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EFFECT OF TEMPERATURE AND LIGHT INTENSITY ON THE GROWTH, CHLOROPHYLL *A* CONCENTRATION AND MICROCYSTIN PRODUCTION BY *MICROCYSTIS AERUGINOSA*

Pavlova V.[1*], S. Furnadzhieva[2], J. Rose[3], R. Andreeva[2], Zl. Bratanova[1], A. Nayak[3]

[1]National Center of Public Health Protection, Akad. Ivan Ev. Geshov Blvd. 15, BG-1431, Sofia, Bulgaria

[2]Institute of Plant Physiology, Acad. G. Bonchev Str., Bl. 21, BG-1113 Sofia, Bulgaria

[3]Michigan State University, 13 Natural Resources Bldg, East Lansing, MI 48824, USA

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Summary. This paper presents research on the concentration of microcystins produced by *Microcystis aeruginosa* (Kützing, UTEX 2667). The relationship between the production of microcystins, the algae growth and the content of chlorophyll *a* was investigated at five temperatures (range 20-38°C) and two light intensities – 8000 lx and 2x8000 lx. ELISA method was used for measuring the toxin level. Optimal conditions for the production of microcystins at 25-26°C were observed. Neither of the two light intensities impacted the amount of microcystins. HPLC-DAD quality analysis for determination of microcystins in the algal biomass was performed. The results showed presence of microcystin-LR and six unknown peaks possessing characteristic microcystin-like UV-spectra, which are of interest for future investigations.

Key words: cyanobacteria; *Microcystis aeruginosa*; microcystins; ELISA; HPLC.

Abbreviations: ELISA – Enzyme-Linked ImmunoSorbent Assay; HPLC-DAD – High Performance Liquid Chromatography with Diode Array Detector; UV – Ultraviolet.

INTRODUCTION

The cyanoprocaryotes (cyanobacteria) are distributed globally. Their ability to bloom in water is mainly a result of eutrophication of water bodies, the safety of which is connected to the presence of toxin producing algal species (Oliver and Ganf, 2000). Cyanobacteria can produce a broad spectrum of toxins – cyanotoxins which may adversely affect aquatic and the terrestrial wildlife as well as humans. If the water inhabitants come in contact with the polluted water or if the animals consume or inhale the toxins, neurological and gastrointestinal symptoms and even death can result (Sivonen and Jones, 1999). The cyclic heptapeptide

*Corresponding author: vera_pavlova@abv.bg

hepatotoxins, microcistins, are frequently reported in water (Sivonen and Jones, 1999) and are isolated from several species of freshwater genera including *Microcystis, Planktothrix (Oscillatoria), Anabaena and Nostoc*. Microcystin-LR is considered to be one of the most important toxins and World Health Organization suggests provisional guideline values of 1 μg/L in drinking water (WHO Guidelines, 1998) and 20 μg/L in bathing water (Chorus, 2005). Many studies across the globe have reported on the occurrence of microcystins in surface water (Bláha and Maršálek, 2003; Pavlova et al., 2006; Sedmak and Kosi, 1997; Sivonen and Jones, 1999). However, there is still uncertainty regarding what environmental factors play a role in any given bloom or species in the initiation of toxin productions. The influence of temperature, light intensity, pH, the ratio N:P on and relationship to the content of chlorophyll *a* and to the production of cyanotoxins has been investigated, but the data are discrepant (Almeida et al., 2006; Codd, 2000; Hobson et al., 1999; Lee et al., 2000; Orr et al., 2004; Wiedner et al., 2003).

Studies that have investigated the genetic capacity for microcystin production have revealed no clear indication of recombination across the genera, while frequent recombination events both within and between *mcyB* and *mcyC* sequences were detected between strains from the same genus, except for *mcyC* from *Planktothrix* (Tooming-Klunderud et al., 2008). The authors demonstrated the remodeling of *mcyB* and *mcyC* genes including evidence for positive selection suggesting that the microcystin variant profile of a given strain is likely to influence the ability of the strain to interact with its environment.

