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Impact of multiple anthropogenic stressors on freshwater: how do glyphosate and the invasive mussel *Limnoperna fortunei* affect microbial communities and water quality?

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Abstract The study of the joint effect of multiple anthropogenic stressors is important because the emerging consequences are often unpredictable on the basis of knowledge of single effects. We explored the joint impact of glyphosate and the invasive golden mussel *Limnoperna fortunei* on freshwater phytoplankton, bacterioplankton and periphyton, and on the physical and chemical properties of the water. We manipulated both stressors simultaneously in a 25-day experiment using outdoor mesocosms; we assayed technical-grade glyphosate acid at four concentrations: 0, 1, 3 and 6 mg gly L^{-1} under scenarios with and without mussels. The addition of the glyphosate significantly increased total phosphorus according to the concentration used; the high clearance rate of *L. fortunei* significantly decreased phytoplanktonic abundance leading to low values of turbidity. The mussel significantly stimulated the development of filamentous green algae (metaphyton). Interestingly, the combined effect revealed that *L. fortunei* accelerated the dissipation of glyphosate, which showed a 4-fold decrease in its half-life; this promoted the rapid bioavailability of glyphosate-derived phosphorus in the water. The interaction had a synergistic effect on soluble reactive phosphorus concentrations and was directly dependent on the concentration of glyphosate. A synergistic effect was also observed on bacterioplankton, water turbidity and metaphyton, thus inducing enhanced and rapid eutrophication. The ability of mussels to reduce glyphosate in water may be valued as positive, but our results allow us to predict that the invasion of *Limnoperna fortunei* in natural freshwater systems contaminated by glyphosate will accelerate the negative impact of the herbicide associated with eutrophication.

Keywords Glyphosate · *Limnoperna fortunei* · Anthropogenic stressors · Outdoor mesocosms · Eutrophication · Synergism

Introduction

Anthropogenic stressors act on the environment at different scales, and their simultaneous interaction affects ecosystems (Matthaei et al. 2010). In this sense, the study of the joint effect of multiple anthropogenic stressors is important because the emerging consequences are often unpredictable on the basis of knowledge of single effects (Townsend et al. 2008). This information is valuable for the understanding of the actual anthropogenic impact on ecosystem goods and services, and the estimation of associated costs. The interaction of multiple stressors may have additive effects or generate complex outcomes, including synergistic or antagonistic responses of the different ecosystem variables (Folt et al. 1999; Piggott et al. 2012). Freshwaters appear to be at particular risk of multiple-stressor effects, due to the numerous uses of water and the fact that controls and environmental protection are

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often unusual (Ormerod et al. 2010). Moreover, they act as conduits or sinks that accumulate and concentrate contaminants (Kelly et al. 2010). Invasive species and contamination were regarded by the Millenium Ecosystem Assessment (2005) as anthropogenic stressors strongly affecting freshwater ecosystems.

Glyphosate [N-(phosphonomethyl)glycine] is a nonselective, broad spectrum, post-emergent agrochemical used worldwide, mainly for weed control (Goldsborough and Brown 1988). Glyphosate's primary mode of action in plants and several microorganisms is the disruption of aromatic amino acid biosynthesis, through the inhibition of the enzyme 5-enolpyruvyl shikimic acid-3-phosphate synthase (EPSPS), which halts the production of chorismate (Amrhein et al. 1980). In 2011, the worldwide application of glyphosate products was estimated to be about 650,000 tons (CCM International 2011). In South America, shallow water bodies receive a significant amount of this herbicide. It is particularly the case for those related to the basin of the Paraná-Uruguay-de La Plata rivers, due to both intensive cultivation of GM soybean and no-till practice, which rocketed in the mid-1990s. Glyphosate may reach aquatic systems either by accidental or wind driven drift of the herbicide spray, or through transport in surface runoff and suspended particulate matter (Feng et al. 1990). Glyphosate is usually assumed to be safe and non-toxic to the environment due to its fast biodegradation and/or adsorption by soil particulates. Nevertheless, some studies have demonstrated important off-target displacements of glyphosate, producing structural and functional changes in the freshwater biota, consistent with decreased water quality (Pérez et al. 2007; Vera et al. 2010).

On the other side, the golden mussel *Limnoperna fortunei* (Dunker) is a freshwater mytilid bivalve native to mainland China which constitutes a major fouling pest in Asia. It was accidentally introduced into the Paraná-Uruguay drainage basin through the ballast water of commercial ships (Boltovskoy and Cataldo 1999), under conditions of increased economic globalization and trade between continents during the early 1990s (Karatayev et al. 2006). Along its entire range, *L. fortunei* has become a major nuisance for industrial and power plants that use river water. Today, after only 21 years of its first record in South America (Pastorino et al. 1993), this mussel largely dominates the benthic fauna of almost the entire Paraná-Uruguay-de la Plata river system (Argentina, Uruguay, Brazil and Paraguay), reaching densities of over 200,000 ind m^{-2} (Boltovskoy et al. 2006). Adults show high filtration rates with values of about 100 mL ind^{-1} h^{-1} (Cataldo et al. 2012a), thus exerting a negative impact on the structure and function of the ecosystem (Boltovskoy et al. 2009; Cataldo et al. 2012b; Sylvester et al. 2005). These findings constitute strong evidence that *L. fortunei* invasion is an important stressor and force of change for freshwater communities.

In South America, glyphosate and *L. fortunei* were introduced simultaneously but their combined effect on freshwater has never been tested at the ecosystem scale. In a laboratory experiment, Di Fiori et al. (2012) demonstrated that *L. fortunei* is capable of degrading glyphosate and hypothesized that its occurrence in waters contaminated with this herbicide would speed up the bioavailability of phosphorus for autotrophs, facilitating water eutrophication.

The objective of the present study was to experimentally explore the joint impact of glyphosate and *L. fortunei* on structural features of freshwater phytoplankton, bacterioplankton and periphyton, and the physical and chemical properties of the water. The two stressors were manipulated simultaneously in a 25-day experiment using outdoor mesocosms, to evaluate their single and combined effects on the microscopic communities and the water quality of the systems. We tested four concentrations of glyphosate in scenarios with and without mussels to analyze the potential concentration-dependent responses. We hypothesized that the effect of these agents on the physical, chemical and biological variables depends on whether they act alone or in combination.

Materials and methods

Experimental design

We carried out an experiment using three outdoor pools (3000 L) containing eight 70-L polythene bags (mesocosms) each, during February 2011. Six months before the beginning of the experiment, the pools were filled with underground water, and bottom not rooted aquatic plants as propagule source were added to generate the environmental conditions of natural systems (phytoplankton chlorophyll $a \approx 10$ μg L^{-1}). Eight periphytometers (special devices holding artificial substrata for periphyton analysis) were placed inside each pool and left for substantial colonization for 30 days before the experiment began.

At t_0, we filled the mesocosms with water from each pool and placed a colonized periphytometer inside each mesocosm (Fig. 1).

