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Detection of glyphosate and its metabolite aminomethylphosphonic acid: Risk assessment for the aquatic organisms

Određivanje glifosata i metabolita aminometilfosfonske kiseline: Procena rizika na akvatične organizme

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ABSTRACT

This paper delves into a thorough risk assessment of glyphosate, a widely used herbicide, and its metabolite, aminomethylphosphonic acid (AMPA), within the aquatic ecosystems of the Danube-Tisa-Danube (DTD) network, which represents large land drained system between Serbia, Hungary, and Romania. The primary objective is to employ a Toxicity and Exposure Ratio (TER) framework, encompassing both acute and chronic considerations, to comprehensively evaluate the potential risks these agrochemicals pose to aquatic organisms in the intricate hydro system of the DTD. The study integrates field data, laboratory experiments, and mathematical modeling to quantify glyphosate and AMPA exposure levels in the DTD channels. Furthermore, it explores the toxicological impacts of these substances on a diverse range of aquatic organisms, such as fish, invertebrates, and amphibians, considering acute effects arising from short-term exposure and chronic effects resulting from prolonged interactions. This research aims to provide a holistic understanding of the risk landscape associated with glyphosate and AMPA in the DTD hydro system by employing the TER approach. The findings contribute valuable insights into the potential ecological implications of herbicide usage in this critical water network, aiding regulatory decision-making and facilitating the development of targeted mitigation strategies to safeguard aquatic biodiversity and ecosystem health.

Keywords: risk assessment, herbicides, water, pesticides, aquatic organisms, ecotoxicology

ABSTRAKT

U okviru rada je urađena procena rizika ostataka glifosata, široko korišćenog herbicida i njegovog metabolita, aminometilfosfonske kiseline (AMPA), na akvatične organizme ekosistema mreže Dunav-Tisa-Dunav (DTD), koja predstavlja veliki hidrološki sistem između Srbije, Mađarske i Rumunije. Primarni cilj je da se odredi odnos toksičnosti i izloženosti (TER), koji obuhvata akutna i hronična razmatranja, kako bi se sveobuhvatno procenili potencijalni rizici koje ove agrohemikalije predstavljaju za vodene organizme u hidrosistemu DTD. Studija integriše podatke dobijene sa terena, laboratorijske eksperimente i matematičko modeliranje za kvantifikaciju nivoa izloženosti glifosatu i AMPA u DTD kanalima. Štaviše, istražuje toksikološke uticaje ovih supstanci na različite vodene organizme, kao što su ribe, beskičmenjaci i vodozemci, uzimajući u obzir akutne efekte koji proističu iz kratkotrajne izloženosti i hronične efekte koji su rezultat produženih interakcija. Ovo istraživanje ima za cilj da pruži holističko razumevanje rizika do kojeg mogu da dovedu prisutni ostaci glifosatom i AMPA u DTD hidrosistemu primenom TER pristupa. Dobijeni rezultati predstavljaju dragocen uvidu u potencijalan ekološki uticaj primene herbicida u hidrološkoj kanalskoj mreži, pomažući u donošenju regulatornih odluka i olakšavajući razvoj ciljanih strategija za ublažavanje uticaja kako bi se zaštitili akvatični biodiverzitet i zdravlje ekosistema.

Ključne reči: procena rizika, pesticidi, voda, akvatični organizmi, ekotoksikologija

INTRODUCTION

In recent decades, the widespread use of herbicides has become integral to modern agricultural practices, aiding in the control of unwanted vegetation and ensuring optimal crop yield (Perotti et al., 2020). Among these herbicides, glyphosate has emerged as one of the most extensively employed and controversial substances globally (Klingelhöfer et al., 2021). As a broad-spectrum herbicide, glyphosate is utilized in various formulations and has proven effective in controlling a broad range of weeds (Agarski et al., 2023; Travlos et al., 2017). However, concerns have been raised regarding its potential impact on non-target organisms, mainly freshwater fish inhabiting aquatic ecosystems affected by agricultural runoff (Lacroix and Kurrasch, 2023).

Glyphosate, a key component in popular herbicides such as Roundup (Nerozzi et al., 2020), is known for its systemic mode of action, inhibiting the activity of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) crucial for the synthesis of aromatic amino acids in plants (Boocock and Coggins, 1983). While its primary target is plant life, glyphosate's widespread use has led to its presence in water bodies through runoff, posing potential risks to aquatic ecosystems (Bursić et al., 2023). Moreover, glyphosate undergoes degradation in the environment, giving rise to a primary metabolite known as aminomethylphosphonic acid (AMPA) (Ojelade et al., 2022). Both glyphosate and AMPA have been detected in surface waters, raising questions about their ecological impact, especially on freshwater fish populations (Singh et al., 2020).

