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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*.

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) surfarctant 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.