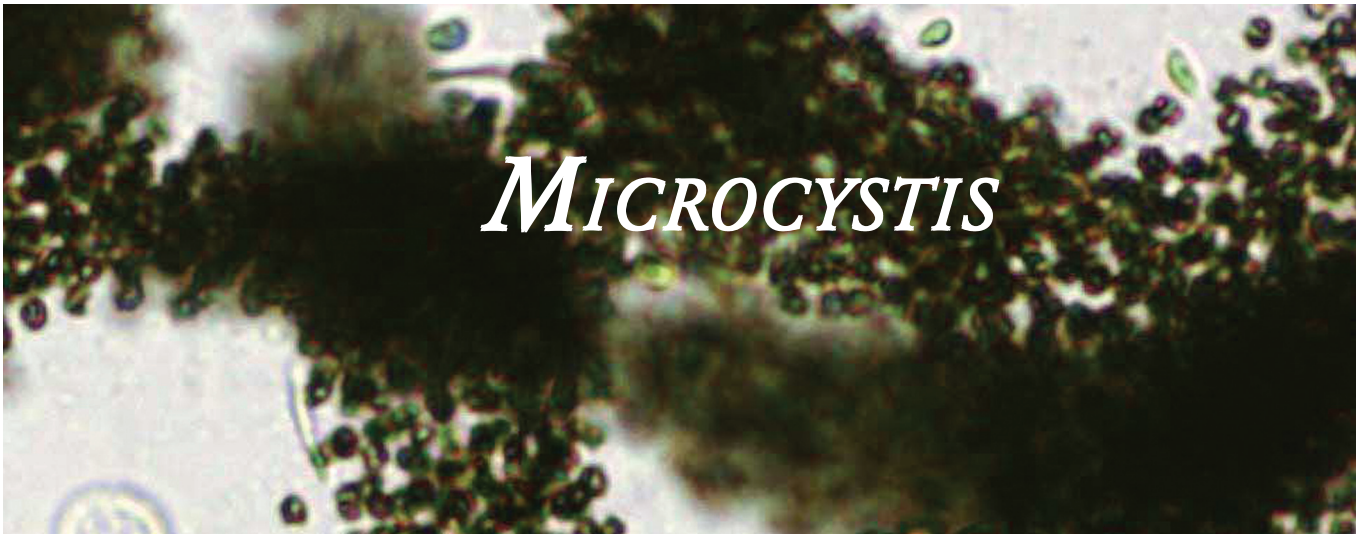
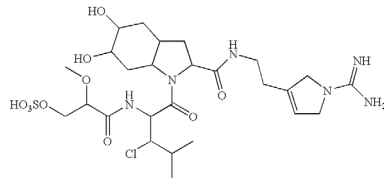


NEW FINDINGS IN PEPTIDE PRODUCTION, TAXONOMY AND AUTECOLOGY



LENKA ŠEJNOHOVÁ

I wish to dedicate this Ph. D. Thesis to all

Who learn me about the science

Oldřich Benada
Michal Bílý
Jana & Luděk Dobroruka
Markéta & Tomáš Hajnyš
František Hindák
Eva Jedličková
Tomáš Kalina
Jaroslava & Jiří Komárek
Jaromír Lukavský
Eliška & Blahoslav Maršálek
Petr Marvan
Magdalena Meszárosová
Yvonne Němcová
Jiří Neustupa
Eva Nováková
Sylvie Nováková
Karel Prášil
Petr Pumann
Petr Šípek
Magda & Pavel Škaloud
Jarda Škopek
Marie & Jiří Váňa
Martin Welker
Eliška Zapomělová

And many other colleguaes

... And especially to all Who show me whats about the life

My family from Ústí nad Orlicí
My grandparents from Řetová and Řetůvka
Šípek family from Dvůr Králové
Brigitte and Gerhard Weidner from Berlin
Lucko family from Eichhorst
Rajavat family from Golakabas in India
Adhikary family from Utkal in India
and
Petr Rímský

ISBN:	978-80-86188-27-0
Title:	*Microcystis*. New findings in peptide production, taxonomy and autecology by cyanobacterium *Microcystis*.
Title in Czech:	*Microcystis*. Nové poznatky v produkci peptidů, taxonomii a autekologii sinice *Microcystis*.
Author:	Lenka Šejnohová
Layout and design:	Petr Rímský
Cover pictures:	Microscopic image of *Microcystis aeruginosa* photographed by L.Šejnohová
Publisher:	Institute of Botany, Czech Academy of Sciences (www.ibot.cas.cz)
Published in:	Průhonice, Czech Republic
Published on:	June 2008
Number of printed copies:	50
PDF source:	www.sejnohova.cz

The studies described in this thesis were performed at the Department of Experimental Phycology and Ecotoxicology, Institute of Botany, Czech Academy of Sciences, Květná 8, 603 00 Brno, Czech Republic (www.cyanobacteria.net, www.sinice.cz).

The investigations reported in this thesis were supported by:

- Centre for Bioindication and Revitalization, Czech Academy of Sciencies (www.ibot.cas.cz/cbr)
- The project PEPCY, funded by the European Commission (www.pepcy.de)
- The Ministry of Education and Youth of the Czech Republic the grant no. 0021622412
- The project no. AV0Z60050516 of the Academy of Sciences of the Czech Republic
- Association Flos Aquae, Kunešova 6, Brno-Chrlice, Czech Republic (CZ)
- Department of Botany and Zoology, Faculty of Science, Masaryk University in Brno, CZ

Masaryk University in Czech Republic, Faculty of Science
Department of Botany and Zoology
&
Institute of Botany
Czech Academy of Sciences

Microcystis

New findings in peptide production, taxonomy
and autecology by cyanobacterium *Microcystis*

Nové poznatky v produkci peptidů, taxonomii
a autekologii sinice *Microcystis*

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Ph. D. Thesis

Lenka Šejnohová
Brno 2008

Supervisor:
Assoc. Prof. Blahoslav Maršálek, Ph. D.

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Structure and purpose of this thesis

The present study is being submitted as Ph.D. thesis at Department of Botany and Zoology, Faculty of Science, Masaryk University in Brno, Czech Republic. According to the valid "Educational and examinatorial rules" Faculty of Science, the thesis is composed from an introduction and a set of published scientific papers and manuscripts of scientific papers.

In Introduction, Chapter 1, I shortly summarize the current knowledge about the cyanobacterial taxonomy, toxicology and autecology. The largest part of this Chapter is concern on *Microcystis* genus.

Chapter 2 (*Šejnohová, L.; Neméthová, D., Welker, M., Maršálek, B. (submitted): Cell diameter by the bloom-forming cyanobacterium Microcystis correlatates with the occurence of specific toxic oligopeptides within the season. - Journal of Phycology*) analyzed the relationship between morphology and oligopeptides occurrence by *Microcystis* genus. This study represents the first work tested direct the relationship between individual morphological features of colony and individual peptides by using multivariate analysis. The novel method, mass spectrometry (MALDI-TOF MS), analyzed single colonies were used. We hypothesis that occurrence of peptides in *Microcystis* colony could be potentially used in polyphasic taxonomy on subgeneric level.

The idea that *Microcystis* reinvasion should be result of endogenous timing is studied in Chapter 3 (*Šejnohová, L.; Maršálek, B., Drábková, M. (submitted): Reinvasion of Microcystis colonies (Cyanobacteria) - changes in cell numbers, form of colony and ultrastructure in different conditions. - Hydrobiologia*). We compared the changes of *Microcystis* cell numbers, size of colony and ultrastructural changes in sediment in reservoir and stable condition of refrigerator.

In Chapter 4 (*Welker, M.; Šejnohová, L.; Neméthová, D.; van Dohren, H., Jarkosvký, J., Maršálek, B.: Seasonal shifts in chemotype composition of Microcystis sp. communities in the pelagial and the sediment of a shallow reservoir. - Limnology and Oceanography 52(2007): 609-619*), we investigate dynamics, diversity and co-occurence of oligopeptides by *Microcystis* community within the season. The term chemotype applied in this study refers thus to colonies having the same peptide pattern in mass spectra. The main aim of this paper was to determined the seasonal changes of chemotypes in the pelagial and sediment with a view to the reinvasion and sedimentation.

In Chapter 5 (*Welker, M.; Maršálek, B.; Šejnohová, L.; van Dohren, H.: Detection and identification of oligopeptides in Microcystis (cyanobacteria) colonies: Toward an understanding of metabolic diversity. - Peptides 27 (2006): 2090-2103*), we report on the mass spectral analysis of cyanobacterial peptides in individual colonies of *Microcystis* genus. The purpose of this paper was to describe a high diversity of potentially bioactive compounds by *Microcystis* genus. We alert that beside the

microcystins also aeruginosins, anabaenopeptins, microginins and microviridins could be more frequent cyanotoxins in *Microcystis* community.

In general discussion, in Chapter 6, the main results of the topics investigated in this thesis are placed in perspective with current knowledge and where need, further discussed. Additionally, the questions arising from this work and the taxonomical implications of my findings are presented.

Aims of the present study

The central aim of this dissertation was to contribute to ongoing research on the bloom forming cyanobacteria with a view to the taxonomy and autecology of the most frequent genus *Microcystis,* namely to:

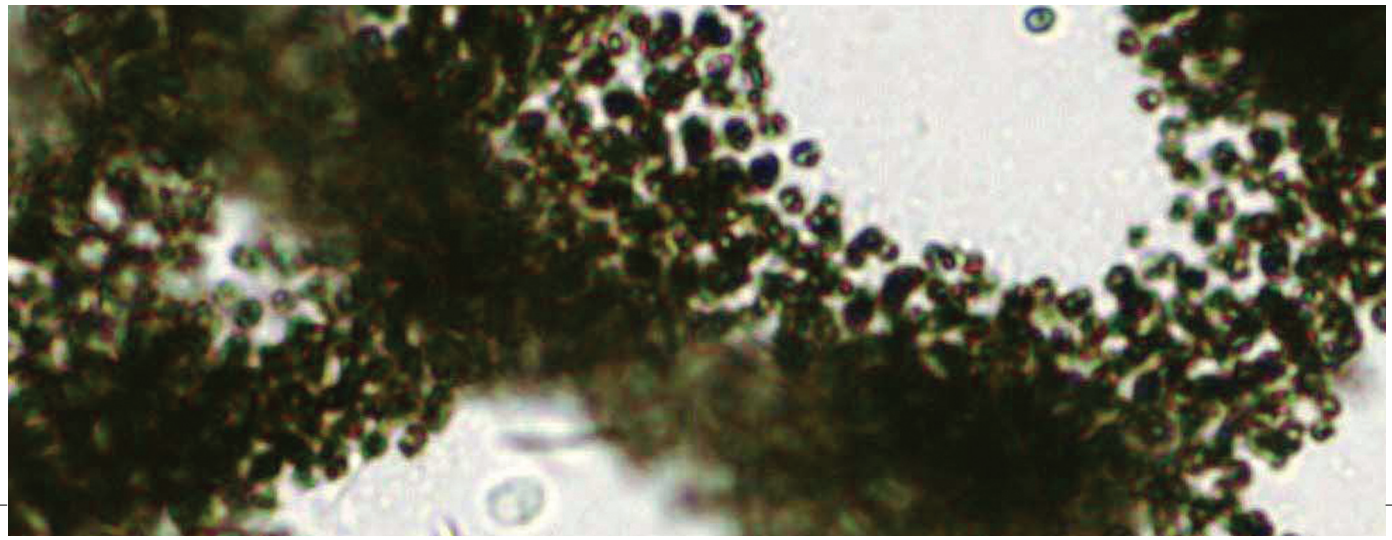
1) Cyanobacterial taxonomy

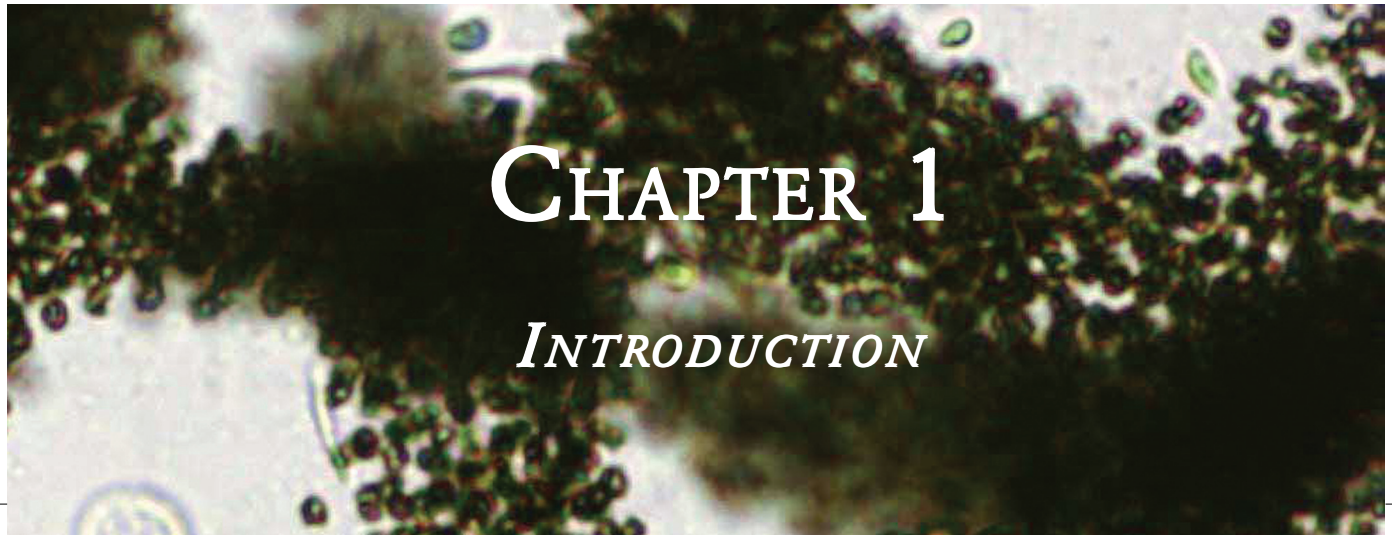
a) To test the relationship between oligopeptides occurence and individual morphological characters within *Microcystis* genus on single-colonies by mass spectrometry (MALDI-TOF MS) and multivariate analysis. We hypothesis that occurrence of peptides in *Microcystis* colony could be potentially used in polyphasic taxonomy on subgeneric level (Chapter 2).

b) Desription of the whole spectrum of oligopeptides by *Microcystis* and investigation of their seasonal shift (Chapter 4, Chapter 5)

2) Autecology

Investigation of *Microcystis* ultrastructure and number of cells in sediment during the reinvasion in the different parts of reservoir and compare it with the simultaneous observation in the in stable condition of refrigerator. There is a question about the exo- and endogenous timing during the *Microcystis* reinvasion (Chapter 3).





Chapter 1

Introduction

1.1. Cyanobacteria

1.1.1. The origin and importance of cyanobacteria

The cyanobacteria are the most primitiv photosynthetic organisms, with the long evolutionary history extend at least 3,5 bilion years back in time. The illustrations of the cyanobacteria ancient origin are musroom rocks called stromatolites, which were most abundat during the „Age of Cyanobacteria", Proterozoic Era, 2500-570 milion years ago. These rocks are characterized by the specific structure: the layers of filamentous or spherical cyanobacterial cells alternate with calcareous deposition or trapped sand/silt. Stromatolites have been described in geological strata that date from earliest history into the recent age. One of the most well-known living stromatolites can be seen in the Shark Bay, Western Australia (Cronberg and Annadotter, 2006). The recent use of the genetic analysis on DNA from the present-day stromatolites showed only a single cyanobacterial strain in each sample, and successfully examined internal core samples at least 10 years old (Neilan and Burns, 2002).

Because of the cyanobacteria ability to perform oxygenic photosynthesis (plant-like) is thought to have converted the early reducing atmosphere in an oxidizing one, which dramatically changed the life forms on Earth and provoked an explosion of biodiversity. The other grate contribution of the cyanobacteria is the origin of algae and higher plants chloroplasts through endosymbiosis. (http://en.wikipedia.org). Nowadays, cyanobacteria have a crucial role as a significant component of the nitrogen cycle and the primary producer in many areas of the ocean. In rice field, species capable to fixing nitrogen gas are used during the cultivation as nitrogen fertilizer (van den Hoek et al., 1995). Cyanobacteria have also the important place in biotechnology, where the filaments of *Arthrospira (Spirulina) maxima* are cultivated and used in the health food due to their richness in proteins, essential fatty acids (linoleic and gamma-linoleic acid), vitamins (provitamin A, vitamin B12, ...) and minerals (iron source) (Anagnostidis and Komárek, 1999).

1.1.2. Ecology

Cyanobacteria are present in almost every conceivable habitat, from oceans to fresh water to bare rock to soil, where they play an important role as primary producents. Due to their ability to fix nitrogen in aerobic conditions they are often found also as endosymbionts in lichens, plants, various protists, or sponges and provide energy for the host. It has been suggested that high pH is the most important environmental factor for their occurrence even the reason remains still unexplained (Komárek, 2003). Cyanobacteria are conspicuous in many extreme environments such as hot springs, soda lakes and biotopes exposed to high solar irradiance. Several cyanobacterial strains have a high tolerance to ultraviolet-B and -C radiation, an ability to probably evolved in the early

Precambrian when the levels of ultraviolet radiation were high (Cronberg and Annadotter, 2006). In phytoplankton communities, cyanobacteria form an important part due to their ecophysiological properties (Chapter 1.2.).

1.1.3. Cell ultrastructure and morphology

Cyanobacteria are prokaryotes, therefore their cytology is very simple. They have no nucleus and organelles (mitochondria, chloroplast). Cyanobacteria possess a single circular chromosome, which has been completely sequenced in several species (Kaneko et al. 1996). Some also carry plasmids, small circular strands of DNA, which do not appear to have a role in toxicity (Schwabe et al., 1988). The protein-synthesizing organelles of cyanobacteria are the ribosomes of prokaryotic type. Cyanobacterial photosynthesis proceeds on highly organized system of internal thylakoids membrane containing chlorophyll -a and phycobilisomes attached to surface of thyllakoid membrane. Phycobilisomes contain phycocyanin and phycoerythrin and act as light harvesting antennae for the photosystems. Thylakoid arrangement is the most important uniform feature of the inner cell structures which is usable for taxonomic classification. Cell inclusions, such as polyphospahte granules, carboxysomes and cyanophycin granules, are important ultrastructural features. Their quantity is dependent upon environmental factors. It is not clear, whether their position in some species is stable and characteristic. The last specific feature occur mainly in planktic types are aerotopes. Aerotopes are composed from groups of gas vesicles aggregated paralely int oclustres recognizable in a light microscope as light points in cells.

The cell wall is uniform gram-negative, composed of three obligatory layers and one obligatory crystalline S-layer. The mucilage on cyanobacterial surface are not integral parts of cell wall.

A few cyanobacterial genera live solitary as unicellular (*Cyanobium, Cyanothece, Synechococcus, Synechocystis*), in all others the cells aggregate into various kinds of colonies or may form filaments. Besides the vegetative cells, the normal, photosynthetic cells that are formed under favorable conditions, we found several specialized cell types in filaments (Fig. 1). *Akinetes* are a very large spherical to oval-shaped cell with granular contents. They form resting cells when the environmental conditions become harsh and the filaments dieds, regenerating when the conditions are favourable. *Heterocysts* are the specialist nitrogen-fixing cells, which may occure terminally or within the filament. They are large, clear, highly refractive cells, which have a thickened cell wall and do not possess photosynthetic membranes. Within the heterocysts the enzyme nitrogenase reduces molecular nitrogen to ammonia, which is incorporated into the amido group of glutamine. By non-heterocytous filamentous we could find the enzym nitrogenase in special cells sometimes joined together and forming distinct segments of trichomes, *diazocytes* (Komárek and Anagnostidis, 2005). Some cyanobacteria

appear to be able to fix atmospheric nitrogen without visible heterocysts or diazocytes, which may relate to the anaerobic condititons in which th organisms can survive (Falconer 2005). Many filamentous cyanobacteria also form short section of cells called *hormogonia,* separating from the original trichome after the fragmentation or after formation of necridic cells or heterocysts and serving for reproduction. Within certain limits, character of hormogonia is constant in various species (Komárek and Anagnostidis, 2005).

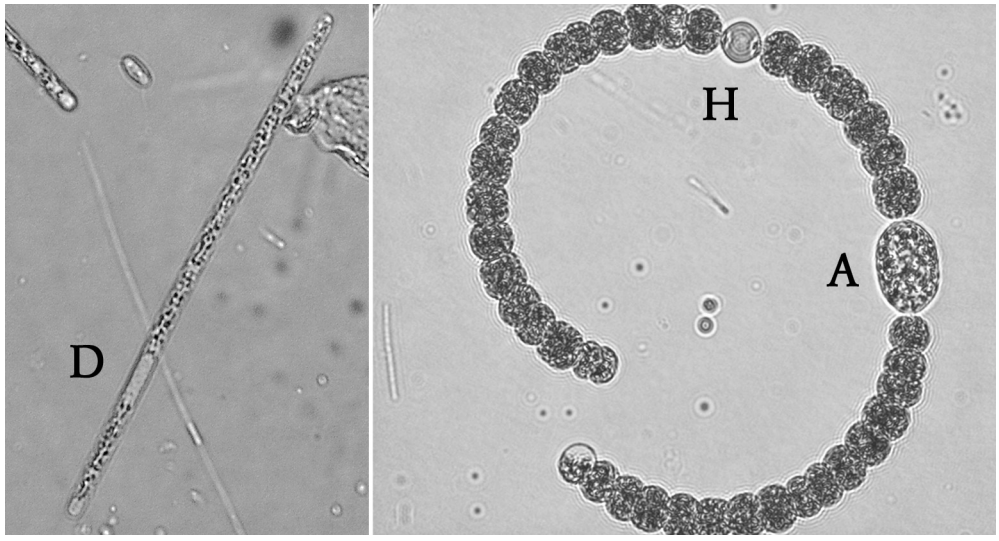


Fig. 1. Specialization of cells by filamentous cyanobacteria: D - diazocytes by Planktothrix, H- heterocyte and A - akinete by Anabaena. Photograph by L. Šejnohová

1.1.4. Classification system past and present

The traditional system of cyanobacteria was based mainly on morphology (Desikachary 1959, Geitler 1932, Rippka et al. 1979, Starmach 1966). The main criteria used for clasification were phenotypic markers as type of thallus and type of divission. The polarity of cells, form and structure of a colony, formation of various types of mucilaginous strands layers, and the position of cells in a colony are particularly important (Anagnostidis and Komárek, 1999). Consequently, cyanobacteria were classifed to two non-filamentous groups/sections (Chroococcacean, Pleurocapsalean) and three or four filamentous groups/sections (Oscillatorian, Nostocacean, Rivularean, Stigonematalean).

At present, the taxonomic classification of cyanobacteria is under large revision. The main purpose of modern „polyphasic" approach is to combined molecular, cytomorphological and ecological-biochemical stable characters for the biological diversity understanding. The new *supraspecific classification* compiled by Komárek and Anagnostidis (2005) divede the non-filamentous species to one single order Chroococcales with seven clearly distinguishable families and to three filamentous orders - Oscillatoriales, Nostocales and Stigonematales. Oscillatoriales is

characterized by simple filaments without branching or false branching and without heterocytes. Order Nostocales comprises filamentous forms with sometimes false branching but with always differentiated cells named heterocytes. Order Stigonematales seems to be morphologically most developed with true branching, heterocysts and complicated life cycles. According to molecular studies based on 16S ribosomal RNA sequences, order Chroococcales and Oscillatoriales are paraphyletic, Nostocales and Stigonematales are monophyletic (Litvaitis, 2004).
The definition of families differ according to affiliation to orders and in dependence on research forwardness: within Chroococales primary criterion is type of cell division, within Oscillatoriales thylakoid arragnement in cells, within Nostocales and Stigonematales type of branching and frequency of heterocysts and akinetes. The good situation is on the genera level, where the genotypic characterisation by 16S ribosomal RNA corresponds almost perfectly to revised morphological traditional genera (Komárek and Anagnostidis, 2005). However, classification based solely on the analysis of single sequences could be incorrect due to the horizontal gene transfer (HGT)(Young, 2001).
The problems arise with *the species concept*, which is most complicated and at present is under intensive discussion. Traditional morphological features used in the characterization of intrageneric diversity, such as shape of cells, ability to form colony, presence of sheets, pigmentation and type of branching, have been frequently found to be unstable and variable. According to molecular research, species delimitations by traditional morphological criteria have not been commonly supported. The ecophysiological and biochemical analysis have also demonstrated that the enormous number of single deviations and or wide variability at he strain-level within each genetically stable genotype (genus) prohibits the definition and delineation of suitable clusters (species). Although molecular analysis is modern tools for classification of organisms, the system cannot be based on them only. We must also take into account, that there are known to exist stabilized clearly recognizable, distinct morpho- and ecotypes, which are still important within the genera (Komárek and Anagnostidis 2005). It appers, that numbers of species will be reduced during the revision of cyanobacteria, however the new system should respect stabilized and repeatedly occur morphotypes. It will be really mistake to fuse several existing species to category genus sp. and throw away the data discovered by lineage. In conclusion, at present the category morphospecies (morphotype) is used instead the therm species for ecological and toxicological studies in the field.

1.2. Bloom forming cyanobacteria

The term "bloom forming cyanobacteria" is nowadays understood to refer to the planktonic blue-green algae with the ability to the accumulation at the surface of lakes and reservoir (Fig. 2). This group of cyanobacteria can form intensive scums in warm, nutrient-enriched fresh and brackish waters. This phenomenon is world-wide and described by various synonyms: water blooms, bloom formation etc. In temperate regions, blooms develop mostly during the hot weather in summer and autumn; in the tropics, they can form in any time of year.

The first attestable description of a water-bloom was at Llangorse Lake, Breconshire, Great Britain by Giraldius Cambrensis in 1188. The bloom has recorded also in Shropshire and Cheshire (Great Britain), where the inhabitants have spoken as that of "breaking of the meres" (Reynolds and Walsby, 1975). In 1476, the red-brown bloom occurred in Murtensee (Switzerland) was attributed by local inhabitants to the blood of Burundian soldiers who had drowned there during a battle.

During the end of the 20th century, intensity of surface blooms seems to have been increasing. It is cause ecological, hygienic and water-managing problems. Some hard developed population influence negatively physical and chemical parameters of water and cause lost of ecosystem diversity (Zurawell, 2005).

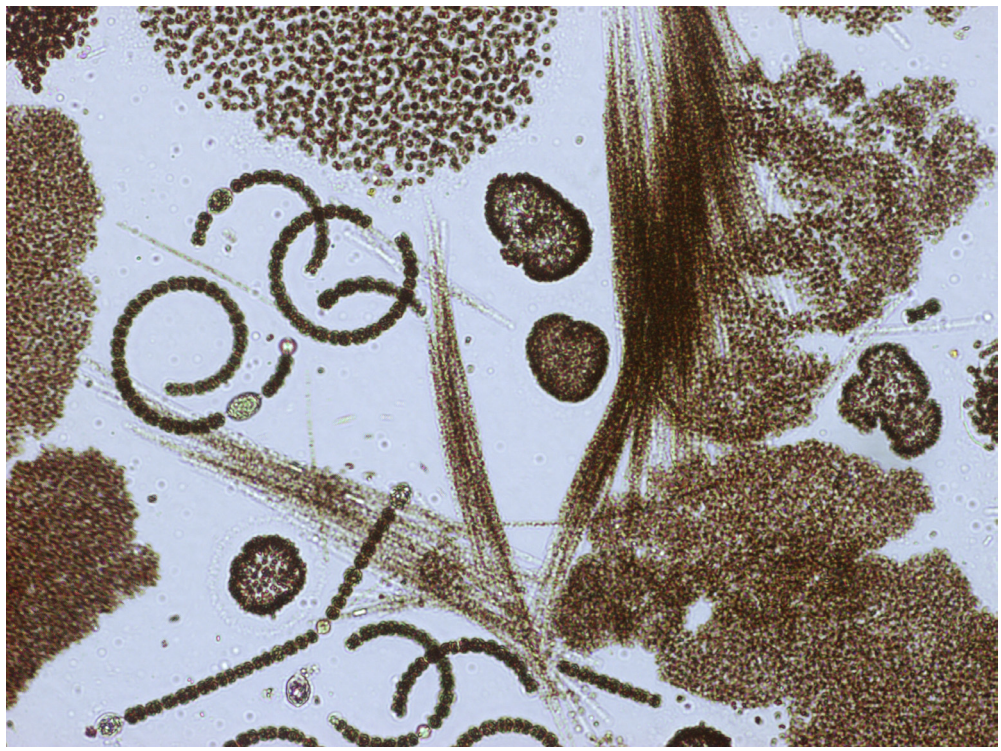
1.2.1. Taxonomy

There are three main groups of bloom forming cyanobacteria, which differ with their ecological strategies: i) those capable of buoyancy and fixing molecular nitrogen ii) those capable of buoyancy regulation but without the ability to fix N_2 and iii) those not capable of neither buoyancy regulation nor N_2-fixation (Anagnostidis and Komárek, 1985).

Table 1.

Ecological strategies	**Taxonomical group**	**Genera**
Buoyancy, fix N	Nostocales	*Anabaena,*
		Aphanizomenon
		Cylindrospermopsis
		Gloeotrichia
		Raphidiopsis
Buoyancy, not fix N	Chroococcales	*Microcystis*
		Woronichinia
	Oscillatoriales	*Planktothrix*
Not buoyancy, not fixing N	Chroococcales	
	Oscillatoriales	
	Nostocales	

Figure 2. A surface bloom at Brno reservoir, The Czech Republic, summer 2005. Photograph by L. Šejnohová



1.2.2. Factors affecting bloom formation

Factors leading to the cyanobacterial dominance have been discussed by several authors, who supposed that the mass growth of cyanobacteria is caused by a complex of environmental factors, which partly act synergistically, more than by one dominant factor (Blomqvist et al., 1994; Varis, 1993). The latest review (Hyenstrand et al., 1998) states that the cyanobacterial success may explain at least nine following theories:

TN/TP ratio. The low total nitrogen to total phosphorus ratio hypothesis was first applied to explain the success of nitrogen-fixing (Flett et al., 1980; Schindler 1977) and also non-nitrogen fixing cyanobacteria (Smith, 1983). A comparison between the optimum N:P rations for eukaryotic algae (16-23 molecules N:1 molecule of P) with the optimum rates for bloom forming cyanobacteria (10-16 molecules N: 1 molecule P), shows that the ratio is lower for cyanobacteria (Schreurs 1992). McQueen and Lean (1987) noted that the probability for a cyanobacterial bloom was highest when this quotient was below 5:1. However, some other factor(s) mustbe involved in regulation cyanobacterial success (Hyenstrand et al., 1998).
Experimental data also have shown further advantage of cyanobacteria: the higher affinity for nitrogen or phosphorus than for many other photosynthetic organisms. Consequently, cyanobacteria can out-complete other phytoplankton groups under conditions of nitrogen or phosphorus limitation. In addition to their high nutrient affinity, cyanobacteria have a substantial storage capacity for phosphorus (Mur et al., 1999).

Low light. Like algae, cyanobacteria contain chlorophyll a as a major pigment for harvesting light and conducting photosynthesis. However, they also contain other photosynthetic pigments such as phycobiliproteins which provide for cyanobacteria special advantages. First, these unique pigments harvest light with the lower wavelengths (500-650 nm) in green, yellow and orange part of the spectrum. In this way, cyanobacteria are able to live in the deeper part of the water column, where the light with the longer wavelengths is less transmitted and hardly used by other phytoplankton groups (chlorophyll absorbing the light in 680-700 nm). The second advantage of phycobiliproteins is the 95% efficient of energy transfer by means of their geometrical arrangement to unique macromolecular structure knows as the phycobilisome. The Figure 3 shows the subunits of phycobilisome: allophycocyanin (light blue), phycocyanin (blue) and sometimes phycoerythrine

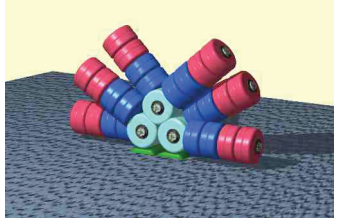


Figure 3. Phycobilisome structure (source Cyano-site)

(red), the green elements represent two PSII reaction centers that associate with each phycobilisome. In some species of cyanobacteria, when both phycocyanin and phycoerythrin is present, the phycobilisome can undergo significant restructuring as responce to light color. In green light increase amount of red colored phycoerythrin, this absorbs better green light. In red lihgt, this is replaced by blue colored phycocyanin, which absorbs better red light. This process is know as chromatic adaptation, is reversible and enable to cyanobacteria to maximally utilize all available light. Moreover, cyanobacteria require little energy to maintain cell function and structure (Gons, 1977; Van Liere et al., 1979). Thus, they are able to harvest light energy efficiently and can maintain a relatively higher growth rate than other phytoplankton organisms when light intensities are low.
On the other hand, many cyanobacteria are sensitive to prolonged periods of high light intensities. The growth of *Planktothrix* agardhii is inhibited when exposed for extended periods to light intensities above 180 uE m2/s. Long exposures at light intensities of 320 uE m2/s are lethal for many species (Van Liere and Mur, 1980). Nevertheless, species form surface blooms seem to have a higher tolerance for high light intensities because of an increase in carotenoid production which protects the cells from photoinhibition (Paerl et al., 1983).

High pH/low CO2. This theory suggests that cyanobacteria can outcompete other algae in water with a high pH or low CO2 content (King, 1970; Shapiro, 1973; Shapiro, 1984; Shapiro, 1990). This hypothesis was recently evaluated by unique experiments in Squaw Lake in Wisconsin (Shapiro, 1997). Results showed that cyanobacteria became abundant also in high C02 concentration and thus their initiation is not caused by high pH or low CO2 levels. However, according to kinetic studies he concluded that cyanobacteria ensure their dominance by reducing concentrations of CO2 to levels so low that other algal species have difficulties to utilize them (Hyenstrand et al., 1998). This supports the idea that cyanobacteria posses beside the cyanobacterial RubisCo the carboxysomes as the mechanism for inorganic carbon fixation.

Buoyancy. Buoyancy is the upward force enables the cells or colony of cyanobacteria to float. This force ensures the gas-filled vesicles (GV), which have a density of about one tenth more than of water (Walsby and McAllister, 1987). Thus GV can give cyanobacterial cells a lower density than water (Mur et al., 1999). By means of buoyancy cyanobacteria gain a competitive advantage by being able to regulate their position in the water column (Hyenstrand et al., 1998). For more information see Chapter *1.3.4. Ultrastructure.*

Temperature. The elevated water temperature is the next theory illustrates the

cyanobacterial success (Hyenstrand et al., 1998). Maximum growth rates are attained by most cyanobacteria at temperatures above 25°C (Robats and Zohary, 1987). These optimum temperatures are higner than for green algae and diatomes (Mur et al., 1999).

Resistance to zooplankton grazing. Cyanobacteria have long been reported to be an inadequate food for zooplankton (Lefevre, 1950; Bogatova, 1965). Many recent studies (Lampert, 1987) confirmed this with the description of mechanisms, why cyanobacteria are either not eaten or are pure food for zooplankton. Cyanobacteria are unattractive for zooplankton because of their shape, size and production of toxins. Large colonies of *Aphanizomenon, Anabaena* and *Microcystis* cannot be handled by zooplankton. Small colonies and filaments may cause inhibition of the feeding process by mechanical interference. At least, toxic population of cyanobacteria has a toxic effect on zooplankton. These mechanisms may reduce zooplankton growth, reproduction and survival and thus, its grazing on cyanobacteria.

Storage (phosphorus) strategy from the bottom. The storage strategy hypothesis is based on the ability of cyanobacteria to bring an internal nutrient reserve, mainly phosphorus, acquired in the sediment into the pelagic zone (Hyenstrand et al., 1998). This theory explains the sudden increase of cyanobacterial biomass (Hansson, 1996; Shapiro, 1997) and is most definitely key feature enhancing cyanobacterial development (Pettersson et al., 1993).

Inorganic nitrogen strategy. The inorganic nitrogen hypothesis suggests that cyanobacteria for development use different form of nitrogen than eukaryotes. An ammonium-nitrogen favors the development of non-nitrogen-fixing cyanobacteria, whereas nitrate-nitrogen favors the development of eukaryotic phytoplankton. Nitrogen scarcity favors the development of nitrogen-fixing species (Blomqvist et al., 1994). The importance of inorganic nitrogen for the development of cyanobacteria was provided during the field study (Toetz, 1981). Cyanobacteria did not bloom in the lake with the low supply of ammonium, while nitrate was major nitrogen source and the other groups of phytoplankton were dominant. An importance of ammonium-nitrogen for cyanobacterial dominance was showed on the whole-lake experiments when nitrogen-fixing cyanobacteria were replaced by chlorophytes, cryptophytes and euglenophytes after the additions of nitrate to sediments (Leonardson and Ripl, 1980). The later comprehensive study from lakes with the different trophic status (Blomqvist et al., 1994) showed that cyanobacteria are favored by ammonium-nitrogen but disfavored by nitrate-nitrogen. Nevertheless, nitrates seem to be main important form of nitrogen in the relationship low proportion TN/TP (McQueen and Lean; 1987).

Requirements for trace elements. TThe trace element hypothesis was originally put forward to explain the success of nitrogen-fixing cyanobacteria. This theory states that levels of bioavailability trace metals (e.g. iron) must be high enough to meet the demands associated with nitrogen fixation (Murphy and Lean, 1976). However, recently study (Kudo and Harrison, 1997) showed that also non-nitrogen cyanobacteria are able to grow faster on ammonium than on nitrate in a low-iron environment.

1.2.3. Cyanobacterial toxins

The cyanotoxins belong to a diverse group of chemical substances, each of which shows specific toxicity. Mechanisms of their toxicity are very diverse and range from hepatotoxic, neurotoxic and dermatotoxic (Sivonen and Jones, 1999).

Clasiffication of cyanotoxins and principles of their toxicity. Cyanobacterial toxins can be devided according to chemical structure into three broud groups:

1) The oligopeptides are the best known and most frequent hepatotoxic cyanobacterial toxins occured almost by all bloom forming genera. They consist of between two and twelve amino acids bond to linear or cyclic forms. Into linear type we classify an aeruginosins and microginins, into cyclic cyanopeptides microcystins, nodularins, cyanopeptolins, anabaenopeptins and microviridins.

Aeruginosins (Fig. 4A) are the linear tetra-peptides with the unique moiety 2-carboxy-6-hydroxyoctahydroindole (Choi) and C-terminal Arg-derivate. The toxic effect of aeruginosins is based on protease inhibition - trypsin (Ploutno et al., 2002) and trombin (Kodani et al., 1998; Murakami et al., 1995). Consequently, they may lead to deep vein trombosis, myocardial infarction, and stroke (Radau et al., 2003). It seems that aeruginosins could be most frequent in some *Microcystis* community that microcystins (Welker et al., 2007).

Microginins (Fig. 4B) are the linear peptides with a characteristic N-terminal 3-amino-2-hydroxydecanoic acid (Ahda). Some 40 structural variants are known. Some of microginins are leucine aminopeptidase (Ishida et al., 1997;1998) and zinc metalloprotease inhibitors (Ishida et al., 2000).

Microcystins (Fig. 5A) are monocyclic heptapeptides with the characteristic feature, unusual ß-amino acid Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4E, 6E-dienoic acid). General structure of microcystins is a cyclo (-D-alanine[1] - L-X[2] D-erythro-ß-methylaspartic acid[3] - L-Y[4] - Adda[5] - D-glutamate[6] - N methyldehydroalanine[7]). The molecular weight of microcystins varies in the range of 909 to 1115 (Duy et al., 2000). The main structural variations in microcystins are observed in the L-amino acid residues X and Y, which are indicated by two-letter suffix in the name. For example, microcystin-LR contains leucine (L) and arginine (R) in these positions. Nevertheless, modifications of

the other parts of molecule exist and more than 70 structural variants of microcystins have been identified so far (Codd et al., 2005). However, different structural variants are not equally prevalent in the environment. For instance, microcystin-RR, -YR and -LR can be found very frequently in environmental samples (de Figueiredo et al., 2004), whereas others are detected only rarely and usually in the trace amounts (Lawton & Edwards, 2001). Microcystins are synthesised by ancient biochemical pathways involving unique mixed polyketide synthases and nonribosomal peptide synthetases (Nishizawa et al., 2000; Tillett et al., 2001).

