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Pilot application of drone observations and pigment marker detection by HPLC in studies of cyanobacterial harmful algal blooms in Bulgarian inland waters

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Abstract. This paper describes the first use of aerial observations by a drone as an additional means for choosing sampling points during field studies of cyanobacterial harmful algal blooms (CyanoHABs) in selected Bulgarian waterbodies and the use of HPLC analysis of marker pigments for the fast determination of phytoplankton composition and biomass. The selection of waterbodies was based on the authors' personal expertise and data collected over a 25-year period. In all sites chosen by drone, there were high levels of cyanobacteria and cyanotoxins were present: microcystins (MC-LR, MC-RR, MC-YR in Durankulak Lake and MC-LR and MC-RR in the Sinyata Reka Reservoir), cylindrospermopsin (in the Vaya Lake and in the Mandra Reservoir) and saxitoxins (in Durankulak Lake). The finding of cylindrospermopsin is the first in Bulgaria, the detection of saxitoxins is the first for Durankulak Lake and the microcystins records are the first for Sinyata Reka Reservoir. Considering the high total number of wetlands in Bulgaria, many of which are lowland, small and shallow and therefore vulnerable to CyanoHABs, we recommend further use of drones and HPLC in monitoring, which should speed up detection and reduce sampling efforts while enabling valuable information to be gathered.

Additional keywords: cyanobacteria, cyanoprokaryotes, cyanotoxins, cylindrospermopsin, microcystins, phytoplankton, saxitoxins.

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Introduction

The phylum Cyanoprokaryota (Cyanobacteria), known also as blue–green algae, represents a group of peculiar prokaryotic phototrophs without developed plastids that are widespread in all types of aquatic and aeroterrestrial habitats (Graham *et al.* 2009; Whitton and Potts 2012). Some cyanoprokaryotes are hazardous, causing environmental and human health problems during their mass development, which is commonly referred to as a blooms (Paerl 2001; Merel *et al.* 2013). Their negative effects are due mostly to the production of strain-specific, gene-controlled toxic metabolites (cyanotoxins), which have adverse acute or chronic health effects on mammals (including humans) and other organisms (including plants; Codd *et al.* 1989, 1999, 2005*a*, 2005*b*; Carmichael 1994; Codd 1995; World Health Organization 1998; Chorus and Bartram 1999; Maršálek *et al.* 2000; Meriluoto and Codd 2005; Mitrovic *et al.* 2005; Metcalf and Codd 2012; Merel *et al.* 2013; Walker 2015; Liyanage *et al.* 2016). Therefore, the common term 'harmful algal blooms' (HABs) is used for blooms dominated by cyanoprokaryotes (abbreviated as CyanoHABs, C-HBs or cHAbs; e.g. Paerl *et al.* 2011; Carmichael and Boyer 2016). These blooms are perceived as one of the most dangerous threats for our future, ironically referred to as the 'blue–green future' (Elliott 2012).

Despite this general knowledge, which has high public recognition, and despite considerable advances in the detection and analysis of cyanotoxins, with vast amounts of data collected, numerous problems still remain. A lot of work has to be done to discover and better understand the taxonomy and biology of different causative agents, toxins and toxic effects, toxin cell quotas, all driving forces, risk assessments and the socioeconomic and ecological costs of CyanoHABs, which are largely unmeasured (e.g. Roelke and Buyukates 2001; Van Dolah *et al.* 2001; Mowe *et al.* 2015; Carmichael and Boyer 2016).

At the same time, the scientific tools, devices and methods used in CyanoHAB studies are constantly improving. HPLC determination of marker pigments is a useful tool for the rapid assessment of phytoplankton biomass and composition at the phylum and class levels (e.g. Jeffrey *et al.* 1997; Wright and Jeffrey 2006). Originally developed in marine studies, HPLC determination of marker pigments has been successfully used in studies of estuaries (e.g. Paerl *et al.* 2003) and fresh waters, in ecological studies (e.g. Descy *et al.* 2000; Schlüter *et al.* 2006) and, in combination with microscopic assessment of dominant taxa and determination of functional groups (Reynolds *et al.* 2002), to assess lake status (Sarmento and Descy 2008) and estimate cyanobacterial biomass (Descy 2017). An example of the successful application of the pigment technique for the quantification of cyanoprokaryotes and cyanobacteria can be found in Van Wichelen *et al.* (2010), where cyanobacterial contribution as determined by HPLC with CHEMTAX software (CSIRO Marine Laboratories, Hobart, Tas., Australia; Mackey *et al.* 1996) was well correlated with *Microcystis* spp. biomass in a hypertrophic lake. Recently, studies of CyanoHABs, as well as the monitoring and removal and wetland assessments of CyanoHABs, have made use of available optical remote sensing, geographic information system (GIS)-based methods and modern field sampling devices (e.g. Gons *et al.* 2005; Williams 2014; Boon *et al.* 2016; Jung *et al.* 2017; Ragueno *et al.* 2017). However, no single method seems sufficient for the accurate monitoring of blooms. It is broadly accepted that all approaches need to be tailored for specific waterbodies using methods based on economic feasibility, speed, sensitivity and field applicability, with an emphasis on early-warning systems for the detection of toxigenic algal populations (Codd *et al.* 2005*b*; Srivastava *et al.* 2013). Beyond doubt, the timing and speed of the invention, development and application of these techniques differs and is primarily related to the economic development of the affected countries.

Bulgaria is an Eastern European country with a temperate climate, positioned in a biodiversity hot spot of the Balkan peninsula, with numerous waterbodies, including important drinking water reservoirs, recreational sites and protected areas (Michev and Stoyneva 2007). Although Bulgaria has a high number of waterbodies (~8900), they are small and their surface area of ~112 000 ha covers less than 0.1% of the entire country (Michev and Stoyneva 2007). The waterbodies in Bulgaria are situated primarily in the lowlands (Michev and Stoyneva 2007). Most are shallow and therefore quite vulnerable to human impact caused primarily by strong agricultural activities in the country; 340 are included in the Red List of Bulgarian wetlands (Michev and Stoyneva 2007). Algological studies in Bulgaria started at the end of 19th century (Petkoff 1898) and, since the first decades of the 20th century, blooms of blue–green algae were identified in different waterbodies with suggestions as to their toxic character (Stoyneva 2014; Descy *et al.* 2018; Dimitrova *et al.* 2018 and references therein). Since the beginning of the 21st century, 61 of the 115 waterbodies studied have been found to be susceptible to CyanoHABs, with more than 42 toxin-producing taxa found, in addition to the detection of microcystins, nodularins and saxitoxins by HPLC, ELISA and cytotoxicological tests (Stoyneva-Gärtner *et al.* 2017; Descy *et al.* 2018). All these results were based on standard phytoplankton sampling and conventional microscopy, and indicated the need for further permanent monitoring and studies based on modern sampling and research methods (Stoyneva-Gärtner *et al.* 2017; Descy *et al.* 2018). Therefore, the aim of the present study was to use aerial observations from a drone equipped with a camera as an additional way of choosing sampling sites during field studies of CyanoHABs in Bulgarian inland waters, in combination with HPLC analysis of marker pigments for fast determination of phytoplankton.

