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MICROCYSTIS SPECIES AND THEIR TOXIGENIC STRAINS IN PHYTOPLANKTON OF TEN BULGARIAN WATERBODIES

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Abstract

Uzunov B., Stefanova K., Radkova M., Descy J.-P., Gärtner G., Stoyneva-Gärtner M., 2021: *Microcystis* species and their toxigenic strains in phytoplankton of ten Bulgarian waterbodies. – Botanica, 27(1): 77–94.

The summer phytoplankton structure of ten Bulgarian waterbodies was studied by HPLC analysis of marker pigments, light microscopy (LM) and PCR amplification of *mcyB* and *mcyE* gene sequences. The aim was to detect biodiversity and spread of toxigenic strains of potential microcystin producers and the important bloom-forming genus *Microcystis* in particular. The screening was done in three waterbodies, where *Microcystis* had already been found (Lakes Ezerets and Durankulak and Reservoir Koprinka), three waterbodies from which it had not been reported (Reservoirs Shilkovtsi, Zhrebchevo, Suedinenie) and four reservoirs that were sampled for the first time (Malka Smolnitsa, Plachidol 2, Preselka, Duvanli). LM and HPLC data similarly showed that cyanoprokaryotes contributed significantly to the total phytoplankton composition (29%) and biomass (15–87%) in nine sampled waterbodies. *Microcystis aeruginosa*, *M. natans*, *M. smithii, M. wesenbergii, Microcystis* spp., *M.* cf. *comperei* and *M. pseudofilamentosa,* were identified using LM (the last two tropical species were found for the first time in the country). Despite the low contribution of *Microcystis* to the phytoplankton diversity (1–4 taxa per sample) and to the total phytoplankton biomass (< 0.01–0.5%), 57 toxigenic strains of this genus were revealed by PCR, most of which demonstrated high similarity with NCBI *M. aeruginosa* and *M. wesenbergii* strains.

Keywords: algal blooms, alien species, cyanobacteria, cyanotoxins, lakes, microcystins, reservoirs.

INTRODUCTION

Scientific knowledge about Cyanoprokaryota/Cyanobacteria (also known as blue-green algae) and environmental factors promoting their mass development (blooms), as well as their toxic metabolites (cyanotoxins) and primary toxin exposure vehicles (drinking water, recreational water activities, freshwater seafood) has rapidly advanced in recent times (Merel et al., 2013; Ibelings et al., 2014; Descy et al., 2016; Meriluoto et al., 2017; Flores et al., 2018). The most common and most frequently studied cyanotoxins are the cyclic heptapetides microcystins, which occur in about 280 variants and are produced by several cyanoprokaryote genera (Pelaez et al., 2010; Catherine et al., 2017; Meriluoto et al., 2017; Bouaicha et al., 2019; Le Manach et al., 2019; Svirčev et al., 2019; Massey et al., 2020; Stoyneva-Gärtner et al., 2021). One of these genera, *Microcystis* Kütz., is widely spread in the freshwater phytoplankton and contains more than 50 species (Komárek et al., 2014; Le Manach et al., 2019; Guiry & Guiry, 2021) described according to the rules of the International Code of Nomenclature for Algae,

Fungi and Plants (Turland et al., 2018). However, opinions on the taxonomic validity of its species distinguished mainly by morphological criteria (morphospecies) differ from author to author (Komárek et al., 2014; Guiry & Guiry, 2021). These differences show the need for more polyphasic data with the maximum possible delimitation of the morphospecies in separate classification units (taxa) (Kormas et al., 2011; Komárek, 2016, 2018). There are also different opinions on the toxigenic potential of all *Microcystis* species, for some of which the ability for microcystin production is still unknown or has been questioned (Gkelis & Zaoutsos, 2014; Šejnohová & Maršálek, 2012; Köker et al., 2017; Radkova et al., 2020; Stoyneva-Gärtner et al., 2021 and references therein). The importance of studies of *Microcystis* and its blooms is growing considering that like other cyanoprokaryotes, they are favoured by anthropogenically enhanced eutrophication (Šejnohová & Maršálek, 2012; Zanchett & Oliveira-Filho, 2013; Komárek et al., 2014; Descy et al., 2016; Genuário et al., 2016; Le Manach et al., 2019; Wilhelm et al., 2020; Guiry & Guiry, 2021), and are likely to expand their distribution, frequency and abundance with global warming (Paerl & Huisman, 2008; Carey et al., 2012; Lehman et al., 2013; Wilhelm et al., 2020). Moreover, there is accumulating evidence that an increase of nutrient concentrations and water temperature may promote the potentially toxic *Microcystis* genotypes (Vézie et al., 2002; Davis et al., 2009; Joung et al., 2011; Srivastava et al., 2015; Scherer et al., 2017; Jankowiak et al., 2019).

The present contribution is a continuation of our previous polyphasic studies of the bloom-forming genus *Microcystis* in Bulgaria (Radkova et al., 2020; Stoyneva-Gärtner et al., 2021). It presents new data on the diversity and spread of some *Microcystis* species and their strains containing microcystin-synthase genes (*mcy* genes). The paper reflects the contribution of *Microcystis* to the total phytoplankton of ten different Bulgarian waterbodies. They include two lakes and eight reservoirs important for public health and national security due to their use as drinking water supply or use for irrigation of vineyards and arable lands and their importance as recreational sites or use for sport-fishing and fish-breeding (Michev & Stoyneva, 2007). In addition, some of these wetlands are significant for biodiversity conservation, and two of them are designated as Critically Endangered in the first Red List of Bulgarian Wetlands (Michev & Stoyneva, 2007). From two of the waterbodies (coastal Lakes Durankulak and Ezerets), *Microcystis* has frequently been reported as a common phytoplankter (Descy et al., 2018) and recently in Durankulak its toxigenic strains have been revealed by polyphasic approach (Radkova et al., 2020; Stoyneva-Gärtner et al., 2021). In one of the large inland reservoirs (Koprinka), *Microcystis* was recorded at the end of the 80s. In contrast, from three other inland reservoirs (Shilkovtsi, Suedinenie and Zhrebchevo), *Microcystis* was not reported. Four other waterbodies (inland small Reservoirs Duvanli, Malka Smolnitsa, Plachidol 2 and Preselka) were sampled for the first time (Michev & Stoyneva, 2007; Stoyneva-Gärtner et al., 2017b).

This sampling design was applied because of three main reasons:

1) The polyphasic approach in cyanoprokaryote studies combines genetic evaluation with diagnostic criteria from morphological and ecological analysis (Komárek, 2016), and, therefore, its application on the background of total phytoplankton composition and abundance, combined with environmental data, has been encouraged in studies on toxin producers (Rantala et al., 2006; Radkova et al., 2020; Stoyneva-Gärtner et al., 2021);

2) Despite that presence of toxin-producing cyanoprokaryotes, especially during blooms, leads to loss of ecosystem services (Wilhelm et al., 2020), imposes a potential hazard to humans and wildlife, and in this way concerns also the national security, yet in Bulgaria, freshwater ecosystems are under no special monitoring for cyanotoxins (Stoyneva-Gärtner et al., 2017a);

3) Cyanoprokaryote blooms (and this of *Microcystis* in particular) and their content are a strongly variable phenomenon, mainly depending on local conditions (Ibelings et al., 2014; Hartnell et al., 2020; Wilhelm et al., 2020), including the land use (Srivastava et al., 2015), which shows the need for accumulating of data from more waterbodies in each country. This is valid especially for Bulgaria, where all waterbodies cover < 1% of the country's territory and, due to their shallow character, are prone to cyanoprokaryote blooms (Michev & Stoyneva, 2007).

