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NATIONAL CENTER OF PUBLIC HEALTH AND ANALYSES

Mariya Angelova Mitreva

HYGIENIC AND ANALYTICAL ASPECTS OF THE STUDY OF THE SEASONAL DYNAMICS OF MICROCYSTINS IN SURFACE WATERS INTENDED FOR DRINKING PURPOSES

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LIST OF ABBREVIATIONS

Abbreviation	Full term
DAD	Diode Array Detector
ELISA	Enzyme-Linked Immunosorbent Assay
HPLC	High-Performance Liquid Chromatography
LOQ	Limit of Quantification
MC–LR	Microcystin–LR
MC–RR	Microcystin–RR
MC–YR	Microcystin–YR
SPE	Solid-Phase Extraction
UV	Ultraviolet

TABLE OF CONTENTS

INTRODUCTION 6
1. AIM AND OBJECTIVES 8
2. MATERIALS AND METHODS 9
3. RESULTS AND DISCUSSION 9
3.1. Iskar Reservoir 10
3.1.1. Iskar Reservoir – Water Intake Tower 10
3.1.2. Iskar Reservoir – Shtarkelovo Gnezdo 13
3.1.3. Iskar Reservoir – Sailing Base 15
3.2. Kokalyane Dam 16
3.3. Studena Reservoir 18
3.4. Krasava Reservoir 23
3.5. Summary 25
4. CONCLUSIONS 28
5. RECOMMENDATIONS 30
5.1. Monitoring of vulnerable water bodies 30
5.2. Preventive monitoring of potentially at-risk reservoirs 30
6. CONTRIBUTIONS 31
7. PUBLICATIONS 32
8. PARTICIPATION IN SCIENTIFIC FORUMS 33

INTRODUCTION

Cyanobacteria, also known as blue-green algae, represent one of the oldest groups of prokaryotic, autotrophic microorganisms. Extensive analyses based on molecular, physiological, paleontological, and geochemical data indicate that cyanobacteria originated between 2.54 and 2.1 billion years ago. These widely distributed prokaryotes employ diverse metabolic strategies to survive and adapt to varying environmental conditions. Cyanobacteria, as Gram-negative autotrophic bacteria, possess a wide range of metabolic pathways and adaptive mechanisms, including nitrogen fixation, chromatic adaptation, and the ability to form symbiotic relationships with eukaryotic hosts in association with other microorganisms such as fungi and protists. Some cyanobacteria are capable of performing oxygenic photosynthesis using sulfide as an electron donor. In addition, under conditions of oxygen and light deficiency, these organisms can carry out fermentation. Over evolutionary time, cyanobacteria have developed the ability to produce a wide variety of secondary metabolites, enabling them to persist under diverse environmental conditions.

In aquatic environments, cyanobacteria occur in various habitats: dispersed or aggregated in the water column, at the water surface, on bottom sediments, or attached to shoreline substrates and aquatic vegetation. Under favorable conditions, they may form mass populations such as blooms, scums, and biofilms. These proliferations can occur even in pristine water bodies unaffected by human activity, where nutrient enrichment results from natural geological processes. However, it is now widely recognized that the increase in trophic status of water bodies (eutrophication) is strongly influenced by anthropogenic pressures. These include nutrient enrichment from domestic, agricultural, and industrial sources, increased erosion within the catchment area, and intensified water abstraction. Under favorable conditions of temperature, light penetration, pH, and water residence time, rapid cyanobacterial growth and bloom formation may occur.

In addition to their adverse impact on ecosystem biodiversity, cyanobacterial mass occurrences may negatively affect the availability, safety, and cost of water resources intended for drinking purposes. Human activities that may be adversely affected include drinking water supply, irrigation of agricultural crops and livestock, aquaculture, recreation, and tourism.
Current requirements for drinking water quality in Europe are defined in Directive (EU) 2020/2184 of 16 December 2020 on the quality of water intended for human consumption. Among the emerging contaminants that will be subject to mandatory monitoring from 2026, and for which risk assessment is required, is microcystin-LR. This compound is a representative

of the microcystins—a group of cyanotoxins produced by cyanobacteria. The maximum allowable concentration in drinking water, according to the Directive, is 1 μg/L. The Directive has been transposed into Bulgarian national legislation through amendments to Ordinance No. 9/2001 on the quality of water intended for drinking purposes (State Gazette No. 30/2001, last amended SG No. 9 of 23.01.2026).

1. AIM AND OBJECTIVES

Aim

To develop, validate, and apply analytical methods for the determination of microcystins in surface waters intended for drinking purposes, as well as to characterize the status of selected reservoirs in the region of Sofia and its surroundings by investigating the occurrence of these toxins and assessing their relationship with environmental factors.

Objectives

To achieve the stated aim, the following main objectives were defined:

1. To develop and validate analytical methods for the qualitative and quantitative determination of microcystins in water samples.
2. To determine the concentrations of microcystins in surface waters intended for drinking purposes.
3. To investigate the temporal dynamics of cyanobacterial species composition and the occurrence of microcystins in selected reservoirs in the Sofia region.
4. To assess the influence of environmental factors on the production of microcystins under real environmental conditions.

2. MATERIALS AND METHODS

Contemporary analytical methods were applied in this study, with a primary focus on high-performance liquid chromatography with diode array detection (HPLC-DAD) for the qualitative and quantitative determination of microcystins. A competitive indirect ELISA (ABRAXIS commercial kits) was used for screening purposes, and the results were compared with the chromatographic data.

The concentrations of key nutrients—total nitrogen and total phosphorus—were determined using established titrimetric and spectrophotometric methods in accordance with standard procedures.

Hydrobiological analysis was applied as a complementary approach for the characterization of phytoplankton communities.

Statistical analysis of the results was performed using correlation and regression analysis (Pearson), with a significance level of $p < 0.05$.

3. RESULTS AND DISCUSSION

A chromatographic method for the determination of microcystins (–LR, –RR, and –YR) in water by HPLC-DAD was developed and validated, providing reliable separation and quantification under optimized gradient elution conditions with UV detection at 238 nm. The method has been included in the scope of accreditation of the “Health” Testing Center at the National Center of Public Health and Analyses (NCPHA). Analytical competence was confirmed through successful participation in an international interlaboratory comparison test, with very good z-scores obtained for microcystins (–LR, –RR, and –YR), namely –0.49, 0, and 1.67, respectively.

Within the framework of the study conducted over the period 2023–2025, systematic monitoring of the seasonal dynamics of microcystins was carried out in major drinking water sources in the Sofia region (Iskar Reservoir, Kokalyane Dam, Studena Reservoir, and Krasava Reservoir). A total of 99 water samples were analyzed, collected from different sampling sites and depths.

The application of appropriate sample preparation procedures, including filtration and solid-phase extraction (SPE), enabled the concentration and reliable determination of both extracellular and intracellular microcystins. The obtained results provide a basis for assessing the dynamics of toxin occurrence and their relationship with environmental factors in the studied water bodies.

A particularly significant outcome is the first-time dynamic monitoring of microcystins in surface waters intended for drinking purposes in Bulgaria, which is of direct relevance for the implementation of the new requirements of European legislation on water quality.

3.1. Iskar Reservoir

3.1.1. Iskar Reservoir – Water Intake Tower

The results of the analyses of samples for the presence of microcystins and nutrients, collected in 2023 and 2024, are summarized in **Table 1.**

Table 1. Iskar Reservoir – Water Intake Tower (2023–2024). Results of analyses for the determination of microcystins and nutrients.

