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The toxicity of Roundup® 360 SL formulation and its main constituents: Glyphosate and isopropylamine towards non-target water photoautotrophs

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ABSTRACT

The toxicity of commercial formulation of Roundup® 360 SL, widely used, nonselective herbicide and its main constituents, glyphosate (PMG), equimolar (1:1) isopropylamine salt of glyphosate (GIPA) and isopropylamine (IPA) was examined towards eight aquatic microphotoautotrophs; seven cyanobacterial strains representing either saline or freshwater communities, and common eukaryotic algae *Chlorella vulgaris* Beijerinck. Autotrophs were cultured 21 days in their appropriate standard media supplemented with various amounts of Roundup®, glyphosate, GIPA and IPA. The determination of the growth of examined photoautotrophs was performed by time-course measurements of total chlorophyll content in experimental cultures. The growth rates related to corresponding concentrations of chemicals, the EC_{50} values and generation doubling time were determined in order to present the toxicity Roundup® 360 SL formulation and its main constituents. Market available formulation of Roundup® was found to possess toxicity significantly higher than this, attributed to its main constituents; however both these compounds, isopropylamine and glyphosate, also inhibited the growth of examined strains in a dose-dependent manner. Notably, the interpretation of toxicity of the examined substances was found to be significantly dependent on the method of EC_{50} calculation. The choice of molar or weight concentration of substances tested separately and in specific formulation was found to be essential in this matter. Due to these findings the EC_{50} values were calculated based either on molar or on weight concentrations. Considering Roundup® 360 SL formulation, these values ranged from 10^{-3} up to 10^{-1} mM and they were one order of magnitude lower than those found for isopropylamine. Quite surprisingly the minimum EC_{50} values found for glyphosate did not reach micromolar concentrations, whereas most of the EC_{50} values revealed to IPA did not exceed this range. Notably, in all the cases except for *Synechocystis aquatilis* Sauvageau, isopropylamine alone was indicated as more toxic than glyphosate.

1. Introduction

The worldwide success of Roundup® as the most frequently used organophosphorus broad-spectrum and biodegradable herbicide is beside any discussion. However, during the past decades information regarding its environmental concerns increased significantly. All these results were collected either from agricultural practice or from specially arranged experiments. In the first approach – agricultural practice – Roundup® was treated as a homogenic ready-to-use substance and all effects of its non-target toxicity were attached to this market available formulation (Marques et al., 2008). In the second approach, Roundup® is treated as the mixture of isopropylamine salt of N-(phosphonomethyl)glycine (glyphosate) as principal ingredient, water, colorant(s) and some surfactants, e.g. polyoxyethylene tallow amine, POEA, added in order to improve herbicide transport into the plant tissue. This attempt allows for the exploration of hypotheses about the toxicity of market available formulation(s) of herbicide as resulted from specific activity of ingredients. Hence the results reported by Pereira et al. (2009) indicated higher antialgal toxicity of Spasor® formulation than that of sole glyphosate, its active ingredient.

Glyphosate is a broad-spectrum herbicide used in agriculture, forestry and for aquatic weeds control. Microbial biodegradation of glyphosate occurs mainly in soil, and to a much lower extent in aquatic sediment and water, with the major metabolite being aminomethylphosphonic acid (AMPA). Glyphosate is chemically stable in water and does not undergo photochemical degradation. The mobility of glyphosate in soil indicates its potential for the contamination of groundwater. Glyphosate can enter surface and subsurface waters after its direct use near aquatic environments or by runoff or leaching from terrestrial applications (Abrantes et al., 2009). The process of biodegradation of mentioned phosphonate, also in natural aquatic ecosystem, was proved to

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be a reason for decreasing toxicity of this substance (Vera et al., 2010); however, there is information about moderate, season-dependent responses of natural riverine microbial communities to glyphosate exposure (Pesce et al., 2009).

