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The dominance of invasive algae *Raphidiopsis raciborskii* in lowland reservoirs in Bulgaria

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Abstract

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The aim of this publication is to warn for the presence, among the dominant algae, of blooms of the invasive *Raphidiopsis raciborskii* in six lowland reservoirs in Southeastern Bulgaria and for its increasing distribution in the country. Cyanoprokaryotic *R. raciborskii* has been broadly studied for toxicity and its invasive potential, which can seriously affect the health of the organisms and the environment they inhabit. Its increasing geographical distribution makes it a cosmopolitan species. The expansion of the range of *R. raciborskii* may be a result of the physiological plasticity, as well as the existence of different ecological types. It is known that this alga tolerates major changes in temperature and light conditions through a variety of life strategies, and the species abundance in the communities, where *R. raciborskii* is invasive, can be seriously disturbed. In the studied reservoirs, 34 Cyanoprokaryotic taxa is identified, 20 of which are dominant and at least 18 are known to be potential producers of cyanotoxins. *Raphidiopsis raciborskii* is among the most abundant algae in the studied reservoirs and in Troyan Reservoir, its blooms continue throughout the summer of 2019, with a share of total the algae biomass reaching 89.3%.

Keywords: invasive *Raphidiopsis raciborskii;* blooms; dominant algae; cyanotoxins; reservoirs

Introduction

Cyanoprokaryotes are ancient photoautotrophic microorganisms found in different habitats. They are responsible for causing blooms in water bodies and have the potential to produce cyanotoxins, that threaten the health of living organisms (Svirčev et al., 2019). *Raphidiopsis raciborskii* (Woloszynska) Aguilera, Berrendero Gómez, Kastovsky, Echenique & Salerno is a widespread species that is frequently found in freshwater around the world. Its presence causes particular concern because the strains in some geographical areas are capable of producing toxins with negative effects on human and animal health. The studies of this species have increased rapidly in recent decades, especially in the Southern hemisphere, where toxic strains predominate (Burford et al., 2016). Nowadays, is tropical species has often been reported in temperate regions (Padisak, 1997; Wiedner et al., 2002;Saker et al., 2003; Manti et al., 2005; Kokociński et al., 2010; Kokociński & Soininen, 2012; Tokodi et al., 2020). In many European countries, *R. raciborskii* is considered to be a species belonging to the group of nitrogen fixing cyanoprokaryotes. It is a typical example of an invasive species that is constantly expanding his areal, and is among the species that easily stand out as foreign to Bulgarian algal flora (Stoyneva, 2014; 2015; Stoyneva-Gärtner et al., 2017). In Bulgaria, it was first reported in the Danubian Lake Srebarna (Draganov & Stoyneva, 1992; Stoyneva, 1995; 1998a; 1998b; 2003). Later *Raphidiopsis raciborskii* was found in the coastal Lake Vaya, and the registered higher biomass values confirm the frequent presence of this invasive species in the recent years (Stoyneva, 2003; Dimitrova et al., 2014; Pavlova et al., 2015; Stoyneva-Gärtner et al., 2017).The presence of *R.*

raciborskii in some Bulgarian reservoirs is reported by Stoyanov et al., 2012; 2013. The aim of this article is to warn about the blooms and the presence among dominant algae of the invasive *Raphidiopsis raciborskii* in lowland reservoirs in Bulgaria and to analyze some of the main reasons for its widespread distribution.

Material and Methods

The study was conducted in six lowland reservoirs in the Southeastern part of the country: Troyan (TR) IBW2247, Daskal Atanasovo (DAR) IBW2219, Mechka (MR) IBW1584, Pustren (PR) IBW1828, Kirilovo 1 (KR) IBW1757 and Malazmak (MaR) IBW2407 (Michev & Stoyneva, 2007) (Figure 1). These water bodies are used as important water resources in irrigation, aquaculture and for recreational purposes. Twenty-four water samples for phytoplankton analysis were collected by Niskin-Type water sampler 5 L model (Hydro-Bios Apparatebau GmbH, Germany).Water temperature (WT, T°C), dissolved oxygen (DO) and oxygen saturation (O_2%) were measured *in situ* with an oxygen meter (WTW OXY 1970i). Electrical conductivity (Cond.) and pH were measured with WTW conductivity meter (Cond3310/SET) and WTW pH-meter (315/SET) respectively. The depth of the euphotic layer was determined by measuring the water transparency (Sd) with a 20 cm diameter Secchi disk. The phytoplankton samples were collected and processed by standard methods of fixation with formalin to final concentration 4% and further sedimentation (ISO5667-1:2006/AC:2007;ISO5667-3: 2003/AC:2007). Microscope work has been done on Bürker chamber. The species composition was determined by light microscope (Carl Zeiss, Axioscope 2 plus) with magnification 400x using standard taxonomic literature with critical use of AlgaeBase (Guiry & Guiry, 2020). The main counting unit was the cell and the biomass was estimated by the method of stereometrical approximations (Rott, 1981, Deisinger, 1984). Counting is carried out individually (cell, filament or colony). The total biomass of each sample was assessed and it was defined as the amount of biomass of all species summarized in separate taxonomic groups. Dominant species were determined according to the percentage of individual species to the total biomass.

