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Assessment of cyanoprokaryote blooms and of cyanotoxins in Bulgaria in a 15-years period (2000-2015)

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ABSTRACT

The scientific and public awareness of hazardous photosynthetic prokaryotes (cyanobacteria/cyanoprokaryotes) and especially the contamination of drinking-water reservoirs with cyanotoxins is world-wide increasing. Recently much more attention has been paid to the events and results of mass proliferation of these toxic organisms even in South-East European countries in spite of the fact that, as a rule, they are not controlled by national legislation.The present paper presents a summary of results of such studies carried out in summer-autumn periods of the last 15 years (2000-2015) in Bulgarian water bodies differing by location, morphometry and trophic status, incl. drinking-water reservoirs, recreational lakes and sites of nature conservation importance. A multivariate analysis allowed to outline the distribution patterns and environmental drivers of the planktonic cyanoprokaryote assemblages in relation with the available data on the water bodies, highlighting species composition and abundance of the main taxa, including potentially toxic species. Samples analysis by HPLC-DAD and/or LC/MS, ELISA and *in vitro* cytotoxicity tests allowed detection of microcystins, nodularins and saxitoxins. Toxin concentration ranged between 0.1 and 26.5 µg L^{-1} in water samples and between 10.9 and 1070 µg g^{-1} (d.w.) in concentrated (net) samples. Despite the fact that microcystins were not found in all studied water bodies and that the recorded levels were still lower in comparison with some other European countries, the fact that cyanotoxins were detected in 16 water bodies (incl. 3 drinking-water reservoirs) could serve as an alert for the need of recognition of cyanotoxins as a new health risk factor in the country. Therefore, permanent monitoring with identification of toxins in water bodies at risk and activities for limitation and control of toxic blooms are urgently needed, in combination with increase of the attention to the effects of cyanotoxins on both human health and health of aquatic ecosystems in Bulgaria.

Key words: Microcystins; nodularins; saxitoxins; reservoirs; lakes; health risk.

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INTRODUCTION

Current estimates suggest that between 4000 and 5000 phytoplankton species have been described from inland waters (Reynolds, 1996, 2006). Among them are the prokaryotic phototrophs named Cyanoprokaryota (Cyanobacteria, Cyanophyta or Blue-green algae), which are also quantitatively amid the most important organisms on Earth (Whitton and Potts, 2012). Their appearance, traced back to the Early Archaean 3900 Ma ago (Graham *et al.,* 2009), became a crucial step in the evolution of life in water and, subsequently, on land. The later origin and spread of their heterocytous forms, capable of nitrogen fixation, fits well with the timing of Great Oxidation Event about 2400 Ma ago (Schopf, 2012). Since then cyanoprokaryotes are the only nitrogen fixing organisms that also produce oxygen through photosynthesis, with increasing at steady pace number of known non-heterocytous species that possess this ability (Stal, 2012). Considering their additional attribute of buoyancy regulation through gas vacuoles, it is to understand why these peculiar organisms for years remained a *fascinating topic* (Reynolds, 2006) in biology, with increasing findings of their great potential as providers of ecosystem services. Cyanoprokaryotes are primary colonizers of various (even extreme) habitats, important basis of numerous food chains and have been repeatedly reported for their applications in biotechnology, food industry and pharmacy (Whitton, 2012). However, during the last decades the recognition of the group turned in an almost *universal contempt* (Reynolds, 2006) due to their potential toxicity, key role in many harmful blooms and general assuming hazardous for human and ecosystem health (Carmichael 1994; Codd 1994, 1995; Chorus and Bartram, 1999; Codd *et al*., 1999, 2005a, 2005b; Maršálek *et al*., 2000; Huisman *et al*., 2005; Meriluoto and Codd, 2005; Metcalf and Codd, 2012; Walker, 2015). The situation in Bulgaria is not an exception in this case. Moreover, the country is of intermediate position on the Balkan Peninsula - a great European hotspot of biodiversity (Griffiths *et al*., 2004). The studies of its water inhabitants have been conducted since more than hundred years and 216 taxa (incl. 170 cyanoprokaryotes and algae), new for the science, have been described (Michev and Stoyneva, 2007). However, the inventory assessment of Bulgarian water bodies (WBs) brought to the recognition of anthropogenically fast eutrophication with nuisance blooms in rapidly increasing numbers of WBs. This situation was considered by the authors as *alarming.* Since then the number of studies and publications on the cyanotoxins in Bulgarian waters, pioneered by Pavlova *et al*. (2006), increased and proved their existence in different sites, some of which of high conservational, drinking-water, sport fishing or recreational importance. A part of the results has been published in Bulgarian language, which, in spite of being accepted as one of the official languages of the European Union, remains exotic and not understandable for the majority of scientists. Therefore, the aim of the present paper is to summarize data on cyanotoxins and water blooms in Bulgaria gathered during the last 15 years' period and to assess them on the background of cyanoprokaryote distribution, diversity and abundance in relation to potential toxin producers and driving environmental variables.

METHODS

Study area

The study area covers practically the whole territory of Bulgaria (111,000 km^2), a part of the eastern Balkan Peninsula with The Danube and Black Sea as north and east borders (Fig. 1). One-third of the country territory is occupied by plains, while the other is covered by hills, plateaus and higher mountains. Its peak Mousala (2925 m) is the highest

Fig.1. Map of Bulgaria with indication of location of the 120 water bodies studied in 2000-2015. Numeration follows Tab. 1.

point of the Balkan Peninsula, while the lowest parts of Bulgaria are at sea level. The climate is temperate continental with a Mediterranean influence in its southern (mainly SW) part with a significant amount of snowfall during winter. Temperature amplitudes vary in different areas (from -38.3°C to 45.2°C) and precipitation range is from 500 mm in plains to more than 2500 mm in the mountains, being about 630 mm per year on average. Due to the interaction of climatic, hydrological, geological and topographical conditions, Bulgaria is one of the countries with highest biodiversity in Europe (Peev *et al*., 2013). According to the Appendix № XI, map A of Water Framework Directive 2000/60/EC (European Commission, 2000) Bulgaria belongs to the ecoregions № 12 Pontic province and № 7 Eastern Balkans, the borders of which have been updated by Cheshmedjiev *et al.* (2010b). The results from the first Inventory of Bulgarian wetlands and their biodiversity lead to the conclusion that the country is characterized by great diversity and number of WBs, which cover ca. 1% of its territory (Michev and Stoyneva, 2007). The term *wetlands* was consistently used by these authors for all types of WBs, following the definition of Ramsar Convention on wetlands, signed in 1971.

Data collection on planktonic cyanoprokaryotes

This study compiles and evaluates the currently available data on phytoplankton and relevant environmental data on Bulgarian WBs (mainly reservoirs and lakes) gathered during the period 2000-2015 (Stoyneva, 2003, 2010, 2014, 2015; Beshkova and Botev, 2004; Kalchev *et al*., 2004; Traykov, 2005; Pavlova *et al.,* 2006, 2013a, 2014, 2015; Beshkova *et al.,* 2008a, 2008b, 2012; Pavlova, 2007; Tsanev and Belkinova, 2008; Teneva *et al.,* 2009, 2010a, 2010b, 2011, 2014; Cheshmedjiev *et al.,* 2010a, 2013; Stoyanov *et al.,* 2012, 2013, 2016; Stoyanov, 2014 Stoyneva *et al.,* 2013, 2015; Belkinova *et al.,* 2014; Dimitrova *et al.,* 2014a, 2014b; Dochin and Stoyneva, 2014, 2015; Dochin, 2015; Georgieva *et al.,* 2015).

The total number of WBs investigated in the above mentioned studies is 115 (Tab. 1, Fig. 1). In addition, five recreational lakes have been checked for cyanotoxins, but data on their phytoplankton composition have not been published (Pavlova, 2007; Pavlova *et al.,* 2013a). They are added in Tab. 1 and Fig. 1 with numbers 116-120. In Tab. 1 we provide the commonly used categories (reservoirs, lakes, swamps, *etc*.) and the unique number of each WB according to the DataBase of Bulgarian wetlands inventory (IBW - Michev and Stoyneva, 2007), where more details on the origin, location, morphometry, trophic status, history, way of exploitation, conservation, *etc.* could be found. Since most investigated WBs are reservoirs, in the text bellow, in an operational way, we assign all WBs to two types – reservoirs (R) and other, mainly natural WBs (OWB). According to the geographical location and vertical position all studied WBs could be grouped in the following 8 groups (phyla) of the hierarchical Bulgarian wetland classification (Michev and Stoyneva, 2007): *Black Sea coastal surface lowland WBs* (*CV.I,* 0-200 m asl) – 11 (3 R, 8 OWB); *Black Sea coastal surface low mountain WBs* (*CV.IV*; >1000 m asl) – 1 R; *Inland surface lowland WBs* (*LV.I*; 0-200 m asl) – 41 (35 R, 6 OWB); *Inland surface plain WBs* (*LV.II*; 200-600(700) m asl) – 24 R; *Inland surface kettle WBs* (*LV.III*; 500-1000 m asl– 11 (8 R, 3 OWB); *Inland surface low mountain WBs* (*LV.IV*; >1000 m asl) – 10 R; *Inland surface middle mountain WBs* (*LV.V*; 1000-1800 m asl – 3 (2 R, 1 OWB); *Inland surface high mountain and alpine WB*s (*LV.VI*; >1800 m asl – 19 (2 R, 17 OWB). Among the WBs studied 36 are of conservational importance (Tab. 1): 21 are included in the Red List of Bulgarian wetlands as critically endangered (CR) – 9, endangered (EN) – 6 and vulnerable (VU) – 6 (Michev and Stoyneva, 2005, 2007); 36 belong to protected areas with different International and National status (Michev and Stoyneva, 2007); 17 are special subjects of National Action Plan for conservation of wetlands of high significance of Bulgaria for 2013-2022 (Vassilev *et al.,* 2013).

Phytoplankton sampling and laboratory processing

Phytoplankton sampling procedures and measurements of the main environmental variables (water temperature - t, pH, electric conductivity - cond, Secchi depth - SD, total phosphorus - TP and total nitrogen -TN) were based on International and Bulgarian standards (*e.g*., CEN EN 15204, 2006; State Order N 4/14.09.2012, 2013; Belkinova and Gecheva, 2013 and references therein). Some studies included more environmental data (chlorophyll *a*, dissolved oxygen, saturation, NO_3, PO_4, *etc.*) with tools and protocols described in detail in the relevant publications, but all of them generally followed the same design. More differences concern the microscopic processing of the samples because the cited standards provide possibilities to choose between inverted microscopy and standard microscopy in combination with different types of counting chambers. In the works of the authors of this paper standard light microscopy was used in combination with Thoma or Burker counting chambers. Always cell was the main counting unit. The biomass estimations were based on the measurement of the dimensions of each cell according to the method of stereometrical approximations (Rott, 1981) instead of the often recommended and broadly applied by other Bulgarian authors usage of average cell sizes. The reasons for choosing this more time-consuming way of work were described in Stoyneva *et al.* (2015) but for the purpose of the present paper they could be briefly summarised as follows: i) necessity of cell measurements for correct taxa identification; ii) differences in cell size during the cell division process; 3) variations in cell size of the same taxon in different WBs,

Tab. 1. Bulgarian WBs studied in the period 2000-2015. The names are provided according to the Bulgarian Wetlands Inventory (IBW) through transliteration of their vernacular names; in brackets the synonyms used in the literature.

