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First report of cyanobacterial bloom of *Microcystis viridis* (A. Braun) Lemmermann in Slovenia

Prvi opis cvetenja cianobakterije *Microcystis viridis* (A. Braun) Lemmermann v Sloveniji

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Abstract. The presence of the cyanobacterial bloom of *Microcystis viridis* (A. Braun) Lemmermann is reported for the first time in Slovenia. After field sampling, and detailed microscopic observations, species analysis, chlorophyll content analysis, and cyanobacterial cyclic peptides were determined, the latter by high performance liquid chromatography (HPLC). Cells were found in colonies with limited amounts of more or less refractive mucilage. The average diameter of a cell was 4–7 µm. Three microcystins, two anabaenopeptins and planktopeptin BL 1125, were identified. The content of cyclic peptides in the bloom was in the range of 2.3–6.6 mg g^{-1} of cellular dry weight. *M. viridis* was dominant in the cyanobacterial bloom, other species being *Micorcystis wesenbergii, Microcystis aeruginosa, Anabaena flos-aque, Anabaena spiralis, Aulacoseira granulata, Closterium* sp.*, Euglena* sp.*, Pediastrum duplex, Scenedesmus quadricauda, Staurastrum gracile, Trachelomonas volvocina, Trachelomonas hispida* and *Tetraedron limneticum*. In keeping with previous studies the content of cyclic peptides in the cyanobacterial bloom was high enough to cause bloom lysis. This fact was also confirmed by field observation; not only bloom composition change, but after 8 days there was no visible cyanobacterial bloom on the Boreci reservoir surface, although no heavy rain or wind was observed during this period. The discovery of *M. viridis* bloom in Slovenia is very important, since toxic bloom constitutes a threat all over the World.

Key words. cyanobacteria, cyanobacterial bloom, *Microcystis viridis*, microcystin, cyclic peptides

Izvleček. V Sloveniji prvič poročamo o prisotnosti cianobakterijskega cveta *Microcystis viridis* (A. Braun) Lemmermann. Po vzorčenju, temeljitem mikroskopskem pregledu, analizi vrstnega sestava in analizi vsebnosti klorofila smo določili prisotnost cianobakterijskih cikličnih peptidov s pomočjo tekočinske kromatografije visoke ločljivostji (HPLC). Celice so v kolonijah obdane z sluzjo, ki lomi svetlobne žarke. Povprečni premer celic je bil 4–7 µm. Identificirali smo tri mikrocistine, dva anabaenopeptina in planktopeptin BL 1125. Vsebnost cikličnih peptidov v cvetu je bila 2.3–6.6 mg g^{-1} suhe celične mase. V cianobakterijskem cvetu je prevladovala vrsta *M. viridis*, ostale vrste pa so bile *Micorcystis wesenbergii, Microcystis aeruginosa, Anabaena flos-aque, Anabaena spiralis, Aulacoseira granulata, Closterium* sp.*, Euglena* sp.*, Pediastrum duplex, Scenedesmus quadricauda, Staurastrum gracile, Trachelomonas volvocina, Trachelomonas hispida* in *Tetraedron limneticum.* Na podlagi rezultatov prejšnjih raziskav lahko zaključimo, da je vsebnost cikličnih peptidov dovolj visoka, da lahko povzroči lizo cveta. To dejstvo je bilo potrjeno tudi z opazovanji v naravnem okolju v okviru te raziskave. Ni prišlo samo do spremembe

vrstne sestave cveta, temveč do izginotja cianobakterijskega cveta na površini jezera Boreci po osmih dneh, čeprav v tem obdobju ni bilo močnega deževja, niti vetra. Odkritje pojavljanja cvetenja *M. viridis* v Sloveniji je izrednega pomena, saj strupen cianobakterijski cvet predstavlja grožnjo po celem svetu.

Ključne besede. cianobakterije, cianobakterijski cvet, *Microcystis viridis*, mikrocistin, ciklični peptidi

Introduction

About 60 % of cyanobacterial samples investigated worldwide contain toxins (Guidelines 2003). The toxicity of a single bloom can, however, change in both time and space. Demonstration of toxicity of the cyanobacterial population in a given lake or reservoir does not necessarily imply an environmental or human hazard as long as the cells remain thinly dispersed. Mass developments and, especially, surface scums pose the major risk.