Materials and methods

The study was conducted from 20 to 27 June 2018 in nine shallow (mean depth 0.5–2 m) lowland waterbodies, situated in central and eastern Bulgaria (Fig. 1; Table 1). This early-summer period was chosen because of an atypical extremely dry and warm spring in the country (with temperatures up to 31°C in April and May). However, unexpectedly, the sampling days were preceded by strong rainfalls and floods along the Black Sea coast. The waterbodies and the main sampling sites were chosen according to all results from previous studies, which indicated a threat of CyanoHABs, as summarised by Stoyneva-Gärtner *et al.* (2017) and Descy *et al.* (2018) and based on 25 years of expertise of the authors in studies on phytoplankton and cyanotoxins in most of these waterbodies (Stoyneva 2000*a*, 2000*b*, 2003, 2014, 2016; Pavlova *et al.* 2006, 2007, 2014; Dimitrova *et al.* 2014*a*, 2014*b*; Stoyneva *et al.* 2015; Stoyneva-Gärtner *et al.* 2017). Detailed descriptions of the morphometry, historical development, use, conservation status and biodiversity of each of the waterbodies are provided in the Database of Bulgarian wetlands in the Inventory of Bulgarian wetlands and their biodiversity (Michev and Stoyneva 2007). Table 1 provides the unique inventory number for each waterbody from this database (IBWXXXX).

After reaching the target site at the shore of the chosen waterbody, before sampling a drone equipped with a camera was sent to observe and document the whole waterbody and possible hot spots indicated by colour differences. The drone used was DJI Mavic Pro (Model M1P GL200A; SZ DJI Technology, Shenzhen, PR China). The records were stored as photographs and videos. Decisions were made on the basis of aerial photographs obtained by remote sensing without any attempt to correct data for surface reflection etc. Spots or areas of different colour were chosen for sampling (Fig. 2) or, in case of visible water homogeneity, sampling was done at the same sites as in previous studies (Stoyneva 2000*a*, 2000*b*, 2003, 2014; Pavlova *et al.* 2006, 2007, 2013, 2014, 2015; Dimitrova *et al.* 2014*a*, 2014*b*; Stoyneva *et al.* 2015; Stoyneva-Gärtner *et al.* 2017). All sampling sites were reached by inflatable boat, with engine and oars, used according to site characteristics.

Water transparency was measured using a Secchi disc (in accordance with the requirements of Bulgarian monitoring legislation: State Order for characterisation of the surface waters of the Minister of Environment and Waters of Bulgaria N 4/14.09.2012). The site coordinates, altitude, water temperature, pH, dissolved oxygen (DO), total dissolved solids (TDS) and conductivity were measured *in situ* using an Aquameter AM-200 and Aquaprobe AP-2000 (Aquaread Water Monitoring Instruments, Broadstairs, UK).