MATERIALS AND METHODS

Sites and sampling

The sampling was carried out between 14th and 21st August 2019 in ten waterbodies in Central and North-Eastern Bulgaria (Fig. 1, Table 1). In Table 1, due to the significant variation in names of reservoirs in Bulgaria, we provide the unique number from the Inventory of Bulgarian Wetlands (Michev & Stoyneva, 2007), where detailed descriptions of the waterbodies are available. However, it is worth mentioning that:

1) All studied reservoirs are inland plain waterbodies (except the lowland reservoir Suedinenie), while Lakes Durankulak (=Durankulashko Ezero) and Ezerets (=Ezeretsko Ezero) are coastal lowlands;

2) Coastal Lakes Durankulak and Ezerets are protected areas and are included in the Red List of Bulgarian Wetlands (Michev & Stoyneva, 2007) in the category of Critically Endangered;

3) Reservoirs Koprinka (854 ha), Suedinenie (159 ha), Shilkovtsi (=Yovkovtsi; 553 ha) and Zhrebchevo (1851 ha) are included in Appendix 1, "List of complex and significant reservoirs" of the Water Act (State Gazette 67/1999) (Michev & Stoyneva, 2007);

4) Reservoirs Malka Smolnitsa, Plachidol 2, Preselka and Duvanli (=Duvanlii) are small reservoirs (9, 4, 15 and 27 ha, respectively);

5) Reservoirs Malka Smolnitsa, Plachidol 2, Preselka and Duvanli were sampled for the first time.

Before sampling, we observed each waterbody by a drone DJI Mavic 2 Enterprise Dual Pro, which could measure the surface water temperature and was supplied by a photo camera. In this way, spots with visible differences in colour were chosen for the sampling of cyanoblooms. In cases of visible water homogeneity, the sites from our previous studies were repeated (Stoyneva-Gärtner et al., 2019; Radkova et al., 2020; Stefanova et al., 2020; Stoyneva-Gärtner et al., 2021) or, for the newly sampled small shallow reservoirs, the most accessible site was se-

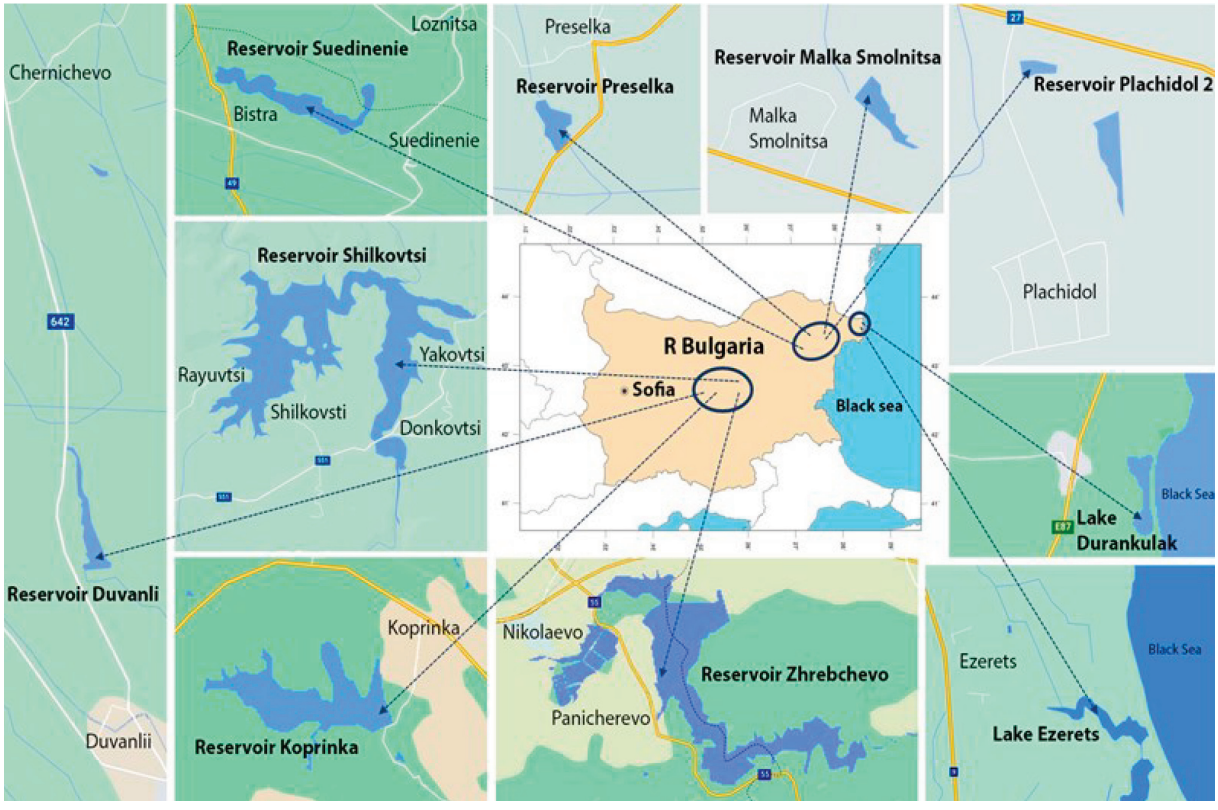

Fig. 1. Map of R. Bulgaria indicates the studied waterbodies (modified after (http://www.ginkgomaps.com and Google Maps)

Table 1. Sampling sites (organised by the order of sampling, 14–20 August 2019) and their main environmental parameters. Legend: IBWXXXX – number of the waterbody in the Inventory of Bulgarian Wetlands (Michev & Stoyneva, 2007); Alt – altitude (m a.s.l.); WT – water temperature (°C); SD – Secchi depth (m); CN – conductivity (S m^{-1}); TD – total dissolved solids (µg L^{-1}); DO – oxygen concentration (mg L^{-1}); TP – total phosphorus (mg L^{-1}); TN – total nitrogen (mg L^{-1})

Waterbody	Alt	Latitude / Longitude	WT	pH	SD	CN	TD	DO	TP	TN
Res. Shilkovtsi (IBW2105)	410	42°55.2320' / 25°47.6743'	27.2	8.88	0.5	0.000746	479	7.48	0.03	0.1
Res. Suedinenie (IBW2642)	133	43°20.0734' / 26°33.6368'	28.1	7.61	0.5	0.000739	481	6.77	0.10	0.3
Res. Preselka (IBW3078)	281	43°25.3767' / 27°16.6214'	24.1	9.00	0.5	0.000138	282	10.05	0.60	2.8
Res. Malka Smolnitsa (IBW3107)	211	43°36.2606' / 27°44.5367'	25.2	9.08	0.3	0.000755	490	7.05	0.60	0.63
Res. Plachidol 2 (IBW5073)	220	43°33.3504' / 27°52.6338'	24.6	9.04	0.5	0.001225	793	9.13	0.20	0.4
Lake Ezerets (IBW0233)	6	43°35.2681' / 28°33.2096'	25.9	8.58	1.5	0.001669	1739	8.58	0.10	0.1
Lake Durankulak (IBW0216) – western part	2	43°40.0006' / 29°32.6166'	26.5	8.89	0.6	0.000974	631	7.86	0.30	0.66
Res. Zhrebchevo (IBW2545)	253	42°36.6024' / 25°51.2345'	27.6	7.70	0.7	0.000358	233	8.01	0.10	0.18
Res. Koprinka (IBW2062)	450	42°37.0172' / 25°19.4795'	27.2	8.22	2.5	0.000250	163	7.21	0.10	0.16
Res. Duvanli (IBW1483)	250	42°23.1851' / 24°43.1000'	26.3	8.76	0.4	0.004050	291	7.09	0.10	0.25

lected. The phytoplankton sampling was performed from inflatable boats. Data on geographical coordinates, altitude, water temperature, pH, total dissolved solids, oxygen concentration, and conductivity were taken by the water monitoring instruments Aquameter AM-200 and Aquaprobe AP-2000. Water transparency was measured by Secchi disk. Aqualytic AL410 Photometer from AQUALYTIC® was used for *ex situ* measurement of total nitrogen (TN) and total phosphorus (TP) (Stoyneva-Gärtner et al., 2019; Radkova et al., 2020; Stefanova et al., 2020; Stoyneva-Gärtner et al., 2021). All obtained data are shown in Table 1.

The surface water layer (0–20 cm) samples for phytoplankton identification and counts (0.5–1 L in volume, depending on the trophic state) were fixed immediately to 2% final formalin concentration and transported to the lab, where they were further concentrated by sedimentation to a volume of 50 ml (Rott, 1981; Stoyneva-Gärtner et al., 2019, 2021; Radkova et al., 2020). Within a few hours after collection, the water samples for pigment analyses and PCR-studies (each in volume 0.5 L) were filtered under a mild vacuum through Macherey-Nagel GF5 filters (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) with porosity 0.4 µm and Whatman 0.45 µm cellulose filters Whatman NC45 ST/ Sterile EO (Merck KGaA, Darmstadt, Germany), respectively. The filters were immediately placed in 15 mL sterile plastic tubes (Falcon) and preserved in dry ice to transport to the labs and further treatment.

