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Biochemical responses of the golden mussel *Limnoperna fortunei* under dietary glyphosate exposure

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ABSTRACT

The aim of this study was to analyze the biochemical alterations in the golden mussel *Limnoperna fortunei* under dietary glyphosate exposure. Mussels were fed during 4 weeks with the green algae *Scenedesmus vacuolatus* previously exposed to a commercial formulation of glyphosate (6 mg L^{-1} active principle) with the addition of alkyl aryl polyglycol ether surfactant. After 1, 7, 14, 21 and 28 days of dietary exposure, glutathione-S-transferase (GST), catalase (CAT), superoxide dismutase (SOD), acetylcholinesterase (AChE), carboxylesterases (CES) and alkaline phosphatase (ALP) activities, glutathione (GSH) content and damage to lipids and proteins levels were analyzed. A significant increase (72%) in the GST activity and a significant decrease (26%) in the CES activity in the mussels fed on glyphosate exposed algae for 28 days were observed. The ALP activity was significantly increased at 21 and 28 days of dietary exposure (48% and 72%, respectively). GSH content and CAT, SOD and AchE activities did not show any differences between the exposed and non exposed bivalves. No oxidative damage to lipids and proteins, measured as TBARS and carbonyl content respectively, was observed in response to glyphosate dietary exposure. The decrease in the CES activity and the increases in GST and ALP activities observed in *L. fortunei* indicate that dietary exposure to glyphosate provokes metabolic alterations, related with detoxification mechanisms.

1. Introduction

The water bodies in Argentina are increasingly exposed to several agrochemicals. This occurs as a consequence of the expansion of the agricultural frontier and a growing trend in the use of different agrochemicals in current agricultural practices (Altieri and Pengue, 2006). Agrochemicals can be distributed in different aquatic compartments (water column, suspended particles, sediments, biota) (Warren et al., 2003) and they interact with the biota of all trophic levels, producing adverse effects over short and long term. According to their chemical nature, agrochemicals can also be bioaccumulated and transferred through the trophic chains (Nfon et al., 2008).

The use of glyphosate (N-phosphonomethyl glycine) for crop production is world-wide spread, both in industrialized and developing countries (Benbrook, 2016). For example, the global use of this herbicide reached about 127,000 t in USA and 700,000 t worldwide in 2012 (Van Bruggen et al., 2018). Similarly, glyphosate is the most widely used herbicide in Argentina. During 2013 its consumption was 182,484,206 L, representing 65% of the total pesticides used in the country (Aparicio et al., 2015). Glyphosate is highly soluble in water (12 g L^{-1}, log K_{ow}: −3,2) with a half-life ranging from 4 to 5 days to 60 days in water and from a few days to several months in the soil,

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depending on the environmental conditions (Székács and Darvas, 2012; Vereecken, 2005).

Glyphosate has a powerful herbicidal action and its primary mechanism of action is the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Sergiev et al., 2006), which is only present in plants and microorganisms. This inhibition results in the arrest of protein synthesis and the death of the target organisms. Glyphosate is an organophosphonate, therefore it has a C-P bond which makes it thermally and chemically stable (Kertesz et al., 1994). Its microbiological degradation occurs mainly in the soil, whereas in water column and aquatic sediments it occurs to a lesser extent (Lipok et al., 2010).

In general, the regulation of pesticide use is based on the effects of the active principles that exert herbicidal, insecticidal or fungicidal actions (Séguin et al., 2017). Nevertheless, in the field, glyphosate is applied as formulations containing adjuvants and inerts, some of which also have toxic effects on the organisms. In particular, in the central Pampean region of Argentina, one of the most applied formulations is Glifosato Atanor® (48% glyphosate as isopropylamine salt) with 2.5% of the surfactant Impacto® (alkyl aryl polyglycol ether) (Romero et al., 2011).

Microalgae can accumulate and bioconcentrate xenobiotics, biotransform them (sometimes generating more toxic metabolites) and/or adsorb them on their cell walls (Okay et al., 2000). Algae are the basis of food webs of aquatic ecosystems, so they act as a point of entry of pollutants into aquatic organisms that feed directly or indirectly on them (Okay et al., 2000).

Bivalves are organisms that filter large amounts of water for nutritional and respiratory requirements. They are placed in the first levels of many aquatic food webs and play an important ecological role, regulating the turbidity of the water column, recycling nutrients and organic matter and controlling phytoplankton biomass (Binelli and Provini, 2003). Mussels are considered excellent biomonitors of pollution due to their feeding habits, sedentary lifestyle, abundance, wide distribution in different water bodies, tolerance to a wide range of environmental conditions, and tendency to accumulate contaminants in their tissues (Belaich et al., 2006). Then, as filter feeding organisms, bivalves could be exposed to pollutants directly or by dietary exposure. Although glyphosate has a high water solubility and low octanol/water partition coefficient, it could potentially bioccumulate in bivalves, as it has already been reported for other aquatic animals (CCME, 2012; Contardo-Jara et al., 2009).