Cyanopeptolins or micropeptins or aeruginopeptins (Fig. 5C) could be defined as (a) cyanobacterial 19-membered cyclic depsipeptides cyclized by an ester-linkage of the hydroxyl-group of threonine with the carboxy terminus of the C-terminal amino acid of a proposed linear precursor, (b) an unusual 3-amino-6-hydroxy-2-piperidone unit, and (c) a cis-configurated amide linkage between the amino acids in position 3 and 4 (Weckesser et al., 1996). These compounds are inhibitors of serine proteases - trypsin (van Elert et al., 2005; Czarnecki et al., 2006). According to latest studies cyanopeptolins also display cytotoxic activity and promote cell differentiation (www.pasteur.fr).

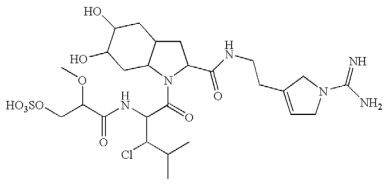
Anabaenopeptins, cyclic depsipeptides (Fig. 5D), were described from the cyanobacterium *Anabaena flos-aquae* (Harada et al., 1995). Some of these compounds were found as elastase and trypsin inhibitors (Grach-Pogrebinsky et al., 2003).

Microviridins are tricyclic depsipeptides (Fig. 5E) which were described from the cyanobacterium *Microcystis viridis* (Ishitsuka et al., 1990). The laboratory experiments identifed microviridin J as a source of a fatal molting disruption in *Daphnia* species feeding on *Microcystis* cells. The molting disruption was presumably linked to the inhibitory effect of microviridin J on daphnid proteases (Rohrlack et al., 2004).

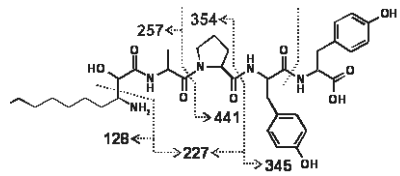
2) Alkaloids toxins are diverse, both in their chemical structures and in their toxicities. In general, they are a broad group of heterocyclic nitrogenous compounds usually derivates of amino acids. They could be with the neurotoxic (anatoxins and saxitoxins occured by *Aphanizomenon, Anabaena, Lyngbya* and *Cylindrospermopsis* genera), cytotoxic (cylindrospermopsin by *Cylindrospermopsis* and *Aphanizomenon* genera) or dermatotoxic effect (aplysiatoxins and lyngbyatoxins from benthic genera *Lygbya. Oscillatoria*, *Schizothrix*). To date no alkaloids have been found by *Microcystis* genus (Sivonen and Jones, 1999).

3) Lipopolysacharids (LPS) are an integral component of the cell wall of all Gram negative bacteria, including cyanobacteria, and can elicit irritant and allergenic responses in human and animal tissues. LPS are generally found in the outer

Figure 4. The structure of linear peptide toxins.

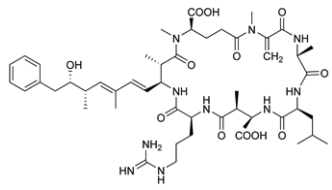


A) Aeruginosin

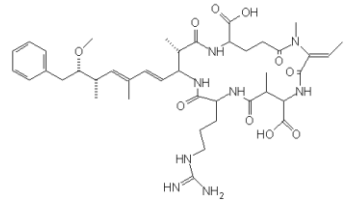


B) Microginin

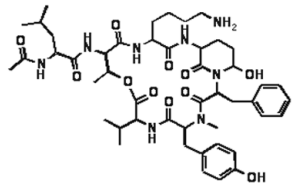
Figure 5. The structure of cyclic peptide toxins.



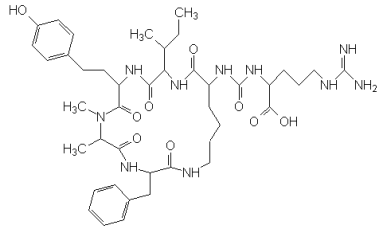
A) General structure of microcystins (MCYST)



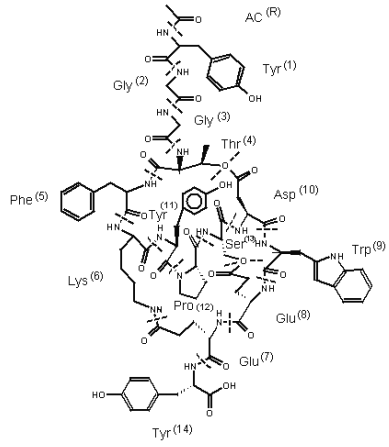
B) Nodularins



C) Cyanopeptolin



D) Anabaenopeptin



E) Microviridin

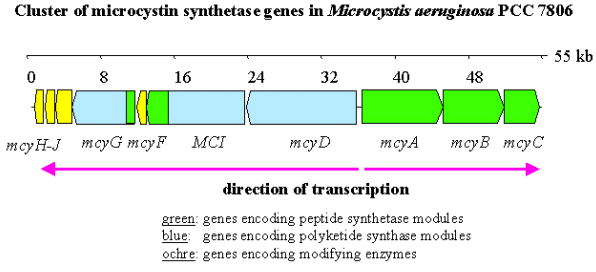
memrane of the cell wall, where they form complexes with proteins and phosphoholipids (Sivonen and Jones, 1999). To date, we know only that cyanobacterial LPS could be different according to genera (Kerr et al., 1995). The few results also indicated that cyanobacterial less toxic than the LPS of other bacteria, such as *Salmonella* (Keleti and Sykora, 1982; Raziuddin et al., 1983).

Molecular biology of cyanobacterial toxins. Until today, only a few genes involved in the biosythesis of cyanobacterial toxins have been discovered, all belonging to the group of peptides (Borner and Dittmann, 2005). At present, only the microcystin and nodularin synthesis are know in detail from the whole spectrum of toxic cyanobacterial oligopeptides.

Microcystin biosynthesis included two stages: non-ribosomal (NRPS) way for the peptide backbone (from acetate, pyruvate, L-glutamic acid, L-phenylalanine, L-methionine etc.) and a polyketide pathway (PKS) for the characteristic Adda moiety ((2S, 3S, 8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid). Non-ribosomal peptide synthetase (NRPS) represent a family of large, multifunctional modular enzymes, in which each module is responsible for the activation, modification and condensation of an individual amino acid (Borner and Dittman, 2005). These enzymes form large complex serving as a templates for the biosynthesis of peptides as an alternative way to peptide formation on ribosomes (Marahiel et al., 1997; von Dohren et al., 1997). The microcystin synthetase (*mcy*) genes have been characterized recently not only for *Microcystis,* but also for *Planktothrix* (Christiansen et al., 2003) and *Anabaena* (Rouhiainen et al., 2004).

The *mcy* gene cluster (Fig. 6.) coding enzymes involved in microcystin biosynthesis differs between the genera, but it seems, that it is identical in the same morphospecies. By *Microcystis aeruginosa mcy* gene cluster spans 55kb of the chromosomal DNA and comprises of 10 genes embedded in two bidirectionally transcribed operons (Nishizawa et al., 2000; Tellett et al., 2000). McyA-C encode three NRPS comprising 5 modules, whereas mcyD-J encode polyketide synthases (mcyD), hybrid enzymes (mcyE, G) and additional tailoring enzymes (mcyF, I, J) as well a component of a putative ABC transporter (mcy H). More than 65 structural variants of microcystin have been described. Usually, the individual strains produce more than one microcystin variant (Sivonen and Jones, 1999). Mutation of *mcy*

Figure 6



genes has led to mutant clones that were no longer capable of producing any kind of microcystins. This demonstrates that one *mcy* gene cluster is responsible for the synthesis of all micrystin variants (Dittmann et al., 1997; Nishizawa et al., 1999; Tillett et al., 2000). The existence of genes for microcystin biosynthesis is detect by the help of *mcy*-specific primers in PCR studies. Thus, it was recognized that PCR with *mcy*-specific primers provided a way of dicriminating between potentially harmful cyanobacteria in water bodies (Quellette and Wilheim, 2003).

Dynamics of cyanobacterial toxins. Studies on the regulation of microcystin production, not to mention the synthesis of other cyanobacterial toxins, are still at their beginning. In microcystin-producing cyanobacteria, genes encoding for the microcystin synthetase enzyme complexes are always present and are apparently expressed constitutively. Only a few examples exist of non-microcystin-producing species with microcystin synthetase genes present (Neilan et al., 1999; Pan et al., 2002). The key cyanobacterial genera known for their potential ability to produce toxic substances include *Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya, Microcystis, Nodularia, Nostoc* and *Planktothrix* (Carmichael, 2001).

The concentration of microcystins has revealed a high variability in space and time and may range over more than 5 orders of magnitude. Concequently, four sources of variation may be responsible for this variability in microcystin concentrations (Kardinaal and Visser, 2005):

1) Variability in cyanobacterial biomass - higher biomass of microcystin-producing cyanobacteria will result in higner microcystin concetration.

2) Methodological variability - microcystin can be expressed per volume of water, per unit of dry weight, per unit of chlorophyll a, or per biovolume.

3) Physiological variability - impact of environmental factors like phosphorus (Sivonen, 1990; Rapala et al., 1997; Oh et al., 2000), nitrogen (Sivonen, 1990; Orr and Jones, 1990; Long et al., 2001), light conditions (Sivonen, 1990; Utkilen and Gjolme, 1992; Weidner et al., 2003), temperature (van der Westhuizen and Eloff, 1985; Rapala et al., 1997), pH (Song et al., 1998), iron (Utkilen and Gjolme, 1995) and also growth rate (Long et al., 2001). The majority of studies indicate that cyanobacteria produce the highest amount of toxins under most favourable conditions for growth (Sivonen and Jones, 1999). These optimal conditions may differ among various cyanobacterial taxa (e.g. *Planktothrix* prefers low light, whereas *Aphanizomenon* prefers high light). Also, nutrient-rich conditions generally result in a higher microcystin production (Kardinaal and Visser, 2005).

4) Variability in cyanobacterial species and genotype composition - the microcystin content can be quite variable among species and even among different

genotypes within the same species (Fastner et al., 1999; Rohlack et al., 2001). As a result, changes in the species composition of cyanobacteria, and also changes in genotype composition within the same species, may lead to coconsiderable changes in microcystin concentration (Kardinaal and Visser, 2005). Consequently, cyanobacterial strains can be either toxic or nontoxic, even within species. These toxic and nontoxic strains coexist in the field (Fastner et al. 2001; Kurmayer et al., 2002; Janse et al., 2004). Strain composition may change during the season and thus the the microcystin dynamics in lakes result succession of certain cyanobacterial genotypes that differ in their ability to produce microcystins (Welker et al., 2003; Kardinaal and Visser, 2005). The factors driving population dynamics of these toxic and nontoxic strains are not yet well understood (Tonk, 2007).

Function of cyanobacterial toxins. Until today, research on the function of cyanotoxins has mainly focused on microcystins. As microcystins, exhibiting protease inhibition, also other peptides (cyanopeptolins, aeruginosins, anabaenopeptins, microviridins, microginins) could play the similar role and function in ecosystems. It has been suggest that microcystins may have an ecological function by reducing grazing pressure from predators such as zooplankton (Koski et al., 1999; Rohlack et al., 1999), or by suppressing competing phototrophic organisms (Gross, 2003, Babica et al., 2007). The other hypotheses suppose that these peptides play the role in cyanobacterial physiology (for instance as metal ion chelators - Utkilen and Gjolme, 1995) or as intraspecific signaling molecules (Ditmann et al., 2003). What function that is, is thus far still unknown (Tonk, 2007).

1.2.4. Impact on biota and human

The main mode of action of microcystins is binding to catalytic units of protein phosphatases 1 and 2A and inhibition of their function (MacKintosh et al. 1990). It can lead into hyperphosphorylation of structural proteins and cell necrosis, mitochondrial permeability transition, mitochondrial membrane potential alteration, formation of reactive oxygen species and oxidative stress induction, oxidative damage of DNA, modulation of apoptosis, changes in cell proliferation and cytokinesis (Falconer 2006). However, the exact mechanism of microcystin toxicity remains to be elucidated (Babica, 2007).

Effects on aquatic bacteria. The impact of cyanobacterial toxins on bacteria is not fully understood and the scientific literature gives number of contradictory results (Sivonen and Jones, 1999). It appears that an extract of *Microcystis aeruginosa* or pure microcystin-LR have any biocidal effect on *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* or *Pseudomonas hydrophila* (Foxall and Sasner, 1988). However, it has been suggested that the negative effect may be related to the presence of

unidentified LPS endotoxins in the cell wall of the cyanobacterial cells (Sivonen and Jones, 1999). From the ecological point of view microcystins could not be toxic to all bacteria because several species are known to degrade microcystins (Jones et al., 1994).

Effects on fytoplankton and plants. Microcystins act as potent inhibitors of protein phosphatases not only in animals, but also in plants (MacKintosh et al. 1990). In plants, protein phosphatases regulate important cellular processes (Luan 1998; Luan 2000; Luan 2003) and their inhibition by microcystins can result in harmful effects on the whole photoautotrophic organisms.

Effects on zooplankton. There is dramatic variation among zooplankton genera, species and clone in their response to toxic cyanobacteria (DeMott et al., 1991). Thereby, cyanotoxins may influence the structure of zooplankton community, especially during times when cyanobacteria are dominant within the phytoplankton (Sivonen and Jones, 1999). The feedback from zooplankton is the graze of the non-toxic cyanobacterial species (DeMont et al., 1991). Consequently, the toxicity of a *Microcystis* population could increase by the feeding activity of zooplankton over a longer period (Benndorf and Henning, 1989). The bioacumulation of microcystins in zooplankton was observed (Watanabe et al., 1992).

Effects on fish and other animals. Mass developments of microcystin-producing cyanobacteria caused many incidents of animal poisonings and deaths (Duy et al. 2000; Briand et al. 2003). Early publications from Denmark (1833), Australia (1878) and Poland (1884), report farm livestock deaths, and bird-kills after drinking from blooms or scums, with fish-kills also at some sites (Sivonen and Jones, 1999). Numerous later examples of animal deaths related to cyanobacterial blooms over the past century are by Yoo et al. (1995). Poisonings have most typically occurred during drinking (e.g. cattle (Saker et al., 1999) and flamingo deaths (Chittick et al., 2002) or via the food chain (Eriksson et al., 1986). Microcystins bioaccumulate in common aquatic vertebrates and invertebrates, including fish (Carbis et al., 1997) and mussels (Eriksson et al., 1989; Falconer et al., 1992; Prepas et al., 1997). Differences in senzitivity may be pronounced between species: goldfish were found to be nearly 30 times less susceptible to microcystin than mice (Sugaya et al., 1990). Pathological symptoms ascribed to toxic cyanobacterial blooms include damage to the liver, heart, kidney, gills, skin and spleen (Garcia, 1989; Rabergh et al., 1991). Moreover, laboratory studies indicate that dissolved mirocystins may affect fish embryos (Oberemm et al., 1997) and behaviour of fish (Baganz et al., 1998). However, the toxins are likely to be among a range of toxicants and physiological stress that are presented to the fish by cyanobacterial blooms, especially during bloom senescence. These include oxygen deficit, high pH and ammonia release due to bloom proteolysis

(Codd et al., 2005). Consequently, toxic cyanobacteria affect wildlife organisms and might have negative impact on aquatic ecosystems (Wiegand & Pflugmacher 2005; Zurawell et al. 2005).

Effects on human. There are also several examples of negative human health outcomes associated with the occurrence of microcystin-producing cyanobacteria in recreation water bodies, drinking water reservoirs or in reservoirs supplied haemodialysis units (Kuiper-Goodman et al. 1999; Codd et al. 2005). Microcystins primarily act as hepatotoxins in mammals, since hepatocytes highly express organic anion transport proteins, which are responsible for active cellular uptake of microcystins. However, organic anion transporters are present also in other organs than liver, e.g., in the gastrointestinal tract, the kidney or the brain (Hagenbuch & Meier 2003). In fact, microcystins might affect multiple organs and tissues. They have been showed to induce renal damage (Nobre et al. 1998; Nobre et al. 1999; Milutinovic et al. 2002; Milutinovic et al. 2003) or neurological symptoms (Azevedo et al. 2002; Maidana et al. 2006). Microcystins affected erythrocytes (Sicinska et al. 2006) or immune cells (Hernandez et al. 2000; Chen et al. 2004; Teneva et al. 2005). There is increasing evidence on tumour promotion properties of microcystins from laboratory experiments (Falconer & Buckley 1989; Falconer 1991; Nishiwaki-Matsushima et al. 1992; Ohta et al. 1993; Ito et al. 1997; Sekijima et al. 1999; Humpage et al. 2000; Dietrich & Hoeger 2005). Potential health risk might result from the consumption of contaminated food (due to the bioaccumulation of microcystins in fish tissue or in vegetables irrigated with microcystin-contaminated water) or the consumption of contaminated food supplements made from cyanobacteria (Dietrich & Hoeger 2005). Besides acute poisonings, the chronic exposure of humans to low concentrations of microcystin in drinking water can lead to liver or colorectal cancer promotion (Yu 1995; Zhou et al. 2002). Special attention given to microcystins is reflected by WHO guideline value set for concentration of microcystin-LR in drinking water (1 μg/L)(WHO 1998). Consequently, limits for microcystin concentrations have been implemented in legislations of many countries as well, and numerous studies have addressed risk assessment and management of these cyanotoxins (Codd et al. 2005b; Dietrich & Hoeger 2005; Chorus 2005).

Figure 7 Information board about the mass development of cyanobacteria in the basin for stork in the National Zoological Park at New Delhi, India. Photograph by L. Šejnohová

Figure 8. Effects of blooms on vertabrates (reservoir Nové Mlýny, Czech Republic). Photograph by J. Kohoutek

1.3. Genus *Microcystis*

The first records of *Microcystis* causing water-bloom were recorded from early history (Reynolds and Walsby, 1975). The attention to this genus has risen with construction of artificial water bodies and eutrophication of landscape. *Microcystis* develops in stagnant freshwaters and also in coastal waters (Parra et al., 1980) all over the world with the exception of circumpolar areas (Komárek and Komárková, 2002).

1.3.1. Morphology in light microscopy

The genus *Microcystis* is characterised by coccal cels irregularly or in three-dimensional perpendicular rows agglomerated in colonies with colorless mucilage. Gas vesicles gathered in aerotopes during the vegetative stage are visible in light microscopy as dark points (Anagnostidis and Komárek, 1999). Cells are spherical, after division hemispherical. To date, this morphological features are used for the genus and species delimitation (more Chapter 1.3.3.)

1.3.2. Reproduction

Cell division by *Microcystis* genus proceeds by binnary fission in three perpendicular planes in regular cubic arrangement. Daughter cells grow into the original form

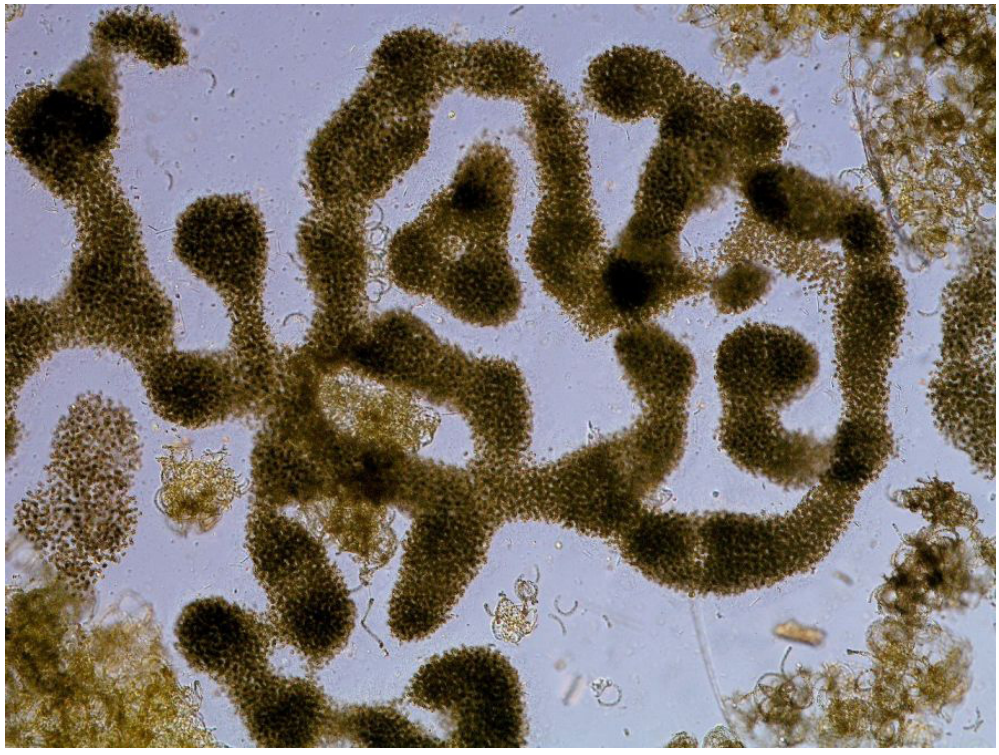


Figure 9. Typical holes-forming colony of Microcystis aeruginosa, rybník Svět, Czech Republic, Photograph by L. Šejnohová

and size befor next division. Reproduction is known only by disintagration of colonies into small clustrers or even into solitary cells. By *Microcystis flos-aquae* also nanocyte are known (Anagnostidis and Komárek, 1999).

1.3.3. Taxonomy

Nowadays the genus *Microcystis* is well delimited by morphological features (see above Chapter 1.3.1.) as well as by molecular data (Janse et al., 2003).
However, the subgeneric classification is defined chiefly by morphological characteristics, hence the term "morphospecies". According to form of colony, structure of mucilage, diameter of cells, density and organisation of cells in colony, pigment content (PC: PE ratio), and life cycle eight morphospecies have been described in the Czech Republic (the most frequent forms are demonstrated on Fig. 9, 10). Neverthelees, occurrence of transient forms of colonies in the field (Komárek and Komárková, 2002) and change or loss of colony in culture (Kato et al., 1991; Komárek, 1991; Otsuka et al., 2000) leads doubts about the validity of *Microcystis* morphospecies delimitation. Therefore a lot of studies deal with the question of *Microcystis* subgeneric classification (sensu stricto, only morphospecies with gas vesicles) use the different approaches and give different results: 1) comparision of 16S rRNA genes (Neilan et al., 1997; Otsuka et al.,

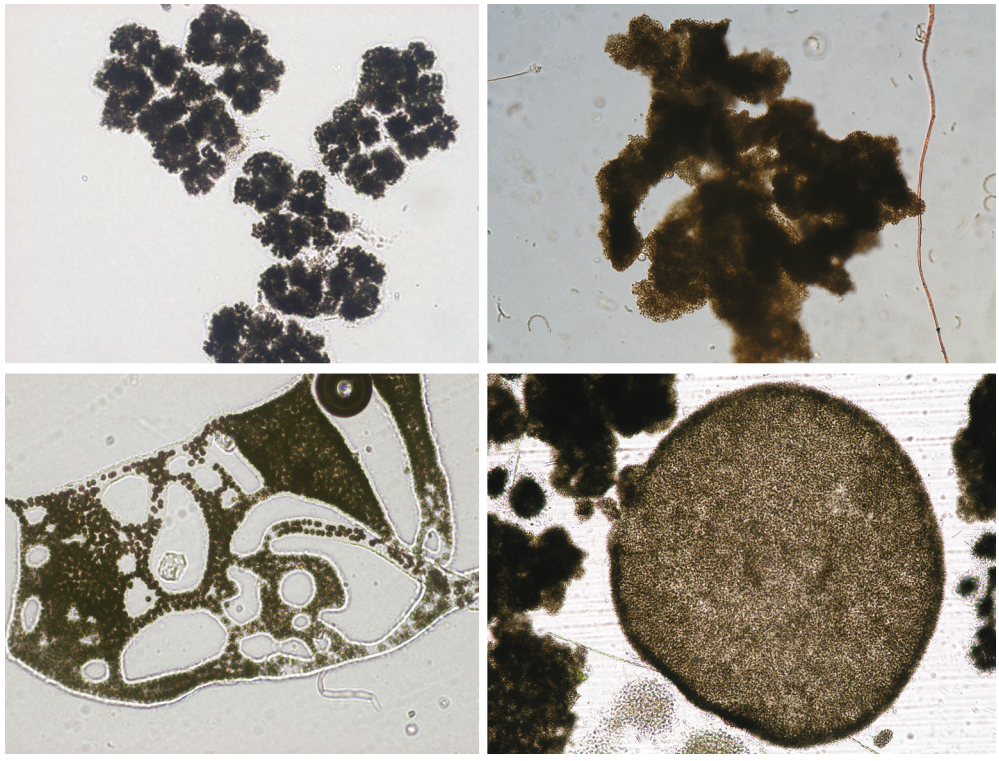


Figure 10. Further morphotypes of Microcystis: M. viridis (left up), M. ichthyoblabe (right up), M. wesenbergii (left down), M. flos-aquae (right down). Photograph by L. Šejnohová

Table 2. Delimitaion of eight *Microcystis* morphospecies occured in the Czech Republic according to the different approaches

Morphospecies	Cell diameter (um)	ITS+IGS	16S rRNA	Allozymes	Mcyst -synthetase	Peptide production
Microcystis aeruginosa	4-6.5 clear delimit.	delimit.	delimit.	delimit	delimit.	mainly microcystins (Fastner et al., 2001)
M. botrys	4.9-6					
M. ichthyoblabe	2-3.2 clear delimit.			delimit.		anabaenopeptins, microviridins, unknow peptides (Fastner et al., 2001, Sejnohova et al., 2008)
M. flos-aquae	3.5-4.8					similar peptide groups as *M. ichthyoblabe* (Sejnohova et al., 2008)
M. natans	1-2(3)					
M. novacekii	2.4-6					specific aeruginosisn and cyanopeptolins (Sejnohova et al., 2008)
M. viridis	4-7.9			delimit.		mainly microcystins and cyanopeptolins (Welker et al., 2007, Sejnohova et al., 2008)
M. wesenbergii	(4)6-8.5 clear delimit.	delimit.		delimit.	delimit.	no oligopeptides (Welker et al., 2004, 2006, Sejnohova et al., 2008) cyanopeptolins, unknow peptides (Fastner et al., 2001)

delimit. - delimitation of morphospecies by method mentioted in the column, morphospecies marked bold -
(sources: cell diameter - Anagnostidis and Komárek, 1999; Komárek and Komárková, 2002; ITS+IGS - Sanches et al. (2005); 16S rRNA - Neilan et al. 1997, Otsuka et al. (1998); Allozymes - Kato et al. (1991)

1998) and DNA-DNA hybridization showing high similarity (70-99%) among individual morphospecies, therefore there is an opinion that all morphospecies belong to one single type *Microcystis aeruginosa* (Otsuka et al., 2001) 2) comparison of 16S-23S rDNA internal transcribed spacer (ITS) dividing the *Microcystis* genus into three clusters irrespective of morphological findings (Otsuka et al., 1999); 3) comparison of genes encoding the main light-harvesting accessory proteins suggest the occurrence of more genotypes within a single morphospecies (Bittencourt-Oliveira et al., 2001); 4) Mcyst-synthetase, mcy genes or Mcysts production (Watanabe, 1996; Kurmayer et al., 2002; Via-Ordorika et al., 2004) and allozyme divergence (Kato et al., 1991) confirm the division of genus *Microcystis* into some morphospecies. Also a pioneering study concerning peptide diversity within a natural *Microcystis* population from a single lake indicated a correlation between specific taxa and oligopeptide patterns (Fastner et al., 2001). *M. aeruginosa* was found to contain mainly Mcysts, occasionally accompanied by aeruginosins. In contrast, Mcysts were not detected of *M.* ichthyoblabe; these colonies contained anabaenopeptins, microginins and unknown peptides. Colonies of *M. wesenbergii* produced mainly cyanopeptolins and unknown peptides (Fastner et al., 2001). A further study from six water bodies around Berlin (Welker et al., 2004) showed similar patterns of peptide co-production as mentioted above, but after the clustering of peptides no clear correlation between clusters (chemotypes) and morphospecies could be established. The Table 2 shows the delimitation of individual *Microcystis* morphospecies according to different taxonomical approaches.

1.3.4. Ultrastructure

The ultrastructure has been described with use of material from cultures (Jost and Zehnder, 1966; Jost and Jones, 1970) and from natural sources (Belikova, 1978; Reynolds et al., 1981) on various *Microcystis* morphospecies. The details of *Microcystis* cell are very simple and could changing during the various phases in growth and part of annual cycle (Reynolds et al., 1981) (see below the chapter 1.3.6.). It seems that ultrastructure features are same for all morphospecies. Some difference between morphospecies was found only in the structure of the proteinaceous surface in S-layer of cell wall with using a freeze-fracturing preparation, but this feature may be dependent on environmental conditions and it is not constant (Šmajs and Šmarda, 1999).

Cell wall. On the tripartite plasmalemma lie the cell wall which is consist from three uniform peptidoglykan layers and outer proteinaceous S-layer, which is responsible for protective coats, molecular sieves and molecule and ion traps, promoters for cell adhesion and surface recognition and as frameworks, determining and maintaining cell shape or envelope rigidity (Sleyter et al., 1988).

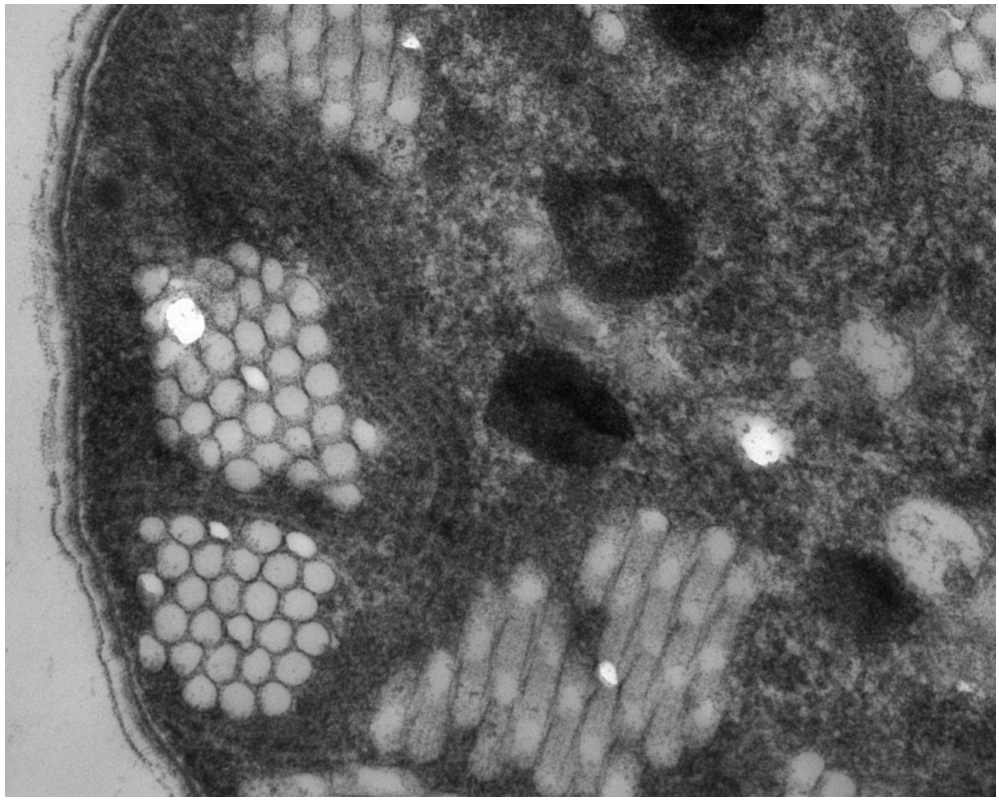


Figure 11. Ultrasctructure of Microcystis cell, aerotops, Photograph by O. Benada.

The S-layer is the simplest biological membrane forming two-dimensional crystalline arrays of identical single protein (or glycoprotein) subunits. The S-layer protein comprises up to ten percent of the total protein of the bacterial cell (Šmarda, 1991). The cell wall surface is surrounded by polysaccharide mucilage with associated heterotrophic bacteria (Brunberg and Bostrom, 1992).

Intracellular granules. The intracellular granules are not surrounding by a membrane and they contain mostly reserve substances. The main substances of reserve in the genus *Microcystis* are:
1) Glycogen granules (cyanophycean starch), which are first visible product of photosynthesis in cyanobacteria (Lang, 1968) and they are often with the close association with regions of the thylakoids. These reserves are present as "carbohydrate contents" and they are use for long-term store of energy. It occurs as white irregularly spherical form with the measurement 16x33nm, which are invisible in the light microscope.
2) Cyanophycin granules are unique to cyanobacteria (Lawry and Simon, 1982) and are use to store nitrogen and perhaps energy. Cyanophycin is a co-polymer of aspartate and arginine, which are essential component in the synthesis of gas vesicles and during nitrogen starvation is firstly degraded (Whitton, 2000).

Cyanophycin is accumulated when cells are starved of light, phosphorus or sulphur, and when grown at low temperatures (Allen, 1984). These granules are slightly angular and can be seen with light microscope (up to 500 nm across), even specific staining at the bounary between the centroplasm and chromatoplasm.
3) Phycocyanin is at first a major pigment component of the light-harvesting antenna, but under condition of nitrogen limitation it acts as a nitrogen reserve. During nitrogen starvation is degraded and than the cells are bleaching (Tandeau de Marsac and Houmard, 1993). When nitrogen is available phycocyanin is synthesized after the formation of cyanophycin (Allen, 1984).
4) Polyphosphate granules (volutin, PP) are form of highly polymerized polyphosphate and are use for the storage of phosphorus. *Microcystis* has a high Vmax for phosphorus uptake, a low minimum P content and a large capacity to accumulate phosphorus (Kromkamp et al., 1989a). The range volutin granules size in *Microcystis* is 100 to 400 nm in diameter (Jacobson and Halmann, 1982), stain with toluidine blue and dissolve in slightly acid enviroments. These granules contain metals, mostly potassium, calcium, magnesium and some heavy-metals too. For their formation is necessary ATP (Kromkamp, 1987).
5) Poly-B-hydroxybutyric acid (PHB) is a lipid polymer which forms vesicles about 200 nm in diameter and is abundant in *Microcystis* cell. It may be distinguished from polyphosphate by the presence of a 3 nm limiting monolayer (Reynolds et al., 1981).
6) Carboxysomes, formerly polyhedral bodies, are specialized cellular structures for reserve of the enzym RuBisCO which catalyses the photosynthetic fixation of CO2. With the low CO2 concentration elevated their numbers (Whitton, 2000). Under TEM these granules apper as black polyhedral bodies each 200-300 nm in diameter.

Gas vesicles (GV, Fig. 11). One of the diagnostic features in the genus *Microcystis* is presence of gas vesicles (GV). Gas vesicles, formerly termed as gas vacuoles (Anagnodtidis, 1961), are clearly unusual oval hollow gas-filled structures with conical end caps in certain prokaryotes from aquatic habitats (Walsby, 1972). Firstly GV have been observed using the light microscope in the cyanobacterial genera *Gleotrichia, Microcystis, Aphanizomenon and Nostoc* in 1895 (Ahlborn, 1895; Klebahn, 1895). The era of electron microscopy brought a surprising discovery. It was revealed on the extremely halophilic bacterium Halobacterium halobium, that "gas vacuoles" contain up of numerous, cylindrical gas vesicles which are integrated parallel in bundles so-called "aerotopes" (Houwink, 1956). The first similar results on cyanobacteria were published later in 1965 on the genus Aphanizomenon (Bowen and Jensen, 1965). In the following years were published many ultrastructure studies on the genus *Microcystis* focused on GV from material in cultures (Jost and Zehnder, 1966; Smith and Peat, 1967; Jones and Jost, 1970)

and whereupon from natural sources (Belikova, 1978; Reynolds et al., 1981). All investigation on structure and function of gas vesicles was reviewed in 1994 (Walsby, 1994).

The main function of GV is provide the buoyancy cell in response to changing environmental conditions in water column (Reynolds and Walsby, 1975). There have been described three mechanisms to regulation of buoyancy, which is dependent upon the previous nutrient or light history of the cell (Brookes and Ganf, 2001). In the beginning of 1920s the other idea suggest, that the GV are light shielding organelles, which can protection the cells with their reddish colour and with the increased their numbers during high light intensities (Walsby, 1972). The considerations that these organelles store gases from metabolism have been rejected long-ago (see below).

Before using electron microscope it was a matter of dispute, which material formed GV and what is their content. Gas vesicles under the light microscope seem to distinguish them from the other granules with their clearly bright, refractile qualities and reddish appearance (Walsby, 1972). First electron microcopy studies of the GV membranes have showed an unusual composition: they contain no lipid and are largely protein (Stoeckenius and Kunau, 1968). The membrane appear as three-layered and thickness is thinner than the typical cell unit membranes. In the halobacteria is GV membrane 8nm wide (Stoeckenius and Rowen, 1967), but in *Microcystis* only 3 nm (Jones and Jost, 1970).

In the genus *Microcystis* there are at least two types of GV membrane proteins:
GvpA - the main hydrophobic small protein arranged in a linear crystalline array along ribs. This protein forms the hollow shell of GV, is in all cyanobacterial genus as a main component of GV and must be responsible for many of GV properties (Walsby, 1994).
GvpC - the large hydrophilic protein on the outer surface, which is a minor component. The function is strengthening the GV, without this protein GV are weaker and collapse at a much lower critical pressure (Walsby and Hayes, 1988).

GV grows from a small biconical structure which is forming by the complicated process assembly of proteins encoding by gene in nucleoplasm, which can be incorporated into the plasmid. (see below). The biconical initials first enlarges to a critical diameter and then lengthens by extension of the cylinder that forms between the terminal cones (Waaland and Branton, 1969). The same type of formation proceed after collapse by impact of pressure but the new GV cannot be reerected directly from collapsed GV, but general de novo with recycling of some protein from destroyed membranes (Hayes and Walsby, 1984). The production of GV is very fast, in *Microcystis* GV may reach to their maximum length 600nm only during 12h.

The GV in all species of cyanobacteria have the same basic morphology: hollow cylinders with conical ends but vary in dimensions. Length varies widely also within the one cell, ranging from 0,1 μm to more than 1 μm, but the diameter is more constant (Šmajs et al., 1999). In each species the width is fairly uniform, only partly is forming of GV dependent on surrounding conditions (Walsby, 1994). The contradictory results showed, that width it is neither species, nor genus or family specific (Šmarda and Šmajs, 1996). The width of the GV is genetically controlled by means of natural selection. The survival phenotypes requires inheritance through genotypes that encode the phenotype in the next generation (Walsby, 1994). The critical collapse pressure of GV should be inversely related to their width (Walsby, 1971). Wider GV are more fragile and narrower ones less fragile, but wider GV providing buoyancy with the highest efficiency, narrower GV are to bee anticipated in organisms possessing high osmotic pressure. The genus *Microcystis* from culture and from freshwater reservoir has narrower GV (65nm) than genera *Planktothrix* (Šmarda and Šmajs, 1996) and *Anabaena* (Walsby and Bleything, 1988). The orientation of GV to the parallel bundles might increase gas permeability in cells (Walsby, 1994).

The GV are gas-filled and provide cells with buoyancy (Klebahn, 1895). The considerations that these organelles store gases such as nitrogen (Klebahn, 1922) or the other products of fermentation (Kolkwitz, 1928) have been rejected long-ago. The vesicle surface is freely permeable to gas therefore the internal gas composition is equal to that of the external gas composition in solution. GV are permeable only to small molecules as H2, N2, O2, CO2, CO, CH4 and Ar, because a diameter of pores in gas vesicle membrane is 0,36 nm. However, the liquid from protoplast cannot seep into the GV while the inner surface of membrane is hydrophobic (Walsby, 1969). The time required for equilibration of gas across the wall of GV are only in milliseconds or less with dependence on actual pressure (Walsby, 1984). In genus *Microcystis* the gases can diffuse inside the GV 1000x more quickly than by lipid-monolayer (Walsby, 1994).

The surface of GV is constantly exposes to source of pressure. The net pressure (pn) is given by total outer pressure (ph+pf +pt) minus the gas pressure inside the GV (pg).

$$pn = ph + pf + pt - pg$$

ph is the hydrostatic pressure of water column (0,01MPa/m), pf is the pressure of gas in the overlying gas phase (the atmospheric pressure is about 0,1MPa) and pt is additional pressure, which may be generate of cell surface (cell turgor pressure).

