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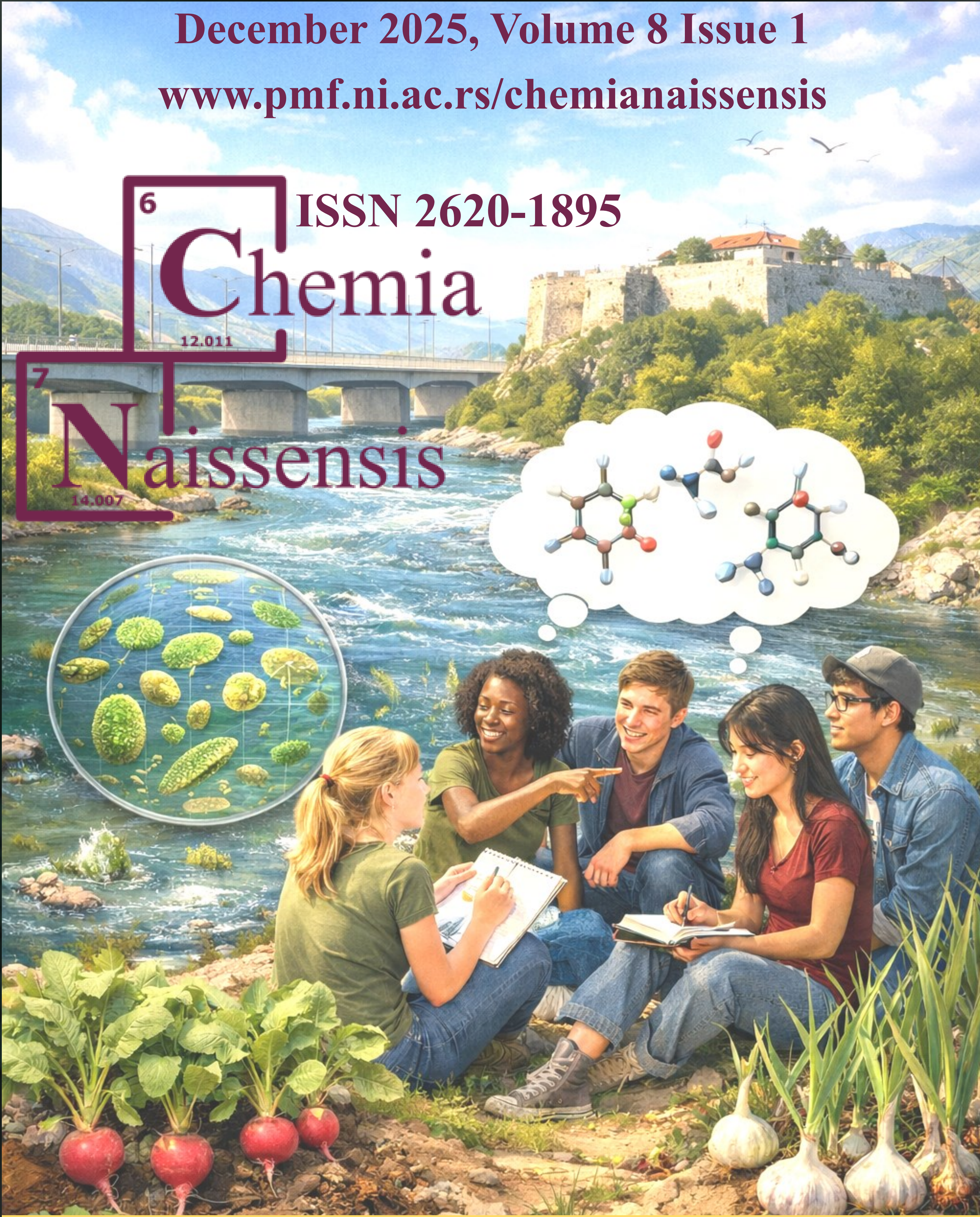

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CONTENT

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Headspace profile of fresh garlic bulbs, stems, leaves and commercial garlic granules

Running title: Headspace Volatile Profile of Garlic Tissues and Granules

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ABSTRACT

The composition of headspace volatile (HSV) components was determined by direct HS-GC/MS analysis of fresh garlic leaves, stems, and bulbs, as well as granules obtained by extraction of dried bulbs. In all tested samples of fresh garlic parts, the most abundant HSV constituent was diallyl disulfide (65.6 – 46 %), while in granules, diallyl disulfide (27.1 %) and diallyl trisulfide (29.5 %) were the most abundant in approximately equal amounts. It has been shown that automated direct HS-GC/MS analysis can be used to determine volatile constituents of fresh garlic parts and commercial garlic granules.

Keywords: garlic, headspace, GC-MS, sulfides, disulfides, trisulfides

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Introduction

Garlic (*Allium sativum* L.) is used all over the world not only as food and spice but also for the prevention and treatment of numerous disorders of the human body. El-Saber Batiha et al. (2020) have very comprehensively analyzed published research on the composition and activity of garlic bulbs' components and extracts. Research has shown that the most important chemical constituents are: sulfur-containing compounds such as alliin, allicin, ajoenes, vinyl dithiins, and sulfides. Extracts and individual components reported to possess immunomodulatory, anti-inflammatory, anticarcinogenic, antioxidant, antidiabetic, renoprotective, anti-atherosclerotic, antibacterial, antifungal, antiprotozoal, and antihypertensive activities. The question arises: can anything new be discovered about the chemical composition of garlic? Several papers have been published describing different headspace methods for the analysis of volatile components of garlic bulbs÷ derivatization (Warren et al., 2013), solid phase extraction (Najman et al., 2022; Keleş et al. 2014; Clemente et al., 2011) and loop system method (Molina-Calle et al., 2016). ResearchGate and ScienceDirect index databases listed only one paper related to the analysis of garlic granules by direct HS analysis (Jovanović et al., 2020). With the aim of supplementing the data on the composition of volatile constituents of garlic of individual garlic organs and the possibility of using the direct HS method for their analysis, this paper presents the results of direct HS analysis of volatile components of fresh garlic leaves, stems, bulbs and granules in an automated process without any prior treatment such as derivatization, solid-phase adsorption, or sampling HS components via a so-called loop.

Experimental

Five whole fresh garlic plants and a garlic granule were purchased at a local market. Fresh garlic is divided into bulbs, stems, and leaves, then chopped into similar-sized pieces with a knife. Five hundred mg of each plant part and granules were weighed, and each one was separately placed in a 20 mL HS vial. Only one batch was measured per sample, and each batch was injected only once. The samples were immediately subjected to the following automated HS-GC-MS program:

In the thermostat of the instrument Agilent 7890 gas chromatograph with 7000B GC-MS-MS triple quadrupole system, operating in MS1 scan mode, and equipped with a fused-silica capillary column Agilent HP-5 MS (30 m × 0.25 mm i.d. × 0.25 μm film thickness), they were heated at 70 °C for 20 minutes with shaking for 5 seconds and pausing for 2 seconds. After that, the 500 μL of volatiles with a split ratio 2:1, was injected into the GC. The GC was operated under the following conditions: injector temperature 250 °C; GC-MS interface temperature 300 °C; oven temperature programmed 50 °C for 2 minutes, then to 150°C at 5 °C/min, to 200 °C at 10°C/min (carrier gas He, 1.0 mL/min, constant flow mode). MS conditions were as follows: ionization voltage of 70 eV; acquisition mass range 40-440; scan time 0.32 seconds. HSV were identified from TIC by comparison of their linear retention indices relative to C_8-C_{40} n-alkanes recorded on the same column/temperature program with literature values - NIST Chemistry WebBook and Adams 2007, and their mass spectra with those of standards from Wiley 6, NIST02, Adams by the application of the AMDIS software (the Automated Mass Spectral Deconvolution and Identification System, Ver. 2.7, distributed within software package for 7890-7000 BGC-MS/MS triple quadrupole system). The percentage composition was computed from the TIC peak areas.

Results and Discussion

Fourteen organosulfur compounds were identified using the direct HS-GC/MS method (Table 1). The identified compounds are formed through a series of reactions from S-alk(en)yl-L-cysteine via S-alk(en)yl-L-cysteine-S-oxides, among which alliin is the most abundant. The allinase enzyme is activated during chopping and transforms allin into intermediates that condense in the intracellular aqueous environment into thiosulfinates, among which allicin predominates. These unstable compounds produce a large number of volatile sulfur compounds, among which the most common are diallyl mono-, di- and trisulfides (Figure 1).

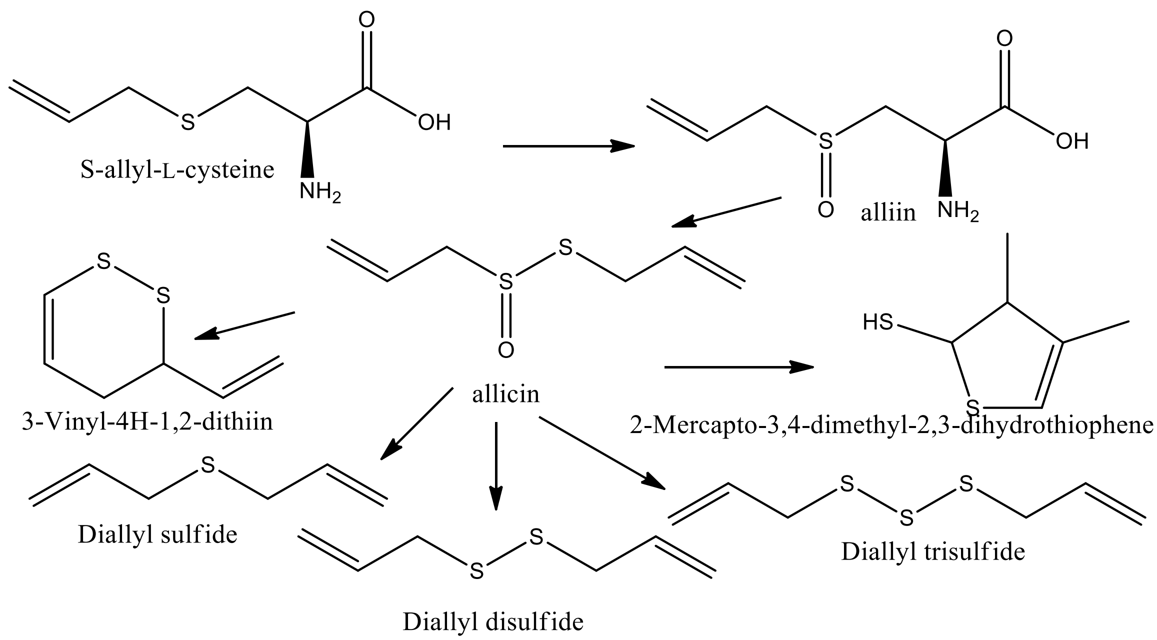

Figure 1. The reaction sequence of the formation of garlic volatile organic compounds

The distribution of organosulfur compounds in leaves differs from their content in the stem and bulbs, which are similar to each other (Table 1). Namely, sulfides and disulfides are more abundant in leaves, while trisulfides are found only in traces. Diallyl disulfide is the most abundant constituent in all three organs examined. Its content accounts for about half of the total content in the stem and bulb and about two-thirds of the content of HSV (headspace volatiles) in the leaves. The abundance of constituents in garlic granules differs significantly from the abundance of the same components in the bulb. The comparison was made only with garlic bulbs, given that the manufacturer's declaration states that the granules were obtained by grinding dried garlic bulbs. The sulfide content is about 8 times higher, the trisulfide content is about 5 times higher, while the disulfide content is about 2.5 times lower. The results obtained are in agreement with those from direct HS analysis on the same apparatus, although the experiment differs in that the previous analysis was performed by adding 1 mL of water to a vial containing 0.2 g of granules (Jovanović et al., 2020). Observed differences in abundance between fresh and dried bulbs indicate that at oven-drying temperatures, thiosulfinates are transformed more rapidly into stable sulfides and trisulfides than into disulfides. The different compositions of fresh and dried bulbs also determine different tastes. Namely, fresh bulbs have a pungent, spicy, and earthy flavor, while granulated garlic is milder and more balanced, with a savory and aromatic taste.

Jimenez-Amezcua et al. (2025) found that the garlic diallyl sulfide content decreases with aging time under controlled temperature (<65 °C) and humidity (60–90 %) conditions from 5.5 $\mu g\ g^{-1}$ to 1 $\mu g\ g^{-1}$ for 40 days, which is not in accordance with our results (2.2 % fresh bulbs, 7.9 % granules). The diallyl disulfide content also decreases from 38 $\mu g\ g^{-1}$ to 10 $\mu g\ g^{-1}$, which is in agreement with the results of this work (46 % fresh bulbs, 27.1 % granules). For diallyl trisulfide an increase from 23 $\mu g\ g^{-1}$ to 30 $\mu g\ g^{-1}$was observed after 14 days of aging, and then its content dropped to 7.2 $\mu g\ g^{-1}$after 40 days of aging. The results of our samples show an increase from 5.3 % for fresh bulbs to 29.5 % for granules. Radulovic et al. (2015) examined *Allium ursinum* L. essential oils of fresh, air-dried, and oven-dried aerial parts. In this work, it was published that fresh aerial part oil contains 0.6 % sulfides, 1 % disulfides and 0.7 % trisulfides. The sulfides, disulfides, and trisulfides contents of air-dried samples were 60.3 %, 21.0 %, and 27.1 %, respectively. The oven-dried samples contained 28.2% sulfides, 34.6 % disulfides and 57 % trisulfides.

Table 1. The chemical composition (%) of the garlic HSV

RI	RN	Compound	Leaves	Steam	Bulbs	Bulbs*	Granules	Granules**
864.6	859	Diallyl sulfide	5.8	2.5	2.2	6.7	17.9	2.3-26.6
915.3	919	Allyl methyl disulfide	13	12.3	16.4	0.04	6.8	3.8-9.0
929.4	932	Methyl propyl disulfide	t	t	0.5	-	t	-
937.5	940	Methyl (*E*)-1-propenyl disulfide	0.9	1.3	1.8	0.33[b]	t	0.0-0.2
967.6	972	Dimethyl trisulfide	t	t	t	0.18	0.9	0.6-6.5
1079.5	1077	Diallyl disulfide	65.6	48.7	46	24.8	27.1	10.8-28.6
1095.2	1097	Allyl propyl disulfide	5.4	4.9	4.7	-	1.0	-
1105.1	-	Allyl (*E*)-1-propenyl disulfide	9.1	19.1	19.5	-	-	-

1116.0	1110.3	(*Z*)-1-propenyl propyl disulfide	t	0.5	t	-	-	-
1127.2	1117.6	(*E*)-1-propenyl propyl disulfide	t	0.7	0.6	-	-	-
1138.6	1144	Allyl methyl trisulfide	t	1.3	1.8	4.6	7.9	11.3-27
1174.4	-	2-Mercapto-3,4-dimethyl-2,3-dihydrothiophene	t	0.5	0.5	-	-	-
1212.1	-	3-Vinyl-4H-1,2-dithiin (Syn 3-Vinyl-1,2-dithiacyclohex-5-ene)	t	T	t	0.55	0.7	0-0.8
1301.8	1304	Diallyl trisulfide	t	7.5	5.3	18.5	29.5	23.2-59.9
		Sulfides	5.8	2.5	2.2	6.7	17.9	10.6±10.0
		Disulfides	94.0	87.5	89.5	26.87	34.9	25.0±6.2
		Cyclic disulfides	t	t	t	0.55	0.7	0.4±0.4
		Trisulfides	t	8.8	7.1	23.8	38.3	61.6±10.9

RI- Experimental linear retention indices relative to C_8–C_{40} alkanes on the HP-5MS
NI-retention indices from NIST Chemistry WebBook relative to HP-5MS
t -trace (≤0.1 %).
[b] -In the cited paper, it is not stated which stereoisomer it is

[*] Molina-Calle, M., Priego-Capote, F., & de Castro, M. D. (2016). HS-GC/MS volatile profile of different varieties of garlic and their behavior under heating. Analytical and bioanalytical chemistry, 408(14), 3843–3852. https://doi.org/10.1007/s00216-016-9477-0
[**] Jovanović, S. Č., Jovanović, O. P., Mitić, Z. S., Petrović, G. M., & Stojanović, G. S. (2020). Chemical composition and distribution of the headspace volatiles in commercial culinary herbs and spices: Chemometric approach. Journal of the Serbian Chemical Society, 85(8), 1001–1010. https://doi.org/10.2298/JSC191121007J

Molina-Çalle et al. (2016) examined the HSV constituents of fresh white, purple, and Chinese garlic varieties at an equilibrium temperature of 103 °C using headspace sampling via a loop. Within the organosulfur compounds, they found that diallyl disulfide and diallyl trisulfide were major garlic volatiles which represented a relative concentration of 26.4 % and 25.3 %, respectively, for Chinese garlic, 24.8 % and 18.5 %, respectively, for White garlic, and 26.4 % and 25.3 %, respectively, for Purple garlic. It can be assumed that the different experimental conditions are the cause of the not-so-good agreement of diallyl disulfide and diallyl trisulfide contents in their results and the results presented in this paper (diallyl disulfide 46 % and diallyl trisulfide 5.3 %).

Conclusion

By direct automated HS-GC/MS analysis, without pre-treatment, except for the chopping of individual organs of garlic, 14 organosulfur compounds were identified. It has been shown that their distribution in leaves is different from that in the stem and bulb. Furthermore, the content of trisulfides and sulfides is much higher in garlic granules compared to their content in the bulb from which the granules are obtained. It has also been shown that the composition of HSV constituents depends on the sampling method used, i.e. whether HSV constituents are injected directly from a thermostated vial or via a loop.

Acknowledgement

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Conflict-of-Interest Statement

The author declare no conflicts of interest.

References

Clemente, J. G., Williams, J. D., Cross, M., & Chambers, C. C. (2011). Analysis of garlic cultivars using head space solid phase microextraction/gas chromatography/mass spectroscopy. The Open Food Science Journal, 6, 1-4. https://doi.org/10.2174/1874256401206010001

El-Saber Batiha, G., Magdy Beshbishy, A., Wasef, L., Elewa, Y. H. A., A Al-Sagan, A., Abd El-Hack, M. E., Taha, A. E., Abd-Elhakim, Y., & Prasad Devkota, H. (2020). Chemical Constituents and Pharmacological Activities of Garlic (*Allium sativum* L.): A Review. Nutrients, 12(3), 872-893. https://doi.org/10.3390/nu12030872).

Jiménez-Amezcua, I., Luz Sanz, M., Díez-Municio, M., Parker, J. K., Oruna-Concha, M. J., & Soria, A. C. (2025). Changes in the bioactive content and aroma profile of aged garlic with processing conditions. LWT - Food Science and Technology, 227,118003. https://doi.org/10.1016/j.lwt.2025.118003.

Jovanović, S. Č., Jovanović, O. P., Mitić, Z. S., Petrović, G. M., & Stojanović, G. S. (2020). Chemical composition and distribution of the headspace volatiles in commercial culinary herbs and spices: Chemometric approach. Journal of the Serbian Chemical Society, 85(8), 1001–1010. https://doi.org/10.2298/JSC191121007J

Keleş, D., Taşkin, H., Baktemur, G., Kafkas, E., & Büyükalaca, S. (2014). Comparitive study on volatile aroma compounds of two different garlic types (Kastamonu and Chinese) using gas chromatography mass spectrometry (HS-GC/MS) technique. African journal of traditional, complementary, and alternative medicines: AJTCAM, 11(3), 217–220. https://doi.org/10.4314/ajtcam.v11i3.30

Molina-Calle, M., Priego-Capote, F., & de Castro, M. D. (2016). HS-GC/MS volatile profile of different varieties of garlic and their behavior under heating. Analytical and Bioanalytical Chemistry, 408(14), 3843–3852. https://doi.org/10.1007/s00216-016-9477-0

Najman, K., Król, K., & Sadowska, A. (2022). The Physicochemical Properties, Volatile Compounds and Taste Profile of Black Garlic (*Allium sativum* L.) Cloves, Paste and Powder. Applied Sciences, 12(9), 4215-4234. https://doi.org/10.3390/app12094215

Radulović, N., Miltojević, A., Stojković, M., & Blagojević, P. (2015). New volatile sulfur-containing compounds from wild garlic (*Allium ursinum* L., Liliaceae). Food Research International, 78, 1-10. https://doi.org/10.1016/j.foodres.2015.11.019.

Warren, J. M., Parkinson, D. R., & Pawliszyn, J. (2013). Assessment of thiol compounds from garlic by automated headspace derivatized in-needle-NTD-GC-MS and derivatized in-fiber-SPME-GC-MS. Journal of Agricultural and Food Chemistry, 61(3), 492–500. https://doi.org/10.1021/jf303508m)

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Hedspejs profil svežih lukovica belog luka, stabljika, listova i komercijalnih granula belog luka

Tekući naslov: Profil isparljivih jedinjenja u tkivima i granuloma belog luka

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SAŽETAK

Sastav isparljivih komponenti u hedspejsu (HSV) određen je direktnom HS-GC/MS analizom svežih listova, stabljika i lukovica belog luka, kao i granula dobijenih ekstrakcijom osušenih lukovica. U svim ispitivanim uzorcima svežih delova belog luka najzastupljenija HSV komponenta bila je dialildisulfid (65,6–46%), dok su u granulama dialildisulfid (27,1%) i dialiltrisulfid (29,5%) bili najzastupljeniji u približno jednakim količinama. Pokazano je da se automatizovana direktna HS-GC/MS analiza može koristiti za određivanje ispaljivih jedinjenja svežih delova belog luka i komercijalnih granula belog luka.

Ključne reči: beli luk, hedspejs, GC-MS, sulfidi, disulfidi, trisulfidi

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Responses of radish (*Raphanus sativus* L. var. Saxa) to salt stress during early vegetative development

Running title: Radish Responses to Salt Stress during Early Growth

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ABSTRACT

Increased soil salinity is a threat to plant growth and development. Plant species, including different genotypes within the same species, vary in their tolerance to stress. By monitoring quantitative morphological growth parameters under stress conditions, insight can be gained into plant stress resistance. In this context, the present study examined the effects of two types of salts, NaCl and K_2SO_4, at concentrations ranging from 0.025 M to 0.25 M, on the germination and early vegetative development of radish (*Raphanus sativus*, variety Saxa). Results showed that the highest biomass yield was recorded in plants grown under non-stress conditions, indicating optimal growth. In contrast, the lowest biomass was observed under severe sodium salt stress (0.2 M NaCl), although the smallest seedlings were found under strong potassium salt stress (0.15 M K_2SO_4). Notably, plant growth appeared more sensitive to salt-induced stress than seed germination. Under the highest stress intensity for both salts, germination was completely inhibited.

Keywords: salt stress, Raphanus sativus, germination, seedling growth

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Introduction

During ontogeny, plants are continuously exposed to a range of environmental factors, which can become stressful when their intensity exceeds species-specific optimal thresholds. The impact of these stressors on plant growth and development depends on their intensity and duration. Plant responses vary by species and can manifest resistance, tolerance, or varying degrees of sensitivity. Sensitivity levels also fluctuate with developmental stage and species-specific traits. When adverse conditions persist, stress factors can inhibit growth, delay development, and ultimately reduce yield (Stikić & Jovanović, 2012).

