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Bacterial composition of the biofilm on valves of *Limnoperna fortunei* and its role in glyphosate degradation in water

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ABSTRACT: Knowledge of the anthropogenic drivers of environmental change is important for understanding ecosystem dynamics. We studied 2 of these factors — an invasive mollusk (golden mussel *Limnoperna fortunei*) and the herbicide glyphosate — focusing on the effects of the mussel, the herbicide, and their interaction on the abundance and composition of different groups of biofilm bacteria present on the mussel's valves. We carried out semi-static experiments to assess changes in nutrient and glyphosate concentrations in the presence or absence of the mussel. The catalyzed reporter deposition-fluorescence *in situ* hybridization technique was used to evaluate changes in biofilm bacteria growing on whole mussels or dissected valves. When the mussel was exposed to glyphosate, there was a significant decrease in the concentration of the herbicide, a significant increase in the concentration of its major metabolite (aminomethylphosphonic acid), and a significant increase in the concentration of nutrients. These results may be explained by the capacity of biofilm bacteria associated with *L. fortunei* to degrade glyphosate. After exposure to the herbicide, the analysis of 5 groups of *Eubacteria* (i.e. *Alpha-*, *Beta-*, and *Gammaproteobacteria*, *Actinobacteria*, and *Bacteroidetes*) showed a significant increase in the abundance of *Gammaproteobacteria*, suggesting that they would participate in glyphosate transformation in water. This study represents a starting point for investigating the bacterial component of the biofilm present on the valves of *L. fortunei*. Moreover, this invasive mussel is a promising tool for glyphosate degradation. However, the liberation of nutrients as a consequence of the degradation of herbicide mediated by the presence of *L. fortunei* may accelerate eutrophication processes in freshwater ecosystems.

KEY WORDS: *Limnoperna fortunei* · Glyphosate degradation · *Gammaproteobacteria* · Biofilm · Catalyzed reporter deposition-fluorescence *in situ* hybridization · CARD-FISH

1. INTRODUCTION

Economic globalization and technological development have produced adverse effects on ecosystem structure and function. Anthropogenic drivers of ecosystem change include climate change, pollution, introduction of exotic species, and habitat fragmentation (Millennium Ecosystem Assessment 2005). In particular, the expansion of the agricultural frontier (i.e. more soil being used for agriculture) at a global

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scale has intensified the use of fungicides, fertilizers, and herbicides (Foley et al. 2005) which constitute major contamination sources. Glyphosate is among the most used herbicides worldwide (Barja & dos Santos Afonso 2005, Liphadzi et al. 2005), with 650 million t of glyphosate being applied in 2011 (CCM International 2011). In Argentina, 197 million kg of glyphosate were applied in 2012 (CASAFE 2013), mainly in soybean crops, which covered about 19 792 100 ha during 2014–2015 (Ministerio de Agricultura, Ganadería y Pesca de la Nación Argentina 2015).

The link between agricultural activity and water availability facilitates the transfer of this herbicide to freshwater systems through runoff (Aparicio et al. 2015). Glyphosate may reach aquatic systems by accidental or wind drift, through spraying of agricultural fields using aircraft or self-propelled sprayers, and via leaching and surface runoff (Feng et al. 1990, Borggaard & Gimsing 2008, Aparicio et al. 2015).

Glyphosate contaminates water bodies, deteriorates water quality (Pérez et al. 2007), and causes eutrophication due to increased availability of nutrients (mainly phosphorus) resulting from its degradation (Pérez et al. 2007, Vera et al. 2010). Moreover, it induces alterations in the structure of microbial communities (Relyea 2005a, Pérez et al. 2007), such as increased abundance of *Picocyanobacteria* (Pizarro et al. 2016b), and may exert a negative impact on filtering organisms like crustaceans and mollusks, sediment dwellers, fish, and amphibians, mainly by affecting filtration capacity and reproductive cycles (Folmar et al. 1979, Liong et al. 1988, Relyea 2005b, Relyea et al. 2005). The herbicide appears to have different physiological and behavioral effects on microbial communities (Kreutzweiser et al. 1989, Tsui & Chu 2003), protozoans (Bonnet et al. 2007), invertebrates (Trumbo 2005, Pérez et al. 2007), amphibians (Thompson et al. 2004, Relyea & Jones 2009, Moore et al. 2012), fish (Folmar et al. 1979, Wan et al. 1989, Modesto & Martinez 2010, Menezes et al. 2011, Glusczak et al. 2011), and birds (Oliveira et al. 2007).

The Asian native golden mussel *Limnoperna fortunei* (Dunker, 1857) was most likely introduced into the Río de la Plata River estuary by ocean-going vessels entering the Buenos Aires Port in the 1990s (Pastorino et al. 1993). Since then, it has successfully spread out due to its high growth rate, early sexual maturation, high fecundity rate, and high adaptation capacity (Darrigran & Pastorino 1993, Darrigran & Ezcurra de Drago 2000, Darrigran & Damborenea 2006).

