Review of toxicity of cyanobacteria in Slovakia

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Although the dominance of cyanobacterial species forming water blooms in Slovakia is well documented for many years, the toxicity of their populations has not been monitored. The results of analyses of hepatotoxic microcystins identified and quantified by HPLC with diode array detector are reported, as well as a rapid clean-up method based on centrifugal ultrafiltration of crude cyanobacterial extract, which can replace conventional time consuming solid-phase extractions. The toxicity of cyanobacterial biomass was confirmed by bioassay using the crustacean species Thamnocephalus platyurus. Level of microcystin-LR ranged from 0 up to 1445.5 µg/g d.w. Variability in microcystin content within different localities was noticed. Not only common bloom-forming species (Microcystis flos-aquae, M. aeruginosa, Planktothrix agardhii or Anabaena circinalis), but also Cylindrospermopsis raciborskii and Raphidiopsis mediterranea were identified to be dominant species in some cyanobacterial blooms in W Slovakia. Blooms dominated by cyanobacteria were observed in lakes, fishponds, and water reservoirs. Increased attention should be paid to the effects of cyanobacterial toxins on human health and health of aquatic ecosystems in general.

Key words: cyanobacteria, cyanobacterial blooms, microcystin, toxins, Microcystis, Cylindrospermopsis, Anabaena, Raphidiopsis, Slovakia.

Introduction

Cyanobacteria became to be dominant in water reservoirs with high nutrient levels. Many of water bloom forming cyanobacteria were reported to produce toxins (cyanotoxins). The latest review of cyanotoxins is presented in the monograph of the WHO (CHORUS & BARTRAM eds., 1999). Cyanobacterial toxins became serious problem world-wide, and the levels of the most widely studied cyanotoxins, hepatotoxic microcystin-LR, has been reported from several countries (SIVONEN et al., 1990; VASCONCELOS, 1994; MARŠÁLEK et al., 1996; SEDMAK & KOSI, 1997, etc), but no information on the concentrations of microcystins in waters of Slovakia has been published.

Cyanobacteria produce a broad spectrum of biotoxins, which fall into three groups of chemical structures: peptides (both cyclic and linear), alkaloids and lipopolysaccharides. From the toxicological point of view, they possess several activities (hepatotoxicity, neurotoxicity, immunotoxicity, genotoxicity and mutagenity, tumor promoting activity, embryotoxicity, dermatotoxicity, or cytotoxicity (for review see SIVONEN & JONES, 1999). Microcystins, having clastogenic and tumour promoting activity, are the most widely studied cyanotoxins. The provisional safety level for drinking waters was derived for microcystin-LR by World Health Organisation (WHO 1998). Standardized instrumental methods of detection and quantification of microcystins, thus became highly important. Because crude extracts of cyanobacterial biomass
contain high amounts of proteins, they should be pre-purified prior to conventional HPLC analyses. Recently employed methods of sample pre-purification use clean-up by solid-phase extraction (SPE) C-18 columns (MARTIN et al., 1990; LAWTON et al., 1994).


The species composition and microcystin-LR levels in cyanobacterial blooms in selected localities in W Slovakia, as well as rapid clean-up method, which can replace time consuming SPE are reported in this study.

