

PHYSIOLOGICAL AND ECOTOXICOLOGICAL EFFECTS OF GLYPHOSATE ON THE GREEN ALGAE *RAPHIDOCELIS SUBCAPITATA*

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Abstract: Glyphosate is the world's most widely used herbicide, yet its effects on aquatic ecosystems – especially microalgae – remain poorly understood. This study investigated the physiological responses (photosynthesis, chlorophyll-*a* and pheophytin content, nitrogen and phosphorus uptake rates, optical density, protein content, and stress enzyme activity) of *Raphidocelis subcapitata*, a standard OECD test species, to a range of glyphosate concentrations (0.45–7.2 µg L⁻¹) over 14 days. Results showed that glyphosate significantly influenced several parameters, including reductions in photosynthetic activity and nutrient uptake rates, alongside increased oxidative stress responses at higher concentrations. At low concentrations (≤ 0.9 µg L⁻¹), signs of hormesis were observed, with elevated photosynthesis and chlorophyll-*a* content. Principal component analysis revealed clear separation of treatment groups based on glyphosate levels and exposure time. The findings supported species-specific and dose-dependent effects of glyphosate on freshwater algae, and emphasised the ecological vulnerability of shallow water bodies, such as Lake Balaton, to herbicide contamination. The results highlight the necessity for a stricter monitoring system, and further research on the chronic low-level impacts of glyphosate, as well as the development of environmentally safer alternatives. Present paper was inspired by Professor Judit Padisák, who has been a committed and unwavering advocate of Lake Balaton research from the beginning of her scientific career to the present day.

Key words: glyphosate, herbicide, microalgae, physiology, toxicology

INTRODUCTION

Glyphosate (N-(phosphonomethyl)glycine) is a synthetic amino acid derivative patented by Monsanto (America) in 1971 and firstly marketed as the active ingredient of Roundup in 1974 (Tórkés 2024). Glyphosate is a non-selective total herbicide affecting nearly all green plants both monocotyledonous and dicotyle-

donous, annual and perennial weeds (KLÁTYIK *et al.* 2023, MALIK *et al.* 1989). The active ingredient glyphosate is an absorbable (systemic) agent that the plant absorbs through its green parts containing chlorophyll. Glyphosate is absorbed through leaves and stems, crossing cuticular and symplastic barriers, then moves via both phloem (mainly toward growing sinks) and to a lesser extent xylem toward roots and reproductive structures (MARTINEZ *et al.* 2018). The symptoms of the effect appear after 1–2 weeks in the form of aging, dwarf growth, and browning of leaves and shoots. The process is irreversible, the affected plant dies (DE FREITAS and SILVA *et al.* 2021).

By 2007, it had become one of the most widely used active ingredients in the world due to its efficiency and relative cheapness (TÖKÉS 2024). Approximately 72% of global pesticide usage was glyphosate and glyphosate-based herbicides; the weight of active ingredient applied globally is 800,000 tonnes/year (KLINGERHÖFER *et al.* 2021). According to the Hungarian Central Statistical Office, Hungarian farmers used 422 tonnes of glyphosate in 2019. This amount was applied across approximately 272,000 hectares of farmland (www1). As a result of the large-scale usage, traces of it have been widely detected in a variety of areas, such as food, rain and airborne dust, even further away from its area of use (KISVARGA *et al.* 2023). In its study, the first breakthrough result was achieved in 2009, when the toxic and endocrine-disrupting effects of glyphosate-based herbicides in human cells were demonstrated (AGRO NAPLO 2016). Since then, research on the negative effects of glyphosate has expanded considerably, demonstrating its toxicity to crops, soil, wildlife, livestock, and the entire ecosystem. Moreover, studies have reported serious adverse health effects even below the recommended level for agricultural use. This information led the WHO International Agency for Research on Cancer (IARC) to classify glyphosate as a probable human carcinogen in 2015 (IARC 2015), but its use is still authorised in the European Union (with the sole exception of Austria).