Di Fiori et al. (2012) demonstrated under experimental conditions that microorganisms present on *L. fortunei* valves lead to a decrease in glyphosate concentration in water through mineralization. Degradation processes of glyphosate and other xenobiotics occurring in natural environments are mediated by bacteria within a polysaccharide matrix known as biofilm (Denyer et al. 1993, Costerton et al. 1994). It has been suggested that biofilm composition changes in response to different glyphosate concentrations, favoring bacteria that can metabolize the herbicide (Bricheux et al. 2013). Glyphosate-degrading bacteria use it as a source of carbon, nitrogen, and phosphorus for metabolic processes (Cuervo 2007, Bazot & Lebeau 2008, Krzysko-Lupicka & Sudol 2008). The most commonly cited glyphosate-degrading bacteria belong to the class *Gammaproteobacteria* and include *Pseudomonas* sp. 4ASW (Dick & Quinn 1995), *P. aeruginosa*, *P. fluorescens*, *Burkolderia gladioli*, and *Flavimonas oryzihabitans*, as well as to the following consortia: *P. fluorescens* + *P. aeruginosa* and *B. gladioli* + *P. fluorescens* + *F. oryzihabitans* (Martínez et al. 2012). Some *Actinobacteria* (Pipke et al. 1987, Forlani et al. 1999) and *Betaproteobacteria* (Martínez et al. 2012, Jacobsen & Hjelms 2014) have also been reported to be glyphosate degraders. Bacteria degrade glyphosate through 2 routes: the most important one produces aminomethylphosphonic acid (AMPA) (Cox 1995), which is then degraded to carbon dioxide and ammonia, while the alternative route yields inorganic phosphate and sarcosine, with the subsequent formation of glycine (Dick & Quinn 1995).

The objective of this study was to analyze changes in the abundance and composition of biofilm bacteria associated with *L. fortunei* valves and to assess their role in the degradation of glyphosate in water.

2. MATERIALS AND METHODS

Bioassays were carried out under controlled laboratory conditions (25 ± 1°C) using 18 experimental units during June 2013. The effect of mussels was evaluated at 3 levels: whole mussels (M), empty valves (V), and absence of mussels (controls, C); glyphosate concentration was evaluated at 2 levels: with glyphosate at 20 mg l^{-1} (G) (technical-grade glyphosate acid, 95% purity, Chemical Abstracts Service: 1071-83-6) and without glyphosate. Thus, we conducted the following treatments: C, G, M, V, MG, VG, which were tested in 7 d bioassays performed in triplicate in 25 l containers provided with constant aeration.

Each experimental unit of the M and MG treatments consisted of 10 randomly chosen individuals (mean size, corresponding shell length ± SD, 23.7 ± 3.0 mm). Each experimental unit of the V and VG treatments had 10 randomly chosen pairs of empty valves of the same mean size and from the same mussel group used in the M and MG treatments. *Limnoperna fortunei* specimens were collected manually from the Lower Delta of the Paraná River (34.429° S, 58.547° W), immediately transported to the laboratory, and placed in aquaria containing dechlorinated and aerated tap water at 25 ± 1°C. Only mussels with opened valves, extended siphons, and showing active filtration were selected for the bioassays. For the V and VG treatments, soft tissue was carefully removed without damaging the biofilm. The mussels were not fed during the experiment. The physical and chemical characteristics of the water from the site where mussels were collected (pH: 7.1; conductivity: 120 µS cm^{-1}; dissolved oxygen: 6.2 mg l^{-1}; soluble reactive phosphorus: 0.080 mg l^{-1}, nitrate + nitrite: 0.090 mg l^{-1}, ammonium: 0.007 mg l^{-1}) are within the ranges reported for the Lower Delta of the Paraná River (De Cabo et al. 2003, Di Fiori et al. 2012). The glyphosate concentration used in the assay (20 mg l^{-1}) corresponds to a worst-case field scenario of direct herbicide application (e.g. aerial spraying) (for further explanation, see Vera et al. 2010).

Measurements were carried out at the beginning (T_i) and end (after 7 d, T_f) of the experiment. Physical and chemical variables of the water (temperature, pH, conductivity, and dissolved oxygen) were measured with a field multiparametric sensor (Hach® Sension 156 meter). Samples were first filtered through 0.7 µm pore size glass fiber filters (Whatman® GF/F) (APHA 2005). Dissolved inorganic nutrient concentrations were then measured with a spectrophotometer (Hach® DR/2010). Soluble reactive phosphorus ($P\text{-}PO_4^{3-}$) was determined by the ascorbic acid method, nitrate + nitrite ($N\text{-}NO_3^-$ + $N\text{-}NO_2^-$) by the cadmium reduction method (Mackereth et al. 1978), and ammonium ($N\text{-}NH_4^+$) by the salicylate method (APHA 2005), with a detection limit of 0.001 mg l^{-1}.

The analyses of glyphosate and its main metabolite AMPA were performed using an HPLC-UV system (Jasco Analytical Instruments) with a Microsorb C18 5 µm column (Varian) after a derivatization step with fluorenylmethyloxycarbonyl chloride (Sancho et al. 1996, Stalikas & Konidari 2001), by means of mobile phases of ammonium acetate (5 mM) and acetonitrile as described by Gattás et al. (2016). Products were detected at 265 nm. Calibration curves were performed simultaneously for both analytes using the same initial water of the bioassays. The limits of detection and quantification of the resulting procedure were 0.1 and 0.3 mg l^{-1}, both for glyphosate and AMPA. All reagents were of analytical grade (Sigma-Aldrich).

The soft-tissue dry weights of the mussels used in the M and MG treatments were calculated after they were oven-dried at 60°C to constant weight.

To obtain the samples of biofilm bacteria at T_i and T_f, the 10 pairs of valves from each experimental unit of treatments V and VG were carefully scraped and then rinsed with Milli-Q water (100 ml per treatment). These biofilm samples were fixed with 37% formaldehyde and stored at 4°C during 24 h; later, they were sonicated with a sonicator (SONICS Vibra Cell, model VCX 130PB, frequency: 20 KHz) to disaggregate clumps of bacteria in the biofilm. Finally, the samples were filtered onto 0.2 µm pore polycarbonate white filters (Millipore GTTP), which were stored at −20 ± 1°C until processing.