Currently, on the European Union market, 526 active pesticide substances are registered with approved status, including 134 with herbicidal action, among which glyphosate is included. The authorization for the use of glyphosate is constantly renewed (EU Pesticides Database, 2022). Currently in force is EU Regulation 2022/2364, which approves the use of this herbicide until December 15, 2023. Its future fate depends on the decisions of the European Food Safety Authority (EFSA) and the European Chemical Agency (ECHA). The latest information regarding the fate of glyphosate issued on November 16, 2023, indicates that the European Commission has announced it will renew the permit for the use of glyphosate based on safety assessments by EFSA and ECHA, with some changes, i.e., new conditions and restrictions such as maximum application concentration (EU, 2023).

The significance of understanding the potential risks associated with glyphosate and AMPA exposure to freshwater fish lies in the pivotal role these organisms play in maintaining ecological balance (Bai and Ogbourne, 2016). Fish species dwelling in freshwater ecosystems contribute to nutrient cycling, and control of aquatic invertebrate populations, and serve as indicators of environmental health (Chagnon et al., 2015). Therefore, any adverse effects on these organisms have the potential to ripple through the entire ecosystem, impacting biodiversity and ecosystem services.

The basic canal network of the Danube-Tisa-Danube (DTD) hydrosystem consists of 20 canals, with the total length of the primary canal network being 929 km. The organizational structure of the hydrosystem is not included, but its integral part is the Karas River (Milovanov, 1972). The primary canal network of the DTD hydrosystem serves multiple purposes. It enables drainage by accepting excess water from about 760,000 ha and discharging surplus water from the canals into the Danube and Tisa rivers. Irrigation, and water supply for fisheries, industry, and settlements are also purposes of the DTD canals and navigation. Through the DTD hydrosystem, 1,060,000 ha of land is drained. From the Republic of Serbia, 1,060,000 ha of land is drained, from the Hungarian territory 159,000 ha, and from the Romanian territory 285,000 ha of land. The DTD hydrosystem accepts 512 m^3/s of water, and irrigation is possible on 510,000 ha. The length of navigable canals of the DTD hydrosystem is 600.6 km (Horvat et al., 2021).

This article aims to comprehensively explore the current knowledge regarding the ecotoxicological risk assessment and safety evaluation of glyphosate and its metabolite AMPA on freshwater fish. By synthesizing existing research findings, we seek to provide a nuanced understanding of the potential ecotoxicological consequences of glyphosate exposure in aquatic environments. Additionally, we will delve into the methodologies employed in assessing these risks, critically evaluating the strengths and limitations of current research approaches. Ultimately, this exploration is important for informing regulatory decisions, guiding sustainable agricultural practices, and safeguarding the integrity of freshwater ecosystems.

MATERIALS AND METHODS

Sample collection

The Danube-Tisa-Danube (DTD) canal water sampling was conducted from July 2017 to July 2018. Water sampling was conducted following the guidelines for collecting samples from rivers and streams outlined in SRPS EN ISO 5667-6:2017. Samples were collected monthly by the Environmental Protection Agency from a total of eight locations (Sombor, Bač, Bačko Gradište, Doroslovo, Novi Sad 1, Novo Miloševo, Melenci, and Vrbas 2) from the canal network of the DTD (Table 1 and Figure 1). The sampling depth was 50 cm.

All samples were collected using a telescopic sampler and stored in dark plastic bottles of 0.5 L capacity. The samples were immediately transported to the Laboratory for Biological Research and Pesticides at the Faculty of Agriculture, University of Novi Sad, and kept in a freezer until the analysis. The total number of samples was 144. Most sampling locations covered agricultural areas, except for the Novi Sad 1 location in the industrial part of the city and Vrbas 2 in a residential area.

Preparation of basic and working solutions

The 6 mol/L HCl solution was prepared by dissolving 50 mL of concentrated HCl (12 M) in 50 mL of water (in a 1:1 ratio), while the 6 M KOH solution was prepared by dissolving 33.6 g of KOH in 100 mL of water. Borate buffer pH 9 was prepared by dissolving 3.1 g of H_3BO_3, 3.75 g of KCl, and 0.8 g of NaOH in 80 mL of water. The pH of the solution was adjusted to 9 using 1 M NaOH, then transferred to a standard 100 mL flask and topped up with water to the mark. This borate buffer solution remained stable at room temperature for 6 months. The derivatization solution, specifically the 6.5 mM FMOC-Cl, was prepared by dissolving 168 mg of FMOC-Cl in 100 mL of acetonitrile, and the 0.1 M EDTA solution was prepared by dissolving 37.2 g of EDTA in 1000 mL of water.