Results and Discussion

During the study, in the summer of 2019, *R. raciborskii* is identified in six reservoirs in Bulgaria: TR, DAR, MR, PR, KR and MaR. Its presence is established in 66.7% of the samples. Representatives of the division Cyanoprokaryota dominate in the studied reservoirs, with 34 taxa identified (Table 1).

Among all samples with cyanoprokaryote dominance, only two samples make an exception. The first is from TR at the end of August, when the share of cyanoprokaryotes is

Fig. 1. Location of the studied reservoirs

29.1% from blooms of *Peridinium* sp., and the second is from KR at the end of September, with a share of 4%. Blooms of *R. raciborskii* are detected in TR throughout the study period. Its biomass varied from 26.5% to 89.3% in August. In July, *R. raciborskii* (51%) dominated with *Aphanizomenon flosaquae* Ralfs ex Bornet & Flahault (44.3%), at the end of August with *Peridinium* sp. (68.6%), and at the end of September with *Planktolyngbya limnetica* (Lemmermann) Komárková-Legnerová & Cronberg (21.3%), (Figure 2). The biomass of *R. raciborskii* ranged from 2.671 mg.l^{-1} in the end of September to 10.768 mg.l^{-1} at the beginning of July. In DAR, *R. raciborskii*is found in two samplings as a subdominant species in August with a share of the total phytoplankton biomass (6.6-6.7%). At the end of August, its maximum biomass reached 0.771mg.l^{-1}. During the same month, the main dominant species are *Dolichospermum planctonicum*

Table 1. List of identified algae from division Cyanoprokaryota

Taxon	Reservoir					
Cyanoprokaryota	TR	PR	KR	MaR	DAR	MR
Anabaena sp.			x	x		
Anabaenopsis arnoldii Aptekar	x		x			
Anabaenopsis sp.			x			
Anathece clathrata (West & G.S.West) Komárek, Kastovsky & Jezberová		x	x	xx		x
Aphanizomenon flosaquae Ralfs ex Bornet & Flahault		xx	xx	xx	x	xx
Aphanizomenon gracile Lemmermann	xx	xx	x		x	x
Aphanizomenon sp.	x		x	xx		
Aphanocapsa delicatissima West & G.S.West			x	x	x	
Aphanocapsa elachista West & G.S.West					x	
Aphanocapsa sp.			x	xx	x	
Aphanothece sp.		x				
Chroococcus turgidus(Kützing) Nägeli			x		x	x
Cuspidothrix issatschenkoi(Usachev) P.Rajaniemi, Komárek, R.Willame, P. Hrouzek, K.Kastovská, L.Hoffmann & K.Sivonen		xx	xx	xx	xx	xx
Dolichospermum flosaquae (Brébisson ex Bornet & Flahault) P.Wacklin, L.Hoffmann & J.Komárek		x	x	x		
Dolichospermum planctonicum (Brunnthaler) Wacklin, L.Hoffmann & Komárek		xx		x	xx	
Dolichospermum spiroides (Klebhan) Wacklin, L.Hoffmann & Komárek		x	xx	xx	xx	x
Gloeotrichia sp.						x
Limnococcus limneticus (Lemmermann) Komárková, Jezberová, O.Komárek & Zapomelová				x		
Merismopedia sp.	x		x			
Merismopedia tenuissima Lemmermann			x	x	xx	
Microcystis aeruginosa (Kützing) Kützing	xx	xx	x	xx	x	
Oscillatoria limosa C.Agardh ex Gomont	x	x	x	x	x	
Planktolyngbya limnetica (Lemmermann) Komárková-Legnerová & Cronberg	xx	xx	xx	xx	xx	
Planktolyngbya sp.		xx	x		x	xx
Planktothrix agardhii (Gomont) Anagnostidis & Komárek	x	xx	x	xx	xx	
Pseudanabaena catenata Lauterborn			xx	x	xx	
Pseudanabaena cf. *galeata* Böcher			x		x	
Pseudanabaena limnetica (Lemmermann) Komárek	xx	xx	xx			
Pseudanabaena mucicola (Naumann & Huber-Pestalozzi) Schwabe				xx		
Pseudanabaena sp.	xx		x	xx	x	xx
Raphidiopsis mediterranea Skuja					x	
Raphidiopsis raciborskii (Woloszynska) Aguilera, Berrendero Gómez, Kastovsky, Echenique & Salerno		xx	xx	xx	xx	xx
Rhabdoderma lineare Schmidle & Lauterborn in Schmidle					x	
Woronichinia sp.				x		

