

STUDY OF SEASONAL DYNAMICS OF MICROCYSTINS AND NUTRIENTS IN STUDENA RESERVOIR IN 2023

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Abstract

This paper is a synthesis of data from studies conducted in 2023 on the seasonal dynamics of the presence of microcystin-LR and its homologues in water samples from Studena reservoir. A total of ten samples were collected from June to October 2023. The species composition of phytoplankton and the nutrients total nitrogen and total phosphorus were determined. The presence of microcystins was detected in three samples from the months of September and October (microcystin-LR concentration 0.38 and 0.75 µg/L in the water sample and in a scum at the reservoir wall in October; microcystin-RR 0.10 µg/L in September and 0.55 and 0.70 µg/L in the water sample and in a scum at the reservoir wall in October; microcystin-YR 0.27 and 0.48 µg/L in the water sample and in a scum at the reservoir wall in October). Trace amounts of microcystin-LR equivalents other than microcystin-RR and microcystin-YR were observed in water samples in June and July.

The phytoplankton communities in the reservoir are persistently inhabited by blue-green algae. The presence of microcystins at concentrations close to the permissible level in the autumn sample indicates that at the time of sampling the bloom of cyanobacteria that produced the microcystins had passed and the toxins released remained in the water.

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Due to the near acceptable concentration of MC-LR in the autumn sample, serious consideration should be given to monitoring the reservoir during periods favourable for bloom formation.

Key words: microcystins, drinking water, phytoplankton

Introduction. Studena reservoir is located at the western end of Vitosha mountain, on the upper reaches of the Struma River. It is located in the land of the villages of Studena, Kladnitsa and Bosnek in the municipality of Pernik [1]. It was put into operation on December 5, 1953 [2]. In 1992 the spillway of the reservoir was upgraded, which brought the volume of the reservoir to 25.2 million m³ [2]. It is used for drinking and domestic water supply of the city of Pernik, as well as for irrigation and industrial purposes.

In addition to its natural catchment, including the Struma River and its high tributaries, the reservoir also collects water from the Vladaia Canal (which brings water from the northeastern part of Vitosha mountain, with its main intakes on the Vladaiska and Rudarska rivers) and the Palakaria Canal (from the southern slopes of Vitosha mountain and the Palakaria River) [2]. Blue-green algae, also known as Cyanoprokaryota/Cyanobacteria, have existed on earth since the beginning of life. Cyanobacteria are prokaryotes that photosynthesize and contain chlorophyll-*a*. Some of them produce cyanotoxins. Cyanotoxins adversely affect the health of humans and animals when water bodies used for recreation or drinking water supply are contaminated with them. A wide variety of cyanobacteria can produce cyanotoxins. The most common genera are *Microcystis*, *Anabaena*, *Planktothrix* (*Oscillatoria*) and *Aphanizomenon*. Among the cyanotoxins, microcystins, cylindrospermopsin, anatoxins, and saxitoxins are most commonly found [3].

The current requirements for drinking water quality in Europe are laid down in the new Directive (EU) 2020/2184 of 16 December 2020 on the quality of water intended for human consumption. Among the new pollutants that will be mandatory for monitoring from 2026 and for which the risk of contamination of water bodies should be assessed is microcystin-LR. It is representative of microcystins, which are a group of cyanotoxins produced by blue-green algae. Its maximum permissible concentration in drinking water according to the new directive is 1 µg/L [4]. The European Directive has been transposed into our national legislation in an amendment to Ordinance No. 9/2001 on the quality of water intended for drinking purposes (promulgated in SG No. 30 of 2001, as amended in SG No. 43 of 16 May 2023) [5].

Microcystins (MCs) are cyclic heptapeptide toxins produced by some species of cyanobacteria – *Microcystis*, *Oscillatoria*, *Anabaenopsis*, *Aphanocapsa*, etc. [6]. Their cyclic structure makes them resistant to extreme pH values, high temperatures and sunlight. More than 270 variants of varying toxicity have been isolated [7]. The most toxic isomer is the known hepatotoxin microcystin-LR (MC-LR). Microcystin-LA has similar toxicity, followed by microcystin-RR (MC-RR) and

microcystin-YR (MC-YR) [8]. Adverse health effects in humans occur due to physical contact, inhalation and contaminated drinking water or food. The World Health Organization recommendation is for a microcystin content in drinking water of no more than 1 µg/L [3], which is also reflected in the new drinking water directive.

