

## Rare occurrence of nine *Microcystis* species (Chroococcales, Cyanobacteria) in a single lake (Lake Dojran, FYR Macedonia)

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### ABSTRACT

Investigations carried out on Lake Dojran, FYR Macedonia, during the spring-autumn seasons in 2015 have been focused on detecting the degree of eutrophication in the lake, successive algal flora changes in the plankton communities and eventual presence of cyanotoxins (free microcystins) in the water. The obtained results revealed a co-existence of nine *Microcystis* species in the lake (*M. aeruginosa*, *M. botrys*, *M. flos-aquae*, *M. ichthyoblabe*, *M. novacekii*, *M. protocystis*, *M. smithii*, *M. viridis* and *M. wesenbergii*), with domination of the pan and neo-tropical species *M. protocystis*, again confirmed in a European lake. Results also corroborate the necessity to change the accepted *morphospecies* concept into separation of *Microcystis* taxa as distinct species which are clearly delimited according to their constant morphological features. Toxicity analyses demand for a specific and targeted investigation, since the toxin production and presence depends on many factors, and the toxin dynamics including the highest peaks may be easily overlooked if other issues are in the focus of the performed monitoring. Detected values for free microcystins in the water reached 2.84 µg L<sup>-1</sup> microcystin-LR equivalents.

*Key words:* Lake Dojran; nine *Microcystis* species; *morphospecies* vs *species* concept.

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### INTRODUCTION

Dojran Lake is situated on the border between two countries, FYR Macedonia and Greece; 2/3 of the open lake waters belonging to the former while the same amount of the catchment area belongs to the later country (Fig. 1). It is considered as a remnant of a much larger tectonic/volcanic Peonic Lake (Plio-Pleistocene) (Stojanov and Micevski, 1989).

Located at 144 m asl, Dojran Lake has been labeled as a stable eutrophic lake (Stojanovski *et al.*, 1996) with intensive primary production (up to 760 g m<sup>-2</sup> as periphytic growth on *Phragmites* spp.; Stojanov, 1986), rich with various microflora and a high fish productivity – the richest lake with fish in south-east Europe. Nevertheless, due to irrigation purposes the lake has been subjected to an intensive water outlet through deepening of the former channel in Greece (Griffiths *et al.*, 2002; Zacharias *et al.*, 2002) what has produced a huge oscillation and decrease in water level which has culminated in 1993, with a total water column decrease of 7 m. This situation has forced an accelerated eutrophication process in this small and quite shallow lake that has been recorded in the chemical, biological and stable isotope records of the surface sediments (Franke *et al.*, 2013).

While the rapid decrease of water level due to abstraction combined with a period of prolonged dry conditions

(1988-1998) has devastated the lake ecosystem, the anthropogenic pressure has also intensified in form of ever increasing waste water input and agricultural diffuse pollution. Consequently, the ecological status of the lake has turned rapidly towards hypertrophy, clearly reflected and documented in its microflora communities (Stojanovski *et al.*, 1997). Namely, at the first instance in period 1988-1990 the hitherto scarce populations of the periphytic *Gloeotrichia natans* Rabenhorst ex Bornet & Flahault have exploded into mass domination in periphytic and planktic (in their later stages) communities with extensive biomass. This event has caused a rapid depletion of oxygen in the whole water column and a mass extinction of the bottom dwelling mollusks [*Dreissena presbensis* (*D. polymorpha*)], but also a massive succession in microflora assemblages towards domination of *Asterionella gracillima* (Hantzsch) Heiberg, *Dolichospermum flos-aquae* (Brébisson ex Bornet & Flahault) P.Wacklin, L.Hoffmann & J.Komárek and *Dolichospermum sigmaideum* (Nygaard) Wacklin, L.Hoffmann & Komárek (Stojanovski *et al.*, 1997). Many of detected taxa belonging to blue-green algae (like *Chroococcus minutus* (Kützinger) Nägeli, *Merismopedia glauca* (Ehrenberg) Kützinger, *M. punctata* Meyen and many others) and green algae (like various *Pediastrum*, *Scenedesmus*, *Ankistrodesmus*, *Golenkinia*, *Tetraedron*, *Crucigenia*, or desmid taxa) have disappeared from the system, while the diatom community has evidently shifted towards taxa composition in-

dicative for higher levels of trophic and salinity. Many of the persistent and alarming scientific publications and public information (Stojanovski *et al.*, 1997) or propositions for helping the Dojran Lake and minimizing the lethal effects of the forced ecocide (Stojanovski *et al.*, 1995; Krstić *et al.*, 1996) have been in vain.

For a long time, the lake Dojran has been left without any monitoring system in place and with a very doubtful attempt to bring additional water supply originating from River Vardar by means of artificial system of channels. As a result, the water level rose at 2.5 m below the optimum while the ecological parameters of the water got worse due to heavy impact of the River Vardar's highly polluted waters. The deep layer of organic mud, at places reaching to 7 m in depth, and the continuous input of untreated waste waters have resulted in dominating cyanobacterial blooms consisted of *Microcystis aeruginosa* (Kützing) Kützing, *Microcystis ichthyoblabe* (G.Kunze) Kützing, *Microcystis wesenbergii* (Komárek) Komárek ex Komárek and *Planktolyngbya contorta* (Lemmermann) Anagnostidis & Komárek, with some remnants the previous microflora like *Aphanizomenon flos-aquae* Ralfs ex Bornet & Flahault, *Dolichospermum scheremetieviae* (Elenkin) Wacklin, L.Hoffmann & Komárek, *Aphanocapsa elachista* West & G.S.West or *Pediastrum boryanum* (Turpin) Meneghini still present in very limited numbers (Krstić, 2011). At this point the dominant cyanobacterial community has produced the

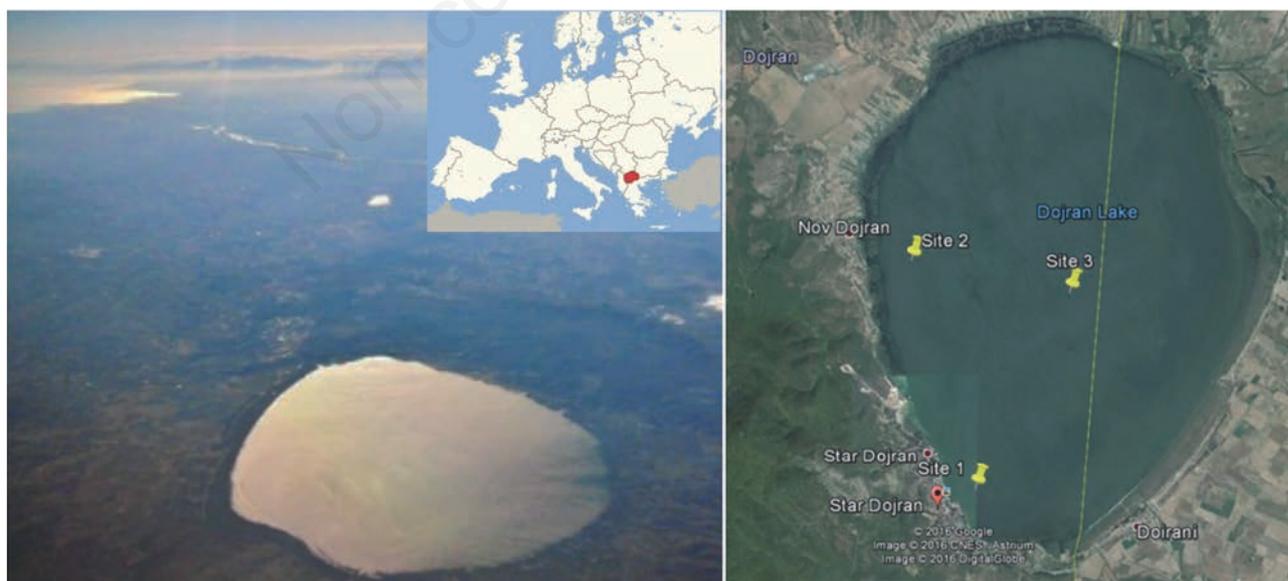
highest hitherto detected dissolved microcystins concentration in a grab water sample for FYR Macedonia detected by ELISA - 270  $\mu\text{g L}^{-1}$  microcystin-LR equivalents.

Therefore, during 2015, we aimed to investigate the intensity of the eutrophication processes and the present microalgal communities in Dojran Lake, with a special focus on the taxonomically important genus *Microcystis* and the morphological characters of the detected *Microcystis* species. The results of the determined cyanobacterial community structure, plankton species composition and successions in relation to water quality parameters, as well as the detected free microcystins concentrations in the water are presented in this study.

## METHODS

Filed sampling campaigns were conducted on 6<sup>th</sup> of May, 5<sup>th</sup> of July and 28<sup>th</sup> of September 2015, on three sampling sites (Fig. 1): two near shore locations in vicinity of the major settlements and one pelagic open water site near the border with Greece.

Field measurements included basic physicochemical parameters (temperature, pH, dissolved oxygen, oxygen saturation, conductivity) by means of portable equipment Senso Direct 150 multimeter, while the measurements of basic nutrients and chlorophyll *a* were performed according to laboratory protocol standard methods (APHA, 1998)



**Fig. 1.** Aerial photograph of Dojran Lake (41°12'N, 22°44'E). The lake water mirror, final part of River Vardar prior to confluence in Thessaloniki bay and the bay itself are visible. Locations of the sampling sites on Lake Dojran (43.1 km<sup>2</sup>, max. depth 8 m, N-S length 8.9 km, E-W width 7.1 km): Site 1: 41°10'55.67"N 22°43'46.93"E, (Star Dojran, Dock of City Beach); Site 2: 41°12'52.77"N 22°43'3.27"E (Nov Dojran, Kaldra); Site 3: 41°12'36.41"N 22°44'50.80"E (Pelagic zone, the deepest zone of the lake).

using spectrophotometer Lovibond Tintometer®. The measurement of the basic physicochemical parameters, nutrients and chlorophyll *a* was performed on an integrated water sample using Ruttner water sampler. For the measurement of dissolved nutrients, the water sample was firstly filtrated through 0.45 µm membrane filters. The maximal depth was measured with weighted marked line.

