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Diversity of the Summer Phytoplankton of 43 Waterbodies in Bulgaria and Its Potential for Water Quality Assessment

Maya P. Stoyneva-Gärtner [1], Jean-Pierre Descy [2], Blagoy A. Uzunov [1,*], Peter Miladinov [3], Katerina Stefanova [4], Mariana Radkova [4] and Georg Gärtner [5]

[1] Department of Botany, Faculty of Biology, Sofia University, 8 Blvd. Dragan Zankov, 1164 Sofia, Bulgaria
[2] Unité d'Océanographie Chimique, Université de Liège, Sart Tilman, 4000 Liège, Belgium
[3] Department of Library and Information Studies, Faculty of Philosophy, Sofia University, 125 Blvd. Tzarigradsko Shousse, 1113 Sofia, Bulgaria
[4] AgroBioInstitute, Bulgarian Agricultural Academy, 8 Blvd. Dragan Zankov, 1164 Sofia, Bulgaria
[5] Institut für Botanik, Universität Innsbruck, Sternwartestrasse 15, 6020 Innsbruck, Austria
* Correspondence: buzunov@uni-sofia.bg

Abstract: The general awareness of the threats on biodiversity and water quality raised the number of studies that use phytoplankton in assessment procedures. Since most metrics require obtaining mean values, this paper presents data that may help speed up field work and find indicators for a rapid water quality assessment based on single samplings, allowing simultaneous work on many sites. The phytoplankton from 43 Bulgarian waterbodies collected during three summer campaigns (2018, 2019, 2021) at sites selected after drone observations was studied by conventional light microscopy (LM) and an HPLC analysis of marker pigments. Our results allowed us to recommend drones and the HPLC application as reliable methods in rapid water quality assessments. In total, 787 algae from seven phyla (53 alien, new for Bulgaria) were identified. Chlorophyta was the taxonomically richest group, but Cyanoprokaryota dominated the biomass in most sites. New PCR data obtained on anatoxin and microcystin producers confirmed the genetic diversity of *Cuspidothrix* and *Microcystis* and provided three new species for the country's toxic species, first identified by LM. A statistical analysis revealed significant correlations of certain algal phyla and classes with different environmental variables, and their species are considered promising for future search of bioindicators. This is especially valid for the class Eustigmatophyceae, which, as of yet, has been almost neglected in water assessment procedures and indices.

Keywords: algal blooms; anatoxin; bioindication; *Cuspidothrix*; cyanobacteria; cyanoprokaryotes; drone; Eustigmatophyceae; green algae; HPLC; microcystin; *Microcystis*

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1. Introduction

Since mankind has existed, water has been one of the most important and precious resources of our planet. It is commonly recognized that the lifestyle, agriculture and industry of the modern society, experienced during the last century, led to climate changes and nutrient enrichment of waters, which, in turn, caused a considerable impact on the aquatic habitats. These changes provoked the interest of the scientific community with an increasing intensity of studies on all characteristics of water regarding its use, united by the term "water quality", and its assessment and management [1–5]. Since the end of the 19th century, they have been related with the inhabitants of aquatic biotopes and their potential role in bioindication (for historical details see [6]). Although today, different approaches serve to assess water quality, the use of primary producers with a short life cycle, such as phytoplankters, has a long and worldwide-known tradition [6–11]. The methodological tools applied involve certain indicator species or different functional groups, but also the total composition and indices based on diversity, sensitive to the number of species or to their quantitative role [6–23]. Over the years, the research has also focused on so-called algal

blooms, toxic compounds and their producers with increasing number of records [4,24,25]. Phytoplankton, with its different characteristics, has been used in the methods aiming at the assessment of the ecological status in the European Union's Water Framework Directive (WFD) [26]. However, most of the proposed metrics require obtaining the mean values from more than one sampling per year or season [27], which is not applicable in single, "snapshot" samplings, when numerous waterbodies are investigated in a short time.

At the same time, since the end of the 20th century, there has been an increasing general awareness of the importance of biodiversity and its threats [28,29], which intermingles with different problems related with water quality. Numerous comparative studies have revealed a significant among-lake variation, that has not completely been explainable by available environmental data. This may suggest the influence of unmeasured drivers of the phytoplankton community and the recognition of the fact that current phytoplankton structure in a certain waterbody represents a biological response to previous environmental conditions [30–35]. Moreover, waterbodies normally contain a bulk of rare species, which keep such ecological memory and can become dominants in changed conditions [36]. Considering all these aspects, the comparison of phytoplankton data from different geographic regions and the types of waterbodies with taxonomically well-defined taxa can possibly lead to novel successful combinations of tools based on phytoplankton in order to outline reliable indicator species in rapid but effective methods for water quality assessment even in cases of single samplings.

The present study provides new data on the summer phytoplankton in 43 standing waterbodies of Bulgaria, a country with more than 10,000 wetlands, most of which are still poorly studied [37]. The work was done within the framework of three complementary projects, oriented towards harmful algal blooms of cyanoprokaryotes/cyanobacteria, which produce different toxic compounds (cyanotoxins) in relation to public health and national security in the country. Some data have been published and demonstrated the broad distribution of different cyanotoxins and their producers (i.e., microcystins, anatoxins, saxitoxins, cylindrospermopsin, microviridins) at the studied sites [38–44]. In the present paper, they are completed with new data on anatoxin and microcystin producers in the country. The simultaneous application and comparison of the results from the conventional light microscopic (LM) work and HPLC marker pigment analysis demonstrate the similarity in the results obtained on the relative algal contribution to the phytoplankton biomass [38,40–42]. These allow us to encourage a broader application of HPLC in the methodology of a fast water quality assessment in order to avoid the time- and effort-consuming counting of the phytoplankton by relevant experts. In addition, concerning the improvement of the sampling methodology, it has to be noted that all the results we obtained over these three years prove the usefulness of its application in the studies of biodiversity and water quality assessment with modern remote vehicles, drones. They ensure a fast orientation for the selection of sampling sites, which allows us to save time and efforts but also fuel for vehicles (cars, boats) during field studies [38,45]. Our results demonstrate the great biodiversity of the phytoplankton in all waterbodies but also its variability from site to site, with more than half of the species found in a single waterbody. This great diversity, on one hand, shows the phytoplankton sensitivity to water quality, but on the other hand, it hinders the consideration of certain indicator species for its rapid assessment. Therefore, we provide statistical data that demonstrate a more specific distribution of three phyla and four classes according to the environmental variables such as altitude, water conductivity, water hardness, and chlorophyll *a* concentration as a robust measurement for the trophic status [46,47], which can serve as a grounded basis for future work for bioindicator selection. Eustigmatophyceae is considered one such promising group, almost neglected in accepted phytoplankton metrics.

2. Materials and Methods

2.1. Sampling Sites

The study is based on phytoplankton samples from 43 selected waterbodies in Bulgaria (35 inland and 8 coastal) collected during three summer sampling campaigns in June 2018, August 2019 and August 2021 (Table 1). Detailed descriptions of the type, morphology, hydrology, history of development, physicochemical parameters, biota, use, conservation status and protection measures with references to previous studies and publications are available in the Database of the Inventory of Bulgarian wetlands, IBW [37]. Therefore, the identification numbers of the studied waterbodies are provided in Table 1. In order to help the reader, here, we provide some important notes: (i) three general categories of surface waterbodies were studied such as natural lakes, large reservoirs (>100 ha) and small reservoirs (<100 ha), the latter quite widespread and commonly known in Bulgaria as microreservoirs; (ii) in addition to the core group of coastal lakes and reservoirs, which have been studied for years due to their global conservational importance [37,48,49], a set of 20 small reservoirs of local importance, used mainly for irrigation and as fishponds, was sampled for the first time; (iii) the sampled waterbodies were situated from the sea level up to 1550 m a.s.l. and were selected in accordance mainly to their use by people (for drinking water, irrigation, fishing and fish-farming, recreation) and potential threat from harmful algal blooms; (iv) the sampling in the summer of 2020 was impossible due to the restrictions caused by the COVID-19 pandemic; (v) different months for sampling were chosen because of the different meteorological conditions in the years 2018, 2019 and 2021, with extremely high temperatures and dryness in April–May 2018 and strong rains in May–July 2019 [40].

Table 1. Sampling sites in Bulgarian waterbodies and their environmental parameters during summer sampling campaigns in 2018, 2019 and 2021. Legend: WBN—name of the waterbody, IBW—identification number in the Inventory of Bulgarian Wetlands [37], Abbr—abbreviation of the name, Type—type of waterbody: M (small reservoir/"microreservoir", <100 ha), R (large reservoir, >100 ha) and L (natural lake), Alt—altitude above the sea level (m), WT—water temperature (°C), CN—conductivity (S m^{-1}), TDS—total dissolved solids (µg L^{-1}), DO—oxygen concentration (mg L^{-1}), TP—total phosphorus (µg L^{-1}), TN—total nitrogen (mg L^{-1}). Waterbodies are presented according to their geographical location in counterclockwise order, starting from South-Western Bulgaria. Asterisks indicate the waterbodies which were sampled for the first time.

	WBN and IBW	Abbr	Type	Year	Alt	Latitude	Longitude	WT	pH	CN	TDS	DO	TP	TN
1	* Hadzhidimovo	Hdz	M	2021	156	41°29.8933	23°50.1890	29.1	9.5	300	192	17.00	0.1	0.1
2	* Dubnitsa (IBW3698)	Dbn	M	2021	600	41°33.8500	23°50.7500	25.2	9.6	246	159	9.21	0.1	0.1
3	* Ablanitsa (IBW6013)	Abl	M	2021	682	41°32.8594	23°55.5869	27.2	8.8	242	157	8.54	1.0	0.5
4	* Satovcha 2 (IBW1197)	Stv	M	2021	1017	41°36.8222	23°58.1446	27.4	8.70	272	176	9.00	0.5	0.1
5	Dospat (IBW3155)	Dsp	R	2021	1214	41°39.1495	24°89.5596	25.9	9.9	81	52	8.73	0.1	0.5
				2021	1212	41°39.1493	24°89.5918	25.6	9.5	83	52	8.70	0.3	0.5
6	Golyam Beglik (IBW1314)	GBg	R	2021	1540	41°48.8927	24°07.8725	22.0	9.1	99	63	8.92	1.5	1.0
7	Shiroka Polyana (IBW3144)	SPl	R	2021	1550	41°46.1776	24°08.8201	25.3	8.9	66	42	8.70	0.5	0.5
8	Beglika (IBW3141)	Bgl	M	2021	1535	41°49.7963	24°07.8196	21.7	9.1	242	157	9.11	1.0	0.8
9	* Chetiridesette Izvora (IBW1523)	CIz	M	2021	246	42°00.5510	24°56.2819	28.7	7.5	402	263	8.66	1.0	0.5
10	* Mechka (IBW1584)	Mck	M	2021	319	41°55.8970	25°06.1595	27.1	9.0	241	154	8.50	1.5	1.0
11	* Byalata Prust-Mezek	BPM	M	2021	167	41°45.1080	26°05.2403	29.7	8.5	291	188	9.37	2.0	1.0
12	* Birgo (Shtit)	Brg	M	2021	215	41°49.7743	26°22.1889	27.3	8.0	594	385	8.75	1.5	1.8

