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Cytotoxicity of water from five Bulgarian wetlands contaminated by toxigenic cyanobacteria and cyanotoxins

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Microscopic photosynthetic cyanoprokaryotes/cyanobacteria, or blue-green algae, produce various numbers of bioactive compounds, including different cyanotoxins which are hazardous for the ecosystem and human health. Cyanoprokaryotes are widely spread on the Earth and in Bulgaria specifically, where during the last two decades their toxins were found in different wetlands. However, only few studies conducted in Bulgaria mention cytotoxic effects of waters contaminated with cyanotoxins and up-to-now only three types of cell lines were used in the tests. Therefore, the present study was focused on the cytotoxic effect of waters from five chosen Bulgarian wetlands (two reservoirs and three lakes) with proved development of toxigenic cyanoprokaryotes. Moreover, for the first time in the country, the cytotoxicity was tested on the Hs27 human skin cells line. MTT test was performed to measure the cell viability upon exposure to increasing concentrations of water samples in culture medium. During the study three important results, which generally correspond to the cyanoprokaryote composition, biomass and detected cyanotoxins, were obtained: 1) applied water samples exhibited their effect after 24 hours of exposure; 2) at the lowest concentration of 1% cytotoxic effects were not observed; 3) at concentration of 8% in the culture medium, all water samples decreased cell viability by more than 50% compared to non-treated cells. These results allow to suppose the strong adverse effect of cyanoprokaryotes and their metabolites (mainly cyanotoxins) which should be considered as a risk factor for animal and human health in the studied water bodies.

Keywords: cyanobacteria, cyanoprokaryotes, cyanotoxins, health risk, toxigenic algae

INTRODUCTION

Cyanoprokaryotes/Cyanobacteria (known also as blue-green algae) are photosynthetic prokaryotic organisms, which develop as single cells, colonies or filaments rapidly growing in all types of aquatic, aeroterrestrial and extremophilic habitats. During the last decades the expansion of cyanoprokaryote growth at high blooming densities is increasing because of human activities, growing human population, globalization and climatic changes leading to global warming [1]. Cyanoprokaryotes form a high number of bioactive molecules, and certain species produce cyanotoxins as defense mechanisms against different ambient stress factors [2, 3]. Currently more than 120 different cyanotoxins are known, classified in three major groups by their chemical structure (alkaloids, cyclic peptides and lipopolysaccharides) or in three main groups according to the main target of their activity (hepatotoxins, neurotoxins and dermatotoxins) [2, 4]. The cyclic heptapeptides microcystins are the most widespread hepatoxins in water blooms and therefore are best known and commonly studied [5]. Nowadays another cyanotoxin – the neurotoxin cylindrospermopsin – attracts the attention of the research community because of its extracellular character and diverse multiple effects [2].

Humans' intoxication with cyanotoxins is possible *via* different pathways such as bathing and recreational activities with contaminated water, aerosolization or consumption of contaminated food [2, 6-9]. In the period 1960-2016, cyanotoxin poisonings of animals and humans were registered in different world's regions (Australia, Brazil, Canada, China, Namibia, Portugal, Serbia, Sri Lanka, Sweden, UK, and USA) [5]. Besides direct acute cases, some of which lethal [8, 9], experiments demonstrated that chronic exposures to low concentrations of cyanotoxins, and of microcystins in particular, could increase the risk for carcinogenesis because of their potential long-term adverse effects, and the International Agency for Research on Cancer classified them as a possible human carcinogen [10]. The effects of microcystins on different types of cell cultures were

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investigated and the observed mechanisms of cytotoxicity were summarized [11].

Among them, for example, was the ability of microcystins to cause apoptosis or necrosis in varying concentrations applied to different cell types in *in vitro* cell culture studies [12, 13]. However, it is important to recall that the harmful effects of cyanoprokaryotes cannot be attributed only to the known cyanotoxins and in this respect different *in vitro* tests for cytotoxicity of complex water samples are useful [2].