Number	Name	Type	GL	AZ	PA	RL	NAP	IBWnumber
1	Aheloy*	R	CV.IV.	3				IBW3032
2	Aleksandrovo	R	LV.I.	3				IBW2017
3	Aleksandur Stamboliyski*	R	LV.I.	3				IBW2056
4	Blato Alepu*	S	CV.I.	1	x	CR		IBW1770
5	Musalensko ezero 3 (Alekovo ezero)	L	LV.VI.	13	x	VU		IBW0078
6	Antimovo*	R	LV.I.	3				IBW 2818
7	Asenovets	R	LVII	4				IBW2549
8	Asparuhov val*	R	LV.I.	2				IBW 3674
9	Atanasovsko ezero*	L	CV.I.	-1	x	VU	x	IBW1900
10	Baniska	R	LV.I.	3				IBW9042
11	Batak*	R	LV.V.	8				IBW1316
12	Bebresh*	R	LV.II.	5				IBW2397
13	Belmeken	R	LV.VI.	10				IBW1187
14	Blatse do AEC-Belene	TS	LV.I.	1	x			IBW4638
15	Beli Iskur*	R	LV.VI.	10	x			IBW1180
16	Beli Lom	R	LV.II.	5				IBW2810
17	Bezbozhsko ezero 1 (Ezero Bezbog)*	L	LV.VI.	11	x			IBW0442
18	Balastrierni ezera Bistratsite/Bistraka	L	LV.III.	5				IBW4563
19	Bistritsa*	R	LV.III.	6				IBW1067
20	Blatse do AEC-Kozloduy	T	LV.I.	1				
21	Borovitsa	R	LV.IV.	7				IBW1580
22	Boyka	R	LV.II	4				IBW2573
23	Vaya (Burgasko ezero)*	L	CV.I.	1	x	CR	x	IBW0191
24	Barzina*	R	LV.I.	3				IBW1276
25	Nevenino ezero 1 (Chernoto ezero)*	L	LV.VI.	12	x			IBW0371
26	Chirpan*	R	LV.I.	3				IBW1704
27	Choklyovo Blato*	S	LV.V.	7	x	VU	x	IBW 0003
28	Gergiysko ezero 2*	L	LV.VI.	12	x			IBW0480
29	Gorni Dabnik*	R	LV.I.	3				IBW 5606
30	Daskal Atanasovo	R	LV.I.	3				IBW2219
31	Devets (Monchovets)	R	LV.II	4				IBW10869
32	Dospat*	R	LV.V.	5				IBW3155
33	Drenovets	R	LV.I.	3				IBW1128
34	Dabnika	R	LV.II.	5				IBW5393
35	Durankulashko ezero (Durankulak)*	L	CV.I.	1	x	CR	x	IBW0216
36	Dyakovo*	R	LV.IV.	6				IBW1033
37	Blato Dyuleva Bara	T	LV.I.	1	x	EN	x	IBW0154
38	Eleshnitsa*	R	LV.I.	2				IBW3023
39	Enitsa	R	LV.I.	3				IBW1444
40	Ivaylovgrad*	R	LV.I.	3				IBW2271
41	Iskur*	R	LV.III.	7				IBW1200
42	Hr. Smirnenski na reka Lom*	R	LV.I.	3				IBW1135
43	Hr. Smirnenski na reka Yantra (Hr. Smirneski/Gabrovo)*	R	LV.II.	5				IBW2080
44	Kamenets	R	LV.I.	3				IBW2162
45	Karaisen	R	LV.I.	3				IBW5113
46	Musalensko ezero 7 (Karakashevo ezero)	L	LV.VI.	13	x	VU		IBW0080
47	Seyatchi (Kavacite, Popovo)*	R	LV.II.	4				IBW2606
48	Kamchiya*	R	LV.IV.	5				IBW2745
49	Koprinka*	R	LV.II.	5				IBW2062
50	Kovachitsa	R	LV.I.	3				IBW1160
51	Krapets*	R	LV.III.	5				IBW2000
52	Kremensko ezero 2*	L	LV.VI.	12	x			IBW9088

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Tab. 1. Continued from previous page.

Number	Name	Type	GL	AZ	PA	RL	NAP	IBWnumber
53	Krichim	R	LV.I.	3				IBW1366
54	Krushovitsa 3 (Krushovitsa)	R	LV.I.	1				IBW1452
55	Kula	R	LV.II.	4				IBW1105
56	Kardzhali*	R	LV.II.	5				IBW1668
57	Musalensko ezero 1 (Ledeno ezero)	L	LV.VI.	13	x	VU		IBW0076
58	Lazhenska bara (Ladzhenska bara)	R	LV.I/	2				IBW2166
59	Mandra*	R	CV.I.	1	x	EN	x	IBW1720
60	Marichino ezero 2 (Gorno Marichino ezero)	L	LV.VI.	12	x			IBW0085
61	Marichino ezero 3 (Dolno Marichino ezero)	L	LV.VI.	12	x			IBW0086
62	Murtvo blato	TS	LV.I.	1	x	EN	x	IBW0158
63	Ezero Momin brod*	L	LV.I.	1				IBW8307
64	Novo Zhelezare	R	LV.II.	4				IBW1475
65	Ognyanovo*	R	LV.III.	6				IBW2340
66	Ogosta*	R	LV.I.	3				IBW 1137
67	Ovcharitsa	R	LV.I.	3	x		x	IBW2317
68	Ovchi kladenets	R	LV.I.	3				IBW2367
69	Pchelina*	R	LV.III.	6				IBW1039
70	Blato Peschina (Pischina)	T	LV.I.	1	x	EN	x	IBW0156
71	Poletkovtsi 2 (Poletkovtsi)*	R	LV.II.	4				IBW1103
72	Pomoriysko ezero*	L	CV.I.	-1	x	VU	x	IBW 0189
73	Popovo ezero 2 (Popovo ezero)*	L	LV.VI.	11	x			IBW0447
74	Poroy*	R	CV.I.	2				IBW3038
75	Pyasuchnik 1 (Pyasuchnik)*	R	LV.II.	4			x	IBW5834
76	Rabisha*	R	LV.II.	4				IBW1102
77	Rasovo 2 (Rasovo)	R	LV.I.	3				IBW1158
78	Redzhepsko ezero 2 (Redzhepsko ezero)	R	LV.VI.	12	x			IBW0342
79	Dragash Voyvoda	R	LV.I.	1	x			IBW3935
80	Onogour (Efreytor Bakalovo)	R	LV.I.	3	x			IBW5667
81	Bunderishko ezero 9 (Ribno ezero)*	L	LV.VI.	11	x			IBW0400
82	Shablensko ezero (Shabla)*	L	CV.I.	1	x	CR	x	IBW0219
83	Shablenska tuzla	L	CV.I.	-1		EN		IBW0218
84	Kayabash 2 (Golyamo Skalensko ezero)	R	LV.IV.	5				IBW2659
85	Kayabash 1 (Malko Skalensko ezero)	R	LV.IV.	5				IBW2658
86	Hisar 12 (Sinyata reka)*	R	LV.II.	5				IBW1893
87	Sopot*	R	LV.II.	5				IBW1437
88	Blato Srebarna*	L	LV.I.	1	x	EN	x	IBW0208
89	Srechenska bara*	R	LV.IV.	5				IBW3668
90	Stoychovtsi (Stoykovtsi)	R	LV.IV.	8				IBW3237
91	Studena*	R	LV.IV.	7				IBW 1060
92	Studen kladenets*	R	LV.II.	4				IBW1763
93	Suedinenie*	R	LV.I.	3				IBW2642
94	Telish*	R	LV.I.	3				IBW1413
95	Ticha*	R	LV.I.	3				IBW2700
96	Toshkov chark	R	LV.V.	9	x			IBW1315
97	Trakiets	R	LV.II.	4				IBW1677
98	Tri kladentsi*	R	LV.I.	3				IBW1275
99	Tsonevo*	R	LV.I.	2				IBW3022
100	Varnensko ezero*	L	CV.I.	1	x	CR		IBW0203
101	Vlahinsko ezero 1*	L	LV.VI.	12	x			IBW0475
102	Vucha	R	LV.I.	3				IBW 3143
103	Valchovets	R	LV.I.	2				IBW2129
104	Yarlovets (Yarlovtsi)	R	LV.IV.	7				IBW 1038
105	Yasna polyana*	R	CV.I.	2				IBW2887

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Tab. 1. Continued from previous page.

Number	Name	Type	GL	AZ	PA	RL	NAP	IBWnumber
106	Yastrebino*	R	LV.II.	5				IBW2602
107	Shilkovtsi (Yovkovtsi)*	R	LV.II.	5				IBW2105
108	Zhrebchevo*	R	LV.II.	4				IBW2545
109	Lomtsi	R	LV.II.	4				IBW2772
110	Pancharevo	R	LV.III.	6				IBW1088
111	Zhernov	R	LV.I.	2				IBW4639
112	Ezero Bliznaka	L	LV.VI.	11	x	CR	x	IBW0350
113	Ezero Bubreka	L	LV.VI.	11	x	CR	x	IBW0349
114	Ezero Okoto	L	LV.VI.	12	x	CR	x	IBW0348
115	Ezero Sulzata	L	LV.VI.	12	x	CR	x	IBW0347
116	Balastrierni ezera Dolni Bogrov	L	LV.III.	6				IBW0708
117	Botunets	R	LV.III.	6				IBW1792
118	Ezero 1 v kvartal Druzhba	L	LV.III.	6				IBW5284
119	Krasava	R	LV.IV.	7				IBW1049
120	Rudnichno ezero Kutina 1	L	LV.III.	6				IBW0705

*Denote WB included in the dataset for the Principal component analysis (PCA) and Redundancy analysis (RDA) for this paper. R, reservoir; L, lake; S, swamp; T, temporary swamp. For each WB, the main group of geographic location and vertical position (GL) is shown (CV.I – LV.VI) and altitude zone (AZ) in 14 elevation classes (-1 -13); for details on GL and AZ see the text. The conservation value of the WB is provided as protected area (PA), National Action Plan for conservation of wetlands of high significance of Bulgaria for 2013-2022 (NAP) and Red List of Bulgarian Wetlands (RL); CR, critically endangered; EN, endangered; VU, vulnerable. IBW number is the number of WB in the Bulgarian Wetlands Inventory (Michev and Stoyneva, 2007), where more details on them could be found.

or even in the same water body due to different temperature, nutrient content or grazing pressure (Stoyneva *et al.,* 2007). We believe that the results obtained in this way reflect the real biomass for a given time and site, especially when the samples are analysed by the same person.

