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Short-term effects of glyphosate and Roundup Transorb® formulation on the cyanobacteria *Synechococcus elongatus*

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Abstract

Glyphosate is an active ingredient used in herbicide formulations worldwide. However, besides glyphosate, these formulations have other components to facilitate glyphosate absorption by plants. These include the surfactants such as polyoxyethylene amine (POEA) present in the Roundup Transorb® (RT) formulation. Glyphosate formulations are potentially more toxic to non-target organisms than the pure active ingredient. In this work, we evaluated the toxicity (72 h) of pure glyphosate and RT for the cyanobacterium *Synechococcus elongatus*, based on biomass growth and cell viability. The formulation proved more toxic than pure glyphosate for both parameters analysed, with an IC_{50} (Inhibition concentration mean) based on biomass measured by optical density (750 nm) that was sixty times lower. Cell viability was not as sensitive as the biomass because, of the few cells left in the culture, most were viable. This indicates that there is a variation in tolerance between the cyanobacteria present in the inoculum. Thus, cell viability may underestimate the results of glyphosate and RT toxicity and be useful only in low concentrations of exposure.

Keywords: Biomass, cell viability, herbicide, optical density, SYTOX Green.

INTRODUCTION

Glyphosate is a molecule with herbicidal function (Amrhein *et al.*, 1980), exercised by inactivating the enzyme 5-enolpyruvylchiquimato-3-phosphate synthase (EPSPS) that participates in the metabolism of the shikimate pathway, that when inactivated, prevents the synthesis of essential aromatic amino acids from vegetables and some microorganisms (Herrmann & Weaver, 1999). It is the most used active ingredient in herbicides worldwide (Benbrook, 2016) and for some years it has been detected in the aquatic environment in extremely variable concentrations, which can reach 0.7 mg L^{-1} (Peruzzo *et al.*, 2008; Van Bruggen *et al.*, 2018). For these reasons, several studies have evaluated the toxicity of this molecule and its formulations in non-target organisms, such as fish (Zhang *et al*., 2017; Zheng *et al*., 2021), molluscs (Sandrini *et al*., 2013; Séguin *et al*., 2017), crustaceans (Hansen & Roslev, 2016; Banaee *et al*., 2019), microalgae (Iummato *et al*., 2019) and cyanobacteria (Wu *et al*., 2016; Ye *et al*., 2019). This toxicity can manifest itself at molecular (Hashim *et al*., 2021), physiological (Wu *et al*., 2016), behavioural (Sánchez *et al*., 2021) and reproductive (Muller *et al*., 2021) levels, among others (Corrales *et al*., 2021; Sylwestrzak *et al*., 2021). Usually, the toxicity of commercial glyphosate formulations is greater than the pure active component (Annett *et al*., 2014; Bridi *et al*., 2017; Bonfanti *et al*., 2018; Szepanowski *et al*., 2019). This is associated with the presence of the other components present in these formulations as

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surfactants used to facilitate herbicide penetration in the cell wall and cell membranes of plants, such as POEA, present in some glyphosate-based formulations, like RT, a formulation composed of glyphosate isopropylamine salt (IPA) at 648 g L^{-1} (480 g L^{-1} glyphosate acid equivalent) and 594 g L^{-1} of other ingredients considered "inert" (Lipok *et al.*, 2010; Bridi *et al.*, 2017; Janssens & Stoks, 2017; Turhan *et al.*, 2020).

Although some studies have evaluated glyphosate toxicity and some glyphosate-based formulations for phytoplankton (Lipok *et al.*, 2010; Qiu *et al.*, 2013; Wu *et al.*, 2016; Lam *et al.*, 2019; Ye *et al.*, 2019; Sabio y García *et al.*, 2020), little is known about the comparative effects of pure glyphosate and its formulations on cyanobacteria. Cyanobacteria are photosynthetic prokaryotes that are found at the base of the food chain and contribute to the fixation of carbon and nitrogen, in addition to being physiologically like plants (glyphosate target organisms) (Whitton, 2012). Therefore, an imbalance in the cyanobacterial community, whether in abundance or diversity, caused by pesticide pollution can lead to immeasurable damage to aquatic ecosystems. In this context, the objective of this work was to analyze and compare the effects of exposure to pure glyphosate and RT on the growth and cell viability of the freshwater cyanobacteria *Synechococcus elongatus*, a species found in several aquatic ecosystems and a good model in ecotoxicology studies, because it is easy to cultivate in the laboratory, presents rapid response to contaminants, well-known biology, the sequenced genome (Holtman *et al*., 2005; Chen *et al*., 2008) and great ecological importance. Some other studies used this cyanobacterium as a model organism to assess the toxicity of heavy metals (Selim & Haffner, 2020), UV filters (Vicente et al., 2019) and pesticides (González-Barreiro et al., 2004).