Table 1. *Cont.*

	WBN and IBW	Abbr	Type	Year	Alt	Latitude	Longitude	WT	pH	CN	TDS	DO	TP	TN
13	* Studena (Fishera)				282	41°54.2136						1		
	(IBW2421)	Std	M	2021			26°24.5964	29.3	9.0	652	423	3.35	1.0	0.3
14	* Mogila (Kaynaka)				166	42°29.8310								
	(IBW2626)	Mgl	M	2021			26°36.1043	29.2	9.5	682	442	15.75	4.0	1.0
15	* Hadzhi Yani (Lozenets)				12	42°12.0333								
	(IBW2893)	HYn	M	2021			27°47.3000	26.1	7.5	751	488	8.42	1.5	0.8
16	Mandra (IBW1720)	Mnd	R	2018	12	42°24.0643′	27°26.1120′	25.9	8.3	649	421	6.81	3.0	3.0
				2018		42°24.0670′	27°19.1310′	26.2	8.2	663	461	5.89	6.0	4.0
				2018		42°26.1420′	27°26.5860′	24.9	8.5	639	415	7.91	4.0	3.3
				2019		42°24.0295′	27°19.1194′	25.88	7.9	676	436	7.93	0.7	0.5
				2019		42°25.9303′	27°26.7652′	27.2	8.5	578	375	7.87	1.5	1.8
				2021		42°24.2370	27°19.1205′	27.3	9.0	513	333	9.32	7.0	4.0
				2021		42°25.9282	27°26.7675′	27.3	9.0	513	333	10.70	7.5	4.0
17	Uzungeren (IBW0710)	Uzn	L	2018	7	42°26.1782′	27°27.1998′	25.9	8.1	1458	9351	7.83	5.0	2.8
				2019		42°26.1551′	27°27.2235′	27.6	8.5	1748	1132	9.70	0.4	0.3
				2021		42°26.1532′	27°27.2214′	28.1	9.0	18520	12000	11.21	5.5	4.0
18	Burgasko Ezero (Vaya) (IBW0191)	BEz	L	2018	0	42°30.5940′	27°22.0750′	26.9	9.7	2588	1682	12.51	13	5.4
				2018		42°28.4540′	27°25.4820′	28.28	8.9	1183	768	11.94	11	3.7
				2018		42°29.1850′	27°26.5310′	23.7	9.5	1024	665	7.01	12	4.6
				2019		42°30.5940′	27°22.0750′	27.9	9.2	490	170	7.69	0.5	0.3
				2021		42°30.7934′	27°24.2425′	26.6	9.0	4421	2873	1.26	12	5.3.
19	Poroy (IBW3038)	Por	M	2018	41	42°43.0190′	27°37.3160′	25.10	8.3	762	495	9.45	1.0	2.8
				2019		42°43.3403′	27°37.5255′	27.5	8.1	644	416	7.60	0.1	0.3
				2021		42°43.4683′	27°36.8757′	26.1	9.0	792	514	11.68	2.1	1.5
20	Aheloy (IBW3032)	Ahl	M	2018	144	42°42.8230′	27°30.9740′	25.4	8.5	614	399	8.92	1	4.1
21	* Yunets	Ynt	M	2021	79	42°55.6700′	27°45.3074′	27.4	8.5	965	765	11.00	2.5	1.8
22	Tsonevo (IBW3022)	Tsn	R	2019	75	43°01.8055′	27°24.3965′	24.8	8.8	355	231	8.20	0.1	0.1
				2021		43°01.8278′	27°24.3954′	26.6	8.0	417	272	10.65	0.1	0.1
23	Eleshnitsa (IBW3023)	Els	M	2019	44	43°00.3344′	27°28.0744′	26.7	8.4	532	347	6.78	0.1	0.3
24	Ezerets (IBW0233)	Ezr	L	2018	0	43°35.2770′	28°33.2290′	26.4	8.4	1084	10	9.94	0.5	5.3
				2019	6	43°35.2681′	28°33.2096′	25.9	8.6	1669	1739	8.58	0.1	0.1
25	Shabla (IBW0219)	Shb	L	2018	0	43°33.8180′	28°34.1860′	27.1	8.5	1087	706	9.97	0.1	5.1
				2019		43°33.8212′	28°34.8204′	25.9	8.7	1842	1196	9.64	0.1	1.0
26	Durankulak (IBW0216)	Drn	L	2018	4	43°40.3240′	28°32.0470′	24.03	8.5	1111	722	7.35	21	2.8
				2018		43°40.3340′	28°32.0220′	24.7	8.2	1094	711	7.79	20	4.0
				2018		43°40.5300′	28°32.9930′	24.6	8.5	1075	698	6.19	24	3.9
				2018		43°40.6950′	28°32.6000′	26.5	8.5	1087	706	9.60	20	3.2
				2019		43°40.0006′	29°32.6166′	26.5	8.9	974	631	7.86	0.3	0.7
				2019		43°40.5355′	28°33.0806′	26.7	8.9	1048	680	6.04	0.3	0.6
				2021		43°40.6935′	28°32.6000′	25.5	9.0	2960	736	10.70	14	4.5
				2021		43°40.5300′	28°33.0826′	25.5	9.0	3008	1952	7.40	11	2.0

Table 1. *Cont.*

	WBN and IBW	Abbr	Type	Year	Alt	Latitude	Longitude	WT	pH	CN	TDS	DO	TP	TN
27	* Plachidol 2 (IBW5073)	Plc	M	2019	220	43°33.3504′	27°52.6338′	24.6	9.0	1225	793	9.13	0.2	0.4
28	* Malka Smolnitsa (IBW3107)	MSm	M	2019	211	43°36.2606′	27°44.5367′	25.2	9.1	755	490	7.05	0.6	0.6
29	* Preselka (IBW3078)	Prs	M	2019	281	43°25.3767′	27°16.6214′	24.1	9.0	138	282	10.05	0.6	2.8
30	* Izvornik 2 (IBW3082)	Izv	M	2019	255	43°27.3838′	27°21.1110′	24.5	9.4	389	253	13.26	9.0	4.8
31	* Fisek (IBW2674)	Fsk	M	2019	182	43°18.8453′	26°44. 3765′	27.2	8.7	690	397	7.52	0.2	0.1
32	* Shumensko Ezero (IBW2754)	SEz	M	2019	152	43°14.8140′	26°57.5675′	25.2	8.5	471	445	6.32	0.2	0.5
33	* Kriva Reka (IBW3071)	KRk	M	2019	133	43°22.6573′	27°10.9807′	23.7	8.4	662	428	6.24	1.0	9.0
34	Suedinenie (IBW2642)	Sdn	R	2019	133	43°20.0734′	26°33.6368′	28.1	7.6	739	481	6.77	0.1	0.3
35	* Nikolovo (IBW3176)	Nkl	M	2021	89	43°50.9768	26°05.1796	26.0	9.8	2156	1400	11.88	11	2.0
36	Shilkovtsi (Iovkovtsi) (IBW2105)	Shl	R	2019	410	42°55.2320′	25°47.6743′	27.2	8.9	746	479	7.48	0.03	0.1
37	Koprinka (IBW2062)	Kpr	R	2019	450	42°37.0172′	25°19.4795′	27.2	8.2	250	163	7.21	0.1	0.2
38	Zhrebchevo (IBW2545)	Zhr	R	2019	253	42°36.6024′	25°51.2345′	27.6	7.7	358	233	8.01	0.1	0.2
39	Al. Stamboliyski (IBW2056)	ASt	R	2019	190	43°07.0000′	25°07.3936′	29.4	8.9	670	433	9.82	1.4	3.5
40	Krapets (IBW2000)	Krp	M	2019	410	43°04.0316′	24°52.3835′	28.7	8.3	870	564	7.74	0.1	1.0
41	Sopot (IBW1437)	Spt	R	2019	376	40°00.7017′	24°52.6045′	29.0	8.3	779	490	3.44	0.1	0.1
42	* Duvanli (IBW1483)	Dvn	M	2019	250	42°23.1851′	24°43.1000′	26.3	8.8	4050	291	7.09	0.1	0.3
43	Sinyata Reka (IBW1890)	SRk	M	2018	317	42°28.1480′	24°42.2170	27.4	9.7	470	305	9.36	25	4.8
				2018		42°28.1473′	24°42.2175	26.7	9.4	468	306	9.21	27	4.3
				2019		42°28.1518′	24°42.0159′	28.2	10.4	490	317	14.76	1.0	0.2

The sampling was preceded by a drone sent to observe in real time the whole water area of each waterbody (Figure 1) and to identify the sites with algal blooms [38–45]. In cases of visible water homogeneity, the sites from our previous studies were repeated, or new sites were selected in cases of waterbodies sampled for the first time. Two types of drones (each supplied by a photo camera) were used: DJI Mavic Pro, Model: M1P GL200A (SZ DJI Technology Co., LTD, Shenzhen, China) in 2018 and DJI Mavic 2 Enterprise Dual Pro (DJI Technology Co, LTD, Shenzhen, China) in 2019, 2021 because the latter had the ability to measure the surface water temperature [38–45].

The sampling was conducted from inflatable boats and by motorboats in the large reservoirs. Aquameter AM-200 and Aquaprobe AP-2000 from Aquaread's water-monitoring instruments, 2012 Aquaread Ltd., were applied for in situ measurement of the coordinates and the altitude of each site, as well as the water temperature, pH, water hardness (expressed by total dissolved solids), oxygen concentration, chlorophyll *a* and conductivity (Table 1). Total nitrogen (TN) and total phosphorus (TP) were measured ex situ with an Aqualytic AL410 photometer from AQUALYTIC®, Dortmund, Germany—Table 1.

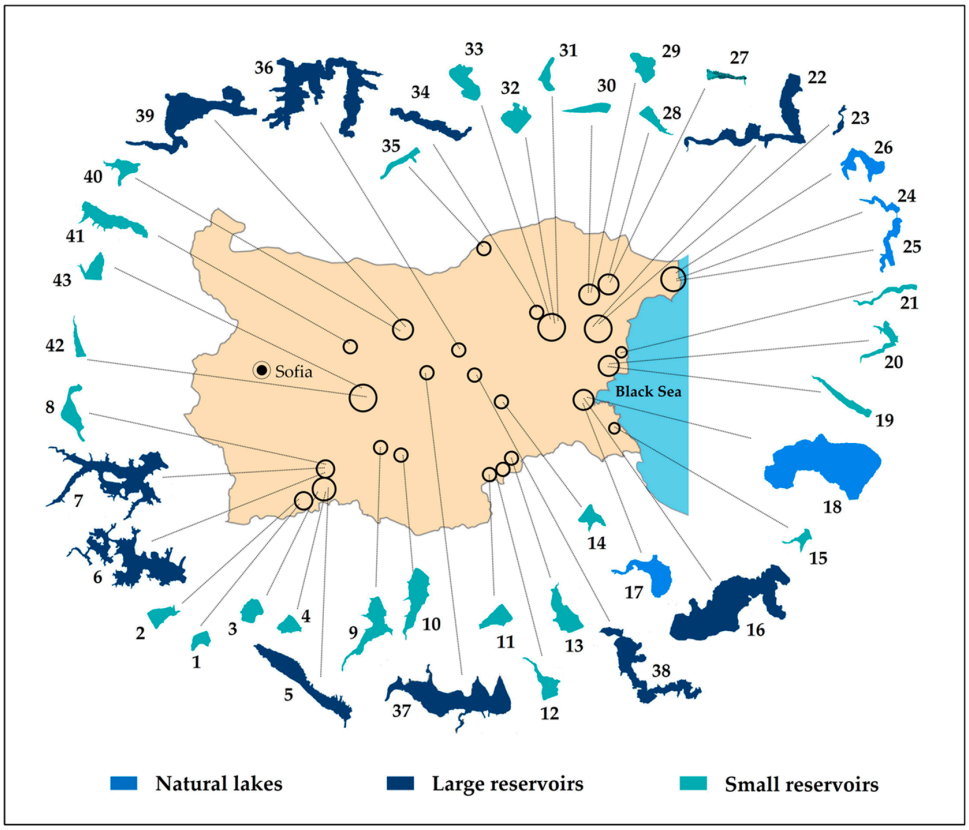


Figure 1. Map of Bulgaria (modified after [50,51]) with locations of the studied waterbodies and indication of their type. The waterbodies are represented by numbers that follow those in Table 1.

2.2. Algal Identification and Counting by Light Microscopy

At each site, a water sample was collected for algal determination and counting by light microscopy (LM). The samples were taken from the surface layer (0–50 cm) in a volume of 0.5 L in case of visible blooms and of 1–1.5 L in cases of bright color of the water. The samples were immediately fixed with 2–4% formalin and transported to the lab, where they were sedimented to 30 mL for at least 48 h [38–43].

The taxonomic LM work was performed twice for all samples: (i) almost immediately after the collection on a Motic BA microscope with a Moticam 2000 camera, supported by the Motic Images 2 Plus software program; (ii) some months later, all samples were processed in a repetitive and comparative way on a Motic B1 microscopes supplied by a Moticam 2.0 MP camera with the Motic Images 3 Plus software program. Here, we note that the identification and counting was done by the same person (MPSG), which ensured the consistency of the LM data.

The algal identification was done on nonpermanent slides under 100× magnification with the application of immersion oil and was based on the standard European taxonomic literature ([52–56], etc.) consulted with recent data from AlgaeBase [57]. With the lack of general consensus on common algal classification system, the phytoplankton composition was represented in the following main phyla: Cyanoprokaryota (blue-green algae), Chlorophyta, Streptophyta, Pyrrhophyta, Euglenophyta, Cryptophyta and Ochrophyta (yellow-brown algae), the last subdivided in the following classes: Bacillariophyceae (diatoms), Chrysophyceae (golden algae), Synurophyceae (silica-scaled chrysophytes), Xanthophyceae (yellow-green algae), Eustigmatophyceae and Raphidophyceae [58].

Algae were counted on a Thoma blood-counting chamber, with a minimum of four iterations for each sample with the cell taken as the main counting unit and a further estimation of the biomass [10,38–42]. The relative abundance of the species was expressed according to the following modification of the Starmach scale [59] in comparison with

species' contribution to the biomass [60]: "rare species" were those seen as single specimens in the whole microscopic slide (<0.5% of the biomass), "occasional species" those represented by up to five specimens (<5% of the biomass), "common, or abundant species" those seen with 6 to 30 specimens in a slide (5–20% of the biomass), whereas dominants and subdominants were evaluated among the most numerous species which contributed to >20 and >25% of the biomass, respectfully.

2.3. Analysis of Phytoplankton Marker Pigments

For the estimation of the general phytoplankton composition and relative phytoplankton biomass, HPLC was applied for marker pigment analysis following the standard operational procedure SOP5 described by [61]. Phytoplankton samples in a volume of 0.5–1 L were filtered at the earliest possibility after collection through 0.45 cellulose filters Whatman NC45 ST/Sterile EO (Merck KGaA, Darmstadt, Germany). Pigments were extracted by two 15 min sonications in ice, separated by an overnight stay in darkness, at a temperature of 4 °C and the final application of 90% acetone. Afterwards, the samples were transported to the lab in plastic tubes in a box with dry ice. During this transportation, only 1 of 70 samples, the tube from the reservoir Sopot, was destroyed and, therefore, pigment data for this reservoir are not provided in the paper.