Glyphosate

We assayed technical-grade glyphosate acid (gly) (≥95 % purity; CAS: 1071-83-6) at four concentrations: 0, 1, 3, and 6 mg $glyL^{-1}$. These nominal concentrations of glyphosate were chosen for being similar to that recommended for aquatic and terrestrial weed control (3.7 mg $glyL^{-1}$, Giesy

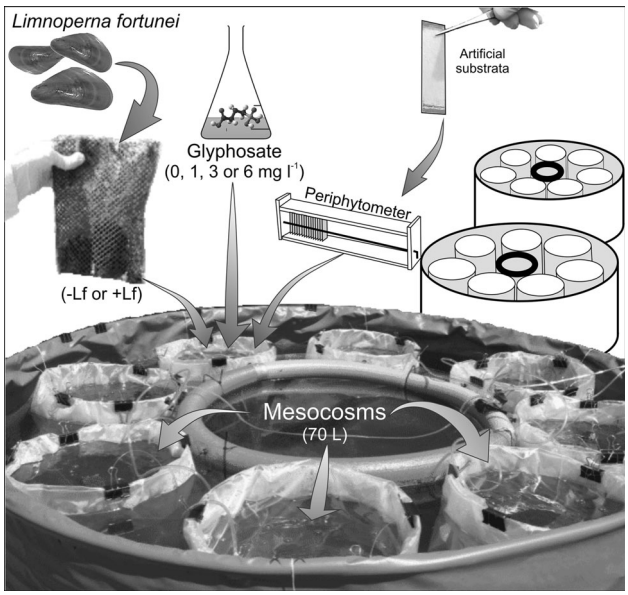

Fig. 1 Scheme of the experimental design showing the outdoor mesocosms, a cage with *Limnoperna fortunei*, a periphytometer and an artificial substratum for periphyton colonization

et al. 2000), and lie within the range of "worst-case" scenarios of glyphosate contamination in freshwater reviewed by Relyea (2006) (from 1.4 (Canadian government) to 10.3 mg $glyL^{-1}$ (Mann and Bidwell 1999)).

Limnoperna fortunei

Individuals of *L. fortunei* (Lf) were manually collected on the banks of the Río de La Plata river located near Buenos Aires City, Argentina. They were placed in aquaria containing dechlorinated water with continuous air supply and fed baby fish food daily. Groups of 350 ± 15 live individuals (≈120 g wet weight) were enclosed in small cages of plastic mesh with 5-mm pore size. One cage with mussels was randomly assigned to each of four microcosms in the pool (+Lf), while the other microcosms remained without mussels (−Lf).

At t_0, the different concentrations of glyphosate were added to randomly selected mesocosms at each pool and the cages with *L. fortunei* were submerged to about 20 cm depth. Water measurements, samples and artificial substrata were taken from each mesocosm at baseline and at days 1, 7, 14 and 25 (t_0 to t_4, respectively).

Physical and chemical response parameters

Water temperature, pH, conductivity and dissolved oxygen were measured in situ with a Hach® sension 156 meter, while nephelometric turbidity was measured with a 2100P Hach® turbidimeter. Water samples for chemical analysis were poured into polypropylene containers and immediately transported to the laboratory. Water was immediately filtered through Whatman® GF/F filters. Soluble reactive phosphorus (SRP) was determined by the molybdate-ascorbic method; Total phosphorus (TP, from unfiltered water samples) was converted to SRP after acid digestion with potassium persulfate; SRP and TP were analyzed following APHA (2005). Total nitrogen (TN) was estimated from unfiltered samples by Cd reduction (Mackereth et al. 1978) after digestion with potassium persulfate (APHA 2005).

Glyphosate was determined by ion chromatography using a DIONEX DX-100 chromatograph with a conductivity detector and a 25-ml sample loop (Pessagno et al. 2008). A DIONEX AS-4 was used as an analytical chromatographic column with an experimental error below 5 %; a mixture of $NaOH/Na_2CO_3$ 4 mM/9 mM was chosen as eluent with a flow rate of 2 mL min^{-1}. The glyphosate dissipation rate (k) and half-life in water were estimated using $C_t = C_0e^{-kt}$, where C_t is the concentration at time t and C is the initial concentration (Di Fiori et al. 2012).

Biological response parameters

Phytoplankton

Before the onset of the experiment (t_0), qualitative samples were taken from each pool by means of a 15-µm pore net. On every sampling date, subsurface water samples for quantitative analysis were taken using 100 to 200-mL flasks and fixed with 1 % acidified Lugol's iodine solution. Counts of micro (>20 µm) and nanophytoplankton (2–20 µm) were made using the inverted microscope technique (Utermöhl 1958) at ×400 magnification, with a counting error of <15 %, estimated according to Venrick (1978). The number of dead algae per sample was also estimated; algae were considered dead when they had disorganized chloroplasts and/or broken frustules at the microscope level (Vera et al. 2010).

Samples for chlorophyll *a* (Chl-*a*) analysis were taken in polypropylene flasks and transported to the laboratory under cold and dark conditions. The Chl-*a* concentration was estimated from a known water volume, vacuum filtered under low light conditions through Whatman® GF/F filter paper and stored at −20 °C. Pigments were extracted with ethanol (60–70 °C) and determined spectrophotometrically according to Nusch (1980).

Bacterioplankton

Water samples were preserved in 2 % ice-cold glutaraldehyde and filtered through 0.2-µm-pore size black polycarbonate filters (Isopore GTPB 02500; Isopore,

Billerica, Massachusetts, USA) stained with DAPI (Porter and Feig 1980); bacteria were quantified using an epifluorescence microscope (Olympus BX40) and a minimum of 20 fields and 400 individuals were counted on each slide (error <15 %) (Vera et al. 2012).

Periphyton

Each periphytometer included vertical artificial substrata consisting of rectangular strips of clear polycarbonate with a total area of 29.1 cm^2. The following variables were measured: chlorophyll *a* concentration (P-Chl *a*), dry weight (DW), ash-free dry weight (AFDW) and habit of algal taxa. For P-Chl *a* measurements, the periphyton was scraped from one substratum and pigment was quantified as described for phytoplankton. DW was estimated from the material on another substratum, which was filtered through Whatman® GF/C filters pre-combusted to 550 °C for 2 h prior to use and later weighing the material dried at 60 °C on a stove up to constant weight. AFDW was determined as the mass difference after 2 h of calcination (550 °C) of dry samples (APHA 2005). The autotrophic index (AI) was estimated as the AFDW: chlorophyll *a* ratio. AI values between 100 and 200 indicate high proportion of autotrophs and AI values higher than 200 indicate high proportion of heterotrophic, nonchlorophyllous organisms or organic detritus (APHA 2005). Differences in percentage of filamentous and coccoid algae were analyzed for t_0 and t_4 from scrapped periphytic material. Samples were obtained from one substratum and fixed with 1 % acidified Lugol's iodine solution. Filamentous and coccoid algae were counted using the inverted microscope technique (Utermöhl 1958) at ×400 magnification with a counting error of <15 %, estimated according to Venrick (1978). All the periphytic variables were expressed on an area basis.

Limnoperna fortunei

At the end of the experiment, the cages with *L. fortunei* were removed; organisms were counted and measured using a digital caliper (Sylvac Brand) with an accuracy of 0.01 mm. Dead organisms (i.e. open shells, no soft tissue) per mesocosm were counted at t_4.

Data analysis

For all variables, except for periphytic algal abundance, differences between treatments were assessed using repeated-measures analysis of variance (RM ANOVA), with two factors: glyphosate concentration, 4 levels; *L. fortunei*, 2 levels and five sampling times (t_0–t_4). RM ANOVA was followed by post hoc Tukey's multiple comparisons tests ($P < 0.05$). For dissipation rate constant of glyphosate and abundance periphytic algae (at t_4), differences between treatments were assayed using one-way analysis of variance (one-way ANOVA). Prior to each analysis, Kolmogorov–Smirnov tests (with Lilliefors' correction), Levene median tests and Mauchley Sphericity tests were run to test normality, homoscedasticity and sphericity, respectively. Whenever the data did not conform, the values were transformed (square-root or log) as necessary.