Phytoplankton species composition and abundance assessment by conventional light microscopy (LM)

The taxonomic work on the phytoplankton species composition was done using Motic B1 microscope under magnification 100× and immersion. The microphotographic documentation was done by a Moticam 2.0 mp camera supplied by Motic Images 2 Plus software programme. For algal counts from each site, at least four non-permanent slides were examined on Thoma counting chamber, with the abundance of each species estimated in both cell numbers and biomass, the last by using the stereometrical approximations and subsequent weight recalculation (Rott, 1981; Stoyneva et al., 2007; Stoyneva-Gärtner et al., 2019; Radkova et al., 2020).

The identification of phytoplankters followed standard taxonomic sources with updates from Algaebase (Guiry & Guiry, 2021). The morphological diagnostic features used for species identification in the blue-green algal genus *Microcystis* were the traditionally used in phycological literature shape of the colony, presence/absence of subcolonies, density of cells in the colony, structure of the mucilage sheath, cell size and pigmentation, presence/absence of gas vesicles (Geitler, 1931, 1942; Gollerbakh et al., 1953; Starmach, 1966; Komárek & Komárková, 2002; Komárek & Anagnostidis, 1998). Following the instructions in Komárek & Anagnostidis (1998), the ecological characteristics of the species were taken into account as an integral part of the species characterisation. The list of potential microcystin-producers followed mainly Catherine et al. (2013), Bernard et al. (2017), Lyon-Colbert et al. (2018) and Chapman & Foss (2020).

Phytoplankton composition assessment by HPLC marker pigment analysis

This procedure was applied for the determination of the general phytoplankton composition based on the chlorophyll *a* biomass of the main algal groups, namely green algae (from both phyla Chlorophyta and Streptophyta), cryptophytes, pyrrhophytes (dinoflagellates), euglenophytes and cyanoprokaryotes (two pigment types, cyanoprokaryotes T1 and T2), and two classes of phylum Ochrophyta – golden algae (Chrysophyceae) and diatoms (Bacillariophyceae).

All steps of the performed HPLC analysis and subsequent application of CHEMTAX (Mackey et al., 1996; Wright & Jeffrey, 2006; Sarmento & Descy, 2008; Descy, 2017) followed the standard procedures and coincided entirely with the detailed description provided in our former papers (Stoyneva-Gärtner et al., 2019, 2021).

Molecular-genetic studies

In the lab, the metagenomic DNA was isolated from the *ex situ* filters obtained after sampling in the field, following GeneJET™ Plant Genomic DNA Purification Mini Kit (Thermo Scientific, Waltham, MA, USA). In our previous work, we have performed the sequence analysis of PCR amplified fragments of the target *mcyB* and *mcyE* genes, known for their reliable microcystin-detective biomarker character (Stoyneva-Gärtner et al., 2021; and references therein). All amplifications were carried out in the thermal cycler (QB-96 apparatus, Quanta Biotech), in a final volume of 25 µl, using MyTaqHS Mix PCR. Still, here we point again the synthetase-gene-specific pairs of primer applied and the relevant annealing temperatures: for the PCR amplification of *mcyB* gene – MIf GCAGCGAACTCTTGAAGGGTTTATG and MIr GCGGATTCTGTGCAGCTTGTTCTTC, 850 bp, 55°C (Foulds et al., 2002) and for the *mcyE* gene – HEPF (5'TTTGGGGTTAACTTTTTT3GGGCAT AGTC-3') × HEPR (5'AATTCTTGAGGCTGTAA ATCGGGTTT-3') 470 bp, 57°C (Jungblut & Neilan, 2006).

The obtained *mcyE* and *mcyB* sequences were assembled, manually edited, analysed using Vector NTI 11.5 software, and used for Basic Local Alignment Search Tool (BLAST) search (BLAST, 2021) in the National Centre for Biotechnology Information (NCBI) genetic database (NCBI, 2021). Then, Mega 6.06 software was applied to construct a phylogenetic tree based on the Neighbour-joining method with 1000 bootstrap replication (Tamura et al., 2013). There, the accession numbers of 37 (out of the obtained 51) *mcyE*-based strains (MW602416-MW602424, MW602426-MW602453) and six *mcyB*-based strains (MW602454-MW602459), newly submitted by us to NCBI, are shown in brackets. In the text, the taxonomic identification number in NCBI (NCBItxidXXXXXX) is also indicated.

RESULTS

Phytoplankton species composition and abundance, obtained by light microscopy (LM)

A total of 212 species were identified using LM in the phytoplankton of the ten studied waterbodies. They belonged to seven algal phyla, namely Cyanoprokaryota, Chlorophyta, Streptophyta, Ochrophyta, Cryptophyta, Pyrrhophyta and Euglenophyta (Fig. 2). Cyanoprokaryotes comprised 29% of the total biodiversity and were represented by 62 taxa from 33 genera. Some species from the genera *Aphanothece*, and *Microcystis* (Chroococcales), *Merismopedia*, *Limnothrix*, *Jaaginema*, *Pseudanabaen*a, *Snowella*, *Synechococcus* and *Synechocystis* (Synechococcales), *Anagnostidinema*, *Geitlerinema*, *Oscillatoria* and *Planktothrix* (Oscillatoriales), *Anabaena* s.str.,

Aphanizomenon s.str., *Chrysosporum*, *Dolichospermum* and *Raphidiopsis* (incl. former *Cylindrospermopsis*) (Nostocales) are known as microcystin producers (Catherine et al., 2013; Bernard et al., 2017; Lyon-Colbert et al., 2018; Chapman & Foss, 2020).

Cyanoprokaryotes have an essential contribution to the total phytoplankton biodiversity, occupying the second position after green algae (from both divisions Chlorophyta and Streptophyta) or Ochrophyta (mainly diatoms) in almost all studied waterbodies (Figs 3, 4). The single exception is the drinking-water reservoir Shilkovtsi, in which the representatives of this taxonomic group were not detected (Figs 3, 4).

The total number of phytoplankton species in the studied waterbodies ranged between 16 (Reservoir Shilkovtsi) and 65 (Reservoir Duvanli) (Fig. 4). The number of cyanoprokaryotes was between two (Lake Ezerets) and 21 (Reservoir Duvanli), considering that they were not observed in Shilkovtsi (Fig. 4). *Microcystis* was represented in all waterbodies (except Shilkovtsi) with a low number of taxa ranging between 1 (Reservoir Suedinenie and Lake Ezerets) and five (Reservoir Duvanli) (Fig. 4, Table 2).

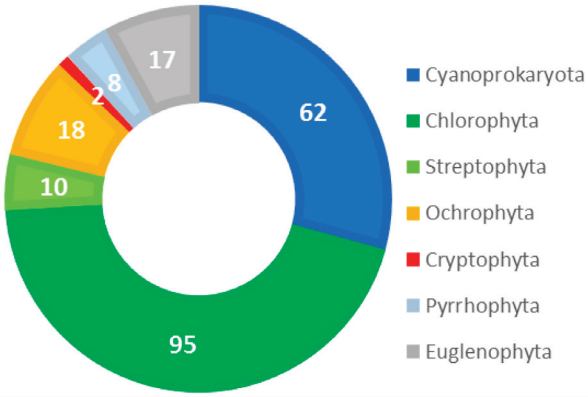

Fig. 2. General phytoplankton species composition in ten Bulgarian wetlands sampled in August 2019. The number of identified taxa in each phylum is indicated

Cyanoprokaryotes comprised from 15 (Lake Ezerets and Reservoir Koprinka) to 89% (Reservoir Malka Smolnitsa) of the total phytoplankton biomass, except in Reservoir Shilkovtsi (Fig. 5). It has to be noted that their blooms dominated by a single species were not detected, and *Microcystis* did not appear as dominant, codominant or subdominant. In Reservoirs Duvanli, Malka Smolnitsa and Preselka, the phytoplankton was polydominated with the contribution of filamentous cyanoprokaryotes (mainly heterocytous *Anabaenopsis*, *Raphidiopsis*, *Sphaerospermopsis* and non-heterocytous *Pseudanabaena* and *Planktolyngbya*).

