those recorded in C, G, and R. No interactions between stressors were detected, and the effect of mussels was registered from day 13 onward (RM ANOVA, $p < 0.05$ in all cases).

Metaphyton

Metaphyton only developed in treatments with mussels. It was detected from day 13 onward, reaching a maximum coverage of 31.7 % (M), 37.4 % (MG), and 40.1 % (MR) at day 20. At day 34, there were significant differences in coverage between M (26.3 %), MG (30.8 %), and MR (25.6 %) with respect to C, G, and R (Kruskal-Wallis test, $p = 0.012$) (Fig. 4b). However, no significant differences in coverage percentage of metaphyton were found among M, MG, and MR. Mussels induced significant differences in Chl-*a* concentration, with values in M and MR (39.5 and 46.2 μg L^{-1}, respectively) being significantly higher than in MG (23.7 μg L^{-1}) (Kruskal-Wallis test, $p = 0.001$) (Fig. 4c).

Grazing rates of *Limnoperna fortunei*

There was no significant mortality of mussels at day 34, with a survival percentage of 98.4 ± 0.6 % (M), 97.0 ± 1.7 % (MG), and 97.4 ± 2.1 % (MR). No significant differences in mussel grazing rate on the phytoplanktonic community were found among treatments (M $29.20 \times 10^3 \pm 10.31 \times 10^3$ cells ind^{-1} h^{-1}; MG $26.71 \times 10^3 \pm 13.83 \times 10^3$ cells ind^{-1} h^{-1}; MR $24.53 \times 10^3 \pm 8.30 \times 10^3$ cells ind^{-1} h^{-1}). Mussel grazing rate on picoplankton was slightly lower in MR

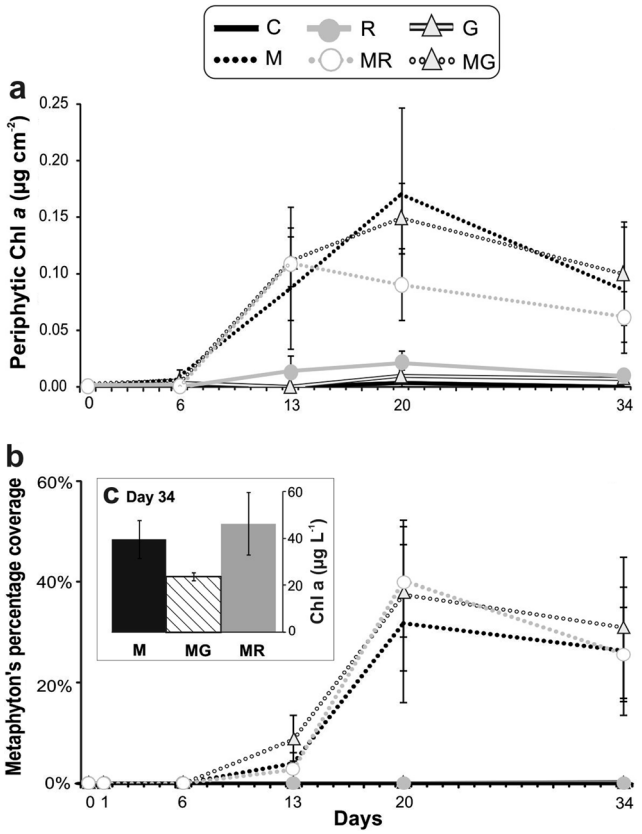

Fig. 4 Mean **a** periphytic Chl-*a* (µg cm^2) recorded in mesocosms 6 days after the beginning of the experiment and at days 13, 20, and 34, **b** percentage coverage of metaphyton recorded in mesocosms throughout the experimental period, and **c** Chl-*a* concentration of metaphyton (µg L^{-1}) at the end of the experiment (day 34) in mesocosms with mussels. *C* control, *G* glyphosate, *R* Roundup Max®, *M* mussels, *MG* mussels + glyphosate, *MR* mussels + Roundup Max®. *Bars* denote SE

($2.63 \times 10^6 \pm 1.11 \times 10^6$ cells ind^{-1} h^{-1}) than in M ($4.03 \times 10^6 \pm 1.09 \times 10^6$ cells ind^{-1} h^{-1}) and MG ($4.25 \times 10^6 \pm 7.20 \times 10^5$ cells ind^{-1} h^{-1}), without significant differences among them.