Materials and methods

Cyanobacterial samples for toxicity testing

Twelve field samples of cyanobacterial water-blooms from various freshwater reservoirs in W Slovakia were selected according to the dominating cyanobacteria. One sample (No. 1) was taken from a maturing basin of the curative peloid at Piešťany Spa; a typical Oscillatorietum was developed with the dominance of Oscillatoria limosa and O. princeps. A gravel pit lake at Senec (No. 2) is one of the largest man-made lakes in Slovakia and it is widely used for recreation purposes; a water bloom of Microcystis aeruginosa has been intensively developed for last years. A gravel pit lake at Janíčkov dvor (No. 3) is a small reservoir in which water blooms have been developed only slightly; in contrast to lake at Senec, the dominant species was Cylindrospermopsis raciborskii. Forest fishponds in Bratislava-Železná Studienka (Nos 4-8) are relatively small reservoirs, but with an intensive water blooms from early spring to late autumn; dominant species in analyzed samples were Microcystis aeruginosa and M. flos-aquae in the fishpond No. 1, Anabaenopsis elenkinii in the fishpond No. 3. A village fishpond at Gajary (Nos 9, 10) is a typical strongly eutrophized pool; intensive water blooms were formed mainly by Anabaenopsis elenkinii. An ox-bow near Stupava (No. 11) is a dead-arm of the Morava river (rkm 12), in which a heavy bloom of Anabaena circinalis was developed. The last sample was collected from a village pool at Modra which was eutrophized similarly like in Gajary; the dominant species of a water bloom was Anabaenopsis elenkinii (see Tab. 1).

Cyanobacterial biomass of selected samples were split in two portions: 100 mL of the biomass was separated for the taxonomic analyses and the second portion of water blooms concentrated by plankton net was frozen and thawed twice. Samples were then stored at –20 °C. The biomass of the samples was disintegrated by glass homogenizator and ultrasonic bath prior to toxicity testing. The sample was then filtered (Whatman GF/C) to obtain crude extract. Crude filtrate was used for biotests, as well as for HPLC analyses.

Extraction procedures and sample preparations for HPLC

Three extraction procedures based on recommendations of previous reports (KRISHNAMURTI et al. 1986; MARTIN et al. 1990; LAWTON et al. 1994; FASTNER 1998) were compared and their use for pre HPLC clean-up step was evaluated. Extractions were carried out as follows: 10 mg of freeze-dried biomass of each respective sample was either (1) twice extracted with 1 ml of redistilled deionized water (milliQ quality), extracts were pooled; or (2) twice extracted with 1 ml of 75% methanol, extracts were pooled and concentrated by evaporation; or (3) extracted sequentially in two steps with 1 mL of water, followed by 1 mL of water/methanol/butanol (75/20/5% v.v.). Each extraction step was performed by 5 min extensive vortexing followed by extraction in ultrasonic bath (15 min). Prior to HPLC analyses, pooled extracts were either: (1) blown to dryness by nitrogen (methanol) or lyophilized (water, water/methanol/butanol) and after dissolution in water pre-purified on SPE columns (Waters, SEP-Pack, 100 mg); or (2) directly ultrafiltered by centrifugation through low binding cellulose membrane in single use
filter units (Millipore Ultrafree-MC 10,000 NMWL). Ultrafiltration removed high molecular weight fraction (MW>10,000 – mostly proteins).

**HPLC analyses**

Both SPE-extracts and ultrafiltrates were analyzed by HPLC for microcystin-LR. Waters 600E HPLC solvent delivery system, equipped with Waters 717plus autosampler was used. Analytical column: 150 x 4.6 mm Supelcosil ABZ+Plus, 5 µm (Supelco). Gradient elution: mobile phases - (A) 0.05% tetrafluoroacetic acid (TFA); (B) acetonitrile + 0.05% TFA. Gradient composition: 0 min 80% A and 20% B, 20 minutes: 52% A and 48% B. Flow rate 1.0 mL min, temperature 25°C. Analytically pure microcystin – LR (Calbiochem-Novabiochem Corp., La Jolla, CA) was used as an external analytical standard. Photodiode array detector (Waters PDA 996, wavelength 200 – 350 nm) was employed for scanning UV absorbance and evaluation of spectra.

**Data processing**

All treatments were carried out in four replicates. Results – content of microcystin-LR in each respective sample treatment – are expressed as mean ± standard deviation in µg/g d.w. Comparisons between extraction efficacies of three solvents and differences between two clean-up procedures (SPE and ultrafiltration) were evaluated by ANOVA. P values less then 0.05 were considered statistically significant. All calculations were performed with Microsoft Excel® and GraphPad Prism® software.