Several biochemical markers are used to evaluate the toxicity of pesticides on bivalves. These biomarkers include parameters related to the oxidation of biomolecules, and to changes in the levels of antioxidant, detoxification and general metabolic enzymes. Thus, the lipid and protein oxidation markers (thiobarbituric reactive substances –TBARS-, and carbonyl contents), the levels of the antioxidant metabolite reduced glutathione (GSH), and the activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) are commonly evaluated in pesticide exposed bivalves (Mottier et al., 2015). Other enzymes that could be altered in bivalves exposed to pesticides are the detoxification enzymes glutathione-S-transferase (GST) and carboxylesterases (CES), the general metabolism enzyme alkaline phosphatase (ALP) and the organophosphate sensitive enzyme acetylcholinesterase (AChE) (Castro et al., 2017; Galloway et al., 2002).

The golden mussel *Limnoperna fortunei* (Bivalvia, Mytilidae) is a freshwater bivalve from rivers and streams in China and Southeast Asia, and it was first detected in Argentina in 1991 (Darrigran and Ezcurra, 2000). This species has a high reproductive potential and adaptability and it has no natural predators or pathogens in the aquatic systems of Argentina (Darrigran and Damborenea, 2006; Darrigran and Ezcurra, 2000). Thus, L. *fortunei* has expanded to the main river basins, reaching densities higher than 200,000 individuals/m^2 (Boltovskoy et al., 2006), and occurring in wetland areas impacted by glyphosate.

Although glyphosate is promoted as harmless for animals, several ecotoxicological studies show that glyphosate could be toxic to bivalves and other aquatic organisms (Modesto and Martinez, 2010; Mottier et al., 2015). Inhibition of CES and AChE activity and alteration of oxidative stress parameters have been reported in bivalves exposed to glyphosate acid or their formulations (Abdel-Nabi et al., 2007; Iummato et al., 2013; Mottier et al., 2015).

Aquatic organisms, mainly those that filter large amounts of phytoplankton, face the impact of the contaminants that enter through their food. In general, few researches have addressed the study on the effects of this via of pesticide exposure. Additionally, there are no studies on enzymatic and oxidative stress responses of a freshwater mussel with dietary exposure to glyphosate. Therefore, the aim of this study was to evaluate biochemical alterations, mainly related to oxidative stress and detoxification responses, in the golden mussel L. *fortunei* after dietary exposure to glyphosate.

2. Materials and methods

2.1. Chemicals

The commercially available herbicide used in this study was Glifosato Atanor® (48% p/v isopropylamine salt of N-phosphonomethyl glycine, Atanor, Munro, Buenos Aires province, Argentina) and the surfactant was alkyl aryl polyglycol ether 50% (p/v) Impacto® (Agroasist S.R.L., Argentina).

2.2. Organisms

The BAFC CA4 strain of *Scenedesmus vacuolatus* (Chlorophyceae, Chlorophyta) is currently kept in the Culture Collection of the Laboratorio de Biología de Protistas, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires.

Specimens of L. *fortunei,* of 25.7 ± 2.2 mm shell length, were collected manually on the banks of the Río de la Plata (34° 29' 5.55"S, 58° 28' 49.67" W, Buenos Aires, Argentina), and were transported to the laboratory. Specimens of this size would correspond to adult animals of approximately 1–1.5 years old (Boltovskoy and Cataldo, 1999). Prior to the experiments, the specimens of *L. fortunei* were acclimatized for 4 weeks in 20 L tanks (200 individuals per tank). Tanks were filled with aerated dechlorinated tap water and the mussels were maintained at 20 ± 1 °C and 12:12 photoperiod. During the acclimation period, mussels were fed twice a week with 2×10^5 *S. vacuolatus* cells mL^{-1} for 24 h.

2.3. Algal cultures

S. vacuolatus cultures were prepared in 500 mL flasks containing 300 mL of Bold´s basal medium (BBM, Bischoff and Bold, 1963) with Glifosato Atanor® (6 mg L^{-1} active principle) and 2.5% surfactant Impacto® (treated cultures) or without glyphosate nor surfactant (control cultures). Cells from an exponential phase culture were used as inoculum to achieve an initial cell density of 30,000 cells mL^{-1}. The flasks were incubated at 23 ± 1 °C, under continuous agitation and illumination (80 μmol photons m^{-2} s^{-1}). After 96 h, the cells from both culture groups (control and treated) were harvested, washed and suspended in dechlorinated water to obtain a concentrated cell suspension. The cell density from the different suspensions was determined by cell counting in a Neubauer's chamber, using a Leica light microscope at 400×.