MATERIAL AND METHODS

Cyanobacteria and culture conditions

Axenic cultures of cyanobacteria *S. elongatus* (PCC 7942) were obtained from the culture bank of the Molecular Biology Laboratory of the Institute of Biological Sciences of the Federal University of Rio Grande (FURG), Brazil. The cyanobacteria cultures were grown in sterile BG-11 culture medium (Rippka, 1979) at pH 7.5 ± 0.1 and kept in a BOD at 34 °C and under constant lighting of 8.000 lx. All manipulation was carried out in a laminar flow cabinet previously sterilised for 20 min with UV. The materials used for cyanobacterial manipulation and in the experiments (e.g., glassware and tips) were sterilised in UV or autoclaved before use.

Exposure to herbicides

A pre-culture of *S. elongatus* was prepared by adding 15 mL of a concentrated inoculum (OD_{750} = 1) in 135 mL of BG-11 medium. The pre-culture was prepared three days before starting the exposures to achieve an exponential growth phase during the exposure period. Toxicity tests were performed (OECD 201) exposing the cyanobacteria from pre-culture to pure glyphosate (Gly) at 40; 60; 80; 100 and 120 mg L^{-1} glyphosate (Glyphosate Pestanal®, 98.6%, Sigma-Aldrich®) or the RT formulation at 0.75, 1.5, 3, 6 and 12 mg L^{-1} glyphosate (Roundup Transorb®, Monsanto do Brazil Ltda.). Herbicides were dissolved in Milli-Q water and then added to the BG-11 medium. A control group (Ctr), without glyphosate or RT in the medium, was also kept during the test. The proportion of Milli-Q water and BG-11 culture media were the same in all treatments. The final concentration of *S. elongatus* in the flasks (Erlenmeyer with 30 mL) was 10% (3 mL). Three replicates were used for each concentration tested. Cyanobacteria biomass values based on the optical density (OD) were measured daily during the test, and after 72 h of exposure, the number of cells and viability were immediately analysed. The OD was measured in 1 mL aliquots of each replicate using a spectrophotometer (BioMate 3, Thermo Scientific) at a wavelength of 750 nm (Lee *et al*., 1991). Temperature and luminosity were kept as in the cultivation regimen during the experimentation.

Growth analysis and IC_{50} (Inhibition concentration mean)

The growth of cyanobacteria was estimated by subtracting the initial OD from the final OD for each sample. The inhibition of cyanobacteria growth relative to the Ctr groups was calculated using the OD measurements at the end of the test (72 h), as shown in equation 1. These data were used to estimate the concentration that inhibits 50% of cyanobacteria biomass growth (IC_{50}).

(Equation 1) $$Growth\ inhibition\ (\%) = \left(\frac{DC-DT}{DC}\right) \times 100$$

where DC is the density of control and DT is the density of treatment with Gly or RT at 72 h of the test.

Cell viability analysis

For the cell viability, aliquots of 9.5 µL were collected from three replicates of each experimental group. Then, 0.5 µL of SYTOX® Green fluorescent was added (Invitrogen ™, 50 µM stock solution diluted in DMSO), achieving a final concentration of 2.5 µM. SYTOX® Green is a dye capable of penetrating the membranes of non-viable cells and staining nucleic acids and emitting green fluorescence (Sato *et al.*, 2004). Samples with dye were incubated for 5 min at room temperature (20 °C) in the dark. At the end of the incubation period, slides were prepared using 10 µL of the sample. Cells were observed under a fluorescence microscope (Olympus BX53) equipped with a digital camera (Olympus DP72). Viable cells were analysed using a filter with a wavelength between 510 and 550 nm (red) and non-viable cells were analysed using a filter with a wavelength between 450 and 480 nm (green). Viable and non-viable cells were counted from images of three different areas of each slide using the free public software Image J (US National Institutes of

Health, Bethesda, MD, USA). Cell viability was expressed as a percentage of cells coloured in red on each slide.

