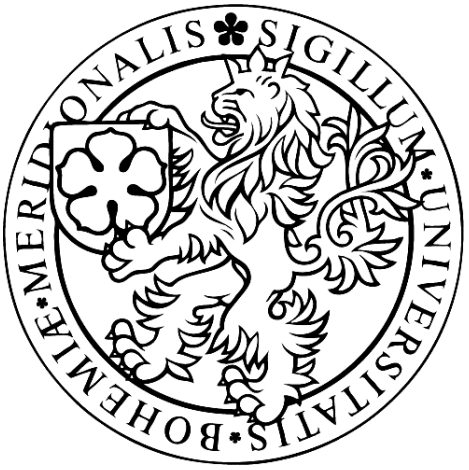
UNIVERSITY OF SOUTH BOHEMIA
FACULTY OF SCIENCE



Cytotoxicity and secondary metabolites production in cyanobacteria

Pavel Hrouzek

Ph.D. Thesis

Supervisor: Ing. Jiří Kopecký
Institute of Microbiology,
Section of Phototrophic Microorganisms, CAS

Consultant: Prof. Jiří Komárek
University of South Bohemia,
Faculty of Science

ČESKÉ BUDĚJOVICE 2010

Hrouzek, P. 2010: Cytotoxicity and secondary metabolites production in cyanobacteria – 136 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

Annotation

Cyanobacteria are well-known producers of secondary metabolites of different chemical structures and a wide range of biological functions. In the present thesis, the cytotoxic activity of cyanobacteria, originating from different habitats, was studied in order to reveal whether cytotoxicity is an environmentally dependent characteristic. In addition, the data were compared with the toxicity of these extracts to the model invertebrate *Artemia salina*. The obtained data suggest that cytotoxic cyanobacteria are favoured under some conditions and thus more frequent in particular localities. The majority of the studied extracts and fractions exhibited cytotoxitity to the Sp/2 cell line not accompanied by toxicity to *A. salina*. Moreover, in most of the strains with both activities to *A. salina* and Sp/2 cells the toxic effect was caused by an identical fraction. This result suggests that the toxic effect of the cyanobacterial secondary metabolites is mostly affecting basal cell metabolism rather than targeting specific organisms. In one of studied strains, *Cylindrospermum* sp. C24/1989, novel lipopeptides puwainaphycin F and G have been detected, isolated and their structure and biological effect have been characterized. Both of these structures interfere with eucaryotic membranes and cause a Ca^{2+} leakage into the cell. Subsequently, an enhanced tyrosine phosohorylation and relocalization of f-actin in the cell was observed. Lastly, the correlation between metabolite production and the reconstructed phylogeny was studied in planktonic *Dolichospermopsis* strains. Most of the detected compounds were found to be randomly disspersed across the reconstructed phylogeny and thus cannot be considered as good chemotaxonomic markers. This result also hampers the possible detection of toxic cyanobacteria by morphological methods or molecular detection based on the 16SrDNA gene.

Finnancial support

This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (Project FRVŠ no. 3491/2005 and MŠM 6007 665 808, MSMT 1MO571, MŠMT ME874) and by European territorial cooperation BIOPHARM M00140.

Declaration

I declare that this dissertation was fully worked out by myself using the cited literature only. I declare that in accordance with the Czech legal code § 47b law No. 111/1998 in valid version I consent to the publication of my dissertation in an edition made by removing marked parts archived by Faculty of Science in an ellectronic way in the public acces section of the STAG database run by the University of South Bohemia in České Budějovice on its webpages.

Prohlašuji, že jsem svoji disertační práci vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.
Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné databázi STAG provozované Jihočeskou universitou v Českých Budějovicích na jejích internetových stránkách.

České Budějovice, 15th April 2010 Pavel Hrouzek

Acknowledgement

I would like to express many thanks to my supervisor Jiří Kopecký, for his guiding during my PhD study at the Institute of Microbiology. I am very grateful for his advice, help and all other support of my work. My deep thanks also go to Jiří Komárek who inspired me and provoked my fascination in cyanobacteria and phycology during the first years at the University of South Bohemia. I wish to express many thanks to Alena Lukešová, not only for all the strains she provided for my study, but for her kindness, good advice and help. For support and inspiration I am very grateful to Ondřej Prášil, Dalibor Štys and Jiří Masojídek. My thanks also go to Blahoslav Maršálek for his help in the direction of my thesis. Finishing of this work wouldn't have been possible without the help of my colleagues Petr Tomek, Daniel Hisem, Jana Tomšíčková, Lada Samcová and Pavel Souček. I would also like to thank Eliška Zapomělová and Klára Řeháková for inviting me to collaborate with them on planktonic cyanobacteria. I am very grateful for spending time in different laboratories and for having the possibility to get different points of view; namely, I would like to express many thanks to Kaarina Sivonen, Pirjo Wacklin and Stefano Ventura. I am also very glad to have had the possibility to collaborate with, and to be taught about new methods by Jan Černý and Marek Kuzma. All my colleagues from Institute of Microbiology are greatly acknowledged for the nice atmosphere and „scientific discussions" by the coffee – Michal Koblížek, Zuzana Čuperová, Eva Kotabová, Roman Sobotka, Katja Boldareva, Radek Kaňa, Eva Žižková, Jiří Šetlík, Eva Hojerová, Ola Kapuscik, Monika Hlavová, Markéta Foldýnová and Jana Kopečná.

Finally, I would like to send my warm thanks to family, my parents Petr and Dagmar and my brother Petr, for all the help, support and nice time. My greatest thanks are sent to Ema for her understanding and patience.

List of original articles

I **Hrouzek P.,** Tomek P., Lukešová A., Urban J., Voloshoko L., Pushparaj B., Ventura S., Lukavský J., Štys D. & Kopecký J. 2010. Cytotoxicity and secondary metabolites production in terrestrial *Nostoc* strains, originating from different climatic/geographic regions and habitats: Is their cytotoxicity environmentally dependent? *Environmental Toxicology* (accepted: DOI: 10.1002/tox.20561)

II. **Hrouzek P.,** Kuzma M., Černý J., Novák P., Fišer R., Šimek P., Štys D., Lukešová A., & Kopecký J.: Cyanobacterial cyclic peptides Puwainaphycins F and G are causing cytotoxic effect via cell membrane permeabilization and subsequent actin relocalization. (*manuscript*)

III. Hisem D., **Hrouzek P.,** Tomek P., Tomšíčková J., Zapomělová E., Skácelová K., Lukešová A. & Kopecký J.: Cyanobacterial cytotoxicity to mammal cell lines versus toxicity to brine shrimp. *Toxicon* (*submitted*)

IV. Rajaniemi P., **Hrouzek P.**, Kaštovská K., Willame R., Rantala A., Hoffmann L., Komárek J. & Sivonen K. 2005. Phylogenetic and morphological evaluation of the genera *Anabaena, Aphanizomenon, Trichormus* and *Nostoc* (Nostocales, Cyanobacteria). *International Journal of Systematic and Evolutionary Microbiology* 55: 11-26.

V. Zapomělová E., **Hrouzek P.**, Řezanka T., Jezberová J., Řeháková K., Hisem D. & Komárková J.: Are fatty acid profiles and secondary metabolites good chemotaxonomic markers of genetic and morphological clusters of *Dolichospermum* spp. and *Sphaerospermopsis* spp. (Nostocales, Cyanobacteria)? (*manuscript*).

VI. Zapomělová E., Jezberová J., **Hrouzek P.,** Hisem D., Řeháková K. & Komárková J. 2009. Polyphasic characterization of three strains of *Anabaena reniformis* and *Aphanizomenon aphanizomenoides* (Cyanobacteria) and their reclassification to *Sphaerospermum* gen. nov. (incl. *Anabaena kisseleviana*. *Journal of Phycology* 45(6): 1363-1373.

VII. Zapomělová E., Hisem D., Řeháková K., **Hrouzek P.,** Jezberová J., Komárková J., Korelusová J. & Znachor P. 2008. Experimental comparison of phenotypical plasticity and growth demands of two strains from the *Anabaena circinalis*/*A. crassa* complex (cyanobacteria). *Journal of Plankton Research* 30 (11): 1257-1269.

Authors contribution to the articles

I P. Hrouzek performed the cultivation, extract preparation and cytotoxicity testing in half of the studied strains. He also performed the HPLC-MS analysis, processed the data, conducted the statistical analysis of the data, and wrote the paper.

II The author performed the cytotoxicity experiments with the strain *Cylindrospermum* sp. C24/1989 and detected the cytotoxic fraction by activity-guided fractionation. Furthermore, he performed large-scale cultivation, isolation and purification of both puwainaphycin variants by preparative HPLC. He assisted in immunofluorescence visualization of the cell componens. Finally, the author tested the IC_{50} value and the membrane permeabilization by the LDH test and compile the paper.

III This paper is based on the results of the bachelor thesis of Daniel Hisem, who was supervised by P. Hrouzek. P. Hrouzek performed the fractionation and cytotoxicity testing of the obtained fraction. Together with D. Hisem he wrote the paper.

IV P. Hrouzek evaluated the morphology of the strains included in this study. He also performed statistical analysis of the morphological data and participated in writing of the paper.

V The author performed the HPLC-MS analysis together with D. Hisem, analyzed the data and wrote the part concerning secondary metabolites.

VI The author performed the HPLC-MS analysis of *A. renirormis* and *Aph. aphanizomenoides*, evaluated the data and prepared them for publication. He also tested the cylindrospermopsin production in the studied strains and wrote the results and discussion parts concentrated on the secondary metabolite production.

VII The author performed the HPLC-MS analysis, evaluated the data and prepared them for publication.

Content

General Introduction

The study of cyanobacterial secondary metabolites has become a fascinating field of microbiology during the last few decades. It has attracted the attention of many research groups, and hundreds of different compounds with various biological effects have been described (Carmichael, 1992; Namikoshi and Rinehart, 1996; Sivonen, 1996; Welker and von Döhren, 2006). Cyanobacteria have been refered to produce different forms of cyclic and linear peptide, alkaloides, macrolactones and heterocyclic compounds (Carmichael 1986, 1990, 1992, Fujii et al. 2002, Knűbel *et al.* 1990, Patterson and Carmeli 1992, Rodey et al. 1999, Sivonen 1996, Welker and von Döhren 2006). From the above cited, peptides are the most important secondary metabolites produced by cyanobacterial cells in high concentrations. These structures consist of standart as well as modified D- and L-aminoacid forms. The majority of cyanobacterial oligopeptides are assumed to be synthesized by a non-ribozomal synthetic pathway placed on the inner membrane of the cyanobacterial cell (Ditman et al. 1997, Tillet et al 2000, Welker and von Döhren, 2006), but the synthesis of peptidic structures by cyanobacterial ribozomes with post-translation modification has also been suggested (Schmidt et al. 2005). Recently, strong evidence for the ancient origin of this synthetic system has been brought forward (Rantala el al. 2004). Presently around 600 variants of cyanobacterial peptides have been described (Welker and von Döhren, 2006), but screening studies have increasingly indicated that this number is only a small fraction of the real peptide diversity in cyanobacteria (e.g. Welker et al. 2006). Peptides can be classified, based on their common characteristics of chemical structure, into six classes – aeruginosins, microginins, anabaenopeptins, cyanopeptolins, microcystins, microviridins and cyclamides (Welker and von Döhren, 2006). However, at least one third of all described structures do not fit into any of these classes. The enormous diversity of cyanobacterial peptides can probably be considered as due to the modular structure of the non-ribozomal synthetic pathway. Peptides are synthesized by large multidomain synthetases bounded in the membrane, the final aminoacid sequence then depending on the position of the enzyme. Every change in function of a particular enzyme then results in the production of a different peptide variant. Despite the production of cyanobacterial peptides being widely studied from many different points of view, the primary function of cyanobacterial peptides remains unresolved.

As mentioned above, several different biological consequences have been reported for peptides and other secondary metabolites produced by cyanobacteria. One of the first reported effects of cyanobacterial compounds on living organisms was the hepatotoxicity of microcystins (Miura et al. 1988, Falconer et al. 1994). This heptapeptide causes liver necrosis by way of inhibiting protein phosphatases and inducing apopotosis in human hepatocytes (Botha et al. 2004, MacKintosch et al. 1990). This effect was later proved for other type of microcystins. The second compound affecting human health is the alkaloid anatoxin-a (and related compounds) that interferes with the sodium channels and enhances neuron stimulation leading to the suffocation of the organism (Carmichael et al. 1975, 1979). Besides these effects important for the human population, there is a large body of evidence of different biological activities including antibacterial (Jaki et al. 1999),

antifungal (Bonjouklian et al. 1991, Kajiyama et al. 1998), and virostatic activity (e.g. Knűbel *et al.* 1990), enzyme inhibition (Murakami et al. 1994, 1995, 1997, Reshev and Carmeli 2001) as well as inhibition of the cell cultures of mammal cells (Barchi et al 1983, Patterson *et al.* 1993, Rodney et al. 1999, Trimurtulu et al. 1994). Thus, some authors defined two basic groups of cyanobacterial secondary metabolites based on their biological effects (e.g. Maršálek et al. 1996): biotoxins, causing death to higher organisms; and cytotoxins, compounds causing inhibition to cell cultures or single cell organisms. It is clear that the cytotoxic effect can, in certain conditions, lead to biotoxicity. This is the case with the cytotoxic alkaloid cylindrospermopsin inhibiting proteosythesis in liver cells, which can lead to liver destruction and the subsequent death of all organisms (Ohtani et al. 1992). However, there exists another compound with a clearly higher toxicity to individual cells that affects whole mammal organisms at low levels. Such compounds, called cytotoxins, are showing much promise in the field of pharmacology as potential cancer treatment agents. Cytotoxic compounds are very heterogeneous regarding their chemical structures and modes of action. One of the most effective cyanobacterial compounds, isolated from cyanobacteria of the genus *Nostoc,* is depsipeptide cryptophycin (Trimurtulu et al. 1994). This causes tubulin depolymerization in different types of cancer cells at nanomolar concentrations (Smith *et al.* 1994), which leads to the blockage of the cell cycle at the G2 phase and subsequent apoptosis; specific activity against solid tumors for this compound has also been reported (Teicher *et al.* 2000). Actin depolymerization is the functional mechanism of the macrolidic cytotoxic compound tolytoxin (Patterson *et al.* 1993). Apart from these effects, several cyanobacterial compounds have been proved to interfere with the DNA replication process (Teneva *et al.* 2003).

The production of cyanobacterial metabolites can be examined from various perspectives. First, screening studies can be targeted towards the isolation of novel compounds for applications in pharmacology or biotechnology. Second, significant amounts of such compounds can be released by living or decaying cyanobacterial cells, and thus affects the environment. Accordingly, knowledge about the function and fate of these compounds within the environment is also important. Last but not least, knowledge of the distribution of particular compounds' production among cyanobacteria of different genotypes, morphotypes and ecology is extremely important for hydrobiology and water quality monitoring, as well as theoretical diversity studies and chemotaxonomy.

The first part of this presented work is concentrated on the interaction of cyanobacterial metabolites with mammal cells in vitro and the characterization of its effects. Special attention is given to the question of whether cytotoxins are part of a cyanobacterial adaptation to environmental conditions, or whether this bioactivity is only coincidental. In the second part of this thesis, correlation between secondary metabolite production and cyanobacterial phylogeny is tested.

Results

This thesis is based on results of five accepted scientific articles, one article submited to international journals and two manuscript prepared for submision.

I

Extensive selection of cyanobacterial strains (82 isolates) belonging to the genus *Nostoc*, isolated from different climatic regions and habitats, were screened for both their secondary metabolite content and their cytotoxic effects to mammalian cell lines. The overall occurrence of cytotoxicity was found to be 33%, which corresponds with previously published data. However, the frequency differs significantly among strains, which originate from different climatic regions and microsites (particular localities). A large fraction of intensely cytotoxic strains were found among symbiotic strains (60%) and temperate and continental climatic isolates (45%); compared with the less significant incidences in strains originating from cold regions (36%), deserts (14%), and tropical habitats (9%). The cytotoxic strains were not randomly distributed; microsites that clearly had a higher occurrence of cytotoxicity were observed. Apparently, certain natural conditions lead to the selection of cytotoxic strains, resulting in a high cytotoxicity occurrence, and vice versa. Moreover, in strains isolated from a particular microsite, the cytotoxic effects were caused by different compounds. This result supports our hypothesis for the environmental dependence of cytotoxicity. It also contradicts the hypothesis that clonality and lateral gene transfer could be the reason for this phenomenon. Enormous variability in the secondary metabolites was detected within the studied *Nostoc* extracts. According to their molecular masses, only 26% of these corresponded to any known structures; thus, pointing to the high potential for the use of many terrestrial cyanobacteria in both pharmacology and biotechnology.

II

Puwainaphycins F and G, which cause unique cytoskeletal changes in mammalian cell lines and subsequent cell death, have been isolated from the cyanobacterium *Cylindrospermum* sp. C24/89. Puwainaphycin F has been shown to be a cyclic peptide (valyl-2aminobut-2(E)-enoyl-asparaginyl-2aminobut-2(E)-enoyl-asparaginyl-alanyl-threonyl-Nmethylasparaginyl-prolyl) containing the β-amino acid unit (2-hydroxy-3-amino-tetradecanoic acid). It differs from previously described variants of the puwainaphycins, at five amino acids as well as in the β-amino acid unit. The rapid interaction of this compound with the plasma membrane leads to an elevation of the concentration of intracellular Ca^{2+}, with kinetics comparable to the well-established calcium ionophore ionomycin. Subsequently, the induction of tyrosine phosphorylation was followed by the unique transformation of the actin cytoskeleton into ring structures around the nuclei being observed. All of these alterations in the cellular morphology and physiology after ca. 10 hrs finally results in necrotic cell death. Puwainaphycin G, congener of puwainaphycin F, differs in the substitution of an

asparaginyl moiety for a glutaminyl in the third position; but exhibited the same biological function and moderate toxicity as did puwainaphycin F.

III

Heterocytous cyanobacteria originating from different habitats have been screened for toxicity to brine shrimp *Artemia salina* and the murine lymphoblastic cell line Sp/2. Methanolic extracts of biomass and cultivation media were tested for toxicity and selected extracts were fractionated to determine the active fraction. We found a significant toxic effect to *A. salina* and Sp/2 cells in 5.2% and 31% of studied extracts, respectively. Only 8.6% of all tested strains were highly toxic to both *A. salina* and the Sp/2 cell line. Based on these data, we conclude that it is impossible to monitor cytotoxicity using only the brine shrimp bioassay, since cytotoxicity is a more frequent feature in comparison with toxicity to *A. salina*. It seems that in most cases the toxic effect of cyanobacterial secondary metabolites is targeted at some basal metabolic pathways present in eucaryotic cells rather than being a specific mechanism against a complex organism. Only in two of all tested strains was toxicity to *A. salina* recorded not accompanied to murine cell line toxicity. Moreover, in most of the selected strains exhibiting activity to *A. salina* and Sp/2 cells, the toxic effect to *A. salina* and Sp/2 cell line was caused by an identical fraction. These findings lead us to the conclusion that cyanobacterial metabolites can secondarily act as a defensive mechanism against grazing, although they are almost certainly not synthesized specifically against herbivores**.**

IV

The heterocytous cyanobacteria form a monophyletic group according to 16S rRNA gene sequence data. Within this group, phylogenetic and morphological studies have shown that genera such as *Anabaena* and *Aphanizomenon* are intermixed. Moreover, the phylogeny of the genus *Trichormus*, which was recently separated from *Anabaena*, has not been investigated. The aim was to study the taxonomy of the genera *Anabaena*, *Aphanizomenon*, *Nostoc* and *Trichormus* belonging to the family *Nostocaceae* (subsection IV.I) by morphological and phylogenetic analyses of 16S rRNA gene, rpoB and rbcLX sequences. New strains were isolated to avoid identification problems caused by morphological changes of strains during cultivation. Morphological and phylogenetic data showed that benthic and planktic *Anabaena* strains were intermixed. In addition, the present study confirmed that *Anabaena* and *Aphanizomenon* strains were not monophyletic, as previously demonstrated. The evolutionary distances between the strains indicated that the planktic *Anabaena* and *Aphanizomenon* strains as well as five benthic *Anabaena* strains in cluster 1 could be assigned to a single genus. On the basis of the 16S rRNA, rpoB and rbcLX gene sequences, the *Anabaena*/*Aphanizomenon* strains (cluster 1) were divided into nine supported subclusters which could also be separated morphologically, and which therefore might represent different species. *Trichormus* strains were morphologically and phylogenetically heterogeneous and did not form a monophyletic cluster. These *Trichormus* strains, which were representatives of three distinct species, might actually belong to three genera

according to the evolutionary distances. *Nostoc* strains were also heterogeneous and seemed to form a monophyletic cluster, which may contain more than one genus. It was found that certain morphological features were stable and could be used to separate different phylogenetic clusters. For example, the width and the length of akinetes were useful features for classification of the *Anabaena*/*Aphanizomenon* strains in cluster 1. This morphological and phylogenetic study with fresh isolates showed that the current classification of these anabaenoid genera needs to be revised.

V

The genera *Dolichospermum* (Ralfs ex Bornet et Flahault) Wacklin et al. 2009 and *Sphaerospermopsis* Zapomělová et al. 2010 represent highly diversified group of planktonic cyanobacteria that have been recently separated from the traditional genus *Anabaena* Bory ex Bornet et Flahault 1888. In this study, morphological diversity, phylogeny of 16S rRNA gene, production of fatty acids and secondary metabolite profiles were compared among 33 strains of 14 morphospecies isolated from the Czech Republic. Clustering of the strains based on 16S rRNA gene sequences corresponded to wider groups of species in terms of morphology. On the contrary, the overall secondary metabolite and fatty acid profiles were neither correlated to each other, nor to 16S rRNA phylogeny and morphology of the strains suggesting that these compounds are not good chemotaxonomic tools for the cyanobacterial genera studied. Nevertheless, a minor part of the detected secondary metabolites (19% of all compounds) were present solely in the closest relatives and can be thus considered as autapomorphic features.

VI

Occurrences of rare cyanobacteria *Anabaena reniformis* Lemmerm. and *Aphanizomenon aphanizomenoides* (Forti) Horecká et Komárek were recently detected at several localities in the Czech Republic. Two monoclonal strains of *An. reniformis* and one strain of *Aph. aphanizomenoides* were isolated from distant localities and different sampling years. They were characterized by a combination of morphological, genetic, and biochemical approaches. For the first time, partial 16S rRNA gene sequences were obtained for these morphospecies. Based on this gene, all of these strains clustered separately from other planktonic *Anabaena* and *Aphanizomenon* strains. They appeared in a cluster with *Cylindrospermopsis* Seenaya et Subba Raju and *Raphidiopsis* F. E. Fritsch et M. F. Rich, clustered closely together with two *An. kisseleviana* Elenkin strains available from GenBank. A new generic entity was defined (*Sphaerospermum* gen. nov., with the type species *S. reniforme*, based on the traditional species *An. reniformis*). These results contribute significantly to the knowledge base about genetic heterogenity among planktonic *Anabaena*–like and *Aphanizomenon*–like morphospecies. Accordingly, the subgenus *Dolichospermum*, previously proposed for the group of planktonic *Anabaena*, should be revaluated. Secondary metabolite profiles of the *An. reniformis* and *Aph. aphanizomenoides* strains differed considerably from 17 other planktonic *Anabaena* strains of eight morphospecies isolated from Czech water bodies. Production of puwainaphycin A was

found in both of the *An. reniformis* strains. Despite the relatively short phylogenetic distance from *Cylidrospermopsis*, the production of cylindrospermopsin was not detected in any of our strains.

VII

Two cyanobacterial strains were isolated in 2004 from different localities in the Czech Republic. Field morphology of the strain 04-26 (Jesenice reservoir) matched with the species description of *Anabaena crassa* (Lemm.) Kom.-Legn. et Cronb. 1992, whereas the strain 04-28 (Hodějovický fishpond) was identified as *A. circinalis* Rabenh. ex Born. et Flah. 1888. Both these strains, exposed to various experimental conditions (temperature, light intensity, nitrogen and phosphorus concentration), displayed highly similar morphologies and spanned the morphological variability of both of the above-mentioned species. Significant relationships between environmental conditions (temperature, phosphorus) and morphological characteristics (vegetative cell and heterocyte dimensions, trichome coiling parameters) have been recorded for the first time within the genus *Anabaena*. The strains studied differed in their temperature and light growth optima and in secondary metabolite contents. However, both were identical (100% similarity) in their 16S rRNA gene sequence and showed 99.9–100% similarity to the published 16S rRNA sequences of *A. circinalis* strains from northern Europe.

General discussion

The origin of cyanobacteria has been dated back to 3.5 billion years ago (Schopf 1993). Fossil records suggest that the morphology of these organisms have not undergone any dramatic changes since those times. Unfortunately, we have very limited information regarding the biochemical and physiological properties of the ancient ancestors of this remarkable group. However, evolutionary models allow us to estimate the biochemical characteristics of these ancient cyanobacteria. Today's cyanobacteria are well-known producers of different forms of small peptides (Sivonen 1996, Van Wagoner et al. 2007, Welker and von Döhren 2006). Molecular studies suggest a congruent phylogenetic pattern of 16SrDNA reconstucted phylogeny and mcy genes responsible for the production of the cyanopeptide microcystin (Rantala et al. 2004). These findings lead to the conclusion that the huge synthetic apparatus producing cyanobacterial peptides is very ancient and must have played an important role during the evolutionary history of the cyanobacterial group. Surprisingly, this synthetic machinery is continuously producing enormous quantities of peptides of fascinating structural diversity. Up to now, no satisfactory explanation of the primary function of cyanobacterial peptides has been proposed and generally accepted.

In the present study, the cytotoxicity of cyanobacterial secondary metabolites has been evaluated. Cytotoxic compounds in general are defined as substances inhibiting or stopping the growth of individual cells (e.g. cell cultures). In most cases these compounds usually exhibit a wide range of effects on unicellular organisms and invertebrates (Patterson

and Carmeli 1992, Rodney et al., 1999, Berry et al., 2004; Biondi et al., 2004). Approximately 30% of cyanobacterial extracts have been reported to cause damage to mammal cells in vitro (**Paper I**, Surraka et al., 2005, Piccardi et al., 2000). These effects can be caused due to the presence of specific toxins affecting the cell metabolism, by the presence of more compounds with a synergic effect, or even simply by changing the medium composition. However, within the present study eight cyanobacterial strains with a significant cytotoxic effect have been fractionated and in five cases a single compound was found responsible for the toxic effect (**Paper I and III**). This suggests that most cytotoxic activities within a cyanobacterial extract can be probably attributed to the presence of cytotoxic compounds rather then to additive effects and cultivation media changes. The high estimated number of cytotoxic compounds agrees well with the diversity in cytotoxic structures that have already been described (e.g. Barchi *et al*. 1983, Patterson and Carmeli 1992, Rodney *et al.* 1999, Trimurtulu *et al.* 1994).

There remains the question whether cytotoxicity provides some advantage to cyanobacteria in the natural environment. If cyanobacteria produce cytotoxic compounds with respect to some environmental condition (e.g. competition, predation or parasitic pressure, etc.) then in particular habitats where these stimuli occur cytotoxic cyanobacteria should be preferred and thus become more frequent. In the opposite case, when any cytotoxic effect is coincidental, the cytotoxic cyanobacteria should be randomly dispersed across all habitats. In order to test this hypothesis, cyanobacteria originating from different microhabitats (i.e. particular localities) were screened for cytotoxicity to the Sp/2 cell line. Eight microsites were selected for the comparison. From the obtained distribution, it could be clearly seen that in most of the studied microsites either cytotoxic or noncytotoxic strains were found. This distribution is in disagreement with the hypothesis that cytotoxicity is not dependent on environmental conditions and that it occurs randomly (**paper I**). Moreover, in selected microsites, cytotoxicity was caused by different compounds and thus lateral gene transfer or clonality of the isolates could not be considered the reason for the high cytotoxicity occurrence in these microsites. Based on the potential of its synthetic apparatus, cytotoxic metabolites are probably synthesized by the cyanobacterium with respect to their cytotoxic function. This result would lead us to conclude that the production of cytotoxic compounds is an environmentally dependent character, and that strains producing cytotoxic compounds are favoured under specific conditions (**paper I**). Nevertheless, it is clear that a more comprehensive study including more strains as well as manipulative experiments would be required to confirm these results.

One of the possible reasons for the high cytotoxicity occurrence in some habitats could be enhanced predation pressure; as discussed above, an inhibitory effect for most of the cytotoxic compounds to invertebrate has been proven (Patterson and Carmeli 1992, Rickards et al., 1999, Berry et al., 2004; Biondi et al., 2004). Furthermore, a direct link betweeen the production of the glucosidase di-(hydroxymethyl) dihydroxypirolidine (DMDP) and grazer pressure has been recorded in the peryphytic *Cylindrospermum* sp. strain (Jüttner and Wessel, 2003). Bioactive secondary metabolites were regarded as an evolutionary response to the pressure of competing organisms or grazers when the genus *Nostoc* was studied (Dodds et al., 1995; Piccardi et al., 2000). Nevertheless, the synthetic

pathway of cyanobacterial peptides is at least older then the history of the eukaryotic lineage (Rantala et al. 2004), and thus the primary function of these structures as defence molecules against grazers is not possible. On the other hand, given the long coevolution of cyanobacteria and grazers these specific mechanisms could have evolved. In a comparison of cyanobacterial extracts toxicity to the Sp/2 cell line and to the model invertebrate *Artemia salina*, it was observed that most of the extracts exhibit some cytotoxicity to the mammal cell without being accompained by a toxicity to *A. salina* (**paper III**). In contrast, only four out of 65 cyanobacterial strains produced compounds with significant activity to *A. salina* and no activity to Sp/2 cells. It has therefore been suggested that the toxic effect is most frequently targeted at some basal metabolic pathways present in most eukaryotic cells, rather then being a specific mechanism against a complex organism. This suggestion is strongly supported by the fractionation of the extracts being found to be toxic to both *A. salina* and Sp/2 cell lines. In four of the six fractionated extracts, the toxic effects to mammal cells and A. *salina* were caused by identical compounds, suggesting that the functional mechanism is probably the same for both cell and complex organism (**paper III**). Despite the primary function of cyanobacterial secondary metabolites not being a defence against grazers, and also taking into account the data here presented, it seems that no specific toxicity mechanisms against grazers have evolved, even though cyanobacteria possess cytotoxic compounds that can affect metabolic activity or even prove lethal to invertebrate grazers. Subsequently these compounds can be expected to be more frequent in habitats with a higher grazing pressure. In order to find out whether the production of compounds toxic to *A. salina* is somehow linked to cyanobacterial ecology, the presence of a compound toxic to *A. salina* in biomass and extracellular extracts of cyanobacteria originating from different habitats has been compared. While a relatively high occurrence of toxicity to *A. salina* was found in the biomass of strains originating from soil, few soil strains were found to produce compounds toxic to *A. salina* extracellularly. However, the opposite situation was observed in planktonic strains. Only one out of 30 biomass extract of planktonic strains caused lethality to *A. salina*, but 50% of the media (extracellular) extracts from plantonic strains were positive for *A. salina* toxicity (**paper III**). This result can have a simple ecological explanation. In the planktonic environment, extracellular production make sense since there exists an easy diffusion of compounds towards their intended target the grazer. Whereas within the soil it will be more advantageous to store defence compounds within the biomass of the organism.

