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Effect of glyphosate on the growth, morphology, ultrastructure and metabolism of *Scenedesmus vacuolatus*

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ABSTRACT

The effects of a commercial glyphosate formulation on the oxidative stress parameters and morphology (including the ultrastructure) of the phytoplanktonic green microalga *Scenedesmus vacuolatus* were evaluated. After 96 h of exposure to increasing herbicide concentrations (0, 4, 6, 8 mg L^{-1} active ingredient) with the addition of alkyl aryl polyglycol ether surfactant, the growth of the cultures decreased (96 h-IC50- 4.90 mg L^{-1}) and metabolic and morphology alterations were observed. Significant increases in cellular volume (103–353%) and dry weight (105%) and a significant decrease in pigment content (41–48%) were detected. Oxidative stress parameters were significantly affected, showing an increase in the reactive oxygen species (ROS) and reduced glutathione (GSH) contents, oxidative damage to lipids and proteins and a decrease in the activities of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) and the detoxifying enzyme glutathione-S-transferase (GST). Cells exposed to glyphosate formulation were larger and showed an increase in vacuole size, bleaching, cell wall thickening and alteration of the stacking pattern of thylakoids. The results of this study showed the participation of oxidative stress in the mechanism of toxic action of the commercial glyphosate formulation on *S. vacuolatus* and the relation between the biochemical, morphological and ultrastructure alterations.

1. Introduction

The contamination of the freshwater environments is a matter of concern. Different chemical compounds enter water bodies and affect the aquatic biota. In particular, the agrochemicals can reach water bodies in different ways (aerial drift, runoff and intentional fumigation, among others) and affect non-target biota (Tang et al., 2012; Van Bruggen et al., 2018).

The green microalgae and higher plants share metabolic pathways, such as the photosynthetic way and aromatic amino acid biosynthesis through the shikimate pathway (Tohge et al., 2013). This similarity may determine that green algae are susceptible to the action of herbicides (Lipok et al., 2010). In the aquatic environment, microalgae play a relevant role in the primary production and recycling of nutrients, being the basis of many food webs in the aquatic ecosystem. Thus, a negative impact on microalgae can have adverse consequences on the whole aquatic ecosystem. Moreover, microalgae can accumulate and bioconcentrate xenobiotics, biotransform them (sometimes generating more toxic metabolites) and/or adsorb them on the cell walls. Therefore, microalgae often act as an input of pollutants to aquatic trophic

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chains (Torres et al., 2008).

The microalgae are considered excellent biomonitors of pollution due to their high sensitivity to different contaminants and to changes in the conditions of the water bodies related to their presence (Bellinger and Sigee, 2010). Besides, microalgae have a short life cycle, which enables the evaluation of the effects of exposure to contaminants in the short term, giving an early warning against a polluting situation (Torres et al., 2008).

It has been shown that different pollutants induce oxidative stress in the organisms. That condition occurs when the prooxidant compounds (reactive oxygen species, ROS) overcome the antioxidant defenses, such as reduced glutathione (GSH) and catalase (CAT) and superoxide dismutase (SOD) enzymes, and produces damage to lipids, proteins and DNA. Therefore, parameters related to oxidative damage and antioxidant response can be used as pollutant effect / exposure biomarkers (Gomes et al., 2017; Torres et al., 2008). Other biomarkers that can be analyzed in the algae exposed to contaminants are the activity of the detoxifying enzyme glutathione-S-transferase (GST), the pigment content and morphological parameters, such as cell size and cellular vacuolation. Additionally, the analysis of cellular ultrastructure allows a more exhaustive approach of the effect of contaminants on the microalgae.

Glyphosate is the most used herbicide in the world and it is applied in the field as different formulations (glyphosate as active ingredient with the addition of adjuvants). In Argentina, the commercial formulation Glifosato Atanor® is widely used, and it is applied in combination with the surfactant Impacto® (alquil aryl polyglycol ether) (Romero et al., 2011), which improves the efficiency of the herbicide treatment.

Glyphosate is highly soluble in water (11.6 g L^{-1}) (Székács and Darvas, 2012) and its primary mechanism of action is the inhibition of 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS). This enzyme is involved in the synthesis of aromatic aminoacids in plants and some microorganisms, but not in animals (Sergiev et al., 2006), so glyphosate is consider toxic to the former and innocuous for the latter.

Previous research recorded different alterations in microalgae exposed to glyphosate and glyphosate formulations (Factor 540® and Glifosato Atanor®): growth inhibition, decreases in the chlorophyll content, and increases in cellular vacuolation, cell size and protein content (Romero et al., 2011; Smedbol et al., 2018; Vendrell et al., 2009; Wong, 2000). Besides, it has been proposed that glyphosate induces oxidative stress in plant cells (Gomes and Juneau, 2016; Gomes et al., 2016; Percival, 2017) and in the green microalgae *Chlorella kessleri* (Romero et al., 2011).

Scenedesmus vacuolatus (formerly *Chlorella fusca* var. *vacuolata*) is a cosmopolitan green microalga, present in the phytoplankton of different freshwater bodies. The effects of several contaminants (including herbicides and metals) on this microalga have been studied, recording alterations in the growth rate, photosynthesis, protein content, chlorophyll content and increases in lipid peroxidation and antioxidant defenses (Sabatini et al., 2009; Vallotton et al., 2008).

The herbicides may have different effects on phytoplankton that may cause a decrease in the viability of their populations. The aim of this study was to evaluate the effects of a recommended agrochemical mixture (Glifosato Atanor® with the addition of surfactant Impacto®) on a phytoplanktonic green microalgae, *S. vacuolatus*. We hipothesize that this mixture provokes ultrastructural and metabolic alterations related to oxidative stress on the cells at concentrations that provide environmentally relevant levels of glyphosate. We analyzed biomass, growth, pigment content, cell morphology and ultrastructure, ROS levels, oxidative damage parameters (lipid peroxidation and protein oxidation), antioxidant defenses (GSH, CAT, SOD) and the activity of the detoxifying enzyme GST.

2. Materials and methods

2.1. Chemicals

The commercial glyphosate-based formulation used in this study was Glifosato Atanor® (48% w/v isopropylamine salt of N-phosphonomethyl glycine, Atanor, Munro, Buenos Aires province, Argentina) and the surfactant was alkyl aryl polyglycol ether (nonylphenol) 50% (w/v) Impacto® (Agro Asist S.R.L., Argentina).

