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СТРУКТУРА НА ФИТОПЛАНКТОНА И МОНИТОРИНГ НА ЦИАНОТОКСИНИ В ЯЗОВИР «ТРАКИЕЦ»

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PHYTOPLANKTON ASSEMBLAGES AND MONITORING OF CYANOTOXINS IN TRAKIETS RESERVOIR

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Abstract
The cosmopolitan distribution of Cyanoprokaryota and the formation of "water blooms" during the summer months significantly increase the risk of contamination of water basins with cyanotoxins. Yet, *Cyanoprokaryota*, often detected in the Bulgarian water bodies, are not studied in toxicological aspects. The aim of our study was to evaluate the diversity, distribution and quantitative development of the phytoplankton as well as the presence of cyanotoxins in the public reservoir Trakiets. We have collected water and phytoplankton samples at different time points from Trakiets reservoir used as fishponds. All water samples were analyzed for presence of cyanotoxins by ELISA, and tested for cytotoxicity on cell cultures *in vitro*. Physicochemical parameters, including water temperature, pH, total nitrogen and total phosphorus were measured on field. Algae, belonging to six divisions (*Cyanoprokaryota, Chlorophyta, Xantophyta, Dinophyta, Euglenophyta* and *Bacillariophyta*) were identified. Potentially toxic cyanoprokaryotes *Aphanizomenon flos-aquae* and *Microcystis aeruginosa* were detected in July together with *Anabaena affinis* in August and *Oscillatoria sp*. in September. The water sample collected in August contained 0.01 ng/ml of STXs. The total microcystins/nodularins concentration in the water samples collected during the study period was 0.09 $\mu g.L^{-1}$, 0.18 $\mu g.L^{-1}$ and 0.14 $\mu g.L^{-1}$ in July, August and September, respectively. Distinct responses depending on the cell line and the exposure period were detected after exposure of the cells to water samples. The cell viability was affected in all cell cultures after 24 and 48 h of exposure.

Introduction
Many of the blue-green algae (Cyanoprokaryota) produce secondary metabolites known as cyanotoxins (van Apeldoorn et al., 2007). These substances cause direct or indirect intoxication in

animals and humans and lead to damage of the liver and the nervous system as well as to development of tumors (Falconer, 1999; Ito et al., 2000; Lakshmana Rao et al., 2002). Very often this type of intoxications are fatal due to rapid intoxication or accumulation of the effects over time (Jochimsen at al., 1998). The cosmopolitan distribution of Cyanoprokaryota and the formation of "water blooms" during the summer months, significantly increases the risk of contamination of water basins with cyanotoxins (Chorus and Bartram, 1999). Many of the water basins in Bulgaria are used as a source of drinking water, irrigation water and fishing. Taking in account the aforementioned risks of contamination, there is an urgent need for continuous monitoring of water quality in terms of pollution with cyanotoxins. Most of the fresh water basins in Bulgaria, including dams, are relatively well studied in terms of the phytoplankton composition, but the data for presence of cyanotoxins are limited. There is only one report about microcystin contamination of water samples from 15 Bulgarian reservoirs and lakes (Pavlova et al., 2006). Data showed that the concentration of total microcystins (MC-LR, MC-RR and MC-YR) in the biomasses ranged from 8 to 1070 $\mu g.g^{-1}$(d.w.). Linking the phytoplankton assemblages of a water basin with the presence of cyanotoxins and water quality will give an opportunity to establish a good system for monitoring of the phytoplankton communities and their toxic potential.
The aim of our study was to evaluate the diversity, distribution and quantitative development of the phytoplankton as well as the presence of cyanotoxins in the public reservoir Trakiets.

Materials and Methods

Site description and physicochemical water quality analysis

Reservoir Trakiets is located in the Haskovo region of South-Central Bulgaria (geographic coordinates 41°85'96''N and 25°40'58''E). Initially, the dam was designed as a facility for irrigation. Until 2005 it provided about 30% of the drinking water for Haskovo town and surrounding villages. After that it is used mainly for fish farming and irrigation.
Physicochemical parameters, including water temperature, pH, total nitrogen and total phosphorus were measured on field by a portable photometer pHotoFlex® (WTW GmbH, Weilheim, Germany) or in the laboratory.

Sample collection and phytoplankton analysis

For qualitative analyses of the phytoplankton, samples were collected three times (July, August and September, 2008) from the surface layer (0.5 m) with a 20 μm mesh size net at a fixed collection station and preserved in 4% formaldehyde. For phytoplankton counting, samples were collected with Meyer bottles of 1L and preserved with Lugol solution. Phytoplankton analyses were performed from both, fresh and conserved (in 4% formaldehyde) samples, with an inverted microscope (PZO Poland) according to Lund et al. (1958), using sedimentation chambers for phytoplankton identification and cell density estimation.
Water samples for chemical and toxicological analysis were collected at the same time as the phytoplankton samples from the same sampling point.

