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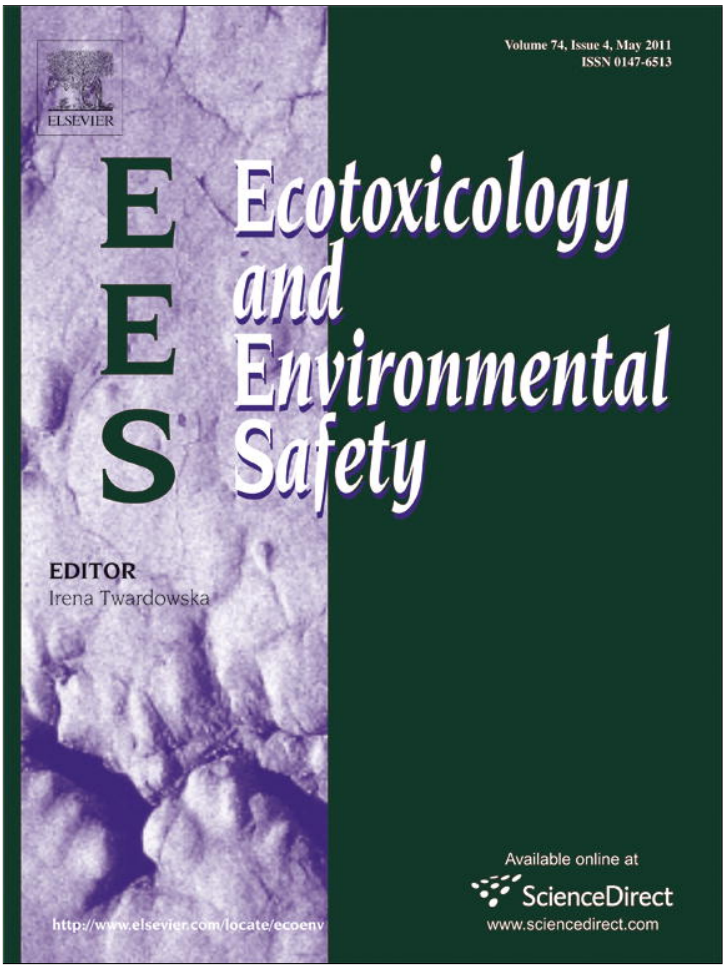

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Oxidative stress induced by a commercial glyphosate formulation in a tolerant strain of *Chlorella kessleri*

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ABSTRACT

We studied the toxicity of a glyphosate formulation and provide evidence of metabolic alterations due to oxidative stress caused in a *Chlorella kessleri* tolerant strain by exposure to the herbicide. After 96 h of exposure to increasing concentrations of the herbicide (0–70 mg L^{-1}) with alkylaryl polyglycol ether surfactant, growth was inhibited (EC50-96 h 55.62 mg L^{-1}). Glyphosate increased protein and malondialdehyde content which was significantly higher than in the control at 50–70 mg L^{-1}. Superoxide dismutase and catalase activities and reduced glutathione levels increased in a concentration-dependant manner. Morphological studies showed increases in vacuolisation and in cell and sporangia sizes. The glyphosate formulation studied has a cytotoxic effect on *C. kessleri* through a mechanism that would involve the induction of oxidative stress. Upon glyphosate exposure, oxidative stress parameters such as SOD and CAT activities and MDA level could be more sensitive biomarkers than usually tested growth parameters in *C. kessleri*.

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1. Introduction

Recently, there has been increased interest in the effects of different contaminants on aquatic organisms, due to the increase in contamination of aquatic environments by heavy metals and organic compounds (hydrocarbons and pesticides). Herbicides that are used in agricultural activities are transported to bodies of water by drift, runoff and leaching to groundwater, and increase the risk of exposure for non-target organisms (Amorós et al., 2007). Phytoplankton are one of the first aquatic communities to respond to variations in water quality (McCormic and Cairns, 1997), and any impact at this level could affect organisms from higher trophic levels, resulting in important consequences to the aquatic ecosystem (De Lorenzo et al., 2001). Because of their role in the maintenance of aquatic food chains, phytoplanktonic microalgae are important models for studying the toxicity of aquatic pollutants *in vivo*. *Chlorella* (Chlorophyta) is one of the most widely distributed green microalgae and species of this genus are often used in toxicity tests due to their sensitivity to different pollutants, their relatively short life cycle and the ease with which they can be cultured in the laboratory (Lewis, 1995).