Considering the results on toxigenic strains obtained by PCR analysis, we present the LM data on the genus *Microcystis*. Based on the traditional diagnostic features (Geitler, 1931, 1942; Gollerbakh et al., 1953; Starmach, 1966; Komárek & Komárková, 2002; Komárek & Anagnostidis, 1998), we identi-

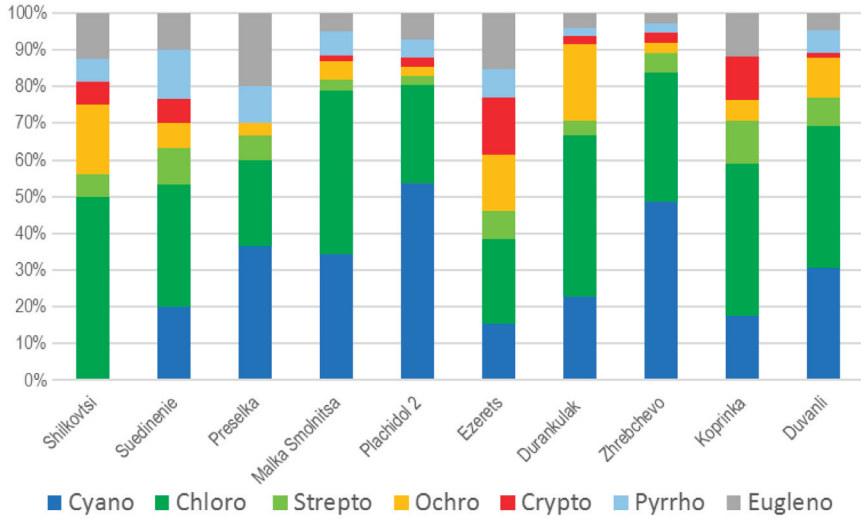

Fig. 3. The relative contribution of different taxonomic groups to the phytoplankton biodiversity according to the number of species obtained by light microscopy in the studied Bulgarian waterbodies (August 2019). Legend: Cyano – Cyanoprokaryota, Chloro – Chlorophyta, Strepto – Streptophyta, Ochro – Ochrophyta, Crypto – Cryptophyta, Pyrrho – Pyrrhophyta and Eugleno – Euglenophyta

Table 2. Distribution of *Microcystis* taxa identified by LM and their contribution to the total phytoplankton biomass in the studied Bulgarian waterbodies (WBs) in August 2019: MA – *Microcystis aeruginosa*, MC – *Microcystis* cf. *comperei*, MN – *Microcystis natans*, MP – *Microcystis pseudofilamentosa*, MS – *Microcystis smithii*, MSp – *Microcystis* sp., MW – *Microcystis wesenbergii*, SS/DC – separate cells or disintegrated colonies, TTs – colonies with transitional morphology (for details see the text of the paper), n.d. – not detected

Waterbody	MA	MC	MN	MP	MS	MW	MSp	SS/DC	TTs
Res. Shilkovtsi	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Res. Suedinenie	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< 0.05%	n.d.
Res. Preselka	n.d.	n.d.	n.d.	n.d.	n.d.	< 0.5%	< 0.05%	< 0.05%	n.d.
Res. Malka Smolnitsa	n.d.	n.d.	n.d.	< 0.1%	< 0.1%	< 0.05%	n.d.	< 0.05%	< 0.05%
Res. Plachidol 2	n.d.	n.d.	n.d.	n.d.	n.d.	< 0.1%	n.d.	< 0.1%	n.d.
Lake Ezerets	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< 0.05%	n.d.
Lake Durankulak	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< 0.05%	< 0.05%
Res. Zhrebchevo	< 0.5%	n.d.	< 0.05%	n.d.	n.d.	n.d.	n.d.	< 0.05%	n.d.
Res. Koprinka	< 0.5%		n.d.	n.d.	n.d.	n.d.	n.d.	< 0.05%	n.d.
Res. Duvanli	n.d.	< 0.01%	< 0.1%	n.d.	n.d.	< 1%	n.d.	< 0.1%	< 0.05%

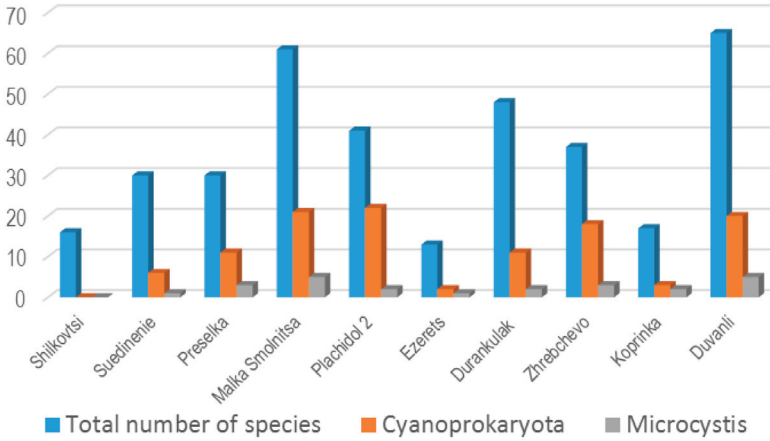

Fig. 4. Biodiversity in the studied Bulgarian waterbodies (August 2019) obtained by light microscopy and expressed as the total number of species, number of Cyanoprokaryota species and number of *Microcystis* morphospecies

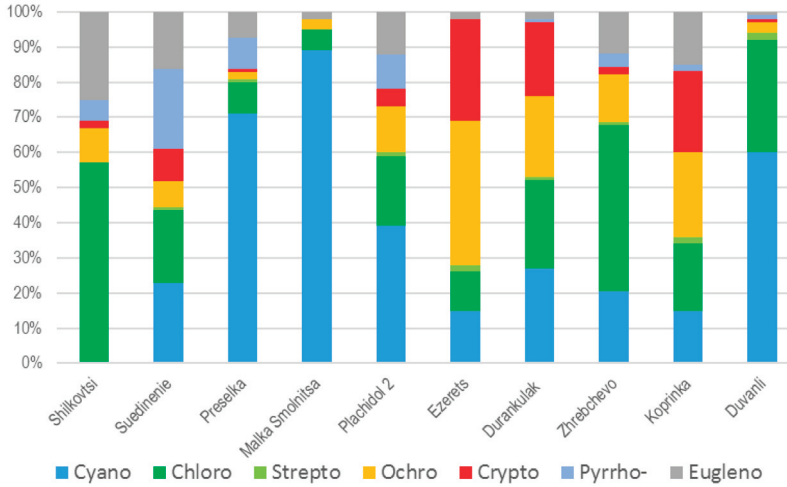

Fig. 5. The relative contribution of different taxonomic groups to the total phytoplankton biomass, obtained by light microscopy, in the phytoplankton of the studied Bulgarian waterbodies (August 2019). Legend: Cyano – Cyanoprokaryota, Chloro – Chlorophyta, Strepto – Streptophyta, Ochro – Ochrophyta (mainly diatoms), Crypto – Cryptophyta, Pyrrho – Pyrrhophyta and Eugleno – Euglenophyta

fied six species*: M. aeruginosa* (Kütz.) Kütz., *M.* cf. *comperei* Komárek, *M. natans* Lemmerm. ex Skuja, *M. smithii* Komárek & Anag., *M. pseudofilamentosa* W. B. Crow, *M. wesenbergii* (Komárek) Komárek, distinguished one more morphospecies, which could not be referred to any known species and presented here as *Microcysti*s sp. (Figs 6–9; Table 2). Regarding *M. comperei*, we would like to note that few colonies were found in the samples from Reservoir Duvanli, with smaller dimensions (2.5–3 μm in diameter) than indicated for the species (4.5–5.2 μm) (Komárek, 1984; Komárek & Anagnostidis, 1998), but very consistent with the other species features and with the records, published from Yukatan (Tavera et al., 2013 (p. 42, Fig. 62)). These colonies slightly resemble the initial stages of *M. smithii*, but the scattered cells are more typical for this species. Therefore, until more data are accumulated, we refer to the material from Duvanli as *M.* cf. *comperei*. In some samples, we also found separate cells, disintegrated colonies, or initial colonies and colonies with transitional morphology, for which species identification was not possible (Figs 6–8; Table 2). *Microcystis* distribution was different in the studied waterbodies, and its contribution to the total phytoplankton biomass was extremely low – between < 0.05% and 0.5% (Table 2).

The highest diversity of colonies and single cells was observed in Reservoirs Duvanli, Malka Smolnitsa, Plachidol 2 and Preselka.