Most research on glyphosate focuses on higher plants and animals (KLÁTYIK *et al.* 2023), while much less information is available on its effects on aquatic organisms and ecosystems (KLÁTYIK *et al.* 2024a). Glyphosate is a water-soluble and stable compound, which makes it relatively easy to release into groundwater and surface waters. A growing number of studies have shown its presence in various water resources (CARLES *et al.* 2019, MAGGI *et al.* 2020, FELTRACCO *et al.* 2022). In most surface waters, glyphosate is found at a low level. Reported averages in rivers and lakes are often between 0.1–10 µg L⁻¹ depending on season and agricultural intensity (reviewed by KLÁTYIK *et al.* 2024a). Although ambient concentrations are typically lower than lethal doses, the long-term ecological consequences of chronic low-level exposure, such as species composition changes, biodiversity loss, are of concern. Small water bodies and sensitive ecosystems

where degradation is slower and biota are less tolerant are especially vulnerable. For instance, Lake Balaton is the largest shallow lake in Central Europe, whose railway network is very well developed; four railway lines provide a circular route running directly along the shoreline (with the exception of two cities, namely Tihany and Szigliget) for approximately 194 kilometres. Weed control is a part of the railway track maintenance process, which extends the entire area of Lake Balaton. Various chemical mixtures are used for this, but one of their main ingredients is glyphosate. Consequently, there is a high risk of this pesticide entering the lake (e.g. through runoff), which has already been detected in the lake with a maximum of $2.0 \mu\text{g L}^{-1}$ (TÓTH *et al.* 2022). Since Lake Balaton is of particular importance from both nature conservation and an economic point of view, thus gaining a better understanding of the effects of glyphosate on ecological processes taking place in it and predicting possible future changes are extremely important for our present and future.

Overall, the effects of glyphosate on aquatic organisms are species-specific and dose-dependent, and it is therefore important to study different groups of organisms. Regarding the phytoplankton, glyphosate exerts diverse and concentration-dependent effects (reviewed by KLÁTYIK *et al.* 2024a). Reported impacts range from growth inhibition, ultrastructural damage, and oxidative stress to shifts in community composition. Sensitive taxa, such as green algae and diatoms, frequently show reduced chlorophyll-*a*, carotenoids, and photosynthetic efficiency at concentrations as low as $1\text{--}10 \mu\text{g L}^{-1}$. Other taxa, particularly some cyanobacteria and dinoflagellates, can tolerate or even utilise glyphosate as a phosphorus source under nutrient-limited conditions, in some cases, can have stimulation of growth and toxin production. This dual role means glyphosate can alter community composition rather than uniformly suppress phytoplankton. Our knowledge about glyphosate is still controversial and incomplete, which are particularly true for algae, even though their importance is very high thanks to the many ecosystem services they provide to humans (e.g. LENGYEL *et al.* 2023, NASELLI-FLORES and PADISÁK 2023). Therefore, our aim is to better understand the effects of glyphosate on aquatic algae through the investigation of a green algal species. *Raphidocelis subcapitata* is well justified for standardised toxicological assessment of glyphosate, because it is a sensitive, well-established model organism representing primary producers. The OECD 201 guideline explicitly frames green algae and cyanobacteria as primary producers suitable for 72-h growth tests, and *R. subcapitata* is a common default because it grows fast, is easy to culture, and yields reproducible concentration–response curves – hence its widespread use for hazard screening and cross-study comparability.

MATERIAL AND METHODS

Algal culturing

The species *Raphidocelis subcapitata* (Korshikov) Nygaard, Komárek, J. Kristiansen et O. M. Skulberg (Fig. 1a) originated from the algal culture collection of the University of Pannonia Center of Natural Sciences (Veszprém, Hungary). The monoculture of *Raphidocelis subcapitata* was maintained in liquid medium (Fig 1b, OECD 2011). The algal culture was grown at room temperature under photosynthetic active radiation (PAR) of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ (provided by 'daylight' tubes) and a 14:10 light:dark cycle.

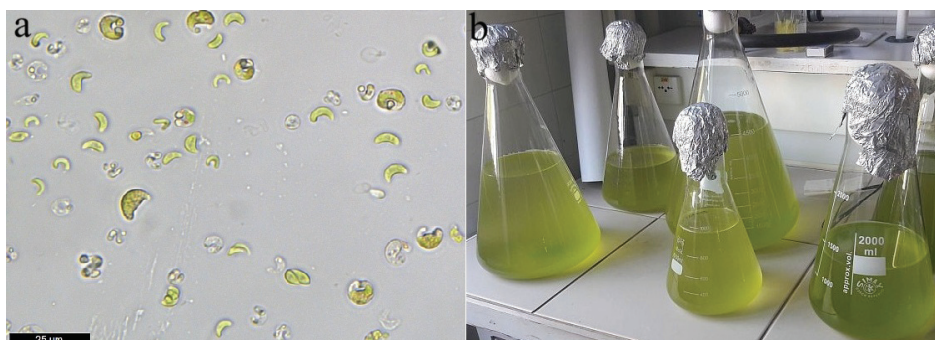


Fig. 1. Light microscopic picture (a, scale bar: 25 μm) and Batch cultures (b) of *Raphidocelis subcapitata*.