Drinking water is an important route of exposure [9]. Cyanotoxins can also accumulate in some foods of aquatic origin. Therefore, country or region specific assessments should be made as to whether food can contribute significantly to exposure – especially molluscs, fish and shellfish from ponds with blooms. Recreation in ponds with cyanobacterial blooms may also periodically expose humans to high concentrations of cyanotoxins.

Studies of cyanobacterial blooms have attracted the attention of scientists around the world for decades. In Bulgaria, the first study on the presence of microcystins in Bulgarian water bodies intended for drinking water supply and recreation was conducted by a research team of the National Center for Public Health and Analyses (NCPHA) in the period 2004–2005 [10, 11]. In 2022, a summary of the results of analyses of cyanotoxin content, nutrients, and species composition of phytoplankton of water samples collected from Studena reservoir in the period 2017–2022 was made [12].

The purpose of this article is a summary of data from studies supported by the project “Seasonal dynamics of microcystin-LR, a priority according to the new European Directive on the quality of drinking water”, funded by the National Science Fund through the Contract KP-06-M69/5 from 15.12.2022 from the competition for financial support for project of junior basic researchers and postdocs. The research is related to the seasonal dynamics of microcystins and nutrients content in water samples from Studena reservoir taken during the summer and autumn season of 2023.

Materials and methods. The water samples from Studena reservoir considered in this paper were taken mainly from three points: from the surface near the tower intake site; a few meters from the wall; and from a quay 500 m from the water tower. A total of ten samples were taken, in the months from June to October 2023, for analysis of microcystins, nutrients (total nitrogen and total phosphorus), and algal species composition.

Analysis of phytoplankton communities. For the hydrobiological analyses, 1 L water samples collected from specific points in the water body were preserved with an appropriate fixative [13]. In this case, the fixative used was a formaldehyde solution (formaldehyde 35–38%, Sigma Aldrich: 10 mL per 1 L sample). Next, the samples were concentrated by sedimentation. The aim of this procedure is to “capture” all the phytoplankton cells present in each 1 L sample and separate them into a smaller water volume of 10 mL for analysis – the so-called concentrated sample. Microscopic analysis is carried out on the samples thus prepared to determine the species composition and quantitative abundance

of the different species in the phytoplankton community. Microscopic analysis was performed with a Carl Zeiss Laboval 4 Microscope at a magnification of 400 (10×40). Biological identifiers and atlases were used for species identification and a 0.9 mm^3 Burkner chamber was used for quantification. The final result expresses the total number of cells or the number of cells per mass species (at bloom). The saprobial assessment was performed by applying the Pantle–Buck method based on the indicator value (s) and the relative frequency of occurrence (h) of the microalgal species in the samples [14].

Determination of microcystins by liquid chromatography (HPLC).

Water samples for the determination of microcystins shall be stored frozen in 1.5 L plastic bottles. Thaw before processing (freeze and thaw twice in the presence of massive blooms) to ensure lysis of the cyanobacterial cell walls, then filter with a glass-fibre filter (glass microfibre filter GF/A, 47 mm-Cytiva).

The filtered samples were extracted by solid-phase extraction with cartridges (Oasis HLB 60 μm (LP) 6 mL, Waters Corporation, USA). Elution was performed with methanol (HPLC purity) and evaporated to dryness by a gentle stream of nitrogen. Add 0.5 mL of 50% (v/v) methanol. The final extracts were filtered with PTFE filters (0.45 μm , Alltech).

Chromatographic determination was carried out with an Agilent 1200 diode array liquid chromatograph. The analytical column was a Supelcosil ABZ+Plus column ($150 \times 4.6 \text{ mm}$, 5 μm , Supelco), thermostated at 25°C . A gradient of acetonitrile (HPLC grade) and ultrapure water (0.055 μS) was used with an addition to both solvents of 0.1% trifluoroacetic acid (spectroscopically pure): 20% acetonitrile at 0 min to 46% at 20 min, chromatography time 25 min at 1 mL/min flow, 238 nm wavelength. Certified reference materials for microcystins -LR and -RR and reference material for microcystin-YR (Abraxis, Eurofins) were used for calibration. The HPLC method is accredited in-laboratory methodology according to EN ISO/IEC 17025:2018.

Determination of nutrients. Determination of total nitrogen was performed after mineralization of the sample with concentrated sulphuric acid in a thermoblock (Digester unit DS6, type 2006, FOSS TECATOR, Sweden). The ammonium sulphate obtained in this process is decomposed with sodium base to ammonia, which is trapped in boric acid solution in a Kjeltrec 2100 distillation apparatus (Kjeltrec 2100 type distiller, FOSS TECATOR, Sweden). The ammonium borate is determined by reverse titration with 0.01 mol/L (0.01N HCL) hydrochloric acid solution. A 10 cm^3 burette class “AS”, tolerance $\pm 0.02 \text{ cm}^3$, ISOLAB, Germany, was used. Calculate total nitrogen, N with a limit of quantification of 0.5 mg/L.