Soil salinization represents a major environmental challenge, with over 955 million hectares globally affected and approximately 20% of irrigated land classified as saline (Metternicht & Zinck, 2003). Saline soils are present across all climate zones and are characterized by high concentrations of soluble salts such as NaCl, Na_2SO_4, $NaHCO_3$, Na_2CO_3, $MgCl_2$, $MgSO_4$, K_2SO_4, $CaCO_3$ and $CaSO_4$, which exert osmotic and ionic stress on plants. These stresses negatively impact various physiological and biochemical processes, thereby inhibiting plant growth and development (Metternicht & Zinck, 2003; Cuartero et al., 2006). Projections indicate that by 2050, up to 50% of arable land may become saline, posing a substantial threat to global crop production and food security. Plant responses to salinity are highly variable and species-specific, influenced by salt type, concentration, and developmental stage. Tolerance mechanisms vary across species, underscoring the need for targeted studies on the effects of salinity and other abiotic stressors to inform sustainable agricultural practices and enhance crop resilience in agroecosystems. Therefore, the action of each stress factor should be given special attention to each plant species.

Raphanus sativus L. (Brassicaceae) is a root vegetable with a long-standing role in human nutrition, valued for its distinctive pungent flavor and high nutritional content. The edible roots and leaves are commonly consumed fresh or cooked and represent a rich source of calcium, potassium, phosphorus, carbohydrates, and vitamins A, C, and B (Larry, 1977). In addition to its dietary relevance, radish exhibits medicinal potential, further underscoring its agronomic importance (Ghosh et al., 2014). The presence of bioactive compounds—particularly phenolics, flavonoids, and antioxidants—has been associated with various health benefits, including confirmed anticancer properties of certain secondary metabolites (Cartea & Velasco, 2007; Barilliari2008).

This study investigates the effects of salt stress induced by different concentrations of NaCl and K_2SO_4 on seed germination and early seedling growth of Raphanus sativus. The cultivar 'Saxa', characterized by its round red roots and broad adaptability, was selected due to its suitability for cultivation under diverse environmental conditions.

Experimental

Commercial seeds were surface sterilized by immersion in 25% sodium hypochlorite solution containing two drops of liquid detergent for 30 minutes, followed by three rinses with sterile distilled water. Germination tests were conducted using the standard Petri dish method. Seeds were placed on two layers of filter paper, moistened with either distilled water (control) or saline solutions representing salt-stress treatments. Two salt types, NaCl and K_2SO_4, were applied at five concentrations (0.025 M, 0.05 M, 0.1 M, 0.15 M and 0.2 M). Petri dishes were incubated in a growth chamber under controlled conditions at 20 °C, with a photoperiod of 16 hours of light and 8 hours of dark.

Seed germination was monitored over a period of ten days, after which the germination percentage (GP) and sensitivity index (SI) were calculated. The germination percentage was determined using the formula GP = (N/S) × 100, where N is the number of germinated seeds, and S is the total number of seeds sown. The sensitivity index (SI) was calculated as SI = e/d, where e represents the germination percentage in the control, and d the germination percentage under salt treatment. After the tenth day, growth parameters were assessed, including root length (mm), shoot length (mm), and seedling fresh and dry weight (g).

The experiment was conducted in triplicate, and all obtained data were statistically analyzed using analysis of variance (ANOVA), followed by the LSD (Least Significant Difference) test at a significance level of $p < 0.05$. Statistically significant differences between means are indicated by different letters.

Results and Discussion

The germination percentage was evaluated ten days after the experiment was initiated. In the control treatment (distilled water), radish seeds exhibited the highest germination rate (86.6%) (Table 1). Exposure to salt stress resulted in a concentration-dependent decline in germination, with more pronounced effects observed under NaCl treatment. At a concentration of 0.15 M, germination in NaCl solution was approximately 2.5 times lower compared to K_2SO_4. In a 0.2 M NaCl solution, germination remained equally low, whereas in the corresponding K_2SO_4 solution, no germination occurred despite visible seed imbibition. At the highest tested concentration (0.25 M), seed germination was completely inhibited under both salt treatments.

Table 1. Seed germination and sensitivity index under salt stress conditions

Treatment		GP	SI
Control	dH_2O	86.6	
NaCl (M)	0.025	33.3	2.6
	0.05	60	1.4
	0.1	43.3	2
	0.15	6.66	13.03

	0.2	6.66	13.03
	0.25	0	-
K_2SO_4 (M)	0.025	70	1.2
	0.05	80	1.08
	0.1	60	1.44
	0.15	16.66	5.19
	0.2	0	-
	0.25	0	-

The germination percentage (GP) is calculated as GP = (N/S) × 100, where N is the number of germinated seeds and S is the total number of seeds sown. The sensitivity index (SI) is calculated as SI = e/d, where e is the germination percentage in the control treatment and d is the germination percentage under salt treatment.

Different radish varieties such as *R. sativus* L. var. Red Bombay, Tasakisan Mula-1, and Druti also showed that germination was inhibited as the NaCl concentration increased (Ghosh et al. 2014). *R. sativus* L. var. *longipinnatus* Bailey also showed a significantly low percentage of germination at the highest NaCl concentration (Jungklang, 2018).

Comparison of the sensitivity index (SI) for seed germination under salt stress revealed that germination is more adversely affected by NaCl than by K_2SO_4 (Table 1). The lowest SI value was observed under low-intensity potassium salt stress (0.05 M), whereas the highest SI value (13.03) was recorded under high NaCl stress. These results indicate that radish seeds exhibit approximately 60% greater sensitivity to NaCl-induced stress compared to potassium salt stress.

Seedlings exhibited the greatest growth, with the longest root and shoot lengths under control conditions (Table 2). Exposure to salt stress significantly reduced root length, even at the lowest NaCl concentration tested. A pronounced inhibition of root growth was observed under high NaCl stress (0.2 M). Although mild K_2SO_4 stress also negatively affected root elongation, its impact was less severe than that of NaCl stress. However, under higher potassium salt concentrations, seedlings developed the shortest roots, measuring approximately 11 mm.

The greatest shoot length (Table 2) was observed in seedlings exposed to 0.025 M K_2SO_4, slightly exceeding that of the unstressed control. In contrast, NaCl at the same concentration caused a 19.6% reduction in shoot length, indicating that salt tolerance or sensitivity varies with salt type. Under higher potassium salt stress, shoot length was significantly reduced, reaching a minimum of 9.5 mm.

Table 2. Seedling growth under salt stress condition

Treatment		Root length (mm)	Shoot length (mm)	Fresh weight (mg)	Dry weight (mg)
Control	dH_2O	79.67 ± 7.38^{d}	37.54 ± 2.53^{d}	78.0 ± 9.0^{e}	13.3 ± 0.2^{e}
NaCl (M)	0.025	33.37 ± 7.53^{b}	30.17 ± 3.94^{cd}	25.0 ± 3.0^{c}	1.0 ± 0.8^{a}
	0.05	42.05 ± 4.76^{c}	28.44 ± 2.47^{c}	25.0 ± 3.0^{c}	8.7 ± 0.5^{b}
	0.1	$43.50 \pm 3,72^{c}$	32.93 ± 3.65^{d}	40.0 ± 6.0^{d}	12.8 ± 0.8^{d}
	0.15	44.00 ± 4.05^{c}	18.36 ± 2.23^{b}	18.0 ± 2.0^{b}	10.3 ± 0.6^{c}
	0.2	18.50 ± 4.50^{a}	13.50 ± 0.65^{a}	14.0 ± 2.0^{a}	8.5 ± 0.6^{b}

K_2SO_4 (M)	0.025	66.61 ± 7.63^{c}	39.84 ± 2.91^{d}	56.0 ± 8.0^{d}	11.0 ± 0.1^{a}
	0.05	49.17 ± 4.69^{c}	28.88 ± 2.63^{c}	53.0 ± 6.0^{d}	12.0 ± 0.1^{ab}
	0.1	27.33 ± 5.45^{b}	18.30 ± 2.88^{b}	24.0 ± 4.0^{c}	1.3 ± 0.3^{b}
	0.15	11.00 ± 1.00^{a}	9.50 ± 2.50^{a}	17.0 ± 2.0^{b}	1.2 ± 0.1^{ab}
	0.2	-	-	-	-

Values represent means ± SE, n = 3. Means within the same column marked with different letters are significantly different according to Tukey's HSD test at $P \leq 0.05$.

The highest biomass accumulation was recorded in seedlings grown under non-stress conditions, reflecting optimal growth. Although the smallest seedlings were observed under severe potassium salt stress (0.15 M K_2SO_4), the lowest biomass values were found under intense sodium salt stress (0.2 M NaCl). Water uptake by plants occurs when the root system's water potential is lower than that of the surrounding substrate.

Excessive concentrations of dissolved salts in the substrate decrease the water potential at the root-soil interface, thereby restricting water uptake by the roots (Zelm et al., 2020). This salt-induced osmotic stress results in a water deficit within the plant, leading to reduced cell turgor pressure, impaired hydraulic conductivity, and decreased relative water content (RWC). Consequently, water-use efficiency (WUE) is negatively affected (Jia et al., 2001; Huang et al., 2017). Collectively, these physiological disruptions contribute to a significant decline in plant biomass accumulation.

When comparing seedlings subjected separately to potassium and sodium salt stress, the highest biomass yield was observed under low potassium salt stress, whereas under sodium salt stress, the highest biomass was recorded at moderate stress intensity (0.1 M NaCl). Literature reports on Raphanus sativus L. var. longipinnatus Bailey indicate that exposure to 0.1 M NaCl stress also resulted in maximal fresh biomass, aligning with our findings. Jungklang (2018) further documented significant reductions in all measured growth parameters at 0.2 M NaCl, consistent with our results. Despite using different radish varieties, the concordance between these results suggests that, notwithstanding genetic variability, similar physiological and biochemical mechanisms govern the response to salt stress of equal intensity across varieties.

Radish seed germination exhibited greater resilience to salt stress compared to seedling growth. Salt-induced osmotic stress increases the osmotic pressure of the soil solution, leading to water deficit that adversely impacts cellular processes such as cell division and elongation, photosynthesis, transpiration, and dry matter allocation (Lu et al., 2023; Zhao et al., 2021; Arif et al., 2020). Additionally, ion accumulation within cells disrupts nutrient uptake, further inhibiting growth. Collectively, these physiological disturbances under saline conditions negatively affect plant development and ultimately reduce crop yield.

Conclusion

Salt stress negatively influences *R. sativus* seedlings, especially at high concentrations. Measured morphological parameters indicate that plant growth is more severely reduced as salt stress intensity increases. Low doses of salt may be stimulative for seedling development, depending on the type of salt. Seed germination is less susceptible to the presence of salt than the growth of sprouts and roots. The inhibitory effect of salt on early vegetative development affects biomass production, and a decrease in yield would negatively affect agroeconomic profit.

Radish seed germination and seedling growth are adversely affected under saline conditions. Varying concentrations of sodium and potassium salts reduce overall plant growth, with the inhibitory effects most pronounced at higher salt concentrations. While seed germination exhibits a relatively lower sensitivity to salinity compared to seedling growth, it is more susceptible to the effects of sodium salts than to those of potassium salts.

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Conflict-of-Interest Statement

The authors declare no conflict of interest.

References

Arif, Y., Singh, P., Siddiqui, H., Bajguz, A., Hayat, S. (2020). Salinity induced physiological and biochemical changes in plants: An omic approach towards salt stress tolerance. Plant Physiol Biochem. Nov; 156:64-77. doi: 10.1016/j.plaphy.2020.08.042

Barillari, J., Iori, R., Papi, A., Orland, M., Bartolini, G., Gabbanini, S., . . . Valgimigli, L. (2008). Kaiware Daikon (Raphanus sativus L.) extract: a naturally multipotent chemopreventive agent. Journal of Agricultural and Food Chemistry, 56(17), 7823-7830.

Cartea, M. E., & Velasco, P. (2007). Glucosinolates in Brassica foods: Bioavailability in food and significance for human health. Phytochemistry reviews, 7(2), 213-229.

Cuartero, J., Bolarín, M. C., Asíns, M. J., & Moreno, V. (2006). Increasing salt tolerance in the tomato. Journal of Experimental Botany, 57(5), 1045–1058.

Ghosh, P., Dash, P. K., Sarker, R., & Mannan, M. A. (2014). Effect of salinity on germination, growth and yield of radish (*Raphanus sativus* L.) varieties. International Journal of Biosciences, 5(1), 37-48.

Huang, L., He, B., Han, L., & Liu, J. (2017). A global examination of the response of ecosystem water-use efficiency to drought based on MODIS data. Science of the Total Environment, 601, 1097-1107.

Jia, W., Zhang, J., & Liang, J. (2001). Initiation and regulation of water deficit-induced abscisic acid accumulation in maize leaves and roots: cellular volume and water relations. Journal of Experimental Botany, 52(355), 295-300.

Jungklang, J. (2018). Effects of sodium chloride on germination, growth, relative water content, and chlorophyll, proline, malondialdehyde and vitamin C contents in Chinese white radish seedlings (*Raphanus sativus* L. var. *longipinnatus* Bailey). Maejo International Journal of Science & Technology, 12(2), 89-100.

Larry, K. H. (1977). Commercial vegetables crops development of Horticulture. Washington State University, Pullmar, Washington. American Journal of Horticulture, 99(2), 164.

Lu, Y., & Fricke, W. (2023). Salt Stress—Regulation of Root Water Uptake in a Whole-Plant and Diurnal Context. International Journal of Molecular Sciences, 24(9), 8070. https://doi.org/10.3390/ijms24098070

Metternicht, G., & Zinck, J. (2003). Remote sensing of soil salinity: potentials and constraints. Remote Sensing of Environment, 85, 1-20.

Stikić, R., & Jovanović, Z. (2012). Fiziologija stresa biljaka. Beograd: Univerzitet u Beogradu Poljoprivredni fakultet.

Zhao, S., Zhang, Q., Liu M., Zhou, H., Ma, C., Wang, P. (2021). Regulation of Plant Responses to Salt Stress. Int J Mol Sci. 28, 22(9), 4609. doi: 10.3390/ijms22094609.

Zelm, E. v., Zhang, Y., & Testerink, C. (2020). Salt tolerance mechanisms of plants. Annual review of plant biology, 71(1), 403-433.

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Odzivi rotkvice (*Raphanus sativus* L. var. Saxa) na stres izazvan solju tokom ranog vegetativnog razvoj

Tekući naslov: Reakcije rotkvica na stres izazvan solju tokom ranog rasta

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SAŽETAK

Povećan salinitet zemljišta predstavlja ozbiljnu pretnju rastu i razvoju biljaka. Biljne vrste, kao i različiti genotipovi unutar iste vrste, razlikuju se u stepenu tolerancije na stres. Praćenjem kvantitativnih morfoloških parametara rasta u stresnim uslovima može se dobiti uvid u otpornost biljaka na stres. U tom kontekstu, ova studija je ispitivala efekte dva tipa soli, NaCl i K_2SO_4, u koncentracijama od 0,025 M do 0,25 M, na klijanje i rani vegetativni razvoj rotkvice (Raphanus sativus, sorta Saxa). Rezultati su pokazali da je najveći prinos biomase zabeležen kod biljaka gajenih u nestresnim uslovima, što ukazuje na optimalan rast. Suprotno tome, najniža biomasa uočena je pri jakom stresu izazvanom natrijumovom solju (0,2 M NaCl), iako su najmanje biljke zabeležene pod snažnim stresom od kalijumove soli (0,15 M K_2SO_4). Važno je istaći da je rast biljke pokazao veću osetljivost na stres izazvan solima u poređenju sa klijanjem semena. Pri najvišem intenzitetu stresa za obe soli, klijanje je bilo potpuno inhibirano.

Ključne reči: stres izazvan solju, Raphanus sativus, klijanje, rast klijanaca

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Improvement of students' achievements in organic stereochemistry by active learning using information and communication technologies

Running title: Active Learning and ICT in Organic Stereochemistry Education

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ABSTRACT

Stereochemistry remains a challenging topic for undergraduate chemistry students, often leading to misconceptions and learning difficulties. To improve students' understanding, traditional teaching methods should be complemented by innovative strategies such as interactive learning tools, digital resources, and hands-on activities. This study aimed to identify the main difficulties students face when learning stereochemistry and to evaluate the effectiveness of different pedagogical interventions.

A pre-test assessing key stereochemistry concepts was administered to 24 second-year students at the Institute of Chemistry, Faculty of Science and Mathematics in Skopje, Republic of Macedonia. The results revealed significant challenges, particularly in identifying chiral centers, determining *R/S* configurations, recognizing meso compounds, and understanding *E/Z* isomerism. Students also struggled with the spatial representation of molecules and applying stereochemical rules to structural representations. Targeted teaching activities, including web-based tutorials, physical molecule models, and the HyperChem Professional computer program, were introduced to address there difficulties.

A post-test was then conducted to measure learning gains. Statistical analysis using a paired-samples *t*-test indicated a significant improvement in students' stereochemical knowledge ($p < 0.001$), with an average increase of 30.95% in test scores. These findings demonstrate the effectiveness of interactive and hands-on approaches and underscore the value of multifaceted teaching strategies that integrate digital tools, laboratory activities, and visualization techniques to

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reinforce student learning and bridge the gap between theoretical and practical aspects of stereochemistry.

Keywords: active learning, chemistry teaching, learning difficulties, misconceptions, organic stereochemistry

Introduction

Organic chemistry is a major branch of chemistry, forming the basis for developments in biochemistry, pharmacy, and medicine. and playing a key role in the discovery of new materials with numerous practical applications. As a teaching subject, it covers a wide range of abstract and complex topics because the molecular structure of organic compounds and many phenomena occur at the molecular level and are invisible to the naked eye. To acquire conceptual knowledge, students must learn in all three domains of learning: macroscopic, microscopic, and symbolic (Brown et al., 2012). The gradual introduction of chemical concepts – starting with macroscopic observations before moving on to molecular explanations and symbolic representations – is consistent with the constructivist approach (Taber and Watts, 2000). Despite this, many students find it difficult to represent molecular structures and processes symbolically because they struggle to understand the real structures, molecular interactions, and rearrangements during chemical reactions.

Numerous studies have highlighted that even fundamental terms and concepts in organic chemistry, such as nomenclature, properties, and classification of organic compounds, can be difficult for students to master (Donkoh, 2017; Uchegbu et al., 2017; Anderson & Bodner, 2008; Domin et al., 2008; Hassan et al., 2004). Donkoh (2017) emphasizes the need for curriculum reform to provide a stronger foundation in organic chemistry, while Eticha & Ochonogor (2015) and Dwyer & Childs (2017) report that students often struggle to identify functional groups and write reactions. Laboratory work plays a crucial role in overcoming these difficulties (Uchegbu et al., 2017). However, challenges such as bond polarity, functional group recognition, and stereochemistry remain significant (Hassan et al., 2004; Donkoh, 2017; Nartey & Hanson, 2021; Salame et al., 2019; Salame et al., 2020; Amsad et al., 2019). These challenges highlight the need for targeted teaching strategies that emphasize conceptual understanding, problem-solving, and critical thinking, rather than rote memorization. There are several terms in the literature that refer to misconceptions, including "misconceptions" (Bekkink, 2016; Uce & Ceyhan, 2019) and "alternative concepts" (Garnett et al., 1995; Talanquer, 2008). Misconceptions are defined as ideas, opinions, or understandings that are incorrect because of an incomplete or faulty understanding of a particular term or concept (Leonard et al., 2014). In chemistry, these misconceptions can arise from a variety of sources, including prior knowledge, teaching methods, and the abstract nature of certain chemical concepts. Therefore, it is important to recognize the difficulties and misconceptions that arise in the study of organic chemistry, particularly in the teaching content related to organic stereochemistry. The understanding of organic stereochemistry reflects students' conceptual knowledge of organic chemistry, and it is important to pay more attention to this part. By detecting these errors in time and finding ways to correct and overcome them, teachers enable students to further develop their knowledge on a solid and reliable foundation. Moreover, this approach encourages students to develop their critical and logical thinking.

Stereochemistry is among the most complex topics in organic chemistry, primarily due to the challenges it poses in visualizing three-dimensional (3D) molecular structures and accurately translating them into symbolic representations. This abstract nature is compounded by the fact that 3D molecular arrangements can significantly influence molecular properties and biological activities. As Johnstone (1991) notes, mastering conceptual knowledge in stereochemistry requires harmonizing the macroscopic, microscopic, and symbolic domains of learning. A significant challenge lies in translating 2D structural formulas into 3D models, particularly for molecules with multiple chiral centers (Kumi et al., 2013; Olimpo et al., 2015).

Recent studies have sought to address these difficulties through targeted education strategies, with growing research interest in stereochemistry education (Boukhechem et al., 2011; Milne et al., 2024; Ping et al., 2022; Kusumaningdyah et al., 2023; Barrientos et al., 2024). For example, Schmidt (1992) found that students often restrict their understanding of isomerism to compounds with identical functional groups, a limitation that Miu (2019) addresses by proposing more efficient learning strategies. Studies on stereochemistry education explore various approaches to mitigate difficulties. Researchers such as Obumnenye and Ahiakwo (2013), Durmaz (2018), and Collini et al. (2024) emphasize teaching stereochemical configurations, simplifying 3D structure representation (Tuckey et al., 1991; Salah and Alain, 2016), and utilizing programming tools (Kurbanoglu et al., 2006; Rius et al., 2011).

Web-based tutorials, as demonstrated by Burrmann and Moore (2013) and Iyamuremye et al. (2024), support learning by offering interactive stereochemistry tasks. Innovative teaching methods like games have shown promise in engaging students. Ippoliti et al. (2022) developed "*R/S* Chemistry", a game-based learning tool, while Da Silva Junior et al. (2019) introduced an innovative teaching method using a card game designed to help students learn stereochemical concepts effectively. These methods foster interactive learning environments, enhancing conceptual understanding and problem-solving skills.

Addressing specific challenges, Salah and Alain (2016) advocate testing students' ability to transition between 3D models and 2D representations using tools like Newman and Fischer projections. Similarly, Mistry et al. (2018) recommend web-based tools for constructing 3D models, exploring molecular conformations, and determining the R/S configurations of stereocenters using Cahn-Ingold-Prelog rules. Burrmann and Moore (2013) propose tutorials that extend these capabilities to include *E/Z* isomerism for alkenes. Ultimately, adopting diverse teaching approaches, hands-on experiments, interactive tools, and gamification can help overcome the complexities of stereochemistry, improving students' engagement and comprehension. Similarly, Ippoliti et al. (2022) developed "*R/S* Chemistry", a free, game-based learning tool that engages students in practicing stereochemistry tasks within an interactive environment. Both approaches aim to enhance student engagement and foster active participation in the learning process.

As can be seen from all this, the problems associated with the study of organic stereochemistry are becoming increasingly important. We have confirmed this through our previous research on the frequency of organic stereochemistry questions in international chemistry competitions (Naumoska & Aleksovska, 2023). Additionally, in our previous research, we investigated high school students' conceptual understanding of this topic and found that students struggle with these concepts, leading to a large number of misconceptions (Naumoska & Aleksovska, 2023). To understand the main reasons for these problems, their impact on the study of organic stereochemistry among undergraduate students, and potential solutions, we conducted the research described in the following section.

Experimental

The aim of the research

The research aimed to evaluate undergraduate chemistry students' knowledge and identify their difficulties and misconceptions regarding key organic stereochemistry concepts after attending the course on this topic. The course utilized PowerPoint presentations, animations, and demonstrations of various molecular models, presented by the teacher. It focused on challenges in understanding conformations and stereochemistry of alkanes and cycloalkanes, recognizing geometric isomerism in alkenes, determining the *R/S* configuration of chiral centers, and identifying meso compounds.