The data mentioned above suggest that cytotoxicity is probably an environmentally-dependent character and that production of cytotoxic compounds is more frequent in certain habitats. These compounds can work in defence against herbivore grazing, although they are certainly not synthesized specifically against herbivore grazers.

In one of the strains included in this study, *Cylindrospermum* sp. C24/89, the isolation and elucidation of the structure of the active compound was undertaken. Within an extract of this strain, a fraction causing significant inhibition to the HeLa, Sp/2 and YAC-1 cell, as well as significant mortality to *A. salina*, was found (**paper II, III**). In the mass spectrum of this active fraction, two molecular ions corresponding to the presence of compounds with molecular weights of 1146.6512 Da and 1160.6672 Da was detected. By a

combination of NMR and MS experiments, the structures of these compounds were deduced to be cyclic peptides containing a β-amino acid unit. Similar structural skeletons had been previously discovered and denominated as puwainaphycins A-E (Gregson et al. 1992, Moore et al. 1989). The newly-isolated variants have been therefore described as puwainaphycin F (MW=1146.6512) and puwainaphycin G (MW=1160.6672). Puwainaphycin F was found to have the sequence valyl-2aminobut-2(*E*)-enoyl-asparaginyl-2aminobut-2(*E*)-enoyl-asparaginyl-alanyl-threonyl-Nmethylasparaginyl-prolyl-2-hydroxy-3-amino-tetradecanoic acid. Puwainaphycin G differs from the F variant only by the subsitution of glutaminyl for asparagynil at the third position (**paper II**). Both these structures differ from other congeners (Gregson et al. 1992) in 5 amino-acid positions as well as the β-amino acid unit. Puwainaphycin C has been previously reported to have a cardiotonic activity in isolated mouse atria (Moore et al. 1989); however, data about its toxicity as well as about the function mechanism are missing. Both newly-isolated variants puwainaphycins F and G interfered with plasma membrane and caused fast calcium ion leakage into the cell comparable with that of the established ionophore ionomycin in a concentration of 10 μM (**paper II**). Subsequently, the activation of tyrosine phosphorilation and relocalization of F-actin into ring-like structures around the nucleus was observed. Disruption of the plasma membrane integrity continued and at longer exposure times (30 min. – 10 hrs.) and leakage of intracellular lactate dehydrogenase could also be detected, which suggested vast membrane damage. Finally, the cells died by necrotic cell death after 10 hrs exposure in a concentration of 10 μM (**paper II**). Identical biological effects were observed for both studied puwainaphycin variants. The interaction of these structures with the plasma membrane could occur by way of the long non-polar chain present in the molecule, but this hypothesis needs to be tested. It is also probable that the observed Ca^{2+} leakage must have been caused at a specific place in the membrane, because of the very specific response in the cell (ring-like actin relocalization). Only in a few cyanobacterial cytotoxins has the mode of action been explained. The interaction of a cyanobacterial secondary metabolite with cytoskeletal structures (e.g. cryptophycin and tolytoxin), different eukaryotic enzymes (microcystin, nodularin, abaenopeptins, microviridins, aeruginosins), as well as DNA (e.g. tubercidin), has been proved (Barchi et al. 1983, MacKintosch et al. 1990, Murakami et al. 1994,1995,1997, Patterson et al. 1993, Smith et al. 1994). The observed membrane permeabilization caused by puwainaphycins F and G is the first report of an interaction of cyanobacterial secondary metabolites with eukaryotic plasma membrane. The pharmacological potential of these compounds as an anti-cancer drug is low, because of their high IC_{50} value and high toxicity to primary human cells (tested on human skin fibroblasts). However, we can conclude that puwainaphycin F and G can potentially mimic physiological Ca^{2+} signalling events and can be used as a biochemical tool for the study of cell biochemistry.

The last topic within this dissertation is the study that was conducted of the relationship between cyanobacterial phylogeny/geographical origin and total secondary metabolite content. The congruence of secondary metabolites production with phylogeny was tested in planktonic *Dolichospermum* (*Anabaena*) strains. This group of planktonic cyanobacteria is a well-known producer of neurotoxic as well as hepatotoxic secondary

metabolites (e.g. Beltram and Neilan 2000, Fujii et al. 2002, Sivonen 1996); the question as to whether defined taxonomical units can be characterized by their production of particular secondary metabolites is thus important from a hydrobiological point of view. As in many other bacterial groups, a discordance between traditionally defined species and their reconstructed phylogeny has been found (Gugger et al. 2002, **paper IV**). All plaktonic *Anabaena* isolates were grouped in a well-supported cluster sharing 98.6% in 16SrDNA when isolates from lake Tuusulajärvi (Finland) were studied (**paper IV**). Surprisingly, in some subclusters of this group a clustering of the *Anabaena* strain with strains of the easily-distinguishable genus *Aphanizomenon* were proved (**paper IV**). Since several morphological characters have been recognized in support of these results the planktonic *Anabaena* and *Aphanizomenon* strains have been transferred into the genus *Dolichospermum* (Wacklin et al. 2009). The basal subcluster of this large group involving strains of *Aphanizomenon issatschenkoi* have been transferred into the new genus *Cuspidothrix* because of its distinct morphology (**paper VII**). Recently, planktonic *Anabaena reniformis* strains with densely-coiled trichomes and rounded akinetes have been found to fall out of the main *Anabanena* cluster and move to the vicinity of the genus *Cylindrospermopsis*. Thus a new genus *Sphaerospermopsis* (firstly *Sphaerospermum*), containing the strains of *A. renifromis* and *Aphanizomenon aphanizomenoides*, has been formed (**paper VI**). Total secondary metabolite profiles have been compared in 33 isolates of *Dolichospermum* and *Sphaerospermopsis* strains. Extracts of these strains were analyzed by means of HPLC-MS and each compound detected as a molecular ion within a certain retention time was taken into consideration as a biochemical marker. A similar approach was sucessfully applied to an analysis of whole bacterial cell spectra by means of MALDI-TOF MS (Holland et al 1996). Most of the detected compounds (67%) were randomly dispersed across the NJ tree based on 16S rRNA gene sequences and thus did not reflect phylogenetic relations. A minor portion of the compounds were found to be produced by some strains within the monophyletic cluster (15%). Finally, 19% of detected metabolites were found to be produced by closely-related strains, and not by the other strains tested, and thus can be considered as an autapomorphy suitable for taxonomic purposes (**paper V**). One of the most important congruences found between metabolite production and phylogenetic analysis was the different metabolite composition of *Sphaerospermum reniformis* strains from all other planktonic *Dolichospermum* strains (**paper VI**), which thus supports the delimination of this genus. The congruence between neurotoxin production and a reconstructed phylogeny has also been recorded in planktonic *Dolichospermum* (*Anabaena*) strains isolated from lake Tuusulajärvi; however, it is possible that the distribution observed may only be reliable for this particular lake and no general conclusion should therefore be construed.

Nevertheless, most of the compounds detected exhibit a random distribution across a reconstructed phylogenetic tree and thus total secondary metabolite content in general appears not to be a reliable chemotaxonomic tool in cyanobacteria. This result also hampers the possible detection of toxic cyanobacteria by morphological methods or molecular detection based on the 16SrDNA gene. As suggested by Thacker and Paul (2004), who found a similarly low consistency between 16S rRNA gene phylogeny and chemical traits in

the cyanobacterial genera *Lyngbya* and *Symploca*, a divergence in chemosynthetic genes may not be reflected in 16S rRNA gene sequences if the ribosomal sequences are relatively more conserved. Earlier studies have demonstrated differences in toxin production among genetically similar strains and vice versa (Lyra et al. 2001, Baker et al. 2002, Gugger et al. 2002b) and also substantially different total secondary metabolite content in closely-related (100% 16SrDNA similarity) *Dolichospermum* strains (**paper VIII**). The observed and referred random distribution of secondary metabolites across the phylogenetic spectrum is probably the result of the ancient origin of the chemosynthetic apparatus, at least as far as cyanobacterial peptides are concerned. By the extinction of some synthetic genes in certain lineages or by mutation in a particular enzyme included in this machinery, such a random distribution could be easily obtained. Since in the synthesis of cyanobacterial peptides the co-linearity rule is applied, most of the non-synonomous mutation will easily lead to the production of different compounds. The fact that the synthetic apparatus of secondary metabolites is rapidly evolving is supported by the result that most of the compounds detected in *Dolichospermum* strains were observed in just one of the studied strains (144 of total 170 detected compounds). Furthermore, frequent lateral gene transfer has been verified for the biosynthesis gene cluster of the low-molecular-weight peptide cyanobactin (Leikoski et al. 2009). However, the role and frequency of lateral gene transfer in the distribution of chemosynthetic genes needs to studied in much greater detail since the cyanobactin operon is quite small in comparison with other synthethases and thus easily transferable. The random distribution of this secondary metabolite production has been found not only across the phylogenetic spectrum but also for strains originating from different geographical areas in studies involving the genus *Nostoc* (**paper I**). The production of identical compounds has been detected in strains originating from distant regions; it means in strains which would presumably not have any possibility to share genetic material. Thus it is still questionable as to what degree the random distribution of a particular compound is given by lateral gene transfer or the ancient origin of synthethases.

References

Baker J.A., Entsch B., Neilan B.A. and McKay D.B. 2002. Monitoring changing toxigenicity of a cyanobacterial bloom by molecular methods. *Appl. Environ. Microbiol.* 68:6070–6076.

Barchi J.J., Bortin T.R., Furusawa E., Patterson G.L.M. and Moore R.E. 1983. Identification of a cytotoxin from *Tolypothrix byssoidea* as tubericin. *Phytochemistry* 22: 2851-2852.

Beltram E.C. and Neilan B.A. 2000. Geographical segregation of the neurotoxin-producing cyanobacterium Anabaena circinalis. *Appl. Environ. Microbiol.* 66:4468-4474.

Berry J.P., Gantar M., Gawley R.E., Wang M. and Rein K.S. 2004. Pharmacology and toxicology of pahayokolide A, a bioactive metabolite from a freshwater species of *Lyngbya* isolated from the Florida Everglades. *Comp. Biochem. Physiol. C.* 139:231-238.

Biondi N., Piccardi R., Margheri M.C., Rodolfi L., Smith G.D. and Tredici M.R. 2004. Evaluation of *Nostoc* strain ATCC 53789 as a potential source of natural pesticides. *Appl. Environ. Microbiol*. 70:3313-3320.

Bonjouklian R., Smitka T.A., Doolin L.E., Molloy R.M., Debono M. and Shaffer S.A. 1991. Tjipanazoles, new antifungal agents from the blue-green algae *Tolypothrix tjipanasensis*. *Tetrahedron* 47:7739-7750.

Botha N., Gehringer M.M., Downing T.G., Venter M. and Shephard E.G. 2004. The role of microcystin-LR in the induction of apoptosis and oxidative stress in a CaCo2 cells. *Toxicon* 43: 85-92.

Carmichael W.W., Biggs D.F. and Gorham P.R. 1975. Toxicology and pharmacological action of *Anabaena-flos aque* toxin. *Science* 187:542-544.

Carmichael W.W., Biggs D.F. and Gorham P.R. 1979. Pharmacology of anatoxin-a produced by freshwater cyanophyte *Anabaena-flos aque* NRC-44-1. *Toxicon* 17:229-236.

Carmichael W., Hones C., Mahmood N. and Theiss W. 1985. Algal toxins and water-based diseases. *Critical Rev. Environ. Contr.* **15**: 275-313.

Carmichael. W.W. 1986. Algal Toxins. *Advances in Bot. Res.* 12:47-101.

Carmichael W.W. 1990. Natural toxins from cyanobacteria. *Marine Toxins* 418:87-106.

Carmichael W.W. 1992. Cyanobacterial secondary metabolites - a review. *J. App. Bacteriol*. 72: 445-459.

Dittmann E., Neilan B.A., Erhard M., von Döhren H. and Börner T. 1997. Insertional mutagenesis of a peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. *Mol. Microbiol.* 26: 779–787.

Dodds W.K., Gudder D.A. and Mollenhauer D. 1995. The ecology of *Nostoc*. *J. Phycol.* 31:2-18.

Falconer R.I., Beresford A.M., Steffensen D.A. Choise M. and Coverdale O.R. 1994. Evidence of liver damage by toxin from bloom of blue-green alga *Microcystis aeruginosa. J. Med. Aust.* 1: 511-514.

Fujii K., Sivonen K., Nakamo T. and Harada K. 2002. Structural elucidation of cyanobacterial peptides encoded by peptide synthetase gene in *Anabaena* species. *Tetrahedron* 58: 6863-6871.

Gregson J.M., Chen J.L., Patterson G.M.L. and Moore R.E. (1992): Structures of puwainaphycines. *Tetrahedron* **48:** 3727-3734.

Gugger M., Lyra C., Henriksen P., Couté A., Humbert J.F. and Sivonen K. 2002a. Phylogenetic comparison of cyanobacterial genera *Anabaena* and *Aphanizomenon*. *I. J. Syst. Evol. Microbiol.* 52:1-14.

Gugger M., Lyra C., Suominen I., Tsitko I., Humbert J.F., Salkinoja-Salonen M.S. and Sivonen K., 2002b. Cellular fatty acids as chemotaxonomic markers of the genera *Anabaena, Aphanizomenon, Microcystis, Nostoc* and *Planktothrix* (Cyanobacteria). *Int. J. Syst. Evol. Micr.* 52:1007–1015.

Holland R.D., Wilkes J.G., Rafii F., Sutherland J.B., Persons C.C., Voorhees K.J. and Lay J.O., 1996. Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.*10:1227–1232.

Jaki B., Orjala J.and Sticher O. 1999. A novel extracellular diterpenoid with antibacterial acivity from the cyanobacterium *Nostoc commune. J. Nat. Prod.* 602: 502-503.

Jüttner F. and Wessel H.P. 2003. Isolation of di(hydroxymethyl)dihydroxypyrrolidine from the cyanobacterial genus *Cylindrospermum* that effectively inhibits digestive glucosidases of aquatic insects and crustacean grazers. *J. Phycol.* 39:26-32.

Knűbel G., Larsen K.L., Moore R.E., Levine I.A. and Patterson G.L.M. 1990. Cytotoxic, antiviral indolocarbazoles from a blue-green alga belonging to the *Nostocaceae*. *The Journal of Antibiotic* 10:1236-1239.

Kajiyama S.I., Kanzaki H., Kawazu K. and Kobayahi A. 1998. Nostofungicine, an antifungal lipopeptide from the field-grown terrestrial glue-green alga *Nostoc commune*. *Tetrahedron Lett.* 39: 3737–3740.

Leikoski N., Fewer D.P. and Sivonen K. 2009. Widespread Occurrence and Lateral Transfer of the Cyanobactin Biosynthesis Gene Cluster in Cyanobacteria. *Appl. Environ. Microbiol*. 75:853–857.

Lyra C., Suomalainen S., Gugger M., Vezie C., Sundman P., Paulin L. and Sivonen K., 2001. Molecular characterization of planktic Cyanobacteria of *Anabaena*, *Aphanizomenon*, *Microcystis*, and *Planktothrix* genera. *Int. J. Syst. Evol. Microbiol.* 51:513–526.

MacKintosch C, Beattie K.A., Klumpp S., Cohen P. and Codd G.A. 1990. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett.* 264: 187-192.

Maršálek B., Keršner V. and Marvan P. (ed.) 1996. Vodní květy sinic. [Water-blooms of the cyanobacteria] (in Czech). *Nadatio flos aque.* Brno: 1-140.

Miura G.A., Leclaire RD, Templeton C.B., Pace J.G. 1988. Enhanced hepatotoxicity of microcystin in fasted rats. *Faseb J.* 2:1351-1351.

Moore R.E., Bornemann V., Niemczura W.P., Gregson J.M., Chen J.L., Norton T.R., Patterson M.L. and Helms G. 1989. Puwainaphycin C, a cardioactive cyclic peptide from the blue-green alga *Anabaena* BQ-16-1. Use of two-dimensional 13C-13C and 13C-15N correlation spectroscopy in sequencing of amino acid units. *J. Amer. Chem. Soc.* 111: 6128-6132.

Murakami M., Okita Y., Matsuda H., Okino T. and Yamaguchi K. 1994. Aeruginosin 298-A, trombin and trypsin inhibitors from the blue-green alga *Microcystis aeruginosa* (NIES-298). *Tetrahedron Lett.* 35:3129-3132.

Murakami M., Ishida K., Okino T., Okita Y., Matsuda H. and Yamaguchi K. 1995. Aeruginosins 98-A and B, trypsin inhibitors from the blue-green alga *Microcystis aeruginosa* (NIES-98). *Tetrahedron Lett.* 36:2785-2788.

Murakami M., Sun Q., Ishida K., Matsuda H., Okino T. and Yamuguchi K. 1997. Microviridins, elastase inhibitors from the cyanobacterium *Nostoc minutum* (NIES-26). *Phytochemistry* 45: 1997-1202

Namikoshi M. and Rinehart K.L. 1996. Bioactive compounds produced by cyanobacteria. *J. Ind. Microbiol. Biotechnol.* 17: 373-384.

Ohtani I., Moore R.E. and Runnegar M.T.C. 1992. Cylindrospermopsin: a potent hepatotoxin from blue-green alga *Cylindrospermopsis raciborskii. J. Amer. Chem. Soc.* 114: 7941-7942.

Patterson G.M. and Carmeli S. 1992. Biological effects of tolytoxin (6-hydroxy-7-O-methyl-scytophycin b) a potent bioactive metabolite from cyanobacteria. *Ach. Microbiol.* 157:406-410

Patterson G.M.L., Smith C.D., Kimura L.H., Britton B.A. and Carmeli S. 1993. Action of tolytoxin on cell morphology, cytoskeletal organization, and actin polymerization. *Cell. Motil. Cytoskeleton* 24: 39-48.

Piccardi R., Frosini A., Tredici M.R. and Margheri M.C., 2000. Bioactivity in free-living and symbiotic cyanobacteria of the genus *Nostoc. J. Appl. Phycol*. 12:543-547.

Rajaniemi P., Komárek J., Willame R., Hrouzek P., Kaštovská K., Hoffmann L. and Sivonen K. 2005. Taxonomic consequences from the combined molecular and phenotype evaluation of selected *Anabaena* and *Aphanizomenon* strains. *Arch. Hydrobiol. Suppl.* 117:371-391.

Rantala A., Fewer D.P., Hisbergues M., Rouhiainen L., Vaitomaa J., Börner T. and Sivonen K. 2004. Phylogenetic evidence for the early evolution of microcystin synthesis. *Proc. Natl. Acad. Sci. USA.* 101:568-573.

Reshef V. and Carmeli S. 2001. Protease inhibitors from a water bloom of the cyanobacterium *Microcystis aeruginosa. Tetrahedron* 57:2885-2894.

Rodney W.R., Rothschild J.M., Willis A.C., Chazal N.M., Kirk J., Saliba K.J. and Smith G.D. 1999. Calothrixin A and B, novel pentacyclic metabolites from *Calothrix* cyanobacteria with potent activity against malaria parasites and human cancer cells. *Tetrahedron* 55:13513-13520.

Schmidt E.W., Nelson J.T., Rasko D.A., Sudek S., Eisen J.A., Haygood M.G. and Ravel J. 2005. Patellamide A and C biosynthesis by a microcin-like pathway in *Prochloron didemni*, the cyanobacterial symbiont of *Lissoclinum patella*. *Proc. Nat. Acad. Sci. USA* 102:7315–7320.

Schopf J.W. 1993. Microfossil of the early Archean Apex chert: new evidence of the antiquity of life. *Science* 260:640-646.

Sivonen K. 1996. Cyanobacteria toxins and toxin production. *Phycologia* 35:12-24.

Smith C.D., Zhang X.Q., Moorbery S.L., Patterson G.M.L. and Moore R.E. 1994. Cryptophycin - a new antimicrotubule agent active against drug-resistant cells. *Cancer Res*. 54:3779-3784.

Surakka A., Sihvonen L.M., Lehtimaki J.M., Wahlsten M., Vuorela P. and Sivonen K. 2005. Benthic cyanobacteria from the Baltic Sea contain cytotoxic *Anabaena*, *Nodularia*, and *Nostoc* strains and an apoptosis-inducing *Phormidium* strain. ***Environ. Toxicol.*** 20:285–292.

Teicher B.A., Forler P., Menon K., Phares V., Amsrud T. and Shih C. 2000. Cryptophycin 52 and cryptophycin 55 in sequential and simultaneous combination treatment regimens in human tumor xenografts. *In Vivo* 14:471-480.

Thacker R.W. and Paul V. J. 2004. Morphological, Chemnical, and Genetic Diversity of Tropical Marine Cyanobacteria Lynbya spp. and Symploca spp. (Oscillatoriales). ***Appl. Env. Microbiol.*** 70: 3305–3312.

Teneva I., Asparuhova D., Dzambazov B., Mladenov R. and Schirmer K. 2003. The freshwater cyanobacterium *Lyngbya aerugino-coerulea* produces compounds toxic to mice and to mammalian an fish cells. ***Environ. toxicol.*** 18:9-20.

Tillett D., Dittmann E., Erhard M., von Döhren H., Börner T. and Neilan B.A. 2000. Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system. ***Chem. Biol.*** 7:753–764.

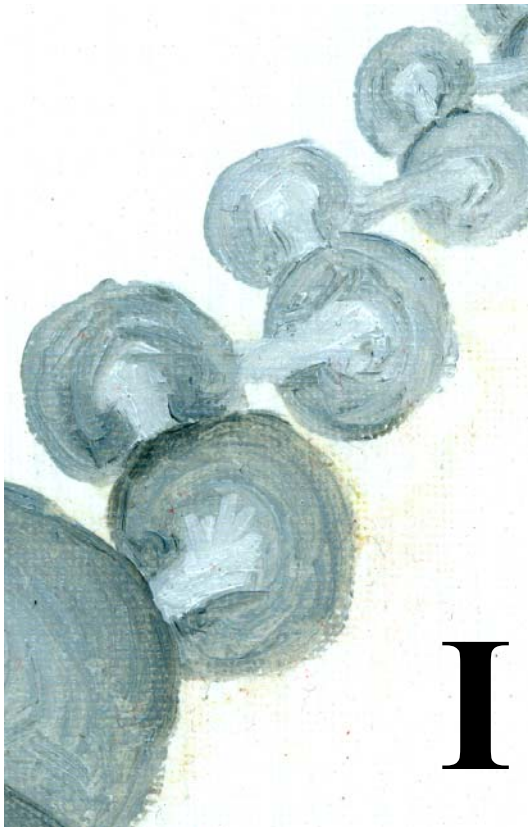
Trimurtulu G., Ohtani I., Patterson M.L., Moore R.E., Corbett T.H., Valeriote F.A and Demchik L. 1994. Total structures of cryptophycins, potent antitumor depsipeptides from the blue-green alga *Nostoc* sp. strain GSV 224. ***J. Amer. Chem. Soc.*** 116:4729-4737.

Van Wagoner R.M. and Drummond A.K. 2007. Biogenetic diversity of cyanobacterial metabolites. ***Adv. Appl. Microbiol.*** 61:89-217.

Wacklin P., Hoffmann L. and Komarek J. 2009. Nomenclatural validation of the genetically revised cyanobacterial genus *Dolichospermum* (RALFS ex BORNET et FLAHAULT) comb. nova. *Fottea* 9: 59-64.

Welker M. and von Dohren H., 2006. Cyanobacterial peptides – Nature's own combinatorial biosynthesis. ***Fems Microbiol. Rev.*** 30:530–563.

Welker M., Maršálek B., Sejnohová L and vön Dohren H. 2006. Detection and identification of oligopeptides in Microcystis (cyanobacteria) colonies: Toward an understanding of metabolic diversity. *Peptides* 27:2090-2103.



I

Hrouzek, P., Tomek, P., Lukešová, A., Urban J., Voloshoko L., Pushparaj, B., Ventura, S., Lukavský, J., Štys, D. & Kopecký, J. (2010):

Cytotoxicity and secondary metabolites production in terrestrial *Nostoc* strains, originating from different climatic/geographic regions and habitats: Is their cytotoxicity environmentally dependent?

Environmental Toxicology
(Accepted: DOI: 10.1002/tox.20561)

Cytotoxicity and Secondary Metabolites Production in Terrestrial *Nostoc* Strains, Originating From Different Climatic/Geographic Regions and Habitats: Is Their Cytotoxicity Environmentally Dependent?

Pavel Hrouzek,[1,2,3] Petr Tomek,[1,4] Alena Lukešová,[5] Jan Urban,[3] Ludmila Voloshko,[6] Benjamin Pushparaj,[7] Stefano Ventura,[7] Jaromír Lukavský,[8] Dalibor Štys,[3] Jiří Kopecký[1,3]

[1]Institute of Microbiology, Academy of Sciences of the Czech Republic, Opatovický Mlýn, 379 81 Třeboň, Czech Republic

[2]Department of Botany, Faculty of Sciences, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic

[3]Institute of Physical Biology, University of South Bohemia, Zámek 136, 373 33 Nové Hrady, Czech Republic

[4]Department of Molecular Biology and Biochemistry, Faculty of Sciences, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic

[5]Institute of Soil Biology, Biology Centre AS CR, v.v.i., Na Sádkách 7, 370 05 České Budějovice, Czech Republic

[6]Department of Microbiology, Saint-Petersburg State University, Oranienbaumskoye shosse 2, 198504 St. Petersburg, Russia

[7]Institute of Ecosystem Study, CNR, via Madonna del Piano 10, 50019 Sesto Fiorentino, Italy

[8]Centre for Bioindication and Revitalisation, Department of Plant Ecology, Institute of Botany, Academy of Sciences of Czech Republic, Dukelská 135, 37982 Třeboň, Czech Republic

Received 14 June 2009; revised 6 November 2009; accepted 15 November 2009

ABSTRACT: Extensive selection of cyanobacterial strains (82 isolates) belonging to the genus Nostoc, isolated from different climatic regions and habitats, were screened for both their secondary metabolite content and their cytotoxic effects to mammalian cell lines. The overall occurrence of cytotoxicity was

Correspondence to: P. Hrouzek, Institute of Microbiology, Department of phototrophic microorganisms, Třeboň, Czech Republic; e-mail: hrouzekp@gmail.com

Contract grant sponsor: Ministry of Education, Youth and Sports of the Czech Republic.
Contract grant numbers: FRVŠ no. 3491/2005MŠM 6007 665 808, MSMT 1MO571.
Contract grant sponsor: Institutional Research Concept.
Contract grant number: AVOZ50200510
Contract grant sponsor: ISB.
Contract grant number: AV0Z60660521
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/tox.20561

found to be 33%, which corresponds with previously published data. However, the frequency differs significantly among strains, which originate from different climatic regions and microsites (particular localities). A large fraction of intensely cytotoxic strains were found among symbiotic strains (60%) and temperate and continental climatic isolates (45%); compared with the less significant incidences in strains originating from cold regions (36%), deserts (14%), and tropical habitats (9%). The cytotoxic strains were not randomly distributed; microsites that clearly had a higher occurrence of cytotoxicity were observed. Apparently, certain natural conditions lead to the selection of cytotoxic strains, resulting in a high cytotoxicity occurrence, and *vice versa*. Moreover, in strains isolated from a particular microsite, the cytotoxic effects were caused by different compounds. This result supports our hypothesis for the environmental dependence of cytotoxicity. It also contradicts the hypothesis that clonality and lateral gene transfer could be the reason for this phenomenon. Enormous variability in the secondary metabolites was detected within the studied *Nostoc* extracts. According to their molecular masses, only 26% of these corresponded to any known structures; thus, pointing to the high potential for the use of many terrestrial cyanobacteria in both pharmacology and biotechnology. © 2010 Wiley Periodicals, Inc. Environ Toxicol 00: 000–000, 2010.

Keywords: cytotoxicity; cyanobacteria; *Nostoc*; distribution; bioprospection

INTRODUCTION

The study of secondary cyanobacterial metabolites has become a fascinating field of microbiology during the last several decades. It has attracted the attention of many research groups, and hundreds of different compounds with different biological effects have been described (Carmichael, 1992; Namikoshi and Rinehart, 1996; Sivonen, 1996; Welker and von Döhren, 2006). The production of different cyanobacterial metabolites can be examined from two different perspectives. First, screening studies are targeted toward the isolation of novel compounds for applications in pharmacology or biotechnology. Second, significant amounts of such compounds are released by living or decaying cyanobacterial cells, and thus affect the environment. Accordingly, knowledge about the function and fate of the compounds within the environment is also important. Are the biological activities, which are the object of our interest, part of cyanobacterial adaptation to environmental conditions, or is this bioactivity effect coincidental?

Cyanobacteria play an important role in various terrestrial habitats. In some, they are the only primary producers, and thus the ecosystem is dependent on their supply of biomass (Lukešová, 1993; Dodds et al., 1995). Within these habitats they are exposed to enormous grazing and parasitic pressures from microorganisms and fungi, as has been demonstrated by numerous studies (Birkemoe and Liengen, 2000; Pajdak-Stos et al., 2004; Darby et al., 2007). Apart from this role, they are also important producers of different secondary metabolites. A wide range of compounds including peptides, alkaloids, macrolactones, and heterocycles have been reported to be produced by cyanobacteria (Carmichael, 1992; Namikoshi and Rinehart, 1996; Sivonen, 1996). The most abundant compounds among cyanobacteria are different types of linear and cyclic peptides (Welker and von Döhren, 2006). They exhibit unique amino acid compositions, and are synthesized via a nonribozomal synthetic pathway (Kleikauf and von Döhren, 1996; Welker and von Döhren, 2006); which has been confirmed to be a primeval biochemical process (Rantala et al., 2004).

Cyanobacterial metabolites exhibit a wide range of biological effects. Some of these substances possess potent cytotoxic or cytostatic activities to different mammalian cell lines, making them interesting for pharmaceutical research and applications. In the cyanobacterial depsipeptide cryptophycin, specific activity against solid tumors was found (Teicher et al., 2000). In general, cytotoxins are very heterogeneous regarding their chemical structures and modes of action. The induction of oxidative stress (Ding and Ong, 2003; Botha et al., 2004), actin depolymerization (Patterson et al., 1993; Smith et al., 1994), or interference with DNA replication process (Patterson and Carmeli, 1992; Teneva et al., 2003) are the most commonly described effects for cyanobacterial cytotoxins. Most of these compounds affect a wide range of organisms. Tolytoxin, one of the most effective cytotoxins, causes profound injury to nearly all eukaryotic organisms, including fungi and different invertebrates (Patterson and Carmeli, 1992). A similar wide-ranging effect can also be found in other cytotoxins such as calothrixins, cryptophycins, and pahayakolide (Rickards et al., 1999; Berry et al., 2004; Biondi et al., 2004).