Sample derivatization and chromatographic method

Derivatization of samples was performed according to the method described by Ibáñez et al. (2006) and Hanke et al. (2008) to determine glyphosate and AMPA in natural waters. Specifically, 100 mL of filtered water sample was acidified to pH 1 with 6 M HCl and immediately transferred to a smaller plastic container with a 250 mL lid. The samples were left to stand for 1 hour. Subsequently, the samples were neutralized with 6 M KOH to pH 6-7, after which 10 mL of borate buffer (pH 9) and 10 mL of

Table 1. Data on sample locations

Station	Name of the water body	Coordiantes (Gauss-Krüger projection)
Sombor	DTD Vrbas-Bezdan canal	5073582/7347246
Bač	DTD Bački Petrovac-Karavukovo canal	5028554/7362001
Bačko Gradište	DTD Bečej-Bogojevo canal	5047950/7424125
Doroslovo	DTD Odžaci-Sombor canal	5052669/7358076
Novi Sad 1	DTD Novi Sad-Savino Selo canal	5016000/7407550
Novo Miloševo	DTD Kikindski canal	5069562/7451150
Melenci	DTD Banatska Palanka-Novi	
Bečej canal	5044463/7448738	
Vrbas 2	DTD Vrbas-Bezdan canal	5048238/7395450