The plankton material was collected by means of a planktic net (pore size 10 µm) by slowly dragging using a motor boat, or by means of vertical column mixing. Collected material was preserved in 4% formaldehyde or in Lugol's solution for further processing in the laboratory (WHO, 1999). All plankton material was analyzed by standard light microscopy, but for better visualization of the mucilaginous envelope, the material was also stained with China Ink and analyzed in details. Microphotographs were taken both from native materials and stained colonies with Nikon eclipse E800M LM with Nikon Coolpix 4500 camera. The identification of the species was performed using standard literature (Komárek and Anagnostidis, 1998; Šejnohová and Maršálek, 2012) as well as the main report for European *Microcystis* morphospecies (Komárek and Komárková, 2002).

For the first and second campaign we have also performed toxin analyses. For this purpose, 50 mL of the integrated water sample were filtered *in situ* through glass-fiber filters 47 mm (GF/C) using vacuum filtration device. The filtrated samples were transported on cold and kept frozen at -20°C till the day of the analysis. The quantitative detection of free microcystins in the samples was performed using the Microcystins-ADDA ELISA kit from Abraxis (Product No. 520011) according to the manufacturer's instructions. All analyses were performed in triplicate. The assay was calibrated with microcystin-LR and the results were expressed as microcystin-LR equivalents.

## RESULTS

### Basic physicochemical parameters

The results obtained from the basic physicochemical analyses of the lake's water as well as the principal nutrients are presented on Tab. 1. These results clearly reflect the seasonal changes in the water chemistry and they also point out several important trends:

- The water temperature was almost constant in the whole period indicating the shallowness of the lake and the intensity of the mixing of water column, but also the favorable conditions for an intensive cyanobacteria development throughout the investigated period;
- As expected, the measurements for dissolved oxygen and pH showed an opposite trend, oxygen a tendency of depletion towards the summer months while the pH a trend of increasing towards increased alkalinity. The conductivity also had an increasing trend towards autumn;
- All of the measured nutrients showed marked increase of their concentrations towards the autumn, the total N being the most pronounced one, but total P had also very obvious increase towards summer and autumn period. These findings directly corroborate the intensive tourist pressure on the lake during summer and lack of any waste water treatment activities (also presented in the detected nitrite values);
- Chlorophyll *a* concentrations revealed two distinct peaks, one in July when the increased algal concentration was recorded in a near shore location (site 1) and the second one in September which was almost double in value, but during this month the intensive presence of significant algal biomass was recorded in all three sampling stations.

It seems that the pollution pressure was intensive, con-

**Tab. 1.** Basic physicochemical parameters of the Dojran Lake water in all three seasons and sampling sites.

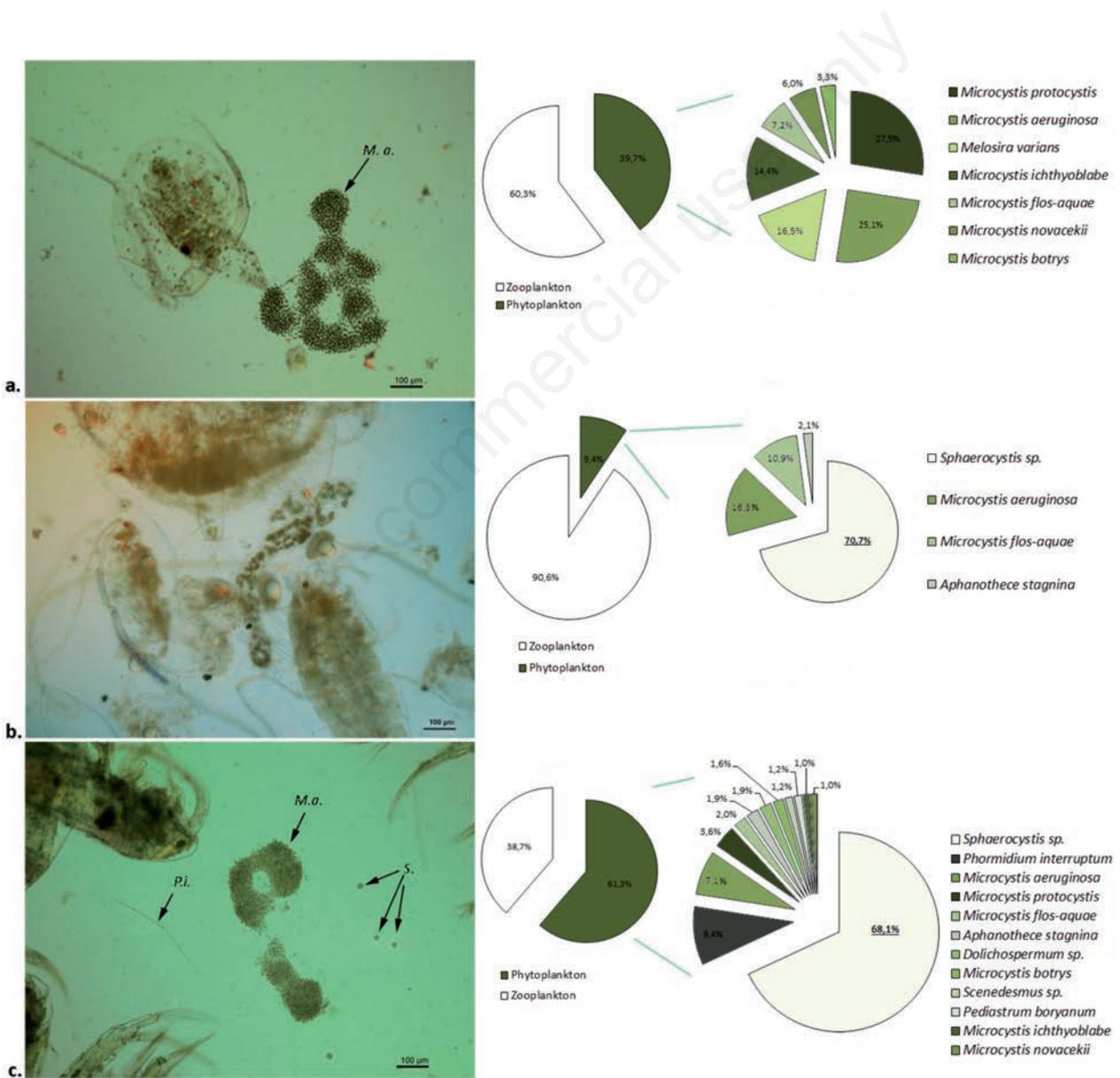
	1 <sup>st</sup> sampling (May)			2 <sup>nd</sup> sampling (July)			3 <sup>rd</sup> sampling (September)		
	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3
Maximal depth (m)	4	5.5	8	5	4.8	8	5	4	8
Temperature (°C)	22.6	24.7	22.9	24	25	24	23.4	25.6	23.5
D.O. (mg L <sup>-1</sup> )	9.4	9.2	9.2	4.5	2.5	5.7	10.7	6.1	8.1
pH	7.64	7.88	8.04	8.23	8.60	9.27	9.39	8.78	8.86
Conductivity (µS)	582	646	638	750	722	686	705	725	735
Secchi depth (m)	4	4	4	3	2.2	2.7	1.2	0.9	1.2
Chl <i>a</i> (µg L <sup>-1</sup> )	2	0.9	0.332	11.88	3.88	4.26	13.4	22.7	17.1
Total P (µg L <sup>-1</sup> )	90	150	170	120	210	200	280	380	150
Total N (µg L <sup>-1</sup> )	600	1900	800	600	6400	1100	980	730	1320
Ammonia (µg L <sup>-1</sup> )	50	20	20	60	<20	<20	140	180	50
Nitrite (µg L <sup>-1</sup> )	<10	12	<10	<10	<10	<10	19	36	<10
Nitrate (mg L <sup>-1</sup> )	<1	<1	<1	<1	<1	<1	2.5	1.5	<1
Potassium (mg L <sup>-1</sup> )	7.5	9.5	8.8	9.5	7.0	7.2	11	8.9	6

stant and prolonged during the investigated period (Tab. 1), with a clear increase of the observed parameters over the summer-autumn period. This ecological situation should have been reflected in the plankton communities and algal flora successions what has been documented in this study.

**Dynamics of plankton communities and *Microcystis* spp. dominance**

Our results concerning the planktic communities for the first sampling in May are shown in Fig. 2. On the first site, a domination of the zooplankton was recorded. The phyto-

plankton was composed of *Microcystis* species in association with *Melosira varians* C.Agardh. We did not detect any green algae. Six *Microcystis* species were determined, with co-dominance of *M. protocystis* Crow and *M. aeruginosa*. Site 2 showed almost complete domination of the zooplankton (90.6%). In the phytoplankton, high domination of *Sphaerocystis* sp. (probably *S. schroeteri*) was detected and low abundance of the genus *Microcystis*. Two *Microcystis* species were identified: *M. aeruginosa* and *M. flos-aquae* (Wittrock) Kirchner. However, we have detected domination of the phytoplankton in Site 3, with massive presence of *Sphaerocystis* sp. and low abundance of the genus *Microcystis*. Six *Microcystis* species were identified: *M.*