*Abbreviations:*TR – Troyan Reservoir; PR – Pustren Reservoir; KR – Kirilovo 1 Reservoir; MaR – Malazmak Reservoir; DAR – Daskal Atanasovo Reservoir; MR – Mechka Reservoir: Legend: xx – dominant species; x – common species.

(Brunnthaler) Wacklin, L.Hoffmann & Komárek (61.4%), *Dolichospermum spiroides* (Klebhan) Wacklin, L.Hoffmann & Komárek (38.3%), and *Pl. limnetica* (21%). *R. raciborskii* is identified in only one sample (36%) in MR at the end of August and dominate with low total biomass together with *Cuspidothrix issatschenkoi* P.Rajaniemi, Komárek, R.Willame, P. Hrouzek, K.Kastovská, L.Hoffmann & K.Sivonen (40%).The above-mentioned species is registered three times in PR among the dominant species biomass in July (11.5%) and twice in August (1.91; 25%).

In July, *R. raciborskii* codominated with *C. issatschenkoi* (56%), *Aph. flosaquae* (23.3%) and *Pseudanabaena limnetica* (Lemmermann) Komárek (25.4%) and again with *C. issatschenkoi* (31.9%) at the end of August. The biomass at the beginning of August reached 4.559mg.l^{-1}. During the study period, from July to September, in KR *R. raciborskii* is detected in all samples, with a share of the total algal biomass varying from 3.1 to 17.1%. In July, it codominated with *Aph. flosaquae* (46.2%) and *D. spiroides* (27.7%). In August, *R. raciborskii* is among the dominant species (11.1-17.1%), together with *Pseudanabaena catenata* Lauterborn (44.7%), *Ps. limnetica* (42.1%) and *Planktothrix agardhii* (Gomont) Anagnostidis & Komárek (33.1%). The maximum value of the biomass is established at the end of August -1.906 mg.l^{-1}. In MaR, *R. raciborskii* is identified twice in August (17.2%) and September (1%). In both cases, it is described in bloom of *P. agardhii* (33.1%; 51%), (Figure 2). The highest biomass values of *R. raciborskii* (1.935mg.l^{-1}) are registered again at the end of August.

In all studied reservoirs, during 2019, the dominant cyanoprokaryotes are 20 species and, at least 18 of them are known as potential cyanotoxins producers. The highest number of cyanoprokaryotes (25) are found in KR, (21) in MaR, and (21) in DAR. TR and MR reservoirs have the lowest species abundance, with 10 registered taxa and PR – with 15. The species *Aph. flosaquae* and *R. raciborskii*, as well as species from other 11 genera of the Cyanoprokaryota division, are more widespread in the country. The genera *Aphanizomenon*, *Cylindrospermopsis*, *Microcystis* and *Planktothrix* have often been reported as toxic bloom forming species in eutrophic waters (Stoyneva, 2015; Stoyneva-Gärtner et al., 2017). Three of the dominant species identified by us are present in the six studied reservoirs: *R. raciborskii*, *C. issatschenkoi* and *Pl. limnetica*. The species *Aph. flosaquae*, *D. spiroides*, *P. agardhii*, and *M. aeruginosa* are dominant in most samples. *Pseudanabaena limnetica*, *D. planctonicum* and *Aphanizomenon gracil*e Lemmermann are registered in at least two of the reservoirs. According to Burford et al. (2016) *R. raciborskii* has a high adaptive ability to light, which gives it an advantage in different light conditions. In temperate regions, this species develops in warm waters with temperatures above 23-25°C, and this is probably related to the development of its akinets (Padisak, 1997; Briand et al., 2002). *R. raciborskii* is known for high tolerance for temperature and light regime as part of his survival strategy (Antunes et al., 2012). Cyanoprokaryotic blooms are often associated with an external source of biogenes, and with changes in the temperature and light regime in relation to meteorological conditions (Bouvy et al., 1999; 2000).