Discussion

Our experiment shows different results in the combined effect of *L. fortunei* and the herbicide depending on whether it was Roundup Max® or technical-grade glyphosate. The joint effect of Roundup Max® and *L. fortunei* was synergistic, antagonistic, or additive depending on the variables analyzed. Interestingly, *L. fortunei* seemed to play a leading role in many interactions.

The application of herbicides resulted in drastic changes in physicochemical parameters, especially for Roundup Max®. The higher levels of TN in R and MR at the beginning of the experiment may be attributed to the surfactants present in the commercial formulation, as also the monoammonium salt of glyphosate. In treatments with Roundup Max®, there was a decreasing trend in TN in water throughout the experiment, in agreement with the results of Vera et al. (2012), who treated outdoor mesocosms with another commercial formulation (Glifosato Atanor®). The explanation for this trend may be related to the incorporation of nitrogen from the water column by other aquatic communities, such as periphyton and metaphyton (see below). However, in the joint treatment of Roundup Max® and mussels, we observed a significant increase in TN 1 week after the beginning of the experiment. This was consistent with an increase in ammonium concentration, which was probably due to an increase in the metabolic rate of *L. fortunei* as a stress response to the commercial formulation. There are some other mechanisms mediated by the mussels that may explain the increased ammonium in the water column, such as the remobilization of glyphosate or surfactants adsorbed to particles, the mineralization of nutrients, and the degradation of glyphosate or adjuvants that the commercial formulation may contain. However, we consider that the stress response from mussels is the only mechanism that would be the responsible of an increase in TN in the water column, since there cannot be more nitrogen than what the aquatic medium initially contained, unless it comes from an external source or that comes from the own metabolism of the mussels. Although glyphosate alone may have no direct effect on TN, the hypothesis of a stress response is reinforced by the increase in TN observed in the *L. fortunei*-technical-grade glyphosate treatment at day 6. The active ingredient and the commercial formulation may induce *L. fortunei* to release nitrogen to the water as a consequence of increased metabolic rates. This may result in higher levels of nitrogen in the system than those expected from a "normal" metabolism and herbicide input. In line with this, mussels alone or in combination with both herbicides triggered an increase in ammonium during the first week, also attributable to a rise in the metabolic rate, in agreement with other experimental studies using *L. fortunei* alone (Cataldo et al. 2012a; Frau et al. 2012) and also combined with technical-grade glyphosate (Di Fiori et al. 2012).

In regard to ammonium concentrations, mussels interacted synergistically with Roundup Max® and antagonistically with technical-grade glyphosate 6 days after the beginning of the experiment. Although ammonium levels were lower in the latter than in treatments with mussels alone, we found differences with respect to initial values. Then, ammonium concentration decreased over time in all treatments, possibly because it was consumed by other aquatic communities (see below). On the other hand, the differential effect of the commercial formulation compared with technical-grade glyphosate may be due to the ammonium supplied by the adjuvants and by the monoammonium salt of glyphosate. This is a reasonable explanation for the higher than expected levels of ammonium found in the mussels-Roundup Max® treatment.

During the first 6 days of the experiment, TN increased in the presence of *L. fortunei* regardless of the form of herbicide used. This may be a stress response of *L. fortunei* driven by the herbicides. Iummato et al. (2013), who analyzed the effect of glyphosate on *L. fortunei*, detected oxidative stress in mussels exposed to 3 and 6 mg L^{-1} glyphosate and an increase in the activity of enzymes involved in general metabolism and detoxification processes. The lack of published data about the joint effect of *L. fortunei* and Roundup Max® prompted us to carry out evasion and toxicity tests with golden mussels to assess the concentration of Roundup Max® at which its filtering activity and survival are not affected (unpublished data). In these preliminary experiments, mussels spawned a few hours after a first exposure to Roundup Max® at concentrations between 6 and 8 mg L^{-1}, which supports the idea that in the presence of the formulation, mussels may have a stress response. Cataldo et al. (2005) reported that *L. fortunei* exposed to a sublethal dose of the molluscicide agent Clamtrol CT2 also underwent spawning as a stress response.