Quantitative analysis of total bacteria in the biofilm was performed using DAPI staining, and the different bacterial groups were then quantified by the catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) technique (Pernthaler et al. 2002, 2004). Group-specific horseradish peroxidase (HRP)-labeled rRNA probes were used for hybridization. These probes allow the detection and emission of fluorescence signals to visualize and quantify bacterial cells. Hybridized samples were examined with an epifluorescence microscope (Olympus BX40F4). We used probes of the bacterial groups most commonly found in water bodies as follows: *Eubacteria*, probe EUB338-II-III (Amann et al. 1990, Daims et al. 1999); *Actinobacteria*, probe HGC69a (Amann et al. 1995); *Bacteroidetes*, probe CF319a (Manz et al. 1996); *Alphaproteobacteria*, probe ALF968 (Neef1997); *Gammaproteobacteria*, probe GAM42a (Manz et al. 1992); *Betaproteobacteria*, probe BET42a (Manz et al. 1992). The probes for *Actinobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* were competitive and not labeled with HRP. After hybridization, we carried out tyramide signal amplification, and all bacteria were stained with DAPI. The combination of HRP-labeled probes and tyramide signal amplification results in a bright and stable signal. To quantify the different bacterial groups from each sample, sections of filters hybridized with the different probes were observed with an epifluorescence microscope (Olympus BX40F4) under blue light and UV excitation. Results were expressed as cells mm^{-2} by calculating the planar area of valves using the program Surfer 8®.

Glyphosate, AMPA, and dissolved inorganic nutrient concentrations were statistically analyzed using repeated-measures ANOVA (RM ANOVA) (Zar 1996), after testing the assumptions of the model. The effects of treatments on each bacterial group were analyzed with a 2-factor ANOVA and multiple post hoc comparisons (Tukey test). A principal component analysis (PCA) was used to order the treatments (M, MG, V, and VG) based on the abundance of the bacterial groups. RM ANOVA was conducted with the SPSS statistical package®, and PCA with infoStat® statistical software.

3. RESULTS

No mortality of mussels was observed over the 7 d experiment for any of the treatments.

3.1. Physical and chemical parameters in water

No significant differences in pH (6.9 ± 0.4, ±SD) were observed either among the experimental units within the same treatment or among treatments. Oxygen levels remained above 70% saturation in all experimental units, while conductivity ranged between 255 and 269 µS cm^{-1} during the experimental period.

3.2. Concentration of glyphosate and AMPA in water

At the beginning of the experiment, no significant differences in glyphosate concentration were found among treatments G, VG, and MG (mean 20.4 ± 0.7 mg l^{-1}, RM ANOVA, $p > 0.05$). At T_f, glyphosate concentration did not vary significantly with respect to T_i in treatment G (21.1 ± 0.6 mg l^{-1}), but decreased significantly in treatments VG and MG ($p < 0.05$ in both cases). At T_f, glyphosate concentration was significantly lower in treatment MG (9.20 ± 1.05 mg l^{-1}) than in treatment VG (11.40 ± 0.07 mg l^{-1}) ($p < 0.05$; Fig. 1A). Glyphosate concentrations remained below the detection limit in treatments C, V, and M at both T_i and T_f. Glyphosate dissipation (between T_f and T_i) were 47.8 ± 1.9% and 58.7 ± 1.2% in treatments VG and MG, respectively.

At T_i, AMPA was not detected in any of the experimental units, while its concentration increased significantly at T_f (RM ANOVA, $p < 0.05$) in treatments MG and VG with respect to the glyphosate control (G), where this metabolite was not detected. The concentration of AMPA was significantly higher in treatment VG (3.7 ± 0.153 mg l^{-1}) than in treatment MG (2.7 ± 0.115 mg l^{-1}) (Tukey test, $p < 0.05$; Fig. 1B).

3.3. Nutrients

At T_i, phosphate concentrations ($P\text{-}PO_4^{3-}$) did not differ significantly among treatments (0.107 ± 0.012 mg l^{-1}; RM ANOVA, $p > 0.05$). At T_f, this variable was significantly higher in treatment MG with respect to T_i and the rest of the treatments (0.21 ± 0.01 mg l^{-1}, $p < 0.05$; Fig. 2A).

At T_i, no significant differences in nitrates ($N\text{-}NO_3^-$) were observed among treatments with glyphosate (G, VG, MG; 0.060 ± 0.010 mg l^{-1}; RM ANOVA, $p > 0.05$) and among treatments without glyphosate (C, M, V; 0.060 ± 0.011 mg l^{-1}; $p > 0.05$). At T_f, the concentration of this nutrient was not significantly different between treatments C and G (0.057 ± 0.012 and 0.067 ± 0.058 mg l^{-1}, respectively; $p > 0.05$ in both cases), and between V and VG (0.417 ± 0.029 and 0.407 ± 0.035 mg l^{-1}, respectively; $p > 0.05$ in both cases), but they differed significantly between these pairs of treatments ($p < 0.05$). The