Results on general phytoplankton composition from HPLC analysis of marker pigments

According to HPLC data on marker pigments, the majority of phytoplankton in the studied waterbodies consisted of Cyanoprokaryota, green algae (Chlorophyta and Streptophyta) and Ochrophyta (mainly Bacillariophyceae and less Chrysophyceae) and a smaller part Cryptophyta, Euglenophyta and Pyrrhophyta (Fig. 10**)**.

The chlorophyll *a* values, measured by HPLC, indicated the meso- to hypertrophic status of the stud-

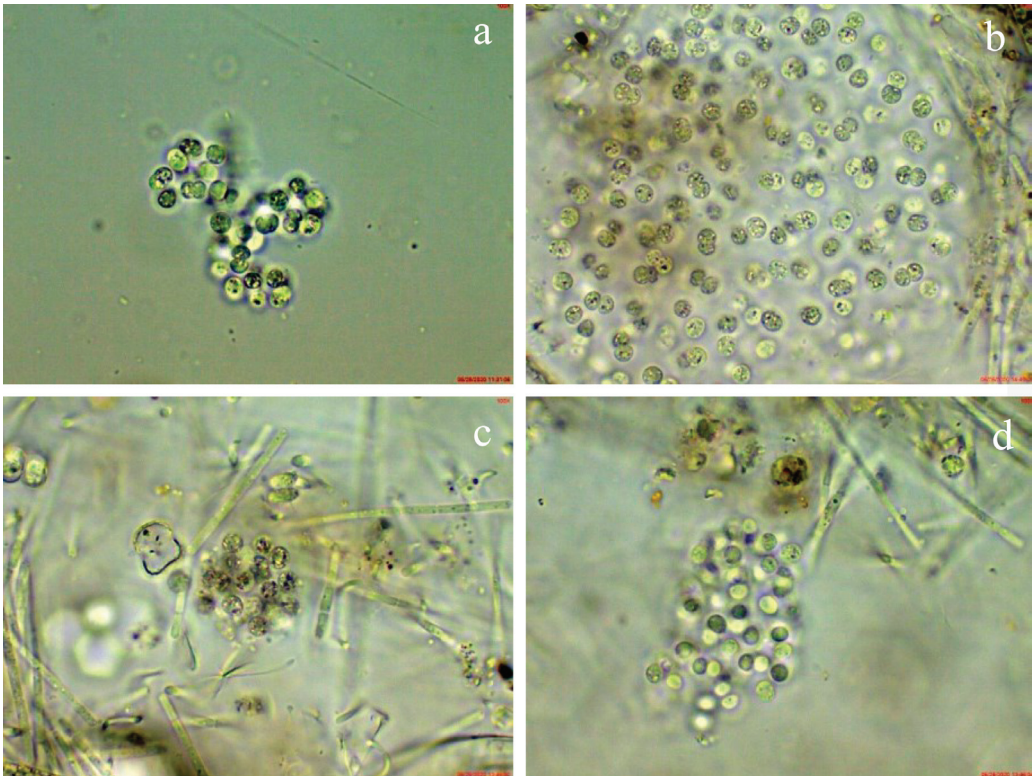

Fig. 6. *Microcystis* from different Bulgarian waterbodies (under immersion and objective 100×). Sample from Reservoir Preselka: (a) *Microcystis* sp. Samples from Reservoir Malka Smolnitsa: (b) *M. smithii* (c) Initial colony with densely packed cells and without clearly visible mucilage (which could be tentatively referred to *M. aeruginosa*); (d) *M. pseudofilamentosa.* The scale bar on all photos is 10 μm

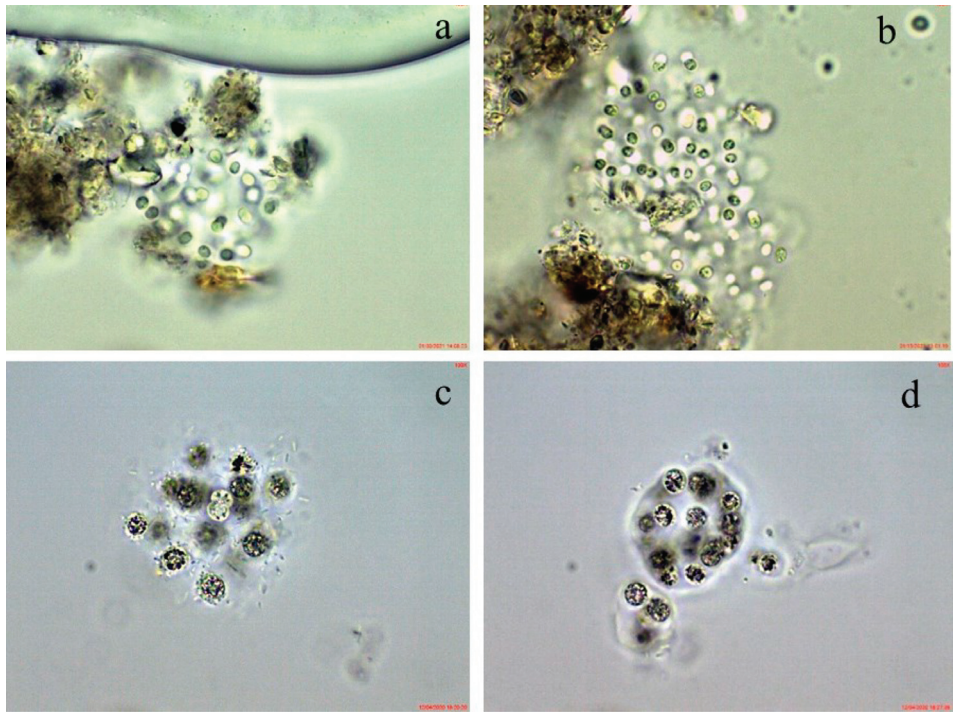

Fig. 7. *Microcystis* from different Bulgarian waterbodies (under immersion and objective 100×). Samples from Reservoir Zhrebchevo: (a, b) *M. natans*. Samples from Reservoir Plachidol 2: (c) initial (?) colony, which could be tentatively related to *M. aeruginosa*; (d) *M. wesenbergii.* The scale bar on all photos is 10 µm

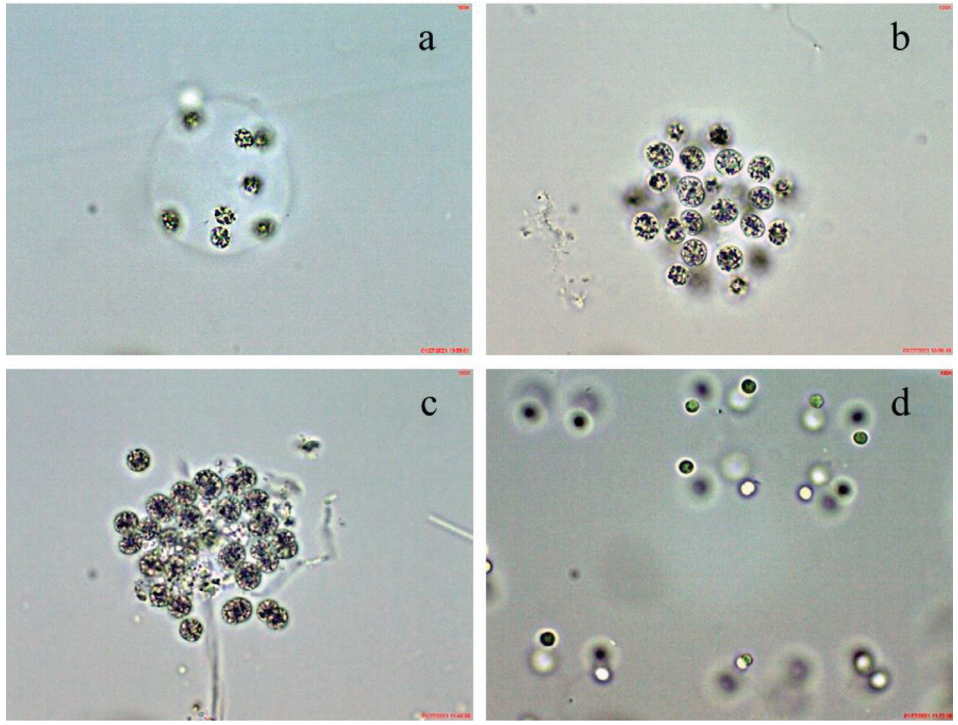

Fig. 8. *Microcystis* from different Bulgarian waterbodies (under immersion and objective 100×). Samples from Reservoir Duvanli: (a) *M. wesenbergii* – juvenile, but typical colony with a small number of cells; (b, c) initial colonies with invisible mucilaginous margin, transitional between *M. wesenbergii* and *M. aeruginosa*; (d) disintegrating colony. The scale bar on all photos is 10 µm