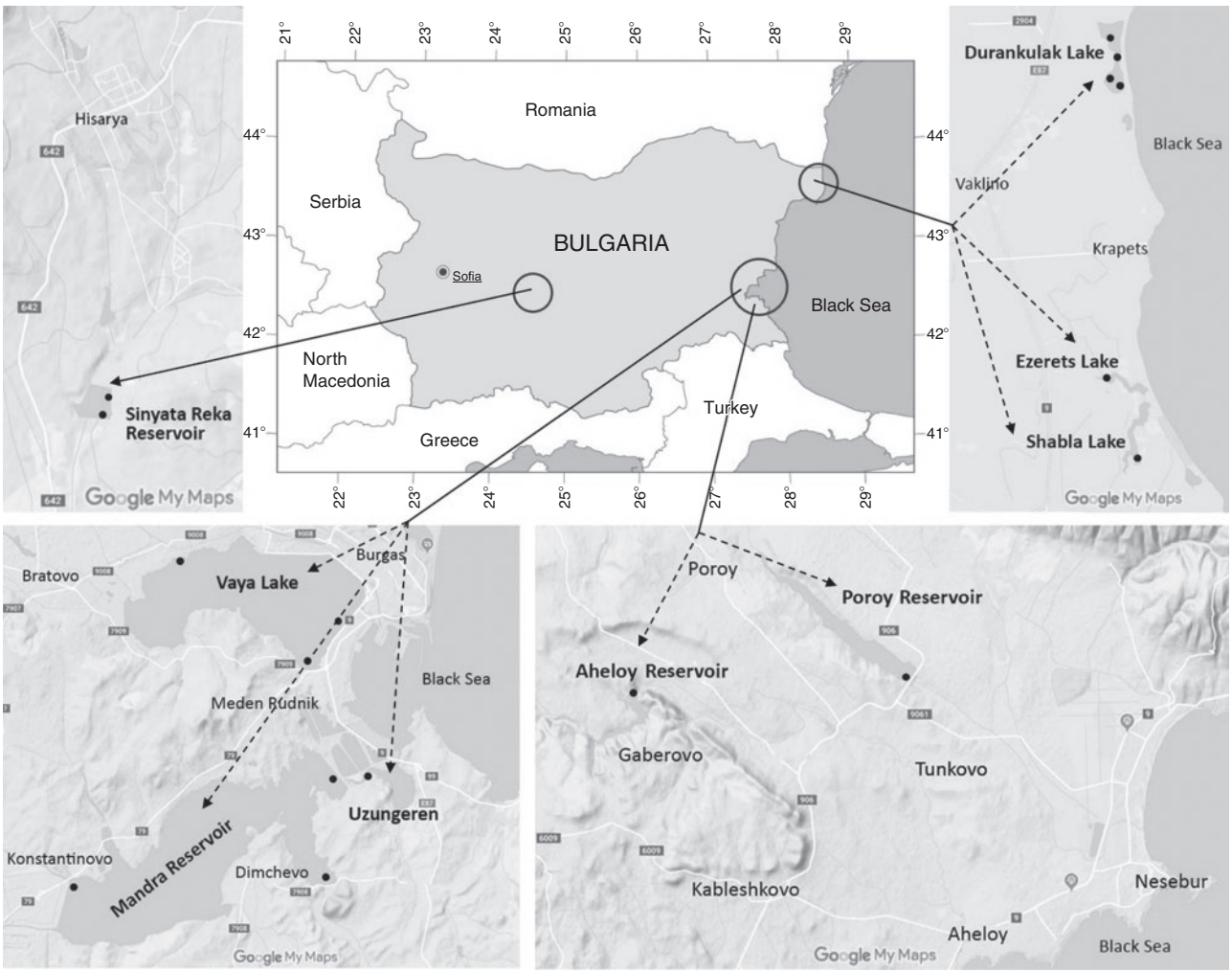

Fig. 1. Map of Bulgaria showing the sampling sites (modified after http://www.ginkgomaps.com, accessed 28 September 2018).

Total nitrogen (TN) and total phosphorus (TP) were measured *ex situ* using an Aqualytic AL410 Photometer from (AQUALYTIC, Dortmund, Germany). The TN : TP ratio was used to assess nutrient limitation according to Forsberg *et al.* (1978) and Overbeck (1988), where values above 12 indicate P limitation, values below 7 indicate N limitation and values between 7 and 12 indicate that either of the nutrients may be limiting.

Following the basic guidelines for the detection and monitoring of toxic cyanobacteria (Salmaso *et al.* 2017), HPLC analysis was used to estimate biomass by marker pigment analysis, as described in detail by Descy (2017, SOP5). Water samples were filtered through Macherey-Nagel GF5 filters (porosity 0.7 μm, Macherey-Nagel GmbH & Co. KG, Düren, Germany) and placed in 8 mL of 90% acetone (HPLC grade) in centrifugation tubes. For pigment extraction, samples were subjected to two 15-min periods of sonication (in a sonication bath containing melting ice) separated by overnight incubation at 4°C. Pigments were analysed using a Waters (Milford, MA, USA) HPLC system equipped with diode array detection. Calibration was made using commercial external standards of carotenoids and chlorophylls (DHI, Hoersholm, Denmark). Chromatograms were processed and quantified using Empower software (Sean O'Sullivan, Otago University, Auckland, New Zealand) and pigment concentrations were processed using CHEMTAX software (CSIRO Marine Laboratories; Mackey *et al.* 1996), enabling estimation of the contribution of phytoplankton classes and phyla to chlorophyll (Chl)-*a*. Data processing followed a procedure similar to that of Sarmento and Descy (2008), enabling estimation of the relative proportion of green algae, chrysophytes, diatoms, cryptophytes, dinoflagellates and cyanoprokaryotes or cyanobacteria in the biomass, expressed per unit volume (μg Chl-*a* L^{-1}). These taxonomic groups were separated according to the pigments, indicated in the initial ratio matrix (Table 2) used in the CHEMTAX processing, similar to that of SOP5 (Descy 2017).

Total Chl-*a* was used as a proxy of total algal biomass in the evaluation of trophic status according to the Open-Boundary System of the Organization for Economic Cooperation and Development (Vollenweider and Kerekes 1982; Vollenweider 1993).

Table 1. Sampling sites and their environmental parameters

ASL, above sea level; CND, conductivity; DO, dissolved oxygen; IBW, Inventory of Bulgarian Wetlands (Michev and Stoyneva 2007) SA, site abbreviation (asterisks indicate that the site was chosen based on drone observations); SD, Secchi depth (m); TDS, total dissolved solids; TN, total nitrogen; TP, total phosphorus; TTB, transparent to the bottom; W, water temperature

Waterbody (IBW number)	SA	Sampling date	Longitude	Latitude	Altitude (m ASL)	WT (°C)	pH	SD (m)	CND (μS)	TDS (μg L^{-1})	DO (mg L^{-1})	TP (mg L^{-1})	TN (mg L^{-1})	TN : TP
Sinyata Reka Reservoir (IBW1793)	SR1*	21 June 2018	42°28.1480′	24°42.217′	317	27.4	9.72	0.5	470	305	9.36	2.50	4.8	1.92
	SR2	21 June 2018	42°28.1473′	24°42.2175′	317	26.7	9.36	0.6	468	306	9.21	2.20	4.3	1.95
Vaya Lake (IBW0191)	VA1*	22 June 2018	42°30.5940′	27°22.075′	−2	26.9	9.65	0.25	2588	1682	12.51	1.30	5.4	4.15
	VA2	22 June 2018	42°28.4540′	27°25.482′	0	28.28	8.86	0.25	1183	768	11.94	1.10	3.7	3.36
	VA3*	23 June 2018	42°29.1850′	27°26.531′	6	23.7	9.50	0.25	1024	665	7.01	1.20	4.6	3.83
Mandra Reservoir (IBW1720)	MN1	23 June 2018	42°24.0463′	27°26.1120′	12	25.88	8.28	0.4	649	421	6.81	0.90	3	3.33
	MN2*	23 June 2018	42°24.0670′	27°19.1310′	13	26.2	8.20	0.2	663	461	5.89	1.30	4	3.08
	MN3	23 June 2018	42 26.1420′	27°26.5860′	9	24.9	8.48	0.3	639	415	7.91	1.40	3.3	2.36
Uzungeren Lake (IBW0710)	UZ	23 June 2018	42°26.1782′	27°27.1998′	7	25.9	8.06	0.4	1438	9351	7.83	0.50	2.8	5.60
Aheloy Reservoir (IBW3032)	AH	24 June 2018	42°42.8230′	27°30.9740′	144	25.4	8.51	1.10	614	399	8.92	0.10	2.8	28.00
Poroy Reservoir (IBW3038)	PR	24 June 2018	42°43.0190′	27°37.3160′	41	25.10	8.33	1.2	762	495	9.45	1.60	4.1	2.56
Ezerets Lake (IBW0233)	EZ	25 June 2018	43°35.2770′	28°33.2290′	−2	26.4	8.35	TTB	1084	10	9.94	0.50	5.3	10.60
Shabla Lake (IBW0219)	SH	25 June 2018	43°33.8180′	28°34.1860′	−2	27.1	8.46	TTB	1087	0706	9.98	0.10	5.1	51.00
Durankulak Lake (IBW0216)	DR1	25 June 2018	43°40.3240′	28°32.0470′	6	24.03	8.54	1	1111	722	7.35	1.70	2.8	1.65
	DR2*	25 June 2018	43°40.3340′	28°32.0220′	6	24.7	8.21	1	1094	711	7.79	1.70	4	2.35
	DR3	25 June 2018	43°40.5300′	28°32.9930′	4	24.6	8.49	1	1075	698	6.19	1.50	3.9	2.60
	DR4	25 June 2018	43°40.6950′	28°32.6000′	3	26.5	8.53	1	1087	706	9.60	1.30	3.2	2.46