Sampling site/ Sampling month	**ELISA** *LOQ 0.15 µg/L*		**MC–RR, –YR, –LR, µg/L** *LOQ 0.2 µg/L*		**Total N mg/L** *LOQ 0.5 mg/L*		**Total P mg/L** *LOQ 0.02 mg/L*	
	2023	**2024**	**2023**	**2024**	**2023**	**2024**	**2023**	**2024**
Iskar Reservoir – Water Intake Tower 0 m June	< LOQ	< LOQ	< LOQ	< LOQ	0.78 ± 0.08	1.35 ± 0.14	0.031 ± 0.003	0.208 ± 0.021
Iskar Reservoir – Water Intake Tower −25 m June	< LOQ	< LOQ	< LOQ	< LOQ	0.70 ± 0.07	0.63 ± 0.07	0.028 ± 0.003	0.026 ± 0.003
Iskar Reservoir – Water Intake Tower 0 m July	0.30 ± 0.07	< LOQ	LR eqv ~ 0.2	< LOQ	1.46 ± 0.15	0.53 ± 0.06	0.044 ± 0.004	0.022 ± 0.002
Iskar Reservoir – Water Intake Tower −25 m July	0.26 ± 0.06	< LOQ	LR eqv ~ 0.2	< LOQ	1.05 ± 0.11	0.54 ± 0.05	0.051 ± 0.005	0.036 ± 0.004
Iskar Reservoir – Water Intake Tower 0 m August	< LOQ	< LOQ	< LOQ	< LOQ	1.26 ± 0.13	0.66 ± 0.07	0.026 ± 0.003	0.047 ± 0.005
Iskar Reservoir – Water Intake Tower −25 m August	< LOQ	< LOQ	< LOQ	< LOQ	1.01 ± 0.10	0.58 ± 0.06	0.057 ± 0.006	0.168 ± 0.017
Iskar Reservoir – Water Intake Tower 0 m September	< LOQ	< LOQ	< LOQ	< LOQ	0.98 ± 0.10	0.74 ± 0.08	0.023 ± 0.002	0.034 ± 0.003
Iskar Reservoir – Water Intake Tower −25 m September	< LOQ	< LOQ	< LOQ	< LOQ	0.88 ± 0.09	0.53 ± 0.05	0.026 ± 0.003	0.073 ± 0.007

Iskar Reservoir – Water Intake Tower 0 m October	< LOQ	< LOQ	< LOQ	< LOQ	1.06 ± 0.11	1.01 ± 0.10	0.037 ± 0.004	0.064 ± 0.006
Iskar Reservoir – Water Intake Tower −25 m October	< LOQ	< LOQ	< LOQ	< LOQ	0.56 ± 0.06	0.74 ± 0.07	0.026 ± 0.003	0.052 ± 0.005

The results of the conducted analyses (**Table 1**) indicate an extremely low level of toxin occurrence in the area of the water intake tower of Iskar Reservoir during the period 2023–2024. In most of the analyzed samples, concentrations were below the limit of quantification (LOQ), with only two out of a total of twenty samples (both from July 2023) showing trace amounts of microcystin-LR equivalent (~ 0.2 μg/L)—a compound with a similar spectral profile but a different retention time compared to the target analytes. The detected values are close to the LOQ and significantly below the guideline value for drinking water (1 μg/L), indicating the absence of a significant toxicological risk for water supply.

The observed vertical and seasonal dynamics of nutrients are characterized by variations in total nitrogen in the range of 0.53–1.46 mg/L and total phosphorus in the range of 0.022–0.208 mg/L. The detected trace amounts of microcystins coincide with the highest measured values of total phosphorus, suggesting a possible relationship between phosphorus enrichment and the initiation of toxin release.

Correlation analysis shows a moderate positive relationship between microcystins and total nitrogen in 2023, while the relationship between microcystins and total phosphorus is less pronounced. However, these relationships are not statistically significant ($p > 0.05$), although they indicate a tendency for increasing microcystin concentrations with higher nutrient levels. In 2024, all measured concentrations of microcystins were below the LOQ and were calculated as LOQ/2, resulting in an insufficient variability and preventing correlation analysis between microcystins and nutrients. The results obtained by ELISA and HPLC are comparable, and paired t-test analysis shows no statistically significant differences, confirming the reliability of the applied analytical methods.

The area of the water intake tower is characterized by a stable oligotrophic to oligo-mesotrophic status, with limited development of cyanobacteria and absence of toxic blooms during the study period, indicating a relatively stable ecological condition of the reservoir.

Figure 1 presents a chromatogram of a sample from Iskar Reservoir – water intake tower (0 m), July 2023, showing MC–LR eqv ~ 0.2 μg/L.

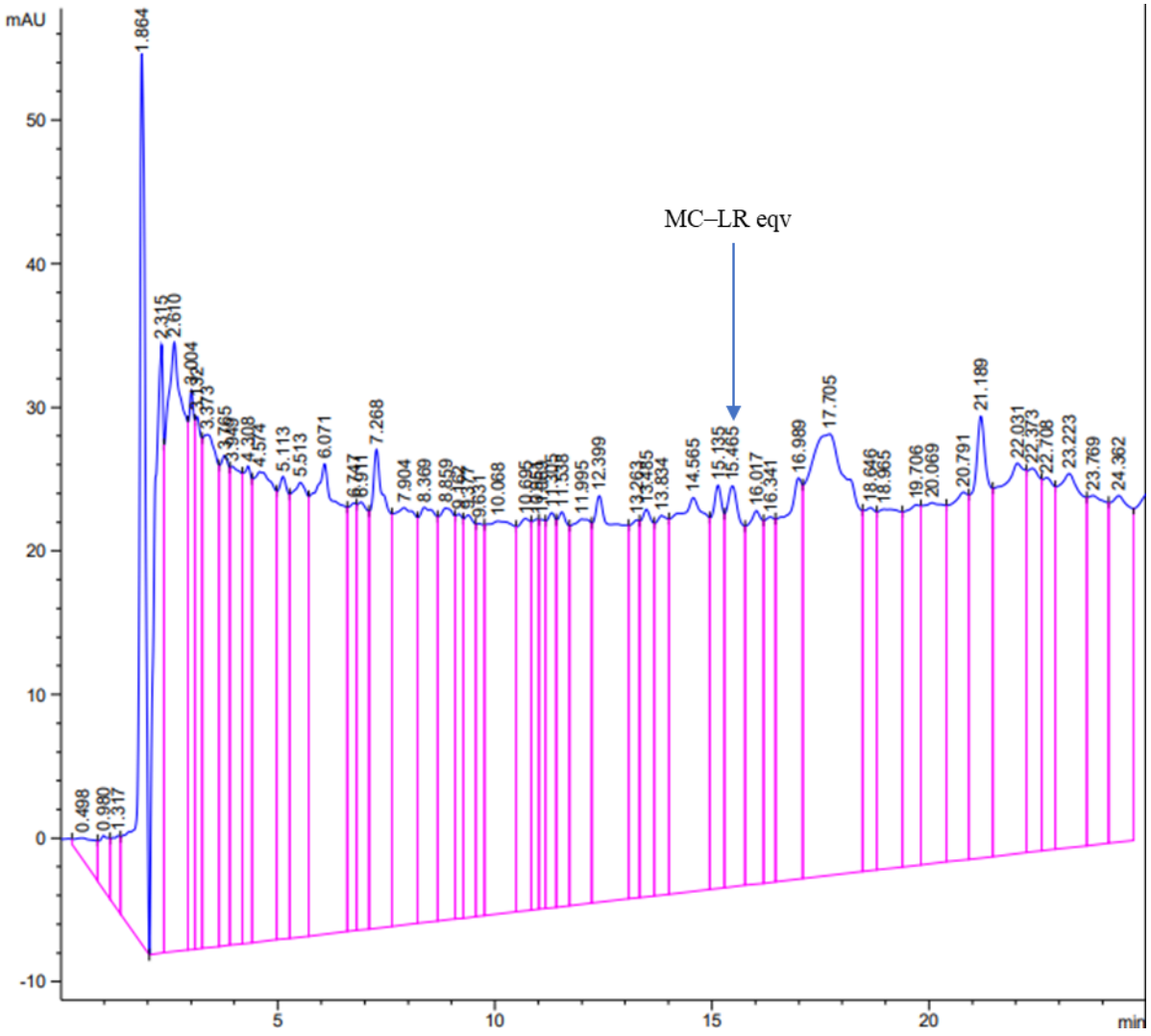

Figure 1. Chromatogram of a sample from Iskar Reservoir – water intake tower (0 m), July 2023, MC–LR eqv ~ 0.2 µg/L.