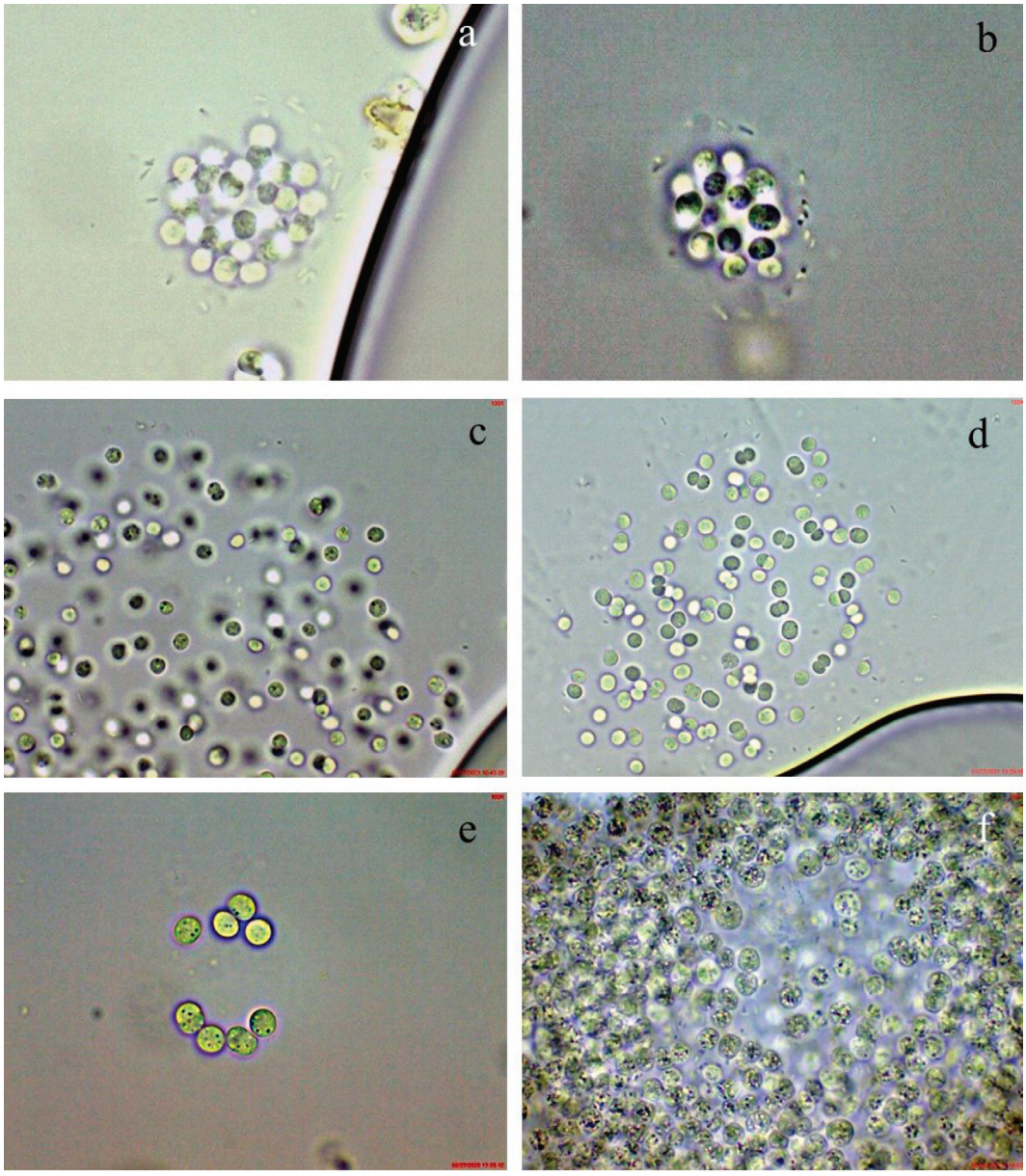

Fig. 9. *Microcystis* from different Bulgarian waterbodies (under immersion and objective 100×). Samples from Reservoir Duvanli: (a, b) *M.* cf. *comperei*; (c) *Microcystis natans*; (d) *Microcystis* cf. *natans.* Sample from Lake Durankulak (western part): (e) juvenile colony could be tentatively related to *M. wesenbergii* due to the lack of thickened mucilage outline. Sample from Reservoir Koprinka: (f) *M. aeruginosa*. The scale bar on all photos is 10 µm

ied waterbodies, in agreement with the Secchi disk depth and TP data (Table 1). The chlorophyll *a* values ranged from 1.5 (Reservoir Koprinka) to 26 µg L^{-1} (Reservoir Duvanli) and were significantly higher only in Reservoirs Preselka and Malka Smolnitsa, where they were 113 and 101 µg L^{-1}, respectively. In the last three reservoirs, cyanoprokaryotes had the highest contribution to the phytoplankton (Fig. 10).

Results from PCR analysis for microcystin-producing strains

The generic microcystin-synthetase gene E (*mcyE*) was successfully amplified in nine from the ten metagenomic DNA samples, and 51 sequences were obtained. They and their corresponding high homologous (98–100%) NCBI (NCBI, 2021) sequences are presented on the constructed *mcyE* phylogenetic tree (Fig. 11).

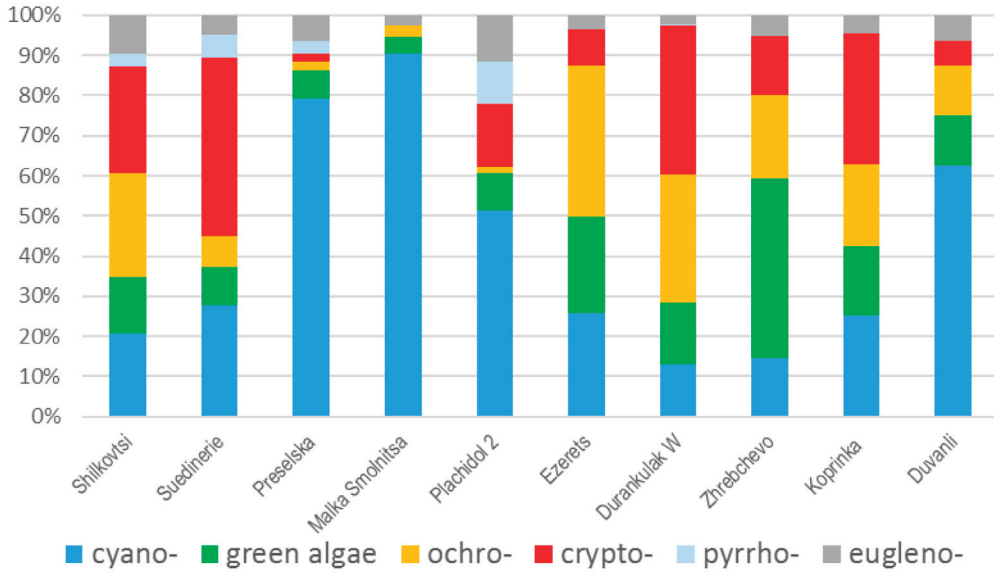

Fig. 10. General phytoplankton composition according to the pigment markers analysed by HPLC (expressed as percentage contribution to chlorophyll *a*, calculated using CHEMTAX) in the ten studied Bulgarian waterbodies (August 2019). Legend: cyano – cyanoprokaryotes, green algae – chlorophytes and streptophytes, ochro – ochrophyta (incl. diatoms and golden algae), crypto – cryptophytes, pyrrho – pyrrhophytes and eugleno – euglenophytes

The primary grouping was around the NCBI strains of two *Microcystis* species – *M. aeruginosa* and *M. wesenbergii* (Fig. 11). Detailed analysis showed that eight sequences from Lake Durankulak, Reservoirs Preselka and Koprinka were 100% homologous with *M. aeruginosa* (and two sequences from Reservoirs Koprinka and Zhrebchevo (Kop 1 and Zhr 1) were 100% homologous with *M. wesenbergii* NIES-107 (NCBI:txid315483). Other 18 strains from Lakes Durankulak and Ezerets and from Reservoirs Duvanli, Koprinka, Malka Smolnitsa, Preselka and Zhrebchevo had high homology with NCBI *M. aeruginosa* strains. Two strains from Preselka (Pre 1 and Pre 4) showed high similarity with *M. viridis* NIES-102 (NCBI:txid213615). The rest of the strains had similarity with cultured and uncultured *Microcystis* NCBI strains, which were not supplied by species names (Fig. 11). Despite general grouping in a single cluster, the obtained *mcyE* sequences were diverse, spread in three subclusters and demonstrated the rich biodiversity of toxigenic *Microcystis* strains in the studied waterbodies.