**Results and discussion**

**Dominant and trace cyanobacterial species in water blooms of Slovakia**

The dominant cyanobacterial species in collected water blooms were *Microcystis aeruginosa*, *M. flos-aquae*, *Planktothrix agardhii*, *Anabaena circinalis* and *Anabaenopsis elenkinii*. However, *Cylindrospermopsis raciborskii* or *Raphidiopsis mediterranea*, seems to be expanding increasingly. The last two mentioned species are known from Slovakia for many years (HINDÁK, 1988a). Virtually nothing is known about the toxicity of *Raphidiopsis mediterranea* or endogloeic cyanobacteria *Pseudanabaena mucicola* or *Aphanothece desikacharyi* (HINDÁK, 1996a), and other trace cyanobacterial species presented commonly in water blooms not only in Slovakia. As we analyse water blooms as a mixture sample, we cannot assess how the trace species contribute to the total toxicity and microcystin content. Generally, these cyanobacterial species could be the source of the serious underestimation of cyanotoxicity health risk.

List of samples and dominant cyanobacterial species is described in Table 1. However, we have made evaluation only of 12 selected samples which were collected mostly during one vegetation season only; the exceptions were fishponds at Železná Studienka and a village pool at Gajary.

**Toxicity of cyanobacterial water bloom – forming species from Slovakia**

Two of the most frequent dominant species forming water blooms also in Slovakia, *Microcystis aeruginosa* and *Planktothrix agardhii*, are known as microcystin producers. Molecular mode of microcystin toxicity is the formation of adducts with protein phosphatases 1 and 2A (YOSHIDA et al., 1998), resulting in inhibition of these importants. In experimental animals, these occur in the cytoplasm and nuclei of hepatocytes in the centrilobular regions (YOSHIDA et al. 1998), and are followed by intrahepatic haemorrhage, apoptosis, progressive liver necrosis, hepatomegaly and death if the dose is sufficient. Apoptosis seen after oral dosing is considered one of the distinguishing features in the microcystin-induced hepatotoxicity (YOSHIDA et al., 1997).

Of considerable concern are the several lines of evidence indicating that may pose a carcinogenic risk to humans. In epidemiological studies in China, an elevated incidence of
hepatocellular carcinoma in certain areas has been associated with the regular ingestion of \textit{Microcystis}-contaminated surface water (YU, 1995).

One of the most interesting cyanobacterial dominant species seems to be \textit{Cylindrospermopsis raciborskii} occurring also in water blooms in Slovakia (HINDÁK, 1988a). Studies on the mechanism of action of cylindrospermopsin have shown that in mouse hepatocytes \textit{in vivo} the toxin disrupts protein synthesis (TÉREO et al., 1994), and in rat hepatocytes \textit{in vitro} the toxin is metabolised by cytochrome P-450 whereupon a metabolite acts to inhibit glutathione synthesis; pharmacological inhibition of cytochrome P-450 protected against the toxicity of cylindrospermopsin (RUNNEGAR et al., 1995). This is then a very different mechanism to the action of microcystins and nodularin. Several groups have dosed mice intraperitoneally with purified cylindrospermopsin or \textit{C. raciborskii} extract and reported on the progressive necrosis in liver and kidney (HARADA et al., 1994).

Of particular relevance to human exposure, laboratory studies in mice have been recently conducted using oral administration of a \textit{Cylindrospermum raciborskii} extract containing 0.2% cylindrospermopsin; the toxin was shown to be bioavailable by this route (SEAWRIGHT et al., 1999). Animal lethality could be effected with a single gavage dosing of the aqueous extract, the median lethal dose being in the range of 4.4 to 6.9 mg toxin-equivalent.kg$^{-1}$ (SEAWRIGHT et al., 1999). This oral administration resulted in the same liver pathology changes as those caused by i.p. dosing, with a NOAEL for repeated oral dosing of about 0.1 mg.kg$^{-1}$ (MOORE et al., 1998). At minimally toxic doses the liver was the primary target organ with some evidence of spleen lymphophagocytosis, while at higher doses effects included thymic atrophy, renal ischaemic acute tubular necrosis, subepicardial and myocardial haemorrhage, adrenal cortex sinusoid congestion, and microscopic ulcerations of the oesophageal part of the gastric mucosa with moderate stomach wall oedema. No pathological alterations were seen in the ileum and duodenum (SEAWRIGHT et al., 1999).