Statistical analysis

Data were expressed as mean ± standard error (SE). Differences between treatments relative to the Ctr group were determined by one-way analysis of variance (ANOVA) followed by the post hoc Bonferroni test, with a significance level of 95% ($p < 0.05$). The assumptions of normality and homogeneity of data variations were previously tested using the Kolmogorov-Smirnov test and Bartlett's test, respectively. The IC_{50} was estimated by the Trimmed Spearman-Karber software (Hamilton *et al*., 1978).

RESULTS AND DISCUSSION

Figure 1 shows the growth patterns of *S. elongatus* cultures exposed to Gly and RT over the 72 h of the test (Figure 1A and Figure 1B, respectively) expressed as OD. At concentrations of 40, 60 and 80 mg L^{-1} of pure glyphosate, the growth of *S. elongatus* was like the Ctr group, suggesting that this species was quite tolerant to this compound. This may be associated with its ability to degrade glyphosate, using phosphorus (P) present in the glyphosate molecule as a nutrient for its growth (Lu *et al*., 2020). However, according to Powell *et al.* (1991), this tolerance of cyanobacteria may be also related to an overproduction of the enzyme EPSPS or the production of another enzyme tolerant to glyphosate. In contrast, only the treatment with the lowest glyphosate concentration in the RT formulation (0.75 mg L^{-1}) did not affect the growth profile of *S. elongatus* when compared to the Ctr group. The treatments with RT at 1.5, 3, 6 and 12 mg L^{-1} of glyphosate inhibited the growth pattern and in the highest concentrations (6 and 12 mg L^{-1} of glyphosate) the OD values were lower for all exposure times (24, 48 and 72 h), than for the initial value of the experiment, without any growth during the experiment (Figure 1B). This means that the exposure not only inhibited the growth of the strain but also led to the death of the cells present in the initial inoculum. Another aspect is that glyphosate or RT exerts toxicity in the first 24 h of exposure, with no recovery over time.

The greater toxicity of RT was reflected in the values of IC_{50}, which were sixty times lower in RT exposed organisms, being 109.44 mg L^{-1} (confidence interval of 106.66–112.28 mg L^{-1}) of glyphosate for Gly treatments and 1.73 mg L^{-1} (confidence interval of 1.68–1.78 mg L^{-1}) of glyphosate for RT treatments. Considering biomass growth, Figure 2A shows a significant effect of pure glyphosate only in the two highest concentrations (100 and 120 mg L^{-1} Gly), with the greatest inhibition of 70% at the highest concentration tested (120 mg L^{-1} Gly). For RT, significant growth inhibition was observed earlier, reaching levels of no growth at the highest concentrations (Figure 2 B). Lam *et al.* (2019) found similar results for cyanobacterium *Microcystis aeruginosa* exposed to the formulation Roundup Custom® (RC), at a concentration of 0.7 mg L^{-1} glyphosate. However, contrary to us, they did not observe a reduction in growth after exposure to 7 mg L^{-1} of glyphosate, which may be related to the lower sensitivity of *M. aeruginosa* to glyphosate or less toxicity for the RC formulation. These variations among glyphosate-based formulation toxicity values have been reported not only for cyanobacteria but also for fish (Sánchez *et al.* (2017 and 2019) and has been attributed to the type of glyphosate salt and to the other components that differ among the formulations.

Despite the drop in cell viability, this value was not as significant as the reduction in the number of cells counted per slide (Figure 3) or the growth. It seems that the few cells that were left over at the end of the test were viable. Possibly,