In Bulgaria, a pilot assessment of cyanotoxins as potential risk factor for cancer was made [14] following the survey on studies on cyanoprokaryotes conducted during 15 years (2000-2015) in 120 wetlands which demonstrated occurrence of blooms, toxigenic species and cyanotoxins [15]. The presence of cyanotoxins (microcystins LR, LA, RR, YR, nodularins, and saxitoxins) in 16 of these 120 wetlands was proved by using ELISA, HPLC or *in vitro* cytological tests with the latter method applied only in studies of six, mainly small, reservoirs [15]. Up-to-now three types of cell lines were used for *in vitro* detection of water toxicity in Bulgarian water bodies: HeLa (human cervical epithelial adenocarcinoma), 3T3 (mouse embryonic fibroblasts) and FL (normal amniotic human cells) (for details and references see [15]) and no studies on dermatotoxicity had been made. Therefore, the aim of the present study was to apply for the first time in Bulgaria a cell line of human skin fibroblasts (Hs27) for *in vitro* measurement of the changes of cell viability in respect to increasing concentrations of water samples collected from five different wetlands (2 reservoirs and 3 lakes) in which toxigenic cyanoprokaryote species and cyanotoxins were found [16-18].

MATERIALS AND METHODS

Water samples

Water samples were collected in June 2018 from the reservoirs Mandra and Sinyata Reka, and from the lakes Durankulak, Vaya and Uzungeren after application of a drone for finding of spots of blooming algae (for sampling details see [16]) – Table 1. Additional samples from Durankulak and Mandra were collected in the same way and from the same places in August 2019, and were labelled with Arabic number 2. All studied wetlands were chosen because of their different classification types in the Inventory of Bulgarian wetlands (IBW) and their biodiversity [19], their diferent usage and conservation importance (except Sinyata Reka, for details see [19]) and because of proved presence of harmful cyanoprokaryotes and their cyanotoxin metabolites by different methods (light microscopy, chemical analyses and molecular-genetic studies) [16-18]. For this study, identification of phytoplanktonic cyanoprokaryotes in all samples was done using conventional light microscopy according to standard taxonomic manuals and toxigenic genera were identified after [2].

Table 1. Main types and usage of the studied wetlands according to the Inventory of Bulgarian wetlands [19], where IBW is the relevant number in [19].

Wetland	IBW	Type	Position	Usage
Durankulak (DRK)	IBW0216	freshwater lake	coastal lowland in North-East Bulgaria	irrigation, recreation, sport fishing, industrial yield of crayfish
Mandra (MND)	IBW1720	large reservoir	coastal lowland in South-East Bulgaria	irrigation, fishing
Vaya (VA)	IBW0191	lake with varying halinity	coastal lowland in South-East Bulgaria	recreation, sport fishing
Uzungeren (UZNG)	IBW0710	lake with varying halinity	coastal lowland in South-East Bulgaria	irrigation, recreation, sport fishing
Sinyata Reka (SNR)	IBW1793	small reservoir	Inland kettle in Central Bulgaria	fish-breeding; irrigation

Cell line

Human skin cell fibroblasts (Hs27) were obtained from the American Type Culture Collection (ATCC). Cells were raised in 75 cm^2 flasks at 37°C in a humidified chamber with 5% CO_2 atmosphere. Complete nutrient medium comprised phenol of red-containing Dulbecco's Modified Eagle's medium (DMEM, Lonza) with

4.5 g L^{-1} glucose, L-glutamine and supplemented with fetal bovine serum (FBS, Sigma-Aldrich) to a 10% final concentration and penicillin/streptomycin mixture to final concentrations of 1%.

Experimental procedure

All water samples were filtered through 0.2 μm filter. Each water sample was added to the cell growing medium without any supplements to reach following concentrations: 1 v/v %; 2 v/v %; 4 v/v %; 8 v/v %; 16 v/v %. Hs27 human cells were collected and seeded in 12 well flasks at a density of 6.5×10^4 cells per well. Each water sample at all five concentrations was separately applied to Hs27 cell line after overnight incubation in two replicates.