A principal components analysis (PCA) was run using Statistica 10 (StatSoft Inc., Tulsa, OK, USA) to examine the relationships among limnological and phytoplankton variables and to establish a classification of the lakes according to the main environmental gradient.

For detection of microcystins, nodularins, saxitoxins and cylindrospermopsins, the recommendations of Ballot *et al.* (2017), Catherine *et al.* (2017) and Kokociński *et al.* (2017*a*) were followed. Owing to possible variations in methods, more details are provided below, with a note that the methods used are completely compatible with those from our previous studies (for details, see references in Stoyneva-Gärtner *et al.* 2017). For microcystin and nodularin determination, stored water samples were frozen and thawed three times to achieve cell lysis. Next, samples were filtered through 0.45-μm nylon membrane filters (Alltech Associates Inc., Deerfield, IL, USA). Microcystins and nodularin were extracted from water samples by solid-phase extraction with Empore Extraction Disks C-18 (Varian, Darmstadt, Germany). Toxins were eluted with methanol. Eluates were dried by a gentle stream of nitrogen, redissolved in 500 μL of 50% methanol (v/v), filtered through 0.22-μm polytetrafluoroethylene (PTFE) syringe filters (ALBET LabScience, Dassel, Germany) and analysed by HPLC according to ISO 20179:2005 (International Organization for Standardization 2005).

The HPLC system for quantitative and qualitative analyses included an Agilent 1200 Series coupled with a diode array detector (DAD; Agilent Technologies, Santa Clara, CA, USA). Toxins were analysed on a Supelcosil ABZ+Plus column (150 × 4.6 mm, 5 μm; Supelco, Inc., Bellefonte, PA, USA). The binary gradient of the mobile phase consisted of Milli-Q water + 0.1% trifluoroacetic acid (TFA) (A) and acetonitrile + 0.1% TFA (B), with a linear increase from 20% B at 0 min to 46% B at 25 min and stop time at 30 min; the flow rate was 1 mL min^{-1} and samples were run at temperature 25°C. Chromatograms were recorded at 238 nm and toxins were identified by the retention time and characteristic ultraviolet (UV) absorption spectra from 200 to 300 nm.

Purified microcystins MC-LR, MC-RR, MC-YR and nodularin (Abraxis, Inc., Warminster, PA, USA) were used as external standards.

Cylindrospermopsin and saxitoxins were detected using specific antibodies in stored frozen water samples by ELISA. Cylindrospermopsin, when present in a sample, and a horseradish peroxidase (HRP)-conjugated cylindrospermopsin analogue compete for the binding sites of rabbit anti-cylindrospermopsin antibodies in solution. The anti-cylindrospermopsin antibodies are then bound by a secondary antibody (goat anti-rabbit) immobilised on the wells of the microtitre plate. Saxitoxin, when present in a sample, and a saxitoxin–enzyme conjugate compete for the binding sites of rabbit anti-saxitoxin antibodies in solution. The saxitoxin antibodies are then bound by a secondary antibody (sheep anti-rabbit) immobilised on the plate. After a washing step and the addition of substrate solution, a colour signal (blue) is generated. The intensity of the blue colour is inversely proportional to the concentration of cylindrospermopsin or saxitoxins present in the sample. The colour reaction is stopped after a specified time and the colour is evaluated using an ELISA