A stock solution of Glifosato Atanor® with 2.5% w/v of surfactant was prepared. Different amounts of the herbicide stock solution were mixed into Bold´s basal medium (BBM), pH 6.5 (Bischoff and Bold, 1963), to achieve concentrations of 4, 6, and 8 mg L^{-1} of glyphosate (as isopropylamine salt). The addition of glyphosate did not alter the pH of the BBM medium.

The analysis of initial glyphosate concentrations in the culture medium was carried out by HPLC-UV chromatography after a derivatization step with FMOC-Cl, as described in Gattás et al. (2016). The limit of detection and quantification of the resulting procedure were 0.2 and 0.7 mg L^{-1}, respectively, and the percent variation coefficient was of 4.3%. All reactives were of analytical grade (Sigma-Aldrich).

A full description of the analyses in this section can be obtained from Supplementary material.

2.2. Algal strain and experimental design

The BAFC CA4 strain of *S. vacuolatus* (Chlorophyceae, Chlorophyta) is currently maintained 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.

The experimental design was carried out according to algal growth inhibition test standards (USEPA, 2002), with modifications. The bioassays were performed in 500 mL flasks containing 300 mL of BBM medium with different concentrations of the herbicide (0, 4, 6 and 8 mg L^{-1} glyphosate) and with an initial cell density of 3×10^4 cells mL^{-1}. The cultures were maintained on an orbital shaker (145 rpm) at 23 ± 1 °C, with continuous cool-white fluorescent light illumination (80 μmol photons m^{-2} s^{-1}). Three replicates were used per treatment and the bioassays were repeated three times Two controls were performed in all bioassays: control culture (C) without glyphosate Atanor® nor surfactant Impacto® and the surfactant control (SC) containing only the maximum surfactant concentration used (0.52 mg L^{-1} alkyl aryl polyglycol ether). Additional details on the experimental design are presented in Supplementary material.

After 96 h, the cell number was determined by counting in a Neubauer chamber, using a Leica DM 500 light microscope at 400×. The counting of at least 25 squares ensured an error of less than 10% (Venrick, 1978).

Growth rate (r) was estimated as: $r = (\ln N_f - \ln N_i)/t_f - t_i$, where N_f = final cell density, N_i = initial cell density (cells per mL), and $t_f - t_i$ = test duration in days.

The 96 h-IC50 value was estimated by Linear Interpolation Method (USEPA, 2002).

2.3. Dry weight and cellular volume

Algal dry biomass was measured by filtration of 40 mL of each culture through a preweighed Whatman GF/C glass fibre filter, and dried at 80 ± 1 °C to constant weight. In order to analyze the herbicide effect on the cell mass, results were expressed as μg dry weight per 10^6 cells.

The diameter of at least 100 randomly chosen cells was measured and the volume of each cell was calculated applying the equation for volume of a sphere (Hillebrand et al., 1999). In order to analyze the herbicide effect on cell´s volume, results were expressed as volume (in

μm^3) ± SD

2.4. Pigment content

Cells from 10 mL of each culture were harvested by centrifugation at 3000 × *g*, resuspended in 80% acetone and sonicated in an ultrasonic homogenizer Cole Parmer CP600 4710. Chlorophyll *a*, chlorophyll *b* and carotene concentrations in the acetonic extracts were spectrophotometrically determined using the equations of Lichtenthaler (1987). In order to analyze the herbicide effect on the cell pigment content and pigment balance, the results were expressed as μg of total chlorophyll (*a* + *b*) per 10^6 cells and the ratio of carotene/chlorophyll *a*.

Additional details on procedure for pigment content determination are presented in Supplementary material.

2.5. Reactive oxygen species content

The ROS content was determined according to Wang and Joseph (1999). Cells from 30 mL of each culture were incubated with 0.0375 mM 2′,7′-dichlorodihydrofluorescein diacetate (H_2CDF-DA), and the fluorescence was measured in a microplate reader FLUOstar OPTIMA, using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The ROS level of the samples was calculated, as equivalents of H_2O_2, from the corresponding calibration curve. In order to analyze the herbicide effect on ROS level, results were expressed as μmol ROS per mg proteins. Protein content was determined by Bradford (1976) method.

Additional details on procedure for reactive oxygen species content determination are presented in Supplementary material.

2.6. Oxidative damage

Determination of the lipid peroxidation level was performed by measuring the thiobarbituric acid reactive substances (TBARS) according to Vavilin et al. (1998), as previously described in Romero et al. (2011). The TBARS content was estimated as malondialdehyde (MDA) equivalents, using the extinction coefficient of MDA–thiobarbituric acid complex (ε: 0.157 μM^{-1} cm^{-1}). In order to analyze the herbicide effect on cell´s TBARS content, results were calculated using the Hodges et al. (1999) equations and were expressed as nmol TBARS per 10^6 cells.

Protein oxidation was quantified as the content of carbonyl groups according to Reznick and Packer (1994). Protein carbonyls concentration was estimated from the absorbance measurement at 370 nm, using an extinction coefficient ε = 0.022 $\mu M^{-1} cm^{-1}$. In order to analyze the herbicide effect on protein´s carbonyl content, results were expressed as nmol carbonyl per mg proteins.

Additional details on procedures for lipid and protein oxidation determination are presented in Supplementary material.

2.7. Reduced glutathione content

Reduced glutathione (GSH) levels were measured according to Okamoto et al. (2001) as previously described in Romero et al. (2011). The content of GSH was determined in the supernatant by reaction with 5,5′-dithiobis-2-nitro-benzoic acid (DTNB) and measurement of the absorbance at 412 nm. A freshly prepared solution of GSH was used to generate a standard curve. In order to analyze the herbicide effect on cell´s GSH content, results were expressed as nmol GSH per 10^6 cells.

Additional details on procedure for reduced glutathione content determination are presented in Supplementary material.