The pigment analysis was performed on a Waters HPLC system equipped with a photodiode array detector. Pigment concentrations were determined from calibration with chlorophyll and carotenoid standards (DHI, Denmark), and CHEMTAX was used for the calculation of the contribution of the main phytoplankton groups [38,40–42,61–64]. The initial table of pigments, applied as a matrix, is provided in [38].

The chlorophyll *a*, measured by HPLC, was compared with its field measurement and used as an expression of total algal biomass for the assessment of the trophic status according to the OECD System [46] and of the ecological status according to the intercalibrations related with the WFD [47].

2.4. Molecular-Genetic Analysis

- Molecular-genetic analysis for the identification of anatoxin producers

Anatoxin-A (ATX) and its analogues, anatoxins (ATXs), are alkaloid neurotoxins released by more than 40 species of Cyanoprokaryota [65,66]. They are produced by eight ATX synthetase genes (*ana* genes) [67], among which *anaB-anaG* genes are common for different producing genera [68]. Therefore, the *anaC* gene was selected for the amplification by the set of the following primer sequences, F-ATGGTCAGAGGTTTTACAAG and R-CGACTCTTAATCATGCGATC [69], of the material extracted from the samples collected in 2021 in order to complete our data, obtained after the analysis of the samples from 2018 and 2019 [43].

DNA was extracted from the field samples through filtration performed on 0.45 cellulose filters Whatman NC45 ST/Sterile EO (Merck KGaA, Darmstadt, Germany). The extracted DNA was amplified following the procedure MyTaqHS Mix (Bioline), which included the 12.5 µL Tag Mix, 10 pmol (1 µL) primers (both straight and inverted) and 50 ng total DNA. A specified program was used for the incubation of the reaction mixtures in a QB-96 Thermal Cycler: 35 cycles of denaturation (each 10 s at 95 °C), annealing at 55 °C for 30 s, an extension for 30 s at 72 °C, followed by a final extension for 5 min at 72 °C.

GeneJET™ Thermo Scientific and Clone JET PCR kits (Thermo Fisher Scientific, Waltham, MA, USA) were used for the purification and cloning of the *anaC* PCR products, and the recombinant sequences were sent to Macrogen Inc. (Seoul, Republic of Korea) for Sanger sequencing with the same pJET primers. All resulting data were manually edited and initially analyzed using the Vector NTI 11.5 (Thermo Scientific) software package. The Mega 6.0. program [70], a BLAST [71] search in the National Centre for Biotechnology Information (NCBI) GenBank database [72] and the neighbor-joining method with 1000 bootstrap values were used for organizing the *anaC* sequences in a phylogenetic tree. The

obtained sequences were deposited in the NCBI GenBank [72] under the accession numbers OQ3119995–OQ3200013 and OQ355032.

- Molecular-genetic analysis for the identification of microcystin producers

Microcystins are the best-known and most-studied cyanotoxins, produced by Cyanoprokaryota, considered as being the most widely spread toxins in freshwaters [25,73]. In this study, the amplification of the *mcyA* gene from the microcystin synthetase *mcyA-J* gene cluster [74] was applied to the samples from 2018 in order to complete our earlier investigations, in which the *mcyB* and *mcyE* genes were used [39,40,42]. The amplified region was 510 bp long, described from the toxic strains *M. aeruginosa* UWOCCPCC 7806 and *M. aeruginosa* UWOCCPCC 7820 [75]. The amplification was accomplished by the set of forward primer mcyA-102F-CGATGAACAAATCGGGCAATGGCA and reverse primer u-620R-TGCAAGTTTCGCACATCTCCAAGG following [76,77].

A specified manufacturer program was used for the incubation of the reaction mixtures in a QB-96 Thermal Cycler starting with the denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation (each 30 s at 95 °C) and 30 s of annealing at 52 °C, an extension at 72 °C for 30 s with a final extension step lasting 5 min at 72 °C. The cloning and further steps coincided with those described above for anatoxin. The obtained sequences were deposited in the NCBI GenBank database [72] under the accession numbers OM525685-OM525722, and ON075818-ON075819.

2.5. Statistical Analysis

The statistical analysis was conducted using the records of all identified species organized by main taxonomic groups (phyla and classes) and their abundance (rare, occasional, abundant, subdominant and dominant species) in the studied waterbodies. All records were encoded for use by statistical software. Data processing was done by the cross-disciplinary tool SPSS version 19, developed by IBM [78] using descriptives, frequencies and crosstabs, which aimed to prove the relations between the taxonomic groups and environmental parameters.

The environmental parameters were grouped in the following categories regarding the water quality in accordance with their distribution in drinking and natural waters [4,37,79]: (i) water hardness: 0–4 °dh—very soft water, 4–8 °dh—soft water, 8–12 °dh—middle hard water, 12–18 °dh—rather hard water, 18–30 °dh—hard water, >30 °dh—very hard water, considering that 1 °dh = TDS/10; (ii) CN: <10 μS cm^{-1} (distilled water, uncontaminated freshwater), <800–10 μS cm^{-1} (drinking water), 800–2000 μS cm^{-1} (water for irrigation and freshwater streams), >2000 μS cm^{-1} (industrial and wastewater); (iii) pH: >6—acid water, 6–7—neutral water, >7—alkaline water; (iv) TN: <0.3 mg L^{-1}, 0.4–7 mg L^{-1}, 7–10 mg L^{-1}, >10 mg L^{-1}; (v) TP: <10 μg L^{-1}—oligotrophic, 10–35 μg L^{-1}—mesotrophic, 35–100 μg L^{-1}—eutrophic, >100 μg L^{-1}—hypertrophic; (vi) chlorophyll *a:* <1.5 μg L^{-1}—oligotrophic, 1.5–10 μg L^{-1}—mesotrophic, 10–25 μg L^{-1}—eutrophic and >25 μg L^{-1}—hypertrophic waters. In addition, the altitude was considered, classified after [37], as follows: 0–200 m a.s.l.—lowland, 200–500 m a.s.l.—plain, 500–1000 m a.s.l.—kettle, and >1000 m a.s.l.—mountain waterbodies.

The statistical error was estimated by Pearson chi-square values and the correlations were determined according to a comparative analysis in crosstabs [80]. The strength of the relations between two discrete variables was measured by Cramér's V, with a value between 0 and +1, as an effective size measurement for the chi-square test of independence [78,80].

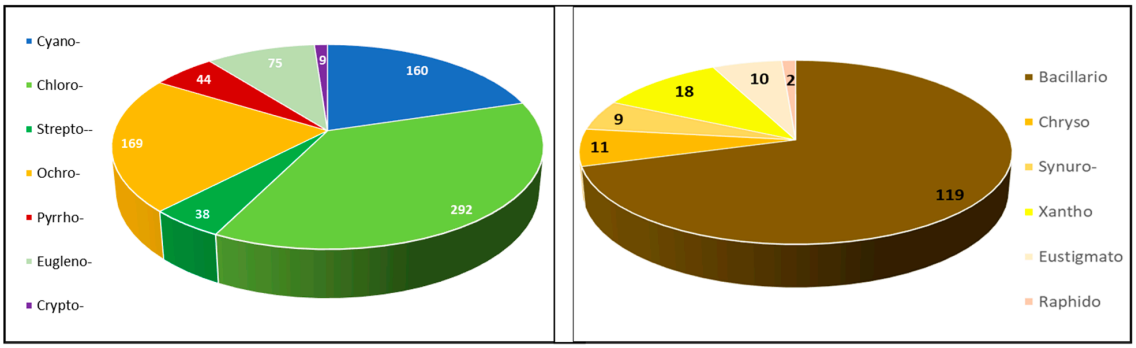
On the basis of the statistical tests, graphs were created using Microsoft®® Excel®® from Microsoft 365 MSO (Version 2212 Build 16.0.15928.20196) 64-bit.

3. Results

3.1. Total Biodiversity of the Phytoplankton

The total biodiversity of the phytoplankton comprised 787 species from seven phyla (Figure 2a). Green algae were represented by the highest number of species (330) with a predominance of taxa from the phylum Chlorophyta (292) and less from the second green

phylum—Streptophyta (38). Cyanoprokaryota, represented with 160 species, occupied the second place in the total taxonomic structure, followed by Ochrophyta, Euglenophyta, Pyrrhophyta and Cryptophyta (Figure 2a). Among Ochrophyta (169 taxa), diatoms (class Bacillariophyceae) were the most diverse (119), while all other classes of this large phylum (Chrysophyceae, Synurophyceae, Xanthophyceae, Eustigmatophyceae, Raphidophyceae) contained much less species (Figure 2b).



(**a**) (**b**)

Figure 2. Total biodiversity (expressed as number of species) of the summer phytoplankton of 43 Bulgarian waterbodies (abbreviations follow those in Table 1): (**a**) Biodiversity in the main taxonomic phyla: Cyano—Cyanoprokaryota, Chloro—Chlorophyta, Strepto—Streptophyta, Pyrrho—Pyrrhophyta, Eugleno—Euglenophyta, Ochro—Ochrophyta and Crypto—Cryptophyta; (**b**) biodiversity in different classes of the phylum Ochrophyta: Bacillario—Bacillariophyceae, Chryso—Chrysophyceae, Synuro—Synurophyceae, Xantho—Xanthophyceae, Eustigmato—Eustigmatophyceae and Raphido—Raphidophyceae.

In almost all waterbodies, chlorophytes were the main contributors to the biodiversity, followed by cyanoprokaryotes (Figure 3). An exception was the phytoplankton of the small mountain reservoir Beglika, in which algae from these two phyla were not found by conventional LM. Cyanoprokaryotes were not found by LM in two other waterbodies—in the large reservoir Suedinenie and in the small reservoir Krapets (Figure 3).

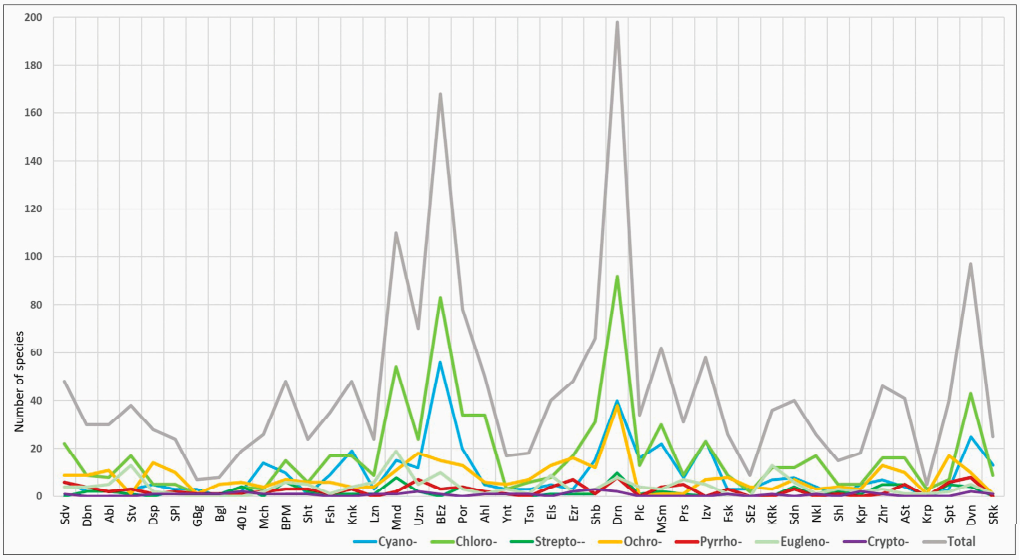
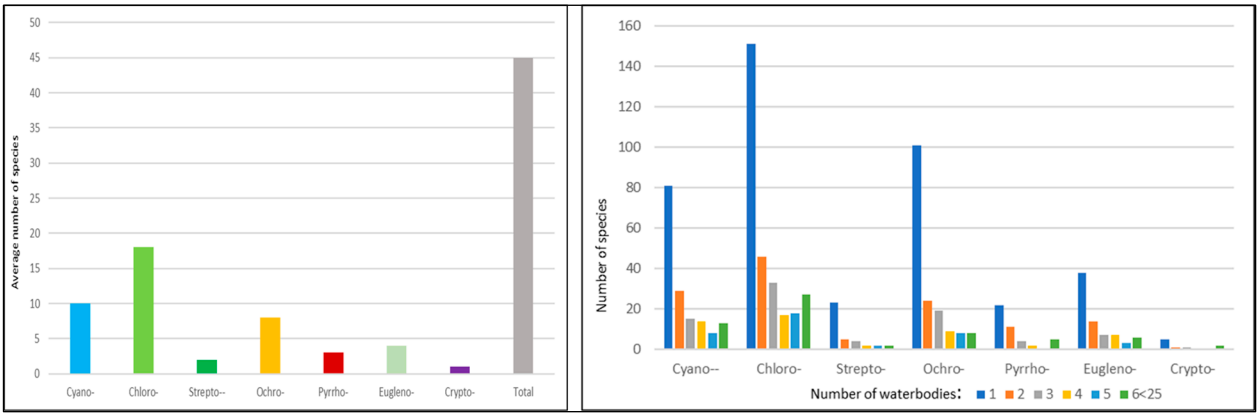


Figure 3. Total number of species in comparison with number of species in the main taxonomic phyla in the summer phytoplankton of 43 Bulgarian waterbodies (abbreviations follow those in Table 1): Cyano—Cyanoprokaryota, Chloro—Chlorophyta, Strepto—Streptophyta, Pyrrho—Pyrrhophyta, Eugleno—Euglenophyta, Ochro—Ochrophyta and Crypto—Cryptophyta.