Results

Limnoperna fortunei

The percentage of dead mussels (<2 %) was non-significant at t_4 for all treatments. Moreover, at t_4 valve length differences were not significantly different for any of the treatments, thus indicating absence of growth.

Glyphosate dissipation

The concentration of glyphosate decreased gradually in all herbicide treatments during the experimental period. In treatments without *L. fortunei* (Fig. 2a), the decrease was significant at t_1 for 3−Lf and 6−Lf (RM ANOVA $P < 0.01$ for each comparison), with final mean concentrations of 1.75 ± 0.03 mgL^{-1} and 3.43 ± 0.6 mgL^{-1}, respectively, representing a dissipation of 41 and 43 % from t_0, respectively. The dissipation was lower for 1−Lf, reaching a mean of 0.43 ± 0.1 mgL^{-1} at t_4. Treatments with *L. fortunei* led to dramatic dissipation of glyphosate, with a decay of 100 % for 1+Lf and 86 % for 3+Lf and 6+Lf (Fig. 2b). Mean glyphosate concentrations at t_4 were 0.41 ± 0.13 mgL^{-1} and 0.84 ± 0.10 mgL^{-1} for 3+Lf y 6+Lf, respectively, with significant differences between them (RM ANOVA $P < 0.05$). Assuming first-order kinetics, the estimated dissipation rate constant (k) was significantly higher (one-way ANOVA $p < 0.01$) in presence of *L. fortunei*. Mean half-lives were 33.4 ± 4.3 d (−Lf) ($k = 0.022 \pm 0.002$ mgd^{-1}) and 8.7 ± 0.6 d (+Lf) ($k = 0.081 \pm 0.004$ mgd^{-1}). Mussels reduced glyphosate half-life by ≈74 %.

Table 1 shows the mean values of the physical, chemical and biological variables measured for all treatments at t_0 and t_4. For better understanding of effects, some variables were plotted considering the following treatments: "Lf": 0−Lf and 0+Lf; "Gly": 0−Lf; 1−Lf; 3−Lf and 6−Lf and "GlyxLf": 0+Lf; 1+Lf; 3+Lf and 6+Lf (Figs. 2 and 3).

Physical and chemical response parameters

Initial mean dissolved oxygen concentration (DO) ranged between 8.8 ± 0.17 and 9.1 ± 0.26 mgL^{-1}, with values at t_4 being higher in mesocosms with *L. fortunei* (RM

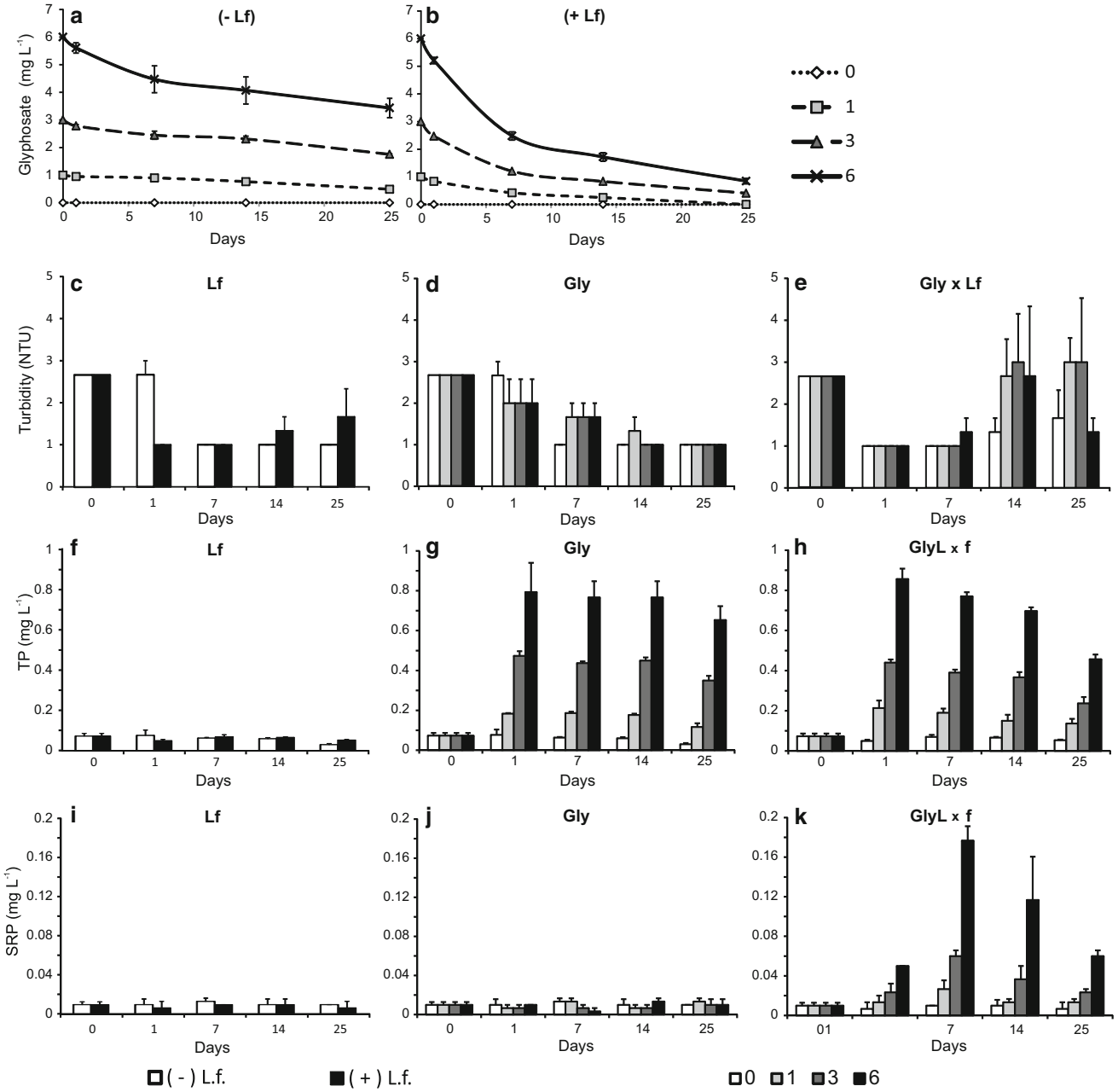

Fig. 2 **a**-**k** Averages ± 1SD of glyphosate in mesoscosms **a** without *L. fortunei* (−Lf) and **b** with (+Lf) for glyphosate concentrations (0, 1, 3 and 6 mg L^{-1}) throughout the study period. Averages +1SD c-e) of turbidity; **f**–**h** total phosphorus (TP) and **i**–**k** soluble reactive phosphorus (SRP). *Lf Limnoperna fortunei* effect, *Gly* glyphosate effect, *Gly* × *Lf* Glyphosate–L. *fortunei* interaction. n = 3 for all cases