2.8. Enzyme activities

Cells from 150 mL of each culture were harvested by centrifugation at 3000 × *g* for 30 min, washed with 0.134 M potassium phosphate buffer (pH 6.5) and resuspended in 0.5 mL of the same buffer containing protease inhibitors (0.2 mM benzamidine and 0.5 mM PMSF). Then, cells were disrupted by sonication using a Cole Parmer CP600 4710 Ultrasonic homogenizer. The samples were centrifuged at 10,000 × *g* for 30 min, and the supernatants were employed as enzyme sources. All procedures were carried out at 4 °C.

SOD (EC 1.15.1.1) activity was measured according to the method of Beauchamp and Fridovich (1971), measuring the photochemical reduction of nitrobluetretrazolium (NBT) The absorbance was measured at 560 nm and. Results were expressed as specific activity (units of SOD per mg protein). One SOD unit was defined as the amount of enzyme necessary to inhibit by 50% the NBT reduction rate.

CAT (EC 1.11.1.6) activity was determined spectrophotometrically by monitoring the decomposition of hydrogen peroxide at 240 nm, according to Aebi (1984). Results were expressed as specific activity (units of CAT per mg protein), using an extinction coefficient of 40 M^{-1} cm^{-1}. One CAT unit was defined as the amount of enzyme necessary to decompose 1 μmol of H_2O_2 per min.

GST (EC1.11.1.9) activity was determined according to the method of Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The absorbance of GS-DNB complex was measured at 340 nm (extinction coefficient GST-CDNB: 9.6 mM^{-1} cm^{-1}). Results were expressed as specific activity (units of GST per mg protein). One GST unit was defined as the amount of enzyme required to catalyze the formation of 1 nmol of GS-DNB per min.

The protein content was estimated according to Bradford (1976) using bovine serum albumin as a standard.

Additional details on enzyme activities determination procedures are presented in Supplementary material.

2.9. Morphology and ultrastructure

The morphology of *S. vacuolatus* was evaluated in vivo from cultures grown in BBM liquid medium that contained 0, 4, 6 or 8 mg L^{-1} of glyphosate. Lugol staining was applied to highlight the cell starch granules. The observation and photomicrography were performed with a Leica DM 500 light microscope equipped with a Leica ICC50 digital camera.

The ultrastructure from control and treated cells was analyzed by transmission electron microscopy. The ultrathin sections were observed and photographed with a Zeiss EM 109 T electron microscope equipped with a Gatan ES100W digital camera at the Instituto de Biología Celular y Neurociencias (LANAIS - MIE), Facultad de Medicina, Universidad de Buenos Aires.

The number of nuclei and the cell wall thickness of at least 40 randomly chosen control and trated (8 mg L^{-1}) cells were measured in the ultrathin sections photographed. The percentage of cells with more than one nucleus and the average cell wall thickness were calculated.

Additional details on procedure for ultrathin section preparation are presented in Supplementary material.

2.10. Statistical analysis

The results from the different treatments were compared with the control without glyphosate Atanor nor surfactant Impacto by one-way analysis of variance (ANOVA) followed by a Dunnet's post hoc test. The significance threshold was set at 0.05. Normality and homoscedasticity were tested using the probes of Shapiro-Wilk and Bartlett, respectively. GraphPad Prism version 5.00 and Infostat 2014 software were used for statistical analysis.

3. Results

3.1. Growth and pigments

The exposure of the cultures to increasing concentrations of the

Table 1
Effects of glyphosate on cell density, growth rate, dry weight, cell volume and pigments of *S. vacuolatus* cultures after 96 h of exposure.

Glyphosate Concentration (mg L^{-1})	Cell density (10^6 mL^{-1})	Growth rate ($days^{-1}$)	Cell volume (μm^3 10^6 $cells^{-1}$)	Dry weight (μg 10^6 $cells^{-1}$)	Total chlorophyll ($a+b$) (μg 10^6 $cells^{-1}$)	Carotene/chlorophyll *a*
SC	0.403 ± 0.022	0.649 ± 0.014	312.197 ± 42.351	46.936 ± 13.215	0.311 ± 0.023	0.204 ± 0.022
0	0.422 ± 0.031	0.661 ± 0.019	433.987 ± 48.307	44.384 ± 9.181	0.383 ± 0.054	0.195 ± 0.044
4	0.261 ± 0.095**	0.531 ± 0.088	883.930 ± 124.051***	59.215 ± 2.546	0.297 ± 0.054	0.212 ± 0.054
6	0.162 ± 0.026**	0.420 ± 0.043**	1429.750 ± 283.352***	71.600 ± 19.684	0.224 ± 0.098*	0.279 ± 0.025
8	0.096 ± 0.034**	0.279 ± 0.103***	1966.518 ± 178.551***	91.385 ± 19.743***	0.196 ± 0.076*	0.395 ± 0.083***

Data are expressed as means ± SD. Asterisks denote significant differences compared to the control -without glyphosate nor surfactant- (* $p < 0.05$; ** $p < 0.01$; ** $p < 0.001$). SC: surfactant control.

commercial glyphosate formulation with the addition of alkyl aryl polyglycol ether surfactant had increasing inhibitory effects on the algal growth. The cell density decreased significantly in all the glyphosate concentrations tested ($F_{(4,10)} = 25.06$, p-value < 0.001). The culture growth rate (r) showed a significant decrease at 6 mg L^{-1} and 8 mg L^{-1} ($F_{(4,10)} = 18.82$, p-value < 0.001). The 96 h-IC50 estimated by the Linear Interpolation Method was 4.90 (4.26–5.41) mg L^{-1}(Table 1).

The commecial glyphosate formulation with the addition of surfactant alkyl aryl polyglycol ether provoked a significant increase ($F_{(4,10)} = 25.06$, p-value < 0.001) in the cell volume at all tested concentrations. The cell dry weight showed a significant increase at 8 mg L^{-1} of glyphosate ($F_{(4,10)} = 5.38$, p-value = 0.014). (Table 1).

A significant decrease ($F_{(4,10)} = 3.81$, p-value = 0.0392) in the total chlorophyll content per cell was observed in *S. vacuolatus* exposed to 6 and 8 mg L^{-1} of glyphosate, whereas a significant increase ($F_{(4,10)} = 8.217$, p-value = 0.0033). in the carotene/chlorophyll *a* ratio was recorded at 8 mg L^{-1} of glyphosate.

No significant differences between the surfactant control and the bioassay control were found in any of the parameters analyzed (Table 1).