Viability of treated cells was estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (commonly abbreviated as MTT assay) [20, 21]. The assay is based on the ability of viable cells to reduce the yellow MTT to purple insoluble formazan [20, 21]. At each well 100 μL of MTT solution in phosphate buffer saline (pH=7.4) at a concentration of 2 mg mL^{-1} was added 20 hours after the start of water treatment. After a 4-h incubation the medium was removed and 1 mL of dimethyl sulfoxide was added to each well for cell lysis. After thorough mixing, 100 μL were transferred to 96-well plates and the absorbance was measured at 550 nm wavelength. Afterwards Synergy 2 plate reader (BioTek) was used. Viability of treated cells was presented in percentage of the viability of the non-treated cells, which is considered 100%. All the treatments were performed in duplicate.

Statistical analyses were performed using Microsoft Excel Office software with calculated standard deviation (SD) and probability threshold (*p*) value less than 0.05 considered as significant.

RESULTS AND DISCUSSION

All results obtained during this study are represented on Fig. 1 as mean values and show the general decrease of the viability of the studied Hs27 cells with increase of the sample concentrations. The calculated SD was less than 0.5-1% of the measured values and therefore is not specifically indicated on the presented graph. The statistically significant changes ($p<0.05$) in the cell viability were recorded in 20 (or 57%) from 35 studied samples and are shown in Table 2. The lowest applied concentration of 1% did not induce changes in the cell viability, which remained 100% (except for the cell treated with water from Vaya, where the viability had fallen to 95%) indicating that at this concentration there were no cytotoxic effects. However, the double increase of the sample concentration (up to 2%) reduced cell viability below 83% compared to non-treated cells, with statistically significant slight decrease to 95% only in the samples treated by water from Uzungeren. At 4% concentration statistically significant decrease of cell viability was detected only for samples treated by water from Vaya, Mandra 1 and Mandra 2. The decrease of the Hs27 cell viability compared to non-treated cells was much better pronounced when the applied concentrations increased to 8%: the cell viability significantly decreased by more than 50% (68-53%) in all studied samples. It decreased to the lowest values when Hs27 cells were treated with the highest concentration of 16% with samples from Durankulak 1, Sinyata Reka and Uzungeren – 25, 36 and 37%, respectively, where Durankulak 1 shows the strongest cytotoxicity. According to these results it is possible to state that the effective concentration causing 50% inhibition (IC_{50}) was 8-16% and the lowest observable effect level (LOEL) was at 2-4% concentrations.

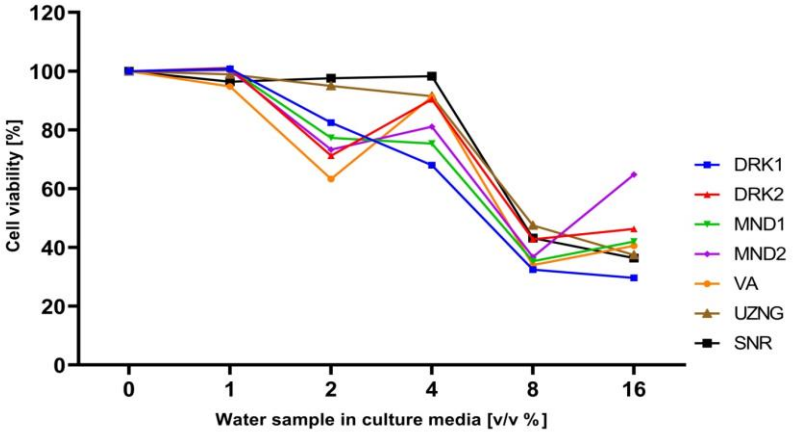

Fig. 1. Cell viability of HS27 fibroblasts treated with water collected from wetlands in Bulgaria. For each sample mean values with standard deviation are represented. Legend: DRK1 – Durankulak 1, DRK2 – Durankulak 2, MND1 - Mandra 1, MND2 - Mandra 2, VA – Vaya, UZNG – Uzungeren, SNR – Sinyata Reka.

Table 2. Statistically significant ($p<0.05$) changes of cell viability after application of water samples from five studied water bodies in different concentrations (from 0 to 16%) vs. untreated control cells. Legend: DRK1 – Durankulak 1, DRK2 – Durankulak 2, MND1 - Mandra 1, MND2 - Mandra 2, VA – Vaya, UZNG – Uzungeren, SNR – Sinyata Reka.