By applying direct sequencing based on *mcyB* marker, six sequences from Lake Ezerets and Reservoirs Duvanli, Koprinka, Malka Smolnitsa, Preselka and Zhrebchevo were obtained and included in the constructed phylogenetic tree with *Fischerella* as outgroup (Fig. 12). They belonged to a single cluster and were affiliated with a single *Microcystis viridis* Nies-102 strain and six *M. aeruginosa* strains registered in NCBI (NCBI, 2021). However, the *mcyB* sequences from Lake Durankulak and Reservoirs Plachidol 2 and Suedinenie were not clear and were not included in this tree.

DISCUSSION

The results on general phytoplankton composition revealed by marker pigment analysis (Fig. 10) are consistent with the taxonomic groups in phytoplankton species composition (Figs 3, 4) and their contribution to the biomass (Fig. 5) identified by LM. The single discrepancy in the results concerned the drinking water Reservoir Shilkovtsi in which cyanoprokaryotes were not found by LM. It could be explained by finding by HPLC of cyanoprokaryote pigments of "*Synechococcus* pigment type" (T1) typical for picoplankters, which could not be seen by LM (Figs 4, 5).

Generally, both HPLC data on marker phytoplankton pigments and LM counts demonstrated the high proportion of cyanoprokaryotes in the quantitative structure of the summer phytoplankton of the other studied waterbodies (Figs 5, 10). These data fit with the well-established notion that cyanoprokary-

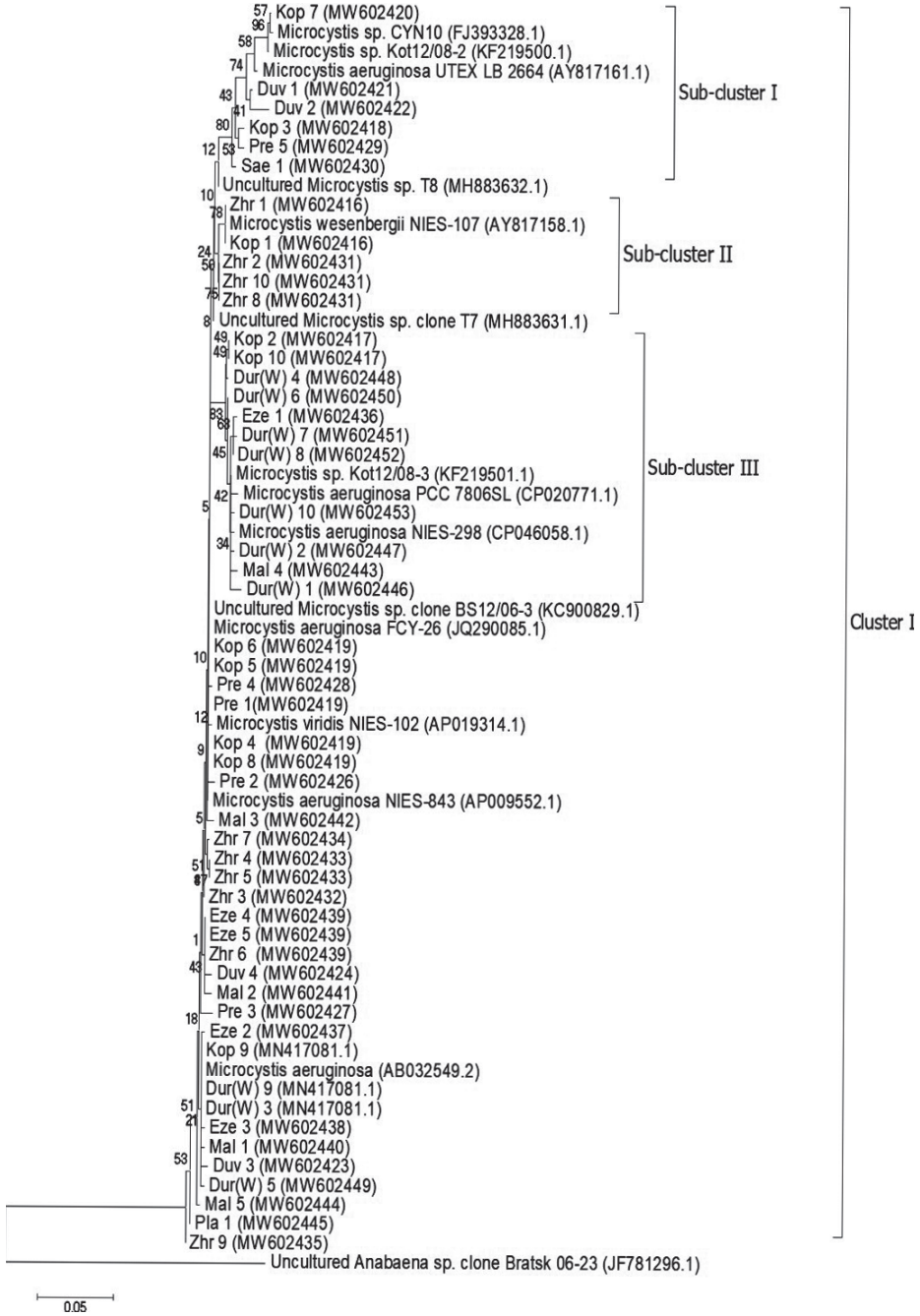

Fig. 11. Neighbour-joining phylogenetic tree constructed using nucleotides sequences from nine library samples and closest sequences in NCBI database with their accession number there, and with Uncultured *Anabaena* sp. JF781296.1 as outgroup. Bootstrap values are shown at branch points (percentage of 1050 resamplings). Legend: Duv – Reservoir Duvanli; Dur (W) – Lake Durankulak, western part; Eze – Lake Ezerets; Kop – Reservoir Koprinka; Mal – Reservoir Malka Smolnitsa; Pre – Reservoir Preselka; Sae – Reservoir Suedinenie; Zhr – Reservoir Zhrebchevo. For the identical sequences (IS), obtained during this study, only one accession number received from NCBI is provided: 1) IS from Koprinka and Zhrebchevo (Kop 1 and Zhr 1) have accession number MW602416; 2) IS from Koprinka and Preselka (Kop 4, Kop 5, Kop 6, Kop 8 and Pre 1) have accession number MW602119; 3) IS from Koprinka (Kop 2 and Kop 10) have MW602417; 4) IS from Zhrebchevo (Zhr 2, Zhr 8, Zhr 10) have MW602431, and Zhr 4 and Zhr 5 have MW602433); 5) IS from Ezeretz and Zhrebchevo (Eze 4, Eze 5 and Zhr 6) have accession number MW602439; 6) IS from Durankulak (Dur (W) 3 and Dur (W) 9) have accession number MN417081.1, because these sequences are identical with the strain Dur2_10 MN417081.1 submitted earlier by us (Radkova et al., 2020)

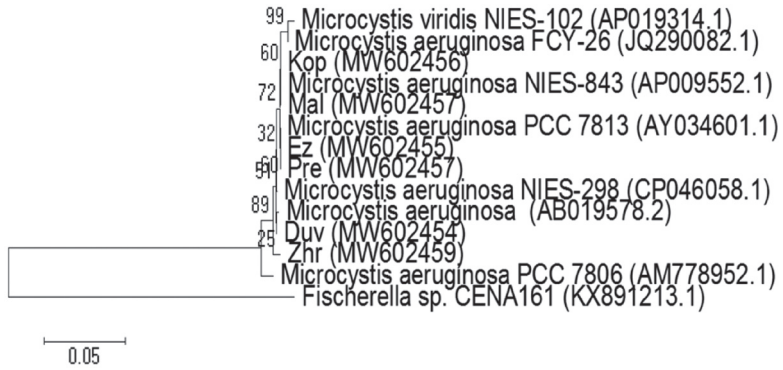

Fig. 12. Neighbour-joining phylogenetic tree based on nucleotides sequences from direct sequencing of PCR products and *mcyB* marker, constructed after BLAST search in NCBI database with *Fischerella* sp. KX891213.1 as outgroup. Bootstrap values are shown at branch points (percentage of 1050 resampling). Legend: Duv – Reservoir Duvanli; Ez – Lake Ezerets; Kop – Reservoir Koprinka; Mal – Reservoir Malka Smolnitsa; Pre – Reservoir Preselka; Zhr – Reservoir Zhrebchevo

ote bloom occurrence is more widespread during periods when light intensities and water temperatures are higher (Turner et al., 2018). This general result is following the statement for the summer cyanoprokaryote dominance as a common feature of eutrophic waterbodies worldwide (Reynolds, 2006; Lymperopoulou et al., 2011; Whitton, 2012) and in Bulgaria in particular (Stoyneva-Gärtner et al., 2017b, 2019, 2021; Descy et al., 2018).