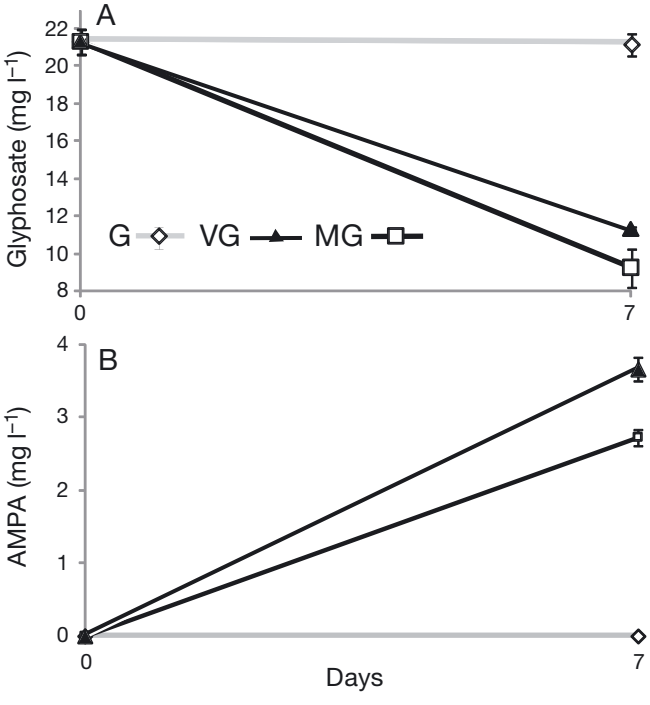

Fig. 1. Mean concentration of (A) glyphosate and (B) aminomethylphosphonic acid (AMPA) at the beginning and end of the experiment in the treatments glyphosate (G), *Limnoperna fortunei* mussel + glyphosate (MG) and empty valves + glyphosate (VG). Bars indicate SD. Treatments are described in detail in Section 2

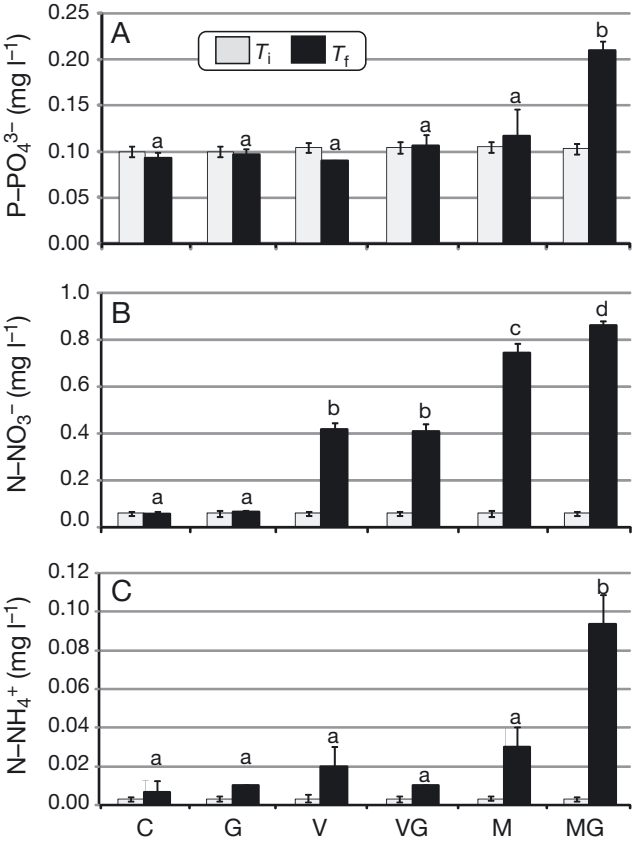

Fig. 2. Mean concentration of nutrients in the different treatments (C: no-mussel controls; G: glyphosate only; V: empty valves; VG: empty valves + glyphosate; M: mussels only; MG: mussels + glyphosate) at the beginning (T_i) and end (T_f) of the experiment for (A) phosphates (P-PO_4^{-3}), (B) nitrates (N-NO_3^-), and (C) ammonium (N-NH_4^+). Different letters indicate significant differences between treatments at T_f ($p < 0.05$, repeated-measures ANOVA). Bars indicate SD

concentration of nitrates was significantly higher in treatments M and MG than in the other treatments (0.743 ± 0.011 and 0.860 ± 0.001 mg l^{-1}, respectively; $p < 0.05$ in both cases). Treatment MG showed the highest nitrate concentration ($p < 0.05$; Fig. 2B). At T_i, there were no significant differences in ammonium (N-NH_4^+) among treatments, whereas at T_f a significant increase in treatment MG was found (0.09 ± 0.02 mg l^{-1}) compared to T_i and the other treatments at T_f ($p < 0.05$; Fig. 2C).

3.4. Biofilm bacterial groups on valves of *Limnoperna fortunei*

Hybridization efficiency using CARD-FISH was evaluated as the proportion of bacteria hybridized with a general *Eubacteria* probe (EUB 338-I-III), compared with total DAPI cell counts. The percentages of hybridization were 84% for treatment VG, 78% for M, 75% for MG and 64% for V. At T_i, hybridization was 82%.

At T_i, mean density of hybridized bacteria was 6.60 ± 0.4 × 10^8 bacteria cm^{-2}. The most abundant group corresponded to *Alphaproteobacteria* (53.4%) followed by *Betaproteobacteria* (15.8%) and *Gammaproteobacteria* (12.1%), while *Bacteroidetes* showed the lowest abundance (6.9%).

