Ecotoxicology and Environmental Safety 81 (2012) 106–113

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

# Impact of the invasive mussel *Limnoperna fortunei* on glyphosate concentration in water

Eugenia Di Fiori [a,b], Haydée Pizarro [a,b], María dos Santos Afonso [c], Daniel Cataldo [a,b,d,*,1]

[a] Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (C1428EHA), Buenos Aires, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina
[c] Departamento de Química Inorgánica, Analítica y Química Física, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (C1428EHA), Buenos Aires, Argentina
[d] Museo Argentino de Ciencias Naturales "Bernardino Rivadavia", Buenos Aires, Argentina

ARTICLE INFO

*Article history:*
Received 6 March 2012
Received in revised form
27 April 2012
Accepted 29 April 2012
Available online 16 May 2012

*Keywords:*
Glyphosate
*Limnoperna fortunei*
Microcosms
Valvae
Freshwater

ABSTRACT

The use of glyphosate has increased dramatically during the past years around the world. Microbial communities are altered when glyphosate reaches water bodies. The freshwater golden mussel *Limnoperna fortunei* is an invasive species that has rapidly dispersed since it was introduced in Argentina two decades ago. Mussels alter aquatic conditions through their filtrating activity by increasing water clarity and nutrient recycling. We aim to evaluate the potential capacity of the golden mussel to reduce glyphosate concentration in water, in laboratory conditions. Firstly, the evasive response of mussels to glyphosate (10, 20, and 40 mg $l^{-1}$) was evaluated and a toxicity test was carried out for these concentrations. A three-week experiment was then performed to assess glyphosate variation under mussel presence for two mussel sizes. Finally, mussels' role on glyphosate concentration was evaluated considering different mussel parts (living organisms and empty shells) through another three-week experiment. Laboratory experiments were performed in triplicate using 2–l microcosms. An initial glyphosate concentration between 16 and 19 mg $l^{-1}$ was used, and when mussels or valvae were added, 20 organisms per aquaria were used. Samples were obtained at days 0, 1, 2, 4, 8, 14, and 21. Glyphosate decreased by 40% under large mussel presence in both experiments, and was reduced by 25% in empty shell treatments. We believe that part of the herbicide that disappears from the water column is adsorbed in valvae surface, while another proportion is being mineralized by microbial communities in shells' biofilm. The mechanisms by which living mussels increase glyphosate dissipation would be degradation, possibly mediated by bacteria associated to mussel's metabolism. Glyphosate half-life depended on mussel and valvae presence and varied with mussel size. *L. fortunei* presence (either alive or as empty valvae) alters glyphosate concentration in water. We provide preliminary observations from laboratory experiments, with strong potential ecological consequences, about two stressors that could be acting jointly on the environment.

© 2012 Elsevier Inc. All rights reserved.

## 1. Introduction

As tolerant crop species arose through genetic engineering techniques, the use of agrochemicals increased exponentially in order to enhance crop production and economic benefits. Among herbicide tolerant crops, the glyphosate-tolerant ones are the most widely grown around the world (Liphadzi et al., 2005). Glyphosate is a phosphonate compound used as a non-selective, broad spectrum, post emergent herbicide for weed control of perennial and annual plants (Barja and dos Santos Afonso, 1998). It penetrates plants through leaves and stems, and acts by inhibiting the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase, essential in the biosynthetic pathway of vital aromatic amino acids (phenylalanine, tyrosine, and tryptophan) (Salisbury and Ross, 1994; Williams et al., 2000). Although glyphosate was believed to be "environmentally friendly" and harmless for non-target organisms, in the last decade several investigations have indicated that glyphosate, including its commercial formulations containing additives, affects not only plants, but also terrestrial and aquatic animals and microorganisms as well (Paganelli et al., 2010; Pérez et al., 2007; Relyea, 2005a). In Argentina, the expansion of the soybean crop began in the early 1970s; by 2006, half of

*Abbreviations:* SM, Small mussel treatments; LM, Large mussel treatments; C, control treatments (without mussel)

* Correspondence to: Universidad de Buenos Aires, Departamento. de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, 1428 EHA Buenos Aires, Argentina. Fax: +54 11 4576 3384.
*E-mail address:* daniel@ege.fcen.uba.ar (D. Cataldo).
[1] Postal address: Depto. Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, (1428 EHA) Buenos Aires, Argentina.

0147-6513/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ecoenv.2012.04.024

the total cultivated area of the country was destined to this crop (Aizen et al., 2009), which represents 17 million hectares of which more than 98% corresponds to the glyphosate-tolerant variety (Trigo and Cap, 2006). Glyphosate is not only used for soybeans, but also for other crops like maize, cotton and canola, as well as for chemical fallow. This herbicide can reach water bodies through direct spraying during crop dusting as well as by drainage after intense rains. The side effects of glyphosate in water bodies include changes in microbial community structures (Pérez et al., 2007; Relyea, 2005a) and can have potential negative effects on aquatic filtrating organisms such as crustacean and mollusk, as well as on organism feeding on sediment such as fish and amphibians (Folmar et al., 1979; Liong et al., 1988; Relyea et al., 2005; Relyea, 2005b).

The freshwater golden mussel *Limnoperna fortunei* is an invasive species from Asian rivers which was accidentally introduced in South America two decades ago, through ships' ballast water (Pastorino et al., 1993). Today, this species dominates the benthic fauna of almost all the Rio de la Plata basin (Argentina, Uruguay, Brazil, and Paraguay), with densities of more than 200,000 organisms/$m^2$ (Boltovskoy et al., 2006). *L. fortunei* is one of the filtering organisms with the highest clearance rate, of up to 350 ml $h^{-1}$ ind $^{-1}$ (Cataldo et al., 2012; Sylvester et al., 2005). Another invader, present in North America since the late 1980s, is the zebra mussel *Dreissena polymorpha*, which affects trophic interactions and food availability for benthic and pelagic species in freshwater, as well as other processes such as oxygen availability, sedimentation rates and pollutants dynamics (Karatayev et al., 1997). Though taxonomically distant, *L. fortunei* shares similar ecological characteristics (Cataldo et al., 2003; Karatayev et al., 2007) but its capability of affecting pollutants dynamics is still unknown.

Our aim is to evaluate the potential of the invasive golden mussel as a biological agent, capable of reducing the levels of glyphosate in water, discriminating the different compartments that could be participating in herbicide dissipation (living soft tissue and empty valvae), with their associated microbial communities. We believe that glyphosate decreases more rapidly with the presence of *L. fortunei*, and that this rate depends on the mussel size.

## 2. Materials and methods

Mussels used were manually collected on the banks of the Rio de la Plata river located near Buenos Aires City, Argentina (34°36.522'S, 58°20.658'W), during a low water period, and brought to the laboratory within 2 h. The organisms collected were carefully separated from the substrate and arranged in aquaria with dechlorinated water over a week for acclimation to laboratory conditions: temperature of $24 \pm 1$ °C, natural light illumination and constant aeration. Mussels were fed a daily diet of baby fish food. Subsequently, the organisms were transferred to a tray with clean dechlorinated water and those with a rapid response, detected by the extension of their siphons and an active filtration, were selected for the experiment.