The determination of total phosphorus was carried out according to the procedure described in BDS EN ISO 6878:2005 “Water quality – Determination of phosphorus – Ammonium molybdate spectrometric method” [15] using a Specol 11 spectrophotometer, Carl Zeiss Jena. The limit of quantification is 0.02 mg/L.

Results and discussion. The results of the analyses of water samples from Studena reservoir collected in 2023 for the presence of microcystins, nutrients (total nitrogen and total phosphorus) and determination of phytoplankton species composition are summarized in Table 1.

The present study has established that the phytoplankton community of Studena reservoir is species-poor and consists of single representatives of blue-green, green, diatom, euglenophyte and pyrrhophyte microscopic algae. In the study period (June – October 2023) two species dominate, the diatom alga *Lindavia comta* (Kützing) T. Nakov & al. and the blue-green algae *Aphanocapsa elachista* West & G.S. West. A bloom of the alga *Lindavia comta* (Kützing) T. Nakov & al. was observed in the summer sampling of 23.08.2023 at both sites – water tower and quay. Diatom algae are one of the most common phytoplankters in standing water bodies and the species mentioned above is also well represented in aquatic biocenoses. These results are in agreement with our previous studies on the reservoir (2017–2022) [12].

It is noteworthy that the species *Aphanocapsa elachista* West & G.S. West of blue-green algae is one of the permanent members of the phytoplankton community inhabiting the reservoir. No cyanobacterial blooms were observed during the period analysed.

According to the calculated saprobic indices, ranging from 1.14 to 1.90 in 2023, Studena reservoir maintains an oligosaprobic status transitioning to betame-saprobic, which means that it is moderately polluted with organic compounds and is in a good condition with respect to dissimilation processes. The trophic status of the reservoir is “moving” from oligotrophic to oligo-mesotrophic, which is typical of non-highland reservoirs. A similar conclusion was reached in a study of the reservoir conducted between 2011 and 2015 [16] and in a study from 2017–2022 [12]. The reservoir was in a beta-mesosaprobic – oligosaprobic state, proving that this has been the constant equilibrium state of the reservoir over the last 13 years.

The results obtained for microcystin content (MC-RR, MC-YR and MC-LR) from this study indicate that trace amounts of microcystins that are not among the standard mixture but have a characteristic spectrum (LR-equivalents) were observed in the water tower and quay samples in June and July 2023. In September, traces of MC-LR were detected in the sample from the quay.

In October, however, worrying amounts of microcystins were detected in the samples from the water tower and from a scum next to the reservoir wall formed near the tower, which in the sample from the scum approached the maximum value for MC-LR allowed under Directive (EU) 2020/2184 of 16.12.2020 on the quality of water intended for human consumption and Ordinance No 9/2001 on the quality of water intended for drinking and drinking purposes (promulgated in SG No. 30 of 2001, as amended in SG No. 43 of 16 May 2023) – 1 µg/L.

In the sample from the water tower, the content of MC-LR was 0.38 µg/L with a total content of the three microcystins of 1.2 µg/L, i.e. MC-LR was about

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Studena reservoir 2023. Results of analyses for phytoplankton identification, cyanotoxin content and nutrients

No.	Sampling site/ Date of sampling	Species (according to https://www.algaebase.org/search/species/detail/?species_id=358)	TCN (Total Cells Number)/L PBSITS (Pantle- Buck's Saprobio- logical Index and Trophic State)	MC-RR, YR, LR µg/L LOD 0.1 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
1.	Studena water tower 15.06.2023	Cyanophyta <i>Aphanocapsa elachista</i> West & G.S. West <i>Oscillatoria tenuis</i> C. Agardh ex Gomont Bacillariophyta <i>Lindavia comta</i> (Kützing) T. Nakov & al.	TCN/L 3.6×10^5	< LOQ (LR eqv ~ 0.2)	0.86
			PBSITS: 1.7 β -mesosabrobic/ oligo-mesotrophic state		
2.	Studena water tower 20.07.2023	Cyanophyta <i>Aphanocapsa elachista</i> West & G.S. West Euglenophyta <i>Trachelomonas volvocina</i> (Ehrenberg) Ehrenberg Bacillariophyta <i>Lindavia comta</i> (Kützing) T. Nakov & al. <i>Nitzschia sigmaidea</i> (Nitzsch) W. Smith <i>Epithemia</i> spp.	TCN/LTCN/L 1.13×10^6	< LOQ (LR eqv ~ 0.2)	1.51
			PBSITS: 1.6 β -mesosabrobic/ oligo-mesotrophic state		
					0.26