Our previous work of the microalgal flora and microcystin content in some Bulgarian water bodies found that *Microcystis aeruginosa* is a dominant species in three of the investigated lakes with algae blooms and high microcystins concentrations (Pavlova et al., 2006). The goal of this study was to further examine the effect of a range of temperatures and light intensities on the growth, chlorophyll *a* concentration and the production of microcystins in a controlled laboratory culture of *Microcystis aeruginosa* to shed light on the inconsistencies reported in literature.

MATERIALS AND METHODS

The strain *Microcystis aeruginosa* (Kützing, UTEX 2667) was used for addressing the key research questions for this current study and was cultivated using a block with a temperature gradient (Dilov, 1985) with temperature investigated in the range 20 - 38°C. The light was continuous with an intensity of 8000 lx and 2x8000 lx. Aeration was carried out by bubbling 100 L gas - air mixture (enriched with 2% CO_2) per one liter of suspension per hour. The cultivation was carried out with a continuously growing density of the algae for a period of 96 hours using the medium Allen Arnon (Allen and Arnon, 1955). Chlorophyll *a* was measured spectrophotometrically after extraction with hot methanol from three parallel samples and the average concentrations were calculated according to a formula which is cited in MacKinney, 1941. The algal growth was measured by dry weight from three parallel samples as absolutely

dry substance (average ADS). The content of microcystins was determined by the ELISA method. Multiskan RC (Labsystems) coupled with ELISA Quanti Plate Kit was used. The sample pretreatment was carried out according to the manufacturer's instructions with measurement of three parallel samples. The average concentrations were calculated. The absorption was detected at 450 nm and additionally at 600, 630 and 650 nm as a comparison.

For quality determination of microcystins the same strain was cultivated at 25 - 26°C and the biomass was used for HPLC-DAD analysis according to Pavlova et al., 2006.

RESULTS AND DISCUSSION

Light and temperature are the main factors influencing photosynthesis. Figure 1 presents the growth of *Microcystis aeruginosa* (Kützing, UTEX 2667) at five