The gas inside GV is usually in equilibrium with the gas dissolved in the suspending water with the concentration c therefore pg balances the atmospheric pressure.

Consequently if the over pressure raises, the pressure transmit immediately to GV through the suspending water, and it may collapse them (Walsby, 1994). The gas from collapsed GV diffuses away rather than escaping as a bubble (Walsby, 1971).

The GV are rigid structures, which can undergoes little decrease in volume, when is pressure low, but at higher pressure they collapse irreversibly and then the cell loss the buoyancy (Walsby, 1971). The critical pressure in which GV collapse occurs varies widely in different cyanobacteria and it is dependent on elastic compressibility, which is determined by properties of protein from their membrane. The GV cannot collapse with the higher volume of compressibility, but the low volume is important in natural habitat for faster change in buoyancy (Walsby, 1994).
The median critical pressure is an inverse correlation between GV width and strength, in *Microcystis* is higher (0,77MPa), than in the another genera forming water blooms (*Anabaena, Aphanizomenon* 0,60MPa) (Walsby and Bleything, 1988).

Genes encoding the GV synthesis (gvp) has recently described in *Microcystis aeruginosa* (Mlouka et al., 2004b). In the cultures under standard growth conditions are forming the GV-deficient mutants, which can not synthesis GV proteins, although they have gene gvpA encoding the major GV protein. It was showed, that inactivation of genes gvp cause insertion sequences (IS), which is the mobile DNA elements (Mlouka et al., 2004a).

1.3.5. Autecology

Nutrients. Laboratory experiments have demonstrated that growth of cyanobacteria can be described as a function of the concentration of the limiting nutrient, especially phosphorus and nitrogen. The uptake rate (V) of the limiting nutrient into the cells can be described by the Michaelis-Menten as a function of the external nutrient concentration (s):

$$V = Vmax\ s/(Km + s)$$

Km ... half saturation constant for uptake

During stead-state growth the uptake rate of a nutrient is dependent upon the internal content according to:

$$q = \mu Q$$

where is: q...steady-state uptake rate, μ...the growth rate, Q...internal nutrient content

In general, micro-organisms respond to a limiting nutrient concentration by increasing Vmax (Rhee, 1980). A sudden increase of a nutrients leads to a rapid accumulation of them in the cells and the cells respond by decreasing up to an inhibition the uptake rate, which is define by the inhibition constant (k). This inhibition kinetics can be of ecological significance because species with a low constant can store more phosphate than species with a high k. For *Microcystis* constant k was measured as 0.8h. compare with *Oscillatoria* 1,1h. (Kromkamp et al., 1989a). With these attributes *Microcystis* does seem to be a storage specialist (Kilham and Hecky, 1988).

Phosphorus. The polyphosphate granules (volutin) were intensive syntheses, when the cells were transferred from medium almost without phosphorus (1,5mg orthophosphate/L) after 8 days to standard culture medium (10-320mg). In this case the synthesis of volutin was 25x higher than in the culture which was cultivated for the same time in standard culture (Jacobson and Halmann, 1982). Using flow cytometry it was showed that forms of GV in *M. aeruginosa* is significantly dependent on the total phosphorus. P-starved population produces less GV volume per cell, did not display large variability in the rate of density change of GV and cell photosynthetic rate is lower than in P-replete culture (Brookes et al., 2000). In P-limited (0μM) culture was minimal change in buoyancy, although there was a dilution of GV volume and increased carbohydrates. In the highest treatment (10μM) was observed the greatest buoyancy loss (Brookes and Ganf, 2001). In the phosphate limited conditions *Microcystis* outcompeted *Oscillatoria* due to the lower constant of inhibition k (see above) and the Vmax (110 and 30μgP/mg on proteins, respectively). With these storage attributes *Microcystis* can form 6x more new cells than Oscillatoria. *Microcystis* with the adaptation to phosphate limitation by increasing its phosphate uptake capacity and decreasing its light harvesting capacity might be an important organism in waters with a fluctuating P-supply (Kromkamp et al., 1989a). In the P-limited culture needed the cells of *Microcystis* after P-pulse at least 10h light for start synthesis of GV (Kromkamp et al., 1989b).

Nitrogen. The cells in N-limited (0μM, nitrate) culture suffered a dilution in GV and increased the carbohydrate contents, which resulted in a loss of buoyancy. When N was not limited (100μM) increased GV volume, metabolized carbohydrate more efficiently than N-limited culture and retained positive buoyancy (Brookes and Ganf, 2001). With the various supply rates of NH4 in the mixed chemostat cultures *Microcystis* was dominant only at the slow dilution rate (0,1d-1), at the higher (0,3d-1, 0,8d-1), was dominant *Scenedesmus quadricauda* (Chlorophyta) (Takeya et al., 2004).

Carbon. Most cyanobacteria have as a source of carbon a carbon dioxide, which is fixed to glycogen. Mobilizations of glycogen is caused by enzyme, which increases in the dark and thus it keeps a constant turnover of the glycogen, indicative of a strict regulation of carbohydrate storage (Kromkamp, 1987).

Light. The influence of constant high irradiance (90μmol/photons/m2/s) on mutant culture without GV in 25°C after 12 hours causes greatly decreased frequent of thylakoids and increased number of poly-β-hydroxybutyric acid (PHB), lipids bodies, the thickness of peptidoglykan layer, whereas cyanophycin granules observed only scarce. After 30 days intracellular reserves (PHB, glycogen granules) completely filled the cells, thylakoids were scare and culture was bleach. The floating was induced in the GV-free culture by PHB and lipid droplets (Canini et al., 2003). These conclusions were confirmed with the others experiments, when the cells in cultures exposed to high irradiance lost buoyancy by accumulation of carbohydrates which overcame the lift produced by GV (Thomas and Walsby, 1985b).

Light limitation has the opposite effect to nutrient limitation. When light is in short supply, gas vesicles tend to accumulate (Deacon and Walsby, 1990). The other responds of *Microcystis* cells to low light is an increase in pigmentation (Zevenboom and Mur, 1984). Thus when the cells are gives to high light intensities from light-limited conditions, than start the synthesis of the high level of polysaccharide (Kromkamp and Mur, 1984). The cells incubated in the dark following light exposure formed more GV if their previous irradiation was high and they have carbohydrate contents (Brookes and Ganf, 2001). During growth with short photoperiods (8h.) cyanobacteria are able to sustain relatively high growth rates and the loss of polysaccharide was minimized with the losses respiratory. The reduction of light intensity in the nutrient-limited cultures lowered the polysaccharide contents (Kromkamp, 1987). In the cultures growth in L:D cycle with the constant content of phosphate were detected diurnal fluctuations in buoyancy release only by carbohydrate contents whereas the number of GV was constant (Kromkamp et al., 1988). Light dependent formation of GV proteins was observed after the phosphate pulls in P-starved culture at a photon flux density of 50μmol/m2/s. GV formation did not occur in the dark. The length of the light period correlated with the increase in gas vacuolation (Kromkamp et al., 1989b). When a buoyant culture was transferred from photon flux density of 15 to 200μmol/m2/s the turgor increased from 2,5 to 4,3 bar, but these turgor was not enough for the GV collapse (Kromkamp et al., 1988). *M. aeruginosa* has weak darkness tolerance in 25°C, after 20 days darkness decreased initial cell number to only 1%. In contrast Scenedesmus (Chlorophyta) and Melosira (Bacillariophyta) retained their biomass even after 20 days. After restarting light-dark cycle, however, *Microcystis* increased exponentially and reached maximum biomass levels similar as *Scenedesmus* and *Melosira* (Furusato et al., 2004). The surviving of *Microcystis* cells in darkness is dependent on temperature too.

Temperature. Darkness tolerance in *Microcys*tis should be prolong in colder conditions (Furusato et al., 2004). Acclimate to temperature fluctuation (range

of 14-30°C) was more rapidly by *M. viridis* than *Selenastrum capricornutum* (Chlorophyta) (Fujimoto et al., 1994). In light-limited culture at 20 and 28°C was observed floating cells of M. *aeruginosa* during the entire light period whereas at 15°C the buoyancy was loss at the start of light period. *Microcystis* cultivated in light-dark cycle (18/6) with high photon flux (50μmol/m2/s) lost buoyancy in 8°C and 20°C during light period due to accumulation of carbohydrate (Thomas and Walsby, 1986). These results support resume, that the rate of photosynthesis (Pmax), specific respiration rate (Rest) and growth rate are temperature independent in the light-limited cultures, whereas in the light-saturated *Microcystis* cultures these characteristics are temperature dependent (Robarts and Zohary, 1987).

The buoyancy decreased at low temperature and mainly caused by an increased polysaccharide (decrease respiration rate of polysaccharide) and reduction in the GV volume. The light harvesting efficiency is independent of the temperature, thus the sudden drop in temperature causes a buoyancy loss. The maximal photosynthetic rate was temperature dependent, but that it was also dependent on the speed of growth temperature. The temperature dependency was greater at higher growth temperatures. It seems that *Microcystis* is a genus which will be develop in the temperate climates later in the growth season as the water temperature rises (Kromkamp et al., 1989b). These results correspond with meaning that *Microcystis* mass development is limited by low temperatures more severely than the other genera of bloom-forming cyanobacteria, with the sharp declines in growth rate below a critical temperature of about 15°C (Robarts and Zohary, 1987). The optimal temperature for photosynthesis in *Microcystis* was measured in 20°C and was the same as in genera *Anabaena* and *Aphanizomenon*. However, in the in 15°C *Microcystis* showed greatest drop to 4% of maximum photosynthesis, in contrast *Anabaena* and *Aphanizomenon* maintained rates of 76% and 48%, respectively (Konopka and Brock, 1978).

Pressure. The critical pressure which is necessary to collapse the GV in *Microcystis* ranged from 6-11 bar with the median critical pressure (causing 50% collapse) occurring at 7,5 bar (0,75 MPa) (Thomas and Walsby, 1985a). The turgor pressure increased as soon as the light period starts to a maximum in about 1-2h. and with increases the light intensity (Kromkamp and Mur, 1984).

Buoyancy regulation. The success of water-bloom forming cyanobacteria is often attributed to their ability to regulate buoyancy. There were described three mechanisms by which GV cyanobacteria can regulate their buoyancy (Whitton, 2000).

1) Changes in cell density through alterations in cellular composition.

Nutrient uptake has a feedback to the amount of accumulated nutrients in the

cell to the granules. Besides a storage function these granules are apply as a load for the sink during conditions of energy stress (Kromkamp, 1987). Buoyancy is regulate mainly with accumulates of carbohydrate (CHO) in response to the light, but the other cell constituents (starch and volutin) may also affect cell buoyancy (Kromkamp and Mur, 1984; Brookes and Ganf, 2001). CHO reserves are accumulation through photosynthesis and reduction through respiration (Walsby, 1994). In M. *aeruginosa* cell of 5μm diameter accumulating carbohydrate of density 1600kg/m3 (Kromkamp and Walsby, 1990). Only little influence on changes in buoyant density have ribosomes and small proteins (Walsby, 1980).
2) Changes in degree of GV by turgor pressure
Turgor pressure is generated within the cell as a result of photosynthesis, the magnitude of the increase depending upon the previous light history. When turgor pressure rise with the increase of irradiance, GV can collapse (Whitton, 2000).
3) Changes in degree of GV by synthesis and subsequent dilution by growth
The synthesis of GV is regulated at two levels. First is the molecular control through gene expression, second is physiological control by availability of energy and structural components (Whitton, 2000).

1.3.6. Life cycle

The annual cycle of *Microcystis* genus from temperate regions has been described on morphospecies *M. aeruginosa* (Reynolds et al., 1981). In this comprehensive study, authors followed the nomenclature of Kondrateva (1968), where only two separate species were recognized, *Microcystis aeruginosa* and *M. wesenbergii.* Within the species *M. aeruginosa*, Reynolds et al. (1981) distinquished the six morphological categories (stati) of colonies differ by size, shape, cell arrangement and cell ultrastructure (Tab. 4). The occurrence of individual stati depend on the phase of life cycle. The annual cycle includes overwintering in upper layers of sediment, reinvasion into water column in spring months, bloom and sinking of the population in autumn (Fig. 12).

Overwintering. Colonies of *Microcystis* overwinter in the lake sediment as vegetative cells not in special akinetes. Their photosynthetic apparatus evidently retained for one or more years. The ability to have survived long periods of darkness is presumably based upon endogenous respiration of stored carbohydrate (Reynolds et al., 1981). The amount of viable vegetative stocks depends upon the environmental conditions at the bottom and upon the physiological stage of cells (Brunberg, 1995). These factors also influence the relative concentration of the various morphological stati of colony (Reynolds et al., 1981). The overwintering phase includes the following stati: Ia, II+III, IV, Vb (Tab. 3). The volume of gas vezicles in the cells is relative high, nevertherless, colonies are negatively buoyant.

The explanation of loss buoyancy is the accumulation of storage products (polyphospate bodies, carbohydrates, proteinaceous structured granules) elevates mean colony density above that of the water (Reynolds et al., 1981). The number of survival colonies increased with the dark and the low oxygen conditions (Brunberg and Blomqvist, 2002; Cáceres and Reynolds, 1984). The benthic biomass may substantially exceed the maximum pelagic biomass (Bostrom et al., 1989), thus indicating that *Microcystis* colonies are able to survive for longer periods and accumulate at the bottom (Brunberg and Blomqvist, 2002).

Reinvasion. The onset of *Microcystis* bloom often coincides with the increasing of light (Caceres and Reynolds, 1984; Walsby, 1969) and temperature (Trimbee and Harris, 1984), anoxic conditions (Reynolds et al., 1981), low T:P ratio (Oliver, 1994; Stahl-Delbanco et al., 2003) and CO2 limitation (Klemer et al., 1996) over lake sediments. These environmental factors iniciate an anctive buoyancy change consist in the increasing of gas vezicles by simultaneous descreasing of carbohydrates in cells. According the latest studies the recruitment of *Microcystis* is also result of passive process, as bioturbation or wind-mixing (Verspangen et al., 2004). After the reinvasion almost 20% from the overwintering benthic stock remained in the sediment (Ihle et al., 2005) and could play an important role as a inoculum for the further recruitment if they remain vital an if they are able to leave the sediment (Preston et al., 1980; Reynolds et al., 1981; Reynolds and Rogers, 1976; Trimbee and Harris, 1984; Verspagen et al., 2004). During the reinvasion the number of gas vezicles in cells incrase by simultaneous descreasing of carbohydrates. The reinvasion stadium includes morphological st. IV and Vb of colonies (Tab. 3 and 4) that characterize by series of cell divisions gives rise to the cell clusters (Reynolds et al., 1981).

Bloom (Planktonic growth). The inicial stadium of planktonic growth is characterized by an exponentical encrease in the number of cells. The gas vezicles occupy the majority of cell volume (Reynolds et al., 1981). Colony morphology during the actively incerasing population varied between a preponderance of morphological stati Va and VI colonies to one of almost complete dominance by st. Ib colonies (see Tab. 3 and 4).

Sinking (Autumnal population decline). The loss of buoyancy in autumn is related to events accompanying autumnal overturn, such an increase in the ballast of storage products (Reynolds et al., 1981). The changes in carbohydrates levels suggest differences in relative rates of respiration and photosynthesis at lower temperatures. The study at lower temperatures concluded that the accumulation of carbohydrate at reduced temperature was the result of a lowered rate of protein synthesis during the light period (Visser et al., 1995). Another explanation for

the autumnal sedimentation is co-precipitation with the suspended particles (Oliver et al., 1995; Verspangen et al., 2004). In this case, sedimentation took place at all times of the year (Visser et al., 2005). The autumnal *Microcystis* population is formed by st. II and III colonies (Tab. 3 and 4).

Figure 12. Life cycle of Microcystis aeruginosa according to Reynolds et al. (1981)

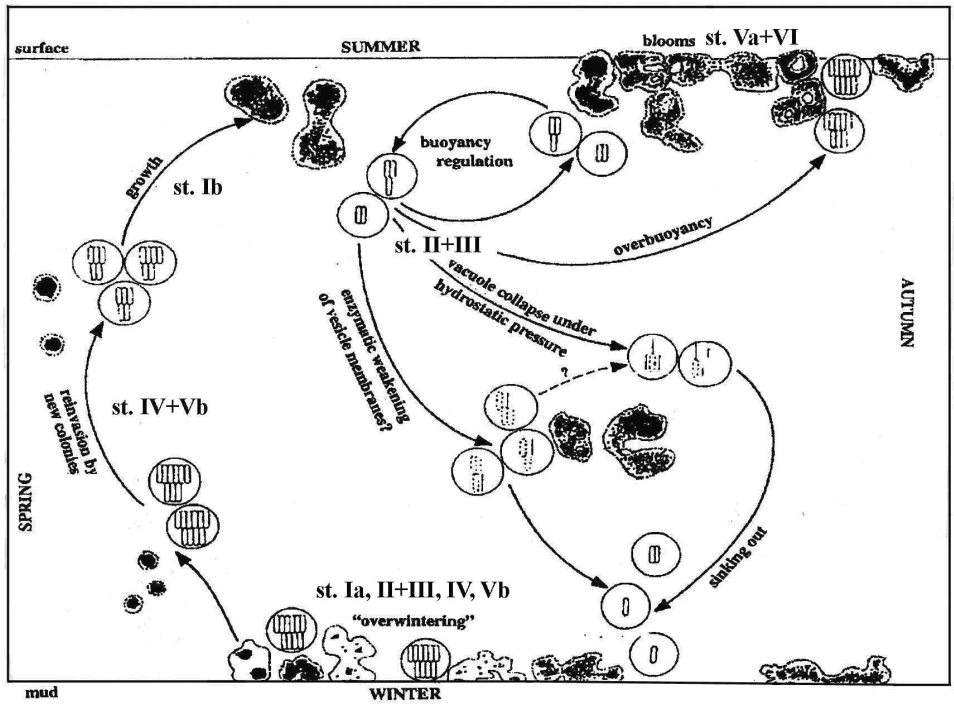
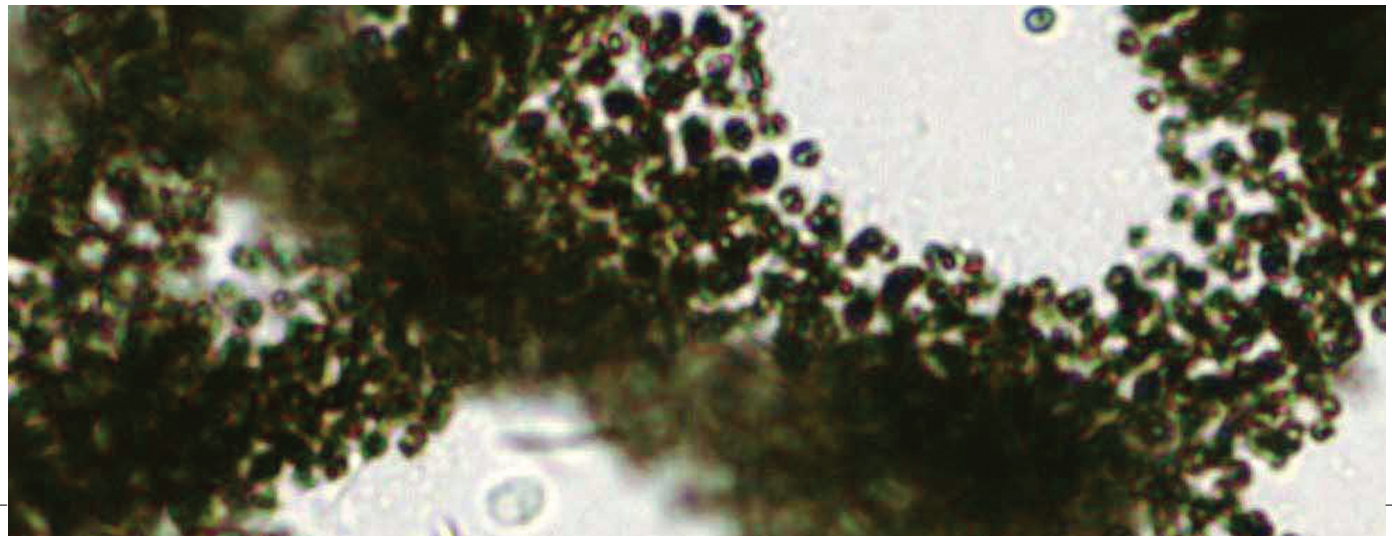


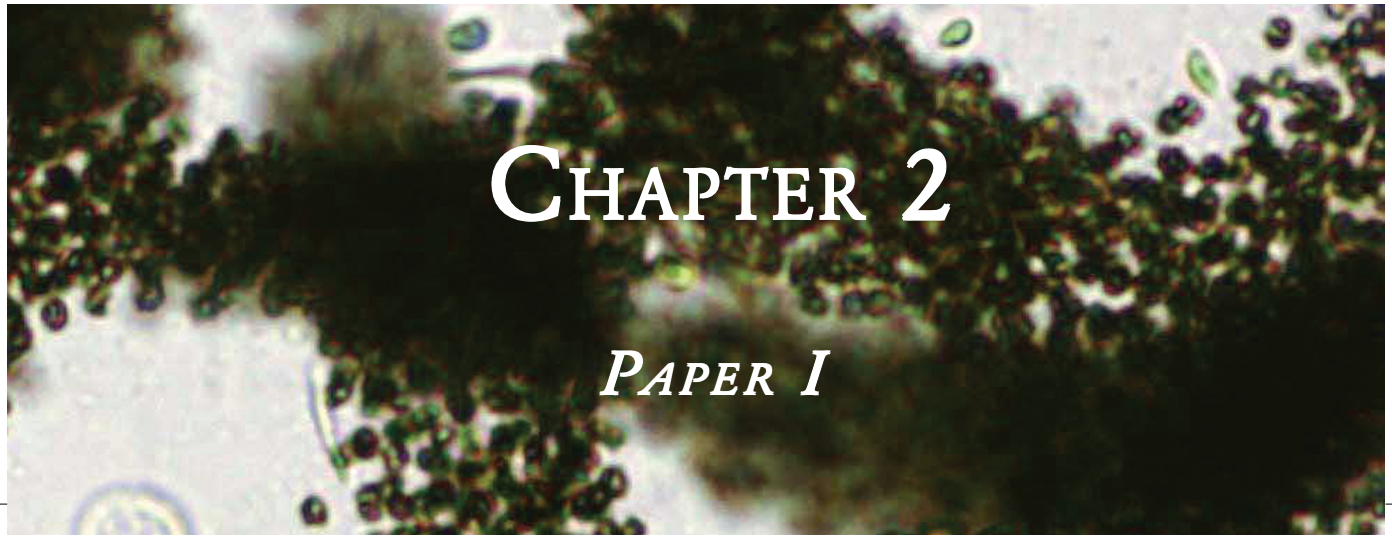
Table 3. Phases of Microcystis life cycle and occuerrence of individual morphological stati

Type of subpopulation	**Phase of annual cycle**	**Morphological stati of *Microcystis* colony according to REYNOLDS et al. (1981)**
pelagial	growth	Ib
pelagial	blooms	Va, VI
pelagial	regulation of buoancy	II+III
(late) pelagial	collapse and decline of gas vesicles, accumulation of storage products	II+III
(late) pelagial	sinking out	II+III
benthic	overwintering stocks	Ia, II+III, IV, Vb
(reinvaded) benthic	reinvasion	IV, Vb

Table 4. Description of six morphological stati by Microcystis colony according to Reynolds et al. (1981)

Status	Form of colony	Size of colony (µm)	Cell density organization	Cell density organization (in cells)	Size of mucilage	Physiological status	Subpopulation
Ia	elliptical or less regular mucilaginous bodies (cultivation invokes st. IV)	40-1000	diffuse, all the cells to be individually viewed	<1/ 1000 μm^3	large with the numbers of bacteria	senescent or moribund empty cells	benthic (high bacterial activity)
Ib	irregular ovoid or cylindrical colonies	max. 250	same as st.Ia	<1/ 1000 μm^3 (20-200live cells /col.)	no record	no senescent cells	early pelagic exponential growth
II **III**	irregular elliptical, high variability, fenestrated (II) striped (III) colony	40-300	cells compactly arranged, individual cells are indistinguishable	>3/1000 μm^3	10-45 µm	active (cultivation invokes st.Va)	late pelagic, benthic
IV	similar as st. Ia (by disintegration arise st. Vb)	no record	life cells form aggregations within the	20-200 live cells./col.	no record	stati for overwintering or for reinvasion	(reinvaded) benthic
Va	ovoid or spherical form	80-300 (570)	velmi kompaktní	3-5/ 1000 μm^3	3µm (active growing pop.) 35µm (end of season – September)	active	pelagic (large part)
Vb	spherical, small	40	no record	<100 cells/col.	2-5 µm	active-for regeneration of summer pelagial population	spring, early summer reinvaded benthic and early pelagic
VI	lobe formation, dividing colonies	no record	no record	no record	no record	active	pelagic





CHAPTER 2

PAPER I

Cell diameter by the bloom-forming cyanobacterium *Microcystis* correlates with the occurrence of specific toxic oligopeptides within the season

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Journal of Phycology (submitted)

The author's contribution in the article: Lenka Šejnohová participated in the experimental designing, performed part of the morphological analyses, evaluated data and interpreted results. She wrote parts of the article and finalized the entire manusript.

Abstract

We have clarified the relationship among four types of variables by *Microcystis* colonies: morphological features, oligopeptides presence, sampling date and sampling origin using canonical correspondence analysis (CCA). We sampled a large set of 833 single *Microcystis* colonies from pelagial and sediment in two water bodies throughout one season. We detected and identified 99 oligopeptides by MALDI-TOF MS.

Colonies with any mass signals for oligopeptides were characterized by cells 7.4±0.8 μm in diameter (*M. wesenbergii)* within the whole season in pelagial and sediment as well. Colonies with smaller cells than 6 μm were positive for oligopeptides according to the following patterns. Non-seasonal peptides were related entirely to cells with 3.3±0.7 μm (*M. ichthyoblabe, M. flos-aquae*) - aeruginosin 98-A, anabaenopeptin B, cyanopeptolin 962, microginin FR1 in pelagial; cyanopeptolins -972C, 1000 A, -1034A and 1006 D in sediment; anabaenopeptin F, microcystilide A and microviridin 1777 in both origin. Colonies with 5±0.4 μm cells (*M. aeruginosa, M. novacekii, M. viridis*) contained mainly seasonal peptides - cyanopeptolins 986 A and -1014 A in spring and cyanopeptolins 959, -1063, aeruginosin 602, Mcyst WR in autumn. We open up the discussion about the use of absence/presence of selected oligopeptides in cyanobacterial taxonomy.

2.1. Introduction

The genus *Microcystis* (Chroococcales) is the important bloom-forming cyanobacteria responsible for poisonings of domestic livestock, wild animals, fish, and humans (Zurawell et al., 2005). For the long time, the toxicity of *Microcystis* genus has been attributed to the best studied toxins microcystins (Mcysts) inhibit protein phosphate PP2A. Nevertheless, in recent years there is increase of studies ilustrated that genus *Microcystis* produce a large variety of "new" bioactive oligopeptides (Fastner et al., 2001a; Welker et al., 2003; Welker et al., 2006a; Welker and von Dohren, 2006) with the wide spectrum of effects on other organisms. Aeruginosins (Murakami et al., 1995), cyanopeptolins (Ploutno et al., 2002; Bister et al., 2004; von Elert et al., 2005; Czarnecki et al., 2006) and microviridins (Rohrlack et al., 2003) inhibit the trypsin activity; microginins inhibit aminopeptidases (Ishida et al., 1997); anabaenopeptins display no animal toxicity or inhibition of protein phosphatase (Harada et al., 1995). The occurrence and sources of microcystins variability within *Microcystis* genus is well studied on the space and time level (Kardinaal and Visser, 2005), however the knowledge about the patterns of other oligopeptide occurrence and their co-occurrence with Mcysts remind unexplored.

A pioneering study concerning whole oligopeptide diversity within a natural *Microcystis* population from a single lake (Fastner et al., 2001a) indicated a correlation between some specific taxa and oligopeptide patterns. *M. aeruginosa*

was found to contain mainly Mcysts, occasionally accompanied by aeruginosins. In contrast, Mcysts were not detected by *M. ichthyoblabe*; these colonies contained anabaenopeptins, microginins and unknown peptides. Colonies of *M. wesenbergii* produced mainly cyanopeptolins and unknown peptides. A single-sampling from the six water bodies around Berlin (Welker et al., 2004) showed similar patterns of peptide co-production as mentioned above, nevertheless no clear correlation between chemotypes (clusters of peptides) and morphospecies could be established by using PCA (principal component analysis). The latest research concern on seasonal change of peptides within *Microcystis* population showed 37 chemotypes by K-means clutering and one chemo-morphotype relationship (Welker et al., 2006b) were established.

Our study addresses the question whether the oligopeptides patterns are related to indvidual morphological features (not morphospecies, but to non-affected input data), the period of season or the origin of colonies. For this purporse we used larger set of single colonies (n=833) and multivariate approach as a valuable tool for visualising trends in natural population.

2.2. Materials and methods

2.2.1. Sampling, isolation and determination of colonies

In the period July-November 2004, the pelagial colonies were sampled monthly from Brno reservoir and once in September from Brilicky rybnik with a plankton net (mesh 42 μm) by vertical hauls. Sediment samples were taken from Brno reservoir twice in the season during the expected reinvasion of colonies in July and then after the sinking of the population in November from surface layer of 0-10 cm in shallow bay (depth 2-4 m) and from a deep area (14 m). Both artificial water bodies are situated in the Czech Republic (Central Europe) and are considered as hyperthrophic (Brno reservoir: N 49°, E 16°30', 259 ha, lake volume 15Mm3, maximum depth 19 m, the Svratka river, TP 170 μm/L, TN 2,5 mg/L; Brilicky pond: N 49°, E 15°00', 20 ha, maximum depth 4 m, TP 250 μm/L, TN 3,5 mg/L). Both lakes suffer from extensive cyanobacterial blooms of *Microcystis*. Samples were rediluted and colonies were selected randomly under the binocular microscope and carefully washed several times in droplets of sterile water. Each colony was then observed and measured under the light microscope at 400x magnification; the morphological features important for the taxonomic determination (Komárek and Komárková, 2002) were recorded (Table 2). Colonies processed in this manner were documented with microphotos and then placed on a stainless steel template for MALDI-TOF mass spectral analysis.

2.2.2. Mass spectrometry.

Dried colonies were extracted directly on the template by placing, dependent on the colony size, a droplet of 0.1 to 0.5 μL of a matrix solution directly on the colony. The matrix solution was 20 mg of 2,5-dihydroxy benzoic acid dissolved

in 1 mL of a mixture of water:acetonitril:ethanol (1:1:1) acidified with 0.1 % v/v trifluoro acetic acid. MALDI-TOF mass spectral analyses were carried out on Voyager-DE PRO Biospectrometry workstation as described previously (Welker et al., 2004). Metabolites detected with intense mass signals were further characterized by post-source-decay fragmentation (PSD). Detection and identification of oligopeptides was described in detail previously (Welker et al., 2006a).

2.2.3. Mass spectral data processing and statistical analysis

A presence/absence data set of individual peptides was generated for each colony and data of all colonies were unified in a data matrix finally containing 99 peptides in 833 colonies. With the aim to assess the relationshipship between peptides in colonies, the characteristics and origin of the colonies and sampling date, we used a multivariate statistical method canonical correspondence analysis (CCA) and the program CANOCO version 4.5 (ter Braak and Smilauer, 2002). The matrix of explanatory variables included 33 parameters characterising the colonies (Table 2). The forward selection of explanatory variables with the Monte Carlo permutation test was used in order to select variables significantly influencing the occurrence of peptides. Variables were taken as significant if the permutation test derived $P < 0.05$. Colonies from pelagial and sediment were analysed separately.

2.3. Results

From a total 833 *Microcystis* colonies analysed by MS, we detected 99 oligopeptides. One third of them (Table 2, full index see Welker et al., 2006a) occurred with the best fit frequency (>5%, CCA). Eleven (Fig. 1A) and five (Fig. 2A) characteristics of *Microcystis* colony used as explanatory variables in CCA (Table 2) were confirmed by the Monte Carlo permutation test to have a significant effect ($P < 0.05$) on peptides occurrence in pelagial and sediment, respectively. Neither the size of the colonies (range 200-3500 μm), nor their form (Table 2) statistically influenced the peptide distribution in the colonies. All colonies with distinct and distant mucilage not overlapping the cells 7.4±0.8 μm in diameter, determined as *M. wesenbergii* (n=94), gave no peptide mass signal neither in the pelagial nor in sediment.

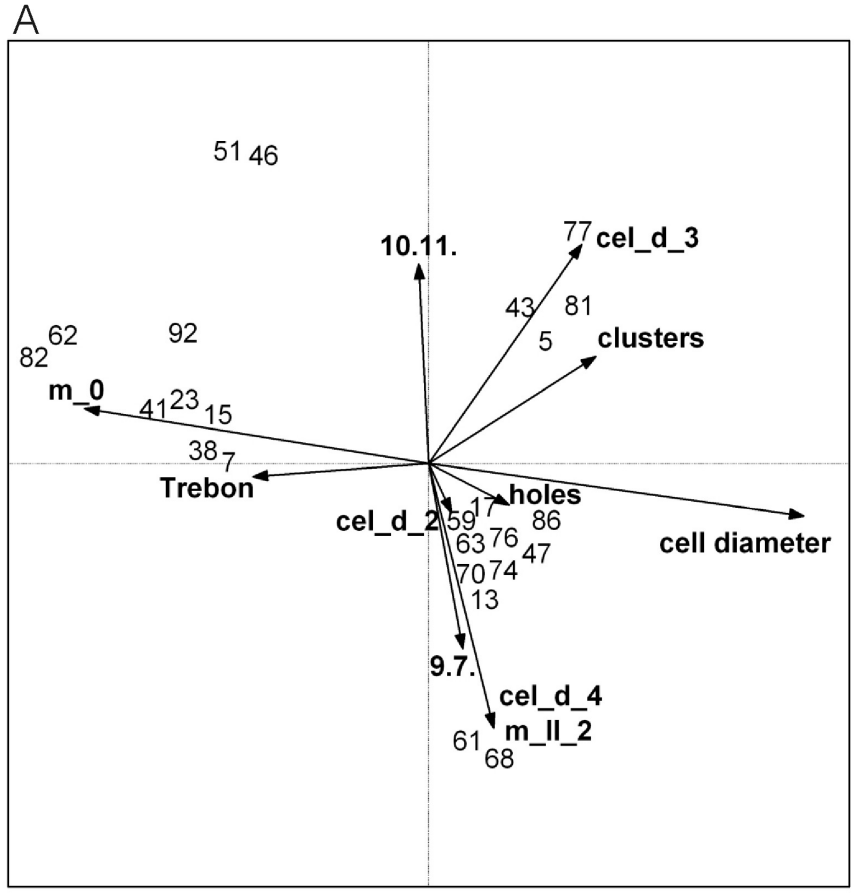
2.3.1. Pelagial

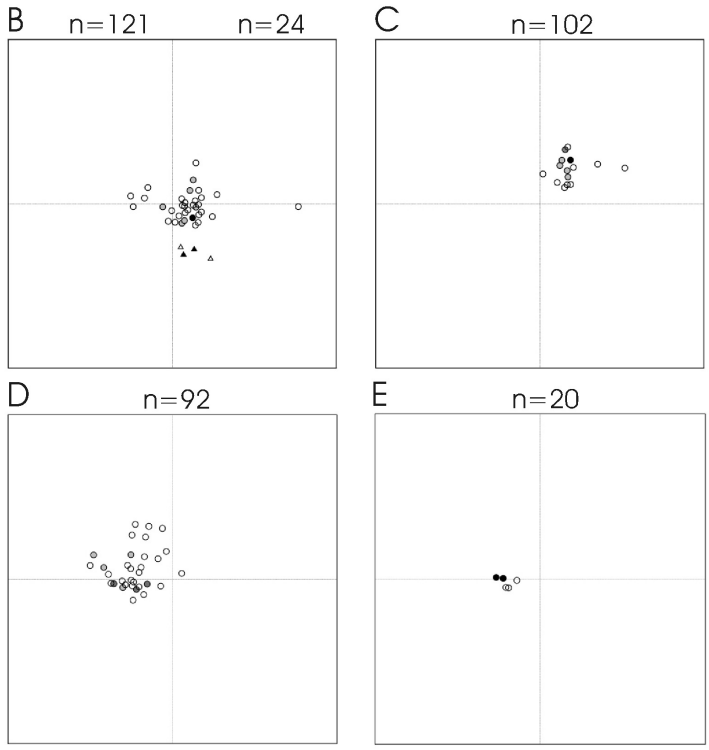
From 443 pelagic *Microcystis* colonies we analysed 84 peptides, from which 25 best fitted into ordination space (>5%; Table 1). The most important variables correlated with the peptide distribution in pelagial (Fig. 1A) were cell diameter (correlation with first axis 0.894), indistinct mucilage (correlation with first axis -0.818) and season (date 9.7. and date 10.11. correlation with second axis 0.4372 and 0.4711, respectively).

The smaller cell size (3.3±0.7 μm) and invisible mucilage (m_0) corresponded

Fig. 1. Pelagial - A. Canonical Correspondence Analysis (CCA) ordination plot as a biplot of 11 significant explanatory variables (arrows; for abbreviations see Table 2) and peptides (numbers=ID; for names see Table 1) in Microcystis colonies from two water bodies in the Czech Republic during the period July-November 2004. Of the 84 peptides included in the analysis, only peptides with best fit (>5%) are shown (A). Small figures (B-E) show the position of single colonies with regard to morphospecies in the same ordination space: M. aeruginosa-circle and M. viridis-triangl n=24 (B), M. novacekii (C), M. ichthyoblabe (D), M. flos-aquae (E) - n show the count of colonies; the percentage of colonies within the morphospecies is marked by colour of dots: <2% white, <10% light grey, <20% dark grey, >20% black. M. wesenbergii is not illustrated, because none of its colonies contained any peptide.

The first two canonical axes accounted for 5.9% of the variance of peptides presence and 44% of the peptide-explanatory variables (morphology and course of season) relationship. The eigenvalues of the two first canonical axes are $\lambda 1 = 0.581$ and $\lambda 2 = 0.436$.





mainly to the high frequency of occurrence (at least 38%) of microginin FR1 (ID 23), anabaenoptins F (ID 41) and - B (ID 38), cyanopeptolins 964 (ID 62), microcystilide A (ID 82) and aeruginosin 98 A (ID 7) independent on the season. Colonies showing these features were determined generally as M. ichthyoblabe (Fig. 1D) or *M. flos-aquae* (Fig. 1E). *M. ichthyoblabe* colonies contained Mcysts in 30%, however any Mcysts were found by *M. flos-aquae*.

In contrast, larger cells with 5±0.4 μm in diameter were characterized by significantly higher Mcysts annual frequency ($\div 2 = 25.33$; df=1; $P < 0.001$; 52.7% large and 24.5% small cells) and by presence of different peptides than microginins and anabaenopeptins. Colonies with cubic-arragement with mucilage clearly overlaping the cells (Fig. 1A cel_d_4, m_II_2; determinated as *M. viridis* Fig. 1B triangles) sampled in the spring were positive all for Mcysts, cyanopeptolins 986A and - 1014. Hole-type colonies with not densely regularly agglomerated cells (Figs. 1A cel_d_2; determinated as *M. aeruginosa* Fig.1B) produced mainly wide spectrum of Mcysts with high frequency (75%). Colonies forming clusters with densely and irregularly agglomerated cells (mainly determined as *M. novacekii*) occurred in autumn differed by presence of aeruginosin 602 (ID 5), cyanopeptolin 959 (ID 43), Mcyst WR (ID 81) and were characterized by low frequency of Mcysts (14.7%). When regarding whole *Microcystis* population significant decrease of Mcysts frequency was found ($\div 2 = 74.26$; df=3; $P < 0.001$) from spring (65%) to autumn (10%) in pelagial.

Table 1: The frequency of peptide occurrence with the best fit (CCA, > 5%, Fig. 1 and 2) from pelagic (25 peptides) and sediment samples (23 peptides) of Microcystis colonies (Table 2) with regard to cell diameter and morhospecies. 833 colonies were isolated from Brno reservoir and Brilicky pond during the period July-November 2004; peptides, which exceeded 30% are marked bold, smaller number font indicates additional peptides.