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3.	Studena quay 20.07.2023	Cyanophyta <i>Aphanocapsa elachista</i> West & G.S. West Bacillariophyta <i>Lindavia comta</i> (Kützing) T. Nakov & al. <i>Asterionella formosa</i> Hassall Euglenophyta <i>Trachelomonas</i> spp.	TCN/L 1.7 × 10 ⁶	< LOQ (LR eqv ~ 0.2)	0.74
			PBSITS: 1.4 oligosaprobic/ oligotrophic state		0.19
			Taq Man Cyano 16S		
4.	Studena water tower 23.08.2023	Cyanophyta <i>Aphanocapsa elachista</i> West & G.S. West Euglenophyta <i>Trachelomonas volvocina</i> (Ehrenberg) Ehrenberg Chlorophyta <i>Coenococcus planktonicus</i> Korshikov <i>Chlorella vulgaris</i> Beijerinck Bacillariophyta <i>Asterionella formosa</i> Hassall <i>Lindavia comta</i> (Kützing) T. Nakov & al.-blooming	1.7 × 10 ⁶ /L number of cells of the flowering species <i>Lindavia comta</i> . in the sample	< LOQ	1.05
			PBSITS: 1.9 β -mesosaprobic/ oligo-mesotrophic state		0.08
5.	Studena quay 23.08.2023	Bacillariophyta <i>Lindavia comta</i> (Kützing) T. Nakov & al. -blooming <i>Asterionella formosa</i> Hassall	1.3 × 10 ⁶ /L number of cells of the flowering species <i>Lindavia comta</i> in the sample	< LOQ	1.03
			PBSITS: 1.14 oligosaprobic/ oligotrophic state		0.07

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6.	Studena water tower 13.09.2023	Cyanophyta <i>Aphanocapsa elachista</i> West & G.S. West Bacillariophyta <i>Lindavia comita</i> (Kützing) T. Nakov & al. Euglenophyta <i>Trachelomonas volvocina</i> (Ehrenberg) Ehrenberg	TCN/L 3.2×10^5	< LOQ	1.05
			PBSITS: 1.7 β -mesosaprobic/ oligo-mesotrophic state		0.02
7.	Studena quay 13.09.2023	Cyanophyta <i>Aphanocapsa elachista</i> West & G.S. West <i>Merismopedia tranquilla</i> (Ehrenberg) Trevisan Chlorophyta <i>Scenedesmus bijugus</i> (Turpin) Lagerheim Bacillariophyta <i>Lindavia comita</i> (Kützing) T. Nakov & al.	TCN/L 4.3×10^5	– RR 0.10 ± 0.01	1.13
			PBSITS: 1.5 oligosaprobic/ oligotrophic state		0.02
8.	Studena water tower 12.10.2023	Cyanophyta <i>Aphanocapsa elachista</i> West & G.S. West Bacillariophyta <i>Lindavia comita</i> (Kützing) T. Nakov & al. <i>Navicula</i> spp. Euglenophyta <i>Trachelomonas volvocina</i> (Ehrenberg) Ehrenberg	TCN/L 3.8×10^5	– RR 0.55 ± 0.09 – YR 0.27 ± 0.06 – LR 0.38 ± 0.05	1.06
			PBSITS: 1.6 β -mesosaprobic/ oligo-mesotrophic state		0.03

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9.	Scum at the reservoir wall 12.10.2023			– RR 0.70 ± 0.08 – YR 0.48 ± 0.06 – LR 0.75 ± 0.09	
10.	Studena quay 12.10.2023	<i>Cyanophyta</i> <i>Aphanocapsa elachista</i> West & G.S. West <i>Bacillariophyta</i> <i>Lindavia comta</i> (Kützinger) T. Nakov & al. <i>Fragilaria crotonensis</i> Kitton <i>Stephanodiscus astraea</i> (Kützinger) Grunow <i>Euglenophyta</i> <i>Trachelomonas volvocina</i> (Ehrenberg) Ehrenberg <i>Dinophyta</i> <i>Ceratium hirundinella</i> (O.F. Müller) Dujardin <i>Chlorophyta</i> <i>Chlorella vulgaris</i> Beijerinck <i>Cosmarium baileyi</i> Wolle	TCN/L 9.8 × 10 ⁵	< LOQ	1.05
			PBSITS: 1.8 <i>β</i> -mesosabrobic/ oligo-mesotrophic state		0.02

32% of the total content of the three toxins.