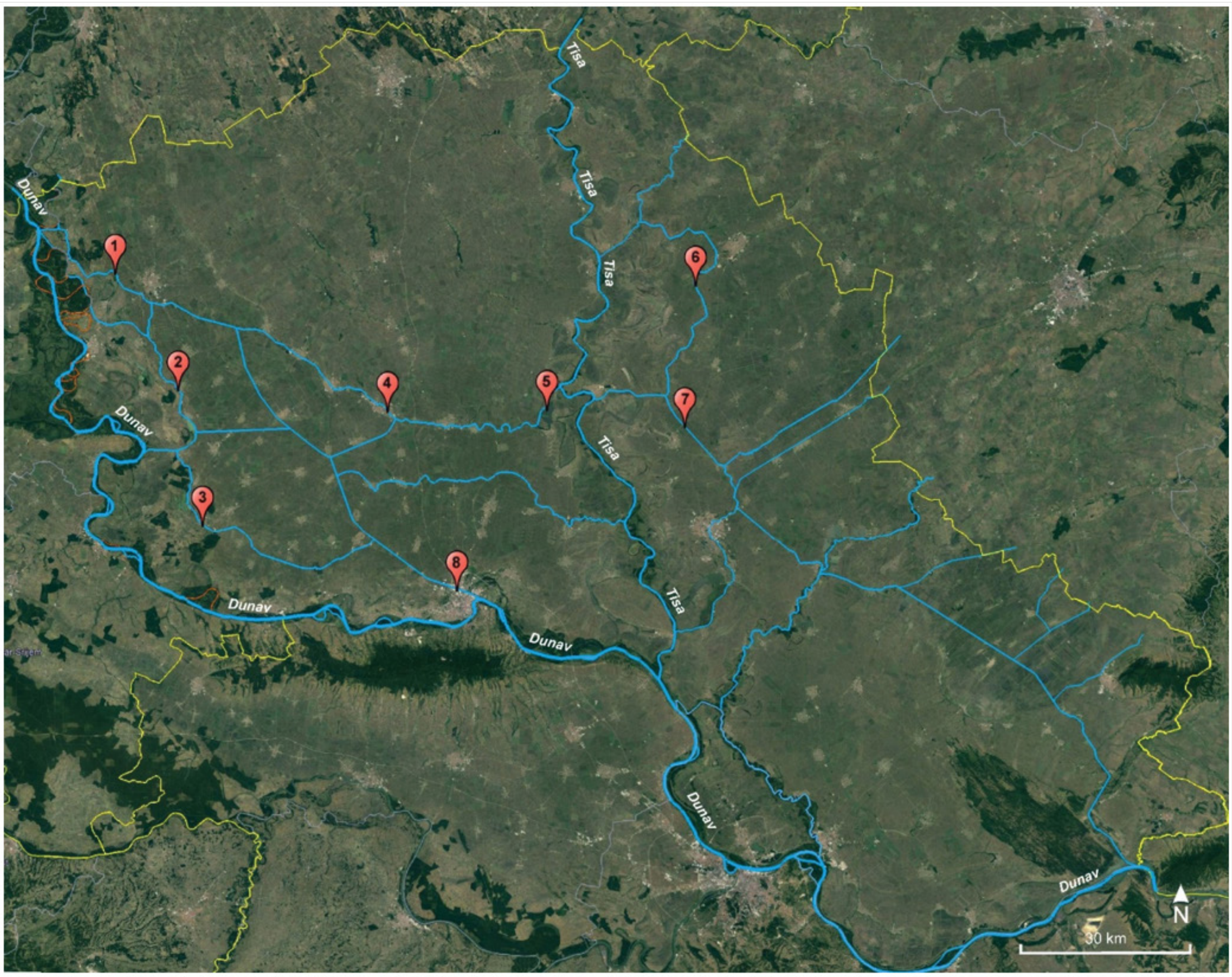

Figure 1. Data on sample locations with Gauss-Krüger projection coordinates: 1. Sombor; 2. Doroslovo; 3. Bač; 4. Vrbas 2; 5. Bačko Gradište; 6. Novo Miloševo; 7. Melenci; 8. Novi Sad 1

6.5 mM FMOC-Cl were added. Ibáñez et al. (Ibáñez et al., 2006) applied this acidification and neutralization step, followed by derivatization with FMOC-Cl, to enhance the extraction yield. Without this step, extraction yields were around 15%. A possible explanation is the slow kinetic interaction between glyphosate and some matrix components, which may act as chelating agents, rendering glyphosate inaccessible for derivatization and analysis.

Plastic containers were then placed in an ultrasonic bath for 10 minutes. The prepared samples were stored in the dark overnight at room temperature. The next day, the samples were acidified with formic acid (pH to 3) to stop derivatization and filtered through filter paper into Erlenmeyer flasks. To the filtered derivatized samples, 100 mL of deionized water and 4 mL of 0.1 M EDTA were added and mixed on a vortex for 1 minute.

SPE columns (Bond Elut PLEXA) were set up on a vacuum manifold (Supelco Manifold) and activated by passing 5 mL of methanol (2×2.5 mL) and then 5 mL of water. After activating the SPE columns, the entire volume of samples was passed through the column using a vacuum of 5 bars. Column rinsing was done by passing 3 mL of dichloromethane twice. Elution of compounds of interest was performed by passing 6 mL of methanol (2×3 mL), with a plastic vial of 15 mL placed under each column. The obtained extract was evaporated under a stream of nitrogen and re-dissolved in the mobile phase. After filtering through a 45 µm filter, the sample was analyzed using LC-MS/MS (Table 2).

Table 2. Conditions for LC-MS/MS analysis

Liquid chromatography	
Instrument	Agilent 1260
Autosampler	1260 ALS, model G1329B
Injected sample volume	$V_{inj,pest}$ = 10 µL
Injection type	With rins
Binary pump	1260 QuatPump, model G1311B
Mobile phase	A: 10mM ammonium formate u MeOH
	B: 10mM ammonium formate in water
Flow rate	0.3 mL/min
Gradient	0 min – 70% B
	5 min – 70% B
	10 min – 10% B
	15 min – 5% B
	18 min – 5% B
	20 min – 70% B
Analysis duration	20 min
Return to initial conditions time	5 min
Column thermostat	1260 TCC, model G1316A
Column temperature	35 °C
Mass spectrometer	
Instrument	Agilent 6410B Triple Quad LC/MS
Ion source	Agilent ESI
Ionization type	(-) ESI
Drying gas flow	10 L/min
Gas temperature	350 °C
Nebulizer pressure	40 psi
Mass measurement range	m/z 15 – 1650
Capillary voltage	Negative 4000 V
Fragmentation voltage	100 V

Validation parameters, limit of detection, and limit of quantification

The validation parameters - linearity, extraction yield, repeatability, limit of detection (LOD), and limit of quantification (LOQ) - were determined following the guidelines outlined in the SANTE/11312/2021 documents. Deionized water (blank samples) was used to establish the validation parameters of the method. Blanc water samples were spiked with specific volumes of working solutions standard of glyphosate and AMPA to achieve the desired concentrations for method validation.

Linearity, for which glyphosate and AMPA were determined using FMOC (derivatization), was tested at four calibration levels in the 0.01 to 0.1 µg/L range. While derivatization can indeed be a time-consuming procedure, the benefits it offers in terms of improved sensitivity, selectivity, and accuracy often outweigh the additional time and effort required. Moreover, advancements in derivatization techniques and automation have helped to streamline the process, making it more efficient and practical for routine glyphosate and AMPA analysis.