3.1.2. Iskar Reservoir – Shtarkelovo Gnezdo

The results of the analyses of samples for the presence of microcystins and nutrients, collected in 2023 and 2024, are summarized in **Table 2.**

Table 2. Iskar Reservoir – Shtarkelovo Gnezdo (2023–2024). Results of analyses for the determination of microcystins and nutrients.

Sampling site/ Sampling month	**ELISA** *LOQ 0.15 µg/L*		**MC–RR, –YR, –LR µg/L** *LOQ 0.2 µg/L*		**Total N mg/L** *LOQ 0.5 mg/L*		**Total P mg/L** *LOQ 0.02 mg/L*	
	2023	**2024**	**2023**	**2024**	**2023**	**2024**	**2023**	**2024**
Shtarkelovo Gnezdo 0 m June	< LOQ	< LOQ	< LOQ	< LOQ	1.32 ± 0.13	0.77 ± 0.08	0.053 ± 0.005	0.022 ± 0.002
Shtarkelovo Gnezdo −20 m June	< LOQ	< LOQ	< LOQ	< LOQ	1.01 ± 0.10	0.77 ± 0.08	0.062 ± 0.006	0.023 ± 0.002
Shtarkelovo Gnezdo 0 m July	0.21 ± 0.05	< LOQ	LR eqv ~ 0.2	< LOQ	1.04 ± 0.10	0.56 ± 0.06	0.035 ± 0.004	0.026 ± 0.003
Shtarkelovo Gnezdo −20 m July	0.23 ± 0.06	< LOQ	LR eqv ~ 0.2	< LOQ	1.83 ± 0.18	0.57 ± 0.06	0.038 ± 0.001	0.058 ± 0.006
Shtarkelovo Gnezdo 0 m August	0.28 ± 0.07	< LOQ	LR eqv ~ 0.2	< LOQ	1.28 ± 0.13	0.63 ± 0.06	0.091 ± 0.009	0.067 ± 0.007
Shtarkelovo Gnezdo −20 m August	< LOQ	< LOQ	< LOQ	< LOQ	0.99 ± 0.10	0.57 ± 0.06	0.136 ± 0.014	0.144 ± 0.014
Shtarkelovo Gnezdo 0 m September	< LOQ	< LOQ	< LOQ	< LOQ	1.11 ± 0.11	0.71 ± 0.07	0.022 ± 0.002	0.023 ± 0.002
Shtarkelovo Gnezdo −20 m September	< LOQ	< LOQ	< LOQ	< LOQ	1.31 ± 0.13	0.58 ± 0.06	0.023 ± 0.002	0.035 ± 0.004
Shtarkelovo Gnezdo 0 m October	< LOQ	< LOQ	< LOQ	< LOQ	1.47 ± 0.15	0.56 ± 0.06	0.036 ± 0.004	0.036 ± 0.004
Shtarkelovo Gnezdo −20 m October	< LOQ	0.15 ± 0.04	< LOQ	-LR 0.20 ± 0.02	1.28 ± 0.13	0.51 ± 0.05	0.037 ± 0.004	0.187 ± 0.019

The results of the study (**Table 2**) show a different pattern compared to the water intake tower.

In 2023, trace amounts of microcystin-LR equivalent were detected in three out of ten analyzed samples (July and August). The concentrations were close to the limit of quantification and significantly below 1 µg/L.

Particularly notable is the result from July 2023 at a depth of 20 m, where:

- the highest concentration of total nitrogen for the period was recorded (1.83 mg/L);

- trace amounts of toxin were detected.

This coincidence suggests a possible relationship between increased nitrogen enrichment and the initiation of toxin release, albeit at minimal levels.

Total phosphorus concentrations in 2023 ranged from 0.022 to 0.136 mg/L, with the highest values recorded in the deeper layer in August.

In 2024, no microcystins were detected in the surface layer. Only one sample from October 2024 at a depth of −20 m showed traces of MC–LR.

A characteristic feature of 2024 is lower nitrogen enrichment (0.51–0.77 mg/L), while phosphorus concentrations exhibited local increases up to 0.187 mg/L. Despite the relatively high total phosphorus concentrations in some samples, no development of toxin-producing cyanobacterial blooms was observed.

Correlation analysis indicates weak relationships between microcystin concentrations and nutrient levels, with low correlation coefficients that are not statistically significant ($p > 0.05$). The results obtained by ELISA and HPLC are comparable and show no significant differences, confirming the reliability of the applied methods.

Compared to the water intake tower area, the Shtarkelovo Gnezdo site is characterized by higher variability of nutrients and more frequent detection of trace toxin levels, identifying it as a more sensitive zone. Nevertheless, no development of toxic cyanobacterial blooms was observed, and the ecosystem maintains a stable oligo-mesotrophic status, typical of deep mountain reservoirs without a persistent trend toward eutrophication.

3.1.3. Iskar Reservoir – Sailing Base

The results of the analyses of samples for the presence of microcystins and nutrients, collected in 2023 and 2024, are summarized in **Table 3.**

Table 3. Iskar Reservoir – Sailing Base (2023–2024). Results of analyses for the determination of microcystins and nutrients.

Sampling site/ Sampling month	**ELISA** *LOQ 0.15 µg/L*		**MC–RR, –YR, –LR µg/L** *LOQ 0.2 µg/L*		**Total N mg/L** *LOQ 0.5 mg/L*		**Total P mg/L** *LOQ 0.02 mg/L*	
	2023	**2024**	**2023**	**2024**	**2023**	**2024**	**2023**	**2024**
Iskar Reservoir – Sailing Base June	< LOQ	< LOQ	< LOQ	< LOQ	0.79 ± 0.08	1.18 ± 0.12	0.023 ± 0.002	0.048 ± 0.005
Iskar Reservoir – Sailing Base July	0.24 ± 0.06	< LOQ	LR eqv ~ 0.2	< LOQ	1.52 ± 0.15	0.51 ± 0.05	0.063 ± 0.007	0.032 ± 0.003
Iskar Reservoir – Sailing Base August	0.26 ± 0.06	< LOQ	LR eqv ~ 0.2	< LOQ	1.58 ± 0.16	0.96 ± 0.10	0.066 ± 0.007	0.034 ± 0.003
Iskar Reservoir – Sailing Base September	< LOQ	< LOQ	< LOQ	< LOQ	0.90 ± 0.09	1.01 ± 0.10	0.025 ± 0.003	0.036 ± 0.004
Iskar Reservoir – Sailing Base October	< LOQ	< LOQ	< LOQ	< LOQ	0.88 ± 0.09	1.13 ± 0.11	0.041 ± 0.004	0.062 ± 0.006

The results of the study (**Table 3**) indicate low concentrations of microcystins in the area of the sailing base during the period 2023–2024. In 2023, trace amounts of microcystin-LR equivalent (~ 0.2 µg/L) were detected in some samples (July–August), while in 2024 no toxins were detected. The measured values are significantly below the guideline value for drinking water and do not pose a risk to water supply.