In our work, we asked if the distribution of cytotoxicity and the production of a particular compound have a specific pattern across different geographical regions and microsites (habitats with homogenous conditions in a particular locality). These data could provide greater insights into whether the cytotoxicity is a result of coincidence or is environmentally dependent. Finally, such data can help to predict good sample collecting sites for strains with potential for pharmaceutical or biotechnological purposes.

MATERIALS AND METHODS

Cyanobacterial Strains and Extraction

In total, 82 cyanobacterial original isolates were incorporated into the study. For complete strain information and deposition see Table I. Strains were classified into the climatic zones according to newest upload of Köppen-Geiger climate classification (Peel et al., 2007). Cyanobacteria were grown on liquid Alen-Arnold medium in 200 mL tubes, bubbled with CO_2 enriched air (1.7%) for 10–14 days. Biomass was harvested by centrifugation in 50 mL glass cuvettes (3000 rpm, 15 min.), stored at −40°C, and lyophilized. 200 mg of lyophilized biomass was transferred into 10 mL glass test tubes and extracted, using 70% methanol, for 2 h. Test tubes were centrifuged (3000 rpm, 15 min); the supernatant was then transferred to an evaporating vessel and dried via a rotation vacuum evaporator. The solid extract was re-suspended in 1 mL of 70% methanol.

Cell Line Maintenance and Preparation of the Experiment

Two mammalian cell lines were selected for the cytotoxicity assay - YAC-1 (murine lymphoblast, induced by Moloney leukemia virus), and Sp/2 (murine myeloma cells). The cells were cultivated in RPMI 1640 medium (Sigma R-8005) with the addition of 5% fetal calf serum (PAA A15-04), 1% glutamine (Sigma G-5763), and 1% antibiotic-antimycotic solution (Sigma A-7292); all in plastic tissue culture flasks at 37°C. Prior to the experiments, cells were dyed with Trypan blue, in order to estimate viability, and counted in a Bürkers plate chamber in a light microscope. Only cell cultures with a higher viability than 90% were used for the experiment. The cell suspension was centrifuged (1000 rpm, 10 min, 4°C), and an adequate amount of fresh RPMI medium was added, in order to obtain a concentration of 1.5×10^5 cells per well (200 μL RPMI).

MTT Test

Cyanobacterial extract of 10 μL was added to the wells in triplicates, and triplicates treated only by 70% methanol were left as the controls. The plate was kept in an incubator (37°C) until the 70% methanol was evaporated; then the cell suspension was added and incubated (37°C and 3.5% CO_2) for 12 h. Cell viability, after exposure, was estimated by MTT assay (Mosman, 1983); 10 μL of MTT solution (4 mg/mL) was added and the plates incubated for 4 h. After incubation, the plates were centrifuged (3000 rpm, 10 min.), and the supernatant removed. DMSO of 200 μL was added to dissolve formazan crystals. Test and background absorbencies were measured at 590 and 640 nm, respectively. The survival of cell lines was evaluated as the ratio of treated wells' absorbance to that of the control wells, and expressed as a percent.

Extract Analysis

Extract composition was analyzed using an HP 1100 Agilent mass spectrometer with an HP 100 MSD SL-Ion trap. The extract was subjected to separation on a reversed phase column (Zorbax XBD C8, 4.6 × 150 mm, 5 μm) at 30°C, eluted by gradient MeOH/H_2O + 0.1% HCOOH (30–100% MeOH for 30 min, 100% for 5 min), with a flow rate of 0.6 mL/min. The obtained total ion chromatograms were evaluated and molecular ions were detected based on signal intensity, the presence of sodium and potassium adducts, and the distribution of isotopomeres. The molecular masses of confirmed compounds were compared with known secondary cyanobacterial metabolites.

Activity-Guided Fractionation

In selected strains originated from one particular locality (such localities are marked as microsites), an activity-guided fractionation was performed in order to reveal the bioactive fraction responsible for the cytotoxic effect, and in order to reveal whether in strains from a particular microsite the cytotoxicity is caused by an identical compound. The extracts were separated on a preconditioned analytical column (Zorbax XBD C8, 4.6 × 150 mm, 5 μm) using a standard analytical gradient (30–100% MeOH for 30 min, 100% for 5 min.) to obtain identical retention times. Each extract was fractionated 5–8 times. Fractionation was performed based on peak retention times (obtained in prior analyses), and also based on the on-line UV absorption. Final fractions were evaporated and re-suspended to obtain the same concentration as in the crude extracts. The obtained fractions were subjected to a bioassay, carried-out in the Sp2 cell line, and their inhibition values determined by a MTT test.

Statistical Analysis

Differences in the number of secondary metabolites between tropical and temperate *versus* continental climate isolates were tested by the *t* test for independent samples. The difference in cytotoxicity values between the *Nostoc* strains (originating from different climatic zones) and symbioses was tested by ANOVA with posthoc comparisons (Fisher LSD test). The hypothesis of a random distribution of cytotoxic strains among the microsites and climatic zones was tested by the χ-square test. Comparison of similarities between strains, based upon secondary metabolite content, was done using cluster analysis, complete linkage, and Euclidean distance. All the computations were done using the 1999 Edition of Statistica for Windows.

TABLE I. Strains under study

Scientific name	Strain—collection	Habitat	Place of isolation	Citation
Tropical climates (Group A)				
Nostoc entophytum	Zehnder/76[a]	Stone surface	Yaounde/Cameroon	n.a.
Nostoc sp.	CALU 994[b]	Soil	Guinea	n.a.
Nostoc sp.	CALU 983[b]	Soil	Guinea	n.a.
Nostoc sp.	CALU 979[b]	Soil	Guinea	n.a.
Nostoc sp.	CALU 992[b]	Soil	Guinea	n.a.
Nostoc sp.	CALU 993[b]	Soil	Guinea	n.a.
Nostoc sp.	CALU 996[b]	Soil	Guinea	n.a.
Nostoc sp.	CALU 995[b]	Soil	Guinea	n.a.
Nostoc sp.	CALU 981[b]	Soil	Guinea	n.a.
Nostoc sp.	CALU 982[b]	Soil	Guinea	n.a.
Nostoc sp.	CALU 984[b]	Soil	Guinea	n.a.
Nostoc sp.	CALU 997[b]	Soil	Guinea	n.a.
Nostoc sp.	RQ II[a]	Tree bark	Paranapiciaba-Sao Paulo/Brazil	n.a.
Nostoc sp.	BROMEL[a]	Tree bark	Paranapiciaba-Sao Paulo/Brazil	n.a.
Nostoc sp.	BRIII[a]	Tree bark	Paranapiciaba-Sao Paulo/Brazil	n.a.
Nostoc sp.	BRV[a]	Tree bark	Paranapiciaba-Sao Paulo/Brazil	n.a.
Nostoc sp.	BRVI[a]	Tree bark	Paranapiciaba-Sao Paulo/Brazil	n.a.
Nostoc sp.	BRIIB[a]	Tree bark	Paranapiciaba-Sao Paulo/Brazil	n.a.
Nostoc sp.	Cam2SO1[c]	Rice field	Cambodia	Papaefthimiou et al., 2008
Nostoc sp.	Lukešová 6/96[d]	Forest soil after fire	Sao Carlos/Brazil	Hrouzek et al., 2005
Nostoc sp.	Lukešová 2/89[d]	Humid forest soil	Havana/Cuba	n.a.
Nostoc sp.	TH1SO1[c]	Dry rice field, soil	Thailand	Papaefthimiou et al., 2008
Nostoc sp.	TH2S22[c]	Dry rice field, soil	Thailand	Papaefthimiou et al., 2008
Arid climates (Group B)				
Nostoc calcicola	Badour 1963/23[a]	Desert soil	Egypt	n.a.
Nostoc indistinguendum	Flechtner CM1-VF10[a]	Desert soil	Clark Mts./USA	Řeháková et al, 2007
Nostoc punctiforme	Flechtner ICM-VF1Y[a]	Desert soil	Clark Mts./USA	Řeháková et al, 2007
Nostoc sp.	Hindák 06/9[a]	Desert soil near high salinity lake	Chott-el-Djerib/Tunis	n.a.
Nostoc sp.	Lukešová 32/03[d]	Desert soil	Minia/Egypt	n.a.
Nostoc sp.	CALU 129[b]	Soil of cotton field	Tashkent/Uzbekistan	n.a.
Nostoc sp.	CALU 542[b]	Soil	Kazachstan	n.a.
Temperate and continental climates (Group C and D)				
Nostoc calcicola	Lukešová 1/86[d]	Mineral soil-oak forest	Netolice/Czech Republic	Papaefthimiou et al., 2008
Nostoc calcicola	Lukešová 9/89[d]	Garden, soil	Č. Budějovice/Czech Republic	n.a.
Nostoc calcicola	Lukešová 3/97[d]	Fallow, soil	Č. Budějovice/Czech Republic	n.a.
Nostoc calcicola	Desortová 67/2[a]	Soil	Krkonoše Mts./Czech Republic	n.a.
Nostoc edaphicum	DAEON	Soil	Russia	n.a.
Nostoc ellipsosporum	Lukešová 51/91[d]	Arable field, soil	Nezamyslice/Czech Republic	Rajaniemi et al., 2005
Nostoc ellipsosporum	Lukešová 52/91[d]	Arable field, soil	Nezamyslice/Czech Republic	n.a.
Nostoc microscopicum	Zehnder 76/210[a]	Limestone	Ruinestein/Switzerland	n.a.
Nostoc muscorum	Lukešová 2/91[d]	Arable field, soil	Nezamyslice/Czech Republic	Papaefthimiou et al., 2008

TABLE I. (*Continued*)

Scientific name	Strain—collection	Habitat	Place of isolation	Citation
Nostoc muscorum	Lukešová 1/85[d]	Meadow, soil	Jevany/Czech Republic	Hrouzek et al., 2003
Nostoc muscorum	Lukešová 1/87[d]	Abandoned field, soil	Dlouhá ves/Czech Republic	Papaefthimiou et al., 2008
Nostoc muscorum	Lukešová 14/86[d]	Abandoned filed, soil	Dlouhá ves/Czech Republic	Rajaniemi et al., 2005
Nostoc sp.	CALU 453[b]	Soil near bog	Kamchatka Peninsula/Russia	n.a.
Nostoc sp.	CALU 374[b]	Periphyton	Karelia Region/Russia	n.a.
Nostoc sp.	Lukešová 116/96[d]	Dump after coal mining, soil	Sokolov/Czech Republic	n.a.
Nostoc sp.	CALU 760[b]	Periphyton	Pskov/Russia	n.a.
Nostoc sp.	Lukešová 5/96[d]	Dump after coal mining, soil	Sokolov/Czech Republic	Hrouzek et al., 2005
Nostoc sp.	Lukešová 18/89[d]	Garden, soil	Č. Budějovice/Czech Republic	n.a.
Nostoc sp.	OSNI32s01[e]	Periphyton	Poodří/Czech Republic	n.a.
Nostoc sp.	Lukešová 6/99[d]	Dump after uranium mining, soil	Ronneburg/Germany	n.a.
Nostoc sp.	Lukešová 5/99[d]	Dump after uranium mining, soil	Ronneburg/Germany	n.a.
Nostoc sp.	Lukešová 7/99[d]	Dump after uranium mining, soil	Ronneburg/Germany	n.a.
Nostoc sp.	OBU36s07[c]	Rock surface	The Burren/Ireland	n.a.
Nostoc sp.	CALU 545[b]	Soil	Kirov region/Russia	n.a.
Nostoc sp.	CALU 546[b]	Soil	Kirov region/Russia	n.a.
Nostoc sp.	CALU 1191[b]	Soil	Rostoc city/Russia	n.a.
Nostoc sp.	CALU 521[b]	Soil	Kiev/Ukraine	n.a.
Nostoc sp.	CALU 327[b]	Soil	Czech Republic	n.a.
Nostoc sp.	SAG 5979	Field soil	France	n.a.
Nostoc sp.	TO1SO1[c]	Soil	Tuscany/Italy	Papaefthimiou et al., 2008
Nostoc sp.	CALU 543[b]	Soil	Kirov region/Russia	n.a.
Polar climates (Group E)				
Nostoc sp.	Lukešová 20/97[d]	Deglaciated soil	King George Island/Anctarctica	n.a.
Nostoc sp.	Lukešová 19/96[d]	Deglaciated soil	King George Island/Anctarctica	n.a.
Nostoc sp.	Lukešová 24/97[d]	Deglaciated soil	King George Island/Anctarctica	n.a.
Nostoc sp.	Kaštovská 2002/14[a]	Fertilized vegetation	Stuphallet/Svalbard isl.	n.a.
Nostoc sp.	EPN16bSO1 +	Soil	Edmonson Point/Antarctica	n.a.
Nostoc sp.	117SO1 +	Flooded soil	Cape King/Antarctica	n.a.
Nostoc sp.	88SO1 +	Flooded soil	Cape King/Antarctica	n.a.
Nostoc sp.	99s05 +	Lake water	Mt. Murray Lake/Antarctica	n.a.
Nostoc sp.	43s01 +	Lake water	Tarn Flat/Antarctica	n.a.
Nostoc sp.	8s01 +	moss cover	ICARO 2/Antarctica	n.a.
Nostoc sp.	72EPs01 +	soil	Edmonson Point/Antarctica	n.a.
Symbiotic strains				
Nostoc sp.	Cr4 +	symb. of *Cycas revoluta* (Greenhouse)	Rome/Italy	Vagnoli et al., 1992
Nostoc sp.	De +	symb. of *Dioon edule* (Greenhouse)	Rome/Italy	Vagnoli et al., 1992
Nostoc sp.	Gm1 +	symb. of *Gunnera manicata* (Greenhouse)	Luca/Italy	Vagnoli et al., 1992
Nostoc sp.	Mm1 +	symb. of *Macrozamia* sp. (Greenhouse)	Rome/Italy	Vagnoli et al., 1992
Nostoc sp.	Lp1 +	symb.of *Lepozamia* sp. (Greenhouse)	Rome/Italy	Vagnoli et al., 1992
Nostoc sp.	OGU36S01 +	symb. of *Gunnera manicata*	Achill island/Ireland	Papaefthimiou et al., 2008
Nostoc sp.	OGU36S02 +	symb. of *Gunnera manicata*	Achill island/Ireland	Papaefthimiou et al., 2008
Nostoc sp.	Cc3 +	symb.of *Cycas cycrinalis* (Greenhouse)	Bologna/Italy	Vagnoli et al. 1992
Nostoc sp.	AI3 +	symb. of *Anthocerros leavis* (Greenhouse)	Naples/Italy	Vagnoli et al., 1992
Nostoc sp.	CALU 268[a]	symb. of *Gunnera chilensis* (Greenhouse)	Praha/Czech Republic	n.a.

Nostoc isolates are grouped into climatic zones (taken from Peel *et al.* 2007). Strains are deposited in the following collections:
[a]CCALA, the collection of phototrophic microorganisms, Institute of Botany, Třeboň, Czech Republic.
[b]The culture collection of St. Petersburg State University, St. Petersburg, Russia.
[c]The algal collection of the Institute of Ecosystem study, Florence, Italy.
[d]The collection of soil algae and cyanobacteria of the Institute of Soil Biology ASCR, České Budějovice, Czech Republic.
[e]The collection of the Institute of Hydrobiology, České Budějovice, Czech Republic.

TABLE II. Cytotoxicity of *Nostoc* strains studied to the murine cell lines Sp2 and YAC-1

Scientific name	Strain	Clim. reg.	No. met.	YAC-1 viab.		Sp2 viab.	
				Mean ± SD	Cyt.	Mean ± SD	Cyt.
Nostoc entophytum	Zehnder/76	A	1	105 ± 7.1		100 ± 2.5	
Nostoc sp.	CALU 994	A	12	104 ± 6.9		102 ± 3.5	
Nostoc sp.	CALU 983	A	6	97 ± 1.5		107 ± 9.5	
Nostoc sp.	CALU 979	A	7	103 ± 7.6		99 ± 1.7	
Nostoc sp.	CALU 992	A	5	103 ± 4.4		97 ± 6.7	
Nostoc sp.	CALU 993	A	3	99 ± 1.2		102 ± 2.6	
Nostoc sp.	CALU 996	A	4	96 ± 4.0		118 ± 27.4	
Nostoc sp.	CALU 995	A	11	99 ± 1.2		109 ± 9.0	
Nostoc sp.	CALU 981	A	4	107 ± 8.2		100 ± 12.7	
Nostoc sp.	CALU 982	A	1	111 ± 6.0		111 ± 6.7	
Nostoc sp.	CALU 984	A	5	94 ± 4.0		100 ± 9.0	
Nostoc sp.	CALU 997	A	8	110 ± 14.8		97 ± 2.1	
Nostoc sp.	RQ II	A	8	113 ± 10.1		93 ± 3.1	
Nostoc sp.	BROMEL	A	5	103 ± 16.5		101 ± 8.5	
Nostoc sp.	BRIII	A	15	129 ± 19.5		88 ± 2.6	
Nostoc sp.	BRV	A	16	120 ± 6.6		110 ± 4.5	
Nostoc sp.	BRVI	A	17	144 ± 33.9		127 ± 14.9	
Nostoc sp.	BRIIB	A	16	118 ± 9.3		118 ± 7.2	
Nostoc sp.	Cam2SO1	A	5	42 ± 27.3	++	52 ± 5.1	+
Nostoc sp.	Lukešová 6/96	A	3	104 ± 9.0		99 ± 4.0	
Nostoc sp.	Lukešová 2/89	A	7	99 ± 1.2		94 ± 4.1	
Nostoc sp.	TH1SO1	A	8	68 ± 29.4	+	101 ± 16.3	
Nostoc sp.	TH2S22	A	9	104 ± 19.5		92 ± 13.0	
Nostoc calcicola	Badour 1963/23	B	4	88 ± 4.6		100 ± 3.5	
Nostoc indistinguendum	Flechtner CM1-VF1	B	5	91 ± 4.8		80 ± 5.9	
Nostoc punctiforme	Flechtner ICM-VF1Y	B	3	74 ± 1.8	+	87 ± 7.2	
Nostoc sp.	Hindák 06/9	B	0	81 ± 5.9		95 ± 4.7	
Nostoc sp.	Lukešová 32/03	B	9	110 ± 9.5		98 ± 3.3	
Nostoc sp.	CALU 129	B	3	93 ± 2.9		98 ± 2.5	
Nostoc sp.	CALU 542	B	12	104 ± 10.4		100 ± 2.0	
Nostoc calcicola	Lukešová 1/86	C+D	7	57 ± 2.1	+	68 ± 2.9	+
Nostoc calcicola	Lukešová 9/89	C+D	8	51 ± 4.0	+	48 ± 2.9	++
Nostoc calcicola	Lukešová 3/97	C+D	2	97 ± 5.2		103 ± 3.5	
Nostoc calcicola	Desortová 67/2	C+D	5	100 ± 4.1		100 ± 2.4	
Nostoc edaphicum	DAEON	C+D	6	97 ± 5.4		95 ± 4.1	
Nostoc ellipsosporum	Lukešová 51/91	C+D	3	59 ± 18.2	+	61 ± 4.2	+
Nostoc ellipsosporum	Lukešová 52/91	C+D	6	62 ± 7.1	+	71 ± 4.7	+
Nostoc microscopicum	Zehnder 76/210	C+D	3	94 ± 4.1		80 ± 6.3	
Nostoc muscorum	Lukešová 2/91	C+D	9	60 ± 4.0	+	62 ± 3.6	+
Nostoc muscorum	Lukešová 1/85	C+D	7	62 ± 7.6	+	55 ± 6.2	+
Nostoc muscorum	Lukešová 1/87	C+D	8	99 ± 4.0		112 ± 11.2	
Nostoc muscorum	Lukešová 14/86	C+D	9	35 ± 3.5	++	47 ± 10.1	++
Nostoc sp.	CALU 453	C+D	9	94 ± 3.8		102 ± 2.9	
Nostoc sp.	CALU 374	C+D	6	100 ± 0.3		96 ± 4.0	
Nostoc sp.	Lukešová 116/96	C+D	4	117 ± 29.4		99 ± 5.0	
Nostoc sp.	CALU 760	C+D	7	78 ± 2.9		79 ± 3.8	
Nostoc sp.	Lukešová 5/96	C+D	13	59 ± 4.7	+	48 ± 3.8	++
Nostoc sp.	Lukešová 18/89	C+D	4	134 ± 38.7		146 ± 40.9	
Nostoc sp.	OSNI32s01	C+D	2	105 ± 10.5		100 ± 0.4	
Nostoc sp.	Lukešová 6/99	C+D	12	59 ± 6.6	+	46 ± 7.8	++
Nostoc sp.	Lukešová 5/99	C+D	8	46 ± 13.0	++	52 ± 7.6	+
Nostoc sp.	Lukešová 7/99	C+D	4	28 ± 1.9	++	34 ± 5.0	++
Nostoc sp.	OBU36s07	C+D	10	47 ± 23.0	++	58 ± 12.8	+

TABLE II. (*Continued*)

Scientific name	Strain	Clim. reg.	No. met.	YAC-1 viab.		Sp2 viab.	
				Mean ± SD	Cyt.	Mean ± SD	Cyt.
Nostoc sp.	CALU 545	C+D	8	73 ± 3.1	+	62 ± 9.6	+
Nostoc sp.	CALU 546	C+D	13	114 ± 17.1		104 ± 4.6	
Nostoc sp.	CALU 1191	C+D	5	97 ± 2.0		133 ± 35.1	
Nostoc sp.	CALU 521	C+D	7	101 ± 6.0		110 ± 13.2	
Nostoc sp.	CALU 327	C+D	7	114 ± 5.0		105 ± 4.4	
Nostoc sp.	SAG 5979	C+D	7	98 ± 2.9		95 ± 8.1	
Nostoc sp.	TO1SO1	C+D	13	55 ± 4.9	+	60 ± 8.0	+
Nostoc sp.	CALU 543	C+D	6	103 ± 2.8		108 ± 8.5	
Nostoc sp.	Lukešová 20/97	E	7	97 ± 2.9		101 ± 2.3	
Nostoc sp.	Lukešová 19/96	E	4	91 ± 4.7		100 ± 5.5	
Nostoc sp.	Lukešová 24/97	E	6	79 ± 5.4		103 ± 8.7	
Nostoc sp.	Kaštovská 2002/14	E	3	96 ± 5.0		84 ± 6.2	
Nostoc sp.	EPN16bSO1	E	3	93 ± 4.9		98 ± 3.0	
Nostoc sp.	117SO1	E	3	78 ± 10.2		72 ± 8.5	+
Nostoc sp.	88SO1	E	6	100 ± 6.3		108 ± 5.9	
Nostoc sp.	99s05	E	4	55 ± 5.8	+	50 ± 0.3	+
Nostoc sp.	43s01	E	8	75 ± 5.0	+	72 ± 4.9	+
Nostoc sp.	8s01	E	4	96 ± 6.3		94 ± 8.0	
Nostoc sp.	72EPs01	E	4	40 ± 8.6	++	26 ± 11.5	++
Nostoc sp.	Cr4	Sy	8	71 ± 23.2	+	95 ± 19.1	
Nostoc sp.	De1	Sy	14	15 ± 8.8	++	36 ± 7.6	++
Nostoc sp.	Gm	Sy	16	99 ± 22.6		86 ± 20.6	
Nostoc sp.	Mm1	Sy	9	58 ± 2.6	+	49 ± 5.9	+
Nostoc sp.	Lp1	Sy	7	59 ± 3.0	+	61 ± 10.1	+
Nostoc sp.	OGU36S01	Sy	10	75 ± 15.5	+	69 ± 20.0	+
Nostoc sp.	OGU36S02	Sy	11	73 ± 10.8	+	75 ± 9.2	+
Nostoc sp.	Cc3	Sy	9	83 ± 14.3		103 ± 13.6	
Nostoc sp.	Al2	Sy	6	95 ± 6.0		92 ± 10.0	
Nostoc sp.	CALU 268	Sy	4	102 ± 19.1		109 ± 9.5	

Mean values and standard deviations of cell viability, after exposure to the extract, are shown for each extract. Extracts which exhibited a strong toxicity (51–100% inhibition) are marked by ++. Strains with a moderate toxic effect are marked by +. The climatic regions of the strains' origin are noted in the column **clim. reg**. (climatic region): **A**—tropical climates, **B**—arid climates, **C + D**—temperate and continental climates, **E**—polar climates. Symbiotic strains are marked by **Sy**. Number of metabolites detected by HPLC-MS analysis is shown in the column **no. met**.

Chromatogram Analysis

Chromatograms obtained by HPLC-MS analysis were analyzed by Expertomica Metabolite Profiling Software (Urban et al. 2009) (http://bieng.over.cz/software.html) in order to reveal molecular ions of low intensity, and for filtration of noise in the chromatograms obtained. The instrument's output was divided into three parts by the software: (a) useful signal; (b) random noise; and (c) systematic noise of the instruments, related to the experiment. Because the noise presented have to correspond to some probability distributions, it is possible to approximate these distributions, based on a probabilistic approach to LC-MS data analysis. Understanding the measurement is more straightforward, according to the estimated probability; this allows quantifying the probability that the output is a useful signal, at each point in time and mass.

RESULTS

Frequency and Distribution of Cytotoxicity

The investigation of the 82 terrestrial cyanobacterial isolates led to our finding 27 extracts with cytotoxic activity (Tab. II). Ten of these extracts caused very potent cytotoxic effects, inhibiting the growth of 51–85% of experimental cell lines, at a concentration equivalent to 0.5 mg LyB/mL (mg of Lyophilized biomass per ml). In another 17 extracts, a moderate cytotoxic effect, resulting in an inhibition between 25 and 50% at the same concentration, was recorded. The highest occurrence of cytotoxicity [Fig. 1(A), Table III] was observed among strains originating from symbiotic associations, and among free-living isolates from temperate and continental climates (60 and 45%, respectively). While only one of the symbiotic strains

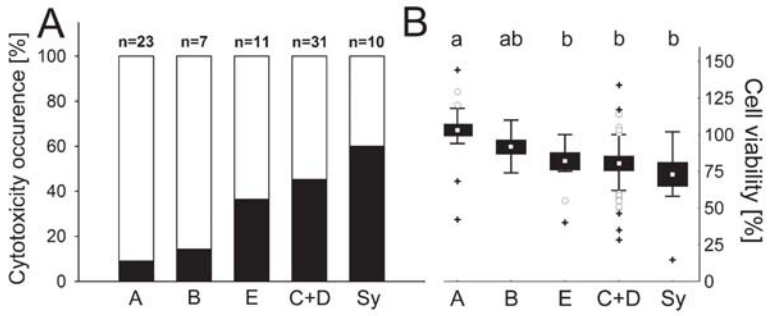


Fig. 1. Comparison of cytotoxicity occurrence and the mean inhibition values in Nostoc strains, originating from different climatic zones. A—Cytotoxicity occurrence (black portion of the column), expressed in percent. The number of the strain tested is shown above a column. B—Mean values and ranges of inhibition for strains, originating from different climatic zones. The different letters above the graph categorize the significant difference in the mean inhibition value, at the 5% significance level (tested by Fisher LSD test). Mean value (white square), standard error of the mean (box), nonoutlier maximum (whiskers), outliers (empty circles), and extremes (black crosses) are all plotted in the graph. Labels: A—tropical climates, B—arid climates, C + D—temperate and continental climates, E—polar climates, Sy—symbiotic strains.

exhibited a strong cytotoxic effect (*Nostoc sp*. De), an inhibition higher than 50% was observed in five strains from isolates from both temperate and continental climates (Table II). A lower occurrence of cytotoxicity was found in isolates originating from polar (frequency 36.4%) and arid regions (frequency 14.3%). Within these groups, a strong inhibition was only found in an extract of the Antarctic strain *Nostoc sp*. 72Eps01. The lowest occurrences of cytotoxicity were found in tropical isolates. Two out of 23 terrestrial tropical *Nostoc* strains tested inhibited mammalian cell lines (frequency 9.1%); and only the Cam2SO1 isolate (originating from Cameroon) exhibited a strong cytotoxic effect (42 and 52% inhibition to YAC-1 and Sp2, respectively). Statistical analysis of the data, when tested by ANOVA (SS = 9702, $F = 4.542$, $P = 0.00204$), confirmed a significant difference in the mean values of cytotoxicity among isolates originating from different climatic zones. The mean inhibition value of tropical isolates was significantly lower, when compared with the mean inhibitions of polar, temperate/continental, and symbiotic isolates ($P = 0.0006$, 0.0139, 0.0009, respectively—tested by the Fisher LSD test). However, the difference from arid climate isolates was not significant. The differences among the mean inhibition values of polar, temperate/continental and symbiotic isolates were not significant. For the results of the Fisher LSD test see Figure 1(B). Similar results were obtained when the cytotoxicity occurrence, expressed as the number of positive strains, was tested by the χ-square test against the hypothesis that cytotoxic strains occur randomly. The differences in cytotoxicity occurrence among isolates from different climatic zones were insignificant, when all groups were tested together; but with the probability value lying near the 5% threshold value ($P = 0.074$). The difference between cytotoxicity occurrence in temperate/continental isolates and tropical isolates was significant with the χ-square test ($\chi = 5.512$, $df = 1$, $P = 0.0188$), when tested separately.

The occurrence of cytotoxicity was also compared among strains isolated from different microsites. Eight microsites were selected for comparison (see Table III).

TABLE III. The number of cytotoxic versus non-cytotoxic strains within the studied microsites

Label	Location	Habitat	Cytotoxic	Noncytotoxic
m1	King George Island/Antarctica	Deglaciated soil	0	3
m2	Nezamyslice/Czech Republic	Soil, arable fields	3	0
m3	Dlouhá ves/Czech Republic	Soil, abandoned fields	1	1
m4	Eastern Thuringia nr. Ronneburg/Germany	Dumps after uranium mining	3	0
m5	Sokolov/Czech Republic	Dumps after coal mining	1	1
m6	Kirov Region/Russia	Agricultural field	1	2
m7	Guinea	Rice fields soil	0	11
m8	Sao Paulo/Brazil	Tree bark	0	6

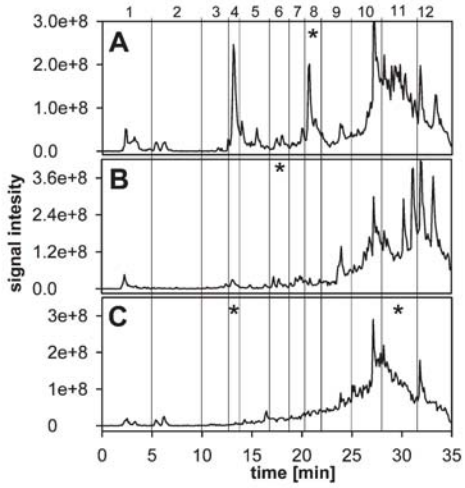


Fig. 2. Total ion chromatograms of strains isolated from microsite 4 (mine tailings in Ronneburg, Germany). Different secondary metabolite compositions and different chromatographic fractions were found, responsible for the cytotoxic effects. Extracts were fractionated into 12 identical fractions. Cytotoxic fractions are marked with asterisk. Total ion chromatograms are shown for each strain (A) *Nostoc sp*. Lukešová 6/99, (B) *Nostoc sp*. Lukešová 5/99, (C) *Nostoc sp*. Lukešová 7/99.