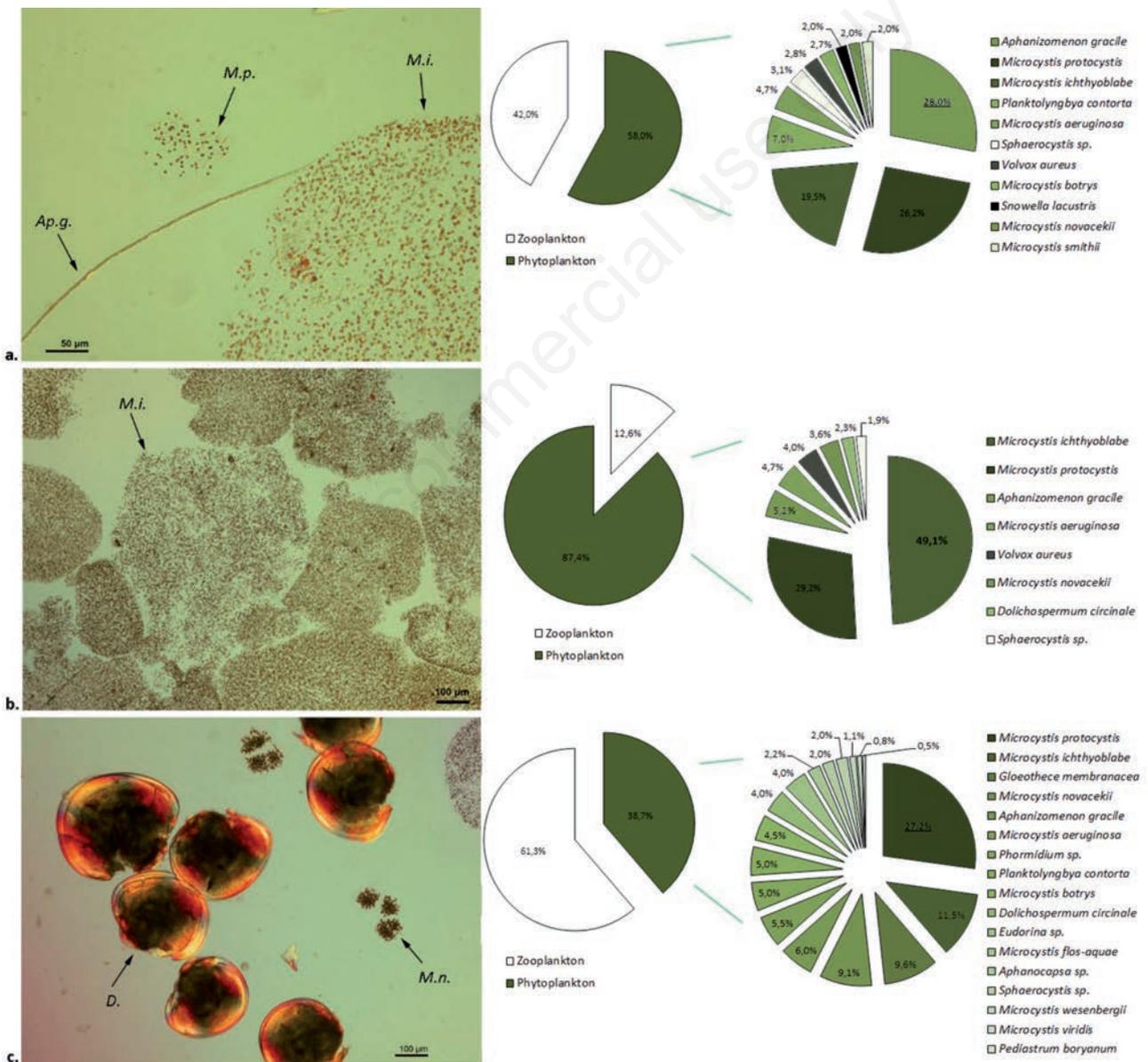


**Fig. 2.** First sampling campaign conducted in May, 2015. a) Site 1. b) Site 2. c) Site 3. M.a., *Microcystis aeruginosa*; P.i., *Phormidium interruptum*; S., *Sphaerocystis* sp.

*aeruginosa*, *M. protocystis*, *M. flos-aquae*, *M. botrys* Teiling, *M. ichthyoblabe* and *M. novacekii* (Komárek) Compère in association with *Phormidium interruptum* Kützing ex Forti, *Aphanothece stagnina* A. Braun and low presence of *Pediastrum boryanum* and *Scenedesmus* sp.

Our results from the second sampling in July are shown on Fig. 3. On Site 1 we have detected domination of the phytoplankton with co-dominance of *Aphanizomenon gracile* Lemmermann, *M. protocystis* and *M. ichthyoblabe*. Six *Microcystis* species were present on this site from which *M. aeruginosa*, *M. botrys*, *M. novacekii* and *M.*

*smithii* Komárek & Anagnostidis were present with low abundance in association with colonies of *Volvox aureus* Ehrenberg. Presence of the endogloecic *Pseudanabaena mucicola* (Naumann & Huber-Pestalozzi) Schwabe was detected in the mucilage of *Microcystis* spp. The second site (Site 2) was characterized by massive domination of the phytoplankton (completely opposite from the first sampling in May), with *M. ichthyoblabe* as clearly dominant species, with fully developed, and sometimes macroscopic (gigantic) colonies. *M. protocystis* was also well developed and the rest two *Microcystis* species (*M. aeruginosa* and

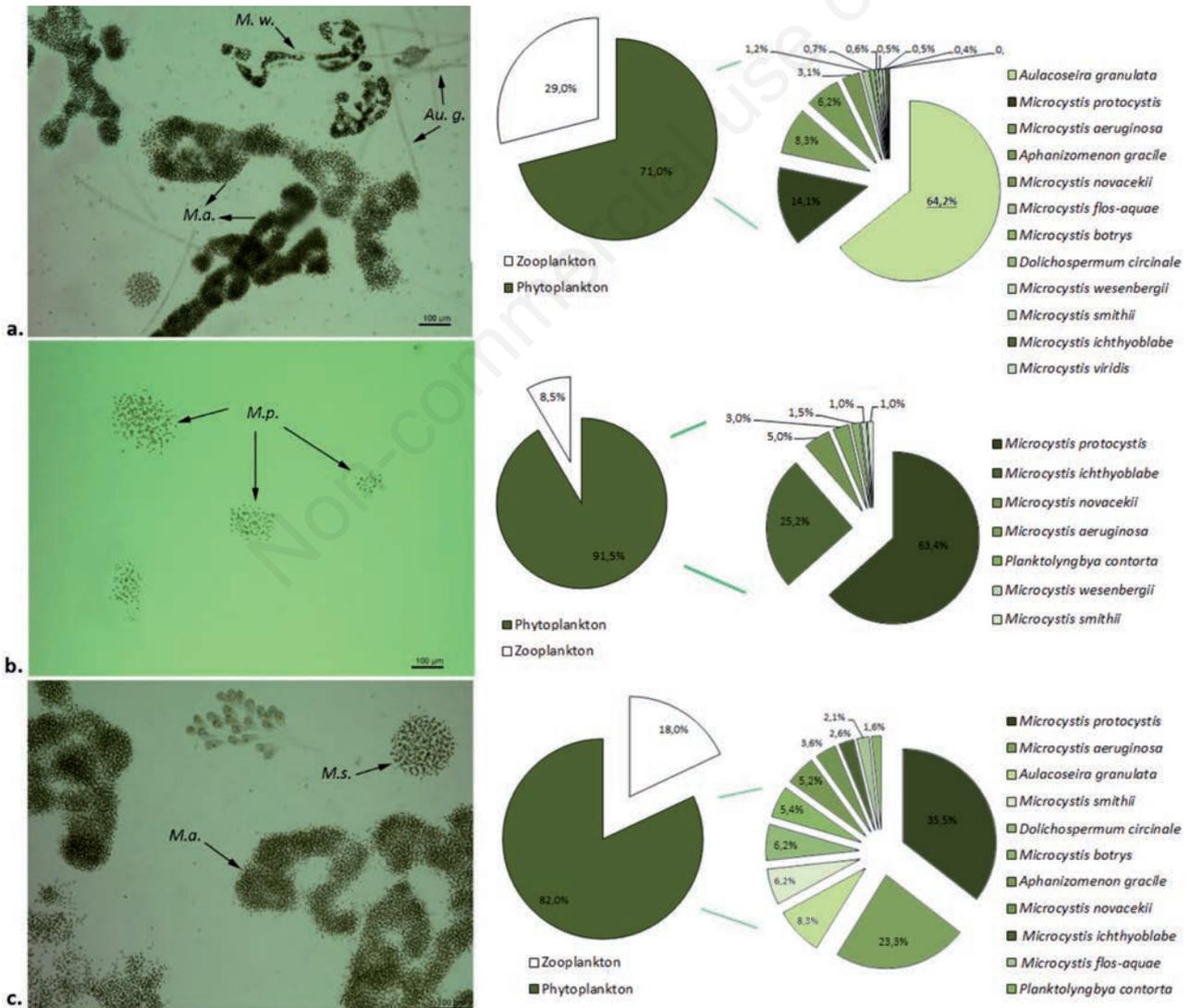


**Fig. 3.** Second sampling campaign conducted in July, 2015. a) Site 1. b) Site 2. c) Site 3. M.p., *Microcystis protocystis*; Ap.g., *Aphanizomenon gracile*; M.i., *Microcystis ichthyoblabe*; D., *Dreissena* sp. larvae; M.n., *Microcystis novacekii*.

*M. novacekii*) were poorly present. *Microcystis* species were detected in association with *Aphanizomenon gracile* and *Volvox aureus*. Our results have also shown high presence of the endogloecic *Pseudanabaena mucicola* in the mucilage of the *Microcystis* spp. However, on the third site (Site 3), we have determined clear domination of the zooplankton (61.3%), because of the massive presence of the *Dreissena* sp. larvae. The phytoplankton was not dominant, but nevertheless quite rich with cyanobacterial taxa. Eight *Microcystis* species were identified. *M. protocystis* showed dominance, with *Microcystis ichthyoblabe* as sub-dominant. The rest *Microcystis* spp. (*M. novacekii*, *M. aeruginosa*, *M. botrys*, *M. flos-aquae*, *M. wesenbergii* and *M. viridis* (A. Braun) Lemmermann) were not abundant. The detected *Microcystis* species were identified in coin-

cidence with several other cyanobacteria: *Gloeothece membranacea* (Rabenhorst) Bornet, *Aphanizomenon gracile*, *Phormidium* sp., *Planktolyngbya contorta* and *Dolichospermum circinale* (Rabenhorst ex Bornet & Flahault) P. Wacklin, L. Hoffmann & J. Komárek. The high presence of the endogloecic *Pseudanabaena mucicola* was again confirmed.