To overcome the detected difficulties and misconceptions, the study also sought to develop targeted instructional methods that actively involve students in the learning process. To enhance understanding, theoretical exercises for students, performed individually or in pairs, were developed using web-based tutorials, programming software HyperChem Professional, and molecular models. The final goal was to foster conceptual change, ultimately improving students' progress in organic stereochemistry.

Research questions included:

1. Are there objective difficulties and misconceptions among students about organic stereochemistry topics?
2. What causes these difficulties and misconceptions?
3. Does the intervention help students overcome these challenges and improve their understanding?

Based on these research questions, the following hypotheses are proposed:

H1: Undergraduate students exhibit significant conceptual difficulties and misconceptions when learning organic stereochemistry, particularly in identifying chiral centers, assigning *R/S* configurations, and recognizing meso compounds.

H2: These difficulties are primarily caused by limited spatial visualization skills, the abstract nature of stereochemical concepts, and insufficient use of interactive or tactile learning tools.
H3: The implementation of targeted pedagogical interventions (including digital tutorials, physical molecular models, and stereochemistry simulation software) leads to statistically significant improvement in students' understanding of stereochemistry.

Research sample

In order to investigate the possible existence of difficulties and misunderstandings, i.e., misconceptions, related to the concepts of stereoisomerism of alkanes and cycloalkanes, *E/Z* and *R/S* stereoisomerism, and determination of meso compounds, a study was conducted at the Institute of Chemistry (Faculty of Science and Mathematics, Skopje, Republic of Macedonia) in the period between November 2023 and February 2024. A total of 24 undergraduate students aged 19–20 years participated in this study. It is important to note that this study was conducted with a relatively small sample (24 students) and lacked a control group. Although this allows for a thorough analysis of conceptual understanding and the effectiveness of the interventions applied, it still limits the generalisability of the results. Therefore, further research with larger samples and control groups is recommended to confirm and expand upon these results.

Design of the research

The pre-test and post-test design is a widely used method in educational research to evaluate the effectiveness of an applied intervention. This design was employed to assess students' understanding of organic stereochemistry topics and address difficulties and misconceptions. The research followed several steps:
1) Administering a pre-test to assess students' knowledge;
2) Analyzing the pre-test data, particularly focusing on the most frequent incorrect answer for each question;
3) Identifying difficulties and misconceptions;
4) Implementing the intervention;
5) Administering a post-test;
6) Analyzing the post-test data and comparing it with pre-test results using JASP.
When composing the questions, care was taken to ensure they covered the intended concepts and objectives to be tested, as well as the different domains of Bloom's Taxonomy. To address these varied domains, the questions were assigned different point values (2, 4, and 6 points). Misconceptions were identified through distractor analysis, focusing on the incorrect options chosen by a significant proportion of students. Particular attention was given to distractors selected by more than 20% of students, as these indicate the presence of systematic misconceptions. To ensure content validity, the test questions were reviewed and approved by a professor and an assistant from the Institute of Chemistry.

The pre-test questions were divided into three categories, with each category consisting of four multiple-choice questions. The first four questions were aimed at testing knowledge of the stereoisomerism of alkanes and cycloalkanes. These questions aimed to assess students' ability to apply Newman projection formulas when naming alkanes, to represent structures in their most and least stable conformations, and to graphically represent the change in potential energy as a function of angle of rotation. The second set of questions aimed to assess students' ability to recognize the presence of geometric isomerism in alkenes based on a given structural formula, but also to correctly identify cis/trans, i.e., *E/Z* isomers. The last four questions aimed to assess students' ability to identify chiral centers and determine the *R/S* configuration as well as recognize meso compounds based on a given structural formula.

Intervention phase

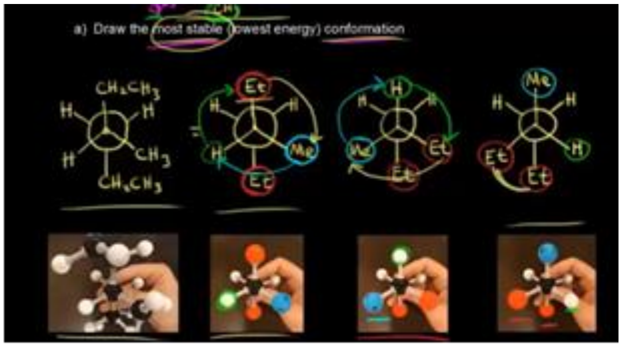
The intervention phase incorporated multiple strategies to support students in mastering stereochemistry concepts. These included:

I. **Web-based tutorials**: The free Khan Academy website (www.khanacademy.org) offers a series of tutorials on cycloalkane stereochemistry, Newman projection formulas, conformational analysis of alkanes, and determination of *R/S* configuration. Selected tutorials have been integrated into theoretical exercises to deepen these topics. The free Chem Tube3D website (www.chemtube3d.com) provided students with opportunities to explore and practice various examples of *R/S* configuration determination.

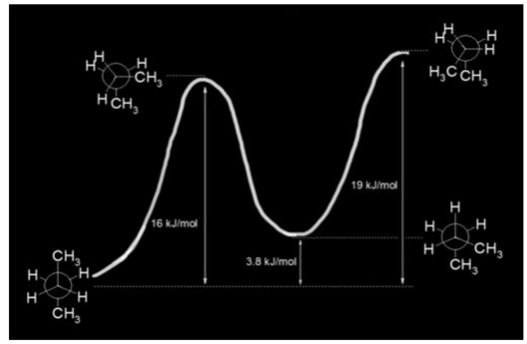
II. **Computer programs**: The HyperChem Professional program was used to facilitate the determination of stereochemical configurations and recognition of meso compounds.

III. **Molecular models**: While engaging with tutorials and computer programs, students used molecular models to assemble the structures displayed on the screen.

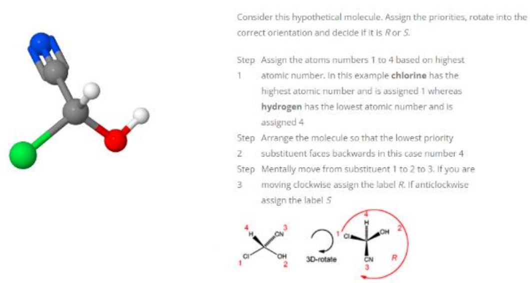
Figure 1 illustrates the various activities carried out during the intervention phase. Figure 1a and Figure 1b show web-based tutorials that helped students better understand the Newman projection formulas and the graphical representation of changes in potential energy as a function of angle of rotation. The remaining images (Figures 1c–1f) depict activities conducted using the ChemTube3D website, physical molecular models, and HyperChem Professional software. Using these tools, students practised determining geometric isomerism in alkenes, identifying chirality and achirality in molecules, assigning *R/S* configurations, and recognizing planes of symmetry in meso compounds.



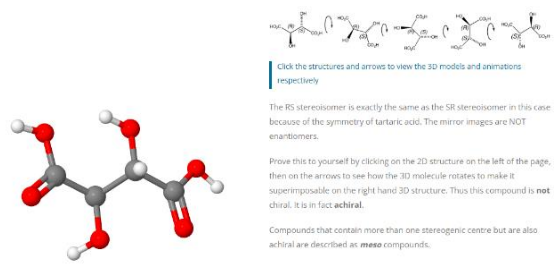
a) Represent structures in their most/least stable conformations



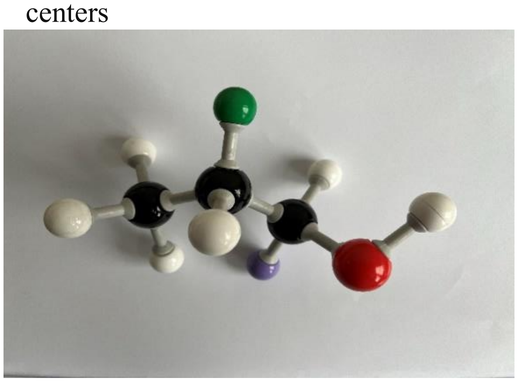
b) Graphical representation of potential chair energy versus angle of rotation

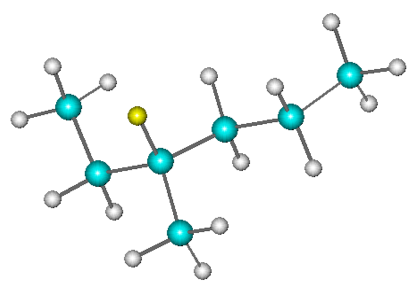


c) Determining the *R/S* configuration of chiral centers



d) Recognizing meso compounds





e) Determining the *R/S* configuration of chiral centers using physical molecular models and HyperChem Professional (f)

Figure 1. Activities used during the intervention

After the intervention, a post-test was administered, similar to the pre-test, consisting of 12 questions divided into three categories, with four questions in each category. The purpose of the post-test was to assess whether the intervention helped students overcome difficulties and misconceptions regarding topics in organic stereochemistry. Both tests are provided as supplementary materials.

Results and Discussion

As already mentioned, the students' acquired knowledge in stereochemistry was first assessed using a pre-test. The results of the pretest showed that, with the exception of questions 5 and 7 (each worth 2 points), more than half of the students answered all other questions incorrectly (Table 1). Particular attention was paid to the incorrect answers (distractors), selected by more than 20% of the students, as these indicated the presence of misconceptions regarding specific stereochemical content. According to Gilbert (1977), conceptual understanding can be categorized into four levels:

- Satisfactory Conceptual Understanding (SCU): correct answers given by 75% or more of the students.
- Roughly adequate performance (RAP): Correct answers in the range of 50–74%.
- Inadequate Performance (IP): Correct answers in the range of 25–49%.
- Fairly Inadequate Performance (QIP): Correct answers selected by less than 25% of students.

Table 1. Number of correctly answered (C) and incorrectly answered (I) questions on the pre-test

Number of questions		1	2	3	4	5	6	7	8	9	10	11	12
Number of answers	C	9	4	3	11	13	10	14	8	9	6	7	6
	I	15	20	21	13	11	14	10	16	15	18	17	18
Percentage of correct answers		37.5	16.7	12.5	45.8	54.1	41.7	58.3	33.3	37.5	25	29.2	25
Standard deviation		0.484	0.373	0.331	0.498	0.498	0.493	0.493	0.471	0.484	0.433	0.455	0.433

The results presented in Table 1 support the first hypothesis, which states that students exhibit significant conceptual difficulties in stereochemistry. The low percentage of correct answers across several items (particularly those involving chiral centers, *R/S* configurations, and meso compounds) indicates that these topics pose substantial challenges. The variability in responses, reflected in the standard deviations, further confirms that students have differing levels of understanding, consistent with the hypothesis. Namely, the standard deviations of the responses (correct–incorrect) range from 0.331 to 0.523, which is within the expected range for binary data of this type. These values indicate that students exhibited varying levels of understanding across the questions. Moreover, the distribution suggests that the items were well constructed, neither too difficult nor too easy, allowing for meaningful differentiation in student performance. To identify the reasons behind the low performance, we analyzed the answers for each question.

The purpose of the first question was to assess how well students could apply the rules for naming organic compounds using a given Newman projection formula. Only 37.5% of students answered correctly, while 30% chose the distractor (c). Students often make mistakes when naming compounds using the Newman projection formula because it requires a strong spatial understanding of molecular geometry. Errors occur in identifying the correct relative positions of substituents (e.g., staggered vs. eclipsed conformations) and translating this arrangement into the correct nomenclature. Confusion also arises when priorities are overlooked in naming substituents or assigning configurations. The difficulties in visualizing the 3D structures represented by the Newman projection are likely the primary reasons for the high percentage of incorrectly selected answers, along with probably insufficient understanding of systematic naming rules. These difficulties are consistent with previous reports indicating that students often struggle to visualize three-dimensional structures and correctly apply stereochemical rules (Mistry et al., 2020; Boukhechem et al., 2011; Burrmann & Moore, 2013; Tuckey et al., 1991).

The second and third question assesses of chair conformations of cyclohexane, axial and equatorial positions of substituents, and conformational stability. For the second question, in order to choose the correct structure, the students had to draw all four possibilities offered by their names. A very low percentage of correct answers was obtained here, i.e., a full 16.7% for the second and 12.5% for the third question. For both questions, students chose the incorrect answer- c. Regarding the second question, it is clear that students have difficulties drawing the chair conformation, i.e., the symbolic representation of the structure. They mismatched the *cis/trans* with axial/equatorial positions of the substituents. Students often made errors when selecting the most stable chair conformation because they had difficulty visualizing the 3D spatial arrangement of the substituents, which is evident in the third question. Key errors include misidentifying axial and equatorial positions, failing to recognize steric hindrances caused by large groups in axial positions, and overlooking 1,3-diaxial interactions. In addition, a limited understanding of conformational energy differences between chair inversions can lead to incorrect decisions. These challenges are compounded by the lack of practice in analyzing and predicting the stability of conformations based on substituent interactions.

The final question in this category assessed students' ability to match conformers to specific points on a graph showing the change in potential energy as a function of angle of rotation. The students achieved significantly better results (45.8% correct answers) in comparison with the previous two questions. Students often have difficulty with such diagrams because they cannot relate the molecular conformations to the energy trends. Identifying energy minima (staggered conformations) and maxima (eclipsed conformations) can be particularly challenging. Again, the main difficulty arises because of the lack of ability to visualize the 2D presentation.

The second group of questions was designed to investigate how well students can recognize and correctly identify geometric isomerism in alkenes. The students performed best on this part of the test, but the results are still unsatisfactory. To this end, four questions were designed for each of the two tests, asking students to use structural formulas to determine whether geometric isomerism was present and, if so, whether it was cis/trans or *E/Z*. In addition, for some questions, students were presented with pre-labelled *E/Z* isomers and had to determine which labels were correct or incorrect. In these questions, students often made mistakes when using the *E/Z* system to name alkenes because they had difficulty determining the priority of substituents on each carbon of the double bond according to the Cahn–Ingold–Prelog (CIP) priority rules. Students often struggle with naming structures containing multiple bonds, particularly those involving C-O and C-N bonds, as seen in the fifth and sixth questions. Additionally, they face challenges in recognizing geometric isomerism in alkenes and identifying isomers of a given formula. This difficulty stems from a failure to recognize the limited rotation around a double bond, a key characteristic of isomerism that is essential for understanding geometric isomerism. They may overlook the presence of different substituents on either side of the double bond, which is necessary for geometric isomerism. Confusion arises when multiple substituents or chains are present, making it difficult to identify isomers and assign the correct *cis/trans* or *E/Z* configuration. These challenges reflect common misconceptions reported in the literature, highlighting that students frequently struggle with geometric isomerism and the application of the *E/Z* system (Durmaz, 2018; Miu, 2019; Salame et al., 2019).

The students' scores on the seventh question were the highest on the test. However, some students still thought that geometric isomerism is possible only when two hydrogens are bonded to the same carbon atom in a C=C bond, so they didn't consider tetrasubstituted C=C bonds with four different substituents. The eighth question was actually assessing the ability of the students to recognize the type of isomerism. Surprisingly, the score was low (33% of correct answers), and 42% of the students incorrectly believed that the two compounds were *cis/trans* isomers. This means that they had serious misconceptions in recognizing types of isomerism.

The last group of questions was designed to assess students' ability to identify chiral centers, determine *R/S* configuration, and recognize meso compounds. In the ninth question of the pre-test, 29% of students incorrectly thought that compound V contained a chiral center (answers a and c). This highlights the difficulties students faced in identifying chiral centers, such as recognizing tetrahedral carbon atoms bonded to four different groups. These difficulties often stemmed from limited spatial visualization skills, unfamiliarity with chirality rules, and misconceptions about symmetry or what "different groups" were. Overcoming these problems required focused practice and a solid foundation in stereochemical principles. In the tenth question, students had to identify which structure was different from the others. In order to answer correctly, they had to apply the *R/S* nomenclature system to all given compounds and determine their configurations. However, only 25% of the students gave the correct answer. This shows that a significant proportion of students had difficulty distinguishing the structures based on their symbolic presentation using perspective formulas. Since only a small proportion of students answered this question correctly, it is suggested that many students had difficulty effectively applying the *R/S* system, which is essential for understanding stereochemistry. This difficulty could be due to the challenge of prioritizing substituents around chiral centers, determining the correct orientation of molecules, or unfamiliarity with how to systematically analyze stereoisomers.

Similarly, in the penultimate question, only 29% of students answered correctly. In this case, the students were asked to find which of the given structures has the *S*-configuration. The low achievement level again indicates that many students had difficulty using *the R/S* nomenclature system to assign the correct configuration to the chiral centers. Identifying the configuration can be particularly challenging, especially when dealing with complex molecules with multiple substituents. This difficulty primarily stemmed from a lack of the ability to visualize the three-dimensional arrangement of atoms around the chiral center and the challenge of following the steps required to prioritize the substituents according to the Cahn-Ingold-Prelog rules. Overall, this difficulty emphasized the need for further instruction and practice in the correct application of stereochemical conventions.

In the final question, only 25% of students correctly identified the structure representing a meso compound. This suggested that most students had difficulty understanding the key features of meso compounds, which contain chiral centers but are achiral due to an internal plane of symmetry. Meso compounds present a unique challenge because, while they exhibit stereoisomerism, their internal symmetry renders them optically inactive. Identifying these compounds requires a thorough understanding of molecular symmetry and the specific arrangement of substituents around chiral centers. The low success rate on the corresponding questions indicates that many students have difficulty distinguishing meso compounds from other stereoisomers, such as enantiomers, or recognizing the symmetry responsible for achirality, even when chiral centers are present. This emphasized the need to better communicate the characteristics of meso compounds, their identification and the evaluation of symmetry in molecules.

The pre-test results indicated that serious intervention was necessary to improve the students' knowledge. To achieve this goal, students were directly and actively involved in the learning process. They were taught using computer tutorials and programs, while simultaneously using molecular models and working individually or in pairs. Previous studies support the effectiveness of such approaches, demonstrating that integrating ICT tools, interactive molecular models, and guided hands-on activities can enhance students' conceptual understanding and spatial reasoning in stereochemistry (Burrmann & Moore, 2013; Rius et al., 2011; Kusumaningdyah et al., 2024; Da Silva Junior et al., 2019).

These findings are consistent with Hypothesis 2, which attributes students' difficulties in stereochemistry to limited spatial reasoning skills and the abstract nature of the concepts involved. The low performance on items requiring identification of chiral centers and assignment of *R/S* configurations suggests that traditional instruction may not sufficiently support the development of three-dimensional thinking.

After various instruments were used to eliminate difficulties and misconceptions, the post-test, which consisted of questions that checked the same concepts as the pre-test was conducted. The results obtained were compared with the results of the pre-test using the *t*-statistic (paired samples *t*-test) from the statistical software package JASP. The results obtained were shown in Table 2.

Table 2. Comparison of Pre-Test and Post-Test Results: N-Number of students; $\bar{x}$- Average success of the group; s-Standard deviation; $\Delta\bar{x}$- Difference between the average successes; t: t-test value; t_{crit}-Critical value of the t-test; df-Degrees of freedom; p- p-value

Test	N	$\bar{x}$ /%	s	$\Delta\bar{x}$ /%	Cohen's d	t	t_{crit}	df	p
Pre-test	24	34.72	12.07	30.95	2.46	13.4	2.07	23	0.001
Post-test	24	65.67	13.1						

The results presented in Table 2 strongly support Hypothesis 3. As shown in Table 2, students performed better on the post-test than on the pre-test. The statistical significance of this improvement can be determined from the t-test result at the 5% significance level. This test compares the means to evaluate the validity of the null hypothesis, which assumes no significant difference between the two sample means. The results show that $t > t_{crit}$ and $p < 0.001$, indicating a statistically significant difference and suggesting that the improvement is likely due to the applied activities. In addition to the statistically significant improvement, the magnitude of the learning gains was assessed using Cohen's d, which was calculated as 2.46. According to established guidelines, this represents a very large effect size (Cohen, 1988), indicating that the intervention had a substantial and meaningful impact on students' understanding of stereochemistry. These findings are consistent with previous studies demonstrating that targeted instructional strategies, including visualisation tools and structured exercises, can significantly improve students' performance and conceptual understanding in stereochemistry (Barrientos et al., 2024; Mistry et al., 2020; Kusumaningdyah et al., 2024). Figure 2 shows the group's average results for each pre-test and post-test question.

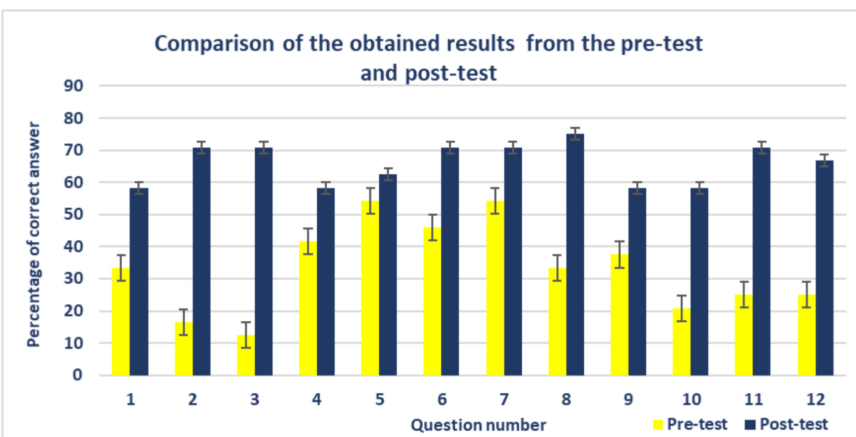


Figure 2. Comparison of the results achieved from the pre-test and post-test

As can be seen in Figure 2, the results of the post-test show a remarkable improvement in the students' performance, with error bars representing the standard deviation,demonstrating that the applied activities effectively improved their understanding of stereochemistry. Specifically, the activities helped students gain a better understanding of isomerism in alkenes and gave them the tools to better analyze and interpret stereochemical concepts.

To assess whether and to what extent students' performance improved after the intervention, a detailed comparison of results before and after the test was conducted for each student. Figure 3 not only highlights the extent of improvement but also provides valuable insight into the intervention's overall effectiveness in addressing the challenges and misconceptions associated with stereochemical concepts. The comparative approach ensures a clear assessment of progress and identifies areas of significant conceptual change.

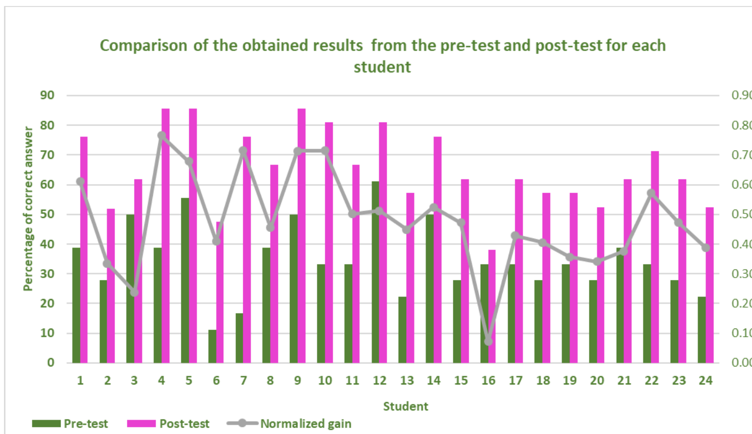


Figure 3. Comparison of the results achieved from the pre-test and post-test for each student Figure 3 shows that all 24 students demonstrated a significant improvement in their performance on the post-test, after the applied intervention, compared to their performance on the pre-test. The normalized gain (g) calculated for each student further quantifies this improvement, showing that most students achieved moderate to high gains, while only a few achieved lower gains. This consistent progress across the group underlines the effectiveness of the intervention strategies used. The activities implemented during the intervention appear to have successfully addressed and corrected the difficulties and misconceptions associated with stereochemical concepts. Furthermore, this finding underscores the value of targeted instructional approaches and the application information and communication technologies (ICT) to improve student understanding and facilitate conceptual change in difficult areas of organic chemistry.