The trace amounts detected in 2023 coincide with the highest measured nutrient concentrations at this site:

Total nitrogen: 1.52 mg/L (July) and 1.58 mg/L (August);

Total phosphorus: 0.063 mg/L (July) and 0.066 mg/L (August).

This relationship supports the trend observed at other sites, linking increased nutrient enrichment with episodic toxin production.

Correlation analysis indicates a positive relationship between microcystin concentrations and nutrient levels in 2023; however, this relationship is not statistically

significant ($p > 0.05$), likely due to the limited number of observations. In 2024, due to the lack of variability in microcystin values, correlation analysis could not be performed. A statistically significant relationship was observed between the two nutrients—total nitrogen and total phosphorus ($r = 0.989$, $p = 0.001$), indicating a similar dynamic pattern of their variation within the water column.

For samples from the Iskar Reservoir – sailing base, paired t-test analysis shows no statistically significant difference between the results obtained by ELISA and HPLC.

No development of toxic cyanobacterial blooms was observed. The area is characterized by a stable oligo-mesotrophic status and limited phytoplankton development, which may be associated with more active hydrodynamic processes, sediment resuspension, and increased levels of abiotic suspended matter.

Compared to the other studied sites of Iskar Reservoir, the sailing base is characterized by moderate nutrient enrichment and the lowest level of toxin production, identifying it as a more hydrodynamically active zone with a low risk of cyanotoxin contamination.

3.2. Kokalyane Dam

The results of the analyses of samples for the presence of microcystins and nutrients, collected in 2023 and 2024, are summarized in **Table 4**.

Table 4. Kokalyane Dam (2023–2024). Results of analyses for the determination of microcystins and nutrients.

Sampling site/ Sampling month	**ELISA** *LOQ 0.15 µg/L*		**MC–RR, –YR, –LR µg/L** *LOQ 0.2 µg/L*		**Total N mg/L** *LOQ 0.5 mg/L*		**Total P mg/L** *LOQ 0.02 mg/L*	
	2023	**2024**	**2023**	**2024**	**2023**	**2024**	**2023**	**2024**
Kokalyane Dam June	< LOQ	< LOQ	< LOQ	< LOQ	0.86 ± 0.09	1.20 ± 0.12	0.045 ± 0.005	0.025 ± 0.003
Kokalyane Dam July	0.24 ± 0.06	< LOQ	LR eqv ~ 0.2	< LOQ	1.62 ± 0.16	1.01 ± 0.10	0.043 ± 0.004	0.026 ± 0.003
Kokalyane Dam August	0.28 ± 0.07	< LOQ	LR eqv ~ 0.2	< LOQ	1.75 ± 0.17	1.02 ± 0.10	0.052 ± 0.005	0.041 ± 0.004
Kokalyane Dam September	< LOQ	< LOQ	< LOQ	< LOQ	1.46 ± 0.15	0.58 ± 0.05	0.028 ± 0.003	0.043 ± 0.004
Kokalyane Dam October	< LOQ	< LOQ	< LOQ	< LOQ	0.73 ± 0.07	0.66 ± 0.06	0.027 ± 0.003	0.046 ± 0.005

The results of the study (**Table 4**) indicate low concentrations of microcystins in Kokalyane Dam during the period 2023–2024, with most values below the limit of quantification. In 2023, trace amounts of microcystin-LR equivalent were detected in individual

samples (July and August), while in 2024 no toxins were detected. The measured concentrations are significantly below the guideline value for drinking water and do not pose a risk to water supply.

The observed nutrient concentrations show moderate variability, with the detected trace amounts of microcystins coinciding with the highest values of total nitrogen, supporting the trend of a relationship between nitrogen enrichment and the initiation of toxin release.

Correlation analysis indicates a positive but not statistically significant relationship between microcystins and nutrients in 2023, while in 2024 the lack of variability in microcystin concentrations does not allow correlation analysis to be performed. No correlation is observed between the two nutrients (total nitrogen and total phosphorus), indicating weaker synchrony in their dynamics at this site.

Variance analysis demonstrates good agreement between the results obtained by ELISA and HPLC, confirming the reliability of the applied analytical methods.

Kokalyane Dam is characterized by a lower temperature regime, more limited phytoplankton diversity, absence of cyanobacterial blooms, and episodic occurrence of trace toxin levels only in 2023. As a result, a stable oligo-mesotrophic status is observed, with dominance of non-toxic phytoplankton groups and absence of toxic blooms.

As part of the drinking water supply system, the site is characterized by a low risk of cyanotoxin contamination during the study period.

The integrated analysis of the studied sites of Iskar Reservoir (water intake tower, Shtarkelovo Gnezdo, and sailing base) and Kokalyane Dam shows that the reservoir system maintains a stable oligo-mesotrophic status during the period 2023–2024, without signs of accelerated eutrophication or development of toxic cyanobacterial blooms. In all investigated zones, microcystins were detected only as trace amounts and episodically, mainly during the summer months of 2023, coinciding with increased nutrient concentrations, but remaining significantly below drinking water guideline values. A clear spatial heterogeneity is observed: the water intake tower is characterized by the lowest trophic enrichment, Shtarkelovo Gnezdo by higher sensitivity to nutrients, the sailing base by more active hydrodynamic processes, and Kokalyane Dam by limited phytoplankton development due to its lower temperature regime.

The overall correlation analysis indicates a positive but not statistically significant relationship between microcystin concentrations and nutrients in 2023, with a more pronounced association with total nitrogen, while in 2024 the lack of variability in microcystin values prevents correlation analysis.

The obtained results demonstrate that despite local variations in nutrient enrichment, the ecosystem maintains its stability and no risk to drinking water quality is observed.

3.3. Studena Reservoir

The results of the analyses of samples for the presence of microcystins and nutrients, collected during the period 2023–2025, are summarized in **Table 5**.

Table 5. Studena Reservoir (2023–2025). Results of analyses for determination of microcystins and nutrients.