Laboratory essays to analyze the interaction of mussels with glyphosate were carried out in two stages. Firstly, we analyzed the effect of glyphosate on mussels through a toxicity test and evasive response study of the mussels exposed to herbicide. Secondly, we evaluated the potential of *L. fortunei* to decrease the concentration of glyphosate in water, analyzing the effects of the sizes and the different compartments of the mussels (soft tissue vs. valvae) that could be involved in the dissipation of glyphosate in the water column by means of two different three-week experiments.

### 2.1. Evasive response of L. fortunei and toxicity test

For the evasive response, the filtering activity of 30 organisms of *L. fortunei* was analyzed at different herbicide concentrations: 0, 10, 20, and 40 mg $l^{-1}$ glyphosate (acid, 95% pure) during 2 h, registering every 10 min the number of mussels with opened valvae and extended siphons. For the toxicity test, ten organisms were exposed during 10 days to 0, 10, 20, and 40 mg $l^{-1}$ glyphosate, in triplicate, in 2–l microcosms with dechlorinated tap water and continuous aeration. Water was renewed during the first three days to ensure a constant exposure to glyphosate concentration in each treatment for an acute toxicity test. Dead organisms (i.e. opened valvae organisms without soft tissue) per microcosm were registered for days 1– 3, 6, 8–10.

### 2.2. First experiment: effect of mussel size

With the purpose of analyzing the effect of body size, a three-week experiment was carried out to evaluate glyphosate decrease in water with and without *L. fortunei* presence for small (mean length of $13 \pm 1$ mm) and large (mean length of $22 \pm 1$ mm) mussels. The individuals were measured to the nearest 0.01 mm with a digital caliper. Twenty organisms of each size were disposed in 2–l microcosms with dechlorinated tap water, continuous aeration and $18.8 \pm 0.3$ mg $l^{-1}$ glyphosate. Mussels were starved for 24 h before the experiment was run in order to eliminate any contamination from external sources due to feces and pseudofeces excretion. A high glyphosate concentration was used to test the possibility of mussels feeding on glyphosate. Moreover, this concentration would simulate a worst-case scenario of glyphosate concentration found in natural water bodies just after a direct application of the herbicide, an agricultural practice very common in Argentina and other countries of the region (Vera et al., 2010). Treatments consisted of (1) microcosm with small size mussels (SM); (2) microcosm with large size mussels (LM) and (3) microcosm without mussels (control) (C), each in triplicate with the addition of $18.8 \pm 0.3$ mg $l^{-1}$ herbicide at $24\ ^{\circ}C \pm 1$ °C. Mussels were not supplied with any other food source during the experience. Water samples were collected at days 0, 1, 2, 4, 8, 14, and 21 in order to quantify glyphosate concentration in water for every treatment.

### 2.3. Second experiment: effect of different L. fortunei compartments

A second long-term experiment was carried out afterwards including more control treatments and separating the different compartments of the mussel that could be participating in glyphosate dissipation: living soft tissue and empty valvae with their associated microbial communities. This new experiment lasted 3 weeks and was performed using only large size *L. fortunei* organisms (LM), under the same conditions as before: twenty organisms per 2–l microcosms with constant temperature and aeration supply. Empty valvae were obtained from living organisms of the same size that were collected and kept in aquaria prior to experiment and were dissected on the same day the experiment was initiated so as to conserve the microbial community living on their shelves. With the aim of comparing mussels' behavior in dechlorinated tap water (TW) with mussels' behavior when using water obtained from their natural environment (river water, RW), experiments were performed in triplicate for each type of water. RW was obtained from the same site where the mussels were captured. Four treatments where then performed with each type of water: (1) mussel (Muss): 20 LM organisms; (2) glyphosate (Gly): $15.8 \pm 0.3$ mg $l^{-1}$ glyphosate; (3) glyphosate and valvae (Gly+Val): $15.8 \pm 0.3$ mg $l^{-1}$ glyphosate and 20 pair of LM valvae without soft tissue; and (4) glyphosate and mussel (Gly+Muss): $15.8 \pm 0.3$ mg $l^{-1}$ glyphosate with 20 LM. Experimental and sampling conditions were exactly the same as described in the first experiment. In this case, besides glyphosate, dissolved inorganic nutrients (nitrogen (N) as nitrate+nitrite ($NO_2$, $NO_3$), N as total ammonia ($NH_4$) and soluble reactive phosphorous (P) as ($PO_4$)) were also quantified in water samples for every sampling day.

### 2.4. Sample analysis

Soluble reactive phosphorus (ascorbic method), nitrate+nitrite (cadmium reduction method) and ammonia (salicylate method) were analyzed with a HACH DR/2010 (HACH Company, USA) spectrophotometer using the corresponding kits of HACH® reagents, with a detection limit of 0.001 mg $l^{-1}$. Glyphosate was determined with ion chromatography using a DIONEX DX-100 chromatograph with a conductivity detector and a 25 µl 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 $Ct = C_0 e^{(-kt)}$, where $Ct$ is the concentration at time $t$ and $C$ is the initial concentration. At the end of the experiment, dry weight of mussels was quantified for each microcosm (at 60 °C until constant weight was achieved) in order to estimate the excretion rate of soluble reactive phosphorus expressed as micromoles of phosphate excreted per gram of soft mussel tissue dry weight per day, with a digital balance KENR model ARS 120-4 (error 0.0001 g).

### 2.5. Statistical analysis

For the two long-term experiments, inorganic nutrients as well as glyphosate concentration data were analyzed using Repeated Measures ANOVA, where assumptions of the model were previously analyzed. In the first experiment,

factors were evaluated with *a-priori orthogonal* contrasts. Paired comparisons were used for the second experiment due to significant interaction between factors, and the corresponding model assumptions were correctly evaluated. In this experiment, nutrient variables had to be Log-transformed in order to fulfill the Sphericity requirement. In order to analyze differences between treatments for mussel soft tissue weight and for excretion rate, one-way ANOVA was used with *a-priori* and *a-posteriori* comparisons, according to the hypothesis that were handled. For all these statistical tests, the SPSS® Software was used.

## 3. Results

### 3.1. Evasive response of L. fortunei and the toxicity test

There were no significant differences between the behavior of mussels exposed to glyphosate concentrations of up to 18.8 mg $l^{-1}$ and mussels that remained in freshwater conditions (control, *C*) ($p=0.36$). In all cases (0, 10, 20, and 40 mg $l^{-1}$ glyphosate), the animals remained with opened valvae and extended siphons and developed a continuous filtering activity most of the time. In fact, during the ten-day toxicity test, only two of the 120 organisms used in the experiment died: one organism at 10 mg $l^{-1}$ in day 1, and another organism at 40 mg $l^{-1}$ in day 2. Furthermore, during the two long-term (three-week) experiments with herbicide addition, no dead organisms were registered.

