Dissertation thesis in Environmental Chemistry

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# Modern methods for sampling and detection of cyanobacterial toxins – microcystins

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## Abstract

Microcystins are toxic oligopeptides produced by various species of cyanobacteria. Presence of these contaminants in the aquatic environment can inadvertly influence many ecosystem functions. Last not least, recreational and drinking water use can be affected too. Massive occurence of cyanobacteria is very often connected with process of anthropogenic eutrophication of surface waters. And this phenomenon became global problem. Regarding the serious consequences of microcystin contamination there is the necessity of appropriate sampling and detection methods.

Presented dissertation thesis is focused on the development of sensitive and robust methods for sampling and instrumental analyses of microcystins. Among others, development of passive sampling technique is a part of this work. The key factors influencing sampling process were evaluated. The comparison with conventional grab sampling under natural conditions is also presented. Achieved results reveal the advantages of passive sampling approach in monitoring of cyanobacterial toxins. And the present study demonstrate that the modification of POCIS passive sampler is suitable for monitoring of occurrence and retrospective estimations of microcystin water concentrations, especially with respect to the control of drinking water quality

Analyses of microcystins in complex environmental matrices comprise further part of this work. Particularly evaluation and optimization of toxin extraction from sediments is discussed. Attention is devoted also to comparison of analytical techniques used for detection of microcystins in sediments. Our results demonstrate the suitability of the methods described here for studying the occurrence and fate of MCs in the aquatic environment

Determination of microcystins and their metabolites in tissue samples is the next topic compiled within the dissertation thesis. The development of analytical method based on liquid chromatographic separation with single and tandem mass spectrometric detection is presented. Different results obtained by these two methods are discussed. Screening of fish from natural reservoirs was also performed as a part of our work. This study provide first comparison of liquid chromatography with single (LC-MS) and tandem mass spectrometric detection (LC-MS/MS) for analyses of microcystins in complex fish tissue samples.

## Abstrakt (in Czech)

Microcystiny jsou toxické oligopeptidy produkované některými druhy sinic. Přítomnost těchto látek může negativně ovlivnit některé funkce vodních ekosystémů, včetně jejich vodárenského a rekreačního využití. Masivní růst cyanobakterií je skloňován zejména ve spojení s fenoménem antropogenní eutrofizace povrchových vod, jež je v současné době celosvětovým problémem. Vzhledem k závažnosti důsledků které mohou tyto toxické látky vyvolat, je nezbytné sledovat jejich výskyt v různých složkách životního prostředí. Ke splnění tohoto požadavku je třeba mít spolehlivé metody pro jejich vzorkování a detekci.

Předkládaná disertační práce je zaměřena na vývoj citlivých a robustních vzorkovacích a instrumentálně analytických metod pro stanovení microcystinů. Součástí této práce byl mimo jiné vývoj techniky pasivního vzorkování, včetně kompletního zhodnocení klíčových faktorů ovlivňujících vzorkovací proces. Prezentovány jsou také výsledky využití tohoto přístupu v reálných přírodních podmínkách a jejich srovnání s konvenčně využívaným přístupem. Dosažené výsledky nastiňují nesporné výhody využití techniky pasivního vzorkovaní pro microcystiny.

Dalším tématem, které je zpracováno v rámci předkládané disertační práce, je stanovení microcystinů v komplexních environmentálních matricích. Konkrétně je práce zaměřena na zhodnocení účinnosti a optimalizaci extrakce toxinů z různých typů sedimentů. Pozornost je věnována také porovnání analytických technik pro stanovení toxinů v sedimentech.

Determinace microcystinů a jejich metabolitů ve vzorcích tkání je posledním řešeným problémem. V textu je zachycen vývoj metody založené na kapalinově chromatografické separaci spojené s jednoduchou a tandemovou hmotnostní detekcí. Jsou zde prezentovány a diskutovány rozdílné výsledky dosažené pomocí těchto dvou přístupů. V závěru je prezentována studie zaměřená na potenciální kontaminaci svaloviny sladkovodních ryb. Tato část přináší první kritické srovnání výše zmíněných metod využívaných ke stanovení microcystinů ve tkáních a dalších komplexních matricích.

## List of original articles and the author's contribution

This thesis is based on the publications listed below. Full list of the author's papers is provided in the Curriculum vitae section.

J. Kohoutek, P. Babica, L. Blaha and B. Marsalek (2008). "A novel approach for monitoring of cyanobacterial toxins: development and evaluation of the passive sampler for microcystins." Analytical and Bioanalytical Chemistry 390(4): 1167-1172.

- Jiří Kohoutek performed the experiments, analysed the samples using HPLC, evaluated and interpreted data, and prepaired the manuscript.

J. Kohoutek, B. Marsalek and L. Blaha (2010). "Evaluation of the novel passive sampler for cyanobacterial toxins microcystins under various conditions including field sampling." Analytical and Bioanalytical Chemistry 397(2): 823-828.

- Jiří Kohoutek performed laboratory and field experiments, analysed the samples using HPLC, evaluated and interpreted data, and prepaired the manuscript.

P. Babica, J. Kohoutek, L. Blaha, O. Adamovsky and B. Marsalek (2006). "Evaluation of extraction approaches linked to ELISA and HPLC for analyses of microcystin-LR, -RR and -YR in freshwater sediments with different organic material contents." Analytical and Bioanalytical Chemistry 285(8): 1545-1551.

- Jiří Kohoutek participated in the experimental design, performed the sediment extractions and part of the final HPLC analyses.

J. Kohoutek, O. Adamovský, M. Oravec, Z. Šimek, M. Palíková, R. Kopp and L. Bláha (2010). "LC-MS analyses of microcystins in fish tissues overestimate toxin levels – critical comparison with LC-MS/MS." Analytical and Bioanalytical Chemistry 398: 1231-1237

- Jiří Kohoutek performed part of the analyses using LC-MS, evaluated and interpreted data, and prepaired the manuscript.

# Table of content

# 1. Objectives and aims

Pollution of the environment by various compounds is one of the key problems of present days. Thousands of chemical substances are released into all environmental compartments. Majority of these chemicals are released as a result of various human activities but there are also natural contaminants produced by other living creatures. Anyway, the quantity of these natural contaminants (such as cyanobacterial toxins addressed in the present thesis) is in many cases influenced also by activity of man. Many of these chemicals exhibit toxic effects and their presence in the environment, even in very low concentrations, may inadversely influence other living organisms and the environment itself.

The existence of suitable techniques for determination of such compounds is essential for estimation of environmental risks and correct decision making process. As many of these chemicals are present at very low concentrations and their distribution in environmental compartments as well as spatial and temporal variation on particular site is quite large, the development of precise and accurate determination technique is a challenge for environmental analysis. There are urgent demands of selective and sensitive determination protocols and techniques for numerous environmental contaminants. Current advances in the field of detection methods enabled the determination of various chemicals at very low and previously unexpected levels. Hand in hand with detection of trace levels of contaminants goes also appropriate sampling and sample preparation. Without these two steps even the most accurate and precise determination technique is almost useless.

The primary goal of this study was to contribute to ongoing research of microcystin - a peptide toxin produced by toxic cyanobacteria in water blooms, namely to the development of complex methods for its determination in various environmental samples. The work is focused especially on the development of efficient and effective sampling method based on the use of passive samplers and on the development of instrumental analytical techniques for determination of microcystins in complex environmental matrices (including animal tissues) by liquid chromatography with mass-spectrometric detection.

# 2. Microcystins, their origin, occurence and fate

Microcystins are group of peptidic compounds produced by cyanobacteria (blue-green algae). Cyanobacteria are able to synthesize a wide spectra of chemically unique compounds and many of these chemicals exhibits various kinds of bioactivity. There are structures that acts as hormones, antibiotics, allelochemicals and toxins [1]. Some of these chemicals acts like selective cytostatics [2]. The others shows potent virocide, fungicide or antibiotic mode of action. Many cyanobacterial secondary metabolites are strong inhibitors of various enzymes (for instance, microcystins inhibits some proteinphosphatases, microginins some proteases) [3, 4].

Microcystins are probably the most often produced, abundant and studied cyanotoxins. Structurally they are cyclic heptapeptides (Fig.1) characterized by unique amino acid Adda ((2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid), glutamate, and an aspartate derivative in positions 5, 6, and 3 of the ring. The aspartate derivative is refered to as D-erythro-β-methyl-iso-aspartate (DmiA). Other D-amino acids in most structural variants are D-Ala in position 1 and D-Glu in position 6.

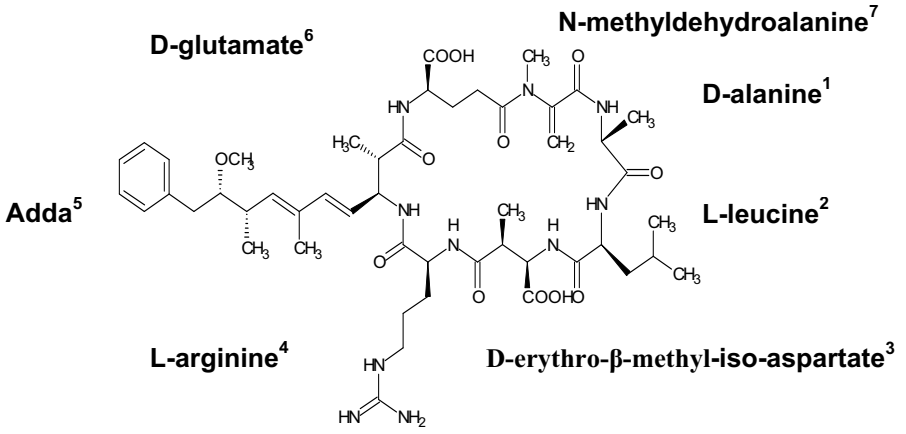


**Fig. 1:** Microcystin-LR ( the microcystin chemical structure can be generalized as cyclo-D-Ala[1]-X[2]-D-MeAsp[3]-Z[4]-Adda[5]-D-Glu[6]-Mdha[7] where X and Z denote the highly variable second and fourth positions)

Two positions show high variability, namely positions 2 and 4, whereas all other positions are more conserved. For this reason the nomenclature of microcystins has been revised in an early stage naming variants according to the two most variable positions by applying the one-letter code for amino acids, e.g. microcystin-LR for the variant with leucine in position 2 and arginine in position 4 [5, 6].

Nearly 90 structural variants have already been characterised but, considering all possible variability in the amino acid sequence, it is probable that new variants can be found. Nonetheless, despite the structural variability in field samples as well as in isolated strains a few variants (namely MC-RR, LR and YR) are dominant and most structural variants occurr only in low concentrations [7, 8].

From the point of physico-chemical properties microcystins are rather hydrophobic molecules. The grade of hydrophobicity/hydrophility is affected mainly by particular functional moieties (amino, carboxylic, etc.) present in the molecule. From three most abundant microcystins (MC-RR, -YR, -LR) the most hydrophilic is structural variant –RR, the least –YR (pH=7). The ionization of carboxylic groups occurs in pH range 3.3-3.4 (in natural conditions microcystins exists as neutral molecules or anionts). They are soluble in water (solubility of MC-RR is greater than 1g/L), non-volatile and stable [9, 10]. Microcystins can be decomposed under UV light with wavelenght close to their absorption maximum ($\lambda$=238-254nm) by isomerisation of the double bond of the Adda moiety [11].

Microcystins are toxic to various organisms. In vertebrates they affect mainly liver tissue. After intraperitoneal injection of radioactivelly labeled microcystins, 70% of toxins was located in liver [12]. The half life of microcystins in blood plasma (mouse) is 6.9 minutes while the concentration in liver stays unchanged for 6 days [13]. Toxicity of microcystins is caused by their covalent binding with catalytic subunit of enzymes proteinphosphatases 1 and 2A [14]. This leads to the inhibition of the enzymes which are neccessary in regulation processes, signaling and maintenance of homeostasis. Their inhibition leads to hyperphosphorylation of proteins and to damage of cytoskeletal structures and lysis of hepatocytes. Microcystins can affect also kidneys, lungs and intestine [15-17].

Microcystin-LR is highly toxic. The acute $LD_{50}$ value is 25 - 150μg/kg (brown rat) body weight for intraperitoneal exposure [18] and 5000-10900μg/kg (mouse) for oral application [19]. $LD_{50}$ values of particular structural variants differ. MC-RR is ten times less toxic than microcystin-LR.

**Cyanobacteria - microcystin-producing organisms**

As mentioned above microcystins are produced exclusively by cyanobacteria – a very old group of prokaryotic microorganisms. The class includes about 150 genera and about 2000 species but genes of *mcy*-operon encoding enzymes for microcystin biosynthesis have been identified only in genera *Microcystis*, *Planktothrix*, *Anabaena* and *Nostoc* [6].

Cyanobacteria are very unpretentious and extremely adaptive oxygenic photoautotrophic organisms. The ability of oxygenic photosynthesis is unique among the prokaryotic organisms and their light-gathering systems are very efficient [20]. Many cyanobacteria are able to assimilate atmospheric nitrogen [21] [22]. These characteristics determine their occurence and domination in suitable conditions which are very often created by activity of man. Especially the phenomenon of anthropogenic eutrophication (i.e., increased input of nutrients, mainly phosphorus and also nitrogen) of surface waters is connected with cyanobacteria occurence. While cyanobacterial populations are usually limited by lack of nutrients in clean oligotrophic aquatic ecosystems, massive water blooms and production of cyanotoxins have become a serious problem in eutrophicated (rich of nutrients) waterbodies. The nutrient pollution results principally from unsufficiently treated waste water, runoff from fertilized agricultural areas, manure and various complex effluent from livestock industries [23]. In Europe, Asia and America, more than 40% of freshwater reservoirs are eutrophic and offer appropriate conditions for cyanobacterial mass development [24]. Besides eutrophication, other environmental factors enhancing bloom formations are also low turbulence, stagnant water conditions, higher pH values and higher temperature [25, 26]. Furthermore, consequences of global climate changes (elevated temperature, increased atmospheric concentrations of carbon dioxide, raised solar radiation) have also been discussed in connection with cyanobacterial ecology and growth [27, 28].

Water blooms formed by planktonic cyanobacteria or mats of benthic species have severe impacts on ecosystem functioning, e.g., disturbances of interorganismal relationships, changes of biodiversity, decreasing of light and oxygen concentrations. The occurrence of massive cynobacterial populations can create a significant water quality problem, especially as many cyanobacterial species are capable of synthesizing a wide range of secondary metabolites [29]. It has been estimated that 25 to 75% of cyanobacterial blooms are toxic [30]. In the Czech Republic more than 80% of major reservoirs including drinking water supplies contained microcystins in considerable concentrations [31-33].

**Environmental occurrence and effects of microcystins**

Cyanotoxins are known to occur in various environmental compartments and thus can be detected in number of environmental samples. Microcystins are often detected in water (fresh, brackish and treated), in sediments and in various kinds of biological materials (animal and plant tissues). The sources of exposure from human health point of view are drinking and recreational water use [34-47]. To minor extent also contaminated food resources (especially shellfish and fish) and food supplements [48-56]. Contaminated food resources represent health risk especially in regions with very long lasting and heavy cyanobacterial contamination. Drinking of contaminated water as well as recreational and other utilisation of water present the main health risk.

Numerous incidents of animal and human poisonings[18, 57-61] associated with cyanobacterial blooms were reported. Consequently, the World Health Organization (WHO) proposed the value of tolerable daily intake (TDI, 0.04μg/kg of body weight) for MC-LR to restrict health risks. This value was also used for calculation of maximal allowable concentration in drinking water (1.0μg/L) [18, 62].

Cyanobacterial secondary metabolites represent important group of chemical compounds from viewpoints of ecotoxicology, toxicology and environmental chemistry. The fact that these compounds pose considerable health risk elicit the need for reliable and sensitive methods of detection and monitoring. Special attention should be dedicated to ensure water quality. Therefore, the presented work focuses on questions and problems of sampling

and instrumental analysis of microcystins and tries to draft some practical improvements to contemporary monitoring methods.

# 3. Microcystins – the issue of sampling

## 3.1. Introduction

Comprehensive monitoring of lakes and reservoirs requires extensive resources as the sampling should have coverage in temporal, horizontal and depth dimensions due to high variability of cyanotoxin concentrations (Fig. 2 and 3). In most cases, only a fraction of the waterbodies can be monitored in a satisfactory manner.

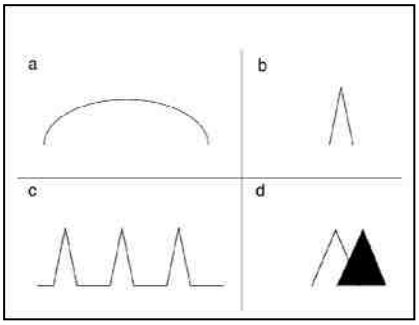


**Fig. 2:** Temporal variation of cyanobacterial blooms: a) persistent, b) intermittent, c) recurrent water bloom. Situation d) represent two toxin (or species) profiles at two locations of waterbody. [63]

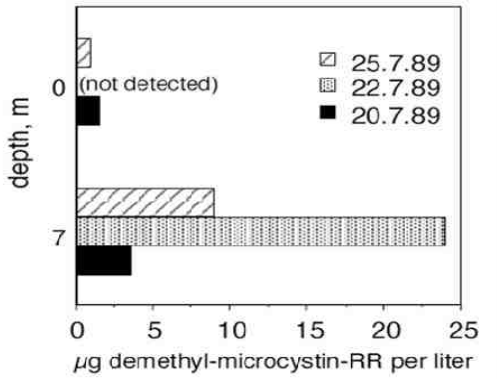


**Fig. 3:** Vertical movement of *Planktothrix* in lake (measured as concentration of dmMC-RR in water) [63]

There are many methods for monitoring of cyanotoxin occurence in water. In routine water monitoring some indirect techniques, including monitoring of presence of cyanobacterial cells in water and monitoring of their photosynthetic pigments (namely chlorophyll-a) can be usefull [63-67]. But these technique can serve only as a first estimation and indication of the presence of possible producers of toxins. Direct techniques are necessarry for determination and quantification of cyanobacterial toxins. The complete procedure of

determination of cyanotoxins in water follow the common sequence of steps starting with sampling and finishing with analytical determination.

Correct sampling is critical step in the whole procedure. The guidelines on sampling of water samples from various sources as well as the preservation and handling of water samples are described in international standard guidelines – namely ISO 5667-3,4,5 and 6 [68-71].

But the limitations in nearly all currently employed sampling techniques and analytical methodology hinder comprehensive exposure assessments. These limitations include the use of single point in time sampling methods and analytical methods that have insufficient sensitivity and selectivity to determine trace levels of contaminants in water. Also the extremes of water quality often preclude correct estimation of contaminant water concentration [72].

### 3.1.1. Passive sampling

Above mentioned limitations led to the development of devices that can provide a spatially and temporally integrated response as a result of passive adsorption. These passive sampling methods and devices have shown to be promising as tools for measuring concentrations of a wide range of priority water pollutants. Passive samplers avoid many of the problems outlined above, since they collect the target analyte *in situ* and without affecting the bulk solution [73].

Passive sampling can be defined in its broadest sense as any sampling technique based on free flow of analyte molecules from the sampled medium to a receiving phase in a sampling device, as a result of a difference between the chemical potentials of the analyte in the two media. The net flow of analyte molecules from one medium to the other continues until equilibrium is established in the system, or until the sampling period is stopped [74]. Sampling proceeds without the need for any energy sources other than the chemical potential difference. The construction of passive sampler is quite simple. Usually it consist of receiving phase enclosed in semipermeable membrane. Single-phase (without the membrane) samplers can be also constructed. The receiving phase can be a solvent, chemical reagent or a porous adsorbent. The receiving phase is exposed to the water phase, but without the aim of quantitatively extracting the dissolved contaminants.

The history and recent development on the field of passive sampling in environmental studies including water, air and soil media have been reviewed in literature [74-77].

### 3.1.2. Theory and principles of passive sampling

Pollutant sorption from water (or other media) to most passive sampling devices follows the general uptake pattern shown in figure 4.

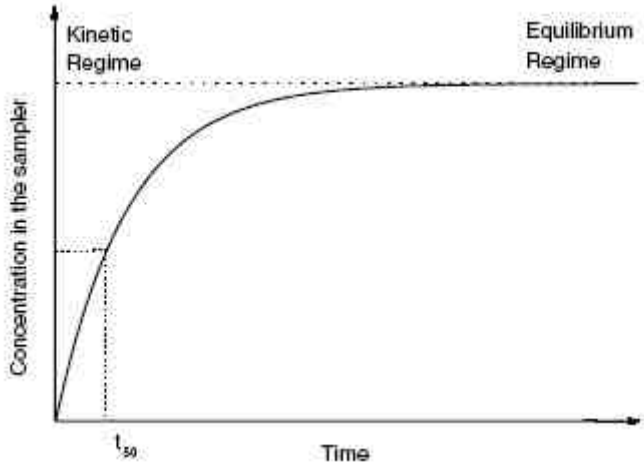


**Fig. 4:** Uptake of analyte by passive sampling device [74].

The exchange kinetics between the sampling device and medium can be described as a first-order reaction shown in equation 1:

$$Cs(t) = Cw\frac{k1}{k2}(1 - e^{-k2t})$$

Where *Cs (t)* is concentration of analyte in the sampler in time *(t)*, *Cw* is concentration of the analyte in the sampled medium and *k1* and *k2* are the uptake and offload rate constants, respectively. Two main accumulation regimes, either equilibrium or kinetic, are utilised in the operation of a sampler during the deployment.

In equlibrium sampling regime the deployment time is long enough to ensure establishment of thermodynamic equilibrium between the receiving phase and sampled medium. In such situation equation 1 reduces to:

$$Cs = Cw\frac{k1}{k2} = CwK$$

Knowledge of the phase partition coefficient *(K)* allows estimation of dissolved analyte concentration. The basic requirement of this passive sampling approach is that the equlibrium is reached after known period of time that must be reasonably short to overcome the influence of analyte concentration fluctuation in the media. The capacity of the sampler have to be low to ensure fast response. Review on equilibrium sampling devices has been published by Mayer et al. [78].

The second principle, the kinetic sampling regime, is employed more frequently in environmental applications. In this regime it is assumed that the rate of mass transfer to the receiving phase is directly proportional to the difference between the chemical activity of an analyte in medium and receiving phase. In this stage the desorption rate of the analyte from receiving phase is negligible and the sampling device works in the linear uptake regime. When this conditions occur the equation 1 can be reduced to:

$$Cs(t) = Cwk1t \text{ or } Ms(t) = CwRst$$

Where Ms(t) is the mass of an analyte accumulated in the sampler in time t, and Rs is the sampling rate. The sampling rate is product of the first-order rate constant k1 and the volume of water that have been in contact with the sampling device. Rs can be also interpreted as the volume of water completely cleared of analyte per unit of time [79, 80]. For most applications sampling rate is independent on concentration in the medium but is characteristic for individual analyte. Rs is affected by water flow, turbulencies, temperature and biofouling (influence of living creatures – especially microorganisms)[80-83].

In most cases sampling rate for individual analytes have to be determined by performing calibration for particular sampling device. In theory, calibration in the kinetic regime requires the reproduction of conditions in the field. Therefore, mainly waterflow and also the temperature of the exposure media

should be reproduced. During the calibration it is essential to ensure constant concentration of analytes in the media. There are several calibration approaches described in the literature [74, 84-89]. A review on the calibration methods for passive sampling devices also has been published recently [90]. Many of these approaches provide a good model on adsorption and flow rate dependencies. The only influence which seems to be hardly met is biofouling. Nevertheless, the variability of potential fouling agents is immense to be assessed and there are records that biofouling has not a major influence on sampling [82].

Since the passive sampling devices are developed for the monitoring of trace contaminants high sampling rate is required. The key parameters are minimal limitation by layers between the receiving phase and medium and low sorbent mass per surface area ratio [74, 76, 77].

Due to integrative nature of sampling these devices used in kinetic regime are applicable for sequestering of trace amounts of water contaminants and for contaminants that occur episodically. They can be also effectively used where concentration of contaminants vary greatly in time and space.

### 3.1.3. Passive sampling of natural compounds

Passive sampling seems to be helpful tool also for monitoring of natural toxins such as microcystin with increasing popularity [91] steming from quite high spatial and temporal variability of algal blooms, which is rather difficult to meet with conventional grab sampling [63].

The first use of integrative passive sampling for algal toxins is described in the work of MacKenzie *et al*. They have developed passive sampler (SPATT bag) based on synthetic resin enclosed in porous sachets and used it for monitoring of group of marine toxins known as paralytic shellfish poisons [92]. The device was designed as early warning of developing algal blooms to protect consumers and prevent the harvesting of contaminated seafood products. This work was continued by other authors. Fux *et al*. evaluated various sorbents in the SPATT system [93]. Rundberget *et al*. redesigned the device and used it for monitoring of various natural toxins on the south coast of Norway [94].

Shea *et al*. described the development of monophasic device for monitoring of brevetoxins, highly toxic compounds produce during red tide

events. Devices constructed of polydimethylsiloxane sheets were succesfully used for integrative sampling [95].

One of the aims of presented work was to evaluate the applicability of passive sampling approach in monitoring of cyanobacterial toxins microcystins. During laboratory and field experiments the viability of passive sampling was proven. We have customized the performance of POCIS sampling device specifically for microcystins. The calibration of sampler was performed under various conditions. Field deployment and simultaneous comparison with grab sampling was also accomplished. Passive sampling approach was proven to be effective, accurate, reliable and user friendly. The details and results of the work are presented in following chapters.

## 3.2. A novel approach for monitoring of cyanobacterial toxins - development and evaluation of the passive sampler for microcystins

**Published as:** Kohoutek, J., Babica, P., Bláha, L., and Maršálek, B. (2008). A novel approach for monitoring of cyanobacterial toxins - development and evaluation of the passive sampler for microcystins. Analytical and Bioanalytical Chemistry 390, 1167-1172.

### Introduction

Passive samplers offer many advantages comparing to traditional sampling methods and can be used for monitoring of various kinds of environmental pollutants [73, 75, 83, 96]. Several sampling approaches have been designed, and the polar organic compound integrative sampler (POCIS) has been suggested to estimate cumulative aqueous exposure to hydrophilic organic chemicals. This sampler was originally designed to mimic exposures of aquatic organisms to dissolved chemicals eliminating the problems of metabolism, depuration of chemicals, avoidance of contaminated areas, and mortalities of organisms. POCIS consists of a solid sequestration medium (sorbent) enclosed within a hydrophilic microporous membrane [79]. Both sorbent medium and membranes are versatile, and they can be changed to target specific chemicals or chemical classes.

So far, two general configurations of POCIS have been typically used, and include a generic design (suitable for virtually all hydrophilic organic contaminants), and the specific design for sampling of pharmaceuticals. The generic configuration contains the triphasic sorbent admixture of Isolute ENV+ polystyrene divinylbenzene and Ambersorb1500 carbon dispersed on S-X3 Biobeads [79]. This mixture showed excellent retention and recovery of many pesticides, natural and synthetic hormones, and many other contaminants [73, 97, 98]. The pharmaceutical sampler is based on the Oasis HLB sorbent and it is suitable for many pharmaceuticals with multiple functional groups [79]. The microporous membrane contains water-filled pores to facilitate transport of the

hydrophilic chemicals, and it may be semipermeable allowing chemicals of interest to pass and accumulate in the sorbent, while excluding particulate matter, biological material and other interfering substances.

The aim of this study was to develop and evaluate the use of passive sampler for microcystins, and compare different configurations (membranes and sorbents materials) to effectively accumulate these hazardous cyanobacterial toxins.

## Material and methods

### Chemicals and reagents

Oasis HLB was purchased from Waters (Milford, MA, USA). Bondesil-LMS was supplied by Varian, Inc. (Lake Forest, CA, USA). Nylon, polyethersulfone, polyester and polycarbonate membranes (90 mm diameter, 0.45 μm pore size) were obtained from Sterlitech co. (Kent, WA, USA). Standards of microcystins (MC-LR, -RR, -YR) were obtained from Alexis Biochemicals (Läufelfingen, Switzerland). The solvents (HPLC grade) were purchased from Sigma-Aldrich (Prague, Czech Republic). Deionized water was prepared with the Millipore Simplicity 185 system (Millipore, Bedford, MA, USA).

### Sampling devices

The POCIS samplers (pharmaceutical configuration) for pilot experiment were obtained from Exposmeter AB (Tavelsjo, Sweden). The in house made passive samplers were later constructed using various membranes (polycarbonate, polyethersulfone, polyester and nylon) and sorbents (Oasis HLB or Bondesil-LMS). Method of the physical compression for sealing the membranes has been used instead of other approaches using adhesives, heat etc. The membranes were kept in the hollow polyethylene screw and nut (30.0 mm i.d., 40.4 mm o.d.). This design ensured the ideal stretch of the membrane, uniform distribution of the sorbent material and provided a very good seal. The dimensions of the holder allowed applications of commercially available 47-mm or 90-mm membranes. Total exchanging surface area of the membrane (both sides counted) was 14.1 $cm^2$.

Microcystin mixture

Microcystins for the experiments were isolated from the natural cyanobacterial water bloom (dominated by *Microcystis aeruginosa*, collected during summer 2003 at Nove Mlyny reservoir, Czech Republic) by repeated extraction with 50% methanol and partial purification with solid-phase extraction on an ODS cartridge (SepPak 35 cc 10 g C-18 cartridge, Waters, Millford, MA, USA). The final extract (in 75% v/v methanol:water) which contained two dominant microcystin variants MC-LR and -RR (HPLC purity of total microcystins was ~75% at 238 nm) was aliquoted and stored at −18 °C.