The LOQ was experimentally determined by enriching blank water samples to achieve a final concentration of 0.01 µg/L for glyphosate and AMPA. The LOD was determined using MassHunter software based on a signal-to-noise ratio of 5. The LOD was calculated based on the ratio of peak area to the standard deviation of noise in the chromatogram for the lowest concentration of the spiked sample.

The LOD represents the minimum concentration that can be detected by the given method but cannot be quantified with satisfactory reliability. The LOQ represents the minimum concentration that can be determined by the given method with acceptable accuracy and precision.

The extraction yield was determined by spiking blank samples in five replicates at two concentration levels (0.01 and 0.1 µg/L). Method repeatability was assessed by preparing one sample in five replicates at the same concentration level. The obtained results were statistically processed using Microsoft Excel 2013, and the %RSD value was compared with the criteria using the Horowitz equation. The obtained values of RSDr, RSDR (%), were compared with the calculated RSD (%), i.e., the theoretical relative standard deviation (AOAC Peer-Verified Methods Program Manual on Policies and Procedures).

Chromatographic separation and MS analysis included the optimization of the mass spectrometer, i.e., adjusting fragmentation and collision energies to obtain ions with the strongest response. Ions providing the strongest signals were selected for quantification (Q), while ions of lower intensity were used for confirmation.

Validated LC-MS/MS method according to the SANTE/11312/2021 document was used for the chromatographic analysis of real water samples. Total ion chromatographic (TIC) and multiple reaction monitoring (MRM) chromatograms of water sample with detected glyphosate and AMPA residues are presented in Figure 2.

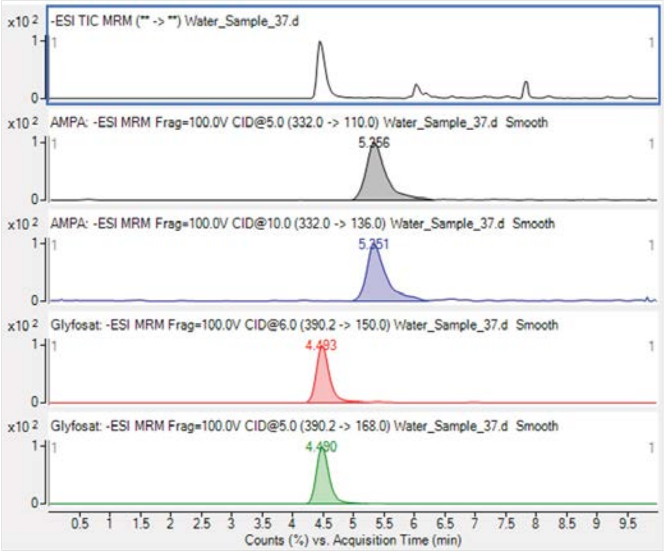

Figure 2. TIC and MRM chromatograms of a sample of glyphosate (0.503 µg/L) and AMPA (0.255 µg/L)

Risk assessment for the aquatic organisms

Within the research related to the risk to aquatic organisms exposed to detected glyphosate concentrations in the DTD hydrosystem, the Toxicity and Exposure Ratio (TER) approach was applied (EFSA Panel on Plant Protection Products and their Residues (PPR), 2013). For acute and chronic toxicity parameters, LC_{50} (EC_{50} or IC_{50}) and NOEC (no observed effect concentration) values from relevant databases and the EFSA systematics for glyphosate were considered. Exposure concentrations of glyphosate measured at monitoring sites in the DTD hydrosystem (Sombor, Vrbas, Bačko Gradište, Novi Sad 1, Bač, Novo Miloševo, Melenci, and Doroslovo) were used. Each TER value lower than the threshold values indicating unacceptable risk was considered in terms of the possibility of scenario modification, especially for fish. For algae and crustaceans, any TER value lower than the threshold is regarded as an unacceptable risk to the organisms in the aquatic environment, i.e., the DTD hydrosystem.

Acute toxicity with a threshold of 100:

$$TER = LC_{50} / PEC$$

Chronic toxicity with a threshold of 10:

$$TER = NOEC / PEC$$

where:

TER – toxicity and exposure ratio;

LC_{50} – 50% lethal concentration, a general indicator of a substance's acute toxicity;

PEC – predicted concentration in the environment;

NOEC – no observed effect concentration.