The LM analysis revealed seven morphospecies, among which *M. aeruginosa* and *M. wesenbergii* were most widespread (Figs 5–9, Table 2). These data are in accordance with our results for nine other sites, sampled during the same campaign in August 2019, which pointed *M. aeruginosa* and *M. wesenbergii* as primary microcystin-producers (Stoyneva-Gärtner et al., 2021). Despite the rich phytoplankton diversity (212 taxa from seven algal phyla – Fig. 2) and finding of species from almost all cyanoprokaryote genera, known as potential microcystin-producers (Catherine et al., 2013; Bernard et al., 2017; Lyon-Colbert et al., 2018; Chapman & Foss, 2020), *mcyB* and *mcyE* genes were identified only in *Microcystis* strains (Figs 11–12). Moreover, data obtained by PCR amplification demonstrated the high biodiversity of potentially toxigenic *Microcystis* strains, the coexistence of several strains in the studied waterbodies, and their unequal geographic distribution in the country. They once more confirmed *M. wesenbergii* as a potential microcystin-producer (Namikoshi et al., 1992; Šejnohová & Maršálek, 2012; Pavlova et al., 2014; Köker et al., 2017; Stoyneva-Gärtner et al., 2017b, 2021; Chapman & Foss, 2020; Radkova et al., 2020) in addition to the best-known producer of this toxin – *M. aeruginosa*. The delimitation of *M. aeruginosa* and *M. wesenbergii* strains by their *mcyE* toxigenic sequences in our study is in agreement with general phylogenetic distinction in two groups based on 16S-23S ITS fragments (> 600 bp long) (Le Manach et al., 2019).

Our results are consistent with the opinions about the infrageneric diversity of *Microcystis* and its uneven geographical distribution, shared in earlier works (Le Manach et al., 2019) and with our results in both previous studies (Radkova et al., 2020; Stoyneva-Gärtner et al., 2021). The PCR analysis on *mcyE* and *mcyB* gene also demonstrated the high similarity of strains from Reservoirs Koprinka, Malka Smolnitsa and Preselka with two genetically identical NCBI strains of *M. viridis,* namely strains *M. viridis* NIES-102 (NCBI:txid213615) and *M. viridis* NIES-843 (NCBI:txid449447), from which only the first is shown on the trees (Figs 11–12). This morphospecies was not identified by LM in any of the studied waterbodies, but in the abovementioned lakes and reservoirs, single cells, juvenile or disintegrated colonies were seen (Table 2), the identification of which was practically impossible.

The results from PCR analysis did not allow us to relate the other five *Microcystis* taxa, which we identified based on morphology, with NCBI strains, which shows the necessity of further studies for revealing presence/absence of toxigenic strains in *M. natans*, *M. novacekii* and *M. smithii.* In the last two species, microcystin-producing strains have been rarely found (Liu et al., 2011; Bernard et al., 2017;

Chapman & Foss, 2020). The existence of such strains in Bulgarian waterbodies has been tentatively supposed only for *M. natans* in our earlier studies (Radkova et al., 2020). We could not refer any of the obtained *mcy* sequences to the two tropical species, *M.* cf. *comperei* and *M. pseudofilamentosa*, found by LM for the first time in Bulgaria during this study. The occurrence of each of them in a single site (Reservoirs Duvanli and Malka Smolnitsa, respectively) in deficient amounts allows us to suggest their alien character for the country.

Despite the finding of toxigenic strains of *Microcystis*, we have to note that in the water samples collected during the same sampling campaign, microcystins (-LR, RR and YY) were not chemically detected (Stoyneva-Gärtner et al., 2021). Considering that amounts and dynamics of cyanotoxins in the water are generally related to the population dynamics of their producers (Kardinaal et al., 2007; Lehman et al., 2013), but in some cases, strong correlations with *Microcystis* biomass have not been found, because not all cells produce toxins (Davis et al., 2009), the explanation can be found in the extremely low contribution of all *Microcystis* cells (both morphologically indistinguishable toxic and non-toxic cells) to the total phytoplankton.

CONCLUSIONS

The present study revealed seven morphospecies of *Microcystis* and 57 toxigenic strains of this genus in nine of ten sampled waterbodies. Although they were detected in low amounts during the single sampling campaign, their presence can serve as an alarm signal for the potential risk for human and environmental health in the investigated reservoirs and lakes. The study highlights the positive effect of applying molecular-genetic methods to detect potential toxin-producers even when they are at low abundance in the phytoplankton. At the same time, the impossibility to refer most of the strains to known *Microcystis* species once more demonstrates the necessity of providing detailed morphological descriptions and illustrative documentation to the strains supplied to NCBI, to get a better view of the taxonomy and global distribution of essential cyanoprokaryotes as *Microcystis.*

The high summer contribution of cyanoprokaryotes to the total phytoplankton diversity and biomass, confirmed by both LM and HPLC analyses, and the unequal spread of toxigenic strains supports all previously expressed opinions on the need for implementing a broader monitoring programme of harmful cyanoblooms in the country.

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MICROCYSTIS RŪŠYS IR TOKSINIAI JŲ KAMIENAI DEŠIMTIES VANDENS TELKINIŲ FITOPLANKTONE BULGARIJOJE

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Santrauka

Darbo tikslas buvo nustatyti potencialių mikrocistinų gamintojų įvairovę, jų toksinių kamienų paplitimą, akcentuojant žydėjimus sukeliančias *Microcystis* genties melsvabakteres. *Microcystis* tyrimai atlikti dešimtyje Bulgarijos vandens telkinių: Ezerets, Durankulako ežeruose ir Koprinka vandens saugykloje *Microcystis* melsvabakterės buvo tirtos anksčiau, Shilkovtsi, Zhrebchevo, Suedinenie vandens saugykloje iki šiol nebuvo rastas; Malka Smolnitsa, Plachidol 2, Preselka, Duvanli vandens saugykloje pirmą kartą buvo tirta fitoplanktono struktūra. Vasaros fitoplanktono struktūra buvo tiriama šviesiniu mikroskopu, pigmentai – aukšto slėgio chromatografijos metodu, o PGR amplifikacijos pagalba nustatyti *mcyB* ir *mcyE* genai. Šviesinės mikroskopijos ir aukšto slėgio chromatografijos tyrimų duomenys parodė, kad devyniuose iš 10-ties tirtų vandens telkinių cianoprokariotai fitoplanktono sudėtyje sudarė reikšmingą gausumo (29%) ir biomasės (15–87%) dalį. Šviesinės mikroskopijos pagalba buvo identifikuotos rūšys: *Microcystis aeruginosa*, *M. natans*, *M. smithii, M. wesenbergii, Microcystis* spp., *M.* cf. *comperei* ir *M. pseudofilamentosa*. Pastarosios dvi tropikams būdingos rūšys šalyje buvo rastos pirmą kartą. Bendroje fitoplanktono įvairovėje ir jos biomasėje *Microcystis* sudarė nedidelę dalį. Molekuliniais tyrimais nustatyti 57 toksiniai šios genties melsvabakterių kamienai, kurių sekos pagal NCBI duomenų bazę buvo artimiausios *M. aeruginosa* ir *M. wesenbergii* rūšims.