3.2. Oxidative stress and damage

The ROS content increased in a concentration dependent manner with the glyphosate treatment, and a significant increase was observed at 8 mg L^{-1} ($F_{(4,10)} = 5.384$, p-value = 0.0142). (Fig. 1).

Cells exposed to glyphosate registered a significant increase in TBARS content from 6 mg L^{-1} ($F_{(4,10)} = 41.29$, p-value < 0.001) with respect to the controls (Fig. 2A). The level of oxidized proteins also increased significantly at 6 mg L^{-1} and 8 mg L^{-1} ($F_{(4,10)} = 9.961$, p-value = 0.0016) (Fig. 2B).

GSH content showed a significant increase ($F_{(4,10)} = 75.79$, p-value < 0.001) with respect to the control in the *S. vacuolatus* cells exposed to 6 mg L^{-1} and 8 mg L^{-1} of glyphosate (Fig. 3).

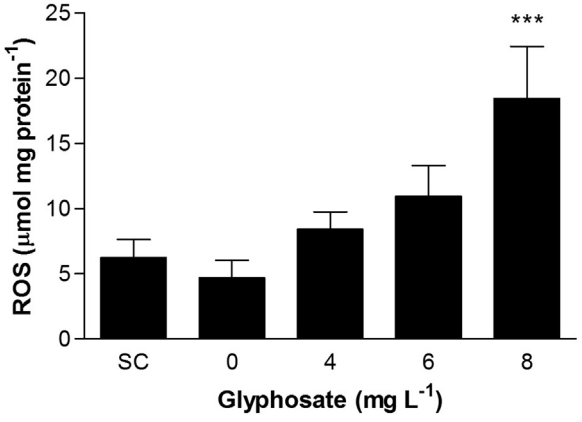

Fig. 1. Reactive oxygen species (ROS) content (µmol mg $protein^{-1}$) in *S. vacuolatus* exposed to glyphosate for 96 h. Data are expressed as means ± SD. Asterisk denotes significant differences with respect to the control (***$p < 0.001$). SC: surfactant control.

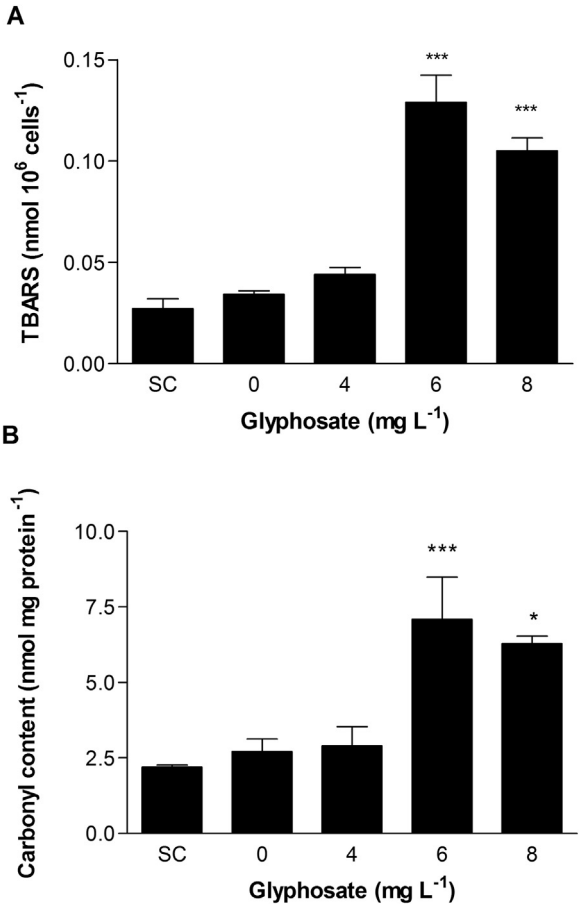

Fig. 2. Thiobarbituric acid reactive substances (TBARS) content in nmol 10^6 $cells^{-1}$ (A) and carbonyl content in nmol mg $protein^{-1}$ (B) in *S. vacuolatus* exposed to glyphosate for 96 h. Data are expressed as means ± SD. Asterisk denotes significant differences with respect to the control (*$p < 0.05$; ***$p < 0.001$). SC: surfactant control.

The activity of the antioxidant enzymes SOD and CAT showed a significant decrease with respect to the control in the *S. vacuolatus* cells exposed to all the glyphosate concentrations ($F_{(4,10)} = 5.602$, p-value = 0.0125 and $F_{(4,10)} = 12.55$, p-value < 0.001 for SOD and CAT, respectively) (Fig. 4A and B).

The activity of the detoxifying enzyme GST was also significantly decreased in the cells exposed to all glyphosate concentrations ($F_{(4,10)} = 6.376$, p-value = 0.0081) (Fig. 4C).

No significant differences between the surfactant control and the bioassay control were observed in any of the parameters analyzed.

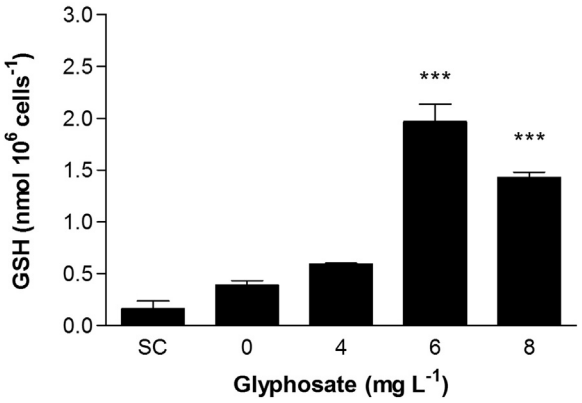

Fig. 3. Reduced Glutathione (GSH) content (nmol 10^6 cells^{-1}) in *S. vacuolatus* exposed to glyphosate for 96 h. Data are expressed as means ± SD. Asterisk denotes significant differences with respect to the control (***p < 0.001). SC: surfactant control.

3.3. Morphological parameters

Cells of *S. vacuolatus* exposed to the surfactant alkyl aryl polyglycol ether did not present any changes in their morphology in comparison with the control cells (Fig. 5A), whereas cells exposed to the commercial glyhosate formulation with the addition of the surfactant presented morphological alterations with respect to the control cells: increase in cell size (Fig. 5B–E), increase in cell vacuolation (Fig. 5B), chloroplast disorganization (Fig. 5C), thickening of the cell wall (Fig. 5D), and accumulation of starch granules and loss of green pigmentation (Fig. 5E).