### 3.2. First experiment: effect of mussel size

Glyphosate concentration in *C* was almost constant throughout the experimental period, with a mean decline of $3 \pm 2\%$ (mean $\pm$ SE) (Fig. 1). In treatments with *L. fortunei* presence, glyphosate concentration decreased by $11 \pm 4\%$ with SM, and by $40 \pm 2\%$ with LM, resulting in glyphosate concentrations at the end of the experiment of $16.8 \pm 0.7$ mg $l^{-1}$ and $11.4 \pm 0.6$ mg $l^{-1}$ for SM and LM respectively. In SM, glyphosate concentration differed significantly from initial values ($p=0.02$) only by the end of the experimental period (day 21). In contrast, in LM microcosms, glyphosate decreased more rapidly, differing from the initial concentration as early as on the second day ($p=0.009$). When comparing SM and LM treatments, glyphosate differed from day 8 onwards between them, being lower in LM than in SM ($p=0.005$). Assuming that glyphosate residue adjusted to a logarithmic function with a first-order kinetic ($\ln \text{glypho} = -0.023 + 18.343$; $R^2 = 0.95$; $p < 0.05$), glyphosate from LM presented an estimated dissipation rate ($k$) of 0.023 mg $day^{-1}$ ($\pm$ 0.002 SE) with half-life in LM of $30.6 \pm 4.5$ days. Half-life could not be calculated for SM and *C*, since the glyphosate concentration only decreased at the most by 11% after three weeks.

### 3.3. Second experiment: effect of different L. fortunei compartments

#### 3.3.1. Glyphosate concentration in water

In the following long-term experiment, in treatments with herbicide addition, glyphosate concentration in water decreased only in treatments with mussel presence as well as in treatments with empty valvae, whereas in aquaria that only contained glyphosate (Gly), the concentration of the herbicide remained constant throughout all the essay (Fig. 2). Glyphosate concentration in tap water treatments with empty valvae and mussel presence decreased from mean value of $16.0 \pm 0.2$ mg $l^{-1}$ to $11.4 \pm 0.9$ mg $l^{-1}$ for Gly+Val, and $9.6 \pm 0.3$ mg $l^{-1}$ for Gly+Muss (mean values $\pm$ SE). This reduction in glyphosate concentration resulted statistically significant with respect to control from 48 h onwards ($p=0.005$ and $p=0.000$ for Gly+$Val^{TW}$ and Gly+$Muss^{TW}$ respectively) (Fig. 2A). On average, the presence of *L. fortunei* produced a glyphosate decrease of $50.2 \pm 3.4$ per gram mussel dry weight per day in tap water. In river water treatments, glyphosate concentration decreased in water during the experiment from a mean value of $15.5 \pm 0.2$ mg $l^{-1}$ to values of $12.5 \pm 0.5$ mg $l^{-1}$ and $9.5 \pm 0.8$ mg $l^{-1}$ in Gly+$Val^{RW}$ and Gly+$Muss^{RW}$ respectively, differing from control treatment from day 8 onwards ($p=0.045$ and $p=0.001$ for Gly+Val and Gly+Muss, respectively) (Fig. 2B). Mussels incubated in river water produced an average decrease of $33.9 \pm 1.1$ of glyphosate per gram mussel dry weight per day. Nonetheless, no differences were detected between equal treatments, which only differed in the type of water: namely control treatments only with glyphosate (Gly$^{TW}$ and Gly$^{RW}$) treatments with empty valvae (Gly+$Val^{TW}$ and Gly+$Val^{RW}$) and treatments with whole mussels (Gly+$Muss^{TW}$ and Gly+$Muss^{RW}$). This indicates that the variation in glyphosate concentration is mainly given by the presence of mussels, while water quality does not affect in a significant way the response of the herbicide to the type of water used (tap or river). The effect of mussels would be then masking possible impacts of aquatic environment in treatments with different water types. Glyphosate concentration decreased more rapidly in mussel treatments than in empty shells, but this difference was statistically significant only at the end of the experimental period (day 21) in river water treatments ($p=0.033$). Respect to glyphosate variation through time, in Gly+$Val^{TW}$ there were statistical differences from day 8 ($p < 0.001$) while in Gly+$Val^{RW}$ there were only statistical differences ($p=0.007$) between days 14 and 21. In Gly+$Muss^{TW}$ there were differences from day 8 onwards ($p < 0.001$) and Gly+$Muss^{RW}$ had an important decline in herbicide concentration between days 4 and 8 ($p=0.001$). Glyphosate half-life was of $28.8 \pm 1.4$ days in microcosms with mussels in tap water, and of $34.4 \pm 5.0$ days in mussel treatment with river water, with an estimated dissipation rate ($k$) of 0.024 mg $day^{-1}$ and of 0.021 mg $day^{-1}$ for Gly+$Muss^{TW}$ and Gly+$Muss^{RW}$ respectively. On the other hand, in valvae treatments, glyphosate half-life was longer than when exposed to living mussel, being of $33.8 \pm 1.5$ day in tap water microcosms, and of $64.8 \pm 4.8$ days in river aquaria. Though half-life was longer in river water treatments compared to tap water ones, these differences were only statistically significant between valvae treatments (Tukey: $p=0.017$ between Gly+$Val^{TW}$ and Gly+$Val^{RW}$ and $p=0.07$ between Gly+$Muss^{TW}$ and Gly+$Muss^{RW}$).

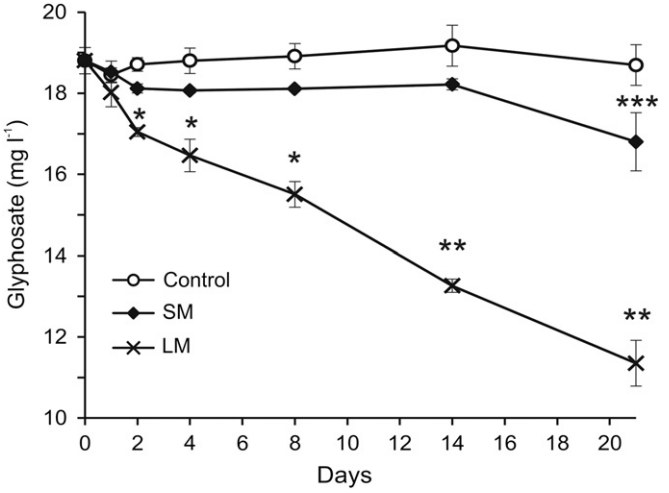


**Fig. 1.** Glyphosate: variation of glyphosate concentration in water along a three-week experiment, in control (circles), small mussel (diamond) and large mussel (crosses) treatments. Symbols represent mean values of three replicates $\pm$ standard error. Asterisks indicate statistical differences between LM treatment and control, whereas double asterisks refer to statistical differences between LM with respect to both control and SM treatments. Three asterisks indicate statistical differences compared to initial values.