The initial glyphosate concentration in the BBM medium solution was analytically determined at INQUIMAE–CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. The determination of glyphosate was performed by HPLC-UV chromatography after a derivatization step with FMOC-Cl (Sancho et al., 1996; Stalikas and Konidari, 2001). The analysis was performed using a HPLC-UV

system (Jasco Analytical Instruments, Easton MD, USA) with a Microsorb C18 5 µm column, 250 × 4.6 mm inner diameter (Varian). The measured glyphosate concentration was 93.3 ± 2.3% of the nominal value. The exposure concentration tested in this study is similar to glyphosate concentrations recorded in several water bodies in Canada, USA and Argentina (CCME, 2012; Ronco et al., 2008; WHO, 2005).

2.4. Filtration rate

The filtration rate experiment was performed in order to evaluate the maximum cell concentration at which L. *fortunei* could filter the control and treated *S. vacuolatus* cells at similar rates. Six mussels (25.5 ± 1.5 mm shell length) were put into individual beakers with 500 mL of dechlorinated tap water, with constant aeration and a temperature of 20 ± 1 °C. After 48 h of acclimation without food, when each individual had its valves open, *S. vacuolatus* from the control and treated concentrated cell suspensions was added at a final concentration of 2×10^5 cells mL^{-1}. Samples were taken at 0, 1, 2, 3, 4 and 24 h and the cell density was estimated by direct counting. Filtration rate was expressed as L/h per dry soft tissue weight (g) (Jorgensen, 1990). The dry soft tissue weight was measured after drying the soft tissues for 48 h at 60 °C, until constant weight. The filtration rate experiment was carried out in triplicate.

2.5. Experimental design

After the acclimation period, animals were measured and placed in 125 mL Erlenmeyers with aerated dechlorinated tap water (six individuals per Erlenmeyer). Then, 120 animals corresponding to the control group were fed with control *S. vacuolatus* cells (i.e. with algal cells grown in BBM without glyphosate), and 120 animals corresponding to the treated group were fed with glyphosate treated *S. vacuolatus* cells (i.e. with algal cells grown in BBM with 6 mg L^{-1} glyphosate). No other possible source of glyphosate was available for the treated mussels. Each group of mussels was fed twice a week with 2×10^5 algal cells mL^{-1} for 24 h, added from the corresponding concentrated cell suspension. This procedure was repeated for 4 weeks. Water was changed before adding the algae. The mussels from four Erlenmeyers (replicates) were sacrificed at the end of each experimental time (1, 7, 14, 21 and 28 days) (Fig. 1), and their total weight and shell length were recorded. Then, the soft body was dissected, weighed and stored at − 80 °C until biochemical analysis. A Condition Index (CI), defined as the soft body wet mass / shell length, was determined.

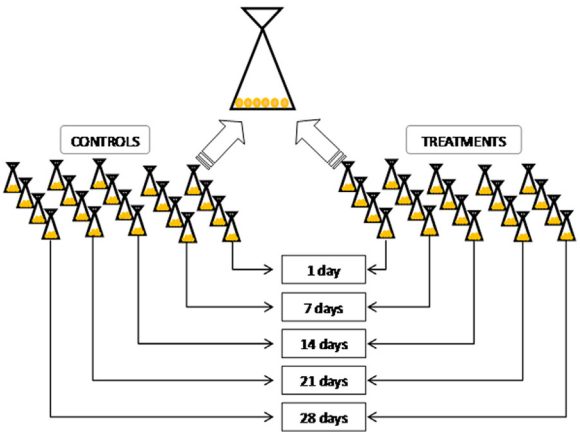

Fig. 1. Scheme of the experimental design showing the flasks containing 6 mussels (circles) each, and the time at which the mussels were sacrificed for biomarker determination. Controls: mussels fed with *S. vacuolatus* cells without any previous exposure to glyphosate. Treatment: mussels fed with *S. vacuolatus* cells previously treated with glyphosate.

2.6. Sample preparation

Unless indicated, the soft bodies were homogenized with 0.154 M KCl (1:5 w/v) containing protease inhibitors (0.2 mM benzamidine and 0.5 mM phenyl methyl sulfonyl fluoride). The homogenates were centrifuged at 11,000 × *g* for 30 min and the supernatant was used for determination of the enzyme activities, GSH and carbonyl content and lipid peroxidation level. All procedures were performed at 4 °C. The total soluble protein content was measured by the method of Bradford (1976), using bovine serum albumin as standard.