Cells of *S. vacuolatus* exposed to the surfactant alkyl aryl polyglycol ether did not present any changes in their ultrastructure with respect to the control cells (Fig. 6A–C), whereas cells exposed to the commercial glyphosate formulation with the addition of the surfactant presented morphological alterations when compared to the control cells: thickening of the cell wall (Fig. 6D), accumulation of starch granules distributed between the chloroplast lamellae (Fig. 6E and F), increase in cell vacuolation (Fig. 6G) and thylakoids distributed without any stacking pattern (Fig. 6D). Moreover, in the cultures exposed to the glyphosate formulation, a high percentage (70%) of cells with two – four nuclei was observed (Fig. 6H), whereas only 8.7% of the cells presented this characteristic in the control cultures.

4. Discussion

The results from this study show that the commercial glyphosate formulation applied together with the surfactant alkyl aryl polyglycol ether alters the growth, metabolism and cellular structure of *S. vacuolatus* through toxic mechanisms that would partly involve an oxidative damage process.

The growth rate of the cultures decreased from 18% to 57% with respect to the controls, depending on the herbicide concentration, and the 96 h-IC50 was 4.9 (4.26–5.41) mg L^{-1}. Data from the literature show a wide variation in the glyphosate IC50 values obtained in green microalgae bioassays. Thus, 96 h-IC50 of 79 mg L^{-1} (Pereira et al., 2009) and 5.81 mg L^{-1} (Tsui and Chu, 2003) were reported for *Selenastrum capricornutum* (=*Pseudokirchneriella subcapitata, currently Raphidocelis subcapitata*) exposed to different glyphosate formulations. On the other hand, a study with the commercial glyphosate formulation used in our study with the addition of alkyl aryl polyglycol ether showed a 96 h-IC50 of 55.62 mg L^{-1} for *C. kessleri* (Romero et al., 2011) and an unique study on *S. vacuolatus* determined an 72 h-IC50 of 46.36 mg L^{-1} for glyphosate acid (Daouk et al., 2013). Similar results were recorded by Vendrell et al. (2009), with 72 h-IC50 ranging from 24.5 mg L^{-1} to 41.4 mg L^{-1} for *S. acutus*, *S. subspicatus*, *C. vulgaris* and

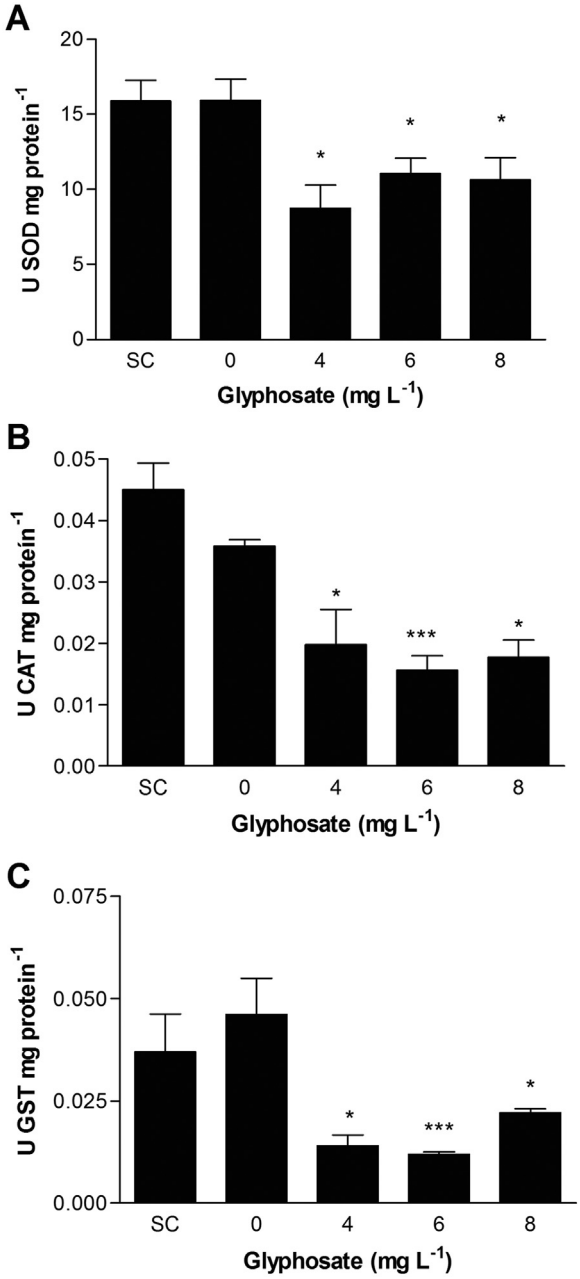

Fig. 4. Superoxide dismutase (SOD) activity in units mg protein^{-1} (A), catalase (CAT) activity in units mg protein^{-1} (B) and glutathione-S-transferase (GST) activity in units mg protein^{-1} (C) in *S. vacuolatus* exposed to glyphosate for 96 h. Data are expressed as means ± SD. Asterisk denotes significant differences with respect to the control (*p < 0.05; ***p < 0.001). SC: surfactant control.

C. saccharophila exposed to analytical grade glyphosate. Factors such as the initial cell density, test volume, exposure time, and algal species together with the specific glyphosate formulation applied can condition the toxic effect. Different species of microalgae may have different sensitivities to herbicides and the results of our study indicate that *S. vacuolatus* is more sensitive than *C. kessleri* to the commercial glyphosate formulation and surfactant applied. On the other hand, the presence and nature of surfactants has been reported to increase the toxicity of glyphosate formulations (Tsui and Chu, 2003), as well as the isopropylamine salt that acompanying the glyphosate (Lipok et al., 2010). A comparison between the 96 h-IC50 value determined for the glyphosate formulation in our strain of *S. vacuolatus* and those from the