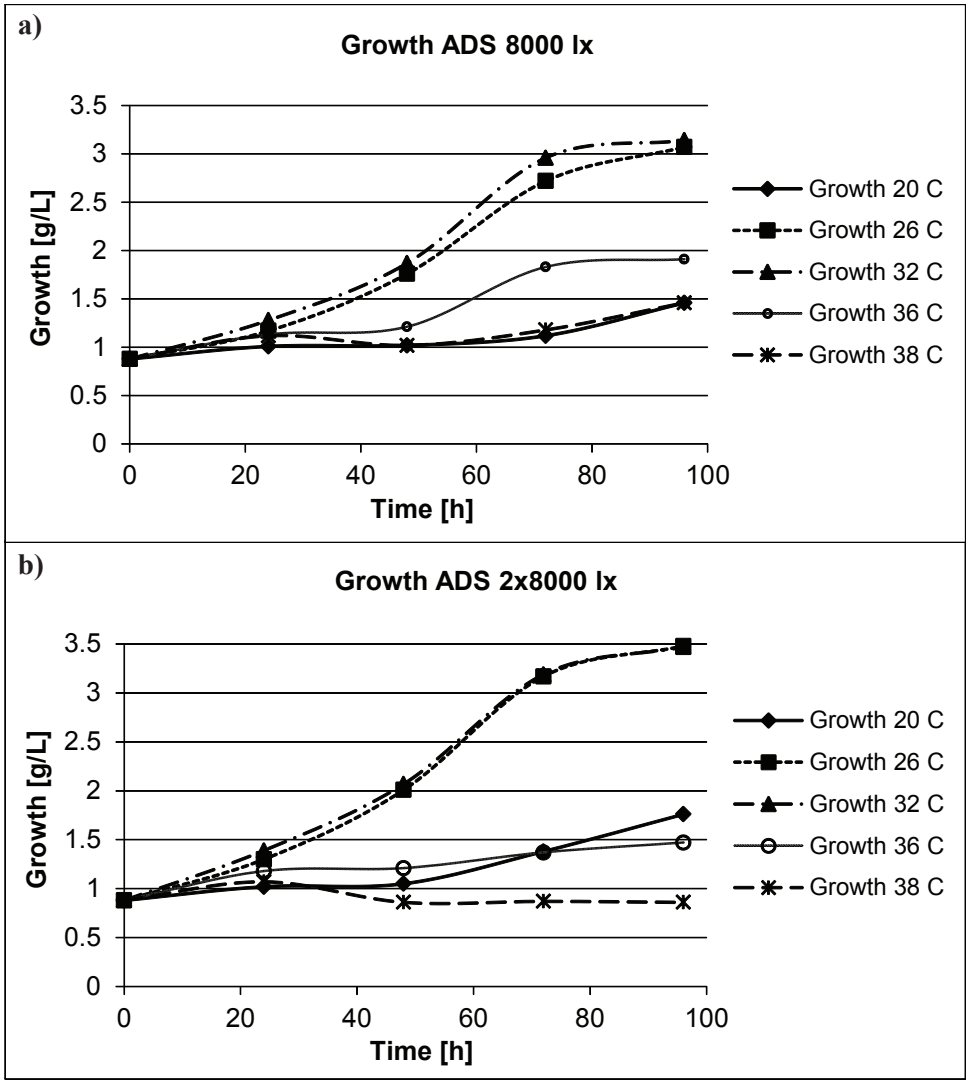

Fig. 1. Growth of *Microcystis aeruginosa* (UTEX 2667) at 8000 lx (a) and 2x8000 lx (b) expressed as absolutely dry substance (ADS).

temperatures and two light intensities. This algal species was able to grow over a broad temperature range of 26°C and 32°C, with lower and higher temperature effecting the ADS at 8000 lx light intensity. The results were similar at 26°C for both light intensities, however, better exponential growth was achieved with 2x800 lx at 26°C, whereas at 32°C under the same light intensity it did not grow as readily. The temperature relationships to the microcystin concentration, the growth and the content of chlorophyll *a* are illustrated in Table 1. Not surprising, all showed a strong relationship to temperature. Investigations by other scientists had given the optimum of growth at 32,5°C and between 32-36°C respectively for the strains *Microcystis aeruginosa* (NRS-1) and *Microcystis aeruginosa* (UV-006) (Gorunova and Demina, 1974; Van der Westhuizen and Eloff, 1985). Thus this strain is able to compete, grow and produce microcystins at much lower temperatures than previously suggested by the literature, suggesting adaptation perhaps to local environmental conditions.

Figure 2 shows the growth of the algae and microcystin production over the temperature gradients used in this

Table 1. Quadratic equations of the temperature dependences. The regression analysis shows correlation between the temperature and the concentration of microcystins, the amount of chlorophyll *a* and the growth respectively.

Temperature dependence	Quadratic equation ($p<0.05$)	R^2
Concentration of microcystins 48 h, 8000 lx	$Y= -4.9788xT^2 + 285.772xT - 3363.8$	0.829
Concentration of chlorophyll *a* 48 h, 8000 lx	$Y= -0.2073xT^2 + 11.9291xT - 147.72$	0.835
Growth (ADS) 48 h, 8000 lx	$Y= -0.0106xT^2 + 0.6067xT - 6.8172$	0.956
Concentration of microcystins 48 h, 2x8000 lx	$Y= -9.3611xT^2 + 522.644xT - 6223.3$	0.884
Concentration of chlorophyll *a* 48 h, 2x8000 lx	$Y= -0.1941xT^2 + 11.0717xT - 135.40$	0.908
Growth (ADS) 48 h, 2x8000 lx	$Y= -0.0152xT^2 + 0.8662xT - 10.222$	0.980
Concentration of microcystins 96 h, 8000 lx	$Y= -33.089xT^2 + 1729.24xT - 18054$	0.963
Concentration of chlorophyll *a* 96 h, 8000 lx	$Y= -0.4532xT^2 + 26.3244xT - 339.25$	0.900
Growth (ADS) 96 h, 8000 lx	$Y= -0.0252xT^2 + 1.4724xT - 18.197$	0.981
Concentration of microcystins 96 h, 2x8000 lx	$Y= -40.123xT^2 + 2091.96xT - 222102$	0.952
Concentration of chlorophyll *a* 96 h, 2x8000 lx	$Y= -0.4641xT^2 + 26.3738xT - 334.52$	0.937
Growth (ADS) 98 h, 2x8000 lx	$Y= -0.0302xT^2 + 1.6969xT - 20.101$	0.966