2.7. Biomarkers

2.7.1. Lipid peroxidation

Determination of the lipid peroxidation level was carried out measuring thiobarbituric acid reactive substances (TBARS) according to Buege and Aust (1978) procedure. Briefly, 175 µL of supernatant were mixed with thiobarbituric acid (TBA) solution and incubated in a boiling bath for 30 min. After cooling, the reaction mixture was centrifuged and the absorbance of supernatant was measured at 535 nm. The TBARS concentration was estimated as malondialdehyde (MDA) equivalents, using the extinction coefficient of MDA–thiobarbituric acid complex (156 mM^{-1} cm^{-1}). Results were calculated as nmol TBARS per mg protein and were expressed as % respect to the control.

2.7.2. Protein oxidation

Protein oxidation was quantified as carbonyl content according to Reznick and Packer (1994). First, nucleic acids from supernatants were removed by precipitation with 10% streptomycin sulfate, and centrifugation at 10,000 × *g* for 15 min. One half of the supernatant was treated with 2.5 M HCl (1:4, v/v) and used as a blank, and the other was treated with 10 mM dinitrophenylhydrazine (DNPH) in 2.5 M HCl (1:4 v/v), and both were incubated 1 h at room temperature. Then, 20% trichloroacetic acid (TCA) was added, the samples were centrifuged at 7000 × *g* for 5 min, and the precipitate was washed with TCA 10% and then with ethanol: ethyl acetate (1:1 v/v) until a colorless supernatant was obtained. The precipitated proteins were dissolved in 6 M guanidine hydrochloride in 0.02 M potassium phosphate buffer (pH 2.3) and incubated for 10 min at 37 °C. Protein carbonyls concentration was estimated from the absorbance measurement at 370 nm, using an extinction coefficient $\varepsilon = 0.022\ \mu M^{-1} cm^{-1}$. Results were expressed as nmol carbonyl groups per mg protein and were expressed as % respect to the control.

2.7.3. Reduced glutathione content

Reduced glutathione (GSH) levels were measured according to Anderson (1985), with modifications. Briefly, an aliquot of supernatant was deproteinized with 5% sulfosalicylic acid, centrifuged for 10 min at 10,000 × *g*, and the supernatant was used as a source of endogenous GSH. The reaction mixture contained: the sample, 0.143 mM sodium phosphate buffer (pH 7.5) containing 6.3 mM EDTA and 6 mM 5,5′-dithiobis-2-nitro-benzoic acid (DTNB). Absorbance at 412 nm was measured after 30 min incubation at room temperature. Results were calculated as nmol GSH per mg protein and were expressed as % respect to the control.

2.7.4. Enzyme activity levels

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured using the procedure of Beauchamp and Fridovich (1971). This method is based on the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture contained**:** enzyme sample, 13 mM methionine, 0.1 mM EDTA, 75 µM NBT, 2 µM riboflavin, and 50 mM potassium phosphate buffer (pH 7.8). The samples were exposed to intense cool-white light for 15 min and then the absorbance was measured at 560 nm. One SOD unit was defined as the amount of enzyme necessary to inhibit by 50% the NBT reduction rate. Results were

calculated as units of SOD per mg protein and were expressed as % respect to the control.

Catalase (CAT, EC 1.11.1.6) activity was measured by monitoring the decomposition of hydrogen peroxide spectrophotometrically at 240 nm, using an extinction coefficient of 40 M^{-1} cm^{-1}(Aebi, 1984). The reaction mixture contained: enzyme sample, 50 mM potassium phosphate buffer (pH7.4) and 10 mM hydrogen peroxide. One CAT unit was defined as the enzyme amount decomposing 1 mmol of H_2O_2 per min. Results were calculated as units of CAT per mg protein and were expressed as % respect to the control.

Glutathione-S-transferase (GST, EC 1.11.1.9) activity was measured by the technique of Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The reaction mixture contained: enzyme sample, 100 mM phosphate buffer (pH 6.5) and 10 mM CDNB. The absorbance of GS-DNB complex was monitored at 340 nm (extinction coefficient GST-CDNB: 9.6 mM^{-1} cm^{-1}). One GST unit was defined as the amount of enzyme required to catalyze the formation of 1 μmol of GS-DNB per min. Results were calculated as units of GST per mg protein and were expressed as % respect to the control.

Acetylcholinesterase (AChE, EC 3.1.1.7) activity was measured in supernatants, obtained by homogenization in 20 mM Tris-HCl buffer (pH 7.5) plus 5 mM EDTA, according to Ellman et al. (1961). The reaction mixture contained: enzyme sample, 100 mM sodium phosphate buffer (pH 8.0), 0.2 mM DTNB and 0.75 mM acetylthiocholine iodide as substrate. The product was monitored continuously at 412 nm (extinction coefficient: 13.6 mM^{-1} cm^{-1}). One AChE unit was defined as the amount of enzyme required to hydrolyze 1 nmol of acetylthiocholine iodide per min. Results were calculated as AChE units per mg protein and were expressed as % respect to the control.