Conclusion

Based on the results, several conclusions can be drawn. The pre-test results demonstrated that when students are passively taught organic stereochemistry through traditional instruction, even when supported by computer animations or molecular models, their achievements remain unsatisfactory. The analysis of the pre-test responses indicated that the main learning difficulties stem from students' limited spatial visualization skills and their inability to connect 3D molecular structures with their 2D representations, such as Newman and perspective formulas. Additionally,

students struggle to apply the Cahn-Ingold-Prelog rules for assigning priorities, especially with complex substituents.
After implementing an intervention based on active learning and ICT (e.g., computer tutorials and programs), students' conceptual understanding of stereochemistry significantly improved. This improvement, confirmed by a statistical analysis, highlights the effectiveness of engaging students directly in visual and manipulative learning activities.
The findings emphasize the importance of incorporating visualization-based and technology-supported methods into the organic stereochemistry curriculum to promote deeper conceptual understanding. Furthermore, the study suggests that teacher-training programs should include targeted preparation to develop students' spatial reasoning skills and to integrate ICT resources effectively into stereochemistry instruction.

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Conflict-of-Interest Statement

The authors declare that they have no conflict of interest.

References

Anderson, T. L., & Bodner, G. M. (2008). What can we do about 'Parker'? A case study of a good student who didn't 'get' organic chemistry. *Chemistry Education Research and Practice*, 9, 93–101. https://doi.org/10.1039/b806223b.

Amsad, L. N., Liliasari, S., Kadarohman, A., & Sardjono, R. E. (2019). Recognizing students' abilities to work through synthesis organic pathways. *Journal of Physics: Conference Series, 042021*, 1157 (4), 1–5. https://doi.org/10.1088/1742-6596/1157/4/042021.

Boukhechem, M.S., Dumon, A., & Zouikri, M. (2011). The acquisition of stereochemical knowledge by Algerian students intending to teach physical sciences. *Chemistry Education Research and Practice*, 12, 331–343. https://doi.org/10.1039/C1RP90040D.

Brown, T. L., LeMay, H. E., Bursten, B. E., Murphy, C. J. & Woodward P. M. (2012). *Chemistry: The Central Science (12th ed.)*. Pearson.

Burrmann N. J., & Moore W. J. (2013). Development of a Web-Based, Student-Centered Stereochemistry Tutorial. *Journal of Chemical Education*, 90 (12), 1622–1625. https://doi.org/10.1021/ed300744s.

Bekkink, M. O., Donders, A. R. T., Kooloos, J.G., de Waal R. M. W., & Ruiter D. J. (2016). Uncovering students' misconceptions by assessment of their written questions. *BMC Medical Education*, 16, 221–227. https://doi.org/10.1186/s12909-016-0739-5.

Barrientos, C., Moris, S., & López J. G. (2024). A teaching strategy for teaching stereochemistry: a proposal for converting from one representation to another. *Educación Química*, 35(4), 94–102. https://doi.org/10.22201/fq.18708404e.2024.4.88280.

Cohen, J. (1988). Statistical Power Analysis for the Behavioral Sciences (2nd ed.). Hillsdale, NJ: Lawrence Erlbaum Associates.

Collini, M. A., Miguel K., Weber, R. & Atkinson M. B. (2024). Investigating changes in students' attitudes towards organic chemistry: a longitudinal study. *Chemistry Education Research and Practice*, 25, 613–624. https://doi.org/10.1039/D3RP00228D.

Domin, D. S., Al-Masum, M. & Mensah, J. (2008). Students' categorizations of organic compounds. *Chemistry Education Research and Practice*, 9, 114–121. https://doi.org/10.1039/B806226A.

Donkoh, S. (2017). What students say about senior high school organic chemistry? *International journal of environmental & science education*, 12(10), 2139–2152. http://www.ijese.net/makale_indir/IJESE_1977_article_5a1b379c0a6c6.pdf.

Dwyer, A. O., & Childs, P. E. (2017). Who says Organic Chemistry is Difficult? Exploring Perspectives and Perceptions. *Eurasia Journal of Mathematics, Science and Technology Education*, 13 (7), 3599–3620. https://doi.org/10.12973/eurasia.2017.00748a.

Durmaz, M. (2018). Determination of Prospective Chemistry Teachers' Cognitive Structures and Misconceptions About Stereochemistry. *Journal of Education and Training Studies*, 6 (9), 13–20. https://doi.org/10.11114/jets.v6i9.3353.

Da Silva Junior, J. N., De Andrade Uchoa, D. E., Lima M. A. S,. & Monteiro, A. J. (2019). Stereochemistry Game: Creating and Playing a Fun Board Game To Engage Students in Reviewing Stereochemistry Concepts. *Journal of Chemical Education*, 96 (8), 1680–1685. https://doi.org/10.1021/acs.jchemed.9b01020.

Eticha, A. T. & Ochonogor, C. E. (2015). Assessment of undergraduate chemistry students' difficulties in organic chemistry. *Proceedings of the ISTE International Conference on Mathematics, Science and Technology Education*, 285–296. https://www.researchgate.net/publication/344179315_ASSESSMENT_OF_UNDERGRADUATE_CHEMISTRY_STUDENTS%27_DIFFICULTIES_IN_ORGANIC_CHEMISTRY.

Gilbert, J. K. (1977). The study of student misunderstandings in the physical sciences. *Research in Science Education*, 7 (1), 165–171. https://doi.org/10.1007/BF02643123.

Garnett, P. J., Garnett, P. J., & Hackling, M. W. (1995). Students' Alternative Conceptions in Chemistry: A Review of Research and Implications for Teaching and Learning. *Studies in Science Education*, 25 (1), 69–96. https://doi.org/10.1080/03057269508560050.

Hassan, A. K., Hill, R. A. & Reid, N. (2004). Ideas Underpinning Success in an Introductory Course in Organic Chemistry. *University Chemistry Education*, 8, 40–51. https://www.rsc.org/images/p2_reid_tcm18-31146.pdf.

Ippoliti, F. M., Nguyen, M. M., Amber, J., Reilly, A. J. & Gar, N. K. (2022). Gaming stereochemistry. *Nature Reviews Chemistry*, 6, 373–374. https://doi.org/10.1038/s41570-022-00395-5.

Iyamuremye, A., Twagilimana, I. & Niyonzima, F. N. (2024). Examining the utilization of web-based discussion tools in teaching and learning organic chemistry in selected Rwandan secondary schools. *Heliyon*, 10 (20), e39356. https://doi.org/10.1016/j.heliyon.2024.e39356.

Johnstone, A. H. (1991). Why is science difficult to learn? Things are seldom what they seem. *Journal of Computer Assisted Learning*, 7 (2), 75–83. https://doi.org/10.1111/j.1365-2729.1991.tb00230.x.

Kurbanoglu, N.I., Taskesenligil, Y. & Sozbilir, M. (2006). Programmed instruction revisited: a study on teaching stereochemistry. *Chemistry Education Research and Practice*, 7, 13–21. https://doi.org/10.1039/B5RP90012C.

Kumi, B. C., Olimpo, J. T., Bertlett, F., & Dixon, B. L. (2013). Evaluating the effectiveness of organic chemistry textbooks in promoting representational fluency and understanding of 2D-3D diagrammatic relationships. *Chemistry Education Research and Practice*, 14 (2), 177–187. https://doi.org/10.1039/C3RP20166J.

Kusumaningdyah, R., Devetak, I., Utomo, Y., Effendy, E., Putri D., & Habiddin, H. (2024). Teaching Stereochemistry with Multimedia and Hands-On Models: The Relationship between Students' Scientific Reasoning Skills and The Effectiveness of Model Type. *Center for Educational Policy Studies Journal*, 14 (1), 171–197. https://doi.org/10.26529/cepsj.1547.

Leonard, M.J., Kalinowski, S.T., & Andrews, T.C. (2014). Misconceptions Yesterday, Today, and Tomorrow. *CBE—Life Sciences Education*, 13 (2), 179–186. https://doi.org/10.1187/cbe.13-12-0244.

Miu, B. (2019). Teaching The Concept Of Isomerism In Organic Chemistry In High School Education. *Education Facing Contemporary World Issues, European Proceedings of Social and Behavioural Sciences*, 363–373. https://www.europeanproceedings.com/article/10.15405/epsbs.2019.08.03.43?utm_source=chatgpt.com.

Mistry, N., Singh, R. & Ridley, J. (2020). A Web-Based Stereochemistry Tool to Improve Students' Ability to Draw Newman Projections and Chair Conformations and Assign *R/S* Labels, *Journal of Chemical Education*, 97, 1157–1161. https://doi.org/10.1021/acs.jchemed.9b00688.

Milne, S. L., Finkenstaedt-Quinn, S. A., Garza, N. F., Zimmerman, S. C., & Shultz, G. V. (2024). Capturing students' identification of the relevance of organic chemistry in writing. *Chemistry Education Research and Practice*, 25, 403–416. https://doi.org/10.1039/D3RP00161J.

Nartey, E. & Hanson, R. (2021). The Perceptions of Senior High School Students and Teachers about Organic Chemis-try: A Ghanaian Perspective. *Science Education International*, 32 (4), 331–342. https://doi.org/10.33828/sei.v32.i4.8.

Naumoska, A. & Aleksovska, S. (2023). Identification of Difficulties and Misconceptions in the Study of Organic Chemistry in High School. *26th Congress of Pure and Applied Chemistry of SCTM, September 2023, Ohrid, Macedonia*, 199–200. https://sctm.mk/conferences/2023-26th-Congress-Book%20of%20Abstracts.pdf.

Naumoska, A. & Aleksovska, S. (2023). Representation of questions from organic stereochemistry and their evaluation in knowledge tests at the International Chemistry Olympiads (in macedonian). *Proceedings of the 4th International Conference on Education in Mathematics, Physics and Related Sciences in Skopje*, N. Macedonia, 109–116. https://drive.google.com/file/d/151rOeDU36JHfXm49_9SUZ41jQsWEb8Jp/view?usp=sharing.

Obumnenye, O. & Ahiakwo, M. J. (2013). Using stereochemistry models in teaching organic compounds nomenclature: effects on senior secondary students' performance in rivers state of Nigeria. *African Journal of Chemical Education*, 3 (2), 91–105. https://drive.google.com/file/d/1mP1KlGVqAWQ_jNrdtwbpBL8uon-GJUUr/view?usp=sharing.

Olimpo, J. T., Kumi, B. C., Wroblewski, R., & Dixon B. L. (2015). Examining the relationship between 2D diagrammatic conventions and students' success on representational translations tasks in organic chemistry. *Chemistry Education Research and Practice*, 16, 143–153. https://doi.org/10.1039/C4RP00169A.

Ping, E., Parrill, F., Church, E. B., & Meadow, S. G. (2022). Teaching stereoisomers throught gesture, action, and mental imagery. *Chemistry Education Research and Practice*, 23 (3), 698–713. https://doi.org/10.1039/D1RP00313E.

Rius, C., Dominguez, H. M. T., Gonzalez-Quezeda, Y., & Pozas-Horcasitas, R. (2011). The use of stereoscopis tools (3D) to teach stereochemistry. *Proceedings of EDULEARN11 Conference*, 289–297.
https://www.researchgate.net/publication/259176906_THE_USE_OF_STEREOSCOPIC_TOOLS_3D_TO_TEACH_STEREOCHEMISTRY.

Schmidt, H. J. (1992). Conceptual difficulties with isomerism. *Journal of Research in Science Teaching*, 29 (9), 995–1003. https://doi.org/10.1002/tea.3660290908.

Salah, B. M., & Alain D. (2016). To what degree does handling concrete molecular model promote the ability to translate and coordinate between 2D and 3D molecular structure representations? A case study with Algerian students. *Chemistry Education Research and Practice*, 17 (4), 862–877. http://dx.doi.org/10.1039/C5RP00180C.

Salame, I. I., Patel, S., & Suleman, Sh. (2019). Examining Some of The Students' Challenges in Learning Organic Chemistry. *International Journal of Chemical Education Research*, 3 (1), 6–14. https://www.researchgate.net/publication/333003516_Examining_Some_of_The_Students'_Challenges_in_Learning_Organic_Chemistry.

Salame, I. I., Casino, P. & Hodges, N. (2020). Examining Challenges that Students Face in Learning Organic Chemistry Synthesis. *International Journal of Chemical Education Research*, 4 (1), 1–9. https://www.researchgate.net/publication/341608142_Examining_Challenges_that_Students_Face_in_Learning_Organic_Chemistry_Synthesis.

Tuckey, H., Selvaratnam, M. & Bradley, J. (1991). Identification and rectification of student difficulties concerning three-dimensional structures, rotation, and reflection. *Journal of Chemical Education*, 68(6), 460. https://doi.org/10.1021/ed068p460.

Taber, K. S., & Watts, M. (2000). Learners' explanations for chemical phenomena. *Chemistry Education Research and Practice*, 1(3), 329–353. https://doi.org/10.1039/B0RP90015J.

Talanquer, V. (2006). Commonsense Chemistry: A Model for Understanding Students' Alternative Conceptions. *Journal of Chemical Education*, 83 (5), 811–816. https://doi.org/10.1021/ed083p811.

Uchegbu, R. I., Ahuchaogu, A. A., & Amanze K. O. (2017). Tertiary institution students' perception of difficult topics in organic chemistry curriculum in Imo State. *AASCIT Journal of Education*, 3, 9–15. https://www.researchgate.net/publication/319357563_1_Tertiary_Institution_Students%27_Perception_of_Difficult_Topics_in_Organic_Chemistry_Curiculum_in_Imo_State.

Uce, M. & Ceyhan, I. (2019). Misconception in Chemistry Education and Practices to Eliminate Them: Literature Analy-sis. *Journal of Education and Training Studies*, 7 (3), 202–208. https://doi.org/10.11114/jets.v7i3.3990.

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Poboljšanje postignuća studenata u organskoj stereohemiji kroz aktivno učenje korišćenjem informaciono-komunikacionih tehnologija

Tekući naslov: Aktivno učenje i IKT u nastavi organske stereokemije

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SAŽETAK

Stereohemija i dalje predstavlja izazovnu oblast za studente osnovnih studija hemije, što često dovodi do pogrešnih shvatanja i teškoća u učenju. Da bi se poboljšilo razumevanje studenata, tradicionalne metode nastave treba dopuniti inovativnim strategijama kao što su interaktivni alati za učenje, digitalni resursi i praktične aktivnosti. Ova studija imala je za cilj da identifikuje glavne poteškoće sa kojima se studenti suočavaju pri učenju stereohemije i da proceni efikasnost različitih pedagoških intervencija.

Pretest koji je procenjivao ključne koncepte stereohemije primenjen je na 24 studenta druge godine na Institutu za hemiju, Prirodno-matematičkog fakulteta u Skoplju, Severna Makedonija. Rezultati su pokazali značajne izazove, posebno u identifikaciji hiralnih centara, određivanju R/S konfiguracija, prepoznavanju mezo-jedinjenja i razumevanju E/Z izomerije. Studenti su takođe imali poteškoće sa prostornom predstavom molekula i primenom stereohemijskih pravila na strukturne formule. Ciljane nastavne aktivnosti, uključujući tutorijale na internetu, fizičke modele molekula i računski program HyperChem Professional, uvedene su kako bi se ove teškoće prevazišle.

Nakon toga sproveden je test radi merenja napretka u učenju. Statistička analiza korišćenjem t-testa za uparene uzorke pokazala je značajno poboljšanje znanja iz stereohemije ($p < 0.001$), uz prosečno povećanje rezultata od 30,95%. Ovi nalazi potvrđuju efikasnost interaktivnih i praktičnih pristupa i naglašavaju vrednost višestrukih nastavnih strategija koje integrišu digitalne alate, laboratorijske aktivnosti i tehnike vizualizacije radi jačanja studentskog učenja i povezivanja teorijskih i praktičnih aspekata stereohemije.

Ključne reči: aktivno učenje, nastava hemije, teškoće u učenju, pogrešna shvatanja, organska stereohemija

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Allelopathic potential of Microcystin-RR at environmentally relevant concentrations: Species-specific growth responses of phytoplankton

Running title: Species-Specific Phytoplankton Responses to Microcystin-RR

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ABSTRACT

Microcystin-RR (MC-RR), a common cyanotoxin frequently detected in freshwater ecosystems, can influence phytoplankton dynamics by altering the growth patterns of coexisting species. While a large body of work has examined the allelopathic effects of microcystin-RR (MC-LR) and other microcystin variants, there are relatively few studies that specifically address the impact of pure MC-RR on phytoplankton species, particularly those that themselves produce this toxin, under environmentally relevant concentrations. This study investigated the effects of an environmentally relevant concentrations of MC-RR (1, 5, and 10 μg L^{-1}) on the growth of five phytoplankton species: three cyanobacteria (*Trichormus variabilis*, *Nostoc* sp., *Microcystis* sp.) and two green microalgae (*Chlorella* sp., *Scenedesmus* sp.), under laboratory conditions. Growth responses were monitored spectrophotometrically to determine chlorophyll *a* concentration over a 14-day period. Additionally, MC-RR was identified and quantified in the treated cyanobacterial cultures using the HPLC-DAD technique. The results demonstrated species-specific responses. MC-RR exhibited a stimulatory effect on both green algae species. In contrast, *T. variabilis* showed progressive growth inhibition, which became statistically significant after day 5. *Nostoc* sp. displayed slight, non-significant inhibition, while *Microcystis* sp. showed tolerance to MC-RR exposure. These findings demonstrate the allelopathic potential of MC-RR, with species-specific effects on growth that reflect differential sensitivity among phytoplankton taxa. The results underscore the ecological relevance of this toxin in shaping phytoplankton community structure and species interactions in freshwater ecosystems.

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Keywords: cyanotoxin, microcystin-RR, allelophathy, phytoplankton, microbial community

Introduction

Allelopathy is a biological phenomenon in which biochemicals produced by one organism have positive or negative effects on other organisms (Cheng and Cheng, 2015). A wide range of organisms, including plants, cyanobacteria, algae, fungi, and soil microorganisms, can synthesize and release bioactive molecules that act as allelochemicals and thereby exert allelopathic effects (Polyak & Sukharevich, 2025; Leao et al., 2010; Revillini et al., 2023). Effects of allelopathy can be either inhibitory or stimulatory, and they can shape species composition and diversity by conferring competitive advantages to certain organisms (Willis, 2007). Allelopathy plays a significant role in shaping the structure and dynamics of aquatic microbial communities (Gross, 2003).

Phytoplankton is a diverse group of photosynthetic prokaryotic and eukaryotic microorganisms which are widely distributed in both aquatic (freshwater and marine) and terrestrial environments, where they contribute significantly to primary production and global carbon cycling (Falkowski, 1994, Kosek et al., 2016). Ecologically, phytoplankton serve as a foundational component of the aquatic food chain (Yarnold et al., 2019), providing essential nutrients for zooplankton and other consumers. As eukaryotic unicellular or colonial organisms, green microalgae (phylum *Chlorophyta*) are characterized by the presence of chlorophylls *a* and *b*, giving them their distinctive green color, and by storing energy in the form of starch within their chloroplasts (Graham et al., 2009). In addition to their ecological roles, microalgae have attracted growing interest for their potential in biotechnology, particularly in the fields of biofuel production, wastewater treatment, and as sources of valuable biomolecules such as antioxidants, pigments, and fatty acids (Srimongkol et al., 2022). Cyanobacteria represent a diverse and widespread group of photosynthetic prokaryotes belonging to the domain Bacteria, that can thrive in a wide range of environmental conditions, from freshwater and marine systems to extreme habitats such as hot springs and deserts (Schirrmeister et al., 2015; Whitton & Potts, 2012). Ecologically, cyanobacteria as well as green microalgae, are primary producers and form the base of many aquatic food webs (Saleem et al., 2025). However, under favorable conditions such as high nutrient availability, warm temperatures, and stable water columns, some cyanobacteria and green microalgae can proliferate excessively, leading to harmful algal blooms (HABs) (Paerl & Otten, 2013). These blooms not only disrupt aquatic ecosystems but can also produce a variety of bioactive and toxic compounds, including microcystins, cylindrospermopsins, and anatoxins, which pose serious risks to animal and human health (Zanchett & Oliveira-Filho, 2013).

In freshwater ecosystems, cyanobacteria are known not only for their primary productivity and ecological plasticity but also for their ability to produce a wide array of secondary metabolites with allelopathic potential. Cyanobacterial allelopathy is widespread and occurs in almost all aquatic habitats (Śliwińska-Wilczewska et al., 2022). Production of allelopathic compounds represents an adaptation performed by some cyanobacteria to get a competitive advantage over other primary producers (Śliwińska-Wilczewska et al., 2021). Cyanobacteria produce potent compounds known as cyanotoxins, the ecological and allelopathic roles of which remain only partly understood (Teneva et al., 2023). Among these, microcystins (MCs), a group of cyclic heptapeptide hepatotoxins, are predominantly recognized for their toxic effects on animals and humans. The cytotoxic effects of cyanotoxins have been well studied in animals and humans, but many questions remain about their effects on coexisting phytoplankton communities (Omidi et al., 2021). However, beyond their toxicological significance, MCs have increasingly been investigated for their ecological roles, including potential allelopathic functions within phytoplankton communities (Wei et al., 2024; Polyak & Sukharevich, 2025).

MCs are heptapeptides formed by L-amino acids, regularly distributed in a cyclic structure based on the general structure: cyclo-[(1)-alanine-(2)-X-(3)- methyl aspartic acid-(4)-*Z*-(5)-Adda-(6)-glutamic acid-(7)-methyl dehydroalanine] (Martínez-Piernas et al., 2025). Since 2010, MC-RR has been found in water bodies and cyanobacterial blooms in Africa, America, Asia, and Europe, and is the most frequent MC congener in lakes, rivers, and reservoirs in China (Junfeng et al., 2010). Microcystin-RR (MC-RR) is one of the most commonly detected microcystin variants in freshwater systems and, as this congener is the second most common cyanotoxin in the environment (Díez-Quijada et al., 2019), it merits particular attention in ecological-toxicological studies. Its occurrence at environmentally relevant concentrations, particularly during and after cyanobacterial blooms, raises important questions regarding its impact on coexisting microorganisms. While MC-RR has been extensively studied in terms of its mode of toxicity and accumulation in higher trophic levels, its effects on aquatic primary producers, especially in the context of interspecific interactions, remain underexplored.

Cyanobacteria and green microalgae often coexist and compete for limited resources in eutrophic waters (Suikkanen et al., 2004). The ability of some cyanobacterial species to influence competitors' growth via allelochemical release may confer a competitive advantage, contributing to bloom dominance and persistence. However, responses to such chemical cues can vary widely depending on the target species, their physiological state, and environmental conditions (Li et al., 2023). Shifts in community structure, driven by group-specific environmental sensitivities, can alter elemental cycling at both local and global scales (Litchman et al., 2015).

This study aims to assess the allelopathic potential of MC-RR at environmentally relevant concentrations by evaluating species-specific growth responses in selected cyanobacteria and green microalgae. The findings are expected to contribute to a better understanding of the ecological functions of MCs in shaping the structure of the phytoplankton community.