3.5. Bacterial composition

The *Eubacteria* (Eub 338-II-III) included all of the group-specific hybridization probes. At the beginning of the experiment, no significant differences were observed in the abundance of *Eubacteria* between treatments (6.5 ± 0.9 × 10^7 cells cm^{-2}; $p > 0.05$). At T_f, the highest bacterial growth was observed in treatment M (2.5 ± 0.6 × 10^8 cells cm^{-2}), followed by MG (9.4 ± 3.3 × 10^7 cells cm^{-2}) and VG (8.6 ± 5.6 × 10^7 cells cm^{-2}). The lowest bacterial growth was recorded in treatment V (8.3 ± 9.7 × 10^7 cells cm^{-2}). However, at T_f, differences in abundance among treatments were not statistically significant ($p > 0.05$). Fig. 3 shows the mean abundance of the bacterial groups for the different treatments at T_i and T_f. At the beginning of the experiment, no significant differences were observed in the abundance of *Alphaproteobacteria* between treatments (1.7 ±0.1 × 10^8 cells cm^{-2}, $p > 0.05$). At the end of the experiment, *Alphaproteobacteria* reached the highest abundance in treatment M (27.1 ± 0.2 × 10^8 cells cm^{-2}) followed by MG (1.9 ± 0.3 × 10^8 cells cm^{-2}) and V (1.7 ± 0.4 × 10^8 cells cm^{-2}). This group dropped to the lowest value in treatment VG (1.3 ± 0.7 × 10^8 cells cm^{-2}), which differed significantly only from that obtained in M ($p < 0.05$; Fig. 3A). Initial mean abundances of *Betaproteobacteria* were 3.5 ± 0.2 × 10^7 cells cm^{-2}. At the end of the experiment, no significant differences in *Betaproteobacteria* were found among treatments ($p > 0.05$). The highest bacterial abundance occurred in the treatments without glyphosate added, i.e. V (1.2 ± 0.8 × 10^8 cells cm^{-2}) and M (1.1 ± 0.3 × 10^8 cells cm^{-2}), while the lowest abundance was recorded in treatments with the herbicide, i.e. MG (0.4 ± 0.1 × 10^8 cells cm^{-2}) and VG: (0.9 ± 0.2 × 10^8 cells cm^{-2}; Fig. 3B). At the beginning of the experiment, no significant differences were observed in abundance of *Gammaproteobacteria* between treatments (1.7 ± 0.1 × 10^8 cells cm^{-2}, $p > 0.05$). At T_f, for *Gammaproteobacteria*, there were significant differences between