Carboxylesterases (CES, EC 3.1.1.1) activity was determined according to Kristoff et al. (2010) in supernatants obtained by homogenization in 20 mM Tris-HCl buffer (pH7.5) plus 5 mM EDTA. The reaction mixture contained: enzyme sample, 100 mM sodium phosphate buffer (pH 8.0) containing 5% (v/v) acetone and 1 mM p-nitrophenyl butyrate as substrate. The product p-nitrophenol (pNP) was continuously recorded at 400 nm (extinction coefficient: 18.6 mM^{-1} cm^{-1}). One CES unit was defined as the amount of enzyme required to catalyze the hydrolysis of 1 μmol of p-nitrophenyl butyrate per min. Results were calculated as CES units per mg protein and were expressed as % respect to the control.

Alkaline phosphatase (ALP, EC 3.1.3.1) activity was determined according to Bowers and McComb (1966) in supernatants obtained by homogenization in 40 mM Tris-HCl buffer (pH 8.6) containing 20 mM KCl, 30 mM $MgCl_2$, 0.2 mM benzamidine and 0.5 mM phenyl methyl sulfonyl fluoride. The reaction mixture contained: enzyme sample, 80 mM Tris-HCl buffer (pH 8.0) containing 40 mM KCl and 60 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.5 mM $MnCl_2$ and 25 mM p-nitrophenyl-phosphate as substrate. The product pNP was recorded at 405 nm (extinction coefficient: 18.8 mM^{-1} cm^{-1}). One ALP unit was defined as the amount of enzyme required to generate 1 nmol of pNP per min. Results were calculated as ALP units per mg protein and were expressed as % respect to the control.

2.8. Statistical analysis

Glyphosate effects at different times on the condition index and biochemical parameters were tested by two-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test. Normality and homoscedasticity were tested by Lilliefors and Bartlett tests, respectively (Sokal and Rohlf, 1999). GraphPad Prism 5 and Statistica 8 software were used for statistical analysis.

3. Results

The filtration rate of *L. fortunei* was 7.45 ± 2.1 L/h per g of dry tissue for mussels fed with *S. vacuolatus* control cells and 6.54 ± 2.05 L/h per g of dry tissue for mussels fed with *S. vacuolatus* treated cells. These rates were not statistically different ($p > 0.05$). After 24 h mussels filtered 100% of the supplied algal cells.

Table 1
Shell length, total weight and Condition Index in *L. fortunei*, at different times of dietary glyphosate exposure.

Time of exposure (days)	Group	Shell length (cm)	Total weight (g)	Condition Index (g/cm)
1	Control	2.46 ± 0.13	1.26 ± 0.16	0.110 ± 0.019
	Treatment	2.47 ± 0.14	1.28 ± 0.17	0.113 ± 0.020
7	Control	2.45 ± 0.19	1.25 ± 0.24	0.109 ± 0.028
	Treatment	2.48 ± 0.19	1.29 ± 0.23	0.113 ± 0.029
14	Control	2.50 ± 0.23	1.31 ± 0.29	0.115 ± 0.032
	Treatment	2.51 ± 0.01	1.33 ± 0.02	0.119 ± 0.002
21	Control	2.79 ± 0.18	1.67 ± 0.23	0.152 ± 0.021
	Treatment	2.68 ± 0.40	1.54 ± 0.50	0.136 ± 0.045
28	Control	2.61 ± 0.21	1.45 ± 0.26	0.131 ± 0.026
	Treatment	2.84 ± 0.25	1.63 ± 0.31	0.148 ± 0.028

Data are expressed as means ± SD.

Mussel mortality throughout the experiment was < 2% in both control and treated group. Moreover, no significant differences were observed ($p > 0.05$) in the length neither of the shell nor in the total weight, nor in the Condition Index (CI) (Table 1).

Glyphosate dietary exposure neither affected ($p > 0.05$) the TBARs levels nor the carbonyl content over 4 weeks. Moreover, there were no significant differences ($p > 0.05$) in the antioxidant defenses (SOD and CAT activities and GSH content), between the control and treated bivalves after 28 days of dietary exposure (Table 2).

The AChE activity did not show significant differences between the control and treated mussels (Fig. 2A). The CES activity was significantly lower (around 26%, $p < 0.05$) in the treated mussels than in the control mussels at day 28 (Fig. 2B).