We hypothesize that MC-RR, at concentrations commonly detected in natural waters, exerts species-specific allelopathic effects on the growth of freshwater phytoplankton. Specifically, exposure to environmentally relevant concentrations of MC-RR will affect the growth rates of cyanobacteria and green microalgae, reflecting species-specific sensitivity. Additionally, we are particularly interested in how cyanobacterial strains that produce MC-RR will respond to the presence of that toxin under experimental conditions. We further posit that cyanobacterial strains will exhibit greater tolerance to MC-RR than green microalgae, suggesting a potential competitive advantage during bloom events.

Experimental

Phytoplankton cultures and cultivation conditions

Chlorella sp., *Scenedesmus* sp., *Nostoc* sp. and *Microcystis* sp. were isolated from a freshwater pond (43°17'51.9"N 21°47'40.8" E) in Southeast Serbia and were cultivated at the Department of Biology and Ecology, Faculty of Science and Mathematics in Niš. They are identified using an identification key to the genus level (Burchadt, 2014). *Trichormus variabilis* 0441 (Kützing ex Bornet & Flahault) (Komárek & Anagnostidis, 1989) (heterotypic synonym *Anabaena variabilis*) was isolated from the Danube River in the Vojvodina region (Serbia). It was cultivated in the Department of Biology and Ecology laboratory in Novi Sad (NSCCC). *T. variabilis* was identified using molecular methods described in the previous study (Stankovic et al., 2022).

All cultures were prepared in 250 ml Erlenmeyer flasks at 24 °C under cool LED lighting (15 W, 6400 K, 1500 lm) with a 16:8 h light: dark cycle and constant aeration. Standard BG11 (Rippka et al., 1979) liquid medium was used to cultivate all species except *A. variabilis* and *Nostoc* sp. These species were cultivated using modified BG11 medium without a nitrogen source.

Exposure conditions to MC-RR and growth influence test

To examine the influence of MC-RR on several phytoplankton species, the effect of different concentrations of MC-RR (1, 5, 10 µg/L) on the growth rate of *A. variabilis*, *Microcystis* sp., *Nostoc* sp., *Chlorella* sp., *Scenedesmus* sp., within two weeks was monitored. Investigated concentrations of MC-RR are based on their ecological relevance: they span from the low-level World Health Organization drinking-water guideline (~1 µg L^{-1}, set for MC-LR) through concentrations commonly encountered during cyanobacterial blooms (5–10 µg L^{-1}). This range allows a clear characterization of dose–response effects, while noting that MC-RR is generally considered to be less toxic than MC-LR. In the experiment, in the sterile glass test tubes 10 ml of liquid medium BG11 were added, 2 ml of a culture of each strain individually, and a specific concentration of MC-RR. Each concentration for each tested strain was set up in triplicate, as were the controls (9 replicates for each species). Controls (without MC-RR) were monitored for assay validity. All tubes were incubated at 21℃ under cool LED lighting for a 16-h photoperiod for 14 days. Phytoplankton growth was monitored spectrophotometrically to determine chlorophyll concentration. In the first seven days the measurements were taken daily to capture the rapid initial growth and transition into the exponential phase; thereafter a single measurement was taken at day

14 to characterize the stationary/plateau phase of the growth curve. During the two weeks of the experiment, 1 ml of the culture, previously vortexed, was placed in cuvettes, and the optical density at 678, 720, and 750 nm was determined spectrophotometrically (Shimadzu UV-1650PC, double-beam). The concentration of chlorophyll *a* was calculated according to the following equation (Stankovic, 2020):

$$Chl\ (\text{m}g\times ml\text{-}1) = 14.96 \cdot (OD678 - OD750) - 0{,}616 \cdot (OD720 - OD750) \qquad \text{(Eq. 1)}$$

MC-RR detection and quantification

To assess whether *T. variabilis*, *Nostoc* sp., and *Microcystis* sp. produce the cyanotoxin MC-RR, 500 mg of lyophilized biomass were extracted with 5 mL of a solvent mixture (75% methanol, 25% water). The suspension was sonicated in an ultrasonic bath for 30 minutes to ensure efficient cell disruption. Following ultrasonication, the samples were centrifuged at 4000 rpm for 10 minutes at 20 °C, and the obtained supernatant was filtered through 0.22 μm membrane filters (Agilent), according to the method described by Minasyan et al. (2018).

Toxin detection and characterization were performed using an Agilent 1200 Series HPLC system (Agilent Technologies, USA), equipped with a photodiode array (DAD) detector, an autosampler, a binary pump, and ChemStation software. Chromatographic separation was achieved on a Supelcosil ABZ Plus analytical column (Supelco, 150 × 4.6 mm, 5 μm). The mobile phases consisted of water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% trifluoroacetic acid (TFA). A linear gradient from 20% to 80% of solvent B was applied over 30 minutes, at a flow rate of 1 mL min^{-1}, with a column temperature of 40 °C and an injection volume of 10 µL. UV absorbance spectra were recorded in the 190–300 nm range using the DAD detector. Toxin identification and quantification were carried out by comparison with certified MC-RR standards (LGC, Germany).

Statistical Analyses

Statistical data processing was performed by STATISTICA 8 software (Statsoft, Inc., Tulsa, OK, USA). An independent t-test was used to determine the significance of the difference in mean values between groups, with statistical significance set at $p < 0.05$.

Results and Discussion

In this study, we observed that exposure to low concentrations of MC-RR (1 µg/L and 5 µg/L) did not lead to any measurable changes in the growth rates of the five tested phytoplankton strains, three cyanobacteria and two green microalgae, compared to control groups. On the other hand, exposure to a concentration of 10 µg/L of the same toxin resulted in a detectable alteration in the growth rate of certain tested strains. Accordingly, only the effect of the 10 µg/L concentration was graphically analysed in the subsequent sections.

Cyanobacterial strains and their responses

The growth dynamics of the three cyanobacterial strains were then investigated in detail to assess the effect of the toxin treatment (Figure 1).

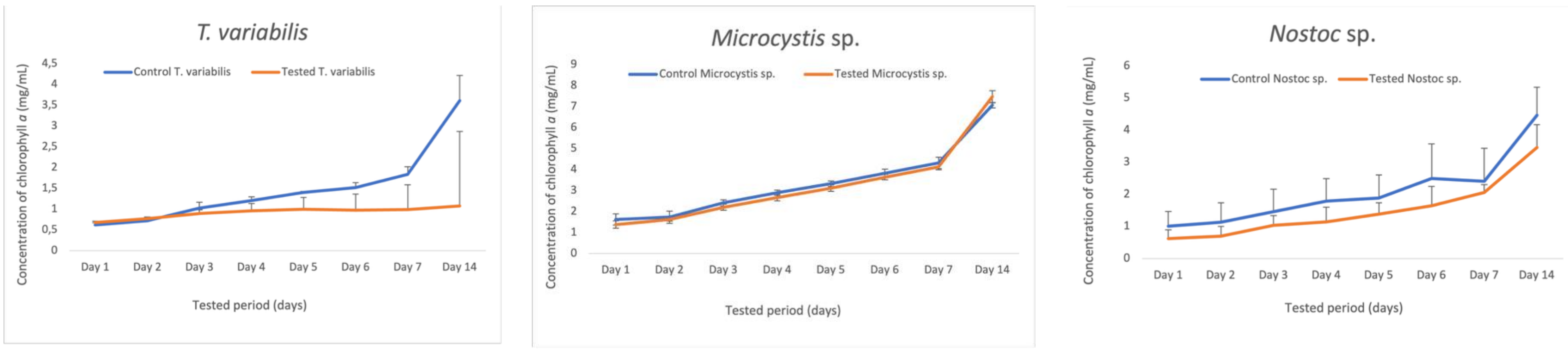

Figure 1. Spectrophotometric comparison of the growth of tested cyanobacteria exposed to MC-RR (10 µg/L), compared to the growth of the same strains in control conditions. Mean chlorophyll-a values (mg/mL) are shown for eight consecutive days (n = 3). Positive error bars represent +SD above the mean

Statistical analysis revealed a significant inhibition of growth in *T. variabilis* from day 5 until the end of the 14-day experiment when exposed to the cyanotoxin MC-RR at a concentration of 10 µg/L (Figure 2). Lower concentrations (1 µg/L and 5 µg/L) produced no measurable changes compared with the control. In contrast, for *Nostoc sp.* and *Microcystis sp.*, the presence of dissolved MC-RR at all three tested concentrations (1, 5, and 10 µg/L) had no significant effect on growth throughout the experimental period. Both strains exhibited growth trajectories similar to their controls, indicating high tolerance to the applied toxin.

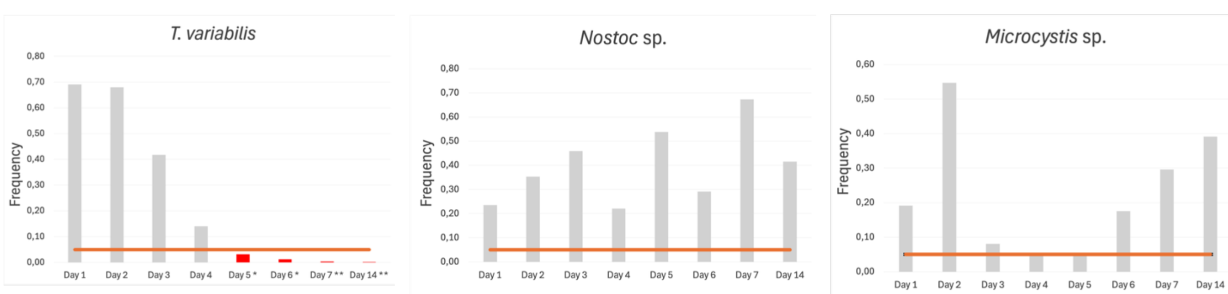

Figure 2. Bars represent the values for daily comparisons between the Control and MC-RR treatments, with significant inhibition highlighted in red. The horizontal line denotes the significance threshold at $p = 0.05$. A single asterisk (*) indicates $p < 0.05$, two asterisks (**) indicate $p < 0.01$, and three asterisks (***) indicate $p < 0.001$

Quantification of intracellular MC-RR by HPLC-DAD confirmed intracellular toxin production in all three cyanobacteria (Figure 3). The measured intracellular concentrations were 16.62 µg/g in *T. variabilis*, 8.13 µg/g in *Nostoc sp.*, and 4.95 µg/g in *Microcystis sp.* These findings suggest that the strains naturally produce MC-RR, reflecting their underlying genetic capacity for its biosynthesis.

The inhibitory response of *T. variabilis* to 10 µg/L MC-RR contrasts with the tolerance observed in *Nostoc sp.* and *Microcystis sp.*, suggesting species-specific sensitivity and possibly differences in detoxification capacity or cellular uptake of the toxin. Previous studies have indicated that cyanobacteria capable of producing MCs often exhibit self-protection mechanisms, including regulated export via ABC transporters, sequestration within specific compartments, and glutathione-dependent conjugation that prevent intracellular accumulation of harmful concentrations (Zilliges et al., 2011; Wei et al., 2024).

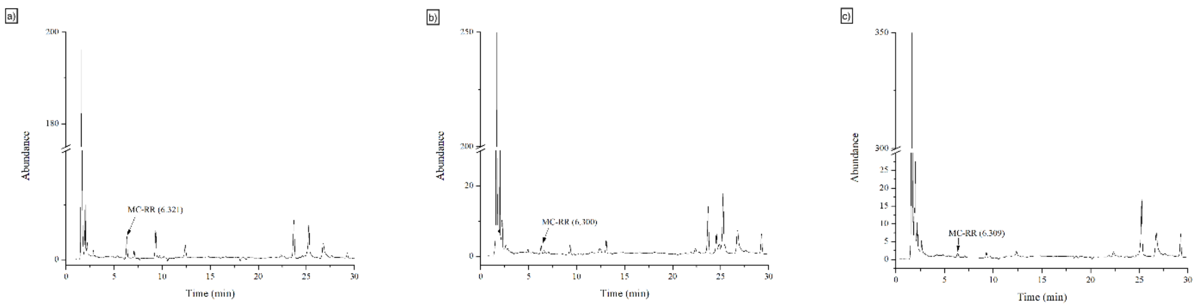

Figure 3. Chromatograms of detected and quantified MC-RR in tested Cyanobacteria (a- *Microcystis* sp., b-*Nostoc* sp., c-*T. variabilis*)

The growth inhibition detected in *T. variabilis* at the highest concentration may indicate that exogenous MC-RR exceeded its physiological tolerance threshold, disrupting protein phosphatase activity, photosynthetic efficiency, or redox homeostasis. MCs, including MC-RR, are known to bind and inhibit serine/threonine protein phosphatases 1 and 2A (MacKintosh et al., 1990; Žegura et al., 2011), thereby impairing signal transduction and increasing reactive oxygen species (ROS) formation. Such stress responses could explain the statistically significant suppression of growth observed after day 5.

By contrast, *Nostoc* sp. and *Microcystis* sp. showed no measurable inhibition, likely reflecting a higher basal tolerance or more efficient detoxification of MC-RR. Both genera are well-known microcystin producers and may sustain growth even in environments containing dissolved toxins (Kaplan et al., 2012; Hu & Rzymski, 2019). This insensitivity to environmentally relevant concentrations (~ 10 µg/L) supports the hypothesis that cyanobacteria are generally resilient to their own toxins, while they possess a genetic background to produce specific toxins. The quantification of intracellular MC-RR in tested cyanobacteria further supports this interpretation. Our findings of strain-specific MC-RR production are consistent with earlier studies indicating that cell quotas may vary by more than an order of magnitude depending on nutrient status, light intensity, and growth phase (Christiansen et al., 2003, Wood et al., 2021).

Taken together, our findings indicate that *T. variabilis* is moderately sensitive to externally applied MC-RR at 10 µg/L, whereas *Nostoc* sp. and *Microcystis* sp. are largely unaffected. All three strains are confirmed MC-RR producers, which implies that physiological regulation of intracellular and extracellular toxin pools plays a central role in determining their tolerance. Similarly, a previous study (Li et al., 2009) showed that MCs can trigger stress responses even in their producer cyanobacteria, as MC-RR induces antioxidant enzyme genes (*sodB*, *katG*) and the heat-shock protein gene *dnaK2* (encoded for Hsp70) in *Synechocystis*. Also, proteomic analyses in *Microcystis aeruginosa* reveal changes in protein profiles linked to toxin production, suggesting a multifaceted regulation of intracellular toxin pools (Alexova et al., 2011; Tonietto et al., 2012). These results highlight the complexity of cyanobacterial interactions with dissolved MCs and underscore that toxin effects are both strain and concentration dependent.

It should be noted that the applied MC-RR concentration in our experiment was measured independently of the live cultures, and we did not monitor real-time synthesis, uptake, efflux, or degradation of MC-RR by the cells during the experiment. Therefore, we cannot exclude the possibility that extracellular and intracellular MC-RR levels changed over the course of the experiment in ways that influenced our observations. For example, cyanobacteria may retain, sequester, or re-bind MCs, or conversely release them under stress or via lysis — dynamics that could modulate toxicity and physiological responses. Indeed, previous studies suggest that microcystin export and protein-bound pools can vary across the growth cycle, indicating complex regulation of toxin partitioning (Wei et al., 2016; Greenstein et al., 2020).

Microalgal strains and their responses

Over a 14-day period, exposure of the green microalgae *Scenedesmus* sp. and *Chlorella* sp. to MC-RR at three concentrations revealed that only 10 µg/L elicited a stimulatory effect on growth (Figure 4). For both microalgae species, the lower concentrations (1 µg/L and 5 µg/L) did not produce statistically significant differences in growth compared to controls over the 14-day period. In contrast, at 10 µg/L, the response diverged. For Scenedesmus sp., a statistically significant stimulation of growth was observed from day 5 onward, and for *Chlorella* sp., stimulation reached significance only at day 14 (Figure 5). Thus, MC-RR at 10 µg/L triggered enhanced biomass production in both tested green microalgae under the culture conditions used.

The stimulatory effect of MC-RR on these green microalgae at 10 µg/L, and the absence of effect at 1 and 5 µg/L, indicate a threshold effect and a non-linear dose-response. That is, below about 10 µg/L, no detectable response occurred, but at this level, the microalgae responded by increasing growth. This pattern is consistent with the phenomenon of hormesis, where a low dose of some environmental agent can cause low stimulation or have a beneficial effect on certain cells, while a high dose can cause inhibitory or toxic effects (Mattson, 2007).

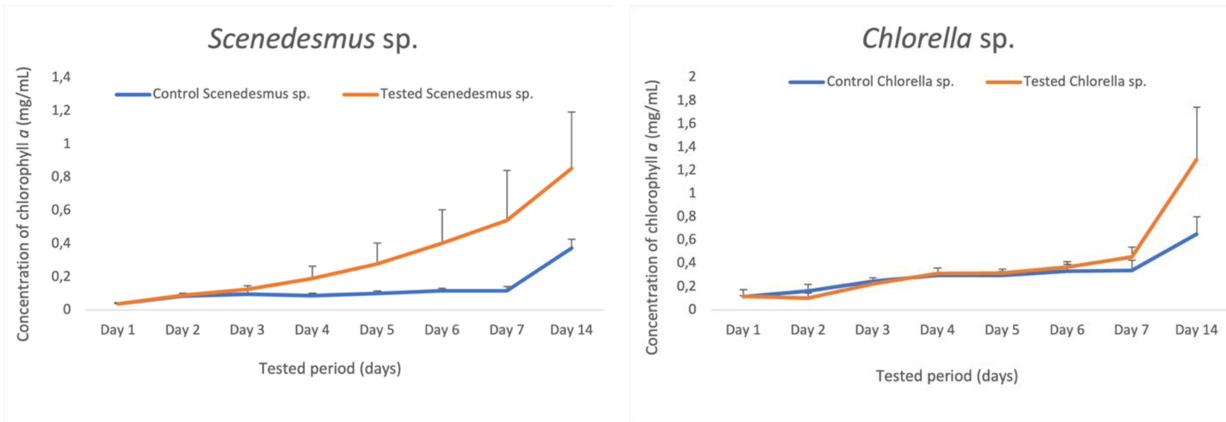

Figure 4. Spectrophotometric comparison of the growth of tested green microalgae exposed to MC-RR (10 µg/L), compared to the growth of the same strains in control conditions. Mean chlorophyll a values (mg/mL) are shown for eight consecutive days (n = 3). Positive error bars represent +SD above the mean. The control culture shows a continuous increase in chlorophyll a content, whereas the MC-RR treatment exhibits reduced growth throughout the experiment

In the literature, the effects of MCs on green algae are variable and often inhibitory. Most previous work on green algae and MCs (especially the variant Microcystin-LR, MC-LR) has focused on inhibitory responses at high doses. For example, exposure of *Chlorella vulgaris* to MC-LR at hundreds of µg/L caused growth inhibition and oxidative stress (Campos et al., 2013; Teneva et al., 2023; Li et al., 2024). However, a review found that some green algae respond to pure cyanotoxins (MC-LR at 5 g/L) or allelopathic compounds under certain conditions with stimulation rather than inhibition (Teneva et al., 2013). The variant MC-RR is less frequently studied than MC-LR, and its specific interactions with phytoplankton remain underexplored.

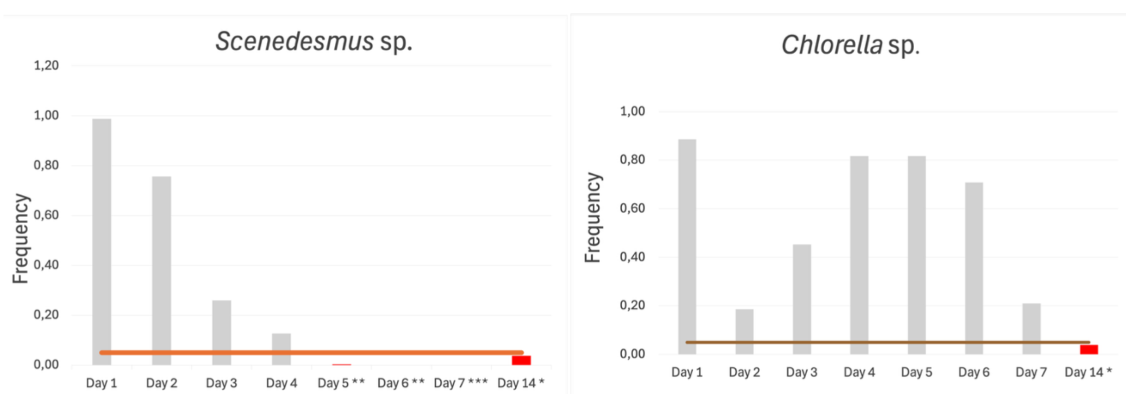

Figure 5. Bars represent the values for daily comparisons between the Control and MC-RR treatments, significant inhibition highlighted in red. The horizontal line denotes the significance

threshold at $p = 0.05$. A single asterisk (*) indicates $p < 0.05$, two asterisks (**) indicate $p < 0.01$, and three asterisks (***) indicate $p < 0.001$

The absence of any effect at 1 and 5 µg/L suggests that these concentrations are below the physiological detection or activation threshold of the microalgae under our culture conditions. The switch to significant stimulation at 10 µg/L is consistent with already mentioned hormetic type dose–response.

The difference in timing between the two species likely reflects intrinsic physiological or ecological differences: *Scenedesmus sp.* responded early (day 5) whereas *Chlorella sp.* only showed stimulation at day 14. This may reflect differences in growth rate, capacity to detect or metabolize MC-RR, or differential regulatory or adaptive mechanisms triggered by toxin exposure.

Our result is consistent with the notion that the effects of dissolved MCs may be species-specific, and that not all phytoplankton will respond adversely at low toxin levels. For instance, Babica et al. (2007) reported that microcystin effects vary greatly depending on both the phytoplankton species and the specific microcystin variant tested; in their study, some species showed resilience at low concentrations. Similarly, Sedmak et al. (2005) demonstrated morphological and physiological changes under microcystin exposure in representative phytoplankton, but typically at higher concentrations than those tested here.

Conclusion

These findings demonstrate the allelopathic potential of MC-RR, with species-specific effects on growth, reflecting differential sensitivity among phytoplankton taxa. The emergence of significant growth alteration at 10 µg/L suggests that this concentration may represent a biologically relevant threshold in ecosystems experiencing blooms. Monitoring concentrations approaching this level should be treated as potentially harmful to sensitive phytoplankton taxa.

Our experimental data demonstrate that while all tested cyanobacterial strains produce MC-RR, their tolerance to externally applied MC-RR varies considerably among strains. Whereas *Nostoc* sp. and *Microcystis* sp. tolerated up to 10 µg/L without inhibitory effects, *T. variabilis* suffered significant growth inhibition at the same concentration. Thus, the hypothesis that MC-RR-producing cyanobacteria will generally exhibit greater tolerance than green microalgae is only partially supported and appears to be highly strain-specific rather than a universal trait. Meanwhile, the green microalgae *Scenedesmus* sp. and *Chlorella* sp. displayed a stimulatory response to 10 µg/L MC-RR, suggesting that under certain conditions MC-RR may even enhance growth in non-cyanobacterial taxa. Results underscore the role of this toxin in influencing phytoplankton community structure and interactions in freshwater ecosystems.

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Conflict-of-Interest Statement

The authors declare no conflict of interest.