This knowledge of the toxicity of cylindrospermopsin, together with the discovery that the toxin is produced also by other cyanobacterial species, namely \textit{Umezakia natans} in Japan (HARADA et al. 1994) and \textit{Aphanizomenon ovalisporum} in Queensland and Israel (MOORE et al., 1998). This toxic \textit{Cylindrospermopsis raciborskii} does not belong to the rare species in Slovakia, and sometimes is a dominant species in waters also in neighbouring countries.

\textit{Toxicity of the samples selected in this study}

Sample 1 was collected from maturing peloid basins in Piešťany, where special mud is used in balneotherapy. Filamentous Cyanobacteria from the genus \textit{Oscillatoria} were assumed to assist in the slime formation, but no microcystins and no toxicity was detected in this sample (Tab. 2). Sample 2 contents no microcystin, but shows relatively high toxicity in bioassay. We presume that this is caused by cylindrospermopsin – the hepatotoxic alcaloid produced by \textit{Cylindrospermopsis}, presented in this sample. It is known, that crustacean species – \textit{Thamnocephalus platyurus} is senzitive to cylindrospermopsin (KOZMA, 1999). Samples No 3 and 6 had the highest content of microcystins and these localities content cyanobacterial blooms in thick scum. As both investigated localities are recreational reservoirs, the impact on human health should be considered. Similar comments should be done in the case of samples 4, 5 and 7, where relatively high content of microcystins estimated by HPLC is confirmed by the bioassay. Interesting results represent sample No. 9. According to HPLC analyse it has only moderate content of microcystin, but represents high toxicity in bioassay. Phycological analyses show that \textit{Anabaenopsis elenkinii} represented 50\% of all biomass, \textit{Raphidiopsis mediterranea} 20\%, and the rest belonged to \textit{Cylindrospermopsis raciborskii}, \textit{Microcystis viridis}, and \textit{Planktothrix agardhii}. The last two species could be responsible for the microcystin content estimated by HPLC, while \textit{Anabaenopsis elenkinii}, \textit{Raphidiopsis mediterranea} and \textit{Cylindrospermopsis raciborskii} can produce alkaloids with both hepatotoxic and neurotoxic activity (SIVONEN & JONES, 1999).
Samples of bloom-forming cyanobacteria in Slovakia were found to contain the microcystins in high concentrations. The toxicity of tested cyanobacteria differs in three order of magnitude and represents high risk for human health (in the case of recreational and drinking water use) and the risk for aquatic health ecosystem (especially for the development of early phase of fish development, adult fish, phytoplankton and zooplankton communities).

Comparison of extraction solvents and sample clean-up procedures

Only non-significant differences were observed when comparing the two pre-HPLC clean-up procedures - conventional SPE and ultrafiltration (Fig. 1). Detailed comparison of three extraction solvents (water, 75% methanol, sequential extraction with methanol-butanol) also showed no significant difference in extraction efficacy. Thus, aqueous extraction seems to be sufficient for quantitative extraction of microcystin-LR from cyanobacterial biomass.

As shown in Fig. 1, an aqueous extraction was quantitatively comparable with other extraction solvents. No significant differences between extraction procedures were observed, neither between samples with varying microcystin content, nor different dominant cyanobacteria. Previously published findings by Fastner et al. (1998) also showed no differences between water and methanol extractions, however 75% methanol was recommended to be the most appropriate solvent for extraction of microcystin variants other than microcystin-LR. For microcystin-LR we recommend aqueous extraction, because water seems to be a better solvent for purposes of the next clean-up step – ultrafiltration through low binding cellulose membranes.