Microcystis has been known to be the major genus among the cyanobacteria to cause blooms in fresh waters worldwide (Carmichael 1992; Guidelines 2003). *Microcystis* blooms frequently occur in the eutrophic waters. In many north-eastern Slovenian lakes and reservoirs, nutrient loading, coupled with year-round warm weather, favours the growth of cyanobacteria, several of which can produce cyanotoxins, especially the potent genotoxins (Žegura & al. 2003) and liver toxins called microcystins (MC). The toxins are of interest due to their threat to humans and animals (Carmichael 1994; Falconer & al. 1994, 1999).

M. viridis has been found in Finnish fresh and coastal waters (e.g. Sivonen & al. 1990), a Swedish lake (Cronberg & al. 1999), a Brazilian reservoir (Figueredo & Giani 2001), China (Song & al. 1998) and Japan (Kameyama & al. 2004). Based on 16S rRNA analyses of *M. viridis* (Lepre & al. 2000) and DNA-DNA homology analysis (Kondo & al. 2000), some authors (Otsuka & al. 2001), under the rules of the bacteriological code, propose the unification of five species of

Table 1: Isolated peptides from cyanobacteria ***Microcystis viridis***.

***Microcystis viridis* strain**	**isolated peptides**	**reference**
M. viridis	cyanoviridin-RR	Kusumi et al., 1987
M. viridis NIES-102	cyanoviridin-RR	Ooi et al., 1989
M. viridis	microcystin-RR, -YR, -LR	Watanabe et al., 1989
M. viridis NIES-102	mikroviridin	Ishitsuka et al., 1990
M. viridis - axenic	microcystin-RR, -YR, -LR, -LA	Kaya & Watanabe, 1990
M. viridis – from bloom	microcystin-RR, -LR	Kaya & Watanabe, 1990
M. viridis NIES-102	hepatotoxic polypeptides	Yusamo & Sugaya, 1991
M. viridis NIES-102	aeruginosin 102-A, 102-B	Matsuda et al., 1996
M. viridis NIES-103	micropeptin 103	Murakami et al., 1997
M. viridis NIES-103	aeruginosin 103-A	Kodani et al., 1998
M. viridis– from bloom	microcystin-RR	Song et al., 1998
M. viridis NIES-102	polypeptide MVL	Yamaguchi et al., 1999
M. viridis NIES-102	microcystin-RR, -YR, -LR	Kameyama et al., 2002, 2004
M. viridis FACHB	cyclic peptides with *mcyB* gene	Pan et al., 2002

cyanobacterial genus *Microcystis: M. aeruginosa, M. ichthyoblabe, M novacekii, M. viridis* and *M. wesenbergii*. It has been recommended that attention should be paid to the occurrence and possibility of toxic blooms of *M. viridis* from the standpoint of water management and public health (Watanabe & al. 1986).

Peptides from *M. viridis* do not differ completely from other peptides from genus *Microcystis* (Table 1). Some *M. viridis* peptides are known to be active as essential intracellular nitrogen compounds in toxic cyanobacteria, substances active against grazing zooplankton (Yasuno & Sugaya 1991), a chymotrypsin inhibitor (Murakami & al. 1997) and as a mannan-binding lectin important for haemagglutination (Yamaguchi & al. 1999). Nevertheless, not much more is known about peptides from *M. viridis* and there is no report to date of *M. viridis* bloom occurrence in Slovenia.

Material and methods

Field sampling

Three sampling points were located in the north-eastern part of Slovenia. Gauss Krüger coordinates for reservoir Boreci (Križevci village) are y = 588239.7; x = 158373,3; z = 182m. For reservoir Podgrad (Podgrad village) y = 574341.4; x = 171416; z = 208m. For reservoir Hotinja vas (Hotinja village) y = 552400.4; x = 147283.2; z = 262m. The majority of results presented in this article are from reservoir Boreci, since the most extensive analyses were performed there. Samples were collected with the planktonic net, separately from the whole water column and surface scum.