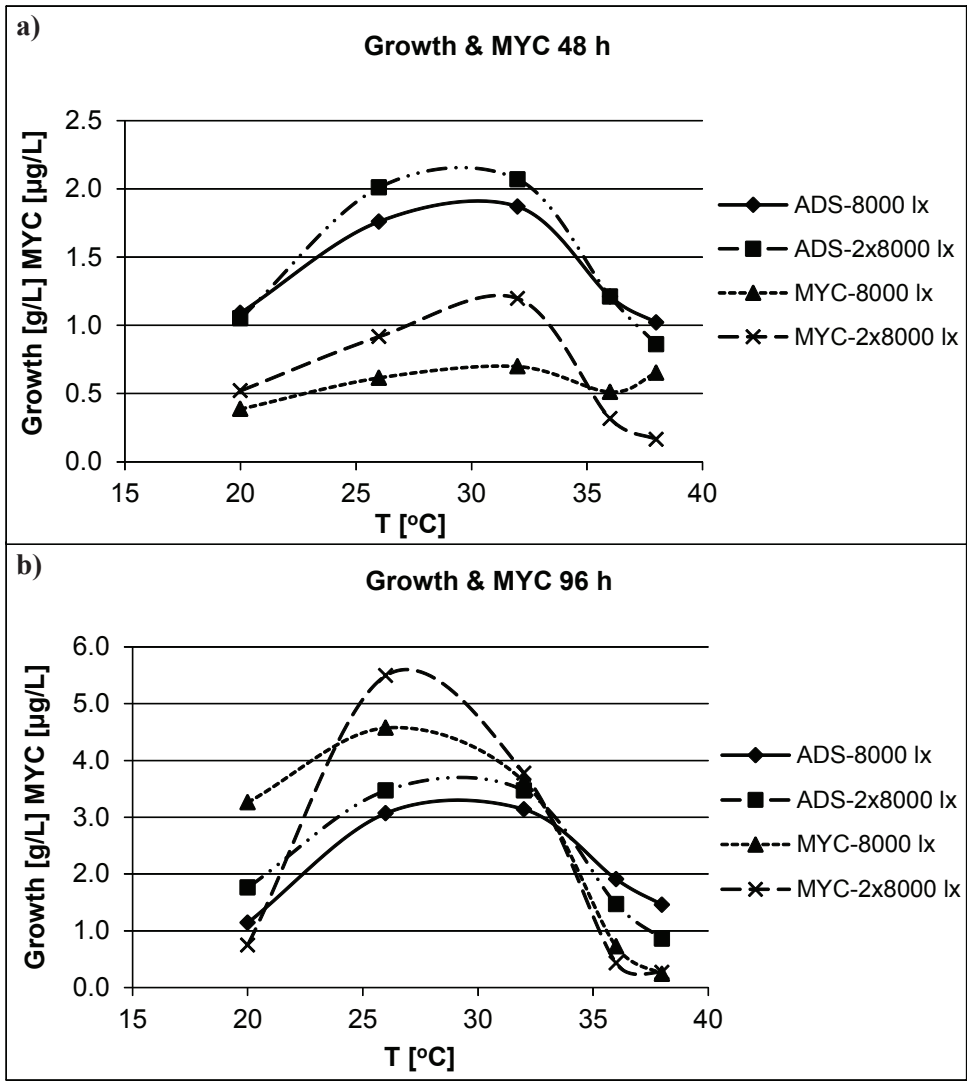

Fig. 2. Comparison of growth and microcystin production (MYC) at 48 h (a) and 96 h (b).

study. Samples for the determination of microcystins and chlorophyll *a* were taken at 48 h and 96 h. Exponential growth occurred between 50 h and 80 h, with maximum biomass achieved due to algal growth by 96 h. Thus, samples were taken slightly before and just prior to reaching the highest algal concentrations. The accumulated algal biomass at optimum temperatures (26°C and 32°C) at 96 h was considerably different than that at 48h. The relationship between of the synthesis of microcystins (total) and the growth of the algae as measured by chlorophyll *a* is shown in Fig. 3. The concentrations of microcystins correlated with the accumulated biomass and content of chlorophyll *a* at 48 h for both light intensities (Figs. 2a and 3a). The same result was obtained by Lyck (2004) in experiments with the strains *Microcystis aeruginosa* (CYA 228). Microcystin toxin production was enhanced at 2x8000 lx (Fig. 2) at both time points (48 and 96 h).