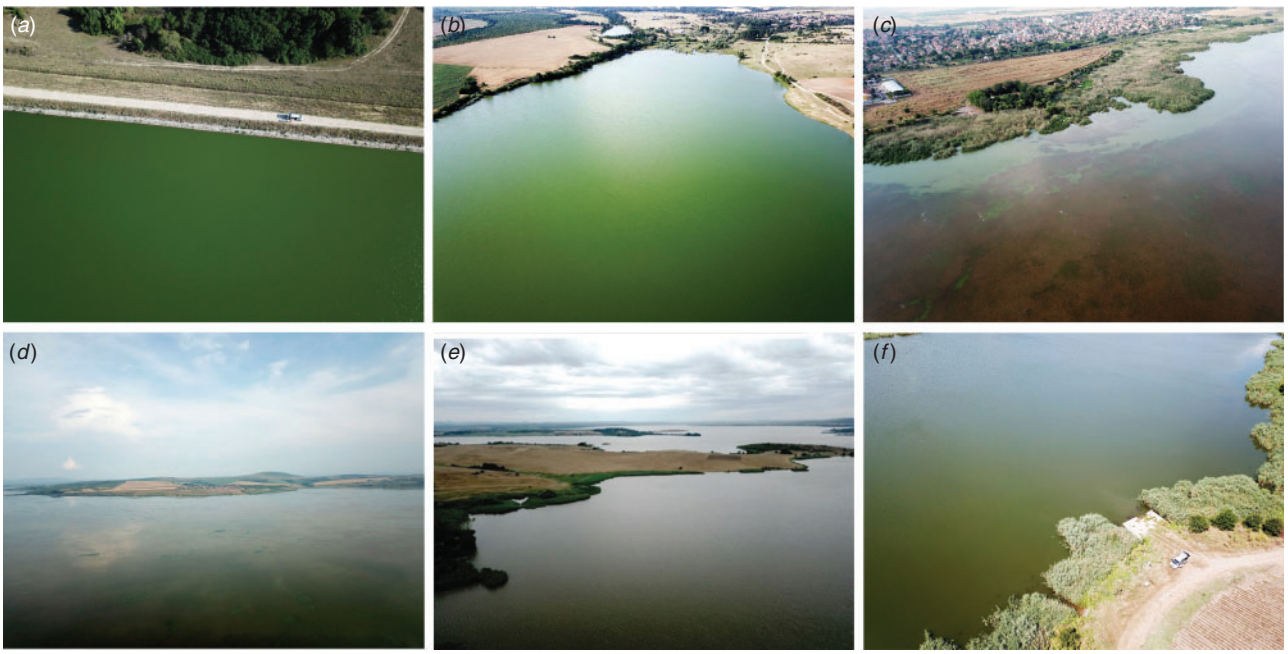

Fig. 2. Aerial photographs taken by drones of some of the Bulgarian water bodies studied (June 2018): (*a*) Site 1 in Sinyata Reka Reservoir; (*b*) Site 2 in Sinyata Reka Reservoir; (*c*) Site 1 of Vaya Lake; (*d*) Site 3 of Vaya Lake; (*e*) Site 2 of Mandra Reservoir; (*f*) Site 3 of Durankulak Lake, showing the minibus parked near the small quay used to access the sampling site.

Table 2. Initial ratio matrix for determination of phytoplankton classes biomass (µg chlorophyll-*a* L^{-1}) using CHEMTAX

The values are, for each phytoplankton class, the concentration of each pigment to chlorophyll a (chl_a); peri, peridinin; fuco, fucoxanthin; neo, neoxanthin; myxo, myxoxanthophyll; viol, violaxanthin; ddx, diatoxanthin + diadinoxanthin; allo, alloxanthin; lut, lutein; zea, zeaxanthin; echi, echinenone; acar, α-carotene; chl_c, chlorophyll-*c*; chl_b, chlorophyll-*b*

	Pigment												
Class	peri	fuco	neo	myxo	viol	ddx	allo	lut	zea	echi	chl_c	chl_b	chl_a
chlorophytes	0.000	0.000	0.033	0.000	0.030	0.000	0.000	0.174	0.023	0.000	0.000	0.273	1.000
chrysophytes	0.000	0.300	0.000	0.000	0.150	0.000	0.000	0.000	0.000	0.000	0.030	0.000	1.000
cryptophytes	0.000	0.000	0.000	0.000	0.000	0.000	0.396	0.000	0.000	0.000	0.100	0.000	1.000
cyanobacteria_T1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.109	0.000	0.000	0.000	1.000
cyanobacteria_T2	0.000	0.000	0.000	0.150	0.000	0.000	0.000	0.000	0.043	0.095	0.000	0.000	1.000
diatoms	0.000	0.563	0.000	0.000	0.000	0.129	0.000	0.000	0.000	0.000	0.200	0.000	1.000
dinoflagellates	0.629	0.000	0.000	0.000	0.000	0.225	0.000	0.000	0.000	0.000	0.150	0.000	1.000
euglenophytes	0.000	0.000	0.030	0.000	0.000	0.450	0.000	0.000	0.000	0.000	0.000	0.200	1.000

reader. The concentrations of the samples are determined by interpolation using a standard curve constructed for each run. For determination of cylindrospermopsin and saxitoxin concentrations in water samples, commercially available ELISA kits were used (Saxitoxin (PSP) ELISA, Microtiter Plate; Abraxis) in combination with a microplate reader (5060-006; LKB, Vienna, Austria).

Results

During the field trip, 17 sites from nine shallow lowland waterbodies were sampled and their main environmental characteristics were measured (Fig. 1; Table 1). Based on drone observations of the water surface and colour, four sites with visible coloured spots were chosen for targeted sampling of CyanoHABs: Sinyata Reka 1, Vaya 1, Vaya 3 and Durankulak 3 (Fig. 2). The Mandra 2 site could be tentatively added to this category because of the strong wind that started blowing approximately 1 h before its sampling. The wind caused water mixing in the shallow Mandra Reservoir (up to 2 m at Sampling sites 1–3), when the Mandra 1 site was sampled (in accordance with our previous studies - Stoyneva 2014, 2016; Stoyneva *et al.* 2015; Stoyneva-Gärtner *et al.* 2017) and the use of a drone was impossible. After the wind stopped, the drone was sent over Mandra 2 and Mandra 3 sites. A slight change in water colour with some tiny greenish glares was seen only at the Mandra 2 site (Fig. 2*e*). A difference in water colour was