ID	m/z	Peptide name	Sediment			Pelagial				
			M.icht. 3.3±0.7	M.aer. 4.8±0.6	M. nov. 5±0.1	M.icht. 3±0.4	M.f-a. 3.9±0.2	M.aer. 5±0.5	M.nov. 5±0.2	M.vir. 5±0.2
5	603	Aeruginosin 602	25.7	61.7	76.5	8.7	5	57.9	84.3	0
7	609	Aeruginosin 98-A	-	-	-	13.0	15	1.7	0	0
13	609	Aeruginosin 89	26.5	32.8	9.4	14.1	5	33.9	2.9	75
15	643	Aeruginosin 101	-	-	-	5.4	15	0.8	0	0
16	653	Aeruginosin 102	5.3	6.7	16.5	4..3	0	14.0	6..9	0
17	671	Aeruginosin 670	24.8	31.7	8.2	7.6	5	33.9	2.9	0
23	728	Microginin FR1	1.8	0	0	44.6	45	0.8	0	0
38	837	Anabaenoptin B	11.5	2.2	0	38.0	75	0.8	0	0
41	851	Anabaenoptin F	14.2	2.2	0	42.4	75	0	0	0
43	880	Cyanopeptolin 959	0	0	8.2	1.1	0	1.7	5.9	0
46	882	Cyanopeptolin 881	-	-	-	3.3	0	0	0	0
47	921	Cyanopeptolin 920	8	31.1	8.2	3.3	0	38.8	2.0	0
51	916	peptide 915	-	-	-	3.3	0	0	0	0
53	955	Cyanopeptolin 972C	28.3	6.7	0	-	-	-	-	-
58	973	Cyanopeptolin 972A	8.8	8.9	3.5	2.2	0	3.3	1.0	0
59	981	[Asp3] Mcyst-LR	1.8	3.9	5.9	18.5	0	48.8	9.8	66.7
60	983	Cyanopeptolin 1000A	31	7.8	0	-	-	-	-	-

61	985	Cyanopeptolin 986A	0.9	2.2	3.5	1.1	0	2.5	0	100	
62	965	Cyanopeptolin 964	0.9	0.6	0	12.0	0	0	0	0	
63	995	Mcyst-LR	54	60.0	30.6	27.2	0	72.7	14.7	100	
65	1007	Cyanopeptolin 1006A	7.1	8.9	3.5	1.1	0	4.1	1.0	0	
67	1011	Cyanopeptolin 1006D	27.4	6.7	0	-	-	-	-	-	
68	1015	Cyanopeptolin 1014A	0	1.1	3.5	1.1	0	2.5	0	100	
70	1024	[Dha7] Mcyst-RR	3.5	15.6	5.9	7.6	0	36.4	2.9	66.7	
72	1029	Mcyst-FR	8.8	0.6	12.9	1.1	0	0.8	4.9	0	
74	1038	Mcyst-RR	19.5	48.3	16.5	16.3	0	54.5	8.8	100	
75	1039	Cyanopeptolin 1034A	30.1	6.7	0	1.1	0	0	0	0	
76	1045	Mcyst-YR	48.7	51.1	25.9	18.5	0	49.6	11.8	87.5	
77	1064	Cyanopeptolin 1063	0	28.9	68.2	0	0	21.5	80.4	0	
78	1049	Mcyst-H4YR	0.9	2.2	15.3	7.6	0	3.3	8.8	0	
81	1068	Mcyst-WR	0	0	8.2	0	0	1.7	5.9	0	
82	1073	Microcystilide A	29.2	6.7	0	10.9	0	0	0	0	
85	1136	peptide 1135	6.2	1.1	0	-	-	-	-	-	
86	1265	peptide 1264	0.9	16.7	3.5	1.1	0	24.0	2.0	0	
92	1778	Microviridin 1777	12.4	5.6	0	8.7	0	0	2.9	0	
		Number of colonies	113	180	85	92	20	121	102	24	

Table 2. Twelve features of Microcystis colony used as explanatory variables in CCA (Fig. 1 and 2). By means of Monte Carlo permutation test eleven and five of them were selected as significant (P < 0.05), in pelagial (P) and sediment (S), respectively.

Characteristics	Explanatory variables	Significant variables
Date of sampling	9.7.2004	9.7. (P, S)
	25.8.4004	
	1.9.2004	
	8.10. 2004	
	10.11. 2004	10.11. (P, S)
Locality	Brno - Stred	
	Brno - Rokle	
	Trebon - Brilicky pond	Trebon (P)
Cell diameter	2.5-9 μm	cell diameter (P, S)
Origin	pelagial	analysed separately - Fig. 1
	sediment	analysed separately - Fig. 2
Mucilage type I.	no	m_0 (P)
	diffuse	
	distinct	
Mucilage type II.	slightly overlapping the cells	
	clearly overlapping the cells	m_II_2 (P)
	distant slime, not overlapping the cells	
Size of mucilage	0-75 μm	size muc (S)
Cell density	densely regularly agglomerated	
	not densely regularly agglomerated	cell_d_2 (P)
	densely irregularly agglomerated	cell_d_3 (P)
	not densely irregularly with clearly cubic arrangement of cells	cell_d_4 (P)
	not densely irregularly with lobates	
	not densely irregularly agglomerated	
Form of colony	irregular	
	spheroidal	
	irregularly spheroidal	
Holes	yes	holes (P)
	no	
Clusters	yes	clusters (P, S)
	no	
Size of colony	(50) 200-3700 μm	

2.3.2. Sediment

From 390 benthic *Microcystis* colonies we analysed 63 peptides, from which 23 best fitted into ordination space (>5%; Table 2). The cell diameter (correlation with first axis 0.961), size of mucilage (correlation with first axis 0.4301; size muc) and date of sampling (date 9.7. and 10.11., correlation with second axis -0.8567 and 0.8567, respectivelly) most correlated with peptide distribution in sediment (Fig. 2A). The cells with 3.1±0.5 μm in dimater were positive for anabaenopeptin F (ID 41), cyanopeptolins 972C (ID 53), -1000A (ID 60), -1006D (ID 67), - 1034A (ID 75), microcystilide A (ID 82), peptide 1035 (ID 85) and microviridin 1777 (ID 92). Colonies producing combinations of these peptides were identified mostly as *M. ichthyoblabe* (Fig. 2D). Within the larger cells (4.9±0.5 μm) we found two peptide groups differed by correlation with morphology and season (Fig. 2A). Presence of aeruginosin 102 (ID 16), Mcyst-RR (ID 70), Mcyst-RR (ID 74), cyanopeptolin 920 (ID 47) and peptide 1265 (ID 86) correlated with the size of mucilage on the colonies (Fig. 2A, size muc) independently of the season. Cyanopeptolins 959 (ID 43), -1063 (ID 77), Mcys-WR (ID 81) and Mcyst-H4YR (ID 78) were found in the colonies forming clusters simultaneously with the september date of sampling. Colonies with these features were identifed as *M. aeruginosa* (Fig. 2B) or *M. novacekii*. In contrary to pelagial, Mcysts occurred without any significant relationship ($x2 = 1.39$; df=1; $P>0.05$) to the cell diameter in sediment (Fig. 2A). The frequency of all Mcysts variants was significantly dependent on the course of the season. In July, Mcysts were detected in 71% of colonies, whereas in November only in 30% ($x2 = 65.86$; df=1; $P < 0.001$).

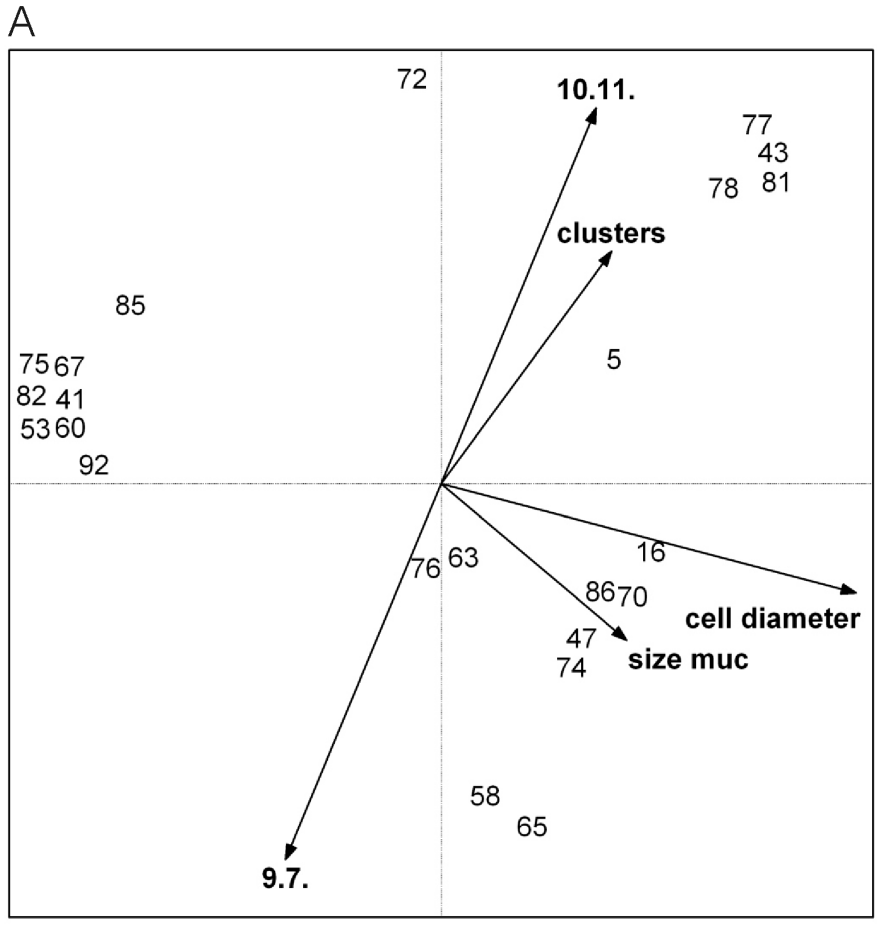
2.4. Discussion

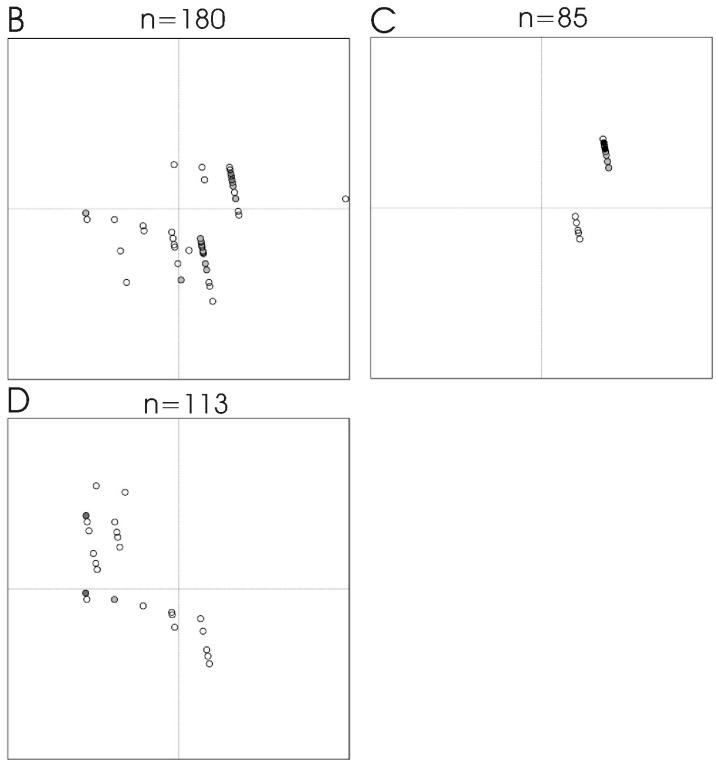
It is well known that some morphological characteristics of *Microcystis* colony may change with time (Kruger et al., 1981; Reynolds et al., 1981; Otsuka et al., 2000). Consequently, the determination of morphotypes can be difficult, influence by the experience of researcher and occasionally incorrect. For these reasons, in our study peptide composition was refered primary to individual morphological characteristics of *Microcystis* colonies (Table 2, Fig. 1 and 2).

Our results show that the oligopeptide occurrence is significantly related mainly to the cell size of *Microcystis*. Within these cell-size categories further effect on peptide composition have sesaon and origin of colonies (Fig. 1 and 2). The small cells (3.3±0.7 and 3.1±0.5 μm, pelagial and sediment) were positive mainly for anabaenopeptins B and -F, microginin FR1 and several variants of cyanopeptolins. These peptides were not found together with Mcysts in large cells (5±0.5 and 4.8±0.6 μm), which is in agreement with the earlier results (Fastner et al., 2001a). Similary, a genetic study on cultures isolated from Lake Wannsee separated *Microcystis* strains into two groups: a larger-cell group produced Mcysts and a small-cell group did not (Rohrlack et al., 2001). Moreover, there is

Fig. 2. Sediment - A. Canonical Correspondence Analysis (CCA) ordination plot as a biplot of 5 significant explanatory variables (arrows; for abbreviations see Table 2) and peptides (numbers; for details see Table 1) in Microcystis colonies from the Brno reservoir in July and November 2004. Of the 63 included peptides in the analysis, only peptides with best fit (>5%) are shown (A). Small figures (B-D) show the position of colonies with regard to morphospecies in the same ordination space: M. aeruginosa (B), M. novacekii (C), M. ichthyoblabe (D) - the percentage of colonies within the morphospecies is marked by colour of dots: <2% white, <10% light grey, <20% dark grey, >20% black. M. flos-aquae and M. viridis are not illustrated, because data originate from one colony only; M. wesenbergii contained any peptide.

The first two canonical axes accounted for 5% of the variance of peptides occurence and 80.6% of the peptide-explanatory variables (morphology and course of season) relationship. The eigenvalues of the two first canonical axes are $\lambda 1 = 0.480$ and $\lambda 2 = 0.266$.





a third group with the largest cells (7.4±0.8 μm, determined *M.wesenbergii*) with any mass signal for oligopeptides.

The observation that the largest cells (*M.wesenbergii*) are without peptides is in agreement with the majority of studies according to which this morphotypes contained little or no Mcysts (Marsalek et al., 2001), gave no PCR product of the mcy genes (Rohrlack et al., 2001; Kurmayer et al., 2002; Via-Ordorika et al., 2004), and generally had any mass signal for peptide metabolites (Welker et al., 2004). In contrast to our results, colonies of *M. wesenbergii* were all found positive for cyanopeptolin in Lake Wannsee (Fastner et al., 2001b) indicating that within this morphospecies different chemotypes could be present. For a *Microcystis* population in Danish lakes dominated by M. wesenbergii hepatotoxicity was reported (Henriksen, 2001), but other morphospecies - especially *M. aeruginosa* were present, so the the occurrence of microcystins can not be related only to *M. wesenbergii*. Nonetheless, in laboratory experiments some Mcyst-positive strains of *M. wesenbergii* were demonstrated (Otsuka et al., 1999), but there could be posibility of the incorect morphotypes determination. The evidence that *M. wesenbergii* differ from other morphotypes might provide also allozyme divergence (Kato et al., 1991).

That the large cells (5±0.5 and 4.8±0.6 μm) are positive mainly for

aeruginosins and Mcysts concluded also former studies (Fastner et al., 2001a; Kurmayer et al., 2002; Via-Ordorika et al., 2004; Welker et al., 2004). However, the usage of Mcysts in chemotaxonomy is disputable because their frequency of occurrence significantly decreased in the dependence on season and origin. These findings supported also studies on Mcysts production affected by enviromental factors (Jungmann et al., 1996; Kaebernick and Neilan, 2001; Carrillo et al., 2003; Kardinaal and Visser, 2005).

The small cells (3.3±0.7 and 3.1±0.5 μm, determined as *M.ichthyoblabe, M.flos-aque*) were characterized by the production of anabaenopeptins and microginins in Brno reservoir (Table 2) as well in lakes around Berlin (Fastner et al., 2001a; Welker et al., 2004). In addition, we detected cyanopeptolins 972C,-100A, -964 and microcystilide A. Only low percentage of these colonies (30.4 %) produced Mcysts with comparison of large cells (75%) in the Brno reservoir. The evidence of importance of Mcysts genes and their expresion besides environmental factors is visible on example of *M. ichthyoblabe*. The genetic studies found mcy synthethase genes in low proportion by *M. ichthyoblabe* (16-20 %) (Kurmayer et al., 2002; Via-Ordorika et al., 2004). Moreover, although this morphotype in Lake Wannsee was positive for mcy genes in 2000 (Kurmayer et al., 2002), the same morphospecies gave no mass signal for Mcysts in the same lake in 1999 (Fastner et al., 2001a). These contradictory results may originate from the development of *M. ichthyoblabe* population dominated by a clone lacking microcystin genes or by inactivation of the mcy operon (Kurmayer et al., 2004). Such an inactivation of oligopeptide production is supported by study investigating Microcystis colonies from various water bodies within Europe that found only 2.5% of colonies containing mcy genes but unable to produce detectable Mcysts (Via-Ordorika et al., 2004).

Previously we defined 37 chemotypes by K-means clustering (Welker et al., 2006b). Due to PCA we found that particular morphotype can be represented by multiple chemotypes in the dependent on season and origin pelagic or benthic colonies. In this paper, we found moreover some peptides repeatedly defined particular morphotypes during the seasonal shift. Typical example are the anabaenoptins, microviridins and microginins, which occurred only in the colonies with the small cells (M.ichthyoblable, M. flos-aquae) independently on the season. Another case is the occurrence of Mcysts which were produced by small (~ 3 μm) such as large cells (~ 5 μm) in the dependent on season. We hypoteses that particular morphotype (or rather cell size group defined in this paper) could be defined by the stable peptides (e.g. anabaenopeptins) and unstable peptides (e.g. microcystins). Consequently, each morphotype seems to be represent by several chemotypes due to unstable parts.

The composition and even concentration of the certain peptides could be connected with their ecological role during the process of colony formation, reinvasion etc. Even we have too limited information concerning this role; we

can see the differences in the composition of peptides withih the same morphotype of the benthic and pelagial origin. As an example we can mentioned the peptides of small benthic cells - cyanopeptolins 972C, -1000A, -1034 (Fig. 2), which never occurred in the small Microcystis cells of the pelagial origin. These peptides can be synthetised as a competitive advantage during the process of colony formation or during the reinvasion. Similar ideas are discussed also by Welker et al. (2006).

Microcystis "species" concept. In the past decade morphological criteria alone were criticised increasingly to be inadequate to characterize *Microcystis* species (and cyanobacterial species in general).
However, our findings support the traditional taxonomic approach, according to which the cell diameter plays an important role on subgeneric level within the genus *Microcystis* (Anagnostidis & Komárek, 1999). We confirm also the previous results (Fastner et al., 2001b) suggesting that several *Microcystis* morphotypes (*M. ichthyoblable, M.aeruginosa and M. wesenbergii* morphospecies) are significantly related to some oligopeptides. Moreover, we enlarge this chemotaxonomical differentiation by the new peptides and findings that Mcysts is not reliable for the distinction of *Microcystis* morphotypes, because their frequency fluctuated depending on season and origin. In general, we conclude that the taxonomic unification of the all morphotypes based on the genomic DNA homologies (Otsuka et al., 2001) seems to be premature. In spite of the well known morphology modification by *Microcystis* colony (Doers & Parker, 1988, Bittencourt-Oliveira, 2000, Otsuka et al., 2000) cell size remain stable within individual morphotypes (Sanchis et al., 2004). We open up the discussion about the integration of absence/ presence of selected oligopeptides in polyphasic taxonomy.

2.5. Acknowledgments

This study was supported by the Ministry of Education and Youth of the Czech Republic the grant no. 0021622412, the project no. AV0Z60050516 of the Academy of Sciences of the Czech Republic and the EU-research project PEPCY.
We thank our colleagues Martina Sadílková and Hana Slováčková for their valuable help during the colonies sampling and isolation. Special thanks to Jiří and Jaroslava Komárek for help during the determination. In addition, we are grateful to Petr Šípek, Lenka Volavková and Jakub Gregor for the linguistic revision.

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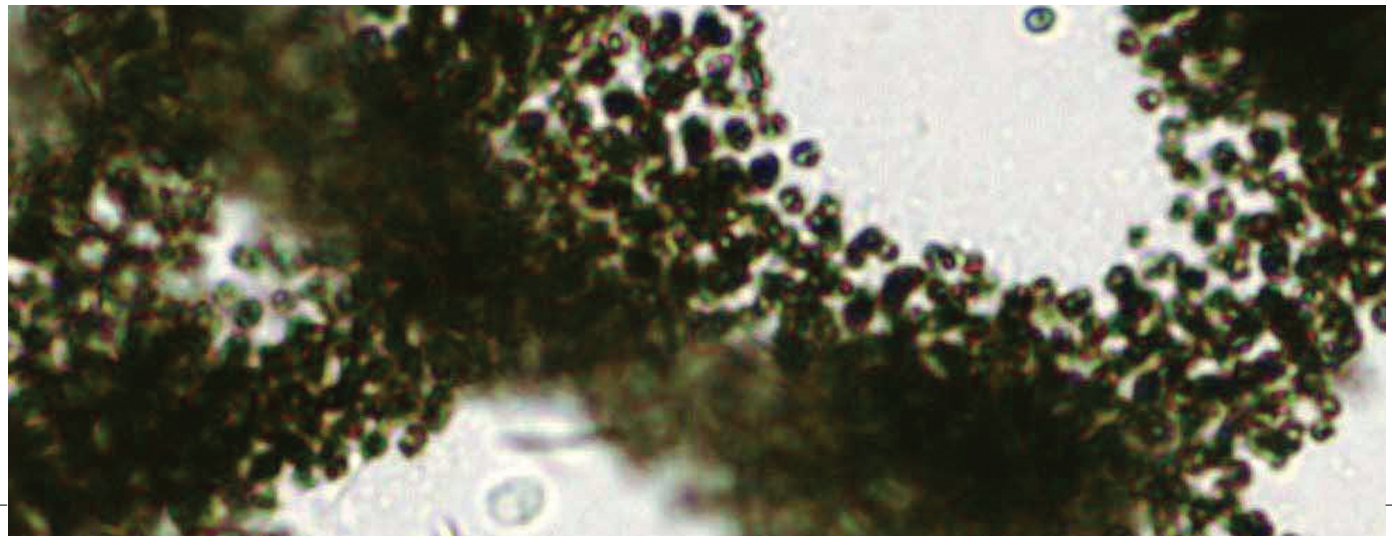

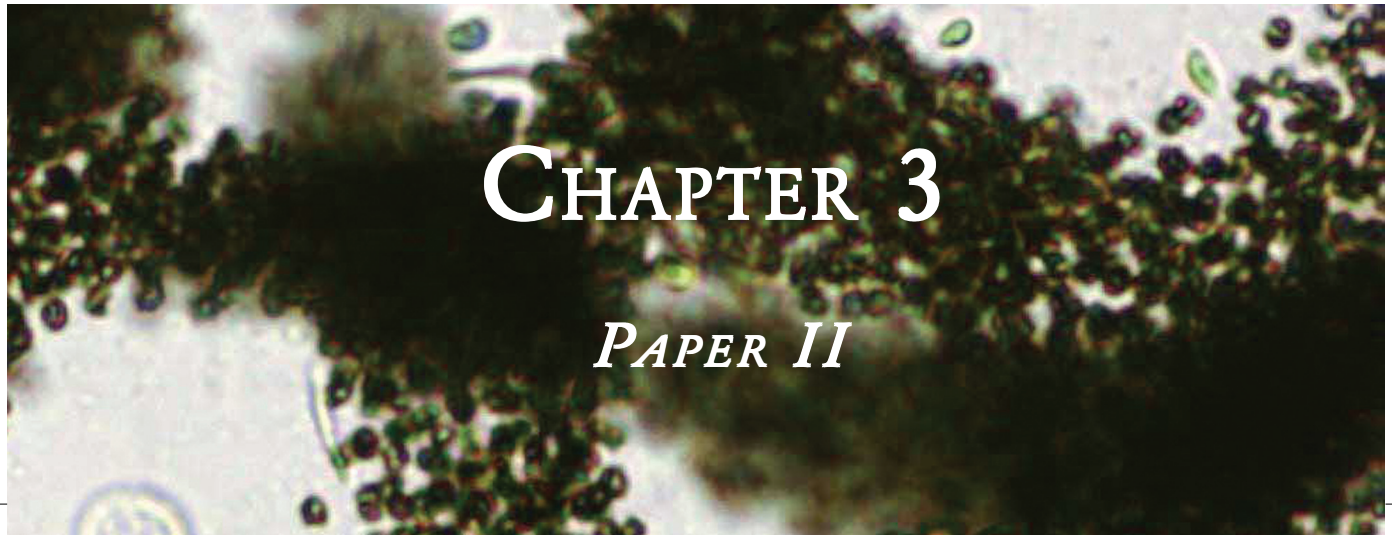

Chapter 3

Paper II

Reinvasion of *Microcystis* colonies (Cyanobacteria) - changes of cell numbers, form of colony and ultrastructure in different conditions

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Hydrobiologia (submitted)

* The author's contribution in the article: Lenka Šejnohová participated in the design and organization of the surveillance and sampling, performed part of ultrastructure, colony size estimating and cell counting. She evaluated data and wrote the article.

Abstract

We observed the quantity of the cell numbers, colony formation and ultrastructure of *Microcystis* benthic population. We compared the results from the shallow and deep parts of reservoir and stable conditions of refrigerator in the time of reinvasion. We found the positive correlation among size of colony, number of *Microcystis* cells and amount of gas vesicles, however only in the reservoir. These changes were started when temperature above sediment exceeds 8-9 °C and oxygen concentration 4 mg/l. Hence, *Microcystis* recruitment seems to be only a result of external factors without a contribution of endogenous timing.

The deep site (8-13m) inoculated the pelagic population by the higher number of cells than the shallow part (2-6m), 56.10^9 /L and 27.10^9 /L, respectively. An upper layer (0-5cm) serves as the main stock of colonies in the deep and shallow areas as well (83% and 65%); the middle layer of sediment (5-10cm) contains an important quantity of inoculum (31%) only in the shallow parts.

3.1. Introduction

The genus *Microcystis* is the most frequent bloom-forming cyanobacteria, which develops in stagnant freshwaters all over the world (Komárek and Komárková, 2002). The life cycle of this genus *Microcystis* from temperate regions has been described on most common morphospecies *M. aeruginosa* (Reynolds et al., 1981). The annual cycle includes bloom, sinking of the population in autumn, overwintering in upper layers of sediment and reinvasion into water column in spring months. Overwintering colonies could resting and sustaining under various environmental conditions (Brunberg, 1995). The benthic biomass may substantially exceed the maximum pelagic biomass (Bostrom et al., 1989), thus indicating that *Microcystis* colonies are able to survive for longer periods and accumulate at the bottom (Brunberg and Blomqvist, 2002). The amount of recruiting colonies dependent on three main factors: the total amount colonies accumulated at the bottom, how long these colonies survive in the sediment and development of environmental factors. After the reinvasion almost 20% from the overwintering benthic stock remained in the sediment (Ihle et al., 2005) and could play an important role as a inoculum for the further recruitment if they remain vital an if they are able to leave the sediment (Preston et al., 1980; Reynolds et al., 1981; Reynolds and Rogers, 1976; Trimbee and Harris, 1984; Verspagen et al., 2004). In spite of the benthic stage is regarded as a physiological rest, no morphological differences are found between pelagic and benthic colonies (Brunberg and Blomqvist, 2002). The onset of *Microcystis* bloom often coincides with the increasing of light (Caceres and Reynolds, 1984; Walsby, 1969) and temperature (Trimbee and Harris, 1984), anoxic conditions (Reynolds et al., 1981) low T:P ratio (Oliver, 1994; Stahl-Delbanco et al., 2003) and CO2 limitation (Klemer et al., 1996) over lake sediments. *Microcystis* displays a range of variability in buoyancy

in response to light which is dependent upon the previous nutrient or light history of cell (Brookes and Ganf, 2001). The impact of these external factors is visible on internal buoyancy changes, through the amounts and densities of the gas vesicles, proteins and carbohydrates, which are designate as an active process (Oliver and Ganf, 2000; Vanrijn and Shilo, 1985; Verspagen et al., 2004). In light, excess of photosynthetic energy is stored as carbohydrate ballast, which makes *Microcystis* colonies sink, up to the point where respiration has sufficiently reduced carbohydrate ballast to make *Microcystis* colonies buoyant again (Kromkamp and Mur, 1984; Thomas and Walsby, 1985). However, no increase in gas-vacuolation was found in colonies in light at low temperature (Thomas and Walsby, 1986; Visser et al., 1995). Because the rate of density and carbohydrates increasing is nonlinear over the response of time, the mathematical modelling of cyanobacterial population development is evolving (Belov and Giles, 1997; Howard, 1997; Howard, 2001; Wallace et al., 2000; Wallace and Hamilton, 1999; Wallace and Hamilton, 2000). Besides the active process also the passive process resulting from resuspension by bioturbation (Stahl-Delbanco and Hansson, 2002) and wind-mixing (Verspagen et al., 2004) play an important role during the recruitment of Microcystis colonies. The wind moreover play an important role during the short term-buoyancy by diurnal cycling of colonies - dawn/dusk migration (Brookes et al., 2003; Oliver and Ganf, 2000). The number of reinvaded colonies is different in the shallow and deep sites of lake. Shallow areas (to 4 m) are more expose by wind-induced mixing, light and temperature changes therefore play an important role during the recruitment of colonies from winter to early summer (Tsujimura et al., 2000; Verspagen et al., 2004). A comparison between shallow (1-2 m) and deep (6-7 m) sites showed that the recruitment from the shallow bay was significantly higher over the entire season, 50% and 8%, respectively (Brunberg and Blomqvist, 2003). In the depth of 70-90 m, no seasonal variation in colony numbers was observe although these colonies were able to grow in cultured conditions. Hence, shallow parts may be crucial by playing an important role as inoculation sites for pelagic populations (Brunberg and Blomqvist, 2003; Verspagen et al., 2004) and deep areas may serve as a long supply accumulating gradually each year (Tsujimura et al., 2000). According to the latest study the majority (65-85%) of benthic population are subjected to decay and the released Mcysts could play a role during the reinvasion of survival colonies (Ihle et al., 2005).

The main purpose of our study was to: (i) observed the changes of *Microcystis* cell numbers, cell ultrastructure, colony formation and microcystins amount in sediment during the reinvasion. (ii) Compare the field results with the data from refrigerator, where was sediment stored for the simulation of conditions on the bottom in the winter. Our hypothesis was to find out whether the reinvasion of colonies is the result of environmental factors whether it depends on endogenous timing, or whether it is the combination of both. The next aim was to shed light

on potential role of microcystins during the colony formation by *Microcystis* recruitment. In addition, we compare the different role of shallow and deep sites.

3.2. Methods

3.2.1. Site description and sampling,

The sediment samples were collected from Brno reservoir, Moravia, Czech Republic (Moravia, N 49°, E 16°30', 259ha, lake volume 15Mm3, maximum depth 19 metre, Svratka River) from three different surface layers 0-5cm, 5-10 and 10-20 cm in the shallow (2-6m) and deep part (8-13m). 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 10m). Brno reservoir suffers from extensive cyanobacterial blooms of *Microcystis* in summer, dominated mainly by *M. aeruginosa* and *M. ichthyoblabe*. In January 2004, we collected sediments from the shallow bay (2-6m) and stored it in refrigerator to simulate winter condition with anoxia, 4°C and darkness.

3.2.2. Colony size estimating and cell counting

Since January 2004, we examined samples from reservoir and refrigerator monthly. Immediately after sampling, we estimated the colony size according the cell numbers. We counted the number of six colony-size categories (50, 100, 200, 500, 1000, > 1000) in 1ml of sediment in Sedgwick-Rafter chamber. Microcystis cells we enumerated from fixed sediments in 2% formaldehyde, which were diluted and sonicated (10min, 100W, power 70%, 20 kHz), filtered on 0, 2 μm polycarbonate filterers and using by epifluorescence microscope.

3.2.3. Electron microscopy

Immediately after the sampling, we rediluted sediment samples and selected colonies randomly under a dissecting microscope, carefully washed thrice in droplets of sterile water and fixed in 2% glutaraldehyd, post-fixed in 1% osmium tetra-oxide in 0,05M phosphate buffer and 1% uranyl acetate in methanol. After dehydration in ethanol, we embedded each colony to the individual block from Spurr medium using propylenoxide. The ultra-thin sections, cut with a glass knife on LKB Ultratome, we post-stained with uranyl acetate and bismuth oxynitrate, and examined with TEM Philips CM 100 at 80kW (with the digital recording of images). The proportion of gas vesicles and reserve granules was estimated "by naked eye".

3.3. Results

3.3.1. Number of *Microcystis* cells

We found the clear differences between the sediment samples from reservoir and refrigerator in the abundance of *Microcystis* cells in the course of season. The

number of cells constantly decreases in the refrigerator during the whole experiment whereas in the reservoir we observed spring and autumn peaks of *Microcystis* biomass (Fig. 1). The first spring peak of benthic *Microcystis* cells (25.10^9 cells/L) were observed during the April in deep part when the temperature exceeds over 8°C and oxygen 4mg/L at the bottom (Fig. 2A). In the same time, the number of *Microcystis* cells declined in the sediment from the shallow bay (Fig. 2B) simultaneously with the first record of *Microcystis* colonies in the pelagial (10^6 cells/L). The second increase of benthic Microcystis biomass was found already in both sites, in the shallow bay ($32\text{-}42.10^9$ /L) during the May/June and in the deep site (65.10^9 /L) in June. After these peaks in June, we detected the main decline of benthic *Microcystis* population (Fig. 2) simultaneously with the main increase of cells abundance in pelagial in July (45.10^6 cells/L) and August (250.106 cells/L). The deep site (8-13m) inoculated the pelagic population by the higher number of cells than the shallow part (2-6m), 56.10^9 /L and 27.10^9 /L. The higher part of the sediment stock reinvaded from the deep than from the shallow parts (84% and 65%). During the summer time, the quantity of benthic *Microcystis* population varied between$10\text{-}20.10^6$ cells/L. The first autumnal peak of benthic *Microcystis* cells was visible in the August only in the deep part, the second peak during the main decline of pelagic population in November in both sites. When we compare the potential function of sediment layers during the *Microcystis* recruitment to the water column, an upper layer (0-5cm) serves as the main stock of colonies in the deep and shallow areas as well (83% and 65%). The middle layer of sediment (5-10cm) contains an important quantity of inoculum (31%) only in the shallow bay (Fig. 2). The deeper layers of sediment (>10cm) contained less than 4% of the total *Microcystis* biomass accumulated in the bottom.

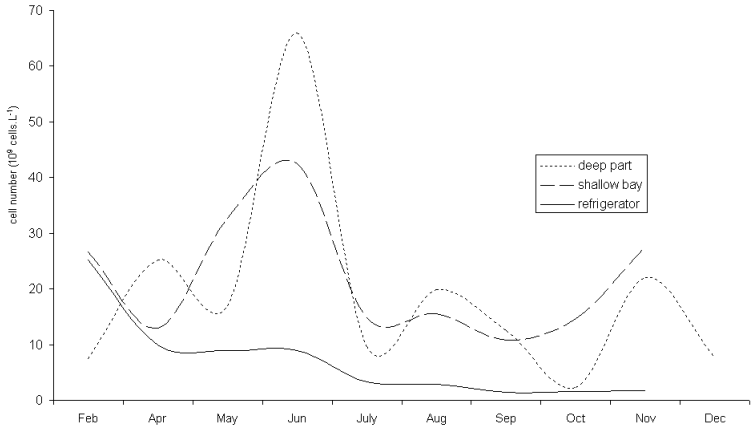
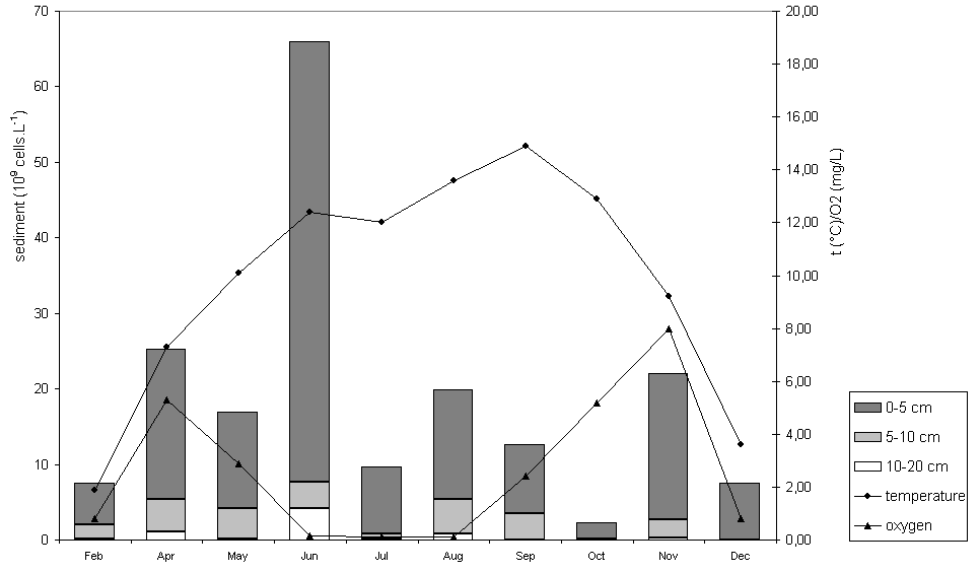


Fig. 1 Changes of benthic Microcystis biomass in the field (shallow and deep site of reservoir) and the stable conditions of refrigerator

Fig. 2 Changes of Microcystis biomass in different layers of sediments in Brno reservoir with regard to oxygen and temperature during the recruitment of colonies.

A) Deep part



B) Shallow bay

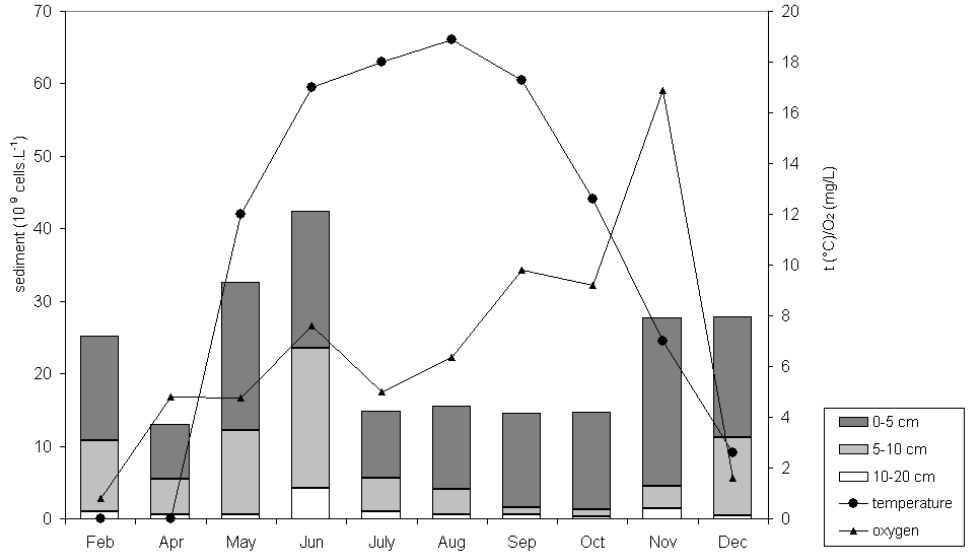
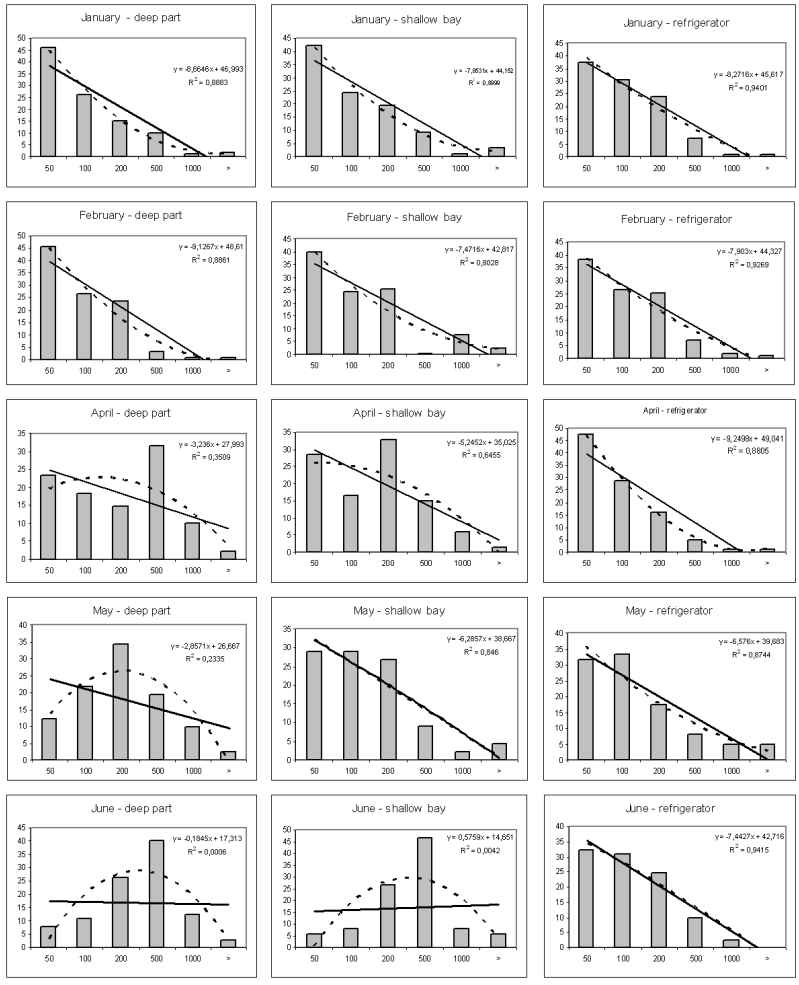


Fig. 3 Colony formation by benthic Microcystis population in the reservoir (shallow and deep par) and stable condition of refrigerator during the time of reinvasion. (broke line - shift of the different sizes of colonies, continues line - regression; axis x show size of colony in number of cells, axis y percent of occurrences).