Clear temperature dependence of the synthesis of microcystins at 26°C was

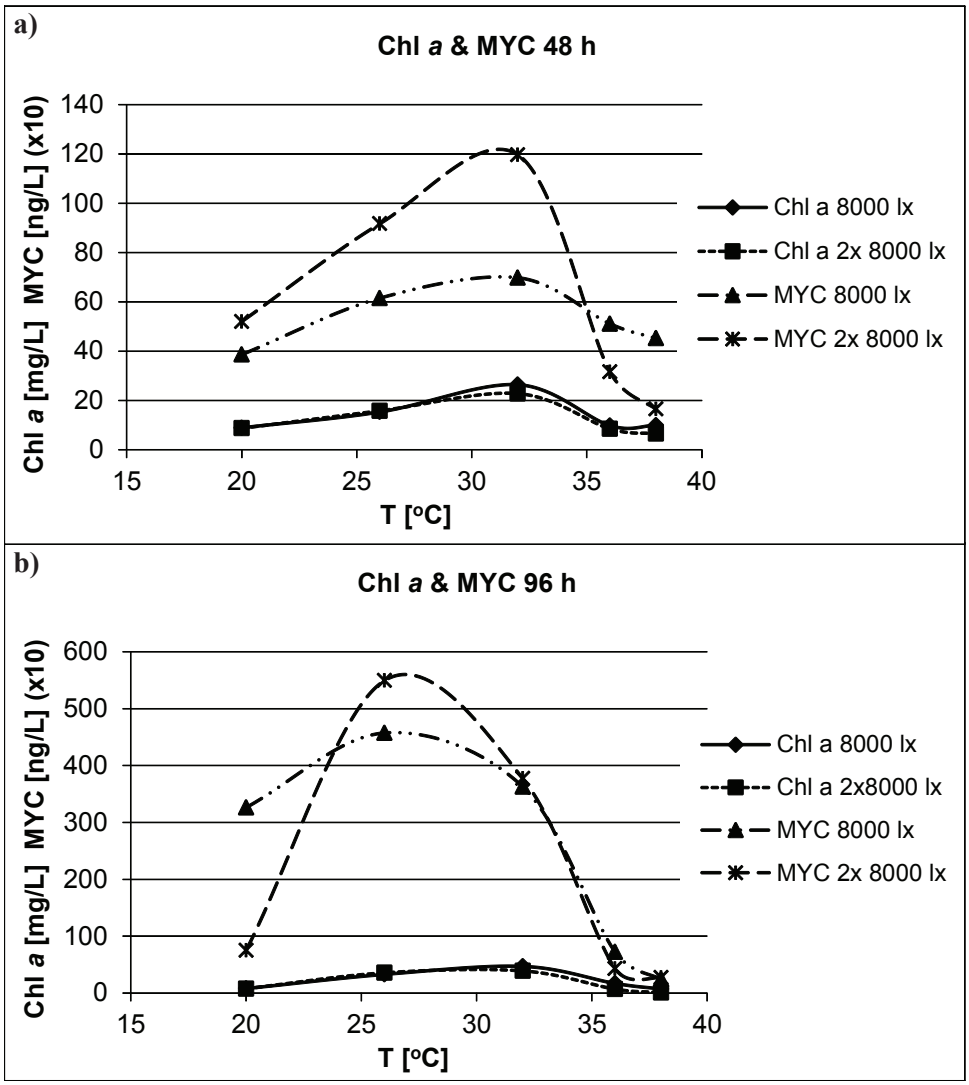

Fig. 3. Comparison of chlorophyll a (Chl a) and microcystin production (MYC) at 48 h (a) and 96 h (b).

observed at both light intensities after 96 h. This maximum did not correspond to the curves of growth and content of chlorophyll *a* (Figs. 2b and 3b). The content of microcystins was increased five times at 96 h compared to 48 h of growth, but biomass and chlorophyll *a* were increased only twice.