Experimental setup

All the experiments were performed under the same conditions used for culturing. Altogether five concentrations of the glyphosate were tested on the basis of its detected level in Lake Balaton (a maximum of $2.0 \mu\text{g L}^{-1}$; Tóth *et al.* 2022): 0 (as control), 0.45, 0.9, 1.8, 3.6, $7.2 \mu\text{g L}^{-1}$. Three parallels were used for each. The dilution series was prepared using ADAMO (360 g L^{-1}), which is a popular herbicide product available in retail stores. During the pilot study, different exposure times were applied; 130 ml of the algal culture was always taken on the zero, 1st, 3rd, 7th and 14th days for the measurements of the endpoints.

Endpoints

Altogether eight parameters were measured at each sampling event.

- The photosynthetic activity ('Ps') was determined by the oxygen yield method. Dissolved oxygen concentrations in Karlsruhe flasks were measured twice in an hour with an LDO sensor (HQ-20, Hach Lange). Net photosynthetic activity normalised to units of chl-a was calculated according to the method by WETZEL and LIKENS (2000).
- The optical density ('OD') was measured by a spectrophotometer (Meter-tech) at 750 nm.
- Immediately after the Ps and OD measurements, 20 ml of the cultures were filtered using a 1.2 μm pore size filter (Prat Dumas). The chlorophyll-*a* ('chl') and pheophytin ('pheo') content of the samples were spectrophotometrically determined after extraction with acetone (WETZEL and LIKENS 2000).
- For measuring peroxidase activity ('POD') and total protein content ('pro') extract was prepared. 100 ml of algae culture was filtered through a 1.2 μm pore size filter (Prat Dumas filter) and the mass was measured with analytical balance. In ice cold mortar the previously measured filters were homogenised in a phosphate buffer (50 mmol L^{-1} , pH 7) with 1 mmol L^{-1} EDTA (ethylene-diamine-tetraacetic acid) and 0.5 mmol L^{-1} PMSF (phenyl-methyl-sulfonyl fluoride). Samples were centrifuged for 20 min at 15,000 $\times$ g at 4 °C and supernatants were collected. For the determination of total protein content, the Lowry method was followed with slight modifications (PETERSON 1983). From extract according to IMBERTY *et al.* (1984) TMB (tetramethyl-benzidine) peroxidase activity was measured with a minor modification. The oxidation rate was calculated by measuring the change of absorbance at A654 nm.
- In the experimental study, nutrient uptake rates ('N rate' and 'P rate') were calculated using the SRP (Soluble Reactive Phosphorus) and NH_4^+ concentrations of the filtered samples. Concentrations of SRP and NH_4^+ were determined using spectrophotometric methods (APHA 1998).

Statistical analyses

Principal Component Analysis (PCA) was performed to examine whether the measured endpoints exhibited any patterns based on the applied glyphosate concentrations and exposure times. Two-way ANOVA tests were used to examine the effect of glyphosate concentrations, exposure time, and their interaction on the measured physiological parameters. All the statistical analyses were performed in R statistical software (4.4.3 version, R CORE TEAM 2024) using the 'vegan' (OKSANEN *et al.* 2024) package.

RESULTS

In general, the measured endpoints changed along wide ranges during the experiments (Table 1). The only exception to this was the OD, which did not show any significant changes. According to the two-way ANOVA tests (Table 1), the glyphosate concentration had significant effects on the chlorophyll-*a* and pheophytin content, the photosynthesis and the nutrient uptake rates. The exposure time had significant correlations with the measured endpoints with the exception of the optical density. Furthermore, ANOVA analysis showed statistically significant interaction between concentration and time in the case of the pheophytin content.