	DRK 1	DRK 2	MND 1	MND 2	VA	UZNG	SNR
1%	0.870304	0.360103	0.499897	0.676256	**0.000970**	0.503676	**0.013847**
2%	0.182717	0.141699	0.100993	0.122090	**0.025245**	**0.000490**	0.238410
4%	0.067736	0.144963	**0.000003**	**0.002156**	**0.000362**	0.101237	0.360264
8%	**0.000009**	**0.000017**	**0.000013**	**0.000787**	**0.000030**	**0.000113**	**0.000124**
16%	**0.000559**	**0.000001**	**0.000713**	0.126381	**0.000225**	**0.000111**	**0.000039**

In addition to the evaluation of the toxic effects, the exposure is important for the risk assessment [2]. In our study, we tested the effect of water samples in condition of 24 hours of exposure. At first glimpse, this result is in disagreement with another study of Bulgarian small reservoirs, where slight cytotoxic effect was recorded only after 48 hours of treatment [22], or with a study on the cytotoxicity of the most dangerous known microcystin MC-LR on cultured cells, where the effect occurred after 72-96 hours [23]. However, considering the differences in the types of the studied cell lines, of the water bodies, their algal biodiversity and detected cyanotoxins, these "discrepancies" are logical and easily explainable. Moreover, it is well-known that different strains of the same cyanoprokaryote species have different biochemical properties and the gene expression of toxic genes can vary depending on the environment [2]. In the same time, our results are in accordance with the detected cytotoxic effects at 24 hours of exposure reported by other authors [24].

Observations from this study concerning exposure time and concentrations correspond well with data available from *in vitro* investigations of other types of cell cultures [2]. For example, low dose of MC-LR after 24 h exposure did not induce apoptosis in the cell line of cultured Chinese hamster ovary, while the application of higher MC-LR concentrations induced apoptosis in a concentration-dependent manner [25].

Although in different amounts, cyanoprokaryotes were found in all five studied wetlands ([16-18], this study - Table 3) and this strongly corresponds to the general result from the present study, which demonstrated cytotoxic effects of the water collected from all of them. However, some differences in the cytotoxic effects of the applied water samples were observed. This result is logical when data on their biomass, algal and cyanotoxin composition are compared (Table 3)

For example, the highest number of toxigenic cyanoprokaryotes (12, among which was the dominant genus) was found in Vaya (Table 3) and this explains the best pronounced effect of the water of this wetland applied in different concentrations (Table 2). By contrast, the number of toxigenic genera was low (3) in the reservoir Sinyata Reka, but there cyanoprokaryotes from one toxigenic genus (*Microcystis*) were dominating the phytoplankton and this is in accordance with the strong effect of significant decrease of the cell viability with the increased sample concentrations (Table 2). Toxins, proved by ELISA and HPLC in these water bodies were also different, with saxitoxins found only in Durankulak 1 and cylindrospermopsin found in Mandra 1 and Vaya, and microcystins proved in Durankulak 1 and Sinyata Reka [16] – Table 3. Here we have to recall that saxitoxins are not specific for cyanoprokaryotes only, but are produced by algae from other taxonomic groups, like dinoflagellates, and therefore the identification of their producers is more complicated [2]. In the samples from Durankulak, collected in both years, such algae were found and their role in the water cytotoxicity is yet to be explored. In the samples from Durankulak 2 and Mandra 2, collected in 2019, microcystins were not found and checking for other cyanotoxins is in progress (V. Pavlova, M. Mitreva – pers. comm.). However, for the same samples the presence of toxigenic cyanoprokaryotes was proved by molecular-genetic methods (M. Radkova, K. Stefanova – pers. comm.). In Table 3 we indicate all recorded cyanoprokaryote genera, including those for which toxicity was not yet proved or was not searched for at global scale, in order to obtain a complete picture of the biodiversity and with the idea to consider them in future investigations. Last but not least, we would like to note that our findings could be related also with the presence of other cyanotoxins than those checked by us

Table 3. Quantitative distribution of cyanoprokaryote genera in the studied Bulgarian wetlands**.** Legend: DRK1 – Durankulak 1, DRK2 – Durankulak 2, MND1 - Mandra 1, MND2 - Mandra 2, VA – Vaya, UZNG – Uzungeren, SNR – Sinyata Reka, TPB – total phytoplankton biomass; d – dominant (>10-25% of TPB), c – common and abundant (5-10% of TPB), o – scarcely occurring (5-0.5% of TPB), r – rare species (<0.5% of TPB); MC – microcystins, SXT – saxitoxins, CYN – cylindrospermopsin, ndm – not detected microcystins. Toxigenic genera are provided after [2], with asterisk (*) are labelled genera published in [17, 18] and cyanotoxins published in [16].