Experimental design

Pilot experiments in the field were performed with commercially available devices (constructed with polyethersulfone membrane and Oasis HLB, total exchanging surface area of the membrane 47,5 $cm^2$, both sides counted). The samplers were exposed for one week in the natural pond (Velke splavisko, Brno, Czech Republic) containing microcystins (about 0.1 μg/L of total microcystins as revealed by HPLC). After 7 days, samplers were collected and analysed as described below. Further, laboratory experiments were constructed with the in house constructed sampling devices (using various combinations of membranes, sorbents and sorbent load) placed in the glass beakers with 1L of water containing mixture of microcystins (5 μg/L of total microcystins). Exposures (media replaced two times a week) were carried out for 14 days under steady conditions at room temperature (22+/-2 °C). Amount of microcystins accumulated in the samplers were determined on days 1, 7 and 14. To study the effect of the surface area-to-sorbent mass ratio on the accumulation of microcystins, samplers with polycarbonate membrane were loaded with three different amounts of sorbent material (Oasis HLB; 2.75, 5.55 and 11.1 mg/$cm^2$ respectively).

Sample preparation and HPLC analyses

Membranes containing enclosed sorbent were extracted two times with 20 mL of methanol and water (90:10 v/v acidified with 0,1% trifluoroacetic acid) for 15 minutes in the ultrasonic bath (Bandelin Electronics, Berlin, Germany). Supernatants were pooled, diluted ten fold with deionized water and MC's were concentrated by solid phase extraction (SPE) on an ODS cartridge (Supelclean LC-18, 3 mL Tube, Supelco, Bellefonte, PA, USA). Microcystins were eluted from the cartridge with methanol/water mixture (90:10, v/v) with 0.1% TFA, and the eluate was evaporated to dryness by rotary vacuum evaporation (45 °C) and then reconstituted with 50% v/v methanol. Extracts were analysed with an HPLC (Agilent 1100 Series, Agilent Technologies, Waldbronn, Germany) on a Supelcosil ABZ Plus, 150×4.6 mm, 5 μm column (Supelco) at 30 °C. The binary gradient of the mobile phase consisted of (A) $H_2O$ + 0.1% TFA and (B) acetonitrile + 0.1% TFA (linear increase from 20% B at 0 min to 59% B at 30 min); the flow rate was 1 mL/min. Chromatograms at 238 nm were recorded with an Agilent 1100 Series PDA detector (Agilent Technologies), and microcystins were identified by retention time and characteristic UV absorption spectra (200–300 nm). Quantification was based on external calibrations of MC-LR, -RR and -YR, respectively.

**Results and discussion**

Pilot experiment

In the pilot study, we have evaluated the general capability of commercial passive sampler to accumulate microcystins in the natural reservoir (Velke Splavisko, Czech Republic). In spite of relatively short sampling period (7 days) and low concentrations of intra- and extracellular microcystins in the reservoir (0.1 μg/L of total microcystins), the toxins were detected in all analysed samples. The average amount of total microcystins found in the samplers was 1.179 ng (s.d.=0.123). This pilot experiment proved the general ability of passive sampling technique to sequestrate microcystins. Our further experiments aimed to determine the most efficient configuration of the sorbent and membrane for the long-term passive sampling of microcystins.

Membrane evaluation

The first step in the sampler optimisation was the evaluation of the diffusion velocity of microcystins through four different commercially available microporous membranes. For these experiments, we have used the Oasis HLB sorbent. Differences in the uptake efficiency for total accumulated microcystins as well as differences between structural variants of microcystins were observed (Fig. 5), the best results were obtained with the polycarbonate membrane.

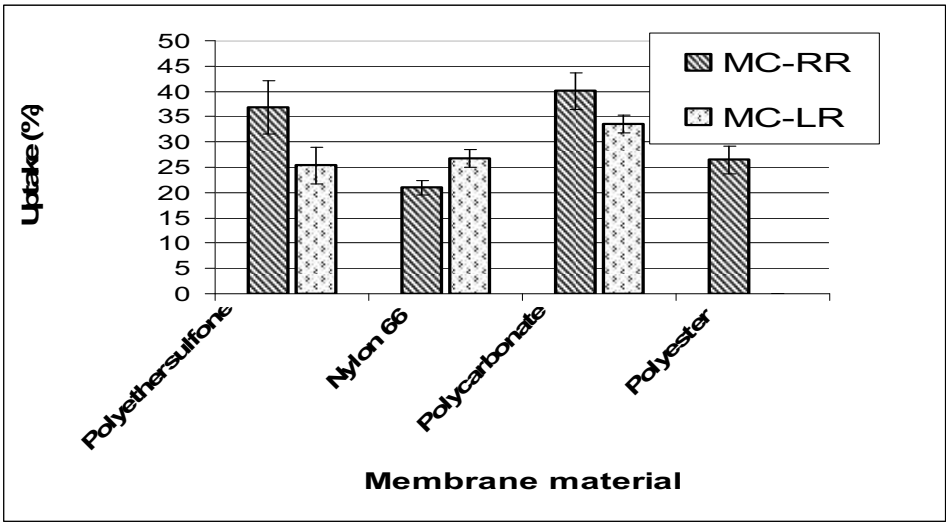


**Fig. 5:** Comparison of microcystins (structural variants MC-RR and MC-LR) uptake (% of total water concentration) into the passive sampler constructed with various membrane following a 14 days sampling period.

There are virtually two possible pathways of migration of the chemicals into the sorbent layer through the membrane, i.e. the dissolution and migration through the polymer matrix of the membrane, and migration through the water-filled pores. These two modes control the intake of analytes into the passive sampler. For chemicals under the membrane control, the sampling rates should remain nearly constant regardless of the turbulence of surrounding environment. On the other hand, sampling rates controlled by the aqueous boundary layer are determined by the velocity of diffusion through the steady

surface layer on the membrane and through the water-filled pores. Turbulences of the surrounding environment can thin the aqueous boundary layer and thereby increase the sampling rate [79]. Assuming the steady exposure conditions used in our experiments, the most important characteristics that affect accumulation are the effective membrane thickness and the open pore volume.

Of the membranes studied, polycarbonate showed the highest microcystin uptake velocity and also similar sampling rates for two different microcystin variants (Fig. 5). Polycarbonate is a thin membrane (5-25 μm according to manufacturers data) assuring fast diffusion and uptake of the analytes with an average flow rate (33 mL/min/cm$^2$ using prefiltered water at 10 psi, manufacturer's data). The membrane is also chemically and mechanically resistant with good tensile strength that ensures stretch and uniform distribution of the sorbent material.

Another studied material, polyethersulfone, is also very durable and resistant with high tensile strength and large open pore volume allowing the highest flow rates from membrane compared (60 mL/min/cm$^2$). It demonstrated good uptake with slightly higher sampling rate for more hydrophilic microcystin-RR (Fig. 5). However, relatively high thickness of this microporous membrane (110-150 μm) may lead to stagnant aqueous boundary layer that extends diffusion pathway for the analytes. Less effective accumulation (higher for microcystin-LR) was determined at the hydrophilic nylon-66 membrane (Fig. 5), which is relatively thick (65-125 μm) with average pore volume (flow rates about 27 mL/min/cm$^2$). It has relatively high tensile strength and durability when dry but it may loose its strength/stretch after longer exposures in water [79]. Naturally hydrophilic and resistant polyester membrane (5-11 μm thin, smooth surface, high tensile strength, typical flow rate of 33 mL/min/cm$^2$) accumulated MC-RR only and no MC-LR was found (Fig. 5).

Based on our experiments, polycarbonate membranes were selected for further calibrations of the microcystin passive sampler.

Sorbent mass per surface area ratios

We have also examined the influence of the mass of sorbent per surface area ratios on uptake of microcystins. According to Alvarez et al. [79], prototype passive sampler configuration consisted of 18 $cm^2$ of exposed membrane surface and 100 mg of sorbent (i.e. sorbent per surface area ratio 5.55 $mg/cm^2$). We have compared three ratios (2.75, 5.55 and 11.10 $mg/cm^2$) using the sampling device constructed of Oasis HLB sorbent and polycarbonate membrane with effective surface area of 14.1 $cm^2$. Results in Fig. 6 demonstrate that the uptake rate declined with the growing mass of sorbent per surface area ratio.

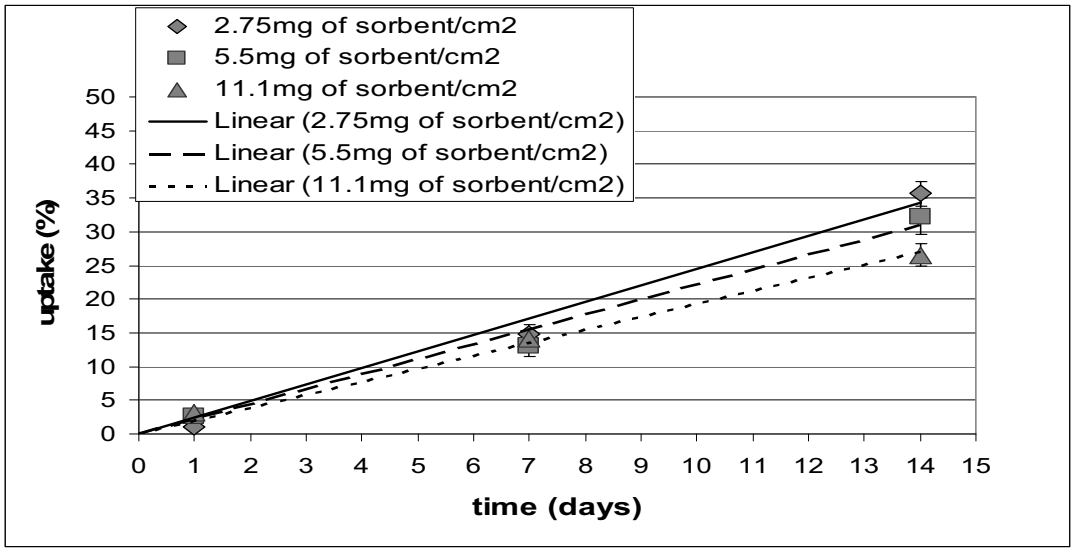


**Fig. 6:** Comparison of microcystin (sum of structural variants MC-RR and MC-LR) uptake rate (% of total water concentration) into passive sampler constructed with various amount of sorbent during 14 days exposure.

This may be related to the changes in the diffusion velocity [78]. Smaller effective thickness (at lower sorbent masses) resulted in higher velocity of the sampled water through the sorbent layer leading to more effective microcystin accumulation. The lowest amount of sorbent (2.75 $mg/cm^2$) was used for further calibration, it is also sufficient to completely cover the membrane area,

and it has enough sorption capacity for long term sampling of microcystins in water.

Sorbent evaluation

Several sorbent materials has previously been used for extraction, concentration and separation of various cyanotoxins, and C-18 based sorbents have become popular [99-101]. Most of these materials could also be employed in the passive samplers but some sorbents (such as C-18) may require the activation usually by organic solvent treatment. This extra step may represent a serious complication in the technical construction of passive samplers. We have studied two different divinylbenzene copolymer-based materials (Oasis HLB and Bondesil-LMS) that may be used directly without activation, and that have been previously used for microcystin extraction [99, 102]. In our experiments, Oasis HLB (without activation by solvents) showed an excellent uptake rates for both microcystin variants (Fig. 7).

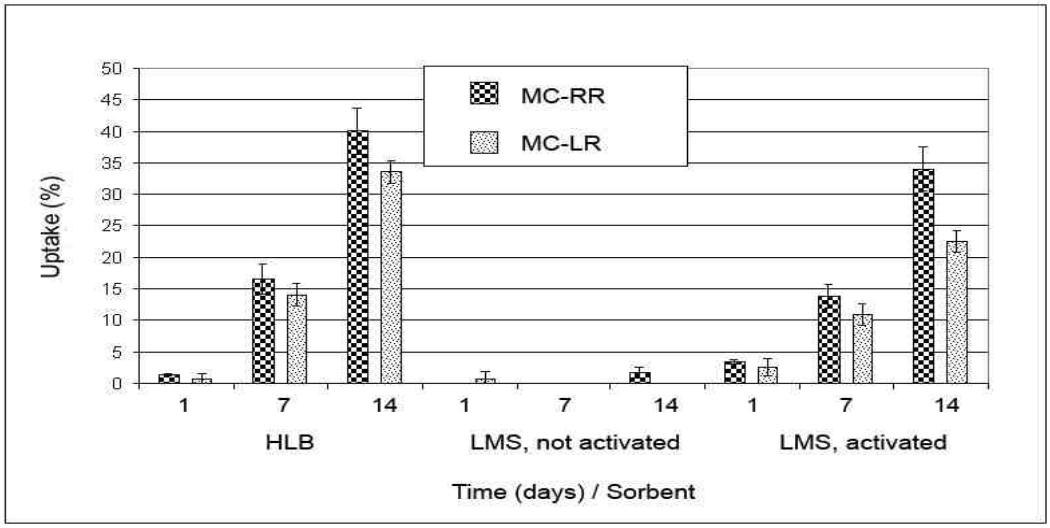


**Fig. 7:** Comparison of microcystins (structural variants MC-RR and MC-LR) uptake rates (% of total water concentration) into the passive sampler (polycarbonate membrane, 2.75 mg of sorbent per $cm^2$) constructed with Oasis HLB and Bondesil-LMS (not activated and activated variant) during 14 days exposure.

Poor uptake with negligible amounts of microcystins in samplers was observed with non-activated Bondesil-LMS. After the pre-activation of the Bondesil-LMS with ethanol, the uptake efficiency improved but it was still lower than that of Oasis HLB (Fig. 7). These data correspond to previous reports [99, 101] that showed good applications of Oasis HLB for concentration of microcystins using solid phase extraction (SPE), and it confirms that this sorbent (which does not require activation) can be used with high efficiency for the construction of passive sampling devices.

An important parameter of the sampler is the sampling rate ($R_s$) expressed as a volume of water completely cleared of the analyte per day [74]. Our studies were performed with the optimised sampling devices (polycarbonate membrane and Oasis HLB, 2.75 mg of sorbent/$cm^2$, total exchange surface of 14.1 $cm^2$) placed in the tap water with microcystins under steady conditions at laboratory temperature (22+/-2 °C). We have used 5 μg/L concentration of microcystins, and it can be considered environmentally relevant as documented by recent monitoring of more than 90 localities in the Czech Republic [32]. The $R_S$ values determined by measuring the microcystin mass accumulated after 1, 7 and 14 days were 0.022 L/d for microcystin-RR and 0.017 L/d for microcystin-LR, respectively. These values are slightly higher than those previously reported for sequestration of pharmaceuticals by passive samplers [79].

## Conclusion

We have evaluated suitability of passive sampling technique to accumulate a group of cyanobacterial toxins – microcystins. A pilot experiment with the commercial sampler exposed to microcystins under natural conditions revealed suitability of this technique for microcystins accumulation. Following, we have compared four different membranes and two sorbents for their efficiency to sample microcystins. Laboratory studies revealed that the best results (highest sampling rates) were obtained with the sampler constructed with the polycarbonate membrane and Oasis HLB sorbent (2.75 mg of sorbent per $cm^2$). The optimised sampler showed very good uptake of microcystins with experimentally determined sampling rates of 0.022 L/d and 0.017 L/d for MC-

RR and MC-LR, respectively. Sampler was able to accumulate about 40% of the total MC in the water during 14 day sampling period.

Current development and field evaluations studies show that passive samplers provide a viable tool for long-term monitoring of various contaminants in all matrices of the environment [74, 96, 103, 104]. Our study for the first time demonstrates that passive sampling approach represents a promising technique also for monitoring of cyanobacterial toxins microcystins. The use of passive samplers in monitoring programs and surveillance studies offers good capability to reflect seasonal, temporal and spatial variations in microcystin distribution without necessity of expensive large-scale active samplings. Passive samplers could be also used for control of microcystin removal in drinking water treatment plants and quality of produced drinking water. In contrast to conventional monitoring (grab sampling of high water volumes followed by laborious concentration using solid phase extraction), passive sampling simulates handling of large volumes of water during a period of several days or weeks. Passive samplers thus provide good detection limits and allow estimation of TWA concentrations for risk assessment. Our further research aims to characterize details in the kinetics of microcystin uptake by passive sampler (long-term experiments to determine complete uptake curve). We also study sampler parameters under environmental conditions characterizing the effects of turbulences, biofouling or biofilm development.

## 3.3. Evaluation of the novel passive sampler for cyanobacterial toxins microcystins under various conditions including field sampling

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### Introduction

In our previous work, we have evaluated the applicability of POCIS sampling technique for monitoring of microcystins. We have have assessed its performance and redesigned the sampler to meet the demands od selectivity and sensitivity. A pilot experiment of the study was made with the commercial sampler under conditions of natural water bloom. The samplers were shown to effectively accumulate microcystins after 7 day exposure. Following, we have tested various combinations of microporous membranes and sorbents for their efficiency to sample microcystins. Laboratory studies revealed that the best results (highest sampling rates) were obtained with the sampler constructed with the polycarbonate membrane and Oasis HLB sorbent (2.75 mg of sorbent per $cm^2$). The optimised sampler showed very good uptake of microcystins with experimentally determined sampling rates of 0.022 L/d and 0.017 L/d for MC-RR and MC-LR, respectively. The aim of our subsequent work presented in this study was to further calibrate and evaluate the sampler, and assess its capability to estimate the time-weighted average (TWA) concentration under natural conditions. The application of the passive sampler for routine monitoring of microcystins in various types of water is discussed.

## Material and methods

### Chemicals and reagents

Oasis HLB was purchased fom Waters (Milford, MA, USA). Polycarbonate membranes (90 mm diameter, 0.45 μm pore size) were obtained from Sterlitech co. (Kent, WA, USA). Polyethylene holders were bought from Atles Trade (Prague, Czech Republic). Standards of microcystins (MC-LR, -RR, -YR) were obtained from Alexis Biochemicals (Läufelfingen, Switzerland). The solvents (HPLC grade) and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Prague, Czech Republic). Deionized water was prepared with the Millipore Simplicity 185 system (Millipore, Bedford, MA, USA).

### Sampling devices

The commercial POCIS samplers (pharmaceutical configuration) and stainless steel holders for field experiment were obtained from Exposmeter AB (Tavelsjo, Sweden). The in house made samplers were constructed using polycarbonate membranes and sorbent Oasis HLB as described previously [105]. Method of the physical compression for sealing the membranes has been used instead of other approaches using adhesives, heat etc. The membranes were kept in the polyethylene holder made of hollow screw and nut (30.0 mm i.d., 40.4 mm o.d.). This design ensured the ideal stretch of the membrane, uniform distribution of the sorbent material and provided a very good seal. The dimensions of the holder allowed applications of commercially available 47-mm or 90-mm membranes. Total exchanging surface area of the membrane (both sides counted) was 14.1 $cm^2$.

### Microcystin mixture

With regard to relatively large amounts of MCs needed for calibration experiments, and high costs of the toxin analytical standards, we have first isolated MCs from the natural cyanobacterial water bloom, and the isolated MC mixture was used for laboratory calibrations. MCs were isolated from the *Microcystis aeruginosa*-dominated water bloom (collected during summer 2003

at Nove Mlyny reservoir, Czech Republic) by repeated extraction with 50% methanol and partial purification with solid-phase extraction on an ODS cartridge (SepPak 35 cc 10 g C-18 cartridge, Waters, Millford, MA, USA). The final extract (prepared in 75% v/v methanol:water) contained two MC variants MC-LR and -RR, which are the most often studied and dominant variants worldwide. HPLC purity of the MCs mixture was ~75% at 238 nm. Extract was aliquoted and stored at −18 °C.

Experimental design

The laboratory experiments were conducted with the in house made sampling devices (constructed from polycarbonate membranes and Oasis HLB sorbent, sorbent load 2.75 mg/cm$^2$ [105]) placed in the glass beakers that contained 1L of water fortified with a mixture of microcystins (nominal concentrations ranged from 0.2 to 5 μg/L of total externally added MCs). Exposures were carried out under static and turbulent conditions. Fortified water was replaced regularly (two times a week during static exposures, and every second day during turbulent exposures) to assure constant concentration of the analytes. Turbulent flow is complex fluid regime and it was not possible to explicitly quantify water velocities. Experimental design used in the previous studies of Alvarez et al. was used [12]. Laboratory exposures as well as field studies were conducted at temperature 22±2°C. This temperature corresponds well to the natural conditions under which MCs occur in waters. No detailed investigations on the influence of temperature were conducted, because temperature is known to have minor influence on the sampling rate in comparison with the flow [79, 106]. Experiments in the field were performed with both in-house made samplers and commercially available POCIS devices (pharmaceutical configuration, constructed with polyethersulfone membrane and Oasis HLB, total exchanging surface area of the membrane 41 cm$^2$, both sides counted). The samplers placed in the stainless steel holders were submerged at depth of approximately 30 cm. Devices were exposed from one to three weeks in the Brno reservoir (Brno, Czech Republic) during cyanobacterial water blooms, which produced microcystins. During the deployment of passive samplers in the field (1 to 3 week exposures), concentrations of dissolved microcystins were determined every 5 days according to the ISO 20179 method [107], volume of

the grab samples was 1 L. For each exposure period (given time point during laboratory and field exposures), three passive samplers were collected and analysed.

Sample preparation and analyses

Exposed samplers were rapidly washed with water and the polyethylene holder was disassembled. Membranes with the enclosed sorbent were transferred into 15 mL centrifuge tube and extracted two times with 5 mL of aqueous methanol (90% v/v acidified with 0.1% trifluoroacetic acid) for 15 minutes in the ultrasonic bath (Bandelin Electronics, Berlin, Germany). After centrifugation (10 min at 2880xg; Hettich Centrifuges, Beverly, MA, U.S.A.), supernatants were pooled, evaporated to dryness by rotary vacuum evaporation (45°C, Heidolph Instruments, Schwabach, Germany) and reconstituted with 500 µL of aqueous methanol (50% v/v). Extracts were analysed by HPLC (Agilent 1100 Series, Agilent Technologies, Waldbronn, Germany) on a Supelcosil ABZ Plus, 150×4.6 mm, 5 µm column (Supelco) at 30 °C. The binary gradient of the mobile phase consisted of (A) H2O + 0.1% TFA and (B) acetonitrile + 0.1% TFA (linear increase from 20% B at 0 min to 59% B at 30 min); the flow rate was 1 mL/min. Chromatograms at 238 nm were recorded with an Agilent 1100 Series PDA detector (Agilent Technologies), and MCs were identified by retention time and characteristic UV absorption spectra (200–300 nm). Quantification was based on external calibrations of MC-LR, -RR and -YR, respectively. Method quantitation limit (MQL) was 0.09 µg/L (S.D.=±0.01) for both structural variants of microcystins, and procedural recoveries were 89% (S.D.=±5%) and 94% (S.D.= ±3%) for MC-RR and MC-LR, respectively.

**Results and discussion**

Most of the conventional techniques for screening of cyanobacterial toxins in water use grab sampling coupled with solid phase extraction. But this technique has some limitations. Grab samples don't provide the complete picture of the pollutant concentration and their capacity is limited. Sample volume sizes are usually 1 or 2 L, thereby limiting the detection limit. On the other hand passive sampling techniques have capacity to handle large volumes

of water during a period of several days or weeks. This results in good detection limits and enables determination of TWA concentration of toxins in water. In our previous study [105] we have solved the problem of optimal configuration of polar organic compound passive sampler (POCIS) for cyanobacterial toxins - microcystins. In this study we present results of further evaluation of microcystin uptake kinetics and results of field deployment of optimised passive sampler.

Passive sampler calibration for different concentrations of microcystins under static and turbulent conditions

In the particular experiment, we have evaluated the dependence of microcystin sampling rates on the concentration of dissolved toxins (MC-LR and MC-RR at 0.2, 1 and 5 μg/L) during both turbulent and static conditions, which were simulated by stirred and nonstirred glass beakers, respectively. The selected microcystins concentrations are environmentally relevant and cover range of concentrations found during cyanobacterial blooms [32].

Analyses after two week exposures (Fig. 8) showed different amounts of microcystins in the samplers deployed in water with variable toxin concentrations, and there were also differencies between static and turbulent conditions.

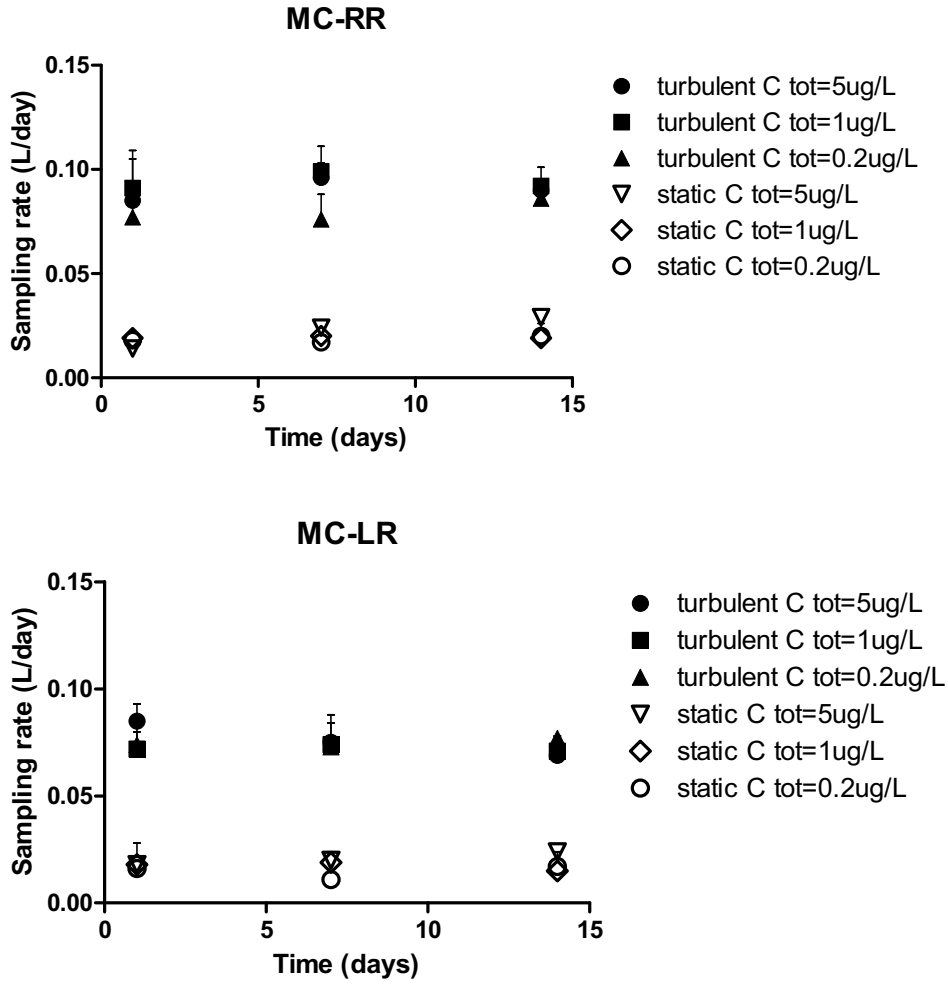


**Fig. 8:** Experimentally determined sampling rates (*R*s) for three different concentrations of microcystin-RR and -LR in the house-made sampler (14.1 cm2 exposed surface area). Study was conducted under static conditions (nonstirred - empty symbols) and turbulent conditions (stirred - filled symbols). Values (expressed as liters of water cleared of analyte per day) represent mean ± average standard deviation of *n*=3 samplers.

Data were used for calculations of the sampling rates (Rs). The Rs values were independent from the microcystin concentrations but expected difference

between stirred and nonstirred variant was observed (Fig.8). The respective $R_S$ values were 0.022 L/d (S.D.=+-0.007) for microcystin-RR and 0.017 L/d (S.D.=+-0.005) for microcystin-LR under static conditions, and 0.090 L/d (S.D.=+-0.019) for microcystin-RR and 0.087 L/d (S.D.=+-0.019) for microcystin-LR under turbulent conditions. These results are in agreement with previous study with number of pharmaceuticals and pesticides [79], which demonstrated influence of turbulencies on the $R_S$ values. The experiment demonstrated general ability of the in-house made passive samplers to sequestrate microcystins without dependency on microcystin water concentrations, and derived Rs characteristics may be directly used for the interpretation of results and estimation of TWA concentration in water.

Long- term sampling and estimation of linear uptake period for microcystins

Time profile of the passive sampling devices operation is gradual in three regimes. The first is the kinetic regime, where the uptake of target compounds into the sampler is linear. Second is the intermediate phase, which is characterised by slowing down the sampling rate and curvilinear uptake kinetics, and the third regime is known as near equilibrium phase, where the sampler reached its capacity and the sampling rate is close to zero. A first-order one-compartment model is often used to fit experimental measurements, and the uptake process could be generalized and described by following equation:

$$Csampler(t) = Cmedium * \frac{k1}{k2} * (1 - e^{-k2*t})$$

where *Csampler(t)* is the concentration of the compound of interest in the sampler as a function of time *(t)*, *Cmedium* is the compound concentration in the environment, and *k1* and *k2* are the uptake and the elimination rate constants, respectively.

During the linear uptake regime (relatively short sampling periods), the *Cmedium* can be deduced from the measured amount of target compound in the passive sampler (*Csampler(t)*) based on the sampling rate. The sampling rate is preferably expressed as total volume of medium cleared of analyte per day, and this principle could be applied to the appropriately calibrated passive sampling techniques during the initial linear uptake.

To characterize the long term-kinetics of the microcystin uptake to passive samplers, a 6-week exposure experiment was designed under stationary/stable conditions (Fig. 9).