Sampling site/ Sampling month	ELISA *LOQ 0.15 µg/L*			MC–RR, –YR, –LR, µg/L *LOQ 0.2 µg/L*			Total N, mg/L *LOQ 0.5 mg/L*			Total P, mg/L *LOQ 0.02 mg/L*		
	2023	**2024**	**2025**	**2023**	**2024**	**2025**	**2023**	**2024**	**2025**	**2023**	**2024**	**2025**
Water intake tower of Studena Reservoir June	0.21 ± 0.05	< LOQ	-	LR eqv ~ 0.2	< LOQ	-	0.86 ± 0.09	0.80 ± 0.08	-	0.092 ± 0.009	0.084 ± 0.008	-
Studena Reservoir shore site June	< LOQ	< LOQ	-	< LOQ	< LOQ	-	0.93 ± 0.09	1.45 ± 0.15	-	0.084 ± 0.008	0.096 ± 0.009	-
Water intake tower of Studena Reservoir July	0.22 ± 0.05	< LOQ	< LOQ	LR eqv ~ 0.2	< LOQ	< LOQ	1.51 ± 0.15	0.63 ± 0.06	0.87 ± 0.09	0.263 ± 0.026	0.021 ± 0.002	0.108 ± 0.011
Studena Reservoir shore site July	0.24 ± 0.06	< LOQ	< LOQ	LR eqv ~ 0.2	< LOQ	< LOQ	0.74 ± 0.07	0.84 ± 0.08	0.82 ± 0.08	0.191 ± 0.019	0.026 ± 0.003	0.085 ± 0.009
Water intake tower of Studena Reservoir August	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	1.05 ± 0.11	1.13 ± 0.11	2.28 ± 0.23	0.084 ± 0.008	0.173 ± 0.018	0.033 ± 0.003
Studena Reservoir shore site August	< LOQ	< LOQ	-	< LOQ	< LOQ	-	1.03 ± 0.10	1.12 ± 0.11	-	0.074 ± 0.007	0.032 ± 0.003	-
Water intake tower of Studena Reservoir September	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	1.05 ± 0.11	0.68 ± 0.07	1.04 ± 0.10	0.026 ± 0.003	0.047 ± 0.005	0.045 ± 0.005
Studena Reservoir shore site September	0.15 ± 0.04	< LOQ	< LOQ	- RR 0.20 ± 0.02	< LOQ	< LOQ	1.13 ± 0.11	1.14 ± 0.11	0.81 ± 0.08	0.022 ± 0.002	0.092 ± 0.009	0.091 ± 0.009
Water intake tower of Studena Reservoir October	1.01 ± 0.25	1.41± 0.35	-	- RR 0.55 ± 0.09 - YR 0.27 ± 0.06 - LR 0.38 ± 0.05	- RR 0.68 ± 0.05 - YR 0.31 ± 0.02 - LR 0.30 ± 0.02	-	1.06 ± 0.11	1.13 ± 0.11	-	0.031 ± 0.003	0.064 ± 0.006	-
Surface bloom near the dam wall October	2.06 ± 0.51	-	-	**- RR 0.70 ± 0.08** - YR 0.48 ± 0.06 **- LR 0.75 ± 0.09**	-	-	-	-	-	-	-	-

Studena Reservoir shore site October	< LOQ	0.30 ± 0.07	-	< LOQ	- RR 0.24 ± 0.02	-	1.05 ± 0.11	1.03 ± 0.10	-	0.027 ± 0.003	0.062 ± 0.006	-

The results of the present study indicate the occurrence of microcystins in a limited number of samples (**Table 5**). In June and July 2023, trace amounts of microcystins (LR equivalents) were detected in samples from both the water intake tower and the shore site. These compounds did not fully correspond to the standard mixture but exhibited a characteristic spectrum. In September 2023 and September 2024, traces of MC–LR were also detected in a sample from the shore site.

The most significant results were recorded in **October 2023**, when:

- a total microcystin concentration of 1.2 μg/L was measured at the water intake tower;
- MC–LR accounted for 0.38 μg/L (~ 32% of the total content);

even higher concentrations, approaching the regulatory limit, were detected in a surface bloom near the dam wall, as shown in the chromatogram in **Figure 2.**

In **2024**, a similar seasonal pattern was observed, but with lower concentrations (MC–LR = 0.30 μg/L).

Nutrient concentrations varied within the following ranges: total nitrogen – 0.63–2.28 mg/L and total phosphorus – 0.021–0.263 mg/L, without a clearly expressed relationship with microcystin concentrations. Correlation analysis for the period 2023–2025 did not reveal a statistically significant relationship between microcystins and nutrients ($p > 0.05$), indicating that toxin production is not directly driven by nitrogen and phosphorus levels, but rather results from a complex interaction of factors, including temperature regime, stratification, light conditions, and local hydrodynamic processes.

Variance analysis demonstrated good agreement between the results obtained by ELISA and HPLC, confirming the reliability of the applied analytical methods.

Studena Reservoir is characterized by a relatively stable oligo-mesotrophic status and a phytoplankton community with low species diversity, dominated mainly by diatoms. The presence of cyanobacteria is limited, and no persistent toxic blooms were observed at the investigated sites.

Comparison with retrospective data indicates an episodic but recurrent pattern of toxin production in the reservoir, with a pronounced peak in 2020 and subsequent detection of measurable concentrations in the present study. Despite the absence of sustained toxic blooms, the observed local increases in microcystins, including in surface accumulations, indicate a potential for the occurrence of risk events.

Overall, Studena Reservoir can be defined as a water source with stable ecological status, but with a demonstrated potential for episodic cyanotoxin production, which necessitates enhanced monitoring, particularly during the late summer and early autumn period.

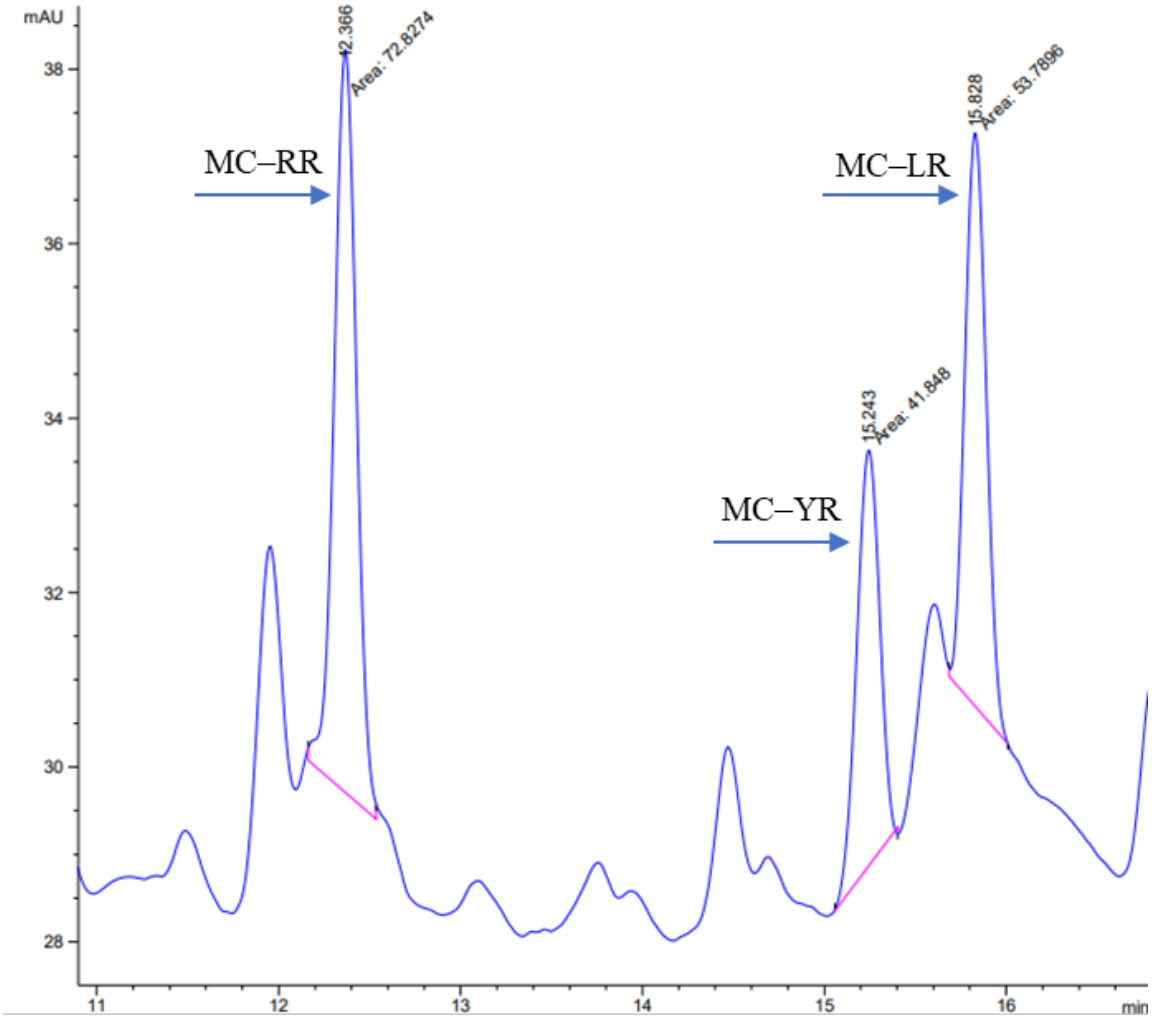

Figure 2. Chromatogram of a sample from a surface bloom near the dam wall, in the vicinity of the water intake tower of Studena Reservoir, October 2023

3.4. Krasava Reservoir

The results of the analyses of samples for the presence of microcystins and nutrients, collected during the period 2023–2025, are summarized in **Table 6**.