References

Alexova, R., Haynes, P. A., Ferrari, B. C., & Neilan, B. A. (2011). Comparative protein expression in different strains of the bloom-forming cyanobacterium *Microcystis aeruginosa*. *Molecular & Cellular Proteomics, 10*(9), M110.003749. https://doi.org/10.1074/mcp.M110.003749

Babica, P., Hilscherová, K., Bártová, K., Bláha, L., & Maršálek, B. (2007). Effects of dissolved microcystins on growth of planktonic photoautotrophs. *Phycologia, 46*(2), 137–142. https://doi.org/10.2216/06-24.1

Campos, A., Araújo, P., Pinheiro, C., Azevedo, J., Osório, H., & Vasconcelos, V. (2013). Effects on growth, antioxidant enzyme activity and levels of extracellular proteins in the green alga *Chlorella vulgaris* exposed to crude cyanobacterial extracts and pure microcystin and cylindrospermopsin. *Ecotoxicology and Environmental Safety, 94*, 45–53. https://doi.org/10.1016/j.ecoenv.2013.04.019

Christiansen, G., Fastner, J., Erhard, M., Börner, T., & Dittmann, E. (2003). Microcystin biosynthesis in *Planktothrix*: Genes, evolution, and manipulation. *Journal of Bacteriology, 185*(2), 564–572. https://doi.org/10.1128/jb.185.2.564-572.2003

Cheng, F., & Cheng, Z. (2015). Research progress on the use of plant allelopathy in agriculture and the physiological and ecological mechanisms of allelopathy. *Frontiers in Plant Science, 6*, 1020. https://doi.org/10.3389/fpls.2015.01020

Díez-Quijada, L., Prieto, A. I., Guzmán-Guillén, R., Jos, A., & Cameán, A. M. (2019). Occurrence and toxicity of microcystin congeners other than MC-LR and MC-RR: A review. *Food and Chemical Toxicology, 125*, 106–132. https://doi.org/10.1016/j.fct.2018.12.042

Díez-Quijada, L., Puerto, M., Gutiérrez-Praena, D., Llana-Ruiz-Cabello, M., Jos, A., & Cameán, A. M. (2019). Microcystin-RR: Occurrence, content in water and food, and toxicological studies — A review. *Environmental Research, 168*, 467–489. https://doi.org/10.1016/j.envres.2018.07.019

Falkowski, P. G. (1994). The role of phytoplankton photosynthesis in global biogeochemical cycles. *Photosynthesis Research, 39*(3), 235–258. https://doi.org/10.1007/BF00014586

Gan, N., Xiao, Y., Zhu, L., Wu, Z., Liu, J., Hu, C., & Song, L. (2012). The role of microcystins in maintaining colonies of bloom-forming *Microcystis* spp. *Environmental Microbiology, 14*(3), 730–742. https://doi.org/10.1111/j.1462-2920.2011.02624.x

Graham, L. E., Graham, J. M., & Wilcox, L. W. (2009). *Algae*. Pearson/Benjamin Cummings.

Gross, E. M. (2003). Allelopathy of aquatic autotrophs. *Critical Reviews in Plant Sciences, 22*(3–4), 313–339. https://doi.org/10.1080/713610859

Greenstein, K. E., Zamyadi, A., Glover, C. M., Adams, C., Rosenfeldt, E., & Wert, E. C. (2020). Delayed release of intracellular microcystin following partial oxidation of cultured and naturally occurring cyanobacteria. *Toxins, 12*(5), Article 335. https://doi.org/10.3390/toxins12050335

Hu, C., & Rzymski, P. (2019). Programmed cell death-like and accompanying release of microcystin in freshwater bloom-forming cyanobacterium *Microcystis*: From identification to ecological relevance. *Toxins, 11*(12), 706. https://doi.org/10.3390/toxins11120706

Kaplan, A., Harel, M., Kaplan-Levy, R. N., Hadas, O., Sukenik, A., & Dittmann, E. (2012). The languages spoken in the water body (or the biological role of cyanobacterial toxins). *Frontiers in Microbiology, 3*, 138. https://doi.org/10.3389/fmicb.2012.00138

Kosek, K., Polkowska, Ż., Żyszka, B., & Lipok, J. (2016). Phytoplankton communities of polar regions – Diversity depending on environmental conditions and chemical anthropopressure. *Journal of Environmental Management, 171*, 243–259. https://doi.org/10.1016/j.jenvman.2016.01.026

Leão, P. N., Pereira, A. R., Liu, W., Ng, J., Pevzner, P. A., Dorrestein, P. C., König, G. M., Vasconcelos, V. M., & Gerwick, W. H. (2010). Synergistic allelochemicals from a freshwater cyanobacterium. *Proceedings of the National Academy of Sciences of the United States of America, 107*(25), 11183–11188. https://doi.org/10.1073/pnas.0914343107

Li, H., Xie, P., Zhang, D., & Chen, J. (2009). The first study on the effects of microcystin-RR on gene expression profiles of antioxidant enzymes and heat shock protein-70 in *Synechocystis* sp. PCC 6803. *Toxicon, 53*(6), 595–601. https://doi.org/10.1016/j.toxicon.2008.11.005

Li, J., Xiao, X., Xian, X., Li, S., Yu, X., & Zhang, X. (2023). Green algae outcompete cyanobacteria in a shallow lake, Longhu Lake. *Water Supply, 23*(7), 2649–2661. https://doi.org/10.2166/ws.2023.154

Li, Z., Zheng, Y., Ma, H., & Cui, F. (2024). Microcystin-LR (MC-LR) inhibits green algae growth by regulating antioxidant and photosynthetic systems. *Harmful Algae, 134*, 102623. https://doi.org/10.1016/j.hal.2024.102623

Litchman, E., de Tezanos Pinto, P., Edwards, K. F., Klausmeier, C. A., Kremer, C. T., & Thomas, M. K. (2015). Global biogeochemical impacts of phytoplankton: A trait-based perspective. *Journal of Ecology, 103*, 1384–1396. https://doi.org/10.1111/1365-2745.12438

Martínez-Piernas, A. B., Badagian, N., Brena, B. M., Pérez-Parada, A., & García-Reyes, J. F. (2025). Identification and occurrence of microcystins in freshwaters and fish from a eutrophic dam through LC-HRMS. *Science of the Total Environment, 959*, 178230. https://doi.org/10.1016/j.scitotenv.2024.178230

Mattson, M. P. (2008). Hormesis defined. *Ageing Research Reviews, 7*(1), 1–7. https://doi.org/10.1016/j.arr.2007.08.007

Minasyan, A., Christophoridis, C., Wilson, A. E., Zervou, S.-K., Kaloudis, T., & Hiskia, A. (2018). Diversity of cyanobacteria and the presence of cyanotoxins in the epilimnion of Lake Yerevan (Armenia). *Toxicon, 150*, 28–38. https://doi.org/10.1016/j.toxicon.2018.04.003

Omidi, A., Pflugmacher, S., Kaplan, A., Kim, Y. J., & Esterhuizen, M. (2021). Reviewing interspecies interactions as a driving force affecting the community structure in lakes via cyanotoxins. *Microorganisms, 9*(8), 1583. https://doi.org/10.3390/microorganisms9081583

Paerl, H. W., & Otten, T. G. (2013). Harmful cyanobacterial blooms: Causes, consequences, and controls. *Microbial Ecology, 65*, 995–1010. https://doi.org/10.1007/s00248-012-0159-y

Perron, M.-C., Qiu, B., Boucher, N., Bellemare, F., & Juneau, P. (2012). Use of chlorophyll a fluorescence to detect the effect of microcystins on photosynthesis and photosystem II energy fluxes of green algae. *Toxicon, 59*(5), 567–577. https://doi.org/10.1016/j.toxicon.2011.12.005

Polyak, Y. M., & Sukharevich, V. I. (2025). Allelopathic properties of cyanobacteria (review). *Inland Water Biology, 18*, 565–574. https://doi.org/10.1134/S1995082925600358

Revillini, D., David, A. S., Reyes, A. L., Knecht, L. D., Vigo, C., Allen, P., Searcy, C. A., & Afkhami, M. E. (2023). Allelopathy-selected microbiomes mitigate chemical inhibition of plant performance. *New Phytologist, 240*(5), 2007–2019. https://doi.org/10.1111/nph.19249

Saleem, A., Anwar, S., Saud, S., et al. (2025). Cyanobacteria diversity and ecological roles:

Insights into cyanobacterial adaptations and environmental implications. *Journal of Umm Al-Qura University Applied Sciences*. https://doi.org/10.1007/s43994-025-00261-2

Schirrmeister, B. E., Gugger, M., & Donoghue, P. C. J. (2015). Cyanobacteria and the Great Oxidation Event: Evidence from genes and fossils. *Palaeontology, 58*, 769–785. https://doi.org/10.1111/pala.12178

Sedmak, B., & Eleršek, T. (2005). Microcystins induce morphological and physiological changes in selected representative phytoplanktons. *Microbial Ecology, 50*(2), 298–305. https://doi.org/10.1007/s00248-004-0189-1

Srimongkol, P., Sangtanoo, P., Songserm, P., Watsuntorn, W., & Karnchanatat, A. (2022). Microalgae-based wastewater treatment for developing economic and environmental sustainability: Current status and future prospects. *Frontiers in Bioengineering and Biotechnology, 10*, 904046. https://doi.org/10.3389/fbioe.2022.904046

Stankovic, R. N. (2020). Phytoplankton influence on benthic macroinvertebrates of freshwater ecosystems in multistress conditions: laboratory testing of the toxic effect of cyanobacteria and green microalgae on individuals of the species Chironomus riparius. Doctoral dissertation. University of Niš, Faculty of Mathematics and Natural Siences

Stanković, N., Jovanović, B., Kokić, I. K., Piperac, M. S., Simeunović, J., Jakimov, D., Dimkić, I., & Milošević, D. (2022). Toxic effects of a cyanobacterial strain on Chironomus riparius larvae in a multistress environment. Aquatic Toxicology, 253, 106321.

Suikkanen, S., Fistarol, G. O., & Granéli, E. (2004). Allelopathic effects of the Baltic cyanobacteria *Nodularia spumigena*, *Aphanizomenon flos-aquae* and *Anabaena lemmermannii* on algal monocultures. *Journal of Experimental Marine Biology and Ecology, 308*(1), 85–101. https://doi.org/10.1016/j.jembe.2004.02.012

Teneva, I., Velikova, V., Belkinova, D., Moten, D., & Dzhambazov, B. (2023). Allelopathic potential of the cyanotoxins microcystin-LR and cylindrospermopsin on green algae. *Plants, 12*(6), 1403. https://doi.org/10.3390/plants12061403

Tonietto, Â., Petriz, B. A., Araújo, W. C., Mehta, Â., Magalhães, B. S., & Franco, O. L. (2012). *Comparative proteomics between natural Microcystis isolates with a focus on microcystin synthesis*. *Proteome Science, 10*, Article 38. https://doi.org/10.1186/1477-5956-10-38

Wei, N., Hu, L., Song, L., & Gan, N. (2016). Microcystin-Bound Protein Patterns in Different Cultures of *Microcystis aeruginosa* and Field Samples. *Toxins*, *8*(10), 293. https://doi.org/10.3390/toxins8100293

Willis, R. J. (2007). *The history of allelopathy*. Springer. https://doi.org/10.1007/978-1-4020-4093-1

Wei, N., Hu, C., Dittmann, E., Song, L., & Gan, N. (2024). The biological functions of microcystins. *Water Research, 262*, 122119. https://doi.org/10.1016/j.watres.2024.122119

Whitton, B. A., & Potts, M. (2012). Introduction to the cyanobacteria. In B. A. Whitton (Ed.), *Ecology of Cyanobacteria II* (pp. 1–13). Springer, Dordrecht. https://doi.org/10.1007/978-94-007-3855-3_1

Wood, S. A., Puddick, J., Hawes, I., Steiner, K., Dietrich, D. R., & Hamilton, D. P. (2021). Variability in microcystin quotas during a *Microcystis* bloom in a eutrophic lake. *PLoS ONE, 16*(7), e0254967. https://doi.org/10.1371/journal.pone.0254967

Yarnold, J., Karan, H., Oey, M., & Hankamer, B. (2019). Microalgal aquafeeds as part of a circular bioeconomy. *Trends in Plant Science, 24*(10), 959–970. https://doi.org/10.1016/j.tplants.2019.06.005

Zanchett, G., & Oliveira-Filho, E. C. (2013). Cyanobacteria and cyanotoxins: From impacts on aquatic ecosystems and human health to anticarcinogenic effects. *Toxins (Basel), 5*(10), 1896-1917. https://doi.org/10.3390/toxins5101896

Zilliges, Y., Kehr, J.-C., Mikkat, S., Bouchard, J., de Marsac, N. T., Börner, T., … & Dittmann, E. (2011). The cyanobacterial hepatotoxin microcystin binds to proteins and increases the fitness of *Microcystis* under oxidative stress. *Science, 334*(6062), 81–85. https://doi.org/10.1126/science.1208583

Žegura, B., Štraser, A., & Filipič, M. (2011). Genotoxicity and potential carcinogenicity of cyanobacterial toxins – A review. *Mutation Research/Reviews in Mutation Research, 727*(1–2), 16–41. https://doi.org/10.1016/j.mrrev.2011.01.002

Śliwińska-Wilczewska, S., Wiśniewska, K., Budzałek, G., & Konarzewska, Z. (2022). Phenomenon of allelopathy in cyanobacteria. In *Cyanobacterial Biology in Freshwater and Marine Systems* (pp. —). Springer. https://doi.org/10.1007/978-981-16-4873-1_11

Śliwińska-Wilczewska, S., Wiśniewska, K., Konarzewska, Z., Cieszyńska, A., Barreiro Felpeto, A., Lewandowska, A. U., & Latała, A. (2021). The current state of knowledge on taxonomy, modulating factors, ecological roles, and mode of action of phytoplankton allelochemicals. *Science of the Total Environment, 773*, 145681. https://doi.org/10.1016/j.scitotenv.2021.145681

Allelopatski potencijal mikrocistina-RR pri ekološki relevantnim koncentracijama: vrste-specifični odgovori fitoplanktona na rast

Tekući naslov: Specifični odgovori fitoplanktona na mikrocistin-RR

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SAŽETAK

Mikrocistin-RR (MC-RR), cijanotoksin koji se često detektuje u slatkovodnim ekosistemima, može uticati na dinamiku fitoplanktona menjajući obrasce rasta koegzistirajućih vrsta. Iako je veliki broj radova proučavao alelopatske efekte mikrocistina-LR (MC-LR) i drugih varijanti mikrocistina, relativno je malo studija koje se posebno bave uticajem čistog MC-RR na fitoplanktonske vrste, naročito onih koje same proizvode ovaj toksin, pri ekološki relevantnim koncentracijama. Ova studija ispitivala je efekte ekološki relevantnih koncentracija MC-RR (1, 5 i 10 µg L^{-1}) na rast pet vrsta fitoplanktona: tri vrste cijanobakterija (*Trichormus variabilis*, *Nostoc* sp., *Microcystis* sp.) i dve vrste zelenih mikroalgi (*Chlorella* sp., *Scenedesmus* sp.) u laboratorijskim uslovima. Odgovori u rastu praćeni su spektrofotometrijski određivanjem koncentracije hlorofila tokom perioda od 14 dana. Pored toga, MC-RR identifikovan je i kvantifikovan u tretiranim kulturama cijanobakterija korišćenjem HPLC-DAD tehnike. Rezultati su pokazali vrste-specifične odgovore. MC-RR je ispoljio stimulativni efekat na obe vrste zelenih algi Suprotno tome, *T. variabilis* je pokazao progresivnu inhibiciju rasta, koja je postala statistički značajna nakon petog dana. *Nostoc* sp. je pokazao blagu, ali neznačajnu inhibiciju, dok je *Microcystis* sp. ispoljio toleranciju na izlaganje MC-RR. Ova saznanja ukazuju na alelopatski potencijal MC-RR, sa vrstama specifičnim efektima na rast koji odražavaju različitu osetljivost među fitoplanktonskim taksonima. Rezultati naglašavaju ekološki značaj ovog toksina u oblikovanju strukture fitoplanktonskih zajednica i interakcija vrsta u slatkovodnim ekosistemima.

Ključne reči: cijanotoksin, mikrocistin-RR, alelopatija, fitoplankton, mikrobna zajednica

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Urbanization and Industrialization in the Nišava District: A GIS Analysis of River Network Change (1983–2050)

Running title: Urbanization Impacts on River Networks in the Nišava District

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ABSTRACT

Urbanization and industrialization have significantly transformed the hydrological systems of southern Serbia, especially in the Nišava and South Morava basins and the city of Niš. Over the past decades, rapid urban growth and industrial activity have altered river morphology, reduced the permanence of tributaries, and reshaped drainage networks. Using GIS and Remote Sensing methods, including high-resolution DEMs, census data, and cartographic archives, this study analyzes urban–river interactions from 1983 to 2023, with projections to 2050.Results show that Niš, strategically located along the Nišava and South Morava corridors, has experienced substantial urban expansion, leading to increased surface runoff, erosion risks, and degradation of riparian ecosystems. The Nišava River has undergone fragmentation of its natural dendritic system, while the South Morava has become a central axis of metropolitan and industrial development. Projections to 2050 highlight intensified risks of floodplain encroachment, declining groundwater recharge, and further deterioration of water quality. The findings emphasize the urgent need for integrated basin management and sustainable urban planning in Niš and its surrounding river valleys. Balancing economic growth with ecological resilience is essential to preserve hydrological stability and ensure long-term sustainability in the region.

Keywords: South Morava River, Nišava River, Niš, Urbanization, GIS analysis, Pollution

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Introduction

Urbanization and industrialization represent two of the most powerful agents of landscape transformation, fundamentally altering hydrological systems and reshaping ecological processes across the globe. Their influence extends beyond built-up environments to encompass entire river basins, where land-cover transitions, increased imperviousness, and intensified anthropogenic activity interact to redefine runoff dynamics, channel morphology, and ecosystem functioning. In transitional economies such as Serbia, these processes are highly concentrated along river corridors, where settlements, industries, and transport routes converge to form critical axes of development (Tomsett and Leyland, 2019; Grill et al., 2015; Shen and Wang, 2023). The Nišava District in southern Serbia epitomizes this interaction: strategically located at the intersection of the Nišava and South Morava valleys and anchored by the city of Niš, it functions as a vital economic, demographic, and ecological corridor linking Central Europe with the Aegean basin. Over the past four decades, this district has experienced rapid urban growth, industrial intensification, and infrastructural expansion, producing far-reaching consequences for river morphology, drainage density, and long-term hydrological resilience.

River networks serve not only as carriers of water, sediments, and nutrients but also as ecological lifelines that sustain biodiversity, agricultural productivity, and groundwater recharge. However, in areas undergoing accelerated land-use change, these networks become vulnerable to fragmentation, simplification, and hydromorphological instability. In the Nišava District, urban expansion has reduced tributary permanence, narrowed riparian zones, and intensified surface runoff, while industrial operations have reshaped sediment loads, modified channel beds, and heightened local flood risks (Du et al., 2019; Zhao et al., 2020; Andersen et al., 2021). Such patterns echo global observations where urban corridors increasingly overlap with ecologically fragile basins, raising concerns about the sustainability of water resources and the capacity of rivers to maintain their natural resilience under sustained anthropogenic pressure. Projections to 2050 indicate that without proactive conservation and regulation, urban encroachment into floodplains and secondary valleys of the Nišava and South Morava will heighten ecological degradation, accelerate channel incision or aggradation, and further constrain groundwater recharge (Redman et al., 2004; Fourqurean et al., 1999).

Previous national-scale studies have emphasized the role of industrial-urban corridors along the main Danube–Sava–Morava axis, where heavy industries, metropolitan expansion, and hydropower development collectively reshape river systems (Chen et al., 2023). Yet, the Nišava District, a dynamic zone connecting the Balkans’ northern and southern hydrological systems, remains comparatively underexplored. Its dual identity as both a southern industrial hub and a hydrologically sensitive region makes it particularly vulnerable to cumulative impacts. Niš, as Serbia’s third-largest city, illustrates the tensions between economic growth and ecological vulnerability. Situated directly on the Nišava River and intimately linked to the South Morava basin, Niš has expanded longitudinally along valley corridors, reinforcing its role as a transportation and industrial center. This expansion, however, has increased exposure to erosion, urban flooding, riparian degradation, and disruption of natural hydrological pathways.

Climatic variability further compounds these challenges. Between 2000 and 2023, Serbia experienced multiple severe droughts, accompanied by declining river discharges and shifting seasonal hydrological regimes in key basins such as the Drina and South Morava (Durlević et al., 2024; Valjarević et al., 2020a). In Niš, summer water demand frequently exceeds sustainable extraction thresholds, with per capita consumption surpassing 1,700 m^3 during the warm season compared to recommended sustainable levels of approximately 1,300 m^3. Such imbalances exert pressure on existing supply systems, particularly suburban settlements dependent on shallow aquifers and riverbed springs. Intensifying hydrometeorological extremes, flash floods, heatwaves, droughts, and sudden high-intensity rainfall events underscore the vulnerability of the Nišava and South Morava basins to compounded natural and anthropogenic stressors.

Adding to this complexity is Serbia's chronically insufficient water management infrastructure. More than 85% of the country's wastewater is discharged untreated into river systems, and even major cities lack fully operational wastewater treatment facilities (Takić et al., 2017; Urošev et al., 2022). Niš and its surrounding municipalities face similar deficiencies, with untreated municipal and industrial effluents contributing to deteriorating water quality, altered nutrient balances, and exacerbation of eutrophication in downstream sections of the Nišava. These trends mirror conditions observed in other Balkan and Eastern European basins, where urban and industrial pollution, combined with insufficient environmental governance, heighten the risk to freshwater ecosystems.

To address these urgent challenges, this study integrates Geographic Information Systems (GIS) and Remote Sensing (RS) to examine urban–river interactions in the Nišava District from 1983 to 2023, with projections to 2050. High-resolution Digital Elevation Models (DEMs), digitized topographic archives, census datasets, CORINE land-cover layers, and hydrological models are combined to reconstruct long-term changes in drainage density, tributary permanence, riparian continuity, and basin morphology. Advanced analytical tools zonal statistics, Kernel Density Estimation (KDE), stream order analysis, landscape fragmentation indices, and Multi-Criteria Decision Analysis (MCDA) are applied to identify spatial hotspots of hydrological disruption, quantify tributary loss, evaluate fragmentation severity, and detect correlations between industrial expansion and river connectivity decline (Yu et al., 2023). Together, these methods provide an integrated spatial temporal framework for understanding basin evolution under sustained anthropogenic pressures and for forecasting future vulnerabilities under scenarios of continued demographic, industrial, and infrastructural development.

By focusing on Niš and its surrounding river basins, this research contributes to broader regional and international debates on sustainable urban planning, watershed governance, and ecological resilience in rapidly changing environments. It positions the Nišava District as both a microcosm of Serbia's industrial hydrological dilemmas and a critical test case for integrated basin-scale decision-making. The findings underscore the necessity of balancing economic development with ecological protection, enforcing stricter wastewater and land-use regulations, restoring riparian buffers, and fostering regional cooperation for transboundary water governance (Zwarteveen et al., 2017). Ultimately, the future hydrological stability of the Nišava and South Morava basins will depend on reconciling urban expansion with ecological sustainability ensuring that southern Serbia's rivers continue to serve not only as economic lifelines but also as essential ecological safeguards.