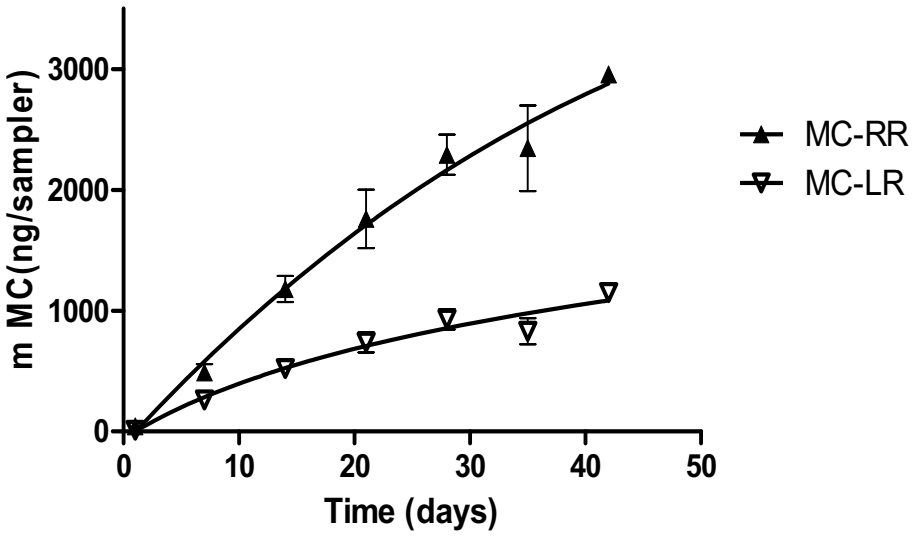


**Fig. 9:** Dependency of the amount of microcystins (MC-RR and MC-LR) retained in passive sampling devices on the exposure time (days) during the long-term sampling experiment. Each value (ng/sampler) represent mean ± average standard deviation of *n*=3 samplers.

As it is apparent, the linear uptake period for both studied microcystins lasted for approximately 28 days (see Fig. 9) with more rapid sampling of MC-RR. This period seems to be shorter than described by Alvarez et al., who studied passive sampling of polar pharmaceuticals and pesticides [79]. This could be explained by higher sampling rate of our device in comparison with commercial POCIS resulting from the smaller effective thickness of the sampler. This was achieved by use of a smaller load of the solid sorbent (i.e. lower sorbent mass per surface-area ratio) which lead to higher diffusion velocity and faster response of the passive sampler to the contaminant concentration in medium [108]. Nevertheless, four weeks of linear uptake regime are highly sufficient for passive sampling of cyanobacterial toxins with

respect to high temporal and spatial variability of cyanobacterial water blooms [32, 41, 109]

Field experiments

Applicability of the optimised passive sampler for microcystin monitoring under natural conditions was assessed in the Brno reservoir, Czech Republic. The experiments (one to three week exposures) were carried out in September 2008 during period of extensive cyanobacterial blooms. TWA concentrations of microcystins in water were calculated using the calculated sampling rate (Rs) obtained under turbulent conditions with respect to its relevancy for the field conditions. Water grab samples were collected and analysed in parallel with passive samplers.

Comparison of the sampling efficiency of commercially available POCIS and in-house made devices is presented in Figure 10.

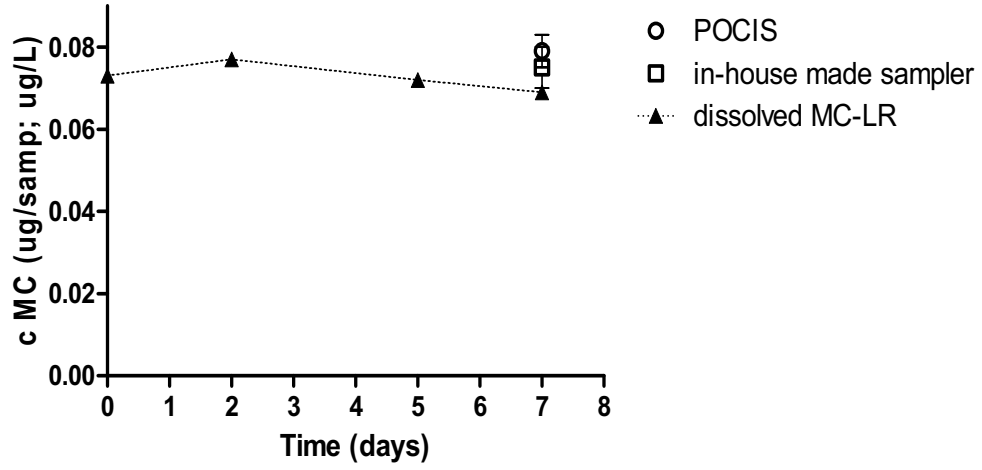


**Fig. 10:** Changes in the extracellular microcystin concentration in Brno reservoir during 7-day sampling period (grab samples - dissolved MC-LR, triangle symbols) and responses, i.e. time-weighted average concentrations (TWA), of two types of passive samplers (POCIS and in-house made devices). Passive samplers were deployed in the water for entire studied period (7 days), TWA concentrations represent the average value (± standard deviation) of $n$=3 samplers.

The estimated concentration of dissolved (extracellular) microcystins at the beginning of the sampling period was approximately 0.07 μg/L, and it remained almost unchanged up to the seventh day of exposure. During the entire 7-day exposure, the amount of retained microcystins was almost the same in both passive samplers, in spite of different surface areas of sampling devices (approximately 41 $cm^2$ for POCIS and 14.1 $cm^2$ for in house made sampler). This may be explained by almost three times higher Rs for the in house made device, which is related to the smaller effective thickness and lower sorbent mass-per surface-area ratio as also described above [108].

Further, performance of passive and active sampling approaches was compared during 3 week experiments (Fig. 11). Analyses of the grab samples collected every 5 days showed microcystin concentrations ranging from 0.2 to 4 μg/L with a significant peak in concentration at day 16. Our relatively frequent grab sampling was able to detect this episodic increase but there was no further information about duration or importance of this event. Such episodes might often be missed during monitoring programmes based on one-month period samplings as traditionally used for example in the Czech Republic [32]. Therefore, it is rather difficult to make correct estimation of the time-weighted average concentration based on the grab samplings, and more sampling points would be needed (even in the frequent sampling design used in the present study).

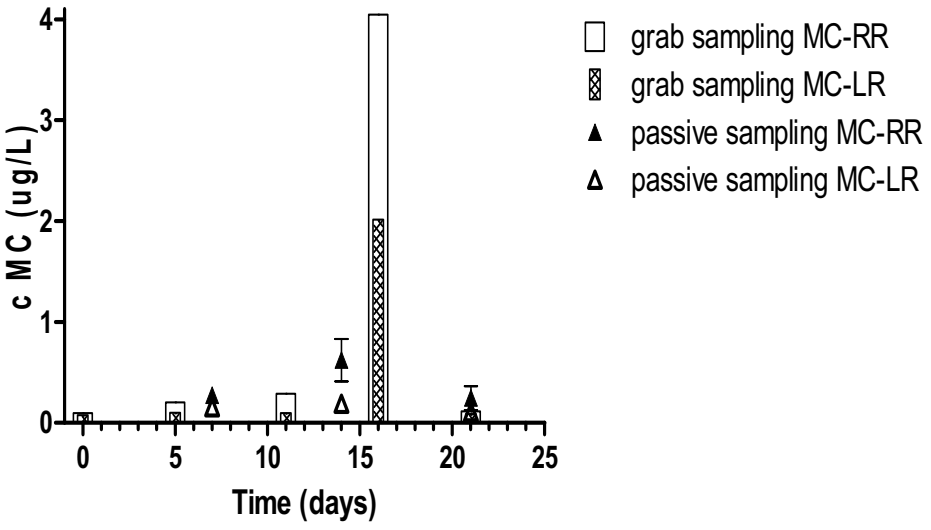


**Fig. 11:** Comparison of water concentrations (microgram/L) of microcystin-RR and -LR in Brno reservoir as determined by active/grab sampling (bars) and passive samplers (time-weighted average concentrations; triangle symbols). Passive samplers were deployed in the water for 7, 14 and 21 days, and corresponding TWA values were calculated. Values represent the average (± standard deviation) of *n*=3 samples for each period.

In contrast, passive sampling provided slightly different image. Concentrations of both microcystins remained in the level of 0.3 μg/L (TWA concentrations ranged from 0.11 to 0.62 μg/L with coefficients of variance not exceeding 30% for MC-RR and 15% for MC-LR, respectively) during the entire 3-week sampling period with a slight increase observed around day 14. This shows that a slight episodic peak observed at grab samples was recorded also by the passive sampler but it seemed to be rather short with minor influence on the overall concentration profile. Because passive samplers operate in the integrative regime they seem to provide more relevant picture of the real situation, which enables correct assessment of potential risks [73, 75, 110, 111].

In summary, sampling rates of microcystins for two different exposure scenarios (stationary/turbulent) were derived in the present study, and these might be used for estimation of TWA concentrations. This study also shows that our modification of the Polar Organic Compound Integrative Sampler is suitable for monitoring of microcystin occurrence and estimating water concentrations under conditions of natural water blooms. It is also a helpful tool for formulation of the time-weighted average (TWA) concentrations, which forms a fundamental part of an ecological risk assessment.

# 4. Microcystins – the issue of determination

## 4.1. Introduction

The existence of efficient and effective analytical methods is a basic presumption for the study of microcystins and for monitoring of their occurence and fate in the environment. The procedure of determination of a chemical in certain sample consist usually of three basic steps, regardless the analyte of interest. Namely, it is the extraction, preconcentration and/or clean-up of the extract and finally analytical determination of the target analyte. In the following chapters development and evaluation of extraction technique for sediments and LC-MS based analytical technique for animal tissues is presented and discussed.

### 4.1.1. Extraction of microcystins from complex samples

Extraction of analytes is essential step which influence the success of analytical determination regardless used analytical method. As mentioned before microcystins can be present in various matrices - from quite simple (water) to highly complex (tissues, sediments). The extraction technique neeeds to be adapted for particular matrix as there are different interactions of analytes with matrix in different types of samples. Also the amount of coextracted compounds play a key role in further analytical determination. In the ideal case only the analytes of interest should be extracted to eliminate the influence of various interferences on the analysis.

The demands and effectivity of extraction procedure vary greatly between particular types of samples. There are very good processed methods for matrices like water or cyanobacterial biomass (e.g. [112-116]). On the contrary, there are samples of sediments and biological samples. The extraction of microcystins from these matrices is quite challenging and in many cases not very effective. Also the amount of coextracted interferencies can be very high and subsequent analysis quite complicated. Microcystins have been detected in the tissues of many organisms including zooplankton, mussels, crustaceans, fish, birds and vertebrates both in laboratory and field experiments. Toxins can

be extracted from fresh [49, 117-121] and lyophilised samples [122-126]. Methanol is often used as extraction solvent [120, 121, 124, 127]. This technique can be combined with subsequent clean-up of raw extract by liquid-liquid extraction with hexane to remove lipidic interferencies [49, 118]. Other authors reported extraction of microcystins with mixture of methanol and water [117, 127]or with phosphate buffer [119]. Also the mixture of methanol:butanol:water can be employed [122, 123, 125, 126, 128]. The efficiency of extraction can be improved by ultrasonication [117, 124, 127, 128]. Mainly because of unclear extraction efficiency and matrix effects there are still several methodological difficulties in the bioaccumulation studies. Typical levels of extractable microcystins detected in tissue materials of aquatic organisms have ranged from undetectable to several tens of μg/g tissue dry weight. The highest levels have been recorded in the hepatic (or hepatopancreatic) tissues of fish and mussels while concentrations in fish muscle have usually been substantially lower or undetectable. It was proposed that a large proportion of the microcystin is bound covalently and irreversibly to the tissue matrix, and cannot be fully extracted from the tissue [120, 129, 130]. The extraction of microcystins from plant samples is comparable to that of animal tissues [131-134]. To be able to determine the toxins in complex tissue extracts highly selective and sensitive analytical methods should be used. One of very appropriate approaches is liquid chromatographic separation with tandem mass-spectrometric detection which will be also discussed in this work.

Sediment is another type of environmental sample where microcystins can be detected. There are reports describing continuously elevated concentrations of MCs in natural reservoirs, including accumulation in freshwater sediments (i.e. persistence for relatively long periods after the previous occurrence of cyanobacteria in the water) [135]. These observations, along with other findings, resulted in a hypothesis that MCs play a role of signaling molecules [136-138], and may participate in the activation of cell growth in early spring and the further colonization of the water column by cyanobacteria. Detailed studies of the fate of MCs in sediments can thus provide valuable information on the natural functions of these unique peptides.

Unfortunatelly, the problem of extraction of microcystins from sediments don´t seem to be completely resolved. Due to their physico-chemical properties microcystins (especially hydrophilic variants such as MC-RR) are strongly

adsorbed onto the mineral particles. They are also known to interact with humic and fulvic substances. These facts contributes to the poor efficiencies of many extraction approaches. Methanol [124, 139] or 5% acetic acid [135] can be used. According to Tsuji et al. [140] the most efficient extraction solvent is 5% acetic acid in methanol acidified with 0.1% TFA. Nevertheless, the extraction yield was still relatively low (approx. 60%), especially for hydrophilic MC-RR, and the work on this topic still continues [141].

The aim of our study was to evaluate different methods for extracting microcystins from sediments. We have worked with sediments which varied in organic carbon content and also in mineral composition. Comparison of results obtained by two widely used MC analytical methods, i.e. HPLC coupled with UV-VIS diode array detection (DAD) and ELISA, was performed. We have also investigated differences between the extraction efficiencies of the dominant MC variants (MC-LR, -RR, -YR). The experiments were designed to reflect natural conditions by using relatively low MC concentrations similar to those previously reported in freshwater sediments. The most efficient method was employed for determination of the seasonal microcystin variability in natural sediments collected at two localities.

## 4.2. Evaluation of extraction approaches linked to ELISA and HPLC for analyses of microcystin-LR, -RR and -YR in freshwater sediments with different organic material contents

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### Introduction

Microcystins are produced in high amounts by many species of cyanobacteria. But their functions and fate in the aquatic environment are not fully understood. Detailed studies of the fate of MCs in sediments can provide valuable information necessary for exploring the natural functions of these unique peptides.

Unfortunately, there is currently a lack of critical comparison between the efficiencies of various extraction and analytical methods, particularly for various aspects of sediment composition such as organic carbon content, which is one of the main factors that affects the sorption of compounds to natural sediments or soils. This study evaluated different methods for extracting MCs from several sediments with different organic carbon contents. We also compared the results from the two most widely used MC analytical methods, i.e. HPLC coupled with UV-VIS diode array detection (DAD) and ELISA, and investigated differences between the extractions of the dominant MC variants (MC-LR, -RR, -YR). These experiments were also designed to reflect natural conditions by using relatively low MC concentrations similar to those previously reported in freshwater sediments [140]. Using the most efficient method, we then analyzed the seasonal MC variability in natural sediments

collected at two localities of interest (six samplings during April to October 2005).

## Experimental

### Chemicals and reagents

Standards of MCs (-LR, -RR, -YR) and monoclonal IgG MC10E7 were obtained from Alexis Biochemicals (Läufelfingen, Switzerland). Anti-Fc-IgG antibody was purchased from ICN Pharmaceuticals (Aurora, OH, USA). Horseradish peroxidase (HRP) and all other chemicals were obtained from Sigma-Aldrich (Prague, Czech Republic). Deionized water was prepared with the Millipore Simplicity 185 system (Millipore, Bedford, MA, USA).

### Sediments

Natural sediments from specific localities (see Table 1 for detailed information) were collected by randomly sampling the surface 5 cm sediment layer; they were then mixed, lyophilized, sieved through the 2 mm opening size, and stored at 4 °C until further processing. Besides model sediments, we also analyzed MCs in sediment samples collected throughout the summer season of 2005 at two specific sites in the Brno reservoir, Czech Republic. The sampling site "Brno reservoir: centre" is located in the middle of the reservoir (~14 m depth), and the second locality "Rokle" is a shallow bay of depth 2–4 m. The distance between the two sites is about 2 km. Two or three random sediment samples were collected at each locality per day of sampling in order to investigate the sampling variability (distance between the collection spots: 10–50 m). Microcystin mixtures for extraction experiments MCs for the experiments were isolated from the natural cyanobacterial water bloom (dominated by Microcystis aeruginosa, collected during summer 2003 at Nové Mlýny reservoir, Czech Republic) using repeated extraction with 50% methanol and partial purification with solid-phase extraction on an ODS cartridge (SepPak 35 cc 10 g C-18 cartridge, Waters, Millford, MA, USA). The final extract, which contained the three dominant MC variants MC-LR, -RR and -YR (HPLC purity of total MCs was ~75% at 238 nm) was aliquoted and stored at −18 °C.

Experimental design

Sediment equivalents (2 g d.m.) were moisturized with 5 mL of water, and the mixture of MCs was added externally to them at final doses of 1.25 μg/g d.m. MC-LR, 3.15 μg/g d.m. MC-RR and 0.7 μg/g d.m. MC-YR (total MC dose: 5.1 μg/g d.m.). Each experimental variant was extracted in duplicate. Sediments with added MCs were kept on the shaker overnight (20 h) to achieve equilibrium sorption to sediment. Suspensions were then extracted twice with 20 mL of appropriate solvent: 5% v/v acetic acid in water [135], or 5% acetic acid in methanol containing 0.1% or 0.2% v/v trifluoroacetic acid (TFA) [140] using an ultrasonic homogenizer (Bandelin Sonopuls HD2070, Bandelin Electronics, Berlin, Germany) for 3 min at 90% of maximum power. Each extraction step was followed by centrifugation (2,800×g, 10 min). Pooled supernatants were diluted ten-fold with deionized water and MCs were concentrated with SPE on an ODS cartridge (Supelclean LC-18, 3 mL Tube, Supelco, Bellefonte, PA, USA). MCs were eluted from the cartridge with 0.1% TFA in methanol, and the eluate was evaporated to dryness by rotary vacuum evaporation (45 °C) and then reconstituted with deionized water (for ELISA) or 50% v/v MeOH (for HPLC analyses). The theoretical limit of detection was 0.0003 μg/g d.m. for ELISA and 0.015 μg/g d.m. for HPLC.

ELISA determination of microcystins

Samples were analysed by direct competitive ELISA, modified from Zeck et al. [142]. Briefly, high protein binding 96-well microplates (Nunc, Wiesbaden, Germany) were pre-incubated overnight with 2,000-fold diluted antimouse anti-Fc-IgG. Free IgG was then removed by washing with phosphate buffer saline (PBS, pH 7.3), and the plates were coated for 1 h with 5,000-fold diluted monoclonal IgG MC10E7 developed against MC-LR. The plate was then washed five times with 0.05% (v/v) Tween-20 in PBS, and nonspecific interactions were blocked by adding 20 μL of the block solution to each well (1% v/v EDTA, 1% v/v bovine serum albumin in 1 M TRIS-HCl, pH 7.4). The filtered samples, standards and controls were immediately added to the wells (200 μL per well) and the plate was incubated for 40 min at room temperature.

Finally, 50 μL of MC-LR conjugated with HRP prepared and purified according to Zeck et al. [143] was added to each well (300-fold dilution of the MC-LR-HRP conjugate was used based on our previously optimized method). The reaction was then incubated at room temperature for another 15 min, the plates were washed five times with 0.05% (v/v) Tween-20 in PBS, and 175 μL of the HRP substrate 3,3′,5,5′-tetramethylbenzidine was added. Development of the coloured product was stopped after 10 min by adding 50 μL of 5% (v/v) sulfuric acid. The absorbance (420 nm with reference 660 nm) was determined with a microplate reader (GENios Spectra Fluor Plus, Tecan Group, Männedorf, Switzerland). Each sample was analysed in three replicates and compared with the 0.125–2 μg/L calibration curve of MC-LR constructed for each individual plate.

HPLC analyses

Extracts of sediments were analysed with an HPLC (Agilent 1100 Series, Agilent Technologies, Waldbronn, Germany) on a Supelcosil ABZ Plus, 150×4.6 mm, 5 μm column (Supelco) at a temperature of 30 °C. The binary gradient of the mobile phase consisted of (A) H2O + 0.1% TFA and (B) acetonitrile + 0.1% TFA (linear increase from 20% B at 0 min to 59% B at 30 min); the flow rate was 1 mL/min. Chromatograms at 238 nm were recorded with an Agilent 1100 Series PDA detector (Agilent Technologies), and MCs were identified by retention time and characteristic UVabsorption spectra (200–300 nm). Quantification was based on external calibrations of MC-LR, -RR and -YR, respectively.

## Results and discussion

The total MC extraction efficiencies for various sediments analyzed with HPLC and ELISA are summarized in Fig. 12. We found no detectable MCs in any of the three experimental sediments prior to external MC addition (Table 1, sediment I–III).

**Table 1:** Basic characteristics of the sediments studied.

| No. | Sediment | % of organic carbon | MC concentrations prior to external addition (µg/g d.m.) | |
|---|---|---|---|---|
| | | | ELISA | HPLC |
| I. | Košetice stream | <0.5% | <LOD | <LOD |
| II. | Dřevnice river: Zlín | 1.25% | <LOD | <LOD |
| III. | Morava river: Kvasice | 5.35% | <LOD | <LOD |
| IV. | Brno reservoir: Rokle 12-X-2005 | 13% | 0.010 | <LOD |
| V. | Brno reservoir: centre 15-VI-2005 | 13% | 0.152 | 0.141 |

LOD: limit of detection (0.0003 µg/g d.m. for ELISA, 0.015 µg/g d.m. for HPLC); MC: microcystin. Since the MC recoveries from the various sediments differed significantly with respect to sediment composition, we only report measured concentrations here (recoveries were not taken into account)

Extraction efficiencies for the procedural blanks (spiked variants without sediments) did not differ among the solvents used or among MC structural variants, and they were (mean±SD of total MCs) 82.8± 8.8% and 78.3±4.1% as analysed with ELISA and HPLC, respectively.

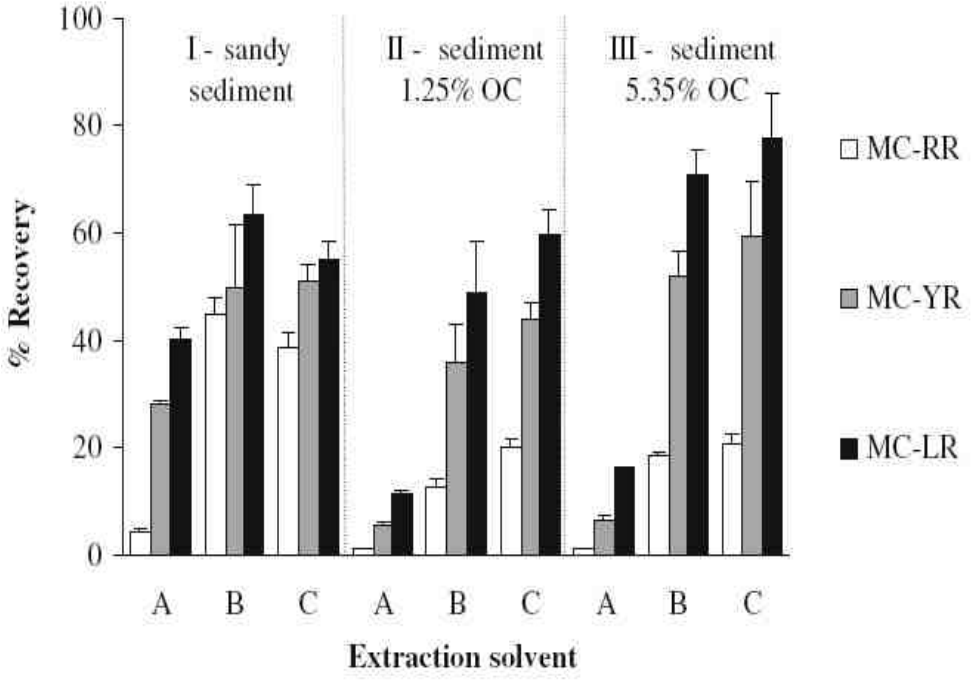


**Fig. 12:** Recovery of different structural variants of microcystin (MC) externally added into three sediments and extracted with different solvents (a: 5% acetic acid, b: 5% acetic acid in 0.1% TFAmethanol, c: 5% acetic acid in 0.2% TFA-methanol). Results of HPLC analyses. OC, organic carbon. Bars represent means of duplicate experiments with standard deviations.

In general, 5% acetic acid in water [135] appeared to be the least efficient extraction solvent regardless of the sediment used (average recovery 4.2–16.4% of total MCs, as analysed by HPLC). On the other hand, methanol with 5% acetic acid and 0.1–0.2% TFA was efficient at extracting about 24.8–50.1% (HPLC) of the externally added MCs from three compared sediments. This extraction method with acidified methanol was also successfully used by other authors, who preferred this procedure over 0.1% TFA in methanol [140]. The higher extraction efficiency with acidified methanol might be explained by the reduced ionization of the carboxylic acid groups in the MC molecules at low pH [144], and correspondingly, the tendency of the MCs to be extracted with less polar solvents such as methanol [116]. Another method for extracting

NODs from sediments using methanol was also employed previously [124, 139], but there is only limited information on its efficiency.

Our comparison revealed a good correlation between the results from the two different analytical methods used (Fig. 12). The extraction efficiencies for spiked sediments extracted by 5% acetic acid in 0.2% TFA–methanol were 32.3–56.8% and 33.1–44.9% as determined with ELISA and HPLC, respectively. However, we observed significant variability in the ELISA results. The average coefficient of variance for repeated independent analyses of the same sample was 29%, in comparison with <5% obtained with HPLC. These observations reflect the basic nature of the ELISA assay, in which the results can easily be affected by sample composition and various experimental conditions [145]. According to these findings, both HPLC and ELISA can be recommended for analyses of total MCs in sediments. Other analytical methods have also been used for MCs analyses in sediments previously. Tsuji et al. [140] suggested the application of complete oxidation of the sediment matrix with ozone, which leads to the formation of MMPB in the presence of MCs. MMPB is a selective derivate of Adda, which is a unique D-amino acid present in the structures of MCs and NODs. MMPB was then extracted, esterified and detected using gas chromatography coupled with mass spectrometry (GC-MS). The authors observed recoveries as high as 90.5–91.7% for MC-LR, -RR or -YR [140]. Nevertheless, this method is not routinely available in many laboratories, in contrast to methods more commonly used for MC analysis, such as HPLC–DAD or ELISA.

We have also studied changes in MC extraction efficiency due to variations in sediment composition. We particularly focused on the total organic carbon content, as it is one of the main factors that affects the sorption of chemicals into sediments or soils. However, we did not observe any clear (negative) correlation between the extraction efficiency and the amount of organic carbon, as expected. The MC extraction efficiency was significantly dependent on other sediment characteristics, and a detailed understanding of these factors will require further research. For example, the number of inorganic clay particles interacting with the ionized molecules could affect the sediment sorption of MCs and NODs [146-148]. The other factor that influences MC sorption could be the proportion of humic and fulvic acids in the total organic carbon content, and their character [9].

We have also investigated differences in the extractions of major MC variants: MC-LR, -RR and -YR (Fig. 13). Recoveries from spiked sediments extracted with 5% acetic acid in 0.2% TFA–methanol were 55.3–77.8% for MC-LR, 20.0–38.8% for MC-RR and 44.1–59.5% MC-YR. Extraction efficiency was therefore strongly dependent on the physicochemical properties of MCs.

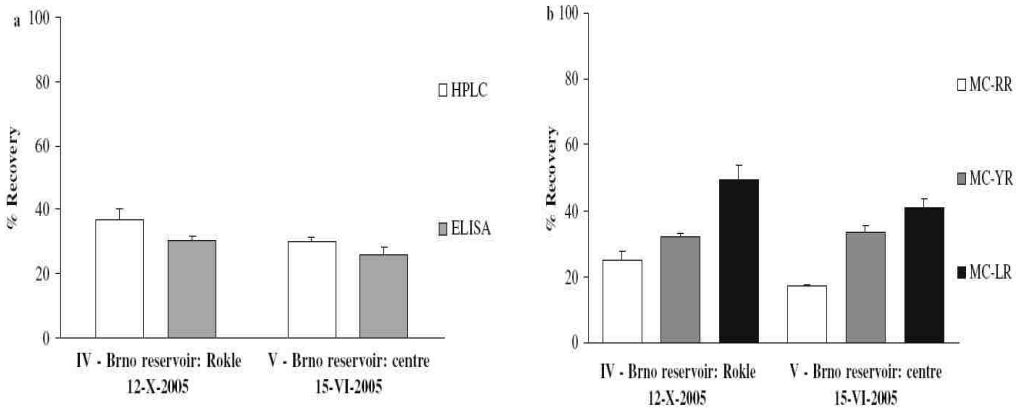


**Fig. 13a, b:** Recovery of externally added microcystin (MC) from two Brno reservoir sediments (extracted with 5% acetic acid in 0.2% TFA–methanol): a) recovery of total MCs, analysed with ELISA and HPLC; b) recovery of different structural variants (analysed by HPLC). Bars represent means of duplicate experiments with standard deviations.

Our analyses revealed a decrease in MC extraction efficiency with increasing hydrophobicity (MC-LR), while there was significant sorption of the more hydrophilic MC-RR onto all of the sediments investigated. These findings reflect the abovementioned independence of total organic carbon content (Fig. 12), and suggest that hydrophilic interactions play a critical role in the sorption of MCs. Our results correspond well with those from the work of Tsuji et al. [140], which also showed lower recoveries for MC-RR than for MC-LR. It is also apparent that the very low recovery of one MC variant (MC-RR) dramatically reduced the total MC recovery rate (compare Figs. 12 and 13). We then applied the most efficient extraction method (5% acetic acid in methanol with 0.2% TFA) to analyses of seasonal variability in the MC content of natural

sediment collected at two selected sites: the Brno reservoir sites “Rokle” and “centre” (Table 1). First, we investigated the MC extraction efficiencies for these two particular sediments using the same design as for the three model sediments (i.e. using external addition of MCs; final dose 5.1 μg/g d.m. of total MCs). Certain concentrations of natural MCs were present in these two sediments prior to external MC dosing (see Table 1). However, they formed only a minor fraction of the externally added MCs, and the starting concentrations were subtracted from the results (Fig. 3). As is clear from Fig. 3a, the extraction efficiencies for both sediments were within a similar range (~30% total MCs), and the recoveries were comparable to those obtained from the Dřevnice river (sediment II, 1.25% of organic carbon), although the organic carbon contents in both sediments differed significantly (Table 1). This observation further suggests the importance of inorganic material in the sorption of MCs onto sediments. Extractions of different MC variants from Brno reservoir sediments (Fig. 3b) also showed a similar trend to that seen for the three model sediments, with the highest efficiencies observed for MC-LR.