ANOVA $P < 0.05$) (Table 1). Neither glyphosate alone nor the Gly-Lf interaction had any effect on DO. Mean values of pH at t_0 ranged between 6.9 ± 0.46 and 7.1 ± 0.28 (Table 1). *Limnoperna fortunei* caused alkalization, with significantly different values at t_4 (7.6 ± 0.95 and 9.1 ± 0.7 for 0−Lf and 0+Lf, respectively) (RM ANOVA $P < 0.01$). The effect of glyphosate on pH was statistically significant from t_1 onward, with 0-Lf showing significantly lower values compared to treatments with herbicide (RM ANOVA $P < 0.05$). The Gly-Lf interaction had no effect on the pH. Water temperature at t_0 varied from 24.8 ± 0.21 to 25.2 ± 0.36 °C for all mesocosms. Values of conductivity at t_0 averaged 140.1 ± 1.0 μS cm^{-1} for all mesocosms; *L. fortunei* clearly decreased conductivity at t_4 (RM ANOVA $P < 0.01$). No effect of glyphosate was evident and the

Table 1 Average ± 1 SD of the physicochemical and biological variables recorded at t_0 and t_4 for treatments without (−) and with (+) *Limnoperna fortunei* and glyphosate concentrations (0, 1, 3and 6 mg L^{-1})

Limnoperna fortunei	Glyphosate (mg L^{-1})							
	0		1		3		6	
	−	+	−	+	−	+	−	+
Dissolved oxygen (mg L^{-1})								
t_0	9.1 ± 0.17	9.1 ± 0.26	9.1 ± 0.11	9.0 ± 0.15	9.0 ± 0.20	8.8 ± 0.17	8.9 ± 0.06	8.9 ± 0.21
t_4	8.8 ± 0.15	9.4 ± 1.21	9.3 ± 0.53	9.9 ± 0.3	9.4 ± 0.23	10.2 ± 1.25	9.2 ± 0.40	10.1 ± 040
pH								
t_0	6.9 ± 0.46	7.0 ± 0.38	7.1 ± 0.25	7.1 ± 0.28	7.0 ± 0.44	6.9 ± 0.63	7.0 ± 0.31	7.0 ± 0.21
t_4	7.6 ± 0.95	9.1 ± 0.70	9.0 ± 0.21	9.1 ± 0.43	8.9 ± 0.10	9.0 ± 0.25	9.0 ± 0.21	8.9 ± 0.0
Water temperature (°C)								
t_0	25.0 ± 0.45	25.0 ± 0.25	25.2 ± 0.35	25.0 ± 0.25	25.2 ± 0.36	24.9 ± 0.11	25.2 ± 0.35	24.8 ± 0.21
t_4	21.7 ± 0.35	22.0 ± 0.32	21.8 ± 0.45	21.8 ± 0.32	21.8 ± 0.26	21.8 ± 0.42	21.9 ± 0.20	21.8 ± 0.20
Conductivity (μS cm^{-1})								
t_0	140 ± 2.38	140 ± 2.12	140 ± 2.03	140 ± 2.52	140 ± 2.21	140 ± 1.78	140 ± 1.94	141 ± 2.18
t_4	141 ± 1.71	128 ± 5.06	140 ± 1.09	132 ± 3.67	139 ± 3.61	130 ± 5.73	138 ± 1.54	128 ± 4.56
Turbidity (NTU)								
t_0	2.7 ± 0.58	2.7 ± 0.58	2.7 ± 0.58	2.7 ± 0.58	2.7 ± 0.58	2.7 ± 0.58	2.7 ± 0.58	2.7 ± 0.58
t_4	1.0 ± 0.0	1.7 ± 1.15	1.0 ± 0.0	3.0 ± 1.0	1.0 ± 0.0	3.0 ± 1.0	1.0 ± 0.0	1.3 ± 0.58
TP (mg L^{-1})								
t_0	0.07 ± 0.02	0.07 ± 0.02	0.07 ± 0.02	0.07 ± 0.02	0.07 ± 0.02	0.07 ± 0.02	0.07 ± 0.02	0.07 ± 0.02
t_4	0.03 ± 0.01	0.05 ± 0.01	0.12 ± 0.03	0.14 ± 0.04	0.35 ± 0.04	0.24 ± 0.05	0.65 ± 0.12	0.46 ± 0.04
SRP (mg L^{-1})								
t_0	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
t_4	0.01 ± 0.0	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.06 ± 0.01
TN (mg L^{-1})								
t_0	4.93 ± 1.93	4.93 ± 1.93	4.93 ± 1.93	4.93 ± 1.93	4.93 ± 1.93	4.93 ± 1.93	4.93 ± 1.93	4.93 ± 1.93
t_4	2.05 ± 1.13	0.77 ± 0.08	1.09 ± 0.69	1.97 ± 0.93	0.90 ± 0.28	1.22 ± 0.39	1.45 ± 1.20	1.63 ± 1.10
Bacterioplankton								
t_0	1.92 ± 1.09	1.83 ± 1.10	1.92 ± 1.09	1.97 ± 0.98	1.92 ± 1.09	1.63 ± 1.12	1.92 ± 1.09	1.99 ± 1.04
Abundance (10^6 ind mL^{-1})								
t_4	7.9 ± 1.58	7.37 ± 3.72	6.16 ± 1.0	14.6 ± 5.3	6.12 ± 2.08	13.5 ± 2.24	6.31 ± 1.91	10.4 ± 4.33
Phytoplankton Chl*a* (μg L^{-1})								
t_0	9.07 ± 4.12	9.07 ± 4.12	9.07 ± 4.12	9.07 ± 4.12	9.07 ± 4.12	9.07 ± 4.12	9.07 ± 4.12	9.07 ± 4.12
t_4	1.63 ± 0.77	6.90 ± 8.24	2.18 ± 1.08	35.9 ± 11.5	5.13 ± 3.33	40.3 ± 59.4	2.50 ± 1.66	7.90 ± 3.49
Phytoplankton								
t_0	18.6 ± 6.54	18.6 ± 6.54	18.6 ± 6.54	18.6 ± 6.54	18.6 ± 6.54	18.6 ± 6.54	18.6 ± 6.54	18.6 ± 6.54
Abundance (10^3 ind mL^{-1})								
t_4	2.4 ± 0.60	1.34 ± 0.44	19.2 ± 9.09	2.0 ± 0.27	16.7 ± 6.91	1.43 ± 0.99	16.5 ± 6.45	1.41 ± 0.86
Periphyton DW (mg cm^{-2})								
t_0	0.19 ± 0.06	0.19 ± 0.08	0.24 ± 0.10	0.22 ± 0.07	0.29 ± 0.13	0.16 ± 0.11	0.18 ± 0.10	0.20 ± 0.13
t_4	0.41 ± 0.13	0.47 ± 0.11	0.65 ± 0.22	0.43 ± 0.21	0.47 ± 0.01	0.34 ± 0.23	0.23 ± 0.07	0.38 ± 0.14
Periphyton AFDW (mg cm^{-2})								
t_0	0.18 ± 0.06	0.18 ± 0.06	0.21 ± 0.07	0.20 ± 0.06	0.26 ± 0.10	0.14 ± 0.09	0.16 ± 0.08	0.18 ± 0.11
t_4	0.36 ± 0.12	0.42 ± 0.09	0.60 ± 0.19	0.39 ± 0.19	0.43 ± 0.22	0.31 ± 0.20	0.20 ± 0.05	0.35 ± 0.12
Periphyton Chl*a* (μg cm^{-2})								
t_0	0.30 ± 0.25	0.19 ± 0.15	0.16 ± 0.14	0.24 ± 0.17	0.27 ± 0.21	0.20 ± 0.19	0.28 ± 0.21	0.17 ± 0.12
t_4	0.33 ± 0.14	1.11 ± 0.63	1.22 ± 0.53	1.31 ± 0.93	0.93 ± 0.10	1.20 ± 0.71	0.65 ± 0.25	1.38 ± 0.35