The results from the third sampling in September are presented in Fig. 4. On the first site the phytoplankton was dominant, with clear dominance of *Aulacoseira granulata* (Ehrenberg) Simonsen in association with massive presence of *Microcystis* colonies. All nine species of the genus *Microcystis* were present, with high abundance of *M. protocystis* and *M. aeruginosa* (always with big and well developed colonies). The rest *Microcystis* spp. (*M. novacekii*,



**Fig. 4.** Third sampling campaign conducted in September, 2015. a) Site 1. b) Site 2. c) Site 3. Au.g., *Aulacoseira granulata*; M.w., *Microcystis wesenbergii*; M.a., *Microcystis aeruginosa*; M.p., *Microcystis protocystis*; M.s., *Microcystis smithii*.

*M. flos-aquae*, *M. botrys*, *M. wesenbergii*, *M. smithii*, *M. ichthyoblabe* and *M. viridis*) showed low abundance. The *Microcystis* spp. were found in coincidence with *Aphanizomenon gracile* and *Dolichospermum circinale*. The second site was characterized by complete domination of the phytoplankton, in which *M. protocystis* showed clear dominance, with *M. ichthyoblabe* as a subdominant species. Six *Microcystis* spp. were present, from which *M. novacekii*, *M. aeruginosa*, *M. wesenbergii* and *M. smithii* were poorly present. Green algae and the diatom *Aulacoseira granulata* were not detected on this site. Site 3 showed domination of the phytoplankton, with massive presence of *Microcystis* colonies. Seven species of the genus *Microcystis* were present, with massive domination of *M. protocystis* and big and well developed colonies of *M. aeruginosa*. The rest of the *Microcystis* species were not present in high abundance (*M. smithii*, *M. botrys*, *M. novacekii*, *M. ichthyoblabe* and *M. flos-aquae*). The colonies of *Microcystis* were in clear association with *Aulacoseira granulata*, as well as with *Dolichospermum circinale* and *Aphanizomenon gracile*. High presence of the endogloecic *Pseudanabaena mucicola* in the mucilage of the *Microcystis* colonies was also detected in all three sites in this campus.

In all, the obtained results from this investigation showed a distinctive pattern of plankton dynamics over the sampling period. Namely, spring samplings revealed the dominance of zooplankton over phytoplankton in near shore locations, while the pelagic waters have clear dominance of phytoplankton taxa with limited presence of six *Microcystis* taxa (*M. aeruginosa*, *M. botrys*, *M. flos-aquae*, *M. ichthyoblabe*, *M. novacekii* and *M. protocystis*). Plankton communities collected in the second sampling campaign in July 2015 had a completely different structure than in May. If the dominance of *Dreissena* sp. larvae in pelagic zone is excluded (due to the period of reproduction of mussels), the plankton was fully dominated by phytoplankton communities, clearly consisted of eight

*Microcystis* taxa (*M. aeruginosa*, *M. botrys*, *M. flos-aquae*, *M. ichthyoblabe*, *M. novacekii*, *M. protocystis*, *M. viridis* and *M. wesenbergii*) where *M. ichthyoblabe* and *M. protocystis* took the dominant role, with significantly declined abundance of *Sphaerocystis* sp. Finally, in September 2015, *M. protocystis* and the well-developed colonies of *M. aeruginosa* have characterized the plankton community, with frequent observations of long *Aulacoseira granulata* chains (replacing the previously detected *Melosira varians*). All nine *Microcystis* taxa (*M. aeruginosa*, *M. botrys*, *M. flos-aquae*, *M. ichthyoblabe*, *M. novacekii*, *M. protocystis*, *M. smithii*, *M. viridis* and *M. wesenbergii*) were detected in the same time.

### Toxin analyses

The levels of free microcystins (expressed as microcystin-LR equivalents) as well as the relations between the concentration of free microcystins and the domination of species are shown on Tab. 2.

## DISCUSSION

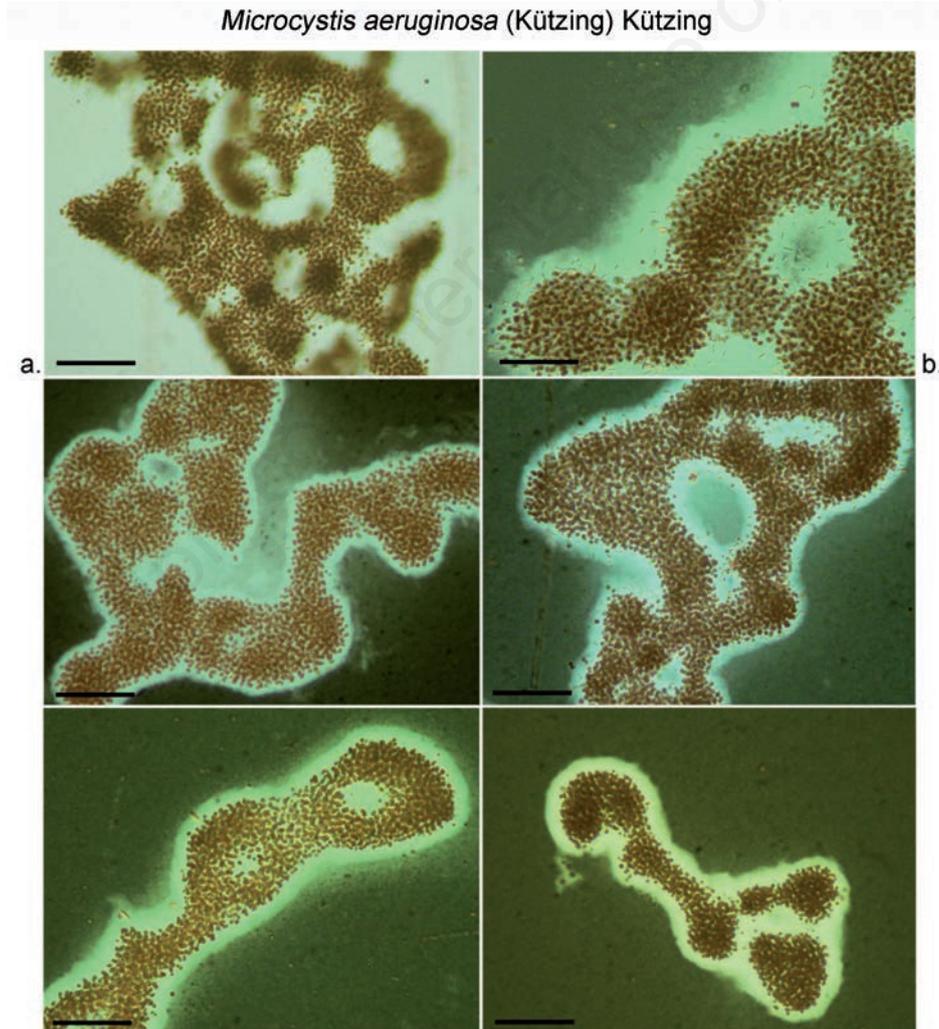
According to Komárek & Komárková (2002) and Šejnohová & Maršálek (2012), the species concept within cyanobacteria, and especially *Microcystis*, is still problematic and doubtful. The wide variations in the basic differential characteristics among species, like form of the colony, mucilage structure, cell diameter, the organization and structure of cells within the colony, pigment content and life cycles have resulted in application of the taxonomic term ‘morphospecies’ which expresses significant overlap of the limiting criteria (Cronberg & Komárek, 1994). Consequently, the numerous unidentifiable colonies, atypical stages or transient forms of *Microcystis* have resulted in a proposition that all main morphospecies (*M. aeruginosa*, *M. ichthyoblabe*, *M. viridis*, *M. no-*

**Tab. 2.** Detected values for dissolved microcystin-LR presence in Dojran Lake sites for the two sampling campaigns in 2015.

		Concentration, MC-LR equiv. ( $\mu\text{g L}^{-1}$ )	Domination of species
1 <sup>st</sup> sampling (May)	Site 1	2.8	Domination of zooplankton; in the frame of the phytoplankton co-dominance of <i>M. protocystis</i> and <i>M. aeruginosa</i>
	Site 2	1.5	Domination of zooplankton and <i>Sphaerocystis</i> , low % of <i>Microcystis</i> spp.
	Site 3	0.5	Domination of <i>Sphaerocystis</i> sp., low abundance of <i>Microcystis</i> spp.
2 <sup>nd</sup> sampling (July)	Site 1	1.3	Co-dominance of <i>Aphanizomenon gracile</i> , <i>M. protocystis</i> and <i>M. ichthyoblabe</i> . Presence of the endogloecic <i>Pseudanabaena mucicola</i>
	Site 2	0.6	Clear domination of <i>M. ichthyoblabe</i> , <i>M. protocystis</i> is subdominant. All the rest are poorly present. High presence of the endogloecic <i>Pseudanabaena mucicola</i>
	Site 3	1.5	Domination of the zooplankton (because of the massive presence of the <i>Dreissena</i> sp. larvae), but quite rich with <i>Microcystis</i> spp. <i>M. protocystis</i> shows dominance and <i>M. ichthyoblabe</i> is subdominant High presence of the endogloecic <i>Pseudanabaena mucicola</i>

*vacekii*, *M. wesenbergii*) should be taxonomically unified (Otsuka *et al.*, 2001) under the Bacteriological Code of nomenclature. Additionally, according to the results provided by molecular sequencing, a lot of *Microcystis* species were found genetically similar, so the question if all of them should be considered as one species was raised. Nevertheless, Šejnohová and Maršálek (2012) point out that studies using molecular and biochemical criteria have been proven contradictory and should be used with caution. Having obtained the presented results on *Microcystis* spp. presence and dominance in Lake Dojran, we believe that new insights can be highlighted on this controversial subject – morphospecies vs species within this cyanobacterial genus. Namely, during the in-

vestigated period we have detected fine and gradual succession of *Microcystis* taxa in this relatively small ecosystem. In general, *M. protocystis* (Plate 6) and *M. aeruginosa* (Plate 1) have dominated in spring and autumn, while in summer *M. ichthyoblabe* (Plate 4) has taken the sub-dominant position, mostly well developed in the site with the highest recorded waste water pressure (Site 2, Fig. 4). In spring, a total of six *Microcystis* taxa were recorded (*M. aeruginosa*, *M. botrys*, *M. flos-aquae*, *M. ichthyoblabe*, *M. novacekii*, *M. protocystis*), while in summer period all nine taxa were present in the lake (*M. aeruginosa*, *M. botrys*, *M. flos-aquae*, *M. ichthyoblabe*, *M. novacekii*, *M. protocystis*, *M. smithii*, *M. viridis* and *M. wesenbergii*). First appearance of *M. viridis* and *M.*