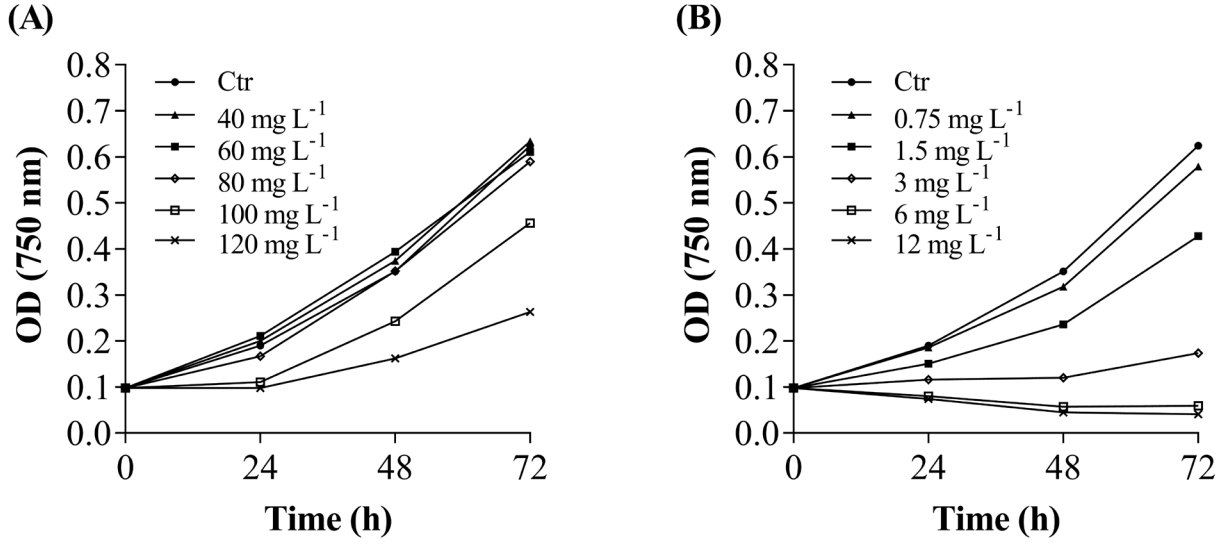

Figure 1. Growth curve of *S. elongatus* cultures during 72 h of exposure to Gly (A) and RT (B). Results are expressed as means of OD ± SE.

the viable cyanobacteria could give rise to a strain more tolerant to glyphosate. Additionally, these results also suggest that cell viability underestimates the toxic effects of pure glyphosate and its RT formulation; therefore, its use as an endpoint for toxicity should be used with caution. Although the mechanisms by which glyphosate affects *S. elongatus* cell viability is not clear, Ye *et al*. (2019) reported that for the species *Microcystis viridis*, glyphosate (2, 5 and 10 mg L^{-1}) causes oxidative damage and a decrease in protein and chlorophyll-a content, leading to apoptosis. Wu *et al.* (2016) also reported the effects observed by Ye *et al.* (2019) but for the cyanobacteria *M. aeruginosa*.

Overall, this work clearly shows that are great differences between the toxicity values of pure glyphosate and RT to *S. elongatus*, with RT being much more toxic. Several studies have reported greater toxicity values related to glyphosate formulations (Qiu *et al.*, 2013; Moraes *et al.*, 2020), and two theories may apply to our results. Firstly, components of the RT formulation other than glyphosate are more toxic than the active ingredient, which has already been observed by some authors, such as Lipok *et al.* (2010), who reported that glyphosate isopropylamine salt (IPA) was more toxic than glyphosate for the growth parameters of some cyanobacteria (*M. aeruginosa*, *Spirulina platensis*, *Arthrospira fusiformis*, *Nostoc punctiforme*, *Anabaena catênula* and *Leptolynbya boryana*). Secondly, components of the RT formulation, such as the POEA, facilitate the penetration of glyphosate in the cyanobacteria, as occurs in plants. This has already been reported by Powell *et al.* (1991), who observed that after exposure to Roundup®, the internal concentrations of glyphosate in the cyanobacteria *Synechocystis* sp. PCC 6803 and *Anabaena variabilis* ATCC 29413 were higher than after exposure to pure glyphosate. An increase in the absorption of the glyphosate molecule by cyanobacteria enhances the toxicity of the compound. Results presented here can contribute to the knowledge of the effects of pure glyphosate and the RT formulation on cyanobacteria as a non-target organism, and they can be useful for risk assessment and to