The average number of species per waterbody was 45, about half of which (20) were green algae (18 chlorophytes and 2 streptophytes), while the other phyla contributed to the phytoplankton with eight to one species on average (Figure 4a).



(**a**) (**b**)

Figure 4. (**a**) Average number of species—total and in the main taxonomic phyla—in the summer phytoplankton of 43 Bulgarian waterbodies; (**b**) distribution of species from main phyla by number of waterbodies (1, 2, 3, 4, 5, and 6–25) in which they were found. For better visibility, the number of species found in more than 5 waterbodies (6 to 25) is summarized in a common group. Legend: Cyano—Cyanoprokaryota, Chloro—Chlorophyta, Strepto—Streptophyta, Pyrrho—Pyrrhophyta, Eugleno—Euglenophyta, Ochro—Ochrophyta, and Crypto—Cryptophyta.

Most of the algal taxa (421, or 53%) were found in a single waterbody and the number of species found in more than five waterbodies was much lower—63, or 8%. The same trend was valid for the species from each of the recorded phyla (Figure 4b). The most widely spread algae belonged to chlorophytes: *Tetraedron minimum* (25 sites), followed by *Coelastrum astroideum* (17), *Nephrochlamys subsolitaria* (14), *Golenkinia radiata* and *Oocystis lacustris* (each in 13 sites), *Monactinus simplex* and *Tetradesmus lagerheimii* (Syn. *Scenedesmus acuminatus*) (each in 12 sites). The most spread cyanoprokaryote was *Planktolyngbya limnetica* (14 sites), followed by *Microcystis wesenbergii* (12 sites), *Microcystis aeruginosa* and *Raphidiopsis raciborskii* (each in 11 sites), *Aphanizomenon klebahnii* and *Coelomoron pusillum* (each in 10 sites). The most widespread species from other taxonomic groups in descending order of findings were the streptophyte *Cosmarium neodepressum* var. *planctonicum* and the pyrrhophyte *Parvodinium elpatiewskyi* (each found in 12 sites), followed by the ochrophytes *Lindavia comta* (11 sites) and *Aulacoseira granulata* (10 sites), as well as the euglenophyte *Trachelomonas volvocina* (10 sites).

Altogether, 79 algae were identified as dominants, codominants or subdominants (Table 2). Among them the most significant was Cyanoprokaryota (33 species of which dominated/codominated in 24 waterbodies and were subdominants in 17), followed by Ochrophyta (14, mainly diatoms) and Chlorophyta (13 taxa), Pyrrhophyta (8 taxa), Euglenophyta (7 species), Streptophyta and Cryptophyta (each with 3 taxa).

According to the available Bulgarian algological literature, out of all 787 species, at least 53 (7%), recorded for first time in the country, can be considered alien. Most of them were observed as rare species in a small number of waterbodies. The exceptions were: (i) the tropical cyanoprokaryotes *Raphidiopsis acuminato-crispa* and *R. gangetica*, which codominated in the small inland reservoir Mechka together with *R. raciborskii,* found earlier in the country [81–91]; (ii) the North-Asian cyanoprokaryote *Aphanizomenon yezoense*, described as being from Japan [92] but currently spread also in Northern and Central Europe [57], which dominated in the small reservoir Studena and was subdominant in the coastal natural lake Durankulak; (iii) the chlorophyte *Tetrallantos lagerheimii*, described as being from Sweden [93] but afterwards recorded on different continents except the Antarctic [57]

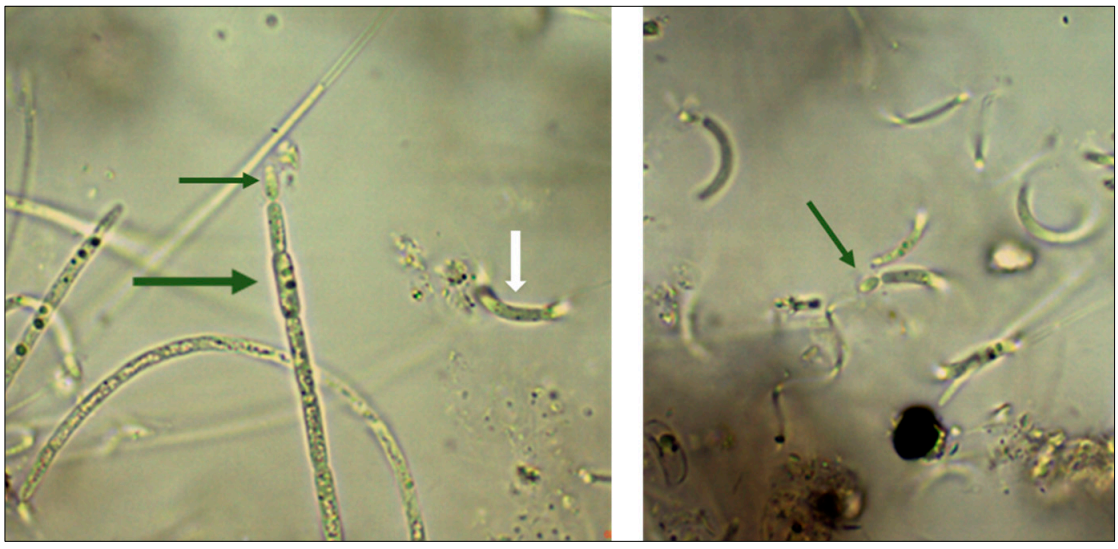
and currently found as dominant in the small inland reservoir Hadzhidimovo (Figure 5). Regarding the non-native, allochthonous species, we would like to note that during this study, the invasive *R. raciborskii* (Figure 5) was found as abundant in 11 waterbodies, where in 9 of them, it was recorded for the first time (i.e., Byalata Prust, Kaynaka, Eleshnitsa, Malka Smolnitsa, Mechka, Preselka, Shabla, Tsonevo and Uzungeren).

Table 2. Dominants, codominants and subdominants in the summer phytoplankton of 43 waterbodies in Bulgaria. The samples obtained in different years are indicated in brackets after the relevant name.

Species	Abundance	Waterbody
Cyanoprokaryota		
Anabaenopsis elenkinii + *Cuspidothrix issatschenkoi*	Codominants	Mogila
Aphanizomenon klebahnii	Dominant/Subdominant	Mandra (2019), Poroy (2019, 2021)/Hadzhi Yani
Aphanizomenon yezoense + *Sphaerospermopsis aphanizomenoides*	Codominants	Studena
Chrysosporum minor + *Raphidiopsis mediterranea*	Codominants	Plachidol 2
Chrysosporum ovalisporum	Dominant	Shabla (2019)
Dolichospermum compactum	Dominant	Izvornik 2
Dolichospermum perturbatum + *Planktothrix isothrix*	Codominants	Burgasko Ezero (2018)
Dolichospermum planctonicum	Dominant/Codominant	Golyam Beglik/Ablanitsa
Dolichospermum scheremetieviae	Dominant	Yunets
Limnothrix redekei	Dominant	Preselka
Limnothrix mirabilis	Codominant	Poroy (2018)
Microcystis wesenbergii	Dominant	Kriva Reka, Nikolovo, Sinya Reka (2018)
Planktothrix isothrix + *Planktothrix suspensa*	Codominants	Burgasko Ezero (2019)
Pseudanabaena limnetica	Codominant/Subdominant	Duvanli, Malka Smolnitsa/Preselka
Raphidiopsis raciborskii	Codominant/Subdominant	Malka Smolnitsa/Byalata Prust, Poroy (2018), Preselka
Raphidiopsis raciborskii + *R. acuminato-crispa* + *R. gangetica*	Codominants	Mechka
Romeria simplex	Codominant	Duvanli
Sphaerospermopsis aphanizomenoides	Dominant	Burgasko Ezero (2021)
Sphaerospermopsis torques-reginae	Dominant	Sinyata Reka (2019)
Anabaenopsis milleri	Subdominant	Izvornik 2
Aphanizomenon yezoense + *Microcystis aeruginosa*+*Pseudanabaena mucicola* + *Synechocystis endobiotica*	Subdominants	Durankulak (2021)
Aphanocapsa delicatissima	Subdominant	Shumensko Ezero
Aphanocapsa holsatica	Subdominant	Durankulak (2018), Hadzhi Yani
Coelomoron pusillum	Subdominant	Kriva Reka
Dolichospermum perturbatum	Subdominant	Izvornik 2
Microcystis aeruginosa	Subdominant	Mandra (2021)
Microcystis sp. (separate cells)	Subdominant	Duvanli
Oscillatoria cf. *simplicissima*	Subdominant	Burgasko Ezero (2021)
Planktolyngbya limnetica	Subdominant	Eleshnitsa
Pseudanabaena mucicola	Subdominant	Nikolovo
Raphidiopsis raciborskii + *Pseudanabaena limnetica*	Subdominants	Shabla (2019)
Chlorophyta		
Binuclearia lauterbornii	Dominant/Subdominant	Sopot, Tsonevo (2019)/Durankulak (2019), Uzungeren (2018)
Gloeocystis sp.	Dominant	Ezerets (2018), Shabla (2018)
Monactinus simplex	Dominant	Hadzhi Yani
Oocystis sp.	Dominant	Al. Stamboliyski, Zhrebchevo
Siderocystopsis pseudoblonga	Codominant	Shilkovtsi
Coelastrum astroideum + *Tetrallantos lagerheimii*	Subdominants	Hadzhidimovo
Didymocystis inconspicua + *Pediastrum duplex*	Subdominants	Poroy (2018)
Elakatothrix lacustris	Subdominant	Al. Stamboliyski
Golenkinia radiata	Subdominant	Plachidol 2
Hariotina polychorda	Subdominant	Suedinenie
Lauterborniella appendiculata + *Lobocystis* sp.	Subdominants	Durankulak (2019)
Scenedesmus ellipticus	Subdominant	Aheloy
Streptophyta		
Cosmarium neodepressum var. *planctonicum*	Dominant	Fisek
Closterium acerosum	Subdominant	Uzungeren (2018)
Cosmarium phaseolus var. *elevatum*	Subdominant	Dubnitsa