Multivariate analyses

Considering the reasons stated above, after analyzing of all available, but quite heterogenous data on the 2000-2015 phytoplankton in Bulgarian WBs (references provided in the "Data Collection" paragraph above), we chose to build a homogenous data set (Stoyneva, 2014, 2015; this study) by including only the WBs (sites) with at last 5 samples processed per site, for which the environmental data listed above were available. WBs in which cyanotoxins have been investigated by the authors of this paper, have been included in the dataset. In addition, WBs were chosen to represent all 8 general groups (from CV.I to LV.VI) proposed in the Bulgarian wetlands classification and the CR, EN and VU conservational threat categories (Michev and Stoyneva 2005, 2007) as well. In this way, the final dataset comprised 61 WBs (marked with asterisk* in Tab. 1) and average values of biomass of 93 species, varieties and forms grouped in 24 genera, environmental data, total cyanoprokaryote biomass (TBC), total phytoplankton biomass (TBS). The dataset also includes average data biomass of other phytoplankton dominant groups, as well as data on WBs morphometry (area and depth), geographic location in the 8 groups of Michev and Stoyneva (2007), exact altitude asl (m) or as 14 elevation classes proposed in the IBW DataBase of Michev and Stoyneva (2007): -1 – below 0; 1-0-50; 2 – 50-100; 3 – 100-200; 4 – 200-300; 5 – 300-500; 6 – 500-700; 7 – 700-1000; 8 – 1000-1100; 9 – 1100-1600; 10 – 1600-2000; 11 – 2000-2300; 12 – 2300-2700; 13 – >2700 m asl.

Multivariate analyses were run using principal components analyis (PCA) and redundancy analysis (RDA), using the R-software (R 3.1.2 version, R Development Core Team, 2010) and the "ADE4" package (Thioulouse *et al.,* 1997). The aims were i) to identify the main environmental gradients among the samples of lakes/reservoirs and ii) to investigate the response of the cyanoprokaryote assemblages (at the genus level) to these gradients. All variables were normalised; the cyanobacterial abundances were transformed using the Hellinger transformation (Legendre and Gallagher, 2001).

Taxonomic sources, terminology and biodiversity assessment

Taxonomic sources include mainly the standardly used volumes of Middle European freshwater flora with some published updates (Komárek 2013; Komárek and Anagnostidis 1999, 2005; Komárek *et al.* 2011, *etc.*) and AlgaeBase (Guiry and Guiry 2016). The Latin names follow the above mentioned botanical sources and the International Code of the Nomenclature for algae, fungi and plants (McNeil *et al.,* 2012). Therefore, the term *cyanoprokaryotes* is used in the paper instead of *cyanobacteria*. For each species and genus, the frequency quotient (FQ) of occurrence in all studied sites was estimated based on its presence / absence in each site. The frequency quotients were grouped in classes with a step of 10% (I class – 0-10%, II class – 10-20%, etc.). The evaluation of cyanoprokaryote diversity was done as comparisons with the recently estimated data on the total algal biodiversity in Bulgaria (Stoyneva, 2014) and the total diversity of Cyanoprokaryota in the country (Stoyneva *et al*., 2016).

Collection and assessment of cyanotoxin data, algal blooms and potential toxin producers

The assessment of cyanotoxins registered in the country in relation to their potential producers is based on the works of Pavlova (2007), Pavlova *et al.* (2006, 2007, 2013a, 2014, 2015), Teneva *et al.* (2009, 2010a, 2010b, 2011, 2014), Stoyanov *et al.* (2012) and Georgieva *et al.* (2015) – Tabs. 2 and 3. For this study the biomass of the species published earlier by Pavlova *et al.* (2006, 2014) with their cell numbers per liter (or milliliter) was estimated (Tabs. 2 and 3). Results on toxin findings were superimposed on the data on phytoplankton blooms according to the references in Tabs. 2 and 3. The cyanotoxin concentrations were evaluated according to the WHO standards (1998, 2003) since their maximum acceptable levels are not indicated in Bulgarian national legislation (Pavlova *et al*., 2013b).

RESULTS

Diversity and abundance of cyanoprokaryotes

A total of 210 taxa (207 species, 1 variety and 2 forms) from 69 genera of Cyanoprokaryota were recorded in the 2000-2015 period. The distribution of species in the three orders Chroococcales, Oscillatoriales and Nostocales clearly shows the Chroococcales (85) as the richest order and the better representation of non-heterocytous filamentous forms (69) in comparison with hetereocytous, filamentous taxa (56). At the genus level, heterocytous taxa (17) were also less numerous than non-heterocytous filamentous (21) and than coccal taxa (31). The most species-rich genera were *Dolichospermum* (11), *Microcystis* (11), *Anabaena* (9), *Aphanocapsa* (9), *Oscillatoria* (9), *Chroococcus* (8), *Phormidium* (8), *Pseudanabaena* (8), *Romeria* (8), *Anabaenopsis* (6) and *Aphanizomenon* (6).

Most of the species were rare (96 were found only in one site) and FQ ranged from 1 to 34%. Among the 19 broadly distributed algae (in ≥11 sites), 14 have been identified at species level (Fig. 2), with *Aphanizomenon flos-aquae* Ralfs ex Bornet & Flahault being the most widespread taxon.

Most of the 69 genera were rare (18 were found in one site), with FQ range from 1 to 43%. The 22 genera with the widest distribution (in ≥12 sites) are shown on Fig. 3: *Aphanizomenon* is outstanding as the most widespread genus in

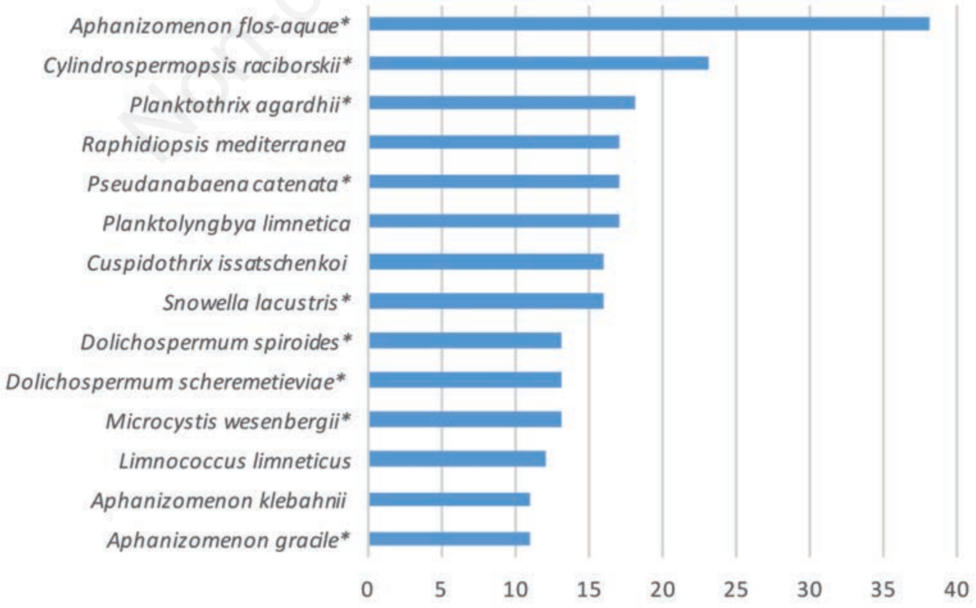

Fig. 2. Distribution of the most widespread cyanoprokaryotes in the phytoplankton of Bulgarian water bodies in the period 2000-2015. 0-40, number of water bodies in which species were found; *species found in the samples containing cyanotoxins.

Tab. 2. Cyanotoxins in Bulgarian WBs, organized according to the first year of finding.

Water body	Main use	Date	Biomass	MC (types)					MC total	NOD		ANT-a	STX	Methods			Source
				LR	RR	YR	LA	YReq		MC/NOD	NOD			HPLC	Elisa	IVCT	
Bistritsa	RS	20.8.2004	1.53*	0.86	x	x			1.64					x			Pavlova *et al.* 2006
		14.10.2013	n.a.	0.6					0.6					x			Pavlova *et al.* 2015
Borovitsa	DR	26.7.2006	not ind.							0.09-0.12		x		x	x		Teneva *et al.* 2010b
		26.9.2006	not ind.	x	x	x	x			0.18			2.5	x	x		Teneva *et al.* 2010b
Vucha	WS	Aug2008	0.11							0.004				x	x	x	Teneva *et al.* 2010a
		Sept2009	0.062							0.005				x	x	x	Teneva *et al.* 2010a
Studen kladenets -dam wall part	IR	July2008	not ind.						0.2-0.4					x	x		Teneva *et al.* 2011
		Aug2008	0.07						0.2-0.4					x	x		Teneva *et al.* 2011
		Sept2008	0.1						0.2-0.4					x	x		Teneva *et al.* 2011
		July2009	0.021						0.2-0.4		x						Teneva *et al.* 2012
		Aug2009	0.011						0.2-0.4		x			x	x		Teneva *et al.* 2011
		Sept2009	0.033						0.2-0.4		x		<0.10	x	x		Teneva *et al.* 2011
Studen kladenets - tail part		July2008	not ind.						0.2-0.4					x	x		Teneva *et al.* 2011
		Aug2008	0.19						0.2-0.4					x	x		Teneva *et al.* 2011
		Sept2008	0.1						0.2-0.4					x	x		Teneva *et al.* 2011
		July2009	0.094								x		<0.10	x	x		Teneva *et al.* 2011
		Aug2009	0.761								x		<0.10	x	x		Teneva *et al.* 2011
		Sept2009	0.453						0.2-0.4		x			x	x		Teneva *et al.* 2011
Trakiets	DR	July2008	0.5	x	x	x	x		0.09	x				x	x	x	Teneva *et al.* 2009
		Aug2008	0.04	x	x	x	x		0.018	x			0.01	x	x	x	Teneva *et al.* 2009
		Sept2009	0.06	x	x	x	x		0.014	x				x	x	x	Teneva *et al.* 2009
Krushovitsa	RS	4.10.2009	2.32						1					x	x	x	Stoyanov *et al.* 2012
Enitsa	RS	4.10.2009	14.57						2					x	x	x	Stoyanov *et al.* 2012
Valchovets	WS	4.10.2009	0										0.02	x	x	x	Stoyanov *et al.* 2012
Pchelina	RS	3.8.2011	0.167		0.5				0.5					x			Pavlova *et al.* 2014
		2.10.2014	6.2	0.17	0.07	0.13			0.37					x			Pavlova *et al.* 2015
Kayabash 2	IR	July2011	30						0.4					x	x	x	Teneva *et al.* 2014
		Sept2011	65.42						0.4					x	x	x	Teneva *et al.* 2014
Studena	DR	28.9.2011		0.1					0.1					x			Pavlova *et al.* 2014
		14.10.2013		0.1					0.1								Pavlova *et al.* 2015
		5.11.2015	0.004*	0.2		0.4			0.6					x			Georgieva *et al.* 2015
Ezero Momin brod	RS	19.8.2012	0.8	1		0.3			1.3					x			Pavlova *et al.* 2015
		21.10.2013	3.71	1	0.4	0.4			1.8					x			Pavlova *et al.* 2015
Durankulashko ezero	RS	18.8.2013	10.5	0.2	0.1	0.9			1.2					x			Pavlova *et al.* 2015
		29.7.2011	7.01	8.3	12.7	5.5			26.5					x			Pavlova *et al.* 2014
		18.8.2013	10.5	0.2	0.1	0.9			1.2					x			Pavlova *et al.* 2015
Net samples / scum "biomasses"																	
Vaya	RS	5.8.2004	68.56*	14	x	x			42					x			Pavlova *et al.* 2006
Vaya -quay		5.8.2004	32.34*	260	x	x			1070					x			Pavlova *et al.* 2006
Durankulashko ezero	RS	3.8.2004	5.43*	260					110					x			Pavlova 2007; Pavlova *et al.* 2006. 2007;
		14.7.2005	n.a.	204	x	x			517					x			Pavlova *et al.* 2007, 2013
		29.7.2011	n.a.	27.5	22.1	x(trace)			49.6								Pavlova *et al.* 2014
		18.8.2013	n.a.	63.5	103.2	47.6			214.3					x			Pavlova *et al.* 2015

To be continued on next page

Tab. 2. Continued from previous page.