One of the herbicides commonly used in agriculture for weed control is glyphosate (N-phosphonomethylglycine). Agricultural areas are extensive worldwide, and different glyphosate-containing formulations are in widespread use (Roundup®, Rodeo®, Avans®, Glypro®, Atanor®, etc.). Particularly in the central Pampean Region of Argentina, one of the most applied formulations is ATANOR® (48% glyphosate as isopropylamine salt) with the addition of 2.5% IMPACTO® (alkylaryl polyglicol ether) surfactant. Glyphosate is a non-selective post-emergent herbicide that inhibits 5-enolpyruvyl-shikimate-3-phosphate synthase (Duke, 1988). This enzyme is crucial in the biosynthesis of the aromatic amino acids that are essentials for protein synthesis. A second mechanism of action for glyphosate has been described in which the synthesis of porphyrins is affected by the inhibition of the δ-aminolevulinate synthetase enzyme (Duke, 1988). It is generally accepted that glyphosate has a low potential as a surface water or groundwater pollutant because of its high adsorption to soil particles (Kd values up to 900 L kg^{-1}) and fast degradation by microorganisms (Borggaard and Gimcing, 2008). Leaching of glyphosate and its degradation product aminomethylphosphonic acid (AMPA) to depths of up to 1 m has been observed. So, glyphosate could represent a potential risk not only for aquatic environments but also for humans (Kjaer et al., 2005).

Glyphosate can enter water bodies bordering fumigated lands through runoff or by drift during aerial application. Additionally,

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glyphosate may enter bodies of water from the washing of fumigation machinery or by intentional application for the elimination of aquatic weeds. In several countries, the herbicide has been found in surface water in considerable concentrations (Kolpin et al., 2006; Struger et al,. 2008). In Argentina, there have been few studies of glyphosate levels in water. Peruzzo et al. (2008) found about 0.7 mg L^{-1} in surface waters of an aquatic system in the Buenos Aires province. Sobrero et al. (2007) consider that the range of concentrations in the field could cover from 0.1 to 80 mg L^{-1}. The low levels may correspond to aquatic environments near croplands where glyphosate is applied, while the highest concentrations might be representative of situations of cleaning machines and/or accidental spills of herbicide.

The toxic effects of glyphosate have been studied in different aquatic organisms (Tsui and Chu, 2003; Relyea, 2005). Studies in microalgae are generally focused on growth parameters (LOEC, NOEC, EC50), content of pigments, photosynthesis and motility (Sáenz et al., 1997; Wong, 2000; Tsui and Chu, 2003; Pettersson and Ekelund, 2006). Surfactants or coadjuvants in the formulations could enhance the toxic effects of the herbicide (Tsui and Chu, 2003).

It has been reported that many pollutants (including herbicides) generate intracellular reactive oxygen species (ROS) (Bagchi et al., 1995). The increased ROS trigger oxidative damage to proteins, nucleic acids and lipids, finally leading to damage of different cellular organelles. The increased ROS also trigger different antioxidant responses that prevent damage of proteins, lipids and DNA. Some of these responses are an increase in the activities of the antioxidant enzymes superoxide dismutase, catalase, ascorbate peroxidase, glutathione peroxidase and others and an increase in non-enzymatic metabolites, such as reduced glutathione, carotenes, α-tocopherol and others (Chaufan et al., 2006; Lei et al., 2006; Sabatini et al., 2009).

Despite recent reports that glyphosate action leads to oxidative stress in maize and rice leaves (Ahsan et al., 2008), in bullfrog tadpoles (Costa et al., 2008) and in the worm *Lumbriculus variegatus* (Contardo-Jara et al., 2009), there are few studies that evaluate oxidative stress parameters in aquatic organisms exposed to glyphosate or its formulations. In addition, there is no information about oxidative stress related to glyphosate in microalgae.

Different species of microalgae may have different tolerances to herbicides and other pollutants (Lei et al., 2006; Vendrell et al., 2009). However, even when a species is tolerant to a given xenobiotic, important metabolic parameters (such as those related to oxidative stress) may still be impaired, which can lead to a decrease in the viability of its population. The BAFC CA 10 *Chlorella kessleri* (Chlorophyta) corresponds to an autochthonous strain isolated from an extreme acidic pond (Juárez and Vélez, 1993) and has shown more tolerance than other microalgae species to chromium, copper and hexachlorobenzene (Schiariti et al., 2004; Chaufan et al., 2006; Juárez et al., 2008). The aim of this work is to study the toxicity of the herbicide glyphosate and to provide evidence of metabolic alterations related to oxidative stress induced in a tolerant strain of *C. kessleri* by exposure to a commercial formulation of glyphosate. For this purpose, we measured parameters related to metabolic damage (biomass, growth rate, chlorophyll content and protein content), lipid peroxidation (malondialdehyde content) and antioxidant response (catalase and superoxide dismutase activities and reduced glutathione level). Additionally, cellular morphology was analysed by light microscopy.