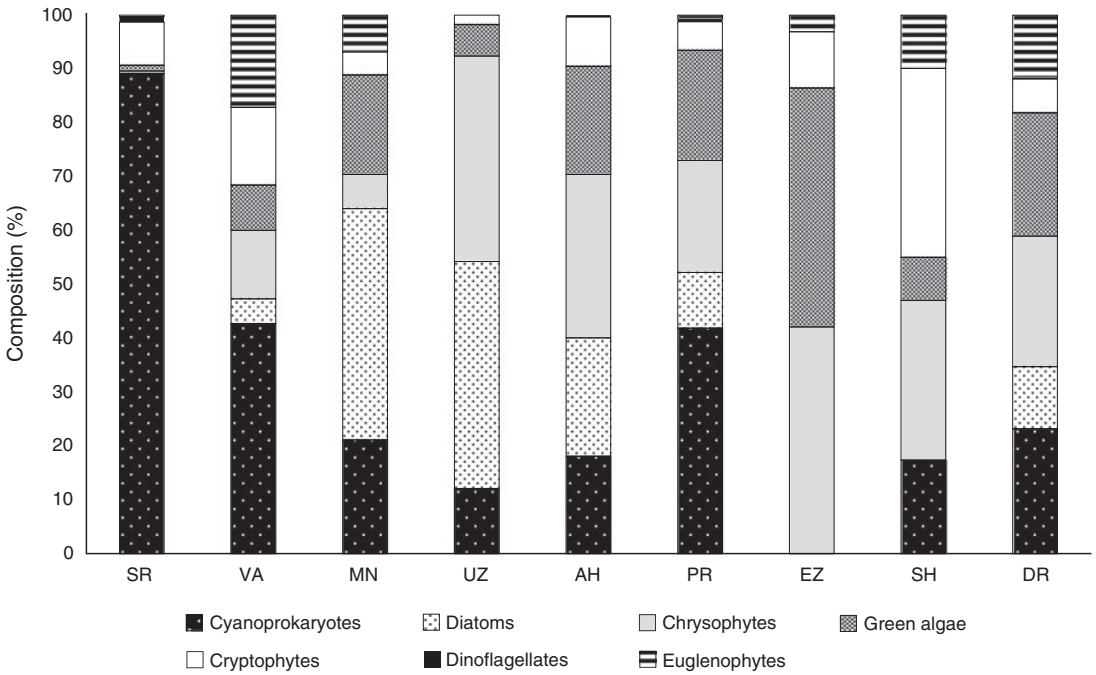

Fig. 3. General phytoplankton composition according to the pigment markers examined by HPLC (expressed as relative percentage abundance of the total biomass) in the Bulgarian water bodies studied. SR, Sinyata Reka Reservoir; VA, Vaya Lake; MN, Mandra Reservoir; UZ, Uzungeren Lake; AH, Aheloy Reservoir; PR, Poroy Reservoir; EZ, Ezerets Lake; SH, Shabla Lake; DR, Durankulak Lake.

visible to the naked eye from the shore at the Durankulak 2 site (also sampled in our previous studies, cited above) without need for a drone.

HPLC data on pigment markers indicate that the phytoplankton of the studied sites primarily comprised cyanoprokaryotes, diatoms and green algae, with a lower contribution of chrysophytes, cryptophytes, euglenophytes and dinoflagellates (Fig. 3).

Phytoplankton composition and abundance varied between the waterbodies studied and between the different sites (Fig. 4). Based on the Chl-*a* concentration, the Poroy and Aheloy reservoirs and the Shabla and Ezerets lakes were eutrophic (10–25 μg L^{-1} Chl-*a*) and all other waterbodies were hypertrophic (>25 μg L^{-1} Chl-*a*), with highest phytoplankton abundance in Sinyata Reka, Vaya, Mandra and Durankulak (Fig. 4). The contribution of Cyanoprokaryota or Cyanobacteria at each site showed a similar distribution pattern to that seen for the waterbodies, being highest in Sinyata Reka Reservoir and lowest in Shabla and Ezerets lakes (Fig. 4). At all sites chosen by drone, and at the Durankulak 2 site, the contribution of both Chl-*a* and cyanoprokaryotes was higher compared with the other sites (Fig. 4).

TN ranged between 2.8 and 5.4 mg L^{-1} and varied slightly between the sites studied in the same waterbody (Table 1). TP ranged between 0.1 and 2.5 mg L^{-1} (Table 1). There was considerable variation in TP values between the waterbodies studied, but only slight variations between different sites in the same waterbody (Table 1).

The TN : TP ratios (Table 1) indicated strong N limitation at almost all sites studied (values between 1.8 and 5.6), with the exception of Shabla, where a TN : TP ratio of 51 indicates strong P limitation, and Ezerets, where a TN : TP ratio of 10.60 indicates that either of the nutrients may be limiting.

The results of the PCA run on environmental variables, Chl-*a* and the biomass of phytoplankton groups are shown in Fig. 5. Chl-*a* and TP were strongly positively correlated with each other, and negatively correlated with Secchi depth, determining the first principal component (PC). Cyanobacterial biomass was strongly correlated with this eutrophication gradient. The second PC was determined primarily by TN, DO and conductivity, which were negatively correlated with diatoms and chrysophytes. Four groups of waterbodies were identified in the lakes ordination (Fig. 5). The grouping of the waterbodies is easy to be explain by their trophic status based on Chl-*a*. The first group contained the hypertrophic inland Sinyata Reka Reservoir and Vaya Lake, both with the highest biomass (mean Chl-*a* 116 and 87 μg L^{-1} respectively) and highest pH values. The second group contained only the large Mandra Reservoir (average Chl-*a* 56 μg L^{-1}), whereas the third group included Uzungeren and Durankulak lakes, with mean Chl-*a* of 65 and 33 μg L^{-1} respectively. The fourth group was formed by all eutrophic waterbodies: the Aheloy and Poroy reservoirs and the two lakes on the north-coast, Shabla and Ezerets (Chl-*a* ranging between 9 and 12 μg L^{-1}).

Toxin analyses revealed the presence of microcystins (MC-RR, MC-YR and MC-LR, but primarily MC-LR) in two of the waterbodies studied (Sinyata Reka and Durankulak), with a difference in their concentrations and type depending on site (Table 3). Cylindrospermopsin was found in Mandra and Vaya, and saxitoxins were recorded in Durankulak, whereas nodularin was not detected in any of the waterbodies (Table 3).