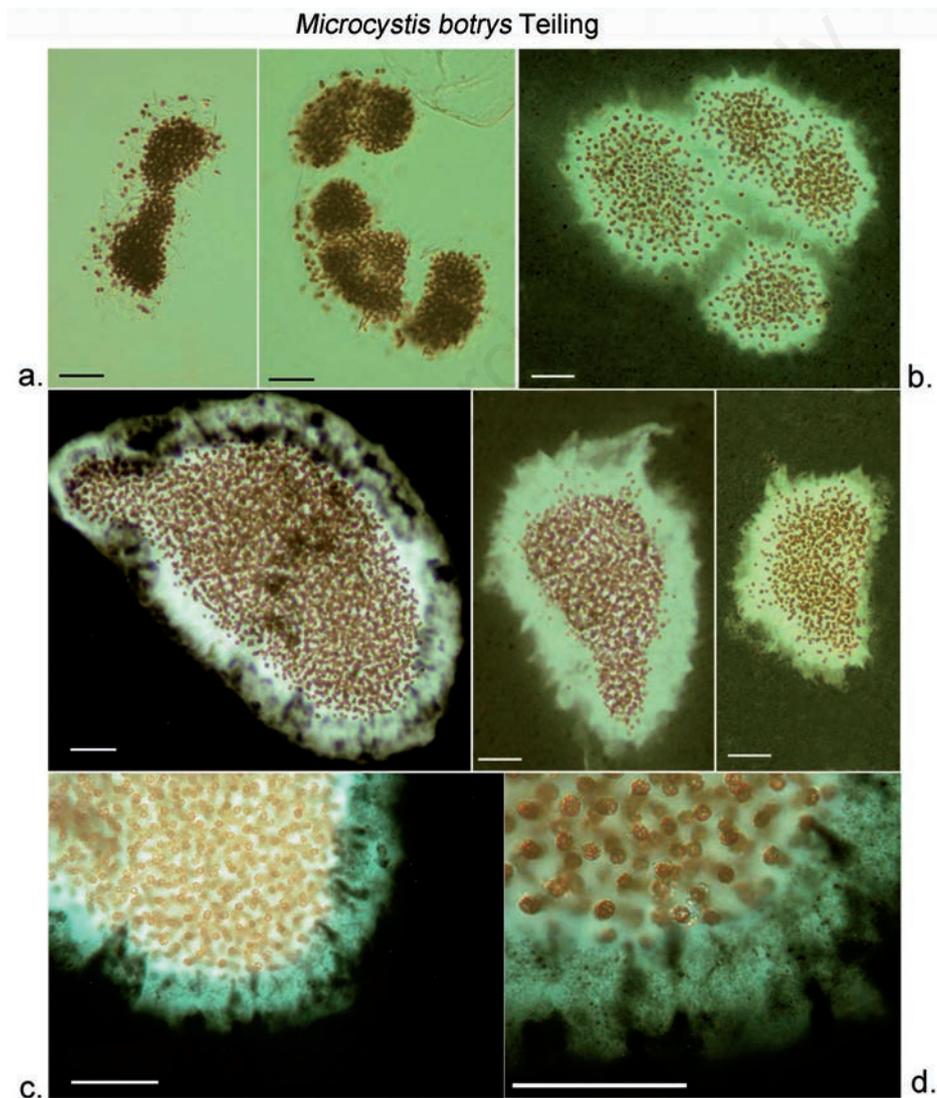


**Plate 1.** a) Colonies of *Microcystis aeruginosa* (magnification: 345x). b) Staining with China ink (magnification: 345x). Scale bars: 100  $\mu\text{m}$ . Diacritical characters: cells medium large (4-7  $\mu\text{m}$ ), green to dark green (sometimes even black because of the huge number of aerotopes), with wide mucilaginous margin around the colony. Colonies up to macroscopic, irregular in outline, lobate and with distinct holes (old colonies).

*wesenbergii* was recorded in the pelagic zone, while *M. smithii* was detected only in the littoral zone. It is important to emphasize that all of the nine detected taxa (Plates 1-9) have been recorded in their full development, meaning that all of the morphological criteria were visible and could be clearly and distinctly characterized without transition. These results inevitably lead to a conclusion that all nine detected *Microcystis* taxa have distinctive features of separate species (Komárková *et al.*, 2005) that have not been transformed over time from another morphospecies. Their ecological preferences might be slightly different

since in our study we have not detected the full development of all nine taxa until the overall domination of high eutrophic conditions and temperature during the summer period, which extended towards autumn (Tab. 1). In this context, the ecology of the different species must represent clear and quite important criterion for taxonomic classification (Komárek, 2013).

The taxonomic confusion in the line morphospecies-species within the genus *Microcystis* could have been a result of several problems associated with important biological features of this complex genus. Namely, the full

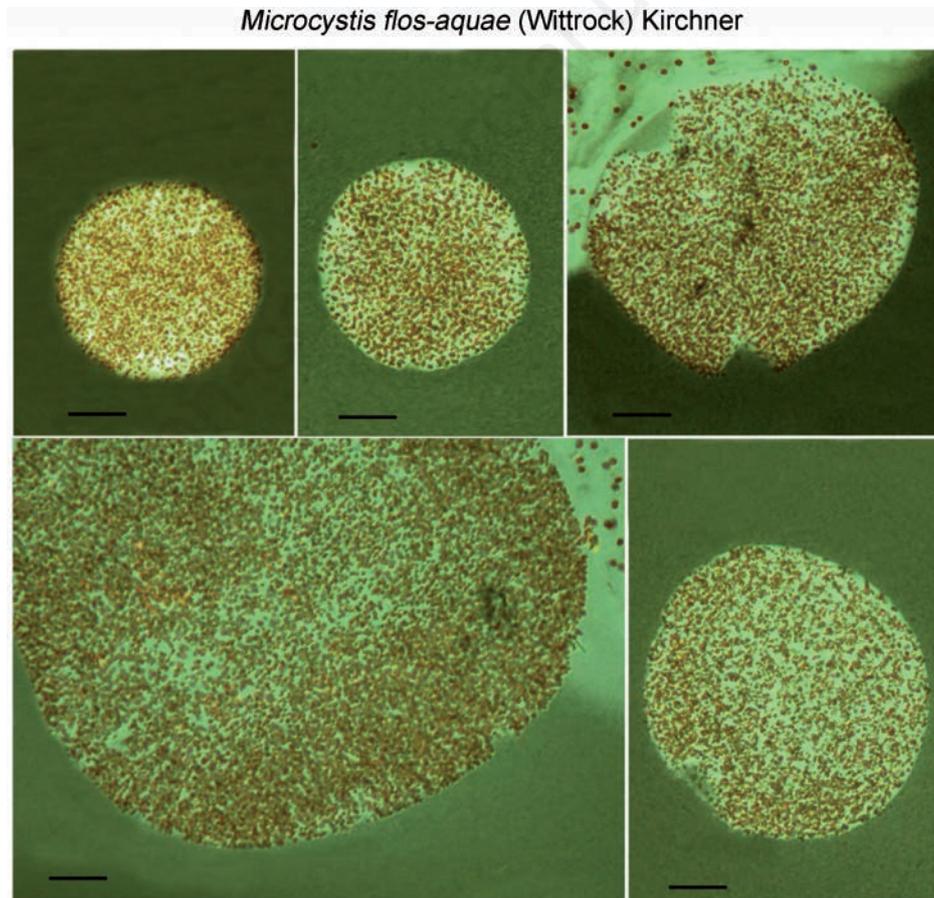


**Plate 2.** a) Colonies of *Microcystis botrys* (magnification: 345x). b) Staining with China ink (magnification: 345x). c) Detail of mucilage (magnification: 690x). d) Detail of mucilage (magnification: 1380x). Scale bars: 50 µm. Diacritical characters: cells medium large (4.9-7 µm). Colonies microscopic, spheroidal or with composed of spherical sub-colonies, never with holes, usually represented as cluster of quite densely agglomerated cells in the center with few “expulsing” solitary cells in the enveloping mucilage. Mucilaginous margin around the cell clusters quite wide, delimited and with radial semi-globose or tubular structures (staining with China ink is needed!). Diacritical character is the radial structure of the mucilage!

development of characteristic colonies might be expected after fulfillment of the optimal environmental conditions. The symbiosis with different heterotrophic bacteria seems to be detrimental for a proper development of the protective mucilage that actually determines the colony form (Xie *et al.*, 2016), which on the other hand is essential for the correct taxonomical determination of the species. The vegetation cycle (the stage of development and decomposition of the colonies) in the time of sampling might also have a critical influence on the final taxonomy. On the technical side, it is important always to use China Ink staining in order to properly identify some of the taxa (ex. *M. botrys* is frequently misinterpreted as *M. aeruginosa* – Sant’Anna *et al.*, 2004).