The data of the monitored *in situ* parameters are presented in Table 2. In the studied reservoirs, the average water temperature, in the summer of 2019, is 26°C and corresponds to the optimum values, known for the development of *R. raciborskii*. In TR, where it dominated throughout the study period, the average temperature is 25.7°C. This is observed in the other studied reservoirs, with the highest temperature differences recorded in the PR. Transparency in TR is almost constant, with an average value, for the study period, of 0.48 cm, and in the other water bodies the values varied. The highest values for transparency are reported in MR, where some of the lowest biomass levels of *R. raciborskii* are found, with the lowest established in DAR and KR reservoirs (Table 2). The high temperatures and pH support the bloom of *R. raciborskii*. It develops during large changes in the electrical conductivity, and thus it is able to tolerate large differences in the ionic composition of the water. The species does not occur at maximum levels of electrical conductivity, despite his high tolerance in terms of ionic composition (Padisák, 1997). The conductivity values in TR are among the lowest found during the study, with an average of 396.8 µS.cm, which correlates with the fact that *R. raciborskii* can tolerate large changes in the ionic composition of water. The highest

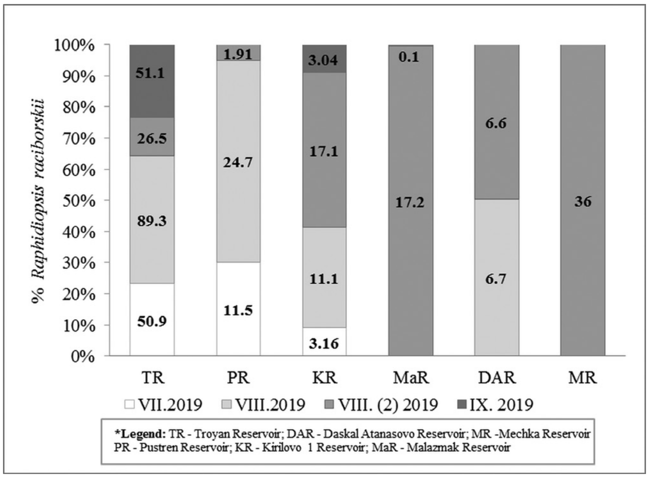

Fig. 2. Presence of *Raphidiopsis raciborskii* in the total biomass of the studied reservoirs

mean levels are recorded in KR and MaR reservoirs, where *R. raciborskii* is subdominant during blooms of *P. agardhii*.

Raphidiopsis raciborskii dominates when the pH ranges between 8.1 and 9.4 during the blooms. This confirms the fact that the carbon indicator is an important factor in its development (Bouvy et al., 1999). Bonilla et al. (2012) reported pH values of 5.49 and 9.91 with an average of 8.2 for the mass development of *R. raciborskii*. In most cases, it develops in levels of pH above 8, which confirms that this cyanoprokaryote can use inorganic carbon with high pH. According to Padisák (1997) *R. raciborskii* develops at pH of 8.0 to 8.7, with a mean value of 8.35. Minimum changes in pH were established in the TR, with an average value of 9.04. In other reservoirs, the average levels are lower (Table 2). The maximum pH values in TR confirm the data published by other authors that the high values of the pH induces blooms of *R. raciborskii*. This species can exist in many communities, without sudden environmental disruptions, and this indicates that it is adaptable to low light due to self-shadowing. Water transparency in the range of 30 cm is typical for the bloom period of *R. raciborskii* (Padisák, 1997). According to Briand et al. (2002), low levels of transparency give a competitive advantage of this species over other cyanoprokaryotes. Vidal & Kruk (2008) report that the light regime in reservoirs is more important than global factors for the development of the morphological characteristics of *R. raciborskii* during its expansion.

In MaR, the aggregated development of *R. raciborskii* and *P. agardhii* is interesting and in both cases, the latter dominates. Kokocinski et al. (2010) report that there are many known examples related to the dominance of *R. raciborskii* and *P. agardhii*, but the data on the co-blooming are few. According to the same study, despite their co-existence, they respond differently to changes in the environment. In low light and low temperatures, *P. agardhii* dominate, while *R. raciborskii* prefers high temperatures and good light. *P. agardhii* prefers dim environment with increased phosphorus concentrations, whereas the abundance of *R. raciborski*i is directly related to the ammonium nitrogen and clearer and warmer water (Kokocinski et al., 2010). The growth rates of *P. agardhii* are significantly higher than those of *R. raciborskii* at 15 and 20°C and at low light intensity, and *R. raciborskii* develops significantly faster at high light intensity and temperatures above 25°C. These two species are common in eutrophic ponds. They have common preferences for the living environment, due to this they can thrive together (Bonilla et al., 2012).