Because of their physiological similarities to terrestrial plants, aquatic phytoplankton seems to be especially vulnerable to the impact of such nonselective herbicide as Roundup® is. The study on the influence of Roundup® on natural marine microbial communities demonstrates that this formulation disturbs this ecosystem even at the dose of 1 μg/L, which is a value typical for those reported in coastal waters during runoff events (Stachowski-Haberkorn et al., 2008). Corresponding results were obtained with respect to freshwater ecosystem during the study on the impact of Roundup® on the periphyton community. In this case the herbicide applied at 8 mg/L of the active ingredient (glyphosate) slightly influenced algae, mainly diatoms; however, the growth of cyanobacterial strains was favoured (Vera et al., 2010). In this case commercial formulation of herbicide delayed periphytic colonization and significantly decreased the total mass and chlorophyll a content in the phytoplankton community. Moreover Roundup® applied in a similar concentration caused significant—about 40-fold—increase in planktonic picocyanobacteria abundance and thus affected the structure of phytoplankton and periphyton assemblages (Perez et al., 2007).

Cyanobacteria, a diverse group of prokaryotes, are the oldest oxygenic photosynthetic organisms sharing the same photosynthetic apparatus as eukaryotic algae and higher plants (Zeng and Vonshak, 1998). These microorganisms, well known to adapt to contaminated environments, comprise a major proportion of the total phytoplankton biomass and show a remarkable tolerance to any kind of stress, including chemical one (Barton et al., 2004; Campanella et al., 2001; Hosetti and Frost, 1998; Lee et al., 2003). A few species have been found to exhibit a remarkable tolerance to glyphosate (Powell et al., 1991), possessing, however, different biochemical bases of this feature (Forlani and Campani, 2001; Forlani et al., 2008). This was also shown for *Spirulina platensis*, one of the few cyanobacteria that do not produce toxins and has become of some commercial value as food additive, especially in developing countries (Dillon et al., 1995; Richmond, 1998).

Green algae are known to be more sensitive to many chemicals in relation to photosynthetic cyanobacteria; however, their ecological position in most aquatic ecosystems and their essential role in nutrient cycling and oxygen production are comparable with the status of cyanobacteria (Sabater and Carrasco, 2001). Because these plants vary in their response to a variety of toxicants (Real et al., 2003; Vendrell et al., 2009), they have been considered as specific indicators of the bioactivity of industrial and urban wastes.

Although some information on the toxicity of pesticides towards cyanobacteria and green algae has been compiled, the data about the toxicity of all main constituents of market formulations of these xenobiotics are extremely rare, especially considering their presence in free form in aquatic environments. Therefore the aim of our work was to examine the acute toxicity of glyphosate herbicide and isopropylamine, its equimolar accompanying compound, as the main constituents of Roundup® 360 SL formulation, towards seven cyanobacterial strains representing saline and freshwater communities and model algae *Chlorella vulgaris*.

2. Materials and methods

Pure N-phosphonomethylglycine was obtained from commercially available formulation Roundup® 360 SL (Monsanto, MO, USA), by dissolving it in water and maintaining the pH of the solution to 1.5–2.0 with hydrochloric acid. This resulted in crystallization of the glyphosate, which was purified by multistep recrystallization. The structure and purity of the compound were confirmed using ^{1}H, ^{13}C and ^{31}P NMR spectroscopy. Also the retention time of this substance was examined using HPLC–UV after its derivatization with p-toluenosulphonyl chloride and was the same as the retention time of pure glyphosate obtained from Monsanto. The isopropylamine (IPA) as standard chemical reagent of 98% purity, and N-(phosphonomethyl)glycine monoisopropylamine salt (GIPA) in the form of solution—(40 wt% in H_2O) were purchased from Sigma-Aldrich. GIPA is also indicated in this paper as equimolar (1:1) isopropylamine salt of glyphosate or simply isopropylamine salt of glyphosate.