Glyphosate effects on GST and ALP activities are shown in Figs. 2C and 2D, respectively. The GST activity was significantly increased (around 72%, $p < 0.001$) after 28 days of dietary exposure in treated mussels, whereas the activity of ALP had a significant increase at 21 and 28 days of dietary exposure (72%, $p < 0.01$% and 48% $p < 0.05$, respectively) in treated mussels with respect to controls.

4. Discussion

Glyphosate based formulations are widely used worldwide and the level of this herbicide has increased in aquatic environments (Van Bruggen et al., 2018). The presence of this herbicide in water bodies can constitute a risk for filter feeding organisms by direct exposure to the surrounding environment and through the consumption of glyphosate contaminated microorganisms. In this study, the impact of the dietary glyphosate exposure was evaluated by feeding *L. fortunei* with algae (*S. vacuolatus*) previously exposed to a glyphosate formulation. Previous assays in our laboratory showed that the 96-h exposure to 6 mg L^{-1} of glyphosate formulation elicited biochemical alterations in *S.vacuolatus*. Although the internal cell glyphosate content in the algal cells could not be measured, increases related to the herbicide exposure both in cell TBARS levels and in GST detoxifying activity, were registered (Iummato, 2016).

Our results showed that 28 days of dietary glyphosate exposure produced several biochemical alterations but did not produce any changes in the *L. fortunei* morphometric parameters such as length, weight and Condition Index. Taking into account the *L. fortunei* life cycle and the experimental design, it is possible that the exposure time has not been enough to elicit morphometric changes in the adult mussels. Some studies have informed morphometric alterations in other bivalves exposed to glyphosate, but in juvenile oysters upon 35–56 days of direct exposure (Mottier et al., 2015; Séguin et al., 2017).

Table 2
Oxidative stress parameters of *L. fortunei* at different times of dietary glyphosate exposure.

Time of exposure (days)	Group	TBARS	Carbonyl groups	SOD	CAT	GSH
1	Control	100.00 ± 26.99	100.00 ± 13.78	100.00 ± 5.73	100.00 ± 8.20	100.00 ± 2.94
	Treatment	111.42 ± 29.01	96.43 ± 15.14	113.92 ± 30.08	119.74 ± 31.59	144.94 ± 38.62
7	Control	100.00 ± 23.41	100.00 ± 13.34	100.00 ± 3.27	100.00 ± 20.77	100.00 ± 21.55
	Treatment	84.87 ± 11.34	106.19 ± 11.14	89.58 ± 29.14	89.64 ± 14.91	115.93 ± 17.00
14	Control	100.00 ± 11.68	100.00 ± 2.26	100.00 ± 22.93	100.00 ± 09.60	100.00 ± 13.29
	Treatment	115.29 ± 48.77	80.96 ± 18.68	84.61 ± 6.45	94.42 ± 19.30	73.01 ± 9.90
21	Control	100.00 ± 7.10	100.00 ± 4.94	100.00 ± 8.46	100.00 ± 6.56	100.00 ± 6.40
	Treatment	81.12 ± 05.97	101.80 ± 9.54	109.47 ± 3.61	72.91 ± 15.85	127.63 ± 38.86
28	Control	100.00 ± 31.45	100.00 ± 28.92	100.00 ± 20.73	100.00 ± 27.79	100.00 ± 34.63
	Treatment	108.24 ± 21.64	117.17 ± 18.11	101.44 ± 12.05	133.88 ± 26.21	114.19 ± 12.07

Data are expressed as % respect to control ± SD.

Although biomarkers related to detoxification mechanisms were altered, the analyzed oxidative stress parameters did not show differences. No significant differences were observed in TBARS and protein carbonyl group levels, GSH content and CAT and SOD activities, between the treated and control mussels throughout the experimental time. Studies in other bivalve species also showed no alterations in the TBARS and GSH levels nor in SOD and CAT activities when they were directly exposed to glyphosate formulations and glyphosate acid technical grade in the medium (Mottier et al., 2015; Séguin et al., 2017). However, in a previous study (Iummato et al., 2013), we found that direct exposure to glyphosate acid in the surrounding environment provoked an increase in the TBARS levels and a decrease in SOD activity of *L. fortunei* after 26 days of treatment. The difference between these results may be due to the type of exposure, which would cause differential effects. The concentration and availability of the glyphosate that the bivalve faces from contaminated microalgae cells could be lower than when the herbicide is in the medium. These differences would determine that, in the present study, there is no damage to lipids and proteins or induction of antioxidant responses. Furthermore, the fact that the difference might also be due to the herbicide applied (glyphosate acid vs glyphosate formulation containing additives) should not be ruled out.

Although our results show that the analyzed oxidative stress parameters were not modified, other biochemical markers were altered by exposure through diet.