All herbicide treatments showed an increase in TP concentration, probably due to the contribution of glyphosate either in the technical-grade or formulated form. Similar results have been reported with Roundup® (Pérez et al. 2007; Vera et al. 2010), Glifosato Atanor® (Vera et al. 2012), and technical-grade glyphosate (Saxton et al. 2011; Pizarro et al. 2015b). Mussels in combination with the active ingredient and Roundup Max® induced a downward trend in TP concentration throughout the experiment. Although part of the phosphorus released from herbicides is possibly consumed by periphyton and metaphyton, it is undoubtedly that herbicide addition contributes to a shift of the system to a more eutrophic state (Austin et al. 1991; Pérez et al. 2007).

It is well known that glyphosate dissipation in nonflowing waters depends on local conditions (Giesy et al. 2000), such as the presence of sediments and their physicochemical characteristics (Goldsborough and Beck 1989), soil composition and phosphate concentration (Gimsing and Borggaard 2002), suspended solids, water temperature, and pH, among others. In our experiment, treatments on the joint effect (MG and MR) showed higher dissipation rate constants and shorter half-lives of glyphosate than treatments with herbicides alone (G and R), as observed in other studies under outdoor mesocosm conditions (Pizarro et al. 2015a) and laboratory conditions, where living mussels increased glyphosate dissipation in water through actively filtering and mineralization of glyphosate, and also the associated microorganisms in the shells' biofilm could break down the herbicide (Di Fiori et al. 2012). No significant differences were found in these variables between herbicides, either alone or in combination with mussels. These results, together with the lack of differences in the dissipation of glyphosate per gram of mussel dry weight per day between treatments, may indicate that the formulation components had no noxious effect on the degrading ability of *L. fortunei*.

Although we observed an accelerated dissipation of glyphosate in the presence of mussels, this may not be true for the adjuvants in the commercial formulation, since we do not know the complete composition.

Water turbidity is strongly affected by the commercial formulation (Pérez et al. 2007; Vera et al. 2012), but not by the active ingredient alone (Pizarro et al. 2015b). In our experiment, increased water turbidity could be related to the growth of phytoplankton, which occurred from the second week to the end of the experiment in the treatment with Roundup Max® only, and to the growth of picoplankton, which doubled its initial abundances by the end of the experiment. In the treatment with the active ingredient only, phytoplanktonic abundance increased to lower levels at the end of the experiment but no changes were detected in water turbidity, and picoplankton abundance was not significantly affected, in agreement with the results of Pizarro et al. (2015b). A sharp increase in water turbidity due to the application of a commercial formulation has been reported by Vera et al. (2010), who observed that a single application of Roundup® shifted the state of outdoor mesocosms from clear to turbid water after 1 year. In contrast, the decreased turbidity observed in the treatments with *L. fortunei* alone or in the combination treatments, was probably due to the filtering activity of the mussels that counteracted the growth of phytoplankton and picoplankton. These results are consistent with those observed in the Río Tercero reservoir (Córdoba, Argentina), where the invasion of *L. fortunei* in 2000 led to an increase in water transparency from 0.25–3 m (Mariazzi et al. 1989, 1992) to 6–9 m (Mariñelarena 2003; Cataldo et al. 2012a).

Nutrients input from glyphosate and the commercial formulation triggered different responses in the biological variables. Phytoplankton was greatly favored by the application of herbicides (mainly Chlorophyceae and Desmidiaceae), within the tested concentrations, unlike what would be expected for the effect of glyphosate on organisms possessing the shikimic acid pathway (Schönbrunn et al. 2001). Some researchers reported that commercial formulations had positive effects on phytoplankton abundances (Vera et al. 2012) while others found negative effects (Pérez et al. 2007). Experiments using technical-grade glyphosate showed positive and negative effects (Saxton et al. 2011) or no effect (Pizarro et al. 2015b) on phytoplankton. In contrast, treatments with mussels alone or in combination with both herbicides impacted negatively on the abundance of all algal groups, probably explained by *L. fortunei* grazing. A decrease in phytoplankton abundance by high clearance rates and filtration activity of mussels has been previously documented (Cataldo et al. 2012a; Sylvester et al. 2005; Boltovskoy et al. 2015; Tokumon et al. 2015). Our experiment provided evidence that the grazing activity of *L. fortunei* on phytoplankton was independent of the presence or absence of herbicides, ruling out a negative effect of the adjuvants. The grazing rates obtained in our experiment are