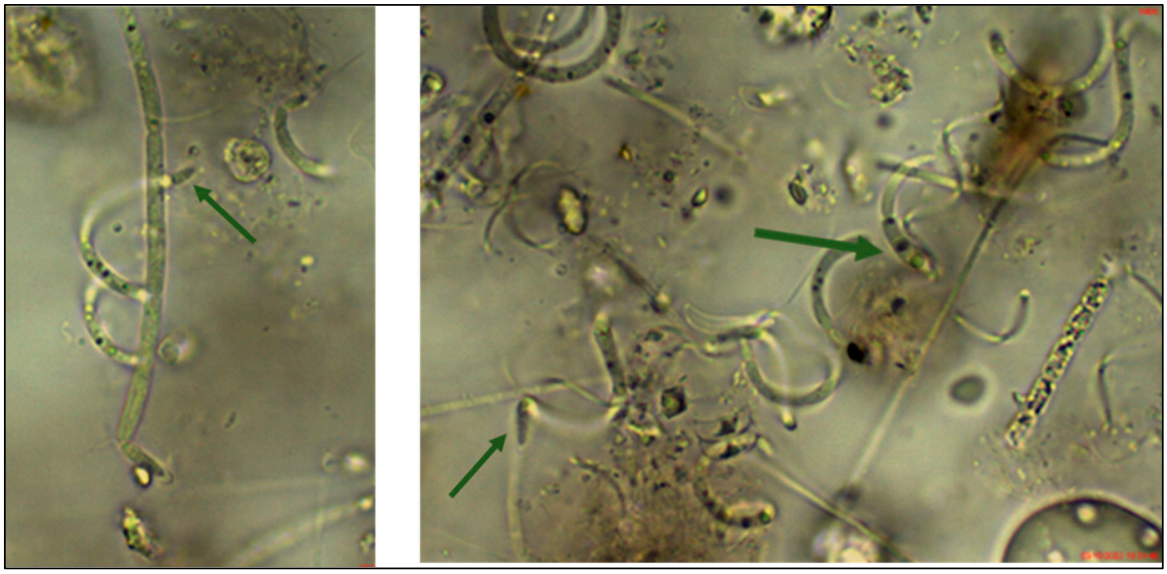
Table 2. *Cont.*

Species	Abundance	Waterbody
Pyrrhophyta		
Ceratium rhomvoides	Dominant	Al. Stamboliyski
Peridinium volzii var. *cinctiforme*	Dominant	Suedinenie
Parvodinium elpatiewskyi	Dominant/Subdominant	Birgo, Dubnitsa/Satovcha 2
Parvodinium cunningtonii	Codominant	Ablanitsa
Parvodinium umbonatum	Dominant	Hadzhidimovo
Sphaerodinium polonicum	Dominant	Duvanli, Eleshnitsa (2019)
Ceratium furcoides	Subdominant	Mandra (2018), Suedinenie
Parvodinium goslaviense	Subdominant	Mechka, Mogila
Euglenophyta		
Euglena adhaerens	Dominant	Uzungeren (2021)
Euglenaria clavata	Dominant	Satovcha 2
Phacus rotundus	Codominant	Hadzhi Yani
Discoplastis spathirhyncha	Subdominant	Kriva Reka
Euglena sp.	Subdominant	Uzungeren (2018)
Trachelomonas hispida	Subdominant	Birgo
Trachelomonas intermedia	Subdominant	Satovcha 2
Ochrophyta		
Bacillariophyceae		
Asterionella formosa	Codominant	Dospat
Coscinodiscus sp.	Dominant/Subdominant	Durankulak (2021)/Mandra (2021), Poroy (2021)
Ctenophora pulchella	Dominant	Shumensko Ezero
Cymbella cf. *cymbiformis*	Dominant	Mandra (2021), Tsonevo (2018)
Fragilaria crotonensis	Codominant	Shilkovtsi
Lindavia comta	Dominant	Beglika, Chetiridesette Izvora
Nitzschia acicularis	Dominant	Uzungeren (2018)
Ulnaria acus	Dominant	Ezerets (2019)
Ulnaria ulna	Codominant	Eleshnitsa (2021)
Stephanocyclus meneghinianus	Subdominant	Koprinka
Chrysophyceae		
Dinobryon bavaricum	Dominant/Codominant	Shiroka Polyana/Eleshnitsa (2021)
Dinobryon korschikovii	Dominant	Sopot
Synurophyceae		
Mallomonas akrokomos	Codominant	Dospat
Xanthophyceae		
Nephrodiella cf. *acuta*	Dominant	Uzungeren (2019)
Cryptophyta		
Cryptomonas erosa	Dominant/Subdominant	Koprinka/Durankulak (2021)
Cryptomonas cf. *ovata*	Subdominant	Shabla (2018)
Cryptomonas sp.	Subdominant	Uzungeren (2019)

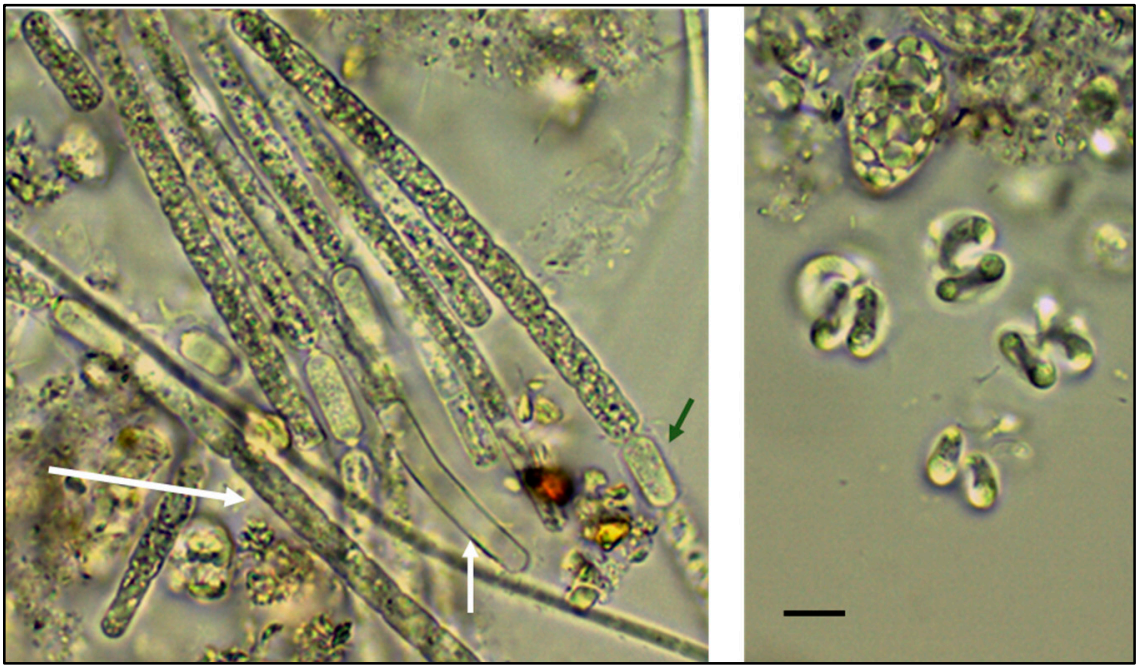


(**a**) (**b**)

Figure 5. *Cont.*



(**c**) (**d**)



(**e**) (**f**)

Figure 5. Alien phytoplankters in Bulgarian waterbodies: (**a**) *Raphidiopsis raciborskii* (straight trichome, thick green arrow points to its akinete, thin green arrow points to the heterocyst) and *Raphidiopsis gangetica* (white arrow points to its coiled trichome) in the inland microreservoir Mechka; (**b**) coiled trichomes of *Raphidiopsis gangetica* (thin green arrow points to the typical rounded heterocyst); (**c**) *Raphidiopsis acuminato-crispa* coiled around the straight trichome of *Raciborskii raciborskii* in Mechka (arrow indicates its pointed heterocyst); (**d**) *Raphidiopsis acuminato-crispa* in Mechka (thin green arrow points to the heterocyst, thick green arrow points to the akinete); (**e**) *Aphanizomenon yezoense* in the coastal lake Durankulak—aggregation of trichomes in a fascicle (long white arrow points to the akinete, short white arrow points to the long transparent apical cell, and green arrow points one of the heterocysts in the fascicle); (**f**) *Tetrallantos lagerheimii*—coenobium of bent cells from the small inland microreservoir Hadzhidimovo, black scale—5 μm, relevant to all figures.

3.2. Phytoplankton Structure according to the Marker Pigment Composition

In the general phytoplankton composition, based on pigment structure (Figure 6), the average relative contribution of the taxonomic groups to the biomass was as follows: cyanoprokaryotes—42%, green algae—10%, ochrophytes—25%, pyrrhophytes—2%, euglenophytes—12%, and cryptophytes—9%.

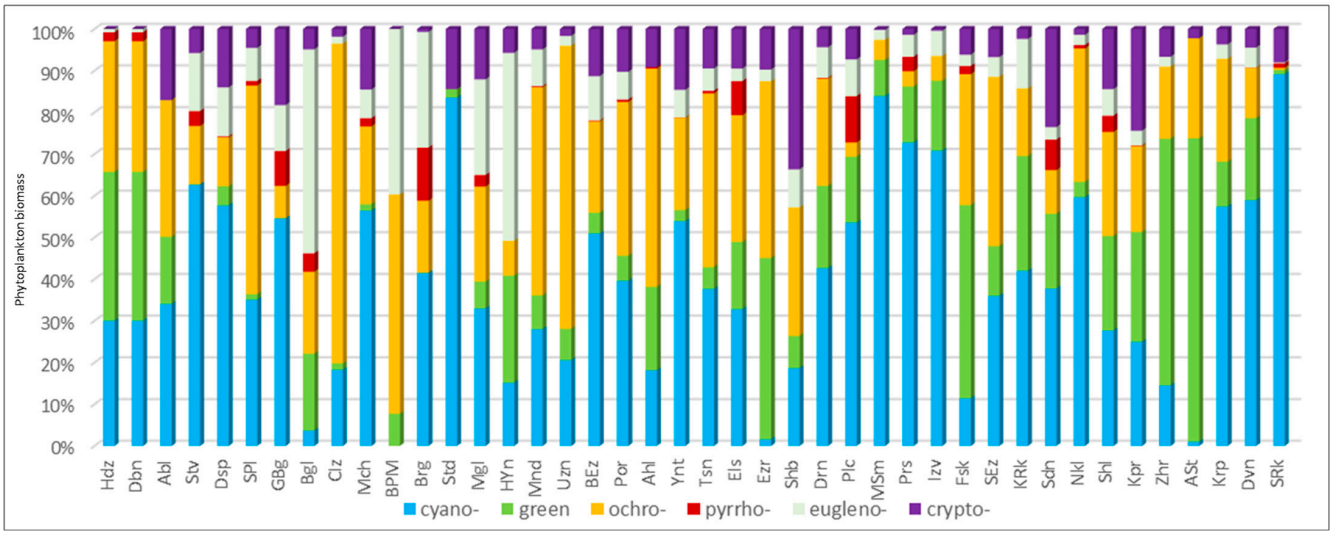


Figure 6. Relative contribution of main taxonomic groups to the phytoplankton biomass (calculated through the chlorophyll *a* concentration) according to the HPLC analysis of marker pigment composition in the studied Bulgarian waterbodies. Legend: cyano—cyanoprokaryotes, green—algae from both phyla Chlorophyta and Streptophyta, pyrrho—pyrrhophytes, eugleno—euglenophytes, ochro—ochrophytes, crypto—cryptophytes. Abbreviations of the names of the waterbodies are in accordance with Table 1.

The values of chlorophyll *a*, measured by HPLC, ranged significantly from 0.199 μg L^{-1} (Tsonevo) to 765 μg L^{-1} (Izvornik 2)—Figure 7. As far as single values can be relied upon, considering the boundary values from the OECD [46] and WFD [47], chlorophyll *a* concentrations indicated the oligotrophic status of seven waterbodies (Beglika, Byalata Prust-Mezek, Dospat, Krapets, Koprinka, Shiroka Polyana, Shumensko Ezero, Tsonevo). Thirteen waterbodies had a mesotrophic status (Ablanitsa, Al. Stamboliyski, Birgo, Dubnitsa, Eleshnitsa, Ezerets, Golyam Beglik, Hadzhidimovo, Mechka, Shabla, Shilkovtsi, Studena and Zhrebchevo). Eight were eutrophic (Aheloy, Chetiridesette Izvora, Durankulak, Fisek, Plachidol 2, Satovcha 2, Suedinenie, Yunets), and thirteen waterbodies were hypertrophic (Burgasko Ezero, Duvanli, Hadzhi Yani, Izvornik 2, Mandra, Mogila, Poroy, Preselka, Kriva Reka, Malka Smolnitsa, Nikolovo, Sinyata Reka and Uzungeren), where strong cyanoblooms were detected (Figure 7).

3.3. Algal Blooms and Toxic Species

According to the drone observations, supported by conventional LM studies and the HPLC analysis of marker pigments, during the three summers of investigation, blooms of cyanoprokaryotes occurred in the microreservoirs Birgo, Duvanli, Izvornik 2, Malka Smolnitsa, Mechka, Mogila, Nikolovo, Plachidol 2, Poroy, Preselka, Sinyata Reka, and Studena, in the large reservoir Mandra, as well as in the coastal lakes Burgasko Ezero and Durankulak (Figures 3, 6 and 7 and details in [38–44]). A relatively high contribution of cyanoprokaryotes to the biomass was detected in the reservoirs Krapets and Dospat (Figure 6), for which chlorophyll *a* data clearly showed a lack of blooms and a low trophic state (Figure 7). Similar was the case of the large mesotrophic reservoir Shilkovtsi, in which blooms were not seen and the relatively high contribution of cyanoprokaryotes was

explained by the identification of marker pigments of *Synechococcus* type T1, typical for picoplankters which cannot be detected by conventional LM [42].