In vitro cytotoxicity tests

Three different commercially available cell lines were used for the *in vitro* tests: HeLa (human cervical epithelial adenocarcinoma, ATCC CCL-2), 3T3 (mouse embryonic fibroblasts, ATCC CCL-92) and FL (normal amniotic human cells, ATCC CCL-62). Cells were cultured in 75 cm2 flasks in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, UK), supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS, PAA Laboratories, Austria), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, Steinheim, Germany), at 37°C with 5% CO_2 in air and high humidity. Cell viability was measured with the trypan blue exclusion test prior to seeding. Cells were plated in 96-well tissue culture plates at a density of $1.5x10^4$ per 200 µL DMEM with 10% FCS. After 24 h (to allow the attachment of the adherent cells) the cultures were exposed to 10%

of water samples. Control wells were prepared by adding equal amounts of Millipore water to the culture medium. The cells were exposed for 24 h or 48 h prior to analysis.
After the desired time of exposure with water samples, 20 µl of MTT solution (5 mg/ml in PBS) were added directly to each well and incubated at 37°C for 3 h. Thereafter, the supernatant was discarded and 0.1 ml of dimethylsulfoxide (DMSO) was added to each well in order to facilitate solubilization of the formazan product. After 15 min at room temperature the plates were shaken, and absorbance was read at 570 nm in a SPECTRAmax PLUS microplate spectrophotometer.

Analysis of cyanotoxins by ELISA

Saxitoxins
The water samples were analyzed by the Ridascreen™ saxitoxin ELISA kit (R-Biopharm, Darmstadt, Germany). This is a competitive ELISA for the quantitative analysis of saxitoxin and related toxins based on the competition between the free toxins from samples or standards and an enzyme-conjugated saxitoxin for the same antibody. The mean lower detection limit of the Ridascreen™ saxitoxin assay is about 0.010 ppb ($\mu g.L^{-1}$).

Microcystins and nodularins
The analysis of the water samples for presence of microcystins and nodularins was performed using the Microcystins ELISA (Abraxis LLC, Warminster, PA). As for the saxitoxin ELISA, this is a quantitative, competitive immunosorbent assay that allows the congener-independent detection of microcystins and nodularins in water samples. The limit of detection of the Microcystins ELISA is 0.10 ppb ($\mu g.L^{-1}$).

Results

Physicochemical parameters
The environmental variables of the investigated reservoir are given in Table 1. The water temperature at the surface ranged from 21.5°C in July to 24°C in August and September. pH values were between 7.7 and 7.1. Total nitrogen (TN) concentrations varied from 0.2 $mg.L^{-1}$ in July to 1.2 $mg.L^{-1}$ in September. Total phosphorus (TP) concentrations were from 0.01 in August to 0.11 $mg.L^{-1}$ in September). TN/TP ratio in August was 30, which indicates phosphorus limitation on a community level. In contrast, in July and September, the TN/TP ratio was <14, which is indicative of nitrogen limitation.

Table 1. *Physicochemical variables of the surface water in Trakiets reservoir.*

No	Sampling site	Sampling time	Temperature (^oC)	pH	TP (mg/L)	TN (mg/L)	N/P
1.	Dam wall	July	21.5	7.7	0.03	0.2	6.67
2.	Dam wall	August	24	7.1	0.01	0.3	30.0
3.	Dam wall	September	24	7.1	0.11	1.2	10.9

Taxonomic composition and structure of the phytoplankton community
In this study, algae belonging to six divisions (*Cyanoprokaryota, Chlorophyta, Xantophyta, Dinophyta, Euglenophyta* and *Bacillariophyta*) were identified. During the study period (July, August and September 2008), *Chlorophyta* represented 55.8% in July, 54.6% in August and 47.9% in September of the total phytoplankton, followed immediately by *Bacillariophyta* (15.6% in July, 14.6% in August and 15.2% in September), *Euglenophyta* (12.9% in July, 15.1% in August and 12.9% in September) and *Cyanoprokaryota* (6.6% in July, 9.1% in August and 9.1% in September) (Fig. 1).