There was no correspondence of microcystin concentrations and the growth at 96 h or the concentrations of chlorophyll *a* at 96 h for both light intensities. Our results confirmed the results of other investigations (Gorunova and Demina, 1974) showing that the maximum toxin production by *Microcystis aeruginosa* (NRS-1) and other *Microcystis* species (*Microcystis viridis)* was reached at 25°C for the same period after protracted cultivation (Song et al., 1998).

At least one study has shown that maximum toxicity was produced at 20°C

for *Microcystis aeruginosa* (UV-006) (Van der Westhuizen and Eloff, 1985) and the authors also concluded that optimal growth was not related to the microcystin. Microcystin production was not essential for the growth of *Microcystis aeruginosa* (PCC 7806) (Hesse et al, 2001). On the other hand, studies with *Microcystis aeruginosa* strains - MASH01-A19 and CYA 228 demonstrated that the process of cell division connected with growth and the microcystin production were tightly coupled (Orr and Jones, 1998; Lyck, 2004).

The ability to use chlorophyll *a* as a proxy for growth and microcystin production is not supported by our results. While some investigations on *strains Microcystis aeruginosa* UTEX 2388 and CYA 228 have associated microcystin production and chlorophyll *a* content (Lee et al., 2000; Lyck, 2004), others have confirmed our conclusion showing the inability to predict microcystin production via the concentration of chlorophyll *a* with *Microcystis aeruginosa* in Bang Phra Reservoir, Thailand (Xialofeng et al.,2002).

Higher light intensity influenced the growth, but while our data showed little statistical correlation between microcystin production and the applied light intensities, there was greater microcystin production at the higher light intensity. Similar results have been obtained for a strain of *Microcystis aeruginosa* obtained from a reservoir (Xialofeng et al., 2002). The interdependence between light and the growth of *Microcystis aeruginosa* (PCC 7806) (Kaebernick et al., 2000) has been observed.

The quality composition of microcystins was determined from the biomass cultivated at 25-26°C. Results of HPLC-DAD analysis of *Microcystis aeruginosa* (UTEX 2667) biomass confirmed the presence of microcystin-LR and six microcystin-LR equivalents. The equivalents were not comparable to those in the standard solution (-LR, -RR and -YR) and were not identified because of lack of standards for them. The unknown peaks possessed characteristic microcystin-like UV-spectra. Fig. 4 shows the chromatogram of the biomass (a) and overview of UV- specters of microcystin-LR and one of the "unknown" microcystin-LR equivalents (b).

The data suggest that *Microcystis aeruginosa* (UTEX 2667) can produce microcystin-LR and other related microcystins (retention time and UV-specter) which could also suggest that they can cause similar health risks. These toxins are concentrated generally in the cells and released in water when the cells are lysed (Chorus, 2005; Codd, 2000).

Microcystin toxins associated with blooms have been shown to be of concern in water samples from 15 Bulgarian reservoirs and lakes (Pavlova et al., 2006). Studies undertaken in 2004 showed that the concentrations of total microcystins (MC-LR, MC-RR and MC-YR) in the biomass ranged from 8 to 1070 μg/g DW. The dissolved microcystin concentration was 1.64 μg/L obtained in one water sample (Pavlova et al., 2006).

An investigation of the phytoplankton diversity, algal biomass, and selected physicochemical parameters in a drinking water reservoir (Borovitsa) located in the Kardzhali region, Bulgaria in 2006 has been recently reported (Teneva et al., 2010). The results demonstrate the presence of anatoxin-a and microcystins/