From the obtained distribution, it can clearly be seen that only in two microsites (mine tailings in Sokolov, Czech Republic, and from abandoned fields in Dlouhá ves, Czech Republic) the number of cytotoxic and noncytotoxic strains were equal. While in the other microsites, either cytotoxic or noncytotoxic strains were found. This distribution is in disagreement with the hypothesis that cytotoxicity is not dependent on environmental conditions and that it occurs randomly, when tested by χ-square test ($\chi = 15.11$, $df = 7$, $P = 0.0345$). This result raises the question of whether the pattern obtained couldn't be caused by either the clonality of isolated organisms or lateral gene transfer. To address this question we fractionated extracts of strains isolated from microsites with a high cytotoxicity occurrence and identified the active fraction or the compound responsible for the cytotoxic effect. Two microsites were selected for these purposes: (1) mine tailings left from uranium mining in Eastern Thuringia near Ronneburg, Germany; and (2) arable fields in Nezamyslice, Czech Republic. In both of these habitats all of the strains exhibited cytotoxic effects.

In cyanobacterial isolates from the uranium mine tailings, different compounds were responsible for the cytotoxic effects; since active fractions exhibited different retention behaviors when obtained using the identical HPLC method on a preconditioned column. The extracts were fractionated into 12 fractions with identical retention times (Fig. 2). In

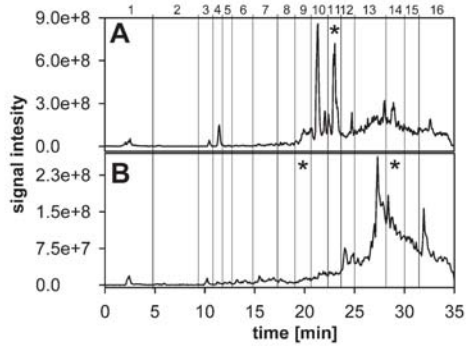


Fig. 3. Total ion chromatograms of strains isolated from microsite 3 (arable fields in Nezamyslice, Czech Republic). Different secondary metabolite compositions and different chromatographic fractions were found, responsible for the cytotoxic effects. Extracts were fractionated into 16 identical fractions. Cytotoxic fractions are marked with asterisk. Total ion chromatograms are shown for each strain (A) *Nostoc muscorum* Lukešová 2/91, (B) *Nostoc ellipsosporum* Lukešová 51/91.

the extract obtained from *Nostoc sp*. Lukešová 6/99, the cytotoxic activity was found in fraction 8, which eluted between 20.4 and 22.0 min [Fig. 1(A)]. Inhibition of this fraction was found to be 40% for the Sp2 cell line. This value was comparable with the cytotoxicity of the crude extract (54% inhibition of the Sp2 cell line); this implies that a single compound was responsible for the cytotoxic effects of this strain. The mass spectrum observed at the maximum chromatographic peak clearly demonstrated peaks at 1081 [M+H$^+$] and 1103 [M+Na$^+$], which correspond to a protonated molecule and its sodium adduct, respectively [Fig. 4(A)]. Based upon the molecular weight, this compound was tentatively identified as a cytotoxic peptide nostopeptolide A1 (Golakoti et al., 2000). In a second strain, *Nostoc sp*. Lukešová 5/99, originating from this microsite, the presence of this compound was not found. A different cytotoxic

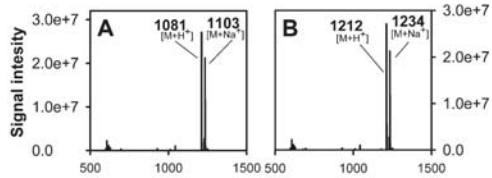


Fig. 4. Mass spectrum of the cytotoxic fractions in selected strains. (A) *Nostoc sp*. Lukešová 6/99 (the molecular ion and its sodium adduct correspond to the presence of Nostopeptolide A1). (B) *Nostoc muscorum* Lukešová 2/91 (the presence of an unknown cytotoxic compound with a molecular weight of 1211 was recorded).

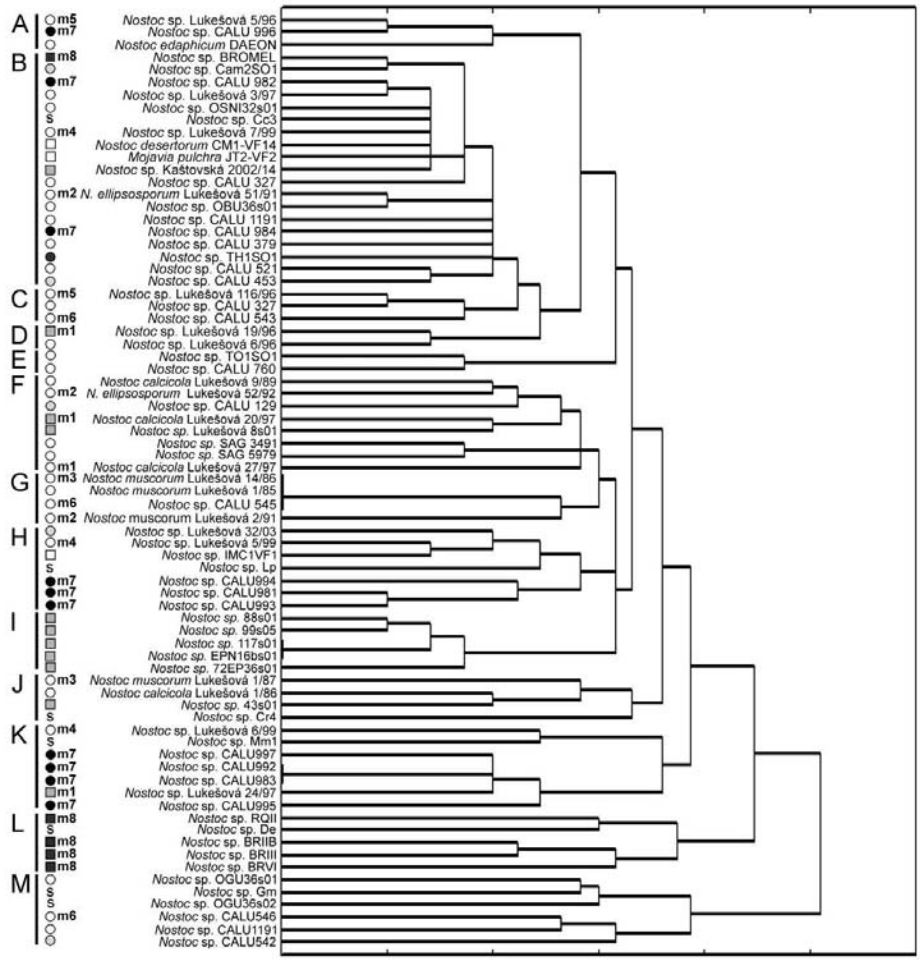


Fig. 5. Similarities among the studied strains, based on extract composition, computed by cluster analysis (Euclidean distance, complete linkage). The strains isolated from different geographic regions and microsites seem to be dispersed randomly, and to intermix with one another. (The only exception seems to be cluster I and L). ○ Europe, ● Asia, □ North America, ■ South America, ● Africa, ■ Antarctica, S Symbiotic. Particular microsites are marked as **m1–m8**.

fraction was detected in the extract. Fraction 6, which eluted between 17.1 and 19.0 min. [Fig. 2(B)] caused a decrease of viability, up to 61% of the Sp/2 cells in an applied concentration (equivalent to 0.5 LyB/mL). In the third strain, isolated from this microsite, *Nostoc sp.* Lukešová 7/99, one fraction exhibited a moderate cytotoxic effect (F11, 28.0–32.0 min., 27% inhibition), plus one fractions with weak cytotoxicity (F3, 12.8–13.7 min., 14% inhibition) was responsible for the resulting strong cytotoxic effect of this strain (app. 70% inhibition to both cell lines) [Fig. 2(C)].

Different compounds were also responsible for the cytotoxic effect in the strains isolated from arable fields (Nezamyslice, Czech Republic). The extracts of these strains were fractionated into 16 fractions, according to the most intensive peaks observed in a total ion chromatogram (Fig. 3). In the extract of the strain *Nostoc muscorum* Lukešová 2/91, only one fraction (F11) exhibited significant cytotoxicity (inhibition of 45%) [Fig. 3(A)]; which is comparable with the mean inhibition of the crude extract of the Sp/2 cell line (53%). The presence of an unknown cytotoxic compound of

TABLE IV. Occurrence of the most common compounds in the studied Nostoc strains, isolated from different geographic regions

m/z	RT (min)	Str. no.	Place of isolation/strains
815	11.0	7	Antarctica (88s01, 8s01,117s01 99s05,72EPs01, EPN16bs01), Ireland (OGU36s02)
865	12.8	9	Antarctica (43s01), Czech Republic (1/86), Egypt (32/03), Germany (5/99), Ireland (OGU36s01), Kazakhstan (CALU542), USA (IMC1-VF1), symbiont (Mm1,Lp1)
1007	14	14	Antarctica (24/97, 19/96), Czech Republic (6/99), Guinea (CALU 983, 992, 995, 996, 997), Italy (TO1SO1), Russia (CALU543, DAEON), Symbiont (Mm1, Cr4, CALU268)
892	15.4	7	Brazil (BRII, BRIII, BRVI, RQII), Czech Republic (1/87), Egypt (32/03), Symbiont (De)
844	17.4	5	Czech Republic (1/86, 52/91), Symbiont (Gm1, OGU36s01, s02)
1035	17.5	7	Antarctica (19/96, 24/97), Guinea (CALU 983,992, 995, 997), Russia (DAEON)
828	20.1	9	Antarctica (43s01), Czech Republic (1/87, 1/86), Kazakhstan (CALU542), Russia (CALU453), Symbiont (Cr4, Gm1, OGU36s01,s02)
1081	21.3	4	Czech Republic (6/99), Guinea (CALU984), Italy (TO1SO1), Russia (CALU543)
856	21.9	8	Guinea (CALU992,997,984), Russia (DAEON), Thailand (TH2S22)
1211	20.8	4	Czech Republic (14/86, 1/85), Russia (CALU545), Thailand (TH2S22)

Retention times (RT) and m/z values are shown for particular compounds.
The number of strains in which particular compound was observed is referred to as str. no.

molecular weight 1211 was confirmed, based upon the presence of molecular ion 1212 [M+H$^+$] and its sodium adduct 1234 [M+Na$^+$] [Fig. 4(B)]. The cytotoxicity of this compound was proven in the pure state, and its unknown origin was confirmed by preliminary NMR analysis (Tomek, personal communication). A completely different extract composition was observed in *Nostoc ellipsosporum* Lukešová 51/91 [Fig. 3(B)]. Its separation revealed two active fractions; however, the cytotoxic compound with molecular weight 1211 was missing in this strain. Instead, fraction 9, which eluted between 18.1 and 20.5 min., exhibited a moderate cytotoxic effect, and resulting in a decrease of the Sp2 cell line growth to 70%. A weak inhibition effect (16%) was recorded for the fraction 14, which eluted at between 28 and 30 min. The toxicity of this fraction could possibly be caused by the presence of pigments and their degradation products (which are commonly eluted in this portion), in which a weak cytotoxic effect has been shown (Sakata et al., 1990; Chernomorsky et al., 1999). Based on the different retention behavior of the cytotoxic fractions, we concluded that the cytotoxicity of the strains isolated from both microsites is caused by different active compounds. Thus, the high occurrence of cytotoxicity in some microsites cannot be caused by clonality or lateral gene transfer.

Extracts Composition and Distribution of Particular Compounds

Analyzing the 70% methanol extracts of the studied strains, we were able to recognize 293 distinct compounds, according to the presence of sodium and potassium adducts and the distribution of isotopomeres. On the basis of their molecular weights, only 77 compounds (26.3%) corresponded to any known compounds of cyanobacterial origins described in the recent literature (data not shown; compared with a private library created from published data).

We did not find any clear geographical pattern in the distribution of the detected compounds. Cluster analysis applied to compounds synthesized by two and more strains revealed that based on the secondary metabolite composition, the strains of different places of isolation clustered randomly (Fig. 5). Strains isolated from de-glaciated soil from Antarctica, clustered into a rather tight group of cluster I. Surprisingly, *Nostoc sp.* 43s01, isolated from the same habitat was linked into cluster J, together with strains of *Nostoc muscorum* and *Nostoc calcicola* isolated from Europe. Cluster L, except the symbiotic strain *Nostoc sp.* De, is also composed of strains from one origin (Nostoc growing on tree bark in a rain forest (Sao Paulo, Brazil)). However, in these strains, nearly identical chromatograms were obtained, which could imply the probable clonality of the isolates. In all of the other obtained groups, strains isolated from different continents and microsites were found which suggest a random distribution of the production of most of these compounds around the world.

This result is even more evident in the distribution of the most common metabolites found. Table IV shows a random distribution of the 10 most abundant substances found and the studied *Nostoc* strains. According to the molecular weight values, we presume that these are unknown variants of cyanobacterial peptides. The most abundant metabolite, of molecular weight 1007, and eluting at 13.9 min., was produced by 14 cyanobacterial strains from different geographical locations and climatic zones: Antarctica, Czech Republic, Guinea, Russia, plus one symbiotic strain isolated from a Cycas symbiont in Italy. Figure 6 shows the total ion chromatograms and mass spectra of selected strains, demonstrating the identity of this compound. A similar broad geographical distribution can be observed in other frequent compounds (Table III).

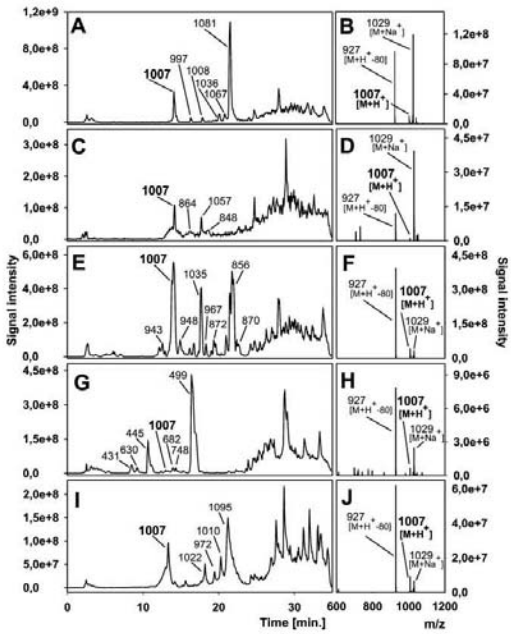


Fig. 6. Production of an identical compound with m/z = 1007, and a retention time of 14.1 min., by strains of different geographical origins. The total ion chromatogram is shown for each strain. The m/z values are shown for the most intense peaks (A) *Nostoc sp.* CALU 543 was isolated from the Kirov region (Russia). (B) *Nostoc sp.* Lukešová 24/97 was isolated from King George Island (Antarctica). (C) *Nostoc sp.* TO1SO1 was isolated from Tuscany (Italy). (D) Symbiotic strain *Nostoc sp.* Mm1 was isolated from Macrozamia, Rome (Italy). (E) *Nostoc sp.* CALU 995 was isolated from rice field soil in Guinea.

DISCUSSION

The overall observed frequency of cytotoxicity in terrestrial isolates (33%) is comparable to other cytotoxicity screening studies, which were based on the screening of a lesser number of isolates. Mian et al., 2003 reported 38% of 22 randomly selected strains of freshwater and terrestrial cyanobacteria to be cytotoxic. The same value was observed by Surraka in benthic cyanobacteria (Surraka et al., 2005), and a frequency of 24% was found in cultured Nostoc strains (Piccardi et al., 2000). Our data show that cytotoxicity strongly depends on the origin of the strain. We found a significant difference between the cytotoxicity frequency of tropical *versus* temperate and continental climate isolates. Non significant differences among other groups, based on posthoc comparison and the comparison of cytotoxicity frequencies, could be connected with the low number of isolates tested from arid, polar, and symbiotic strains. Accordingly, a more comprehensive study, focused upon these groups, is needed. The lower inhibition values and the lower frequency of cytotoxic strains in arid climate isolates is evident (Fig. 1, Table II). The high cytotoxicity occurrence within temperate and continental climatic zones is consistent with recent literature; since many cytotoxins like cryptophycins, nostofungicine, aeruginoguanidines, and calotrixins have been isolated from cyanobacteria originating from a such climate (Kajiyama et al., 1998; Rickards et al., 1999; Ishida et al., 2002; Biondi et al., 2004). The situation in more complicated with the tropical isolates. Mirabimides, indocarbamazoles, and scytophycins, including the very active compound tolytoxin, are the main groups of cytotoxins produced (mainly by Tolypothrix, Scytonema, and Nostoc species from the Hawaiian Islands (Carmeli et al., 1990, 1991; Knübel et al., 1990)). Except for the Hawaiian strains, only in one tropical terrestrial strain (*Hapalosiphon langii*, originating from Papua New Guinea) were a group of cytotoxic indole alkaloids hapalindoles found (Klein et al., 1995). One of the possible reasons for the low number of observed cytotoxic isolates among the tropical isolates could be the decrease of secondary metabolite production during their isolation and maintenance in unnatural conditions. However, at least the number and diversity of metabolites produced by tropical strains did not differ significantly from strains isolated from the temperate and continental zone ($t = -0.57$, $P = 0.57$, Table II). Another surprising finding was the occurrence of high cytotoxicity among symbiotic strains. Although symbiotic cyanobacteria have been intensely studied from different points of view, no systematic study on their cytotoxicity has been published, to date. The intensive production of cyanobacterial peptides by lichen symbionts was reported by Oksanen and others (Oksanen et al., 2004). Moreover, cryptophycins, the most effective of cyanobacterial cytotoxins, have been isolated from the lichen symbiont *Nostoc sp.* ATCC53789 (Biondi et al., 2004).

Despite the fact that our HPLC-MS screening surely had not revealed all compounds, and that molecular weight can not be used for an exact compound identification, it was clearly demonstrated that the production of a particular compound is randomly dispersed across a climatic/geographic region and microsites. In contrast to this, the production of compounds with cytotoxic effects is more frequent in some regions and microsites. We suggest that the production of the cytotoxic compounds is of an environmentally dependent character, and that strains producing cytotoxic compounds are favored under specific conditions. The finding that the cytotoxicity of cyanobacterial strains from particular microsites is caused by different chromatographic fractions supports this idea. Metabolites are synthesized by the cyanobacterium, based on the potential of its synthetic apparatus, and with respect to its cytotoxic

function. Moreover, our results are not consistent with the idea of clonality and lateral gene transfer as the explanation for the high occurrence of cytotoxic strains in some microsites. There is strong evidence that cyanobacterial metabolites can affect grazers via digestive enzyme inhibition or even acute toxicity (Blom et al., 2001; Rohlack et al., 2001; Rohlack et al., 2004; Argaval et al., 2005). As already mentioned, for most of the cytotoxins, a strong toxic effect on invertebrates was reported (Rickards et al., 1999; Berry et al., 2004; Biondi et al., 2004). Subsequently, it is possible that the high occurrence of cytotoxic strains can be linked with high predation pressure in these microsites. This hypothesis will lead to two questions: Why there is a low cytotoxicity occurrence within tropical habitats, in which the high predation pressure is generally known.

The fast growth rate under tropical conditions as the compensation of high predation can be a possible explanation. A second could be why cyanobacterial symbionts produce cytotoxic compounds harmful to invertebrates when they are sheltered by its host plant. Recently, several research studies have revealed a very weak and temporary alliance of nostocacean strains and their partners (Rikkinen et al., 2002; Papaefthimiou et al., 2008). The cyanobacterium could bring the cytotoxicity from the soil environment, where a defense against grazers is comprehensible. Moreover, cytotoxic substances produced by cyanobacterium in the plant tissue can work as a defense of the whole plant organism.

Rantala and others hypothized that the random distribution of cyanopeptides synthesis across the phylogenetic spectrum can be a consequence of lateral gene transfer or the ancient origin of the nonribosomal synthetic pathway (Rantala et al., 2004). Ultimately, Rantala and the others found the phylogenetic congruence between 16S and microcystin gene synthetases, suggesting simultaneous evolution of both genes. Moreover, a molecular clock computation leads to an estimation of the microcystin synthetase age of about 3.5 billion years. This was strong evidence for a confirmation of the ancient origin of the nonribosomal synthetic pathway. However, this conclusion is based solely on evolutionary models. In our study, we have shown that most of the metabolites are synthesized by cyanobacteria from very distant geographical areas, which have no apparent ability to share their genetic material; yet despite this they produce identical compounds. This means that it is highly probable that they share the synthetic machinery from the time of their common ancestor; giving strong support of the results revealed by Rantala et al. (2004).

In conclusion, if we consider that one third of the extracts exhibit a cytotoxic effect, and 70% of observed compounds do not correspond (by molecular weight) to any known structure; we conclude that terrestrial cyanobacteria are an extremely interesting source for novel compounds for pharmacology and biotechnology. Moreover, the number of unknown compounds may be underestimated, since several compounds can have similar molecular weights.

The authors want to thank Lada Samcová and Pavel Souček for their technical support. Special thanks to Michal Koblížek for his critical reading and corrections.

REFERENCES

Agrawal MK, Bagchi D, Bagchi SN. 2005. Cysteine and serine protease mediated proteolysis in body homogenate of a zooplankter. *Moina macrocopa* is inhibited by the toxic cyanobacterium, *Microcystis aeruginosa* PCC7806. Comp Biochem Physiol B-Biochem Mol Biol 141:33–41.

Berry JP, Gantar M, Gawley RE, Wang M, Rein KS. 2004. Pharmacology and toxicology of pahayokolide A, a bioactive metabolite from freshwater species of Lyngbya isolated from Florida Everglades. Comp Biochem Physiol C-Toxicol Pharmacol 139:231–238.

Biondi N, Pikardi R, Margheri MC, Rodolfi L, Smith GD, Tredici M. 2004. Evaluation of Nostoc strains ATCC 53789 as a potential source of natural pesicides. Appl Environ Microbiol 70: 3313–3320.

Birkemoe T, Liengen T. 2000. Does collembolan grazing influence nitrogen fixation by cyanobacteria in the high arctic? Polar Biol 23:589–592.

Blom JF, Robinson JA, Juttner F. 2001. High grazer toxicity of [D-Asp(3) (E)-Dhb(7)] microcystin-RR of *Planktothrix rubescens* as compared to different microcystins. Toxicon 29:1923–1932.

Botha N, Gehringer MM, Downing TG, van der Venter M, Shephard EG. 2004. The role of microcystin-LR in the induction of apoptosis and oxidative stress in CaCo2 cells. Toxicon 43:85–92.

Carmichael WW. 1992. Cyanobacterial secondary metabolites—The cyanotoxins. J Appl Microbiol 72:445–459.

Carmeli S, Moore RE, Patterson GML. 1991a. Tolytoxin and new scytophycins from three species of Scytonema. J Nat Prod 53:1533–1542.

Carmeli S, Moore RE, Patterson GML. 1991b. Mirabimides A-D, new *N*-acylpyrroines from the blue-green algae *Scytonema mirabile*. Tetrahedron 47:2087–2096.

Chernomorsky S, Segelman A, Poretz RD. 1999. Effect of dietary chlorophyll derivatives on mutagenesis and tumor cell growth. Teratog Carcinog Mutagen 19:313–322.

Darby BJ, Neher DA, Belnap J. 2007. Soil nematode communities are ecologically more mature beneath late- than early-successional stage biological crusts. Appl Soil Ecol 35:203–212.

Ding WX, Ong ChN. 2003. Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. FEMS Microbiol Lett 220:1–7.

Dodds WK, Gudder DA, Mollenhauer D. 1995. The ecology of Nostoc. J Phycol 31:2–18.

Golakoti T, Yoshida YW, Chaganty S, Moore RE. 2000. Isolation and structures of nostopeptilides A1. A2 and A3 from cyanobacterium *Nostoc sp*. GSV 224. Tetrahedron 56:9093–9102.

Hrouzek P, Šimek M, Komárek J. 2003. Nitrogenase activity and diversity of six soil Nostoc strains. Arch Hydrobiol/Suppl Algol Stud 108:87–101.

Hrouzek P, Ventura S, Lukešová A, Mugnai MA, Turicchia S, Komárek J. 2005. Diversity of soil Nostoc strains: Phylogenetic and phenotypic variability. Arch Hydrobiol/Suppl Algol Stud 117:251–264.

Ishida K, Matsuda H, Okita Y, Murakami K. 2002. Aeruginoguanidines 98-A—98-C: cytotoxic unusual peptides from the cyanobacterium *Microcystis aeruginosa*. Tetrahedron 58:7645–7652.

Kajiyama SI, Kanzaki H, Kawazu K, Kobayahi A. 1998. Nostofungicine, an antifungal lipopeptide from the field-grown terrestrial glue-green alga *Nostoc commune*. Tetrahedron Lett 39: 3737–3740.

Klein D, Daloze JC, Braekman JC, Hoffman L, Demoulin V. 1995. New hapalindoles from the cyanophyte *Hapalosiphon langii*. J Natl Prod 58:1781–1785.

Kleinkauf H, von Döhren H. 1995. A nonribosomal system of peptide biosynthesis. Eu J Bioch 236:535–551.

Knübel G, Larsen KL, Moore RE, Levine IA, Patterson GLM. 1990. Cytotoxic, antiviral indocarbazoles from a blue-green alga belonging to the nostocaceae. J Antibiotic 10:1236–1239.

Lukešová A 1993. Soil algae in four secondary successional stages on abandoned fields. Arch Hydrobiol/Suppl Algol Stud 71:81–102.

Mian P, Heilmann J, Bürgi R, Sticher O. 2003. Biological screening of terrestrial and freshwater cyanobacteria for antimicrobial activity, brine shrimp lethality, and cytotoxicity. Pharm Biol 41:243–247.

Mosmann T. 1983. Rapid colometric assay for cellular growth and survival: Application to proliferation ans cytotoxicity assay. J Immunol Methods 65:55–63.

Namikoshi N, Rinehart KL. 1996. Bioactive compounds produced by cyanobacteria. J Industr Microbiol 17:373–384.

Oksanen I, Jokela J, Fewer DP, Wahlsten M, Rikkinen J, Sivonen K. 2004. Discovery of rare and highly toxic microcystins from lichen-associated cyanobacterium *Nostoc sp.* strain IO-102-I. Appl Environ Microbiol 70:5756–5763.

Pajdak-Stos A, Fialkowska E, Fyda J. 2004. Vulnerability of *Nostoc muscorum* Agardh (Cyanophyceae) motile hormogonia to ciliate grazing. J Phycol 40:271–274.

Papaefthimiou D, Hrouzek P, Mugnai MA, Lukesova A, Turicchia S, Rasmussen U, Ventura S. 2008. Differential patterns of evolution and distribution of the symbiotic behavior in nostocacean cyanobacteria. Int J Syst Evol Microbiol 58:553–564.

Patterson GML, Carmeli S. 1992. Biological effect of tolytoxin (6-hydroxy-7-*o*-methyl-scytophycin b) a potent bioactive metabolite from cyanobacteria. Arch Microbiol 157:406–410.

Patterson GML, Smith CD, Kimura LH, Britton BA, Carmeli S. 1993. Action of tolytoxin on cell morphology, cytoskeletal organization, and actin polymerization. Cell Motil Cytoskeleton 24:39–48.

Peel MC, Finlaison BL, McMahon TA. 2007. Updated world map of the Köpper-Geiger climate classification. Hydrol Earth Syst Sci 11:1633–1644.

Piccardi R, Frosini A, Tredici MR, Margheri MC. 2000. Biological screening of terrestrial and freshwater cyanobacteria for antimicrobial activity, brine shrimp lethality, and cytotoxicity. J Appl Phycol 12:543–547.

Rajanieni P, Hrouzek P, Kastovska K, Willame R, Rantala A, Hoffmann L, Komarek J, Sivonen K. 2006. Phylogenetic and morphological evaluation of the genera Anabaena, Aphanizomenon, Trichormus and Nostoc (Nostocales, Cyanobacteria). Int J Syst Evol Microbiol 55:11–26.

Rantala A, Fewer DP, Hisbergues M, Rouhiainen L, Vaitomaa J, Börner T, Sivonen K. 2004. Phylogenetic evidence for the early evolution of microcystin synthesis. Proc Natl Acad Sci USA 101:568–573.

Řeháková K, Johansen JR, Casamatta DA, Xuesong L, Vincent J. 2007. Morphological and molecular characterization of selected desert soil cyanobacteria: Three species new to science including *Mojavia pulchra gen. et sp. nov*. Phycologia 46:481–502.

Rickards RW, Rothschild MJ, Willis AC, Chazal NM, Kirk J, Kirk K, Saliba JK, Smith GD. 1999. Calothrixins A and B, novel pentacyclic metabolites from Calothrix cyanobacteria with potent activity against malaria parasite and human cancer cells. Tetrahedron 55:13513–13520.

Rikkinen J, Oksanen I, Lohtander ,K. 2002. Lichen guild share related cyanobacterial symbionts. Science 297:357.

Rohrlack T, Dittmann E, Henning M, Börner T, Christoffersen K. 2001. Effects of cell-bound microcystins on survival and feeding of *Daphnia spp*. Appl Environ Microbiol 67:3523–3529.

Rohrlack T, Christoffersen K, Kaebernick M, Neilan BA. 2004. Cyanobacterial protease inhibitor microviridin J causes a lethal molting disruption in *Daphnia pulicaria*. Appl Environ Microbiol 70:5047–5050.

Sakata K, Yamamoto K, Ishikawa H, Yagi A, Etoh H, Ina K. 1990. Chlorophyllone-A, a new pheophorbide-A related compound isolated from *Rudiatapes phillippinarum* as an antioxidative compound. Tetrahedron Lett 31:1165–1168.

Sivonen K. 1996. Cyanobacterial toxins and toxin production. Phycologia 35:12–24.

Smith CD, Zhang XQ, Moorbery SL, Patterson GML, Moore RE. 1994. Cryptophycin—A new antimicrotubule agent active against drug-resistant cells. Cancer Res 54:3779–3784.

Surakka A, Sihvonen LM, Lehtimaki JM, Wahlsten M, Vuorela P, Sivonen K. 2005. Benthic cyanobacteria from the Baltic Sea contain cytotoxic Anabaena, Nodularia, and Nostoc strains and an apoptosis-inducing Phormidium strain. Environ Toxicol 20:285–292.

Teicher BA, Forler P, Menon K, Phares V, Amsrud T, Shih C. 2000. Cryptophycin 52 and cryptophycin 55 in sequential and simultaneous combination treatment regimens in human tumor xenografts. In Vivo 14:471–480.

Teneva I, Asparuhova D, Dzambazov B, Mladenov R, Schirmer K. 2003. The freshwater cyanobacterium Lyngbya aeruginocoerulea produces compounds toxic to mice and to mammalian an fish cells. Environ Toxicol 18:9–20.

Urban J, Vaněk J, Soukup J, Štys D. 2009. Expertomica metabolite profiling: Getting more information from LC-MS using the stochastic systems approach. Bioinformatics 25:2764–2767.

Vagnoli L, Margheri MC, Allotta G, Materassi R. 1992. Morphological and physiological properties of symbiotic cyanobacteria. New Phytol 120:243–249.