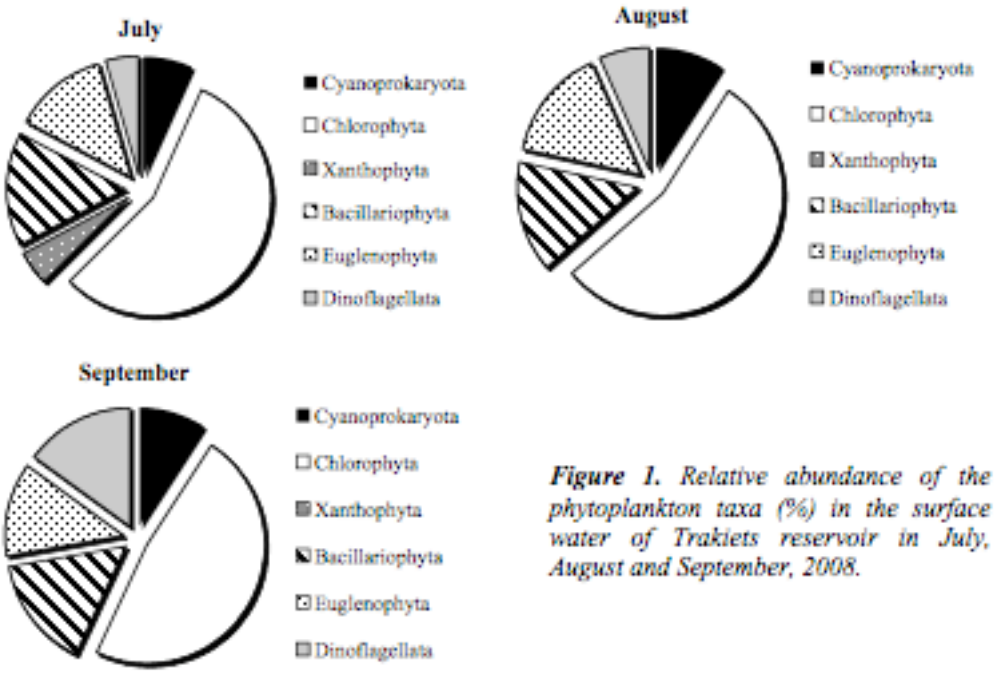

Figure 1. *Relative abundance of the phytoplankton taxa (%) in the surface water of Trakiets reservoir in July, August and September, 2008.*

Potentially toxic cyanoprokaryotes *Aphanizomenon flos-aquae* and *Microcystis aeruginosa* were detected in July together with *Anabaena affinis* in August and *Oscillatoria sp*. in September. More details related to species composition and quantitative characteristics of *Cyanoprokaryota* are given in Table 2.

Table 2. *Species composition and quantitative characteristics of Cyanoprokaryota in Trakiets Reservoir.*

Months	Species composition	Density (10^6/L)	Biomass (mg/L)	Total density	Total biomass
July	*Aphanizomenon flos-aque* *Microcystis aeruginosa*	360 000 280 000	0,03 0,02	640 000	0,05
August	*Anabaena affinis* *Aphanizomenon flos-aque* *Microcystis aeruginosa*	250 000 96 300 56 400	0,03 0,01 -	402700	0,04
September	*Gomphosphaeria lacustris* *Microcystis aeruginosa* *Oscillatoria sp.*	97 000 230 600 128 500	- 0,02 0,01	456 200	0.03

ELISA analyses of water samples for presence of cyanotoxins

Collected water samples from the reservoir were analyzed for presence of cyanotoxins by available ELISA kits for saxitoxins and microcystins. According to the saxitoxin ELISA kit, which has cross reactivity to decarbamoyl saxitoxin, gonyautotoxins II, III, B1, C1 and C2, water sample collected in August contained 0.01 ng/ml of this group cyanotoxins. ELISA analyses showed no presence of saxitoxins in the water samples collected in July and September (Fig. 2a).

The total microcystins/nodularins concentration in the water samples collected during the study period and detected by an ELISA kit, which cross-reacts with microcystin LR, LA, RR, YR and

nodularin, was 0.09 µg.L^{-1}, 0.18 µg.L^{-1} and 0.14 µg.L^{-1} in July, August and September respectively (Fig. 2b).