2. Methods

2.1. Chemicals

The commercially available herbicide used in this study was 48% (p/v) Glyphosate (isopropylamine salt of N-phosphonomethyl glycine) ATANOR® (Atanor, Munro, Buenos Aires province, Argentina) and the surfactant was alkylaryl polyglycol ether 50% IMPACTO® (AGRO ASIST S.R.L., Argentina). All other reagents were of analytical grade.

A stock solution of herbicide Atanor (48% p/v) with 2.5% of surfactant was made. Different amounts of the herbicide stock solution were mixed into Bold's Basal Medium (BBM), pH 6.5 (Bischoff and Bold, 1963), to achieve actual concentrations of 40, 50, 60, and 70 mg L^{-1} of glyphosate, based on data from preliminary bioassays testing a greater range of concentrations. Three replicates were used per treatment and the bioassays were done in triplicate.

Initial glyphosate concentration in culture medium was analytically determined by ion chromatography at INQUIMAE–CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. The ion chromatograph was a Dionex DX-100 equipped with a conductivity detector. Injection volume was 25 µL. A mixture of $NaOH/CO_3^{-2}$ 4 mM/9 mM was chosen as eluent with a flow rate of 2 mL min^{-1}. The suppression was made by an electrochemical system. The actual values were: 90 ± 2.7% of their nominal values.

2.2. Algal strain, culturing and exposure

The BAFC CA10 strain of *C. kessleri* (Trebouxiophyceae, Chlorophyta) was originally isolated from Laguna Verde, Copahue Thermal Complex, Neuquén, Argentina (Juárez and Vélez, 1993) and is currently kept in the Culture Collection of the Phycology Laboratory, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires.

The experimental treatments were prepared according to algal growth inhibition test standards (USEPA, 2002). The bioassays were conducted in 250 mL flasks containing 120 mL of BBM medium with different concentrations of herbicide with initial cell density of 3×10^4 cells mL^{-1}. Cells from an exponential phase culture were used as inoculum. The flasks were incubated at 23 ± 1 °C and kept on an orbital shaker at 210 rpm, with continuous cool-white fluorescent light illumination (80 µmol photons m^{-2} s^{-1}). Two controls were included in all assays: (1) control cultures (C) without glyphosate ATANOR® and without surfactant IMPACTO®; (2) surfactant control (SC) containing only the maximum surfactant concentration used.

After 96 h, the cell number was evaluated by direct counting in Neubauer chamber, using an Olympus light microscope at 400 ×. The counting of at least 25 squares ensured an error of less than 10% (Venrick, 1978). Cell counts were correlated with absorbance at 680 nm, on a Shimadzu UV/visible spectrophotometer (Ma et al., 2006). A strong correlation was confirmed in this experiment, with coefficient correlation r values >0.99 and significance level $P<0.001$ ($C=0.022+9.999\times10^{-8}A$, $r=0.991$, $P=0.000$). Values achieved were expressed as average ± standard deviation. Growth rate (r) was calculated as

$r=(\ln N-\ln N_0)/(\text{days})$, where N = final cell density (cells per mL) and N_0 = initial cell density

The EC50-96 h value was estimated by Linear Interpolation Method (USEPA, 2002).

For determinations of different parameters, cells from 10 mL of each culture were harvested by centrifugation at 3,000 × *g* for 15 min, washed three times with 0.134 M potassium phosphate buffer (pH 6.5) and resuspended in 0.5 mL of the same buffer. Cell for enzymatic activities were obtained from 75 mL of culture. Samples for enzymatic activities were assayed in fresh material and the other samples were stored at −20 °C until they were used (no more than 3 days after cells were collected).

Algal dry biomass was measured by filtration of selected culture volumes (20–40 mL, depending on culture cell density) through a preweighed Whatman GF/C glass fibre filter, and dried at 80 °C to constant weight.

2.3. Pigment content

Cells were thoroughly ground in 80% acetone. After 24 h at 4 °C in the dark, the extracts were clarified by centrifugation for 10 min at 3000 × *g*, and their absorbance was read at 663.2, 646.8 and 470 nm in an UV/vis Shimadzu spectrophotometer. Chlorophyll *a*, chlorophyll *b* and carotene concentrations were calculated using the equations of Lichtenthaler (1987). The results were expressed as µg of carotenes per mg dry weight, µg of total chlorophyll per mg dry weight and the ratio of chlorophyll *a*/chlorophyll *b* (Chl *a*/Chl *b*).

2.4. Lipid peroxidation

Quantification of lipid peroxides through dosage of thiobarbituric acid reactive substances (TBARS) was carried out according to Vavilin et al. (1998). Cells were resuspended in freshly prepared reagent (with and without TBA), incubated in a boiling water bath for 45 min and incubated for an additional 20 min at room temperature. Then, the samples were centrifuged for 10 min at 10,000 × *g*, and the absorbance of cleared supernatant was measured at 440, 532 and 600 nm. The amount of malondialdehyde (MDA) was calculated using Hodges' equations (Hodges et al., 1999). Results were expressed as nmol MDA per mg dry weight.