RESULTS AND DISCUSSION

The use of glyphosate worldwide has led to an increased awareness of its potential harmful effects on the environment and human health (Van Bruggen et al., 2018). Therefore, global contamination has been (and still is) a central issue in the media and on the political stage worldwide regarding the extension (or not) of the approval for glyphosate use. One consequence of the intensive use of glyphosate is environmental contamination, although glyphosate is degraded by soil and aquatic microorganisms. Glyphosate can move through the soil and contaminate surface and groundwater (Carles et al., 2019). According to literature references, the half-life (DT_{50}) of this herbicide in rivers varies from 2.0 to 206.4 days, according to Carles et al. (2019). EFSA (2015) classifies glyphosate as a persistent pesticide due to its half-life in rivers ranging from 13.8 to 301 days. The most commonly detected pesticide compounds in French rivers are glyphosate (in 43% of the analyzed samples) and AMPA (in 63% of the analyzed samples) (Carles et al., 2019). It is also noted that the highest detected concentration of glyphosate was 164 µg/L, and for AMPA, it was 558 µg/L. A characteristic feature of all studies is that the concentrations of the examined substances increased from spring to summer (glyphosate increased first, followed by AMPA), gradually decreasing from late autumn to winter. Besides this phenomenon, it is interesting that the ratio of glyphosate to its metabolite AMPA changed depending on the season but was consistently 2 to 5 times in favor of the metabolite. This ratio is also highlighted in literature data, indicating that the glyphosate to AMPA ratio can vary depending on the season from 2 to 10 times, with more significant differences observed during spring and summer (Agarski et al., 2023; Carles et al., 2019; Desmet et al., 2016).

The results of our study are presented in Table 3.

A scientifically grounded process based on the integration of the toxicity of a substance and the levels of exposure to its action is called toxicological risk assessment. Toxicological risk assessment provides insight into the doses to which a population is exposed to the effects of a toxic substance (Hernández and Tsatsakis, 2017) and whether these concentrations exceed reference levels, allowing the risk of the examined exposure to be characterized as acceptable or unacceptable (Woutersen et al., 2020).

The risk assessment process consists of four phases (Sturla et al., 2014): hazard identification (assessment of whether the substance of interest leads to harmful effects based on *in vivo*, *in vitro*, and *in silico* tests, data from epidemiological studies, or other sources conducted following the principles of good laboratory practice), dose-response assessment (the relationship between doses and harmful effects that occurred, i.e., doses that lead to harmful effects), exposure assessment (assessment of the exposure levels of the population, taking into account population data, exposure routes, sources, and the duration of exposure to the analyzed substances), and risk characterization (risk characterization describing the risk as acceptable or unacceptable) (Hristozov et al., 2016; Seed et al., 2005; Tixier et al., 2002).

Risk assessments for the species of carp (*Cyprinus carpio*) and ide (*Leuciscus idus*), as well as the bluegill (*Lepomis macrochirus*), were conducted using LC_{50} values of 100, 2282, and 30 mg/L, chronic toxicity, i.e. NOEC values (Table 4). The derived Toxicity Exposure Ratios (TER) for all measured concentrations at sampling sites were higher than the threshold value of 10 for acute exposure, indicating an acceptable risk of exposure for carp and ide exposed to measured concentrations of glyphosate.

Table 3. Minimal and maximal values of detected concentrations of glyphosate and AMPA in water samples

Station	Sombor	Vrbas 2	Bačko Gradište	Novi Sad 1	Bač	Novo Miloševo	Melenci	Doroslovo
Glyphosate								
Number of samples (n)	18	18	18	18	18	18	18	18
Positive samples (%)	100	100	100	100	100	100	100	100
Minimal values (µg/L)	0.007	0.009	0.01	0.005	0.008	0.011	0.009	0.007
Maximal values (µg/L)	0.419	0.398	0.407	0.361	0.366	0.503	0.391	0.405
SD	0.13	0.13	0.11	0.11	0.12	0.14	0.13	0.14
AMPA								
Number of samples (n)	18	18	18	18	18	18	18	18
Positive samples (%)	100	100	100	100	100	100	100	100
Minimal values (µg/L)	0.097	0.18	0.079	0.043	0.073	0.048	0.042	0.029
Maximal values (µg/L)	1.605	1.607	1.698	1.388	1.411	2.006	1.669	1.725
SD	0.57	0.51	0.50	0.50	0.55	0.61	0.47	0.48

SD – standard deviation

TER values for chronic exposure for *Cyprinus carpio*, *Leuciscus idus*, and *Lepomis macrochirus* for all measured concentrations were higher than 100, indicating an acceptable risk for the observed fish species exposed to detected glyphosate.