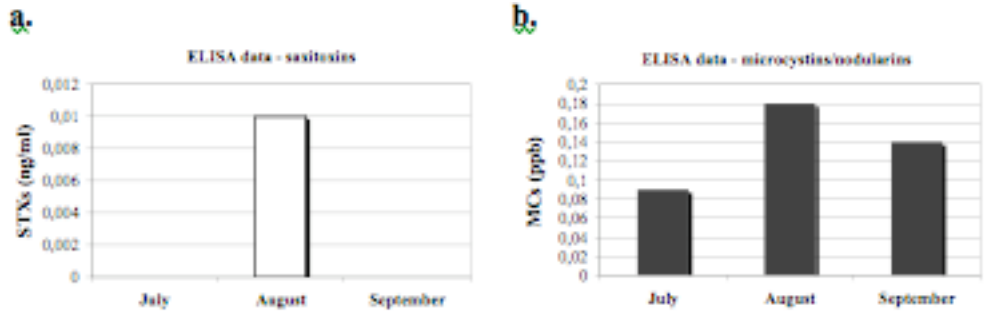

Figure 2. *Presence of cyanotoxins in the water samples tested by ELISA.* ***a.*** *– presence of saxitoxins;* ***b.*** *– presence of microcystins/nodularins.*

Toxicity of water samples in vitro

In order to test the cytotoxic activity of water samples collected during the study period, three different cell lines were used: HeLa (human cervical epithelial adenocarcinoma), 3T3 (mouse embryonic fibroblasts) and FL (normal amniotic human cells).

Distinct responses depending on the cell line and the exposure period were detected after exposure to water samples. The cell viability was affected in all cell cultures after 24 and 48h of exposure to water samples. Relative weak cytotoxic effect (from 5 up to 15% toxicity) were observed after 24 h of exposure in all cell lines, whereas after 48 h all cells revealed different degrees of cytotoxicity up to 30% by HeLa and FL cells (Fig. 3). These differences may be due to the distinct origin of the cells and indicate different degrees or mechanisms of the cellular stress elicited by the samples.

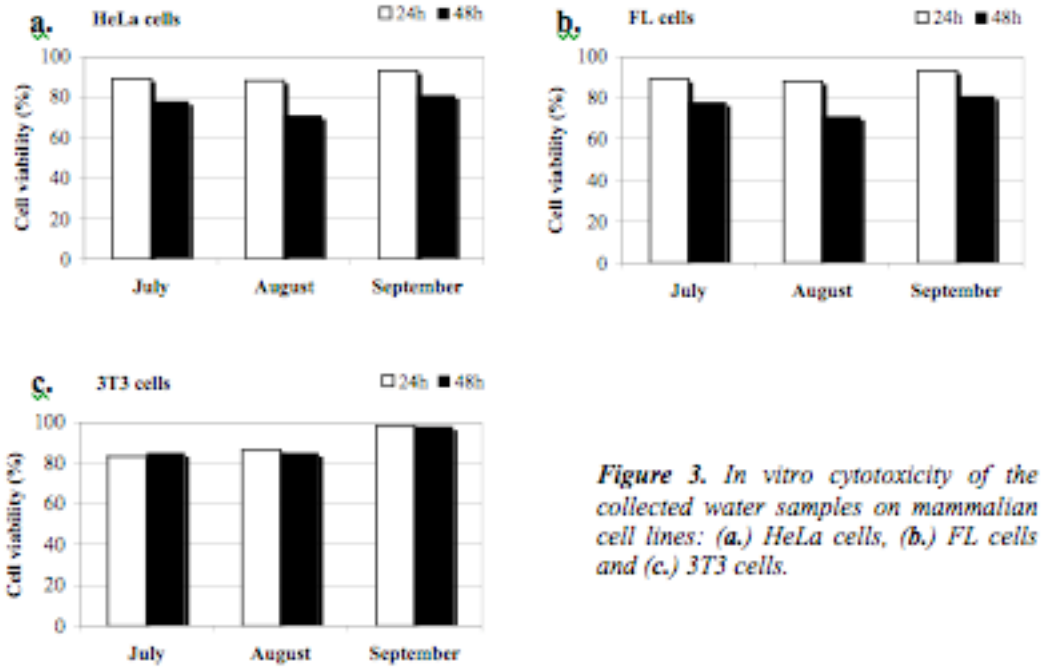

Figure 3. *In vitro cytotoxicity of the collected water samples on mammalian cell lines:* ***(a.)*** *HeLa cells,* ***(b.)*** *FL cells and* ***(c.)*** *3T3 cells.*

In this study, we have investigated the phytoplankton diversity and abundance in Trakiets reservoir with an emphasis on the presence of cyanotoxins and water quality. Although the toxic algal species are object of intensive research worldwide, the aspect of micro algae toxicity is still poorly investigated in Bulgaria. This study underlines that permanent monitoring programs of *Cyanoprokaryota* in Bulgarian reservoirs used as sources of drinking water or for fish farming should be implemented. Indirect exposure and transfer of cyanotoxins through food chains must also be considered.

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