Table 6. Krasava Reservoir (2023–2025). Results of analyses for determination of microcystins and nutrients.

Sampling site/ Sampling month	**ELISA** *LOQ 0.15 µg/L*			**MC–RR, –YR, –LR, µg/L** *LOQ 0.2 µg/L*			**Total N, mg/L** *LOQ 0.5 mg/L*			**Total P, mg/L** *LOQ 0.02 mg/L*		
	2023	**2024**	**2025**	**2023**	**2024**	**2025**	**2023**	**2024**	**2025**	**2023**	**2024**	**2025**
Krasava Reservoir June	0.25 ± 0.06	< LOQ	-	LR eqv ~ 0.2	< LOQ	-	1.17 ± 0.12	1.80 ± 0.18	-	0.021 ± 0.002	0.023 ± 0.002	-
Krasava Reservoir July	0.27 ± 0.07	< LOQ	< LOQ	LR eqv ~ 0.2	< LOQ	< LOQ	1.16 ± 0.12	1.75 ± 0.18	1.81 ± 0.18	0.344 ± 0.034	0.032 ± 0.003	0.122 ± 0.012
Krasava Reservoir August	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	1.35 ± 0.14	1.76 ± 0.18	0.81 ± 0.08	0.246 ± 0.031	0.108 ± 0.011	0.067 ± 0.007
Krasava Reservoir September	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.90 ± 0.09	1.88 ± 0.19	1.96 ± 0.20	0.041 ± 0.004	0.082 ± 0.008	0.035 ± 0.004
Krasava Reservoir October	< LOQ	< LOQ	-	< LOQ	< LOQ	-	1.12 ± 0.11	1.25 ± 0.13	-	0.028 ± 0.003	0.131 ± 0.013	-

The results of the conducted study (**Table 6**) indicate that Krasava Reservoir is characterized by a stable oligo-mesotrophic status and low concentrations of microcystins during the period 2023–2025. In the majority of the analyzed samples, values were below the limit of quantification, with trace amounts of microcystin–LR equivalents (~ 0.2 μg/L) detected only in isolated samples in 2023 (June and July). No microcystins were detected in 2024 and 2025.

Nutrient concentrations varied within the following ranges: total nitrogen – 0.81–1.96 mg/L and total phosphorus – 0.021–0.344 mg/L. The highest value of total phosphorus (0.344 mg/L) coincided with the sample in which trace amounts of microcystins were detected, suggesting a possible local relationship between phosphorus enrichment and toxin production.

Correlation analysis revealed weak to moderate relationships between microcystin concentrations and nutrients, which were not statistically significant in 2023 ($p > 0.05$). In 2024 and 2025, the lack of variation in microcystin values did not allow for correlation analysis. No clear relationship was observed between the two nutrients.

Retrospective data confirm the consistent absence of toxic blooms in the reservoir, with no microcystins detected in previous studies, and only episodic trace amounts observed in the present study. These results indicate a low risk of cyanotoxin contamination during the investigated period. However, given its use for drinking water supply, periodic monitoring during the warm seasons is recommended, particularly under conditions of increased nutrient enrichment and climatic anomalies.

Variance analysis demonstrated good agreement between the results obtained by ELISA and HPLC, confirming the reliability of the applied analytical methods.

Figure 3 presents the chromatogram from July 2023. The detected compound exhibited spectral characteristics similar to MC–LR, but with a different retention time

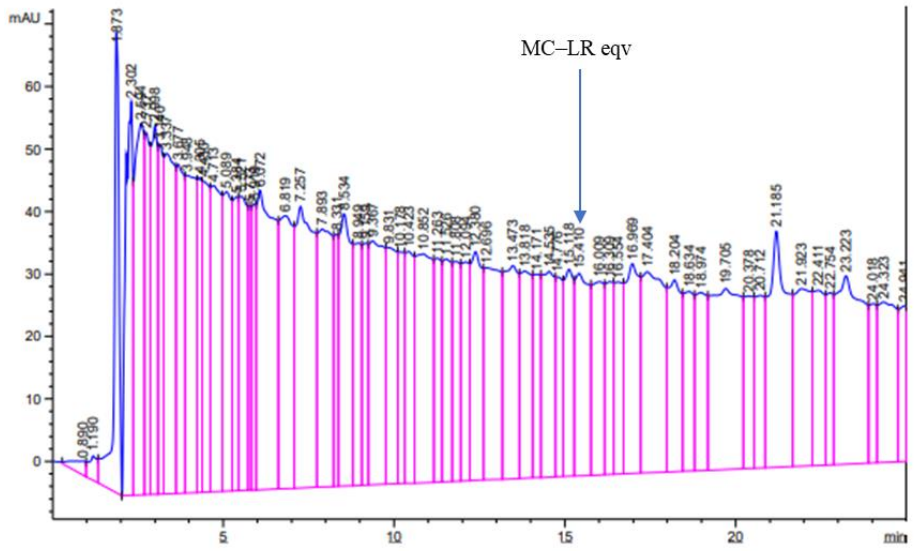

Figure 3. Chromatogram of a sample from Krasava Reservoir, July 2023. MC–LR equivalent ~ 0.2 µg/L.

3.5. Summary

The comprehensive comparative analysis of the Iskar, Studena, and Krasava reservoirs shows that all three water bodies, used for drinking water supply, are characterized by a stable oligo-mesotrophic status and β-mesosaprobic conditions, but exhibit different potentials for cyanobacterial bloom development and microcystin production.

The phytoplankton structure in all reservoirs is dominated by diatoms, with limited presence of cyanobacteria and no formation of persistent toxic blooms. The highest tendency for episodic toxin production was observed in Studena Reservoir, where a surface bloom with significant microcystin concentrations was detected. In contrast, Iskar and Krasava reservoirs demonstrate a more stable ecological structure, with microcystins detected only at trace levels.

The summarized data on positive samples show that microcystin concentrations range between 0.2 and 1.29 µg/L, with the highest values recorded in Studena Reservoir during the autumn period. In Iskar and Krasava reservoirs, the detected concentrations are significantly lower (around 0.2 µg/L) and episodic in nature. Positive samples were mainly recorded during the summer and early autumn, across a wide temperature range (11–28 °C), indicating that temperature is a necessary but not sufficient factor for toxin occurrence.

Nutrient concentrations vary within moderate ranges (total nitrogen: 0.74–1.83 mg/L; total phosphorus: 0.021–0.344 mg/L), with considerable variability in the N/P ratio. In some cases, elevated nutrient levels coincide with the presence of microcystins; however, no consistent relationship was established.

Correlation analysis, both for individual reservoirs and for the combined dataset (n = 99), does not show a statistically significant relationship between microcystin concentrations and nutrient levels. This indicates that microcystin production is not directly determined by nutrient enrichment, but rather results from a complex interaction of environmental factors, including temperature regime, stratification, seasonal mixing, light conditions, and hydrodynamics.