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2.5. Reduced glutathione content

The content of reduced glutathione (GSH) was determined according to Okamoto et al. (2001). Cells were broken by sonication using a Cole Parmer CP600 4710 Ultrasonic homogeniser. Samples were deproteinised by treatment with 5% (p/v) TCA for 30 min and then were centrifuged for 10 min at 10,000 × *g*. The content of GSH was determined in the supernatant by reaction with 5,5′-ditiobis 2-nitrobenzoic acid (DTNB) and measurement of the absorbance at 412 nm. A freshly prepared solution of GSH was used to generate a standard curve. Results were expressed as μmol GSH per mg dry weight.

2.6. Antioxidant enzyme activities

Cells were harvested by centrifugation at 3000 × *g* for 20 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 phenyl methyl sulfonyl fluoride). Then, cells were disrupted by sonication using a Cole Parmer CP600 4710 Ultrasonic homogeniser. The homogenates were centrifuged at 10,000 × *g* for 30 min, and the supernatant was used as the enzyme sample. All procedures were done at 4 °C.

2.6.1. Superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined according to Beauchamp and Fridovich (1971). This method is based on the inhibition of the photochemical reduction of nitro blue tretrazolium (NBT). The reaction was carried out in 50 mM potassium phosphate buffer (pH 7.8), and the absorbance was measured at 560 nm. The method was tested using a standard SOD (SIGMA). Results were expressed as units of SOD per mg dry weight. One unit SOD was defined as the amount of enzyme necessary to inhibit by 50% the NBT reduction rate.

2.6.2. Catalase activity

Catalase (CAT) activity was determined by following the decomposition of hydrogen peroxide spectrophotometrically at 240 nm, in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.4) and 10 mM hydrogen peroxide (Aebi, 1984). Results were expressed as pmol CAT per μg dry weight.

2.7. Protein content

Cells were disrupted by sonication and centrifuged at 10,000 × *g* for 30 min. Total soluble protein content was determined in the 10,000 × *g* supernatant according to Bradford (1976), using bovine serum albumin as a standard. Results were expressed as μg of protein per mg dry weight.

2.8. Morphology

The morphology of *C. kessleri* was analysed from cultures grown in BBM liquid medium containing 0, 40, 50, 60, or 70 mg L^{-1} of glyphosate from the formulation described previously. The observation and photomicrography were done with an Axioplan light microscope equipped with a Karl Zeiss camera.

2.9. Statistical analysis

Results from different treatments were compared statistically by one-way analysis of variance (ANOVA) followed by a Dunnett's post hoc test. The suppositions of normality and homogeneity of variances were tested with Lillieford and Bartlett tests, respectively. Graph Pad Prism 4 software was used for statistical analysis.

3. Results

3.1. Culture growth and EC50-96 h

Effects on the growth of *C. kessleri* cultures exposed to a formulation of glyphosate and surfactant were studied in this work. We observed a diminution in culture growth rate (*r*) when the concentration of the herbicide increased (Table 1). Algal cell density and dry weight were statistically significant diminished with respect to the control values for concentrations of at least 60 mg L^{-1} of glyphosate ($p < 0.01$), where the number of cells was approximately one-third that of the control culture (Table 1). The EC50-96 h estimated by Linear Interpolation Method software was 55.62 (53.08–57.56) mg L^{-1}.

3.2. Protein content

Total protein content was determined after exposure to different glyphosate concentrations. The results showed an increase in protein content in a concentration-related manner (Table 1). Significant differences ($p < 0.01$) were detected at glyphosate concentrations of 60 and 70 mg L^{-1}.

3.3. Pigment content

Pigment contents were studied after exposure to increasing concentrations of glyphosate formulation. Carotene contents ranged from 2.536 to 5.423 μg per mg dry weight, and total chlorophyll

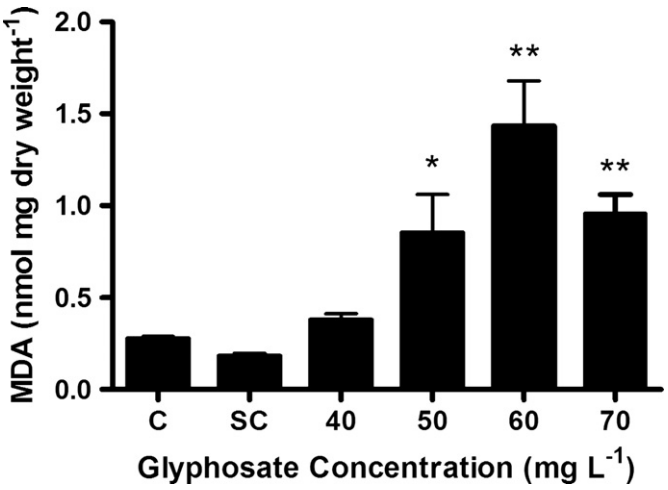

Fig. 1. Effects of glyphosate on MDA content (nmol mg dry weight $^{-1}$) in *C. kessleri* cultures after 96 h of exposure. Data are expressed as means ± SD. Asterisks denote significant differences compared to the control (C): $^{*}p < 0.05$, $^{**}p < 0.01$. C: control; SC: surfarctant control.