Cyanobacterial and algal species

Species were identified using an inverted microscope according to Komarek (1991, 1999–2000), Starmach (1966) and Hindak (1978). The abundance on August the 9th was estimated with several dilutions of original sample and counting with haemocytometer. Samples from other dates did not show dominance of one, but four species, and the abundance with counting could not be estimated precisely enough (symbol + in Table 2). Samples were analysed for composition of plankton species and taxonomic determination under an inverted microscope (Nikon Eclipse TE300). Cells were measured with Lucia (System for Image Processing and Analysis LUCIA 4.6, Laboratory Imaging Ltd.).

Chlorophyll content analysis

Chlorophyll *a* was measured by methanol extraction according to Vollenweider (1969) with a spectrophotometer UV-2101 PC (Shimadzu). The procedure was modified to filtration of 10 ml samples in triplicate.

Cyanobacterial cyclic peptide analysis

The lyophilised bloom material was processed according to Harada & al. (1988) with minor modifications. Dried cyanobacteria (1000 mg) were extracted three times with 5% aqueous acetic acid (3 x 20 ml) for 30 min with stirring. The extracts were centrifuged at 4000 rpm for 10 min. The combined supernatants were applied to preconditioned 500 mg reversed-phase disposable columns (LiChrolut RP-18, Merck). The columns containing the extract were washed with 20 ml of 10 % methanol and the cyclic peptides eluted with 2 ml methanol (LiChrosolv, Merck), evaporated to dryness under nitrogen stream and the residues dissolved in 0.05 M phosphate buffer, pH 3. Samples were analysed by HPLC, using isocratic elution with methanol: phosphate buffer 48:52 (v/v). The HPLC/PDA equipment consisted of a Waters 600 Controller, Waters 616 pump and Waters PDA Detector. Millenium^{32} software (Ver. 3.0, Waters) was used to run the hardware and to process the data.

Identification and visualization of cyclic peptides with a photodiode array detector

The chromatogram was monitored at four wavelength maxima – 238, 225, 220 and 215 nm – in order to locate and distinguish MC from other bioactive cyclic peptides of interest. The wavelengths are characteristic of individual cyclic peptides; MCs have a characteristic absorption at 238 nm, while other isolated cyclic peptides have absorption maxima at lower wavelengths. The depsipeptide planktopeptin BL1125 was detected at 225 nm and anabaenopeptins B and F

Table 2: Bloom sample structure and dominant species (***present in footnote***) on two days in August 2006 from Boreci reservoir.

Bloom sample structure 9. 8. 2006	**Bloom sample structure 17. 8. 2006**
+ *Microcystis viridis 94 %*	+ *Microcystis viridis*
Microcystis wesenbergii 4%	+ *Microcystis wesenbergii*
Microcystis aeruginosa	+ *Microcystis aeruginosa*
Anabaena flos-aque	+ *Anabaena spiroides*
Anabaena spiralis	*Aphanizomenon flos-aque*
Aulacoseira granulata	*Aulacoseira granulata*
Closterium sp.	*Anabaena solitaria*
Euglena sp.	*Woronichinia naegeliana*
Pediastrum duplex	*Dictyosphaerium pulchellum*
Scenedesmus quadricauda	*Euglena* sp.
Staurastum gracile	*Pediastrum duplex*
Trachelomonas volvocina	*Staurastum gracile*
Trachelomonas hispida	*Trachelomonas volvocina*
Tetraedron limneticum	*Trachelomonas hispida*

+ = dominating species

at 215 nm. Both types of non-toxic cyclic peptide have additional characteristic absorption maxima at 278–279 nm that were used to confirm the preliminary identification (Grach-Pogrebinsky & al. 2003). The amounts of the cyclic peptides were calculated from the individual peaks by compari-

Fig. 1: Cyanobacterial bloom in Boreci reservoir, located in Križevci village, 9. 8. 2006, photo: Tina Eleršek.

son of the integrated peak areas with the values from calibration curves standardized by previously isolated cyclic peptides in pure form.