Water body	Main use	Date	Biomass	MC (types)					MC total	NOD		ANT-a	STX	Methods			Source
				LR	RR	YR	LA	YReq		MC/NOD	NOD			HPLC	Elisa	IVCT	
Net samples / scum "biomasses"																	
Mandra	IR. RS	6.8.2004	2.61*	35	x	x			63					x			Pavlova *et al.* 2006
Pchelina	IR	19.8.2004	0.08*	140	x	x			536					x			Pavlova *et al.* 2006
		3.8.2011	n.a.		11.14				11.14					x			Pavlova *et al.* 2014
		31.7.2012	n.a.						x (traces)					x			Pavlova *et al.* 2015
		18.9.2012	n.a.						x (traces)					x			Pavlova *et al.* 2015
		2.10.2014	n.a.	132.8	91.5	71.8			296.1					x			Pavlova *et al.* 2015
Shablensko ezero	RS	3.8.2004	0.62*	40					40					x			Pavlova *et al.* 2006. 2007
		14.7.2005	n.a.	283	x	x		x	1018					x			Pavlova 2007; Pavlova *et al.* 2007
Shablensko ezero - quay		3.8.2004	11.97*	14					14					x			Pavlova *et al.* 2006. 2007
		14.7.2005	n.a.	292	x	x		x	982					x			Pavlova 2007; Pavlova *et al.* 2007
Ezero Momin brod	RS	19.8.2012	n.a.	23		4			27					x			Pavlova *et al.* 2015
Studena	DR	14.10.2013	n.a.	8.1	2	0.8			10.9					x			Pavlova *et al.* 2015

*Biomass estimated for this study; x, presence / not value indicated; IVCT, *in vitro* cytotoxicity; n.a., not analysed; not. ind., no information in the publication; biomass [mg L^{-1}], related to the cyanoprokaryotes in the relevant samples; DR, drinking-water reservoir; WR, reservoir for water suppl; IR, reservoir for irrigation and/or energy supply; RS, recreational site. Concentrations are expressed as µg L^{-1} (water body) or in µg g^{-1} dry weight (biomass).

the country during the analyzed period. In this study, we classified as *picoplankton* single spherical cells of dimensions 0.5-1.5 µm, commonly referred as *Pcy (picocyanobacteria)* in contrast to the second morphological group of colonial picoplankters – *CPcy (colonial picocyanobacteria)* – Stockner *et al.* (2002), Callieri *et al.* (2012).

The number of species and genera per site ranged between (0)1 and 85, and 1 to 35, respectively (Fig. 4), the highest number detected in the shallow lakes Blato Srebarna (site 88: 85/35) and Vaya (site 23: 63/30). The comparison of distribution of species and genera per site, expressed on Fig. 4, shows that in most of the WBs each genus is represented by a single species, which makes the discussion of the distribution on generic level and further PCA analysis quite reasonable.

The contribution of the cyanoprokaryotes to the total phytoplankton biomass in different WBs and in different sampling periods ranged from 0 to 100%, with values exceeding 65-75 mg L^{-1} in summer periods (*e.g.*, in the coastal lake Vaya - Stoyneva, 2003; Pavlova *et al.,* 2006, 2007; Dimitrova *et al.,* 2014a) and reaching 95.9 mg L^{-1} in average for the period 2010-2014 (this study). The assessment of the average cyanoprokaryote biomass in 61 WBs from the dataset shows that in four sites (the lakes Vaya and Blato Srebarna, and the reservoirs Mandra and Tri kladentsi), its values were over WHO's (2003) threshold for medium health risk category (10 mg L^{-1}) in recreational waters and in five other (the lakes Durankulashko ezero and Ezero Momin brod, and the reservoirs Asparuhov val, Chirpan and Pchelina) from the rest 57 WBs, they were over the low health risk category threshold (2 mg L^{-1}), as they were re-estimated from cell numbers in biomass values by Mishke *et al.* (2011).

The single investigation of steady-states, as Sommer and Padisák (1993) have defined them, outlined their presence in the period analyzed for this paper only in the lake Vaya: 3 weeks dominance (89%) of *Microcystis wesenbergii* (Komárek) Komárek in Kondratieva*, Aphanizomenon flos-aquae* and *Dolichospermum spiroides* (Klebahn) Wacklin *et al.* in August–September 2001 and 4 weeks dominance (98%) of *M. wesenbergii* and *Aphanizomenon gracile* (Lemmermann) Lemmermann in August-September 2002 (Stoyneva, 2003). The lake Vaya is the only WB for which the total carbon content was estimated (Dimitrova *et al.,* 2014a). Its mean value of 9.7 mg L^{-1} (2004-2006) together with the average biomass of 46 mg L^{-1} for the same period confirmed the hypertrophic status of the lake. Cyanoprokaryotes dominated constantly in the total carbon content, reaching absolute maxima of 25.1 and 26.9 mg L^{-1} in August 2005 and 2006. Dominance of this group with water blooms (up to 200 mg L^{-1}) was detected also in more recent studies of this shallowest coastal lake and, in parallel, cyanoprokaryote blooms were documented for 19 more Bulgarian WBs: Alepu, Durankulashko ezero,

Tab. 3. Water samples with negative results for cyanotoxins in Bulgarian WBs (2000-2015).

Water body -water samples checked for toxins with negative results/organized by years of the investigations	Date	Methods			Source
		HPLC	Elisa	*In vitro* CT	
Botunets	20.8.2004	x			Pavlova *et al.* 2006
Ezeretsko ezero	3.8.2004	x			Pavlova *et al.* 2006
	2005	x			Pavlova 2007; Pavlova *et al.* 2013a
Choklyovo blato	19.8.2004	x			Pavlova *et al.* 2006
	2005	x			Pavlova 2007; Pavlova *et al.* 2013a
Balastrierni ezera Dolni Bogrov	20.8.2004	x			Pavlova *et al.* 2006
Ezero 1 v kvartal Druzhba	27.8.2004	x			Pavlova *et al.* 2006
Iskur	18.8.2004	x			Pavlova *et al.* 2006
	2005	x			Pavlova 2007; Pavlova *et al.* 2013a
Rudnichno ezero Kutina 1	20.8.2004	x			Pavlova *et al.* 2006
Studena	19.8.2004	x			Pavlova *et al.* 2006
	2005	x			Pavlova 2007; Pavlova *et al.* 2013a
	31.7.2012	x			Pavlova *et al.* 2015
	3.8.2011	x			Pavlova *et al.* 2014
	18.9.2012	x			Pavlova *et al.* 2015
	12.8.2013	x			this study
	2.10.2014	x			Pavlova *et al.* 2015
	4.8.2015	x			Georgieva *et al.* 2015
Yasna polyana	6.8.2004	x			Pavlova *et al.* 2006
	2005	x			Pavlova 2007; Pavlova *et al.* 2013a
Bistritsa	2005	x			Pavlova 2007; Pavlova *et al.* 2013a
	3.8.2011	x			Pavlova *et al.* 2014
	28.9.2011	x			Pavlova *et al.* 2014
	31.7.2012	x			Pavlova *et al.* 2015
	18.9.2012	x			Pavlova *et al.* 2015
	12.8.2013	x			Pavlova *et al.* 2015
	2.10.2014	x			Pavlova *et al.* 2015
	4.8.2015				Georgieva *et al.* 2015
	5.11.2015	x			Georgieva *et al.* 2015
Borovitsa	2005	x			Pavlova 2007; Pavlova *et al.* 2013a
Vaya	2005	x			Pavlova 2007; Pavlova *et al.* 2013a
	3.8.2011	x			Pavlova *et al.* 2014
	2.8.2012	x			Pavlova *et al.* 2015
	16.8.2012	x			Pavlova *et al.* 2015
	21.8.2013	x			Pavlova *et al.* 2015
Durankulashko ezero	2005	x			Pavlova 2007; Pavlova *et al.* 2013a
	12.7.2012	x			Pavlova *et al.* 2015
	8.8.2012	x			Pavlova *et al.* 2015
Krasava	2005	x			Pavlova 2007; Pavlova *et al.* 2013a
Hristo Smirnenski na reka Yantra	2005	x			Pavlova 2007; Pavlova *et al.* 2013a
Yovkovtsi (VT)	2005	x			Pavlova 2007; Pavlova *et al.* 2013a
Mandra	2005	x			Pavlova 2007; Pavlova *et al.* 2013a
Pchelina	2005	x			Pavlova 2007; Pavlova *et al.* 2013a
	28.9.2011	x			Pavlova *et al.* 2014
	31.7.2012	x			Pavlova *et al.* 2015
	18.9.2012	x			Pavlova *et al.* 2015
	12.8.2013	x			Pavlova *et al.* 2015
	14.10.2013	x			Pavlova *et al.* 2015
	4.8.2015				Georgieva *et al.* 2015
	5.11.2015	x			Georgieva *et al.* 2015
Ticha	2005	x			Pavlova 2007; Pavlova *et al.* 2013a
Trakiets	2005	x			Pavlova 2007; Pavlova *et al.* 2013a
Shablensko Ezero	2005	x			Pavlova 2007; Pavlova *et al.* 2013a
Kayabsh 1	July2011	x	x	x	Teneva *et al.* 2014
	Sept2011	x	x	x	Teneva *et al.* 2014
	July2012	x	x	x	Teneva *et al.* 2014
	Sept2012	x	x	x	Teneva *et al.* 2014
Kayabash 2	July2012	x	x	x	Teneva *et al.* 2014
	Sept2012	x	x	x	Teneva *et al.* 2014
Ezero Momin brod	24.5.2013	x			this study

Blato Srebarna and for the reservoirs Acheloy, Boyka, Borovitsa, Barzina, Daskal Atanasovo, Enitsa, Kamenets, Krushovitsa, Kardzhali, Mandra, Ovchi kladenets, Pchelina, Seyachi, Suedinenie, Tri kladentsi, Vucha (Traykov, 2005; Cheshmedjiev *et al.,* 2010a; Stoyanov *et al.,* 2012; Belkinova *et al.,* 2014; Stoyneva, 2014) (Fig. 1). The same authors and also Dochin and Stoyneva (2014, 2015) indicated the "presence of toxic species (*Anabaena, Aphanizomenon, Microcystis, etc.*)" for the following 43 WBs: Choklyovo blato, Durankulashko ezero, Shablensko ezero, Blato Srebarna and for the reservoirs Aheloy, Aleksandrovo, Antimovo, Asparuhov val, Batak, Boyka, Barzina, Daskal Atanasovo, Dospat, Drenovets, Dabnika, Dyakovo, Hristo Smirnenski (na reka Lom), Ivaylovgrad, Kamenets, Krapets, Krushovitsa, Koprinka, Kovachitsa, Kula, Lomtsi, Mandra, Ogosta, Ovcharitsa, Pancharevo, Pchelina, Poletkovtsi, Poroy, Pyasuchnik, Rabisha, Rasovo, Seyachi, Suedinenie, Telish, Tri kladentsi, Valchovets, Vucha, Yastrebino and Zhrebchevo (Fig. 1). Toxic species were indicated as blooming in the reservoirs Kamenets, Beli Lom and Ovchi kladenets but have not been enlisted (Cheshmedjiev *et al.,* 2010a) (Fig. 1).