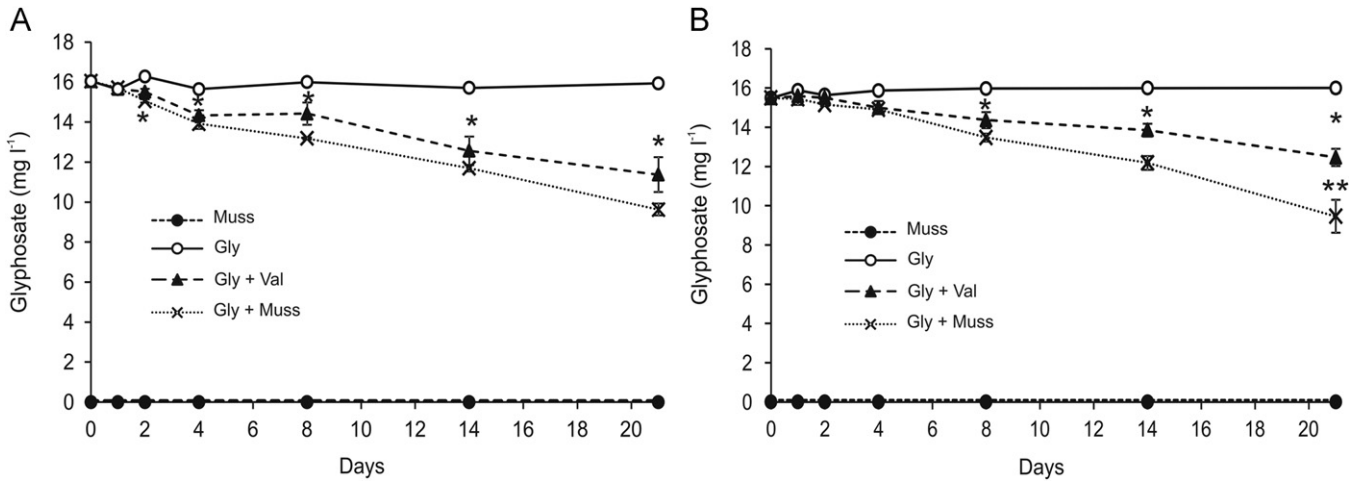


**Fig. 2.** Glyphosate: variation of glyphosate concentration in water along a three-week experiment in tap water (A) and river water (B) microcosms: control treatments with mussels (Muss) are represented with black circles and cut line; control with only glyphosate (Gly) in empty circles and full line; glyphosate and valvae treatments (Gly+Val) in triangles and cut line; glyphosate and mussel treatments (Gly+Muss) in cross and dotted line. Symbols represent mean values of three replicates ± standard error. Asterisks indicate statistical differences of Gly+Muss and Gly+Val respect to control treatment, whereas double asterisks indicate statistical differences between Gly+Muss and Gly+Val.

### 3.3.2. Soluble reactive phosphorous

Initial soluble reactive phosphorous was of $0.09 \pm 0.01$ mg $l^{-1}$ in tap water and of $0.15 \pm 0.03$ mg $l^{-1}$ in river water (Fig. 3). In microcosms without valvae or mussel presence there was no variation in soluble reactive phosphorous (SRP) throughout the experiment. In treatments with valvae a rise in SRP was observed, reaching values of $0.57 \pm 0.03$ mg $l^{-1}$ and $0.53 \pm 0.02$ mg $l^{-1}$ in tap and river water respectively. For both water types, valvae and mussel treatments differed (Gly+Val and Gly+Muss) from day 1 onwards ($p < 0.01$ in all cases). Moreover, SRP concentration increased dramatically with mussel presence, being higher this rise in treatments with glyphosate addition (Gly+Muss), though differences are statistically irrelevant. Final mean values of SRP were as high as $1.70 \pm 0.08$ mg $l^{-1}$ and $2.18 \pm 0.16$ mg $l^{-1}$ in tap water (Muss and Gly+Muss respectively) and of $1.73 \pm 0.15$ mg $l^{-1}$ and $2.45 \pm 0.15$ mg $l^{-1}$ in river water (Muss and Gly+Muss respectively). Changes in SRP through time were detected for all treatments. In aquaria with mussel presence, the SRP increased drastically since the first 24 h of the experience to almost double the initial values ($p < 0.05$ in all cases). In treatments containing empty shells, a more gradual increase in SRP was observed, with statistical differences from initial concentration from day 4 onwards in tap water microcosm ($p = 0.011$) and from day 14 onwards in river water treatment ($p = 0.000$).

### 3.3.3. Nitrate+nitrite and ammonia

Nitrate+nitrite variation during the experiment was similar to what observed with SRP (Fig. 4). In treatments with no mussel or valvae presence ($Gly^{TW}$ and $Gly^{RW}$) nitrate+nitrite concentration was low with no significant variation respect to initial values ($0.33 \pm 0.05$ mg $l^{-1}$ and $0.14 \pm 0.01$ mg $l^{-1}$ for tap and river water respectively). With valvae presence (Gly+Val), the concentration of nitrate+nitrite increased along the experimental period, to final values of $2.90 \pm 0.21$ mg $l^{-1}$ and $3.03 \pm 0.47$ mg $l^{-1}$ in tap and river water respectively. For each water type, statistical differences were found between Gly and Gly+Val treatments only at day 21 ($p = 0.017$ for tap water, and $p = 0.017$ for river water). Treatments with mussel presence (Muss and Gly+Muss) had the largest increase in nitrate+nitrite concentration, and no statistical differences were registered between these. Final values in $Muss^{TW}$ and $Muss^{RW}$ were of $6.90 \pm 0.55$ mg $l^{-1}$ and $7.60 \pm 0.69$ mg $l^{-1}$ respectively, whereas with herbicide addition (Gly+Muss) final concentrations were slightly lower ($6.10 \pm 0.20$ mg $l^{-1}$ and $6.67 \pm 0.49$ mg $l^{-1}$ in tap and river water, respectively).

Unlike what happened with the other variables, ammonia followed a singular pattern (Fig. 5) and varied differently according to the water used. In tap water treatments, Gly as well as Gly+Val treatments had low values of nitrogen from ammonia ($N\text{-}NH_4^+$) during the experience, between 0.01 and 0.09 mg $l^{-1}$. However, in aquaria with valvae there was a slight increase in the first four days (up to $0.09 \pm 0.01$), followed by a rapid decrease of this nutrient. With mussel presence (Muss and Gly+Muss), ammonia values rose in the first 24 h of the experiment and decreased in the following days, boosting again from day 8 onwards with final values of $0.24 \pm 0.02$ and $0.63 \pm 0.16$ mg $l^{-1}$ for Muss and Gly+Muss respectively.

In river water treatments, initial values were higher compared to tap water, of $0.37 \pm 0.01$ mg $l^{-1}$. In Gly and Gly+Val treatments, ammonia decreased rapidly in the first week (during which it continued to be low) and became almost absent in the rest of the experiment. In treatments with mussel presence (Muss and Gly+Muss), ammonia presented a rapid increase in the first 24 h, then declined during the first week, and had a dramatic rise again in the last week of the experience. Final values in treatment with glyphosate addition (Gly+Muss) doubled that without herbicide (Muss) with ammonia concentrations at day 21 of $0.28 \pm 0.16$ and $0.69 \pm 0.19$ mg $l^{-1}$, though these differences were statistically not significant. Ammonia excretion by mussels was higher when glyphosate was present for both types of water, but these differences were statistically irrelevant.

### 3.3.4. Mussel variables

Mussel soft tissue weight varied between 18.4 and 28.2 mg between aquaria, and did not differ significantly between treatments ($p = 0.79$). Mean values (± SE) were of $22.3 \pm 0.1$ mg; $20.0 \pm 1.5$ mg; $20.0 \pm 1.3$ mg and $21.7 \pm 0.6$ mg for treatments $Muss^{TW}$, $Gly+Muss^{TW}$, $Muss^{RW}$ and $Gly+Muss^{RW}$ respectively.