Table 1
Effects of glyphosate on *C. kessleri* cultures after 96 h of exposure.

Glyphosate concentration (mg L^{-1})	Cells (10^6 m L^{-1})	Dry weigth (mg mL^{-1})	Growth rate (*r*)	Total proteins (μg mg dry weight^{-1})	Carotenes (μg mg dry weight^{-1})	Total chlorophyll (μg mg dry weight^{-1})	Chlorophyll a/ Chlorophyll b
C	1.665 ± 0.418	0.062 ± 0.015	0.997 ± 0.059	42.184 ± 9.839	4.575 ± 0.513	30.040 ± 2.210	2.134 ± 0.232
SC	1.387 ± 0.143	0.051 ± 0.005	0.957 ± 0.026	30.625 ± 8.990	2.536 ± 0.504	27.427 ± 2.451	1.788 ± 0.096
40	1.453 ± 0.439	0.054 ± 0.016	0.960 ± 0.070	8.584 ± 13.846	4.553 ± 0.976	30.242 ± 4.499	2.330 ± 0.269
50	1.341 ± 0.487	0.050 ± 0.018	0.930 ± 0.100	51.176 ± 19.517	5.423 ± 0.948	35.091 ± 1.889	1.991 ± 0.345
60	0.480 ± 0.201**	0.015 ± 0.004**	0.650 ± 0.070**	101.321 ± 35.274**	5.079 ± 1.928	29.829 ± 6.764	2.114 ± 0.491
70	0.298 ± 0.065**	0.011 ± 0.002**	0.580 ± 0.060**	97.730 ± 23.528**	3.182 ± 0.795	29.882 ± 4.115	1.363 ± 0.283**

Data are expressed as means ± SD. Asterisks denote significant differences compared to the control (C) ($^{**}p < 0.01$). C: control; SC: surfarctant control.

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ranged from 27.427 to 35.091 μg per mg dry weight (Table 1). Neither showed significant differences ($p > 0.05$) with increasing glyphosate concentration. However, the chlorophyll *a*/chlorophyll *b* (Chl *a*/Chl *b*) ratio decreased by 40% at the concentration of 70 mg L^{-1} ($p < 0.01$) (Table 1).