Environmental gradients and cyanoprokaryotes distribution

The results of the PCA, run on the environmental variables, total phytoplankton biomass (TBS) and total cyanoprokayotes biomass (TBC) of 61 WBs are shown in Fig. 5. The cumulated relative inertia of the two first principal components reached 51.4 %. The main environmental gradient, associated with the first principal component, is determined by altitude and trophic status, with altitude (alt) positively correlated with Secchi depth (SD), and negatively with TN and TP. Unsurprisingly, TBS and TBC is correlated with the nutrient loading. The second principal component is mainly determined by temperature and depth (Fig. 5). Three groups of WBs are identified in this analysis: group 2 is constituted essentially by 3 closely situated coastal shallow WBs (namely Atanasovsko ezero, Vaya and Mandra), which form the well-known geographical group Bourgaski ezera) and group 3, formed by clear, oligotrophic alpine lakes and one high mountain reservoir (reservoir Beli Iskur, Bezbozhsko ezero 1, Nevenino ezero 1, Gergiysko ezero 1, Vlahinsko ezero 1, Bunderishko ezero 9 and

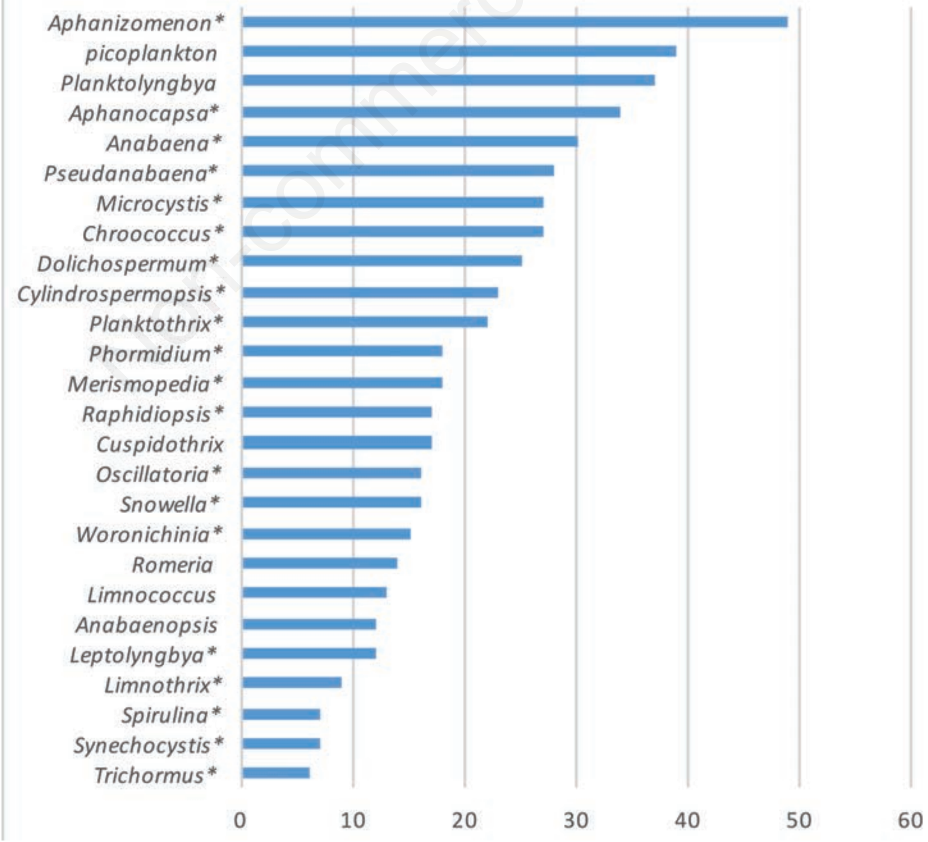

Fig. 3. Distribution of the most widespread genera of cyanoprokaryotes in the phytoplankton of Bulgarian water bodies in the period 2000-2015. 0-40, number of water bodies in which species were found; *genus found in the samples containing cyanotoxins.

Popovo ezero 2). The central group 1 is more heterogenous, comprising mostly mid-altitude WBs with varying depth and nutrient loading, but also some shallow eutrophic lakes (close to group 2, left side of the ordination). The distribution of the WBs in Fig. 5 clearly shows the influence of geographic location on the WBs' characteristics.

The next redundancy analysis (Fig. 6), in which the dependent variables are the biomass data on 24 cyanoprokaryote genera, showed that cyanoprokaryote assemblages responded significantly to the environmental conditions, with TP as the most influential variable, followed by SD, t and TN. Four main groups of genera were identified: two (1 and 2) on the left side of the diagram, associated with eutrophic conditions in low altitude shallow lakes, swamps and reservoirs, one (group 3) of high altitude, clear lakes; and one (4) more heterogeneous group with predominance of high conductivity conditions. Within this last are associated all rare colonial non-toxic species /Rccl/ from genera like *Lemmermanniela, Coelomoron*, *etc*. and *Merismopedia* (mainly *M. tenuissima* Lemmermann) - Mrsp, non-colonial picoplankters of Pcy group – Pcpl) and *Spirulina* and *Glaucospira* species (Spgl) with a single *Phormidium* (Phrm). In group 1 were *Microcystis* - Mcrs; *Anabaena* s.l. (mainly *Dolichospermum*) – Andl, *Aphanizomenon* s.l. – Aphn, *Cylindrospermopsis* (in this case *C. raciborskii* (Wołoszyńska) Seenaya & Subba Raju only) - Clps, *Planktothrix* – Plnt, *Planktolyngbya* s.l. (incl. *Limnolyngbya*) – Plnb, *Romeria* - Rmrr, *Oscillatoria* and *Borzia* - Osbr. Group 2 included *Anabaenopsis* -Anbs, *Pseudanabaena* – Psnb, *Plectonema* – Plct, *Leptolyngbya* – Lptb. Group 3 contains *Aphanocapsa* - Apns*, Chroococcus* - Crcr*, Coelosphaerium* - Clsp*, Snowella* - Snwl, *Woronichinia* - Wrnc) and *Synechocystis* (Sncs).

Cyanotoxins

The analysis of published data on cyanotoxins revealed their presence in 16 WBs – reservoirs Bistritsa, Borovitsa, Enitsa, Kayabash 2, Krushovitsa, Mandra, Pchelina, Studena, Studen kladenets, Trakiets, Vucha, Valchovets and lakes Vaya, Durankulashko ezero, Ezero Momin brod and Shablensko ezero (Tab. 2, Fig. 1). During the summer-autumn period, microcystins LR, LA, RR, YR and similar to YR-type, nodularins, anatoxin-a and saxitoxins (from the decarbamoyl saxitoxin, gonyautotoxins II, III, B1, C1 and C2 group) in different concentrations were proved by High Performance Liguid Chromatography (HPLC, HPLC-DAD and/or HPLC-MS), enzyme-linked immunosorbent assay (ELISA) and *in vitro* cytotests (Tab. 2). Anatoxin was detected only once, in July 2006 in Borovitsa reservoir by HPLC (Teneva *et al.,* 2009). Saxitoxins (STXs) were found in the reservoirs Borovitsa, Studen kladenets, Trakiets and

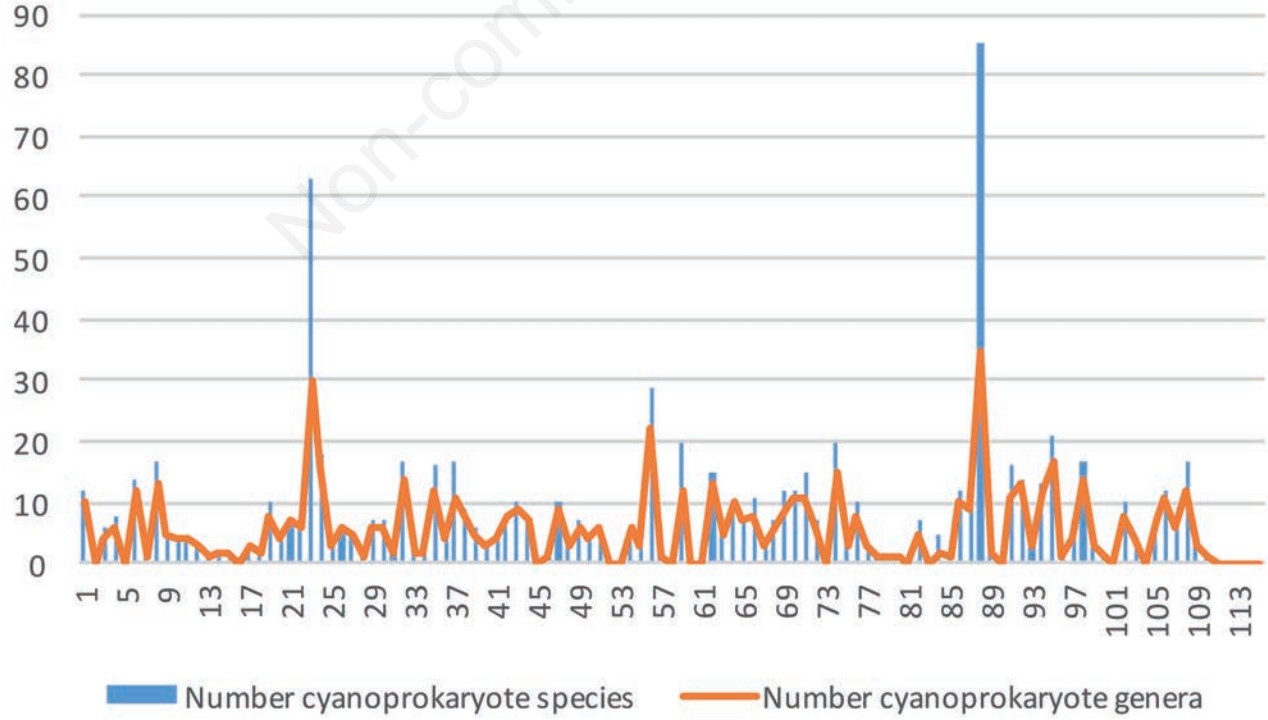

Fig. 4. Distribution of the number of species and genera of cyanoprokaryote phytoplankters in 115 Bulgarian water bodies in the period 2000-2015.