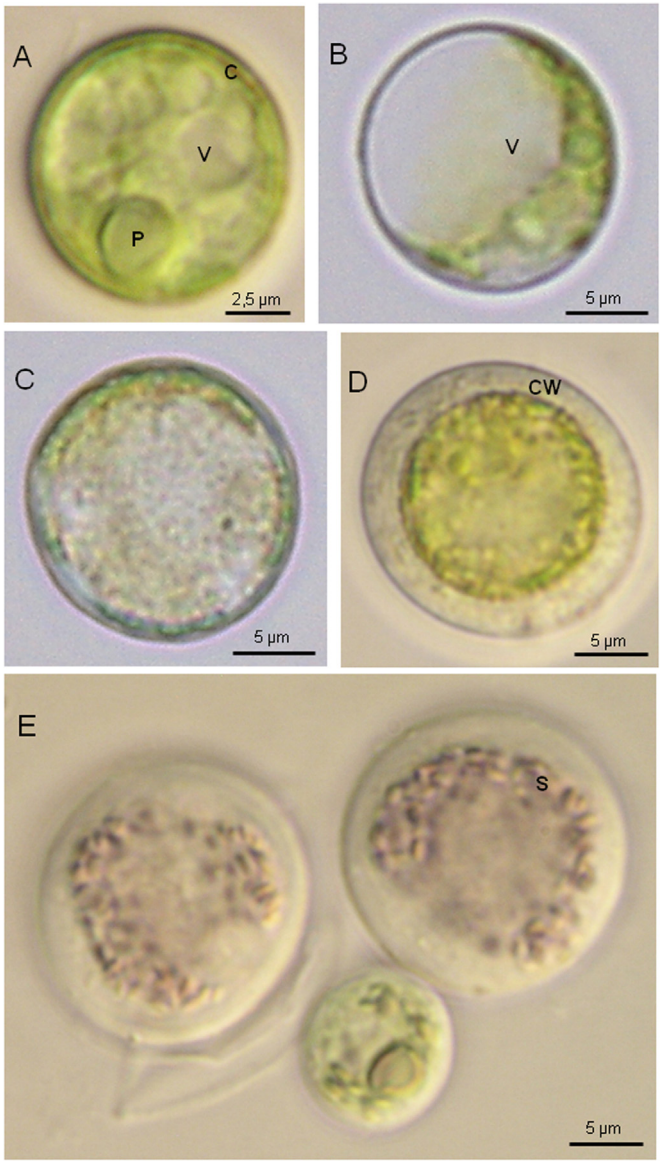

Fig. 5. Effects of the glyphosate formulation with the addition of surfactant alkyl aryl polyglycol ether on *Scenedesmus vacuolatus* cell morphology. A: control cell with vacuoles, pyrenoid and laminar chloroplast. B: cell exposed to glyphosate (4 mg L^{-1}) showing an increase of vacuolation. C: cell exposed to glyphosate (4 mg L^{-1}) showing discoloration. D: cell exposed to glyphosate (6 mg L^{-1}) showing cell wall thickening. E: cells exposed to glyphosate (8 mg L^{-1}) with lugol staining, showing loss of pigmentation, increase of cell size and starch granules accumulation. S: starch, C: chloroplast, P: pyrenoid, CW: cell wall, V: vacuole.

literature data gives evidence of the difference in the sensitivity between species. The 96 h-IC50 determined for *S. vacuolatus* in this study is in the range of concentrations recorded in some water bodies (CCME, 2012; Ronco et al., 2008; WHO, 2005).

The inhibition of chlorophyll production or damage to photosystems have been postulated as a secondary effect of glyphosate in plants (Gomes et al., 2014, 2016). In our study, *S. vacuolatus* exposed to a commercial glyphosate formulation presented a decrease in the chlorophyll *a* + *b* content per cell with respect to the control. Decreases in chlorophyll *a* content have also been reported in other microalga species (*S. acutus* and *S. quadricauda*) and in phytoplankton community exposed to glyphosate (Sáenz et al., 1997; Smedbol et al., 2018; Wong, 2000).

Regarding the carotene/chlorophyll *a* ratio, *S. vacuolatus* cells exposed to the commercial glyphosate formulation with the addition of surfactant alkyl aryl polyglycol ether showed a higher ratio than the control ones. The carotenes are pigments that play a very important role in photosynthetic cells, increasing the efficiency of photosynthesis by absorbing blue-green light and transferring this energy to chlorophyll. Besides, these pigments are essential for assembling and stabilizing the pigment-protein complexes into thylakoidal membranes (Domonkos et al., 2013). In higher plants and algae, the balance of the photosystem components (pigments and proteins) could change according to the physiological and environmental conditions (Basa et al., 2014). The variation in light intensity, temperature, salinity and presence of heavy metals and xenobiotics can induce changes in the composition and levels of photosynthetic pigments. Increases in carotene content and changes in the balance between carotene and chlorophyll *a* have been reported in algae subjected to different stress conditions (nutrient deficiency, UV exposure, high irradiance, oxidative stress, etc.) (Pirastru et al., 2012; Takaichi, 2011; Tsiaka et al., 2013). The increase in carotene/chlorophyll *a* ratio recorded in *S. vacuolatus* in the present study could be related to alterations in the photosystems. Moreover, carotenes are part of the protective system for overexitation, scavenging ROS and protecting the chlorophyll against photooxidation (Okamoto et al., 2001; Salguero et al., 2003). Carotenoids can react with lipid peroxidation products and/or quench singlet oxygen and free radicals, thus serving as a protective mechanism of the photosynthetic apparatus and the concomitant algal growth and survival (Tsiaka et al., 2013). Since ROS can induce carotenogenic responses (Gomes et al., 2017), the carotene level could be reflecting an antioxidant response of *S. vacuolatus* cells to prevent the degradation of chlorophyll and the possible destabilization of pigment-protein complexes into the thylakoidal membranes affected by ROS (Domonkos et al., 2013; Pirastru et al., 2012; Telfer et al., 2008).