## 4. Discussion

### 4.1. Effect of mussel and valvae presence in glyphosate concentration

The golden mussel *L. fortunei* was not adversely affected by the exposure to glyphosate (at least up to 40 mg $l^{-1}$) and responded

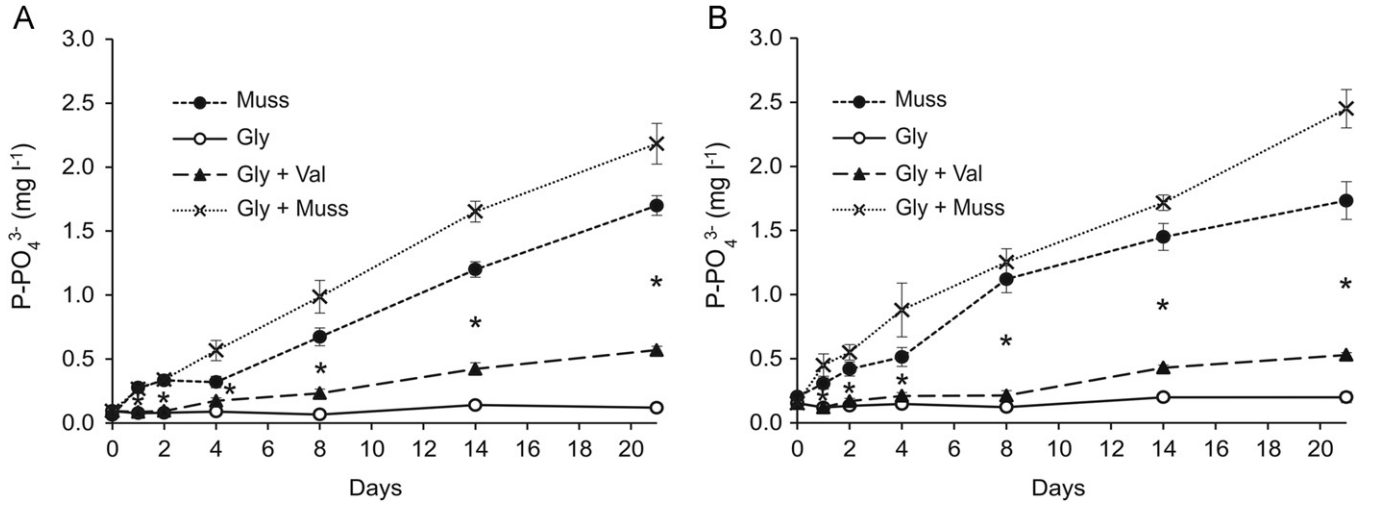


**Fig. 3.** Phosphate: variation of $P–PO_4^{3-}$ in water along a three-week experiment in tap water (A) and river water (B) microcosms: control treatments with mussels (Muss) are represented with black circles and cut line; control with only glyphosate (Gly) in empty circles and full line; glyphosate and valvae treatment (Gly+Val) in triangles and cut line; glyphosate and mussel treatments (Gly+Muss) in cross and dotted line. Symbols represent mean values of three replicates ± standard error. Asterisks indicate statistical differences between Gly+Val and Gly+Muss treatments.

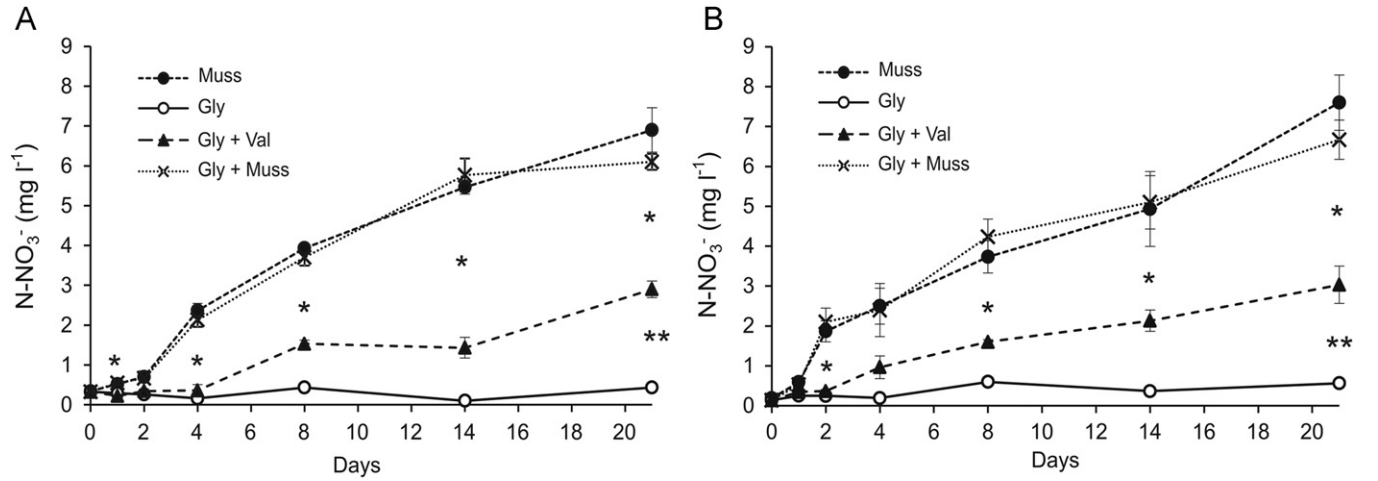


**Fig. 4.** Nitrite and nitrate: variation of $N–NO_3^-$ in water along a three-week experiment in tap water (A) and river water (B) microcosms: control treatments with mussels (Muss) are represented with black circles and cut line; control with only glyphosate (Gly) in empty circles and full line; glyphosate and valvae treatments (Gly+Val) in triangles and cut line; glyphosate and mussel treatments (Gly+Muss) in cross and dotted line. Symbols represent mean values of three replicates ± standard error. Asterisks indicate statistical differences between Gly+Val and Gly+Muss treatments, whereas double asterisks indicate statistical differences between Gly and Gly+Val.

by actively filtering, as detected by their extended siphons, instead of having an evasive behavior (i.e. closing up their valvae). Glyphosate concentration was affected by the presence of living mussels, being reduced after three weeks by 40 ± 2% (LM in first experiment), 40 ± 2% (Gly+Muss$^{TW}$), and 39 ± 5% (Gly+Muss$^{RW}$) in the second experiment (equal to 51 ± 1 and 37 ± 2 mg glyphosate organism$^{-1}$ day$^{-1}$ in tap and river water respectively). The effect of *L. fortunei* on glyphosate concentration under the same experimental conditions is therefore repeatable. In addition, with river water, the decrease in glyphosate concentration is similar to that with tap water, which indicates that water quality does not significantly alter mussel reaction to the xenobiotic. This suggests that the presence of the golden mussel (either alive or its empty shells) has a fast response in reducing glyphosate concentration in water, masking any possible effect water type could have on the herbicide. This promising characteristic of *L. fortunei* as a quick and effective responding agent in aquatic environments susceptible to glyphosate contamination has not been described yet for any other macroorganism (though it has been described for some bacteria and fungi). The exposure of glyphosate to *L. fortunei*'s empty valvae indicates that they too participate in dissipating the herbicide concentration in water, with a mean decline of 29 ± 5% in tap water and of 20 ± 3% in river water treatments (equal to 40 ± 3 and 28 ± 3 mg glyphosate organism$^{-1}$ day$^{-1}$ for tap and river water respectively). Both the shell and the soft mussel tissue would be contributing to reduce glyphosate concentration in water column.