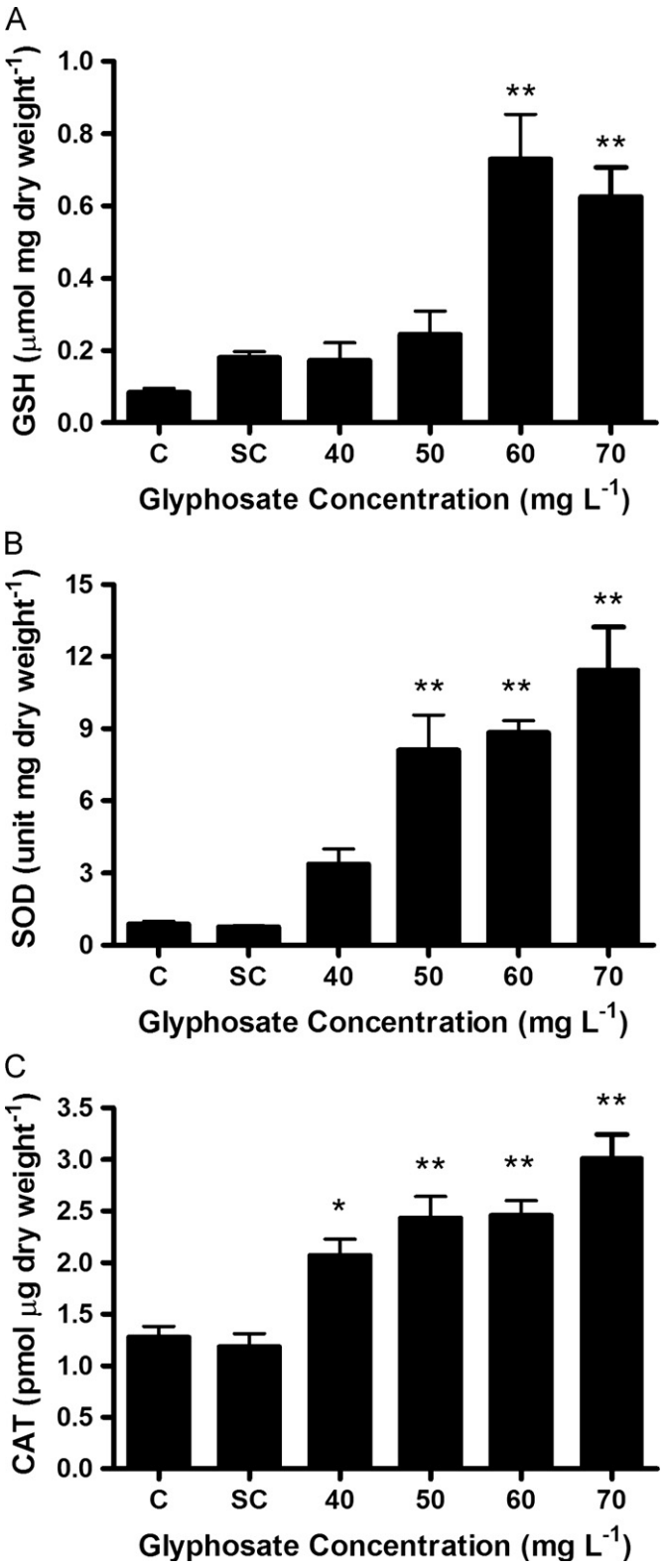

Fig. 2. Effects of glyphosate on antioxidant defences in *C. kessleri* cultures after 96 h of exposure. (A) Reduced glutathione content (μmol GSH mg dry weight^{-1}). (B) Superoxide dismutase activity (unit SOD mg dry weight^{-1}). (C) Catalase activity (pmol CAT μg dry weight^{-1}). All data are expressed as means ± SD. Asterisks denote significant differences compared to the control (C): $^{*}p < 0.05$, $^{**}p < 0.01$. C: control; SC: surfactant control.

3.4. Lipid peroxidation

Lipid peroxidation was determined by evaluating the MDA content. The results showed a concentration-dependant increase in MDA per mg dry weight (Fig. 1). Significant differences ($p < 0.05$) were observed with glyphosate concentrations of 50 mg L^{-1} and higher. At 50 and 60 mg L^{-1}, the content of MDA increased around three times and five times over the control value, respectively.

3.5. Reduced glutathione content

The content of reduced glutathione (GSH) increased with the increase in herbicide concentration (Fig. 2A). Significant differences ($p < 0.01$) were observed at concentrations of at least 60 mg L^{-1} of glyphosate; at this concentration, the content of reduced glutathione was nine times that in the control.

3.6. Antioxidant enzyme activities

Enzymatic defences increased when the herbicide formulation was present in the growth medium of the microalgae (Fig. 2B and C).

Significant differences ($p < 0.05$) in SOD activity were observed at glyphosate concentrations of at least 50 mg L^{-1} (Fig. 2B). Catalase activity also increased significantly ($p < 0.05$) at concentrations of 40 mg L^{-1} and higher (Fig. 2C).

3.7. Morphology

Light microscopy showed several morphological alterations in algal cells exposed to different concentrations of the herbicide (Fig. 3). Cells in control cultures (Fig. 3A) showed the typical size for the species (2.5–8.9 μm). Morphological damage became evident at low concentrations of the herbicide. Increases in the mean size of vegetative cells (Fig. 3B, black arrow) and sporangia (with sporangia sizes of more than 15 μm of diameter) were observed in about 10% of exposed cells. An increased number of large vacuoles (V) inside the cells were also observed in 10–30% of cells exposed at 40 and 70 mg L^{-1} of glyphosate, respectively (Fig. 3B).

4. Discussion

The results obtained show that the ATANOR® glyphosate formulation and the IMPACTO® surfactant (alkylaryl polyglycol ether), when applied together, alter the growth of *C. kessleri* in culture. The exposure of the cultures to increasing concentrations of this formulation had inhibitory effects on the growth, and the EC50-96 h was 55.62 (53.08–57.56) mg L^{-1}.