A risk assessment for water fleas (*Daphnia magna*) based on EC_{50} values of 40 mg/L and NOEC values of 56 mg/L showed that the measured concentrations exceeded the threshold of 10 for acute exposure and 100 for chronic exposure. The obtained data indicate an acceptable risk for water fleas exposed to glyphosate (Table 4).

The considerably shorter table assessing the risk of glyphosate metabolite, namely AMPA, on aquatic organisms (Table 5) points to the insufficient exploration of this topic and opens new avenues for future toxicological analyses. Risk assessments for fish, amphibians, and invertebrates were conducted based on EFSA reports and published results from Tresnakova et al. (2021).

Calculated toxicity exposure ratios for fish, based on an LC_{50} value of 100 mg/L, were higher than the threshold value of 10 for acute exposure at all measurement locations, indicating an acceptable risk of exposure to measured concentrations of AMPA.

Risk assessment for water fleas (*Daphnia magna*) based on an EC_{50} value of 100 mg/L showed that, at all measurement locations, the values exceeded the threshold for acute exposure. The data indicate an acceptable risk for water fleas exposed to the AMPA metabolite.

As for amphibians (*Rana* sp.), based on the prescribed EC_{50} value of 50 mg/L (EFSA Panel on Plant Protection Products and their Residues (PPR), 2013) and calculated acute toxicity exposure ratios, it can be concluded that the detected concentrations of AMPA at all measurement locations do not pose a risk to *Rana* sp.

Table 4. Risk assessment of the impact of measured glyphosate concentrations on harmful effects to aquatic organisms

Station	Sombor	Vrbas 2	Bačko Gradište	Novi Sad 1	Bač	Novo Miloševo	Melenci	Doroslovo
Cyprinus carpio	LC_{50} > 100 mg/L; NOEC > 100 mg/L							
Number of samples (n)	0.24	0.25	0.25	0.28	0.27	0.20	0.26	0.25
Positive samples (%)	0.24	0.25	0.25	0.28	0.27	0.20	0.26	0.25
Leuciscus idus	LC_{50} > 2282 mg/L; NOEC > 2282 mg/L							
Acute TER	5.45	5.73	5.61	6.32	6.23	4.54	5.84	5.63
Chronic TER	5.45	5.73	5.61	6.32	6.23	4.54	5.84	5.63
Lepomis macrochirus	LC_{50} > 30 mg/L; NOEC > 30 mg/L							
Acute TER	0.07	0.08	0.07	0.08	0.08	0.06	0.08	0.07
Chronic TER	0.07	0.08	0.07	0.08	0.08	0.06	0.08	0.07
Daphnia magna	EC_{50} > 40 mg/L; NOEC > 56 mg/L							
Acute TER	0.10	0.10	0.10	0.11	0.11	0.08	0.10	0.10
Chronic TER	0.13	0.14	0.14	0.16	0.15	0.11	0.14	0.14
Rana sp.	LC_{50} > 17.9 mg/L							
Acute TER	0.04	0.04	0.04	0.05	0.05	0.04	0.05	0.04

Table 5. Risk assessment of the impact of measured concentrations of AMPA on harmful effects on aquatic organisms

Station	Sombor	Vrbas 2	Bačko Gradište	Novi Sad 1	Bač	Novo Miloševo	Melenci	Doroslovo
Cyprinus carpio	LC_{50} > 100 mg/L							
Acute TER	0.06	0.06	0.06	0.07	0.07	0.05	0.06	0.06
Daphnia magna	EC_{50} >100 mg/L							
Acute TER	0.06	0.06	0.06	0.07	0.07	0.05	0.06	0.06
Rana sp.	EC_{50} > 50 mg/L							
Acute TER	0.03	0.03	0.03	0.04	0.04	0.02	0.03	0.03

Herbicides are chemicals designed to control or eliminate unwanted plants but can also affect non-target organisms, including aquatic life. Assessing the risks helps to understand the potential ecological impact of these substances on aquatic ecosystems (Cedergreen and Streibig, 2005).

Aquatic ecosystems are often habitats for a diverse range of species (Geist and Hawkins, 2016). Herbicide residues can have varying effects on different organisms, and a thorough risk assessment allows us to identify potential threats to biodiversity, as well as antibiotics and sulfonamides in water and sediment (Pelić et al., 2023; Puvača et al., 2023). This information is vital for the conservation of aquatic ecosystems and the species within them.