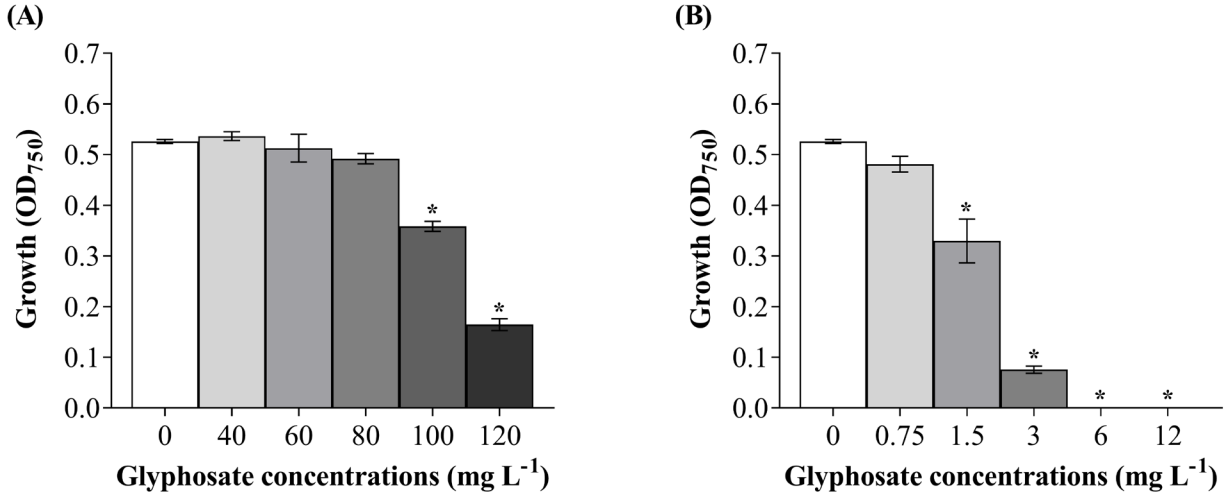

Figure 2. Growth of *S. elongatus* cultures after 72 h of exposure to Gly (A) and RT (B). Data were calculated by subtracting the initial OD value from the experiment from the OD value obtained in each treatment after 72 h of exposure (n = 3). (*) represents a statistical difference relative to the Ctr group ($p < 0.05$).

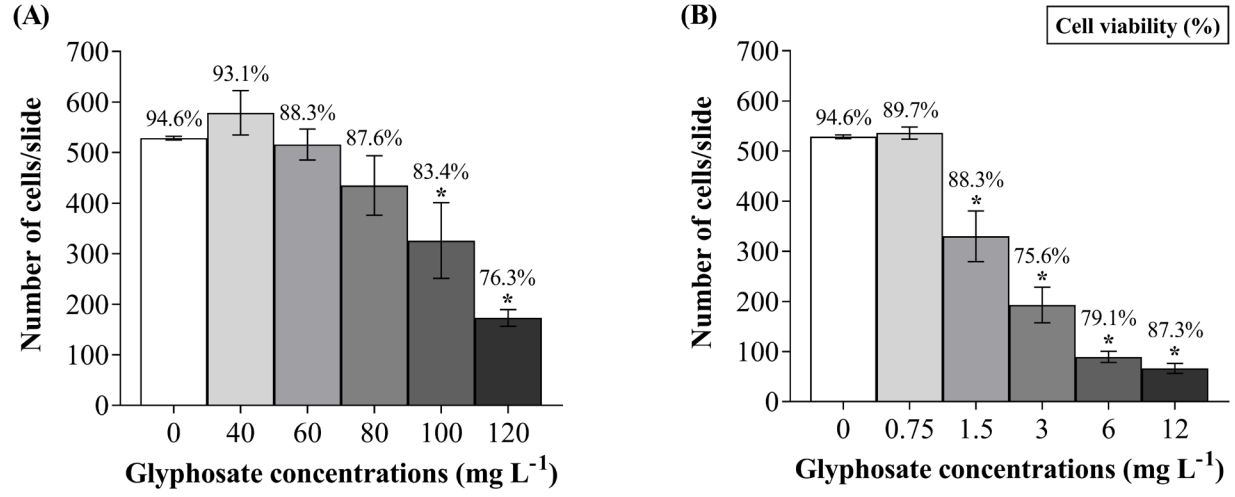

Figure 3. Mean of cells of S. elongatus per slide after 72 h of exposure and corresponding cell viability (%). The number os cells refers to the total number of cells counted from 3 areas randomly chosen on each slide. The percentage of cell viability was obtained considering the number of viable cells relative to the total number of cells in each treatment. (*) represents a statistical difference relative to the Ctr group ($p < 0.05$).

regulate widely commercialised formulations, such as RT, and protecting the environment from potential impacts caused by agricultural activities.

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