	DRK1	DRK2	MND1	MND2	VA	UZNG	SNR
Cyanoprokaryota as % from the total biomass	25%	24%	40%	25%	42%	12%	90%
Toxigenic genera							
Anabaena					o		
Anabaenopsis					c		r
Aphanizomenon	d	o	o	r		o	r
Aphanocapsa		r	r		r		
Aphanothece		r					
Coelosphaerium			r		r		
Cuspidothrix		r			o	o	
Dolichospermum		o			c		
Gomphosphaeria	r				r		
Limnothrix			r			o	
Merismopedia	o	o			r	r	
*Microcystis**	d	d	c		c		d
Oscillatoria					r		
Phormidium		r		r			
Planktothrix			r		d		
Pseudanabaena	r	o	r				o
*Raphidiopsis**					c	d	
Trichodesmium		r					
Woronichinia		o					
picoplancton				d			
Total toxigenic genera	5	11	7	3	12	6	4
Non-toxigenic genera							
Borzia			r				
Chroococcus	r	c			r		
Coelomoron	r						
Cyanobium		r					
Cyanodictyon		o					
Pannus	r	r	r		o		
Planktolyngbya	r	r		r		d	
Romeria					r		
Snowella	r	r					
Synechocystis					r	r	
Total non-toxigenic genera	5	6	2	1	4	2	0
Total genera	10	17	9	4	16	8	4
CYANOTOXINS	MC*, SXT*	ndm	CYN*	ndm	CYN*	ndm	MC*

(microcystins LR, YR, and RR, saxitoxins and cylindrospermopsin), or by different products of cyanoprokaryote metabolism in the tested water samples.

CONCLUSION

Our results on the cytotoxic effects are in general accordance with the previous data obtained by chemical, molecular-genetic and conventional microscopic studies, which showed the presence of

cyanotoxins and cyanoprokaryotic toxin-producers in the studied samples [16-18]. There was a strong agreement between the cyanoprokaryote composition and biomass, with the presence of various toxigenic species, and detected effects on cell viability. The obtained results clearly demonstrated the applicability of human cell line Hs27 for *in vitro* cytotoxic measurements of water samples. This is valid especially for the cases when cyanotoxins have not been chemically proved due to their extreme diversity and impossibility to check all of them during conventional monitoring studies which cover just a small part of all cyanotoxins. Moreover, our results showed that cytotoxic effects occur fast, after 24 hours, even in quite low sample concentration (1-4%) and that 50% decrease of cell viability could be achieved at 8% concentration of the contaminated water. These results inevitably indicate the presence of a serious risk for ecosystem and human health in all five investigated water bodies which are used for recreation, sport fishing, fish-production and irrigation.