comparable to that reported by Cataldo et al. (2012a) from a mesocosm experiment (30.60×10^3 cells ind^{-1} h^{-1}). It is known that *L. fortunei* can feed on organisms of up to 1100 μm (Rojas Molina et al. 2011), but prefer smaller prey (Rojas Molina et al. 2010); in particular for phytoplankton, single cells are more efficiently filtered than colonies or filamentous forms (Gazhula et al. 2012; Cataldo et al. 2012b). *L. fortunei* and both herbicides acted antagonistically on total phytoplankton abundance, suggesting that mussels play a leading role as previously stated by Pizarro et al. (2015a). However, we found no differences in the combination of *L. fortunei* with technical-grade glyphosate or Roundup Max®. In our experiment, mussels caused a significant reduction of Chlorophyceae and Desmidiaceae with respect to the controls, while they were likely to avoid feeding on filamentous forms, which allowed the development of mats of filamentous algae, namely metaphyton. In this line of reasoning, it is possible that the shadow casted by metaphyton is another factor affecting phytoplankton abundances.

The remarkable decrease in the abundance of the picoplanktonic fraction observed throughout the experiment in the presence of mussels (on average 38 % with respect to the controls) suggests that *L. fortunei* grazed upon this community. To our knowledge, there are no studies that examine the effect of *L. fortunei* on picoplankton or the joint effect of *L. fortunei* and herbicides on picoplankton. This is an interesting issue since picocyanobacteria possess a high capacity to use phosphonates as a phosphorus source (Ilikchyan et al. 2009). During our experiment, there was a decreasing trend in bacterioplankton abundances in the treatments with mussels alone or with mussels and herbicides, probably caused by mussel filtration (mean grazing rates of 4.07×10^6 bacteria ind^{-1} h^{-1} in M; 3.98×10^6 bacteria ind^{-1} h^{-1} in MG; and 2.16×10^6 bacteria ind^{-1} h^{-1} in MR). In the treatment with Roundup Max® alone, bacterioplankton abundance first decreased and then increased. The decrease in bacterioplankton abundance may partly be attributed to nutrient competition with phytoplankton, which showed a noticeable increase during the first weeks of the experience. The subsequent increase in bacterioplankton abundance was possibly caused by bacterial activity on the biota (for example on dead algae) and glyphosate degradation (Huang et al. 2005). Bacterioplankton abundance did not vary considerably over time in the treatment with technical-grade glyphosate, suggesting that other factors besides herbicide degradation are involved in its growth. Other authors have proposed an indirect effect of herbicides on picoplankton predators (Bengtsson et al. 2004; Pizarro et al. 2015b). Once again, the effect of the active ingredient differed from that of the commercial formulation, but the treatments involving the combination of stressors did not differ among each other, suggesting that *L. fortunei* has a buffering effect on bacterioplankton growth, through grazing. In treatments with lower picoplanktonic and phytoplanktonic abundances and turbidity (i.e., mussels alone or in combination with herbicides), there was an explosive growth of metaphyton and periphyton.

Unlike previous research pointing to the effect of herbicides on an already established periphytic community (Pérez et al. 2007; Pizarro et al. 2015a), in this experiment, we studied the colonization dynamics of periphyton under different scenarios. It is worthy of mentioning that mussels, either alone or in combination with herbicides, accelerated the settlement of periphytic communities, while no effect was detected over time in the treatments with a single herbicide. Vera et al. (2010) found a delay in the colonization of periphyton and a decrease in chlorophyll *a* concentration with respect to the controls in outdoor mesocosms treated with Roundup®. Differences between this and our study may be explained by differences in the mesocosm dimensions, in the characteristics of the water and the type of formulation applied. Cataldo et al. (2012b) reported enhanced growth of periphyton in mesocosm experiments with *L. fortunei*, which is consistent with our results. Moreover, Pizarro et al. (2015a) observed that the active ingredient and *L. fortunei* acted synergistically on an already established periphytic community (mussels alone and technical-grade glyphosate alone induced a significant increase in periphytic chlorophyll *a*). In the present study, the commercial formulation had no such effect but it is undeniable the crucial role played by mussels in the promotion of periphytic growth and colonization by recycling nutrients from water, which makes them bioavailable. Although we found that periphyton developed in all mesocosms containing mussels independently of herbicide addition, higher biomass levels were first obtained in combined herbicide-mussels treatments, probably due to increased nutrient availability.