Table 1 continued

Limnoperna fortunei	Glyphosate (mg L^{-1})							
	0		1		3		6	
	−	+	−	+	−	+	−	+
AI								
t_0	1458 ± 1504	1499 ± 1437	1915 ± 1374	1240 ± 1091	1559 ± 1456	791 ± 312	783 ± 623	891 ± 107
t_4	1175 ± 851	398 ± 232	443 ± 99	285 ± 84	397 ± 53	208 ± 49	279 ± 65	211 ± 36

n = 3 for all cases

TP total phosphorus, *SRP* soluble reactive phosphorus, *TN* total nitrogen, *DW* dry weight, *AFDW* ash free dry weight, *AI* autotrophic index

Gly-Lf interaction did not show significant differences compared to single-stressor effects (Table 1).

At t_1, *L. fortunei* produced a significant decrease in turbidity (RM ANOVA $P < 0.01$), followed by an increasing trend (Fig. 2c; Table 1). Glyphosate produced a slight, non-significant increase in turbidity at t_1 (Fig. 2d). Despite the lack of significance, a trend toward a synergistic effect of stressors was observed at t_3 for all glyphosate concentrations. At the end of the experiment, there was a decrease in turbidity for 6+Lf, thus indicating an additive effect of stressors, while the synergistic trend continued until t_4 for 1+Lf and 3+Lf (Fig. 2e).

Mean initial value of TP for all mesocosms was 0.07 ± 0.02 mgL^{-1}, a concentration typical of meso-eutrophic waters (Wetzel 2001). There were no significant differences by *L. fortunei* (Fig. 2f; Table 1). In contrast, glyphosate had an increasing effect on TP, reaching mean values of 0.18 ± 0.01, 0.47 ± 0.02, and 0.79 ± 0.15 mgL^{-1} at t_1 for 1−Lf, 3−Lf and 6−Lf respectively, to remain relatively constant thereafter (Fig. 2g) (RM ANOVA $P < 0.05$ for 3−Lf and 6−Lf with respect to 0-Lf for all dates). The trend for the Gly-Lf interaction was similar to that recorded for glyphosate alone (Fig. 2h) but with significant decreases of 23.5 % and 46.5 % from t_1 to t_4 for 3+Lf and 6+Lf (0.24 ± 0.05 and 0.46 ± 0.04 mgL^{-1} at t_4, respectively) (RM ANOVA $P < 0.05$) (Table 1).

Mean concentration of SRP at t_0 was 0.01 ± 0.01 mg $P\text{-}PO_4L^{-1}$ (Table 1). No single-factor effects were found on SRP concentrations throughout the study period (Figs. 2i, j). The Gly-Lf interaction was evident since SRP concentrations varied according to the concentration of glyphosate used (Fig. 2k). The values of SRP peaked at t_2, followed by a gradual decline, with mean values at t_4 of 0.01 ± 0.01, 0.02 ± 0.01 and 0.06 ± 0.01 mg $P\text{-}PO_4L^{-1}$ for 1+Lf, 3+Lf and 6+Lf, respectively (Fig. 2k; Table 1). Differences among treatments were statistically significant (RM ANOVA $P < 0.01$).

Mean concentration of NT at t_0 was 2.9 ± 0.01 mgL^{-1}, which is limiting for phytoplankton development (optimal concentration: ≈0.1 mgL^{-1}, Reynolds 2006). No clear trends were found for single-factor effects or Gly-Lf interaction (Table 1).

Biological response parameters

Mean abundance of bacterioplankton was 1.8×10^6 ± 0.9 $indL^{-1}$. A significant variation in bacterioplankton abundance was found among pools as reflected by the high SD obtained at t_0 and t_4 (Table 1). To avoid this variability, we plotted the abundances of bacteria as a percentage of the initial value for each mesocosm at each sampling date. In this way, we found a trend toward increased bacterioplankton abundance related to t_0, with and without *L. fortunei*, with significant differences from t_2 onward (RM ANOVA $P < 0.05$) (Fig, 3a). This variable did not vary significantly by the addition of glyphosate, regardless of the concentration used (Fig. 3b). The Gly-Lf interaction showed differences compared to the single-factor effects at t_4, when there was a synergistic effect for 1+Lf and 3+Lf (increases of 966 and 1122 %, respectively) and an additive effect for 6+Lf (increase of 667 %) (Fig. 3c) (RM ANOVA $P < 0.05$ with respect to 0+Lf).

Mean phytoplanktonic Chl-*a* concentration was 5.1 ± 1.0 μg L^{-1} at t_0 considering all treatments (Table 1). *L. fortunei* produced a sharp drop in phytoplanktonic Chl-*a* (RM ANOVA $P < 0.05$) at t_1, followed by an increase up to 15.2 ± 0.9 μg L^{-1} (0+Lf) at t_3. Value of 0+Lf was significantly higher than the value of 0−Lf at t_3 (RM ANOVA $P < 0.05$) (Fig. 3d). At t_4, phytoplanktonic Chl-*a* decreased in 0+Lf, reaching a value similar to that in 0−Lf. Glyphosate led to non-significant differences among treatments (Fig. 3e), but there was a gradual decrease up to 1.1–2.5 μg L^{-1} at t_4, except for 3−Lf where values remained unchanged (4–5.5 μg L^{-1}). The interaction effect showed a pattern similar to that obtained with *L.*