Results and discussion

This is the first report of cyanobacterial bloom of *Microcystis viridis* (A. Braun) Lemmermann in Slovenia. Microscopic examination of the phytoplankton samples showed the dominance

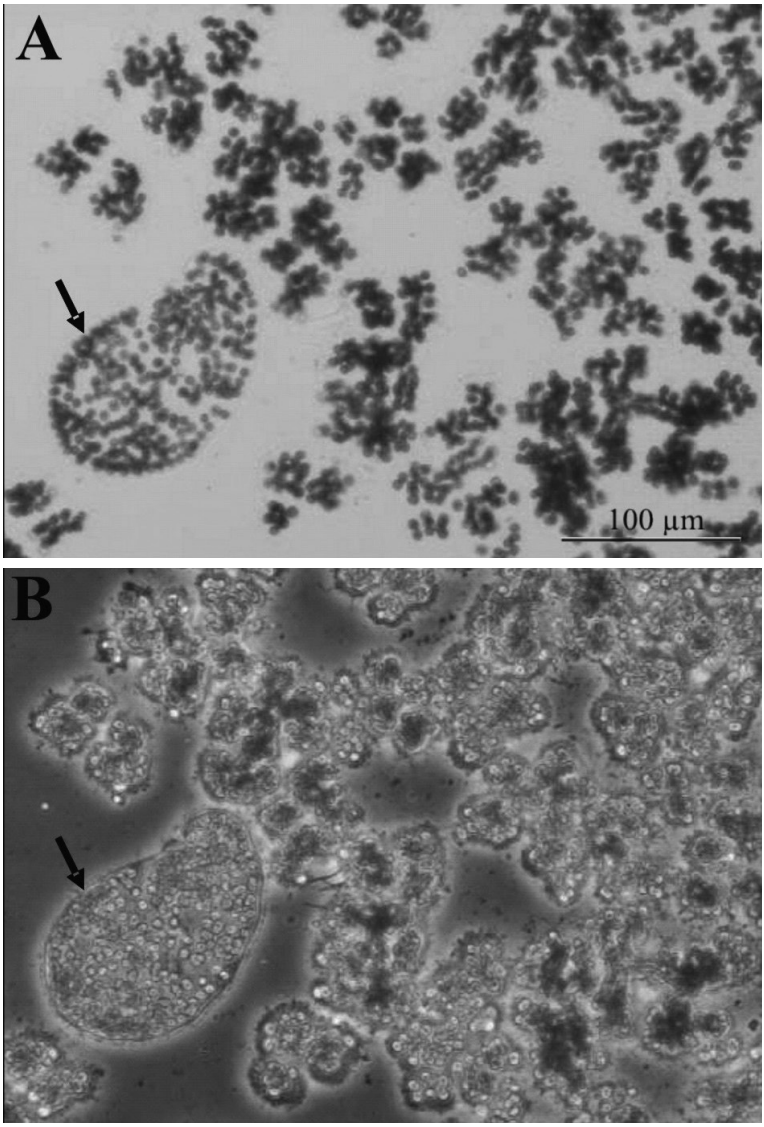

Fig. 2: Colonies of *Microcystis viridis* (right side of figure) and *Microcystis wesenbergii* (indicated by arrows) under (A) light and (B) phase contrast microscope, 200 x magnified, from Locality Boreci reservoir, 9.8.2006, photo: Tina Eleršek.

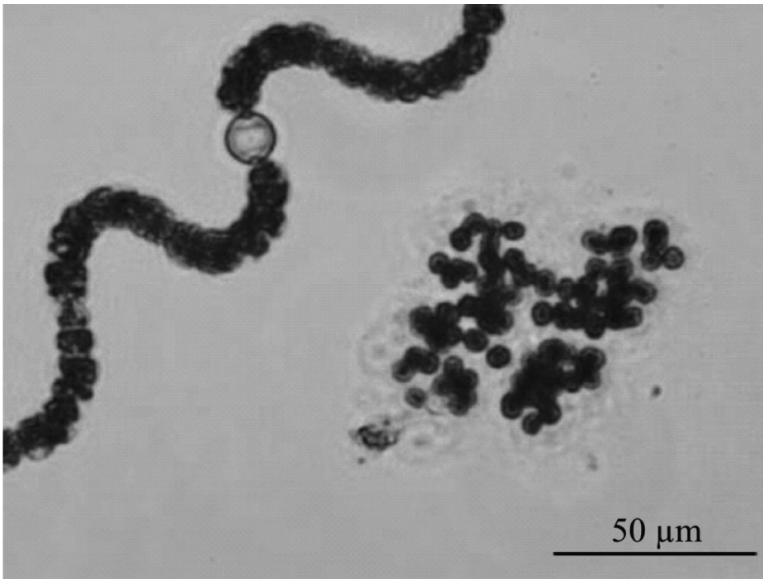

Fig. 3: Colonies of *Microcystis viridis* (right) and *Anabaena spiralis* (left) under phase contrast microscope, 400 x magnified, from Locality Boreci reservoir, 9.8.2006, photo: Tina Eleršek.