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REFERENCES

1. J. Meriluoto, L. Blaha, G. Bojadzija, M. Bormans, L. Brient, G. Codd, Z. Svirčev, *Adv. Oceanogr. Limnol.*, **8(1)**, 161 (2017).
2. J. Meriluoto, L. Spoof, J. Codd, Handbook of Cyanobacterial Monitoring and Cyanotoxin Analysis, John Wiley & Sons, Ltd., Chichester, 2017.
3. L. T. Tan, *Phytochem. Rev.*, **68**, 954 (2007).
4. M. Kaebernick, B. A. Neilan. *FEMS Microbiol*., **35**, 1 (2001).
5. Z. Svirčev, D. Lalić, G. B. Savić, N. Tokodi, D. D. Backović, L. Chen, J. Meriluoto, G. A. Codd, *Arch. Toxicol.*, **93**, 2429 (2019).
6. D. Gutiérrez-Praena, A. Jos, S. Pichardo, I. M. Moreno, A. M. Cameán, *Food Chem. Toxicol.*, **53**, 139 (2013).
7. V. Mulvenna, K. Dale, B. Priestly, U. Mueller, A. Humpage, G. Shaw, G. Allinson, I. Falconer, *Int. J. Environ. Res. Public Health.*, **9**, 807 (2012).
8. E. Jochimsen, W. Carmichael, J. An, D. Cardo, S. Cookson, C. Holmes, M. Antunes, D. de Melo Filho, T. Lyra, V. Barreto, S. Azevedo, W. Jarvis, *N. Engl. J. Med.*, **338**, 873 (1998).
9. S. Azevedo, W. Carmichael, E. Jochimsen, K. Rinehart, S. Lau, G. Shaw, *J. Toxicol.*, **181–182**, 441 (2002).
10. https://monographs.iarc.fr/wp-content/uploads/2018/06/mono94-7.pdf
11. D. Vankova, M. Pasheva, Y. Kiselova-Kaneva, D. Ivanov, D. Ivanova. Mechanisms of Cyanotoxin Toxicity-Carcinogenicity, Anticancer Potential, and Clinical Toxicology, *Med. Toxicol.*, IntechOpen, 2019.
12. J. Fastner, R. Heinze, I. Chorus, *Water Sci. Tech.*, **32**, 165 (1995).
13. M. Piyathilaka, M. Pathmalal, K. Tennekoon, B. De Silva, S. Samarakoon, S. Chanthirika, *Microbiol.*, **161**, 819 (2015).
14. M. P. Stoyneva-Gärtner, B. A. Uzunov, P. Dimitrova, *BioDiscovery*, **20,** e20501, doi: 10.3897/biodiscovery.20.e20501 (2017).
15. M. Stoyneva-Gärtner, J. Descy, A. Latli, B. Uzunov, V. Pavlova, Z. Bratanova, P. Babica, B. Maršálek, J. Meriluoto, L. Spoof, *Adv. Oceanogr. Limnol.*, **8,** 1 (2017).
16. M. Stoyneva-Gärtner, B. Uzunov, J.-P. Descy, G. Gärtner, P. Draganova, C. Borisova, V. Pavlova, M. Mitreva, *Mar. Freshw. Res.* (2019).
17. M. Radkova, K. Stefanova, B. Uzunov, G. Gärtner, M. Stoyneva-Gärtner, *Toxins*, **12**, 39 (2020). doi:10.3390/toxins12010039
18. K. Stefanova, M. Radkova, B. Uzunov, G. Gärtner, M. Stoyneva-Gärtner, *Biotechnol. Biotechnol. Equip.*, in press.
19. T. Michev, M. Stoyneva, (eds.) Inventory of Bulgarian wetlands and their biodiversity, Elsi-M, Sofia, 2007.
20. P. W. Sylvester, Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability, in: D. Satyanarayanajois (ed.), Drug design and discovery, methods and protocols, Springer, Humana Press, 2011.
21. K. Präbst, H. Engelhardt, S. Ringgeler, H. Hübner, Basic colorimetric proliferation assays: MTT, WST, and resazurin, in: D. F. Gilbert, O. Friedrich (eds.), Cell viability assays. Methods and protocols. Springer, Humana Press, 2017.
22. P. Stoyanov, D. Belkinova, R. Mladenow, I. Teneva, PU "P. Hilendarski", Jubilee Proceedings "Biological sciences for a better future", Plovdiv, 2012, p. 237.
23. M. W. Chong, K. D. Gu, P. K. Lam, M. Yang, W. F. Fong, *Chemosphere*, **41**, 143 (2000).
24. M. Chong, B. Wong, P. Lam, G. Shaw, A. Seawright, *Toxicon.*, **40**, 205 (2002).
25. M. Gacsi, O. Antal, G. Vasas, C. Mathe, G. Borbely, M. L. Saker, J. Gyori, A. Farkas, A. Vehovszky, G. Banfalvi, *Toxicol. in Vitro*, **23**, 710 (2009).