Microcystins were detected both in surface waters and in samples taken from greater depths (20–25 m), where light intensity is significantly lower. This suggests vertical distribution of toxins due to mixing processes and cyanobacterial cell lysis. The highest concentrations were recorded in late summer and early autumn, corresponding to the period of stratification breakdown.

Comparative analysis indicates that Studena Reservoir has the highest potential for episodic microcystin production, including concentrations approaching or exceeding the drinking water guideline value (1 μg/L), whereas Iskar and Krasava reservoirs exhibit lower toxicological risk and greater ecological stability.

The comparison of analytical methods demonstrates good agreement between ELISA and HPLC, with ELISA proving suitable for routine screening and HPLC serving as a reliable method for confirmation and identification of individual microcystin variants. **Figure 4** summarizes the positive samples and microcystin concentrations determined by ELISA in the Iskar, Studena, and Krasava reservoirs during 2023–2025.

The obtained results are consistent with international studies showing that cyanotoxin production in moderately trophic water bodies is episodic, difficult to predict, and not always directly related to nutrient concentrations. The observed seasonal patterns and the role of thermal stratification further confirm the importance of hydrophysical factors in the formation of toxic blooms.

In conclusion, the studied Bulgarian drinking water reservoirs—Iskar, Studena, and Krasava—are characterized by good ecological status and low to moderate risk of microcystin contamination. The occurrence of toxic events is driven not solely by nutrient levels, but by the complex interaction of physical, chemical, and biological processes. This highlights the need for dynamic, seasonally oriented monitoring, particularly during high-risk periods.

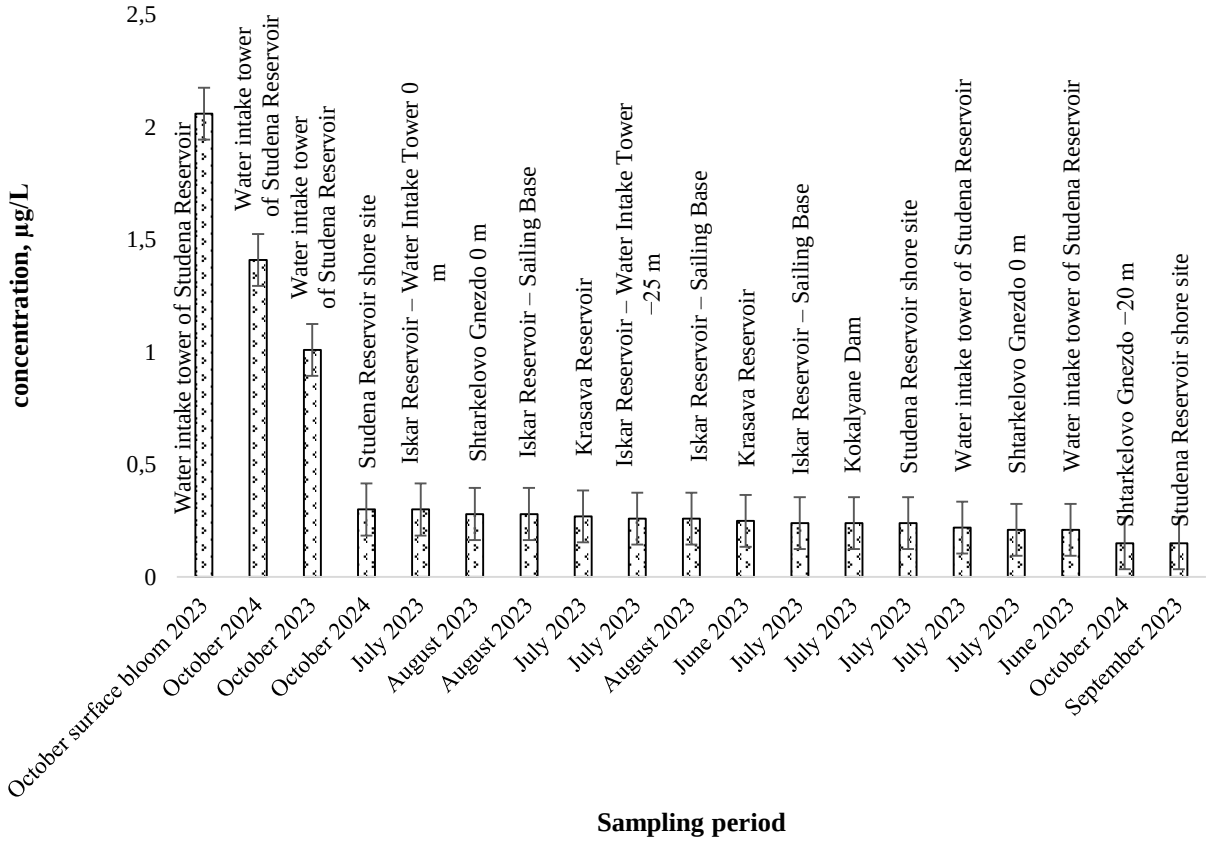

Figure 4. Positive samples and microcystin concentrations determined by ELISA in Iskar, Studena, and Krasava reservoirs.

The research is currently ongoing within the framework of the project “Investigation of cyanotoxins in selected Bulgarian water bodies (CYANOACTION-BG)”, co-funded nationally under the COST Programme – Action CA23160 “Effective Lake Management: Reducing Cyanobacteria by Actions in the Catchment (CYANOACTION)”, under contract with the Bulgarian National Science Fund No. KP-06-COST/3/10.06.2025.

In summary, the results of the present dissertation expand the existing scientific knowledge on the occurrence of microcystins in Bulgarian drinking water reservoirs and identify key environmental factors associated with their presence. They provide a basis for future research and contribute to the improvement of national policies for cyanotoxin monitoring.

4. CONCLUSIONS

Methodological conclusions

1. A chromatographic method for the determination of microcystins (–RR, –YR, and –LR) in water by HPLC was developed and validated, with a limit of quantification (LOQ) of 0.2 μg/L, a limit of detection (LOD) of 0.1 μg/L, a linear range from 0.2 to 1.5 μg/L, and analytical recovery between 66.5% and 123.79%. The method complies with the requirements of Directive (EU) 2020/2184 and Regulation No. 9/2001 on drinking water quality and is included in the scope of accreditation of the Health Testing Center at the National Center of Public Health and Analyses.
2. The microcystin concentrations obtained by ELISA are comparable to those determined by HPLC.

Occurrence and dynamics of microcystins

3. The studied reservoirs—Iskar, Studena, and Krasava—are characterized by predominantly β-mesosaprobic conditions and an oligo-mesotrophic status, with no evidence of persistent mass cyanobacterial blooms during the period 2023–2025.
4. Phytoplankton communities are mainly dominated by diatoms, while cyanobacteria are present in low abundance and do not show a sustained tendency toward dominance.
5. In Studena Reservoir, measurable concentrations of microcystins were detected in 2023 and 2024, including values approaching the regulatory limit for microcystin–LR (1 μg/L), as well as elevated concentrations in surface bloom patches. These data confirm an episodic but recurrent pattern of toxin production.
6. In Krasava Reservoir and at various sites of Iskar Reservoir, only trace amounts of microcystin–LR equivalents were detected in 2023, with no evidence of sustained toxin production in subsequent years.
7. In 2025, no microcystins were detected in the analyzed water samples.

Relationship with environmental factors

8. No consistent linear relationship was established between total nitrogen, total phosphorus, and microcystin concentrations. The observed relationships are weak to moderate and in most cases not statistically significant ($p > 0.05$). The strongest correlations were observed in Iskar Reservoir, while in Studena and Krasava the relationship between microcystins and nutrients is less pronounced, indicating that toxin production is not solely determined by nutrient enrichment.