The variability of MCs concentrations in the Brno reservoir sediments during the summer season 2005 is summarized in Table 2. Concentrations of MCs ranged 0.003–0.380 μg/g d.m. (ELISA) or 0.016–0.474 μg/g d.m. (HPLC), respectively. Concentrations found in Brno reservoir are similar to those from several Japanese lakes, where MCs were detected at concentrations 0.08–2.33 μg/g sediment d.m. by MMPB method [140]. Another study found much lower concentrations of MCs and / or NODs (0.0005–0.0041 μg/g d.m., determined by ELISA) in sediments from prawn farm, New South Wales, Australia [124]. Concentrations of this study expressed as MCs content per volume of fresh sediment (μg/L) were 0.63–96.47 μg/L (ELISA) or 4.67–108.68 μg/L (HPLC), respectively. These values are well comparable with study of Ihle et al. [135] reporting concentrations of MCs from several μg/L up to 300 μg/L (HPLC) in sediments from Quitzdorf reservoir, Germany. Similarly, about 90 μg/L of NOD (ELISA/HPLC) was found in sediment from western Gulf of Finland, Baltic Sea [139].

**Table 2:** Concentrations of microcystins (MCs) in sediments (surface layer 0–5 cm) from the Brno reservoir during 2005 at two different sampling sites, as analysed by ELISA and HPLC

| Date | Locality | no. | Microcystin concentrations in sediments | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | μg/g d.m. | | | | μg/L | | | |
| | | | ELISA | Mean (±SD) | LC | Mean (±SD) | ELISA | Mean (±SD) | LC | Mean (±SD) |
| 6-IV-2005 | Rokle | 1 | 0.056 | 0.047 (±0.009) | 0.041 | 0.049 (±0.021) | 18.93 | 17.58 (±0.97) | 13.74 | 17.88 (±5.63) |
| | | 2 | 0.035 | | 0.029 | | 17.11 | | 14.07 | |
| | | 3 | 0.050 | | 0.077 | | 16.70 | | 25.85 | |
| | Centre | 1 | 0.122 | 0.291 (±0.120) | 0.195 | 0.350 (±0.116) | 25.47 | 69.74 (±31.53) | 40.83 | 82.70 (±29.89) |
| | | 2 | 0.380 | | 0.474 | | 87.27 | | 108.68 | |
| | | 3 | 0.372 | | 0.381 | | 96.47 | | 98.60 | |
| 15-VI-2005 | Rokle | 1 | 0.014 | 0.018 (±0.007) | 0.075 | 0.047 (±0.024) | 4.39 | 5.97 (±2.79) | 23.96 | 15.53 (±8.06) |
| | | 2 | 0.012 | | 0.016 | | 3.64 | | 4.67 | |
| | | 3 | 0.028 | | 0.051 | | 9.89 | | 17.95 | |
| | Centre | 1 | 0.021 | 0.059 (±0.066) | 0.088 | 0.084 (±0.048) | 4.32 | 13.13 (±14.97) | 17.84 | 18.14 (±11.02) |
| | | 2 | 0.152 | | 0.141 | | 34.21 | | 31.79 | |
| | | 3 | 0.004 | | 0.023 | | 0.86 | | 4.80 | |
| 13-VII-2005 | Rokle | 1 | 0.019 | 0.020 (±0.005) | 0.033 | 0.017 (±0.013) | 7.28 | 6.27 (±1.91) | 12.51 | 6.03 (±5.12) |
| | | 2 | 0.026 | | 0.018 | | 7.93 | | 5.58 | |
| | | 3 | 0.015 | | <LOD | | 3.60 | | <LOD | |
| | Centre | 1 | 0.017 | 0.022 (±0.005) | 0.039 | 0.036 (±0.003) | 2.61 | 4.08 (±1.47) | 6.19 | 6.51 (±0.32) |
| | | 2 | 0.027 | | 0.033 | | 5.56 | | 6.83 | |
| 10-VIII-2005 | Rokle | 1 | 0.038 | 0.030 (±0.011) | 0.024 | 0.022 (±0.004) | 12.36 | 9.11 (±3.48) | 7.99 | 6.81 (±1.49) |
| | | 2 | 0.014 | | 0.026 | | 4.29 | | 7.73 | |
| | | 3 | 0.038 | | 0.017 | | 10.69 | | 4.70 | |
| | Centre | 1 | 0.012 | 0.012 (±0.002) | 0.046 | 0.049 (±0.003) | 2.46 | 2.61 (±0.58) | 9.42 | 10.09 (±0.49) |
| | | 2 | 0.015 | | 0.048 | | 3.39 | | 10.58 | |
| | | 3 | 0.010 | | 0.052 | | 1.99 | | 10.27 | |
| 7-IX-2005 | Rokle | 1 | 0.048 | 0.034 (±0.017) | 0.055 | 0.048 (±0.015) | 14.74 | 10.21 (±4.68) | 16.70 | 14.76 (±3.43) |
| | | 2 | 0.042 | | 0.062 | | 12.13 | | 17.63 | |
| | | 3 | 0.010 | | 0.026 | | 3.77 | | 9.94 | |
| | Centre | 1 | 0.021 | 0.042 (±0.035) | 0.114 | 0.094 (±0.028) | 3.05 | 6.63 (±5.61) | 16.53 | 14.56 (±3.73) |
| | | 2 | 0.092 | | 0.113 | | 14.55 | | 17.83 | |
| | | 3 | 0.013 | | 0.054 | | 2.30 | | 9.34 | |
| 12-X-2005 | Rokle | 1 | 0.010 | 0.010 (±0.000) | 0.043 | 0.021 (±0.021) | 3.54 | 3.75 (±0.20) | 15.61 | 7.80 (±7.80) |
| | | 2 | 0.010 | | <LOD | | 3.95 | | <LOD | |
| | | 3 | 0.003 | | 0.063 | | 0.63 | | 11.65 | |
| | Centre | 1 | 0.008 | 0.013 (±0.010) | 0.032 | 0.054 (±0.015) | 1.31 | 2.15 (±1.69) | 5.37 | 9.31 (±2.80) |
| | | 2 | 0.027 | | 0.066 | | 4.51 | | 10.92 | |

The results are expressed both as μg/g sediment d.m. and μg/L of sediment volume to allow comparisons with literature data. Two or three random independent sediment samples were collected and analysed for each locality and sampling date (No. 1, 2, 3 within each sampling date). LOD: limit of detection (0.0003 μg/g d.m. for ELISA, 0.015 μg/g d.m. for HPLC). Since the recoveries of MCs from various sediments differed significantly with respect to the sediment composition, we only report measured concentrations here (recoveries were not taken into account)

In our study, sediment MC concentrations clearly showed spatial variability as well as temporal changes. Concentrations at the "centre" site were generally higher than those at the "Rokle" site. This difference could possibly be attributed to better conditions for overwintering of cyanobacterial cells and slower decay of biomass in deeper sediments (i.e. "centre") [149]. However, substantial variability in MC concentrations was also found among independent samplings performed at the same date and at the same site. For example, a coefficient of variance of up to 114% (μg/L, ELISA results) was observed for repeated samplings at the "centre" site on 15-VI-2005 (Table 2). In general, concentrations of MCs in sediments were higher in spring than in summer and there were slight increases in MC concentrations in September. This seasonal trend was observed at both localities studied, but it was more pronounced at the "centre" site. As Ihle et al. [135] reported, concentrations of MCs in sediments increased in autumn, when the cyanobacterial bloom settles down. The concentrations continued to decrease until the next spring, when cyanobacteria reinvaded the water column or decayed, and the lowest concentrations were observed in summer. The high concentrations of MCs observed in our study during the spring thus seem to correspond to the end of the overwintering phase, followed by a decrease in MC concentration until the summer months (July and August). The correlation between the ELISA and HPLC analyses of the MCs in the sediment samples from Brno reservoir is shown in Fig. 14. Although the results from both methods were in agreement for higher MC concentrations, only weak correlation was observed below 0.1 μg/g d.m., which may reflect the generally higher variability in the ELISA results.

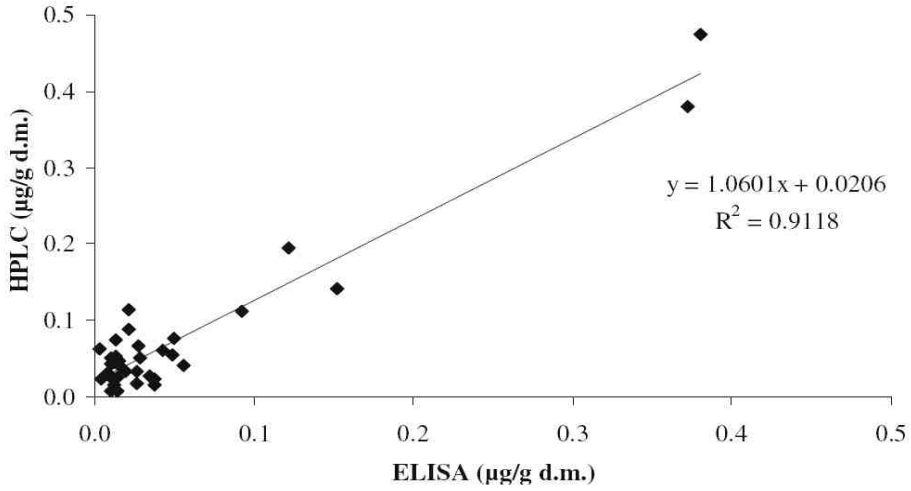


**Fig. 14:** Correlation between the results of ELISA and HPLC analyses of total microcystin content in sediments from Brno reservoir (n=34, two values that were <LOD of HPLC were replaced with 1/2 of LOD). The robustness of the correlation was checked by recalculation for the subset of samples with concentrations <0.2 µg/g d.m., with the following results: y=0.8992x+0.0252, R2=0.543

However, it must also be emphasized here that although the HPLC retention times of the MC peaks corresponded to those of the external MC standards, their UV spectra often did not fit with the characteristics of MCs (particularly for smaller peaks equivalent to concentrations below 0.1 µg/g d.m.). This effect could be caused by coeluting impurities that may affect typical MC UV spectra and complicate quantification. It is important to take this into account as the concentrations of MCs occurring in natural sediments were usually close to the detection limit of our HPLC method. In this case, HPLC analysis may become less selective and ELISA seems to be more reliable for total MC content.

**Conclusion**

Our results revealed several factors that affect MC analyses in sediments. Extraction efficiency depends strongly on the solvent, and the use of 5% acetic acid in 0.1–0.2% TFA– methanol provided the best results. Extraction

efficiency was also affected by the type and composition of the sediment, but did not clearly correlate with the content of total organic carbon. Rather, other characteristics, such as interactions with inorganic (clay) particles seem to substantially affect the sorption of MCs onto sediments. MC structure is another important factor that influences sorption onto sediments, with lower recoveries generally obtained for more hydrophilic variants, such as MC-RR. In spite of the limitations discussed, the methods employed here (especially ELISA for detection at low MC concentrations) can be successfully used to investigate the occurrence, fate and natural ecological role of MCs.

### 4.1.2. Methods of microcystin detection in complex samples

Determination of microcystin in the sample can be based either on physico-chemical or on biochemical properties of the molecule.

**Biochemical methods**

Biochemical methods take advantage of known and specific modes of action (inhibition of enzymes, immunity reaction principles) of microcystin molecule in biological systems. Principle of protein phosphatase inhibition assay (PPIA) is based on measuring of inhibition of release of labeled phosphates from phosphorylated proteins. In the presence of microcystins the reaction speed is slow or even stopped. This technique can be used for detection of toxins in various matrices: in water and biomass [99, 150, 151], plant [132] and animal tissues [120, 128]. Principally slightly different is protein phosphatase binding assay (PPBA) [152-154].

Immunochemical methods using antibodies against MC molecules are another way how to determine these toxins in various samples. The most widely used method is ELISA (enzyme linked immunosorbent assay). This method can be used for determination of microcystins in water and cyanobacterial biomass [99, 155, 156], in plant materials [131, 133], animal tissues [49, 122, 124, 157] and sediments [124, 158].

Although the above mentioned biochemical and immunochemical methods have many advantages like high sensitivity, ease of use and simplicity. They have also some drawbacks such as lower robustness and reproducibility in comparison with instrumental analytical methods. One of the limitations of these methods is also different cross-reactivity and affinity of various MC structural variants to the enzymes/antibodies. These methods can be also easily influenced by many physico-chemical parameters or by sample composition too [145, 159].

## Instrumental analytical methods

Instrumental analytical methods determine microcystins by the physico-chemical characteristics of molecules. Especially in combination with effective separation techniques these methods present selective and sensitive tools for analysis of microcystins.

Reversed phase high performance liquid chromatography (RP-HPLC) is the most often used method for separation of microcystins in mixtures. This technique can be used for determination of toxins in various types of samples. Stationary phases used for this application are usually various kinds of modified silicagel (typically C-18 or C-8) [160]. Separation can be carried out also on monolithic C-18 [161] or amino-endcapped materials [162]. Mobile phases used for separations consists very often of acetonitrile (methanol) and water. Ion pairing reagent (i.e. TFA) is often used to suppress unspecific interactions of free silanol groups and improve separation of compounds with basic substituents [160]. Other mobile phase modifiers (amonium acetate/formate) can be used too. They contribute to better separation of methylated and demethylated variants of microcystins [99] and play important role in some principles of detection. Thin layer chromatography (TLC) can be also used for separation of toxins [114, 163, 164]. Microcystins have been successfuly separated also with capillary zone electrophoresis (CZE) [165-170], micellar electrokinetic chromatography [171, 172] or capillar electrochromatography [173, 174].

Microcystins can be detected with a broad range of detectors. Molecules of microcystins are able to absorb UV light and most of them show typical absorbance spectra with maxima at 238-240nm. Structural variants with aromatic aminoacids (i.e. MC-YR with tyrosine, MC-LW with tryptophane) have different (yet typicall) spectra with prime maxima at 232 and 234nm and secondary maxima at 238nm, respectively. However, spectra of majority of the structural variants are very similar and their identification based on comparison with retention time of standards is required. Nevertheless, diode array detector (contrary to UV detector) allows to identify chromatographic peaks with characteristic spectra and distinguish them from the peaks of interfering

compounds [160]. Another types of detectors that can be coupled with chromatographic separation are fluorescence and chemiluminiscence detectors [175, 176]. In the literature, there are also records on use of electrochemical detector [177, 178]. In the case of fluorescence/luminiscence detection, there is requirement for pre- or post-column derivatisation of microcystins with suitable reagent. On the other hand, these two techniques are more sensitive with lower limits of detection (LOD) than classic photometric approach.

Above mentioned disadvantages of photometric detectors are not an issue in the case of mass spectrometric detection (MS). This method allows identification and confirmation of microcystins without the need of analytical standards. Various ionization and separation techniques can be used for detection of microcystins. In the literature there are reports on successful application of fast atom bombardment (FAB) [179, 180], electrospray [65, 127, 181-188] and matrix assisted – laser desorption ionization (MALDI) [7, 189, 190]. Quadrupole (Q) and time-of-flight (TOF) mass analysers are used predominantly.

Especially the technique of liquid chromatography coupled with electrospray ionization and mass spectrometric detection (HPLC-ESI-MS) is very widespread due to its sensitivity and selectivity. This technique is also capable to identify products of degradation or biotransformation of microcystins [160, 186, 191-195].

Alternative method for detection of microcystins is based on their oxidation, followed by extraction and detection of the specific product of this reaction - 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) [196, 197]. MMPB can be detected by multiple methods. After esterification GC with mass spectrometric [197] or flame ionization detector (FID) can be used [196]. MMPB can be also derivatised and detected by LC with fluorescence detector [196] or without any derivatisation by LC-MS method [183]. MMPB method is quite laborious and only the total amount of microcystins can be detected without any information on present structural variants. On the other hand with this approach it is possible to analyse microcystins even in the most complex matrices like tissues [183] or sediments [140].

The aim of our work in the field of microcystin detection was to develop sensitive, robust and selective method for determination of microcystin in complex samples. We have also tried to compare the performance of single MS and tandem MS methods. There is a number of studies using mass spectrometric detection for analyses of cyanotoxins but no direct comparison of both approaches exists. Many publications, which relied on a single MS for detection and quantification of microcystins, did not consider several drawbacks (particularly insufficient selectivity) of this method. Single MS approach might lead to overestimation of toxin content or false positive results. We have compared performance of LC-MS and LC-MS/MS techniques for a series of tissue samples from fish exposed to cyanobacterial toxins.

## 4.3. LC-MS analyses of microcystins in fish tissues overestimate toxin levels – critical comparison with LC-MS/MS

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### Introduction

Analytical determination of microcystins in complex mixtures based on separation by liquid chromatography with mass spectrometric detection have number of advantages. Especially higher sensitivity and robustness comparing to many instrumental but also biochemical methods. LC-MS seem to be a suitable tool, which minimizes some drawbacks of above mentioned approaches [194, 198, 199]. However, there are also some critical factors strongly influencing precission and accuracy of this method. Although number of studies used mass spectrometric detection for analyses of cyanotoxins, no critical comparison of single MS vs. MS/MS methods for microcystin analyses in the tissues of biota is available. Number of publications, which relied on a single MS for detection and quantification of microcystins, did not consider several drawbacks such as insufficient selectivity, which might result in overestimated or false positive results. In this paper, we compared performance of LC-MS and LC-MS/MS techniques for a series of tissue samples from fish exposed to cyanobacterial toxins microcystins both experimentally (in controlled lab conditions) and collected in the field from fish ponds with natural cyanobacterial blooms.

## Material and Methods

### Chemicals and reagents

Standards of microcystins (MC-LR, -RR, -YR) were obtained from Alexis Biochemicals (Läufelfingen, Switzerland). The solvents (LC/MS grade) were purchased from Sigma-Aldrich (Prague, Czech Republic). Deionized water was prepared with the Millipore Simplicity 185 system (Millipore, Bedford, MA, USA).

### Experimental design

Fish tissues used for the comparative studies of MS and MS/MS method performance were collected from both laboratory experiments and field samplings.

During the short term laboratory experiments, fish were injected with high concentrations of microcystins, and toxin presence in hepatopancreas and its metabolisation was evaluated. For these experiments, common carp (*Cyprinus carpio*; 500±15 g males) were obtained from the commercial aquaculture (Pohorelice fishery, Czech Republic). Experimental fish (N=3) were intraperitoneally injected with the mixture of major microcystin variants at a total microcystin dose 248 μg/kg body weight (injection volume 500 μL). The mixture of microcystins was prepared in the phosphate-buffered saline, PBS pH 7.3 and contained 56% MC-RR, 38% MC-LR and 6% MC-YR. Control fish (N=3) received 500 μL of PBS. After 3h, fish were dissected and samples of hepatopancreas and muscle tissues were collected and stored at -80°C until analysed.

The second series of experiments studied accumulation of microcystins in fish under natural conditions. Fish specimens of common carp (body weight 32±7 g, *n*=10) were kept in two experimental ponds for nine weeks. Pond A contained massive and dense water bloom dominated by *Microcystis aeruginosa* with microcystin concentrations ranging 10.1 - 15.4 μg/L during the whole exposure period. Second pond B also contained some cyanobacteria but they were present in negligible density and maximum concentrations of microcystins in

water reached 2 μg/L. During the experiment, fish were not externally fed, and no mortalities were observed. At the end of the experiment fish were dissected, and tissue samples collected for analyses as described above. Concentrations of dissolved microcystins in ponds were determined by HPLC-DAD method according to the ISO 20179 [107], volume of the grab samples was 1 L. Besides this controlled study, fish of different species randomly caught in various aquaculture ponds in the Czech Republic were also analysed for content of microcystins, and details on environmental conditions and microcystin concentrations are provided in the results section.

Tissue extractions

Tissue extractions were performed by methods described previously [200]. Briefly, frozen sample (0.5 g fresh weight) was homogenised 4-times with methanol (3 mL), sonicated in an ultrasonic bath for 30 min, and centrifuged at 4,000 *g* for 10 min. Supernatants were pooled and repeatedly extracted (three times) with 1 ml of hexane to remove lipids (hexane layers discarded). Methanol extract was evaporated at 50 deg.C, and the residue was dissolved in 300 μL of 50% aqueous methanol (v/v) and analysed. Recovery of the extraction method (~25%; data not shown) was not considered during calculations to remain consistent with the methods and values previously reported in literature [49, 53, 118, 125, 200].

Liquid Chromatography Electrospray Ionization Mass Spectrometry Analyses

The present study compared performance of single MS microcystin analyses, which used SIM (selected ion monitoring mode) with the MS/MS approach using MRM (multiple reaction monitoring mode) with details described below in this paragraph.
Analyses were performed with the HPLC apparatus Agilent 1200 series (Agilent Technologies, Waldbronn, Germany) consisting of a vacuum degasser, a binary pump, an autosampler, and a thermostatted column compartment kept at 30 deg.C. The column was a Supelcosil ABZ+Plus RP-18 endcapped (5 μm) 150 x 4.6 mm i.d. (Supelco). A SecureGuard C18 (Phenomenex, Torrance, CA, USA) guard column was used. The mobile phase consisted of 5 mM

ammonium acetate in water, pH 4 (A) and acetonitrile (B). The binary pump gradient was as follows (0 min - 12.00 min, 32% - 40% B, linear increase; 12.01 min – 20.00 min, 40% - 42% B, linear increase; 20.01 min – 30.00 min, 90% B); the flow rate was 0.4 mL/min. 20 μL of individual sample was injected for the analyses.

The mass spectrometer was an Agilent 6410 Triple Quad mass spectrometer (Agilent Technologies, Waldbronn, Germany) with electrospray ionization (ESI). Ions were detected in the positive mode. The ionization parameters were as follows: capillary voltage, 5.5 kV; desolvation temperature, 350 deg.C; desolvation gas flow, 11 L/min. In selected ion monitoring (SIM) mode following m/z were monitored: MC-RR $[M+2H]^{2+}$ 519.8, MC-YR $[M+H]^{+}$ 1045.5, MCLR $[M+H]^{+}$ 995.5, MC-RR-GSH $[M+2H]^{2+}$ 673.8, MC-LR-GSH $[M+H]^{+}$ 1302.5, MC-RR-CYS $[M+2H]^{2+}$ 580.8, MC-LR-CYS $[M+H]^{+}$ 1116.5. The transitions from the protonated molecular ion to a fragment of amino acid Adda (unusual amino acid present only in microcystins and related nodularins - (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) at m/z 135.2 and fragment at m/z 127.1 were monitored in multiple reaction monitoring (MRM) mode. Collision energies (CE, V) used for fragmentation: MC-RR and respective conjugates, CE= 50V; MC-YR and –LR and respective conjugates, CE= 40V). Quantification of analytes was based on external standards of MC-RR, MC-YR, MC-LR in matrix (final extract of microcystin-free fish tissue). The mixture of microcystins was added into extract prior to analysis. Method detection limit (MDL; per gram of tissue, fresh weight) was 1.2 ng/g (S.D.=5%) for MC-RR and 5.4 ng/g (S.D.= 10%) for MC-YR and –LR in MRM mode. In SIM mode MDL was 3.0 ng/g (S.D.=5%) for MC-RR and 27.0 ng/g (S.D.=7%) for MC-YR and –LR, respectively. Although other approaches such as standard addition method could be used, we have relied on external calibration as it is considered to have higher precision [201-203].

**Results and Discussion**

Number of field studies, which used ELISA and LC-MS for analyses of microcystins in the fish tissues, reported high concentrations ranging 5 - 18000 ng/g (Table 3). However, our previous investigations based on controlled

exposures [200] as well as field studies [204, 205] resulted in systematically lower microcystin concentrations in fish tissue. These findings were surprising because environmental situations and concentrations of microcystins in the water were similar to those reported in the literature. These differences motivated the present study, which aimed to compare single LC-MS method (commonly reported in the literature) with more robust and selective LC-MS/MS.

**Table 3:** Concentrations of microcystins in biological samples reported in literature with indication of respective analytical technique.
* FW - fresh weight, DW - dry weight.

| Fish species | Tissue sample | Concentration (MC-LR/g tissue) | Analyt. tech. | Source |
|---|---|---|---|---|
| *Tilapia* sp. | Muscle | 3-337 ng/g FW* | ELISA | Magalhaes 2001 [118] |
| *Tilapia* sp. | Muscle | 100 ng/g FW | ELISA | Mohamed 2003 [121] |
| *Tilapia* sp. | Muscle | 100 ng/g DW* | ELISA | Soares 2004 [51] |
| *Hypophtalmichtys* sp. | Hepatopancreas | 7000 - 17800 ng/g DW | LC/MS | Xie 2004 [53] |
| *Hypophtalmichtys* sp. | Muscle | 500 - 1700 ng/g DW | LC/MS | |
| *Hypophtalmichtys* sp. *Cyprinus* sp. | Muscle, Hepatopancreas | 1800 - 7700 ng/g DW | LC/MS | Xie 2005 [125] |
| *Hypophtalmichtys* sp. | Muscle | 4.4 - 29 ng/g FW | ELISA | Adamovsky 2007 [200] |
| *Cyprinus* sp. | Muscle | 5.8 – 19 ng/g FW | ELISA | |
| water chestnut (*Trapa natans*) | - | 2 – 5 ng/g FW | LC/MS | Xiao 2009 [206] |

In the first experiment, we investigated general ability of single and tandem MS approaches to analyse microcystins and their metabolites accumulated in fish after intraperitoneal injection. In Figure 15, the chromatograms of tissue extract containing MC-RR obtained in SIM and MRM modes are presented (for MRM with two characteristic transitions; 519.5->135.2, 519.5->127.1).

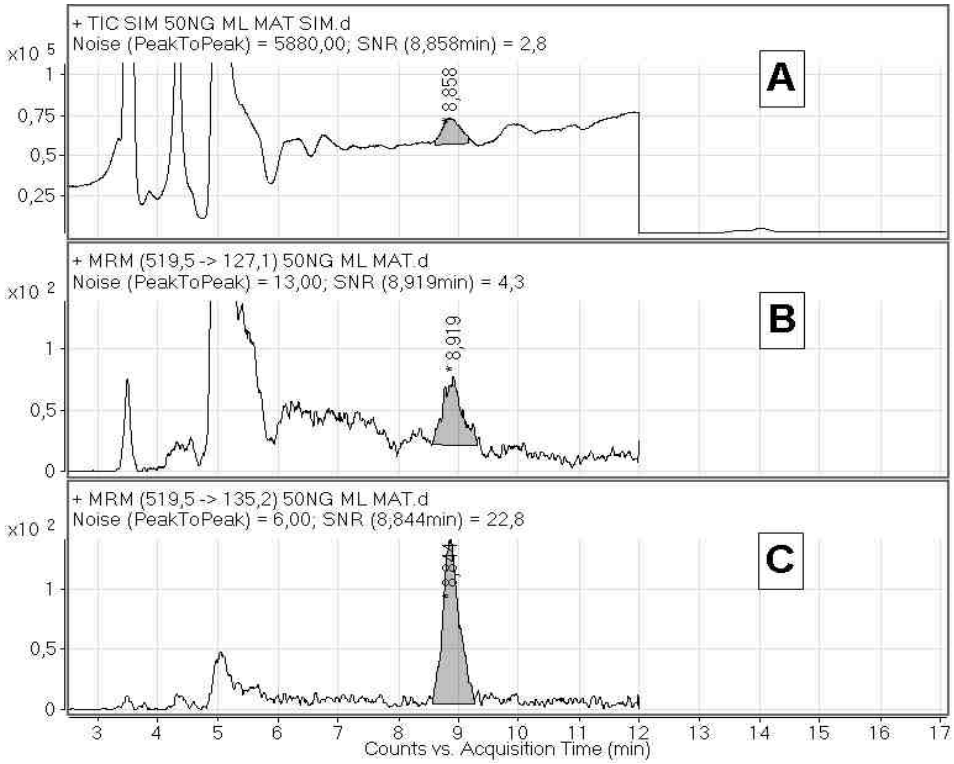


**Fig.15:** Chromatograms of MC-RR obtained in SIM (A) and MRM (two bottom panels with 2 characteristic transitions; B 519.5->127.1, C 519.5->135.2) mode. Note the high noise in SIM chromatogram indicated by low signal to noise ratio (see the values of SNR in the inset of all graphs).

SIM chromatogram has very high background noise limitating thus LOD. On the other hand, MRM chromatograms seem to be more usable, especially transition 519.5->135.2. To be correct, also the qualifier ion (transition 519.5->127.1) should be monitored. However, it is present in very low concentrations when working with natural extracts (i.e. at low - environmentally relevant levels). Consequently, this fragment may be of limited use because of higher background noise. Very similar behaviour shows also MC-YR and –LR. Table 4 shows the obtained results (MRM mode) for all experimental variants (with/without external standards added before the tissue extraction).