The Nišava District is located in southeastern Serbia and covers an area of approximately 2,729 km². It borders the Pirot, Toplica, Jablanica, and Zaječar districts and extends eastward toward the Bulgarian frontier. The district lies within the South Morava basin, with the Nišava River as its dominant hydrological feature. The city of Niš, one of Serbia's largest urban and industrial centers, is situated at the confluence of the Nišava and the South Morava rivers, making this district a crucial node in regional hydrological, economic, and transport networks. The strategic location along the international Corridor X, connecting Central Europe with the Aegean, further emphasizes the Nišava District's importance as both a transit hub and an area of concentrated urban–industrial development (Dimić et al. 2018; Fig. 1).

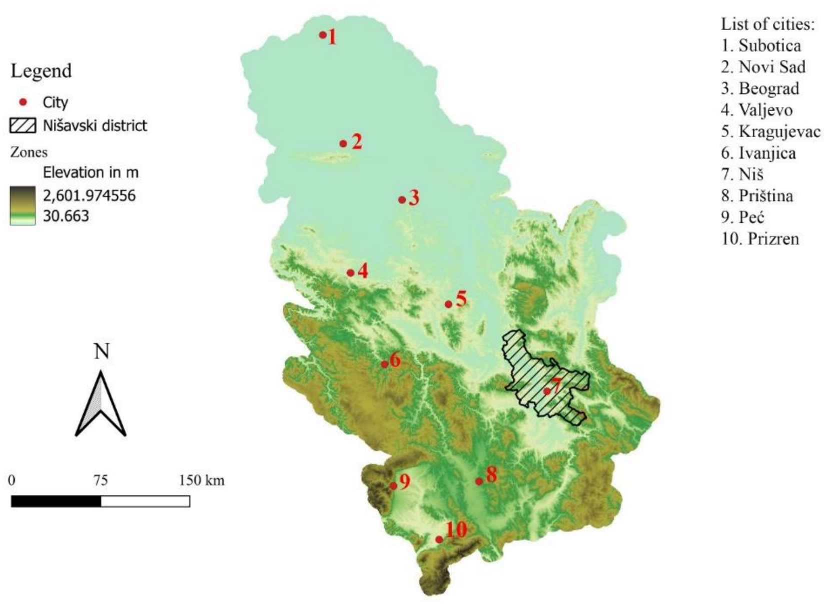

Figure 1. Location of the Nišava District with major urban centers and key physiographic features

This map illustrates the administrative boundaries of the Nišava District in southern Serbia, highlighting its principal urban settlements, major transportation corridors, and the regional river network. The figure provides essential spatial context for understanding the district's role as a strategic corridor for ecological, demographic, and infrastructural connectivity.

Relief is diverse, with elevations ranging from broad alluvial plains in the Nišava and South Morava valleys (180–250 m) to the surrounding mountains, including Suva Planina (1,810 m), the Svrljig Mountains (1,334 m), and Jastrebac (1,491 m). This topographic diversity generates significant spatial contrasts in hydrological regimes. The valley floors accommodate dense urban and industrial activity, while upland zones maintain fragmented rural settlements and forest cover. The climate is predominantly temperate-continental, with hot summers (average July temperatures of 22–24 °C) and moderately cold winters (average January temperatures around 0 °C). Annual precipitation ranges from 600–800 mm in the valleys to over 1,000 mm in mountainous areas. These climatic and geomorphological factors strongly influence runoff, erosion potential, and the vulnerability of local river networks to anthropogenic pressures (Menković et al. 2018; Ilies et al. 2022).

The Nišava River is the main hydrological axis of the district. Originating in western Bulgaria, it flows westward through Dimitrovgrad, Pirot, and Niš before joining the South Morava River. Major tributaries include the Jerma, Temska, and Kutinska rivers, which drain the mountainous terrain and contribute to the basin's dendritic structure. Urban expansion along the Nišava corridor, particularly around the city of Niš, has fragmented the natural fluvial system, altering the permanence of tributaries and increasing surface runoff. The South Morava, into which the Nišava discharges, acts as a collector of industrial effluents and urban wastewater, magnifying cumulative impacts downstream (Kovačević-Majkić and Urošev, 2014).

The Nišava District has experienced accelerated urban growth since the late 20th century, with Niš emerging as the third-largest city in Serbia. Industrial activities include machinery production, electronics, textiles, food processing, and construction materials, much of which is concentrated along river valleys for access to water and transport. The clustering of settlements and factories along the Nišava corridor has produced distinct urban–industrial belts. These belts have increased impervious surfaces, reduced infiltration capacity, and heightened the frequency of flash floods and water quality deterioration. Projections to 2050 suggest further expansion of the Nišava–South Morava urban corridor, reinforcing its role as a metropolitan-industrial hub while simultaneously amplifying hydrological risks (Živanović et al. 2019).

The main environmental challenges in the Nišava District stem from untreated wastewater, riverbank modification, gravel extraction, and deforestation of upland catchments. More than 80% of municipal and industrial wastewater is discharged untreated into rivers, degrading water quality and threatening aquatic biodiversity. Hydropower projects and channelization efforts have also altered natural flow regimes, while climate change intensifies extremes such as droughts, heatwaves, and sudden high-intensity rainfall events. These pressures highlight the Nišava District as both an engine of economic growth and a hydrologically vulnerable region in need of integrated management strategies (Stamenković et al. 2013).

Materials and methods

The methodological framework integrates multi-temporal cartographic, demographic, and remote sensing datasets to analyze the influence of urbanization and industrialization on the river networks of the Nišava District. Historical topographic maps (1:25,000 and 1:50,000 scale) from the Military Geographical Institute were digitized and georeferenced to provide reference conditions for the years 1983, 2000, and 2023. High-resolution Digital Elevation Models (DEMs) derived from the Shuttle Radar Topography Mission (SRTM, 30 m resolution) and Sentinel-1/2 satellite imagery were used to delineate watersheds, extract drainage networks, and identify land-use/land-cover dynamics. Population census data for 1981, 2002, 2011, and 2022 from the Statistical Office of Serbia were rasterized into density surfaces to capture urban expansion trends across the district's municipalities, with emphasis on Niš as the main urban-industrial hub.Industrial activity was mapped from spatial records of major factories, industrial zones, and energy facilities, including historical data on metallurgical, textile, and chemical plants in Niš, Bela Palanka, and Svrljig. These datasets were supplemented with national spatial plans and municipal development strategies to track the spatial evolution of industrial corridors (Zhao et al. 2022).

GIS and Remote Sensing Processing

Hydrological modeling was performed using QGIS 3.28.6 and SAGA GIS. DEMs were preprocessed (sink filling, flow accumulation, and flow direction algorithms) to delineate watersheds, extract stream networks, and calculate slope, aspect, and flow length. The derived hydrographic networks were classified according to Strahler stream order to detect changes in drainage typology, tributary permanence, and fragmentation over time (Valjarević 2025a; Valjarević 2025b)

To assess the interaction between anthropogenic activities and river systems, a buffer analysis was conducted at 500 m, 1 km, and 2 km distances from industrial sites and urban centers. Zonal statistics were applied within these buffers to summarize hydrological attributes such as stream length, drainage density, and slope gradients, enabling a quantitative evaluation of industrial and urban proximity effects.
Kernel Density Estimation (KDE) was used to visualize hydrological "hotspots" where tributary loss, drainage simplification, or river fragmentation coincided with clusters of industrial activity and urban expansion. These heat maps allowed spatial identification of areas most exposed to anthropogenic hydrological stress.

Graduated symbols, choropleth maps, and proportional symbology were used to visualize hydrological change and urban-industrial expansion. Comparative mapping of 1983, 2000, and 2023 allowed temporal tracking of river network modification. Spearman's rank correlation was applied to test the statistical relationship between urban-industrial growth and drainage density decline. Projection scenarios to 2050 were developed using a cellular automata–Markov chain model within SAGA GIS to simulate future land-use dynamics and their potential hydrological implications.

Results and discussion

Between 1983 and 2023, the Nišava District experienced marked urban growth concentrated around the city of Niš. Population density maps derived from census data revealed that Niš expanded from a compact urban core into a sprawling metropolitan area, stretching along the Nišava and South Morava valleys. Secondary centers such as Bela Palanka, Svrljig, and Gadžin Han showed modest demographic increases, though their growth was dwarfed by the metropolitan dominance of Niš. Overall, urban land cover increased by more than 65% across the district, with the most rapid expansion occurring between 2002 and 2022 (Badach et al. 2020).

Industrial zones in the Nišava District followed the same longitudinal corridors as settlement growth. By overlaying industrial site geolocations with buffer analyses, three major industrial clusters were identified:

1. **Niš Industrial Core** – encompassing metallurgy, electronics, textile, and food-processing plants;
2. **Bela Palanka Corridor** – with smaller-scale manufacturing and quarrying;
3. **Svrljig Zone –** characterized by agro-industrial activity and emerging energy facilities.

The buffer analysis demonstrated that 72% of industrial activity lies within 2 km of permanent watercourses, underscoring industries' dependence on rivers for water supply and waste discharge.

Hydrological modeling revealed significant transformations in the Nišava District's drainage systems. In 1983, the Nišava River and its tributaries displayed a predominantly dendritic network with high drainage density and perennial flow in most second- and third-order streams. By 2023, this structure had undergone fragmentation, with drainage density decreasing by 14% across the district and perennial tributaries diminishing by 11%, many downgraded to intermittent or ephemeral streams, particularly near urbanized zones. Stream order simplification was observed in the Nišava Valley east of Niš, where formerly branching tributaries were replaced by parallelized and shortened streams. Kernel Density Estimation (KDE) heat maps further highlighted hydrological "hotspots" around Niš and Bela Palanka, where tributary loss and stream discontinuities strongly overlapped with industrial and settlement expansion zones, emphasizing the profound impacts of urbanization and industrialization on the region's river network (Okabe et al. 2009; Fig. 2).

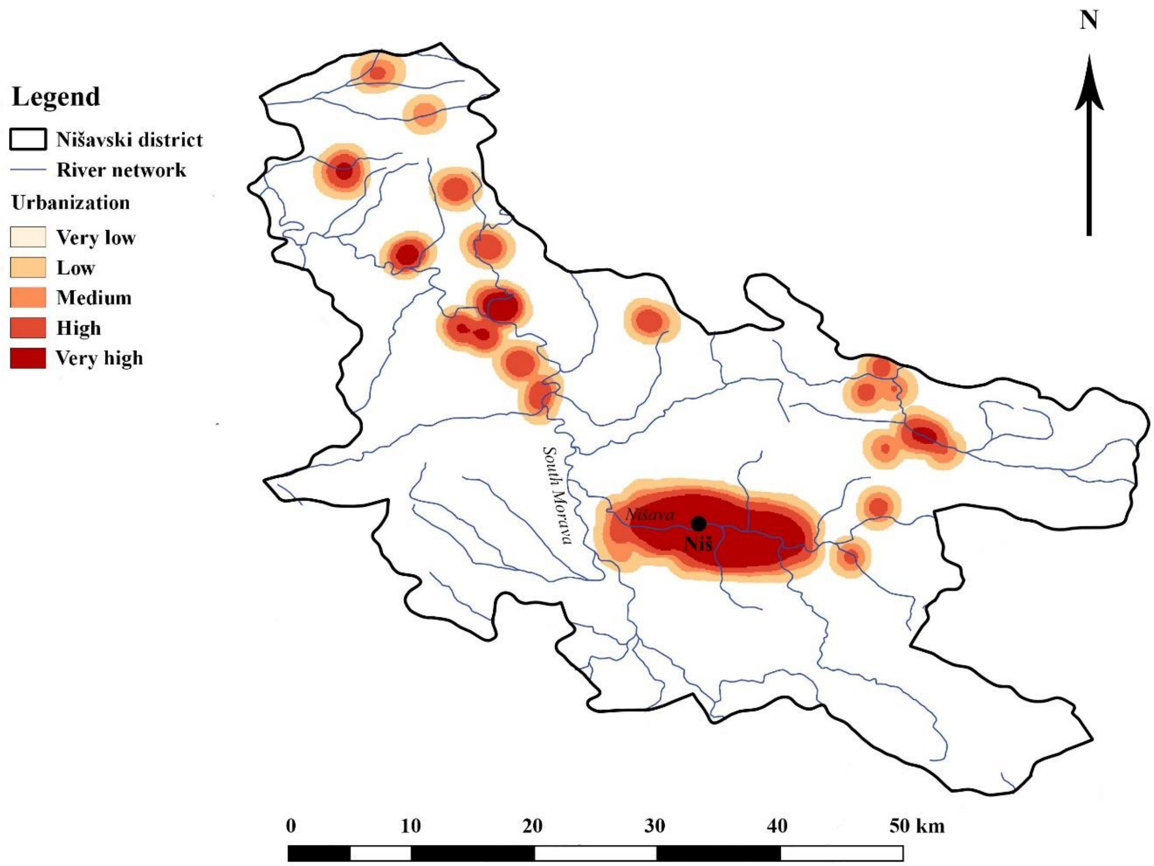

Figure 2. Urbanization patterns and river network configuration in the Nišava District in 1983

This figure shows the spatial distribution of urbanized areas and the structure of the river network during 1983, reflecting the early phase of settlement expansion and landscape transformation. It provides a historical baseline for analyzing subsequent urban growth, hydrological modifications, and long-term environmental change in the district.

The Multi-Criteria Layer Analysis (MCLA) revealed clear spatial patterns of hydrological vulnerability across the Nišava District. High-risk zones were concentrated in the Niš urban-industrial belt, where tributary loss exceeded 20% over the past four decades, in the lower Nišava Valley near Bela Palanka, where quarrying and transport infrastructure drove drainage simplification in small catchments, and at the South Morava confluence, and urban runoff and industrial wastewater jointly reshaped natural flow regimes. Medium-risk zones were mapped in the municipalities of Svrljig and Gadžin Han, where smaller settlements and localized industrial activity are exerting growing pressure on the fluvial system. By contrast, low-risk areas were confined to the surrounding mountainous terrain, where hydrographic networks remain well preserved and urban encroachment is minimal, highlighting the spatial contrasts between disturbed valley zones and more resilient upland catchments (Fig. 3).

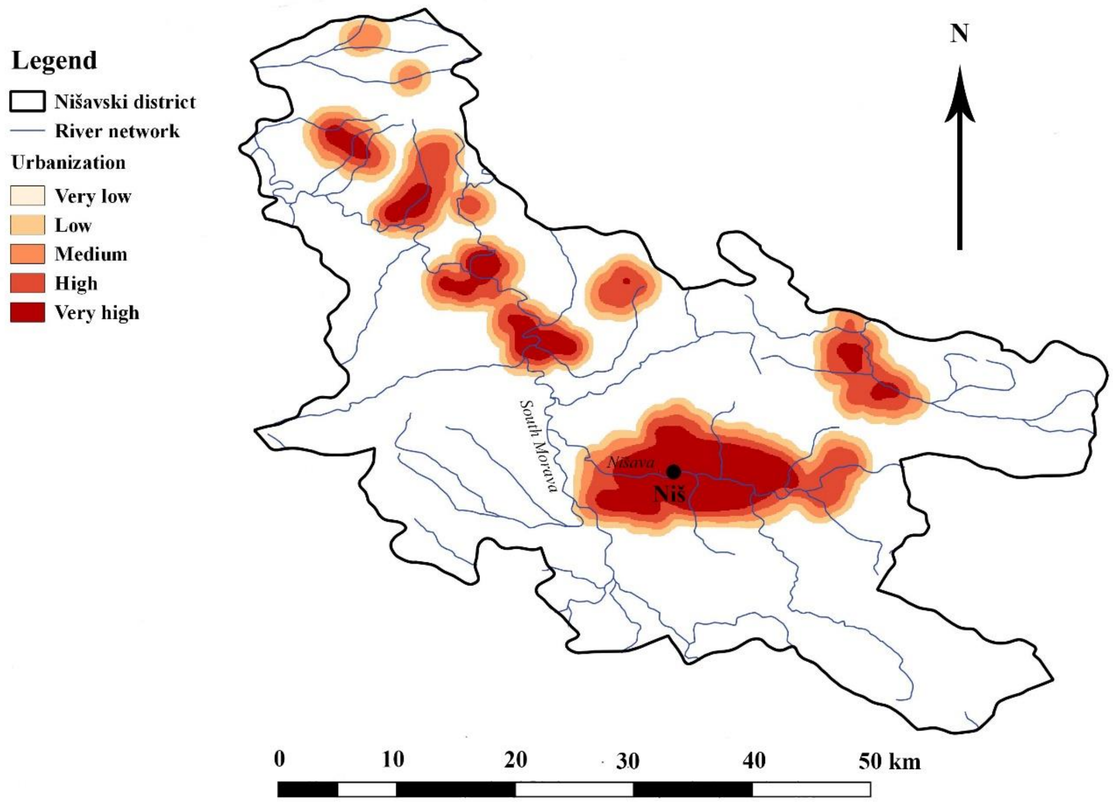

Figure 3. Urbanization patterns and river network configuration in the Nišava District in 2023.

This figure presents the contemporary extent of urban development alongside the current river network, illustrating four decades of spatial expansion, infrastructural growth, and hydrological alteration since 1983. It serves as a key reference for assessing the magnitude and direction of urban-driven landscape and drainage changes within the district.

Simulation modeling using the cellular automata–Markov chain approach projected further hydrological stress by mid-century. If current trends continue, urban expansion in Niš is expected to merge with peri-urban belts along the South Morava corridor, while industrial zones will intensify near transportation hubs. By 2050, drainage density could decline by an additional 8–10%, with perennial tributaries reduced by up to 20% relative to 1983 baselines. The Nišava District is therefore on track to develop into a consolidated urban-industrial corridor with pronounced hydrological instability unless sustainable management measures are adopted (Baykal et al. 2022; Fig.4).

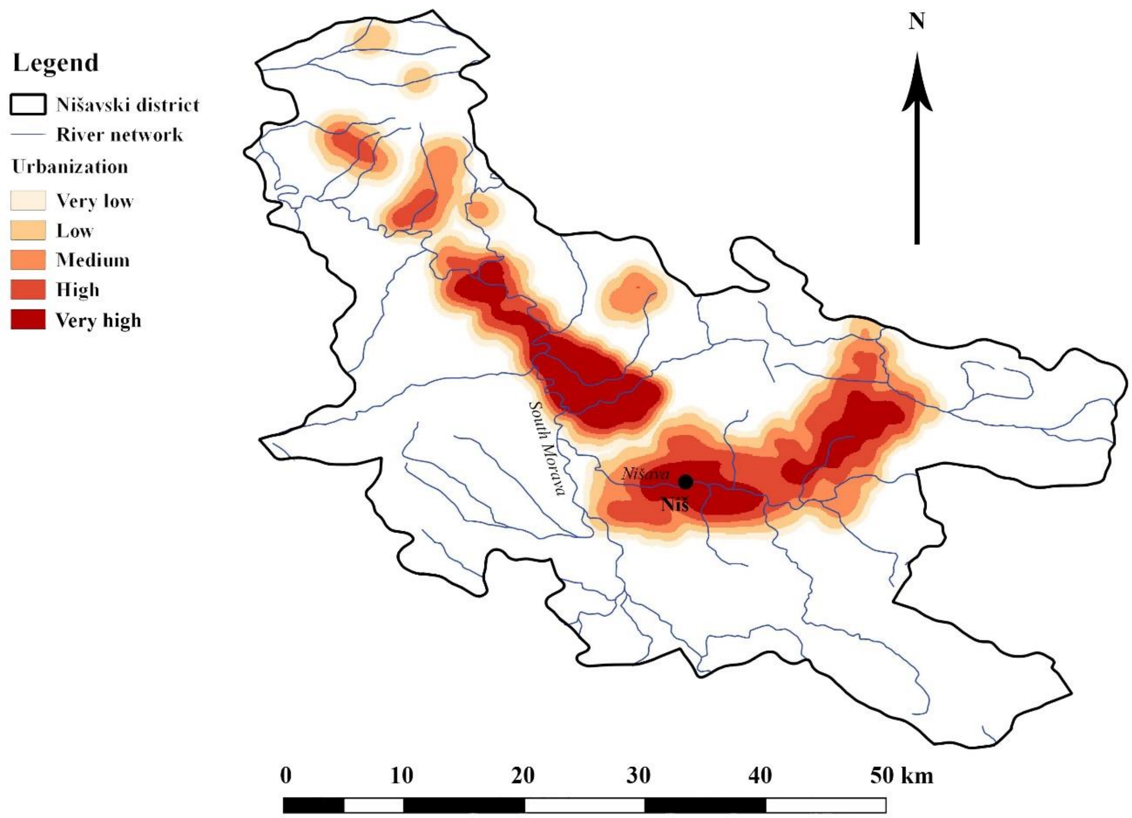

Figure 4. Projected urbanization in the Nišava District and anticipated impacts on the river network by 2050

This figure illustrates modeled urban expansion and the expected spatial reconfiguration of the river network under future development scenarios. It highlights zones of potential hydrological disturbance, increased channel modification, and landscape fragmentation, providing a forward-looking assessment of how continued urban growth may influence drainage patterns and environmental stability by mid-century.

Table 1. Industrial facilities by proximity to rivers in the Nišava District

Municipality	0–500 m	500–2000 m	>2000 m
Niš	18	25	12
Bela Palanka	7	10	5
Svrljig	3	6	4
Gadžin Han	2	4	3

Table 2. Vulnerability index of municipalities in the Nišava District

Municipality	Tributary loss (%)	Drainage density decline (%)	Vulnerability class
Niš	22	18	High
Bela Palanka	15	12	High
Svrljig	9	7	Medium
Gadžin Han	6	5	Medium
Mountain areas	3	2	Low

The results demonstrate that the Nišava District has undergone profound transformations in its hydrological systems due to concentrated urban and industrial development, particularly around Niš. Similar to findings at the national scale the river corridors of the Nišava and South Morava act as both attractors of population growth and as recipients of industrial pressures. The documented reduction in drainage density and tributary permanence highlights how urban sprawl and industrial wastewater discharge collectively erode the resilience of river systems. These changes parallel global observations where rapid urbanization accelerates river fragmentation, alters natural flow regimes, and increases surface runoff (Grill et al., 2015; Du et al., 2019).

The Multi-Criteria Layer Analysis (MCLA) identified Niš and Bela Palanka as high-risk zones, confirming that industrial clustering near rivers magnifies hydrological stress (Valjarević et al. 2020b). Peripheral municipalities such as Svrljig and Gadžin Han remain moderately affected but display emerging vulnerabilities. This spatial polarization reflects broader Serbian patterns, where metropolitan-industrial corridors concentrate growth and risks, while mountainous peripheries preserve ecological stability (Valjarević, 2024). The vulnerability gradient underscores the importance of integrating watershed-scale governance into spatial planning, rather than relying solely on administrative boundaries.

The Nišava case aligns with regional analyses showing that urban-industrial expansion reduces tributary density and increases hydrological simplification (Nawar et al., 2025; Shahid & Mustafa, 2020). However, unlike larger rivers such as the Danube or Sava, the Nišava and its tributaries possess limited self-purification capacity, making them highly sensitive to contamination from industrial effluents and urban runoff. This supports the argument that small- to medium-sized river basins require more stringent protection than large, high-capacity systems.