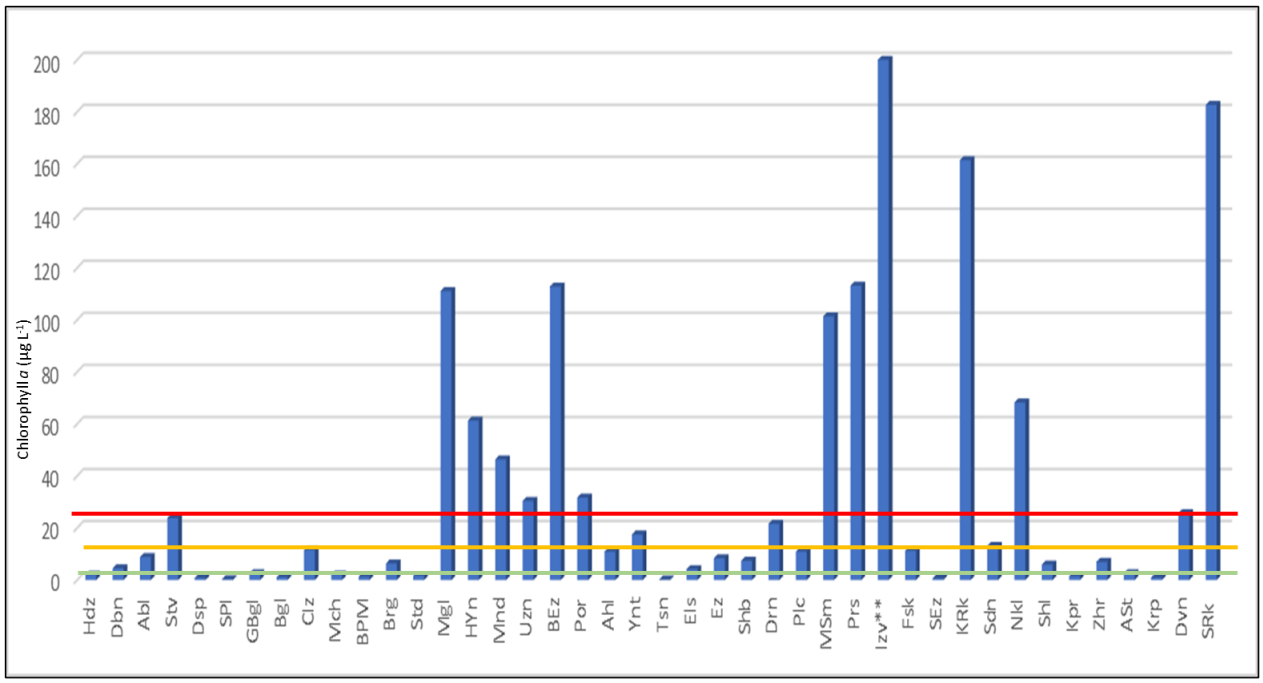


Figure 7. Chlorophyll *a* content (μg L^{-1}) according to the HPLC analysis of marker pigment composition in the studied waterbodies. Green line indicates the upper border of oligotrophic waters (<1.5 μg L^{-1}), yellow line shows the upper border of mesotrophic waters (1.5–10 μg L^{-1}), and the red line indicates the upper border of eutrophic waters (10–25 μg L^{-1}), above which waters were hypertrophic.' The abbreviations of the names of the waterbodies are in accordance with Table 1. Asterisks indicate that the real value of chl *a* in Izvornik 2 was 765 μg L^{-1}.

Most detected blooms, despite their different intensity, supported the development of microcystin-, anatoxin- and microviridin-producing species and of the different cyanotoxins (Table 3) with proved the natural water cytotoxicity [94] and demonstrated the effects of low cylindrospermopsin doses on the gastrointestinal human cells [95]. It has to be noted that toxic cyanoprokaryotes were also found in waterbodies without blooms at the moment of sampling, such as in Ezerets, Koprinka, Uzungeren and Zhrebchevo (Table 3 and Figures 6 and 7). Up to now, in the studied waterbodies, nodularins and their main producer, *Nodularia,* have not been found despite the conducted targeted microscopic, chemical and molecular-genetic analyses [39]. Although cylindrospermopsin was detected in Bulgarian waterbodies [38,96], the molecular-genetic studies also revealed that the identified *Raphidiopsis raciborskii*, *Raphidiopsis mediterranea* and *Chrysosporum bergii* in our study did not contain the *cyrJ* gene responsible for its production [44].

Table 3. Toxins and toxin-producing cyanoprokaryotes in the considered Bulgarian waterbodies, sampled in 2018 and 2019. Legend: CPS—cylindrospermopsin; MC—microcystin, followed by the exact type (LR, RR or YR), MV—microviridin, followed by the letter indicating the specific type (A, B, C, etc.), SXT—saxitoxins; (?)—supposed toxicity based on a comparison of newly obtained genetic sequences with light microscopic data. Waterbodies are arranged by years in alphabetical order.

Waterbody/Year	Toxins	Species	Reference
2018			
Burgasko Ezero	CPS	*Microcystis aeruginosa, Microcystis wesenbergii, Microcystis novacekii; Cuspidothrix issatschenkoi, Cuspidothrix tropicalis*	[38,39,43]
Durankulak	MC-LR, MC-RR, MC-YR, SXT	*Microcystis aeruginosa, Microcystis wesenbergii*	[38,39]
Mandra		*Microcystis novacekii*	[39]
Poroy		*Microcystis novacekii; Cuspidothrix issatschenkoi, Cuspidothrix tropicalis*	[39,43]
Sinyata Reka	MC-LR, MC-RR	*Microcystis wesenbergii; Cuspidothrix issatschenkoi, Cuspidothrix tropicalis*	[38,39,43]
2019			
Burgasko Ezero	MV-CBJ	*Microcystis aeruginosa, Microcystis wesenbergii, Cuspidothrix elenkinii, Cuspidothrix issatschenkoi, Cuspidothrix tropicalis*	[40,41,43]
Durankulak	MC-LR, MV-CBJ	*Microcystis aeruginosa*	[40–42]
Duvanli		*Microcystis aeruginosa, Microcystis viridis* (?), *Cuspidothrix issatschenkoi*	[42,43]
Ezerets		*Microcystis aeruginosa, Microcystis viridis* (?)	[42]
Izvornik 2		*Microcystis wesenbergii*	[40]
Koprinka		*Microcystis aeruginosa, Microcystis viridis* (?), *Microcystis wesenbergii, Cuspidothrix elenkinii*	[42,43]
Malka Smolnitsa		*Microcystis aeruginosa, Microcystis viridis* (?)	[42]
Mandra	MC-LR, MV-CBJ	*Microcystis aeruginosa*	[40,41]
Plachidol 2		*Cuspidothrix issatschenkoi*	[43]
Preselka		*Microcystis aeruginosa, Microcystis viridis*	[42]
Poroy	MV-A, MV/MC19	*Microcystis aeruginosa, Microcystis viridis, Microcystis wesenbergii*	[40,41]
Sinyata Reka	MV-B/C	*Microcystis aeruginosa, Microcystis wesenbergii, Cuspidothrix tropicalis*	[40,41,43]
Uzungeren		*Microcystis aeruginosa*	[40]
Zhrebchevo		*Microcystis aeruginosa, Microcystis viridis* (?), *Microcystis wesenbergii*	[42]

Currently, by combining LM data and molecular-genetic studies based on *anaC* gene with 24 newly obtained sequences, the presence of anatoxin producing *Cuspidothrix* in the 2021 summer samples from Durankulak, Mechka, Nikolovo, Studena and Yunets was proved (Figure 8). A comparison of these results with our data from 2018 and 2019 (Figure 8 and [43]) demonstrated the presence of toxic *Cuspidothrix issatschenkoi* in the samples from Durankulak, Nikolovo and Yunets, and suggested once more the potential toxicity of *Cuspidothrix elenkinii* (found in 2019 in Koprinka [43] and in 2021 in Yunets) and of *Cuspidothrix tropicalis* (found in 2018 in Burgasko Ezero, in 2019 in Sinyata Reka [43] and in 2021 in Studena). In Mechka, rarely, morphologically peculiar young nonheterocytous and sterile trihomes of *Cuspidothrix* were found. Due to a lack of reproductive and resting cells, akinetes, their morphological determination was unreliable. Molecular-genetic data separated the sequences from Mechka from all other identified *Cuspidothrix* strains. In this small reservoir, three different *Raphidiopsis* species (*R. acuminato-crispa*, *R. gangetica*, *R. raciborskii*) codominated and, considering the close phylogenetic position of both genera *Cuspidothrix* and *Raphidiopsis* (for details see [43]), a further analysis of more genes is needed for a clarification of the strains isolated from Mechka. The coincidence with sequences of *Aphanizomenon* sp. in the constructed phylogenetic tree was explained in detail in [43] as caused by the taxonomic separation of the genus *Cuspidothrix* and of its type species *Cuspidothrix issatschenkoi*, in particular, from the genus *Aphanizomenon* [52].

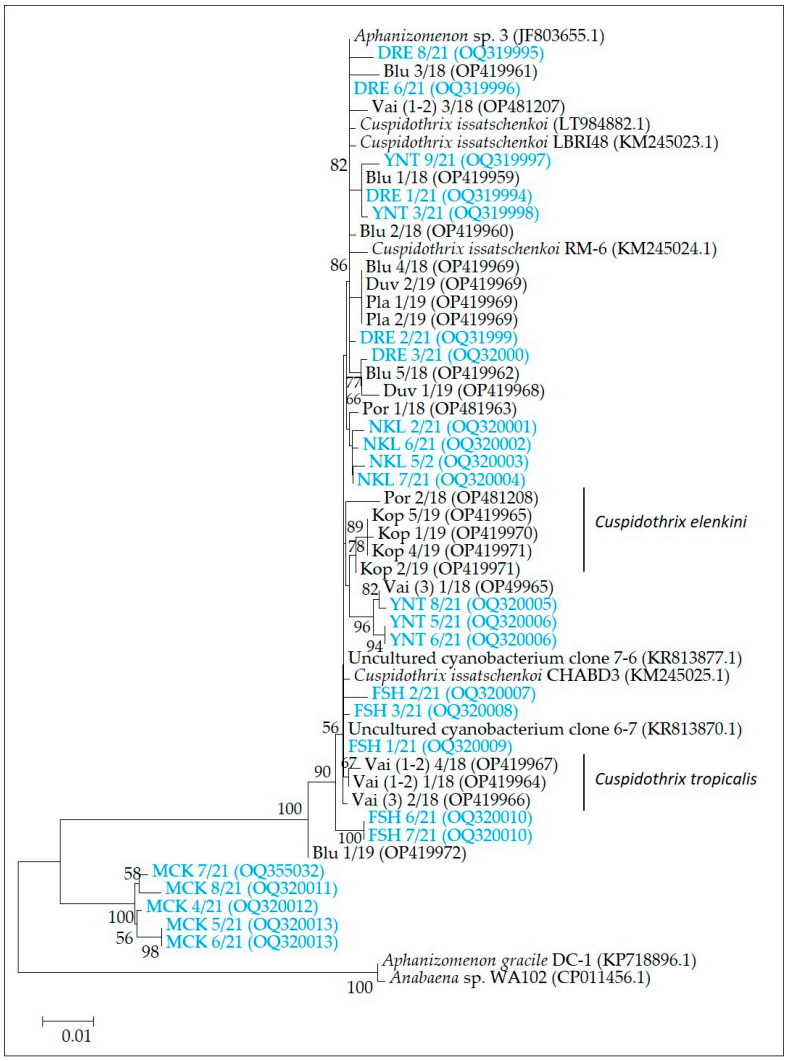


Figure 8. Neighbor-joining phylogenetic tree constructed after processing the samples from the 2021 summer phytoplankton using nucleotide sequences from five library samples indicated in blue and their closest sequences retrieved after a BLAST search [71] of the NCBI database [72]. Bootstrap values are shown at the branch points (percentage of 1000 trials) and an outgroup represented by *Aphanizomenon gracile* DC-1 and *Anabaena* sp. WA102. The 24 newly obtained nucleotides sequences are indicated by the abbreviated name of the waterbody, year of sampling and the relevant accession number in NCBI [72]: OQ3119995-OQ3200013. After the abbreviation, the number of the isolated sequence and after the slash, the year of the collection of the sample, are indicated. For the identical sequences obtained during this study, only one NCBI-derived accession number is provided in each cluster: OQ320003 for the Yunets clones 5 and 6, OQ320010 for the Studena clones 6 and 7, and OQ320013 for the Mechka clones 5 and 6. The following abbreviations are used for the waterbodies: Blu—Sinyata Reka (translated from its Bulgarian name as Blue River), DRE—Durankulak, Duv—Duvanli, FSH—Studena (due to the synonymous name Fishera), Kop—Koprinka, MCK—Mechka, Pla—Plachidol 2, Por—Poroy, Vai—Burgasko Ezero (from the synonymous name Vaya) and YNT—Yunets.

During the PCR amplification of the *mcyA* gene, responsible for the microcystin synthesis, 47 sequences were obtained, 9 of which showed a 100% homology with strains in NCBI [72] and 38 had a 99% homology with them. Molecular-genetic studies based on *mcyA* gene outlined two clusters and four subclusters in the 2018 summer samples (Figure 9), which, in combination with the LM observations, confirmed the presence of microcystin-producing *Microcystis* as follows: *Microcystis aeruginosa and Microcystis novacekii* in Mandra

(cluster I), *Microcytis botrys* in Poroy (subcluster I of cluster II), *Microcystis aeruginosa* in Poroy, Mandra and Durankulak (subcluster II of cluster II), *Microcystis aeruginosa* in Poroy, Burgasko Ezero and Mandra (subcluster III of cluster II), *Microcysis novacekii* in Burgasko Ezero, *Microcystis botrys* in Durankulak, where *Microcystis aeruginosa* also occurred (subcluster IV of cluster II).