**Table 4:** Microcystins determined by tandem MS in common carp (*Cyprinus carpio*; 500±15 g) hepatopancreas three hours after intraperitoneal injection of microcystin mixture into fish (exposed vs. non-exposed). Standards of MCs were further added into samples prior to extraction at indicated doses ("spike" variants).

| **Experimental variant** | **Intraperit. injection** [(1)] | **Pre-extraction addition** [(2)] | **MCs detected in hepatopancreas** | | |
|---|---|---|---|---|---|
| | | | concentration (total MC) | MC congener ratio | MC-GSH conjugates |
| | MC µg / fish | MC µg/g FW | µg/g FW (±S.D.) | MC-RR, YR, LR (%) | µg/g FW |
| **Exposed** | 124 | - | 0.909 (±0.375) | 5, -, 95 | ~ 0.1 * |
| **Exposed +spike** | 124 | 0.424 | 1.062 (±0.341) | 8, -, 92 | ~ 0.1 * |
| **Non-exposed** | - | - | 0.000 (±0.000) | - | - |
| **Nonexposed +spike** | - | 0.424 | 0.124 (±0.019) | 32, -, 68 | - |

(1) i.p. injection mixture containted 56% MC-RR, 6% MC-YR and 38% MC-LR;
(2) MCs externally added into the sample before the extraction (mixture with 27% MC-RR, 29% MC-YR and 44% MC-LR)
* low concentration, close to the limit of quantitation (approximate, based on the MC standard calibration)

The results showed good performance of the tandem MS method to detect microcystins in complex samples with sufficient selectivity and sensitivity. Although no clean up procedure was applied on the raw extracts, chromatograms had relatively low background and allowed detection and quantitation of microcystins in sufficiently low concentrations (MDL was 1.2 ng/g for MC-RR and 5.4 ng/g for MC-YR and –LR). Figure 16 shows MS/MS

chromatogram of hepatopancreas extract with two peaks of MC-RR and MC-LR, and respective conjugates with glutathione.

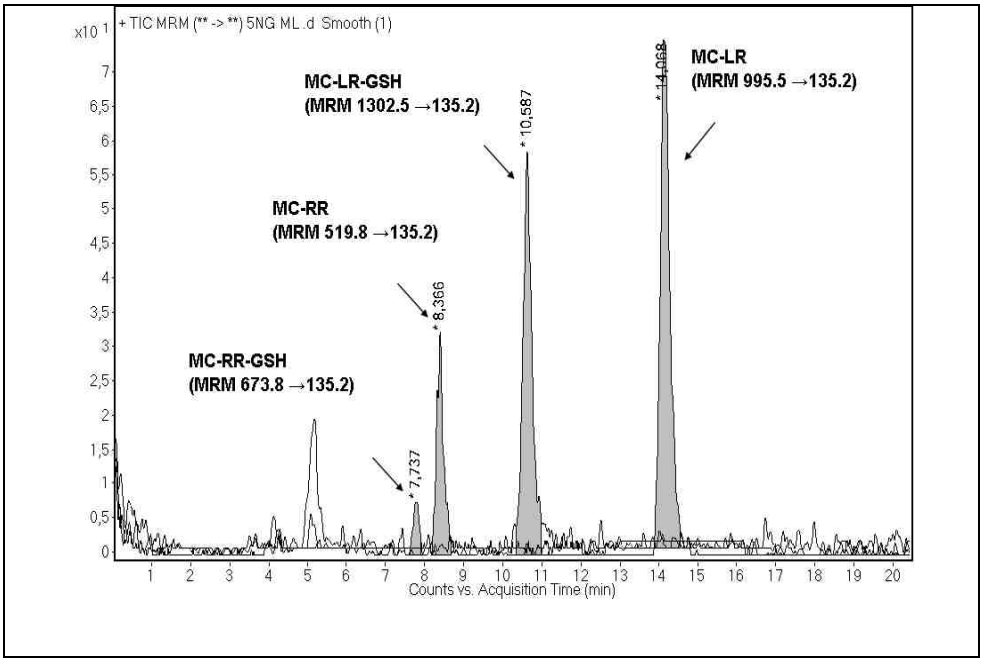


**Fig.16:** Chromatogram of hepatopancreas extract from the tandem MSMS analysis of microcystins (MC; common carp tissues collected 3 hours after intraperitoneal injection of microcystins dosed at 248 μg/kg bw.). Peaks of intact molecules as well as their conjugates with glutathione (GSH) were detected; retention times and characteristic MRM transitions are presented.

In this experiment, no MC-CYS conjugates were detected. As it is apparent, extraction efficiency (variants with external additions of microcystins prior to extraction) was around 25% for total MC, which corresponds to our previous detailed study [200]. Ratio of MC variants detected in the hepatopancreas differed from the original exposure mixtures; no MC-YR was found. This might be affected by number of factors including variable uptake/metabolism of MC variants as well as differences in MS sensitivity but detailed investigation is out of scope of the present work.

In the follow up studies, we compared single MS vs. tandem MS methods by analysing fish caught in natural reservoirs with cyanobacterial blooms. Figure 17 shows concentrations of microcystins in common carp exposed under controlled conditions for 9 weeks in two ponds with different cyanobacterial water blooms and different MC content.

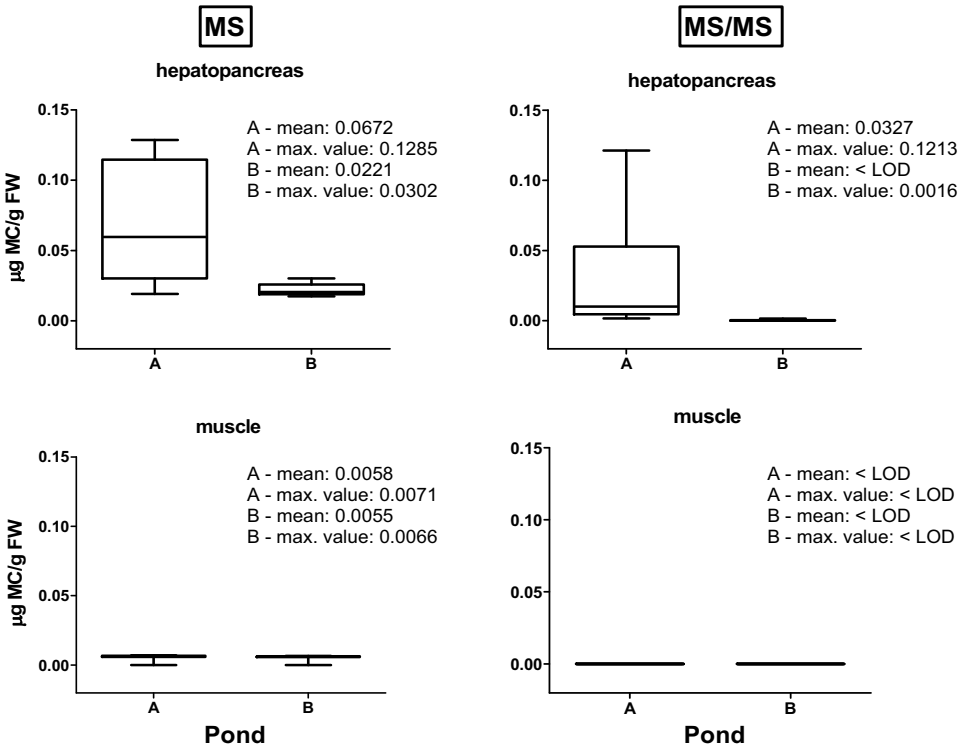


**Fig.17:** Concentrations of microcystins (sum of MC-RR and MC-LR) detected by single and tandem MS (left vs. right panels, respectively) in fish hepatopancreas (upper graphs) and muscles (bottom graphs). Common carp tissues were analysed after 9 week accumulation under natural conditions in two fish ponds. Pond A contained massive water bloom during the whole 9 week experiment (dominated by *Microcystis aeruginosa*; concentrations of dissolved microcystins 10-15 µg/L); pond B contained cyanobacteria in low density with maximum MC concentration 2.2 µg/L. Numerical values at each graph show mean and maximum for each variant (<LOD - less than limit of detection; LOD = 1.2 ng/g for MC-RR and 5.4 ng/g for MC-YR and –LR).

In the single MS (SIM mode), peaks with retention times of MC standards were found in almost all samples, and m/z values previously reported in the literature were observed (519.5 for MC-RR and 995.5 for MC-LR). Hepatopancreas concentrations based on the SIM mode ranged 6.0 – 129.0 ng/g tissue fresh weight (FW). In the muscle samples (i.e. the edible part of fish), MC concentrations were low with maxima around 8.0 ng/g FW. However, SIM mode often determined similar "MC" peaks also in liver and muscle of control non-exposed fish, and these false positives appeared in all experimental variants. More detailed analyses by tandem MS did not confirm MC in all samples. No MC were detected in the muscle samples, and presence of microcystin peaks was confirmed only in hepatopancreas of fish from the pond with high toxin levels. Hepatopancreas concentrations determined by MS/MS ranged 14.0 – 123.0 ng/g FW, which are more than 50% lower values then calculated from single MS in SIM mode. Fish exposed in the field contained only MC-CYS metabolites and in very low concentrations, which were close to the limit of quantification (results not shown).

As a final part of the study, muscle samples (edible parts) of 8 different fish species collected from five reservoirs during 2008 were analysed by LC-MS/MS. Water blooms of toxic cyanobacteria occurred in all reservoirs during the season and characteristics of water blooms and microcystin concentrations are given in Table 5. The study covered representatives of all feeding types of fish including herbivorous (grass carp), omnivorous (bream and common carp), zooplanktivorous (whitefish) and carnivorous (catfish, eel, perch and zander). From total 148 fish investigated, none of them was positive for microcystins MC-RR, -YR or –LR. Our results based on tandem mass spectrometric detection thus indicate lower risk of fish contamination by microcystins, and also lower risks of the toxin transfer to humans.

**Table 5:** Concentrations of microcystins in fish muscles determined by tandem MS/MS in eight fish species collected from five different reservoirs (localities in the Czech Republic) where dense cyanobacterial blooms occurred during 2008 season. LOD was 1.2 ng/g for MC-RR and 5.4 ng/g for MC-YR and –LR.

| Locality | Duration of water bloom (months) | Dissolved MC in water (μg/L) | Fish species | Number of analysed fish | c MC in muscle μg/g FW (±S.D.) |
|---|---|---|---|---|---|
| Novoveský | V. - VII. | 0.638 - 4.485 | asp | 10 | <LOD |
| | | | catfish | 10 | <LOD |
| | | | common carp | 10 | <LOD |
| | | | eel | 1 | <LOD |
| | | | grass carp | 10 | <LOD |
| | | | zander | 10 | <LOD |
| Sykovec | VIII. - IX. | 0.125 - 1.358 | common carp | 10 | <LOD |
| | | | perch | 10 | <LOD |
| | | | whitefish | 10 | <LOD |
| Medlov | VII. - IX. | 0.200 - 0.741 | common carp | 10 | <LOD |
| | | | perch | 10 | <LOD |
| | | | whitefish | 10 | <LOD |
| Plumlov | VI. - VIII. | 0.212 - 0.505 | bream | 18 | <LOD |
| | | | perch | 6 | <LOD |
| Vír | VI. - VIII. | 0.000 - 1.201 | bream | 13 | <LOD |

In summary, our results demonstrate that commonly used methods based on single MS (i.e. monitoring of ions with m/z 519.5 for MC-RR and 995.5 for MC-LR, respectively) may provide false positive responses overestimating thus concentrations of MCs in the tissue samples. This might have important consequences as some authors conclude about high human health risks of MC accumulated in fish [53, 125]. On the other hand, MS/MS analyses resulted in much cleaner chromatograms with very low background noise, which also lowered actual LOD/LOQ in comparison with the SIM analyses. It should be pointed out, that tandem MS/MS also has its limitations in analyses of extremely complex tissue extracts (e.g. modulations of MS signal by components of complex matrices; preferred but not often used quantification using isotopically-labeled standards, etc.), and these will require further research. Nevetheless, our findings show that selective methods such as tandem mass spectrometry should be used for analyses of cyanobacterial toxins in the tissue samples as they provide more reliable results than single MS methods.

# 5. General summary

Occurrence of massive cynobacterial populations can create a significant water quality problems all around the world. Especially with respect to fact that many cyanobacterial species are capable of synthesizing a wide range of secondary metabolites, including potent toxins [29]. It has been estimated that 25 to 75% of cyanobacterial blooms are toxic [30], and microcystins are probably the most often produced and abundant cyanotoxins. They may cause serious health problems [18, 45], and the World Health Organization (WHO) suggested safety guideline value of 1.0 μg/L for drinking waters [62]. This value is a part of many national legislations including the Czech Republic. These evidences highlight the need of microcystin monitoring in the environment.

In the present study, tools that can contribute to the improvement of contemporary monitoring practice have been evaluated. Our effort was focused especially on passive sampling and methods for detection of microcystins in complex environmental samples such as sediments and animal tissues.

Appropriate sampling technique have to be used for monitoring of particular contaminants. Microcystins are toxicants that occur with high spatial and temporal variability [63, 207]. The traditional monitoring programs (based on collection of individual samples at specific single spot and time points) are not fully adjusted for such pollutants and fail in estimation of time averaged concentration (TWA) [72, 74, 97, 106]. However, correct estimation of TWA is essential for evaluation of potential health risks and correct decision making process.

Passive sampling gains increasing popularity in environmetal monitoring because it overcomes some of the drawbacks of traditional sampling techniques. The use of integrative passive samplers enables estimation of TWA concentrations, allows the sequestration of residues from episodic events (commonly not detected with grab sampling), it is not limited to constant water conditions, and it allows accumulation and concentration of trace and ultra-trace contaminant levels over extended time periods [73, 79, 83, 110].

There is a number of passive sampling devices developed for environmental monitoring. We have employed POCIS system which was primarily developed for sampling of polar organic contaminants like pesticides

or pharmaceuticals. This sampler was originally designed to mimic exposures of aquatic organisms to dissolved chemicals eliminating the problems of metabolism, depuration of chemicals, avoidance of contaminated areas, and mortalities of organisms. The POCIS sampler is constructed of a solid sequestration medium (sorbent) enclosed within a hydrophilic microporous membrane [79]. Both sorbent medium and membranes are customizable, and they can be tuned to target specific chemicals or chemical classes.

To fully evaluate POCIS technique applicability for cyanobacterial toxins we have performed a series of experiments targeting all key features of the system (Chapter 3.2 and 3.3). A pilot experiment with the commercial sampler was performed under conditions of natural water bloom to reveal the suitability of this technique for microcystins accumulation. The samplers were shown to effectively accumulate microcystins after 7 day exposure. Following, four different microporous membranes and two sorbents were compared for their efficiency to sample microcystins. Laboratory studies revealed that the best results (highest sampling rates) were obtained with the sampler constructed with the polycarbonate membrane and Oasis HLB sorbent (2.75 mg of sorbent per $cm^2$). The optimised sampler showed very good uptake of microcystins with experimentally determined sampling rates of 0.022 L/d and 0.017 L/d for MC-RR and MC-LR, respectively (Kohoutek et al. 2008, [108]).

Optimized passive sampler was then validated under various exposure scenarios in laboratory and field (Kohoutek et al. 2010, [105]). Calibration of the passive sampler conducted under variable conditions and concentrations of MC revealed linearity of the sampling up to 4 weeks. The sampling rates of microcystins for two different exposure scenarios were derived (e.g. MC-LR: $R_S$=0.017 L/d under static and 0.087 L/d under turbulent conditions). Rs values were further used for calculations of time-weighted average concentrations in natural water. Improved sensitivity and selectivity of the customized sampler was observed in comparison with the commercially available POCIS. Comparisons of grab and passive sampling methods were performed during cyanobacterial water bloom season in the Brno reservoir, Czech Republic in 2008. Data obtained by passive sampling provided more relevant picture of the situation and enabled better assessment of potential risks. The present study demonstrate that the modification of POCIS is suitable for monitoring of occurrence and retrospective estimations of microcystin water concentrations,

especially with respect to the control of drinking water quality (Kohoutek et al. 2010, [105]).

Reliable and precise analytical methods are another presumptions for correct detection and monitoring of pollutants in the environment. That is why the evaluation of analytical techniques was also part of the present work.

The efficiencies of conventional extraction techniques and analytical methods (HPLC–DAD and ELISA) were investigated for analyses of microcystins (MCs) in sediments (Chapter 4.2, [158]). The observations of microcystin accumulation in sediments resulted in a hypothesis that MCs could play a role of signaling molecules [136-138] and may participate in the activation of cyanobacteria growth and further colonization of the water column in spring. Detailed studies of the fate of MCs in sediments should provide information on the natural functions of these peptides. The aim of the experiments was to evaluate different techniques for extraction of microcystins from sediments. Our results showed that the extraction efficiency strongly depends on the extraction solvent (extraction with 5% acetic acid in 0.2% trifluoroacetic acid (TFA)–methanol being the most efficient) for three different sediments (recovery: 33.1–44.9% of total MCs according to HPLC analyses). The recovery of MCs was also affected by the type of sediment but did not correlate with the content of organic carbon. These results suggest that the sorption of MCs onto inorganic materials such as clay minerals is quantitatively more important than interactions of the MCs with organic matter. The extraction efficiency was strongly dependent on the structure of the MCs and its hydrophobicity/hydrophility. Hydrophilic MC-RR gave much lower recoveries (20.0–38.8%) than MC-YR (44.1–59.5%) or MC-LR (55.3–77.8%) from three different types of spiked sediments. Recovery results analysed with HPLC–DAD correlated well with ELISA analyses. The most efficient extraction method was used for analyses of MCs in 34 natural sediment samples collected from Brno reservoir (Czech Republic) from April to October 2005. Concentrations of MCs in sediments ranged from 0.003 to 0.380 μg/g sediment DW (ELISA results) or 0.016–0.474 μg/g DW (HPLC results). Concentrations of microcystins in sediments showed remarkable temporal and spatial variability. The highest concentrations of microcystins were observed in the spring and the lowest concentrations in summer. These findings are in

accordance with those reported in the literature [135]. Our results demonstrate the suitability of the methods described here for studying the occurrence and fate of MCs in the aquatic environment (Babica et al. 2006, [158]).

Several studies reported microcystin accumulation in fish with possible food transfer to humans. But the detection of microcystins in tissues seems not to be completely resolved. There are number of analytical methods applicable for very complex samples but many of them lack sensitivity or selectivity. In chapter 4.3 we provide first comparison of liquid chromatography with single (LC-MS) and tandem mass spectrometric detection (LC-MS/MS) for analyses of microcystins in complex fish tissue samples [208]. Use of traditional single MS (i.e. monitoring of quasimolecular MC ions) was found to provide false positive responses overestimating thus concentrations of MCs in the tissue samples. This finding might have important consequences as some authors report high human health risks of MC accumulated in fish. On the other hand, MS/MS analyses resulted in much cleaner chromatograms with very low background noise, which also lowered actual LOD/LOQ in comparison with the single MS analyses. Concentrations of MCs detected by tandem mass spectrometry in fish from controlled exposure experiments were more than 50% lower in comparison with concentrations provided by single MS. Extensive analyses of edible fish parts - muscles (N=148 fish specimens of eight different species from five natural reservoirs with dense cyanobacterial water blooms) showed negligible MC concentrations (all analyses below limit of detection). Our findings have practical consequences for critical re-evaluation of health risks of microcystins accumulated in fish. It should be pointed out, that tandem MS/MS also has its limitations in analyses of extremely complex tissue extracts (e.g. modulations of ionisation efficiency by components of complex matrices; preferred but not often used quantification using isotopically-labeled standards, etc.), and these will require further research. Nevertheless, our findings show that selective methods such as tandem mass spectrometry should be used for analyses of cyanobacterial toxins in the tissue samples as they provide more reliable results than single MS methods.

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# Appendices

# A novel approach for monitoring of cyanobacterial toxins: development and evaluation of the passive sampler for microcystins

J. Kohoutek, P. Babica, L. Blaha and B. Marsalek

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ORIGINAL PAPER

# A novel approach for monitoring of cyanobacterial toxins: development and evaluation of the passive sampler for microcystins

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**Abstract** We have investigated the ability of an integrative sampler for polar organic chemicals to sequestrate a group of common and highly hazardous cyanobacterial toxins—microcystins. In a pilot experiment, commercially available passive samplers were shown to effectively accumulate microcystins after 7 days' exposure in the field. To find the most efficient configuration for sequestration of microcystins, four different porous membranes (polycarbonate, polyester, polyethersulfone and nylon) and two sorbents (Oasis HLB and Bondesil-LMS) were evaluated in the laboratory experiments, where samplers of different configuration were exposed to microcystins (microcystin-RR and microcystin-LR) for 14 days under steady conditions. We observed differences in sampling rates and amounts of accumulated microcystins depending on the sampler configurations. The samplers constructed with the polycarbonate membrane and Oasis HLB sorbent (2.75 mg/cm$^2$) provided the highest sampling rates (0.022 L/day for microcystin-RR and 0.017 L/day for microcystin-LR). To the best of our knowledge, the present study is the first reporting application of passive samplers for microcystins, and our results demonstrate the suitability of this tool for monitoring cyanotoxins in water.

**Keywords** Passive sampling · Cyanobacteria · Microcystin

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## Introduction

Massive water blooms of cyanobacteria occur in many freshwater lakes, slowly running rivers and shallow coastal water owing to anthropogenic eutrophication [1]. From the viewpoint of recreational and drinking water use, the massive cyanobacterial blooms are a highly undesirable phenomenon with various negative impacts on water quality. Besides the processes related to decay of cyanobacterial biomass (depletion of oxygen, etc.), production of toxic compounds (cyanotoxins) by cyanobacteria is a serious problem [2]. The occurrence of toxic cyanobacterial blooms seems to have an increasing tendency. For example, more than 80% of major reservoirs in the Czech Republic including drinking water supplies contained microcystins in considerable concentrations [3, 4].

The toxins of cyanobacteria are a structurally, functionally and phylogenetically diverse group of compounds with variable toxicological characteristics [5]. The most widely spread and the most often reported toxins are hepatotoxic cyclic oligopeptides—microcystins and nodularins. Microcystins were isolated from planktonic, benthic and soil cyanobacteria *Anabaena*, *Microcystis*, *Oscillatoria* (*Planktothrix*), *Nostoc*, *Anabaenopis*, *Hapalosiphon*, etc. [6, 7].

Microcystins may cause serious health problems as documented by cases of human and animal intoxications as well as by the results of laboratory studies [7, 8]. On the basis of the toxicity data, the World Health Organization suggested the tolerable daily intake value for microcystin-LR is 0.04 μg/kg body weight, and the corresponding safety guideline value 1.0 μg/L is recommended for drinking water [9]. Also the fact that microcystins may pass through drinking water treatment plants into tap water is of great concern [10–12], and monitoring of these toxic contaminants in the aquatic environment is essential.

Traditional monitoring programs of microcystins are based on the collection of individual samples at specific single time points. However, traditional sampling techniques have several drawbacks, such as the need for large volumes to recover sufficient mass of toxin or time- and labour-consuming cleanup prior to instrumental analyses. Further, microcystin concentrations may vary over the time, and episodic peaks of high concentrations may be missed in the traditional monitoring scheme. An increase of the sampling frequency or installing automatic sampling systems may provide a solution, but this can often be complicated, especially in remote areas. Therefore, it is often difficult to formulate the time-weighted average (TWA) concentrations of the contaminants that form a fundamental part of an ecological risk assessment process [13].

Passive samplers offer an attractive alternative to traditional sampling methods. They are promising tools for monitoring of a wide range of priority pollutants, and they help to avoid many of the problems outlined above [14–17]. Depending on the sampler design, the mass of pollutant accumulated by a sampler reflects either the equilibrium or the time-averaged concentration. The use of integrative passive samplers enables estimation of TWA concentrations, allows the sequestration of residues from episodic events (commonly not detected with grab sampling), is not limited to constant water conditions, and allows accumulation and concentration of trace and ultratrace contaminant levels over extended time periods [14, 18].

Several sampling approaches have been designed, and the polar organic compound integrative sampler (POCIS) has been suggested to estimate cumulative aqueous exposure to hydrophilic organic chemicals. This sampler was originally designed to mimic exposures of aquatic organisms to dissolved chemicals, eliminating the problems of metabolism, depuration of chemicals, avoidance of contaminated areas, and mortalities of organisms. The POCIS consists of a solid sequestration medium (sorbent) enclosed within a hydrophilic microporous membrane [18]. Both sorbent medium and membranes are versatile, and they can be changed to target specific chemicals or chemical classes.

So far, two general configurations of the POCIS have been typically used, and include a generic design (suitable for virtually all hydrophilic organic contaminants) and a specific design for sampling of pharmaceuticals. The generic configuration contains the triphasic sorbent admixture of Isolute ENV+ polystyrene divinylbenzene and Ambersorb1500 carbon dispersed on S-X3 BioBeads [18]. This mixture showed excellent retention and recovery of many pesticides, natural and synthetic hormones, and many other contaminants [14, 19, 20]. The pharmaceutical sampler is based on the Oasis HLB sorbent and it is suitable for many pharmaceuticals with multiple functional groups [18]. The microporous membrane contains water-filled pores to facilitate transport of the hydrophilic chemicals, and it may be semipermeable, allowing chemicals of interest to pass and accumulate in the sorbent, while excluding particulate matter, biological material and other interfering substances.

The aim of this study was to develop and evaluate the use of the passive sampler for microcystins, and to compare the ability of different configurations (membranes and sorbents materials) to effectively accumulate these hazardous cyanobacterial toxins.

## Materials and methods

### Chemicals and reagents

Oasis HLB was purchased from Waters (Milford, MA, USA). Bondesil-LMS was supplied by Varian (Lake Forest, CA, USA). Nylon, polyethersulfone, polyester and polycarbonate membranes (90-mm diameter, 0.45-μm pore size) were obtained from Sterlitech (Kent, WA, USA). Standards of microcystins (microcystin-LR, microcystin-RR, microcystin-YR) were obtained from Alexis Biochemicals (Läufelfingen, Switzerland). The solvents (high-performance liquid chromatography, HPLC, grade) were purchased from Sigma-Aldrich (Prague, Czech Republic). Deionized water was prepared with the Millipore Simplicity 185 system (Millipore, Bedford, MA, USA).

### Sampling devices

The POCIS samplers (pharmaceutical configuration) for the pilot experiment were obtained from Exposmeter AB (Tavelsjo, Sweden). The in-house-made passive samplers were later constructed using various membranes (polycarbonate, polyethersulfone, polyester and nylon) and sorbents (Oasis HLB or Bondesil-LMS). Physical compression was used to seal the membranes instead of other approaches using adhesives, heat, etc. The membranes were kept in the hollow polyethylene screw and nut (30.0-mm inner diameter, 40.4-mm outer diameter). This design ensured the ideal stretch of the membrane, uniform distribution of the sorbent material and provided a very good seal. The dimensions of the holder allowed applications of commercially available 47-or 90-mm membranes. The total exchanging surface area of the membrane (both sides counted) was 14.1 $cm^2$.

### Microcystin mixture

Microcystins for the experiments were isolated from the natural cyanobacterial water bloom (dominated by *Microcystis aeruginosa*, collected during summer 2003 at Nove

Mlyny reservoir, Czech Republic) by repeated extraction with 50% methanol and partial purification with solid-phase extraction using an ODS cartridge (Sep-Pak 35 $cm^3$ 10 g C-18 cartridge, Waters, Millford, MA, USA). The final extract (in 75% v/v methanol/water), which contained two dominant microcystin variants microcystin-LR and microcystin-RR (HPLC purity of total microcystins was approximately 75% at 238 nm), was aliquoted and stored at −18 °C.

### Experimental design

Pilot experiments in the field were performed with commercially available devices (constructed with a polyethersulfone membrane and Oasis HLB; the total exchanging surface area of the membrane was 47.5 $cm^2$, both sides counted). The samplers were exposed for 1-week in a natural reservoir (Velke Splavisko, Brno, Czech Republic) containing microcystins (about 0.1 μg/L of total microcystins as revealed by HPLC). After 7 days, the samplers were collected and analysed as described later. Further, laboratory experiments were constructed with the in-house-constructed sampling devices (using various combinations of membranes, sorbents and sorbent load) placed in glass beakers with 1 L of water containing a mixture of microcystins (5 μg/L of total microcystins). Exposures (media replaced two times a week) were carried out for 14 days under steady conditions at room temperature (22± 2 °C). The amount of microcystins accumulated in the samplers were determined on days 1, 7 and 14. To study the effect of the surface area to sorbent mass ratio on the accumulation of microcystins, samplers with the polycarbonate membrane were loaded with three different amounts of sorbent material (Oasis HLB; 2.75, 5.55 and 11.1 mg/$cm^2$ respectively).

### Sample preparation and HPLC analyses

Membranes containing enclosed sorbent were extracted two times with 20 mL of methanol and water (90:10 v/v acidified with 0.1% trifluoroacetic acid, TFA) for 15 min in an ultrasonic bath (Bandelin Electronics, Berlin, Germany). The supernatants were pooled, diluted tenfold with deionized water and the microcystins were concentrated by solid-phase extraction using an ODS cartridge (Supelclean LC-18, 3-mL tube, Supelco, Bellefonte, PA, USA). Microcystins were eluted from the cartridge with a methanol/water mixture (90:10, v/v) with 0.1% TFA, and the eluate was evaporated to dryness by rotary vacuum evaporation (45 °C) and then reconstituted with 50% v/v methanol. Extracts were analysed with an HPLC instrument (Agilent 1100 series, Agilent Technologies, Waldbronn, Germany) on a Supelcosil ABZ Plus, 150 mm×4.6 mm, 5-μm column (Supelco) at 30 °C. The binary gradient of the mobile phase consisted of $H_2O$ and 0.1% TFA (solvent A) and acetonitrile and 0.1% TFA (solvent B) (linear increase from 20% solvent B at 0 min to 59% solvent B at 30 min); the flow rate was 1 mL/min. Chromatograms at 238 nm were recorded with an Agilent 1100 series photodiode array detector (Agilent Technologies), and microcystins were identified by retention time and characteristic UV absorption spectra (200–300 nm). Quantification was based on external calibrations of microcystin-LR, microcystin-RR and microcystin-YR, respectively.