In summary, it seems appropriate to point out the basic morphological and eco-physiological characters that ought to be included in every taxonomical work on this genus. These embrace: i) cell size; ii) pigment content and

cell color; iii) mucilage structure, outline and margins; iv) form of the colonies; v) density and organization of the cells in the colony; vi) toxicity and production of bioactive peptides; and vii) ecological preferences; most importantly, it should be stressed that all of these characters usually appear in strict association in the defined types. For example, *Microcystis ichthyoblabe* (Plate 4) is characterized with small cells (never more than 3.5  $\mu\text{m}$  in diameter), usually more or less reddish and the margin of the mucilage never exceeds the margin of the cell clusters in the colony. Contrarily, *Microcystis aeruginosa* (Plate 1) has always larger cells (4-7  $\mu\text{m}$ ), which are green to dark green, with wide mucilaginous margin around the colony. In all, we believe that the mutual co-existence of nine *Microcystis* taxa in the same lake in their fully developed state is an important finding in regard to the controversial opinion by the molecular biologists (Otsuka *et al.*, 2001). This finding, as well as the observed diacritical characters



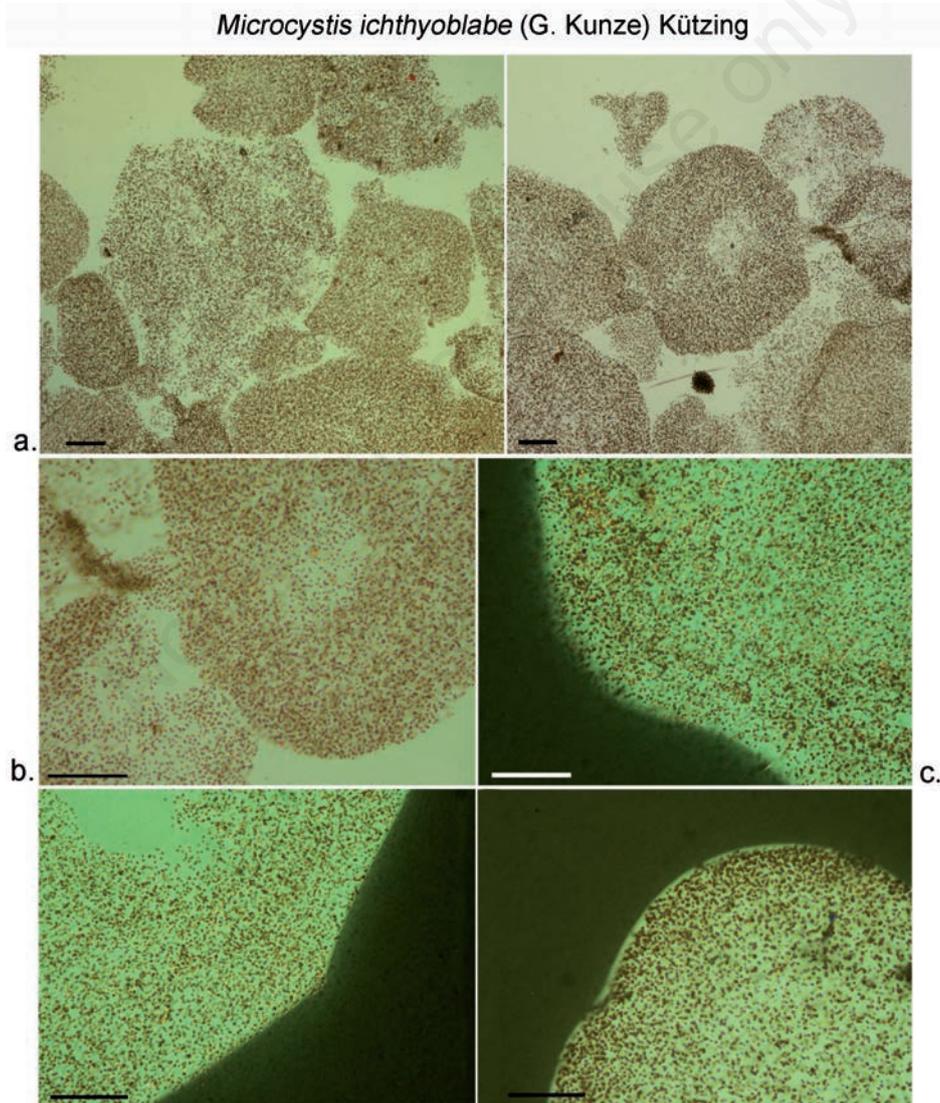
**Plate 3.** Colonies of *Microcystis flos-aquae* stained with China ink (total magnification: 345x; scale bars: 50  $\mu\text{m}$ ). Diacritical characters: cells small (3.5-4.5  $\mu\text{m}$  in diameter), usually more or less reddish. The margin of the mucilage never exceeds the margin of the cell clusters in the colony! Colonies usually microscopic and regularly spherical (in contrast with *M. ichthyoblabe*), quite compact, with very densely agglomerated cells.

that always appear in association in the defined morphotypes, should represent a strong argument against their taxonomic unification.

In the line of previous arguments, we believe that it is important to stress the co-occurrence of nine *Microcystis* morphospecies/species in one small and shallow lake, like Lake Dojran, which is quite rare according to the literature findings. For example, Šejnohová (2008) in her PhD thesis reports that eight morphospecies have been described in the whole Czech Republic. According to Loudiki *et al.* (2002), six *Microcystis* species are present in Morocco. Similarly, six *Microcystis* species were reported in São

Paulo State, Brazil, after careful and several years' biodiversity studies of planktic cyanobacteria of 14 reservoirs (Sant'Anna *et al.*, 2004). Closest to our results is the report for five *Microcystis* morphotypes (*M. aeruginosa*, *M. flos-aquae*, *M. ichthyoblabe*, *M. viridis* and *M. wesenbergii*) in Lake Taihu, China (Hu *et al.*, 2016).

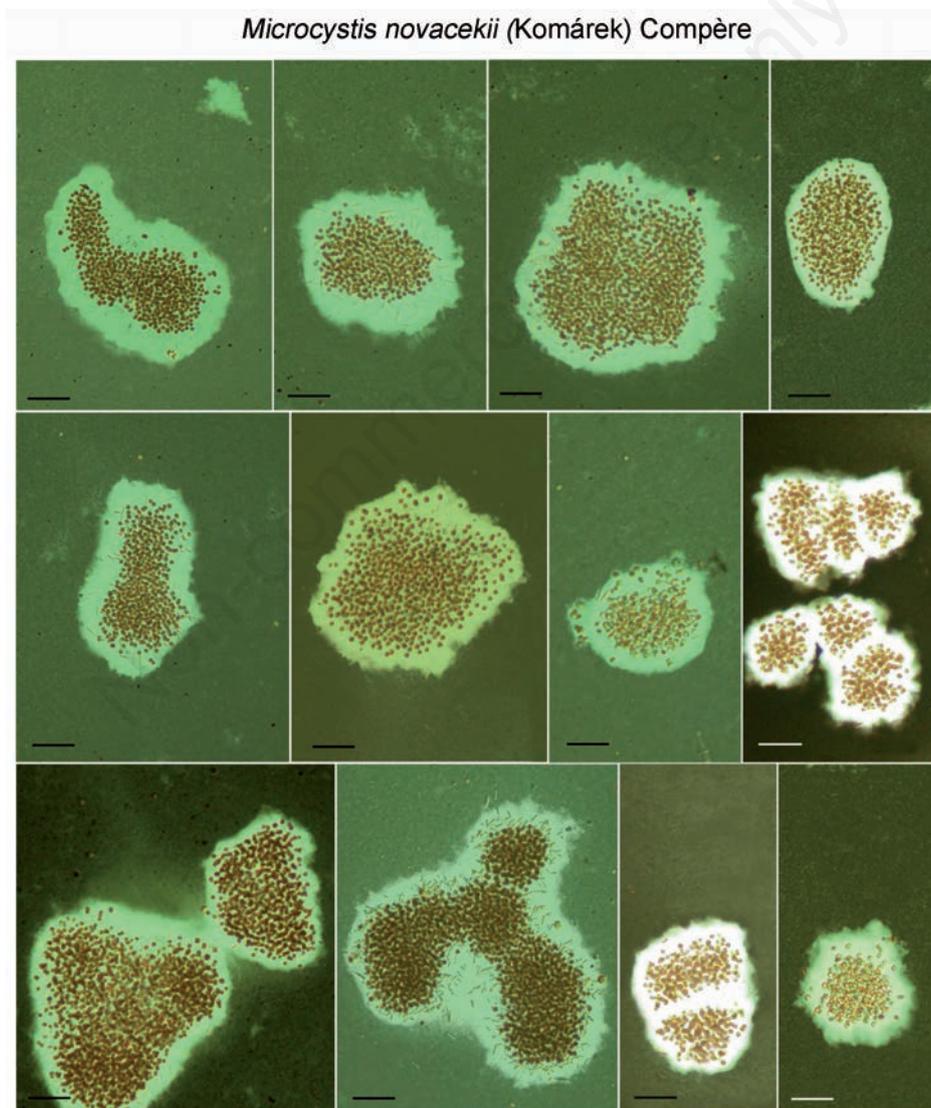
Furthermore, we consider that our results regarding the *M. protocystis* dominance in Lake Dojran deserves a special attention. In our previous paper (Krstić and Aleksovski, 2016), we have reported the presence of *M. protocystis* for the first time in a European lake (Dojran Lake). In the present study, we have performed detailed



**Plate 4.** a) Colonies of *Microcystis ichthyoblabe* (magnification: 138x). b) Colony of *Microcystis ichthyoblabe* (magnification: 345x). c) Staining with China ink (magnification: 345x). Scale bars: 100 µm. Diacritical characters: cells small (never more than 3.5 µm in diameter), usually more or less reddish. The margin of the mucilage never exceeds the margin of the cell clusters in the colony! Colonies up to macroscopic often composed from sub-colonies of cell clusters.

analyses on the same ecosystem, and the presence of this species was again confirmed with a marked dominance. *M. protocystis* was originally described by Crow (1923) and the type locality is Sri Lanka, freshwater plankton of the inland waters of Ceylon. Later, this species was characterized as pan-tropical (Komárek & Anagnostidis, 1999; Komárek & Komárková, 2002) and probably one of the commonest pan-tropical species. Till now, this species was reported as a true element of plankton in karst environments of the Yucatán peninsula, Mexico (López-Adrián & Barrientos-Medina, 2007; Tavera *et al.*, 2013) which are rich in tropical species of cyanobacteria. This

species was also reported on several localities in Brazil: in São Paulo State (Sant'Anna *et al.*, 2004), in the Cordeiro, Camalau and Acauã Reservoirs (Vasconcelos *et al.*, 2013) and in Minas Gerais State (Magalhães *et al.*, 2014). It was also found in the neo-tropical, eutrophic reservoir Riogrande II in Colombia (Palacio *et al.*, 2015), but still, up to our knowledge, it was never reported for Europe. However, in one of these papers the authors suggest that the sparse cell disposition gives *M. protocystis* the aspect of old or senescent colonies of different *Microcystis* species and maybe, for this reason, *M. protocystis* distribution should be wider than normally referred in the