There are two main processes that could be participating in glyphosate dissipation: adsorption of the herbicide to valvae or soft tissue mussel surface, and degradation processes mediated by bacteria from the biofilm attached to valvae and/or associated to the mussel metabolism. In the first case, adsorption is only considered to occur on valvae surface, excluding any significant adsorptive process onto walls of the aquaria containers, given that in the absence of valvae or mussels, glyphosate concentration did not vary much along the three weeks in both experiments. Respect to adsorption to mussel shells, it is well known that glyphosate has affinity to metal ions (Barja and dos Santos Afonso, 1998) and can be easily adsorbed through their phosphate group to cations in solid surfaces. The bivalvae shell is mainly composed

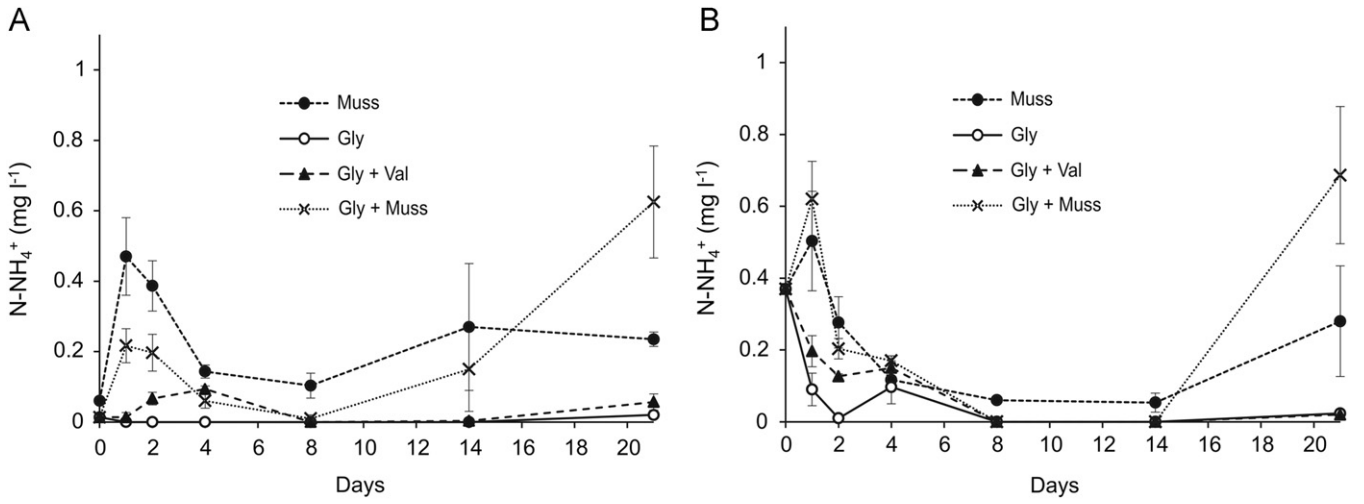


**Fig. 5.** Ammonia: variation of $N–NH_4^+$ in water along a three-week experiment in tap water (A) and river water (B) microcosms: control treatments with mussels (Muss) are represented with black circles and cut line; control with only glyphosate (Gly) in empty circles and full line; glyphosate and valvae treatments (Gly+Val) in triangles and cut line; glyphosate and mussel treatments (Gly+Muss) in cross and dotted line. Symbols represent mean values of three replicates ± standard error.

of two phases: crystalline calcium carbonate in the form of calcite or aragonite, and an organic matrix consisting largely of fibrous protein (Taylor and Layman, 1972). In addition, molluscan shells can contain trace amounts of many other elements as metal cations within calcite and aragonitic lattice structures, as well as anions entrapped in the shell structure as impurities during calcification or adsorbed onto organic matter in the shell (Fritz et al., 1990). Adsorption of glyphosate in shell structure is therefore expected to occur.

On the other hand, mussels supply the environment with associated microorganisms in shells' biofilm that could participate in herbicide degradation. In valvae treatments, degradation of the herbicide would be occurring given that in these treatments phosphate concentration in water increased with time, statistically differing from the initial concentration. As glyphosate is a phosphorous source, the microorganisms in the shells' biofilm would be breaking down the herbicide and providing the environment with dissolved phosphate. As the C–P bond present in phosphonates is resistant to chemical hydrolysis, thermal decomposition and photolysis as well as to the action of phosphatases (Hildebrand, 1983), we consider that this cleavage is being mediated by bacteria and fungi present in the biofilm. Phosphonates are widespread among naturally occurring compounds in all kingdoms of wildlife, but only prokaryotic microorganisms as well as some fungi are able to cleave C–P present in phosphonates, and use it as a phosphorus source (Castro et al., 2007, Kononova and Nesmeyanova, 2002). Nonetheless, degradation is less significant in river water treatments compared to tap water, possibly due to the presence of grazers in river water that would be altering biofilm's structure and function. In addition, the physical and chemical properties of the environment can influence the whole structure of the biofilm or change the biological properties of the cells within the biofilm and this may lead to detachment of microorganisms from the shell (Moore et al., 2000).

### 4.2. Glyphosate half-life

Glyphosate dissipation in natural aquatic environments is site-specific varying according to the chemical and biological characteristics of the water such as pH, temperature, the presence of aluminum and iron ions and on the microbial activity (Barja and dos Santos Afonso, 1998 and 2005). We obtained a half-life of 30.6 ± 2.6 days in the first experiment for LM treatment and of 28.8 ± 1.4 days for the second experiment in $Gly+Muss^{TW}$ treatments (with the same mussel size used in first experiment). Moreover, in the second experiment, when using river water ($Gly+Muss^{RW}$), the half-life, though slightly longer, did not vary much (34.4 ± 5.0 days) respect to same conditions with tap water. In valvae treatments instead, glyphosate half-life was 1.2 and 1.9 times longer for tap and river water respectively. In previous studies during larger-scale experiments, glyphosate half-life was shorter with values around 4.2 days (Vera et al., 2010) to 5.8 and of 7.4 days for more alkaline waters (Pérez et al., 2007). These experiments were carried out in mesocosms of 25 $m^2$ and 1.2 m depth, with added sediments, enhancing glyphosate adsorption to sediment particles and therefore reducing the concentration of the herbicide in the water column. In our experiment, glyphosate persistence was significantly higher, but water in our aquaria had low concentrations of mineral salts and probably a low plankton community biomass that could interact and participate in glyphosate breakdown. Glyphosate half-life is therefore affected by environmental conditions and varies with *L. fortunei* presence, depending on mussel conditions: size and density, and whether organisms are alive or dead (only valvae).

### 4.3. Inorganic nutrients

Soluble reactive phosphorus and nitrate+nitrite concentration in water varied in a similar way, increasing with time under mussel presence and increasing in a lesser extend in empty shell treatments. Mussel presence in aquaria supplies the aquatic environment with phosphate and nitrate due to its basal metabolism, both in treatments with and without glyphosate addition. Whenever the herbicide is present, the rise in phosphate is enhanced both with mussel presence and in valvae treatments as well, mainly given by the breakdown of glyphosate done by microorganisms in valvae's biofilm. Though higher phosphate values are obtained for treatments with glyphosate compared to treatments without glyphosate, no statistical differences are found indicating that although glyphosate is a phosphorus source, the effects of phosphate addition due to herbicide breakdown are masked by the excretion rate of mussels' basal metabolism.