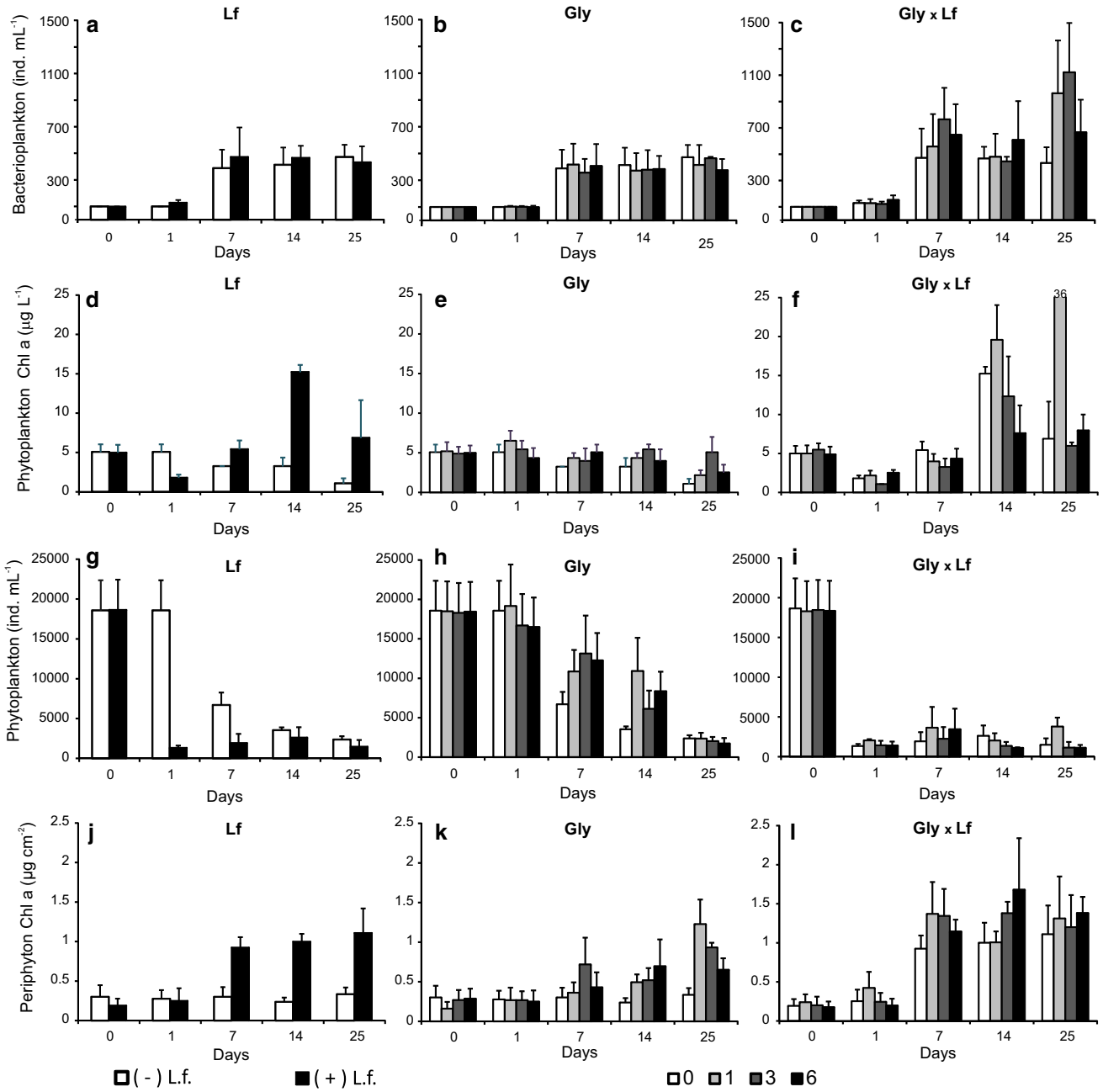

Fig. 3 **a–l** Averages +1SD of **a–c** bacterioplankton; **d–f** phytoplankton chlorophyll *a*; **g–i** phytoplankton abundance and **j–l** periphyton chlorophyll *a,* for glyphosate concentrations (0, 1, 3 and 6 mg L^{-1}) throughout the study period. *Lf Limnoperna fortunei* effect, *Gly* glyphosate effect, *Gly* × *Lf* Glyphosate–*L. fortunei* interaction. n = 3 for all cases

fortunei, with a marked decay at t_1 followed by an increase up to the initial values (Fig. 3f). Then, phytoplanktonic Chl-*a* increased until t_3, reaching 19.6 ± 4.4 µg L^{-1} (1+Lf); 12.3 ± 5.1 µg L^{-1} (3+Lf) and 7.6 ± 3.6 µg L^{-1} (6+Lf). A maximum of 36.0 ± 6.6 µg L^{-1} was recorded in 1+Lf at t_4 which differed significantly from the other treatments (RM ANOVA $P < 0.05$).

Mean phytoplankton abundance was $18.6 \times 10^3 \pm 3.7 \times 10^3$ ind mL^{-1} at t_0; all treatments showed a decreasing trend toward t_4 (Table 1), when 20 ± 5 % of the individuals were dead or injured. The above-mentioned decrease in turbidity caused by *L. fortunei*, which was observed with the naked eye at t_1, was probably due to the decline in phytoplankton abundance (i.e. 93 % of the initial value). Abundances for 0+Lf were statistically lower (RM ANOVA $P < 0.05$) than 0−Lf at t_1 and t_2 (Fig. 3g), with values ≈5000 ind mL^{-1} that remained almost constant toward t_4. Mussels induced a gradual decrease in phytoplankton abundances, with a significant drop at t_1 (RM ANOVA $P < 0.05$); *L. fortunei* decreased the percentage

of dead algae up to 8.2 ± 2.4 % of dead ind mL^{-1} at t_2, reaching values about 2.4 ± 1.7 % toward t_4 (Fig. 3g). Glyphosate caused a lower decrease in phytoplankton abundance compared to the control (0−Lf), with no effect on algal mortality, ranging between 7 and 21 % (Fig. 3h). The interaction effect followed a pattern similar to that observed for *L. fortunei* alone, with a lower decrease in algal mortality (0–2 %) (Fig. 3i).

Periphytic DW was not significantly impacted by single-factor effects. Initial mean value ranged from 0.16 ± 0.11 to 0.29 ± 0.13 $mgcm^{-2}$ at t_0 (Table 1). *L. fortunei* produced a trend toward an increase in DW compared to 0−Lf at t_4 (0.41 ± 0.14 and 0.47 ± 0.11 $mgcm^{-2}$ for 0−Lf and 0+Lf, respectively). Glyphosate also produced a trend toward increasing DW throughout the study period. The interaction effect was similar to the effects of individual stressors (Table 1). Mean AFDW values ranged from 0.14 ± 0.09 to 0.20 ± 0.10 $mgcm^{-2}$ at t_0, with an increasing trend toward t_4 (Table 1). No Gly-Lf interaction or single-factor effects were observed on this variable. Mean periphytic chlorophyll *a* concentration varied from 0.17 ± 0.12 and 0.30 ± 0.25 μg cm^{-2} at t_0. The presence of *L. fortunei* produced significant increases (RM ANOVA $P < 0.01$), reaching values from 1.1 ± 0.63 to 1.38 ± 0.35 μg cm^{-2} at t_4 (Fig. 3j; Table 1). Glyphosate generated a slight increase at t_2 in all treatments; maximum concentrations were found at t_4; with mean values of 1.22 ± 0.53 μg cm^{-2} (1−Lf); 0.93 ± 0.10 μg cm^{-2} (3−Lf) and 0.65 ± 0.25 μg cm^{-2} (6−Lf) (Fig. 3k). There was a trend toward a synergistic effect of the Gly-Lf interaction, which was most clearly evidenced at t_3 (Fig. 3l). Mean initial values of the AI ranged from 783 ± 23 to 915 ± 1374 at t_0 (Table 1), typical of heterotrophic communities. A marked trend toward a decrease in AI (increased autotrophy) was observed under scenarios involving *L. fortunei* and glyphosate separately. The interaction of stressors resulted in a higher degree of autotrophy evidenced at t_4 at higher glyphosate concentrations (Table 1).

The analysis of the habit of periphytic algae always showed the dominance of coccoids over filamentous organisms. *L. fortunei* led to an increase in the relative abundance of filamentous algae throughout the study period, with values between 28 and 37 % (RM ANOVA $P < 0.05$) (Fig. 4a). These results are consistent with the appearance of large clumps of *Oedogonium* spp. in the water column. Such masses constitute the "metaphyton" (Stevenson 1996), composed of microscopic filamentous algae which are attached to substrata during early stages of development. A semi-quantitative scale of abundance based on percentage of total metaphyton coverage was applied to each mescocosms at t_4: (0) absent; (1) up to 25 %; (2) 25–50 %; and (3) more than 50 % of coverage (Fig. 4a, b); a non-parametric Kruskal–Wallis two-way ANOVA was used to test for statistical differences among treatments. Metaphyton development was higher for 0+Lf than for 0−Lf (KW ANOVA $P < 0.01$); glyphosate (1−Lf; 3−Lf; 6−Lf) produced higher coverage than the control (0−Lf) (KW ANOVA $P < 0.01$). Metaphyton coverage was highest under multiple-stressor scenarios (0+Lf vs. 1+Lf; 3+Lf; 6+Lf) (KW ANOVA $P < 0.05$) (Fig. 4a, b).