The literature shows a wide variation in the glyphosate EC50 values obtained in bioassays using green microalgae. Comparisons of the sensitivity of *C. kessleri* to that of other green microalgae are difficult because most of the published studies describe the action of glyphosate acid or isopropylamine salt of glyphosate rather than formulations that include surfactants or coadjuvants. The EC50-96 h obtained for *C. kessleri* was approximately ten times higher than those reported for *Chlorella vulgaris* (4.69 mg L^{-1}) and *Raphidocelis subcapitata* (5.5 mg L^{-1}) using technical grade glyphosate acid (Ma et al., 2002; 2006). On the other hand, Vendrell et al. (2009) reported EC50 values near those reported in our study for *C. vulgaris* and *C. saccharophila* exposed to analytical grade glyphosate (41.7 and 40.6 mg L^{-1}, respectively). Sáenz et al. (1997) and Tsui and

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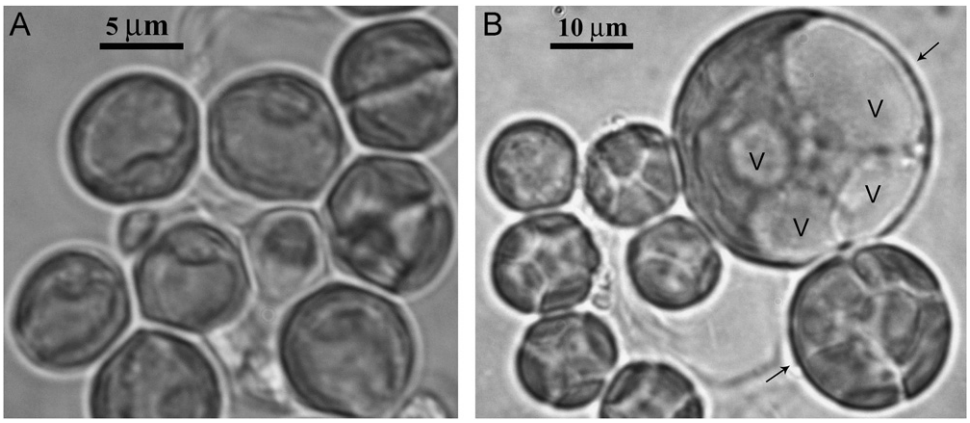

Fig. 3. Effects of glyphosate on *C. kessleri* cells morphology after 96 h of exposure. (A) control and (B) 60 mg L^{-1} of glyphosate formulation. Note the increases in vacuolisation (V) and cell size (black arrow).

Chu (2003) have analysed the effects of the formulations Roundup and Ron-do (both containing surfactant), and they reported EC50-96 h values of 5.81, 9.08 and 9.09 mg L^{-1} for *Selenastrum capricornutum*, *Scenedesmus acutus* and *Scenedesmus quadricauda*, respectively. Taking into account that the presence of surfactants has been reported to increase the toxicity of glyphosate formulations (Tsui and Chu, 2003), the *C. kessleri* strain studied would have a greater tolerance to the herbicide than the other microalgae mentioned. It is known that different species of microalgae may have different sensitivities to herbicides and other toxics (Lei et al., 2006; Vendrell et al., 2009). Additionally, it has been reported that algae adapted to extreme environments are more tolerant of contamination (Rai et al., 1996). The strain of *C. kessleri* studied here has previously shown high tolerance to copper, chromium and hexachlorobenzene (Schiariti et al., 2004; Chaufan et al., 2006; Juárez et al., 2008) and was isolated from a thermal acidic environment (Juárez and Vélez, 1993). Thus, we could expect a certain degree of resistance that would explain, in part, its greater tolerance to glyphosate.

The results of our work suggest that the glyphosate formulation tested promotes harmful effects on the non-target species *C. kessleri* through oxidative stress. Glyphosate inhibits the biosynthesis of the aromatic amino acids essential for protein synthesis (Duke, 1988). However, in this work, we observed an increase in total protein content, suggesting that in *C. kessleri* glyphosate would not inhibit the net synthesis of these macromolecules. Increase in ROS levels triggers signal transduction pathways in which genes encoding proteins involved in toxic metabolization, antioxidant responses and DNA and proteins repair systems, such as Hsp70 stress proteins (Tukaj and Tukaj, 2010) are up-regulated. Additionally, the presence of a more efficient DNA and protein repair system postulated for extreme acidophilic organisms (Baker-Austin and Dopson, 2007) could shed some light on *C. kessleri* response.

Even though there were no significant differences in the content of carotenes, the surfactant control showed a slight reduction in carotene content. A similar effect was observed in 70 mg L^{-1} glyphosate treatment containing the same concentration of the surfactant. This result could suggest that the surfactant alone or combined with high doses of the glyphosate formulation could inhibit carotenes synthesis. A similar result has been reported for other herbicides (Samuel and Bose, 1987), but there is no information for alkylaryl polyglicol ether.