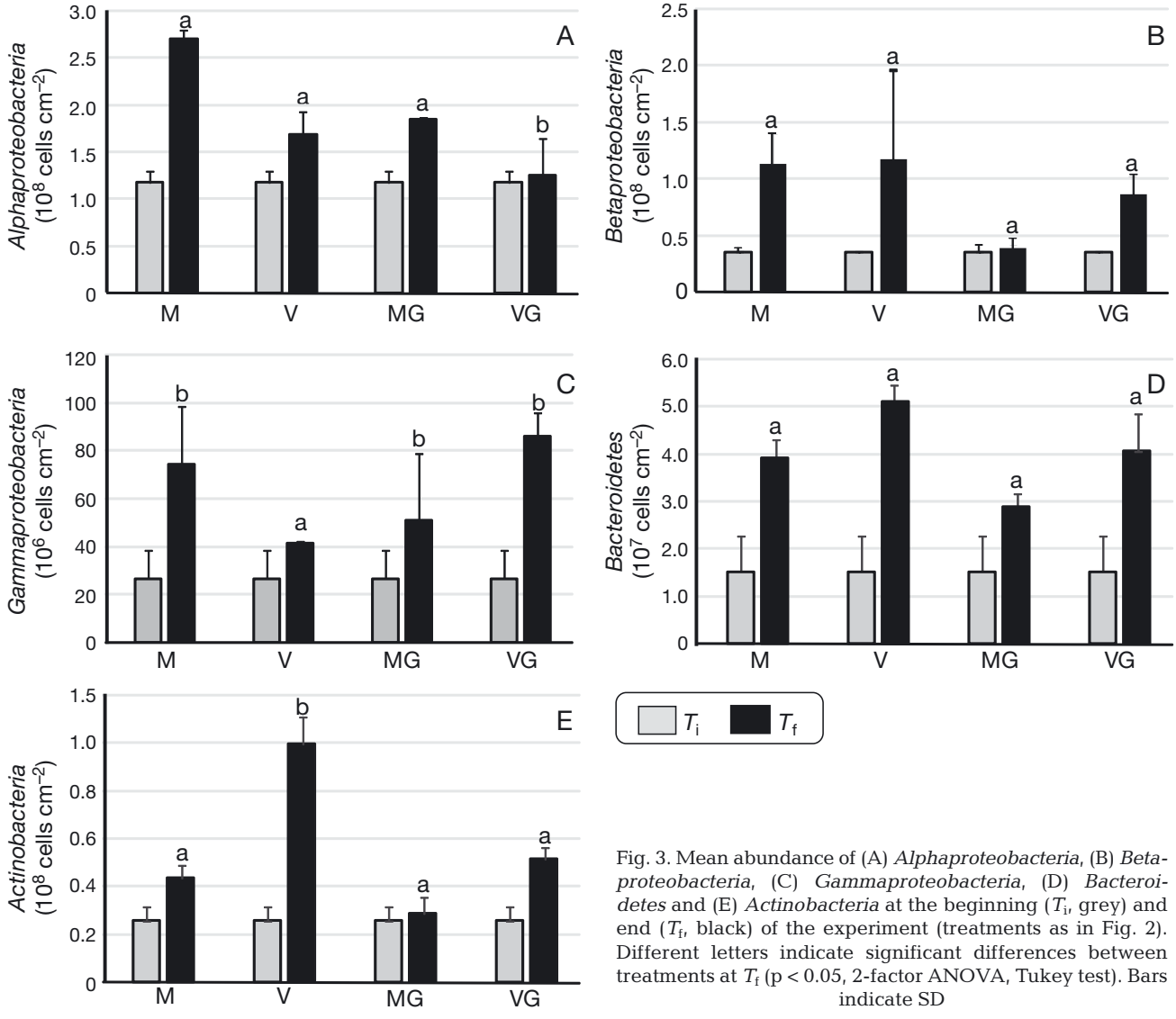

Fig. 3. Mean abundance of (A) *Alphaproteobacteria*, (B) *Betaproteobacteria*, (C) *Gammaproteobacteria*, (D) *Bacteroidetes* and (E) *Actinobacteria* at the beginning (T_i, grey) and end (T_f, black) of the experiment (treatments as in Fig. 2). Different letters indicate significant differences between treatments at T_f ($p < 0.05$, 2-factor ANOVA, Tukey test). Bars indicate SD

V and the other treatments ($p < 0.05$), with the former showing the lowest abundance ($41.6 \pm 0.5 \times 10^6$ cells cm^{-2}), followed by MG ($51.1 \pm 27.5 \times 10^6$ cells cm^{-2}) and M ($74.2 \pm 24 \times 10^6$ cells cm^{-2}), and the highest abundance was observed in VG ($86.1 \pm 9.9 \times 10^6$ cells cm^{-2}; Fig. 3C). For *Bacteroidetes*, no significant differences were found between the treatments at T_i and T_f (in both cases, $p > 0.05$). At T_f, the lowest abundance occurred in MG ($2.4 \pm 0.3 \times 10^7$ cells cm^{-2}), followed by M ($3.9 \pm 0.4 \times 10^7$ cells cm^{-2}) and VG ($4.1 \pm 0.8 \times 10^7$ cells cm^{-2}), while the highest abundance was recorded in V ($4.8 \pm 0.6 \times 10^7$cells cm^{-2}; Fig. 3D). Initial mean abundances of *Actinobacteria* were $2.6 \pm 0.6 \times 10^7$ cells cm^{-2}. At T_f, *Actinobacteria* showed significant differences in abundance between V and the other treatments ($p < 0.05$), with the former having the highest value ($9.9 \pm 1.3 \times 10^7$ cells cm^{-2}). The lowest abundance was recorded for MG ($2.9 \pm 0.7 \times 10^7$ cells cm^{-2}), followed by M ($4.3 \pm 0.5 \times 10^7$ cells cm^{-2}) and VG ($5.2 \pm 0.5 \times 10^7$ cells cm^{-2}; Fig. 3E).

Fig. 4 shows the results of the PCA performed to order the treatments M, V, MG and VG as a function of the abundance of the different bacterial groups during the experiment. The first 2 axes accounted for 83.8% of the total variance (axis 1: 59.3%; axis 2: 24.5%). The first axis showed a direct correlation with *Actinobacteria* (0.54), *Bacteroidetes* (0.54) and *Betaproteobacteria* (0.51), and an inverse correlation with *Gammaproteobacteria* (–0.39). The second axis was directly correlated with *Alphaproteobacteria* (0.82) and *Betaproteobacteria* (0.38), and inversely correlated with *Actinobacteria* (–0.32). The biplot of axis 1 vs. 2 shows that the treatment with valves

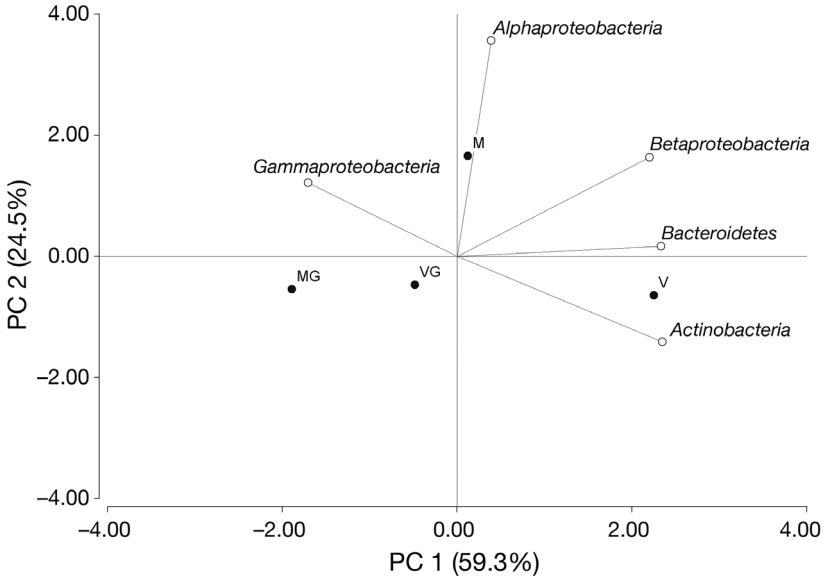

Fig. 4. PCA of the treatments (see Fig. 2), which are ordered according to the abundance of the different groups of bacteria in biofilm of *Limnoperna fortunei* valves. The first 2 axes (PC 1 and PC 2) explain 83.8% of the total variation

alone (V) appears on the right side of the graph (near the center of the upper and lower quadrants), located towards lower abundance values of *Gammaproteobacteria* and higher abundances of *Betaproteobacteria*, *Bacteroidetes*, and *Actinobacteria*. In contrast, treatments with glyphosate in combination with empty valves (VG) or mussels (MG) are placed on the lower left quadrant of the graph toward increasing values of *Gammaproteobacteria*, and, to a lesser extent, *Betaproteobacteria*, *Bacteroidetes*, and *Actinobacteria*, with this trend being more pronounced for MG than for VG. The treatment with mussels alone (M) is positioned in the upper part of the graph, towards increasing values of *Alphaproteobacteria* and, to a lesser extent, *Gammaproteobacteria* and *Betaproteobacteria*.