3.3.2. Colony formation

We found the correlation between the time of the season and size of *Microcystis* colony (from February to June, Fig. 3). In the beginning of the experiment, the most frequent size of colonies was the smallest category contain up to 50 cells (40-45%; Fig. 3) in all observed samples. The significant shift of the colony sizes started in April, when the middle-sized colonies became most frequent in the reservoir (around 200 and 500 cell colony in the shallow and deep parts, respectively). The colony formation was positively correlated with the number of *Microcystis* cells in the sediment in the deep site of reservoir (see Figs. 2, 3 and 4). In the same time, we detected the gradual development of the pelagic population. On the other hand, no colony formation was found in the stable conditions of

refrigerator (percentage among an individual colony sizes stayed constant within the whole experiment, Fig. 3).

3.3.3. Ultrastructure

The ultrastructure of cells during the overwintering and reinvasion is shown in Fig. 4. In February, the interior of cells is occupied approximately by 10 -15 % of gas vesicles and by 30% and 60% of storage granules, in shallow and deep part of reservoir (Fig. 5). The synthesis of new gas vesicles started during March and April, in the shallow and deep parts of reservoir, respectively. In the early phase of the reinvasion, the number of aerotopes per cell was increased and reaches some 20 - 40 % of the cell global inner volume (Fig. 4E-F). We found the positive correlation between the increase of the GV amount and season in both sites of reservoir (Fig. 5). In contrast, the synthesis of GV was negatively correlated with decreasing of the reserve substances, however only in the deep site. In the shallow parts, storage granules filled 20-30% of cell area and this amount did not significantly fluctuated in the course of the season (Fig. 5B). In the summer time, two ultrastructurally distinct benthic colony types can be discerned and exist in parallel. These consist of (i) colonies with the cells with aerotopes occupied 60 - 70 % of the cell area and (ii) aggregates of pallid cells without aerotopes. The ultrastructural seasonal changes were found only in the reservoir population not in the stable condition of refrigerator.

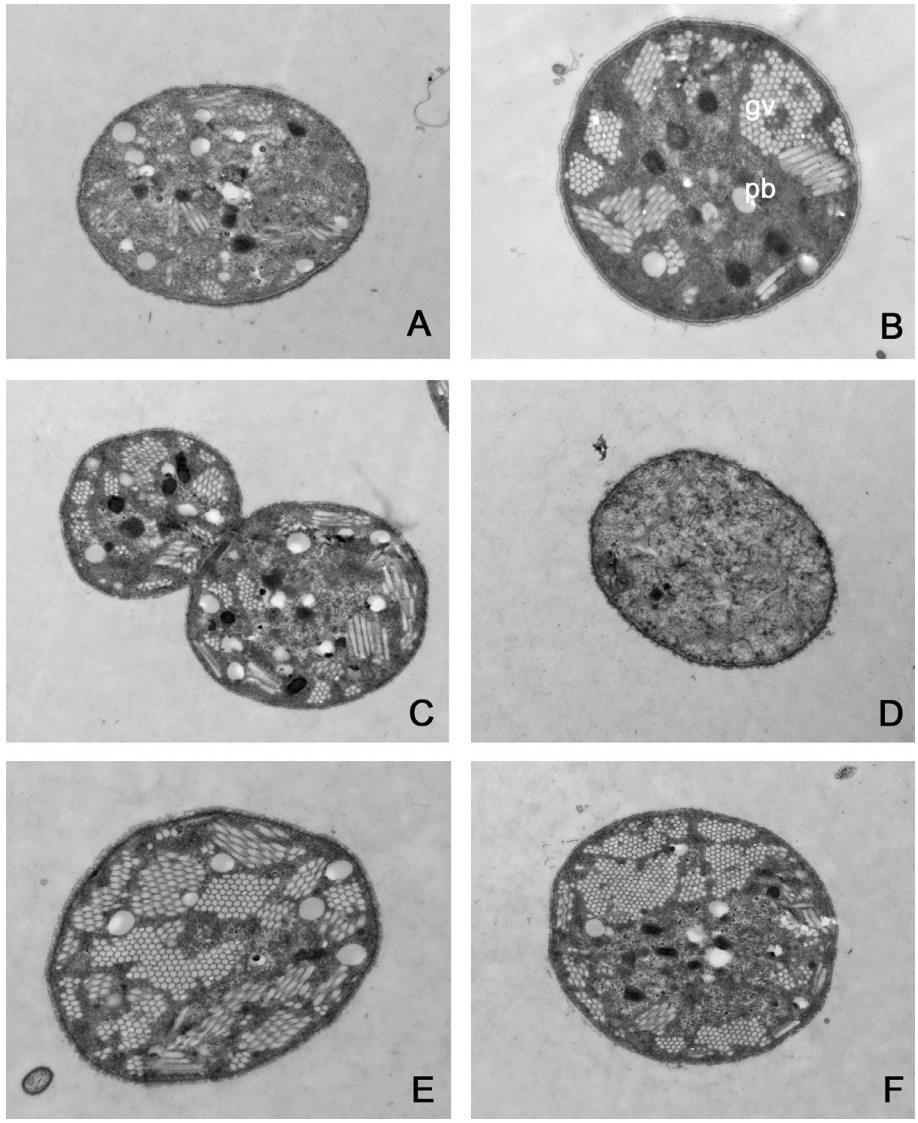
Our study shows a high ultrastructural variability among the colonies within one sample in the reservoir (Fig. 4A-D) which may be attribute to the previous nutrient or light history of the colony (Brookes and Ganf, 2001). Therefore the comparison of the shallow and deep population by ultrastructural features requires the sufficient number of ultrathin sections and colonies from one locality in one time. In addition, the estimation "by naked eye" brings the further potential errors in the results because of the dependence on the observer. Consequently, the use of statistical approach and digital image analysis (method replaces the subjective approximate estimation by an objective evaluation) will be needed to the comprehensive understanding of the ultrastructural changes of *Microcystis* cell during the recruitment.

3.4. Discussion

3.4.1. Importance of shallow and deep parts of reservoir

Our study shows that the shallow (2-6m) and deep parts (8-13m) of the reservoir play the different role for supplement of *Microcystis* pelagic population during the reinvasion. The littoral inoculum provides the first supply of colonies for pelagial during the early spring in the March. This concurs well with the previous studies about the importance of the littoral inocula (Brunberg and Blomqvist, 2002; Brunberg and Blomqvist, 2003; Hansson et al., 1994; Verspagen et al., 2004). In the deep sites, first peak of the cell number was observed one month

Fig. 4 Ultrastructure of Microcystis cell during the reinvasion in shallow site of Brno reservoir. A-D: Benthic cells in February - high ultrastructural variability among colonies from one sample: A-B cells with the arising aerotopes, C - dividing cell with the big amount of storage compounds; D - cell without gas vesicles, E-F: Benthic cells in April. (cb - carboxysome (polyhedral body), gv - gas vesicles, pb - polyphosphate body; phb - poly-ß-hydroxybutyric asid)

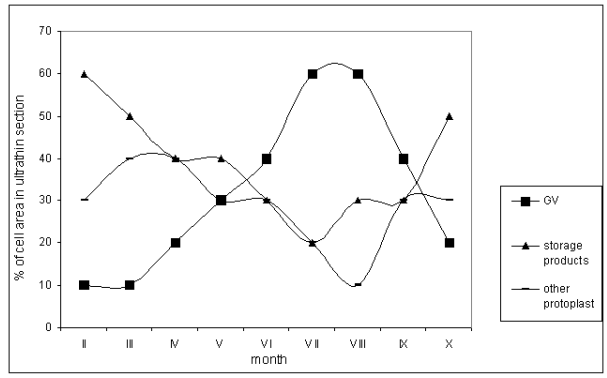


later and this stock serves as a supply for the pelagial during the April/May. The main development of benthic population was recorded in June in both site of reservoir, whereas the deep site supplies pelagial by two times larger stock of *Microcystis* cells than the littoral parts (56.10^9/L and 27.10^9/L, respectively). The recruitment of this "main stock" was timed in the June/July. The deep site provides

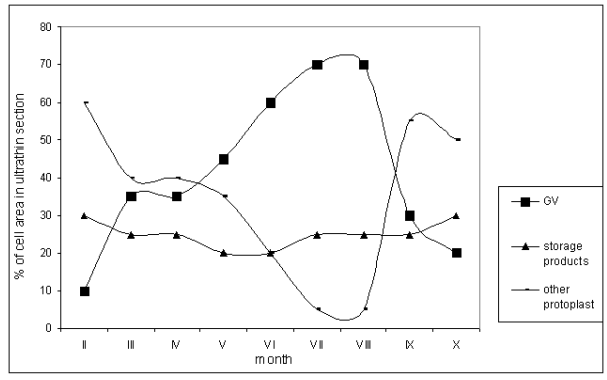
better conditions for the survive of the higher amount of cells in the dark and low oxygen concentrations (Caceres and Reynolds, 1984; Fallon and Brock, 1981; Reynolds et al., 1981). The present study demonstrated that shallow and deep sites of reservoir play the different ecological role during the overwintering and reinvasion of *Microcystis* population. The littoral sites dispose by the smaller cells stock and its main contribution is based on the fast availability during the whole season (Brunberg and Blomqvist, 2002; Preston et al., 1980; Trimbee and Harris, 1984). The deeper sites serves as the larger source of cells, but slowly accessible in the short-term during the spring mixing of the water column. The combination of the unique ecostrategies, different sources of inoculla and fast reorganization of ultrastructure leading to the buoyancy, *Microcystis* population is able to occupy the water column quickly and successfully within the whole season. The hypothesis that shallow parts serve as a fast stock confirm also the ultrastructural results. *Microcystis* cells from littoral contain low amount of storage products within the whole season hence their buoyancy responses on nominal environmental changes

Fig. 5 Seasonal variations of gas vesicles and storage products amount in benthic Microcystis cells in different parts of Brno reservoir (percentage was estimated "by necked eye" on ultrathin sections)

A) Deep part



B) Shallow bay



is very quick. The most amount of *Microcystis* inoculla spent the winter in the upper layer of sediment (to 5cm) from which rise the new dividing cells in the spring reinvaded to the water column. The main importance of the upper layer for the overwintering showed also previous study (Latour et al., 2004; Takamura et al., 1984).

3.4.2. Colony formation by reinvasion

Microcystis genus occurs in the colonial form under the natural conditions, however in the laboratory cultures disintegrate to the single cells. This phenomenon suggests that some environmental factors may be responsible for the typical form of colonies. To date, the following factors affected the colony formation were discussed: water mixing (OBrien et al., 2004), grazing by zooplankton (Burkert et al., 2001; Yang et al., 2006), substances released from zooplankton (Yang et al., 2005a; Yang et al., 2005b) and microcystins. Investigation of microcystins effect on colony formation is of particular importance, especially in the context of ongoing debate on putative microcystin function. Besides other hypothesis, considering microcystin role in nutrient uptake and storage (Utkilen and Gjolme, 1995), regulation of light harvesting (Hesse and Kohl, 2001), allelopathic interactions (Babica et al., 2006a; Babica et al., 2007) or defense against grazer (Jang et al., 2003), possible role of microcystins in cell-cell contacts has been recently discussed as well (Dittmann et al., 2001; Ihle et al., 2005; Kaebernick and Neilan, 2001). The discovery of a surface-exposed protein lectin, microvirin (MVN), was the first evidence of potential cell-cell recognition by *Microcystis* (Kehr et al., 2006). Lectin binding analyses and phenotypic characterizations of MVN-deficient mutants suggest that MVN is involved in cell-cell attachment of *Microcystis*. A binding partner of MVN was identified in the lipopolysaccharide fraction of *M. aeruginosa* PCC7806. MVN is differentially expressed in mutants lacking the hepatotoxin microcystin. Additionally, MVN-deficient mutants contain much lower amounts of microcystin than the wild-type cells. The latest differences in the cellular aggregation of *M. aeruginosa* PCC 7806 and a microcystin-deficient mcyB mutant guided the discovery of a surface-exposed protein that shows increased abundance in PCC 7806 mutants deficient in microcystin production compared to the abundance of this protein in the wild type (Zilliges et al., 2008).

3.4.3. Endogenous timing

The annual cycle of the genus *Microcystis*, especially the gas vesicle formation during the spring period and the disappearance of them during the decline of its pelagic population in autumn, is not well known. The knowledge whether synthesis of gas vesicles is controlled by an endogenous timing or not, belongs to key information concerning the life cycle of *Microcystis*. Presence (or even abundance) of gas vesicles in the cell ultrastructure is of causal importance in the process of reinvasion of the *Microcystis* population from the benthic biotope (from sediment)

into the pelagic one. To date, our knowledge about endogenous timing by cyanobacteria are very limited (Ditty et al., 2003; Lee and Rhee, 1999). According to our results, by *Microcystis* genus, start of the recruitment seems to be only a result of external factors without a contribution of endogenous timing. The ultrastructural changes, increase of cell numbers and colony formation were stimulated only in the wild samples, when temperature above sediment exceeds 8-9 °C and oxygen concentration 4 mg/L. Our results coincided with the previous conclusions when the onset of colonies starts with the increasing of light (Caceres and Reynolds, 1984; Walsby, 1969) and temperature (Trimbee and Harris, 1984) over lake sediments. The impact of these environmental changes is well known through shifts in densities of the gas vesicles, proteins and carbohydrates (Oliver and Ganf, 2000; Vanrijn and Shilo, 1985; Verspagen et al., 2004).

3.5. Acknowledgements

Research has been supported by the Ministry of Education and Youth of the Czech Republic the grant no. 0021622412, the project no. AV0Z60050516 of the Academy of Sciences of the Czech Republic and the research grant of the Grant Agency of the Czech Republic no. 206/03/1215. We would like to thank our colleagues Martina Sadílková for their valuable field and laboratory work during the sediment sampling.

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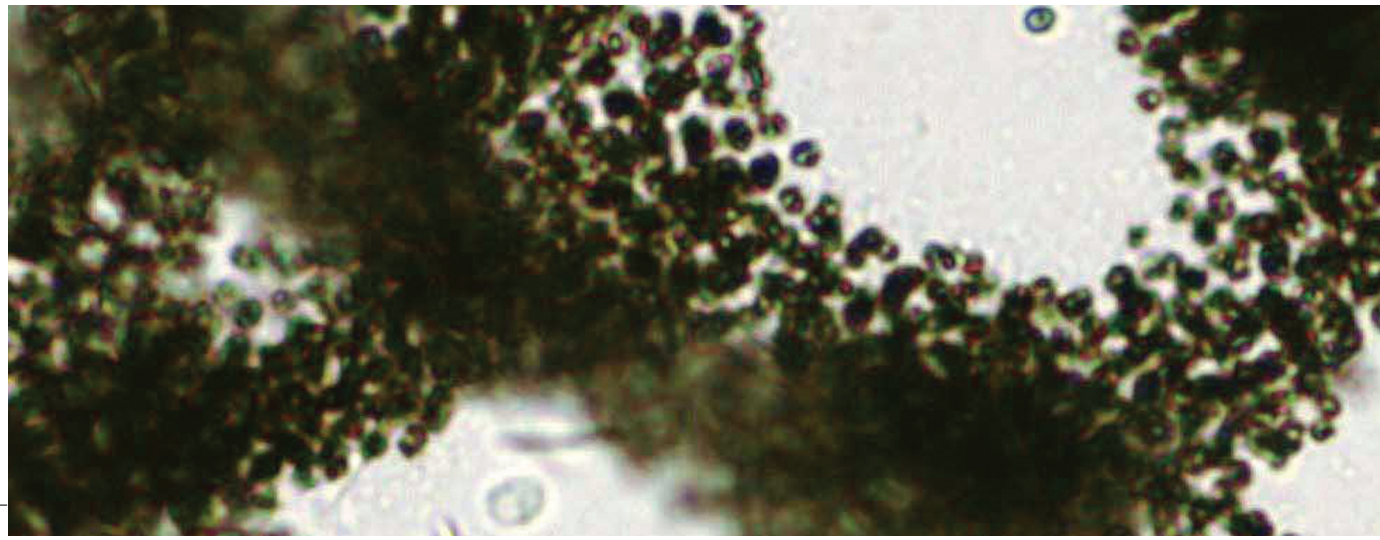
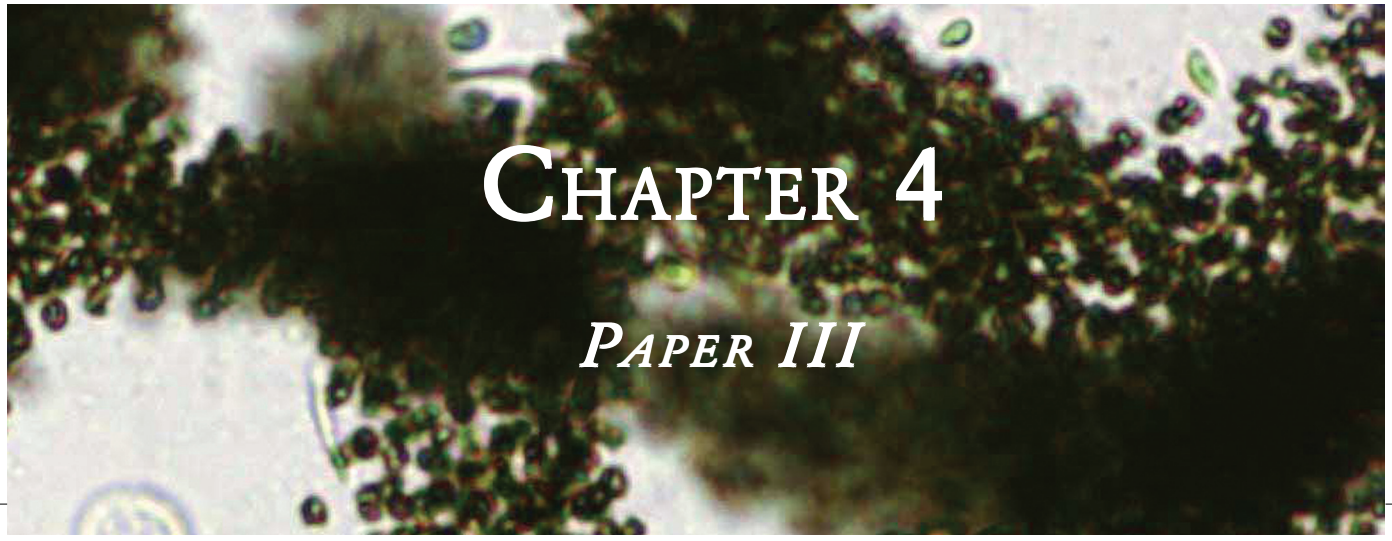




Chapter 4

Paper III

Seasonal shifts in chemotype composition of *Microcystis* sp. communities in the pelagial and the sediment of a shallow reservoir.

Welker M., Šejnohová L., Némethová D., von Dohren H., Jarkovský J., Maršálek B. (2007):

Limnology and Oceanography 52(2): 609-619.

* The author's contribution in the article: Lenka Šejnohová participated in the design and organization of the surveillance and sampling, performed part of colony isolation and morphological analyses. She contributed to the revision and correction of the manuscript.

Abstract

The various oligopeptides produced by individual *Microcystis* clones allow the classification of individual colonies of *Microcystis* in distinct peptide chemotypes. The dynamics and diversity of coexisting chemotypes is regarded as a major factor influencing the microcystin-content of blooms of this potentially toxic cyanobacterial genus. We compared the chemotype composition in planktonic and benthic *Microcystis* communities in Brno reservoir (Czech Republic) from July to November 2004 by single colony mass spectrometry (n=783). Ninety-two peptides were selected to characterize 37 chemotypes as revealed by K-means clustering. In the course of the season the *Microcystis* community became significantly less diverse (linear regression of Shannon indices, $p<0.001$) in the pelagic and in November two chemotypes - both of which did not contain microcystins - accounted for nearly 80 % of the colonies. In contrast, other chemotypes that were dominant in the pelagic in July were no longer encountered after August, while some chemotypes that never accounted for high relative abundances were encountered throughout the season. The shift to some few dominant chemotypes in the pelagic was also reflected by changes in the benthic community where the same chemotypes increased in relative abundances. Nonetheless, chemotypes were identified in the sediment in July and November that were never found in plankton samples. A principal component analysis revealed that communities in the pelagial and the benthal were very different in July but converged during the season due to the deposition of dominant planktonic chemotypes in the sediment. In accordance with the declining percentage of toxin-producing *Microcystis* colonies, the microcystin content of seston samples decreased significantly from 0.9 mg g-1 dry weight to levels below the detection limit (linear regression, $p<0.001$).

4.1. Introduction

Cyanobacterial blooms in water bodies used for recreational purposes and as drinking water resources are of concern to public health due to the ability of some bloom forming genera to produce hepatotoxic peptides, the microcystins. While the modeling of *Microcystis* bloom events can be done with fair accuracy (Roelke and Buyukates 2002) no model has been developed yet predicting microcystin concentrations with sufficient accuracy with respect to the application of safety levels. Microcystins are produced by strains from diverse genera like *Microcystis*, *Planktothrix*, and *Anabaena* but not by all strains of the respective genera (Rantala et al. 2004). The ability of microcystin biosynthesis is not related to phylogenies based on house-keeping genes (Neilan et al. 1997) while evidence is given that the genes required for the biosynthetical pathway are of very ancient origin and that repeated gene loss has led to the recent distribution of these genes (Rantala et al. 2004). Microcystin has been shown to be synthesized by a

non-ribosomal peptide synthetase (NRPS) pathway (Tillett et al. 2000), a pathway by which many peptide antibiotics and other secondary metabolites are produced in heterotrophic bacteria, fungi, and cyanobacteria (von Döhren et al. 1997). Physiological experiments on microcystin production rates have shown that the microcystin content of a particular clonal strain can vary only within a narrow range, for about a factor of three (Orr and Jones 1998), while the microcystin content of field samples can vary for orders of magnitudes (Fastner et al. 1999). When comparing the microcystin content of toxigenic strains it became evident that these can vary also for orders of magnitude (Carrillo et al. 2003), thus suggesting that changes of microcystin content of natural communities rather is the effect of dynamics of individual clones than the result of physiological regulations. Only recently, however, methods have been developed that allow tracking the dynamics of distinct individual (cyano)bacterial clones or groups of clones in natural communities. With quantitative real-time polymerase chain reaction (rt-PCR) the number of microcystin synthetase gene (mcy) copies is estimated and the relative share of toxigenic cells to total cell numbers of *Microcystis*, for example, can then be calculated (Vaitomaa et al. 2003). A distinction between individual toxigenic strains, however, cannot be made and only mcy+ and mcy- genotypes are recorded. A higher resolution, i.e., more distinct groups of strains, is achieved by the application of denaturing gradient gel electrophoresis (DGGE) (Janse et al. 2004). Multiple genotypes based on genes like 16S-23S rRNA intergenic spacer sequences are distinguished and can be followed through the bloom season (Janse et al. 2004).

A third approach relies on the direct detection of secondary metabolites in individual *Microcystis* colonies by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Fastner et al. 2001; Welker et al. 2004). It makes use of the fact that clones of *Microcystis* produce a multitude of oligopeptides, most of which are likely synthesized by NRPS's in analogy to microcystins (Welker and von Döhren 2006), that give individual clones a distinct peptide fingerprint characterizing a peptide chemotype. The term chemotype applied in the present study refers thus to colonies having the same peptide pattern in mass spectra. There is good evidence that chemotypes are stable units representing clonal strains or very closely related clonal strains. Laboratory cultures of *Microcystis*, for example PCC 7806, have been observed for decades producing the same peptides (Bister et al. 2004; Tillett et al. 2000). Further, culture experiments showed that peptides other than microcystins, for example anabaenopeptin and cyanopeptolin variants, also seem to be produced constitutively (Ferreira Ferreira 2006; Repka et al. 2004), i.e., neither complete suppression of peptide production nor a switch to other congeners was observed and relative changes in cell quota are of the same magnitude as observed for microcystins.

While genetic and biochemical aspects of peptide production are known

in principle, the role of the cyanobacterial peptides - including microcystins - has not yet been clearly revealed. Grazing protection (Czarnecki et al. 2006; Ghadouani et al. 2004) and allelopathic competition (Sukenik et al. 2002) in *Microcystis* have been demonstrated, but do not satisfyingly explain the metabolic diversity that has evolved despite high costs to the cells. Besides clonal dynamics in the pelagic, the role of benthic inocula for the population dynamics is potentially of importance for the clonal composition of a *Microcystis* community. The overwintering of colonies in or on the sediment has been recognized decades ago (Preston et al. 1980) but only recently the inoculation from and to the sediment has been subject to quantitative estimates (Brunberg and Blomqvist 2003; Ihle et al. 2005; Verspagen et al. 2005). Likewise, only recently metabolic activity of benthic populations was estimated, showing that in *Microcystis* colonies cell division continues during the winter in the sediment (Latour et al. 2004a).

For the present study the *Microcystis* community in the pelagial and benthal of a shallow, hypertrophic reservoir was sampled over a summer season. Isolated individual colonies were analyzed microscopically and by MALDI-TOF MS to determine chemotype composition and dynamics. The hypotheses we intended to test were as follows: 1. The pelagic *Microcystis* community at the onset of the bloom is influenced by inoculation from the sediment. 2. The pelagic *Microcystis* community shows dynamic changes in chemotype composition during the summer season. 3. The benthic *Microcystis* community at the end of the season reflects the sedimentation of pelagic colonies. 4. The microcystin content of seston samples is correlated to the *Microcystis* community chemotype composition in the pelagic.

4.2. Experimental procedures

4.2.1. Study site

Brno reservoir (N 49°, E 16°30') is an artificial lake dammed up in 1940 in an altitude of 231 m. The reservoir stretches for 10 km in the valley of the Svratka River with a maximum width of 0.8 km in the main basin next to the dam. The maximum and average depths are 19 m and 7.7 m, respectively, and the total volume is some 15x106 m3. The lake area is 2.59 km2 and the annual average water discharge is 8.08 m3 s-1. The trophic state is considered as hypertrophic with annual averages of total phosphorus (TP) of 170 g L-1 and maximum concentrations of chlorophyll a (Chl a)of a few hundred g L-1. The reservoir is used mainly for recreational purposes but also serves as a backup drinking water reservoir.

4.2.2. Sampling

Phytoplankton samples were taken in the central part of Brno reservoir with a plankton net (42 μm) by repeated vertical tows in the uppermost two meter of the water column at a central location. In the laboratory, colonies were selected

randomly from re-diluted samples under a dissecting microscope, carefully washed thrice in droplets of sterile water on the day (and night) of sampling. Microscopical examination included micrography, measuring of cell and colony dimensions, description of colony morphology, and morphospecies determination. Colonies processed in this manner were then placed on a stainless steel template for MALDI-TOF mass spectral analysis and allowed to dry at ambient temperature. Loaded templates were stored dry and cool (4°C) until further processed. An aliquot of the net sample was frozen to be lyophilized later.

Sediment samples were taken with a core sampler at two locations - central (12-14 m deep) and upstream (2-4 m deep) - and the soft sediment was divided immediately after collection in two layers, 0-5 cm depth and 5-10 cm depth. A more refined separation of horizons was not possible due to the soft consistency of the sediments. In the laboratory about 1 mL of sediment was suspended in sterile tap water and colonies were isolated under a dissecting microscope and further processed like pelagic colonies. Isolation was continued until the envisaged number of colonies was isolated. For two sediment samples (central, 5-10 cm) the number of vitally looking colonies was very small and respective samples thus were discarded. The number of colonies isolated from each sample is given in Table 1.

Table 1: List of Microcystis samples taken in Brno reservoir. n refers to the number of individual colonies isolated from a particular sample. "Failed" refers to the number of colonies that were lost during shipping or excluded from the further analysis due to the low quality of the mass spectra.

Sample	date 2004	origin	location	depth	*n*	failed
P1	08 Jul	pelagial	central	0 - 2 m	100	-
P2	31 Aug	pelagial	central	0 - 2 m	100	-
P3	08 Oct	pelagial	central	0 - 2 m	100	7
P4	10 Nov	pelagial	central	0 - 2 m	100	-
S1a	08 Jul	sediment	upstream	0-5 cm	50	1
S1b	08 Jul	sediment	upstream	5-10 cm	100	1
S1c	08 Jul	sediment	central	0-5 cm	50	-
S4a	10 Nov	sediment	upstream	0-5 cm	50	3
S4b	10 Nov	sediment	upstream	5-10 cm	100	4
S4c	10 Nov	sediment	central	0-5 cm	50	-

4.2.3. Mass spectrometry

Dried colonies were extracted directly on the template by placing a droplet of 0.05 to 0.2 μL of a matrix solution - dependent on the colony size - directly on the colony. The matrix solution was 20 mg of 2,5-dihydroxy benzoic acid dissolved in 1 mL of a mixture of water:acetonitril:ethanol (1:1:1) acidified with 0.1 % v/v trifluoro acetic acid. MALDI-TOF mass spectral analyses were carried out on Voyager-DE PRO Biospectrometry workstation as described previously (Welker

et al. 2004). The mass spectra were qualitatively analyzed and only those were used for further procedures in which either peptides (verified by PSD fragmentation) or Chl a derivatives gave unambiguous mass signals (> 1,000 counts).

Mass spectra were screened for known peptide masses (including associated peaks like M+Na+ or M-H2O+H+) and mass signals of yet unknown metabolites. Metabolites that were detected with intense mass signals were further characterized by post-source-decay (PSD) fragmentation (Spengler et al. 1991). Peptide identification and elucidation is described in detail elsewhere (Welker et al. 2006). In colony mass spectra peptides were identified by their mass to the nearest 0.05 Da, by characteristic isotopic distribution, and by characteristic patterns of associated peaks.

4.2.4. Mass spectral data processing and analysis

For each colony a data set of presence/absence of individual peptides was generated and all data for all colonies (including sampling date, morphospecies etc.) were unified in a data matrix finally containing 92 peptides in 783 colonies. A number of colonies without peptides but otherwise positive mass spectral results (see above) were identified as *M. wesenbergii.* The peptide containing colonies were subjected to a K-means clustering procedure, which is a non-hierarchical classification method. K-means clustering divides objects into a previously specified number of groups and is more appropriate than hierarchical clustering procedures in the case of a large dataset like for the present study. The general procedure is to find groups in multivariate space such that within-group similarities and between-group dissimilarities are maximized. The number of groups K must be specified in advance and thus we repeatedly performed the clustering into 2, 3, 4, ... 50 groups (clusters) and calculated the ratio of intra- vs. inter-cluster variability (Ray and Turi 1999). The lowest value (the set of most homogeneous clusters) for the range of 2-50 clusters was obtained for 36 groups of colonies and therefore we took this as the number of peptide chemotypes (CT). One additional group of colonies was formed by the 96 colonies of M. wesenbergii.

After having assigned a chemotype (arbitrary number) to each of the colonies the chemotype composition of each individual sample was expressed as relative frequency of each chemotype. The chemotype diversity of a sample was expressed as Shannon index of diversity of ln-transformed data.

In a principle component analysis (PCA) (Jongman et al. 1997) communities of chemotypes were compared with each set of colonies originating from an individual sample representing one community. The PCA was applied to reduce dimensionality of the data set (37 chemotypes x 10 samples) and was performed on the correlation matrix.

The dependence of microcystin content on the percentage of microcystin-producing colonies was determined by mean of linear regression analysis.

All statistical analyses were performed with the Statistica for Windows software package, version 7.1. (StatSoft Inc.).

4.2.5. HPLC analysis

Some 5 mg (± 0.1 mg) of lyophilized net-samples was extracted sequentially with 70% MeOH, water, and 5 % acetic acid. Combined supernatants were dried in a SpeedVac to be stored at -20 °C until analyzed. Dried extracts were redissolved in 50 % MeOH in a volume resulting in an extract equivalent of 1 mg dry wt per 100 µL final volume. High performance liquid chromatography (HPLC) analysis was carried out on a system and applying conditions described previously (Welker et al. 2003). For the sample taken on 10 November 2004 not enough colonies could be collected with the plankton net to be lyophilized after isolating colonies. The remaining colonies were therefore concentrated on a glass-fiber filter, dried, extracted without a determination of biomass.

Peak fractions of respective extracts were collected manually, dried, and further analyzed by MALDI-TOF MS as described earlier (Czarnecki et al. 2006).

4.3. Results

4.3.1. *Microcystis* abundance

The temporal pattern of *Microcystis* occurrence was in a good agreement with previous findings from eutrophied temperate lakes. In May, diatoms (*Asterionella formosa., Stephanodiscus* sp. and *Navicula* sp.) together with filamentous cyanobacteria (*Anabaena sigmoidea* and *Planktothrix agardhii*), dominated the phytoplankton community with total Chl a concentrations reaching 12 µg L-1. After a pronounced clear-water phase in June, the first *Microcystis* colonies were encountered at the beginning of July with ever increasing abundances peaking in August within the range 130-450 µg L-1 Chl a depending on the sampled location in the reservoir. During the period from July to October, *Microcystis* sp. was the dominant organism. In due course, *Microcystis* abundance declined to finally fall below detection levels in November. By this time, however, colonies could only be collected by repeated net tows and in Lugol-fixed samples of the surface layer (0-2 m) no *Microcystis cells* were encountered. In parallel to the disappearance of *Microcystis* from the water column an increase in diatom abundance was observed together with the occurrence of (few) *Planktothrix* filaments.

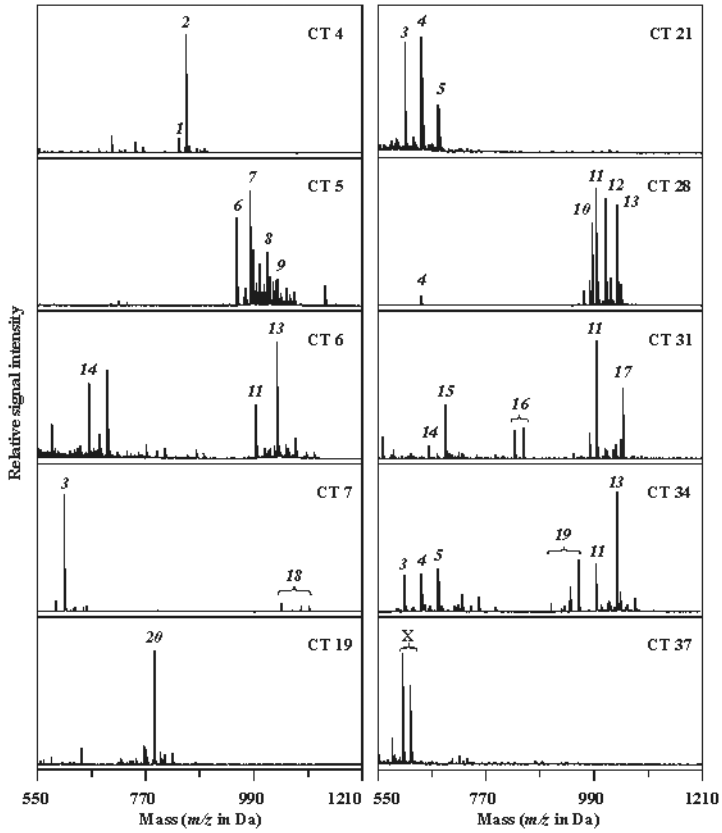
The temperature and nutrient dynamics also followed typical trends with Tmax=23 °C reached in August coinciding with high TP (920 µg L-1) and relatively low TN (1.1 mg L-1) concentrations.

4.3.2. Peptides and chemotypes

From 800 colonies originally isolated the majority gave satisfying mass spectra, i.e., mass spectra in which at least one peptide could be identified or, in the case

of *M. wesenbergii*, Chl a derivatives and repeatedly detected non-peptidic metabolites (Fig. 1). From the pelagic sample collected on 08 October 2004 seven colonies were lost during shipping. All other colonies that failed to give satisfying mass spectra originated from sediment samples collected at the upstream site and were most likely already decaying at the time of sampling. A total of 783 colonies gave mass spectra meeting the above-mentioned criteria and were subjected to the subsequent analytical procedures.

Fig. 1: Partial MALDI-TOF mass spectra of Microcystis sp. colonies representing the main peptide chemotypes encountered in Brno reservoir in July-November 2004. Numbers on each panel with the prefix CT refer to the chemotype number. Numbers in italics above peaks refer to peptides: 1: anabaenopeptin B; 2: anabaenopeptin F; 3: aeruginosin 602; 4: aeruginosin 89; 5: aeruginosin 670; 6: cyanopeptolin 972C; 7: cyanopeptolin 1000A; 8: cyanopeptolin 1006D; 9: cyanopeptolin 1034A; 10: cyanopeptolin 986A; 11: microcystin-LR; 12: cyanopeptolin 1014A; 13: microcystin-RR; 14: aeruginosin 102; 15: aeruginosin 686; 16: cyanopeptolin S; 17: microcystin-H4YR; 18: cyanopeptolin 1063; 19: cyanopeptolin 920; 20: kasumigamide; X: non-peptidic compounds. Parentheses indicate multiple peaks of individual peptides, e.g., sodium adduct peaks. For a full list of detected peptides see Web appendix 1.



In 687 of the colonies at least one peptide was detected. The remaining 96 colonies were identified as *M. wesenbergii* and in the respective mass spectra two compounds were detected that could not be further characterized (Fig. 1, CT 37, see below). Both compounds (M+H = 598.5 and 614.5, respectively) had isotopic patterns not consistent with peptidic compounds and PSD fragmentation did not reveal amino acid fragments. Mass spectra with these characteristics were exclusively encountered for *M. wesenbergii* colonies. In supplementary web appendix 1 peptides were listed that were included in the multivariate statistical analysis. The majority of the peptides listed in the table has been detected before in a variety of different samples (cells, HPLC-fractions, bloom extracts) of *Microcystis.* Nonetheless, for 14 mass signals we could only confirm the peptidic nature of the compounds, and for 15 peptides we could only make a classification with partial elucidation of the structure.