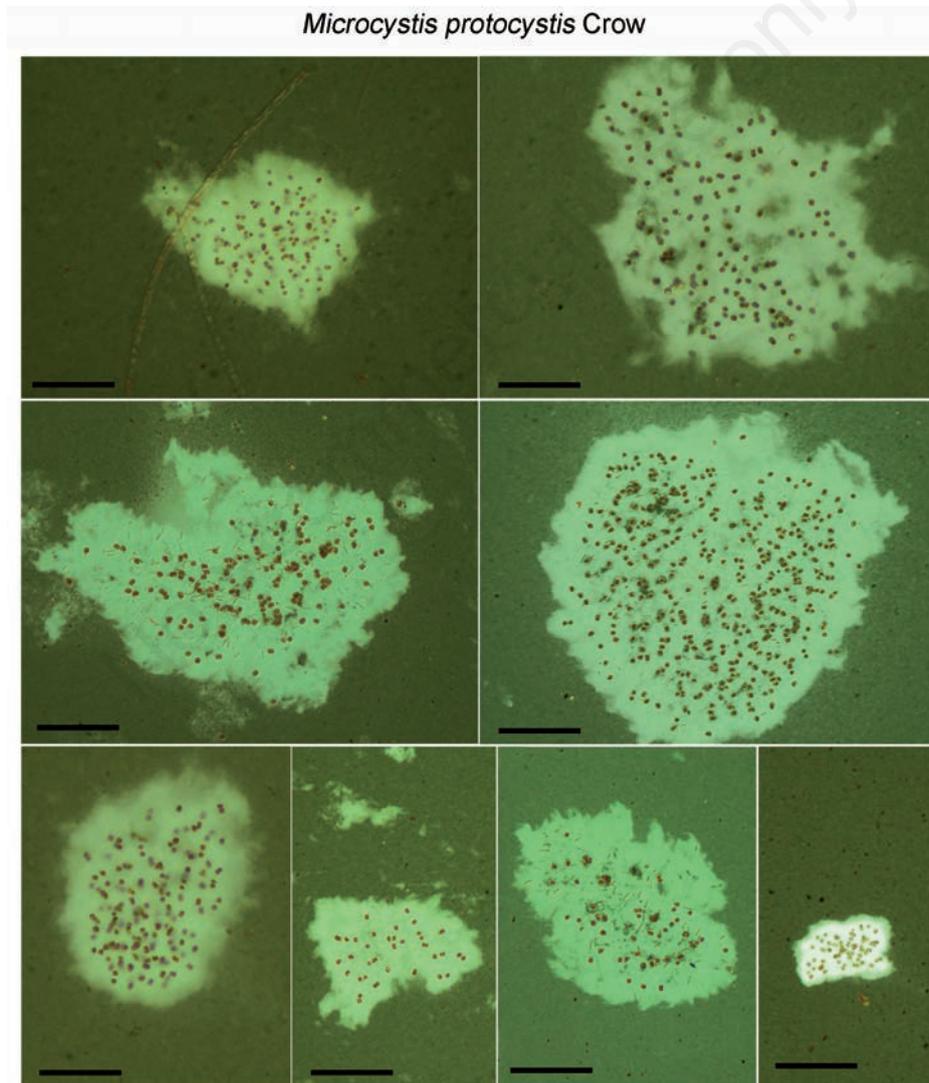


**Plate 5.** Colonies of *Microcystis novacekii* stained with China ink (total magnification: 345x; scale bars: 50  $\mu$ m). Diacritical characters: cells medium large (3-6  $\mu$ m). Colonies microscopic, spheroidal in outline, never with holes, usually represented as cluster of quite densely agglomerated cells in the center with few solitary cells in the enveloping mucilage. Mucilaginous margin around the cell clusters quite wide, delimited and concentrically lamellated (staining with China ink is needed!).

literature (Sant'Anna *et al.*, 2004). It seems obvious that Dojran Lake is experiencing the accelerated eutrophication processes (Svirčev *et al.*, 2014) due to excessive nutrient input, oxygen depletion and the intensive decomposition on the lake's bottom. Additionally, the evident dominance of this pan/neo-tropical species characteristic for Brazil, Columbia and Sri Lanka in a European lake is an important finding which might have a valuable contribution for the global climate change investigations and may be important for the future monitoring programs.

Last but not least, in the list of arguments on the morphospecies/species subject, the issue of toxicity of the specific taxa becomes both taxonomically and ecologically

very important. During our presented investigations, the focus of the research has been pointed to the taxa successions and dominance rather than on the toxicity itself (Tab. 1). The obtained values for this period are quite lower than the values reported in a previous study (Krstić, 2011) when a maximum value of  $270 \mu\text{g L}^{-1}$  has been detected. Detected values for Lake Dojran around  $2\text{-}5 \mu\text{g L}^{-1}$ , similar to the findings in our research, have been also reported in some previous studies (Papadimitriou *et al.*, 2010; Gkelis *et al.*, 2015), what underlines the continuity of predominantly microcystin group of toxins presence in the lake. These discrepancies in detected toxin levels for Lake Dojran, or any other ecosystem, would be primarily a result

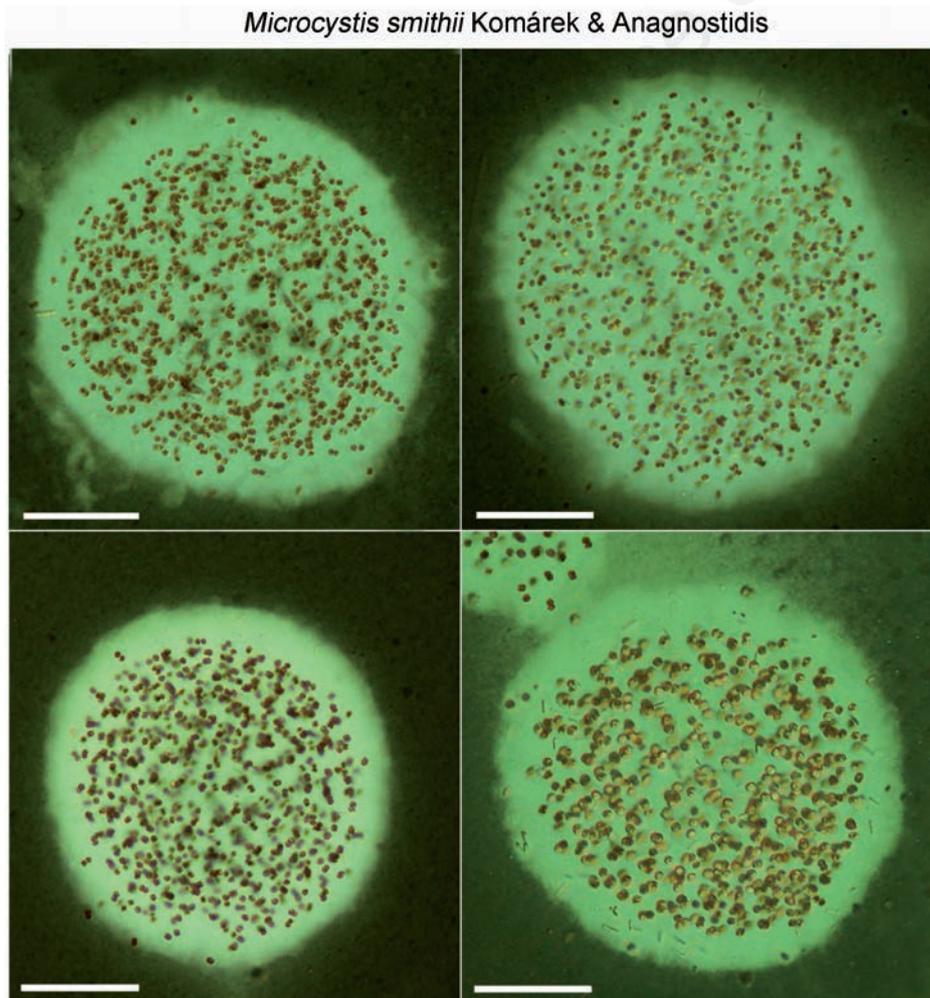


**Plate 6.** Colonies of *Microcystis protocystis* stained with China ink (total magnification: 345x; scale bars: 100  $\mu\text{m}$ ). Diacritical characters: cells medium large (3.5-6.5  $\mu\text{m}$ ) and quite loosely situated in the colony (the distances between adjacent cells is always about 2-4 times the cell diameter). Colonies microscopic, irregular (usually with indefinite nature), always without holes and well provided with mucilage. Mucilaginous margin around the colony wide and quite distinct (after staining with China ink!).