Periphyton and metaphyton developed simultaneously in treatments with mussels alone and in combination with herbicides. The latter community occurs in shallow or ephemeral water bodies mainly associated with eutrophication; it is characterized by filamentous algae which start to develop at the bottom and produce oxygen bubbles through photosynthesis leading to upward growth of entangled, floating algal mats (Hillebrand 1983). Pizarro et al. (2015a) mentioned this process, especially in combined treatments of *L. fortunei* and different concentrations of technical-grade glyphosate. Both periphyton and metaphyton are not readily available for mussels because the former is attached on natural or artificial substrates and the latter comprises large clumped filamentous algae, making them unsuitable for consumption (Horgan and Mills 1997). These authors studied the filtering impacts of the zebra mussel *Dreissena polymorpha*, one of the most studied freshwater invasive species, which shares many biological and ecological traits with *L. fortunei* (Karatayev et al. 2007). They compared the clearance rates of mussels on phytoplankton taxa of different size, and stated that zebra mussels can graze on seston particles smaller than their inhalant siphon.

Therefore, we consider that periphyton and metaphyton growth may have been indirectly enhanced by *L. fortunei* predation on phytoplankton, increased availability of nutrients—resulting from herbicide addition and released by mussels—and lower water turbidity—caused by plankton depletion—with a consequent increase in light penetration. In turn, shading from the metaphyton bloom could affect autotrophic communities (phytoplankton and the picoautotrophic fraction), reinforcing the decreasing trend due to mussel grazing, and may explain the decrease in periphyton abundance by the end of the experiment. Other studies using in situ mesocosms also reported that *L. fortunei* promoted the accelerated recycling of nutrients, decreased phytoplankton abundance, increased water transparency, and exerted different effects on primary producers. In the Río Tercero reservoir, Boltovskoy et al. (2009) observed increased abundance of submerged aquatic macrophytes, and in Salto Grande reservoir, Cataldo et al. (2012b) described changes in algal assemblages, a significant increase in cell density, proportion of colonies, and colony size of cyanobacteria *Microcystis* sp., and an enhanced growth of periphyton. In our experiment, metaphyton was dominant probably because experimental conditions were similar to those of shallow water bodies.

Roundup Max® had a higher effect on total nitrogen, ammonium, and abundances and composition of picoplankton and phytoplankton in comparison with the active ingredient. In stressor combination treatments, mussels played a leading role in affecting most biological response variables (i.e., abrupt decrease in picoplankton and phytoplankton abundance, explosive growth of metaphyton, and increased periphyton colonization). There was a synergistic effect of Roundup Max® and mussels on ammonium concentration, different from the joint effect of *L. fortunei* and technical-grade glyphosate, and an antagonistic effect of both types of combinations on phytoplankton. In absence of mussels, increasing nutrients in water, mainly caused by Roundup Max®, led to more eutrophic conditions and favored picoplankton and phytoplankton growth, which became the main primary producers. In the presence of *L. fortunei*, the effects of higher nutrient availability were counteracted by the filtration activity of mussels, which released nutrients, grazed on seston, and boosted the development of periphyton and metaphyton.

Our study used mesocosms as proxy for natural system conditions, since they were exposed to weather fluctuations and included many aquatic communities interacting with each other.

One point to consider is that although the concentration of glyphosate used in this experiment is within the range of "worst-case" scenarios, it has been recorded in freshwaters from the Pampa plain in Argentina (Ronco et al. 2008). In addition, we chose a commercial formulation widely used in agricultural practice and a mussel density already documented in natural water bodies (Boltovskoy et al. 2009). Although manipulative tests using mesocosms are useful to assess single- and multiple-factor effects on microscopic communities and water quality (Caquet et al. 2000), one must be cautious in extrapolating results from artificial systems to complex, natural freshwater ecosystems. In Argentina, *L. fortunei* and glyphosate coexist in both lotic and lentic water bodies displaying different dynamics. Therefore, further research under different scenarios will provide insight into more realistic situations.

The two stressors considered in our study, invasive species and pollution, are of anthropogenic origin: *Limnoperna fortunei* was introduced in Argentina due to increasing trade between continents and glyphosate is derived from industrial agriculture. It is clear that there is not a unique response of their interaction in natural systems and, as it was demonstrated in the present research, the characteristics of the chemical agent are determinant on the impact of the combined effect with the invaders on natural communities. Therefore, we emphasize the advantages of using a multiple-stressors approach for predicting potential changes in water quality and aquatic communities in freshwaters polluted with herbicides and colonized by *L. fortunei* to avoid unpredictable results (Townsend et al. 2008) and unexpected ecological surprises (Christensen et al. 2006).

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