Comparing efficacy of traditional C-18 SPE clean-up and rapid ultrafiltration, we have obtained systematic higher quantitative efficacy with ultrafiltration, especially at the samples with relatively low microcystin content. Based on our results, we recommend an extraction of cyanobacterial biomass with deionized water followed by fast centrifugal ultrafiltration as the rapid and efficient method for analyses of microcystin-LR in environmental samples of cyanobacterial water blooms. Although microcystin-LR is not the only microcystin form being detected in cyanobacterial water blooms, it usually forms a dominant fraction of total microcystin content (especially in samples dominated by *Microcystis aeruginosa*). Although the use of „microcystin-LR“ equivalents for concentrations of total content of all microcystin variants was discussed and recommended (Falconer, 1999), water quality guidelines of WHO are focused only on single microcystin-LR (WHO 1998). Our present efforts are focused on evaluation of ultrafiltration clean-up method for analyses of other microcystin variants and the total microcystin content, respectively.

Acknowledgements

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References


*Cylindrospermopsis raciborskii* (WOŁOSZYŃSKA) SEENAYYA et SUBBA RAJU. Environ. Toxicol. Water Qual. 14; (in press)


Table 1. List of cyanobacterial sampling sites selected for the study.

<table>
<thead>
<tr>
<th>No.</th>
<th>Location</th>
<th>Sampling date</th>
<th>Species composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Piešťany, peloid</td>
<td>29.7.1995</td>
<td>Microcystis aeruginosa (95%), M. flos-aquae, M. viridis</td>
</tr>
<tr>
<td>2</td>
<td>Senec, gravel pit lake</td>
<td>20.8.1995</td>
<td>Cylindrospermopsis raciborskii, Microcystis flos-aquae, Raphidiopsis mediterranea</td>
</tr>
<tr>
<td>3</td>
<td>Janíčkov dvor, gravel pit lake</td>
<td>21.8.1995</td>
<td>Microcystis aeruginosa + M. flos-aquae (90%), M. wesenbergii, Aphanothece desikacharyi, Pseudanabaena mucicola</td>
</tr>
<tr>
<td>4</td>
<td>Železná Studienka, fishpond No. 1</td>
<td>8.8.1995</td>
<td>Microcystis aeruginosa + M. flos-aquae (90%), Woronichinia naegeliana (9%), highly abundant endogloec species Aphanothece endophytica and Pseudanabaena mucicola</td>
</tr>
<tr>
<td>5</td>
<td>Železná Studienka, fishpond No. 1</td>
<td>29.9.1995</td>
<td>Microcystis aeruginosa (60%), M. wesenbergii (30%), highly abundant endogloec species Pseudanabaena mucicola and Aphanothece endophytica</td>
</tr>
<tr>
<td>6</td>
<td>Železná Studienka, fishpond No. 2</td>
<td>8.8.1995</td>
<td>Microcystis aeruginosa (70%), Woronichinia naegeliana (20%), A. solitaria, M. viridis, M. flos-aquae</td>
</tr>
<tr>
<td>7</td>
<td>Železná Studienka, fishpond No. 2</td>
<td>29.9.1995</td>
<td>Anabaenopsis elenkinii (75 %), Woronichinia naegeliana (20%)</td>
</tr>
<tr>
<td>8</td>
<td>Železná Studienka, fishpond Nr. 3</td>
<td>23.8.1998</td>
<td>Anabaenopsis elenkinii (50%), Raphidiopsis mediterranea (20%), Planktothrix agardhii, Cylindrospermopsis raciborskii, Microcystis viridis</td>
</tr>
<tr>
<td>9</td>
<td>Gajary, village pond</td>
<td>21.8.1995</td>
<td>Anabaenopsis elenkinii (80%), Planktothrix agardhii, Microcystis viridis, M. flos-aquae</td>
</tr>
<tr>
<td>10</td>
<td>Gajary, village pond</td>
<td>12.10.1995</td>
<td>Anabaenopsis elenkinii, Microcystis viridis, M. flos-aquae (5%), Anabaena circinalis</td>
</tr>
<tr>
<td>11</td>
<td>Stupava, ox-bow</td>
<td>21.8.1995</td>
<td>Anabaenopsis elenkinii, Pseudanabaena planctonica, Aphanothece desikacharyi</td>
</tr>
<tr>
<td>12</td>
<td>Modra, village pond</td>
<td>7.10.1995</td>
<td>Anabaenopsis elenkinii, Pseudanabaena planctonica, Aphanothece desikacharyi</td>
</tr>
</tbody>
</table>