Welker M, vön Döhren H. 2006. Cyanobacterial peptides—Nature's own combinatorial biosynthesis. Fems Microbiol Rev 30:530–563.

Letter of acceptance

Dear Mr. Hrouzek,

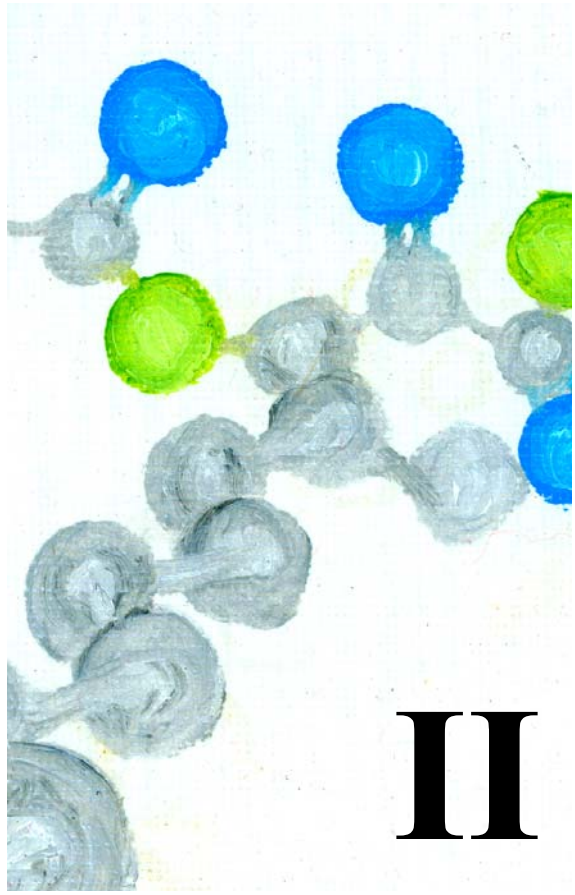
I am pleased to inform you that your paper entitled "Cytotoxicity and secondady metabolites production in terrestrial *Nostoc* strains originating from different climatic/geographic regions and habitats: Is their cytotoxicity environmentaly dependent?" has been accepted for publication in Environmental Toxicology.

If your current manuscript files are not suitable for publication, you will be contacted and directed where to send your printer-ready files.

At this time, please fax a signed copy of the Copyright Transfer Agreement to the Production Editor, Diana Schaeffer, at 717-738-9478.

Sincerely,

Paul B. Tchounwou, Sc.D., F.A.B.I., I.O.M.
Presidential Distinguished Professor & Associate Dean, CSET
Director, NIH-RCMI Center for Environmental Health
Jackson State University
Editor Environmental Toxicology
paul.b.tchounwou@jsums.edu



II

Hrouzek P., Kuzma M., Černý J., Novák P., Fišer R., Šimek P., Štys D., Lukešová A., & Kopecký J.:

Cyanobacterial cyclic peptides Puwainaphycins F and G are causing cytotoxic effect via cell membrane permeabilization and subsequent actin relocalization

Manuscript

Cyanobacterial cyclic peptides Puwainaphycins F and G are causing cytotoxic effect via cell membrane permeabilization and subsequent actin relocalization.

Pavel Hrouzek[1,2,3], Marek Kuzma[4], Jan Černý[5] Petr Novák[4], Radovan Fišer[6], Petr Šimek[7], Dalibor Štys[2], Alena Lukešová[8], Jiří Kopecký[1,2]

[1]*Institute of Microbiology, Academy of Sciences of the Czech Republic, Opatovický Mlýn, 379 81 Třeboň, Czech Republic*

[2]*Institute of Physical Biology, University of South Bohemia, Zámek 136, 373 33 Nové Hrady, Czech Republic*

[3]*Department of Botany, Faculty of Sciences, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic*

[4] *Institute of Microbiology, Academy of Sciences of the Czech Republic, Laboratory of Molecular Structure Characterization, Vídeňská 1083, Prague, Czech Republic*

[5] *Charles University, Faculty of Sciences, Department of Cell Biology, Viničná 7, 12800 Praha 2*

[6] *Charles University, Faculty of Sciences, Department of Genetics and Microbiology, Viničná 7, 12844 Praha 2*

[7] *Institute of Entomology, Biology Centre AS CR, v.v.i., 370 05 České Budějovice, Czech Republic*

[8] *Institute of Soil Biology, Biology Centre AS CR, v.v.i., Na Sádkách 7, 370 05 České Budějovice, Czech Republic*

Abstract: Puwainaphycins F and G, which cause unique cytoskeletal changes in mammalian cell lines and subsequent cell death, have been isolated from the cyanobacterium *Cylindrospermum* sp. C24/89. Puwainaphycin F has been shown to be a cyclic peptide (valyl-2aminobut-2(*E*)-enoyl-asparaginyl-2aminobut-2(*E*)-enoyl-asparaginyl-alanyl-threonyl-Nmethylasparaginyl-prolyl) containing the β-amino acid unit (2-hydroxy-3-amino-tetradecanoic acid). It differs from previously described variants of the puwainaphycins, at five amino acids as well as in the β-amino acid unit. The rapid interaction of this compound with the plasma membrane leads to an elevation of the concentration of intracellular Ca^{2+}, with kinetics comparable to the well-established calcium ionophore ionomycin. Subsequently, the induction of tyrosine phosphorylation was followed by the unique transformation of the actin cytoskeleton into ring structures around the nuclei being observed. All of these alterations in the cellular morphology and physiology after ca.

10 hrs finally results in necrotic cell death. Puwainaphycin G, a congener of puwainaphycin F, differs only in the substitution of an asparaginyl moiety for a glutaminyl in the fourth position; but exhibited the same biological function and moderate toxicity as did puwainaphycin F.

Introduction

The importance of cyanobacterial peptides as agents causing human health problems, as well as important agents affecting ecosystem function, is well documented (MacKintosch et al. 1990, Ohta et al 1994, Yoshizawa et al. 1990). Several unique peptide structures of cyanobacterial origin have been demonstrated to be promising in the field of pharmacology (Teicher et al. 2000, Trimurtulu et al. 1994). Despite the extensive studies into the possible pharmaceutical potential of cyanobacteria in the last decade, the results of the screenings suggest that only a small extent of the structural variability and possible biological functions have been discovered to date (Welker et al. 2006, Hrouzek et al. 2010). Additionally, only a few of the secondary cyanobacterial metabolites have had their exact molecular mechanisms of their biological functions identified. Consequently, more intensive studies are needed to realize the ultimate potential of cyanobacterial metabolites in both pharmacology and biotechnology.

The most common cyanobacterial secondary metabolites are cyclic and linear peptides. They are synthesized by the ancient combinatorial non-ribosomal synthetic pathway, resulting in a striking array of structural variability. Recently, about 600 different cyanobacterial peptides have been described (Welker and von Döhren 2006). They usually contain modified amino acids in both the D and L forms, and also amino acid related carboxyl compounds (Welker and von Döhren 2006). These cyanobacterial peptides are classified into 6 classes, according to the structure and composition of particular amino acid residues (at least 50 unique structures do not fit into any of these categories). Among these, Puwainaphycins A - E, cyclic decapeptides containing the β-amino acid unit, have been isolated from the soil cyanobacterium *Anabaena* sp. (Gregson et al. 1992, Moore et al. 1989). Puwainaphycin C has been characterized as a compound with a strong positive ionotropic effect on isolated mouse atria. Except for this observation, nothing more is known about the function of puwainaphycins within the cyanobacterial cell, or about their interactions with other cell types or organisms.

In this present study, we have isolated two new compounds, Puwainaphycins F and G, and characterized their covalent structures by both NMR and MS techniques and

determined their absolute amino acid configuration by GC-MS analysis. Interactions of these compounds with the HeLa cancer cell line and with primary human skin fibroblasts was studied, and the specific, complex physiological and morphological alterations were characterized.

Experimental section

Culture Conditions and Isolation of Puwainaphycins G and F. The cyanobacterial strain *Cylindrospermum* sp. C24/1989 was isolated from forest soil in Manitoba, Canada. The cyanobacterium was grown in 200 ml tubes on liquid Alen-Arnold medium, and bubbled with CO_2-enriched air for 10 - 14 days prior to their mass cultivation. Mass cultivation was performed in 100 l glass cuvettes under the same conditions. The biomass was harvested by centrifugation in the cuvettes (3000 rpm, 60 min.), stored at -40˚C and then lyophilized. The lyophilized biomass (1 g) was drained into a mortar and extracted with 100 ml of 5% acetic acid; performed in three extraction steps (each 1 hr. apart). The extract obtained (300 ml) was concentrated on a C8 HLB Cartridge (Waters Oasis®) into 1 ml of pure methanol. The concentrate was injected into a prepared Watrex C8 column (250x10mm, 5 µm, R.15.86.S2510), and eluted by a MeOH/H2O gradient (fig. 1). The fraction containing both variants of the puwainaphycins was collected between 32.4' to 34.8'. This mixture was further separated on a normal phase column (Watrex, 250 x 8mm, Reprosil 100, Phenyl 5 µm) and eluted by tetrahydrofuran : methanol (95:5). Retention times for puwainaphycin F and G were 2.5' and 3.5', respectively.

Mass spectrometry: Two mg of Puwainaphycin F **(1)** was dissolved in 1ml of DMSO; afterwards one µl of stock solution was diluted in 1 ml of 0.1% formic acid and 50% MeOH. Mass spectrometry was performed using an APEX-Qe FTMS instrument equipped with a 9.4 T superconducting magnet and Combi ESI/MALDI ion source (Bruker Daltonics, Billerica MA, USA), using electrospray ionization. The flow rate was 1 µl/min and the temperature of the dry (nitrogen) gas was set at 200°C. The Q front-end consists of a quadrapole mass filter, followed by a hexapole collision cell. By appropriately switching the potentials on the exit lenses under the control of the data acquisition computer, the ions could either be accumulated in the hexapole of the Combi ESI source, or in the hexapole collision cell of the Q front end, prior to transfer to the FTMS analyzer cell. The mass spectra were obtained by accumulating ions in the collision hexapole and running the

quadrapole mass filter in the non mass-selective (Rf-only) mode; in order that ions of a broad *m/z* range (150 - 2000) were passed onto the FTMS analyzer cell. The species of interest were isolated in the gas phase by setting the Q mass filter to pass the *m/z* for ions of interest within a 3.0 *m/z* window. After a clean selection of the desired precursor ion had been confirmed, fragmentation was induced by dropping the potential of the collision cell (12V). All MS and MS/MS spectra were acquired in the positive ion mode, with the acquisition mass range 150 - 2000 m/z and 1M data points collected. This results in a maximal resolution of 200,000 at 400 m/z. The accumulation time was set at 0.5s (1.5 s for ms/ms). The cell was opened for 4500 μs, and 8 experiments were collected for one spectrum. The instrument was externally calibrated using triple and double charged ions of angiotensin I, as well as quintuple and quadruple charged ions of insulin. This results in a typical mass accuracy below 1ppm. After the analysis, the spectra were apodized using sin apodization, with one zero fill. The interpretations of the mass spectra were done using the DataAnalysis software package, version 3.4 (Bruker Daltonics, Billerica MA).

NMR experiments: NMR spectra were recorded on a Varian ^{UNITY}*Inova*-600 spectrometer (599.63 MHz for ^{1}H, 150.79 MHz for ^{13}C, and 60.78 MHz for ^{15}N, Varian Inc., Palo Alto, CA, USA) in DMSO-d_6 at 303 K. The residual solvent signal was used as an internal standard (δ_H 2.500 ppm, δ_C 39.60 ppm). ^{1}H NMR, ^{13}C NMR, COSY, TOCSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC, ^{1}H-^{13}C HSCQ-TOCSY, ^{1}H-^{15}N HSQC, and NOESY spectra were measured using the standard manufacturer's software. The ^{1}H NMR spectrum was zero filled to fourfold data points and multiplied by a window function (two-parameter double-exponential Lorentz-Gauss function) before Fourier transformation in order to improve the resolution. The ^{13}C NMR spectrum was zero filled to two-fold data points. Subsequently, the line broadening (1 Hz) was used to improve the signal-to-noise ratio. Protons were assigned by COSY and TOCSY, and the assignment was transferred to carbons by HSQC. The chemical shifts are given on the δ-scale [ppm], coupling constants are given in Hz. The digital resolution allowed us to present the proton and carbon chemical shifts to three or two decimal places, respectively. The carbon chemical shift readouts from HSQC (protonated carbons) and HMBC (quaternary carbons) are reported to one decimal place. The structure of Puwainaphycins F (**1**) and G (**2**) were further elucidated by both NMR spectroscopy and mass spectrometry.

Chiral amino acids analysis: Acid hydrolysis was by 6 M HCl at 110°C, and the derivatization with heptafluorobutyl chloroformate (Šimek et al. 2008) was performed in order to reveal the absolute amino acid configuration. The chirality of the released amino acids (as the corresponding N(O,S)-heptafluorobutoxycarbonyl-heptafluorobutyl derivatives) were determined by gas chromatography, with a flame ionization detector on a 25 m x 0.25 mm ID x 0.12 μm Chirasil-L-Val column (Varian Inc., Palo Alto, CA, USA) using a method described elsewhere (Zahradníčková et al. 2009). The chirality of proline was determined after the derivatization with phosgene on the same Chirasil-L-Val GC column, using the method by Konig et al. 1984.

Bioactivity assays: The cytotoxicity of the cyanobacterial extracts were tested by the addition of 10 μl crude extract dissolved in methanol (at a concentration of 200 mg lyophilized biomass/ml) to three mammalian cell lines (YAC-1, Sp/2, and HeLa). The cells were cultivated in 96-well plates, using RPMI medium with the addition of the cyanobacterial extract; with the viability measured by both the MTT test (Mosman 1983) and Cell Titer Glo (Promega). To determine the IC_{50} value, the HeLa cells were treated by different concentrations of Puwainaphycins G and F for 24 hr. in 96-well plates in RPMI medium; with the viability determined using the MTT test. Briefly, 10 μl of MTT solution, 4 mg/ml, was added to the cell cultures and incubated for 4 hrs. The supernatant was removed and formazan crystals were dissolved in DMSO. The test and reference absorbances were read at 590 and 640 nm. The viability index was expressed as the ratio between the absorbance values of the treated and control wells.

To demonstrate the effects of the pure compounds on the human cell's morphology and physiology (specifically changes in actin cytoskeleton, nucleus, tyrosine phosphorylation, and transferrin mediated endocytosis), immunofluorescence staining was performed. The cells were cultivated in D-MEM medium supplemented with 10% FCS (Gibco, Invitrogen, Carlsbad, CA, USA) grown on glass coverslips (up to 50% density) in 6-well plates (Nunc, Thermo Fisher Scientific, Waltham, MA, USA), treated with puwainaphycins dissolved in the methanol (stock solution 1 mg/ml) for various times and concentrations. Wells containing only methanol (max. 0.1%) were assayed as a blank control; no alterations of cellular morphology and physiology were observed. All samples were tested in triplicate. The treated cells (at various times and concentrations, 37°C, 5% CO_2) were fixed (3.7% paraformaldehyde in PBS, 20´, RT), permeabilized (0.1% Triton X-100 in PBS), blocked (1% BSA in PBS), and then stained with Phalloidin-Alexa567 or

Phalloidin-Alexa488, anti-pTyr-01 antibody (Exbio, Prague, Czech Republic). This was then followed by a secondary antibody, GAM-Alexa488. Transferrin mediated endocytosis was tested using fluorescently-labelled holotransferrin-Dyomics-564 (Exbio, Prague, Czech Republic) diluted to 5 mg/ml various times. Staining of the nuclei were performed by mounting specimens in Mowiol-DAPI. The cells were visualised and the images processed using a Cell^{R} microscope (Olympus, Tokyo, Japan). All fluorescent reagents (except holotransferrin) were purchased from Molecular Probes, Invitrogen (Carlsbad, CA, USA).

Western blotting: The level of tyrosine phosphorylation was measured in HeLa cells (ATCC, VA, USA). The cells were maintained in D-MEM medium, supplemented with 10% FCS at 37°C, under 5% CO_2 atmosphere, in 6-well plates (Nunc). Puwainaphycin F, dissolved in methanol, was added directly into the cultivation medium at a concentration of 10 μM for the appropriate times (1, 2, 5 and 10´, 37°C, 5% CO_2). The cells were lysed directly in the 2 x concentrated Laemly hot sample buffer (100 μl per well, boiled for 2´, sonnified, and loaded onto 4 - 12% gradient gels. The separated proteins were transferred to a nitrocellulose membrane. For the detection of proteins phosphorylated on the tyrosine residues, anti-pTyr-01 antibody (Exbio, Prague, Czech Republic) was used at a dilution of 1:200, in PBS with 2% low-fat milk. This was followed by the goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA), and then exposed to (Kodak) film.

Fluorescence Measurement of Cytosolic Ca^{2+} : HeLa cells grown on glass coverslips were washed in modified HBSS (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 3 mM $MgCl_2$, 10 mM Hepes-Na, 10 mM glucose, pH 7.4). After washing, the cells were loaded with 3M Fura-2 acetoxymethyl ester (Fura-2/AM) for 30 min at 25°C in the dark, rinsed, and allowed to rest in HBSS for 30 min prior to the fluorescence measurements. The ratiometric measurements were performed using a FluoroMax-3 spectrofluorometer, equipped with DataMax software (Jobin Yvon Horriba, France). The observed area of the coverslip was about 10 mm^2, corresponding to approximately 104 cells. Fluorescence intensity of Fura-2 (excitation wavelengths 340 and 380 nm; emission wavelength 510 nm) was recorded every 15 s, and the integration time for each wavelength was 3 s. The measured fluorescence intensity was not corrected for the background intensity (< 10%).

Results and discussion

Isolation and structure elucidation.

The crude extract of the soil cyanobacterium *Cylindrospermum* sp. C24/19 exhibited both strong and sudden inhibitions to murine cell lines YAC-1 (63±5.5%) and Sp/2 (57±3.2%), as well as to human HeLa cancer cells (51±6.4%). Activity-guided fractionation of this extract led to the identification of one cytotoxic fraction containing two molecular ions: 1146 [M+H$^+$] and 1160 [M+H$^+$]. Both compounds were isolated as white amorphous powders by a two-step preparative HPLC fractionation. Separation on a C18 reverse phase column, using a methanol/water gradient, led to one fraction containing both compounds. This fraction was used for the structural elucidation, and further separated using a phenyl column eluted with 95/5 (v/v) tetrahydrofuran/methanol solution (for the complete isolation procedure, see the experimental section).

High-resolution mass spectrometric analysis reveals pseudo-molecular ions [M+H]$^+$ 1146.6512 a.m.u. for Puwainaphycin F **(1)**, and 1160.6672 a.m.u. for Puwainaphycin G **(2)** in the sample; thus indicating two compounds, and fitting well with the NMR spectra containing two sets of signals in the 1:molar ratio. The exact masses match with the molecular formulas of the specific compounds $C_{53}H_{87}N_{13}O_{15}$ (difference: 1 ppm) for (**1**), and $C_{54}H_{89}N_{13}O_{15}$ (difference: 0.6 ppm) for (**2**).

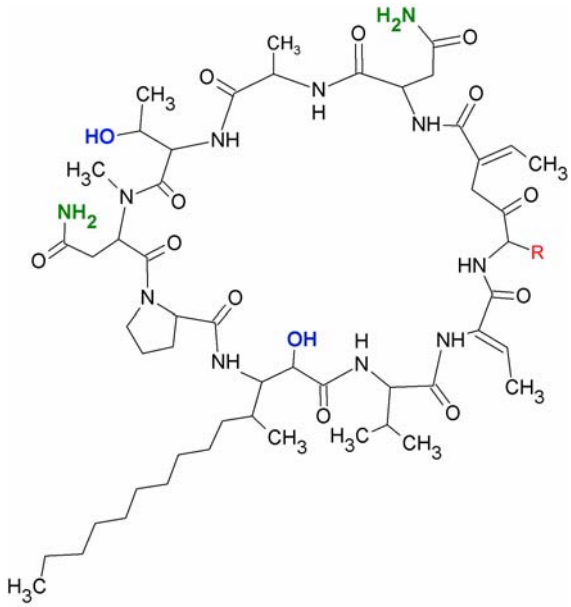


Figure 1: Structure of puwainaphycin F and G. R=CH_2CONH_2 for puwainaphysin F (**1**) and R=$(CH_2)_2CONH_2$ for puwainaphycin G (**2**)

Indirect ^{15}N NMR spectra, detected using ^{1}H-^{15}N gHSQC, identified eight NH and three NH_2 groups. Additionally, one N-Me (δ_H 2.953, δ_C 30.36) was determined. Distinct amino acids were identified by COSY, TOCSY, and ^{1}H-^{13}C HSQC-TOCSY. The amino acid composition of the prevailing peptide (**1**) was: Ala, 2 × Asn, 2 × dThr, MeAsn, Pro, Thr, and Val. Furthermore, one non-amino acid residue, 2-hydroxy-3-amino-tetradecanoic acid (X residue), was characterized. The amino acid sequence was determined by HMBC. It was used by coupling of N-H's to the carbonyls of the preceding amino acids or the X residue. The correlation of N-Me to the previous carbonyl and to its C-α was applied. It was detected following HMBC correlations: 2-NH → 1-CO, 3-NH → 2-CO, 4-NH → 3-CO, 5-NH → 4-CO, 6-NH → 5-CO, 7-NH → 6-CO, 8-NH → 7-CO, 9-Me → 8-CO, 9-Me → 9-Cα, 10-Hα → 9-CO, and 1-NH → 10-CO. The evaluated sequence was confirmed by NOESY. NOE contacts were detected between NH and H-α, or N-Me and H-α. As a result, the sequence obtained was: *cyclo*[X^1-Val^2-$dThr^3$-Asn^4-$dThr^5$-Asn^6-Ala^7-Thr^8-$MeAsn^9$-Pro^{10}]. NOE contacts between H-α to NH of the following residue approved a *trans*-arrangement of the following amidic bonds: 1-2, 2-3, 4-5, 6-7, 7-8, 8-9, 10-1. A NOE correlation between H-9α and H-10α indicates a *cis* peptidic bond between residues 9 and 10.

The MS/MS spectrum of Puwainphycin F (**1**) predominantly contained one series of b_n ions. It was identified as a nearly complete b_n^{10-2} series, specifically: b_1, b_2, b_3, b_4, b_5, b_6, b_7, and b_8. The fragmentation is consistent with the structure revealed by NMR. The monoisotopic mass of the b_8 ion (m/z 454.2038) agrees very well with the tripeptide ion of PXV. This behavior is consistent with that expected, deduced from the NMR-based structure. Puwainaphycin F (**1**) contains one N-methylated amino acid next to the proline residue. N-methylation of linear peptide enhances cleavage at the C-terminus of the methylated residue. B_n ions are preferentially formed. This can be explained by the structure of the oxazolone formed; it carries a methylated nitrogen. The mobile proton transfer of the oxazolone nitrogen is required to form *y*-ions. N-methylation of oxazolone nitrogen prevents this transfer. Therefore, the intensity of the complementary b-ions is more pronounced. Chiral analysis of the hydrolysate amino acid revealed D-Ala to be present in **(1)**; while all other amino acids with chiral atoms were present in their L-conformation (L-Val, L-Asn, L-Thr, L-MeAsp, L-Pro).

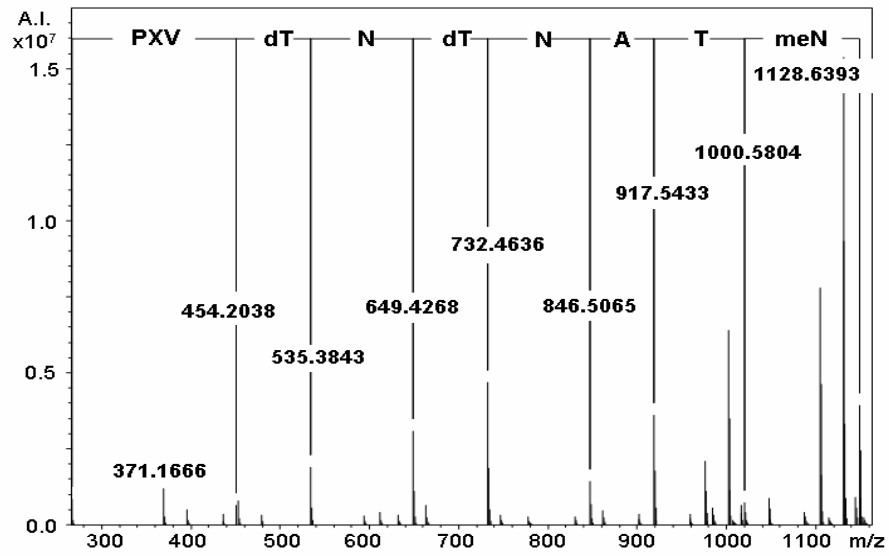


Figure 2: Fragmentation spectrum of Puwainaphycin F

The same approach was applied for the elucidation of the structure of Puwainaphycin G (**2**). A combination of COSY, TOCSY, and HSQC-TOCSY revealed the specific amino acids (*i.e.* Ala, Asn, 2 × dThr, Gln, MeAsn, Pro, Thr, and Val). NMR only allowed us to partially elucidate the structure of the X residue as: $O{=}CCH(OH)CH(NH)CH(CH_3)CH_2(CH_2)_nCH_3$. The length of its aliphatic chain was proofed by MS, and the X residue was resolved as 2-hydroxy-3-amino-tetradecanoic acid. It is the same as is found in the structure of puwainaphycin G. The crucial HMBC correlations for the amino acid sequence are the following: 2-NH → 1-CO, 3-NH → 2-CO, 4-NH → 3-CO, 5-NH → 4-CO, 6-NH → 5-CO, 7-NH → 6-CO, 8-NH → 7-CO, 9-Me → 8-CO, 9-Me → 9-Cα, and 1-NH → 10-CO. The HMBC crosspeak between 10-Hα and 9-CO is missing, with 10-N and 9-CO the only free vacancies in the structure; therefore a cyclic structure can be deduced. Accordingly, the sequence obtained was: *cyclo*[X^1-Val^2-$dThr^3$-Gln^4-$dThr^5$-Asn^6-Ala^7-Thr^8-$MeAsn^9$-Pro^{10}]. The structure was further supported by the detected NOE contacts. The MS/MS spectrum of (**2**) also predominantly contains b_n ions, which belong to the complete b_n^{10-9} series, and also supports the proposed structure of puwainaphycin G (**2**).

The arrangement of the amidic bonds were proven by NOE contacts. It was possible to identify a *trans* peptidic bond between residues: 1-2, 2-3, 4-5, 6-7, 7-8, 8-9, and 10-1. Unfortunately, it was not possible to establish the arrangement on the amidic bond

between residues 9-10. In all of the amino acids, L- form chiral atoms (L-Val, L-Glu, L-Asn, L-Thr, L-MeAsp, L-Pro) have been detected by GC-MS analysis for **(2)**.

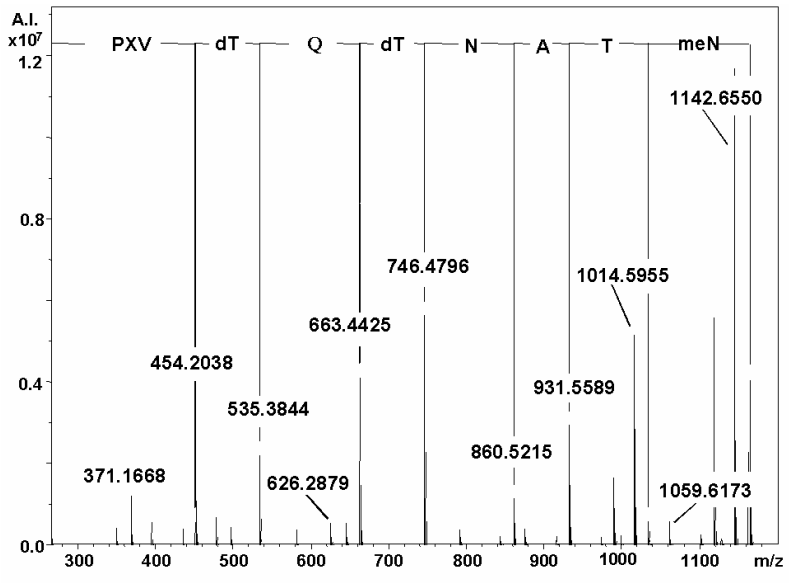


Figure 3: Fragmentation spectrum of Puwainaphycin G

Function of Puwainaphycins F and G in the cell.

Puwainaphycin F (**1**) in a concentration of 10 μM triggers a unique transformation of the actin cytoskeleton in cells, which is followed by a rapid increase in the concentration of intracellular calcium $[Ca^{2+}]_i$ levels and tyrosine phosphorylation. The influx of calcium ions into the cytosol starts within seconds after the addition of puwainaphycin F, and its maximum can be observed after 12 minutes incubation with the compound (Figure V.). The rapid $[Ca^{2+}]_i$ elevation was striking, even when compared with the established calcium ionophore ionomycine (used at a high concentration). Although exhibiting a slightly slower $[Ca^{2+}]_i$ mobilization, the uniqueness of the Puwainaphycin F effect (when compared to that of ionomycine) is its reversibility.

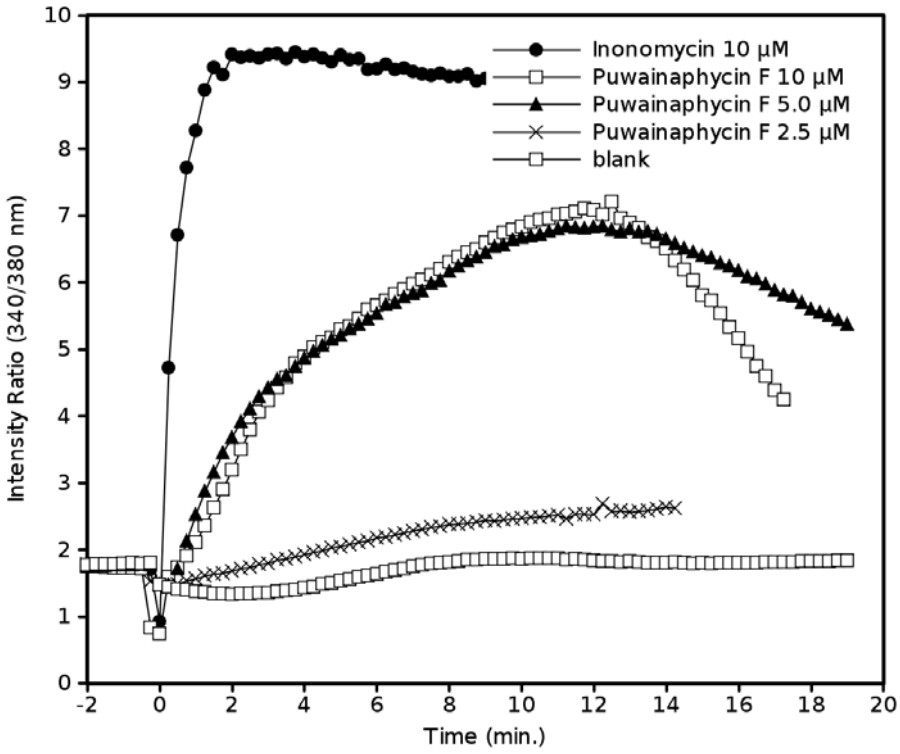
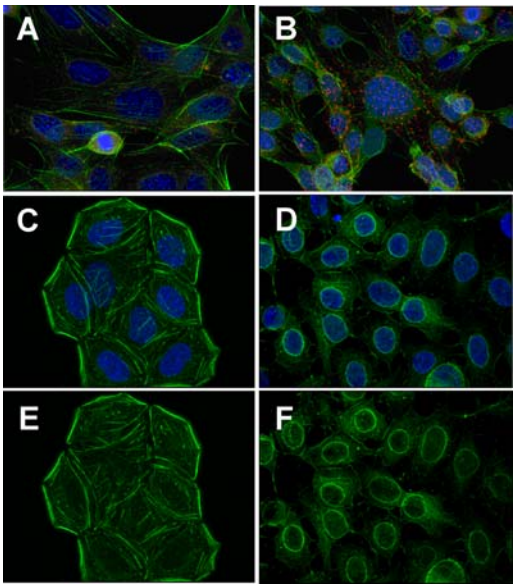


Figure 4: Influx of Ca^{2+} ions into HeLa cells treated with different doses of Puwainaphycin F or conventional ionophore ionomycin in concentration of 10 µM. For measurement of intracellular free Ca^{2+} concentration cells were loaded with 3 µM Fura-2/AM. Time course of calcium entry into cells was determined as ratio of Fura-2 fluorescence excited at 340/380 nm.