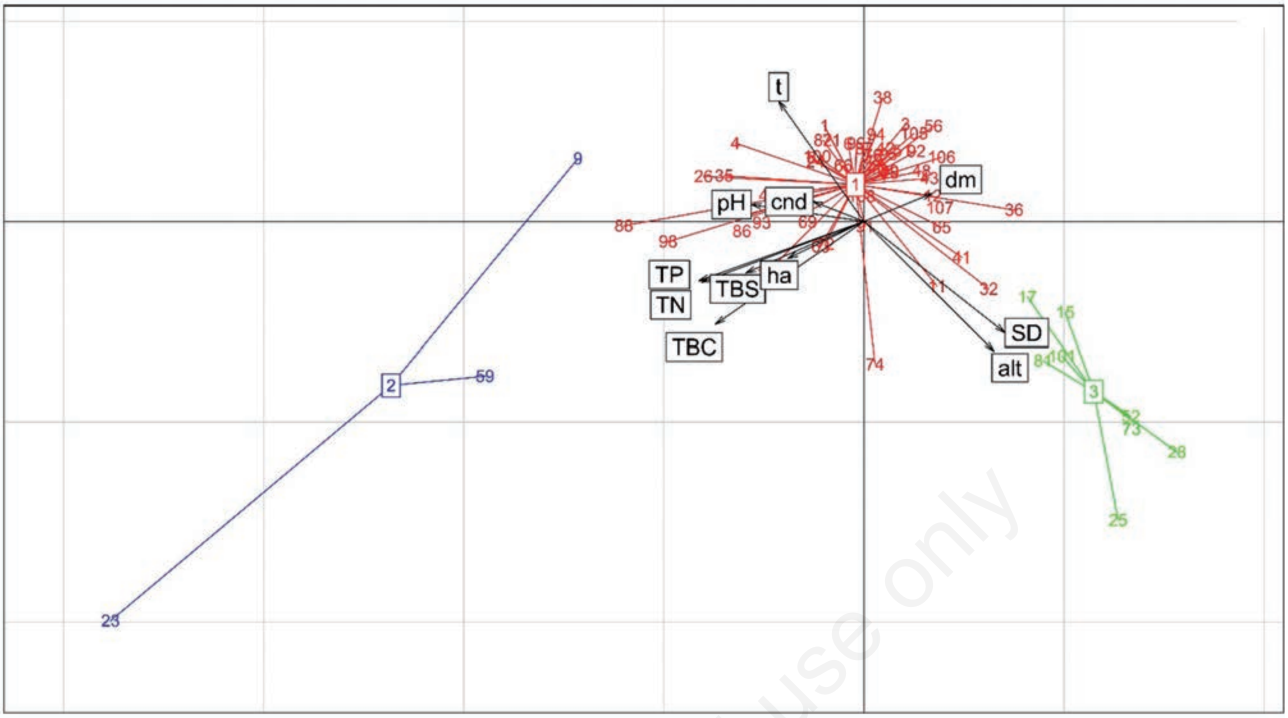

Fig. 5. Results of the principal component analysis on the environmental variables and phytoplankton/cyanoprokaryote biomass of 61 Bulgarian water bodies (WBs); ordination on the two first components (cumulative inertia: 51.4%). Numbers and names of the WBs as in Tab. 1. t, water temperature; cond, electric conductivity; SD, Secchi depth; TP, total phosphorus; TN, total nitrogen.

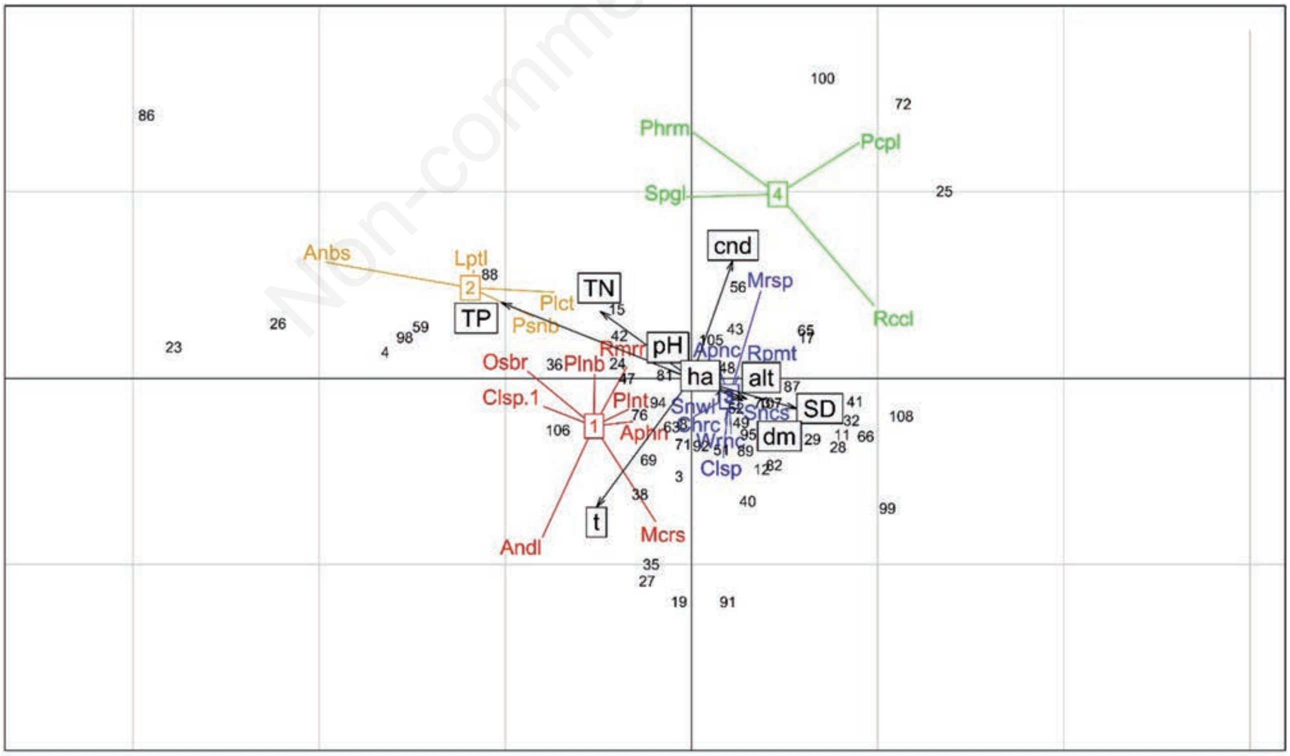

Fig. 6. Results of the redundancy analysis on the environmental variables and cyanoprokaryote genera of 61 Bulgarian water bodies (WBs); ordination on the two first components (cumulative inertia: 45.3%). Numbers and names of the WBs as in Tab. 1. ha, area; alt, altitude; t, water temperature; cond, electric conductivity; SD, Secchi depth; TP, total phosphorus; TN, total nitrogen.

Valchovets by Teneva *et al.* (2009, 2010a, 2011) and Stoyanov *et al.* (2012). Their concentrations varied between 0.1 and 2.5 µg L^{-1}, except in Studen kladenets, where STXs were detected only by HPLC peaks and authors suggest that their concentrations in the water were less than their mean lower detection limit by Ridascreen™ assay – 0.01 µg L^{-1}. In the same reservoir HPLC peaks showed the presence of nodularins, but their amounts were given only as a total of microcystins/nodularins (MC/NOD) detected by ELISA (Teneva *et al*., 2011). In the same way, MC/NOD totals were provided for the reservoirs Borovitsa, Enitsa, Kayabash 2, Krushovitsa, Studen kladenets and Trakiets (Teneva *et al.,* 2009, 2010a, 2011, 2014; Stoyanov *et al.,* 2012). The types of microcystins were indicated in the publications for 10 WBs but only for 9 of them the values have been provided (Tab. 2). According to Pavlova *et al*. (2006, 2007, 2013a, 2014), Pavlova (2007) and Georgieva *et al*. (2015) the concentrations of the different microcystin types ranged as follows: LR – 0.1-8.3 µg L^{-1} in water samples and 14-292 µg g^{-1} in net samples, RR - 0.1-12.7 µg L^{-1} in water samples and 2-103.2 µg g^{-1} in net samples, YR – 0.13-5.5 µg L^{-1} in water samples and 0.8-71.8 µg g^{-1} in net samples. YR equivalent type was detected twice by HPLC-DAD in Shablensko ezero (Pavlova, 2007; Pavlova *et al*., 2007) and LA type was reported once from Borovitsa by Teneva *et al.* (2010b).

Negative results for cyanotoxins have been published by Pavlova (2007), Pavlova *et al.* (2007, 2013a, 2014, 2015), Teneva *et al.* (2014) and Georgieva *et al.* (2015) for 13 WBs and for some samples from 11 WBs with previously detected toxins (Tab. 3).

The distribution of WBs with toxins (16), the WBs with recorded blooms (14), WBs in which toxic species have been found (30) and of the not problematic WBs (54) in the 8 groups of their geographic location and vertical position is shown on Fig. 7.

According to the above cited references, 52 algae and some akinetes have been discovered in the water samples with detected toxins. Among them 45 from 21 genera were identified at species or generic level. The information on the number of their findings in toxic samples, the number of WBs, from which these samples have been collected together with the range of species biomass exactly in these samples, types of toxins, methods used and sources are summarized in Tab. 4. Figs. 2 and 3 illustrate the distribution of these species and genera in Bulgarian WBs during the studied period.

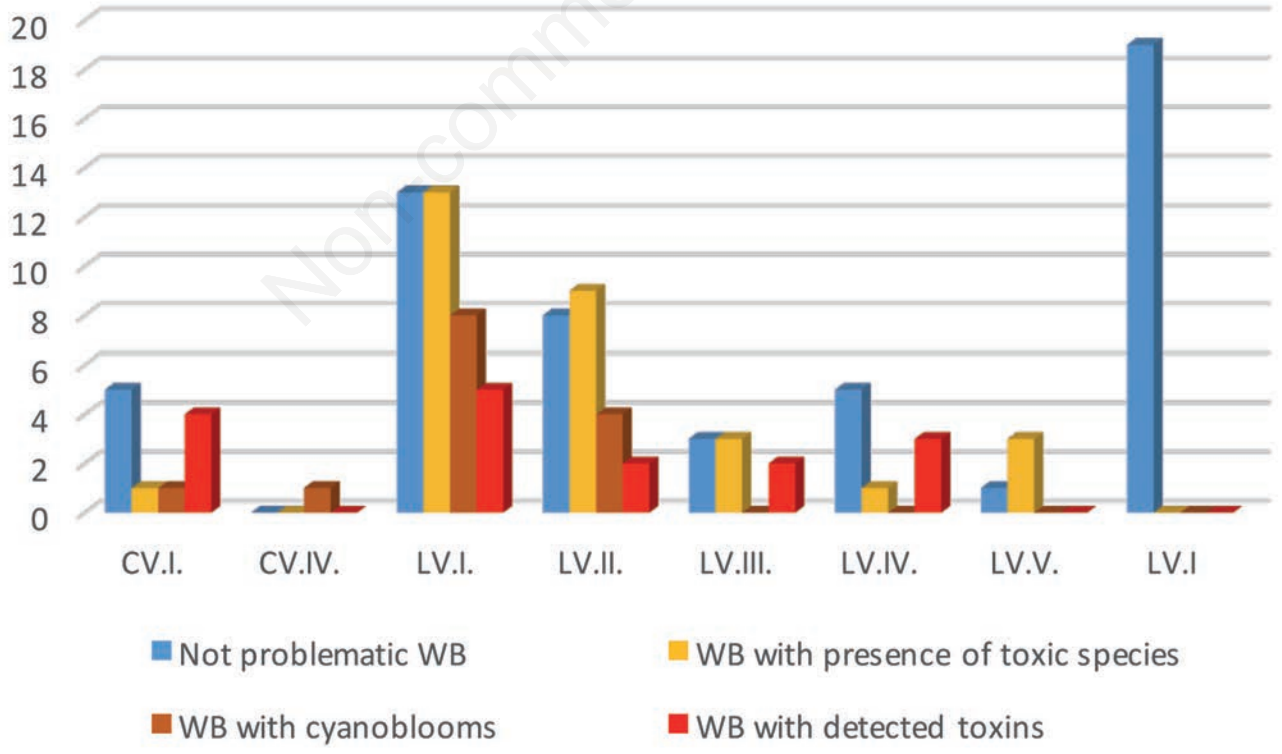

Fig. 7. Distribution of water bodies (WB) with toxins, the WBs with recorded blooms, WBs in which toxic species have been found and of the not problematic WBs in the 8 phyla of their geographic location and vertical position (CV.I-LV.VI). Colours are on conformity with colours on Fig. 1, for the description of CV.I-LV.VI phyla see the text.