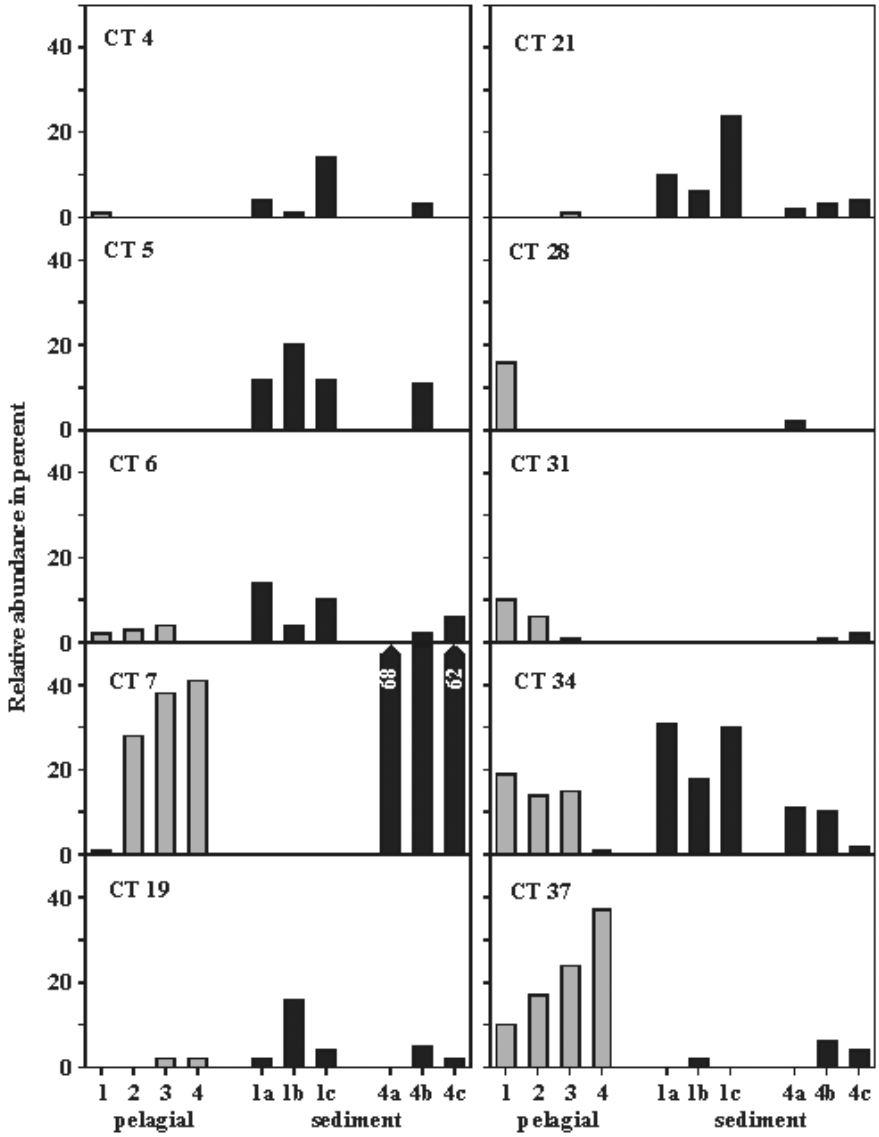
In mass spectral analyses no qualitative difference was observed between colonies of pelagic and benthic origin (Fig. 1). Signal intensities were comparable and no habitat specific background or noise was recorded. Colonies that were subsequently unified to a single chemotype gave very similar mass spectra, i.e., the same pattern of associated peaks and similar relative signal intensities, independent of the origin and the sampling date.

The most frequent peptide was an aeruginosin that could be identified as a chlorine-deficient variant of aeruginosin 89, aeruginosin 602 (603 Da; reference to individual peptides is made by giving the name and detected mass in Da as given in the web appendix 1) that was detected in 47 % of the colonies. Other frequent peptides include three microcystin variants (Mcyst's -LR, -YR, and -RR) that typically were produced by Microcystis. Mcyst-LR was most frequently detected (43% of colonies) while Mcyst's-YR and -RR were detected in about one third of the colonies. One frequent cyanopeptolin congener (cyanopeptolin 1063, 1046 Da) was detected in 28 % of the colonies, but only in one chemotype that was encountered frequently itself.

Based on the peptide presence/absence data matrix and the clustering procedures 37 peptide chemotypes (CT) were established with specific peptide patterns (Fig. 1). Chemotypes differed in the production of individual peptides as well as in the production of peptides in individual combinations. Aeruginosin 602, for example, was detected in colonies that were representatives of five chemotypes, three of which were among the most frequent ones (Fig. 1). In CT 7, the most frequent chemotype representing 28 % of all colonies, aeruginosin 602 was the only aeruginosin detected and occurred together with cyanopeptolin 1063 while in CT 34 aeruginosin 602 was detected together with two chlorinated variants (aeruginosins 89 and 670; 637 and 671 Da, respectively), cyanopeptolin 920 (921 Da), and microcystins LR and RR. In a further chemotype, CT 21, only the three aeruginosins (603, 637, and 671 Da, respectively) could be detected.

Many of the more frequent individual peptides (in more than 5% of colonies) were not restricted to a single chemotype but were found in combinations with other peptides in multiple chemotypes. This was the case for microcystins that were detected in 22 of the 37 chemotypes together with other peptides but never as the sole peptides. Other peptides were restricted to a single chemotype and

Fig. 2: Relative abundance of selected peptide chemotypes (CT) of Microcystis colonies in samples collected in Brno reservoir in 2004. Grey columns represent pelagic samples and black columns benthic samples. Only those out of 37 chemotypes are shown that occurred in a relative abundance of at least 10 % in at least one of the samples as in Fig. 1. For sampling dates and locations see Table 1.



were only found in one particular combination like cyanopeptolin 1063 in CT 7 or a series of cyanopeptolins in CT 5 (cyanopeptolins 972C, 1000A, 1006D, and 1034A with 955, 983, 1011, and 1039 Da, respectively, Fig. 1).

About half of the peptides were encountered only in less than one percent of the colonies. These were either peptides that produced only low intensity mass signals and were thus close to the detection limit (e.g., oscillamide Y (858 Da) and [Asp3,Dha7]Mcyst-LR (1067 Da)) or were produced exclusively by rare chemotypes but then being detected with high signal intensity (e.g., microginin FR9 (751 Da) and microginin 764 (765 Da)).

4.3.3. Morphospecies and chemotypes

Regarding the relationship between the morphospecies of the colonies and the chemotyping based on peptide pattern, a dilemma is evident: some 20 morphospecies are described for temperate lakes (Komárek and Anagnostidis 1999), eight of which have been reported for Czech Republic, while in our samples 37 distinct chemotypes could be detected. Thus, a particular morphospecies can be represented by several chemotypes. In the present study, only two clear relationships were found, firstly, no *M. wesenbergii* colony produced any peptide and secondly, all colonies representing CT 28 were identified as M. viridis. These two morphospecies generally have distinct morphologies, allowing an unambiguous morphospecies determination while for all other morphospecies (*aeruginosa, novaceckii, botrys, flos-aquae, smithii, ichtyoblabe*) numerous colonies had hybrid morphologies, e.g., *aeruginosa/novaceckii* or *aeruginosa/ichtyoblabe*). For the following we therefore focused on the chemotype classification of the colonies and refer to morphospecies only in unambiguous cases.

4.3.4. Chemotype dynamics

The phenology of individual chemotypes in the pelagic was very variable (Fig. 2). About half of the chemotypes (17 out of 37) never reached relative abundances in an individual sample exceeding five percent and some 10 chemotypes made up 60 to 90 percent of colonies in all individual samples. Of these dominant chemotypes, some had their maximum at the onset of the bloom like CT 34, that accounted for 19% of colonies in the pelagic sample of July (P1) declining to a mere percent at the end of the season in November. A similar phenology was observed for CT 31 the relative abundance of which declined steadily. Another chemotype, CT 28, was abundant in July (16%) but vanished completely from the pelagic thereafter and was encountered only in a sediment sample in November. Other chemotypes showed a contrasting phenology with steadily increasing relative abundance. CT 7 started with 1% relative abundance in July (sample P1), soon reaching some 30% to increase to a final 41% in November (P4). A similar, though less dramatic, increase was observed for CT 37 (*M. wesenbergii*) that increased from 10 to 37% relative abundance (in P1 and P4). A third type of phenology

was observed for CT 8 that was encountered in only one sample (10 % in P2).

As a result of the contrasting phenologies the pelagic *Microcystis* community was dominated by two chemotypes (CT 7 and CT 37) at the end of the season, both accounting together for nearly 80% of the colonies in November while the relative abundance of all other individual chemotypes was 4% at highest. In

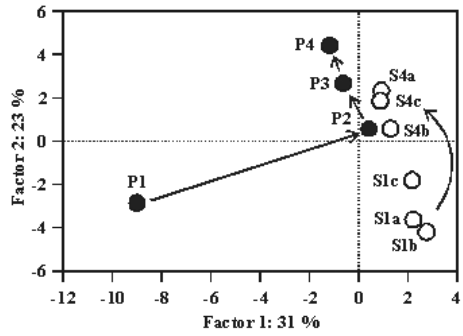


Fig. 3: Ordination plot (factor 1 x factor 2) of a principle component analysis of Microcystis chemotype communities sampled in Brno reservoir July-November 2004. Closed circles represent pelagic samples and open circles represent benthic samples. Next to each data point the sample number is given (see Table 1). Arrows indicate the temporal suite of the samples.

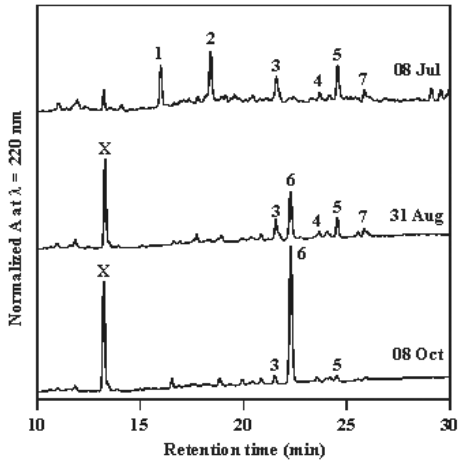


Fig. 4: HPLC chromatograms of seston sample extracts corresponding to three sampling dates in 2004 (Table 1). Numbers above peaks indicate peptides identified by fraction collection followed by mass spectral analysis: 1: anabaenopeptin B; 2: anabaenopeptin F; 3: microcystin-RR; 4: microcystin-YR; 5: microcystin-LR; 6: cyanopeptolin 1063; 7: cyanopeptolin 1014A; X: non-peptidic compound from M. wesenbergii. Chromatograms are normalized and correspond to an extract equivalent of one mg seston per injection.

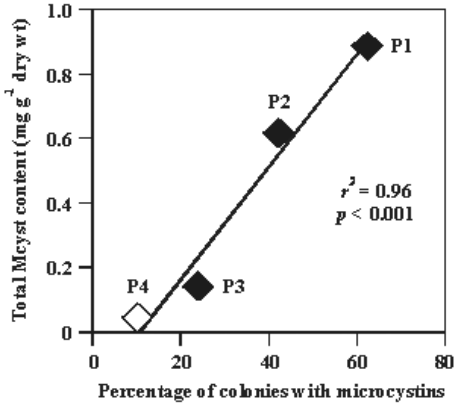


Fig. 5: Dependence of microcystin content of seston on the percentage of microcystin-producing Microcystis colonies in samples taken in Brno reservoir, July-November 2004. Microcystin content was determined by HPLC and summarized for three major structural variants (RR, YR, and LR). Closed diamonds: analysis of lyophilized seston samples; open diamond: analysis and estimate from filter sample. Data points are marked as given in Table 1.

contrast, the sample of July (P1) was more diverse with eight chemotypes with relative abundances of 5% or more. This is also reflected by the Shannon diversity index that declined steadily and significantly ($p<0.001$) from 2.63 in July to 1.58 in November.

In the benthic samples, the *Microcystis* communities showed also a shift in chemotype composition in the course of the season (Fig. 2). Some chemotypes that were dominant in July declined in relative abundance to the end of the year, e.g., CT 5 and CT 6. Others were not or scarcely present in the sediment in July but dominated the community in November. Most decidedly this was the case for CT 7 that has been found in relative abundances of more than 60 % in sediment surface samples (S4a, S4c). Chemotypes encountered exclusively or nearly exclusively in benthic samples were CT 4, CT 5, or CT 21, for example, all of which occurred in high relative abundances in particular samples. The most widely distributed chemotype was CT 34 that was found in all samples and, with two exceptions, in abundances of at least 10 %, both in pelagic and benthic samples.

The comparison of all samples is shown as a factorial PCA plot in Fig. 3. The first principal component (factor 1) was significantly correlated negatively with chemotypes CT 9, CT 13, CT 28, and CT 31 (among others) while no positive correlation was significant. The second principal component (factor 2) was significantly correlated positively with chemotypes CT 7 and CT 37 and negatively with chemotypes CT 5, CT 20, CT 29, and CT 34. The first two factors accounted for 54.5% of the variance in the data set. The position of samples in the PCA ordination space indicates their similarity in chemotype composition, i.e., the closer respective data points were placed to each other, the more resembling was the chemotype composition in respective samples.

The dynamic of the pelagic *Microcystis* community is expressed by a shift of the data points representing samples P1 to P4 over a wide range. The shift is non-circular and no tendency of a 'return' to the origin could be noticed: The community at the end of the season differed markedly from the one at the beginning.

The data points representing the benthic samples showed a less scattered distribution. This was found for sets of samples taken on the same date as well as for the two sets of benthic samples in comparison. The samples from July (S1a-c) were placed close to each other, indicating that the benthic community is rather homogeneous and only a weak tendency of an influence of the location was expressed in a contiguous ordination of the two upstream samples (S1a-b). A similar result could be stated for the benthic samples taken in November, except that the highest similarity was found between the two samples from the sediment surface (S4a and S4c) with a more diverging sample from the deeper layer (S4b). The shift of data points representing benthic samples was much less pronounced

compared to the pelagic samples.

The initial benthic and pelagic communities (P1 and S1a-c) were placed wide apart indicating a rather dissimilar composition. Pelagic and benthic samples placed closest to each other were the benthic samples from November (S4a-c) and the pelagic sample taken during the summer bloom (P2). The dynamics of the benthic community thus could be interpreted as a rapprochement to the pelagic community from an initially larger distance. The pelagic community, in turn, developed further from the point of highest similarity leading again to a higher dissimilarity at the end of the season.

4.3.5. Microcystins and chemotype dynamics

The shift in peptide chemotype composition of the *Microcystis* community was also reflected in HPLC-chromatograms of seston extracts (Fig. 4). In July, the highest peaks were anabaenopeptins B and F and microcystins RR and LR. In later bloom samples anabaenopeptins were no longer detected and microcystin peaks diminished steadily. On the other hand, peaks of cyanopeptolin 1063 and the non-peptidic compound of *M. wesenbergii* (X) occurred with increasing peak heights. Both compounds produced the two major peaks in the chromatogram of the October bloom sample. In Fig. 4 only those peaks were marked that could be clearly identified by mass spectrometry of peak fractions. Besides the major peaks a high number of smaller peaks were found in the chromatograms, likely representing other peptides.

According to the decrease in peak heights of microcystin variants the microcystin content of seston samples declined gradually from 890 mg g-1 dry wt in July to 140 mg g-1 dry wt in October. For the last sampling date no seston sample was available (see above) and only a qualitative analysis was possible that produced negative result. Considering the detection limit of about 5 ng on column it is save to assume a microcystin content below 50 mg g-1 dry wt.

In parallel to the decline in microcystin content the percentage of toxin producing colonies declined from 62 % in July to 24 % in October (10 % in November).

Plotting the microcystin content versus the percentage of microcystin producing colonies in individual samples, revealed a linear relationship (Fig. 5). A linear regression was highly significant ($p<0.001$), also when only the first three samples are considered for which true gravimetric microcystin contents were available ($r^2 = 0.96$, $p = 0.005$).

4.4. Discussion

MALDI-TOF MS and the subsequent statistical analyses proved to be efficient tools to recognize distinct peptide chemotypes in a *Microcystis* community. The similar detectability of peptides in benthic and pelagic colonies supports the

integrity of *Microcystis* cells during the benthic stage (Ihle et al. 2005). The low dropout rate that could be attributed to lysis showed that colonies with an apparently functional photosynthetic apparatus - i.e., green color of the cells - also retain peptides intra-cellularly. The diversity of peptides that can be potentially detected in sediment samples thus depends on the chemotype composition like in pelagic samples.

The number of chemotypes was in a range like it has been estimated previously, as was the number of individual peptides detected and identified in the samples (Fastner et al. 2001; Welker et al. 2004). Both numbers, however, have to be considered as minimum numbers since not all peptides are detected with equal response by MALDI-TOF MS and due to the limited number of colonies that could be analyzed for practical reasons. Individual strains of *Microcystis* often produce some dozens of individual oligopeptides only a few of which are accessible to fast and easy detection, generally the ones that are produced in high cell quota, while other peptides in low cell quota are only detected upon repeated fractionation (Czarnecki et al. 2006; Keishi Ishida, pers. comm.; own unpubl. data) of mass cultured cell material.

The diversity of peptides within an individual strain is considered firstly as a result of presence of specific biosynthetic gene clusters. Secondly, multiple structural variants originate from (sometimes non-stringent) substrate specificities of NRPS-enzymes like adenylation domains, for example (Mikalsen et al. 2003). In natural populations variants of particular gene clusters can be found determining the structural peptide variants produced by respective strains (Mikalsen et al. 2003). Like the mcy-cluster, other cyanobacterial NRPS presumably also have an erratic distribution among *Microcystis* clones (Jan Kehr, Elke Dittmann, Martin Welker, unpubl. data) leading to a practically endless number of peptide fingerprints and chemotypes. The results of the present study showed, on the other hand, that in a given system the number of clones is not endless as could be shown by genetic approaches (Janse et al. 2004).

Changes in community peptide content thus are arguably the result of a change in clonal composition. In the present study, a steady decline in microcystin content of seston samples could be construed as the consequence of a changing chemotype composition. Coincidentally, other peptides typical for particular clones that steadily increased in relative abundance showed increases in respective seston contents as expressed in chromatogram peak heights. Though the driving forces of chemotype (genotype) dynamics are not known, a trend of higher microcystin content at the bloom onset has been observed before (Janse et al. 2005; Kardinaal and Visser 2005; Welker et al. 2003). This observation was interpreted as a potentially better grazing protection through more toxic *Microcystis* cells on average but has not been supported by experiments. Indeed, although intoxication upon ingestion in dependence of actual toxin uptake has been

demonstrated (Rohrlack et al. 1999), it does not explain what the advantage of such a post-mortem grazing protection could be.

Peptide chemotypes can be considered as evolutionary units and the interaction between chemotypes expectedly resembles more the competitive interactions between bacterial species (though not well defined, Cohan 2002) than co-operative interactions between clonal cells (Taga and Bassler 2003). Therefore the term population seemed not to be justified to us to summarize all *Microcystis* colonies in a sample and instead the term community was chosen. Only very few and recent studies deal with dynamics of clones or sub-specific units of prokaryotes in environmental samples, making it difficult to judge whether our findings represent general trends (Janse et al. 2005). On the other hand, the sheer number of different clonal strains of Microcystis that have been isolated worldwide and the varying physiological capacities indicate that mutual influences between clonal strains are likely to happen in every Microcystis bloom.

The seasonal success of particular chemotypes, i.e., increasing relative abundance, can be the results of either bottom-up or top-down regulation (Carpenter and Kitchell 1993). For bottom-up regulation the nutrient and light availability or the ability to exploit resources efficiently, respectively, is crucial and ecophysiological studies have shown that individual *Microcystis* strains differ considerably in their functional responses to variable growth factors (Hesse and Kohl 2001; Schatz et al. 2005). As top-down factor the susceptibility of individual clones to grazing by herbivores - or cyanophage infection (Tucker and Pollard 2005) - is important. Several peptides are discussed as grazing protecting agents like microcystins (Ghadouani et al. 2004), microviridins (Rohrlack et al. 2004), and cyanopeptolins (Czarnecki et al. 2006) acting either as toxins or inhibitors of digestive enzymes. The peptide composition of a clone could thus directly influence the rate by which it is consumed or contribute to a peptide pool that makes it difficult for grazers to adapt to the changing biochemical composition of food particles (Hairston et al. 2001; Sarnelle and Wilson 2005).

A third way of interaction is allelopathy. Several studies have shown that compounds released from cyanobacterial strains can negatively influence the growth of other cyanobacteria and phototrophs in general (Schatz et al. 2005; Sukenik et al. 2002). With regard to the structural diversity of potentially bioactive oligopeptides it is evident that a very complex network of chemical and biochemical interactions could influence the dynamics of metabolically diverse strains (Engelke et al. 2003). We are, however, only beginning to recognize patterns of metabolic diversity, clonal dynamics, and competition.

Surprisingly, benthic inocula apparently did not influence the pelagic chemotype composition at the onset of the bloom and only few chemotypes were found in both compartments, e.g., CT 6, CT 29, CT 34, while others were confined to one habitat at this time. Other chemotypes, in turn, were confined

to one habitat throughout the season, like CT 5 that was found exclusively in sediment samples. From our data we cannot conclude whether this chemotype never was entering the water column or whether it was not successful after inoculating the water column due to inferiority in competition with other chemotypes. The re-invasion of the water column from the sediment seems to be critical for the success of particular *Microcystis* clones: a respective estimation for Quitzdorf reservoir was a meager three percent of benthic *Microcystis* cells exported during a re-invasion phase forming the pelagic inoculum while the majority of the cells was assumed to lyse shortly after entering the water column (Ihle et al. 2005). In contrast, studies by Brunberg and Blomqvist (2003) and Verspagen et al. (2005) came to the conclusion that pelagic inoculation from shallow areas accounted up to half of the original benthic populations. The successful re-invasion of colonies likely depends on many factors determining the growth capacity in the pelagic while the initial inoculation apparently is driven mainly by physical forces, i.e., resuspension by wind-induced mixing (Verspagen et al. 2005). This apparent contradiction can be partly explained by the different approaches yet we rather would expect that successful re-invasions and losses do occur not at average ratios but are strain specifically dependent on actual physico-chemical factors, grazing rates etc. and therefore highly dynamic and highly unpredictable.

Since most studies quantifying the exchange between benthic and pelagic populations did not consider the strain composition no conclusions could be drawn on the success of particular clones. The clonal composition of *Microcystis* populations studied as 16S-23S rRNA ITS clone libraries revealed that the same clones were present in sediment and plankton samples in the course of a season (Humbert et al. 2005). In Brno reservoir particular chemotypes persisted in the sediment for an entire season without successfully entering the pelagic. We could not test whether respective chemotypes would successfully re-invade the water column or whether colonies would simply gradually disappear from the sediment. Chemotypes that were successful in the pelagic, on the other hand, clearly dominated the benthic population at the end of the season. The survival rate of respective chemotypes likely is one crucial step for the determination of the chemotype composition in the following seasons (Latour et al. 2004b). In conclusion, we suppose that benthic and pelagic *Microcystis* communities interact on a multi-seasonal time scale hampering the prediction of chemotype composition and hence toxin contents of eventual blooms.

4.5. Acknowledgements

This study was supported by the EU-research project PEPCY (Bioactive Peptides in Cyanobacteria), by the project AVOZ60050516 granted to the Institute of Botany and by the Ministry of Education and Youth of the Czech Republic

(project IM6798593901; programme 'Research Centres PP2 - DP01'). We thank Hana Slovackova for support in collecting and isolating of Microcystis colonies and Marcel Erhard and Jutta Fastner for providing mass spectral and other supporting data. Two anonymous reviewers contributed significantly to the improvement of an earlier draft of the manuscript and are thanked for their comments.

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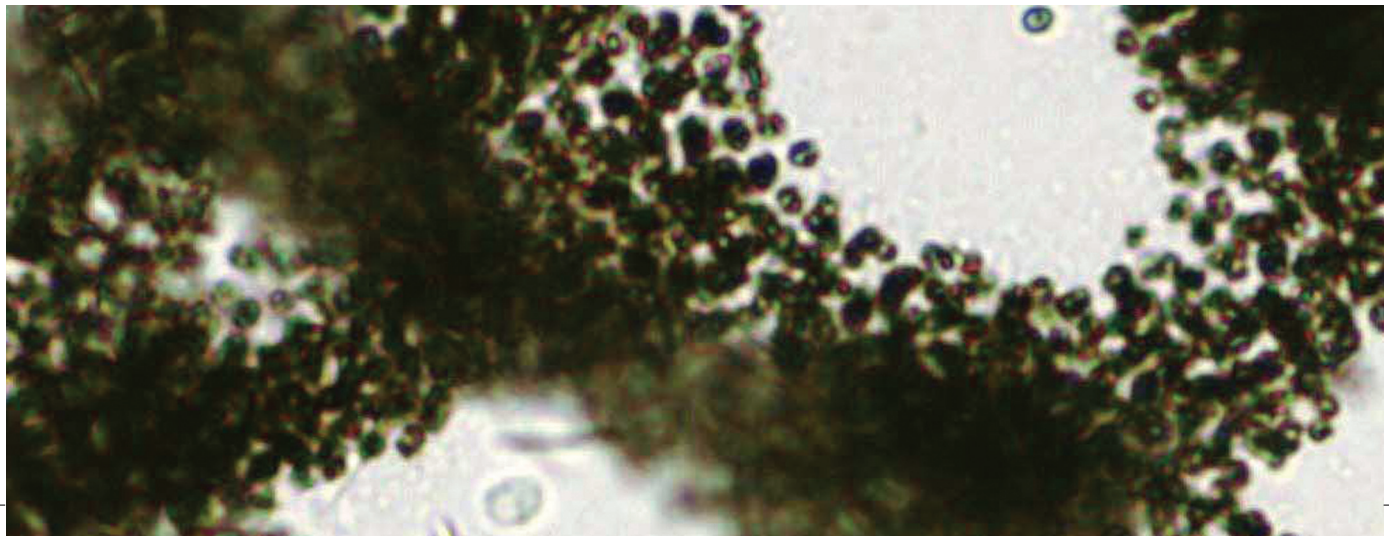
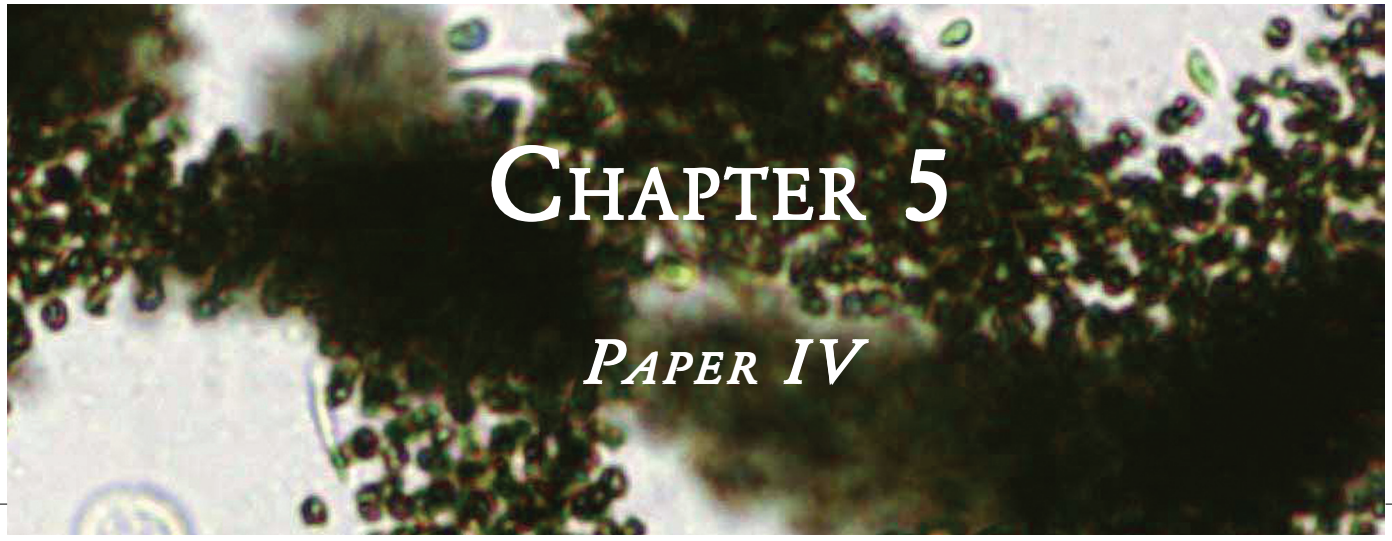




CHAPTER 5

PAPER IV

Detection and identification of oligopeptides in *Microcystis* (cyanobacteria) colonies: Toward an understanding of metabolic diversity.

Welker M., Maršálek B., Šejnohová L., von Dohren H. (2006):

Peptides 27(9): 2090-2103.

* The author's contribution in the article: Lenka Šejnohová participated in the design of study, performed part of sampling and isolation of colonies. She contributed to the revision and correction of the manuscript.

Abstract

Cyanobacteria and particularly *Microcystis* sp. (Chroococcales) are known to produce a multitude of peptide metabolites. Here we report on the mass spectral analysis of cyanobacterial peptides in individual colonies of *Microcystis* sp. collected in a drinking water reservoir. A total number of more than 90 cyanopeptides could be detected, 61 of which could be identified either as known peptides or new structural variants of known peptide classes. For 18 new peptides flat structures are proposed. New congeners differed from known ones mainly in chlorination (aeruginosins), methylation (microginins), or amino acid sequences (cyanopeptolins). The high number of peptides and especially the new peptides underline the capability of *Microcystis* strains as producers of a high diversity of potentially bioactive compounds.

5.1. Introduction

Cyanobacteria of the genus *Microcystis* are notorious for their mass developments in eutrophied inland and brackish waters. In the majority of Microcystis blooms hepatotoxic peptides of the microcystin class can be detected, thus creating a potential health hazard by respective blooms when the infested water is used as source of drinking water or accidentally taken up during recreational activities. Microcystins have been studied intensively during the last two decades with emphasis on genetics (Dittmann et al., 1997), physiology (Wiedner et al., 2003), biochemistry (Nishizawa et al., 2001), toxicology (Dawson, 1998), and ecology (Walls et al., 1997), to name some aspects and studies. One major outcome of these studies was that microcystins are synthesized constitutively thus resulting in fairly stable cell quota but only by strains that possess the respective peptide synthetase gene cluster. The gene cluster coding for the non-ribosomal peptide synthetase (NRPS), mcyA-I (Tillett et al., 2007), has a size of some 60 kbp and seems to be distributed among *Microcystis* clones independently of the phylogeny based on housekeeping genes such as the phycocyanin operon (Neilan et al., 1997). In individual clones a single gene cluster can be responsible for the formation of a multitude of congeners diverging in amino acid composition (e.g. Mcyst-LR and Mcyst-RR) and methylation (Mikalsen et al., 2003).

Besides microcystins, cyanobacteria, and especially *Microcystis*, can produce a high number of oligopeptides that are presumably synthesized by NRPS biosynthetical pathways (Christiansen et al. 2001). Many peptide structures of *Microcystis* and cyanobacteria in general can be classified in types with shared structural properties like microcystins, cyanopeptolins, and aeruginosins. For a number of peptides bioactivity has been reported but respective studies were driven by pharmacological interests and the resulting data can shed only little light on the function of the peptides in cyanobacterial physiology and ecology (Ishida et al. 1999, 2000, Murakami et al. 1995, Reshef and Carmeli 2001). In

fact, no consistent hypothesis has been developed so far to explain the high structural variability and patchy distribution of cyanopeptides. This is partly due to the still very limited knowledge on the occurrence of individual peptides and peptide classes in environmental samples. The diversity of peptide chemotypes has been reported previously (Fastner et al. 2001). Mass spectral analyses of *Microcystis* colonies and strains showed that new structural variants of known peptide classes are frequently encountered (Czarnecki et al. 2006, Welker et al. 2004). In peptide classes for which several congeners have been described, these differ either by exchanges of amino acids or by modifications like chlorination, methylation, or glycosilation.

Several analytical methods have been applied for chemotaxonomic characterization of cyanobacteria (Kansiz et al. 1999, Romano et al. 2000), based on fatty acid compositions, for example (Li and Watanabe 2001). Likewise, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been applied for the rapid typing of micro-organisms (Dieckmann et al. 2005, Hathout et al. 2001). In studies on cyanobacteria, MALDI-TOF MS proved to be a very efficient tool to detect oligopeptides in very small samples and to elucidate flat structures of new congeners without laborious cultivation and isolation procedures (Erhard et al. 1997, Li et al. 1999). Post-Source-Decay (PSD) fragmentation supported by Collision-Induced-Dissociation (CID) has been studied for many different peptides and fragmentation schemes have been developed allowing a reliable reconstruction of amino acid sequences (Harrison and Young 2005, Spengler 1997, van Dongen 1996). Structure elucidation by MALDI-TOF MS is facilitated when respective fragment patterns of similar compounds are available for comparison (Erhard et al. 1999, Yuan et al. 1999). Partial structures or fragments with near identical masses can sometimes not be distinguished and for a full structure elucidation NMR-techniques would then be necessary. This required, however, the isolation of the compounds of interest in the milligram range - and thus in amounts in which many structural variants will probably never be available.

The aim of the present project was to follow changes in peptide chemotype composition during the summer season; a report on the chemotype dynamics will be published elsewhere. Here we report on the detection of cyanopeptides in single colonies of *Microcystis* by MALDI-TOF MS and the identification of known and new structural variants. The present report is focused on the structural variability of peptides in a *Microcystis* population and on the frequency of individual peptides and peptide classes.

5.2. Experimental procedures

Microcystis colonies originated from Brno reservoir near Brno (Czech Republic)

and from Brilicky pond in Trebon (Czech Republic). Colonies were collected with a plankton net from the first two meters of the water column at a central location of Brno reservoir. From Brilicky pond a water sample was taken from the shore and passed through a plankton net. The net samples were stored cool (<8°C) upon return to the laboratory within less than four hours where the isolation of individual colonies was performed immediately under a dissecting microscope. Colonies were picked randomly, checked for pigmentation to ensure the selection of vital cells, and washed thirce in sterile tap water. Colonies were then placed on stainless steel templates for MALDI-TOF MS and allowed to dry at ambient temperature. Isolation and preparation was completed within maximally 12 hours after sampling. A total of 850 colonies were analyzed.

Peptides were extracted directly on the template by applying 0.1 μl of a solution of 20 mg/ml of 2,5-dihydroxy benzoic (DHB) acid in a mixture of aceotintrile:ethanol:water (1:1:1 + 0,1% trifluoro-acetic acid to the dried colonies. The matrix was allowed to crystallize by evaporation at ambient temperature. Mass spectra were obtained on a Voyager DE Pro MALDI-TOF MS apparatus in reflector mode. All samples were first analyzed in positive ion extraction mode and delayed ion extraction (90 nsec). Selected samples were later analyzed in negative ion extraction mode to detect abstractions, mainly of sulphate groups. Acceleration voltage was set at 20 kV, grid voltage at 74 %, and mirror voltage ratio at 1.12 %. Mass spectra were accumulated from at least 100 laser pulses scanning the entire sample spot for a mass range of 450-2000 Da. Since signal intensity was not homogeneous among individual colonies the laser output energy was adjusted manually to result in mass spectra with the most intense mass signals being in a range of 5x103 to 5x104 counts to reach optimal accuracy and resolution. The system was calibrated with polyethylene glycol dissolved in DHB-matrix solution, the accuracy was >25 ppm (0.025 Da for m/z 1000 Da). All mass spectra were recalibrated by using chlorophyll-a derivatives which were identified by their typical isotopic distribution as internal standards (pheophytin-a + H = 871.574 Da, pheophorbide-a + H = 593.269 Da)

Colony mass spectra were first scanned for mass signals of known peptides and respective associated masses, generally sodium adduct peaks (m = 21.98 Da compared to the singly protonated molecular mass), potassium adduct peaks (m = 37.96 Da), and water abstracted peaks (m = 18.01 Da). Further, the mass difference between and the relative intensity of isotopic peaks were analyzed to conclude firstly, the purity of mass signals and secondly, to gain information especially on possible halogenation, e.g. chlorination. Selected mass signals with sufficient peak intensities (ca. 104 counts, not necessarily the protonated molecular ion peak) were further analyzed by post-source-decay (PSD) fragmentation supported by collision induced dissociation (CID) in the lower mass range (Spengler, 1996). Partial fragment spectra were recorded for 12 mirror ratio settings

and assembled by the Data Explorer software. Resulting fragment spectra were analyzed for indicative fragments or series of fragments by comparison to fragment spectra of known peptides. These were either available as purified compounds or in extracts of the samples from which respective compounds have been originally isolated, in most cases from cyanobacterial strains.

Fragment patterns of presumable known peptides were compared to respective theoretical fragment spectra as calculated based on empirical data (Harrison and Young 2005, Spengler 1997) by identifying fragments with matching masses. For putative new structural variants hypothetical molecules were constructed based on data collated on all known structural variants by exchanging amino acids in variable positions by amino acids that have been reported for respective positions (Erhard et al., 1997). Further, modifications like chlorination or methylation were considered. Hypothetical peptides with matching masses and partial structures were then treated as described above. Since structural variants often co-occurred in an individual colony differences in residues were sought to be kept minimal (i.e. a minimum number of amino acid exchanges) considering the biochemical pathway of peptide synthesis that sets limits to the structural variability of congeners in an individual clone.

5.3. Results

More than 150 distinct mass signals in a range of 450-2000 Da were recorded in the *Microcystis* colonies, i.e. with a signal to noise ratio in excess of 10, at least three isotopic peaks, and purity after peak deisotoping. Of these, only a minor part could be directly assigned to known peptides as they have been detected previously on multiple occasions. A larger number of low-intensity mass signals likely also represented peptidic compounds but was excluded from the further analysis for technical reasons. Since the majority of known peptides in *Microcystis* can be grouped in peptide classes we will present the data according to this classification (Welker and von Döhren, 2006). Peptides were detected in all morphospecies with the exception of M. wesenbergii which was found free of (detectable) peptides in more than 100 colony samples.

5.3.1. Microcystins

Microcystins were generally detected as singly protonated molecular ions and associated sodium or potassium adduct peaks were of much lower intensities. In fragment spectra the typical and characteristic fragment in most congeners is m/z 135 Da, a fragment of the Adda side chain. Other typical fragments were m/z 213 and 375 Da corresponding to the fragments Glu-MeDha + H and Adda'-Glu-MeDha + H, respectively, where Adda' denotes the core part of Adda and Me an amino-methylation. In Dha7-variants the respective fragment masses were 14 Da lower (Welker et al. 2002).

In the present colony samples mainly the three congeners typical for most *Microcystis* strains were detected: Mcyst-LR, -RR, and -YR together with their respective demethylated variants (either Asp3 or Dha7 variants) that can not be identified by mass alone. PSD fragmentation clearly indicated [Dha7]Mcyst-RR (M + H = 1024.56 Da) and [Asp3]Mcyst-LR (M + H = 981.54 Da) in the colonies analyzed. Further di-demethylated variants were detected of either Mcyst-LR and Mcyst-RR, but only with low intensities (M + H = 967.53 Da: [Asp3,Dha7]Mcyst-LR and M + H = 1010.54 Da: [Asp3,Dha7]Mcyst-RR). Other congeners were Mcyst-FR (M + H = 1029.54 Da), Mcyst-WR (M + H = 1068.55 Da), and Mcyst-H4YR (M + H = 1049 Da). New congeners were not detected in the colonies analyzed for the present study. In 43 % of the colonies microcystins were detected and 11 variants were encountered.

5.3.2. Aeruginosin type peptides

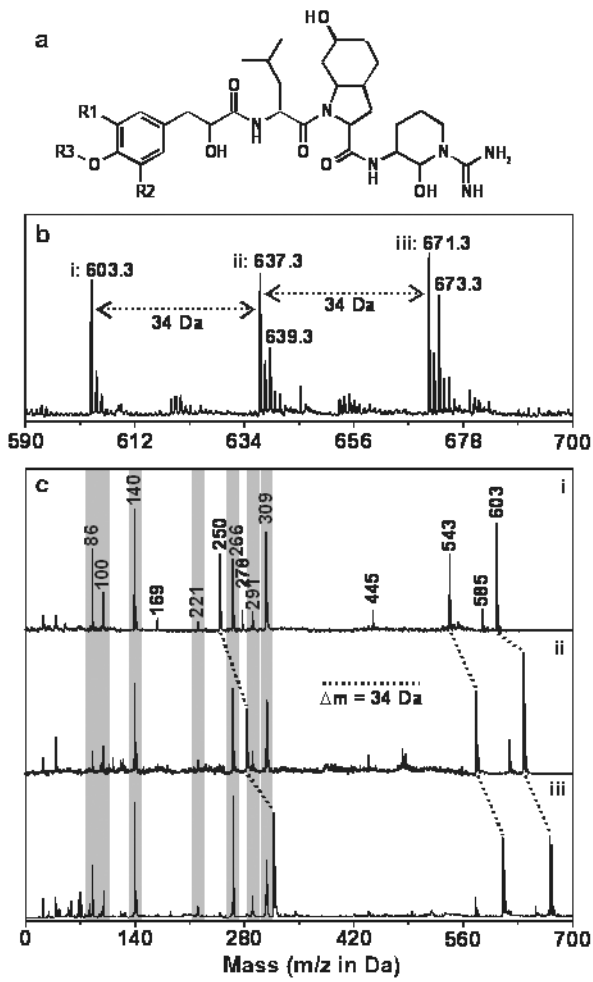
In a number of colonies peptides were identified as aeruginosins, linear tetra-peptides with the unique moiety 2-carboxy-6-hydroxyoctahydroindole (Choi) and a C-terminal Arg-derivative (here and in the following proteinogenic amino acids are abbreviated by the standard three letter code).