4. DISCUSSION

In our study, we explored for the first time the glyphosate degradation by bacteria associated with the valves of *Limnoperna fortunei*. The involvement of biofilm bacteria in this process was suggested by Di Fiori et al. (2012), who found a reduction in herbicide concentration of 40 ± 2% and 29 ± 5% in treatments with mussels and empty valves, respectively. In our study, the reduction in the concentration of glyphosate by 47.8 ± 1.9% compared to the initial concentrations in the VG treatment suggests that the bacterial biofilm may be responsible for the degradation of the herbicide. On the other hand, final glyphosate concentration was lower in the treatment with whole mussels (MG) than with empty valves (VG), while the opposite was obtained for the concentration of AMPA. This suggests the existence of mechanisms other than the degradation to AMPA mediated by the biofilm on valves, which may be related to the activity of the bacterial biota in its pallial cavity and/or digestive system. For this same species, Zhang et al. (2015) also reported a decay in the concentration of the phytoregulator forchlorfenuron of 40% in the presence of whole mussels and 20% in the presence of valves only, and they identified bacterial genes from digestive tract extracts and from the biofilm of the valves related to this process. The authors proposed possible processes of degradation of the forchlorfenuron mediated by bacteria in the digestive tract (in anoxic conditions) and in the valves (in aerobic conditions).

Among the processes involved in the dissipation of glyphosate in water, surface absorption of the herbicide within the experimental units cannot be ignored. On the one hand, absorption that occurs on the walls of aquaria does not seem relevant, given that in the absence of valves or mussels the concentration of the herbicide did not experience significant variations during the experiment. With regard to adsorption to valves, it is known that glyphosate has affinity to metal ions (Barja & dos Santos Afonso 1998) and can be easily absorbed through their phosphate group to cations in solid surfaces (Di Fiori et al. 2012). This process of dissipation through adsorption is important and could partially (but not completely, given AMPA production) explain this decrease of herbicide in our experiment.

The significant increase in the concentrations of phosphates, nitrates, and ammonium observed in the MG treatment at the end of the experiment with respect to the other treatments reflects the release of nutrients into the water column resulting from glyphosate degradation. In particular, the significant increase in the concentration of phosphates suggests the cleavage of the C-P bond and the subsequent release of phosphates (Kononova & Nesmeyanova 2002, Castro et al. 2007), in line with the decrease in herbicide concentration due to glyphosate degra-

dation by bacteria in the digestive tract and in the valves. However, the VG treatment did not show a significant increase in nutrients in response to the degradation of the herbicide mediated by the bacteria present in the valves. In a 24 h laboratory-scale experiment, Zhang et al. (2014) found a decrease in the concentration of nitrates in water in the presence of golden mussels, and they also identified 2 denitrifying bacteria (I-N38 and I-N45) in the shell biofilm, associating the decay with the capability of those microbes to reduce nitrates.

The increase in phosphates observed in the M treatment is in agreement with the study of Cataldo et al. (2012a), who also used *L. fortunei* as their test species. In this treatment, the phosphate increase is due to the impact of the mussel on the phytoplankton. Likewise, increased nitrogen concentration in the MG treatment results from the degradation of the herbicide through a pathway leading to the intermediate formation of AMPA which is then metabolized to CO_2 and NH_4. Most ammonium is converted to nitrate under well-oxygenated conditions. On the other hand, such low nitrate + nitrite concentrations in the field where mussels were collected (0.090 N-NO_3^-+N-NO_2^- mg l^{-1}) could strengthen the AMPA degradation pathway. Microbes can use P but also N from the glyphosate molecule, and in some systems N is certainly a limiting nutrient.

Previous studies in Salto Grande reservoir showed that *L. fortunei* increased ammonium concentration in water (Cataldo et al. 2012b). The joint-action effect of *L. fortunei* and glyphosate on nutrient availability may affect complex interactions among organisms, leading to changes in ecosystem structure and function (Kelly et al. 2010, Cataldo et al. 2012a,b, Boltovskoy et al. 2013, Boltovskoy & Correa 2015). Phosphorus is a major indirect driver of change in phytoplankton and periphyton composition (Pérez et al. 2007). The golden mussel–bacteria system metabolizes glyphosate, with the consequent release of nutrients to the environment (Di Fiori et al. 2012, Gattás et al. 2018), which can thus promote the eutrophication of water bodies invaded by this species. This scenario favors the development of some phytoplankton groups, particularly *Cyanobacteria* (e.g. *Microcystis* sp.) usually associated with eutrophication and a high P:N ratio (Cataldo et al. 2012a).

The hybridization percentages of bacterial groups with the *Eubacteria* probe (EUB I-II-III) were always higher than 60%, indicating a high efficiency of the CARD-FISH technique. These percentages are comparable to those reported from Pampean shallow lakes (Sánchez et al. 2015) and Patagonian lakes (Schiaffino et al. 2016). With regard to the initial bacterial composition, *Alphaproteobacteria* (53.4%) and *Betaproteobacteria* (15.8%) were the most abundant groups, falling within the range reported for freshwater environments (Glöckner et al. 1999, Zwisler et al. 2003, Newton et al. 2007). The dominance of *Alphaproteobacteria* is in agreement with studies performed in Pampean shallow lakes (Sánchez et al. 2015), Patagonian lakes (Schiaffino et al. 2016), and fluvial systems in Italy (Lupini et al. 2011).