Tab. 4. Cyanoprokaryotes found in the samples with detected toxins in Bulgarian WBs.

Taxon	Nf	NWB	Biomass rangee	Microcystins (MC) LR	RR	YR	LA	MCg	Other toxins NOD	ANT-a	STX	Sourc
Microcystis aeruginosa (Kütz.) Kütz.	15	9	x-1.4	x	x	x	x		x		x	Pavlova *et al*. 2006, 2015; Teneva *et al*. 2009, 2010a. 2011. 2014; Stoyanov *et al*. 2012
Aphanizomenon fos-aquae Ralfs ex Born. et Flah.	10	7	x-2.41-Dom	x	x	x			?	x	x	Pavlova *et al*. 2006; Teneva *et al*. 2009, 2010a, b, 2014; Stoyanov *et al*. 2012
Microcystis wesenbergii (Kom.) Kom. In Kondratieva	10	5	0.2-11.97*	x	x	x						Pavlova *et al*. 2006,2014, 2015
Pseudanabaena catenata Lauterb.	6	1	x-0.05	x		x			x		x	Teneva *et al*. 2011
Microcystis flos-aquae (Wittr.) Kirchn.	6	5	0.01-7.2	x	x	x						Pavlova *et al*. 2006, 2015; Stoyanov *et al*. 2012
Synechococcus elongatus (Näg.) Näg.	6	3	0.04-Dom	x	x	x			x		x	Teneva *et al*. 2010a,b, 2011
Dolichospermum affine (Lemm.) Wacklin *et al.*	4	2	x-0.03	x	x	x	x		x		x	Teneva *et al*. 2009; 2011
Dolichospermum scheremetievii (Elenk.) wacklin *et al.*	4	1	0.02-0.09					x	x		x	Teneva *et al*. 2011
Anabaena sp.	4	4	0.003-8.28	x	x	x						Pavlova *et al*. 2006; Teneva *et al*. 2011
Chroococcus minutus (Kütz.) Näg.	4	2	0.001-	x	x	x			x	x	x	Teneva *et al*. 2010b, 2011
Snowella lacustris (Chod.) Kom. et Hind.	4	3	x-0.02	x	x	x		x	?		x	Teneva *et al*. 2009, 2010a, b
Dolichospermum spiroides (Kleb.) Wacklin *et al.*	3	3	x- 65.33	x	x	x		3		x		Teneva *et al*. 2010b, 2014; Stoyanov *et al*. 2012
Merismopedia tenuissima Lemm.	3	2	x-0.02	x	x	x						Pavlova *et al*. 2014. 2015
Microcystis natans Lemm. ex Skuja	3	3	0.05*-1.47*	x	x	x						Pavlova *et al*. 2006
Microcystis sp.	3	2	0.1*-not ind.	x								Pavlova *et al*. 2015
Dolichospermum flos-aguae (Bréb. ex Born. et Flah.) Wacklin *et al.*	2	1	x	x	x	x			?		x	Teneva *et al*. 2010b
Dolichospermum solitarium (Kleb.) Wacklin *et al.*	2	1	0.1-0.18					x				Teneva *et al*. 2011
Trichormus variabilis (Kütz. ex Born. et Flah.) Kom. et Anagn.	2	1	x					x	x		x	Teneva *et al*. 2011
Planktothrix agardhii (Gom.) Anagn. et Kom.	2	2	2.32-10.5					x				Stoyanov *et al*. 2012
Phormidium sp.	2	1	x					x				Teneva *et al*. 2011
Spirulina major Kütz. ex Gom.	2	2	x-0.03	x	x	x						Teneva *et al*. 2011; Pavlova *et al*. 2015
Aphanizomenon sp. juv.	1	1	0.098		x							Pavlova *et al*. 2014
Aphanocapsa delicatissima W. et G.S. West	1	1	0.6	x		x						Pavlova *et al*. 2015
Aphanocapsa grevillei (Berk.) Rabenh.	1	1	0.62*	x								Pavlova *et al*. 2006
Aphanocapsa spp.	1	1	0.004*	x		x						Georgieva *et al*. 2015
Chroococcus aphanocapsoides Skuja	1	1	x	x								Pavlova *et al*. 2015
Chroococcus dispersus (Keissl.) Lemm.	1	1	0.02									Teneva *et al*. 2011
Chroococcus sp.	1	1	0.001	x	x	x						Pavlova *et al*. 2014
Cylindrospermopsis raciborskii (Woł.) Seenaya et Subba Raju	1	1	x								x	Stoyanov *et al*. 2012
Leptolyngbya foveolarum (Rabenh. ex Gom.) Anagn. et Kom.	1	1	0.011	x	x	x						Pavlova *et al*. 2014
Limnoraphis hieronomusii (Lemm.) Kom. *et al.*	1	1	x					x	x			Teneva *et al*. 2011
Limnothrix sp.	1	1	49.93*	x	x	x						Pavlova *et al*. 2006
Merismopedia glauca (Ehr.) Kütz.	1	1	x	x		x						Pavlova *et al*. 2015
Merismopedia hyalina (Ehr.) Kütz.	1	1	0.008	x	x	x						Pavlova *et al*. 2014

To be continued on next page

Tab. 4. Continued from previous page.

Taxon	Nf	NWB	Biomass rangee	Microcystins (MC) LR	RR	YR	LA	MCg	Other toxins NOD	ANT-a	STX	Sourc
Microcystis botrys Teil.	1	1	0.6	x	x	x						Pavlova *et al*. 2015
Microcystis firma (Kütz.) Schmidle	1	1	0.04*									Pavlova *et al*. 2006
Microcystis pulverea (Wood) Forti in De Toni	1	1	dom	x	x	x			?		x	Teneva *et al*. 2010b
Oscillatoria annae Van Goor	1	1	x					x	x			Teneva *et al*. 2011
Oscillatoria sp.	1	1	0.03					x				Teneva *et al*. 2009
Planktothrix compressa (Ütermohl) Anagn. et Kom.	1	1	0.07					x				Teneva *et al*. 2011
Pseudanabaena limnetica (Lemm.) Kom.	1	1	0.1					x				Teneva *et al*. 2011
Pseudanabaena mucicola (Naumann et Hub.-Pest.) Bourr.	1	1	0.118	x	x	x						Pavlova *et al*. 2014
Raphidiopsis mediterranea Skuja	1	1	0.001-0.118	x								Pavlova *et al*. 2014
Woronichinia naegeliana (Ung.) Elenk.	1	1	0.01*	x	x	x						Pavlova *et al*. 2006
Woronichinia sp.	1	1	0.48*	x	x	x						Pavlova *et al*. 2006
Unidentified [solitary cells. akinetes. filaments -2. coccal colonial -1]	9	6	x-22.54*	x	x	x						Pavlova *et al*. 2006, 2014, 2015

Nf, number of findings/samples; NWB, number of WBs in which the species was recorded; biomass range, related to the species in the "toxic" samples, MCg, indicated only as microcystins in the publications (without their exact types); x, presence; Dom, dominant species/dominance; *biomass estimated for this study; not ind., not indicated in the publications.

DISCUSSION

The analysis of data on phytoplankton species composition revealed that, in spite of being quite heterogeneous, they are generally available for 111 from 115 purposively studied WBs included in Tab. 1, except concrete data for the reservoirs Stoychovtsi, Karaisen, Luzhenska Bara and Aleksandrovo. However, the occurrence of cyanoprokaryotes in the last reservoir was documented as "presence of toxic species" (Cheshmedjiev *et al*., 2010a; Fig. 1). In all samples proceeded by us, cyanoprokaryotes were not found in 11 alpine lakes (Bunderishko ezero 9, Musalensko ezero 3, Ezero Bliznaka, Ezero Bubreka, Ezero Okoto, Ezero Sulzata, Kremensko ezero 2, Marichino ezero 2, Marichino ezero 3, Popovo ezero 2 and Vlahinsko ezero 1) and in the occasionally sampled reservoirs Yarlovets and Zhernov. Therefore, we argue that 99 WBs contain different number and amount of cyanoprokaryotes. With the total of 210 taxa found, they represent only 3.8% of the algal biodiversity of the country estimated as ca. 5,500 taxa by Stoyneva (2014), but comprise 36.4% from all 576 cyanoprokaryotes (Stoyneva *et al.,* 2016). Coccal cyanoprokaryotes were the richest group both in species (85) and genera (31), while the number of heterocytous species and genera was the lowest. This result is on general conformity with the shallow and eu- to hypertrophic character of most studied WBs, in which, according to our unpublished results, nitrogen was not a limiting factor (N/P ranged between 7 and 110). Similar conclusion could be made according to the data in the publications by Teneva *et al*., 2010a; Belkinova *et al*., 2014; Pavlova *et al*., 2015; Stoyanov *et al*., 2016, *etc*.

The highest taxonomic diversity, expressed as number of species and genera, was detected in two WBs: Blato Srebarna (85/35) and Vaya (63/30) (Fig. 4). Due to their high importance for conservation of rare and threatened species of national, European and global significance (Michev and Stoyneva, 2007; Vassilev *et al.,* 2013), these two shallow lakes have been intensively studied before and during the analyzed period, and always have been outlined for their rich algal diversity (Michev *et al*., 1998; Stoyneva, 1998a, 1998b, 2003, 2014, 2015; Georgiev, 2012; Dimitrova *et al.,* 2014a, 2014b). However, the same authors outlined the negative trends in their development with enhanced eutrophication, cyanoprokaryote blooms (incl. the rare equilibrium states dominated by cyanoprokaryotes) formed by potentially toxic species. The negative effects of increased eutrophication due to long-term cage fish farming were stressed also for the mountain reservoirs Dospat and Kardzhali, where among the newly appeared group of cyanoprokaryotes the harmful *Aphanizomenon flos-aquae, Dolichospermum spiroides* and *Planktothrix rubescens* (De Candolle ex Gomont) Anagnostidis et Komárek participated in the dominant complexes (Dochin and Stoyneva, 2014, 2015; Dochin 2015).

The analysis of the distribution of species and genera points to 2 species (*Aphanizomenon flos-aquae* and *Cylindrospermopsis raciborskii*) and 11 genera as being the most widespread in the country (*Planktothrix, Cylindrospermopsis, Dolichospermum, Chroococcus, Microcystis, Pseudanabaena, Anabaena, Aphanocapsa, Planktolyngbya* and *Aphanizomenon*; Figs. 2 and 3). Except for *Chroococcus, Aphanocapsa* and *Planktolyngbya* they have been commonly pointed as *principally responsible for forming blooms* (*e.g*. Oliver and Ganf, 2002) mainly due to presence of gas vesicles. Among the total of 69 genera found, at least 29 are known as cyanotoxin producers (Metcalf and Codd, 2012; Pettersson and Pozdnyakov 2013). If the genera *Chrysosporum* and *Sphaerospermopsis,* which relatively recently have been separated from *Anabaena* (Komárek, 2013 and references therein), are considered, this number will increase to 31, or 45% from all found in the country. The frequency of findings of all these potentially toxic genera in combination with the spreading of cyanoprokaryote blooms (Fig. 1) are logically related with the findings of different types and amounts of cyanotoxins in Bulgarian WBs (Tab. 2).