The AChE and CES enzymes are considered biochemical markers of exposure to organophosphate compounds (Van Dyk and Pletschke, 2011). The AChE is a serine hydrolase involved in the transfer of nerve impulse, whereas CES enzymes are serine hydrolases that hydrolyze a wide range of exogenous and endogenous esters (Wheelock et al., 2008). Our results showed no alteration in AChE activity, whereas the CES activity diminished significantly in the bivalves exposed to glyphosate contaminated algae. Similarly, in a previous study, we also recorded no alteration in AChE activity and a decrease in the CES activity in L. *fortunei* directly exposed to glyphosate acid (Iummato et al., 2013). In contrast with these results, inhibition of AChE activity in tadpoles (Güngördü et al., 2016; Lajmanovich et al., 2013), fishes (Modesto and Martinez, 2010) and the mussel *Mytilus galloprovincialis* (Matozzo et al., 2018) directly exposed to glyphosate or glyphosate formulations has been reported. Several researchers also reported inhibition of CES activity in tadpoles exposed to Roundup and other glyphosate formulations (Güngördü et al., 2016; Lajmanovich et al., 2013). The AChE is a sensitive enzyme to organophosphate pesticide inhibition. However, several chemical subclasses of organophosphates

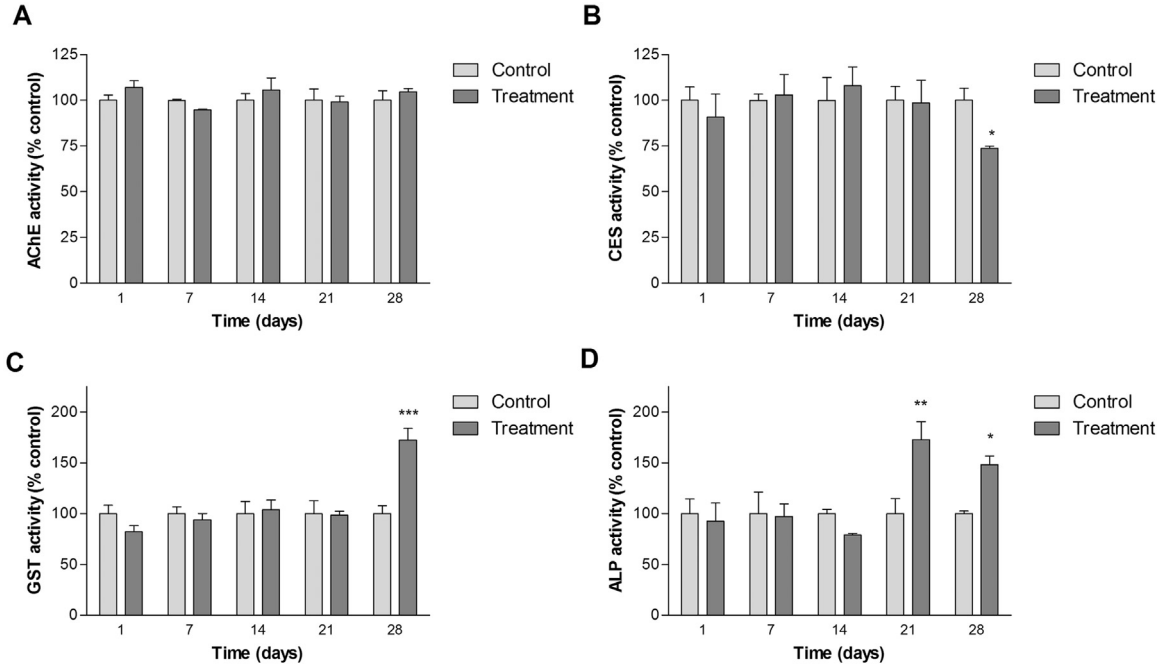

Fig. 2. Enzyme activities. **A:** Acetylcholinesterase (AChE), **B:** Carboxylesterases (CES), **C:** Glutathione-S-transferase (GST) and **D:** Alkaline phosphatase (ALP) in *L. fortunei* at different times of dietary exposure to glyphosate. Data are expressed as % respect to control ± SD. Asterisk denotes significant differences with respect to the control (*p < 0.05; **p < 0.01; *** p < 0.001).

exist, e.g, phosphonothioates, phosphoramidates, phosphonates, etc. Glyphosate is a phophonate compound and the lack of a phosphate leaving group in its molecule, could determine that it does not possess anticholinesterase activity (Gupta, 2006). This could explain the lack of AChE inhibition activity observed in *L. fortunei* exposed to glyphosate diet in this study. The mechanisms behind the inhibition of CES by glyphosate are not yet clear. On the other hand, CES plays an important role in agrochemical detoxification by interacting with three major classes of agrochemicals: organophosphates, carbamates, and pyrethroids (Wheelock et al., 2008). Therefore, the decrease of CES activity caused by glyphosate dietary exposure might increase the sensitivity of *L. fortunei* to environmental contaminants (Iummato et al., 2013).