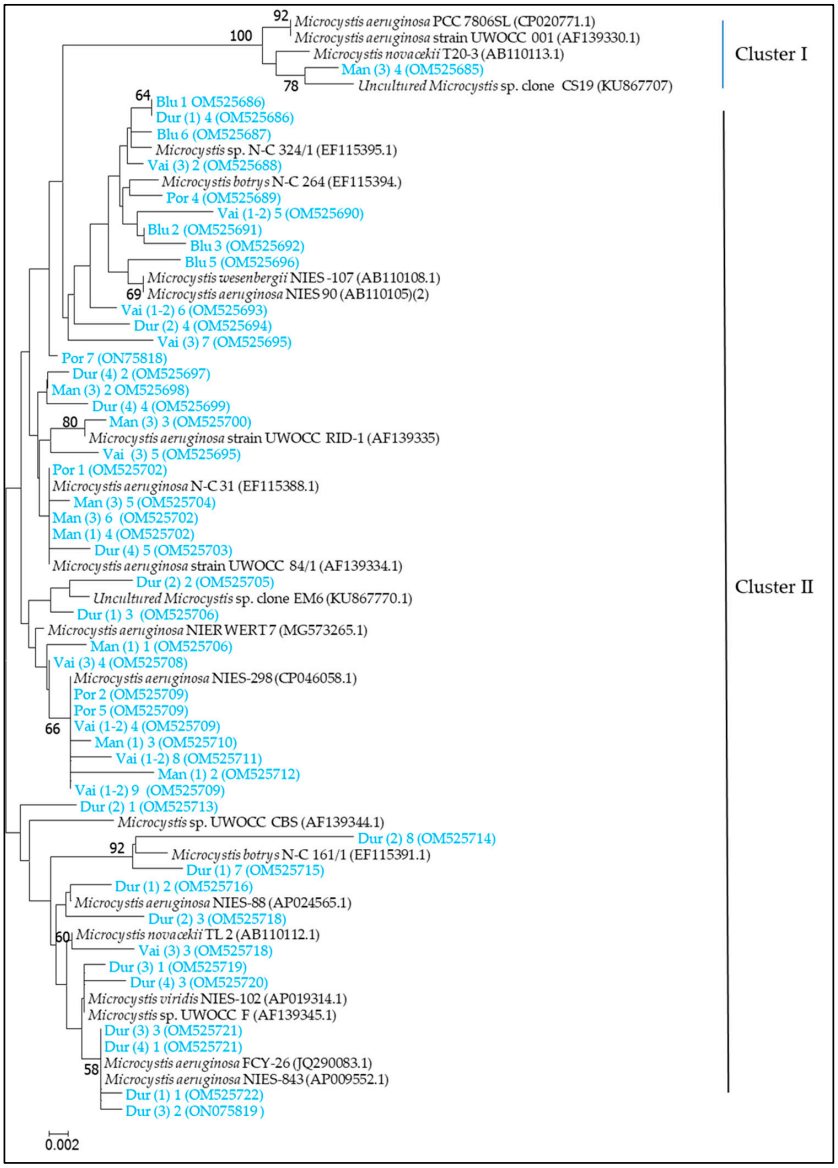


Figure 9. Neighbor-joining phylogenetic tree based on nucleotides sequences from ten library samples and closest sequences retrieved after a BLAST [71] search in the NCBI database [72] with indication of their accession number. Bootstrap values are shown at branch points (percentage of 1000 trials). Legend for the abbreviations of the waterbodies: Man—Mandra; Dur—Durankulak; Vai—Burgasko Ezero (Vaya); Por—Poroy; Blu—Sinyata Reka (Blue River) with Arabic numerals after the abbreviation, indicating the exact site of sampling and number of the sequence. For the identical sequences obtained during this study, only one accession number received from NCBI [72] is provided in each cluster or subcluster: (i) OM525686 is representing the sequences from the reservoir Sinyata Reka and site 4 of lake Durankulak (Blu 1 and Dur (1) 4); (ii) OM525702 is relevant for the sequences obtained from sites 1 and 3 of the reservoir Mandra, Poroy (Man (1) 4, Man (3) 6 and Por 1); (iii) OM525709 is for sites 1–2 from Lake Burgasko Ezero (i.e., Vai (1–2) 4, and Vai (1–2) 9) and for two sequences from the reservoir Poroy (Por 2, Poroy 5); (iv) OM525721 represents the sequences from sites 3 and 4 of Lake Durankulak (Dur (3) 3 and Dur (4) 1).

3.4. Algal Groups and Environmental Variables—Results from Statistical Analysis

In the conducted statistical SPSS analysis [78], the species from each algal group found at certain environmental conditions were expressed as a percentage from all species of the relevant group. The first results from the data processing by the SPSS tool and the application of Cramer's V evaluation [80] of 1996 records of all algal taxa and their abundance showed different but insignificant correlations. Therefore, we decided to exclude all rare species, the presence of which in the waterbodies was considered as nonrepresentative due to their finding in single specimens and in single sites. The resulting correlations obtained in this way were of moderate significance (0.2 < effect size field) except those with pH, which showed low confidence. Most probably, the lack of strong significance in this case was due to the targeted sampling in mostly eutrophic and hypertrophic waters with an alkaline character. The results presented below concern only taxonomic groups that were significantly correlated with other environmental parameters (TN, TP, trophic status, water hardness, conductivity and altitude). They were obtained after conducting the SPSS analysis based on the common, abundant, subdominant and dominant species from all algal groups with the subsequent exclusion of classes and phyla that showed correlations of low confidence.

The significant negative correlations were found between four taxonomic groups and the exact chlorophyll *a* values, considered as a proxy of the trophic status; the occurrence of species from Euglenophyta, Streptophyta and Eustigmatophyceae increased with the rising trophic status, whereas Chrysophyceae demonstrated a clear preference for a lower trophicity (Figure 10).

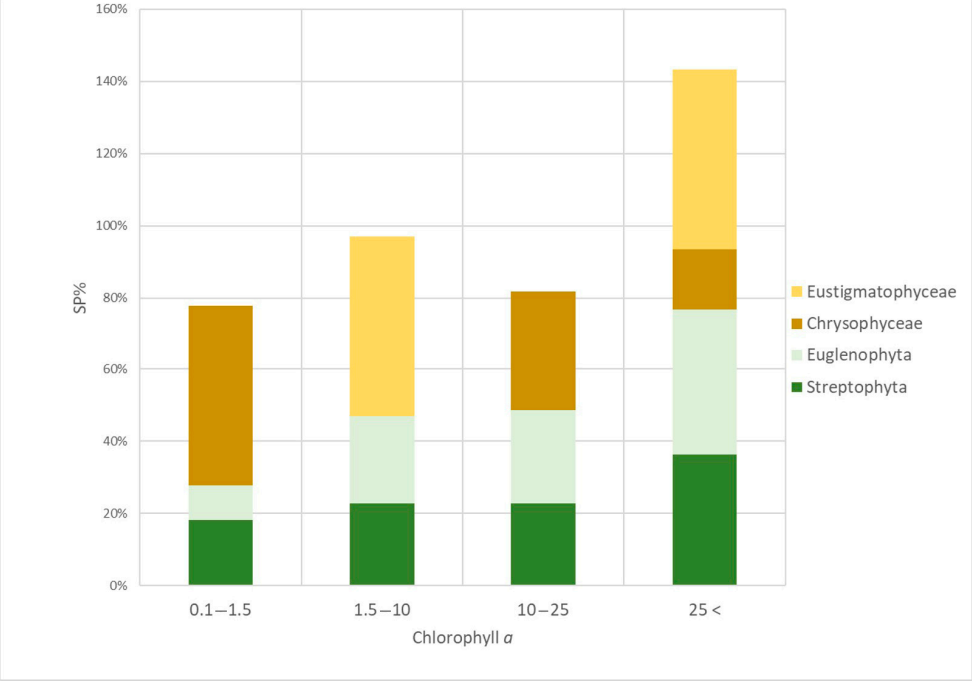


Figure 10. Cumulative species number in main taxonomic groups, expressed as a percentage of the total within the relevant phyla and classes (SP%), in waterbodies with different chlorophyll *a* concentration (μg L^{-1}) as an expression of the trophic state [37].

The occurrence of all main algal groups was correlated with the TN concentrations, and the SPSS analysis revealed the preference of most of the identified species for high water quality conditions with TN below 7 mg L^{-1} [4], with Eustigmatophyceae in particular concentrated in waters with a TN range of 0.4–7 mg L^{-1}, and only Cyanoprokaryota, Euglenophyta and Bacillariophyta were spread in waters with TN values ranging between 7 and 10 mg L^{-1} (Figure 11).

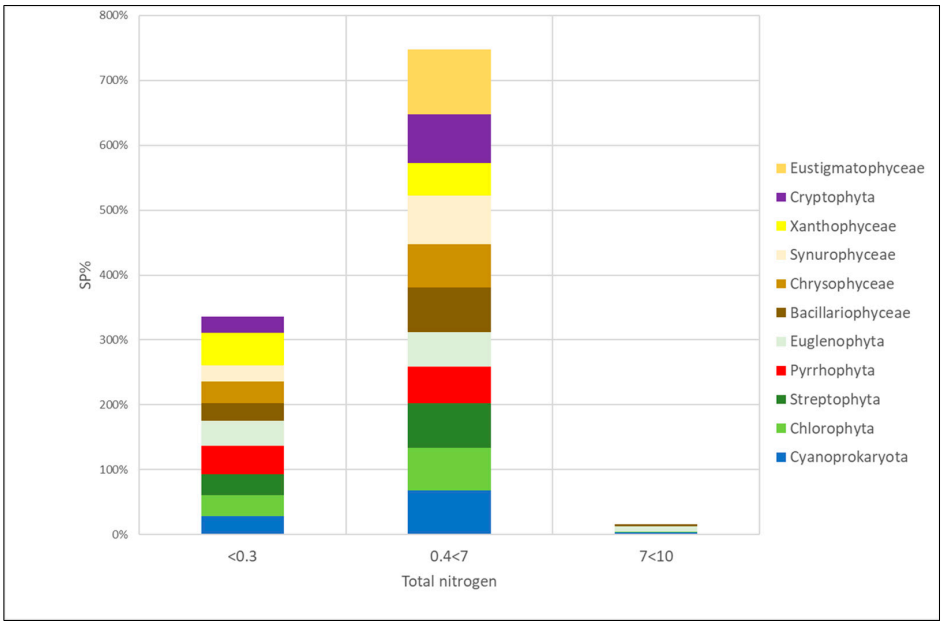


Figure 11. Cumulative species number in main taxonomic groups, expressed as a percentage of the total within the relevant phyla and classes (SP%), in waterbodies with different concentrations of total nitrogen [4,37].

According to the SPSS analysis, the identified taxonomic groups were significantly reverse-correlated with different concentrations of the other important nutrient, TP, except Eustigmatophyceae (Figure 12).

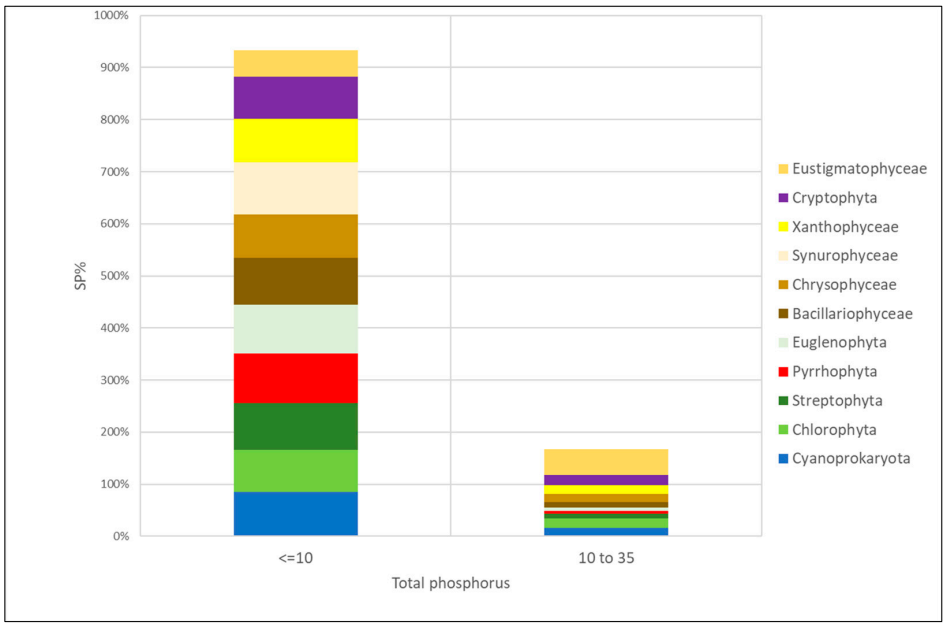


Figure 12. Cumulative species number in main taxonomic groups, expressed as a percentage of the total within the relevant phyla and classes (SP%), in waterbodies with different concentrations of total phosphorus (μg L^{-1}).

Although most species from all groups were found in lowland and plain waterbodies (0–500 m a.s.l.), the distribution of the following taxonomic groups was more specific according to the altitude location: xanthophyceans and eustigmatophyceaens were spread only in the lowland waterbodies (0–200 m a.s.l.), while pyrrhophytes and streptophytes

occurred in all altitude groups but had a preference for lowland and plain waterbodies (Figure 13).