Table 2. Toxicity of the selected samples.

<table>
<thead>
<tr>
<th>No.</th>
<th>Locality</th>
<th>Sampling date</th>
<th>MicLR [µg/g d. w.]</th>
<th>Toxicity to T. platyurus [LC50 mg.mL⁻¹]</th>
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<tbody>
<tr>
<td>1</td>
<td>Piešťany</td>
<td>29.7.1995</td>
<td>0</td>
<td>n.d.</td>
</tr>
<tr>
<td>2</td>
<td>Janíčkov dvor</td>
<td>21.8.1995</td>
<td>0</td>
<td>0.87</td>
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<tr>
<td>3</td>
<td>Senec</td>
<td>20.8.1995</td>
<td>1445.2</td>
<td>0.31</td>
</tr>
<tr>
<td>4</td>
<td>Železná Studienka 1</td>
<td>8.8.1995</td>
<td>588.4</td>
<td>0.96</td>
</tr>
<tr>
<td>5</td>
<td>Železná Studienka 1</td>
<td>29.9.1995</td>
<td>408</td>
<td>1.16</td>
</tr>
<tr>
<td>6</td>
<td>Železná Studienka 2</td>
<td>8.8.1995</td>
<td>1440.5</td>
<td>0.30</td>
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<tr>
<td>7</td>
<td>Železná Studienka 2</td>
<td>29.9.1995</td>
<td>859</td>
<td>0.91</td>
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<tr>
<td>8</td>
<td>Železná Studienka 3</td>
<td>23.8.1998</td>
<td>42</td>
<td>1.88</td>
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<tr>
<td>9</td>
<td>Gajary</td>
<td>21.8.1995</td>
<td>26.4</td>
<td>0.66</td>
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<tr>
<td>10</td>
<td>Gajary</td>
<td>12.10.1995</td>
<td>18.2</td>
<td>1.33</td>
</tr>
<tr>
<td>11</td>
<td>Stupava</td>
<td>21.8.1995</td>
<td>16</td>
<td>2.05</td>
</tr>
<tr>
<td>12</td>
<td>Modra</td>
<td>7.10.1995</td>
<td>0</td>
<td>2.67</td>
</tr>
</tbody>
</table>

Table 3. List of cyanobacterial sampling sites selected for the comparison of extraction procedures of microcystin-LR (MCYST-LR) from cyanobacterial biomass.

<table>
<thead>
<tr>
<th>No.</th>
<th>Dominant species</th>
<th>microcystin-LR (µg/g d.w.)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Anabaena spp</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>Anabaena spp.</td>
<td>42</td>
</tr>
</tbody>
</table>
Fig. 1. Comparison of extraction procedures of microcystin-LR (MCYST-LR) from cyanobacterial biomass in samples Nos 1-10 (Tab. 3). Three extraction procedures (water; 75% methanol, and sequential procedure) for two modifications of pre-HPLC sample clean-up (conventional SPE and centrifugal ultrafiltration) are compared. Bars represent means ± standard
deviation of microcystin-LR (in µg per gram of biomass dry weight – µg/g d.w., number of replicates n=4).
A - samples with relatively low amounts of microcystin-LR (< 100 µg/g d.w.);
B - samples with high amounts of microcystin-LR (> 100 µg/g d.w.).