In more than 100 colonies a series of three mass signals with an increment of 34 Da were indicative of non-, mono-, and dichlorinated variants of a peptide (Fig. 1). This was also supported by the detection of pseudo-molecular peaks (m+2 Da) with increasing relative intensity, corresponding well to theoretical isotopic patterns of chlorinated peptides. In PSD fragment spectra of all three peptides an intense mass signal was detected at m/z 140 Da, the Choi-immonium ion which is indicative of aeruginosins. A comparison to known aeruginosins showed accordance only for one compound: the peptide with M + H = 637.31 Da had a mass and isotopic distribution corresponding to the sulphate-deficient aeruginosin 89 (SO3,ClHpla-Leu-Choi-Argininal; (Ishida et al. 1999)). Since abstraction of sulphate in positive ion extraction mode is common (Czarnecki et al. 2006, Ishida et al. 1999) we analyzed a number of colonies in negative ion extraction mode and detected respective M-H–ions in a number of colonies but not in all those in which aeruginosins were detected in positive mode.

Comparison of the PSD fragment spectra showed that the aeruginosin with M + H = 637 Da indeed was a sulphate deficient aeruginosin 89 while the two other variants were the unchlorinated variant (M + H = 603.35 Da; aeruginosin 602) and the dichlorinated variant (M + H = 671.27; aeruginosin 670), respectively. As shown in Fig. 1 and Tab. 1 part of the fragment mass signals were identical in all variants while other fragments were shifted for 34 Da in concordance to successive chlorination. The chlorination could thus clearly be located at the Hpla where it has been reported before. Aeruginosin 602 was the most frequent peptide, detectable in 45 % of the colonies. In the example shown in Fig. 1 all

three aeruginosins were detected with nearly identical peak intensities. In other colonies, however, only one or two variants were detectable. Repeated analyses clearly showed that, for example, in many colonies only one variant - aerugnosin 602 - was present while neither chlorinated nor sulphated variants were produced. A peptide with M + H = 645.36 Da could also be identified as an aeruginosin (aeruginosin 644) with a number of fragments identical to fragments of aeruginosin 602: m/z 140, 250, 266, 291, and 309 Da (Tab. 1). A derivatization compared to

Fig. 1: Identification of aeruginosin congeners. a) flat structure of aeruginosin 602, R1,2: Cl or H, R3: SO3 or H in structural variants; b) partial MALDI-TOF mass spectrum of a Microcystis colony in positive ion extraction mode, molecular and pseudo-molecular ion peaks are indicated c) PSD/CID fragment spectra of the three aeruginosins aeruginosin 602 (i), aeruginosin 89 (ii), and aeruginosin 670 (iii), grey bars indicate identical fragments and dotted lines mass differences of 34 Da. For assignment of fragment masses see table 1.



aeruginosin 602 could be located at the argininal from fragments m/z 350 Da corresponding to (Choi-AcArginina l- NH2 + H) and m/z 184 Da corresponding to argininal+42 - NH2 + H. One possible derivatization could be an acetyl unit (C2H3O) corresponding to a mass difference of 42 Da.

A further series of chlorinated variants was detected in other colonies: three aeruginosins with masses of M + H = 575, 609, and 643 Da, respectively, also with increasing intensity of the pseudo-molecular peak with increasing

Table 1: Fragment mass assignment of PSD fragment spectra shown in fig. 2 for three aeruginosin variants. The molecular ion was detected as sulphate-deficient ion in positive ion extraction mode. For aeruginosin 89 two stereo-isomers have been described differing in configurations of argininal; a differentiation of stereo-isomers is not possible by mass spectral analyses. The second column gives the fragment type according to the scheme in Spengler (Spengler, 1997). For abbreviations see text.

		aeruginosin 602 (dechloro–aeruginosin 89)	aeruginosin 89	aeruginosin 670 (chloro–aeruginosin 89)
fragment	scheme	Hpla–Leu–Choi–Argal	*Cl*Hpla–Leu–Choi–Argal	*Cl*$_2$Hpla–Leu–Choi–Argal
M + H		603	637	671
M - H_2O + H		585	619	653
M - CH_3N_2 - H_2O + H§		543	577	611
M - Argal	b_3	445		
Choi–Argininal - NH_2 + H	$'y_2$	309	309	309
Choi–Argininal - NH_2 - H_2O + H	$''y_2$	291	291	291
Choi–Argininal - CH_3N_2 - H_2O + H§	$'''y_2$	266	266	266
Hpla–Leu + H	b_2	278		
$R_{1,2}$–Hpla–Leu - CO + H$^{\$}$	a_2	250	284	318
(Leu–Choi) fragment	$'b_3$	221	221	221
Choi + H		169		
Choi-immonium ion		140	140	140
Argal-fragment		100	100	100
Leu-immonium ion		86	86	86

§ CH3N2 is the ureido-group of argininal
$ R1 and R2 are either a hydrogen or a chlorine in the non-, mono-, or di-chlorinated variant, respectively

molecular mass. The mass of the latter one corresponds to the mass of aeruginosin 101 (Ishida et al. 1999) lacking a sulphate group (Cl2Hpla-Leu-Choi-Agmatine). PSD analysis of the aeruginosin M + H = 643.28 Da gave a consistent pattern, although a distinction between Leu and Ile could not be made. The same applies to the mono-chlorinated variant (M + H = 609.32 Da) that corresponds to aeruginosin 98-A - SO3 ((Murakami et al. 1995), ClHpla-Ile-Choi-Agmatine) as well as to a mono-chlorinated variant of aeruginosin 101 (ClHpla-Leu-Choi-Agmatine). Finally, an aeruginosin with M + H = 575.40 Da showed a similar fragmentation. All three variants, M + H = 575, 609, and 643 Da, respectively, shared fragments of m/z 86 Da (Leu or Ile immonium ion), m/z 140 Da (Choi immonium ion), m/z 298 Da (Choi-Agmatine + H), m/z 281 Da (Choi-Agmatine - NH2 + H), m/z 263 Da (Choi-Agmatine - NH2 - H2O + H), and m/z 256 Da (Choi-Agmatine - CH3N2 + H; where CH3N2 is the ureido group of Agmatine). Fragments associated with Hpla showed increasing masses corresponding to the degree of chlorination from m/z 250 Da (Hpla-Leu/Ile - CO + H) to m/z 284 Da in aeruginosin 98A to m/z 318 Da in aeruginosin 101.

An aeruginosin with nearly the same mass as aeruginosin 98-A - SO3 (M + H = 609.34 Da) was identified as microcin SF608 (Banker and Carmeli 1999) by comparison of fragment spectra with one obtained from the purified compound. In the mass spectra microcin SF608 lacked the isotopic distribution of a mono-chlorinated peptide and in the PSD spectrum a Phe-immonium ion (m/z 120 Da) was detected with high intensity.

In two further aeruginosins a Tyr immonium ion (m/z 136 Da) was detected replacing the Leu/Ile immonium ion. Other fragments with m/z 309, 291, and 266 Da, respectively, were indicative of the sequence (Choi-Argininal). One congener thus could be identified as aeruginosin 102 (Matsuda et al. 1996) lacking the sulphate group (M + H = 653.33 Da; Hpla-Tyr-Choi-Argininal) which was confirmed by analysis of *Microcystis* NIES 102 cells. The second Tyr-containing aeruginosin was the respective chlorinated variant (M + H = 687.29 Da; ClHpla-Tyr-Choi-Argininal) as indicated by a mass shift of the fragment Hpla-Tyr (m/z 300 Da) for 34 Da as well as by the isotope pattern of the precursor ion while the Tyr immonium ion was still detected.

Nine structural variants of aeruginosins could be identified. One or more variants were detected in 61% of the *Microcystis* colonies.

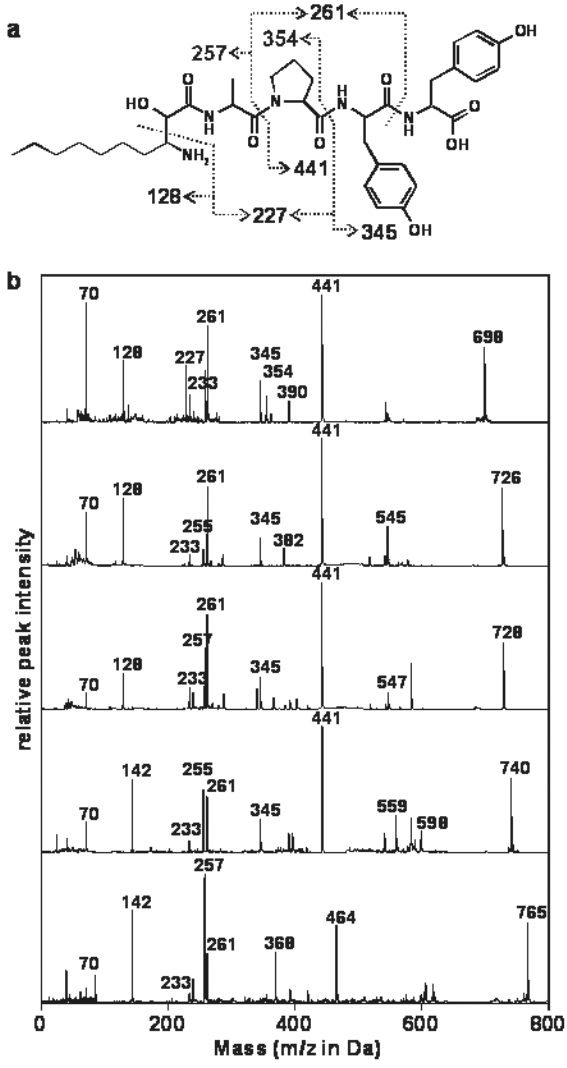
5.3.3. Microginin type peptides

Microginins are linear peptides with a characteristic N-terminal 3-amino-2-hydroxydecanoic acid (Ahda). Fragment spectra of microginins characteristically show a fragment of m/z 128 Da from the side chain of Ahda (Fig. 2, cleavage of the C2-C3-bond). Some 40 structural variants are known, where most congeners are build of four amino acids and Ahda, but variants with four and six units are

also known (Ishida et al. 1998, Sano and Kaya 1995). The C-terminal or the two C-terminal amino acid(s) are Tyr or Hty in most congeners.

In the *Microcystis* colonies a number of masses were detected that correspond to masses of known microginins or derivatives thereof. In Fig. 3 PSD fragment spectra are shown of five microginins that possess common substructures and assigned fragments are listed in table 2. In three of the PSD spectra a fragment of

Fig. 2: Mass spectral analysis of microginins. a) flat structure of microginin T2 with indication of fragmentation and respective masses; b) PSD/CID fragment spectra of five microginin type peptides detected in Microcystis colonies. M + H = 698 Da: microginin T2, M + H = 726 Da: microginin FR5, M + H = 728 Da: microginin FR3, M + H = 740 Da: microginin FR6, M + H = 765 Da: microginin 764 (methyl-microginin FR9). For assignment of fragment masses see table 2 and text.



Ahda was detected (m/z 128 Da) while in two PSD spectra this fragment was not detected but instead a fragment of 142 Da, corresponding to a N-methylated side chain of Ahda (Reshef and Carmeli, 2001), then labeled MeAhda.

One peptide (M + H = 698.38 Da) was identified as microginin T2 (Kodani et al. 1999), that was originally isolated from bloom material of lake Tegamura, Japan (Fig. 2). Three other congeners (M + H = 726, 728, and 740 Da, respectively) showed a set of identical fragments (261, 345, and 441 Da) corresponding to fragments of the substructure Pro-Tyr-Tyr while in the PSD of a fifth congener the latter two fragments were exchanged by fragments with masses elevated for 23 Da. This difference corresponds to the mass difference between Tyr and Trp, thus we propose a Trp at the C-terminus of the respective congener (M + H = 765.42 Da).

The congeners with M + H = 726.41 and 728.39 Da, respectively, correspond to microginin T2 with the second amino acid (Ala) exchanged for Val and Thr, respectively. Such congeners have been identified previously in a bloom of lake Auensee, Germany, (U. Neumann, & J. Weckesser, unpub.) and named microginins FR5 and FR3, respectively.

The fourth congener (M + H = 740.42 Da) differs for 14 compared to microginin FR5 and was identified as microginin FR6. For an increase in molecular mass for 14 Da (one methyl group, CH2) from microginin FR5 to methyl-microginin FR5 several possibilities well in agreement with structural properties of known microginin congeners have generally to be considered. In the present case, however, the methylation could be unambiguously located at the Ahda unit due to a shift in the fragment mass from 128 Da (C8H18N) to 142 Da (C9H20N) while other fragment masses were identical when compared to microginin FR5 (see table). A respective MeAhda-variant of microginin FR3 - microginin FR4, M + H = 742.40 Da - was identified, too.

The fifth congener shown in the fig 2 also was a MeAhda variant with Trp as C-terminal amino acid. The proposed structure MAhda-Thr-Pro-Tyr-Trp is a methylated variant of microginin FR9 (Ahda-Thr-Pro-Tyr-Trp; M + H = 751.40 Da) that was identified in the colonies, too.

Further microginin variants were detected that are derivatives of cyanostatin B (Ahda-Tyr-MeIle-Pro-Tyr; M + H = 754.44 Da) that was identified by comparison of a PSD spectrum made from the original Loch Rescobie bloom material (Sano et al., 2005). The PSD of cyanostatin B showed one intensive fragment peak, m/z 476 Da corresponding to Ahda-Tyr-MeIle+H. In a congener with M + H = 712.39 Da also a very intensive fragment peak was found at m/z 434 Da, 42 Da lower compared to 476 Da thus indicating a modification within the respective sequence. This could be achieved by an exchange of MeIle to MeAla (Ahda-Tyr-MeAla-Pro-Tyr = 711 Da) or exchanges of Tyr to Hty or MeTyr and MeIle to Ala (Ahda-Hty-Ala-Pro-Tyr = 711 Da). The detection of a fragment with 150 Da

(absent in cyanostatin B) indicates the presence of at least one Hty or MeTyr, while the Ahda side chain is unaltered. Therefore, we propose the latter sequence as the one we detected in the *Microcystis* colony. An unambiguous decision, however, was not possible due to very low intensities of other fragment peaks.

A more informative PSD spectrum could be obtained from a microginin with the mass of M + H = 768.45 Da. The mass difference when compared to cyanostatin B indicated a single methylation and indeed, the Ahda side chain fragment was methylated while a series of other fragments (e.g. Ahda'-Tyr-MeIle + H = 349 Da and MeIle-Pro-Tyr + H = 405 Da) were found for both variants.

Table 2: Assignment of fragment masses from MALDI-TOF Post-Source-Decay fragment mass spectra of microginin type peptides detected in colonies of Microcystis sp. (see fig. 1). Masses are given in Dalton rounded to the nearest full number. Ahda': core fragment of Ahda after abstraction of the side chain (m = 58 Da). The second column gives the fragment type according to the scheme in Spengler (Spengler, 1997).

		Microginin T	Microginin FF	Microginin FF	Microginin FF	Microginin 7t *Me*-FR9
fragment	scheme	Ahda–Ala–Pro–Tyr–Tyr	Ahda–Val–Pro–Tyr–Tyr	Ahda–Thr–Pro–Tyr–Tyr	*Me*Ahda–Val–Pro–Tyr–Tyr	*Me*Ahda–Thr–Pro–Tyr–Trp
M + H		698	726	728	740	765
Pro–Tyr–Trp + H	y_3					464
Pro–Tyr–Tyr + H	y_3	441	441	441	441	
*Me*Ahda'–Val–Pro–Tyr–Tyr + H					598	
*Me*Ahda–Val–Pro–Tyr + H	b_4				559	
Ahda–Thr–Pro–Tyr + H	b_4			547		
Ahda–Val–Pro–Tyr + H	b_4		545			
Ahda'–Thr–Pro–Tyr + H	$'b_4$			420		420
Ahda'–Val–Pro–Tyr + H	$'b_4$		418		418	
Ahda'–Ala–Pro–Tyr + H	$'b_4$	390				
*Me*Ahda–Val–Pro + H	b_3				396	
Ahda–Val–Pro + H	b_3		382			
Ahda–Ala–Pro + H	b_3	354				
Tyr–Trp + H	y_2					368
Tyr–Tyr + H	y_2	345	345	345	345	
Pro–Tyr + H	$''b_4$	261	261	261	261	261
Pro–Tyr - CO + H	$'a_4$	233	233	233	233	233
Ahda–Thr + H	b_2			287		
Ahda–Val + H	b_2		285			
Ahda'–Thr–Pro + H	$'b_3$			257		257
Ahda'–Val–Pro + H	$'b_3$		255		255	
Ahda'–Ala–Pro + H	$'b_3$	227				
MAhda-fragment					142	142
Ahda-fragment		128	128	128		
Pro immonium		70	70	70	70	70

The proposed amino acid sequence hence is MeAhda-Tyr-MeIle-Pro-Tyr.

A further pair of microginins was detected in several colonies that also differed in the methylation of Ahda. The methylated congener could be identified as microginin 478 (M + H = 770.47 Da; (Ishida et al., 2000)) while the un-methylated variant has not been reported before (microginin 755; M + H = 756.46 Da).

A further peptide, M + H = 772.5 Da, showed an Ahda-fragment in the fragment spectrum. Two microginin variants with a similar mass have been described previously, microginin 91E (Ishida et al., 2000) and microginin 99A (Ishida et al., 1998a), but both of them are chlorinated at the Ahda or Ada, respectively, side chain. Chlorination was neither indicated by pseudo-molecular peaks nor by respectively increased fragment masses.

Microginins occurred in 14 identified variants but were detected only in 11% of the colonies.

5.3.4. Anabaenopeptin type peptides

Anabaenopeptins are cyclic hexapeptides consisting of a cyclic part of five amino acids with ring closure between the -amino group of a conserved Lys and a side chain built of one amino acid linked to the carboxy-group of Lys via an ureido bond. All amino acids except Lys are variable and one amino acid in the ring is always N-methylated leading to the general structure X0-CO- [Lys-X1-X2-MeX3-X4] where brackets indicate the cyclic part of the peptide and X0 to X4 variable amino acids. The most common anabaenopeptins are anabaenopeptins B and F (M + H = 837.47 Da and M + H = 851.49 Da, respectively) that have been detected in strains and samples of *Anabaena*, *Microcystis*, and *Planktothrix*.

In the *Microcystis* colonies of Brno reservoir and Brilicky pond these two peptides were detected in some 50 colonies with high intensities. Other variants like anabaenopeptin A (M + H = 844.42 Da) and oscillamide Y (M + H = 858.45 Da) were detected only with low intensities. This may lead to an underestimation of their presence as evidenced by LC-MS/MS analyses: although their absolute concentration, e.g. in lake seston samples, is comparable, the response in MALDI-TOF MS is much lower compared to anabenopeptins B and F (Jutta Fastner, pers. comm.). This was most probably caused by the high proton affinity of Arg in the side-chain of anabaenopeptins B and F compared to a Tyr in anabaenopeptin A and oscillamide Y. Similar effects have been observed with microcystin variants (Yuan et al. 1999).

A fifth structural variant was detected with M + H = 821.47 Da, corresponding to a loss of one oxygen atom in comparison to anabaenopeptin F. In the PSD spectrum a number of shared fragment masses indicated indeed a difference of one oxygen. The mass difference could be clearly located in the ring at the homo-tyrosine moiety that is replaced by a homo-phenylalanine

([Phe-MeAla-Hph-Val-Lys]-CO-Arg).

In nine percent of the colonies anabaenopeptins were detected (five structural variants in total), one third of which were colonies from Brilicky pond.

5.3.5. Cyanopeptolin type peptides

A number of peptides were detected and fragmented from the colony samples that could be identified as members of the cyanopeptolin class of cyanobacterial peptides. In this class of peptides many positions in the cyclic part are variable (Welker and von Döhren 2006) and the side chain can be of variable length and composition. Cyanopeptolins have been shown to be protease inhibitors (Czarnecki 2006, Matern 2003). They are identified from fragment spectra not from a single and unique fragment but from series of fragments that are associated with the conserved amino-hydroxy piperidone (Ahp) unit common to all cyanopeptolins. In *Microcystis* most congeners known so far have either one of the following partial amino acid sequences: Ahp-Leu-MeTyr, Ahp-Phe-MeTyr, Ahp-Leu-MePhe or Ahp-Phe-MePhe. Respective series of fragment peaks are listed in table 3. In a few cases MeTrp or a derivative of it - kynurenine - has been found instead of MeTyr or MePhe (Banker and Carmeli 1999). Besides the fragments of the partial structure associated with Ahp, in most PSD spectra a mass signal of the cyclic part of the peptide is detected with high intensity (Welker et al. 2004).

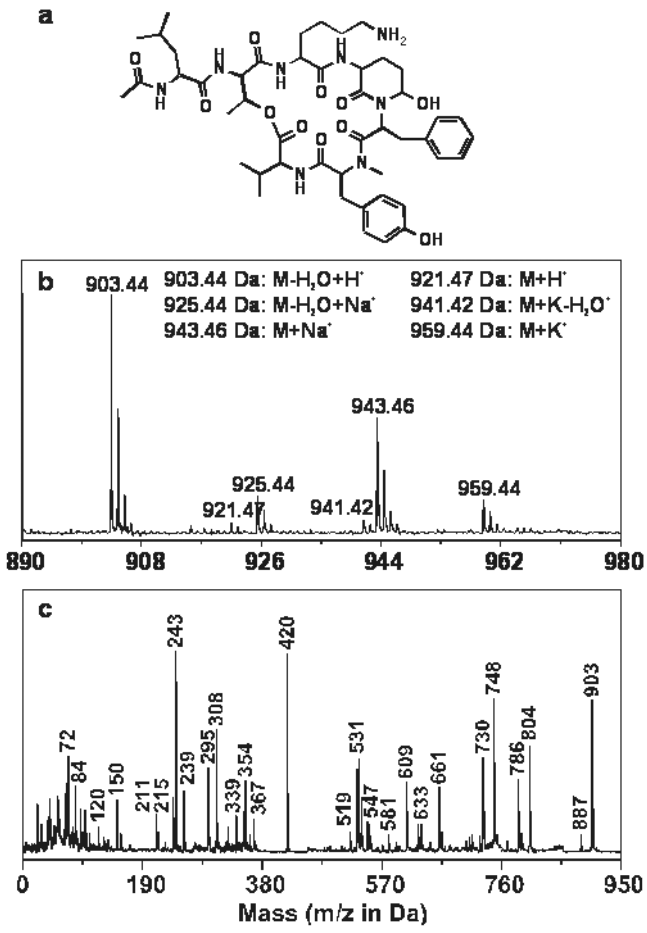
In the present set of sampled colonies a number of cyanopeptolins were identified that have been detected in cyanobacterial samples - mainly *Microcystis* - before (table 4). New congeners were identified by signature fragments but the structure could not be fully elucidated in all cases or a hypothetical structure was not well supported by fragment spectra.

Concerning variability in the side chain two main types can be distinguished in mass spectra. One side chain type consists of a glyceric acid (GA) with one or two sulphate groups. Since sulphate groups are easily abstracted in positive ion extraction mode, the intact molecule can only be detected in negative ion extraction mode while in positive ion extraction mode the sulphate groups are only stabilized in sodium and potassium adduct peaks similar to respective aeruginosins. One known congener of this type - cyanopeptolin S ([Arg-Ahp-Leu-MePhe-Ile-O-Thr]-GA-SO3, (Jakobi et al. 1995)) - was detected in several colonies. Characteristically, the most intense mass signal was the sulphate deficient peak M - SO3 + H = 846.46 Da, followed by M - SO3 - H2O + H = 828.45 Da. In negative ion extraction mode the native compound was detected with high signal intensity (M-H = 924.42 Da) when respective colonies were analyzed. Two further cyanopeptolins with this type of side chain were identified. In cyanopeptolin 911 (detected as m/z 832.45 Da) the Ile in the ring is replaced by Val ([Arg-Ahp-Leu-MePhe-Val-O-Thr]-GA-SO3) while in cyanopeptolin 959

(detected as m/z 880.45 Da) Leu is replaced by Phe ([Arg-Ahp-Phe-MePhe-Ile-O-Thr]-GA-SO3).

The second type of side chain is built of one or two amino acids and a fatty acid in many congeners. Respective molecules are generally detected as intact molecules or as water abstracted derivatives. Water abstraction likely depends on the amino acid N-terminal of Ahp to a high degree. With Lys in that position water abstraction seems to be very likely, while occurring much less intense when Arg occupies this position (pers. observ., unpublished). With Tyr at the respective position a shift of intensity to the sodium (and potassium) adduct peak has been observed - this depends, however, strongly on the salt concentration in the samples.

Fig. 3: Detection and identification of a new cyanopeptolin type peptide detected in Microcystis colonies. a) proposed flat structure, b) positive ion extraction mode MALDI-TOF MS showing related mass signals, and c) PSD/CID fragment spectrum of the M-H2O+H+ precursor ion. For fragment mass assignment see text.



Thus, even in mass spectra of pure compounds the peak with the highest intensity is not necessarily the M + H mass peak and instead M - H2O + H, M + Na, and M + K can have higher peak intensities. In peptide mixtures like *Microcystis* samples this can lead to complex mass spectra with a number of apparent molecular ion peaks exceeding the number of actually present congeners. In the colonies repeatedly series of peaks were detected with the following pattern: X Da, X - 18 Da, X + 22 Da, X + 38 Da and X + 4 Da that were identified as the molecular ion (M + H), the water abstracted ion (M - H2O + H), the sodium adduct (M + Na), the potassium adduct (M + K), and the water abstracted sodium adduct (M - H2O + Na), respectively.

Table 3: Fragment masses indicative of four partial structures of cyanopeptolin type peptides. Numbers are m/z in rounded Dalton. An 'Me' preceding an amino acid indicates an -amino methylation.

	partial sequence			
fragment	Ahp–Leu–*Me*Phe	Ahp–Leu–*Me*Tyr	Ahp–Phe–*Me*Phe	Ahp–Phe–*Me*Tyr
Ahp–Phe–*Me*Tyr - H_2O				420
Ahp–Phe–*Me*Phe - H_2O			404	
Ahp–Leu–*Me*Tyr - H_2O		386		
Ahp–Leu–*Me*Phe - H_2O	370			
Phe–*Me*Tyr - H_2O				308
Phe–*Me*Phe - H_2O			292	
Leu–*Me*Tyr - H_2O		274		
Leu–*Me*Phe - H_2O	258			
Ahp–Phe - H_2O			243	243
Ahp–Phe - H_2O - CO			215	215
Ahp–Leu - H_2O	209	209		
Ahp–Leu - H_2O - CO	181	181		
*Me*Tyr		150		150
*Me*Phe	134		134	

Table 4: Summary of cyanopeptides identified in Microcystis originating from Czech water bodies by MALDI-TOF PSD/CID mass spectral analyses of single colonies. Protonated, monoisotopic molecular masses (M + H) are given in Da together with schematic structures. Names of known peptides are given with an initial upper case. New structures are provisionally named by the peptide class and a suffix referring to the molecular mass. All amino acids are given in three letter codes. Further residues are: Hpla: hydoxyphenyl-lactic acid; Choi: 2-carboxy-6-hydroxyoctahydroindole; Agm: agmatine; Argal: argininal; Ahda: 3-amino-2-hydroxydecanoic acid; Ahipa: 4-amino-3-hydroxy-5-indolylpentanoic acid; Ahp: 3-amino-6-hydroxy-2-piperidone; GA: glyceric acid; Ac: acetyl ; HA: hexanoic acid; OA: octanoic acid; Adda: 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; Dha: dehydro-alanine. Modifications are indicated as Me: amino methylation; me: C-methylation; Cl: chlorination. Brackets indicate a cyclic part of respective peptides. The frequency (F %) refers to the percentage of colonies in which particular peptides were detectable.

M + H	name	F %	amino acid sequence/structure	comment	reference
561.35	Aeruginosinamide	0.7	$(Pren)_2$Ile–Val–Pro–*me*thiazole		(Lawton et al., 1999)
575.36	Aeruginosin 98–B[a]	4.2	Hpla–Leu–Choi–Agm		(Murakami et al., 1995)
603.35	aeruginosin 602	44.4	Hpla–Leu–Choi–Argal	dechloro-aeruginosin 89	this study
609.32	Aeruginosin 98–A[a]	2.0	*Cl*Hpla–Leu–Choi–Agm	dechloro-aeruginosin 101	(Murakami et al., 1995)
609.34	Microcin SF608	0.2	Hpla–Phe–Choi–Agm		Banker et al., 1999
637.31	Aeruginosin 89[a]	20.8	*Cl*Hpla–Leu–Choi–Argal		(Ishida et al., 1999)
639.32	kasumigamide C	0.6	βAla–Ahipa–Arg–phSer		this study
643.28	Aeruginosin 101	1.1	Cl_2Hpla–Leu–Choi–Agm		(Ishida et al., 1999)
653.33	Aeruginosin 102[a]	7.2	Hpla–Tyr–Choi–Argal		(Ishida et al., 1999)
671.27	aeruginosin 670[a]	17.3	Cl_2Hpla–Tyr–Choi–Argal	*Cl*-aeruginosin 89	this study
687.29	aeruginosin 686[a]	4.7	*Cl*Hpla–Tyr–Choi–Argal	*Cl*-aeruginosin 102	this study
698.38	Microginin T2	0.4	Ahda–Ala–Pro–Tyr–Tyr		(Kodani et al., 1999)
712.39	microginin 711	0.8	Ahda–Hty–Ala–Pro–Tyr		this study
714.26	Microginin	0.1	Ahda–Ala–Val–*Me*Tyr–Tyr		(Okino et al., 1993a)
726.41	Microginin FR5	1.4	Ahda–Val–Pro–Tyr–Tyr		U. Neumann & J. Weckesser, unpubl.
728.39	Microginin FR3	6.4	Ahda–Thr–Pro–Tyr–Tyr		U. Neumann & J. Weckesser, unpubl.
740.42	Microginin FR6	0.5	*Me*Ahda–Val–Pro–Tyr–Tyr	*Me*-microginin FR5	U. Neumann & J. Weckesser, unpubl.
742.40	Microginin FR4	0.5	*Me*Ahda–Thr–Pro–Tyr–Tyr	*Me*-microginin FR3	U. Neumann & J. Weckesser, unpubl.
751.40	Microginin FR9	0.1	Ahda–Thr–Pro–Tyr–Trp		U. Neumann & J. Weckesser, unpubl.
754.44	Cyanostatin B	0.7	Ahda–Tyr–*Me*Ile–Pro–Tyr		(Sano et al., 2005)
756.45	microginin 755	0.4	Ahda–Val–*Me*Val–Tyr–Tyr	*de*Me-microginin 478	this study
758.43	microginin 757	0.4	Ahda–Thr–*Me*Leu–Tyr–Tyr		this study
765.42	microginin 764	0.1	*Me*Ahda–Thr–Pro–Tyr–Trp	*Me*-microginin FR9	this study
768.45	microginin 767	0.1	*Me*Ahda–Tyr–*Me*Ile–Pro–Tyr	*Me*-cyanostatin B	this study
770.47	Microginin 478	0.4	*Me*Ahda–Val–*Me*Val–Tyr–Tyr		(Ishida et al., 2000)
771.38	kasumigamide B	2.4	Pla–βAla–Ahipa–Arg–Phe	deoxy-kasumigamide	(Welker et al., 2004)
787.38	Kasumigamide	3.0	Pla–βAla–Ahipa–Arg–phSer		(Ishida et al., 2000)
821.47	anabaenopeptin 820	0.1	[Phe–*Me*Ala–Hph–Val–Lys]–CO–Arg	[Hph$_4$]anabaenopeptin F	this study
837.47	Anabaenopeptin B	8.2	[Phe–*Me*Ala–Hty–Val–Lys]–CO–Arg		(Murakami et al., 1997)
844.42	Anabaenopeptin A	0.2	[Phe–*Me*Ala–Hty–Val–Lys]–CO–Tyr		[19]
851.49	Anabaenopeptin F	8.9	[Phe–*Me*Ala–Hty–Ile–Lys]–CO–Arg		(Shin et al., 1998)
858.45	Oscillamide Y	0.2	[Phe–*Me*Ala–Hty–Ile–Lys]–CO–Tyr		(Sano and Kaya, 1995)
912.41	cyanopeptolin 911[a]	0.1	[Arg–Ahp–Leu–*Me*Phe–Val–O–Thr]–GA–SO_3		this study
921.51	cyanopeptolin 920[b]	14.9	[Lys–Ahp–Phe–*Me*Tyr–Val–O–Thr]–Leu–Ac		this study
926.43	Cyanopeptolin S[a]	1.6	[Arg–Ahp–Leu–*Me*Phe–Ile–O–Thr]–GA–SO_3		(Jakobi et al., 1995)
957.54	Cyanopeptolin A	0.6	[Arg–Ahp–Leu–*Me*Phe–Val–O–Thr]–Asp–HA		(Martin et al., 1993)
959.55	Micropeptin B[b]	0.8	[Lys–Ahp–Leu–*Me*Tyr–Val–O–Thr]–Glu–HA		(Okino et al., 1993b)
960.41	cyanopeptolin 959[a]	1.9	[Arg–Ahp–Phe–*Me*Phe–Ile–O–Thr]–GA–SO_3		this study
967.54	[Asp^3,Dha^7]Mcyst–LR	0.4	[Ala–Leu–Asp–Arg–Adda–Glu–Dha]		[20]
973.48	cyanopeptolin 972A	4.3	[Arg–Ahp–Leu–*Me*Tyr–Val–O–Thr]–Asp–HA		(Welker et al., 2004)

a In some cases the native compound is sulphated either at Hpla or GA; detected as M - SO3 +H in positive ion extraction mode

b Fragmented as M - H2O + H

c Fragmented as M + Na or M + K, respectivel[y]

A respective example is given in fig. 3, representing a new congener named cyanopeptolin 920. The fragment pattern, obtained from the M - H2O + H precursor ion, showed the series of fragments indicative of the partial structure Ahp-Phe-MeTyr (150, 215, 243, 308, and 420 Da). As immonium ions or fragments thereof the amino acids Phe (120 Da), Lys (84 Da), and Val (72 Da) were detected. Other fragments were assigned as follows: 804 Da: M - Val - H2O + H; 748 Da: the peptide ring [Lys-Ahp-Phe-MeTyr-Val-O-Thr] - H2O + H; 661 Da: MeTyr-Val-O-Thr-Leu-Ac + H; 609 Da: Phe-Ahp-Lys-Thr-Leu-Ac - 2H2O + H; 581 Da: MeTyr-Val-O-Thr,Lys-Leu - 2H2O + H; 547 Da: Lys-Ahp-Phe-MeTyr - H2O + H; 367 Da: Lys-Thr-Leu-Ac - H2O + H; 295 Da: Thr-Lys-Ahp - H2O - CO + H; 239 Da: Thr-Leu-Ac - H2O + H. Further mass signals were fragments with a further water or a carbonyl group abstracted (m = 18 and 28 Da, respectively).

In a number of more than 40 colonies four cyanopeptolins were detected that all produced a similar set of apparent molecular ion peaks, hampering a complete separation by the timed ion selector for PSD fragmentation. Nonetheless, all were unambiguously cyanopeptolins as indicated by respective fragment series and putative structures were M + H = 973.56 Da: [Lys-Ahp-Leu-MeTyr-Ile-O-Thr]-Glu-HA; M + H = 1001.59 Da: [Lys-Ahp-Leu-MeTyr-Ile-O-Thr]-Glu-OA; M + H = 1007.55 Da: [Lys-Ahp-Phe-MeTyr-Ile-O-Thr]-Glu-HA; M + H = 1035.58 Da: [Lys-Ahp-Phe-MeTyr-Ile-O-Thr]-Glu-OA. Cyanopeptolins with respective exchanges of hexanoic acid to octanoic acid have been observed in other colonies producing micropeptins B and A or cyanopeptolins 986A and 1014A, respectively (table 4). The exchange of Leu to Phe also has been observed for other congeners, e.g. in cyanopeptolins 972A and 1006A that were co-produced by several colonies. Another structural variant that was detected in more than 200 colonies was also fragmented from the M - H2O + H precursor (m/z 1046 Da). A series of fragments indicated a partial structure Ahp-Leu-MeTyr. Immonium ion mass signals indicated the presence of Tyr and Gln (m/z 136 and 101 Da, respectively), while a signal with high intensity corresponding to the peptide ring [Tyr-Ahp-Leu-MeTyr-Ile-O-Thr] - H2O was recorded (m/z 762 Da) as it has been in other congeners with that partial structure. A side chain would be 284 Da and a matching sequence could be Gln-Leu-Ac. A fragment corresponding to M - Leu-Ac - H2O (m/z 890 Da) was detected and a further one with a mass difference of 128 Da compared to the ring would match to a Gln residue. Three-membered side chains were recorded for cyanopeptolins of *Microcystis* before (Ishida et al. 1998b) and based on the fragment spectral data (not shown completely) we propose the structure of cyanopeptolin 1063: [Tyr-Ahp-Leu-MeTyr-Ile-O-Thr]-Gln-Leu-Ac; M + H = 1064.56 Da.

Cyanopeptolins were the most diverse peptide class in the present study with 16 structural variants and also among the most common ones being detected in 60% of the colonies.

5.3.6. Other peptides

Further peptides that can not be grouped in one of the classes above were also frequently detected. The linear tetra-peptide aeruginosinamide (M + H = 561.35 Da) could be identified in several colonies. The most significant and intense fragments were m/z 492 Da (M - prenyl + H), m/z 223 Da (diprenyl-Ile - CO + H), and m/z 212 Da (the C-terminal Pro-thiazole part of the molecule).

A highly derived peptide is aeruginoguanidin 98-C that was detected as a di-sulphate deficient fragment at m/z 804.5 Da (M + H = 998.40 Da). As the fragmentation was rather unpredictable the peptide could only be identified by comparison with data on the strain from which the compound was originally isolated (NIES 98). Other peptides with similar fragmentation patterns were detected at m/z 808 and 818 Da, the latter likely representing a methylated variant of aeruginoguanidin 98-C. A further characterization, however, was not possible.

Another linear peptide, kasumigamide (M + H = 787.38 Da; (Ishida et al., 2000), was identified, often together with an oxygen deficient variant (M + H = 771.38 Da) that likely lacks the hydroxy-group of the phenyl-serine (thus having a Phe at the C-terminus). In PSD spectra of both variants a fragment with m/z 607 Da was detected that corresponds to the precursor molecule lacking the C-terminal moiety, i.e. Pla-âAla-Ahipa-Arg + H. A further co-occurring peptide with a mass of M + H = 639.33 Da could be identified as kasumigamide lacking the N-terminal phenyl-lactic acid (Pla). This peptide was not detected in all colonies producing kasumigamide and in the fragment spectrum of kasumigamide a respective fragment mass was detected only weakly. Therefore we conclude that the new kasumigamide is not an analytical artifact but synthesized in vivo, probably as a result of truncated synthesis (Psurek et al. 2006).

The largest peptide that could be identified was kawaguchipeptin A (M + H = 1421.69 Da), a cyclic undecapeptide with two prenyl-Trp moieties (Ishida et al. 1996). Respective fragment spectra were of only moderate quality like it is often the case for large peptides, especially when they are cyclic.

A number of other mass signals in colony mass spectra could be characterized as peptides by PSD fragmentation but the respective spectra showed no high similarity to fragment spectra of known peptides and lacked fragments indicative of common peptide classes. In the case of higher masses these represent most likely cyclic peptides (e.g. M + H = 1076.6 Da, M + H = 1544.7 Da, M + H = 1550.8 Da, data not shown) where structure elucidation by mass spectral analysis is hampered by the number of theoretical fragments increasing exponentially with the number of residues. For smaller masses the peptides are likely highly derived, e.g. of mixed PKS/NRPS origin, resulting in fragment spectra that are only to be interpreted in comparison to ones of related compounds.