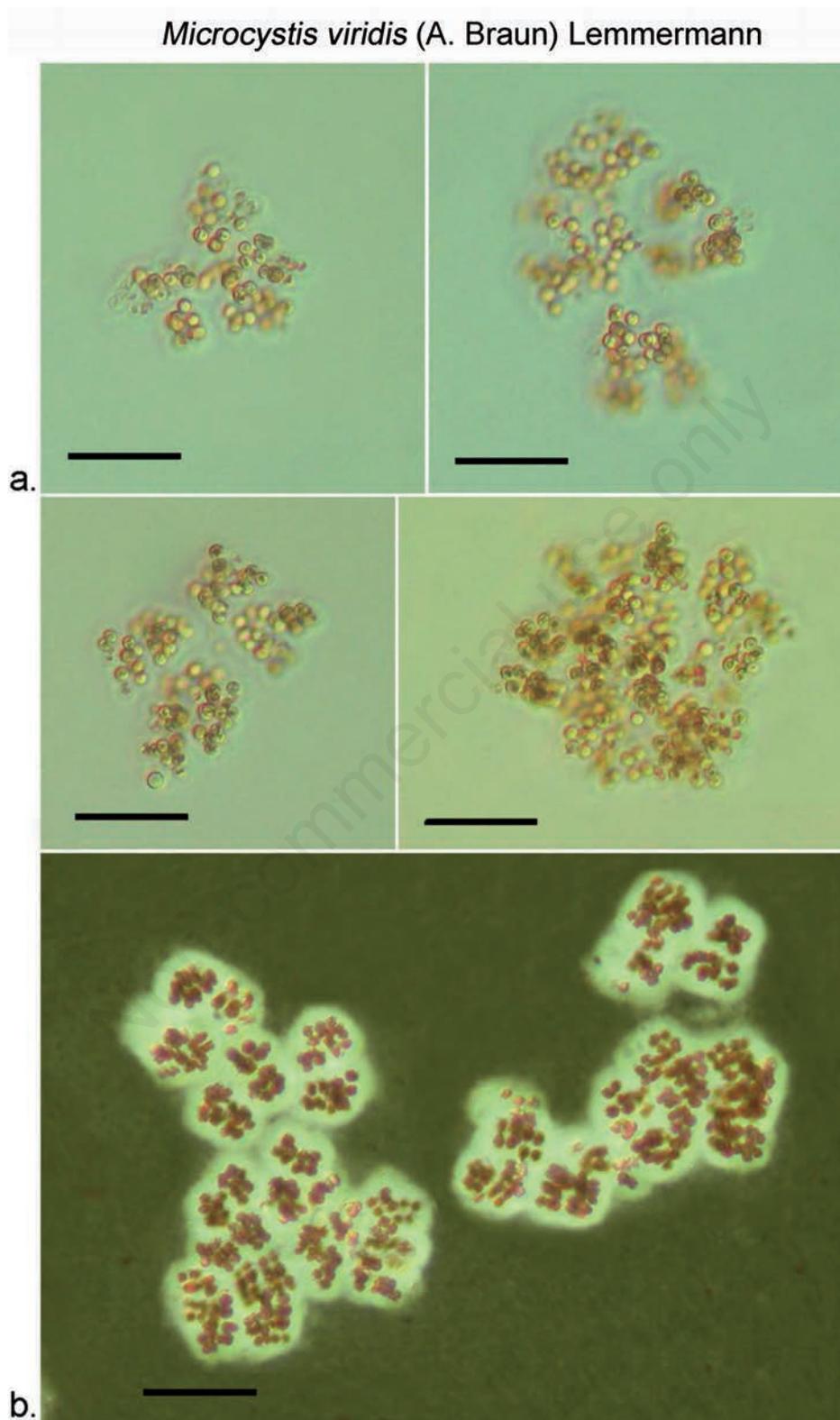
of time of sampling and analyses. As documented in this research, several *Microcystis* taxa dominance successions have been recorded over the investigated period, meaning that taxa with low/high toxin production potency have changed their dominance and consequently the final toxin (microcystins) concentration in water. Also, some of the dominant taxa like *M. protocystis*, or sub-dominant *M. ichthyoblabe*, have not been hitherto reported to produce any toxins or produce other toxins than microcystins, usually neurotoxins. Thus, the detection of toxins in any ecosystem has to be a specific scientific task which demands a toxin orientated sampling frequency, usually with much denser sampling activities, and also analyses of a broad spectrum of toxins in order to reveal the real toxin dynamics and dominance in an ecosystem. In light of pre-

vious findings (Krstic, 2011), it is possible that Dojran Lake undergoes several periods of very high toxin presence (peaks) over the year which may last for couple of days only, and then the ecosystem falls back to the basic microcystins presence of only a few  $\mu\text{g L}^{-1}$ . However, the limitations of the ELISA technique should also be considered (mostly used as a screening tool and without possibility to identify the different microcystin variants) as well as our primary focus on extracellular toxins, so future investigations with complementary methods (*e.g.*, LC-MS) are foreseen.

Having in mind the importance of previous arguments, it seems appropriate to list some of the basic toxin properties of dominant taxa detected in Dojran Lake. Also, the toxin potency and genetic ability of different taxa make



**Plate 7.** Colonies of *Microcystis smithii* stained with China ink (total magnification: 345x; scale bars: 100  $\mu\text{m}$ ). Diacritical characters: cells medium large (3.2-5.6  $\mu\text{m}$ ) and quite loosely situated in the colony (the distances between adjacent cells is always larger than the cell diameter). Colonies microscopic, regularly spherical or spheroidal and always without holes. Mucilaginous margin around the colony wide and quite distinct (after staining with China ink!).



**Plate 8.** a) Colonies of *Microcystis viridis* (magnification: 345x). b) Staining with China ink (magnification: 345x). Scale bars: 50  $\mu\text{m}$ . Diacritical characters: cells medium large (4-7.9  $\mu\text{m}$ ) and usually packed tridimensionally (almost cubic). Colonies microscopic composed of packet-like sub-colonies with not very wide mucilage, but with wavy and more or less refractive margin.

the argument on *morphospecies* even more obsolete since it is very hard to imagine that a taxon genetically equipped for a toxin production will ‘transform’ into a taxon not capable of any toxin production, or vice versa. These basic properties are:

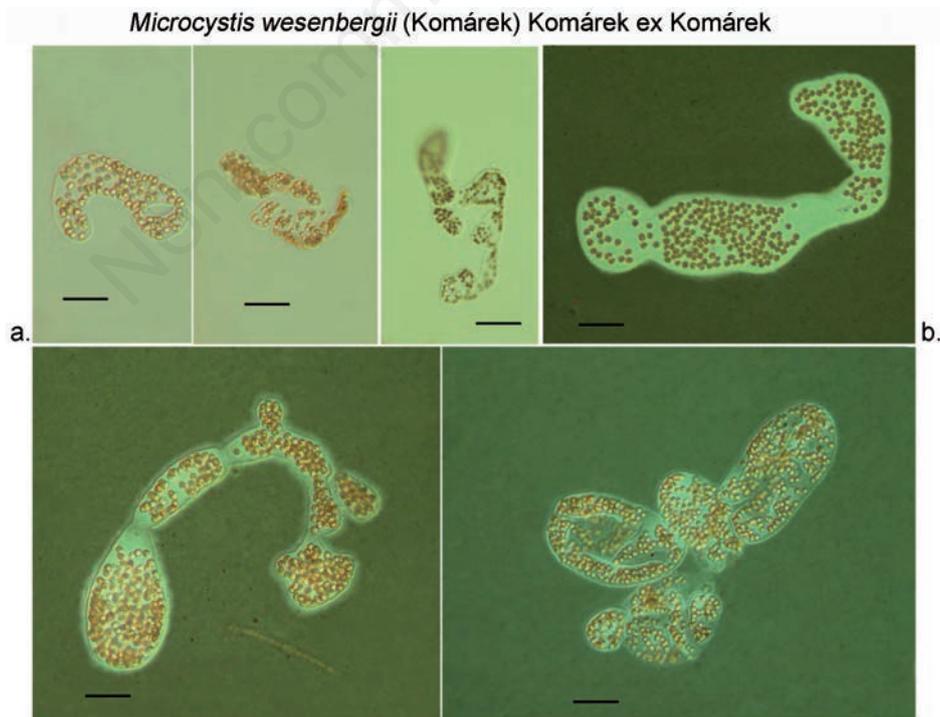
- *M. aeruginosa*: production of neurotoxins and hepatotoxins (Komárek and Anagnostidis, 1998; Komárek and Komárková, 2002), mainly microcystins, occasionally accompanied by aeruginosins (Fastner *et al.*, 2001).
- *M. flos-aquae*: production of isopropylthio-compounds, without neurotoxins (Komárek and Anagnostidis, 1998; Komárek and Komárková, 2002).
- *M. ichthyoblabe*: without ability to produce microcystins; presence of anabaenopeptins, microginins, microviridins and unknown peptides (Fastner *et al.*, 2001, Šejnohová, 2008).
- *M. novacekii*: production of specific aeruginosin and cyanopeptolins, low level of microcystins (Šejnohová, 2008).
- *M. viridis*: strong toxicity (Komárek & Komárková, 2002); production of mainly microcystins and cyanopeptolins, presence of microviridins – serine protease inhibitors (Šejnohová, 2008).
- *M. wesenbergii*: non-microcystin producing (Kurmayer *et al.*, 2002, via-Ordorika *et al.*, 2004); without PCR

product of the *mcy* cluster (Rohrlack *et al.*, 2001, Kurmayer *et al.*, 2002, Via-Ordorika *et al.*, 2004). According to Šejnohová (2008), characterized as non-peptide producing group with large cells and without production of any peptides; however, it is necessary to stress that in contradiction with these results, Fastner *et al.* (2001) report the production of cyanopeptolins by *M. wesenbergii*.

## CONCLUSIONS

In conclusion, seasonal investigations on Lake Dojran during 2015 on presence, dominance, successions and toxin production of *Microcystis* spp. have enabled the following statements:

- Lake Dojran has been confirmed as eu-hypereutrophic lake with parameters which are indicative for an increased or accelerated eutrophy over the sampling period spring-autumn;
- A total of nine *Microcystis* taxa have been observed in co-existence (*M. aeruginosa*, *M. botrys*, *M. flos-aquae*, *M. ichthyoblabe*, *M. novacekii*, *M. protocystis*, *M. smithii*, *M. viridis* and *M. wesenbergii*), which have successively changed in the dominance over the investi-



**Plate 9.** a) Colonies of *Microcystis wesenbergii* (magnification: 345x). b) Staining with China ink (magnification: 345x). Scale bars: 50  $\mu$ m. Diacritical characters: cells large (6-8.5  $\mu$ m). Colonies usually lobate or elongate, with holes when old and often composed of connected spheroidal sub-colonies, with clearly distinct refractive margin of the mucilage.

gated period. *M. protocystis*, *M. ichthyoblabe* and *M. aeruginosa* have been found as the most dominant species in the lake. This is the first report of presence of nine separate *Microcystis* taxa in a single lake, according to literature data, without transitional forms. We have confirmed the presence of *Microcystis protocystis*, a pan-tropical species, in a European lake;

- Elaborated results point out the necessity to change the accepted *morphospecies* concept into separation of *Microcystis* taxa as distinct species which are clearly delimited according to their constant morphological features. These might be a consequence of genetic constitution or a symbiosis with multiple heterotrophic bacteria. Nevertheless, the co-existence of all nine morphologically different species in a single lake strongly corroborates their distinctive species characters;
- Toxicity analyses demand for a specific and targeted investigation, since the toxin production and presence depends on many factors, and the toxin dynamics including the highest peaks may be easily overlooked if other issues are in the focus of the performed monitoring.

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