On the other hand, the increment in phosphorus and nitrate concentration in water in empty valvae treatments, both in tap and river water, indicates that there are living organisms attached to

shells' surface excreting inorganic nutrients to the water. This favors our assumption that there is a microbial community attached to the valvae that would be participating in glyphosate breakdown. The fact that there are no statistical differences between different water types suggests that the degrading microbial community is being provided by the valvae's biofilm and not by the water community. It is important to point out that the biofilm present in mussel's valvae was originated in the natural environment from where the mussels were obtained. The microorganisms present in the biofilm will vary according to the environment where this biofilm is generated and therefore biofilms from different environments will probably respond differently to glyphosate presence.

Ammonia variation in treatments presented a different pattern. Initial ammonia concentration was higher in river water respect to tap water. During the first 24 h, in treatments with mussel presence, ammonia concentration increased, as a result of a higher excretion rate of the mussels. In river water, the concentration lowered to almost zero values, probably due to the fact that there are other microorganisms that use ammonia as a nutrient. After one or two weeks (depending on water type) ammonia concentration started to increase again in treatments with mussel presence, probably due to a higher metabolic rate of the organisms once adapted to aquaria conditions. However, the slight increase observed in ammonia concentration in empty valvae treatments for both types of water, though statistically not significant respect to control treatment, again supports the hypothesis of the presence of living microorganisms on shells' biofilm.

### 4.4. Mussel variables

There were no differences in the mass variables of mussel soft tissue in treatments with glyphosate addition respect to treatments without glyphosate. This indicates that the energy provided by the herbicide was not enough to cover the basal metabolism of the mussels, or that the experimental period was not enough as to detect differences in mass variables.

## 5. Conclusions

Water bodies are susceptible to being contaminated by the enormous amounts of glyphosate sprayed on soils, affecting non-target aquatic organisms (Pérez et al., 2007). Serious problems of water eutrophication are described as a result of the use of Roundup® (commercial formulation of glyphosate) due to the phosphorus addition mediated by the glyphosate (Vera et al., 2010). Glyphosate concentrations found in natural water bodies have still not been well registered; yet Relyea (2006) reviewed the available information on the subject and determined a range of glyphosate of 1.4–10.3 mg $l^{-1}$. According to Peruzzo et al. (2008), these concentrations in Argentina are found to be between 0.1 and 0.7 mg $l^{-1}$, but Berkovic et al. (2006) found herbicide concentrations in water bodies as high as 10.9 mg $l^{-1}$.

The golden mussel *L. fortunei* rapidly invaded the large rivers of the region (Parana and Uruguay rivers), altering the ecosystem with their high filtrating activity, plus their associated community of microorganisms. It has been shown that microbial communities contribute extensively to the attenuation, mineralization and transport of both organic and inorganic contaminants in the environment (Wolfaardt et al., 2000). In this work we provide evidence that indicates that *L. fortunei*, instead of being altered under glyphosate exposure, responds by actively filtering and that their presence acts as a rapid mineralizator of glyphosate releasing inorganic nutrients, partly due to the associated microorganisms in shells' biofilm. These results have implications of strong ecological effects on the environment: on the one hand, the faster dissipation of glyphosate in waters with the presence of *L. fortunei* would diminish the reported impacts of the xenobiotic on non-target aquatic organisms (Austin et al., 1991; Goldsborough and Brown, 1988; Relyea 2005a,b); on the other hand, in waters contaminated with glyphosate, the presence of the golden mussel would speed up the bioavailability of phosphorus for autotrophs, facilitating water eutrophication.

Our observations are a first step towards a promising use of the golden mussel as a biological agent that may moderate the direct effects of glyphosate discharges in freshwater ecosystems. More studies in larger scale, within a biological control plan, are needed in order to better understand the consequences of the interaction between glyphosate and *L. fortunei* in natural conditions. There are studies which use *L. fortunei* as a biomarker of pollution in natural waters by measuring physiological alterations after exposure (Contardo-Jara et al., 2009; Villela et al., 2007), but there is no study considering the golden mussel as a possible bioremediator. In this work, we propose this new point of view and believe it is of great interest, not only for Argentinean water bodies but also for many other countries where glyphosate is being indiscriminately used and where filtrating mussels, such as *L. fortunei*, are widespread.

## Acknowledgments

This work was supported by Agencia Nacional de Promoción Científica y Tecnológica, PICT 2006-1104. and PICT 2010-0908.

## References

Aizen, M.A., Garibaldi, L.A., Dondo, M., 2009. Expansión de la soja y diversidad de la agricultura argentina. Ecol. Aust. 19, 45–54 (Asociación Argentina de Ecología).
Austin, A.P., Harris, G.E., Lucey, W.P., 1991. Impact of an organophosphate herbicide (Glyphosate®) on periphyton communities developed in experimental streams. Bull. Environ. Contam. Toxicol. 47, 29–35.
Barja, B.C., dos Santos Afonso, M., 1998. An ATR-FTIR study of glyphosate and its Fe(III) complex in aqueous solution. Environ. Sci. Technol. 32, 3331–3335.
Barja, B.C., dos Santos Afonso, M., 2005. Aminomethylphosphonic acid and glyphosate adsorption onto goethite: a comparative study. Environ. Sci. Technol. 39, 585–592, http://dx.doi.org/10.1021/es035055q.
Berkovic, A., Marino, D., Lespade, P., Ronco, A., 2006. Análisis de pesticidas asociados al cultivo de soja en aguas y sedimentos de un sector productivo de la pampa ondulada. Dissertation. XXVI Congreso Argentino de Química, Facultad de Química, Bioquímica y Farmacia. Universidad Nacional de San Luis, Argentina.
Boltovskoy, D., Correa, N., Cataldo, D., Sylvester, F., 2006. Dispersion and ecological impact of the invasive freshwater bivalve Limnoperna fortunei in the Río de la Plata watershed and beyond. Biol. Invasions 8, 947–963, http://dx.doi.org/10.1007/s10530-005-5107-z.
Castro Jr., J.V., Peralba, M.C., Ayub, M.A., 2007. Biodegradation of the herbicide glyphosate by filamentous fungi in platform shaker and batch bioreactor. J. Environ. Sci. Health B 42 (8), 883–886.
Cataldo, D., Boltovskoy, D., Pose, M., 2003. Toxicity of chlorine and three non-oxidizing molluscicides to the invasive pest mussel *Limnoperna fortunei*. J. AWWA 95 (1), 66–78.
Cataldo, D., O'Farrell, I., Paolucci, E., Sylvester, F., Boltovskoy, D., 2012. Impact of the golden mussel (*Limnoperna fortunei*) on phytoplankton and nutrient cycling. Aquatic Invasions 7, 91–100.
Contardo-Jara, V., Galanti, L.N., Amé, M.V., Monferrán, M.V., Wunderlin, D.A., Wiegand, C., 2009. Biotransformation and antioxidant enzymes of *Limnoperna fortunei* detect site impact in water courses of Córdoba. Argentina Ecotoxicol. Environ. Saf. 72, 1871–1880, http://dx.doi.org/10.1016/j.ecoenv.2009.07.001.
Folmar, L.C., Sanders, H.O., Julin, A.M., 1979. Toxicity of the herbicide glyphosate and several of its formulations to fish and aquatic invertebrates. Arch. Environ. Contam. Toxicol. 8, 269–278.
Fritz, L.W., Ragone, L.M., Lutz, R.A., Swap, S., 1990. Biomineralization of barite in the shell of the freshwater Asiatic clam *Corbicula fluminea* (Mollusca: Bivalvia). Limnol. Oceanogr. 35 (3), 756–762.
Goldsborough, L.G., Brown, D.J., 1988. Effect of glyphosate (roundup formulation) on periphytic algal photo-synthesis. Bull. Environ. Contam. Toxicol. 41, 253–260.
Hilderbrand, R.L., 1983. The Role of Phosphonates in Living Systems. CRC Press Inc., Boca Raton, FL, USA.