The projection to 2050 suggests the emergence of a consolidated urban-industrial corridor linking Niš with the South Morava Valley. Such development may enhance regional connectivity but will likely intensify hydrological instability unless sustainable measures are implemented. Without adequate intervention, tributary loss could exceed 20% relative to baseline conditions, further reducing biodiversity, groundwater recharge, and flood-regulation capacity. This scenario reflects the paradox observed across Southeast Europe: economic efficiency is gained at the expense of cumulative ecological

The findings emphasize the urgent need for:

1. **Expansion of wastewater treatment infrastructure** – Serbia currently operates far fewer facilities than required, and the Nišava District is particularly under-served.
2. **River buffer protection zones** – strict enforcement of 500–1000 m protective belts could mitigate industrial and urban encroachment.
3. **Integration of vulnerability mapping into planning** – MCLA-based tools should be adopted for regional decision-making to prioritize areas for intervention.
4. **Restoration of degraded tributaries** – ecological engineering, reforestation, and river rehabilitation programs are critical for restoring drainage density.

The Nišava District represents a microcosm of the wider Serbian and Balkan dilemma: balancing economic modernization with ecological sustainability. The district's experience shows that uncontrolled urban-industrial expansion in hydrologically sensitive valleys leads to structural degradation of rivers. However, the same corridors could become models for sustainable development if integrated basin management, clean technologies, and ecological restoration are prioritized.

The projected scenarios for 2050 further emphasize the urgency of intervention. If current urbanization trends persist, the Nišava District is likely to evolve into a continuous urban–industrial corridor connecting Niš with the wider South Morava basin. Such a configuration may support economic growth and regional connectivity but will likely exacerbate hydrological instability, reduce groundwater recharge, intensify flood risk, and accelerate the simplification of the drainage network. The cellular automata–Markov chain model suggests that tributary loss may reach up to 20% relative to 1983, a threshold associated with significant ecological impairment in comparable European basins.

Climate variability adds another layer of complexity. Increased heatwaves, droughts, and flash-flood events reported in Serbia since 2000 enhance the sensitivity of the Nišava basin to both natural and anthropogenic stressors. Reduced baseflows during summer months, combined with increased surface runoff from expanding urban areas, may alter seasonal river regimes and reduce water availability for both ecosystems and human consumption.

Importantly, the results point toward several actionable strategies. First, expanding wastewater treatment infrastructure must be prioritized, as untreated discharges remain a major driver of ecological degradation. Second, enforcing protective riparian buffer zones—particularly the 500–1,000 m belts identified in the analysis could substantially reduce tributary fragmentation and preserve floodplain function. Third, the MCLA-based vulnerability maps produced in this study should be integrated into municipal planning processes to guide zoning, industrial expansion, and ecological restoration. Finally, tributary rehabilitation through reforestation, reconnection of cut-off channels, and bank stabilization could help restore drainage density and enhance basin resilience.

Comparable dynamics are evident in Central Europe. The urban-industrial expansion in the Upper Silesian Coal Basin (Poland-Czech Republic) has long been associated with extensive hydrological alteration, including the diversion of smaller tributaries, depression of the groundwater table, and increased sediment transport from excavated areas. These examples reinforce the findings from the Nišava District, where quarrying, transport infrastructure, and industrial clustering have produced visible impacts on drainage structure and hydrological connectivity (Grizzetti et al., 2017).

Western European experience further illustrates the long-term consequences of unregulated industrial–urban growth. In northern Italy's Po Valley, decades of intensive development along river corridors led to the simplification of fluvial networks and a decline in ecological integrity, prompting widespread restoration initiatives, including re-meandering, riparian reforestation, and floodplain reconnection. Similar interventions may eventually be required in the Nišava basin if current trajectories persist, especially given the projected decline in tributary permanence by 2050.

A key difference, however, is that many Western European basins benefit from stronger wastewater management systems and more mature regulatory frameworks. In contrast, as highlighted in this study, over 80% of the wastewater in the Nišava District remains untreated, placing local tributaries under significantly greater ecological stress than their counterparts in countries with advanced environmental governance. This comparison underscores the need for Serbia and other Western Balkan nations to accelerate the development of wastewater infrastructure and adopt integrated watershed-management principles aligned with EU Water Framework Directive standards.

Overall, the Nišava District represents a microcosm of the broader challenges facing Southern and Eastern European basins where rapid urban and industrial development intersect with fragile hydrological systems. This study contributes valuable empirical evidence demonstrating how spatially concentrated human activities can compromise river basin sustainability. At the same time, it highlights clear opportunities for implementing integrated watershed management strategies that balance economic priorities with ecological protection and long-term hydrological stability (Valjarević, 2025d).

Despite its comprehensive GIS-based framework, this study has several limitations. First, the resolution of available DEMs (30 m SRTM) may underestimate micro-scale tributary networks, especially in mountainous areas. Second, industrial pollution datasets were incomplete, with some smaller facilities lacking publicly accessible discharge records, limiting the precision of water quality correlations. Third, census data were available only at decadal intervals, making it difficult to fully capture short-term fluctuations in urban growth and migration. Finally, while the cellular automata Markov chain model provides robust projection scenarios, its accuracy depends on the assumption that past trends will continue, which may not account for abrupt policy shifts or major infrastructure projects. Addressing these constraints in future research will require higher-resolution satellite products, continuous hydrological monitoring, and integration of field-based ecological assessments.

Future research on the Nišava District should build upon the methodological framework and empirical findings of this study by integrating higher-resolution datasets, advanced modeling techniques, and interdisciplinary approaches. Several key directions can substantially strengthen future understanding of urban-river interactions and support the development of sustainable basin management strategies. First, hydrological monitoring should be enhanced by using high-resolution Digital Elevation Models (DEMs), such as LiDAR and TanDEM-X, which can capture micro-tributaries, subtle channel changes, and erosion processes that remain undetectable in 30 m-resolution products. These data would improve the accuracy of stream-order classification, drainage density measurements, and hydrological connectivity assessments, particularly in narrow valleys and steep upland segments where current models likely underestimate tributary complexity.

Second, future studies should incorporate continuous water quality monitoring and improved pollution datasets, including real-time sensors, industrial discharge registries, and in situ sampling campaigns. The current lack of detailed wastewater data limits the ability to quantify relationships between industrial activity and ecological degradation. Enhanced datasets would allow researchers to integrate nutrient loads, heavy metals, and dissolved oxygen into spatial models, enabling basin-wide assessments of ecological health and biogeochemical resilience.

Third, climate hydrology integration should be deepened by coupling regional climate models (RCMs) with hydrological forecasting tools to evaluate long-term effects of droughts, heatwaves, extreme rainfall, and seasonal regime shifts. Ensemble projections combining CORDEX, ERA5-Land, and local meteorological observations could help identify future thresholds of flood risk, groundwater recharge decline, and ecosystem stress. Such modeling is essential given the documented increase in hydrometeorological extremes and anticipated climatic variability in southern Serbia.

Fourth, socio-ecological and governance dimensions should be expanded in future analyses. Urban growth patterns in Niš, Bela Palanka, and smaller municipalities are strongly shaped by demographic trends, economic transitions, and spatial planning practices. Incorporating household water use data, economic indicators, and land-market dynamics could clarify how social drivers interact with hydrological processes. Additionally, stakeholder-based research involving municipalities, industries, and local communities can illuminate governance barriers to implementing riparian buffer protections, wastewater infrastructure, and integrated basin management.

Fifth, future research should examine restoration potential and ecological rehabilitation scenarios. Modeling the effects of reforestation, riparian buffer expansion, wetland reconstruction, and sustainable urban drainage systems (SUDS) could identify cost-effective strategies for restoring tributary permanence, improving water quality, and reducing flood vulnerability. These nature-based solutions are increasingly relevant as the Nišava basin transitions into a consolidated urban-industrial corridor.

Sixth, machine learning and artificial intelligence (AI) approaches offer valuable new tools for predicting hydrological disruption and land-use change. Deep-learning models such as U-Net, Random Forest, and LSTM networks can improve future urban growth simulations, classify river fragmentation hotspots more precisely, and integrate multi-source datasets from satellite imagery to socioeconomic variables into unified predictive frameworks.

Finally, a priority for future work is the creation of a long-term, integrated river-basin observatory for the Nišava and South Morava valleys. Such a platform would combine hydrological sensors, remote-sensing products, ecological surveys, urban planning data, and historical archives, enabling real-time monitoring and supporting adaptive management strategies. Establishing this observatory would allow researchers and policymakers to track hydrological changes continuously, evaluate the effectiveness of interventions, and build a comprehensive evidence base for sustainable regional development.

Conclusion

This study revealed that the Nišava District, and particularly the urban-industrial hub of Niš, has experienced profound hydrological transformations over the past four decades. Using GIS and Remote Sensing techniques, we demonstrated that rapid urbanization and industrial clustering have directly contributed to tributary loss, drainage density decline, and stream network simplification. These changes underscore the critical role of anthropogenic pressures in reshaping small and medium-sized river systems, which lack the buffering capacity of larger basins.

The spatial analysis highlighted three primary hotspots of hydrological stress: the Niš metropolitan zone, the Bela Palanka corridor, and the South Morava confluence. In these areas, industrial activities, urban sprawl, and inadequate wastewater management collectively intensified river fragmentation and ecological vulnerability. By contrast, mountainous peripheries such as Svrljig and Gadžin Han retained relatively stable hydrographic structures, though signs of emerging risk are already visible.

Projections to 2050 indicate that without intervention, the Nišava District will evolve into a consolidated urban-industrial corridor with further reductions in tributary permanence and a loss of ecological services such as groundwater recharge, biodiversity support, and flood regulation. This trajectory mirrors broader patterns across Serbia and Southeast Europe, where economic integration along river corridors comes at the cost of hydrological resilience.

To reverse these trends, integrated basin-scale governance is essential. Expanding wastewater treatment infrastructure, enforcing protective river buffers, restoring degraded tributaries, and embedding vulnerability maps into spatial planning should be treated as urgent priorities. The Nišava District thus exemplifies both the risks of unmanaged urban-industrial growth and the opportunities for sustainable transformation. If guided by evidence-based policies, the region could transition from a corridor of hydrological stress into a model of balanced development that safeguards rivers as vital ecological and economic lifelines.

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Conflict-of-Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Andersen, L. M., Carstensen, J., Conley, D. J., & Hasler, B. (2021). Urban and agricultural pressures on aquatic ecosystems in Europe: Increasing nutrient loads and decreasing resilience. *Ambio*, 50(2), 273–287. https://doi.org/10.1007/s13280-020-01337-2

Badach, J., Voordeckers, D., Nyka, L., & Van Acker, M. (2020). A framework for air quality management zones – useful GIS-based tool for urban planning: Case studies in Antwerp and Gdańsk. *Building and Environment*, 174, 106743. https://doi.org/10.1016/j.buildenv.2020.106743

Baykal, T. M., Colak, H. E., & Kılınc, C. (2022). Forecasting future climate boundary maps (2021–2060) using exponential smoothing method and GIS. *Sci Total Environ*, 848, 157633. https://doi.org/10.1016/j.scitotenv.2022.157633

Chen, J., & Tang, X. (2023). Towards sustainable cities: Studying evaluation index of water environment carrying capacity. *Water Resour Manage*, 37, 5919–5938. https://doi.org/10.1007/s11269-023-03635-5

Dimić, V., Milošević, M., Milošević, D., & Stević, D. (2018). Adjustable model of renewable energy projects for sustainable development: A case study of the Nišava District in Serbia. *Sustainability*, 10(3), 775. https://doi.org/10.3390/su10030775

Du, Y., Cai, W., Xu, Y., Wang, Y., & Zhang, X. (2019). Urban expansion and its impact on surface runoff in the Yangtze River Delta. *Land Use Policy*, 86, 366–377. https://doi.org/10.1016/j.landusepol.2019.05.010

Durlević, U., Valjarević, A., Novković, I., Vujović, F., Josifov, N., Krušić, J., & Ivanović, M. (2024). Universal snow avalanche modeling index based on SAFI–Flow-R approach in poorly-gauged regions. *ISPRS Int J Geo-Inf*, 13(9), 315. https://doi.org/10.3390/ijgi13090315

Fourqurean, J. W., & Robblee, M. B. (1999). Florida Bay: A history of recent ecological changes. *Estuaries*, 22, 345–357. https://doi.org/10.2307/1353203.

Grizzetti, B., Pistocchi, A., Liquete, C., & others. (2017). Human pressures and ecological status of European rivers. Sci Rep, 7, 205. https://doi.org/10.1038/s41598-017-00324-3.

Grill, G., Lehner, B., Thieme, M., Geenen, B., Tickner, D., Antonelli, F., & Zarfl, C. (2019). Mapping the world's free-flowing rivers. *Nature*, 569(7755), 215–221. https://doi.org/10.1038/s41586-019-1111-9

Ilies, G., Ilies, M., Hotea, M., Bumbak, S. V., Hodor, N., Ilies, D. C., & Vasić, P. (2022). Integrating forest windthrow assessment data in the process of windscape reconstruction: Case of the extratropical storms downscaled for the Gutai Mountains (Romania). *Front Environ Sci*, 10, 926430. https://doi.org/10.3389/fenvs.2022.926430

Menković, L., Košćal, M., Milivojević, M., & Đokić, M. (2018). Morphostructure relations on the territory of the Republic of Serbia. *Glasnik Srpskog geografskog društva*, 98(2), 1–28. https://doi.org/10.2298/GSGD1802001M

Okabe, A., Satoh, T., & Sugihara, K. (2009). A kernel density estimation method for networks, its computational method and a GIS-based tool. *Int J Geogr Inf Sci*, 23(1), 7–32. https://doi.org/10.1080/13658810802475491

Redman, C., Grove, J., & Kuby, L. (2004). Integrating social science into the Long-Term Ecological Research (LTER) network: Social dimensions of ecological change and ecological dimensions of social change. *Ecosystems*, 7, 161–171. https://doi.org/10.1007/s10021-003-0215-z

Shen, J., & Wang, Y. (2023). An improved method for the identification and setting of ecological corridors in urbanized areas. *Urban Ecosyst*, 26, 141–160. https://doi.org/10.1007/s11252-022-01298-5

Stamenković, S. M., Ristić, S. S., Djekic, T. L., Mitrovic, T. U., & Baošić, R. (2013). Air quality indication in Blace (Southeastern Serbia) using lichens as bioindicators. *Arch Biol Sci*, 65(3), 893–897. https://doi.org/10.2298/ABS1303893S

Takić, L., Mladenović-Ranisavljević, I., Vasović, D., & Đorđević, L. (2017). The assessment of the Danube River water pollution in Serbia. *Water Air Soil Pollut*, 228(10), 380. https://doi.org/10.1007/s11270-017-3551-x

Tomsett, C., & Leyland, J. (2019). Remote sensing of river corridors: A review of current trends and future directions. *River Res Appl*, 35, 779–803. https://doi.org/10.1002/rra.3479

Urošev, M., Milanović Pešić, A., Kovačević–Majkić, J., & Štrbac, D. (2022). Hydrological characteristics of Serbia. In Manić, E., Nikitović, V., & Djurović, P. (Eds.), *The geography of Serbia* (pp. 145–164). Springer. https://doi.org/10.1007/978-3-030-74701-5_6

Valjarević, A. (2025b). Ecological impact of oil fields on Venezuela's river systems: Water stress and environmental challenges. *J South Am Earth Sci*, 105675. https://doi.org/10.1016/j.jsames.2025.105675.

Valjarević, A. (2024). GIS-based methods for identifying river networks types and changing river basins. *Water Resour Manage*, 38, 5323–5341. https://doi.org/10.1007/s11269-024-03916-7

Valjarević, A., Filipović, D., & Milanović, M. (2020b). New updated world maps of sea-surface salinity. *Pure Appl Geophys*, 177, 2977–2992. https://doi.org/10.1007/s00024-019-02404-z

Valjarević, A., Filipović, D., Valjarević, D., et al. (2020a). GIS and remote sensing techniques for the estimation of dew volume in the Republic of Serbia. *Meteorol Appl*, 27, e1930. https://doi.org/10.1002/met.1930

Valjarević, A., Morar, C., Brasanac-Bosanac, L., Cirkovic-Mitrovic, T., Djekic, T., Mihajlović, M., & Kaplan, G. (2025c). Sustainable land use in Moldova: GIS & remote sensing of forests and crops. *Land Use Policy*, 152, 107515. https://doi.org/10.1016/j.landusepol.2025.107515

Valjarević, A., Šiljeg, A., Šiljeg, S., Vujović, F., & Sahay, A. (2025a). GIS-based water stress analysis in North African drylands. *J Arid Environ*, 230, 105427. https://doi.org/10.1016/j.jaridenv.2025.105427

Valjarević, A. (2025d). GIS and remote sensing methods in predicting the dissipation time of rural settlements under the influence of climate change in the Republic of Serbia. *J Urban Manag*, 14(2), 500–511. https://doi.org/10.1016/j.jum.2024.12.002.

Yu, Z., Lu, M., Xu, Y., Wang, Q., Lin, Z., & Luo, S. (2023). Network structure and stability of the river connectivity in a rapidly urbanizing region. *Sci Total Environ*, 894, 165021. https://doi.org/10.1016/j.scitotenv.2023.165021

Zhao, Q., Yu, L., Du, Z., Peng, D., Hao, P., Zhang, Y., & Gong, P. (2022). An overview of the applications of earth observation satellite data: Impacts and future trends. *Remote Sens*, 14(8), 1863. https://doi.org/10.3390/rs14081863

Zhao, Y., Zhang, R., Jiang, W., & Hu, M. (2020). Influence of land use and urbanization on runoff characteristics in the Beijing metropolitan area. *Water*, 12(10), 2821. https://doi.org/10.3390/w12102821

Zwarteveen, M., Kemerink-Seyoum, J. S., Kooy, M., Evers, J., Guerrero, T. A., Batubara, B., Biza, A., Boakye-Ansah, A., Faber, S., Cabrera Flamini, A., Cuadrado-Quesada, G., Fantini, E., Gupta, J., Hasan, S., ter Horst, R., Jamali, H., Jaspers, F., Obani, P., Schwartz, K., Shubber, Z., Smit, H., Torio, P., Tutusaus, M., & Wesselink, A. (2017). Engaging with the politics of water governance. *WIREs Water*, 4, e1245. https://doi.org/10.1002/wat2.1245

Urbanizacija i industrijalizacija u Nišavskom okrugu: GIS analiza promena rečne mreže (1983–2050)

Tekući naslov: Uticaj urbanizacije na rečne mreže u Nišavskom okrugu

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SAŽETAK

Urbanizacijai industrijalizacija značajno su transformisale hidrološke sisteme južne Srbije,posebno u slivovima Nišave i Južne Morave, kao i u gradu Nišu. Tokom poslednjih decenija, brzi urbani rast i industrijska aktivnost izmenili su morfologiju reka, smanjili postojanost pritoka i preoblikovali drenažne mreže. Korišćenjem GIS i metoda daljinske detekcije, uključujući visokorezolutivne DTM-ove, podatke popisa i kartografske arhive, ova studija analizira interakcije između urbanih i rečnih sistema u periodu od 1983. do 2023. godine, sa projekcijama do 2050. Rezultati pokazuju da je Niš, strateški smešten duž koridora Nišave i Južne Morave, doživeo značajnu urbanizaciju, što je dovelo do povećanog površinskog oticanja, rizika od erozije i degradacije priobalnih ekosistema. Nišava je pretrpela fragmentaciju svog prirodnog dendritskog sistema, dok je Južna Morava postala centralna osa metropolitanskog i industrijskog razvoja. Projekcije do 2050. godine ukazuju na intenziviranje rizika od zauzimanja poplavnih zona, smanjenja dopune podzemnih voda i daljeg pogoršanja kvaliteta vode. Nalazi naglašavaju hitnu potrebu za integrisanim upravljanjem slivovima i održivim urbanističkim planiranjem u Nišu i okolnim rečnim dolinama. Uravnoteženje ekonomskog rasta sa ekološkom otpornošću ključno je za očuvanje hidrološke stabilnosti i obezbeđenje dugoročne održivosti regiona.

Ključne reči: Južna Morava, Nišava, Nip, urbanizacija, GIS analiza, zagađenje

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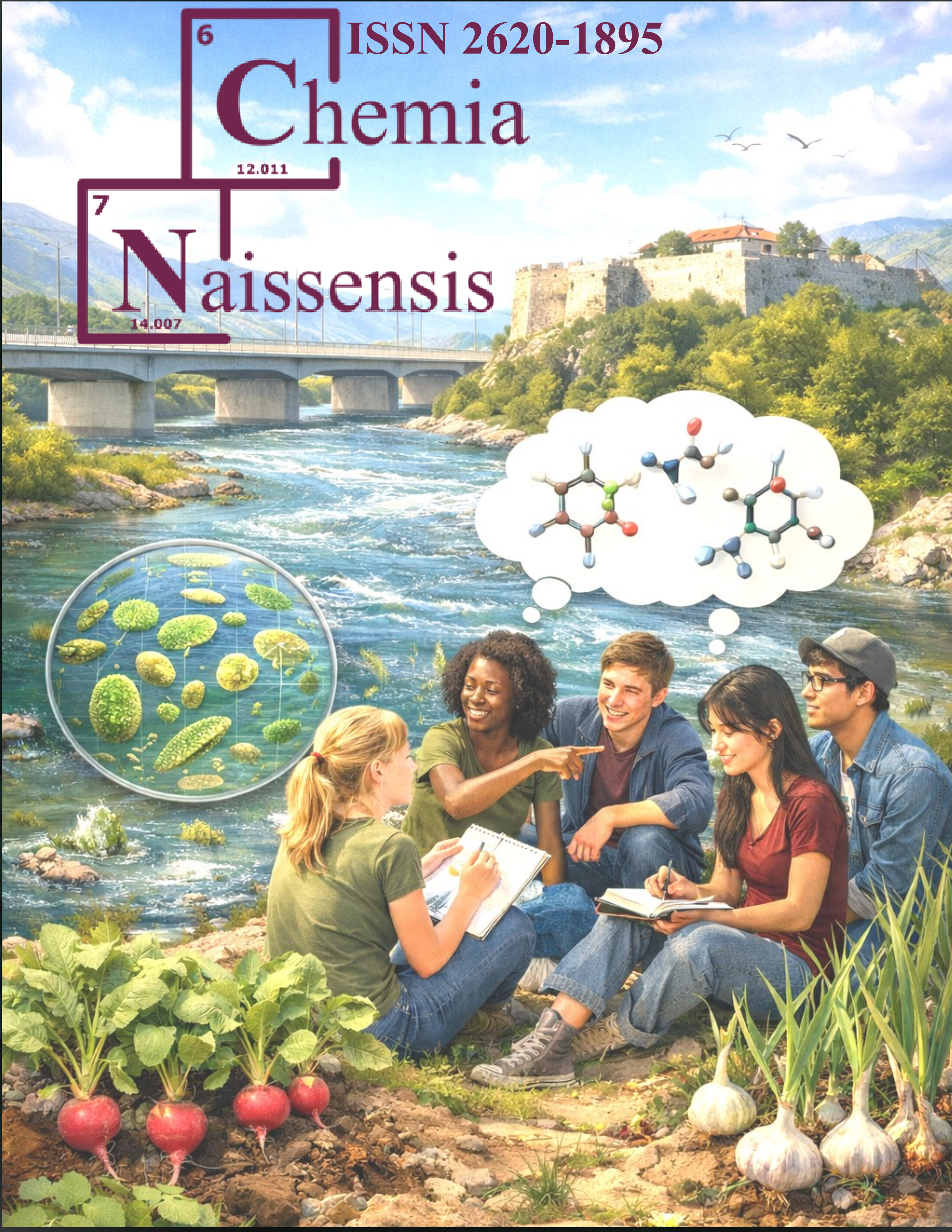
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