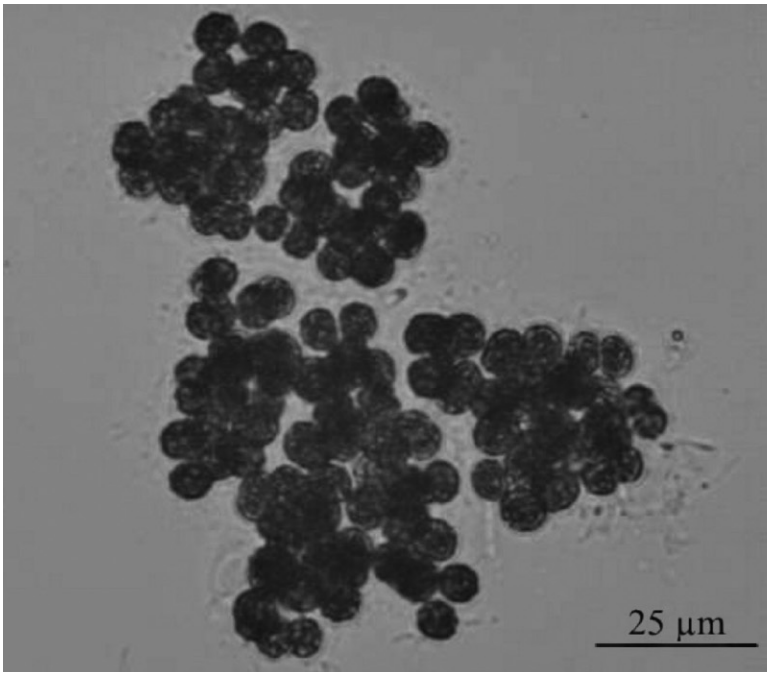

Fig. 4: Colonies of *Microcystis viridis* under phase contrast microscope, 600 x magnified, from Locality Boreci reservoir, 9.8.2006, photo: Tina Eleršek.