In general, glyphosate treatments (VG and MG) showed a greater proportion of *Gammaproteobacteria* and a lower proportion of *Betaproteobacteria*, *Bacteroidetes*, and *Actinobacteria* than the other treatments (Fig. 4). The availability of organic and inorganic nutrients interacted positively or negatively with the different bacterial groups present in the biofilm, affecting their abundance and composition. The extent and nature of the interaction depends on the ability of biofilm bacteria to assimilate nutrients (Atlas & Bartha 2005). For example, high nutrient concentrations favor the abundance of *Gammaproteobacteria* and some *Bacteroidetes*, while *Alphaproteobacteria* are adapted to low nutrient concentrations (Alonso & Pernthaler 2006a,b). In the VG treatment, *Gammaproteobacteria* showed a greater bacterial abundance than its control (V), which, together with the decrease in glyphosate (up to 50.22%) and the production of AMPA, provides additional evidence that this group is present in the biofilm and is involved in the degradation of glyphosate in water. In this sense, several studies indicate that bacteria of this group degrade glyphosate (e.g. Pernthaler & Amann 2005). Indeed, different studies of soil bacteria have reported that *Pseudomonas* sp., belonging to the class *Gammaproteobacteria*, can grow on culture media supplemented with glyphosate, using it as a phosphorus source through the cleavage of the C-P bond (Moore et al. 1983, Jacob et al. 1988, Liu et al. 1991, Dick & Quinn 1995, Martínez et al. 2012). *Pseudomonas* sp. also showed the ability to degrade glyphosate at different growth phases of maize *Zea mays* both *in vitro* and *in vivo* (Travaglia et al. 2015). Another member of the *Gammaproteobacteria*, *Enterobacter cloacae* K7, is able to cleave the C-P bond of glyphosate in the soil to yield glycine in crops of sunflower *Helianthus annuus* (L.) and sorghum *Sorghum saccharatum* (L.) (Kryuchkova et al. 2014).

In the treatment with mussels alone (M), the increase in *Gammaproteobacteria* abundance raises the possibility that *L. fortunei* excretes a metabolite promoting its growth. Some members of the *Gamma-*

proteobacteria have shown high growth rates when cultured in phosphorus- and nitrogen-rich media (Gasol et al. 2002, Šimek et al. 2006). In this context, the high metabolization rate of nutrients by this invasive mollusk (Cataldo et al. 2012a,b) is expected to enhance the abundance of this bacterial group. However, some genera of *Gammaproteobacteria* may be unable to degrade glyphosate, necessitating the hybridization with probes specific for glyphosate-degrading genera such as *Pseudomonas*, which has been widely cited as playing a key role in this process (Moore et al. 1983, Jacob et al. 1988). These interaction effects warrant further investigation considering that microorganisms compete directly for food resources (i.e. available nutrients) and that metabolic products accumulated in the medium may induce growth-inhibition processes (Atlas & Bartha 2005).

With regard to the class *Betaproteobacteria*, differences in abundance were not significant among treatments. Some *Betaproteobacteria* have been recognized as having a great capacity to degrade glyphosate in soil, such as *Burkholderia* sp. (Martínez et al. 2012 Jacobsen & Hjelms 2014). However, in our experiment, *Betaproteobacteria* abundance was not influenced by the presence or absence of glyphosate. Nevertheless, the possibility of competition among bacterial groups for nutrients cannot be ruled out, with *Gammaproteobacteria* more likely to succeed under conditions of high nutrient concentration (Gasol et al. 2002, Šimek et al. 2006).

In treatment V, abundance of *Actinobacteria* was significantly higher than that of the other bacterial groups and did not experience higher abundance in the treatments with glyphosate, in disagreement with studies reporting that this class was capable of degrading this herbicide (Forlani et al. 1999, Arango et al. 2014). As mentioned above, the microorganisms associated with *L. fortunei* may mineralize organic matter and release nutrients, and this process can favor the specific bacterial groups in biofilms on mussels.

L. fortunei has multiple impacts on ecosystems, strongly affecting phytoplankton, zooplankton, fish, and nutrient mineralization (Cataldo et al. 2005, 2012a, Rojas & de Paggi 2008, Boltovskoy et al. 2009, Paolucci et al. 2010a,b, Rojas et al. 2011, 2012, Boltovskoy & Correa 2015). This epifaunal species can attach to any hard surface, allowing it to occupy a vacant niche in freshwater bodies. This ability has led to a remarkable increase in the biomass and composition of the accompanying fauna and to an exponential increase in the surface available for biofilm bacteria, which together with the nutrients excreted by the mussel improve the conditions required for the development of glyphosate-degrading bacteria in water. Our study represents an initial step to study biofilm bacteria on the valves of *L. fortunei* and highlights its potential as a tool for glyphosate biodegradation in environments where it has been introduced. However, this mussel, glyphosate, and their interaction have been reported to accelerate eutrophication processes in freshwater ecosystems (Di Fiori et al. 2012, Vera et al. 2012, Cataldo et al. 2012a,b, Gattás et al. 2016, Pizarro et al. 2016a). The analysis of the relationship between bacterial groups allows us to conclude that *Gammaproteobacteria* was most likely linked to glyphosate degradation. It would also be interesting to study the interaction between bacterial groups and the most often applied glyphosate formulations in Argentina, namely Atanor® and Roundup®, comparing results with those obtained from the active ingredient.

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