Table 1. Statistical parameters of the measured endpoints, and its significant relations with the glyphosate concentration, the exposure time and its interaction according to the two-way ANOVA analysis (CV = coefficient of variation, C = concentration, T = exposure time, I = interaction).

endpoints	unit	min	max	mean	CV	ANOVA		
						C	T	I
POD	nmol (min mg protein) ⁻³	22023.0	162299.1	51341.8	58.3		+	
protein	µg L ⁻¹	20.0	49.6	32.8	23.6		+	
OD	nm	0.197	0.211	0.197	4.4			
chl- <i>a</i>	µg L ⁻¹	0.53	1.87	1.11	30.8	+	+	
pheophytin	µg L ⁻¹	0.00	0.71	0.12	159.7	+	+	+
photosynthesis	mg C mg Chl- <i>a</i> ⁻¹ h ⁻¹)	404.3	4153.3	1441.5	75.0	+	+	
P rate		0.0	92.3	24.8	129.8	+	+	
N rate		12.6	54.5	22.5	55.7	+	+	

According to the results of the principal component analysis (PCA), the first two axes together explained 81.47% of the variance in the measured physiological parameters. The samples also showed a clear separation on the basis of the glyphosate concentration (Fig. 2a) and the exposure time (Fig. 2b). The nutrient depletion rates, chlorophyll-*a* content, and photosynthetic activity were negatively associated with glyphosate concentration and exposure time. As regards the chlorophyll-*a* content, photosynthetic activity, and nutrients uptake, increases in its amount were observed at the lower glyphosate concentrations (0.45 and 0.9 µg L⁻¹) compared to the control. In contrast, glyphosate treatment (concentration and exposure time) showed a positive correlation with POD enzyme activity and pheophytin content.

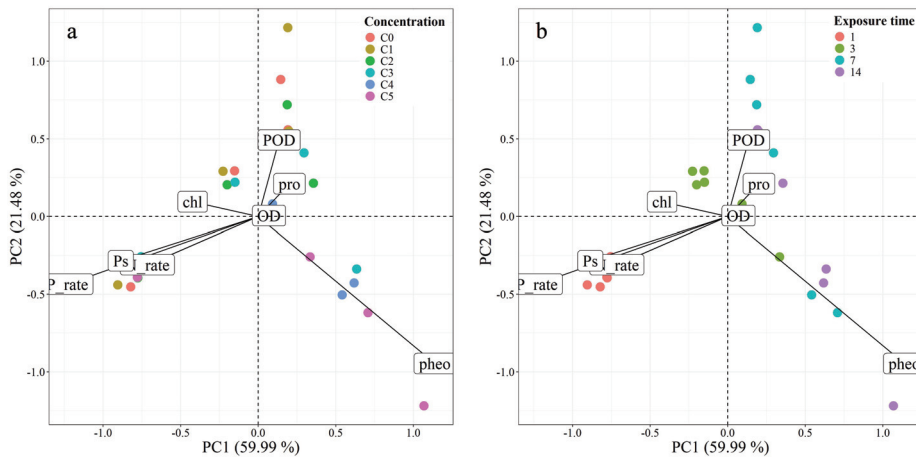


Fig. 2. The biplots of PCA (Principal Component Analysis) on the basis of the glyphosate concentrations (a) and exposure times (b) (C0 = control; C1 = $0.45 \mu\text{g L}^{-1}$; C2 = $0.9 \mu\text{g L}^{-1}$; C3 = $1.8 \mu\text{g L}^{-1}$; C4 = $3.6 \mu\text{g L}^{-1}$; C5 = $7.2 \mu\text{g L}^{-1}$; pro = protein; Ps = photosynthesis; pheo = pheophytin; chl = chlorophyll-*a*; POD = enzyme activity; OD = optical density).

DISCUSSION

Glyphosate is the most popular herbicide used in both agriculture and households. Today, it has become the most widely used herbicide, which means that traces of it can be found in many different fields: in food, animals, rain, and different waterbodies. Although algae provide extremely important ecosystem services (e.g. NASELLI-FLORES and PADISÁK 2023), there is still limited knowledge about the effects of glyphosate on them. Furthermore, species differed in their sensitivity against this herbicide (KLÁTYIK *et al.* 2024b), therefore it is in our interest to learn as much as possible about the effects of glyphosate on different algal species.

It is well known that glyphosate affects nearly all green plants (KLÁTYIK *et al.* 2023, MALIK *et al.* 1989). Primary target of glyphosate is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme in the Shikimate Pathway, where a key enzyme is inhibited (DUKE and POWLES 2008, STEINRÜCKEN and AMRHEIN 1980). Since the shikimate pathway is also present in microbes, glyphosate can also have significant impacts on aquatic microalgae, as it was shown in the case of the studied *Raphidocelis subcapitata*. Beside its concentration, the exposure time and their interaction were also determinant factors in many measured physiological characteristics.