## Results and discussion

### Pilot experiment

In the pilot study, we evaluated the general capability of a commercial passive sampler to accumulate microcystins in a natural reservoir (Velke Splavisko). In spite of the relatively short sampling period (7 days) and the low concentrations of intracellular and extracellular microcystins in the reservoir (0.1 μg/L of total microcystins), the toxins were detected in all samples analysed. The average amount of total microcystins found in the samplers was 1.179 ng (standard deviation 0.123). This pilot experiment proved the general ability of the passive sampling technique to sequestrate microcystins. Our further experiments aimed at determining the most efficient configuration of the sorbent and the membrane for the long-term passive sampling of microcystins.

### Membrane evaluation

The first step in the sampler optimization was the evaluation of the diffusion velocity of microcystins through four different commercially available microporous membranes. For these experiments, we used the Oasis HLB sorbent. Differences in the uptake efficiency for total accumulated microcystins as well as differences between structural variants of microcystins were observed (Fig. 1); the best results were obtained with the polycarbonate membrane.

There are two possible pathways of migration of the chemicals into the sorbent layer through the membrane, i.e. the dissolution and migration through the polymer matrix of the membrane, and migration through the water-filled pores. These two modes control the intake of analytes into the passive sampler. For chemicals under the membrane control, the sampling rates should remain nearly constant regardless of the turbulence of the surrounding environment. On the other hand, the sampling rates controlled by the aqueous boundary layer are determined by the velocity of diffusion through the steady surface layer on the

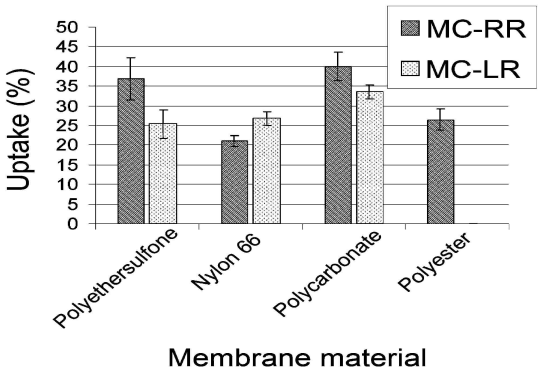


**Fig. 1** Comparison of microcystin (structural variants microcystin-RR and microcystin-LR) uptake (percentage of total water concentration) into the passive sampler constructed with various membranes following a 14-day sampling period. *MC-RR* microcystin-RR, *MC-LR* microcystin-LR

membrane and through the water-filled pores. Turbulence of the surrounding environment can thin the aqueous boundary layer and thereby increase the sampling rate [18]. Assuming the steady exposure conditions used in our experiments, the most important characteristics that affect accumulation are the effective membrane thickness and the open pore volume.

Of the membranes studied, the polycarbonate membrane showed the highest microcystin uptake velocity and also similar sampling rates for two different microcystin variants (Fig. 1). The polycarbonate membrane is a thin membrane (5–25 μm according to the manufacturer's data), ensuring fast diffusion and uptake of the analytes with an average flow rate (33 mL/min/cm$^2$ using prefiltered water at 10 psi, manufacturer's data). The membrane is also chemically and mechanically resistant, with good tensile strength that ensures stretching and uniform distribution of the sorbent material.

Another material studied, polyethersulfone, is also very durable and resistant, with high tensile strength and large open pore volume, allowing the highest flow rates for the membrane compared (60 mL/min/cm$^2$). It demonstrated good uptake with a slightly higher sampling rate for the more hydrophilic microcystin-RR (Fig. 1). However, the relatively high thickness of this microporous membrane (110–150 μm) may lead to a stagnant aqueous boundary layer that extends the diffusion pathway for the analytes. Less effective accumulation (higher for microcystin-LR) was determined for the hydrophilic nylon-66 membrane (Fig. 1), which is relatively thick (65–125 μm) with an average pore volume (flow rates of about 27 mL/min/cm$^2$). It has relatively high tensile strength and durability when dry but it may lose its strength/stretching after longer exposures in water [18]. The naturally hydrophilic and resistant polyester membrane (5–11-μm thick, smooth surface, high tensile strength, typical flow rate of 33 mL/min/cm$^2$) accumulated microcystin-RR only and no microcystin-LR was found (Fig. 1).

On the basis of our experiments, polycarbonate membranes were selected for further calibrations of the microcystin passive sampler.

## Sorbent mass to surface area ratios

We also examined the influence of the mass of sorbent per surface area on the uptake of microcystins. According to Alvarez et al. [18], the prototype passive sampler configuration consisted of 18 cm$^2$ of exposed membrane surface and 100 mg of sorbent (i.e. sorbent to surface area ratio of 5.55 mg/cm$^2$). We compared three ratios (2.75, 5.55 and 11.10 mg/cm$^2$) using the sampling device constructed with Oasis HLB sorbent and a polycarbonate membrane with an effective surface area of 14.1 cm$^2$. The results in Fig. 2 demonstrate that the uptake rate declined with the increasing mass of sorbent to surface area ratio. This may be related to the changes in the diffusion velocity [21]. A smaller effective thickness (at lower sorbent masses) resulted in a higher velocity of the sampled water through the sorbent layer, leading to more effective microcystin accumulation. The lowest amount of sorbent (2.75 mg/cm$^2$) was used for further calibration, it was also sufficient to completely cover the membrane area, and it has enough sorption capacity for long-term sampling of microcystins in water.

## Sorbent evaluation

Several sorbent materials have previously been used for extraction, concentration and separation of various cyano-

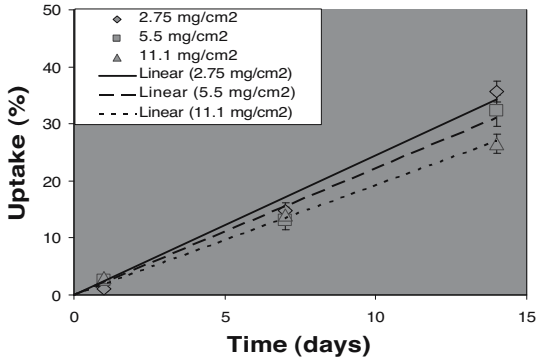


**Fig. 2** Comparison of microcystin (sum of structural variants MC-RR and MC-LR) uptake rate (percentage of total water concentration) into the passive sampler constructed with various amount of sorbent during 14 days' exposure

toxins, and C-18 based sorbents have become popular [22–24]. Most of these materials could also be employed in the passive samplers but some sorbents (such as C-18) may require activation usually by organic solvent treatment. This extra step may represent a serious complication in the technical construction of passive samplers. We studied two different divinylbenzene copolymer-based materials (Oasis HLB and Bondesil-LMS) that may be used directly without activation, and that have been previously used for microcystin extraction [22, 25]. In our experiments, Oasis HLB (without activation by solvents) showed excellent uptake rates for both microcystin variants (Fig. 3). Poor uptake with negligible amounts of microcystins in samplers was observed with nonactivated Bondesil-LMS. After the preactivation of the Bondesil-LMS with ethanol, the uptake efficiency improved, but it was still lower than that of Oasis HLB (Fig. 3). These data correspond to those of previous reports [22, 24] that showed good applications of Oasis HLB for concentration of microcystins using solid-phase extraction, and they confirm that this sorbent (which does not require activation) can be used with high efficiency for the construction of passive sampling devices.

An important parameter of the sampler is the sampling rate, expressed as the volume of water completely cleared of the analyte per day [26]. Our studies were performed with the optimized sampling devices (polycarbonate membrane and Oasis HLB, 2.75 mg sorbent/cm$^2$, total exchange surface of 14.1 cm$^2$) placed in tap water with microcystins under steady conditions at laboratory temperature (22±2 °C). We used 5 μg/L of microcystins, and this can be considered environmentally relevant as documented by recent monitoring of more than 90 localities in the Czech Republic [4]. The sampling rates determined by measuring the microcystin mass accumulated after 1, 7 and 14 days were 0.022 L/day for microcystin-RR and 0.017 L/day for microcystin-LR, respectively. These values are slightly higher than those previously reported for sequestration of pharmaceuticals by passive samplers [18].

## Conclusion

We have evaluated the suitability of passive sampling technique for accumulation of a group of cyanobacterial toxins—microcystins. A pilot experiment with the commercial sampler exposed to microcystins under natural conditions revealed the suitability of this technique for accumulation of microcystins. We compared four different membranes and two sorbents for their efficiency to sample microcystins. Laboratory studies revealed that the best results (highest sampling rates) were obtained with the sampler constructed with the polycarbonate membrane and Oasis HLB sorbent (2.75 mg sorbent/cm$^2$). The optimized sampler showed very good uptake of microcystins, with experimentally determined sampling rates of 0.022 and 0.017 L/day for microcystin-RR and microcystin-LR, respectively. The sampler was able to accumulate about 40% of the total microcystins in the water during the 14-day sampling period.

Current development and field evaluations studies show that passive samplers provide a viable tool for long-term monitoring of various contaminants in all matrices of the environment [17, 26–28]. Our study for the first time demonstrates that the passive sampling approach represents a promising technique also for monitoring of the cyanobacterial toxins microcystins. The use of passive samplers in monitoring programmes and surveillance studies offers a good capability to reflect seasonal, temporal and spatial variations in microcystin distribution without the necessity of expensive large-scale active samplings. Passive samplers could be also used for control of microcystin removal in drinking water treatment plants and quality of the drinking water produced. In contrast to conventional monitoring (grab sampling of high water volumes followed by laborious concentration using solid-phase extraction), passive sampling simulates handling of large volumes of water during a period of several days or weeks. Passive samplers thus provide good detection limits and allow estimation of TWA concentrations for risk assessment.

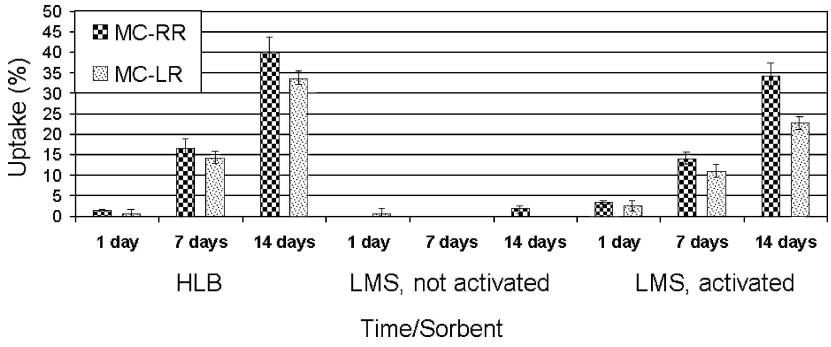


**Fig. 3** Comparison of microcystin (structural variants MC-RR and MC-LR) uptake rates (percentage of total water concentration) into the passive sampler (polycarbonate membrane, 2.75 mg sorbent/cm$^2$) constructed with Oasis HLB and Bondesil-LMS (not activated and activated variants) during 14 days' exposure

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# Evaluation of the novel passive sampler for cyanobacterial toxins microcystins under various conditions including field sampling

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ORIGINAL PAPER

# Evaluation of the novel passive sampler for cyanobacterial toxins microcystins under various conditions including field sampling

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**Abstract** In the present study, we have evaluated the effectiveness of a passive sampler for polar organic chemicals to accumulate a group of widespread and hazardous tumor-promoting toxins produced in cyanobacterial water blooms—microcystins (MC). The previously optimized configuration of the sampler based on polycarbonate membrane and Oasis HLB sorbent (2.75 mg/cm$^2$) was validated under various exposure scenarios in laboratory and field. Calibration of the passive sampler conducted under variable conditions and concentrations of MC revealed linearity of the sampling up to 4 weeks. The sampling rates of microcystins for two different exposure scenarios were derived (e.g., MC-LR: $R_s$=0.017 L/day under static and 0.087 L/d under turbulent conditions). $R_s$ values were further used for calculations of time-weighted average concentrations in natural water. Improved sensitivity and selectivity of the in-house-made sampler was observed in comparison with the commercially available Polar Organic Compound Integrative Sampler (POCIS). Comparisons of grab and passive sampling methods were performed during cyanobacterial water bloom season in the Brno reservoir, Czech Republic in 2008. Data obtained by passive sampling provided a more relevant picture of the situation and enabled better assessment of potential risks. The present study demonstrated that the modification of POCIS is suitable for monitoring of occurrence and retrospective estimations of microcystin water concentrations, especially with respect to the control of drinking water quality.

**Keywords** Passive sampling · POCIS · Microcystin · Cyanobacteria

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## Introduction

Massive water blooms of cyanobacteria occur in many freshwater lakes, slowly running rivers and shallow coastal waters as a result of anthropogenic eutrophication [1]. From the viewpoint of recreational and drinking water use, cyanobacteria are highly undesirable phenomenon with various negative impacts on water quality. Besides the processes related to decay of cyanobacterial biomass (depletion of oxygen, etc.), production of toxic compounds (cyanotoxins) may be a serious problem [2]. Occurrence of toxic cyanobacterial blooms seems to have increasing tendency. For example, more than 80% of major reservoirs in the Czech Republic including drinking water supplies contained microcystins (group of hepatotoxic cyclic heptapeptides) in considerable concentrations [3, 4].

Microcystins may cause serious health problems as documented by cases of human and animal intoxications as well as the results of laboratory studies [5]. Based on the toxicity data, the World Health Organization (WHO) suggested the tolerable daily intake (TDI) value for microcystin-LR being 0.04 μg/kg of body weight, and corresponding safety guideline value 1.0 μg/L is recommended for drinking waters [6]. Some studies demonstrated that microcystins may pass through the drinking water treatment plants into the tap waters [7], and monitoring of these toxic contaminants in the aquatic environment is essential.

Traditional monitoring programs of microcystins are based on collection of individual samples at specific single spot and time points. However, these traditional sampling techniques have several drawbacks such as need of larger volumes to recover sufficient mass of toxin or time- and labor- consuming clean up prior to instrumental analyses. Further, microcystin concentrations may vary over the time, and episodic events may be missed in the traditional monitoring scheme. Although increase of the sampling frequency or installing automatic sampling systems may provide a solution, it may be complicated, especially in remote areas. Therefore, it may be difficult to formulate the time-weighted average (TWA) concentration of the contaminant, which forms a fundamental part of an ecological risk assessment process [8].

Passive samplers offer an attractive alternative to traditional sampling methods. They are promising tools for monitoring of a wide range of priority pollutants, and they help to avoid many of the problems outlined above [9–11]. Depending on the sampler design, the mass of pollutant accumulated by a sampler reflects either the equilibrium or the time-averaged concentration. Thus, the use of integrative passive samplers enables direct estimation of TWA concentrations. This approach also allows the sequestration of residues from episodic events, which may be missed with grab sampling. Use of passive samplers is not limited to constant water conditions, and allows accumulation and concentration of trace and ultra-trace contaminant levels over extended time periods [9, 12].

Several sampling devices have been designed and used for monitoring of various environmental contaminants, and the polar organic compound integrative sampler (POCIS) has been suggested to estimate cumulative aqueous exposure to hydrophilic organic chemicals. The POCIS was originally designed to mimic exposures of aquatic organisms to dissolved chemicals eliminating the problems of metabolism, depuration of chemicals, avoidance of contaminated areas, and mortalities of organisms. POCIS consists of a solid sequestration medium (sorbent) enclosed within a hydrophilic microporous membrane [12]. Both sorbent medium and membranes of different materials can be used and changed to target specific chemicals or chemical classes.

So far, two general configurations of POCIS have been typically used, and include a generic design (suitable for virtually all hydrophilic organic contaminants), and the specific sampler design for sampling of pharmaceuticals. The generic configuration contains the triphasic sorbent admixture of Isolute ENV+polystyrene divinylbenzene and Ambersorb1500 carbon dispersed on S-X3 Biobeads [12]. This mixture showed excellent retention and recovery of many pesticides, natural and synthetic hormones, and many other contaminants [9, 13, 14]. The pharmaceutical sampler is based on the Oasis HLB sorbent and it is suitable for many pharmaceuticals and other compounds with multiple functional groups [12]. The microporous membrane contains water-filled pores to facilitate transport of the hydrophilic chemicals, and it may be semi permeable allowing chemicals of interest to pass and accumulate in the sorbent, while excluding particulate matter, biological material and other interfering substances. The most often used membrane is manufactured from polyethersulfone.

In our previous work, we demonstrated development of a passive sampler for cyanobacterial toxin microcystin [15], and the aim of the present study was to further calibrate and evaluate the sampler, and assess its capability to estimate the TWA concentration under natural conditions. Application of the passive sampler for routine monitoring of microcystins in various types of water is discussed.

## Material and methods

### Chemicals and reagents

Oasis HLB was purchased from Waters (Milford, MA, USA). Polycarbonate membranes (90 mm diameter, 0.45 μm pore size) were obtained from Sterlitech Co. (Kent, WA, USA). Polyethylene holders were bought from Atles Trade (Prague, Czech Republic). Standards of microcystins (MC-LR, -RR, -YR) were obtained from Alexis Biochemicals (Läufelfingen, Switzerland). The solvents (HPLC grade) and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Prague, Czech Republic). Deionized water was prepared with the Millipore Simplicity 185 system (Millipore, Bedford, MA, USA).

### Sampling devices

The commercial POCIS samplers (pharmaceutical configuration) and stainless steel holders for field experiment were obtained from Exposmeter AB (Tavelsjo, Sweden). The in-house-made samplers were constructed using polycarbonate membranes and sorbent Oasis HLB as described previously [15]. Method of the physical compression for sealing the membranes has been used instead of other approaches using adhesives, heat, etc. The membranes were kept in the polyethylene holder made of hollow screw and nut (30.0 mm i.d., 40.4 mm o.d.). This design ensured the ideal stretch of the membrane, uniform distribution of the sorbent material and provided a very good seal. The dimensions of the holder allowed applications of commercially available 47- or 90-mm membranes. Total exchanging surface area of the membrane (both sides counted) was 14.1 $cm^2$.

### Microcystin mixture

With regard to relatively large amounts of MCs needed for calibration experiments, and high costs of the toxin analytical standards, we have first isolated MCs from the natural cyanobacterial water bloom, and the isolated MC mixture was used for laboratory calibrations. MCs were isolated from the *Microcystis aeruginosa*-dominated water bloom (collected during summer 2003 at Nove Mlyny reservoir, Czech Republic) by repeated extraction with 50% methanol and partial purification with solid-phase extraction on an ODS cartridge (SepPak 35 cc 10 g C-18 cartridge, Waters, Millford, MA, USA). The final extract (prepared in 75% *v*/*v* methanol:water) contained two MC variants MC-LR and -RR, which are the most often studied and dominant variants worldwide. HPLC purity of the MCs mixture was ~75% at 238 nm. Extract was aliquoted and stored at −18 °C.

### Experimental design

The laboratory experiments were conducted with the in-house-made sampling devices (constructed from polycarbonate membranes and Oasis HLB sorbent, sorbent load 2.75 mg/cm$^2$ [15]) placed in the glass beakers that contained 1 L of water fortified with a mixture of microcystins (nominal concentrations ranged from 0.2 to 5 μg/L of total externally added MCs). Exposures were carried out under static and turbulent conditions. Fortified water was replaced regularly (two times a week during static exposures, and every second day during turbulent exposures) to assure constant concentration of the analytes. Turbulent flow is complex fluid regime and it was not possible to explicitly quantify water velocities. Experimental design used in the previous studies of Alvarez et al. was used [12]. Laboratory exposures as well as field studies were conducted at temperature 22±2 °C. This temperature corresponds well to the natural conditions under which MCs occur in waters. No detailed investigations on the influence of temperature were conducted, because temperature is known to have minor influence on the sampling rate in comparison with the flow [12, 16]. Experiments in the field were performed with both in-house-made samplers and commercially available POCIS devices (pharmaceutical configuration, constructed with polyethersulfone membrane and Oasis HLB, total exchanging surface area of the membrane 41 cm$^2$, both sides counted). The samplers placed in the stainless steel holders were submerged at a depth of approximately 30 cm. Devices were exposed from 1 to 3 weeks in the Brno reservoir (Brno, Czech Republic) during cyanobacterial water blooms, which produced microcystins. During the deployment of passive samplers in the field (1- to 3-week exposures), concentrations of dissolved microcystins were determined every 5 days according to the ISO 20179 method [17], volume of the grab samples was 1 L. For each exposure period (given time point during laboratory and field exposures), three passive samplers were collected and analyzed.

### Sample preparation and analyses

Exposed samplers were rapidly washed with water and the polyethylene holder was disassembled. Membranes with the enclosed sorbent were transferred into a 15-mL centrifuge tube and extracted two times with 5 mL of aqueous methanol (90% *v*/*v* acidified with 0.1% trifluoroacetic acid) for 15 min in the ultrasonic bath (Bandelin Electronics, Berlin, Germany). After centrifugation (10 min at 2880×*g*; Hettich Centrifuges, Beverly, MA, USA), supernatants were pooled, evaporated to dryness by rotary vacuum evaporation (45 °C, Heidolph Instruments, Schwabach, Germany) and reconstituted with 500 μL of aqueous methanol (50% *v*/*v*). Extracts were analyzed by HPLC (Agilent 1100 Series, Agilent Technologies, Waldbronn, Germany) on a Supelcosil ABZ Plus, 150× 4.6 mm, 5 μm column (Supelco) at 30 °C. The binary gradient of the mobile phase consisted of (A) $H_2O$+0.1% TFA and (B) acetonitrile +0.1% TFA (linear increase from 20% B at 0 min to 59% B at 30 min); the flow rate was 1 mL/min. Chromatograms at 238 nm were recorded with an Agilent 1100 Series PDA detector (Agilent Technologies), and MCs were identified by retention time and characteristic UV absorption spectra (200–300 nm). Quantification was based on external calibrations of MC-LR, -RR, and -YR, respectively. Method quantitation limit (MQL) was 0.09 μg/L (S.D.=±0.01) for both structural variants of microcystins, and procedural recoveries were 89% (S.D.=±5%) and 94% (S.D.=±3%) for MC-RR and MC-LR, respectively.

## Results and discussion

Most of the conventional techniques for screening of cyanobacterial toxins in water use grab sampling coupled with solid-phase extraction. But this technique has some limitations. Grab samples do not provide the complete picture of the pollutant concentration and their capacity is limited. Sample volume sizes are usually 1 or 2 L, thereby limiting the detection limit. On the other hand, passive sampling techniques have capacity to handle large volumes of water during a period of several days or weeks. This results in good detection limits and enables determination of TWA concentration of toxins in water. In our previous study [15], we have solved the problem of optimal configuration of POCIS for cyanobacterial toxins—microcystins. In this study, we present results of further evaluation of microcystin uptake kinetics and results of field deployment of optimized passive sampler.

Passive sampler calibration for different concentrations of microcystins under static and turbulent conditions

In the particular experiment, we have evaluated the dependence of microcystin sampling rates on the concentration of dissolved toxins (MC-LR and MC-RR at 0.2, 1, and 5 μg/L) during both turbulent and static conditions, which were simulated by stirred and nonstirred glass beakers, respectively. The selected microcystins concentrations are environmentally relevant and cover a range of concentrations found during cyanobacterial blooms [4].

Analyses after 2-week exposures (Fig. 1) showed different amounts of microcystins in the samplers deployed in water with variable toxin concentrations, and there were also differences between static and turbulent conditions. Data were used for calculations of the sampling rates ($R_s$). The $R_s$ values were independent from the microcystin concentrations but expected difference between stirred and nonstirred variant was observed (Fig. 1). The respective $R_s$ values were 0.022 L/day (S.D.=±0.007) for microcystin-RR and 0.017 L/d (S.D.=±0.005) for microcystin-LR under static conditions, and 0.090 L/day (S.D.=±0.019) for microcystin-RR and 0.087 L/day (S.D.=±0.019) for microcystin-LR under turbulent conditions. These results are in agreement with a previous study with number of pharmaceuticals and pesticides [12], which demonstrated influence of turbulencies on the $R_s$ values. The experiment demonstrated a general ability of the in-house-made passive samplers to sequestrate microcystins without dependency on microcystin water concentrations, and derived $R_s$ characteristics may be directly used for the interpretation of results and estimation of TWA concentration in water.

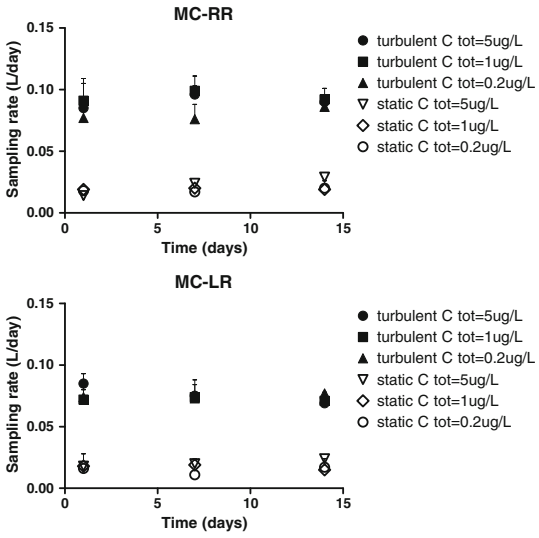


**Fig. 1** Experimentally determined sampling rates ($R_s$) for three different concentrations of microcystin-RR and -LR in the house-made sampler (14.1 $cm^2$ exposed surface area). Study was conducted under static conditions (nonstirred *empty symbols*) and turbulent conditions (stirred *filled symbols*). Values (expressed as liters of water cleared of analyte per day) represent mean ± average standard deviation of $n$=3 samplers

Long-term sampling and estimation of linear uptake period for microcystins

Time profile of the passive sampling devices operation is gradual in three regimes. The first is the kinetic regime, where the uptake of target compounds into the sampler is linear. Second is the intermediate phase, which is characterized by slowing down the sampling rate and curvilinear uptake kinetics, and the third regime is known as near equilibrium phase, where the sampler reached its capacity and the sampling rate is close to zero. A first-order one-compartment model is often used to fit experimental measurements, and the uptake process could be generalized and described by following equation:

$$C_{\text{sampler}}(t) = C_{\text{medium}} \times \frac{k1}{k2} \times \left(1 - e^{-k2 \times t}\right)$$

where $C_{\text{sampler}}(t)$ is the concentration of the compound of interest in the sampler as a function of time ($t$), $C_{\text{medium}}$ is the compound concentration in the environment, and $k1$ and $k2$ are the uptake and the elimination rate constants, respectively.

During the linear uptake regime (relatively short sampling periods), the $C_{\text{medium}}$ can be deduced from the measured amount of target compound in the passive sampler ($C_{\text{sampler}}(t)$) based on the sampling rate. The sampling rate is preferably expressed as a total volume of medium cleared of analyte per day, and this principle could be applied to the appropriately calibrated passive sampling techniques during the initial linear uptake.

To characterize the long-term kinetics of the microcystin uptake to passive samplers, a 6-week exposure experiment was designed under stationary/stable conditions (Fig. 2). As it is apparent, the linear uptake period for both studied microcystins lasted for approximately 28 days (see Fig. 2) with more rapid sampling of MC-RR. This period seems to be shorter than described by Alvarez et al., who studied passive sampling of polar pharmaceuticals and pesticides [12]. This could be explained by higher sampling rate of our device in comparison with commercial POCIS resulting from the smaller effective thickness of the sampler. This was achieved by use of a smaller load of the solid sorbent (i.e., lower sorbent mass per surface area ratio) which lead to higher diffusion velocity and faster response of the passive sampler to the contaminant concentration in medium [15]. Nevertheless, 4 weeks of linear uptake

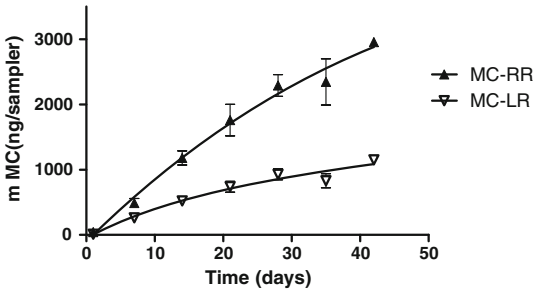


**Fig. 2** Dependency of the amount of microcystins (MC-RR and MC-LR) retained in passive sampling devices on the exposure time (days) during the long-term sampling experiment. Each value (ng/sampler) represent mean ± average standard deviation of $n$=3 samplers

regime are highly sufficient for passive sampling of cyanobacterial toxins with respect to high temporal and spatial variability of cyanobacterial water blooms [4, 18, 19]

## Field experiments

Applicability of the optimized passive sampler for microcystin monitoring under natural conditions was assessed in the Brno 1, Czech Republic. The experiments (1- to 3-week exposures) were carried out in September 2008 during period of extensive cyanobacterial blooms. TWA concentrations of microcystins in water were calculated using the calculated sampling rate ($R_s$) obtained under turbulent conditions with respect to its relevancy for the field conditions. Water grab samples were collected and analyzed in parallel with passive samplers.

Comparison of the sampling efficiency of commercially available POCIS and in-house-made devices is presented in Fig. 3. The estimated concentration of dissolved (extracellular) microcystins at the beginning of the sampling period was approximately 0.07 μg/L, and it remained almost unchanged up to the seventh day of exposure. During the entire 7-day exposure, the amount of retained microcystins was almost the same in both passive samplers, in spite of different surface areas of sampling devices (approximately 41 $cm^2$ for POCIS and 14.1 $cm^2$ for in-house-made sampler). This may be explained by almost three times higher $R_s$ for the in-house-made device, which is related to the smaller effective thickness and lower sorbent mass per surface area ratio as also described above [15].

Further, performance of passive and active sampling approaches was compared during 3-week experiments (Fig. 4). Analyses of the grab samples collected every 5 days showed microcystin concentrations ranging from 0.2 to 4 μg/L with a significant peak in concentration at day 16. Our relatively frequent grab sampling was able to detect this episodic increase but there was no further information about duration or importance of this event. Such episodes might often be missed during monitoring programs based on 1-month period samplings as traditionally used for example in the Czech Republic [4]. Therefore, it is rather difficult to make correct estimation of the time-weighted average concentration based on the grab samplings, and more sampling points would be needed (even in the frequent sampling design used in the present study).