Karatayev, A.Y., Burlakova, L.E., Padilla, D.K., 1997. The effects of *Dreissena polymorpha* (Pallas) invasion on aquatic communities in Eastern Europe. J. Shellfish Res. 16, 187–203.

Karatayev, A.Y., Boltovskoy, D., Padilla, D.K., Burlakova, L.E., 2007. The invasive bivalves *Dreissena polymorpha* and *Limnoperna fortunei*: parallels, contrasts, potential spread and invasion impacts. J. Shellfish Res. 26 (1), 205–213 http://dx.doi.org/10.2983/0730-8000(2007)26[205:TIBDPA]2.0.CO;2.

Kononova, S.V., Nesmeyanova, M.A., 2002. Phosphonates and their degradation by microorganisms. Biochemistry 67 (2), 184–195.

Liong, P.C., Hamzah, W.P., Murugan, V., 1988. Toxicity of some pesticides towards freshwater fishes. Malay. Agric. J. 54, 147–156.

Liphadzi, K.B., Al-Khatib, K., Bensch, C.N., Stahlman, P.W., Dille, J.A., Todd, T., Rice, C.W., Horak, M.J., Head, G., 2005. Soil microbial and nematode communities as affected by glyphosate and tillage practices in a glyphosate-resistant cropping system. Weed Sci. 53 (4), 536–545, http://dx.doi.org/10.1614/WS-04-129R1.

Moore, G.F., Dunsmore, B.C., Jones, S.M., Smejkal, C.W., Jass, J., Stoodley, P., Lappin-Scott, H.M. 2000. In: Editors Allison, D., Gilbert, P., Lappin-Scott, H., Wilson, M. (Eds.), SGM symposium 59: Community structure and co-operation in biofilms. Cambridge Univesity Press, Microbial detachment from biofilms pp. 107–127.

Paganelli, A., Gnazzo, V., Acosta, H., López, S.L., Carrasco, A.E., 2010. Glyphosate-based herbicides produce teratogenic effects on vertebrates by impairing retinoic acid signaling. Chem. Res. Toxicol. 23 (10), 1586–1595 http://dx.doi.org/10.1021/tx1001749.

Pastorino, G., Darrigran, G., Martin, S., Lunaschi, L., 1993. *Limnoperna fortunei* (Dunker, 1957) (Mytilidae) nuevo bivalvo invasor en aguas del Río de la Plata. Neotropica 39, 101.

Pessagno, R.C., Torres Sánchez, R.M., dos Santos Afonso, M., 2008. Glyphosate behavior at soil and mineral-water interfaces. Environ. Pollut. 153, 53–59, http://dx.doi.org/10.1016/j.envpol.2007.12.025.

Pérez, G.L., Torremorell, A., Mugni, H., Rodríguez, P., Vera, M.S., Do Nascimento, M., Allende, L., Bustingorry, J., Escaray, R., Ferraro, M., Izaguirre, I., Pizarro, H., Bonetto, C., Morris, D.P., Zagarese, H., 2007. Effects of the herbicide Roundup on freshwater microbial communities: a mesocosm study. Ecol. Appl. 17, 2310–2322, http://dx.doi.org/10.1890/07-0499.1.

Peruzzo, P.J., Porta, A.A., Ronco, A.E., 2008. Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. Environ. Pollut. 156, 61–66 http://dx.doi.org/10.1016/j.envpol.2008.01.015.

Relyea, R.A., Schoeppner, N.M., Hoverman, J.T., 2005. Pesticides and amphibians: the importance of community context. Ecol. Appl. 15, 1125–1134 http://dx.doi.org/10.1890/04-0559.

Relyea, R.A., 2005a. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecol. Appl. 15, 618–627 http://dx.doi.org/10.1890/03-5342.

Relyea, R.A., 2005b. The lethal impact of Roundup on aquatic and terrestrial amphibians. Ecol. Appl. 15 (4), 1118–1124.

Relyea, R.A., 2006. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecol. Appl. 16, 2027–2034 http://dx.doi.org/10.1890/04-1291.

Salisbury, F.B., Ross, C.W., 1994. Fisiología vegetal. Grupo Editorial Iberoamérica, México, DF.

Sylvester, F., Dorado, J., Boltovskoy, D., Juárez, A., Cataldo, D., 2005. Filtration rates of the invasive pest bivalve *Limnoperna fortunei* as a function of size and temperature. Hydrobiologia 534, 71–80.

Taylor, J.D., Layman, M., 1972. The mechanical properties of bivalve (Mollusca) shell structures. Paleontology 15 (1), 73–87.

Trigo, E.J., Cap, E.J., 2006. Diez años de cultivos transgénicos en la agricultura Argentina. ArgenBio Buenos Aires 53 pp.

Vera, M.S., Lagomarsino, L., Sylvester, M., Pérez, G.L., Rodríguez, P., Mugni, H., Sinistro, R., Ferraro, M., Bonetto, C., Zagarese, H., Pizarro, H., 2010. New evidences of Roundup® (glyphosate formulation) impact on the periphyton community and the water quality of freshwater ecosystems. Ecotoxicology 19, 710–721, http://dx.doi.org/10.1007/s10646-009-0446-7.

Villela, I.V., Marques de Oliveira, I., Coelho Silveira, J., Ferraz Dias, J., Henriques, J.A.P., Silva, J., 2007. Assessment of environmental stress by the micronucleus and comet assays on *Limnoperna fortunei* exposed to Guaíba hydrographic region samples (Brazil) under laboratory conditions. Mutat. Res. 628, 76–86 http://dx.doi.org/10.1016/j.mrgentox.2006.12.001.

Williams, G.M., Kroes, R., Munro, I.C., 2000. Safety evaluation and risk assessment of the herbicide roundup and its active ingredient, glyphosate, for humans. Regul. Toxicol. Pharmacol. 31, 117–165, http://dx.doi.org/10.1006/rtph.1999.1371.

Wolfaardt, G.M., Korber, D.R., Karthikeyan, S., Caldwell, D.E., Biodegradat. 2000 In: SGM symposium 59: Community Structure and Co-operation in Biofilms. Editors Allison, D., Gilbert, P., Lappin-Scott, H., Wilson, M. (Eds.), Cambridge Univesity Press.