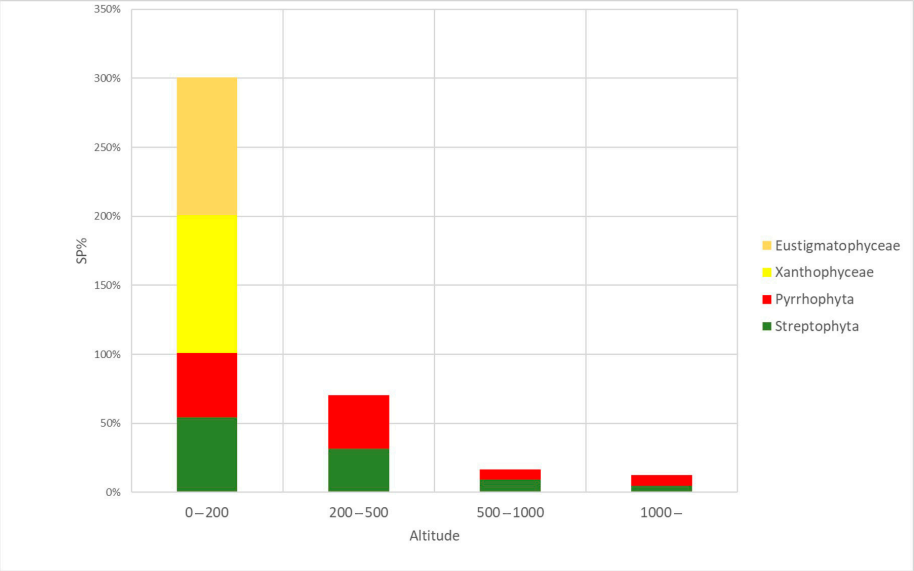


Figure 13. Cumulative species number expressed as percentage of the total within the phyla and classes (SP%), in different waterbodies according to the altitude (m a.s.l.), classified after [37].

Regarding water conductivity, we have to note that during the field studies we did not measure values below <10 µS cm^{-1} (typical for distilled water, Table 1), and the SPSS analysis conducted for the three other conductivity categories allowed us to reveal nine taxonomic groups that showed a significant reverse relationship with this parameter, among which Synurophyceae could be outlined as related with waters of lower conductivity (Figure 14). In this way, only species of Bacillariophyceae and Eustigmatophyceae found in this study could be excluded from the search for potential indicators, as independent from the conductivity of the water.

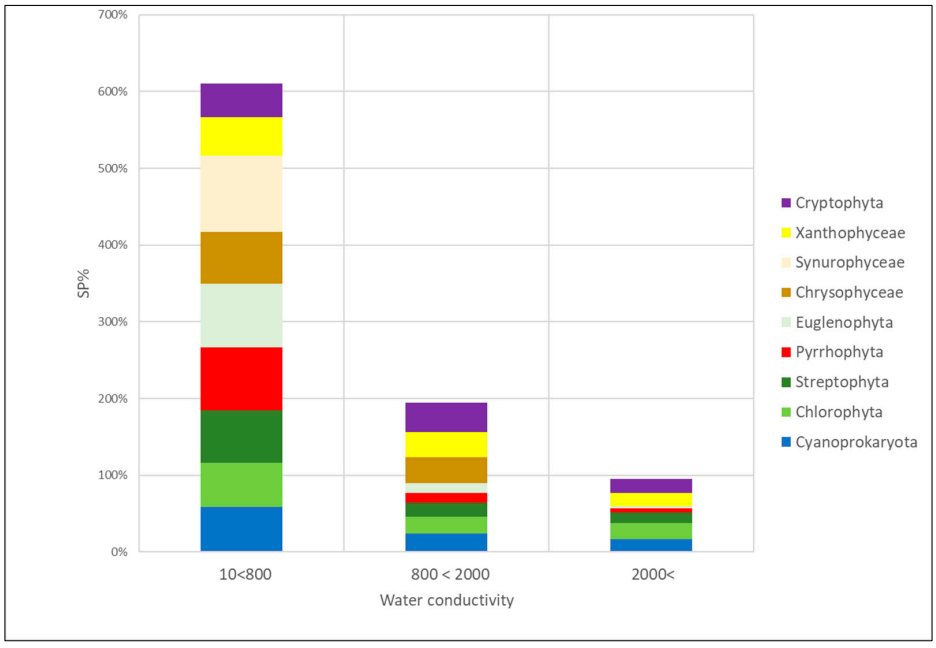


Figure 14. Cumulative species number expressed as a percentage of the total within the phyla and classes (SP%), in waterbodies of different conductivity values (S m^{-1}), classified after [37].

Considering water hardness, the SPSS analysis revealed that the spread of the species of four phyla and three classes was significantly correlated with this variable (Figure 15). The number of species of most algal groups increased with the rise of water hardness, but only Cryptophyta showed a preference for very soft water and Eustigmatophyceae to very hard water (Figure 15).

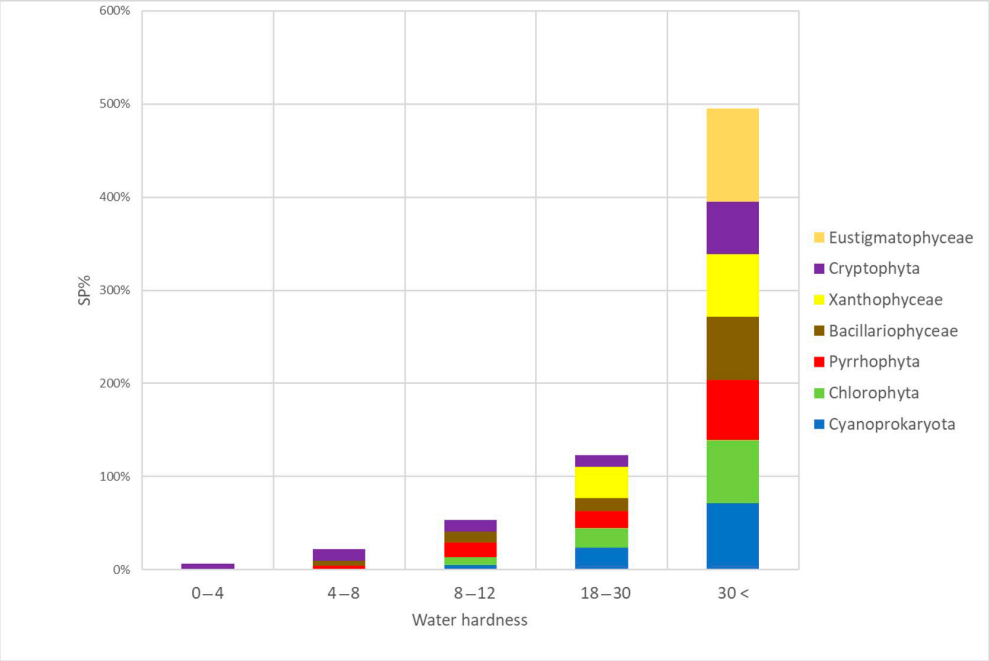


Figure 15. Cumulative species number expressed as a percentage of the total within the phyla and classes (SP%), in different waterbodies according to the water hardness (°dh), classified after [37].

4. Discussion

Results from the present study demonstrated a high phytoplankton diversity in the sampled waterbodies, which comprised 787 species from seven phyla with a clear predominance of the green algae with 330 species, or 42% from all identified taxa. The second taxonomically rich group was Cyanoprokaryota, represented by 160 species. All data obtained by the LM and HPLC studies indicated the generally high contribution of blue-green algae in the summer phytoplankton of the studied waterbodies, especially in eutrophic and hypertrophic ones. A comparison of the results from the LM observations on algal abundance and dominance with the HPLC data (Table 2, Figures 6 and 7) once more demonstrated the reliability of the application of the HPLC analysis of marker pigments in rapid phytoplankton characterization for water quality assessment [38,42,61,62].

Although the use of dominants for indicative purposes has long been debated, focusing on them is supported by the fact that their dynamics is important for the community stability, and they enhance the evaluation of resources availability [34,96–98]. In this study, blue-green algae dominated by 33 species in 60% of the sampled water bodies (Table 2). These data are consistent with the well-known summer dominance of cyanoprokaryotes in nutrient-rich waters (e.g., [11,18,99]). If such dominance in small, shallow, lowland and plain waterbodies can be taken as a normal seasonal event, finding the heterocytous cyanoprokaryote *Dolichospermum planctonicum* as a dominant in the highest (among the studied sites) large oligotrophic mountain reservoir Golyam Beglik (Table 2) can be considered as alarming for the potential decrease of its water quality. This finding is in accordance with previous observations on the enlarged spread of blue-green algae, and of their potentially toxic species in particular in our mountain reservoirs [100–102].

The phytoplankton quantitative structure revealed by the application of the HPLC marker pigment analysis combined with the use of chlorophyll *a* values as a proxy for

trophic status showed that by contrast with the summer dominance of cyanoprokaryotes in nutrient-rich waters, green and most yellow-brown, pyrrhophyte, or euglenophyte algae dominated in the oligo- to mesotrophic waterbodies (Table 2, Figures 6 and 7). Since the water quality in such waters is traditionally considered as being better, we support the use of a lack of cyanoprokaryote dominants to rapidly indicate nonproblematic water quality in the case of single, snapshot samplings. In addition, we confirm our earlier opinion [10] that in water quality assessment and relevant ecological status of the waterbodies both autochthonous and allochthonous species have to be taken into account. This comes from our current results that 53, or 7% of the recorded species were alien, newly recorded in the country. Although most of them were rare, found in single specimens, a few occurred in dominant phytoplankton complexes: the green *Tetrallantos lagerheimii* and the cyanoprokaryotes *Aphanizomenon yezoense*, *Raphidiopsis acuminato-crispa* and *R. gangetica*. Since the last three species belong to well-known cyanotoxin-producing genera [66,73], their abundant development can be problematic, ensuring their future spread in the country, as it was earlier shown for the invasive *Raphidiopsis raciborskii* [89–91] and is supported by the newly obtained data from this study on the increases of its spread and abundance in the country.

The combined LM and molecular-genetic data provided here are in accordance with our previous results on the high genetic diversity of *Microcystis* in Bulgarian waterbodies [39,40,42]. They prove its toxicity, as suggested by us earlier for the species *Microcystis novacekii* in addition to the well-known toxicity of *Microcystis aeruginosa* [39,40,42]. With the current phylogenetic tree, based on the PCR amplification of the *mcyA* gene, we are the first to provide for Bulgaria genetic data on the presence of potentially toxic *Microcystis botrys*, identified also by LM in Durankulak and Poroy, and we genetically confirmed our earlier LM finding of *Microcystis novacekii* in Mandra and Burgasko Ezero [39]. The current PCR data, based on the *anaC* gene amplification from the 2021 summer phytoplankton samples confirmed the presence and relatively broad spread of three potentially toxic *Cuspidothrix* species in our waterbodies (mainly *C. issatschenkoi*, but also *C. elenkinii* and *C. tropicalis*) recorded in 2018 and 2019 [43]. They also indicated this finding in four more waterbodies (Durankulak, Nikolovo, Studena and Yunets) and revealed a yet unidentified *Cuspidothrix* sequence in the small reservoir Mechka. The diversity and wide spread of numerous toxigenic cyanoprokaryote strains has already been stressed as alarming for Bulgarian waterbodies and their water quality ([37,38,49,85,87–89,100,101], among others).

On one hand, the high phytoplankton biodiversity associated with the great variability from site to site (reaching 198 species in Durankulak) showed the phytoplankton sensitivity to water quality, but on the other hand, it complicated the identification of indicator species for its assessment. In order to try to identify taxa that reflected particular environmental parameters, we conducted an SPSS statistical analysis [78,80]. After obtaining the first results based on 1996 records of all taxa and their relative abundance, we had to exclude all rare species, which occurred in single specimens in a single waterbody. In this way, it was possible to demonstrate different responses of the algae from different groups to the environmental variables such as nutrients (TP, TN) and chlorophyll *a* as proxy of the trophic status, water hardness and conductivity, and altitude as well. After the exclusion of some groups whose correlations were statistically insignificant, we outlined that Chrysophyceae showed a preference for a lower trophic status, Bacillariophyceae were indifferent to the water conductivity and occurred in waters of high TN, Cryptophyta preferred more soft water, Eustigmatophyceae were indifferent to the water conductivity but were significantly correlated with the increased trophic status, TP, water hardness and lowland waterbodies, and Euglenophyta preferred waters of higher trophicity and TN concentration. These results may encourage further search for bioindicators from these taxonomic groups, and this is especially valid for Eustigmatophyceae, which showed significant correlations with most variables but up to now was almost neglected in water quality assessments. Most species of this group found in this study were recorded earlier by us as commonly occurring, with an increasing abundance in the summer periods in the coastal lake Durankulak during

its ongoing eutrophication [103,104]. Although they never dominated, we believe that their increasing records and recent outlining by the SPSS analysis will sharpen the attention of phytoplanktonologists to this group.

Last, but not least, all study results strongly supported our earlier opinion about the successful application of remote vehicles in the studies of water quality based on phytoplankton diversity and its blooms in particular [38,45]. The usage of drones allowed us to quickly choose the representative sampling sites, and thus save time, efforts and fuel during the sampling process. Therefore, we strongly recommend the application of this method in future field studies related to rapid water quality assessments.

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Institutional Review Board Statement: Not applicable.

Data Availability Statement: Data sequences from this study have been deposited in the NCBI database with the following numbers.

Conflicts of Interest: The authors declare no conflict of interest.

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