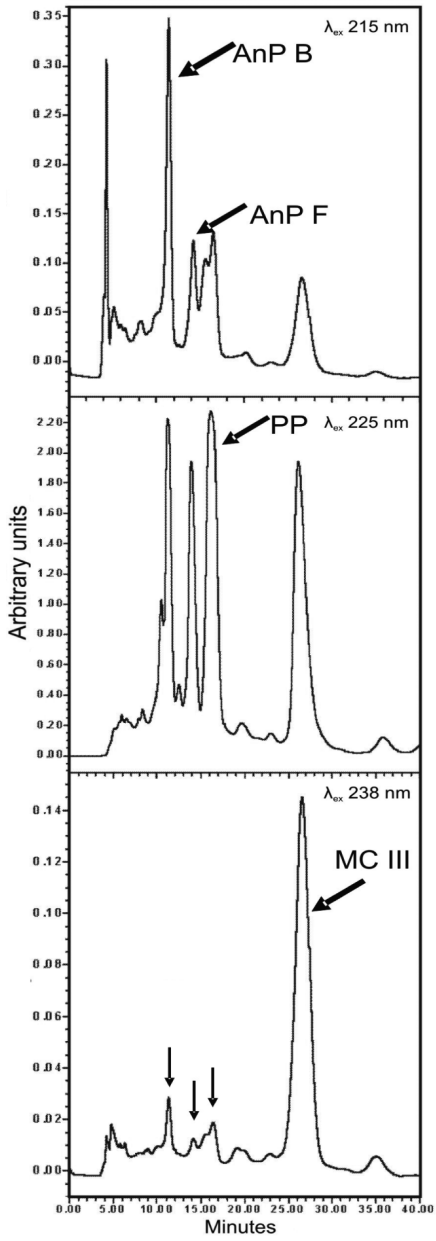

Fig. 5: HPLC chromatogram of *Microcystis viridis bloom* extract run from a preparative column using isocratic elution with methanol: phosphate buffer 50:50 (v/v). The diagrams show the elution pattern monitored at three different wavelengths: 215, 225 and 238 nm. MC is clearly visible at the characteristic λ_{max} of 238 nm, while the other three cyclic peptides are seen only as minor peaks (vertical arrows in the lowest panel). PP BL, AnP B and AnP F are better detected at lower wavelengths (upper two panels). AnP B = anabaenopeptin B; AnP F = anabaenopeptin F; PP = planktopeptin BL 1125, MC = unidentified microcystin

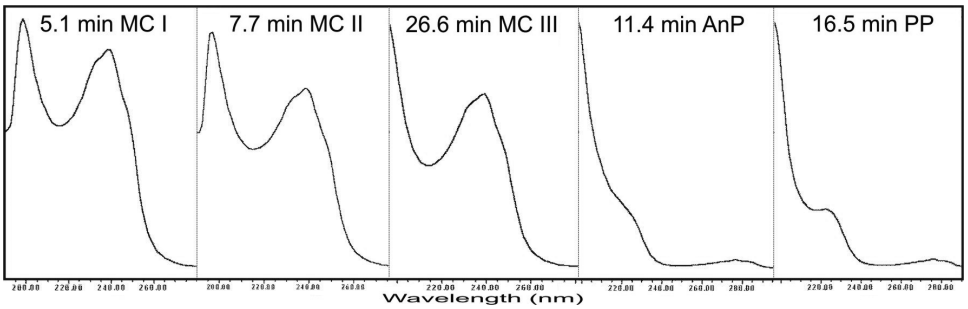

Fig. 6: The typical absorption spectra of five cyclic peptides from *Microcystis viridis bloom* at their characteristic retention time, marked in the upper part. AnP = anabaenopeptin; PP = planktopeptin BL 1125, MC = unidentified microcystin.

of *M. viridis* in the bloom in the first half of the August in reservoir Boreci (Fig. 1). After 8 days, *M. viridis* was found in two neighbouring reservoirs also, Hotinja vas and Podgrad. Changes in bloom sample structure were very fast; in just 8 days we observed different bloom composition (Table 2, Figs. 2 and 3). Cells were found in colonies (Fig. 4) with limited, more or less refractive mucilage, best seen under phase contrast microscopy (e.g. Fig. 2). The average diameter of cells was 4–7 µm. The contents of chlorophyll *a* of cyanobacterial bloom from Boreci reservoir were similar, 320 µg/l (9.8.2006) and 340 µg/l (17.8.2006). HPLC analysis showed that *M. viridis* bloom (from 9.8.2006) contains three MC and three non-toxic cyclic peptides, two anabaenopeptins and plaktopeptin BL1125 (Fig. 5), which have important roles in bloom lysis (Sedmak & Eleršek, 2005, 2006). All the cyclic peptides have characteristic absorption spectra (Fig. 6). Their content varied in the range of 2.3–6.6 mg g^{-1} of cellular dry weight. As found in previous studies (Sedmak & Kosi, 1997; Sedmak & Eleršek, 2005, 2006; Sedmak & al., 2007), the content of cyclic peptides was high enough to cause bloom lysis. Interestingly, this fact was confirmed by field observation; bloom composition not only changed, but, after 8 days, there was no visible cyanobacterial bloom on the reservoir surface, although no heavy rain or wind was detected during this period. The discovery of *M. viridis* bloom in Slovenia is very important, since toxic bloom constitutes a threat all over the World.

Conclusion

The presence of the cyanobacterial bloom of *Microcystis viridis* (A. Braun) Lemmermann is reported for the first time in Slovenia. Cells were found in colonies with refractive mucilage. The content of cyclic peptides (three microcystins, two anabaenopeptins and planktopeptin BL 1125) in the cyanobacterial bloom was high enough to cause bloom lysis. This fact was also confirmed by field observation. The discovery of *M. viridis* bloom in Slovenia is very important, since toxic bloom constitutes a threat all over the World.

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