In the sample from the scum near the water tower, the MC-LR content was 0.75 µg/L with a total content of the three microcystins of 1.93 µg/L, or MC-LR was about 39% of the total content of the three toxins. Determination of the species composition of the phytoplankton did not indicate the presence of cyanobacterial species, which means that the bloom that produced the amounts of microcystins had passed at the time of sampling and the toxins released from it remained in the water.

The results of a study of the microcystin content in Studena reservoir in the period 2017–2022 under the scientific theme of the plan of the National Center for Public Health and Analyses show that in 2020 MC-RR and MC-YR are present in trace amounts in samples from the water tower (MC-RR with concentrations of 0.21 and 0.10 µg/L in summer and autumn, respectively, and MC-YR 0.10 µg/L in both seasons), but MC-LR is present in amounts approaching the maximum allowable concentration for drinking water of 1 µg/L (0.63 and 0.78 µg/L in summer and autumn, respectively). The MC-LR content was 67% in the summer sample and 80% in the autumn sample. The sample taken in autumn from a bloom patch located a few metres from the water supplied area showed the presence of very high amounts of MC-YR and MC-LR (62.17 and 169.47 µg/L respectively), and traces of MC-RR (0.10 µg/L). In this sample the MC-LR content was 73%. Microscopic analysis of the scum indicated the presence of *Coelosphaerium kuetszingianum* Nägeli, a toxic species of the *Microcystaceae* family inhabiting freshwater ponds [12]. In the remaining years of the 2017–2022 period, no microcystins were observed in the sampled water from Studena reservoir.

In the first microcystin surveys conducted in Bulgaria, data showed no microcystins in our drinking water reservoirs for samples collected in 2004 [10]. Subsequent studies of microcystin content in Studena reservoir showed the presence of microcystin-LR 0.1 µg/L in water samples in 2011 and 2013 [17]. In a summary of data on the presence of cyanotoxins in Bulgarian water bodies for the period 2000–2015, 8.1 µg/g dry weight (d.w.), 2.0 µg/g (d.w.) and 0.8 µg/g (d.w.) were found for MC-LR, MC-RR and MC-YR, respectively, in a biomass sample from the bloom in Studena reservoir [18].

In 2015, 0.2 µg/L microcystin-LR and 0.4 µg/L microcystin-YR were detected in water samples from Studena reservoir [16].

The cyanotoxin content of the water samples correspond with slight increases in the concentrations of the nutrients tested – total nitrogen and total phosphorus. The obtained results indicate the presence of total nitrogen in the concentration range from 0.74 to 1.51 mg/L and of total phosphorus from 0.02 to 0.26 mg/L. Similar results for the presence of nutrients were obtained in the study conducted for the period 2017–2022 [12].

Conclusions. The summary and analysis of microcystin data in Studena drinking water reservoir over recent years reveals a notable release of cyanotoxins,

particularly microcystins, that cannot be overlooked. Retrospective data for water samples of Studena reservoir show no microcystins present in 2004, traces in 2011–2013 and low levels in 2015 followed by significant concentrations in 2020 and 2023.

For the nutrients total nitrogen and total phosphorus, the results for 2023 and those obtained in the previous study for the period 2017–2022 show moderate concentrations.

In the study period the months of June to October 2023 the phytoplankton community of Studena reservoir was species poor and consisted of single representatives of blue-green, green, diatom, euglenophyte and pyrrhophyte microscopic algae. The summer sampling of 23.08.2023 revealed a bloom of the diatom alga *Lindavia comta* (Kützing) T. Nakov & al. It should be noted that blue-green algae are constantly present in the phytoplankton communities inhabiting the reservoir. The presence of microcystins at concentrations close to the permissible level in the autumn sample indicates that at the time of sampling the bloom of cyanobacteria that produced the microcystins had passed and the toxins released remained in the water.

Given the reservoir's intended use for drinking water supply, the possibility of monthly sampling during the summer-fall season of 2023 helped to more comprehensively characterize the microcystin situation in its waters. Due to the near acceptable concentration of MC-LR in the fall sample, serious consideration should be given to monitoring the reservoir during periods favourable for bloom formation.

However, in order to have more complete and up-to-date information on the presence of blooms, close contact and cooperation with managers and management staff is needed. Meetings and trainings are needed to make the relevant institutions aware of the nature of the blue-green algal bloom problem so that they can react in a timely manner and refer to competent specialists in the field from the agencies involved in protecting drinking water quality.

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