5.4. Discussion

In 850 *Microcystis* colonies analyzed for the present study a high number of peptide metabolites could be detected and identified. Identification could be achieved either by comparison of actual fragment spectra to spectra obtained from standard material or by calculation of theoretical fragment spectra allowing an error margin of max. 0.5 Da. Although MALDI-TOF MS does not enable the elucidation of full structures including chirality it allows a fast identification of congeners of known peptides and the proposal of an amino acid sequence (Psurek et al. 2006, Spengler 2006). Similar to PSD spectra that can not be analyzed as isolated data, the structures elucidated by mass spectral techniques have to be regarded in the context of known peptides. In aeruginosins, for example, the second position is generally occupied by a D-amino acid with few exceptions (Banker and Carmeli 1999). A corresponding, highly conserved epimerization domain has been identified in two aeruginosin synthetase clusters of *Microcystis* (K. Ishida, E. Dittmann, N. Tandeau de Marsac, M. Welker; unpublished data) and epimerization might be required for the further biosynthesis (Linne 2001, Stachelhaus and Walsh 2000. Therefore, a respective chiral configuration in new aeruginosin variants is likely.

The same peptides could be detected in colonies isolated from net samples and from sediment cores and no obvious difference in mass signal intensities was observed for the two subsets of samples. Therefore it can be argued that peptides, including microcystins, are retained in the cells during the benthic stage in the annual cycle (Reynolds et al. 1981) of Microcystis strains in cell quota that are comparable to those during the pelagic stage (Ihle et al. 2005). When colonies re-enter the pelagic zone (Brunberg and Blomqvist 2003) they apparently are already equipped with the same set of peptides they continue to produce during pelagic growth. The peptide diversity of benthic populations thus is potentially as diverse as in pelagic population.

The total peptide diversity in the recent samples was similar to the findings in previous reports, thus underlining that a high peptide diversity is a common feature of Microcystis populations. The number of individual peptides that can be identified in a single population is the result of two sources of structural diversity: firstly, production of multiple congeners of single peptide classes by individual clones and secondly, the co-existence of many chemotypes, each producing a distinct set of individual peptides.

For microcystin synthesis it has been shown, that a single gene cluster or enzyme complex, respectively, is responsible for the production of peptide congeners that differ in amino acid composition and/or modifications like methylation (Mikalsen et al. 2003). Similar mechanisms are very likely in other peptide biosynthesis pathways allowing a co-production of multiple congeners in individual clones possessing a particular peptide synthetase gene cluster.

Structural variability is achieved by variations in the amino acid backbone and/or by modifications like dehydration or methylation that are assumed to be part of NRPS pathways (Christiansen et al. 2003).

In aeruginosins, for example, the amino acid backbone was identical among co-produced congeners and variation arose mainly from the degree of chlorination (or sulphation). The chlorination is achieved by a putative halogenase the gene of which is inserted in the synthetase gene cluster following the module for Hpla-activation. In strains that do not produce chlorinated aeruginosins the halogenase gene is lacking completely (Cadel-Six et al. 2005). In clones possessing a halogenase in the aeruginosin synthetase a respective chlorination, however, is apparently not performed on each peptide molecule that is formed in the NRPS assembly line. Based on present and published data it is safe to expect a potential chlorination of the Hpla-residue for any known amino acid sequence, i.e. a Cl2-microcin SF608 (M + H = 677.26 Da) very likely is produced by some Microcystis clones although it has not been detected yet. Similar structural variability arises from Hpla-sulphation and by these two modifications, for each amino acid backbone six structural variants can be expected. Further reported modifications are glycolation (Shin et al. 1997), ethylation (Kodani et al. 1998), bromination (Ishida et al. 1999), Choi chlorination, sulphation (Shin et al. 1997), and epimerization (Valls et al. 2003). For the first position this gives, in Microcystis, a number of possible moieties of eight (un-, mono-, and di-chlorinated or brominated multiplied by two for sulphated and non-sulphated Hpla), four amino acids in position two, four Choi variants (native, sulphated, chlorinated, epimerized), and four N-terminal moieties (agmatine, argininol, argininal, ethyl-argininal). This results in 8 x 4 x 4 x 4 = 512 aeruginosin variants in *Microcystis* only with moieties that arguably can be expected. Including structures found in *Planktothrix* (e.g. N-terminal phenyllactic acid) the number multiplies again.

While frequently found in aeruginosins, chlorination was not found in the microginin type peptides in the present study, although it has been reported for several congeners (Ishida et al. 2000, Sano and Kaya 1997). Instead, in single colonies in most cases two congeners were present, that differed in the methylation of the Ahda-moiety. The amino acid sequence seemingly is more strictly determined by individual peptide synthetases. Modifications of the amino acid sequences of co-produced microginins have not been observed for the present study. Generally pairs of microginins differing for 14 Da (e.g. M + H = 726 and 740 Da, M + H = 751 and 765 Da) were detected and the total microginin diversity was mainly the result of multiple co-existing peptide chemotypes. The number of congeners was relatively high with 14 variants in only 89 colonies producing (detectable) microginins. This could be explained regarding the structures of published microginins demonstrating that each individual amino acid position except Ahda can be occupied by a number of amino acids. Such structural variability among

strains is hence arguably achieved rather by the variability of adenylation domains or NRPS modules in NRPS gene clusters (probably through recombination) than through physiological processes (Elsner 1997, Mikalsen 2003, Tanabe 2004). On the other hand, variable amino acid positions within co-produced peptide congeners were frequently found in microcystins and cyanopeptolins produced by an individual clone (Bister et al. 2004, Martin et al. 1993). In microcystins, variable amino acid positions are (mainly) restricted to only two while in cyanopeptolins all positions except two are variable (conserved Ahp and Thr). In cyanopeptolins the co-production of multiple congeners has been reported previously (Martin et al. 1993, Neumann et al. 2003, Okino et al. 1993) and for the present study a number of four congeners at maximum can only be seen as a minimum number due to difficulties in detecting minor compounds with similar or even identical masses (Czarnecki et al. 2006). Although chlorination has been reported in cyanopeptolins (Fujii et al. 1996) when the N-methylated amino acid in the ring is occupied by Tyr and respective variants have been detected in other Microcystis samples (Welker et al. 2004); own data, unpublished), in the colonies studied presently no respective congeners were detected. In analogy to aeruginosins, a Tyr-chlorinated variant would not be an unexpected finding. This could also be said for the other cyanopeptolins the structures of which are proposed here. These new structures rather fill the gaps than represent entirely novel peptides. Typical cyanopeptolins from Microcystis possess four variable positions in the cyclic part that can be occupied by two to four amino acids, e.g. Arg, Lys, and Tyr in position 2 (Bister et al. 2004, Martin et al. 1993, Welker et al. 2006), allowing some 50 peptide rings without any unexpected moieties. Due to the variability of the side chain - mono-, or di-sulphated GA or amino/fatty acids - the number of possible congeners rises quickly to the hundreds, all of which achieved by the variability of amino acid incorporation apparently allowed by homologuous peptide synthetases.

The present mass spectral study underlines that cyanobacteria, and especially strains of the genus Microcystis are a rich source of variable peptide metabolites. The structural diversity achieved in single colonies and the entire population could be attributed to a combinatorial biochemistry in vivo that expectedly offers still many more new peptides to be discovered. Considering all reported amino acid positions and possible derivatizations in particular peptide classes the number of peptide structures that can be expected in Microcystis - or cyanobacteria in general - counts to several thousands.

Structural variations can have pronounced effects on the bioactivity. In cyanopeptolins, for example, the inhibition of daphnid trypsin activity depends mainly on the presence of Lys or Arg in position two of the ring while no inhibitory activity was observed with Tyr in this position (Czarnecki et al. 2006, Yamaki et al. 2005). Even a marginal change, an methyl group at the Dha moiety

in position 7 in microcystins (hence E-Dhb7-mircrocystins) can influence grazer toxicity markedly Blom et al., 2001. Similar structure-activity relationships are known for chlorinated peptides (Eustáquio et al. 2003). Nonetheless, the evolution - and selection - of peptide biosynthesis in cyanobacteria did not lead to a few structures of highly active peptides but apparently to a broad range of diverse peptides.

Many peptides that could be detected in the present set of colonies have been isolated from Microcystis strains of various origins, underlining their potentially ubiquitous occurrence (Czarnecki et al. 2006, Fastner et al. 2001, Via-Ordorika et al. 2004, Welker et al. 2004). Production of oligopeptides apparently is advantageous to Microcystis independent of the geographic location, trophic state, etc. of the actual habitat - otherwise the costly potential likely would have been lost in the very ancient evolutionary line of cyanobacteria. What such an advantage could be, remains, however, still enigmatic.

5.5. Acknowledgements

This study was supported by the EU-research project PEPCY (Bioactive Peptides in Cyanobacteria) and by the Grant Agency of the Czech Republic (project nr. 206/03/1215). We thank Hana Slovackova for support in collecting and isolating of Microcystis colonies and Marcel Erhard, Jutta Fastner, and Jürgen Weckesser for providing reference mass spectra and other supportive information. Keishi Ishida, Shmuel Carmeli, and Rosmarie Rippka generously provided reference peptides and/or reference strain material. Critical remarks and helpful comments by two anonymous reviewers were highly appreciated.

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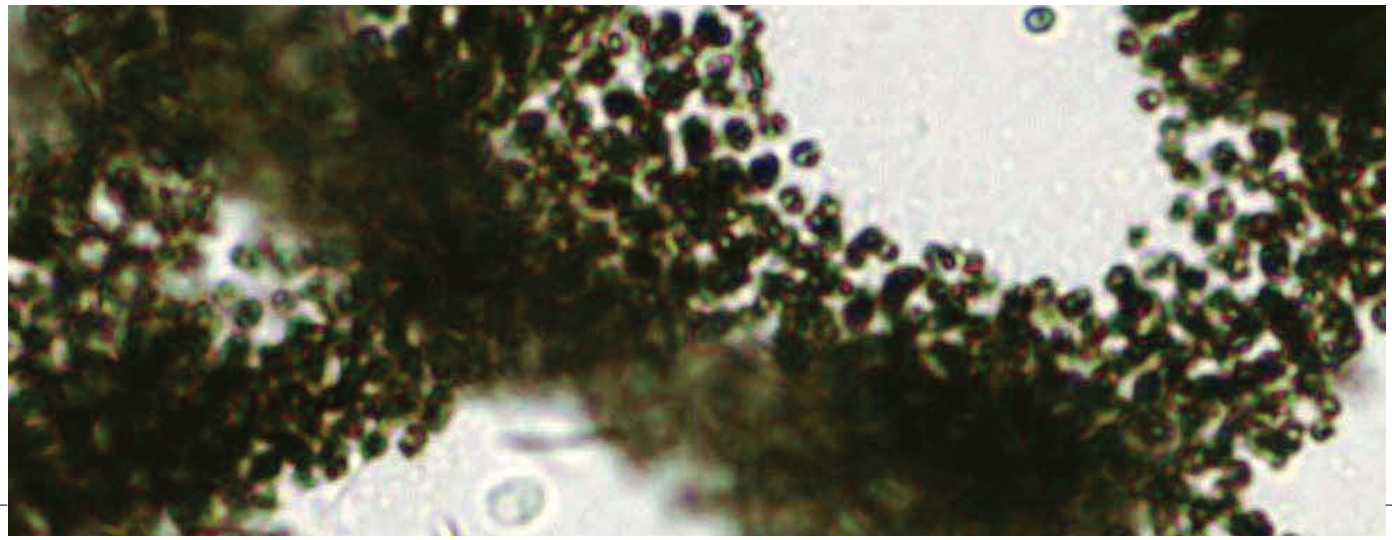
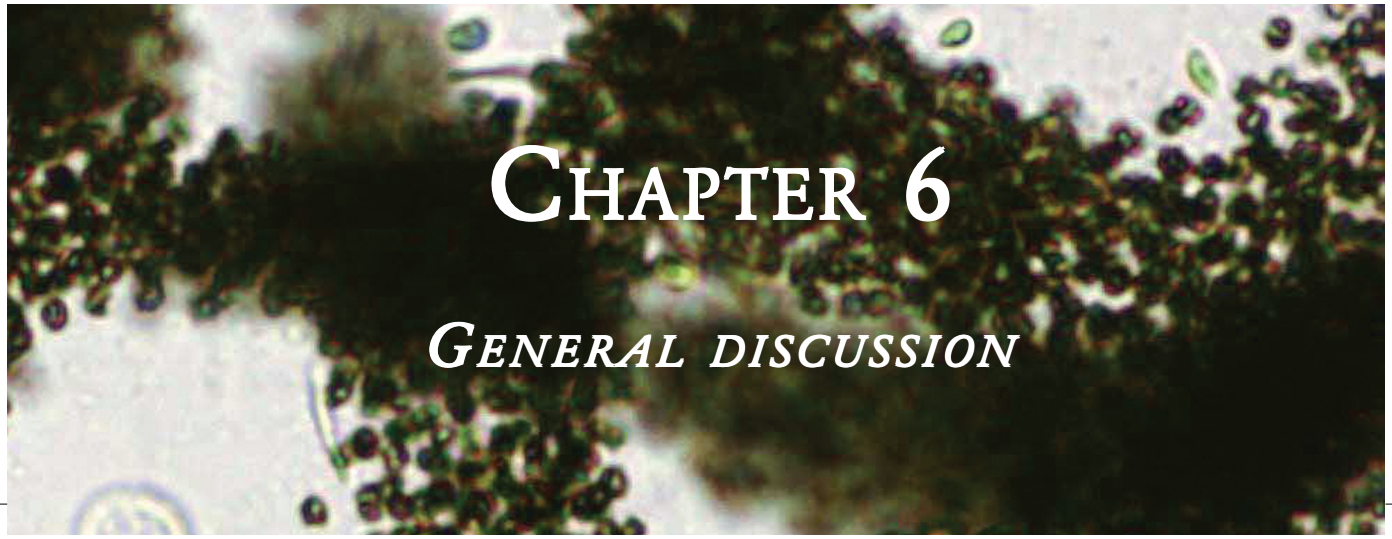




Chapter 6

General discussion

6.1. Introduction

The central aim of this study was to gain insight in the relationship between morphology and peptide presence, autecology during the reinvasion and dynamics of peptide occurrence within the natural *Microcystis* population. In this chapter, the main results presented in this thesis will be evaluated. In particular, focus will be on relationship between the morphological features and occurrence of specific oligopeptides by *Microcystis* within the season. In this consequence, new questions arising from this work and the application of peptides in polyphasic cyanobacterial taxonomy will be discussed.

6.2. Cell diameter in relation to peptide occurrence

We tested firstly the direct the relationship between individual morphological features of *Microcystis* colony and individual peptides by using multivariate analysis (canonical correspondence analysis, CCA). We sampled a large set of 833 single *Microcystis* colonies from pelagial and sediment in two water bodies throughout one season. We detected and identified 99 oligopeptides by the novel method, mass spectrometry (MALDI-TOF MS), analyzed single colonies. We found that within *Microcystis* genus coexist non-peptide and peptide group differed also morphologically. Colonies with any mass signals for oligopeptides were characterized by cells 7.4±0.8 μm in diameter (M. wesenbergii) within the whole season in pelagial and sediment as well. The observation that the largest cells (M.wesenbergii) are without peptides is in agreement with the majority of studies according to which this morphotypes contained little or no Mcysts (Marsalek et al., 2001), gave no PCR product of the mcy genes (Rohrlack et al., 2001; Kurmayer et al., 2002; Via-Ordorika et al., 2004), and generally had any mass signal for peptide metabolites (Welker et al., 2004). In contrast to our results, colonies of M. wesenbergii were all found positive for cyanopeptolin in Lake Wannsee (Fastner et al., 2001b) indicating that within this morphospecies different chemotypes could be present. Colonies with smaller cells than 6 μm were positive for oligopeptides in all cases. The occurrence of peptides was depending on cell diameter (anabaenopeptins, microginins) or season (microcystins). We conclude that occurrence/absence of some specific peptides in *Microcystis* colony could be potentially used in polyphasic taxonomy on subgeneric level.

6.3. Reinvasion of *Microcystis* and colony formation in the different conditions

We found the positive correlation among size of colony and number of *Microcystis* cells, however only in the reservoir not in the stable conditions of refrigerator. These changes were started when temperature above sediment exceeds 8-9 °C and oxygen concentration 4 mg/l. Hence, *Microcystis* recruitment seems to be only a result of external factors without a contribution of endogenous timing.

Our results coincided with the previous conclusions when the onset of colonies starts with the increasing of temperature (Trimbee and Harris, 1984) over lake sediments. The impact of these environmental changes is well known through shifts in densities of the gas vesicles, proteins and carbohydrates (Oliver and Ganf, 2000; Vanrijn and Shilo, 1985; Verspagen et al., 2004).

The deep site (8-13m) inoculated the pelagic population by the higher number of cells than the shallow part (2-6m), 56.109 /L and 27.109 /L, respectively. The deep site provides better conditions for the survive of the higher amount of cells in the dark and low oxygen concentrations (Caceres and Reynolds, 1984; Fallon and Brock, 1981; Reynolds et al., 1981). An upper layer (0-5cm) serves as the main stock of colonies in the deep and shallow areas as well (83% and 65%); the middle layer of sediment (5-10cm) contains an important quantity of inoculum (31%) only in the shallow parts. The main importance of the upper layer for the overwintering showed also previous study (Latour et al., 2004; Takamura et al., 1984).

We conclude that reinvasion process is connected with the colony formation. The possible function in the colony formation by *Microcystis* could play the microcystins. Our conclusions are support by the discovery of a surface-exposed protein lectin, microvirin (MVN), was the first evidence of potential cell-cell recognition by *Microcystis* (Kehr et al., 2006). Lectin binding analyses and phenotypic characterizations of MVN-deficient mutants suggest that MVN is involved in cell-cell attachment of *Microcystis*. The next discovery of a surface-exposed protein that shows increased abundance in PCC 7806 mutants deficient in microcystin production compared to the abundance of this protein in the wild type (Zilliges et al., 2008). In conclusion, the comprehensive study observes the colony formation and microcystins amount is needed.

6.4. Shift of chemotypes within the season

We investigate dynamics, diversity and co-occurence of oligopeptides by *Microcystis* community within the season. The term chemotype applied in this study refers thus to colonies having the same peptide pattern in mass spectra. The main aim of this paper was to determined the seasonal changes of chemotypes in the pelagial and sediment with a view to the reinvasion and sedimentation. We compared the chemotype composition in planktonic and benthic *Microcystis* communities in Brno reservoir (Czech Republic) from July to November 2004 by single colony mass spectrometry (n=783). Ninety-two peptides were selected to characterize 37 chemotypes as revealed by K-means clustering. In the course of the season the *Microcystis* community became significantly less diverse (linear regression of Shannon indices, $p<0.001$) in the pelagic and in November two chemotypes - both of which did not contain microcystins - accounted for nearly 80 % of the colonies. In contrast, other chemotypes that were dominant in the

pelagic in July were no longer encountered after August, while some chemotypes that never accounted for high relative abundances were encountered throughout the season. Nonetheless, chemotypes were identified in the sediment in July and November that were never found in plankton samples. A principal component analysis revealed that communities in the pelagial and the benthic were very different in July but converged during the season due to the deposition of dominant planktonic chemotypes in the sediment. The seasonal success of particular chemotypes, i.e., increasing relative abundance, can be the results of either bottom-up or top-down regulation (Carpenter and Kitchell 1993). For bottom-up regulation the nutrient and light availability or the ability to exploit resources efficiently, respectively, is crucial and ecophysiological studies have shown that individual *Microcystis* strains differ considerably in their functional responses to variable growth factors (Hesse and Kohl 2001; Schatz et al. 2005). As top-down factor the susceptibility of individual clones to grazing by herbivores - or cyanophage infection (Tucker and Pollard 2005) - is important. Several peptides are discussed as grazing protecting agents like microcystins (Ghadouani et al. 2004), microviridins (Rohrlack et al. 2004), and cyanopeptolins (Czarnecki et al. 2006) acting either as toxins or inhibitors of digestive enzymes.

6.5. New peptides within *Microcystis* genus

The purpose of this part was to described a high diversity of potencially bioactive compounds by *Microcystis* genus. We alert that beside the microcystins also aeruginosins, anabaenopeptins, microginins and microviridins could be more frequent cyanotoxins in *Microcystis* community. A total number of more than 90 cyanopeptides could be detected, 61 of which could be identified either as known peptides or new structural variants of known peptide classes. For 18 new peptides flat structures are proposed. New congeners differed from known ones mainly in chlorination (aeruginosins), methylation (microginins), or amino acid sequences (cyanopeptolins). The high number of peptides and especially the new peptides underline the capability of *Microcystis* strains as producers of a high diversity of potentially bioactive compounds.

6.5. Future directions

The research presented within this thesis not only gives new insights in cyanobacterial taxonomy, ecology and toxicology but also raises new questions:

- It is possible to use the oligopeptides in the cyanobacterial taxonomy?
- Is the observation of ultrastructural changes possible method for the prediction of *Microcystis* reinvasion?
- What other combination of chemotypes exist in other reservoirs by *Microcystis* and occurred the chemotypes repeatedly in the similar environmental factors?

- Is the presented diversity of *Microcystis* peptides final or it is only a starting point for the further description on new hundreds of cyanobacterial oligopeptides?

Such questions could be the start for new research in which the further cyanobacterial genera are screened on all type of oligopeptides by MALDI TOF MS.

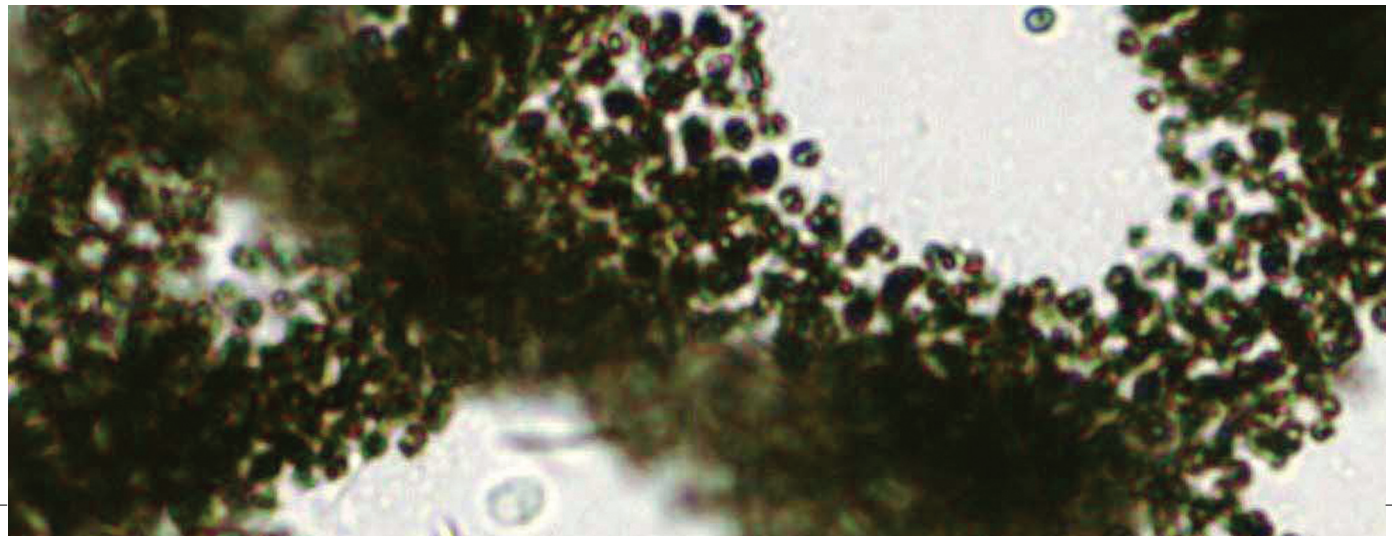
6.6. General conclusions

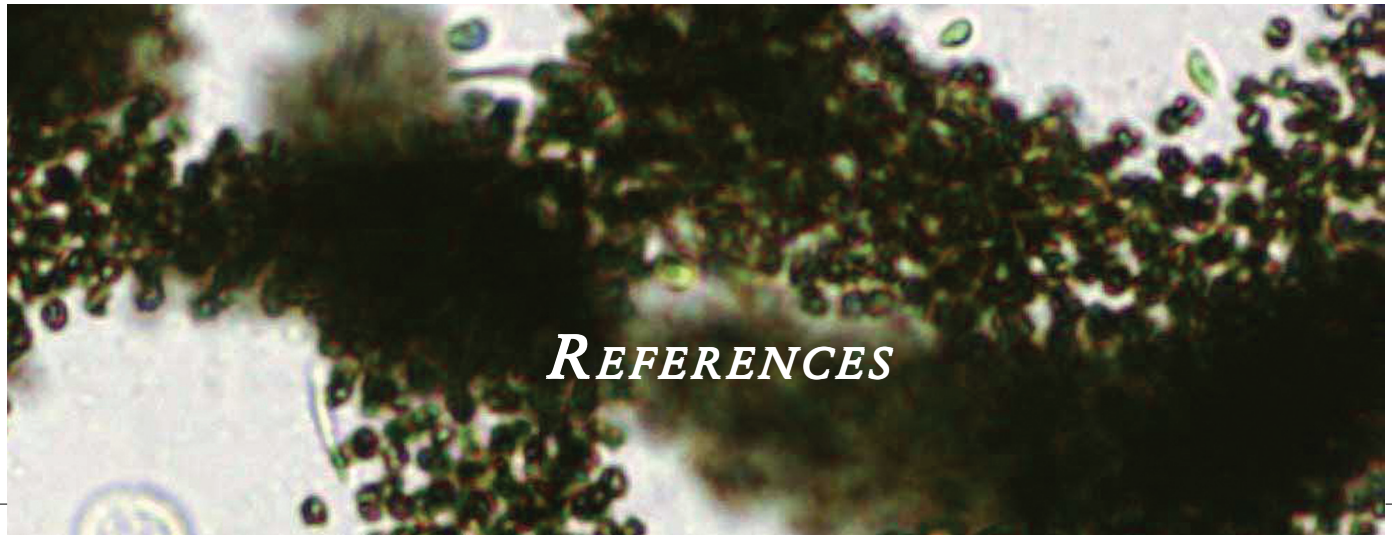
6.6.1. We found that within *Microcystis* genus coexist non-peptide and peptide group differed also morphologically. We conclude that occurrence/absence of some specific peptides in *Microcystis* colony could be potentially used in polyphasic taxonomy on subgeneric level.

6.6.2. The reinvasion of *Microcystis* colonies started when temperature above sediment exceeds 8-9 °C and oxygen concentration 4 mg/l. *Microcystis* recruitment seems to be only a result of external factors without a contribution of endogenous timing. We conclude that start of *Microcystis* reinvasion process is characterised by the colony enlargement (colony formation).

6.6.3. In the course of the season the *Microcystis* community became significantly less diverse in peptide numbers (linear regression of Shannon indices, $p<0.001$). In contrast, other chemotypes that were dominant in the pelagic in July were no longer encountered after August, while some chemotypes that never accounted for high relative abundances were encountered throughout the season. Nonetheless, chemotypes were identified in the sediment in July and November that were never found in plankton samples.

6.6.4. We identified 61 peptides either as known peptides or new structural variants of known peptide classes from 850 colonies from pelagial and sediment as well. We alert that beside the microcystins also aeruginosins, anabaenopeptins, microginins and microviridins could be more frequent cyanotoxins in *Microcystis* community. For 18 new peptides flat structures are proposed. New congeners differed from known ones mainly in chlorination (aeruginosins), methylation (microginins), or amino acid sequences (cyanopeptolins).





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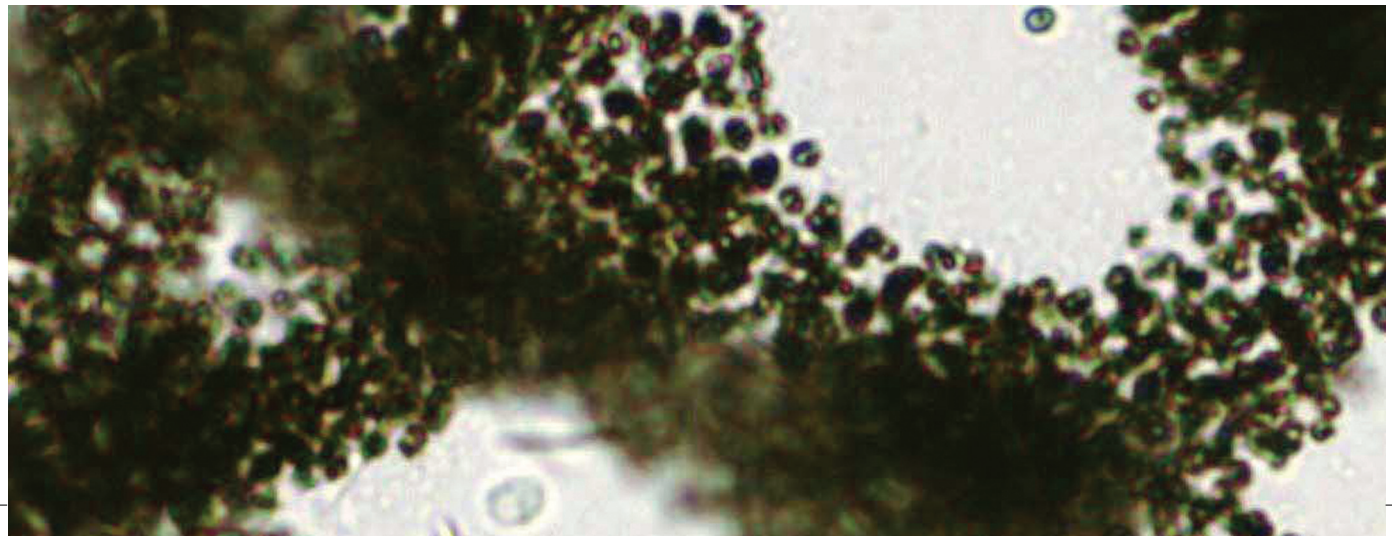
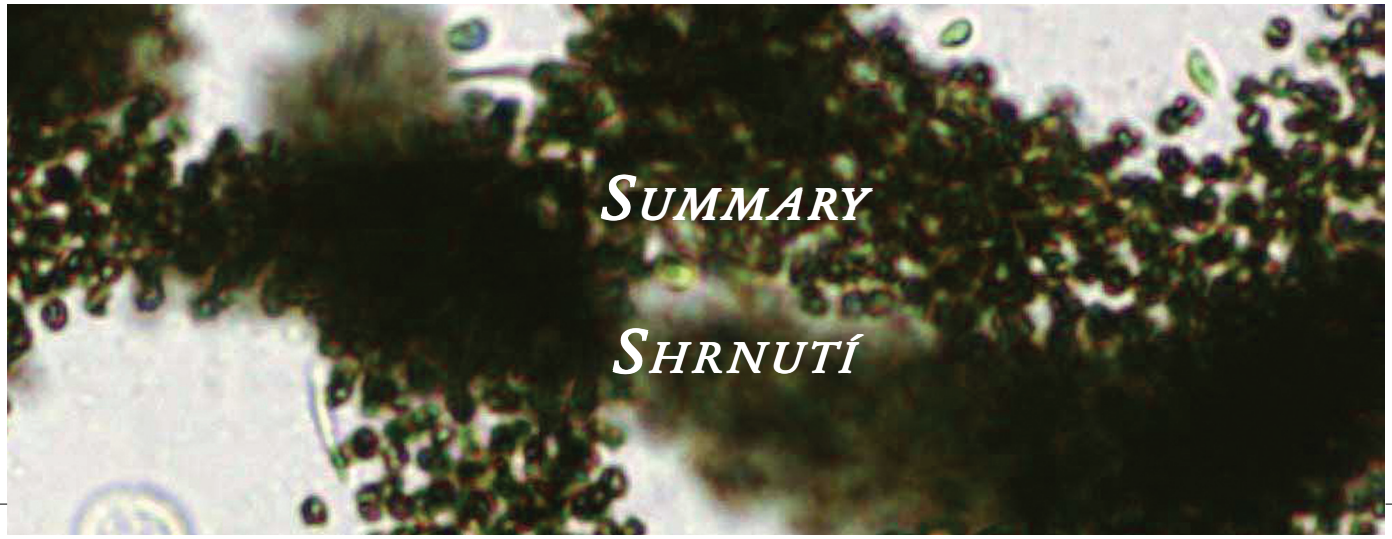




Summary

Shrnutí

Summary

The central aim of this study was to gain insight in the relationship between morphology and peptide occurrence, autecology of colonies during the reinvasion and dynamics of peptide within the natural *Microcystis* population.

We found that within *Microcystis* genus coexist non-peptide and peptide group differed also morphologically. We conclude that occurrence/absence of some specific peptides in *Microcystis* colony could be potentially used in polyphasic taxonomy on subgeneric level.

The increase cell numbers, enlargement of colonies and synthesis of gas vesicles by benthic *Microcystis* population started only in the reservoir not in the stable condition of refrigerator. The changes of *Microcystis* reservoir population were detected when temperature above sediment exceeds 8-9 °C and oxygen concentration 4 mg/L. Hence, *Microcystis* recruitment seems to be only a result of external factors without a contribution of endogenous timing.

In the course of the season the *Microcystis* community became significantly less diverse in peptide numbers (linear regression of Shannon indices, $p<0.001$). In contrast, other chemotypes that were dominant in the pelagic in July were no longer encountered after August, while some chemotypes that never accounted for high relative abundances were encountered throughout the season. Nonetheless, chemotypes were identified in the sediment in July and November that were never found in plankton samples.

We identified 61 peptides either as known peptides or new structural variants of known peptide classes from 850 colonies from pelagial and sediment as well. We alert that beside the microcystins also aeruginosins, anabaenopeptins, microginins and microviridins could be more frequent cyanotoxins in *Microcystis* community. For 18 new peptides flat structures are proposed. New congeners differed from known ones mainly in chlorination (aeruginosins), methylation (microginins), or amino acid sequences (cyanopeptolins).

Shrnutí (Czech)

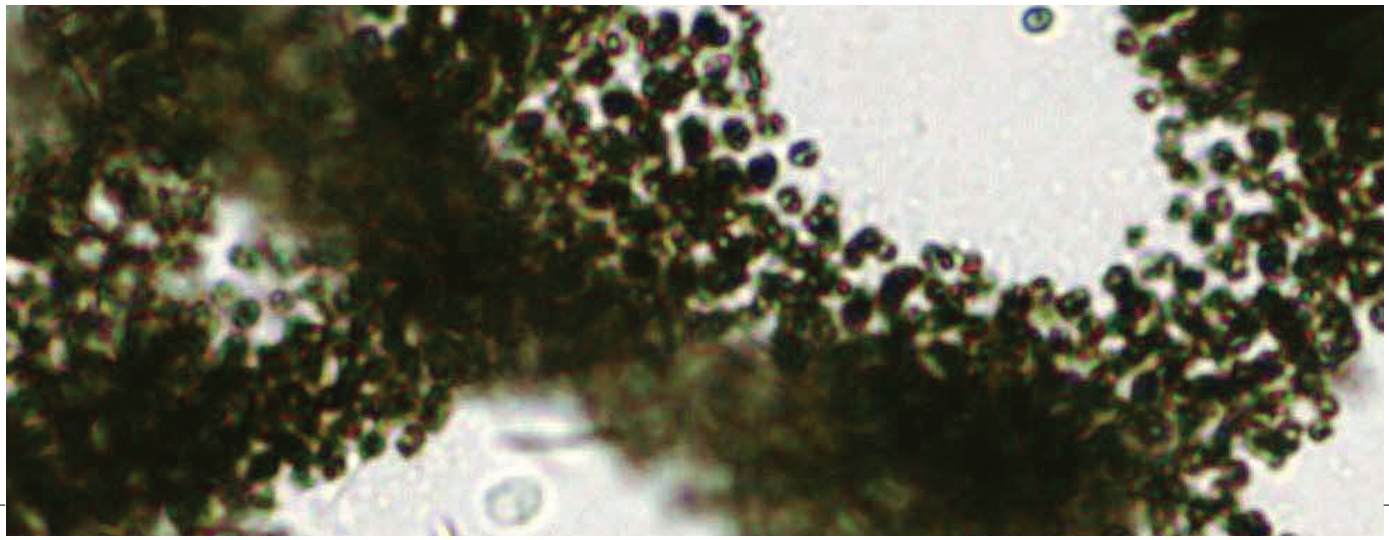
Ústředním cílem této studie bylo získat poznatky ve vztahu mezi morfologií a výskytem oligopeptidů, v autekologii kolonií během reinvaze a dynamice výskytu peptidů v přírodní populaci rodu *Microcystis*.

Zjistili jsme, že v rámci rodu *Microcystis* koexistují non-peptidové a peptidové skupiny lišící se také morfologicky. Docházíme k závěru, že výskyt / absence některých specifických peptidů v rámci rodu *Microcystis* by mohly být potenciálně využity v polyfázickém přístupu v taxonomii sinic na subgenerické úrovni.

Nárůst počtu buněk, tvorbu kolonií a syntézu gas veziklů u bentické populace *Microcystis* jsme zaznamenali pouze v měnících se podmínkách nádrže, ale ne ve stabilních podmínkách v lednici. Změny populace *Microcystis* v nádrži byly detekovány v období, kdy teplota nad sedimentu byla vyšší než 8-9 ° C a koncentrace kyslíku 4 mg /L. Reinvaze *Microcystis* se zdá být pouze důsledkem vnějších faktorů, bez příspěvku endogenního načasování.

V rámci sezony se populace *Microcystis* stala výrazně méně rozmanitá v množství typů peptidů (lineární regrese Shannon indexy, $p < 0{,}001$). Na rozdíl od jiných chemotypes, které byly dominantní v pelagiále v červenci, jsme již od srpna nezaznamenali, zatímco některé chemotypes měly vysoké relativní abundances v celé sezóně. Některé chemotypes byly zjištěny pouze v sedimentu v červenci a listopadu, které nebyly nikdy nalezeny ve vzorcích planktonu.

Identifikovali jsme 61 peptidů, které jsou buď známé peptidy nebo nové konstrukční varianty známých peptidových tříd, z 850 kolonií z pelagiálu a sedimentu. V této práci upozorňujeme na fakt, že vedle microcystinů také aeruginosiny, anabaenopeptiny, microgininy a microviridiny mohou být častějšími cyanotoxiny v populaci *Microcystis*.



List of Publications and Related Outputs

Curiculum vitae

Publications

Scientific papers in journals with IF

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Curriculum vitae Lenka Šejnohová

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Research interest

Autecology and ultrastructure of cyanobacteria and trichal green algae
Use of cyanobacteria and algae for the biomonitoring
Connection between basic research and practice due to lectures specialized on algae and cyanobacteria determination

Study and practice

2001 - B.Sc. in biology, Faculty of Science, Charles University, Prague
2003 - M.Sc. (Mgr.) in biology, specialization: systematics and ecology of nonvascular plants at Department of Botany, Faculty of Sciences, Charles University, Prague, Czech Republic
Since 2003 - Ph.D. in biology, Department of Zoology and Ecology, Programme Hydrobiology, Faculty of Science, Masaryk University in Brno, Czech Republic
Since 2003 - scientist at Department of Experimental Phycology a Ecotoxicology, Institute of Botany, Academy of Sciences, Brno, Czech Republic

Membership in scientific organizations

Czech Phycological Society, World Aquaculture Society

Teaching

2002-2006: Electron microscopy. - Lectures for students of secondary schools, Biological Olympiad, Czech Ministry of Education, Běstvina.
2002-2006: Ecology and taxonomy of algae and cyanobacteria. - Lectures for students of secondary schools, Biological Olympiad, Czech Ministry of Education, Běstvina.
2003-2006: Courses in B.Sc. study, Masaryk University - Determinaton of non-vascular plants.
Since 2003: Lectures specialized on determination of algae and cyanobacteria for practices institution.
Since 2008: Courses in M.Sc. study program, Charles University - Algae and cyanobacteria in water and hygiene practices.

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Abbreviations

VC	vegetative cells
LC	life cycle
MALDI TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
Mcyst	microcystins
WB	water bloom
GV	gas vesicles
IF	impact factor