Photosynthesis, chl-*a* and pheophytin content

Photosynthesis is a frequently used endpoint for testing different abiotic stresses (e.g. LÁZÁR *et al.* 2023, LENGYEL *et al.* 2015), which was also sensitive and suitable for detecting the effects of glyphosate. Similar to other microalgal species (e.g. CHOI *et al.* 2012), photosynthesis of *Raphidocelis subcapitata* was also inhibited by glyphosate (beyond $0.9 \mu\text{g L}^{-1}$). This physiological process can be impacted in several direct and indirect ways: inhibition of plastoquinone biosynthesis, effect on rate of electron transport, and inhibiting the respiratory electron transport chain (reviewed by KLÁTYIK *et al.* 2024b). Furthermore, the reduction in chlorophyll concentration can also lead to reduced photosynthetic activity (GOMES *et al.* 2016). This was supported by the present study, since glyphosate reduced the chlorophyll-*a* content in parallel with the accumulation of pheophytin. However, stimulated photosynthesis was observed at lower glyphosate concentrations ($<0.9 \mu\text{g L}^{-1}$) similar to other studies (e.g. KLÁTYIK *et al.* 2024b). This is a well-known biological phenomenon – called hormesis –, when a harmful parameter shows a stimulating effect at its low concentrations (MATTSON 2008). The observed hormesis phenomenon is quite reliable, since in Lake Balaton and its catchment the glyphosate concentration is under $1 \mu\text{g L}^{-1}$, but sometimes it could peak around $2 \mu\text{g L}^{-1}$ (TÓTH *et al.* 2022). Hormesis is not yet fully understood, but compensation can be a possible explanation; resources can be mobilised in order to achieve a favourable state (CALABRESE *et al.* 2007). However, even low concentrations caused inhibition in the long term.

Nutrient uptake

Phosphorus and nitrogen are essential macronutrients required by algae (ANDERSEN 2005). In the present study, the negative effects of glyphosate were observed on both nutrient uptakes. Rates of phosphorus and ammonium uptake were inhibited at intermediate concentration, which was in accordance with the decline of photosynthetic activity similarly to other studies (e.g. BROWN and LEAN 1995, KRIEGER *et al.* 1998). The reason for this linkage can be that the phosphorus and nitrogen uptake are energetically expensive which is supported by photosynthesis as an energy-producing process (BROWN and LEAN 1995). In the research of FENG *et al.* (2019) after glyphosate exposure oxidative stress in *Scenedesmus vacuolatus* (Shihira et Krauss) E. Kessler, M. Schäfer, C. Hümmer, A. Kloboucek et V. Huss (current accepted name: *Coelastrella vacuolata* (I. Shihira et R. W. Krauss) Hegewald et N. Hanagata) was demonstrated, which can disrupt cellular metabolism and potentially impair nutrient absorption. Similar effects of low glyphosate concentrations were presented in *Chlorella vulgaris* Beijerinck (WANG *et al.* 2022).

Peroxidase activity, protein

Elevated ROS levels activate various antioxidant defences that help protect all molecules sensitive to oxidative radicals especially the ones with unsaturated bonds, e.g. proteins and lipids, from damage. Among these defences are increased activities of key antioxidant enzymes, including superoxide dismutase, catalase, and peroxidase (CHAUFAN *et al.* 2006). POD is the second defence against ROS because it can eliminate the superoxide radicals which were previously converted to hydrogen peroxide (CHO and SEO 2005).