9. The episodic occurrence of microcystins is associated with:

- late summer–early autumn temperature maxima;
- breakdown of thermal stratification;
- seasonal mixing of the water column;
- locally elevated nutrient concentrations.

10. Higher microcystin concentrations observed during the autumn period suggest the release of intracellular toxins as a result of cyanobacterial cell lysis under changing hydrophysical conditions.

Risk assessment

11. Among the studied water bodies, Studena Reservoir shows the highest potential for episodic toxin production, whereas Iskar and Krasava reservoirs demonstrate a more stable ecological status and lower toxicological risk.

12. The obtained results confirm the need for dynamic, seasonally oriented monitoring of drinking water reservoirs, particularly during periods of elevated temperatures and hydrophysical changes, in accordance with European regulatory requirements.

5. RECOMMENDATIONS

5.1. Monitoring of vulnerable water bodies

The conducted study on the seasonal dynamics of microcystins in drinking water reservoirs in the Sofia region during the period 2023–2025 identified Studena Reservoir as a water body with increased vulnerability to episodic cyanotoxin production.

The detected microcystin concentrations, approaching the regulatory limit of 1 μg/L for microcystin–LR, necessitate the inclusion of this parameter in routine monitoring programs for drinking water quality.

It is recommended that monitoring be carried out with increased frequency during the warm months and the early autumn period, when the risk of cyanobacterial events is highest.

5.2. Preventive monitoring of potentially at-risk reservoirs

Under conditions of dynamically changing climate and increasing eutrophication processes, a preventive approach should be applied in the assessment of risks to drinking water sources.

Reservoirs such as Iskar and Krasava, where only trace amounts of microcystins have been detected, should also be included in periodic monitoring during seasons with favorable conditions for cyanobacterial growth and toxin production.

Such an approach would enable early identification of unfavorable trends and timely implementation of management and technological measures to ensure the safety of drinking water.

6. CONTRIBUTIONS

1. An analytical method “Water. Determination of microcystins by HPLC” was developed and validated, implemented as an in-house laboratory method, and included in the scope of accreditation of the Health Testing Center at the National Center of Public Health and Analyses. The method can be applied for monitoring microcystin–LR in accordance with Directive (EU) 2020/2184 and Regulation No. 9/2001 on drinking water quality.
2. For the first time in Bulgaria, a systematic study on the seasonal dynamics of microcystins and major nutrients in reservoirs used for drinking water supply has been conducted.
3. For the first time, the seasonal dynamics of microcystins and nutrients (spring–summer–autumn) were investigated during the period 2023–2024 in the main drinking water source of the capital city—Iskar Reservoir—through sampling at three sites: Iskar Reservoir – water intake tower, Iskar Reservoir – Shtarkelovo Gnezdo, and Iskar Reservoir – sailing base.
4. For the first time, the dynamics of microcystins were assessed at the inlet of the Pancharevo Drinking Water Treatment Plant (Kokalane dam) as part of the Iskar Reservoir water supply system.
5. For the first time, the seasonal dynamics of microcystins and nutrients (spring–summer–autumn) were characterized during the period 2023–2025 in Studena Reservoir, a drinking water source for the city of Pernik and the surrounding region.
6. For the first time, the seasonal dynamics of microcystins and nutrients (spring–summer–autumn) were characterized during the period 2023–2025 in Krasava Reservoir, used for drinking water supply of the town of Breznik and nearby settlements.
7. The obtained results complement and expand long-term studies on cyanotoxins in water bodies in the Sofia region conducted by the National Center of Public Health and Analyses since 2004, enriching the database on the risk of toxin production in drinking water sources.
8. The dissertation provides a scientific basis for the implementation of microcystin–LR monitoring in accordance with Directive (EU) 2020/2184, including the associated homologues microcystin–YR and –RR for a more comprehensive risk assessment.
9. The generated data can be used in the development of risk assessment programs and management strategies for the quality of surface waters intended for drinking purposes.

7. PUBLICATIONS

1. **Mitreva, M.,** & Pavlova, V. (2018). Microcystin–LR: A new contaminant in the European Drinking Water Directive. *Proceedings of the Annual University Scientific Conference of Vasil Levski National Military University*, 382–389, ISSN 2367-7481.

2. **Mitreva, M.,** & Pavlova, V. (2023). Development of a method for determination of microcystin–LR and its analogues by HPLC. *Proceedings of the Annual University Scientific Conference of Vasil Levski National Military University*, 1520–1528, ISSN 2367-7481.

3. **Mitreva, M.,** Karakashkova, P., & Pavlova, V. (2024). Study of the seasonal dynamics of microcystins and nutrients in Kokalyane bent. *Mechanization in Agriculture & Conserving of the Resources*, (4), 159–161, ISSN 2603-3704.

8. PARTICIPATION IN SCIENTIFIC FORUMS

1. **Mitreva, M.,** Pavlova, V. Microcystin–LR: a new contaminant in the European Drinking Water Directive. Oral presentation at the Annual University Scientific Conference of the National Military University "Vasil Levski", 14–15 June 2018, Veliko Tarnovo, Bulgaria.

2. **Mitreva, M.,** Pavlova, V. Development of a method for determination of microcystin–LR and its analogues by HPLC. Oral presentation at the Annual University Scientific Conference of the National Military University "Vasil Levski", 08–09 June 2023, Veliko Tarnovo, Bulgaria**.**

3. **Mitreva, M.,** V. Pavlova. Development of a highly sensitive and reliable analytical method for determination of Microcystin**–**LR in drinking water. Poster presented at 5th International Environmental Chemistry Congress, 30.10–02.11.2023, Antalya, Turkey.

4. Pavlova, V., **Mitreva, M**. Cyanobacterial toxins – a new contaminant for monitoring in the Drinking Water Directive. Oral presentation at an online seminar with international participation during the European Public Health Week, 13–17 May 2024.

5. Pavlova, V., **Mitreva, M.**, Rachinska, M., Sotirova, F. Emerging contaminants in the revised and transposed Directive (EU) 2020/2184 on the quality of water intended for human consumption. Oral presentation at the XI National Conference on Nutrition, 30 May–1 June 2024, Sofia, Bulgaria.

6. **Mitreva**, M., Pavlova, V., Ivanov, Kr., Androv, M., Karakashkova, P., Zhilanov, D., Vlahova, V. Microcystin–LR – a new parameter in the revised Directive (EU) 2020/2184 on the quality of water intended for human consumption. Poster presented at the seminar with international participation *"Instrumental Techniques and Methods for Chemical Analysis – Challenges and New Solutions"*, 5 June 2024, Plovdiv, Bulgaria.

7. **Mitreva, M.,** P. Karakashkova, K. Ivanov, M. Androv, V. Vlahova, D. Zhilanov, M. Rachinska, F. Sotirova, A. Lazarova, V. Pavlova, B. Uzunov. Study of Seasonal Dynamics of Microcystins and Nutrients in Iskar dam. Poster presented at 6th International Environmental Chemistry Congress, 05.11–08.11.2024, Trabzon, Turkey (**1st place poster award).**

8. **Mitreva, M.,** Pavlova, V., Georgieva, V. Investigation of microcystin content in selected water bodies in the Sofia region. Poster presented at the seminar *"Instrumental Techniques and Methods for Chemical Analysis"*, 05 June 2025, Plovdiv, Bulgaria.

9. Pavlova, V., **M. Mitreva.** Microcystin-LR as a New Parameter under the Revised EU Drinking Water Directive (2020/2184), Poster presented at *Twelfth National Conference on*

Chemistry, Chemistry for building a decarbonized society, University of Chemical Technology and Metallurgy, 25−27.09.2025, Sofia, Bulgaria.