We can conclude that Puwainaphycin F can potentially mimic physiological Ca^{2+} signaling events. Changes in tyrosine phosporylation are delayed in 5 - 10 minutes, compared to the calcium influx. A change in the actin cytoskeleton starts 15 minutes after exposure; however, they are most dramatic after 30 - 60 minutes. Tyrosine phosphorylation was first visualized by immunofluorescence of the treated cells, using an anti-phosphotyrosine antibody. A striking localized dotted plasma-membrane pattern of the phosphotyrosine-positive foci was observed in the HeLa cells (not shown) and in primary human fibroblasts (Fig. VII/B). The data were confirmed using immunoblotting, using the same antibody, with an obvious change in the pattern and intensity of the phosphorylated proteins (Fig VI); with the maximum intensity peak after 10 min (corresponding to the peak levels of $[Ca^{2+}]_i$). After that, the signal rapidly declined to almost control levels.



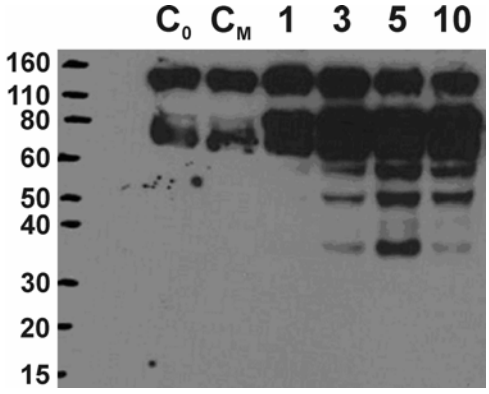


Figure 5: Stimulation of tyrosine phosphorylation in cells treated with Puwainaphycin F in concentration of 10 μM (**C_0** blanc control, **C_M** – cells treated with methanol only, timepoints of 1´ , 3´, 5´ and 10´).

Figure 6: Imunoflorescence staining of nuclei, tytosine phosporilation and f-actin in human skin fibroblasts and HeLa cells. **A** – control human fibroblasts, **B** – human fibroblasts treated with Puwainaphycin F (10μM, 10 min.), f-actin (green, Phalliodin-Alexa488), P-Tyr (red, anti P-Tyr-01), nuclei (blue, DAPI). **C-F** Detection of f-actin in HeLa cells, **C** and **E** control cells, **D** and **F** - cells treated with Puwainaphycin F (10M, 60 min), **C** and **D** – overlay of f-actin (green, phalloidin-Alexa488) and nuclei (blue, DAPI), **E** and **F** – f-actin only.

The subsequent transformation of the actin cytoskeleton is unique and extremely specific. Further, it follows the Ca^{2+} and p-Tyr mediated signaling events. Obviously, the cortical actin continually translocates to the unusual location, *i.e.* to the nuclear envelope (Fig VII/F). This pattern of f-actin localization is unprecedented in animal cells. There are a limited number of actin binding proteins located to the nuclear membrane that are potentially responsible for the unique actin translocation F-actin localization to the nuclear envelope. This is quite normal in plant cells; however, in other cell types it is unusual. Localization of the f-actin resembles the localization of the intermediate filaments; one possible explanation of the actin translocalization is the activation of the actin-IMF crosslinking proteins. One other possible explanation could be the activation (or translocation) of the actin binders at/to the nuclear envelope.

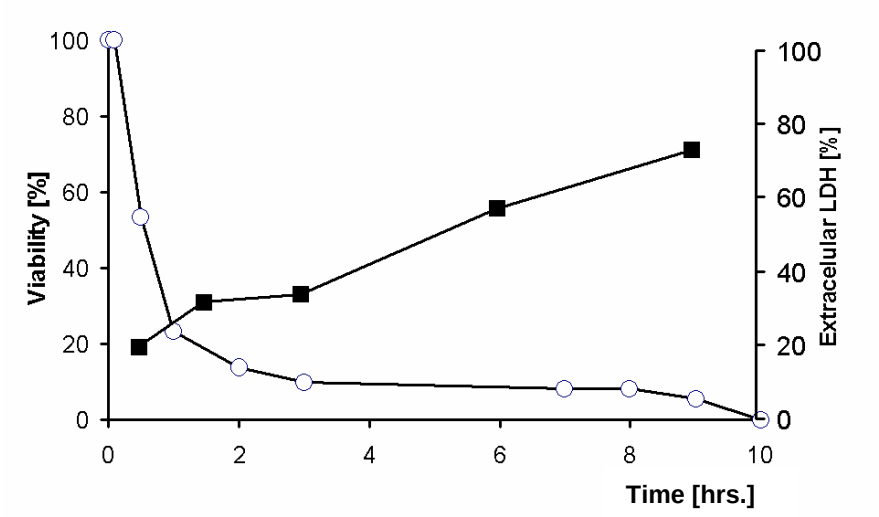


Figure 7: Lactate dehydrogenase (LDH) leakage (full squares) and viabilty of HeLa cells (empty circles) treated by 10 µM puwainaphycin F.

After 60 min the cells start to detach, the nuclei condense, and their overall viability declines. Incubation with both Puwainaphycins leads to a rapid viability decrease within two hours (concentration at 10 µM). With longer exposures, the viability continuously declines; and ends in necrotic cell death of all cells after 10 hrs. The loss of viability at later time points could be explained by the sustained entry of a new toxin into the plasma membrane / cell interior; continuously interfering with the cellular physiology, and leading to the loss of plasma membrane integrity, measured by the entry of fluorescent probes and the release of lactate dehydrogenase (Fig VII). Interestingly, a small subpopulation (less than 5% of cells) was quite resistant to the treatment with Puwainaphycin F; even at the later time points. One possible explanation is a cell cycle dependent resistance, a hypothesis which we plan to test in future experiments.

References

Gregson J.M., Chen J.L., Patterson G.M.L. and Moore R.E. 1992. Structures of puwainaphycines. *Tetrahedron* 48:3727-3734.

Hrouzek P., Tomek P., Lukešová A., Urban J., Voloshko L., Pushparaj B., Lukavský J., Štys D. and Kopecký J., 2010. Cytotoxicity and secondary metabolites production in terrestrial *Nostoc* strains originating from different climatic / geographic regions and habitats: Is their cytotoxicity environmentaly dependent? *Environ. Tox.* (DOI: 10.1002/tox.20561).

König W.A., Steinbach E. and Ernst K. Phosgene 1984. A versatile reagent for enantiomer separation by capillary gas chromatography. *J. Chromatogr. A* 301:129-135.

MacKintosch C., Beattie K.A., Klumpp S., Cohen P. and Codd G.A. 1990. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett.* 264:187-192.

Moore R.E., Bornemann V., Niemczura W.P., Gregson J.M., Chen J.L., Norton T.R., Patterson M.L. and Helms G. 1989. Puwainaphycin C, a cardioactive cyclic peptide from the blue-green alga Anabaena BQ-16-1. Use of two-dimensional 13C-13C and 13C-15N correlation spectroscopy in sequencing of amino acid units. *J. Am. Chem. Soc.* 111:6128-6132.

Ohta T., Sueoka E., Iida N., Komori A., Sugamuna M., Nishiwaki R., Tatematus M., Kim S.J., Carmichael W.W. & Fujiki H. 1994. Nodularin, a potent inhibitor of protein phosphatases 1 and 2A is a new environmental carcinogen in male F344 rat liver. *Cancer res.* 54:6402-6406.

Šimek P., Hušek P. and Zahradničková H 2008. Gas chromatographic-mass spectrometric analysis of biomarkers related to folate and cobalamin status in human serum after dimercaptopropanesulfonate reduction and heptafluorobutyl chloroformate derivatization. *Anal. Chem.* 80: 5776-582.

Teicher B.A., Forler P., Menon K., Phares V., Amsrud T. and Shih C. 2000. Cryptophycin 52 and cryptophycin 55 in sequential and simultaneous combination treatment regimens in human tumor xenografts. *In Vivo* 14:471-480.

Trimurtulu G., Ohtani I., Patterson M.L., Moore R.E., Corbett T.H., Valeriote F.A and Demchik L. 1994. Total structures of cryptophycins, potent antitumor depsipeptides from the blue-green alga *Nostoc* sp. strain GSV 224. *J. Am. Chem. Soc.* **116:** 4729-4737.

Welker M., von Dohren H., 2006. Cyanobacterial peptides – Nature's own combinatorial biosynthesis. *Fems Microbiol. Rev.* 30:530–563.

Welker M., Maršálek B., Šejnohová L. and von Döhren H. 2006. Detection and identification of oligopeptides in *Microcystis* (cyanobacteria) colonies: Toward an understanding of metabolic diversity. *Peptides* 27:2090–2103.

Yoshizawa S., Matsushima R., Watanabe M.F., Harada K.I., Ichihara A., Carmichael W.W. and Fujiki H. 1990. Inhibition of protein phosphatases by microcystin and nodularin associated with hepatotoxicity. *J. Cancer Res. Clin. Oncol.* **116:** 609-614.

Zahradníčková H., Hušek P. and Šimek P. 2009. GC separation of amino acid enantiomers via derivatization with heptafluorobutyl chloroformate and Chirasil-L-Val column. *J. Sep. Sci.* 32:3919–3924.

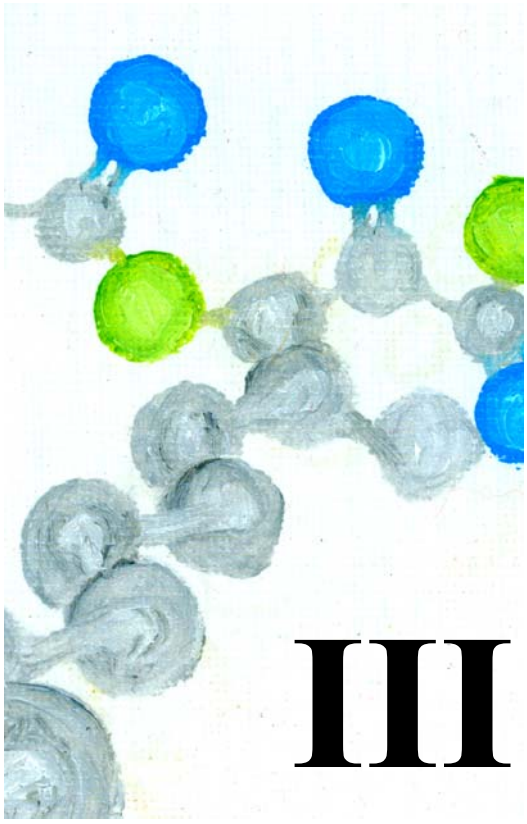
Apendices

Table I. NMR data for Puwainaphycin F

Amino acid residue	Atom #	δ_C	m	δ_H
X[1]	**CO**	169.62	S	-
	α	69.70	D	4.18* (1H, m)
	β	56.18	D	3.93* (1H, m)
	γ	32.20	D	1.66* (1H, m)
	δ	33.43	T	1.62* (1H, m)
				1.17* (1H, m)
		29.71	T	1.286 (2H, m)
		29.27	T	1.25* (2H, m)
		29.14	T	1.150 (2H, m)
		29.10	T	1.24* (2H, m)
		28.77	T	1.26* (2H, m)
		25.45	T	1.18* (2H, m)
		31.35	T	1.23* (2H, m)
		22.15	T	1.26* (2H, m)
		14.01	Q	0.856 (3H, m)
	α-OH	-	-	5.467 (1H, d, J = 4.3 Hz)
	β-NH	-	-	6.830 (1H, d, J = 10.3 Hz)
	γ-CH_3	16.02	q	0.577 (3H, d, J = 6.7 Hz)
Val[2]	**CO**	168.85	S	-
	NH	-	-	6.858 (1H, d, J = 9.5 Hz)
	α	55.82	D	4.29* (1H, m)
	β	32.63	D	1.75* (1H, m)
	γ_1	18.97	Q	0.891 (3H, d, J = 6.8 Hz)
	γ_2	18.64	Q	0.823 (3H, d, J = 6.8 Hz)
dThr[3]	**CO**	163.22	S	-
	NH	-	-	9.152 (1H, s)
	α	132.59	S	-
	β	116.07	D	5.296 (1H, q, J = 7.5 Hz)
	γ	13.02	Q	1.650 (3H, d, J = 7.5 Hz)
Asn[4]	**CO**	171.96	S	-
	NH	-	-	8.708 (1H, d, J = 9.1 Hz)
	α	49.03	D	4.920 (1H, ddd, J = 6.7, 7.7, 9.1 Hz)
	β	38.78	T	2.756 (1H, dd, J = 7.7, 15.0 Hz)
				2.674 (1H, dd, J = 6.7, 15.0 Hz)
	CO	172.19	S	-
	NH_2	-	-	7.544 (1H, s)
				7.071 (1H, s)
dThr[5]	**CO**	162.93	S	-
	NH	-	-	10.147 (1H, s)
	α	129.69	S	-
	β	125.26	D	5.706 (1H, q, J = 7.5 Hz)
	γ	13.27	Q	1.893 (3H, q, J = 7.5 Hz)
Asn[6]	**CO**	170.74	S	-
	NH	-	-	8.382 (1H, d, J = 7.1 Hz)
	α	50.11	D	4.398 (1H, ddd, J = 3.2, 7.1, 7.2 Hz)
	β	35.80	T	2.867 (1H, dd, J = 7.2, 16.5 Hz)
				2.525 (1H, dd, J = 3.2, 16.5 Hz)
	CO	172.78	S	-
	NH_2	-	-	7.544 (1H, s)
				7.013 (1H, s)
Ala[7]	**CO**	172.13	S	-
	NH	-	-	8.250 (1H, d, J = 8.5 Hz)
	α	48.56	D	4.28* (1H, m)
	β	16.93	Q	1.23 (3H, m)
Thr[8]	**CO**	169.98	S	-
	NH	-	-	7.270 (1H, d, J = 8.4 Hz)
	α	54.82	D	4.628 (1H, dd, J = 3.6, 8.4 Hz)
	β	66.43	D	3.90* (1H, m)
	γ	19.62	Q	0.996 (3H, d, J = 6.4 Hz)
	β-OH	-	-	5.066 (1H, d, J = 7.7 Hz)
MeAsn[9]	**CO**	167.84	S	-
	α	49.12	D	5.529 (1H, dd, J = 2.7, 11.7 Hz)
	β	33.97	T	3.012 (1H, dd, J = 11.7, 16.1 Hz)
				1.997 (1H, dd, J = 2.7, 16.1 Hz)
	CO	171.68	S	-
	NH_2	-	-	7.491 (1H, s)
				5.931 (1H, s)
	N-CH_3	30.36	Q	2.953 (3H, s)
Pro[10]	**CO**	171.49	S	-
	α	60.14	D	4.19* (1H, m)
	β	30.32	T	1.92* (2H, m)
	γ	23.82	T	1.91* (1H, m)
				1.83* (1H, m)
	δ	46.85	T	4.090 (1H, m)
				3.252 (1H, m)

Table II. NMR data for Puwainaphycin G

Amino acid residue	Atom #	δ_C	m	δ_H
X[1]	**CO**	169.53	S	-
	α	69.77	D	4.15* (1H, m)
	β	56.06	D	4.32* (1H, m)
	γ	32.13	D	1.67* (1H, m)
	δ	33.43	T	1.62* (1H, m)
				1.18* (1H, m)
		29.74	t	1.286 (2H, m)
		29.27	t	1.25* (2H, m)
		29.08	t	1.150 (2H, m)
		29.06	t	1.25* (2H, m)
		28.74	t	1.26* (2H, m)
		25.45	t	1.25* (2H, m)
	CH_2	31.35	T	1.23* (2H, m)
	CH_2	22.15	T	1.26* (2H, m)
	CH_3	14.01	Q	0.859 (3H, m)
	α-OH	-	-	5.558 (1H, d, J = 3.3 Hz)
	β-NH	-	-	6.77* (1H, d, J = 9.8 Hz)
	γ-CH_3	16.02	q	0.570 (3H, d, J = 6.7 Hz)
Val[2]	**CO**	169.42	S	-
	NH	-	-	6.768 (1H, d, J = 9.8 Hz)
	α	55.73	D	4.330 (1H, dd, J = 7.4, 9.8 Hz)
	β	32.68	D	1.72* (1H, m)
	γ1	18.80	Q	0.888 (3H, d, J = 6.7 Hz)
	γ2	18.70	Q	0.823 (3H, d, J = 6.7 Hz)
dThr[3]	**CO**	163.49	S	-
	NH	-	-	9.321 (1H, s)
	α	132.39	S	-
	β	117.4*	D	5.360 (1H, q, J = 7.5 Hz)
	γ	13.19	Q	1.683 (3H, d, J = 7.5 Hz)
Gln[4]	**CO**	173.46	S	-
	NH	-	-	8.562 (1H, d, J = 9.5 Hz)
	α	50.86	D	4.680 (1H, ddd, J = 4.9, 9.5, 10.6 Hz)
	β	29.06	T	2.168 (2H, m)
	γ	32.01	T	2.38* (1H, m)
				1.98* (1H, m)
	CO	174.12	S	-
	NH_2	-	-	6.86* (1H, s)
				6.719 (1H, s)
dThr[5]	**CO**	163.11	S	-
	NH	-	-	10.244 (1H, s)
	α	129.80	S	-
	β	124.5*	D	5.717 (1H, q, J = 7.5 Hz)
	γ	13.21	Q	1.866 (3H, d, J = 7.5 Hz)
Asn[6]	**CO**	170.51	S	-
	NH	-	-	7.479 (1H, s)
	α	49.43	D	4.40* (1H, m)
	β	35.23	T	3.01* (1H, m)
				2.579 (1H, dd, J = 3.4, 7.0 Hz)
	CO	173.87	S	-
	NH_2	-	-	7.882 (1H, s)
				7.178 (1H, s)
Ala[7]	**CO**	172.08	S	-
	NH	-	-	8.107 (1H, d, J = 8.5 Hz)
	α	48.44	D	4.27* (1H, m)
	β	16.02	Q	1.24* (3H, m)
Thr[8]	**CO**	170.32	S	-
	NH	-	-	7.137 (1H, d, J = 8.1 Hz)
	α	55.25	D	4.544 (1H, dd, J = 3.9, 7.9 Hz)
	β	66.50	D	3.85* (1H, m)
	γ	19.45	Q	1.012 (3H, d, J = 6.5 Hz)
	β-OH	-	-	5.019 (1H, d, J = 8.6 Hz)
MeAsn[9]	**CO**	167.84	S	-
	α	49.16	D	5.55* (1H, m)
	β	33.76	T	2.99* (1H, m)
				1.98* (1H, m)
	CO	171.72	S	-
	NH_2	-	-	7.479 (1H, s)
				5.910 (1H, s)
	N-CH_3	30.39	Q	2.942 (3H, s)
Pro[10]	**CO**	171.31	S	-
	α	59.99	D	4.222 (1H, m)
	β	30.32	T	1.92* (2H, m)
	γ	23.46	T	1.86* (1H, m)
				1.72* (1H, m)
	δ	46.81	T	4.16* (1H, m)
				3.20* (1H, m)



III

Hisem D., **Hrouzek P.,** Tomek P., Tomšíčková J., Zapomělová E., Skácelová K., Lukešová A. & Kopecký J.:

Cyanobacterial cytotoxicity to mammal cell lines versus toxicity to brine shrimp.

Toxicon (*submitted*)

Cyanobacterial cytotoxicity versus toxicity to brine shrimp *Artemia salina*

Daniel Hisem[1, 2], Pavel Hrouzek[1, 3, 5*], Petr Tomek[1, 4], Jana Tomšíčková[1, 2], Eliška Zapomělová[6], Kateřina Skácelová[1, 2], Alena Lukešová[7], Jiří Kopecký[1, 5]

[1]*Institute of Microbiology, CAS, Department of Autotrophic Microorganisms, Opatovický mlýn, CZ-379 81 Třeboň, Czech Republic*

[2]*Department of Plant Physiology, University of South Bohemia, Faculty of Science, Branišovská 31, CZ-37005 České Budějovice, Czech Republic*

[3]*Department of Botany, University of South Bohemia, Faculty of Science, Branišovská 31, CZ-37005 České Budějovice, Czech Republic*

[4]*Department of Molecular Biology and Biochemistry, University of South Bohemia, Faculty of Science, Branišovská 31, CZ-37005 České Budějovice, Czech Republic*

[5]*Institute of Physical Biology, Zámek 136, CZ-37333, Nové Hrady, Czech Republic*

[6]*Biology Centre of AS CR, Institute of Hydrobiology, Na Sádkách 7, CZ-37005, České Budějovice, Czech Republic*

[7]*Biology Centre of AS CR, Institute of Soil Biology, Na Sádkách 7, CZ-37005, České Budějovice, Czech Republic*

Heterocytous cyanobacteria originating from different habitats have been screened for toxicity to brine shrimp *Artemia salina* and the murine lymphoblastic cell line Sp/2. Methanolic extracts of biomass and cultivation media were tested for toxicity and selected extracts were fractionated to determine the active fraction. We found a significant toxic effect to *Artemia salina* and Sp/2 cells in 5.2% and 31% of studied extracts, respectively. Only 8.6% of all tested strains were highly toxic to both *A. salina* and the Sp/2 cell line. Based on these data, we conclude that it is impossible to monitor cytotoxicity using only the brine shrimp bioassay, since cytotoxicity is a more frequent feature in comparison with toxicity to *A. salina*. It seems that in most cases the toxic effect of cyanobacterial secondary metabolites is targeted at some basal metabolic pathways present in eucaryotic cells rather than being a specific mechanism against a complex organism. Only in two of all tested strains was toxicity to *Artemia salina* recorded not accompanied to murine cell line toxicity. Moreover, in most of the selected strains exhibiting activity to *A. salina* and Sp/2 cells, the toxic effect to *Artemia salina* and Sp/2 cell line was caused by an identical fraction. These findings lead us to the conclusion that cyanobacterial metabolites can secondarily act as a defensive mechanism against grazing, although they are almost certainly not synthesized specifically against herbivores.

Keywords: secondary metabolites, brine shrimp bioassay, cytotoxicity, grazing, defense

1. Introduction

The toxicity of cyanobacterial secondary metabolites and their ecological implications has been widely studied over the last few years (e.g. Blom et al., 2006; Ferrao-Filho et al., 2000; Gademann and Portman, 2008; Lürling and Beekman, 2006; Sarnelle and Wilson, 2005; Wilson et al., 2006). Bioactive secondary metabolites were regarded as an evolutionary response to the pressure of competing organisms, such as fungi or grazers, when the genus *Nostoc* was studied (Dodds et al., 1995; Piccardi et al., 2000). This suggestion was then displaced, at least for cyanobacterial peptides, since their synthetic machinery was found to be much older than the evolutionary history of eukaryotic lineage (Rantala et al., 2004). However, the inhibitory or even lethal effects of cyanobacterial metabolites on invertebrates have been shown in numerous studies. For instance, the cyanobacterial peptides microviridin J and microcystin were found to be toxic to eukaryotic organisms such as *Daphnia pulicaria* and *D. galeata* (Rohrlack et al., 1999, 2004). Further, for some non-peptide compounds like cryptophycin, tolytoxin, calothrixins and pahayakolide, strong effects on invertebrates can be found (Berry et al., 2004; Biondi et al., 2004; Rohrlack et al., 1999, 2004, 2005; Agrawal et al., 2005). A direct link betweeen production of di-(hydroxymethyl)dihydroxypirolidine (DMDP) and grazer pressure has been recorded in a peryphytic *Cylindrospermum* sp. strain (Jüttner and Wessel, 2003). Thus a secondary function of cyanobacterial secondary metabolites as a defense mechanism against parasites and grazers is possible. However, there still remains the question as to whether the specific activity of cyanobacterial metabolites against invertebrate grazers has evolved by a long co-evolution of cyanobacteria and grazers, or whether these metabolites interact with the basal metabolism common to most organisms and thus their toxic effect is general.

While there has been much attention paid to the toxicity or digestive enzymes inhibition caused by planktonic cyanobacterial species (Agrawal et al., 2005; Blom et al., 2006; Jüttner and Wessel, 2003; Murakami et al., 1994, 1995; Rohrlack et al., 2004, 2005), few studies have dealt with cyanobacteria from soil and other habitats. Rohrlack et al. (2005) revealed that about 70 % out of 89 planktonic strains of the genus *Planktothrix* produce inhibitors of daphnid trypsin. A comparison of the toxicity of cyanobacteria from different habitats to the brine shrimp *Artemia salina* was made by Piccardi et al. (2000), who studied fifty cyanobacterial strains of the genus *Nostoc* originating from symbioses, the soil environment, and fresh and marine waters. There was a high number of symbiotic and soil strains toxic to *A. salina* compared to fresh or sea-water isolates. According to Falch et al.

(1995), 15 out of the 20 investigated cyanobacterial strains were toxic to *A. salina*. Most toxic were soil, subaerophytic and planktonic strains. A high number within the subaerophytic strains having toxicity is also supported by Jaki et al. (1999). Nevertheless, the toxicity of cyanobacterial strains from other different habitats can generally only be imagined since only a few strains have been studied so far.

For the screening of cyanobacterial toxicity to crustaceans, model organisms such as *Artemia salina* or *Daphnia* spp. are usually used. An *A. salina* assay has also been suggested as a valid method to evaluate the cytotoxic activity of plant extracts (Solis et al., 1993) and as a rapid preliminary screening for toxic cyanobacteria (Lahti et al., 1995). Some published data have suggested a good correlation between the activity in the brine shrimp assay and the cytotoxicity against some tumor cell lines (Anderson et al., 1991), as well as hepatotoxic activity (Kiviranta et al., 1991). The assay is therefore usually used as a low-cost and easily-achievable cytotoxicity test replacing cell lines assays (Piccardi et al., 2000). However, there are several studies from the last few years that present contrary results (Berry et al., 2004; Jaki et al., 1999; Mian et al., 2003).

In the present study, we compare the toxicity of 63 crude extracts of cyanobacteria originating from different habitats to *Artemia salina* and the murine cell line Sp/2 in order to answer the question whether a specific toxicity exists against crustacean grazers or whether the toxic effect is more general. Secondly, we wanted to compare *A. salina* mortality with cell line inhibition values to test the *Artemia salina* test as a substitution for the cytotoxicity assay.

2. Materials and methods

2.1 Cyanobacterial strains, cultivation and extract preparation

A total number of 63 different cyanobacteria were involved in this study: 57 cultured strains of various morphospecies and 6 field samples of Nostoc commune. The strains originated from various different habitats: soil (18 strains), plankton (30 strains), symbiotic associations (7 strains), periphytic (4 strains) and epiphytic strains (4 strains). Soil, symbiotic, peryphitic and epiphytic strains were cultivated in Allen and Arnold medium (Arnon et al., 1974) in 300 mL cylindrical flasks, bubbled with CO_2 enriched air (2%) and illuminated with artificial light of PFD (photon flux density) of 280 $\mu mol.m^{-2}.s^{-1}$ for 2 to 4 weeks and harvested by centrifugation (4500 rpm, 15 min). Planktonic species of the genus Anabaena were cultivated in WC medium (Guillard and Lorenzen, 1972) in 250 mL

Erlenmeyer's flasks, illuminated with artificial light of intensity of PFD of 50 μmol m^{-2} s^{-1} for 3 to 4 weeks. Biomass was harvested by centrifugation in 50 ml glass cuvettes (4500 rpm, 15 min), stored at -80°C and lyophilized. Cultivation medium was separated from biomass after centrifugation, 50 mL of it were filtrated using bacteriological filters (porosity 0.2 μm) and a water-pump to obtain a cell-free medium. 200 mg of lyophilized biomass was transferred into 10 mL glass test tubes and extracted for 2 h using 70% methanol. The test tubes were centrifuged (4500 rpm, 15 min); the supernatant then transferred into an evaporating vessel and dried via a rotation vacuum evaporator. The solid extract was re-suspended in 1 mL of 70% MeOH to get the extract of concentration 200 mg of dry weight per millilitre. To obtain an extract from the medium, 50 mL of filtrated medium was concentrated into 2 mL 100% methanol using solid phase extraction (MCX Cartridge OASIS, Waters) and a vacuum-pump.

2.2 Artemia salina bioassay and cytotoxicity assay

The brine shrimp assay was done according to Lincoln et al. (1996). Cysts were incubated in artificial seawater illuminated by artificial light and gently aerated for 24 h (Metcalf et al., 2002). For toxicity tests, hatched nauplii were diluted to a concentration of 15-20 individuals mL^{-1}. 100 μL of extracts were transferred into a 12-well microtitrate plate and were kept in a laminar box for methanol evaporation. 50 μL of distilled water was added and ultrasonified to improve dissolving of dry extract. 0.95 mL of nauplii (15-20 individuals) were added and numbers of living and dead individuals and unhatched cysts were counted using a stereomicroscope. Individuals were re-counted after 24 and 48 hrs and the percentage mortality calculated. Strains causing a mortality higher than 50% were considered as highly toxic.

Murine lymphoblastic cell line Sp/2 (kindly provided by Dr. Jan Kopecký, Biology Centre of the Academy of Sciences of the Czech Republic, Institute of Parasitology) was used for cytotoxicity testing. The cells were cultivated in RPMI 1640 medium with the addition of 5% fetal calf serum, 1% glutamine, and 1% antibiotic-antimycotic solution; all in plastic tissue culture flasks at 37 °C. Prior to the experiments, cells were dyed with Trypan blue, in order to estimate viability, and counted in a Bürkers plate chamber in a light microscope. Only cell cultures with a higher viability than 90% were used for the experiment. The cell suspension was centrifuged (1000 rpm, 10 min, 4 °C), and an adequate

amount of fresh RPMI medium was added in order to obtain a concentration of 1.5 x 10^5 cells per well (200 μL RPMI).