The GST is a crucial enzyme in the detoxification of xenobiotics. This enzyme detoxifies different endogenous and exogenous compounds, via conjugation with GSH (Jokanović, 2001). In the present study, a significant increase in GST activity was induced in bivalves with dietary exposure for 28 days. Increases in the GST activity have been reported in different freshwater organisms, such as anuran tadpoles (Güngördü, 2013), oligochaetes (Contardo-Jara et al., 2009) and fish (Modesto and Martinez, 2010), directly exposed to glyphosate formulations. Moreover, in our previous study (Iummato et al., 2013), we observed an increase in the GST activity in *L. fortunei* directly exposed to glyphosate acid. In a previous study it was observed that a 96-h exposure to 6 mg L^{-1} glyphosate Atanor® with Impacto® induced an increase in the TBARS content in *S. vacuolatus* (Iummato, 2016). Therefore, algal cells will contain increased lipid peroxidation products, such as malondialdehyde and 4-hydroxy-2-nonenal, that have the potential to covalently modify proteins and DNA and are metabolized via GST (Kalinina et al., 2014). The results from the present study in glyphosate exposed *L. fortunei* through the consumption of herbicide exposed *S. vacuolatus* suggest the participation of GST in the detoxification of glyphosate and/or the toxic metabolites that would be present in the algae. Besides, this detoxifying response could explain, in part, the lack of morphometric alterations, by delaying possible damages to tissues, organs and weight maintenance of bivalves.

ALP activity is considered an indicator of the general metabolic state of the organisms (Van Dyk and Pletschke, 2011) and a biomarker of stress condition in aquatic organisms exposed to several contaminants (Chi et al., 2017). ALP activity can be altered for the presence of organochlorines, organophosphates and carbamates and can be induced or inhibited by these compounds (Van Dyk and Pletschke, 2011). Additionally, ALP can be found in hemocytes and hemolymph and it is involved in bivalve immune response against pathogens and foreign compounds, participating in intracellular and extracellular degradation processes of xenobiotics (Chi et al., 2017). In the present study, *L. fortunei* ALP activity was increased after 21 and 28 days of dietary glyphosate exposure. Similarly, in our previous study on the same species, the ALP activity was increased by direct exposure to glyphosate acid in outdoor microcosms (Iummato et al., 2013). Like the results of the GST enzyme, the ALP activity profile could indicate that, in *L. fortunei*, these enzymes respond after long-term contaminated alga exposure. Alterations in ALP activity have been associated with the response to contaminants exposure. For example, Castro et al. (2017) recorded an induction of ALP activity in hemocytes of the clam *Diplodon chilensis* exposed to the pesticide azinphos-methyl, Aanand et al. (2010) observed an induction of ALP activity in gut and adductor muscle in the mussel *Perna viridis* exposed to metals and Ranilalitha et al. (2014) recorded an increase in ALP activity in the digestive gland of the clam *Anadara rhombea* exposed to pesticide tributyltin chloride. Moreover, Neškovic et al. (1996) found an increase in ALP activity in the liver and heart of the fish *Cyprinus carpio* exposed to 5 and 10 mg L^{-1} of glyphosate acid. The increase in ALP activity of *L. fortunei* with dietary glyphosate exposure could be related with a modulation of the immune response and/or cellular damage, as has been postulated for the clams *D. chilensis* and *A. rhombea* exposed to pesticides (Castro et al., 2017; Ranilalitha et al., 2014)

5. Conclusions

In this research, the feeding of *L. fortunei* during 28 days with algae previously exposed to glyphosate, did not alter the morphometry of the mussels, but provoked significant alterations in various biochemical responses. Considering the analyzed parameters, the results obtained could indicate that no oxidative stress was induced in exposed bivalves, but detoxification responses were altered. The increase in GST activity indicates that the dietary glyphosate exposure activated detoxification mechanisms in *L. fortunei*. The increase in ALP activity, together with the decrease in CES activity observed in the present study, indicate that glyphosate elicited adverse effects on *L. fortunei*. Filter feeding organisms can be exposed to contaminants in their environment directly and also through the food they consume. In this study, we showed that the consumption of contaminated algae impacts on biochemical parameters of the mussels. Since microalgae are the natural food source for bivalves and other filter feeders, our study provides evidence of the impact and risk of glyphosate-contaminated microalgae for the aquatic filter feeding organisms.

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