In our study at low glyphosate concentrations ($<1.8 \mu\text{g L}^{-1}$) POD activity of *Raphidocelis subcapitata* increased until the 7th day, but at higher concentrations ($3.6 \mu\text{g L}^{-1}$) lower activity was detected after 72 h. In the study of TRIPATHI *et al.* (2006) under exposure to low concentrations of toxicants (e.g. heavy metals) led to elevated *Scenedesmus* sp. ascorbate peroxidase (APX) activity after 7 days. Similarly, *Acutodesmus obliquus* (Turpin) Hegewald et Hanagata (current accepted name: *Tetradesmus obliquus* (Turpin) M. J. Wynne) exposed to $0.01\text{--}0.1 \mu\text{M Pb(NO}_3)_2$ exhibited a progressive increase in POD activity over a 7-day period, with peak enzyme levels observed around day 5 and remaining elevated through day 7 (PIOTROWSKA-NICZYPORUK *et al.* 2015). Our findings are similar to ROMERO *et al.* (2011)'s results, such as in the case of *Chlorella kessleri* Fott et Nováková (current accepted name: *Parachlorella kessleri* (Fott et Nováková) Krienitz, E. H. Hegewald, Hepperle, V. Huss, T. Rohr et M. Wolf) also increased enzyme activities were determined over a 96 h exposure window. In the study of WU *et al.* (2016) during the exposure of higher glyphosate concentrations (1, 2, 5, and 10 mg L^{-1}) the peroxidase (POD) activity of *M. aeruginosa* significantly increased for 48 hours. In an experiment with *Navicula* sp. significantly increased POD activity was measured during the initial phase of exposure, potentially due to the presence of oxidative stress induced by exposure to herbicides (ZHENG *et al.* 2024). Overall, these findings suggest that at sublethal concentrations, algae activate and maintain their antioxidant defence systems – particularly peroxidase-related enzymes – throughout the first week of exposure. This enzymatic upregulation appears to be a key adaptive mechanism that allows microalgae to mitigate oxidative damage induced by prolonged but mild toxicant stress. ZHENG *et al.* (2022) showed stress-induced upregulation of antioxidant systems (a selenoprotein, HpMsrA) that improved tolerance to glyphosate. That supports the interpretation that upregulated antioxidant enzymes (including POD) can be protective – but protection has limits: beyond a threshold, damage accumulates.

CONCLUSIONS

Glyphosate has undoubtedly played a key role in the development of modern agriculture, particularly in increasing the efficiency of weed control and promoting soil-conserving farming practices. At the same time, the health and environmental issues associated with it require serious attention. Regarding the aquatic ecosystems, by entering surface water, glyphosate may raise concerns about even drinking water quality. As regards the ecological significance of the observed hormesis, it could improve survival and fitness of populations and help maintain biodiversity in moderately impacted ecosystems. Furthermore, it could affect ecosystem functions and promote functional redundancy, where multiple species can fulfil the same role, enhancing ecosystem stability. Nevertheless, glyphosate could have a negative effect even at very low concentrations, its repeated exposure and chronic presence may result in cumulative ecotoxicological effects. Therefore, it is the responsibility of the scientific community to clarify the real risks of glyphosate through further independent research focusing on long-term effects, different species and regularly monitor the surface waters. Furthermore, developing more sustainable alternatives for agriculture usage and more suitable management plans are highly recommended.

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Összefoglaló: A glifozát a világon a legszélesebb körben használt gyomirtó szer, azonban hatása a vízi ökoszisztémákra – különösen a mikroalgákra – még mindig kevésbé ismert. Jelen tanulmányban a *Raphidocelis subcapitata*, egy standard OECD teszt faj fiziológiai reakcióit (fotoszintézis, klorofill-a és feofitin tartalom, nitrogén- és foszforfelvétel, optikai denzitás, fehérjetartalom, stresszenzimaktivitás) vizsgáltuk különböző glifozát koncentrációk ($0,45\text{--}7,2\ \mu\text{g L}^{-1}$) esetén 14 napon keresztül. Az eredmények azt mutatták, hogy a glifozát jelentősen befolyásolt több paramétert is; többek között csökkentette a fotoszintetikus aktivitást és a tápanyagfelvételt, valamint magasabb koncentrációk esetén fokozta az oxidatív stresszreakciókat. Alacsony koncentrációk esetén ($\leq 0,9\ \mu\text{g L}^{-1}$) a hormézis jelei voltak megfigyelhetők a fotoszintézis és a klorofill-a-tartalom tekin-

tetében. A statisztikai elemzések egyértelműen kimutatták a glifozát koncentrációjának és az expozíciós idő hatásait is. Az eredmények alátámasztották a glifozát fajspecifikus és dóziszfüggő hatását az édesvízi algákra vonatkozóan, valamint hangsúlyozták a sekély víztestek, például a Balaton ökológiai sebezhetőségét a gyomirtó szerekkel való szennyeződés tekintetében. Az eredmények rávilágítanak egy szigorúbb monitoringrendszer szükségességére, a glifozát hosszú távú hatását célzó vizsgálatok szükségességére, továbbá környezetbarát alternatívák kifejlesztésére. A jelen tanulmányt Padišák Judit professzor inspirálta, aki tudományos pályafutása kezdetétől napjainkig elkötelezett és rendíthetetlen támogatója a Balaton-kutatásoknak.

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