In contrast, passive sampling provided slightly different image. Concentrations of both microcystins remained in the level of 0.3 μg/L (TWA concentrations ranged from 0.11 to 0.62 μg/L with coefficients of variance not exceeding 30% for MC-RR and 15% for MC-LR, respectively) during the entire 3-week sampling period with a slight increase observed around day 14. This shows that a slight episodic peak observed at grab samples was recorded also by the passive sampler but it seemed to be rather short with minor influence on the overall concentration profile. Because passive samplers operate in the integrative regime, they seem to provide a more relevant picture of the real

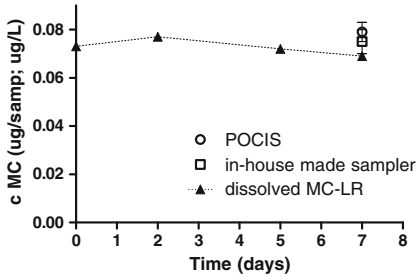


**Fig. 3** Changes in the extracellular microcystin concentration in Brno reservoir during a 7-day sampling period (grab samples—dissolved MC-LR, *triangle symbols*) and responses, i.e., time-weighted average concentrations (TWA), of two types of passive samplers (POCIS and in-house-made devices). Passive samplers were deployed in the water for entire studied period (7 days), TWA concentrations represent the average value (±standard deviation) of $n$=3 samplers

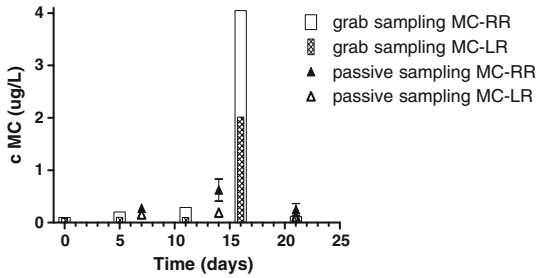


**Fig. 4** Comparison of water concentrations (microgram/L) of microcystin-RR and -LR in Brno reservoir as determined by active/grab sampling (*bars*) and passive samplers (time-weighted average concentrations; *triangle symbols*). Passive samplers were deployed in the water for 7, 14, and 21 days, and corresponding TWA values were calculated. Values represent the average (±standard deviation) of $n$=3 samples for each period

situation, which enables correct assessment of potential risks [8–11].

In summary, sampling rates of microcystins for two different exposure scenarios (stationary/turbulent) were derived in the present study, and these might be used for estimation of TWA concentrations. This study also shows that our modification of the polar organic compound integrative sampler is suitable for monitoring of microcystin occurrence and estimating water concentrations under conditions of natural water blooms. It is also a helpful tool for formulation of the time-weighted average (TWA) concentrations, which forms a fundamental part of an ecological risk assessment.

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# Evaluation of extraction approaches linked to ELISA and HPLC for analyses of microcystin-LR, -RR and -YR in freshwater sediments with different organic material contents

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ORIGINAL PAPER

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# Evaluation of extraction approaches linked to ELISA and HPLC for analyses of microcystin-LR, -RR and -YR in freshwater sediments with different organic material contents

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**Abstract** The efficiencies of conventional extraction techniques and analytical methods (HPLC–DAD and ELISA) were investigated for analyses of microcystins (MCs) in sediments. Our results showed several limitations. First, the extraction efficiency strongly depends on the extraction solvent, and extraction with 5% acetic acid in 0.2% trifluoroacetic acid (TFA)–methanol was confirmed as being the most appropriate for three different sediments (recovery: 33.1–44.9% of total MCs according to HPLC analyses). Second, the recovery of MCs was affected by the type of sediment but did not clearly correlate with the content of organic carbon. These results suggest that the sorption of MCs onto inorganic materials such as clay minerals is probably a more important process than interactions of the MCs with organic sediment matter. Third, the structure of the MCs is another crucial factor that affects the sorption of MCs and their recovery from sediments. Hydrophilic MC-RR gave much lower recoveries (20.0–38.8%) than MC-YR (44.1–59.5%) or MC-LR (55.3–77.8%) from three different types of spiked sediments. Recovery results analysed with HPLC–DAD correlated well with ELISA analyses. Further, extraction with 5% acetic acid in 0.2% TFA–methanol was used for analyses of MCs in 34 natural sediment samples collected from Brno reservoir (Czech Republic) from April to October 2005. Concentrations of MCs in sediments ranged from 0.003 to 0.380 μg/g sediment d.m. (ELISA results) or 0.016–0.474 μg/g d.m. (HPLC results). These values are equivalent to 0.63–96.47 μg/L of sediment (ELISA) or 4.67–108.68 μg/L (HPLC), respectively. Concentrations of sediment MCs showed both temporal and spatial variability, with the highest MC contents observed in the spring (April and May) and the lowest concentrations in July and August. Our results demonstrate the suitability of the methods described here for studying the occurrence, fate and ecological role of MCs in the aquatic environment.

**Keywords** Microcystin · Sediment · ELISA · HPLC–DAD

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## Introduction

Microcystins (MCs) and nodularins (NODs) belong to a diverse group of cyclic oligopeptides produced by cyanobacteria [1, 2]. Over the last few decades, MCs and NODs have become a serious ecological and health issue due to the massive cyanobacterial water blooms that have developed in eutrophied waters worldwide [1, 2]. The toxicity of and risks from some MC variants to vertebrates, including humans, have been studied in detail [3–5], and the World Health Organization recommends a provisional guideline of 1 μg/L for drinking waters for one of the MCs (MC-LR) [6]. On the other hand, the natural functions of these unique non-ribosomally synthesized peptides produced in high amounts in cyanobacterial biomass (up to 10 mg/g d.w.), as well as their fate in the aquatic environment, are poorly understood [7, 8].

MCs are biosynthesized and preferably found inside cyanobacterial cells [9, 10]. The majority of extracellular MCs are probably released after cell lysis and decay, but active transport from growing cyanobacterial cells has also been suggested recently [11].

MCs and NODs are highly stable under laboratory conditions, but they have been shown to be rapidly degraded in the presence of natural microorganisms [12–19] or by indirect photolysis [20, 21]; their fate depends on their structure and is variant-specific [13, 22–26]. However, there are also reports describing continuously elevated concentrations of MCs in natural reservoirs [27], including

overwintering in freshwater sediments (i.e. persistence for relatively long periods after the previous occurrence of cyanobacteria in the water) [28]. These observations, along with other findings, resulted in a hypothesis that MCs play a natural signaling role [29, 30], and may participate in the activation of cell growth in early spring and the further colonization of the water column by cyanobacteria. Detailed studies of the fate of MCs and NODs in sediments can thus provide valuable information on the natural functions of these unique peptides.

Several methods of analysing MCs or NODs in sediments have been proposed, including extraction with various solvents followed by immunochemical analysis [31, 32], or preconcentration by solid-phase extraction (SPE) with subsequent high-performance liquid chromatography (HPLC) analysis [28, 33].

Another method suitable for analysing MCs in sediments has been described by Tsuji et al. [33], and involves the total oxidation of the MCs followed by the extraction, derivatization and mass-spectrometric (MS) detection of the MC-specific fragment 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB).

Unfortunately, there is currently a lack of critical comparison between the efficiencies of various extraction and analytical methods, particularly for various aspects of sediment composition such as organic carbon content, which is one of the main factors that affects the sorption of compounds to natural sediments or soils [34].

This study evaluated different methods for extracting MCs from several sediments with different organic carbon contents. We also compared the results from the two most widely used MC analytical methods, i.e. HPLC coupled with UV-VIS diode array detection (DAD) and ELISA, and investigated differences between the extractions of the dominant MC variants (MC-LR, -RR, -YR). These experiments were also designed to reflect natural conditions by using relatively low MC concentrations similar to those previously reported in freshwater sediments [33]. Using the most efficient method, we then analyzed the seasonal MC variability in natural sediments collected at two localities of interest (six samplings during April to October 2005).

## Experimental

### Chemicals and reagents

Standards of MCs (-LR, -RR, -YR) and monoclonal IgG MC10E7 were obtained from Alexis Biochemicals (Läufelfingen, Switzerland). Anti-Fc-IgG antibody was purchased from ICN Pharmaceuticals (Aurora, OH, USA). Horseradish peroxidase (HRP) and all other chemicals were obtained from Sigma-Aldrich (Prague, Czech Republic). Deionized water was prepared with the Millipore Simplicity 185 system (Millipore, Bedford, MA, USA).

### Sediments

Natural sediments from specific localities (see Table 1 for detailed information) were collected by randomly sampling the surface 5 cm sediment layer; they were then mixed, lyophilized, sieved through the 2 mm opening size, and stored at 4 °C until further processing. Besides model sediments, we also analyzed MCs in sediment samples collected throughout the summer season of 2005 at two specific sites in the Brno reservoir, Czech Republic. The sampling site "Brno reservoir: centre" is located in the middle of the reservoir (~14 m depth), and the second locality "Rokle" is a shallow bay of depth 2–4 m. The distance between the two sites is about 2 km. Two or three random sediment samples were collected at each locality per day of sampling in order to investigate the sampling variability (distance between the collection spots: 10–50 m).

### Microcystin mixtures for extraction experiments

MCs for the experiments were isolated from the natural cyanobacterial water bloom (dominated by *Microcystis aeruginosa*, collected during summer 2003 at Nové Mlýny reservoir, Czech Republic) using repeated extraction with 50% methanol and partial purification with solid-phase extraction on an ODS cartridge (SepPak 35 cc 10 g C-18 cartridge, Waters, Millford, MA, USA). The final extract, which contained the three dominant MC variants MC-LR,

**Table 1** Basic characteristics of the sediments studied

| No. | Sediment | % of organic carbon | MC concentrations prior to external addition (μg/g d.m.) | |
|---|---|---|---|---|
| | | | ELISA | HPLC |
| I | Košetice stream | <0.5% | <LOD | <LOD |
| II | Dřevnice river: Zlín | 1.25% | <LOD | <LOD |
| III | Morava river: Kvasice | 5.35% | <LOD | <LOD |
| IV | Brno reservoir: Rokle 12-X-2005 | 13% | 0.010 | <LOD |
| V | Brno reservoir: centre 15-VI-2005 | 13% | 0.152 | 0.141 |

LOD: limit of detection (0.0003 μg/g d.m. for ELISA, 0.015 μg/g d.m. for HPLC); MC: microcystin. Since the MC recoveries from the various sediments differed significantly with respect to sediment composition, we only report measured concentrations here (recoveries were not taken into account)

-RR and -YR (HPLC purity of total MCs was ~75% at 238 nm) was aliquoted and stored at −18 °C.

### Experimental design

Sediment equivalents (2 g d.m.) were moisturized with 5 mL of water, and the mixture of MCs was added externally to them at final doses of 1.25 μg/g d.m. MC-LR, 3.15 μg/g d.m. MC-RR and 0.7 μg/g d.m. MC-YR (total MC dose: 5.1 μg/g d.m.). Each experimental variant was extracted in duplicate. Sediments with added MCs were kept on the shaker overnight (20 h) to achieve equilibrium sorption to sediment. Suspensions were then extracted twice with 20 mL of appropriate solvent: 5% v/v acetic acid in water [28], or 5% acetic acid in methanol containing 0.1% or 0.2% v/v trifluoroacetic acid (TFA) [33] using an ultrasonic homogenizer (Bandelin Sonopuls HD2070, Bandelin Electronics, Berlin, Germany) for 3 min at 90% of maximum power. Each extraction step was followed by centrifugation (2,800×*g*, 10 min). Pooled supernatants were diluted ten-fold with deionized water and MCs were concentrated with SPE on an ODS cartridge (Supelclean LC-18, 3 mL Tube, Supelco, Bellefonte, PA, USA). MCs were eluted from the cartridge with 0.1% TFA in methanol, and the eluate was evaporated to dryness by rotary vacuum evaporation (45 °C) and then reconstituted with deionized water (for ELISA) or 50% v/v MeOH (for HPLC analyses). The theoretical limit of detection was 0.0003 μg/g d.m. for ELISA and 0.015 μg/g d.m. for HPLC.

### ELISA determination of microcystins

Samples were analysed by direct competitive ELISA, modified from Zeck et al. [35]. Briefly, high protein binding 96-well microplates (Nunc, Wiesbaden, Germany) were pre-incubated overnight with 2,000-fold diluted anti-mouse anti-Fc-IgG. Free IgG was then removed by washing with phosphate buffer saline (PBS, pH 7.3), and the plates were coated for 1 h with 5,000-fold diluted monoclonal IgG MC10E7 developed against MC-LR. The plate was then washed five times with 0.05% (v/v) Tween-20 in PBS, and nonspecific interactions were blocked by adding 20 μL of the block solution to each well (1% v/v EDTA, 1% v/v bovine serum albumin in 1 M TRIS-HCl, pH 7.4). The filtered samples, standards and controls were immediately added to the wells (200 μL per well) and the plate was incubated for 40 min at room temperature. Finally, 50 μL of MC-LR conjugated with HRP prepared and purified according to Zeck et al. [36] was added to each well (300-fold dilution of the MC-LR-HRP conjugate was used based on our previously optimized method). The reaction was then incubated at room temperature for another 15 min, the plates were washed five times with 0.05% (v/v) Tween-20 in PBS, and 175 μL of the HRP substrate 3,3′,5,5′-tetramethylbenzidine was added. Development of the coloured product was stopped after 10 min by adding 50 μL of 5% (v/v) sulfuric acid. The absorbance (420 nm with reference 660 nm) was determined with a microplate reader (GENios Spectra Fluor Plus, Tecan Group, Männedorf, Switzerland). Each sample was analysed in three replicates and compared with the 0.125–2 μg/L calibration curve of MC-LR constructed for each individual plate.

### HPLC analyses

Extracts of sediments were analysed with an HPLC (Agilent 1100 Series, Agilent Technologies, Waldbronn, Germany) on a Supelcosil ABZ Plus, 150×4.6 mm, 5 μm column (Supelco) at a temperature of 30 °C. The binary gradient of the mobile phase consisted of (A) $H_2O$ + 0.1% TFA and (B) acetonitrile + 0.1% TFA (linear increase from 20% B at 0 min to 59% B at 30 min); the flow rate was 1 mL/min. Chromatograms at 238 nm were recorded with an Agilent 1100 Series PDA detector (Agilent Technologies), and MCs were identified by retention time and characteristic UV absorption spectra (200–300 nm). Quantification was based on external calibrations of MC-LR, -RR and -YR, respectively.

## Results and discussion

The total MC extraction efficiencies for various sediments analyzed with HPLC and ELISA are summarized in Fig. 1. We found no detectable MCs in any of the three experimental sediments prior to external MC addition (Table 1, sediment I–III). Extraction efficiencies for the procedural blanks (spiked variants without sediments) did not differ among the solvents used or among MC structural variants, and they were (mean±SD of total MCs) 82.8± 8.8% and 78.3±4.1% as analysed with ELISA and HPLC, respectively.

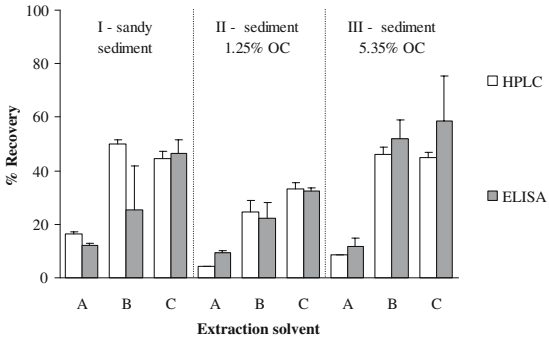


**Fig. 1** Recovery of total microcystins externally added into the three model sediments using different extraction solvents (*a*: 5% acetic acid, *b*: 5% acetic acid in 0.1% TFA–methanol, *c*: 5% acetic acid in 0.2% TFA–methanol). Two different analytical methods were used (ELISA and HPLC). *OC*, organic carbon. *Bars* represent means of duplicate experiments with standard deviations

In general, 5% acetic acid in water [28] appeared to be the least efficient extraction solvent regardless of the sediment used (average recovery 4.2–16.4% of total MCs, as analysed by HPLC). On the other hand, methanol with 5% acetic acid and 0.1–0.2% TFA was efficient at extracting about 24.8–50.1% (HPLC) of the externally added MCs from three compared sediments. This extraction method with acidified methanol was also successfully used by other authors, who preferred this procedure over 0.1% TFA in methanol [33]. The higher extraction efficiency with acidified methanol might be explained by the reduced ionization of the carboxylic acid groups in the MC molecules at low pH [37], and correspondingly, the tendency of the MCs to be extracted with less polar solvents such as methanol [38]. Another method for extracting NODs from sediments using methanol was also employed previously [31, 32], but there is only limited information on its efficiency.

Our comparison revealed a good correlation between the results from the two different analytical methods used (Fig. 1). The extraction efficiencies for spiked sediments extracted by 5% acetic acid in 0.2% TFA–methanol were 32.3–56.8% and 33.1–44.9% as determined with ELISA and HPLC, respectively. However, we observed significant variability in the ELISA results. The average coefficient of variance for repeated independent analyses of the same sample was 29%, in comparison with <5% obtained with HPLC. These observations reflect the basic nature of the ELISA assay, in which the results can easily be affected by sample composition and various experimental conditions [39]. According to these findings, both HPLC and ELISA can be recommended for analyses of total MCs in sediments. Other analytical methods have also been used for MCs analyses in sediments previously. Tsuji et al. [33] suggested the application of complete oxidation of the sediment matrix with ozone, which leads to the formation of MMPB in the presence of MCs. MMPB is a selective derivate of Adda, which is a unique D-amino acid present in the structures of MCs and NODs. MMPB was then extracted, esterified and detected using gas chromatography coupled with mass spectrometry (GC-MS). The authors observed recoveries as high as 90.5–91.7% for MC-LR, -RR or -YR [33]. Nevertheless, this method is not routinely available in many laboratories, in contrast to methods more commonly used for MC analysis, such as HPLC–DAD or ELISA.

We have also studied changes in MC extraction efficiency due to variations in sediment composition. We particularly focused on the total organic carbon content, as it is one of the main factors that affects the sorption of chemicals into sediments or soils [34]. However, we did not observe any clear (negative) correlation between the extraction efficiency and the amount of organic carbon, as expected. The MC extraction efficiency was significantly dependent on other sediment characteristics, and a detailed understanding of these factors will require further research. For example, the number of inorganic clay particles interacting with the ionized molecules could affect the sediment sorption of MCs and NODs [23, 40, 41]. The other factor

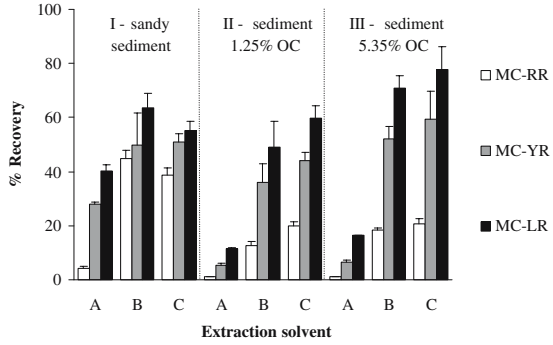


**Fig. 2** Recovery of different structural variants of microcystin (MC) externally added into three sediments and extracted with different solvents (*a*: 5% acetic acid, *b*: 5% acetic acid in 0.1% TFA-methanol, *c*: 5% acetic acid in 0.2% TFA-methanol). Results of HPLC analyses. *OC*, organic carbon. *Bars* represent means of duplicate experiments with standard deviations

that influences MC sorption could be the proportion of humic and fulvic acids in the total organic carbon content, and their character [42].

We have also investigated differences in the extractions of major MC variants: MC-LR, -RR and -YR (Fig. 2). Recoveries from spiked sediments extracted with 5% acetic acid in 0.2% TFA–methanol were 55.3–77.8% for MC-LR,

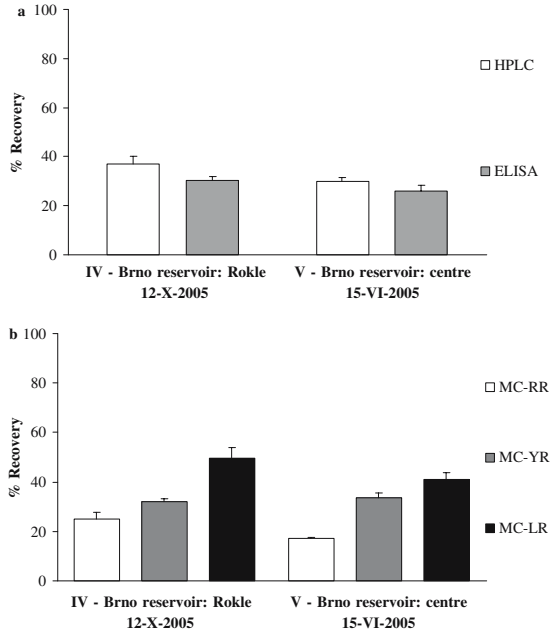


**Fig. 3a, b** Recovery of externally added microcystin (MC) from two Brno reservoir sediments (extracted with 5% acetic acid in 0.2% TFA–methanol): **a**) recovery of total MCs, analysed with ELISA and HPLC; **b**) recovery of different structural variants (analysed by HPLC). *Bars* represent means of duplicate experiments with standard deviations

20.0–38.8% for MC-RR and 44.1–59.5% MC-YR. Extraction efficiency was therefore strongly dependent on the physicochemical properties of MCs. Our analyses revealed a decrease in MC extraction efficiency with increasing hydrophobicity (MC-LR), while there was significant sorption of the more hydrophilic MC-RR onto all of the sediments investigated. These findings reflect the above-mentioned independence of total organic carbon content (Fig. 1), and suggest that hydrophilic interactions play a critical role in the sorption of MCs. Our results correspond well with those from the work of Tsuji et al. [33], which also showed lower recoveries for MC-RR than for MC-LR. It is also apparent that the very low recovery of one MC variant (MC-RR) dramatically reduced the total MC recovery rate (compare Figs. 1 and 2).

We then applied the most efficient extraction method (5% acetic acid in methanol with 0.2% TFA) to analyses of seasonal variability in the MC content of natural sediment collected at two selected sites: the Brno reservoir sites "Rokle" and "centre" (Table 1). First, we investigated the MC extraction efficiencies for these two particular sediments using the same design as for the three model

**Table 2** Concentrations of microcystins (MCs) in sediments (surface layer 0–5 cm) from the Brno reservoir during 2005 at two different sampling sites, as analysed by ELISA and HPLC

| Date | Locality | Sample no. | Microcystin concentrations in sediments | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | μg/g d.m. | | | | μg/L | | | |
| | | | ELISA | Mean (±SD) | HPLC | Mean (±SD) | ELISA | Mean (±SD) | HPLC | Mean (±SD) |
| 6-IV-2005 | Rokle | 1 | 0.056 | 0.047 (±0.009) | 0.041 | 0.049 (±0.021) | 18.93 | 17.58 (±0.97) | 13.74 | 17.88 (±5.63) |
| | | 2 | 0.035 | | 0.029 | | 17.11 | | 14.07 | |
| | | 3 | 0.050 | | 0.077 | | 16.70 | | 25.85 | |
| | Centre | 1 | 0.122 | 0.291 (±0.120) | 0.195 | 0.350 (±0.116) | 25.47 | 69.74 (±31.53) | 40.83 | 82.70 (±29.89) |
| | | 2 | 0.380 | | 0.474 | | 87.27 | | 108.68 | |
| | | 3 | 0.372 | | 0.381 | | 96.47 | | 98.60 | |
| 15-VI-2005 | Rokle | 1 | 0.014 | 0.018 (±0.007) | 0.075 | 0.047 (±0.024) | 4.39 | 5.97 (±2.79) | 23.96 | 15.53 (±8.06) |
| | | 2 | 0.012 | | 0.016 | | 3.64 | | 4.67 | |
| | | 3 | 0.028 | | 0.051 | | 9.89 | | 17.95 | |
| | Centre | 1 | 0.021 | 0.059 (±0.066) | 0.088 | 0.084 (±0.048) | 4.32 | 13.13 (±14.97) | 17.84 | 18.14 (±11.02) |
| | | 2 | 0.152 | | 0.141 | | 34.21 | | 31.79 | |
| | | 3 | 0.004 | | 0.023 | | 0.86 | | 4.80 | |
| 13-VII-2005 | Rokle | 1 | 0.019 | 0.020 (±0.005) | 0.033 | 0.017 (±0.013) | 7.28 | 6.27 (±1.91) | 12.51 | 6.03 (±5.12) |
| | | 2 | 0.026 | | 0.018 | | 7.93 | | 5.58 | |
| | | 3 | 0.015 | | <LOD | | 3.60 | | <LOD | |
| | Centre | 1 | 0.017 | 0.022 (±0.005) | 0.039 | 0.036 (±0.003) | 2.61 | 4.08 (±1.47) | 6.19 | 6.51 (±0.32) |
| | | 2 | 0.027 | | 0.033 | | 5.56 | | 6.83 | |
| 10-VIII-2005 | Rokle | 1 | 0.038 | 0.030 (±0.011) | 0.024 | 0.022 (±0.004) | 12.36 | 9.11 (±3.48) | 7.99 | 6.81 (±1.49) |
| | | 2 | 0.014 | | 0.026 | | 4.29 | | 7.73 | |
| | | 3 | 0.038 | | 0.017 | | 10.69 | | 4.70 | |
| | Centre | 1 | 0.012 | 0.012 (±0.002) | 0.046 | 0.049 (±0.003) | 2.46 | 2.61 (±0.58) | 9.42 | 10.09 (±0.49) |
| | | 2 | 0.015 | | 0.048 | | 3.39 | | 10.58 | |
| | | 3 | 0.010 | | 0.052 | | 1.99 | | 10.27 | |
| 7-IX-2005 | Rokle | 1 | 0.048 | 0.034 (±0.017) | 0.055 | 0.048 (±0.015) | 14.74 | 10.21 (±4.68) | 16.70 | 14.76 (±3.43) |
| | | 2 | 0.042 | | 0.062 | | 12.13 | | 17.63 | |
| | | 3 | 0.010 | | 0.026 | | 3.77 | | 9.94 | |
| | Centre | 1 | 0.021 | 0.042 (±0.035) | 0.114 | 0.094 (±0.028) | 3.05 | 6.63 (±5.61) | 16.53 | 14.56 (±3.73) |
| | | 2 | 0.092 | | 0.113 | | 14.55 | | 17.83 | |
| | | 3 | 0.013 | | 0.054 | | 2.30 | | 9.34 | |
| 12-X-2005 | Rokle | 1 | 0.010 | 0.010 (±0.000) | 0.043 | 0.021 (±0.021) | 3.54 | 3.75 (±0.20) | 15.61 | 7.80 (±7.80) |
| | | 2 | 0.010 | | <LOD | | 3.95 | | <LOD | |
| | Centre | 1 | 0.003 | 0.013 (±0.010) | 0.063 | 0.054 (±0.015) | 0.63 | 2.15 (±1.69) | 11.65 | 9.31 (±2.80) |
| | | 2 | 0.008 | | 0.032 | | 1.31 | | 5.37 | |
| | | 3 | 0.027 | | 0.066 | | 4.51 | | 10.92 | |

The results are expressed both as μg/g sediment d.m. and μg/L of sediment volume to allow comparisons with literature data. Two or three random independent sediment samples were collected and analysed for each locality and sampling date (No. 1, 2, 3 within each sampling date). LOD: limit of detection (0.0003 μg/g d.m. for ELISA, 0.015 μg/g d.m. for HPLC). Since the recoveries of MCs from various sediments differed significantly with respect to the sediment composition, we only report measured concentrations here (recoveries were not taken into account)

sediments (i.e. using external addition of MCs; final dose 5.1 μg/g d.m. of total MCs). Certain concentrations of natural MCs were present in these two sediments prior to external MC dosing (see Table 1). However, they formed only a minor fraction of the externally added MCs, and the starting concentrations were subtracted from the results (Fig. 3). As is clear from Fig. 3a, the extraction efficiencies for both sediments were within a similar range (~30% total MCs), and the recoveries were comparable to those obtained from the Dřevnice river (sediment II, 1.25% of organic carbon), although the organic carbon contents in both sediments differed significantly (Table 1). This observation further suggests the importance of inorganic material in the sorption of MCs onto sediments. Extractions of different MC variants from Brno reservoir sediments (Fig. 3b) also showed a similar trend to that seen for the three model sediments, with the highest efficiencies observed for MC-LR.

The variability of MCs concentrations in the Brno reservoir sediments during the summer season 2005 is summarized in Table 2. Concentrations of MCs ranged 0.003–0.380 μg/g d.m. (ELISA) or 0.016–0.474 μg/g d.m. (HPLC), respectively. Concentrations found in Brno reservoir are similar to those from several Japanese lakes, where MCs were detected at concentrations 0.08–2.33 μg/g sediment d.m. by MMPB method [33]. Another study found much lower concentrations of MCs and / or NODs (0.0005–0.0041 μg/g d.m., determined by ELISA) in sediments from prawn farm, New South Wales, Australia [32]. Concentrations of this study expressed as MCs content per volume of fresh sediment (μg/L) were 0.63–96.47 μg/L (ELISA) or 4.67–108.68 μg/L (HPLC), respectively. These values are well comparable with study of Ihle et al. [28] reporting concentrations of MCs from several μg/L up to 300 μg/L (HPLC) in sediments from Quitzdorf reservoir, Germany. Similarly, about 90 μg/L of NOD (ELISA/HPLC) was found in sediment from western Gulf of Finland, Baltic Sea [31].