Total chlorophyll content per mg dry weight did not show significant differences with any concentration of the formulation applied. Nevertheless, a significant decrease in the Chl *a*/Chl *b* ratio was observed in the presence of the xenobiotic at the highest concentration studied (70 mg L^{-1}). A decrease in the Chl *a*/Chl *b* ratio has been reported in microalgae exposed to different contaminants (Samuel and Bose, 1987; Öncel et al., 2000). Heavy metals, herbicides and other organic compounds could damage thylakoid membranes and/or the synthesis of proteins that assemble chlorophyll molecules into the reaction centre, disturbing structural pigment–protein complexes located in the thylakoid membrane (Pätsikkä et al., 1998; Juarez et al., 2008). The Chl *a*/Chl *b* ratio is an expression of the sensitivity to external factors (either physical or chemical) of the light harvesting complex II (LHC II) enzyme system of chloroplasts (Pintilie et al., 2006). Thus, the decrease observed in *C. kessleri* exposed to 70 mg L^{-1} of herbicide reflects changes in the antenna complex size and therefore alteration in the photosystems (Melis, 1991).

The MDA level is usually used as an index of lipid peroxidation caused by oxidative stress (Janero, 1990). The significant increase in MDA content of *C. kessleri* exposed to 50–70 mg L^{-1} of glyphosate indicates the occurrence of damage to the lipid membranes. Such a mechanism might contribute to the diminution of the Chl *a*/Chl *b* ratio and the possible damage to the antenna complex observed.

GSH constitutes one of the main non-enzymatic antioxidant molecules of the cell, and an increase in its levels is associated with increases in stress-related antioxidant defences (Lei et al., 2006). As reported by Uotila et al. (1995) and Jain and Bhalla-Sarin (2001) for glyphosate-exposed plants, our results showed that the GSH participates in the antioxidant defences of *C. kessleri* upon glyphosate exposure.

Different pesticides are known to elicit ROS production, causing lipid damage and inducing an increase in the activities of antioxidant enzymes (Bagchi et al., 1995). Peixoto (2005) reported that the Roundup formulation depresses the efficiency of the mitochondrial electron transport chain, affecting it at the level of complexes II and III. Thus, the action of glyphosate formulations may be involved in increasing the production of superoxide anions and therefore in triggering oxidative stress and antioxidant responses. In our study, significant increases in SOD and CAT activities were observed when microalgae were exposed to 40–70 mg L^{-1} glyphosate, indicating that the formulation applied induces oxidative stress in *C. kessleri*. These results are in concordance with those reported for rice leaves (Ahsan et al., 2008) and other non-target organisms, such as bullfrog tadpoles (Costa et al., 2008) and *Lumbriculus variegatus* (Contardo-Jara et al., 2009), exposed to glyphosate.

A significant increase in CAT activity was observed at 40 mg L^{-1}, while there was not an increase in MDA content, so the antioxidant response seems to have prevented lipid oxidative damage under this condition. At higher concentrations of glyphosate formulation

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(from 50 mg L^{-1} onwards), increases in other antioxidant defences were observed (SOD activity and GSH content), but these were not enough to neutralise oxidative conditions, thus resulting in metabolic and cellular damages (increases in the MDA level, decreases in the Chl *a*/Chl *b* ratio, reduction in the growth rate and increases in cellular vacuolisation and cell sizes).

Microscopic observations allowed us to determine that glyphosate, besides producing metabolic alterations affects *C. kessleri* morphology. Increases in vacuolisation (both size and number) occurred in a concentration-dependant manner. The presence of giant sporangia (more than 15 μm) can be associated with the inhibition of cell division, preventing the formation of autospores, and the permeabilisation of cell membranes caused by oxidative stress, as has been described for heavy metals and paraquat exposure (Jamers and De Coen, 2010). It has been reported that cells of plants have mechanisms for the exclusion and isolation of xenobiotics (including herbicides) (Coleman et al., 1997; Dixon et al., 1998). These mechanisms imply the conjugation of glutathione to the xenobiotic by glutathione S-transferase and the formation of vacuoles that incorporate these conjugated compounds for their subsequent transformation and liberation. The higher proportion of cells with large vacuoles was observed in the cultures exposed to 70 mg L^{-1} herbicide. However, to confirm whether or not *C. kessleri* presents mechanisms of detoxification by vacuolisation, further studies will be needed.

The response of BAFC CA 10 *C. kessleri* strain against the herbicide concentrations studied in this work may provide evidence of the impact of accidental or intentional spills on water bodies and can be seen as a warning against the indiscriminate use of this agrochemical. This strain showed tolerance to high concentrations of the glyphosate formulation applied. However, despite this tolerance, exposure to the herbicide altered important metabolic parameters. ATANOR® glyphosate and IMPACTO® alkylaryl polyglycol ether surfactant have a cytotoxic effect on *C. kessleri* through a mechanism involving the induction of oxidative stress.

Parameters such as SOD and CAT activities and the MDA level were affected even when there were no significant changes in growth. Thus, these parameters could be more sensitive indicators than growth ones in *C. kessleri* after glyphosate exposure. In addition, the results also suggested that the mechanism in *C. kessleri* would not involve inhibition of protein biosynthesis, in contrast to vascular plants where such inhibition is the specific mode of action of glyphosate.

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