To some extent, the findings from the previous studies are also confirmed by our results. In TR, where *R. raciborskii* is most abundant, the water temperature varied within relative-

Table 2.Physicochemical parameters of water

Parameter	WT				Sd				Cond.				pH				DO				O_2			
Measure	°C				m				µS.cm								mg.l				%			
Reservoir	mean	sd	min	max	mean	sd	min	max	mean	sd	min	max	mean	sd	min	max	mean	sd	min	max	mean	sd	min	max
DAR	27.5	5.1	21	31.4	0.29	0.1	0.2	0.4	601	27.5	576	635	8.3	0.1	8.2	8.4	8.2	0.76	7.1	8.7	105	8.8	97	114
MR	24.9	2.1	24	25.3	1.5	0.3	1.2	1.9	198	0.4	189	203	8.5	0.4	8.1	8.8	6.9	0.62	6.7	7.1	85.6	12	82	88
PR	25.2	5.7	19	31.8	0.45	0.1	0.3	0.5	776	76.3	709	884	8.2	0.5	7.8	8.6	6.1	2.82	3.2	9.1	78	42	38	126
TR	25.7	4.5	20	29.2	0.48	0	0.5	0.5	397	14.1	384	413	9.0	0.1	8.9	9.2	8.6	0.75	7.6	9.4	107	15	93	123
MaR	26.3	1.9	23	27.5	0.38	0.1	0.3	0.5	814	18.7	798	838	8.1	0.3	7.7	8.4	7.0	3.84	2.7	12	85.3	44	33	141
KR	26.5	2.4	23	28.5	0.27	0.2	0.1	0.5	968	56.5	899	1015	8.5	0.3	8.2	8.9	9.2	3.65	5.7	14.1	114	40	74	167

ly wide ranges, while the transparency is almost constant. Regarding *P. agardhii*, during its intensive development in MaR, with *R. raciborskii*, the low transparency and lower temperatures at which *P. agardhii* dominates the community should be noted. A similar dependence is found in late August in KR, where *P. agardhii* is more abundant, at very low light and high temperatures, than *R. raciborskii*.

The ability of cyanoprokaryotes to fix atmospheric nitrogen is a key factor explaining their dominance in freshwater ponds with low nitrogen levels. Although *R. raciborskii* has this ability, some studies question whether this is the cause of its blooms (Moisander et al., 2012). Others report that it is able to use different sources of nitrogen, such as ammonium, nitrate or urea, with preference to ammonium (Saker & Neilan, 2001; Ammar et al., 2014; Stucken et al., 2014). The reaction of *R. raciborskii* to nutrients proves that its competitive advantage lies in its ability to cope with low levels of N and P, and not so much to dominate in conditions with high concentrations of these elements. On the other hand phosphorus is an important factor in its abundant development. It has been found that this species can dominate in waters where dissolved phosphorus concentrations are below the acceptable limits (Padisak, 1997; Istvanovics et al., 2000; Burford et al., 2006). From the conducted study, except the results of the *in situ* physicochemical parameters, we do not have data on the seasonal dynamics of the nutrient in the studied reservoirs. The availability of this information would help to better understand the importance of N and P for the life strategies of the dominance of *R. raciborskii* in the communities in different reservoirs.

Eutrophication accelerates the expansion of the range of *R. raciborskii*, which is a major producer of cylindrospermopsin and saxitoxin in the tropical and subtropical regions. So far, despite numerous studies, there is no European strain of *R. raciborskii* is found, capable to producing toxins (Kokocinski et al., 2017; 2019; Rzymski et al., 2014; 2017a, b; 2018; Falfushynska et al., 2019). The last study of the presence of cyanotoxins and their causative agents in the phytoplankton of different water bodies in Bulgaria, reported that investigated strains of *Raphidiopsis raciborskii* belongs to the non-toxic strains of the European population (Stefanova et al., 2020).

Conclusions

The overall dominance, established in our study in 2019 of six reservoirs and 34 taxa cyanoprokaryotes, of which at least half are known as potential producers of cyanotoxins, confirms their eutrophication. *Raphidiopsis raciborskii* is among the most abundant algae in the studied reservoirs. In Troyan Reservoir, the dominance of the species continues throughout the summer of 2019. This publication warns for the increasing distribution of the dominant invasive species *R. raciborskii* and attempts to analyze some of the main environmental factors, leading to its spread in the lowland reservoirs in the country in recent years. Until now, the negative results regarding the possibility of *R. raciborskii* to produce cyanotoxins in the European region and in the country are to some extent encouraging, but this fact should not prevent us from conducting further research on *R. raciborskii* ecology, life strategies and the main factors contributing for its distribution.

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