In our study, sediment MC concentrations clearly showed spatial variability as well as temporal changes. Concentrations at the "centre" site were generally higher than those at the "Rokle" site. This difference could possibly be attributed to better conditions for overwintering of cyanobacterial cells and slower decay of biomass in deeper sediments (i.e. "centre") [43]. However, substantial variability in MC concentrations was also found among independent samplings performed at the same date and at the same site. For example, a coefficient of variance of up to 114% (μg/L, ELISA results) was observed for repeated samplings at the "centre" site on 15-VI-2005 (Table 2). In general, concentrations of MCs in sediments were higher in spring than in summer and there were slight increases in MC concentrations in September. This seasonal trend was observed at both localities studied, but it was more pronounced at the "centre" site. As Ihle et al. [28] reported, concentrations of MCs in sediments increased in autumn, when the cyanobacterial bloom settles down. The concentrations continued to decrease until the next spring, when cyanobacteria reinvaded the water column or decayed, and the lowest concentrations were observed in summer. The high concentrations of MCs observed in our study during the spring thus seem to correspond to the end of the overwintering phase, followed by a decrease in MC concentration until the summer months (July and August).

The correlation between the ELISA and HPLC analyses of the MCs in the sediment samples from Brno reservoir is shown in Fig. 4. Although the results from both methods were in agreement for higher MC concentrations, only weak correlation was observed below 0.1 μg/g d.m., which may reflect the generally higher variability in the ELISA results. However, it must also be emphasized here that although the HPLC retention times of the MC peaks corresponded to those of the external MC standards, their UV spectra often did not fit with the characteristics of MCs (particularly for smaller peaks equivalent to concentrations below 0.1 μg/g d.m.). This effect could be caused by coeluting impurities that may affect typical MC UV spectra and complicate quantification. It is important to take this into account as the concentrations of MCs occurring in natural sediments were usually close to the detection limit of our HPLC method. In this case, HPLC analysis may become less selective and ELISA seems to be more reliable for total MC content.

## Conclusion

Our results revealed several factors that affect MC analyses in sediments. Extraction efficiency depends strongly on the solvent, and the use of 5% acetic acid in 0.1–0.2% TFA–methanol provided the best results. Extraction efficiency was also affected by the type and composition of the sediment, but did not clearly correlate with the content of total organic carbon. Rather, other characteristics, such as interactions with inorganic (clay) particles seem to substantially affect the sorption of MCs onto sediments. MC structure is another important factor that influences sorption onto sediments, with lower recoveries generally obtained for more hydrophilic variants, such as MC-RR. In

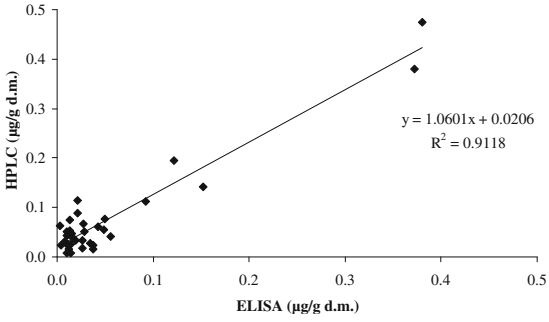


**Fig. 4** Correlation between the results of ELISA and HPLC analyses of total microcystin content in sediments from Brno reservoir ($n$=34, two values that were <LOD of HPLC were replaced with 1/2 of LOD). The robustness of the correlation was checked by recalculation for the subset of samples with concentrations <0.2 μg/g d.m., with the following results: $y$=0.8992$x$+0.0252, $R^2$=0.543

spite of the limitations discussed, the methods employed here (especially ELISA for detection at low MC concentrations) can be successfully used to investigate the occurrence, fate and natural ecological role of MCs.

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# LC-MS analyses of microcystins in fish tissues overestimate toxin levels – critical comparison with LC-MS/MS

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ORIGINAL PAPER

# LC-MS analyses of microcystins in fish tissues overestimate toxin levels—critical comparison with LC-MS/MS

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**Abstract** Microcystins are cyclic peptide toxins with hepatotoxic and tumour-promoting properties which are produced in high quantities in freshwater cyanobacterial water blooms, and several studies have reported microcystin accumulation in fish with possible food transfer to humans. In this study, we provide the first comparison of liquid chromatography with single mass-spectrometric and with tandem mass-spectrometric detection for analyses of microcystins in complex fish tissue samples. Use of traditional single mass spectrometry (i.e. monitoring of ions with *m*/*z* 519.5 for microcystin-RR and *m*/*z* 995.5 for microcystin-LR) was found to provide false-positive responses, thus overestimating the concentrations of microcystins in the tissue samples. More selective tandem mass spectrometry seems to provide more reliable results. The concentrations of microcystins detected by tandem mass spectrometry in fish from controlled-exposure experiments were more than 50% lower in comparison with concentrations obtained by single mass spectrometry. Extensive analyses of edible fish parts—muscles (148 fish specimens from eight different species from five natural reservoirs with dense cyanobacterial water blooms)—showed negligible microcystin concentrations (all analyses below the limit of detection; limit of detection of 1.2–5.4 ng/g fresh weight for microcystin-RR, microcystin-YR and microcystin-LR in multiple reaction monitoring mode). Our findings have practical consequences for critical re-evaluation of the health risks of microcystins accumulated in fish.

**Keywords** Microcystin · Fish tissue · Liquid chromatography–tandem mass spectrometry

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## Introduction

Microcystins are a group of peptide toxins produced by various genera of freshwater cyanobacteria, such as *Anabaena*, *Microcystis* and *Oscillatoria* (*Planktothrix*) [1]. These potent oligopeptides act mainly as inhibitors of serine/threonine protein phosphatases PP1 and 2A, leading to hepatotoxicity and liver tumour promotion. In organisms, microcystins are detoxified mainly by conjugation with cysteine (Cys) and glutathione (GSH) [2]. Microcystins may cause serious health problems, as documented by cases of human and animal intoxications as well as by the results of laboratory studies [3] based on toxicity data. The World Health Organization suggested a tolerable daily intake value (0.04 μg/kg body weight) for one of the microcystin variants microcystin-LR (MC-LR), and the corresponding guideline value of 1.0 μg/L is recommended for drinking waters [4].

Microcystins are produced during massive water blooms of cyanobacteria, which result from worldwide anthropogenic eutrophication of waters [5]. For example, more than

80% of major reservoirs in the Czech Republic (including drinking water supplies and aquaculture ponds) contained microcystins in considerable concentrations [6, 7].

More structural variants of microcystins can be present in one sample and they may represent as much as 1% of the dry cyanobacterial biomass. During massive water blooms, aquatic organisms can be affected by toxins present in water, and microcystin accumulation in biota and its transfer to the food webs—including biomagnification—were previously discussed [8–10]. Some authors reported very high concentrations of microcystins accumulated in fish tissues [10, 11], which might be of toxicological concern for humans. Analyses of microcystins in food and food supplements are thus necessary for critical health risk assessment.

A number of analytical approaches for cyanobacterial toxins (cyanotoxins) in biotic matrices have been described in the literature, including protein phosphatase inhibition assay and immunochemical enzyme-linked immunosorbent assay (ELISA) methods [12, 13]. Further, instrumental analytical methods, namely liquid chromatography (LC), are the most widely used. Microcystins have a characteristic absorption spectrum with a peak at 238 nm, and a number of methods for optical detection in the UV range have been developed. However, biological and traditional instrumental methods may suffer from interferences in the complex matrices, and their application for microcystin analyses in biota is limited. More recent approaches based on LC with mass-spectrometric detection have a number of advantages, including high sensitivity and robustness, which minimizes the above-mentioned complications [14–16].

Although a number of studies used mass-spectrometric detection for analyses of cyanotoxins, no critical comparison of single mass spectrometry (MS) and tandem MS (MS/MS) methods for microcystin analyses in the tissues of biota is available. A number of publications, which relied on a single mass spectrometer for detection and quantification of microcystins, did not consider several drawbacks, such as insufficient selectivity, which might result in overestimated or false-positive results. In this paper, we compare the performance of LC-MS and LC-MS/MS techniques for a series of tissue samples from fish exposed to cyanobacterial microcystins both experimentally (in controlled laboratory conditions) and collected in the field from fish ponds with natural cyanobacterial blooms.

## Material and methods

### Chemicals and reagents

Standards of microcystins (MC-LR, MC-RR, MC-YR) were obtained from Alexis Biochemicals (Läufelfingen, Switzerland). The solvents (LC-MS grade) were purchased from Sigma-Aldrich (Prague, Czech Republic). Deionized water was prepared with a Simplicity 185 system (Millipore, Bedford, MA, USA).

### Experimental design

Fish tissues used for the comparative studies of MS and MS/MS method performance were collected from both laboratory experiments and field samplings.

During the short-term laboratory experiments, fish were injected with high concentrations of microcystins, and the presence of toxin in hepatopancreas and its metabolization were evaluated. For these experiments, common carp (*Cyprinus carpio*; 500±15 g males) were obtained from commercial aquaculture (Pohorelice fishery, Czech Republic). Experimental fish ($N$=3) were intraperitoneally injected with the mixture of major microcystin variants at a total microcystin dose of 248 μg/kg body weight (injection volume 500 μL). The mixture of microcystins was prepared in phosphate-buffered saline, pH 7.3, and contained 56% MC-RR, 38% MC-LR and 6% MC-YR. Control fish ($N$=3) received 500 μL of phosphate-buffered saline. After 3 h, the fish were dissected and samples of hepatopancreas and muscle tissues were collected and stored at −80°C until they were analysed.

The second series of experiments studied accumulation of microcystins in fish under natural conditions. Fish specimens of common carp (body weight 32±7 g, $n$=10) were kept in two experimental ponds for 9 weeks. Pond A contained a massive and dense water bloom dominated by *Microcystis aeruginosa* with microcystin concentrations ranging from 10.1 to 15.4 μg/L during the whole exposure period. Pond B also contained some cyanobacteria but they were present in negligible density and the maximum concentration of microcystins in water reached 2 μg/L. During the experiment, the fish were not externally fed, and no deaths were observed. At the end of the experiment, the fish were dissected and tissue samples were collected for analysis as described above. The concentrations of dissolved microcystins in the ponds were determined by the high performance liquid chromatography (HPLC)–diode-array detector method according to ISO 20179 [17]; the volume of the grab samples was 1 L. Besides this controlled study, fish of different species randomly caught in various aquaculture ponds in the Czech Republic were also analysed for the content of microcystins; details on the environmental conditions and microcystin concentrations are provided in "Results and discussion."

### Tissue extractions

Tissue extractions were performed by methods described previously [18]. Briefly, a frozen sample (0.5 g fresh

weight) was homogenized four times with methanol (3 mL), sonicated in an ultrasonic bath for 30 min and centrifuged at 4,000*g* for 10 min. Supernatants were pooled and repeatedly extracted (three times) with 1 mL of hexane to remove lipids (hexane layers were discarded). The methanol extract was evaporated at 50°C, and the residue was dissolved in 300 μL of 50% aqueous methanol (v/v) and analysed. The recovery of the extraction method (approximately 25%; data not shown) was not considered during the calculations to remain consistent with the methods and values previously reported in the literature [8, 10, 11, 18, 19].

### Liquid chromatography electrospray ionization mass spectrometry analyses

The present study compared the performance of single MS microcystin analyses, which used the selected ion monitoring (SIM) mode, with the MS/MS approach using the multiple reaction monitoring (MRM) mode.

Analyses were performed with an Agilent Technologies (Waldbronn, Germany) 1200 series HPLC apparatus consisting of a vacuum degasser, a binary pump, an autosampler and a thermostatted column compartment kept at 30°C. The column was a Supelcosil ABZ + Plus RP-18 end-capped (5 μm), 150 mm×4.6-mm inner diameter (Supelco). A SecureGuard $C_{18}$ (Phenomenex, Torrance, CA, USA) guard column was used. The mobile phase consisted of 5 mM ammonium acetate in water, pH4 (solvent A), and acetonitrile (solvent B). The binary pump gradient was as follows: 0–12.00 min, 32–40% solvent B, linear increase; 12.01–20.00 min, 40–42% solvent B, linear increase; 20.01–30.00 min, 90% solvent B. The flow rate was 0.4 mL/min. Twenty microlitres of the individual sample was injected for the analysis.

The mass spectrometer was an Agilent Technologies (Waldbronn, Germany) 6410 triple-quadrupole mass spectrometer with electrospray ionization. Ions were detected in the positive mode. The ionization parameters were as follows: capillary voltage, 5.5 kV; desolvation temperature, 350°C; desolvation gas flow, 11 L/min. In SIM mode the following *m*/*z* were monitored: MC-RR $[M+2H]^{2+}$ 519.8, MC-YR $[M + H]^{+}$ 1,045.5, MC-LR $[M + H]^{+}$ 995.5, MC-RR–GSH $[M+2H]^{2+}$ 673.8, MC-LR–GSH $[M + H]^{+}$ 1,302.5, MC-RR–Cys $[M+2H]^{2+}$ 580.8, MC-LR–Cys $[M + H]^{+}$ 1,116.5. The transitions from the protonated molecular ion to a fragment of the amino acid (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (an unusual amino acid present only in microcystins and related nodularins) at *m*/*z* 135.2 and a fragment at *m*/*z* 127.1 were monitored in MRM mode. The collision energies used for fragmentation were as follows: 50 V for MC-RR and respective conjugates; 40 V for MC-YR and MC-LR and respective conjugates). Quantification of analytes was based on external standards of MC-RR, MC-YR and MC-LR in a matrix (final extract of microcystin-free fish tissue). The mixture of microcystins was added to the extract prior to analysis. The method detection limit (MDL; per gram of tissue, fresh weight) was 1.2 ng/g [standard deviation (SD) of 5%] for MC-RR and 5.4 ng/g (SD=10%) for MC-YR and MC-LR in MRM mode. In SIM mode, the MDL was 3.0 ng/g (SD=5%) for MC-RR and 27.0 ng/g (SD=7%) for MC-YR and MC-LR, respectively. Although other approaches such as the standard addition method could be used, we relied on external calibration as it is considered to have higher precision [20–22].

## Results and discussion

A number of field studies which used ELISA and LC-MS for analyses of microcystins in fish tissues reported high

**Table 1** Concentrations of microcystins in biological samples reported in the literature with indication of the respective analytical technique

| Fish species | Tissue sample | Concentration (MC-LR/g tissue) | Analytical technique | Source |
|---|---|---|---|---|
| *Tilapia* sp. | Muscle | 3–337 ng/g FW | ELISA | Magalhaes et al. [11] |
| *Tilapia* sp. | Muscle | 100 ng/g FW | ELISA | Mohamed et al. [9] |
| *Tilapia* sp. | Muscle | 100 ng/g DW | ELISA | Soares et al. [25] |
| *Hypophtalmichtys* sp. | Hepatopancreas | 7,000–17,800 ng/g DW | LC/MS | Xie et al. [19] |
| *Hypophtalmichtys* sp. | Muscle | 500–1,700 ng/g DW | LC/MS | |
| *Hypophtalmichtys* sp., *Cyprinus* sp. and others | Muscle, hepatopancreas | 1,800–7,700 ng/g DW | LC/MS | Xie et al. [10] |
| *Hypophtalmichtys* sp. | Muscle | 4.4–29 ng/g FW | ELISA | Adamovsky et al. [18] |
| *Cyprinus* sp. | Muscle | 5.8–19 ng/g FW | ELISA | |
| Water chestnut (*Trapa natans*) | – | 2–5 ng/g FW | LC/MS | Xiao et al. [26] |

*MC-LR* microcystin-LR, *FW* fresh weight, *DW* dry weight, *ELISA* enzyme-linked immunosorbent assay, *LC* liquid chromatography, *MS* mass spectrometry

concentrations ranging from 5 to 18,000 ng/g (Table 1). However, our previous investigations based on controlled exposures [18] as well as field studies [23, 24] resulted in systematically lower microcystin concentrations in fish tissue. These findings were surprising because the environmental situations and the concentrations of microcystins in the water were similar to those reported in the literature. These differences motivated the present study, which aimed to compare the LC-MS method (commonly reported in the literature) with the more robust and selective LC-MS/MS method.

In the first experiment, we investigated the general ability of the MS and MS/MS approaches to analyse microcystins and their metabolites accumulated in fish after intraperitoneal injection. In Fig. 1, the chromatograms of a tissue extract containing MC-RR obtained in SIM and MRM modes are presented (for MRM with two characteristic transitions; 519.5→135.2, 519.5→127.1). The SIM chromatogram has very high background noise,thus limiting the limit of detection (LOD). On the other hand, the MRM chromatograms seem to be more usable, especially the transition 519.5→135.2.

For correct analysis, also the qualifier ion (transition 519.5→127.1) should be monitored. However, it is present in very low concentrations when working with natural extracts (i.e. at low environmentally relevant levels). Consequently, this fragment may be of limited use because of the higher background noise. MC-YR and MC-LR show very similar behaviour. Table 2 shows the results obtained (MRM mode) for all experimental variants (with/without external standards added before the tissue extraction). The results show the good performance of the MS/MS method to detect microcystins in complex samples with sufficient selectivity and sensitivity. Although no clean-up procedure was applied on the raw extracts, the chromatograms had relatively low background and allowed the detection and quantitation of microcystins in sufficiently low concentrations (the MDL was 1.2 ng/g for MC-RR and 5.4 ng/g for MC-YR and MC-LR). Figure 2 shows the MS/MS chromatogram of a hepatopancreas extract with two peaks of MC-RR and MC-LR, and respective conjugates with GSH. In this experiment, no microcystin–Cys conjugates were detected. As is apparent, the extraction efficiency (variants with external additions of microcystins prior to extraction) was around 25% for total microcystin, which corresponds the finding of to our previous detailed study [18]. The ratio of microcystin variants detected in the hepatopancreas differed from that detected in the original exposure mixtures; no MC-YR was found. This might be affected by a number of factors, including variable uptake/metabolism of microcystin variants as well as differences in MS sensitivity, but detailed investigation is beyond the scope of the present work.

In the follow-up studies, we compared single MS and MS/MS methods by analysing fish caught in natural reservoirs with cyanobacterial blooms. Figure 3 shows the concentrations of microcystins in common carp exposed under controlled conditions for 9 weeks in two ponds with different cyanobacterial water blooms and different microcystin content. In single MS (SIM mode), peaks with retention times of microcystin standards were found in

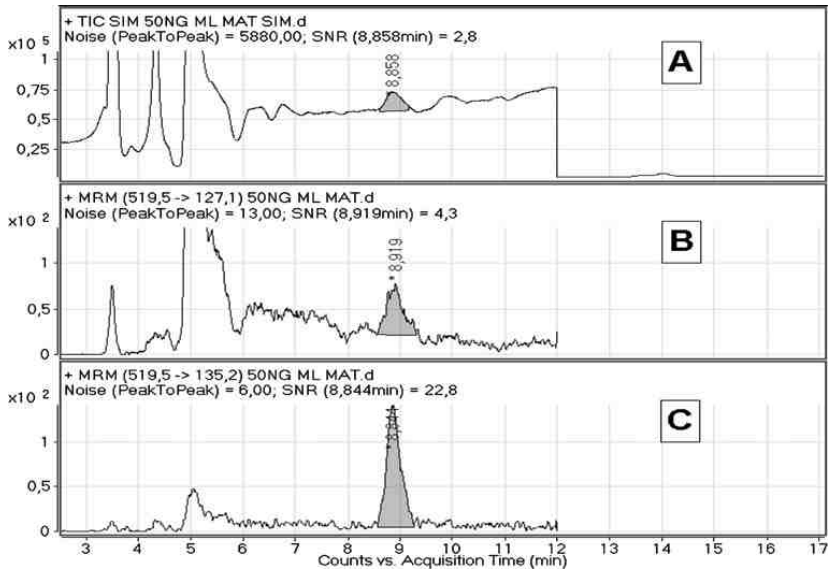


**Fig. 1** Chromatograms of microcystin-RR (MC-RR) obtained in selected ion monitoring (SIM; *A*) and multiple reaction monitoring (MRM) (two characteristic transitions; *B* 519.5→127.1, *C* 519.5→135.2) modes. Note the high noise in the SIM chromatogram indicated by the low signal-to-noise ratio (*SNR*)

**Table 2** Microcystins determined by tandem MS (MS/MS) in common carp (*Cyprinus carpio*; 500±15 g) hepatopancreas 3 h after intraperitoneal injection of a microcystin mixture into fish (exposed vs. non-exposed). Standards of microcystins were further added to the samples prior to extraction at the doses indicated ("spike" variants)

| Experimental variant | Intraperitoneal injection[a] | Pre-extraction addition[b] | Microcystins detected in hepatopancreas | | |
|---|---|---|---|---|---|
| | Microcystin (μg/fish) | Microcystin (μg/g FW) | Concentration (total microcystins; μg/g FW) (±SD) | Microcystin congener ratio (MC-RR, MC-YR, MC-LR; %) | Microcystin–GSH conjugates (μg/g FW) |
| Exposed | 124 | – | 0.909 (±0.375) | 5,–, 95 | ~0.1[c] |
| Exposed + spike | 124 | 0.424 | 1.062 (±0.341) | 8,–, 92 | ~0.1[c] |
| Non-exposed | – | – | 0.000 (±0.000) | – | – |
| Non-exposed + spike | – | 0.424 | 0.124 (±0.019) | 32,–, 68 | – |

*SD* standard deviation, *GSH* glutathione, *MC-RR* microcystin-RR, *MC-YR* microcystin-YR

[a] Intraperitoneal injection mixture contained 56% MC-RR, 6% MC-YR and 38% MC-LR

[b] Microcystins externally added to the sample before the extraction (mixture with 27% MC-RR, 29% MC-YR and 44% MC-LR)

[c] Low concentration, close to the limit of quantitation (approximate, based on the microcystin standard calibration)

almost all samples, and *m*/*z* values previously reported in the literature were observed (519.5 for MC-RR and 995.5 for MC-LR). Hepatopancreas concentrations based on the SIM mode ranged from 6.0 to 129.0 ng/g tissue fresh weight. In the muscle samples (i.e. the edible part of fish), microcystin concentrations were low, with maxima around 8.0 ng/g fresh weight. However, SIM mode often determined similar "microcystin" peaks also in liver and muscle of control non-exposed fish, and these false positives appeared in all experimental variants. More detailed analyses by MS/MS did not confirm microcystins in all samples. No microcystins were detected in the muscle samples, and the presence of microcystin peaks was confirmed only in hepatopancreas of fish from the pond with high toxin levels. Hepatopancreas concentrations determined by MS/MS ranged from 14.0 to 123.0 ng/g fresh weight, which are more than 50% lower than those calculated from single MS in SIM mode. Fish exposed in the field contained only microcystin–Cys metabolites and in very low concentrations, which were close to the limit of quantification (LOQ; results not shown).

As a final part of the study, muscle samples (edible parts) of eight different fish species collected from five reservoirs during 2008 were analysed by LC-MS/MS. Water blooms of toxic cyanobacteria occurred in all reservoirs during the season and the characteristics of the water blooms and the

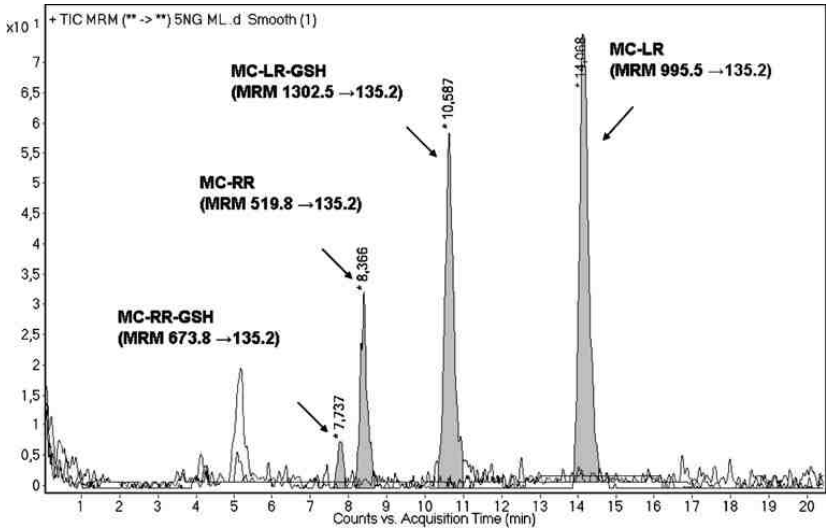


**Fig. 2** Chromatogram of hepatopancreas extract from the tandem mass spectrometry (MS/MS) analysis of microcystins (common carp tissues collected 3 h after intraperitoneal injection of microcystins dosed at 248 μg/kg body weight). Peaks of intact molecules as well as their conjugates with glutathione (*GSH*) were detected; retention times and characteristic MRM transitions are shown. *MC-LR* microcystin-LR, *MC-YR* microcystin-YR

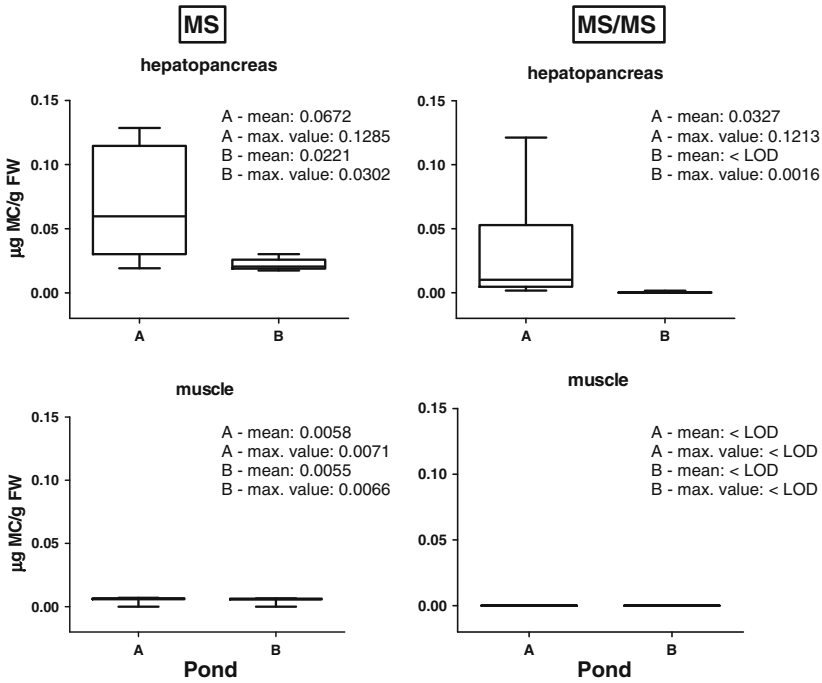


**Fig. 3** Concentrations of microcystins (sum of MC-RR and MC-LR) detected by single mass spectrometry (*MS*) and MS/MS (*left graphs* and *right graphs*, respectively) in fish hepatopancreas (*upper graphs*) and muscles (*bottom graphs*). Common carp tissues were analysed after 9-week accumulation under natural conditions in two fish ponds. Pond A contained a massive water bloom during the whole 9-week experiment (dominated by *Microcystis aeruginosa*; concentrations of dissolved microcystins 10–15 µg/L); pond B contained cyanobacteria in low density with maximum microcystin concentration of 2.2 µg/L. Numerical values on each graph show the mean and the maximum for each variant. Limit of detection (*LOD*) of 1.2 ng/g for MC-RR and 5.4 ng/g for MC-YR and MC-LR

microcystin concentrations are given in Table 3. The study covered representatives of all feeding types of fish, including herbivorous (grass carp), omnivorous (bream and common carp), zooplanktivorous (whitefish) and carnivorous (catfish, eel, perch and zander). Of the total 148 fish investigated, none of them were positive for MC-RR, MC-YR or MC-LR. Our results based on tandem mass-spectrometric detection thus indicate lower risk of fish contamination by microcystins, and also lower risks of toxin transfer to humans.

**Table 3** Concentrations of microcystins in fish muscles determined by MS/MS in eight fish species collected from five different reservoirs (localities in the Czech Republic) where dense cyanobacterial blooms occurred during the 2008 season. The limit of detection (*LOD*) was 1.2 ng/g for MC-RR and 5.4 ng/g for MC-YR and MC-LR

| Locality | Duration of water bloom | Dissolved microcystins in water (µg/L) | Fish species | Number of fish analysed | Microcystins in fish muscle (µg/g FW) (±SD) |
|---|---|---|---|---|---|
| Novoveský | May–July | 0.638–4.485 | Asp | 10 | <LOD |
| | | | Catfish | 10 | <LOD |
| | | | Common carp | 10 | <LOD |
| | | | Eel | 1 | <LOD |
| | | | Grass carp | 10 | <LOD |
| | | | Zander | 10 | <LOD |
| Sykovec | August–September | 0.125–1.358 | Common carp | 10 | <LOD |
| | | | Perch | 10 | <LOD |
| | | | Whitefish | 10 | <LOD |
| Medlov | July–September | 0.200–0.741 | Common carp | 10 | <LOD |
| | | | Perch | 10 | <LOD |
| | | | Whitefish | 10 | <LOD |
| Plumlov | June–August | 0.212–0.505 | Bream | 18 | <LOD |
| | | | Perch | 6 | <LOD |
| Vír | June–August | 0.000–1.201 | Bream | 13 | <LOD |

In summary, our results demonstrate that commonly used methods based on single MS (i.e. monitoring of ions with *m*/*z* 519.5 for MC-RR and *m*/*z* 995.5 for MC-LR) may provide false-positive responses, thus overestimating the concentrations of microcystins in the tissue samples. This might have important consequences as some authors have concluded that microcystins accumulated in fish have high human health risks [10, 19]. On the other hand, MS/MS analyses resulted in much cleaner chromatograms with very low background noise, which also lowered the actual LOD/LOQ in comparison with the SIM analyses. It should be pointed out that MS/MS also has its limitations in analyses of extremely complex tissue extracts (e.g. modulations of the MS signal by components of complex matrices, and preferred but not often used quantification using isotopically labelled standards), and these will require further research. Nevertheless, our findings show that selective methods such as MS/MS should be used for analyses of cyanobacterial toxins in tissue samples as they provide more reliable results than single MS methods.

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