Aquatic organisms are interconnected through food chains. Herbicide residues may accumulate in the tissues of organisms, affecting not only the directly exposed species but also those higher up in the food chain (Ali et al., 2021; Lushchak et al., 2018; Vapa Tankosić et al., 2022). This can have cascading effects on the entire ecosystem. A risk assessment helps in understanding and mitigating these effects.

Some aquatic organisms may be part of human food. If herbicide residues enter or accumulate in these organisms, there is a potential for human exposure by consuming contaminated fish or other aquatic products (Tongo et al., 2022). Risk characterization is the final step to evaluate these risks and take further actions to implement measures to protect human and environmental health (Williams and Paustenbach, 2002).

Many countries have regulations and guidelines regarding the use of herbicides and their impact on the environment (Kristoffersen et al., 2008). Conducting risk assessments is often a legal requirement to ensure that herbicide use complies with environmental protection laws.

Understanding the long-term effects of herbicide residues on aquatic organisms is highly important for sustainable environmental management. By identifying potential hazards, exposure, and risks, regulators and stakeholders can make informed decisions about using herbicides to minimize environmental harm (Jepson et al., 2020).

Aquatic ecosystems contribute significantly to various economic activities such as fisheries and tourism (Lynch et al., 2016). Herbicide-related damage to these ecosystems can have economic repercussions. Assessing the risks allows for the development of strategies to protect these economic interests. Conducting risk assessments contributes to our scientific understanding of the adverse effects of herbicides on aquatic organisms (Beyer et al., 2014). This knowledge can inform future research and the development of more environmentally friendly herbicides.

Glyphosate is a widely used herbicide, and when it enters aquatic environments, it can undergo various transformations, leading to the formation of degradation products, including AMPA (Singh, Kumar, Datta, et al., 2020). Understanding the potential mechanisms of action of glyphosate and AMPA residues on freshwater fishes, such as common carp, involves considering both direct and indirect effects (Gandhi et al., 2021).

Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme in the shikimate pathway, essential for synthesising aromatic amino acids in plants (Boocock and Coggins, 1983). While mammals, including fish, do not have the shikimate pathway, the gut microbiota of fish can be affected (Portune et al., 2016). Disruption of the gut microbiota may indirectly impact the fish by altering nutrient availability and immune function (Xiong et al., 2019).

Glyphosate and its degradation product AMPA have been associated with generating reactive oxygen species (ROS) in various organisms (Gomes et al., 2016). Elevated ROS levels can lead to oxidative stress in fish, causing damage to cellular structures, including proteins, lipids, and DNA (Birnie-Gauvin et al., 2017). This oxidative stress can affect the overall health and survival of the fish (Lushchak, 2016).

Some evidence suggests that glyphosate and its residues may have endocrine-disrupting effects. Endocrine disruption can also interfere with the normal functioning of the endocrine system (Kalofiri et al., 2021). Changes in hormone levels or receptor interactions may lead to reproductive abnormalities. Glyphosate and AMPA may interfere with the activity of certain enzymes in fish. For example, they could affect acetylcholinesterase, an enzyme involved in neurotransmission (Tresnakova et al., 2021). Disruption of enzyme activity can have cascading effects on various physiological processes, including nervous system function (Guilherme et al., 2014).

Exposure to glyphosate and its residues may influence the immune system of fish. Immunotoxic effects could decrease resistance to pathogens, making fish more susceptible to diseases. Impaired immune function can have population-level consequences regarding fish health and survival (Peillex and Pelletier, 2020). It's important to note that the specific effects of glyphosate and AMPA on freshwater fishes can vary depending on factors such as concentration, duration of exposure, and the life stage of the fish. Additionally, the interactions between glyphosate and other environmental stressors can further complicate the assessment of their impact on aquatic organisms (Wall, 2007). Ongoing research is essential to deepen our understanding of these mechanisms and their ecological implications.

CONCLUSIONS

In conclusion, the results shed light on the potential environmental impact of glyphosate and its metabolite AMPA within the intricate waterways of the Danube-Tisa-Danube network. The comprehensive analysis underscores the importance of assessing the risks posed by herbicides in aquatic ecosystems. The findings emphasize the need for vigilant monitoring and mitigation strategies to safeguard the diverse aquatic organisms inhabiting these channels. As the regulatory landscape evolves, particularly in light of the European Union's ongoing evaluations and decisions, continued research and adaptive management practices will be crucial for sustaining the ecological balance of this vital water network.

Finally, this research underscores the interdisciplinary approach required to address the complex challenges associated with sustainable pesticide use.

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