Discussion

Despite the general consensus about the importance of studying the impact of glyphosate on freshwater, only a few investigations have focused on the effect of its interaction with other anthropogenic stressors (e.g. Magbanua et al. 2013). In Argentina, glyphosate became commercially available almost simultaneously with the appearance of the invasive golden mussel *Limnoperna fortunei*. Most studies concerning changes in the structure and function of freshwater ecosystems in the region have examined the effects of each stressor separately. In our research using outdoor mesocosms as proxy of natural environmental conditions, we demonstrated that the impact of their interaction on freshwater is hardly predictable from the knowledge of single-factor effects because they act synergistically on some response parameters.

The herbicide concentration was reduced in all treatments during the experimental period. Laboratory and field studies have demonstrated that the main mechanism of glyphosate removal from water is adsorption to suspended particulates followed by subsequent sedimentation and/or biodegradation (Zaranyika and Nyandoro 1993). Adsorption of glyphosate increases with high amount of suspended solids, decreases with the increase of pH values, inorganic phosphorus content and cation concentration (Glodsborough and Beck 1989). Moreover, the sediment composition also is relevant for the adsorption of the herbicide, as clay minerals rich in Fe^{3+} and Al^{3+} that have greater capability to retain the herbicide (Khoury et al. 2010; Vereecken 2005). Also, the composition and general characteristics of the biota may be determinant for the biodegradation of the herbicide. As a result, different half-life values in water were registered, ranging between 7 and 100 days and even higher (Goldsborough and Brown 1993; Mercurio et al. 2014). Previous experiments carried out in outdoor mesocosms resembling typical water bodies of Pampa plain in Argentina, registered half-life values of glyphosate ranging 4–16 days (Pérez et al. 2007; Vera et al. 2010 and 2012). In the present experiment (in scenarios without *L. fortunei*) the persistence of the herbicide was higher, probably due to the different physical, chemical and biological characteristics of the water. Moreover, the mesocosms were

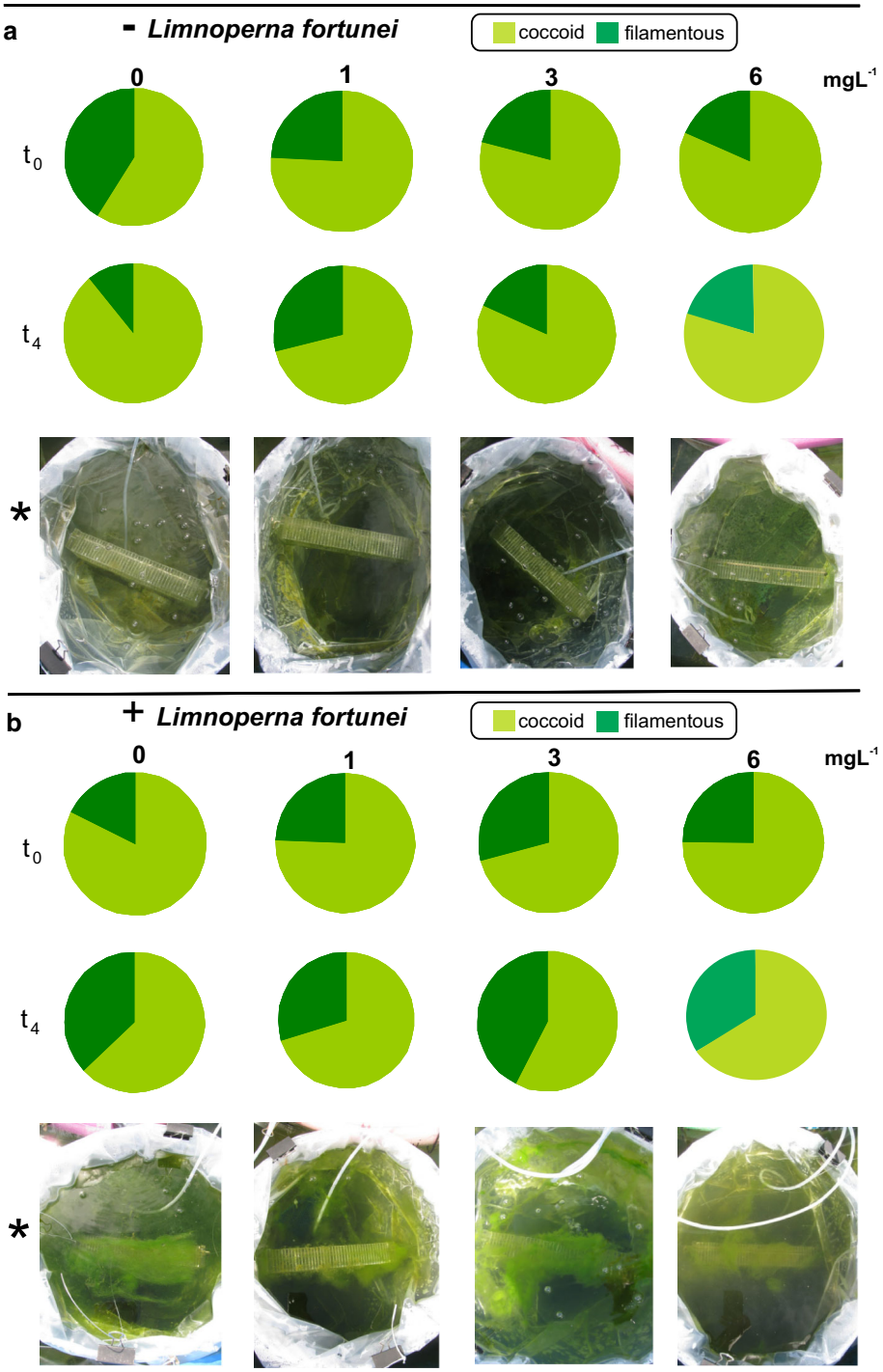

Fig. 4 **a**, **b** Relative abundance of cocooid and filamentous habit in periphyton algae at t_0 and t_4 for glyphosate concentrations in scenarios **a** without (−) and **b** with (+) *Limnoperna fortunei*. *Asterisk* pictures of mesocosms at t_4

designed without bottom sediments, which probably contributed to the difference in the dissipation rate.