In the present study, the results of biochemical parameters related to oxidative damage and antioxidant response showed that oxidative stress is involved in the toxicity of glyphosate formulation in *S. vacuolatus*. It is known that different pesticides induce ROS production, which may cause oxidative damage to macromolecules and provoke an increase in the activities of antioxidant enzymes (Drzeżdżon et al., 2018; Lushchak, 2011). It has been reported that the glyphosate formulation Roundup® depresses the efficiency of the mitochondrial electron transport chain, increasing the production of superoxide anions and therefore triggering oxidative stress and antioxidant responses (Peixoto, 2005). Previous studies conducted in our laboratory on microalgae (Romero et al., 2011), and by other authors on higher plants (Ahsan et al., 2008; Gomes et al., 2016, 2017; Sergiev et al., 2006), recorded increases in ROS, TBARS, GSH and carotene content of cells exposed to glyphosate. In our study, microalgae exposed to 6–8 mg L^{-1} of glyphosate showed significant increases of oxidative stress parameters (ROS, TBARS, carbonyl groups, GSH and carotenes), indicating that the formulation applied would induce oxidative stress in *S. vacuolatus*. On the other hand, in our study the activities of the antioxidant enzymes CAT and SOD showed a significant decrease in *S. vacuolatus* exposed to all the glyphosate concentrations, in accordance with Gomes et al. (2016) that registered the diminution of CAT and SOD activities in leaves of willows exposed to glyphosate. The decrease of SOD and CAT activities observed in *S. vacuolatus* could be explained by the increases in the ROS and TBARS levels and the oxidative damage to proteins observed. The regulation of antioxidant enzymes is complex, and some of them can be inactivated by an excess of ROS, which may react with the protein active sites and alter their function (Kohen and Nyska, 2002). Additionally, some lipid oxidation products, such as 4-hydroxy-2-nonenal and MDA, may modify SOD and CAT functions through adduct formation (Spickett, 2013). Although there was an increase in the GSH and carotene levels in *S. vacuolatus* exposed to the glyphosate formulation, this would not be enough to neutralize the ROS which may attack essential molecules, such as proteins and lipids.

On the other hand, the GSH, TBARS and carbonyl levels in *S.*

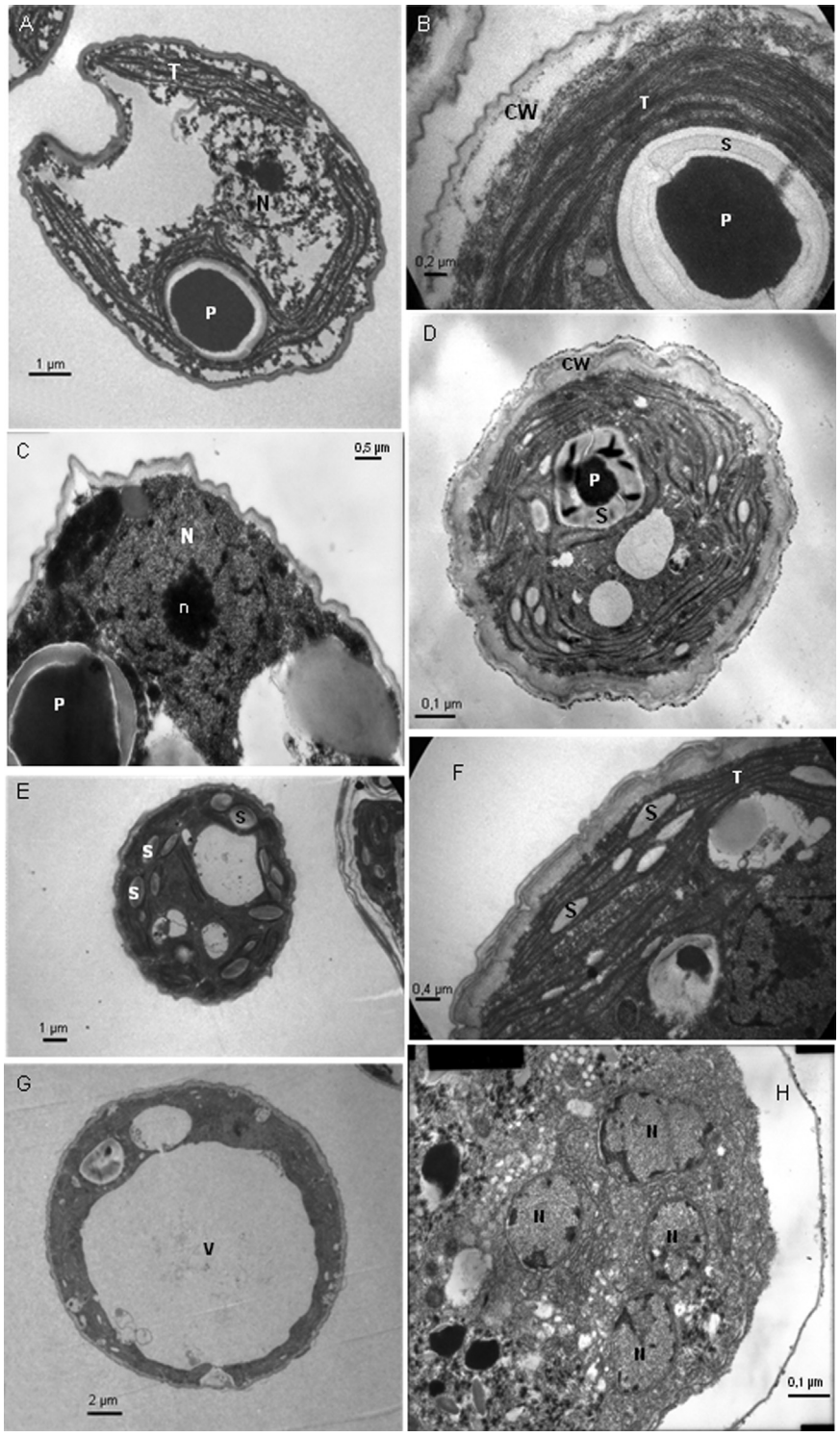

Fig. 6. Effects of the glyphosate formulation with the addition of surfactant alkyl aryl polyglycol ether on *Scenedesmus vacuolatus* cell ultrastructure. A–C: control cells; D–H: cells exposed to 6 mg L^{-1}. A: general view of control cell. B: detail of pyrenoid surrounded by starch granules and 2–4 aligned thylakoids. C: detail of nucleus with central nucleolus. D: cell with thickening of the cell wall and its chloroplast with one pyrenoid surrounded by starch and the thylakoids arranged without their stacking pattern. E: cell with many starch granules. F: detail of starch granules distributed among the thylakoids. G: cell with a large vacuole occupying almost of its entire volume. H: detail of a cell with four nuclei. S: starch, N: nucleus, n: nucleolus, P: pyrenoid, CW: cell wall, T: thylakoids.

vacuolatus exposed to glyphosate formulation showed an apparent diminution from 6 to 8 mg glyhosate L^{-1}. The maximum ROS level observed at 8 mg L^{-1} could explain the diminution in the GSH content which could be being consumed to counteract the pro-oxidant status due to the high ROS level. Additionally, the increase in the carotene content in cells exposed to 8 mg L^{-1} could explain, in part, the slight diminution in oxidative damage (TBARS and carbonyl groups content), since the carotenes have an important role in counteracting the action of ROS (Tsiaka et al., 2013).