Cyanobacterial extract of 10 μL was added to the wells in triplicates, and triplicates treated only by 70% methanol were left as controls. The plate was kept in an incubator (37 °C) until the 70% methanol was evaporated; then the cell suspension was added and incubated (37 °C and 3.5% CO_2) for 12 h. Cell viability after exposure was estimated by MTT assay (Mosmann, 1983); 10 μL of MTT solution (4 mg.mL^{-1}) was added and the plates were incubated for 4 h. The plates were centrifuged after the incubation (3000 rpm, 10 min) and the supernatant was removed. DMSO of 200 μL was added to dissolve formazan crystals. Test and background absorbances were measured at 590 and 640 nm, respectively. The survival of cell lines was evaluated as the ratio of treated wells' absorbance to that of the control wells, and expressed as a percentage.

2.3 Extract analysis

Extract composition was analyzed using an HP 1100 Agilent mass spectrometer with an HP 100 MSD SL-Ion trap. The extract was subjected to separation on a reversed phase column (Zorbax XBD C8, 4.6 x 150 mm, 5 μm) at 30 °C, and eluted by a gradient MeOH/H_2O + 1% HCOOH (30-100% MeOH for 30 min, 100% for 5 min) with a flow rate of 0.6 mL.min^{-1}. The obtained total ion chromatograms were evaluated and molecular ions were detected based on signal intensity, the presence of sodium and potassium adducts, and the distribution of isotopomeres.

2.4 Activity guided fractionation

Six strains were selected for activity-guided fractionation in order to find out which fraction was responsible for the toxic effect. The strains *Cylindrospermum* sp. C24/1989, *Nostoc* sp. 6/99, *Nostoc muscorum* 14/86 and *Nostoc* sp. 5/96 were fractionated using an analytical column, gradient and conditions as discussed above (see also supplementary information). In strains *Nostoc ellipsosporum* 51/91 and *Nostoc* sp. Ds1 the fractionation was performed using preparative HPLC (LabAlliance, Watrex, Prague) on a reverse phase column (C18 Reprosil100, 250x8mm, 5μm, Dr. Maisch GmbH) and a modified gradient (see supplementary information). In these extracts the fractions were collected based on UV absorption in 237 and 220 nm for *Nostoc ellipsosporum* 51/91 and *Nostoc* sp. Ds1,

respectively. Finally, the collected fractions were evaporated and resuspended to obtain the concentration equivalent to the original extract and tested for toxicity to *A. salina* and Sp/2 as mentioned above.

3. Results

A total number of 63 cyanobacterial strains were included in the present study. The investigated cyanobacteria originated from four different habitats (plankton, soil, periphyton, and epiphytic communities), and besides these, strains originating from different symbiotic associations were also studied. For exact information about the origin and each strains' isolation see Table 1.

3.1 Toxicity of biomass extracts to Artemia salina

An overall toxicity causing mortality of *A. salina* $\geq$ 50% was observed in 12.7% of biomass extracts. The highest occurrence of toxicity was found among strains originating from soil (22%). The most active soil isolates were *Nostoc* sp. 5/96 and *N. ellipsosporum* 51/91 that caused 100% mortality, followed by *Nostoc muscorum* 14/86 and *Nostoc commune* NC7 (65% and 64.3% inhibition, respectively). In *Cylindrospermum* sp. C24/89, *Nostoc* sp. 116/96 and *Nostoc* sp. 6/99, mortality slightly above 40% was found. A lower toxicity of 14% was recorded for the symbiotic strains. Nevertheless, the symbiotic strain *Nostoc* sp. Ds1 exhibited a very strong and fast toxic effect manifested by the death of all animals within 24 h. Only one toxic strain was found in both epiphytic and periphytic cyanobacteria; however, the low number of tested strains from these habitats (four from both habitats) did not allow relevant conclusions to be made. From these, the strain *Cylindrospermum* sp. Hrouzek 1/2004 isolated from leaves of water plants caused a strong toxic effect leading to a mortality of 100%. Out of 30 planktonic strains tested, only the strain *Anabaenopsis* cf. *elenkinii* Anaps OLE-03 caused a high mortality of *Artemia salina* (67%). Data are summarized in Figure 1. (Here, Figure 1 should be placed, width 90mm)

Table 1: List of studied cyanobacteria strains. Place of isolation, habitat, *A. salina* mortality (B – biomass, M – medium, + represents mortality ≥ 50%) and Sp/2 cell line inhibition is shown. Different habitats are marked by abbreviations: E (epiphytic), P (periphytic), S (soil), Sy (symbiotic) and Pl (planktonic).

Scientific name	Strain	Place of isolation	Habitat	*A. salina* mortality		Sp/2 inh. [%]
				B	M	mean
Nostoc sp.	BR III[a]	Paranapiacaba-Sao Paulo/Brazil	E	+	-	**12***
Nostoc sp.	RQII[a]	Paranapiacaba-Sao Paulo/Brazil	E	-	-	**7***
Nostoc sp.	BRIIB[a]	Paranapiacaba-Sao Paulo/Brazil	E	-	n.a.	**0***
Nostoc sp.	BROMEL[a]	Paranapiacaba-Sao Paulo/Brazil	E	-	-	**0***
Nostoc sp.	LC17S01	Alberta/Canada	P	-	-	**27**
Nostoc sp.	OSNI 32S01	Sítinový pond/Czech Republic	P	-	n.a.	**0***
Cylindrospermum sp.	Hrouzek 1/2004	Zliv/Czech Republic	P	+	n.a.	**100**
Calothrix sp.	Hrouzek 2/2005	San Monoron-BUSRA/Cambodia	P	-	-	**30**
Nostoc calcicola	Lukešová 2/89[d]	Havana/Cuba	S	-	-	**6***
Nostoc sp.	Lukešová 5/96[d]	Nezamyslice/Czech republic	S	+	-	**52***
Nostoc muscorum	Lukešová 2/91[d]	Nezamyslice/Czech republic	S	+	-	**48***
Cylindrospermum sp.	C 24[d]	Ellesmere island/Canada	S	-	-	**60**
Trichormus variabilis	ISB 13	Dlouhá Ves/Czech Republic	S	-	-	**62,3**
Nostoc sp.	Lukešová 7/99[d]	Germany	S	-	-	**66***
Nostoc sp.	Lukešová 24/97[d]	Germany	S	-	-	**0***
Nostoc sp.	Lukešová 116/96[d]	Sokolov/Czech republic	S	-	-	**1***
Nostoc sp.	Lukešová 6/99[d]	Germany	S	-	+	**54***
Nostoc sp.	Cam2S01[c]	Camerun	S	-	-	**48***
Nostoc ellipsosporum	Lukešová 51/91[d]	Nezamyslice/Czech republic	S	+	-	**39***
Nostoc commune	NC1	Třeboň/Czech republic	S	-	-	**70**
Nostoc commune	NC2	Třeboň/Czech republic	S	-	-	**12**
Nostoc commune	NC3	Nové Hrady/Czech republic	S	-	+	**0**
Nostoc commune	NC4	Nové Hrady/Czech republic	S	-	-	**40**
Nostoc commune	NC 7	České Budějovice/Czech republic	S	+	n.a.	**34**
Nostoc sp.	NC 9	Rožnov p. R.Czech republic	S	-	n.a.	**27**
Nostoc sp.	OBU36S07[c]	The Burren Clare/Ireland	S	-	-	**42***
Nostoc sp.	CC2[c]	greenhouse Pisa/Italy	Sy	-	-	**30**
Nostoc sp.	CR4[c]	greenhouse Florence/Italy	Sy	-	-	**5***
Nostoc sp.	De1[c]	greenhouse Rome/Italy	Sy	-	-	**64***
Nostoc sp.	OGU 36S01[c]	Achill Island/Ireland	Sy	-	-	**31***
Nostoc sp.	Gm1[c]	greenhouse Siena/Italy	Sy	-	+	**14***
Nostoc sp.	Ds1[c]	greenhouse Rome/Italy	Sy	+	-	**54**
Nostoc sp.	Mm1[c]	greenhouse Rome/Italy	Sy	-	-	**0**
A. mendotae x sigmoidea	04 06[b]	Březová/Czech republic	Pl	-	-	**n.a.**
Anabaena mendotae x sigmoidea	04 12[b]	Černiš/Czech republic	Pl	-	-	**70**
A. compacta	04 17[b]	Dubnenský/Czech republic	Pl	-	-	**67**
A. cf. curva	04 19[b]	Hejtman/Czech republic	Pl	-	-	**n.a.**
A. circinalis x crassa	04 22[b]	Husinec/Czech republic	Pl	-	+	**50**
A. lemmermannii	04 24[b]	Husinec/Czech republic	Pl	-	-	**13**
A. circinalis x crassa	04 26[b]	Jesenice/Czech republic	Pl	-	+	**46**
A. cf circinalis	04 28[b]	Hodějovický/Czech republic	Pl	-	-	**29**
A. lemmermannii	04 33[b]	Orlík/Czech republic	Pl	-	-	**n.a.**
A. lemmermannii	04 38[b]	Senecký/Czech republic	Pl	-	-	**17**
A. cf. flos-aquae	04 40a[b]	Skalka/Czech republic	Pl	-	+	**n.a.**
A. lemmermannii	04 42[b]	Svět/Czech republic	Pl	-	+	**57**
Aphanizomenon aphanizomenoides	04 43[b]	Svět/Czech republic	Pl	-	+	**92**
Anabaena affinis	04 44[b]	Svět/Czech republic	Pl	-	+	**66**
A. mendotae x sigmoidea	04 45[b]	Svět/Czech republic	Pl	-	+	**87**
A. cf. spiroides	04 51[b]	Svět/Czech republic	Pl	-	+	**62**
A. cf. flos-aquae	04 52a[b]	Svět/Czech republic	Pl	-	+	**25**
A. cf. flos-aquae	04 53[b]	Švarcenberk/Czech republic	Pl	-	+	**53**
A. circinalis x crassa	04 56[b]	Vajgar/Czech republic	Pl	-	-	**47**
A. cf. flos-aquae	04 57[b]	Vajgar/Czech republic	Pl	-	+	**76**
A. circinalis x crassa	04 59[b]	Valcha/Czech republic	Pl	-	+	**82**
Anabaenopsis cf. elenkinii	Anaps Plást 05[b]	Plástovice/Czech republic	Pl	-	+	**n.a.**
A. compacta	Acom Pěšák 06[b]	Pěšák/Czech republic	Pl	-	+	**43**
A. compacta	Acom Svět 06[b]	Svět/Czech republic	Pl	-	-	**2**
A. lemmermannii - morfotypS	Alem Lipno 05 silná[b]	Lipno/Czech republic	Pl	-	-	**33**
A. lemmermannii - morfotypT	Alem Lipno 05 tenká[b]	Lipno/Czech republic	Pl	-	+	**40**
Anabaenopsis cf. elenkinii	Anaps-Ole 03[b]	Oleksovice/Czech republic	Pl	+	+	**92**
A. eucompacta x reniformis	Anarenif Pěšák 2[b]	Pěšák/Czech republic	Pl	-	+	**77**
A. eucompacta x reniformis	Anarenif Pěšák 4[b]	Pěšák/Czech republic	Pl	-	+	**90**
Anabaena affinis	Staňk 05-11[b]	Staňkovský/Czech republic	Pl	-	+	**37**

*Values of inhibition have been taken from Hrouzek et al., 2010

3.2 Toxicity of media extracts to Artemia salina

Media extracts were available for 58 strains out of the total number 63. Almost forty per cent (37.9%) of all tested media extracts exhibited significant toxicity against *Artemia salina*. This high number can be attributed to the frequent toxicity occurrence found in media of planktonic cyanobacteria (Fig. 1). In contrast to the low incidence of biomass toxicity in planktonic strains, 63.3% of their media extracts exhibited a significant toxic effect. Among these, *Anabaena lemmermanii* (100% inhibition), *Anabaena cf. spiroides* 04-51 (91% inhibition) and *Anabaena circinalis/crassa* 04-22 (86% mortality) caused the strongest effect. The incidence of media toxicity was much lower among cyanobacteria from other habitats: 11.1% in soil strains and 14.3% in symbiotic strains. The medium extract of the symbiotic strain *Nostoc* sp. Gm1 caused significant mortality of *Artemia salina* (50% inhibition) in contrast to its biomass extract with no effect. On the other hand, medium extract of the strain *Nostoc* sp. Ds1 lacked any effect in contrast to its biomass extract (see above). Soil strains *Nostoc commune* NC2 and NC3 exhibited significant medium toxicity (46 and 50%, respectively) with no inhibition found in their biomass extracts. No toxicity of a medium extract was found in epiphytic strains and cyanobacteria isolated from periphyton.

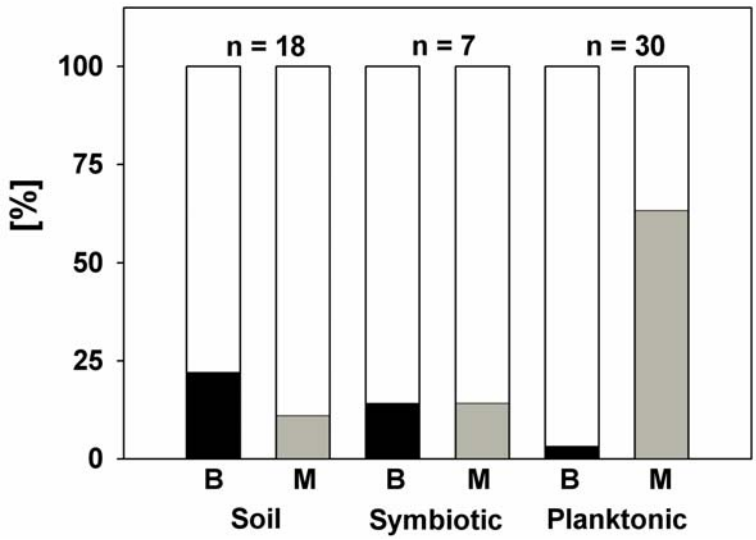


Figure 1: Percentage of biomass (B) and medium (M) extracts causing lethal effects to *Artemia salina* in strains isolated from soil, different symbiotic associations and plankton. Black and grey parts of columns indicate toxicity occurrence in biomass and media respectively, white parts of columns correspond to percentage of non-toxic extracts.

3.3 Comparison of A. salina mortality with Sp/2 cell line inhibition values

The percentage values of *A. salina* mortality (X-axis, Fig. 2) were compared with percentage inhibition values of Sp/2 cell line (Y-axis, Fig. 2) for each extract in order to find whether a correlation between cytotoxicity and toxicity to *Artemia salina* existed. We did not find any significant relationship between toxicity of biomass extracts to Sp/2 cell line and *Artemia salina* and such correlation was also rejected by the linear regression model (R=0.1115 p=0.4047). A high number of extracts (31% of all tested strains) were highly toxic to Sp/2 cell lines and nontoxic to *A. salina* (Fig. 2, area A), while only three strains were toxic to *A. salina* with no activity to the cell line (Fig. 2, area C). *Nostoc* sp. BR III exhibited strong toxicity to *Artemia* while having only a marginal effect to the cell line. In other strains belonging to area C (*Nostoc commune* NC7 and *Nostoc ellipsosporum* 51/91), strong activity to *Artemia* was found; however, it is accompanied by a moderate cytotoxic effect to Sp/2. The activity of strains *Nostoc* sp. Mm1 and *Nostoc* sp. 116/96 (area B) to *Artemia* was apparent; however, toxicity was slightly under the artificial threshold value 50%. In these strains, no effect to Sp/2 cell line was recorded. Other strains grouped in the area B of Figure 2 cannot be considered as significantly toxic due to their low toxic effect to both *A. salina* and Sp/2 cell line. (Here, Figure 2 should be placed, width 140mm)

Five of all the tested strains (*Cylindrospermum* sp. Hrouzek 1/2004, *Anabaenopsis* cf. *elenkinii* Anaps Ole-03, *N. muscorum* 14/86, *Nostoc* sp. Ds1 and *Nostoc* sp. 5/96) were found to cause strong damage to both *Artemia* and cell lines (area D). In *N. ellipsosporum* 51/91, 6/99, *Nostoc commune* NC7 and *Cylindrospermum* sp. C24/89, similar effects with inhibition values near the threshold value were found.

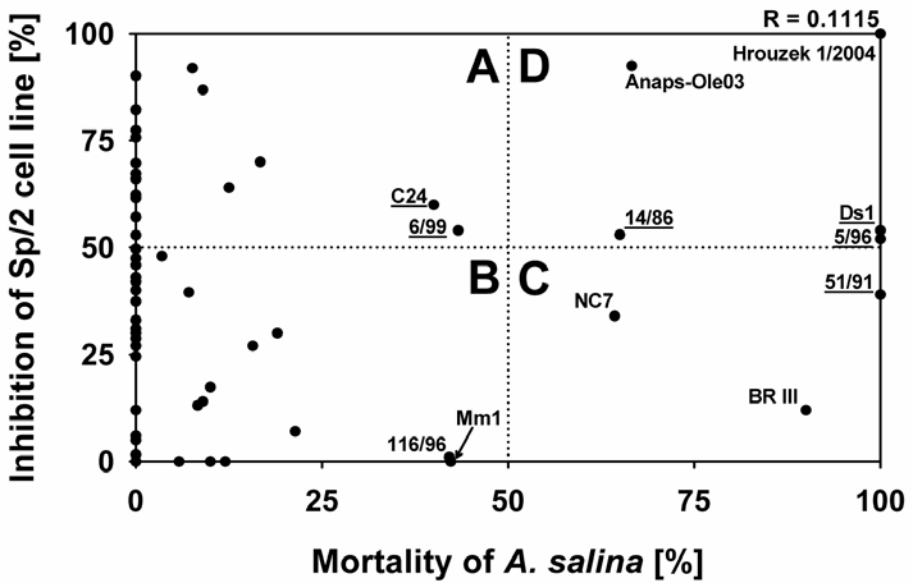


Figure 2: Comparison of effects of biomass extracts of studied strains on *A. salina* mortality and Sp/2 cells inhibition. The non-significant correlation of inhibition values in *A. salina* and Sp/2 cells is obvious from the graph (R = 0.1068, p=0.4047). The high number of strains toxic to Sp/2 cells with no activity to *A. salina* can be seen in Area B. By contrast, only a few extracts causing mortality to *A. salina* were not accompanied by a cytotoxic effect (Area C). The dotted lines represent the borders for both *A. salina* mortality and Sp/2 cell line inhibition values ≥ 50%. Extracts with an inhibition ≥ 50% were considered strongly toxic. The names of considerably toxic strains are given. Underlined strains were selected for fractionation.

3.4 Fractionation of selected strains

Six selected strains were fractionated by analytical and preparative HPLC in order to detect their active compounds. Unfortunately, two strains exhibiting the highest toxicity to both *A. salina* and Sp/2 cells (*Cylindrospermum* 1/2004 and *Anabaenopsis* cf. *elenkinii* Anaps OLE-03), were not studied due to problems with their cultivation and the low amount of extracts obtained. The active fractions of the selected strains were further analyzed to find out their composition. Chromatograms and the active fractions causing toxic effects to Sp/2 cell line and *A. salina* are shown in Figures 3 and 4.

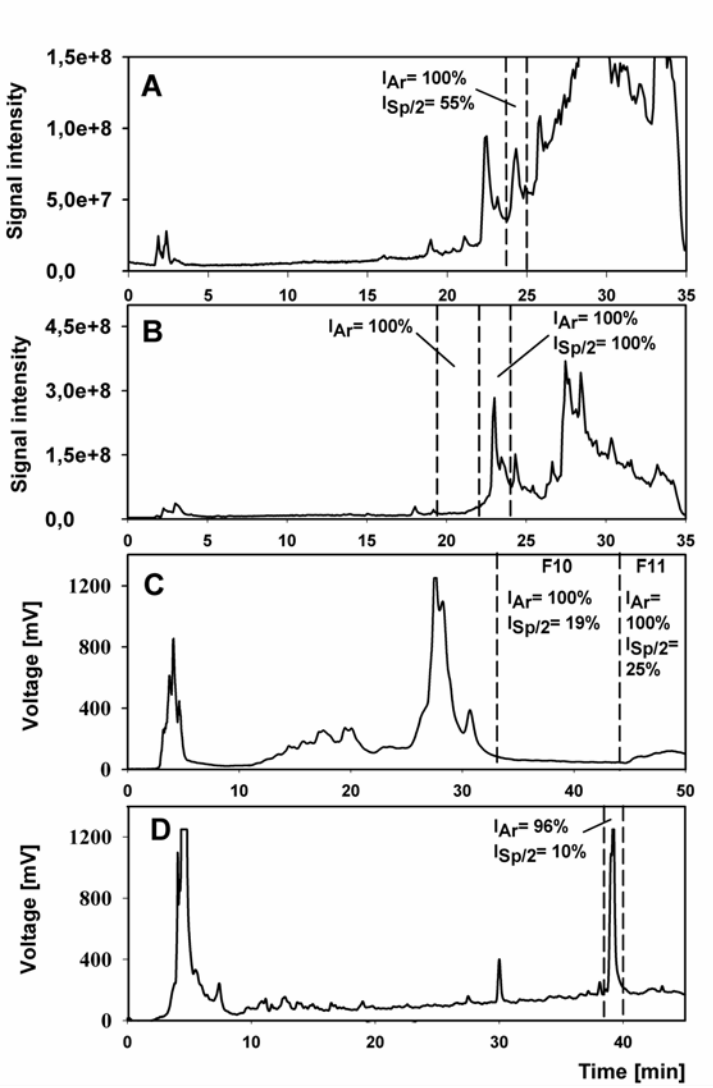


Figure 3: Chromatograms of cyanobacterial strains in which only one fraction toxic to both *A. salina* and Sp/2 cells was observed. The active fraction is marked by dashed lines and the inhibition values of these fractions to *A. salina* **(I_{Ar})** and Sp/2 cells **($I_{sp/2}$)** are shown. **A**: *Cylindrospermum* sp. C 24/89 (Total ion chromatogram), **B**: *Nostoc* sp. 5/96 (Total ion chromatogram), **C**: *Nostoc ellipsosporum* 51/91 (UV absorption at 237 nm), **D**: *Nostoc* sp. Ds1 (UV absorption at 220 nm).

In four of the six studied strains, the toxicity to *Artemia salina* and Sp/2 cell line was caused by an identical fraction or compound (Fig. 3). The toxicity of *Cylindrospermum* sp. C24/89 to both *A. salina* and Sp/2 cells was caused by a compound collected between

23.5' and 25.0' of analytical gradient. Within its mass spectrum, ions corresponding to molecular ion 1146 $[M+H]^+$ and its sodium adduct 1168 $[M+Na]^+$ were detected. The toxicity of this compound was also proved in its pure state obtained by preparative HPLC. In the strain *Nostoc* sp. 5/96, activity in both tests was caused by a compound with a molecular weight of 849.6, which exerted a very strong (100%) inhibition to both *A. salina* and Sp/2 cell lines. Additionally, fraction 3 was also highly toxic to *A. salina* with a mortality of 100% in an extract of this strain. As with the previous two strains, in *Nostoc ellipsosporum* 51/91 toxicity to the murine cell line and *Artemia salina* was also caused by identical fractions. Fractions 10 and 11 with no UV absorption or clear peak in a total ion chromatogram were responsible for 100% mortality to *A. salina* in this strain and caused a moderate effect on the Sp/2 cell line (19 % and 25 % inhibition respectively). The strong toxic effect on *A. salina* was also observed for fraction 10 obtained from an extract of the strain *Nostoc* sp. Ds. The fraction was collected by preparative HPLC based on strong UV absorption at 220 nm and subsequent analysis proved the presence of a compound with a molecular weight of 460.1. The activity of this compound to Sp/2 cells was weak (10%); however, no other fractions of this extract exhibited an inhibition. Thus, it is possible that other components of the extract enhanced the inhibitory effect of this compound and the observed crude extract inhibition was therefore higher (54 %).

Different compounds were responsible for the toxicity to *A. salina* and Sp/2 in the strains *Nostoc muscorum* 14/86 and *Nostoc* sp. 6/99. In *Nostoc muscorum* 14/86, fraction 3 containing a compound of MW = 885.0 (886 $[M+H]^+$; 908 $[M+Na]^+$) caused 61.1% mortality to *A. salin*a, whereas 79% inhibition of the Sp/2 cell line was caused by fraction 9 containing a novel cyclic peptide of MW = 1211 (1212 $[M+H]^+$; 1234 $[M+Na]^+$) (Hrouzek et al., 2010). In the second strain *Nostoc* sp. 6/99, fractions 2 and 5 containing compounds of MW = 1006.9 and 1076.1, respectively, exhibited a significant mortality to *A. salina* (Fig. 4). The cytotoxicity of this strain had been previously found to be caused by a fraction containing Nostopeptolide A1 (MW = 1081) (Golakoti et al., 2000; Hrouzek et al., 2010) and this result was confirmed by our study (Fig. 4).

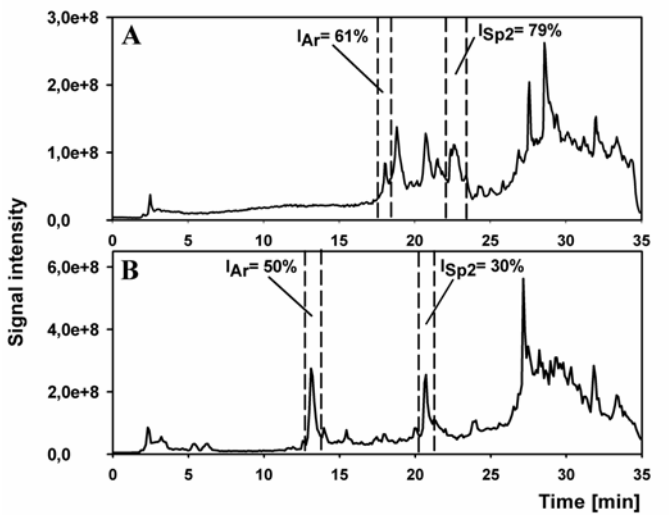


Figure 4: Chromatograms of cyanobacterial strains in which distinct fractions were found to have a toxic effect on *A. salina* and Sp/2 cells. Active fractions are marked by dashed lines and inhibition values of these fractions to *A. salina* **(I_{Ar})** and Sp/2 cells **($I_{sp/2}$)** are shown. **A:** *Nostoc muscorum* 14/86 (Total ion chromatogram), **B:** *Nostoc* sp. 6/99 (Total ion chromatogram).

4. Discussion

Our data demonstrate that 12.7 % of the biomass extracts of all studied strains exerted a toxic effect on *A. salina*. This is in full accordance with Jaki et al. (1999) and Mian et al. (2003), who found toxicity to *A. salina* in 13.9 % and 9.1 % of strains, respectively. A slightly higher number of toxic strains (24 %) was reported by Piccardi et al. (2000) who studied the bioactivities of *Nostoc* strains. On the other hand, Falch et al. (1995) reported that 80 % of all strains were toxic when tested on *A. salina*; however, the strains included in the study were selected because they were previously reported to possess some pharmacological and biological effects, so the frequency of the reported toxic strains therein could be misleading. Based on all the facts above, it seems that the frequency of strains toxic to *A. salina* ranges between 10 to 25 %.

An *Artemia salina* toxicity assay has been suggested as a valid method to evaluate cytotoxic activity (Solis et al., 1993) and thus the method is commonly used as a substitute assay for the screening of cytotoxic compounds. However, Jaki et al. (1999), Mian et al. (2003), and also Berry et al. (2004), all found no correlation between *A. salina* mortality and cell lines inhibition values, and our data are in full accordance with their findings. Only 8.6

% of all tested strains were toxic to both *A. salina* and the Sp/2 cell line. By comparison, 31 % of all extracts exhibited a strong cytotoxic effect and did not cause any mortality to *A. salina*. This result suggests that cytotoxicity is a more frequently occurring feature among cyanobacteria than the toxicity to invertebrates.

The defence mechanisms of cyanobacteria, for example, the production of toxic secondary metabolites as a reaction to the presence of a potential grazer, belong to some of the important questions of cyanobacterial ecology. Many studies have proved the inhibitory effects of cyanobacterial secondary metabolites or extracts to invertebrates (Berry et al., 2004; Biondi et al., 2004; Rohrlack et al., 1999, 2004, 2005; Agrawal et al., 2005). One of the most important questions concerning cyanobacteria-grazer interactions is whether the metabolites synthesized by cyanobacteria are specifically against invertebrates or whether their effect is more general.

A comparison of cytotoxicity occurrence and number of extracts positive to *A. salina* indicates that in most cases the toxic effect is targeted to various basal metabolic pathways present in the eukaryotic cell rather than being a specific mechanism against a complex organism. This result is strongly supported by the fractionation of extracts found to be toxic both to *A. salina* and Sp/2 cell lines. In four of the six fractionated extracts the toxic effects were caused by an identical compound, suggesting that the mechanism of the function is probably the same for both the cell and the complex organism. The activity to *A. salina* that was not accompanied by inhibition of Sp/2 cells was only found in the fractions of strains *N. muscorum* 14/86 and *Nostoc* sp. 6/99 and in three raw extracts (Mm1, BRIII and 116/96). However, it is questionable whether the concentration of these compounds was sufficiently high to manifest the effect in the cells. Moreover, no particular compound toxic to *A. salina* was found in the strain *Nostoc* sp. BR III although the strain as a whole was found highly toxic to *Artemia;* the toxicity of this strain to Sp/2 cells was insignificant. So the mechanism of final toxicity of this extract to *A. salina* could be based on the synergic effect of many compounds present in the strain.

As a second issue concerning cyanobacterial-grazer interactions we tested if the differences in frequency of toxicity to *A. salina* existed among strains isolated from different habitats. For this purpose, mainly soil, planktonic and symbiotic strains were selected. The toxicity of biomass (intracellular) extracts was detected in 22 % of the 18 soil strains. This approximately corresponds to the results of Jaki et al. (1999) who found toxicity in 16.6 % of 30 studied soil strains. On the other hand, our results do not agree with data published by Mian et al. (2003) who did not found any toxic strain, and neither with data of Falch et al.

(1995) and Piccardi et al. (2000), who respectively observed toxicity in 83 % and 75 % of strains. However, Mian et al. (2003), Falch et al. (1995) and Piccardi et al. (2000) studied a low number (8, 12 and 8 resp.) of soil strains and thus reliable conclusions cannot be drawn. Only one out of 30 intracellular extracts (3.3 %) of our planktonic strains exerted a toxic effect to *A. salina*. Though they worked with a low number of strains, Mian et al. (2003) and Piccardi et al. (2000) published very similar results (0 % of toxic strains in both). The occurrence of toxic strains among symbiotic cyanobacteria was over 14 % in the present study, which is slightly lower compared to the results given by Piccardi et al. (2000), who observed toxicity in 6 out of 23 (26 %) studied strains.