The results of multivariate analysis show the consistency of the WBs classification based on geographic position and altitude (as first steps proposed in the hierarchical classification of Bulgarian wetlands by Michev and Stoyneva, 2007), along with in-lake characteristics as depth and nutrient content. Total phytoplankton biomass, total cyanoprokaryote biomass, as well as cyanoprokaryote assemblages showed a strong response to the environmental variables, with an expected major influence of TP.

The redundancy analysis identified four groups of genera in relation to WBs, among which groups 3 and 4 are more heterogenous in comparison with groups 1 and 2. Group 3 contains realtively small colonial embedded in mucilage coccal genera (*Aphanocapsa, Chroococcus, Coelosphaerium, Snowella*, *Woronichinia*) and non-colonial coccal *Synechocystis* which are classical phytoplankters in various types of WBs. Despite their general ubiquity and abundance in a wide spectrum of trophic conditions, these *non-bloom formers* are not a well-known group, particularly in relation to their ecology (Stockner *et al.,* 2002). In our opinion, the presence of all these small coccal genera in group 3 reflects well the heterogenous character of its WBs in terms of geographic location, morphometry and way of use, but united by the prevalence of meso- to oligotrophic conditions.

Within the group 4 with predominantly high conductivity conditions, characterized by extremely low TBC, are associated diverse and most rarely (in this study) distributed smallest coccal cyanoprokaryotes (all rare colonial non-toxic species, *Merismopedia*, non-colonial picoplankters of Pcy group), very thin filamentous rare *Spirulina* and *Glaucospira* species with a single (and most probably benthic) *Phormidium*. All of them are generally considered as *non-blooming* cyanoprokaryotes (Stockner *et al*., 2002). By contrast, most genera well known for their ability to form water blooms due to presence of gas vesicles (Oliver and Ganf, 2002) were in group 1: *Microcystis*; *Anabaena* s.l. (mainly *Dolichospermum*), *Aphanizomenon* s.l., *Cylindrospermopsis* and *Planktothrix*. The other genera in group 1 are filamentous without gas vesicles: classical planktonic thin *Planktolyngbya* s.l., generally short-celled fine *Romeria* and occasionally distributed in this data set short-celled *Oscillatoria* and *Borzia*. As it could be seen, besides the very rare and therefore non-representative Osbr, the "exception" from this *filamentous group 1* is the coccal colonial *Microcystis*. However, the "filamentous" character of another participant in group 1 - *Romeria* - is still not proved and its coccal nature is in discussion (Komárek and Anagnostidis, 2005). Common feature of the *strangers* (*Microcystis, Planktolyngbya* and *Romeria*) in group 1 is the presence of well-developed homogenous colourless mucilage. The irregular presence of gas vesicles in combination with wide, but fine mucilage envelopes is known also for *Anabaenopsis* and *Pseudanabaena*, which are representatives of group 2. The group includes also the thin (1-2 μm) filamentous rarely branched *Plectonema* and straight filamentous *Leptolyngbya* representatives, most of which have not been determined at species level due to their small dimensions and problematic taxonomy (Komárek and Anagnostidis, 2005). The *stranger* in the group is the heterocytous and capable of bloom-forming *Anabaenopsis*. However, a capability for nitrogen fixation was proved for some *Leptolyngbya* species also (*e.g*. Stal, 2012). According to our recent knowledge, very few data confirm the toxic abilities of the genera in group 2 (except for *Anabaenopsis milleri* Voronichin, which was not found in our studies), which comprises coastal and inland lowland lakes and reservoirs with generally hypertrophic character. By contrast, the genera from group 1, and its core representatives (*Aphanizomenon, Cylindrospermopsis, Microcystis* and *Planktothrix*) in particular were repeatedly pointed as bloom-forming and toxin producing genera related mainly with eutrophic conditions and were also the key players in toxic samples, detected in Bulgarian waters (Fig. 3; Tab. 2). They were found among the genera with broadest distribution in the country (Fig. 3). The common traits of the taxa belonging to this group are the affinity for eutrophic conditions and the presence of gas vesicles, which provide buoyancy control of filament and colonies, allowing efficient vertical migration in the water column of relatively shallow lakes which present alternation of periods of stratification and mixing (Reynolds, 2006). Accordingly, the WBs of group 1 are relatively small, inland lowland to low-mountain, eu- to hypertrophic reservoirs.

The samples with detected cyanotoxins were from 16

WBs (Tab. 2) and contained 44 cyanoprokaryotes identified to species or genus level (Tab. 4). Their distribution in Bulgaria, with a few exceptions, is relatively broad (Figs. 2 and 3). The results from this study confirm the trend for their fast spread in the country outlined especially in relation to the invasive species like *Cylindrospermopsis raciborskii* (Stoyneva, 2015; Kokocinski *et al*., in press). Most of these 44 species and 21 genera have been repeatedly reported as real or potential toxin producers but for some of them discussions are still running (*e.g.* for *Microcystis* species as summarized in Šejnohová and Maršálek, 2012). It is out of scope of this study to go deep in the contradictory opinions, which have to be interpreted with caution, moreover for details in relation with findings in Bulgaria we can refer to our previous papers (Pavlova *et al*., 2006, 2014, 2015). Summarizing the results from this study, we could outline that exactly one of the most often discussed species - *M. wesenbergii* - was the most often recorded species in toxic samples (Tab. 4). In the same time, *Pseudanabaena mucicola* (Naumann et Huber-Pestalozzi) Bourrelly*,* which is a relatively regular endophyte in *M. wesenbergii*, was reported only once as associated with cyanotoxin detection. Some of the species in Tab. 4 are identified only at genus level, or are known for problematic identification, and therefore discussion on them is not relevant without proper documentation, which is not available in the published papers. It has to be noted also, that other cyanoprokaryotes, known for their potential to produce toxins, were found in the country. For example, *Nodularia spumigena* Mertens ex Bornet ex Flahault was reported from three temporary WBs on Belene island in The Danube (Beshkova and Botev, 2004), but the study was not supplied with toxin analysis. Obviously, aimed studies on toxin groups other than microcystins, combined with permanent toxin monitoring will reveal more objective picture of presence/absence of different cyanotoxins and species related with them. A co-occurrence of other algae could also have an effect, and even provide a source, for some of the toxins detected (*e.g.* pyrrhophytes for saxitoxins). Since long time it is well-known that cyanoprokaryote dominance (and blooms in particular) is strongly affected by complex interactions between lake morphometry, water temperature, underwater light availability, nutrient supply and total food-web structure and can not be viewed independently from all other members of phytoplankton (Harris, 1986; Reynolds, 1987, 2006; Dokulil and Teubner, 2000 among the many others). Therefore, we would like to outline the necessity for providing data in future publications on the total species composition for the samples and WBs with detected cyanotoxins or harmful blooms.

In the temperate country like Bulgaria it is not surprising that cyanotoxins were detected in the water samples from summer-autumn periods, when the abundance of cyanoprokaryotes is normally the highest for the year. However, the comparison of Tabs. 2 and 3 clearly shows that occurrence of toxins is intermittent in Bulgarian WBs. Conclusions about the correlations between finding of cyanotoxins and relevant species or total sample biomass are hardly possible due to lack of concrete published data for all cases. The only statement which could be done is that cyanotoxins were detected within a broad range of algal abundance (Tabs. 2 and 4). In some cases, *Dolichospermum spiroides* (Klebahn) Wacklin *et al*.*, Limnothrix* sp., *Microcystis wesenbergii, Planktothrix agardhii* (Gomont) Anagnostidis et Komárek*, Anabaena* sp. and some unidentified algae had high biomass values in samples with detected toxins (Tab. 4). But toxins were detected even when species were found in low, even negligible, concentrations (the cases of Borovitsa, Valchovets, Studena, *etc*. in Tab. 2). The topic was discussed by Pavlova *et al.* (2006) and Teneva *et al.* (2009), who supposed that such cyanotoxin quantities were due to blooms, which occurred in the WBs before the sampling. Therefore, it is necessary to outline again that finding of cyanotoxins in waters is not always related with obvious blooms. Moreover, the term *bloom* and its lower borders remained poorly defined (Oliver and Ganf, 2002). However, the result obtained is important for rising the public awareness of the cyanoblooms, which could have long consecutive effect on the ecosystem health and humans.

In spite of heterogenous way of representing the results on cyanotoxin amounts, it could be stated that in most cases, and in the drinking-water reservoirs in particular, the concentration of microcystin LR is lower than WHO's (1998) limit of 1 $\mu g\ L^{-1}$ (Tab. 2). The general amount and types of microcystins and other cyanotoxins are on conformity with the results published for the closest neighboring countries Macedonia, Serbia, Romania, Turkey and Greece, as it was outlined by Pavlova *et al*. (2015). However, the fact of their findings in 16 WBs (among which 3 are important drinking-water reservoirs, 2 are water supply reservoirs, 3 are used for irrigation and 8 are recreational sites for sports and fishing) on the background of more recorded cyanoprokaryote blooms and broad spread of toxic species in Bulgaria (Figs. 1, 2 and 3) is strong enough to alarm both scientists and responsible authorities at national level. Moreover, the analyses of all data clearly show that during the last 15 years cyanoprokaryotes were not restricted in distribution in the lowlands and plains but started to *invade* WBs with higher altitudes, *i.e.* WBs situated in kettles and low mountains (Fig. 7). The outlining of the necessity of stronger recognition of the problem in the country and rising of the public awareness made here is not the first, just the opposite – almost each of the papers on the topic published in the analyzed period had this statement as a main conclusion since there is no doubt that the peculiar and otherwise fascinating group of cyanoprokaryotes is a real hazardous factor for human and aquatic ecosystem health in Bulgaria.

CONCLUSIONS

All results from studies carried in summer- autumn periods of the last 15 years (2000-2015) in 120 Bulgarian WBs different in location, morphometry and trophic status (incl. drinking-water reservoirs, reservoirs for water supply, irrigation and energy production reservoirs, recreational lakes and sites of nature conservation importance), gathered from 35 publications, showed that in 30 of them toxic species were found. Cyanoprokaryote blooms were recorded in 14 WBs and in 16 cyanotoxins (microcystins, nodularins and saxitoxins) were detected, and cyanoprokaryote diversity was quite high (210 taxa of 60 genera). Toxin concentration ranged between 0.1 and 26.5 μg L^{-1} in water samples and between 10.9 and 1070 μg g^{-1} (d.w.) in concentrated (net) samples. Despite the fact that microcystins were not found in all studied WBs and that the recorded levels were still lower in comparison with some other European countries and with WHO's threshold for microcystin LR, the fact of cyanotoxin detection in 3 drinking-water reservoirs and cyanoprokaryote occurrence in low mountain WBs could serve as an alert for the need of recognition of cyanotoxins as a new health risk factor in the country. Therefore, permanent monitoring with identification of toxins in WBs at risk and activities for limitation and control of toxic blooms are urgently needed, in combination with increase of the attention to the effects of cyanotoxins on both human health and health of aquatic ecosystems in Bulgaria. We also stress the need for a more comprehensive monitoring of the problematic WBs and of their watershed – including all key environmental variables (hydrology, nutrient loading and meteorology) and detailed phytoplankton surveys in order to improve water quality management and identify the measures to be taken to reduce the risks associated with cyanoprokaryote blooms.

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