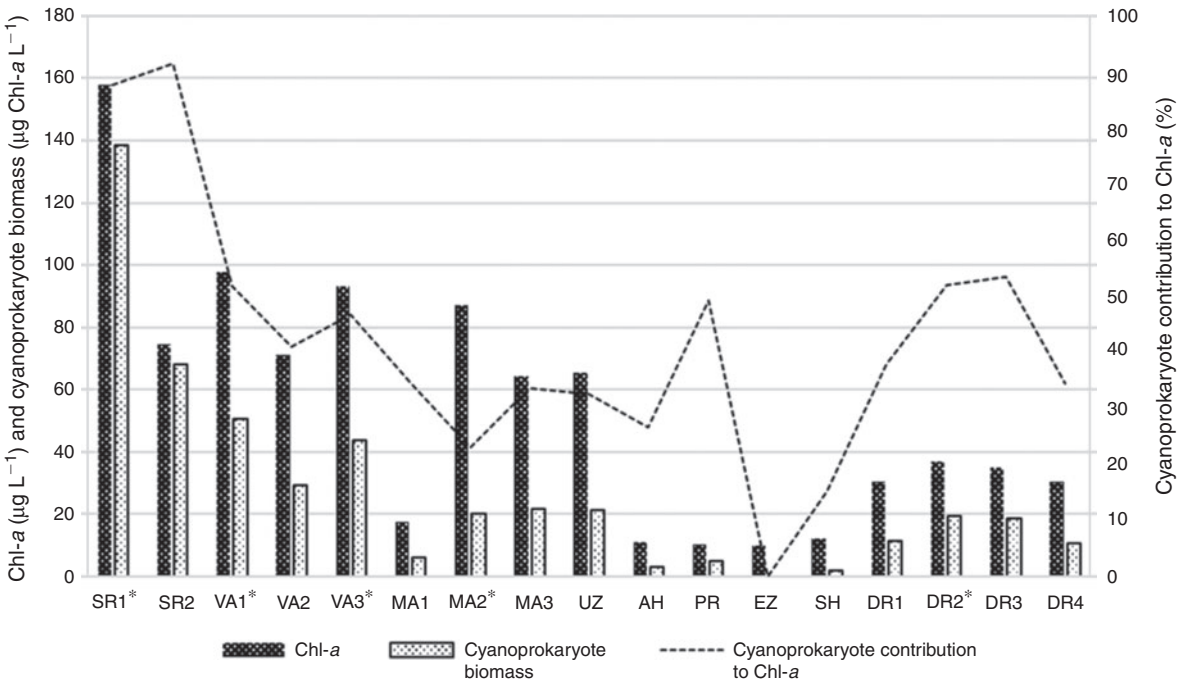

Fig. 4. High performance liquid chromatography (HPLC) data for total chlorophyll (Chl)-*a* and cyanoprokaryote biomass, and cyanoprokaryote contribution to Chl-*a* in numbered sites (asterisks indicate that the site was chosen based on drone observations) in the Bulgarian waterbodies studied: SR, Sinyata Reka Reservoir; VA, Vaya Lake; MN, Mandra Reservoir; UZ, Uzungeren Lake; AH, Aheloy Reservoir; PR, Poroy Reservoir; EZ, Ezerets Lake; SH, Shabla Lake; DR, Durankulak Lake.

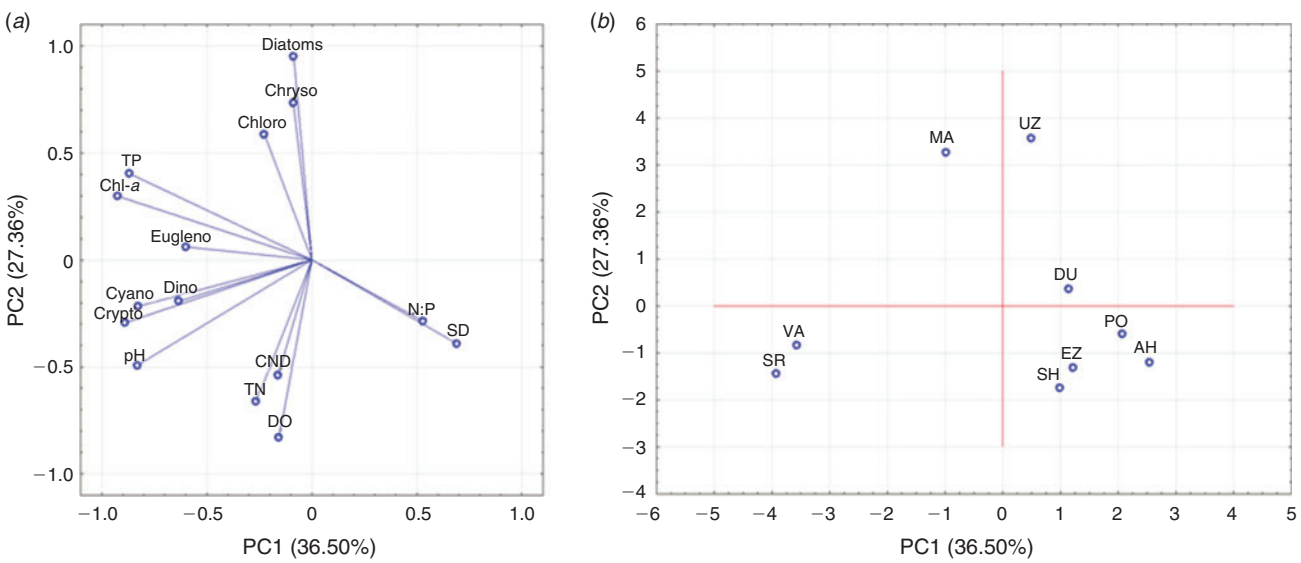

Fig. 5. Results of the principal component analysis on environmental variables and the biomass of phytoplankton groups of nine Bulgarian waterbodies. (*a*) Ordination of variables on the first two principal components (PC1, PC2; cumulative inertia = 63.9%). Values in parentheses in axis titles indicate the percentage variation explained by each PC. The biomass of the main phytoplankton groups was quantified using CHEMTAX software (CSIRO Marine Laboratories, Mackey *et al.* 1996). CND, electric conductivity; SD, Secchi depth; DO, dissolved oxygen; TP, total phosphorus; TN, total nitrogen; N : P, ratio of total N to total P; Chl-*a*, chlorophyll-*a*; chryso, chrysophytes; chloro, green algae; eugleno, euglenophytes; cyano, cyanoprokaryotes; crypto, cryptophytes. (*b*) Ordination of the lakes. SR, Sinyata Reka Reservoir; VA, Vaya Lake; MN, Mandra Reservoir; UZ, Uzungeren Lake; AH, Aheloy Reservoir; PR, Poroy Reservoir; EZ, Ezerets Lake; SH, Shabla Lake; DR, Durankulak Lake.

Table 3. Cyanotoxins detected in Bulgarian waterbodies (June 2018)

Microcystins (MC-RR, MC-LR, MC-YR) were detected using HPCL (limit of detection (LOD) 0.08–0.15 μg L^{-1}), whereas saxitoxins and cylindrospermopsin (CSPM) were detected by ELISA (LOD 0.015 and 0.4 μg L^{-1} respectively). IBW, Inventory of Bulgarian Wetlands (Michev and Stoyneva 2007); SA, site abbreviation (asterisks indicate that the site was chosen based on drone observations). Sampling dates are as provided in Table 1