2.1. The determination of glyphosate and isopropylamine in studied media

The use of commercial formulation of Roundup® required determination of the concentration of glyphosate and isopropylamine in examined media. They were determined according to the procedure reported by Khrolenko and Wieczorek (2005). Briefly, 1 ml samples of each previously sterilized medium supplemented with appropriate, depending on the concentration, amount of Roundup® were mixed with 0.5 ml of 0.4 M phosphate buffer (pH 11) and 0.2 ml of p-toluenesulphonyl chloride solution (10 mg/ml in acetonitrile) and heated in a water bath at 50 °C for 10 min in order to derivatize glyphosate and IPA. After the derivatization process, the volume of 0.25 ml of 1 M HCl solution was added to each sample. Then the 20 μl subsamples were injected on Microsorb-MV 100-5 C18 column followed by Meta Guard Monochrome 5U C18 pre column equilibrated with a mixture of 0.06 M KH_2PO_4 buffer (adjusted to pH 2.3 with H_3PO_4) and acetonitrile (85:15, v/v). Isocratic elution proceeded at 22 °C and at a flow rate of 1 ml/min, monitoring the eluate at 240 nm. The HPLC system used in this work consisted of Hitachi L-7100 (Hitachi Ltd., Tokyo, Japan) solvent delivery module and a Shimadzu SPD-61 UV Spectrophotometric Detector (Shimadzu Corp). The addition of the millimolar solutions of derivatized glyphosate and/or IPA as internal standards to the samples allowed for identification of peaks corresponding to these compounds. Quantification of the tested chemicals was carried out in triplicate, based on the appropriate calibration curve of derivatized media containing known concentrations of each substance.

2.2. Concentrations of tested substances

Due to the average recommended dose of Roundup® 360 SL, which is 4–5 L/ha in agricultural practice, the calculated concentration of this herbicide in 0.01 m thick surface layer of the soil exceeds 44.6 mg/L, which corresponds to 0.06 mM of isopropylamine salt of glyphosate. In cases of orchards or herbicidal treatments on urban and industrial areas, this concentration referred to dose of 6–8 L/ha increases up to about 89 mg/L, which is the equivalent of 0.12 mM of active ingredients. Therefore the final tested molar concentrations of glyphosate, isopropylamine and Roundup® ranging from 0.003 to 10 mM were established as 3×10^{x}, 7×10^{x}, 10×10^{x} mM where $x=(-3,\ -2,\ -1,\ 0)$. In case of Roundup® 360 SL, each examined molar concentration of this formulation has been related to appropriate molar concentration of its active ingredient—equimolar (1:1) isopropylamine salt of glyphosate. The final examined molar concentrations of pure GIPA ranged from 0.003 to 3 mM.

Tested molar concentrations of GIPA, glyphosate and IPA were recalculated into weight ones, considering molar masses of these compounds. Considering Roundup® 360 SL, treated as the mixture of active ingredient GIPA, adjuvant (POEA) and water, additionally the percentage of components and specific gravity of this formulation were taken into account. The collation of examined molar and weight concentrations is presented in Table 1.

2.3. Strains and growth conditions

Strains used in our experiments, however all classified as plankton microorganisms, represent Prokaryota; Bacteria—cyanobacterial strains and Eukaryota; Plantae—*C. vulgaris*. Cyanobacteria naturally occurring in various habitats: hypersaline ponds—*S.* (*Arthrospira*) *platensis* Gomont, *Arthrospira fusiformis* Voronichin and in freshwater ecosystems—*Nostoc punctiforme* Kützing, *Anabaena catenula* Kützing, *Synechocistis aquatilis*, *Microcystis aeruginosa* Kützing, *Leptolyngbya boryana* Gomont. *C. vulgaris* was chosen as common freshwater algae tolerant to pH in the range 5.5–9 (Azov, 1982). It is also important that most of the herein examined cyanobacteria had been successfully studied regarding their tolerance against glyphosate (Forlani et al., 2008).

Before the growth in experimental media, all tested microorganisms were pregrown in their appropriate standard media in order to achieve inocula, which were used to initiate the experimental cultures.

C. vulgaris was grown at 22 ± 1 °C under 16 h days (300 μmol m^{-2} s^{-1} PAR) and 8 h nights in 250 ml Erlenmayer flasks containing 50 ml of modified Bristol medium. Our medium was composed on the faith of 1000 ml of Bristol's solution supplemented with 4 ml of saturated $NaHCO_3$ water solution (about 250 mg/ml) and 18 mg of $FeSO_4\cdot7H_2O$. Subcultures were produced every 24 days, in late log-phase, by transferring 15 ml aliquots to 35 ml of fresh medium.