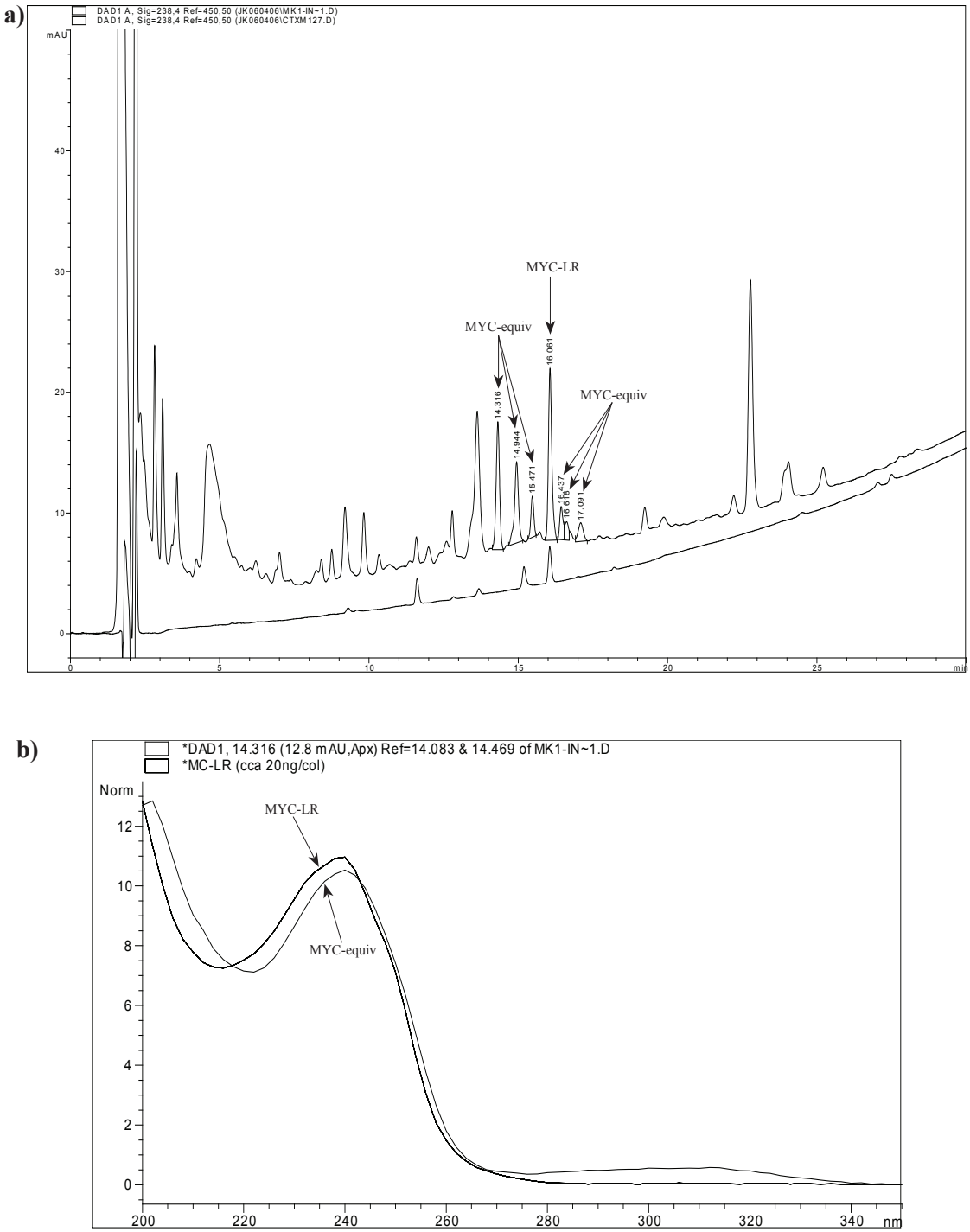

Fig. 4. Chromatogram of *Microcystis aeruginosa* (UTEX 2667) biomass (a) and overview of UV-spectra of microcystin-LR and a microcystin-LR equivalent (b) – retention time 14.316, overview factor 982.5.

nodularins (0.09-0.12 μg/L) (July 2006), and saxitoxins (2.5 μg/L) and microcystins/nodularins (0.18 μg/L) (September, 2006) in the raw water samples. The study underlines that permanent monitoring programs of Cyanoprokaryota in the reservoirs used as sources of drinking water and toxicity assessments should be implemented.While drinking water is of the utmost concern, indirect exposure and transfer of cyanotoxins through food chains must also be considered.

There are very few investigations and reports on the problems associated with cyanobacterial blooms and their toxins in water bodies in Bulgaria. Eutrophication and global climate changes have the potential to increase the numbers, duration and spatial distribution of these blooms. Therefore, investigations of cyanotoxins production in continued field studies are necessary if risks and management strategies have to be identified.

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