Waterbody (IBW number)	SA	Microcystins (μg L^{-1})	Saxitoxins (μg L^{-1})	CSPM (μg L^{-1})
Sinyata Reka Reservoir (IBW1793)	SR1*	MC-RR, 0.09; MC-LR, 0.3	<LOD	<LOD
	SR2	<LOD	<LOD	<LOD
Vaya Lake (IBW0191)	VA1*	<LOD	<LOD	0.1
	VA2	<LOD	<LOD	<LOD
	VA3*	<LOD	<LOD	<LOD
Res. Mandra Reservoir (IBW1720)	MN1	<LOD	<LOD	<LOD
	MN2*	<LOD	<LOD	0.1
	MN3	<LOD	<LOD	<LOD
Uzungeren Lake (IBW0710)	UZ	<LOD	<LOD	<LOD
Poroy Reservoir (IBW3038)	PR	<LOD	<LOD	<LOD
Aheloy Reservoir (IBW3032)	AH	<LOD	<LOD	<LOD
Ezerets Lake (IBW0233)	EZ	<LOD	<LOD	<LOD
Shabla Lake (IBW0219)	SH	<LOD	<LOD	<LOD
Durankulak Lake (IBW0216)	DR1	<LOD	<LOD	<LOD
	DR2	MC-YR, 0.2; MC-LR, 0.2	<LOD	<LOD
	DR3*	MC-LR, 0.1	0.015	<LOD
	DR4	<LOD	<LOD	<LOD

Discussion

The results of this study prove the eutrophic to hypertrophic character of the chosen waterbodies, as reported previously (Stoyneva-Gärtner *et al.* 2017; Descy *et al.* 2018 and references therein). In these conditions, the high relative proportion of Cyanoprokaryota (Cyanobacteria) in the phytoplankton biomass at almost all sites studied is easy to explain and confirms previous knowledge on their widespread distribution, high diversity and abundance in the region studied (Stoyneva 2000*a*, 2003; Pavlova *et al.* 2007; Dimitrova *et al.* 2014*a*, 2014*b*; Stoyneva 2014; Stoyneva-Gärtner *et al.* 2017; Descy *et al.* 2018). The low water transparency and high pH detected during the study (Table 1) correspond to both total phytoplankton and cyanoprokaryote abundance (Fig. 3, 4) and are in general accordance with our previous results for most of these waterbodies obtained after processing by multivariate analyses (Stoyneva 2014; Stoyneva *et al.* 2015; Stoyneva-Gärtner *et al.* 2017). Again, cyanobacterial abundance increased with P concentrations, thus confirming our previous results in Bulgarian wetlands (Stoyneva 2014; Stoyneva *et al.* 2015; Stoyneva-Gärtner *et al.* 2017). Therefore, it is likely that in shallow Bulgarian waterbodies TP is the main cause of cyanobacterial abundance rather than any other measured variable. Although collected during one sampling campaign, the data from this study are in accordance with the knowledge of the general environmental driving forces that allow cyanoprokaryotes to outcompete other phytoplankton in eutrophic to hypertrophic shallow standing waterbodies, such as high temperatures, high pH and mainly high P (Downing *et al.* 2001; Carvalho *et al.* 2013; Descy *et al.* 2016). In contrast, in the present study TN was not correlated with TP and Chl-*a*, and does not appear as a key determinant of cyanoprokaryote biomass.

Considering the studies cited above and the shallow character of the holo- to polymictic lowland wetlands studied, the relatively small variation in spatial distribution of the phytoplankton biomass, as well as the related Chl-*a* content and cyanoprokaryote biomass in the studied waterbodies are to be expected. That was confirmed by the aerial photographs taken in the field by the drone using uncorrected photographs, without any attempt to mathematically correct the data for surface reflections. The four exceptional cases of stronger water colour in single sites at Sinyata Reka, Vaya and Durankulak (Fig. 2*a–d*, *f*) and the less visible spot in Mandra fitted well with the HPLC data on pigment composition and phytoplankton biomass (Fig. 3, 4). The spots observed by drone and confirmed after local sampling and processing of the material collected in the beginning of summer (June 2018) were in accordance with previous knowledge that blooms commonly start at sites offering the best conditions (primarily favourable light and nutrient availability), known as hot spots or fronts of productivity (Oliver and Ganf 2000; Gons *et al.* 2005). A shortcoming of drone application was detected when the Mandra Reservoir was sampled during and after a strong wind, when the water surface and colour looked more homogeneous. However, even in this case, the drone observations helped identify the initial front of a CyanoHAB (Fig. 2*e*).

The toxins detected (Table 3) confirmed our previous findings of microcystins (especially MC-LR, considered the most dangerous type) in Durankulak (Pavlova *et al.* 2006, 2013, 2014, 2015; Pavlova 2007; Stoyneva-Gärtner *et al.* 2017) and are the first reports of microcystins in Sinyata Reka Reservoir. The finding of saxitoxins is the first for Durankulak. During this study the presence of cylindrospermopsin in Bulgaria was proved by reliable methods for the first time. This cyanotoxin was long ago thought to occur in Bulgarian waters

(Pavlova *et al.* 2014; Stoyneva 2014) based on observations of the widening distribution of its main causative agent, namely *Raphidiopsis raciborskii* (Woloszynska) Aguilera, Berrendero Gómez, Kastovsky, Echenique et Salerno (Syn. *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju), in Bulgaria and particularly after the species was detected in Vaya and Mandra (Dimitrova *et al.* 2014*b*; Stoyneva 2016; Kokociński *et al.* 2017*b*; Stoyneva-Gärtner *et al.* 2017). The presence of cylindrospermopsin, produced by heterocytous algae with strong N-fixation ability, is in accordance with the N limitation of the waterbodies studied, and of Mandra and Vaya in particular.

The low concentrations of all toxins found and their detection at separate sites (Table 3) are easily explained by the atypical (for the season) strong rains and floods that started during the sampling week just after a long (also untypical for the country) early period of warm weather and drought in April and May of 2018. Although recorded in low concentrations, almost all toxic substances were found at the sampling sites chosen based on drone observations, which differed visually in terms of water colour and were proven to contain a higher abundance of cyanoprokaryote compared with the other sampling sites (Fig. 2, 4; Table 3).

The results of this study proved the risk in the shallow lowland waterbodies of the occurrence of CyanoHABs and show the considerable potential of using modern remote methods and HPLC in studies and monitoring of CyanoHABs. Despite possible limitations of the field application of a drone because of meteorological conditions (e.g. wind, rain), the results of this study confirm the importance of remote observations for choosing proper sampling sites. Other reasons favouring the use of drones are that they are easy and fast to use and they are small and easy to transport during field trips. Considering the high number of wetlands over Bulgaria (~9000 according to Michev and Stoyneva 2007), we would strongly recommend the further use of drones and the pigment technique in studies of phytoplankton biomass and composition to greatly increase the speed of the field work; these techniques are very efficient in terms of data acquisition and provide valuable information.

Author contribution

The first two authors contributed equally to the preparation of the work and of the manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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