Herbicides can be biotransformed inside the cells. GST is a key enzyme in the detoxification of endogenous and exogenous toxic compounds such as some herbicides (Andrews et al., 2005). Different researchers have shown increases of GST activity in algae exposed to different herbicides (Mofeed and Mosleh, 2013) and in plants exposed to glyphosate (Jain and Bhalla-Sarin, 2001). However, in our study, GST activity decreased in *S. vacuolatus* exposed to all the glyphosate concentrations assayed. This could be due to oxidative damage of this enzyme caused by ROS production induced by glyphosate, as evidenced by the increase in protein oxidation levels.

The analysis by light and electron microscopy showed that the glyphosate formulation applied in combination with the surfactant alkyl aryl polyglycol ether alters the morphology and the subcellular

structure of *S. vacuolatus*. Cells exposed to this herbicide showed wall thickening, discoloration, increased size, alteration in the thylakoids stacking pattern, starch granules accumulation and increased size vacuoles. The walls of the cells exposed to glyphosate reached thicknesses of up to 3.3 times higher than the control cells. This response could be related to the participation of the cell wall as a barrier limiting passive transport of glyphosate into the cells, as it was proposed for *Dictyosphaerium chlorelloides* exposed to heavy metals (Pereira et al., 2013). The cell discoloration is correlated to the decrease in the chlorophyll cell content recorded and it could also be related to alterations in the chloroplast inner membranes as shown by the changes in the thylakoids stacking pattern observed by transmission electron microscopy. This alteration could be due to lipids and proteins damage caused by the increase in the ROS level in the exposed cells (Lee and Hsu, 2013). The disorganization of thylakoidal membranes could lead to the dissociation of the pigment-protein complexes bound to membranes, and then to the degradation and consequent decrease in the chlorophyll content in the exposed cells (Lin et al., 2013). Likewise, the ROS could act directly and oxidize the chlorophyll (Telfer et al., 2008). The inner chloroplast organization provides structural properties for optimal photosynthetic activity and the observed alterations can disrupt normal photosynthesis leading to a decrease in the growth rate. Similar effects on the cellular ultrastructure have been recorded in microalgae exposed to the herbicides glufosinate and S-metolachlor and in plants exposed to the glyphosate formulation Roundup® (Liu and Xiong, 2009; Nuria de María et al., 2005; Qian et al., 2008).

Algae can increase its energy reserves (lipids and carbohydrates) in a situation of stress by toxicants (Geoffroy et al., 2007; Liu and Xiong, 2009; Markou et al., 2012) and an increased number of starch granules was reported in *C. pyrenoidosa* and *C. vulgaris* exposed to other herbicides, such as S-metolachlor and glufosinate (Liu and Xiong, 2009; Qian et al., 2008). In our study, the increment in the *S. vacuolatus* carbohydrate reserve (as the starch granules observed by microscopy) could be result from stress caused by glyphosate exposure and could explain the increment in dry weight observed in the exposed cells. Plants and algal cells have mechanisms for the exclusion and isolation of heavy metals and xenobiotics (including herbicides) that could involve the formation of vacuoles that incorporate the biotransformation products and/or parental compound (Coleman et al., 1997; Perales-Vela et al., 2006). Increases in cellular vacuolation were documented in microalgae exposed to chromium, 2,4-dichlorophenol and glyphosate (Pereira et al., 2013; Romero et al., 2011; Yang et al., 2001). Likewise, glyphosate has been found in vacuoles of exposed plant cells (Morin et al., 1997). The increase in vacuolation observed in our study in glyphosate treated *S. vacuolatus* cells could be related to the presence of vacuoles containing the herbicide and/or products of its biotransformation. However, to elucidate whether or not *S. vacuolaus* presents mechanisms of glyphosate detoxification by vacuolation, further studies will be needed.

Alterations in the cell cycle of microalgae exposed to some herbicides have been documented. Vallotton et al. (2008) and Liu and Xiong (2009) reported inhibition of cell division and an increase in cell size in *S. vacuolatus* and *C. pyrenoidosa* exposed to the herbicide S-metolachlor. Rioboo et al. (2002) reported an uncoupling of the growth phase and the division phase of the cells in *C. vulgaris* exposed to the herbicides isoproturon and terbutryn. Since *S. vacuolatus* reproduction occurs through multiple fission, cells increase their volume and mass and suffer multiple nuclear replication before cytocinesis occurs (Bišová and Zachleder, 2014). In the cultures of *S. vacuolatus* exposed to the glyphosate formulation applied, a higher percentage of cells with two or more nuclei (i.e. cells that have not completed the cytokinesis) was observed. Then, apparent cytokinesis delay, together with an increase in the volume and dry weight of the cells and a decrease in growth rate would provide evidence that the *S.vacuolatus* cell cycle was altered by glyphosate formulation.

5. Conclusions

The commercial glyphosate formulation with the addition of the surfactant alkyl aryl polyglycol ether applied in this study had toxic effects on *S. vacuolatus* by inducing oxidative stress. This agrochemical mixture provoked damages at sub-cellular and cellular levels. At the molecular level, parameters such as ROS, TBARS, carbonyl content, GSH, SOD, CAT and GST were altered, which correlated with the alterations in *S. vacuolatus* cell morphology and ultrastructure. In this regard, we consider that these parameters taken together constitute suitable effect biomarkers of glyphosate in *S. vacuolatus*.

The response of the *S. vacuolatus* strain to the glyphosate formulation used in this study provides evidence of the potential negative impact of this herbicide on the aquatic environment.

The results from this study constitute an additional contribution to the understanding of the toxicity of glyphosate to algae and the possible consequences of the herbicide in aquatic ecosystems.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2019.01.083.

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