The main direct effect of glyphosate on the water consisted of a strong modification in some chemical properties. Taking into account that the herbicide is a phosphonate, the addition of glyphosate resulted in a significant increase of TP in the water column according to its concentration; the measured amounts of glyphosate always sufficed to explain the increase in TP observed in the treated mesocosms. A similar result has been previously reported for assays conducted in outdoor mesocosms using either pure glyphosate or different glyphosate-based formulations (Pérez et al. 2007; Vera et al. 2010 and 2012). The other studied physical and chemical properties of the water were not significantly affected by glyphosate addition, except for pH, which showed a significant increase toward the end of

the experiment probably due to enhanced photosynthesis in the water column. Despite the lack of significance, the phytoplanktonic chlorophyll *a* concentrations showed a clear increasing trend, mainly at a glyphosate concentration of 3 mg L^{-1}. Vera et al. (2012) recorded a similar pattern of phytoplanktonic chlorophyll *a* concentrations following the application of 3.5 mgL^{-1} of glyphosate as active ingredient (Glifosato Atanor®) to outdoor mesocosms like those used by us. The decrease in phytoplankton abundance observed in all treatments was probably due to manipulaton, but it occurred at a slower rate under scenarios with herbicide. Glyphosate is known to inhibit the EPSPS of the shikimate pathway, which also occurs in algae (Toghe et al. 2013). However this impact was not reflected in phytoplankton abundance, in disagreement with results obtained by Vera et al. (op cit.). Moreover, glyphosate promoted the increase in periphytic chlorophyll *a* concentrations, thus leading to a more autotrophic community, in accordance to that observed by Kish (2006) and Vera et al. (2012 and 2014) for periphyton developed on artificial substrata. The effect of glyphosate alone produced an enrichment of the systems and the impoverishment of the water quality, as previously described by Pérez et al. (2007).

On the other hand, *Limnoperna fortunei* induced strong changes in the physical, chemical and biological properties of the water through direct or indirect effects. Mussels are powerful filter feeders, with adults showing a normal clearance rate of 100 mL ind^{-1} h^{-1} (Cataldo et al. 2012a). On this basis, mussels could filter the water volume in each mesocosm about 12 times a day. It is reasonable to assume that such a filtering activity produced a decrease in the suspended matter at the beginning of the experiment, followed by an increase toward its end, probably due to the interference effect of the metaphyton. The high clearance rate of mussels accounted for a significant decrease in phytoplankton abundance, producing low values of water turbidity, as previously reported by Sylvester et al. (2005). Moreover, the remarkable increment in total nitrogen and ammonium concentrations in the presence of *L. fortunei* was a consequence of the ability of mussels to recycle nutrients (Darrigran and Damborenea 2006; Cataldo et al. 2012b). This promoted the development of mats of filamentous green algae (metaphyton), thereby reaching high coverage values. Metaphyton, which is known to occur in stable water columns at high temperatures and high nutrient concentrations (Goldsborough and Robinson 1996), caused changes in the mesocosms, i.e. a significant decrease in phytoplankton abundance. Trochine et al. (2011), who observed the same phenomenon in outdoor mesocosms of 2.8 m^3, stated that filamentous green algae directly suppress phytoplankton growth mostly via the release of allelochemicals; we believe that phytoplankton in our mesocosms was also affected by a shadow effect. Cataldo et al. (2012b) observed that *L. fortunei* induced the proliferation of *Microcystis aeruginosa*, a bloom-forming species of cyanobacteria, in a mesocosm experiment conducted in a large reservoir of the Uruguay River. We hypothesize that the aquatic life history in the water system conditioned the presence of a diversity of propagules, thus determining the development of different species. In turn, this was mediated by the impact of the golden mussel, as could be evidenced by the impoverishment of the water quality. It is clear that *L. fortunei* is not only an aggressive invasive species, but also a very effective ecosystem engineer (Darrigran and Damborenea 2006; Karatayev et al. 2006). The decrease in phytoplankton abundance from t_3 onward was opposed to the pattern observed in phytoplankton chlorophyll *a*, with an increase of the concentrations probably due to the appearance of metaphyton. The presence of large filamentous algae at the end of the experiment could contribute to enlarge the chlorophyll *a* concentration in the water column. This kind of algae would not be consumed by *L. fortunei*, considering that mussels prefer to feed particles smaller than 100 μm as was observed by Cataldo et al. (2012a).

The joint effect of both anthropogenic stressors caused some remarkable and unexpected changes, being the key finding that *L. fortunei* accelerated the dissipation of glyphosate, which showed a four-fold decrease in its half-life compared to its dissipation in scenarios without mussels. The ability of the mussel to degrade glyphosate has already been demonstrated experimentally by Di Fiori et al. (2012). Degradation appears to be the most likely mechanism by which living mussels increase glyphosate dissipation, possibly mediated by bacteria associated to their metabolism, or by microbial biofilm communities (Di Fiori et al. op cit.). Similarly, *Dreissena polymorpha*, another invasive benthic filter-feeding bivalve, can reduce the concentration of several toxic substances in water, such as pharmaceuticals and other drugs; on this basis, *D. polymorpha* would be useful for environmental restoration (Binelli et al. 2014). Although *L. fortunei* is able to degrade glyphosate, it is far from being considered potentially capable of removing glyphosate. Our work demonstrated that *L. fortunei* was able to strongly alter the biogeochemical cycling and the biotic structure of the mesocosms, as well as to modulate the effects of glyphosate. These effects of the alien species were also described by Strayer (2010) for freshwater ecosystems. Mussel-mediated dissipation of glyphosate promoted the rapid bioavailability of the phosphorus supplied by the herbicide to the water, thus producing a faster impoverishment of the water quality. In analyzing the interaction between stressors we found that when *L. fortunei* modulated most of the parameters, there was a synergistic effect on SRP concentrations which depended directly on glyphosate concentration. A

synergistic interaction between stressors was also observed for bacterioplankton, water turbidity and metaphyton development, which in turn promoted more eutrophic conditions.

Our results on the effects of the combined effect of glyphosate and *Limnoperna fortunei* on the freshwater microscopic communities and the water quality are based on mesocosm experiments, used as a proxy of natural conditions. Moreover, the experiment was designed to test the effect of the stressors in quite extreme conditions. On one side, the ratio of number of mussels *versus* volume of water is of particular importance. Mussel densities used in our experiment ($\approx$5 mussels per l of water) are probably higher than those in the field considering the values registered by Boltovskoy et al. (2009) for a reservoir in Argentina. On the other side, we used a concentration of glyphosate included in the range of "worst-case" scenarios of glyphosate contamination in freshwater, as one can obtain by direct application of the herbicide to the water. As such, these conditions obviously are not identical to that in natural systems, for which reason they must be taken with care. However, our results are very promising to verify in future investigations carried out in more realistic conditions.

Extending over an area of about 3,100,000 km^2, the basin of the Rivers Paraná, Uruguay and de la Plata is one of the largest freshwater reservoirs worldwide; here, glyphosate and *Limnoperna fortunei* have coexisted for nearly two decades. In this region, the golden mussel has invaded water bodies that act as sink of material being carried directly or by runoff from adjacent crop fields. Agriculture in Argentina is mainly based on agrochemicals and glyphosate is the most commonly used herbicide in GM-tolerant crops and no-till practice (CASAFE 2013). Under this situation, knowledge of the interactive effects between multiple agents of environmental change in freshwater systems is of great importance because they may bring about new and unexpected responses. We agree with Ormerod et al. (2010) that understanding the nature of multiple-stressor effects on freshwater is a major challenge to identify and prioritize the major management issues and to seek the means to identify, diagnose and tackle multiple-stressor effects. The ability of mussels to reduce glyphosate in water may be valued as positive, but our results allow us to predict that the invasion of *Limnoperna fortunei* in natural freshwater systems contaminated by glyphosate will accelerate the negative impact of the herbicide associated with eutrophication.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors

Informed consent Informed consent was obtained from all individual participants included in the study.

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