As seen from our results the distribution of toxicity to *A. salina* was different in cultivation media (extracellular compounds). The overall occurrence of toxicity in media extracts was lower than in the biomass of soil, subaerophytic, epiphytic and periphytic cyanobacteria. No toxic medium extracts were found in epiphytic and periphytic strains and only a marginal occurrence of toxicity (14.3 % and 11.1 %) was observed in symbiotic and soil strains, respectively. By contrast, a high occurrence of toxic media extracts was found among planktonic strains. However, only one toxic intracellular extract from planktonic strains was found as discussed above. A similar extracellular production of inhibitory compounds was published by Jüttner and Wessel (2003), who found that all five studied strains of *Cylindrospermum* synthesized and excluded zooplankton glucosidases' inhibitor DMDP–di(hydroxymethyl) dihydroxypirolidine. The major part of DMPD (80 %) was found to be extracellular. Such a distribution of toxicity in intra- and extracellular extracts can have an easy explanation ecologically. The diffusion of toxins towards a grazer is easier in a planktonic environment compared with a soil environment, where the intracellular storage of compounds toxic to the grazer will be more efficient. Cyanobacteria with intracellular toxin production will be probably more successful in competition with other soil cyanobacteria and their frequency of occurrence in soils will be consequently higher, which is in accordance with our results. Our data have revealed that planktonic cyanobacteria produce, and are able to release, compounds toxic to *A. salina*. More than 63 % of media extracts of planktonic strains were found to be toxic to *A. salina*, whereas only one strain was found toxic among biomass extracts. In contrast, only 11.1 % of media extracts of all soil strains were toxic to *A. salina*.

Based on our results, we conclude that cyanobacterial secondary metabolites are not synthesized specifically against grazers but are highly toxic in general and interact with basal cell metabolism. However, they can play a role in the defensive mechanisms of

cyanobacteria against grazers and can be more frequent in habitats with a higher predation pressure. Regarding these results, it may not be possible to monitor cytotoxicity using only the brine shrimp bioassay, since cytotoxicity is more a frequent feature compared with the toxicity to *A. salina*.

Acknowledgement

This study was supported by the Ministry of Education, Youth and Sports of the Czech Republic (Project FRVŠ no. 3491/2005 and MŠM 6007 665 808, MŠMT 1MO571; partly by MŠM 6007665801), Institutional Research Concept AVOZ50200510 and the Research plan of ISB AV0Z60660521. Partial financial assistance was provided by the Grant Agency of the Czech Republic (P504/10/1501). We gratefully thank Pavel Souček, Lada Samcová and Lucie Marková for their technical help and practical advice. We also thank Jan Kopecký from the Institute of Parasitology, the Biology Centre of the ASCR, who kindly provided the Sp/2 cell line.

References

Agrawal, M.K., Bagchi, D., Bagchi, S.N., 2005. Cysteine and serine protease-mediated proteolysis in body homogenate of a zooplankter, *Moina macrocopa*, is inhibited by the toxic cyanobacterium, *Microcystis aeruginosa* PCC7806. *Comp. Biochem. Phys. B.* 141: 33-41.

Anderson, J.E., Goetz, C.M., McLaughlin, J.L., Suffness, M., 1991. A blind comparison of simple bench-top bioassays and human tumour cell cytotoxicities as antitumor prescreens. Phytochem. Analalysis. 2, 107–111.

Arnon, D.I., McSwain, B.D., Tsujimot, H.Y., Wada, K., 1974. Photochemical activity and components of membrane preparations from blue-green algae. I. Coexistence of two photosystems in relation to chlorophyll a and removal of phycocyanin. Biochim. Biophys. Acta. 357, 231-245.

Berry, J.P., Gantar, M., Gawley, R.E., Wang, M., Rein, K.S., 2004. Pharmacology and toxicology of pahayokolide A, a bioactive metabolite from a freshwater species of *Lyngbya* isolated from the Florida Everglades**.** Comp. Biochem. Physiol. C. 139, 231-238.

Biondi, N., Piccardi, R., Margheri, M.C., Rodolfi, L., Smith, G.D., Tredici, M.R., 2004. Evaluation of *Nostoc* strain ATCC 53789 as a potential source of natural pesticides. Appl. Environ. Microbiol. 70, 3313-3320.

Blom, J.F., Baumann, H.I., Codd, G.A., Jüttner, F., 2006. Sensitivity and adaptation of aquatic organisms to oscillapeptin J and [D-Asp3, (E)-Dhb7]microcystin-RR. Arch. Hydrobiol. 167, 547-559.

Dodds, W.K., Gudder, D.A., Mollenhauer, D., 1995. The ecology of *Nostoc*. J. Phycol. 31, 2-18.

Falch, B.S., König, G.M., Wright, A.D., Sticher, O., Angerhofer, C.K., Pezzuto, J.M., Bachmann, H., 1995. Biological activities of cyanobacteria: evaluation of extracts and pure compounds. Planta Med. 61, 321-328.

Ferrão-Filho, A.S., Azavedo, S.M.F.O., DeMott, W.R., 2000. Effects of toxic and non-toxic cyanobacteria on the life history of tropical and temperate cladocerans. Freshw. Biol. 45, 1-19.

Gademann, K. and Portman, C., 2008. Secondary metabolites from cyanobacteria: Complex structures and powerful bioactivities. Curr. Org. Chem. 12, 326-341.

Golakoti, T., Yoshida, W.Y., Chagany, S. and Moore, R.E., 2000. Isolation and Structures of Nostopeptolides A1, A2 and A3 from the Cyanobacterium *Nostoc* sp. GSV224. Tetrahedron. 56, 9093-9102.

Guillard, R.R.L. and Lorenzen, C.L., 1972. Yellow-green algae with chlorophyllide C. J. Phycol. 8, 10-14.

Hrouzek, P., Tomek, P., Lukešová, A., Urban J., Voloshko, L., Pushparaj, B., Lukavský, J., Štys, D., Kopecký, J., 2010. Cytotoxicity and secondary metabolites production in terrestrial *Nostoc* strains originating from different climatic/geographic regions and habitats: Is their cytotoxicity environmentaly dependent? Environ. Tox. (In press).

Jaki, B., Orjala, J., Bürgi, H.R., Sticher, O., 1999. Biological screening of cyanobacteria for antimicrobioal and molluscicidal activity, brine shrimp lethality, and cytotoxicity. Pharm. Biol. 37, 138-143.

Jüttner, F., Wessel, H.P., 2003. Isolation of di (hydoxymethyl) dihydroxypyrrolidine from the cyanobacterial genus *Cylindrospermum* that effectively inhibits digestive glucosidases of aquatic insects and crustacean grazers. J. Phycol. 39, 26-32.

Kiviranta, J., Sivonen, K., Niemelä, S.I., Huovinen, K., 1991. Detection of toxicity of cyanobacteria by *Artemia salina* bioassay. Environ. Toxicol. Water Qual. 6, 423–436.

Lahti, K., Ahtiainen, J., Rapala, J., Sivonen, K., Niemela, S.I., 1995. Assessment of rapid bioassays for detecting cyanobacterial toxicity. Lett. Appl. Microbiol. 21, 109-114.

Lincoln, R.D., Strupinski, K., Walker, J.M., 1996. The use of *Artemia* naupli (Brine shrimp larvae) to detect toxic compounds from microalgal cultures. Pharm. Biol. (formely Int. J. Pharmacog). 34, 384-389.

Lürling, M. and Beekmann, W., 2006. Growth of *Daphnia magna* males and females fed with the cyanobacterium *Microcystis aeruginosa* and the green alga *Scenedesmus obliquus* in defferent proportions. Acta Hydrochim. Hydrobiol. 34, 375-382.

Metcalf, J.S., Lindsay, J., Beattie, K.A., Birmingham, S., Saker, M.L., Törökné, A.K., Codd, G.A., 2002. Toxicity of cylindrospermopsin to the brine shrimp *Artemia salina*: comparison with protein synthesis inhibitors and microcystins. Toxicon. 40, 1115-1120.

Mian, P., Heilmann, J., Burgi, H.R., Sticher, O., 2003. Biological screening of terrestrial and freshwater cyanobacteria for antimicrobial activity, brine shrimp lethality, and cytotoxicity. Pharm. Biol. 41, 243-247.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assay. J. Immunol. Methods. 65, 55-63.

Murakami M., Okita Y., Matsuda H., Okino T., Yamaguchi K., 1994. Aeruginosin 298-A, trombin and trypsin inhibitors from the blue-green alga *Microcystis aeruginosa* (NIES-298). Tetrahedron Lett. 35, 3129-3132.

Murakami M., Ishida K., Okino T., Okita Y., Matsuda H.,Yamaguchi K., 1995. Aeruginosins 98-A and B, trypsin inhibitors from the blue-green alga *Microcystis aeruginosa* (NIES-98). Tetrahedron Lett. 36, 2785-2788.

Piccardi, R., Frosini, A., Tredici, M.R. et Margheri, M.C., 2000. Bioactivity in free-living and symbiotic cyanobacteria of the genus *Nostoc*. J. Appl. Phycol. 12, 543-547.

Rantala, A., Fewer, D.P., Hisbergues, M., Rouhiainen, L., Vaitomaa, J., Börner, T., Sivonen, K., 2004. Phylogenetic evidence for the early evolution of microcystin synthesis. PNAS 101, 568-573.

Rohrlack, T., Dittmann, E., Henning, M., Börner, T., Kohl, J.G., 1999. Role of microcystins in poisoning and food ingestion inhibition of *Daphnia galeata* caused by the cyanobacterium *Microcystis aeruginosa.* Appl. Environ. Microbiol. 65, 737–739.

Rohrlack, T., Christoffersen, K., Kaebernick, M., and Neilan, B.A., 2004. Cyanobacterial protease inhibitor microviridin J causes a lethal molting disruption in *Daphnia pulicaria*. Appl. Environ. Microbiol. 70, 5047–5050.

Rohrlack, T., Christoffersen, K., Friberg-Jensen, U., 2005. Frequency of inhibitors of daphnid trypsin in the widely distributed cyanobacterial genus *Planktothrix*. Environ. Microbiol. 7, 1667-1669.

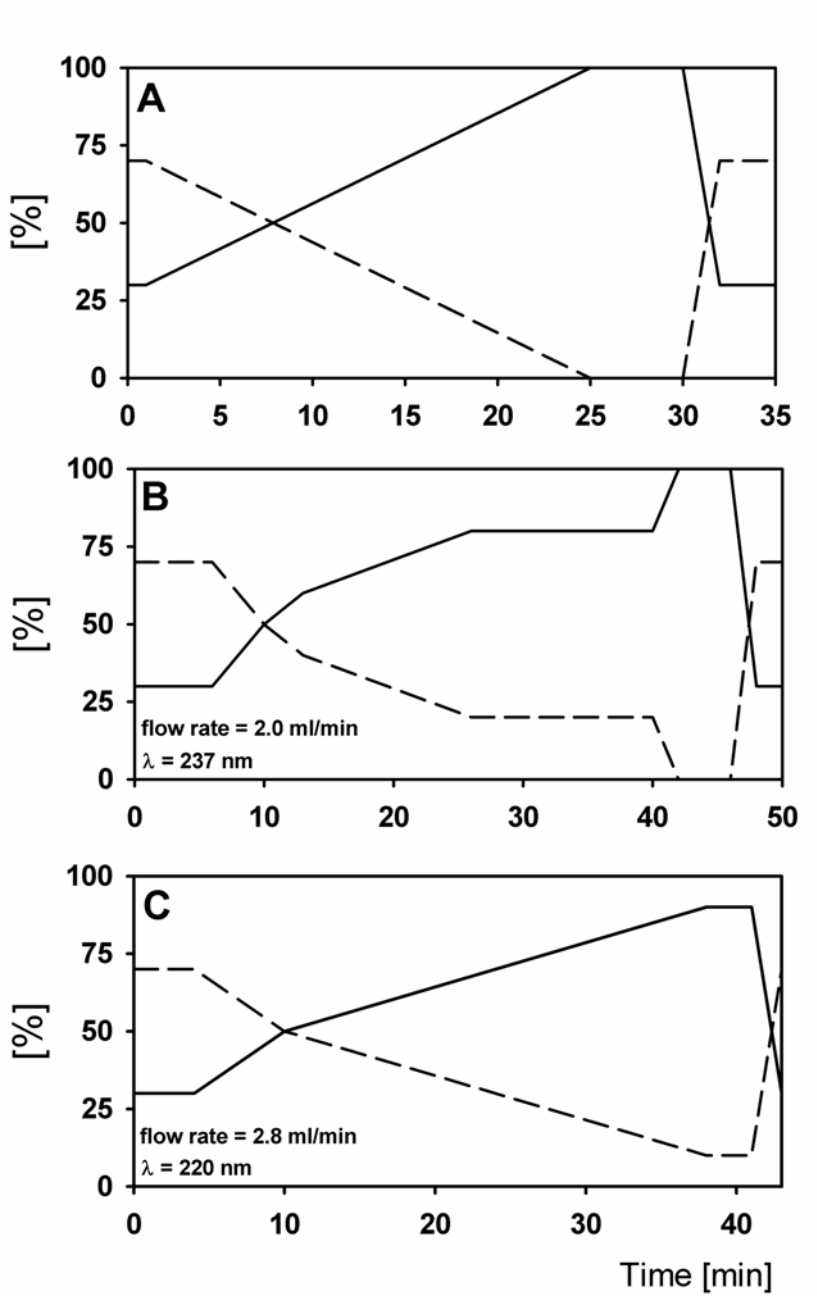
Sarnelle, O. and Wilson, A.E., 2005. Local adaptation of *Daphnia pulicaria* to toxic cyanobacteria. Limnol. Oceanogr. 50, 1565-1570.

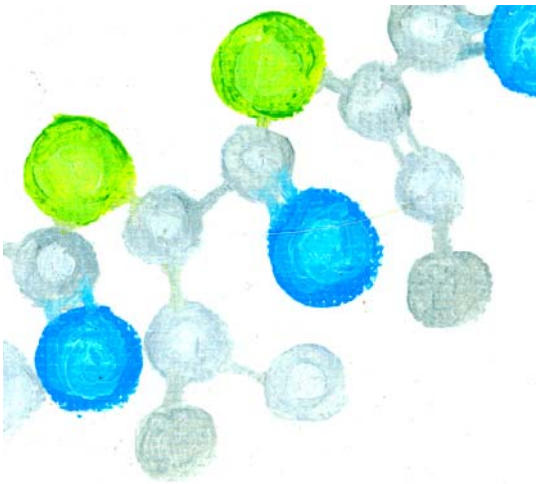
Solis, P.N., Wright, C.W., Anderson, M.M., Gupta, M.P., Phillipson, J.D., 1993. A microwell cytotoxicity assay using *Artemia salina* (Brine shrimp). Planta Med. 59, 250-252.

Wilson, A.E., Sarnelle, O., Tillmans, A.R., 2006. Effects of cyanobacterial toxicity and morphology on the population growth of freshwater zooplankton: Meta analyses of laboratory experiments. Limnol. Oceanogr. 51, 1915-1924.

Apendices

Supplementary data: Separation gradients used for fractionation of the six selected strains. Standard separation gradient used for fractionation of extracts from strains *Cylindrospermum* sp. C 24/1989, *Nostoc* sp. 5/96, *Nostoc muscorum* 14/86, *Nostoc* sp. 6/99. **(A)**. Extracts from strains *Nostoc ellipsosporum* 51/91 and *Nostoc* sp. Ds1 was separated using gradients **B** and **C**, respectively. Percentage of methanol is marked by a solid line, water by a dashed line. Flow rate and monitored absorbance is shown in the lower left corner for B and C.





IV

Rajaniemi P., **Hrouzek P.**, Kaštovská K., Willame R., Rantala A., Hoffmann L., Komárek J. & Sivonen K. (2005):

Phylogenetic and morphological evaluation of the genera *Anabaena, Aphanizomenon, Trichormus* and *Nostoc* (Nostocales, Cyanobacteria)

International Journal of Systematic and Evolutionary Microbiology 55: 11-26.

International Journal of Systematic and Evolutionary Microbiology (2005), **55**, 11–26 DOI 10.1099/ijs.0.63276-0

Phylogenetic and morphological evaluation of the genera *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* (Nostocales, Cyanobacteria)

Pirjo Rajaniemi,[1] Pavel Hrouzek,[2] Klára Kaštovská,[3] Raphaël Willame,[4] Anne Rantala,[1] Lucien Hoffmann,[4] Jiří Komárek[2,5] and Kaarina Sivonen[1]

Correspondence
Kaarina Sivonen
kaarina.sivonen@helsinki.fi

[1]Department of Applied Chemistry and Microbiology, Viikki Biocenter, PO Box 56, FI-00014 University of Helsinki, Finland

[2]University of South Bohemia, Faculty of Biological Sciences, Branišovská 31, Cz-370 05 České Budějovice, Czech Republic

[3]Institute of Hydrobiology, Academy of Sciences of the Czech Republic, Na sádkách 7, Cz-370 05 České Budějovice, Czech Republic

[4]Public Research Center – Gabriel Lippmann, Environment and Biotechnologies Research Unit, 162a avenue de la Faïencerie, L-1511 Luxembourg, Grand-duchy of Luxembourg

[5]Institute of Botany, Academy of Sciences of the Czech Republic, Dukelská 145, Cz-379 82 Třeboň, Czech Republic

The heterocytous cyanobacteria form a monophyletic group according to 16S rRNA gene sequence data. Within this group, phylogenetic and morphological studies have shown that genera such as *Anabaena* and *Aphanizomenon* are intermixed. Moreover, the phylogeny of the genus *Trichormus*, which was recently separated from *Anabaena*, has not been investigated. The aim was to study the taxonomy of the genera *Anabaena*, *Aphanizomenon*, *Nostoc* and *Trichormus* belonging to the family Nostocaceae (subsection IV.I) by morphological and phylogenetic analyses of 16S rRNA gene, *rpoB* and *rbcLX* sequences. New strains were isolated to avoid identification problems caused by morphological changes of strains during cultivation. Morphological and phylogenetic data showed that benthic and planktic *Anabaena* strains were intermixed. In addition, the present study confirmed that *Anabaena* and *Aphanizomenon* strains were not monophyletic, as previously demonstrated. The evolutionary distances between the strains indicated that the planktic *Anabaena* and *Aphanizomenon* strains as well as five benthic *Anabaena* strains in cluster 1 could be assigned to a single genus. On the basis of the 16S rRNA, *rpoB* and *rbcLX* gene sequences, the *Anabaena*/*Aphanizomenon* strains (cluster 1) were divided into nine supported subclusters which could also be separated morphologically, and which therefore might represent different species. *Trichormus* strains were morphologically and phylogenetically heterogeneous and did not form a monophyletic cluster. These *Trichormus* strains, which were representatives of three distinct species, might actually belong to three genera according to the evolutionary distances. *Nostoc* strains were also heterogeneous and seemed to form a monophyletic cluster, which may contain more than one genus. It was found that certain morphological features were stable and could be used to separate different phylogenetic clusters. For example, the width and the length of akinetes were useful features for classification of the *Anabaena*/*Aphanizomenon* strains in cluster 1. This morphological and phylogenetic study with fresh isolates showed that the current classification of these anabaenoid genera needs to be revised.

Published online ahead of print on 6 August 2004 as DOI 10.1099/ijs.0.63276-0.

Abbreviations: ML, maximum likelihood; MP, maximum parsimony; NJ, neighbour joining; PCA, principal-component analysis.

The GenBank/EMBL/DDBJ accession numbers are AJ630408–AJ630458 for the 16S rRNA gene sequences, AJ632022–AJ632070 for the *rbcLX* gene sequences and AJ628068–AJ628134 for *rpoB* gene sequences determined in this study.

A table of complete morphological characters is available as supplementary material in IJSEM Online.

INTRODUCTION

Heterocytous cyanobacteria consistently form a monophyletic cluster among cyanobacteria on the basis of their 16S rRNA gene sequences (Wilmotte, 1994; Turner, 1997, 1999; Wilmotte & Herdman, 2001; Lyra *et al.*, 2001), RFLP and genomic fingerprinting (Lyra *et al.*, 2001) and *nifD* sequences (Henson *et al.*, 2004). This monophyletic cluster contains the orders Nostocales and Stigonematales (Sections IV and V) (Rippka *et al.*, 1979), which were found to be intermixed (Turner *et al.*, 1997, 1999; Gugger & Hoffmann, 2004; Henson *et al.*, 2004). However, the genera *Anabaena* Born. et Flah., *Aphanizomenon* Born. et Flah., *Nostoc* Born. et Flah. and *Cylindrospermopsis* Seen. et Subba Raju clustered together within these orders in a 16S rRNA tree (Gugger & Hoffmann, 2004).

Currently, the genera *Anabaena, Aphanizomenon, Trichormus* (Born. et Flah.) Kom. et Anag. and *Nostoc* belong to order Nostocales, family Nostocaceae by traditional classification (Komárek & Anagnostidis, 1989) and subsection IV.I by bacteriological classification (Rippka *et al.*, 2001a). Identification of these genera is based on morphological features such as morphology of the filament, vegetative cells, heterocytes (heterocysts) and akinetes (Komárek & Anagnostidis, 1989). The form of the colony, shape of terminal cells, presence of sheath and gas vesicles, as well as life cycle, are additional features used for the identification of some genera. Recently, *Trichormus* was separated from the traditional genus *Anabaena* on the basis of akinete development and was transferred into the subfamily Nostocoidae. According to this revision, the species *Anabaena variabilis, Anabaena azollae* and *Anabaena doliolum* belong to the genus *Trichormus* (Komárek & Anagnostidis, 1989). The phylogeny of the *Trichormus* strains has not been studied. The assignment of some species either to *Anabaena* or to *Aphanizomenon* has been discussed (Komárek & Anagnostidis, 1989; Komárek & Kováčik, 1989). Also, the previous phylogenetic studies of Lyra *et al.* (2001), Gugger *et al.* (2002b) and Iteman *et al.* (2002) have shown that the genera *Anabaena* and *Aphanizomenon* are not monophyletic. Furthermore, the separation of the genera *Nostoc* and *Anabaena* has also been discussed in recent years (Henson *et al.*, 2002; Tamas *et al.*, 2000).

Phylogenetic studies of cyanobacteria have demonstrated that genetic relationships sometimes conflict with the morphological classification (Lyra *et al.*, 2001; Iteman *et al.*, 2002; Gugger & Hoffmann, 2004). The comparison of morphological and genetic data is hindered by the lack of cultures of several cyanobacterial morphospecies and inadequate morphological data of sequenced strains. Moreover, some strains may lose some important features such as gas vesicles (Lehtimäki *et al.*, 2000) or form of colony (Gugger *et al.*, 2002b) during long-term laboratory cultivation, which complicates identification. Komárek & Anagnostidis (1989) have estimated that more than 50 % of the strains in culture collections are misidentified. Therefore, new isolates should be studied by combined morphological and genetic approaches.

This study focused on the combined genetic and phenotypic relationships of the four genera *Nostoc, Trichormus, Anabaena* and *Aphanizomenon*. We isolated new *Anabaena, Aphanizomenon* and *Nostoc* strains. Detailed morphological analyses of these strains were carried out at the time of isolation in order to avoid difficulties in identification. The phylogeny of the strains was investigated by sequencing two housekeeping genes, 16S rRNA and *rpoB*, as well as a carbon-fixation-associated gene, *rbcLX*.

METHODS

Strains and cultivation. The 51 *Anabaena, Aphanizomenon, Trichormus* and *Nostoc* strains studied were unialgal, but not axenic (Table 1). The morphology of the strains with a few exceptions was analysed in this study. The morphology of *Nostoc muscorum, Nostoc calcicola, Nostoc ellipsosporum* and *Nostoc edaphicum* was described previously by Hrouzek *et al.* (2003) and *Anabaena compacta* ANACOM-KOR by Zapomělová (2004). The morphology of strain *Anabaena* sp. 277 (Lyra *et al.*, 2001; Gugger *et al.*, 2002a) was re-evaluated because of its clustering with *Aphanizomenon issatschenkoi* strains in the phylogenetic trees.

For morphological studies, the strains were cultivated in $BG11_0$ medium (Stanier *et al.*, 1971) at 18–22 °C under a light intensity of 30 μmol m^{-2} s^{-1}. For DNA extraction, the strains were cultivated in Z8 medium (Zehnder in Staub, 1961; Kotai, 1972) without nitrogen at 18·5–21·5 °C under a light intensity of 10 μmol m^{-2} s^{-1}.

Morphological study. The morphology of cells and filaments was studied using an Olympus CX 40 light microscope with a digital camera. Olympus DP SOFT version 4.0 software was used for image analysis. The following parameters were selected to describe the morphology of the studied strains: length and width of vegetative cells, heterocytes and akinetes; morphology of terminal cell; distance between heterocytes and distance between a heterocyte and the nearest akinete (counted as the number of cells); presence or absence of terminal heterocytes and gas vesicles; and shape of filament and its aggregation in colonies.

Statistical evaluation of morphological data. The mean values of measured morphological parameters were compared with one-way analysis of variance (ANOVA) followed by the Tukey honest significant difference (HSD) test in Statistica for Windows 4. To describe the variability of all morphological data and to evaluate the importance of measured morphological features, principal-component analysis (PCA) was carried out in Canoco for Windows 4.5 (ter Braak & Šmilauer, 1998). The program CanoDraw 4.5 was used for construction of the PCA plot.

DNA extraction. Cells were harvested by filtration through 1 or 5 μm Poretics filters (Osmonic) and stored at −20 °C. DNA was extracted by the modified CTAB method (Hönerlager *et al.*, 1995; Gkelis *et al.*, 2005). Filters containing cells were mechanically lysed using lysis matrix A and a Fast-prep instrument (Bio101) in 500 μl extraction buffer [100 mM Tris/HCl, pH 7·5, 1·5 % (w/v) SDS, 10 mM EDTA, 1 % (w/v) deoxycholate, 1 % (v/v) IGEPAL CA-630 (Sigma), 5 mM thiourea and 10 mM dithiothreitol, according to Hönerlager *et al.*, 1995] for 30 s at speed 5. The extracts were centrifuged at 10 000 ***g*** for 1 min and the DNA-containing supernatants were incubated in 5 M NaCl/10 % (w/v) CTAB at 65 °C for 20 min, followed by chloroform purification and ethanol precipitation.

Table 1. Cyanobacterial strains used in this study, their origin and potential ability to produce microcystins

Taxonomic assignment	Strain	Geographical origin and year of isolation	*mcyE* PCR*
Anabaena			
An. augstumalis	SCMIDKE JAHNKE/4a	Rostock, Germany	−
An. cf. *circinalis* var. *macrospora*	1tu23s3	Lake Tuusulanjärvi, Finland, 2001	−
An. cf. *circinalis* var. *macrospora*	1tu26s10	Lake Tuusulanjärvi, Finland, 2001	−
An. cf. *circinalis* var. *macrospora*	1tu27s5	Lake Tuusulanjärvi, Finland, 2001	−
An. cf. *circinalis* var. *macrospora*	1tu28s13	Lake Tuusulanjärvi, Finland, 2001	−
An. cf. *circinalis* var. *macrospora*	0tu25s6	Lake Tuusulanjärvi, Finland, 2000	−
An. cf. *crassa*	1tu27s7	Lake Tuusulanjärvi, Finland, 2001	−
An. cf. *cylindrica*	XP6B	Sediment, Porkkala, Helsinki, Gulf of Finland, Baltic Sea, 1999	−
An. circinalis	1tu34s5	Lake Tuusulanjärvi, Finland, 2001	−
An. circinalis	1tu30s11	Lake Tuusulanjärvi, Finland, 2001	−
An. circinalis	1tu33s12	Lake Tuusulanjärvi, Finland, 2001	+
An. compacta	ANACOM-KOR[a]†	Water reservoir, Kořensko, Czech Republic, 2002	−
An. flos-aquae	1tu31s11	Lake Tuusulanjärvi, Finland, 2001	+
An. flos-aquae	0tu33s15	Lake Tuusulanjärvi, Finland, 2000	−
An. flos-aquae	0tu33s2a	Lake Tuusulanjärvi, Finland, 2000	−
An. flos-aquae	1tu30s4	Lake Tuusulanjärvi, Finland, 2001	+
An. flos-aquae	1tu35s12	Lake Tuusulanjärvi, Finland, 2001	−
An. lemmermannii	1tu32s11	Lake Tuusulanjärvi, Finland, 2001	−
An. mucosa	1tu35s5	Lake Tuusulanjärvi, Finland, 2001	+
An. oscillarioides	BECID22	Epiphytic, Vuosaari, Helsinki, Gulf of Finland, Baltic Sea, 2001	−
An. oscillarioides	BECID32	Epilithic, Vuosaari, Helsinki, Gulf of Finland, Baltic Sea, 2001	−
An. oscillarioides	BO HINDAK 1984/43	Canada, 1984	−
An. planctonica	1tu33s10	Lake Tuusulanjärvi, Finland, 2001	−
An. planctonica	1tu28s8	Lake Tuusulanjärvi, Finland, 2001	−
An. planctonica	1tu30s13	Lake Tuusulanjärvi, Finland, 2001	−
An. planctonica	1tu33s8	Lake Tuusulanjärvi, Finland, 2001	−
An. planctonica	1tu36s8	Lake Tuusulanjärvi, Finland, 2001	−
An. sigmoidea	0tu36s7	Lake Tuusulanjärvi, Finland, 2000	−
An. sigmoidea	0tu38s4	Lake Tuusulanjärvi, Finland, 2000	−
An. smithii	1tu39s8	Lake Tuusulanjärvi, Finland, 2001	−
Anabaena sp.	1tu34s7	Lake Tuusulanjärvi, Finland, 2001	−
Anabaena sp.	0tu37s9	Lake Tuusulanjärvi, Finland, 2000	−
Anabaena sp.	0tu39s7	Lake Tuusulanjärvi, Finland, 2000	−
An. spiroides	1tu39s17	Lake Tuusulanjärvi, Finland, 2001	−
Aphanizomenon			
Ap. flos-aquae	1tu29s19	Lake Tuusulanjärvi, Finland, 2001	−
Ap. flos-aquae	1tu37s13	Lake Tuusulanjärvi, Finland, 2001	−
Ap. flos-aquae	1tu26s2	Lake Tuusulanjärvi, Finland, 2001	−
Ap. gracile	Heaney/Camb 1986 140 1/1	Freshwater, Lough Neagh, Ireland, 1986	−
Ap. gracile	1tu26s16	Lake Tuusulanjärvi, Finland, 2001	−
Ap. issatschenkoi	0tu37s7	Lake Tuusulanjärvi, Finland, 2000	−
Nostoc			
N. calcicola	III[b]	Field, České Budějovice, Czech Republic, 1989	−
N. calcicola	VI[b]	Field, Dobré Pole, Czech Republic, 1998	−
N. edaphicum	X[b]	Field, Chelčice, Czech Republic, 1989	−
N. ellipsosporum	V[b]	Field, Nezamyslice, Czech Republic, 1990	−
N. muscorum	I[b]	Field, Dlouhá Ves, Czech Republic, 1986	−
N. muscorum	II[b]	Field, Jevany, Czech Republic, 1985	−
Nostoc sp.	1tu14s8	Lake Tuusulanjärvi, Finland, 2001	−

Table 1. cont.

Taxonomic assignment	Strain	Geographical origin and year of isolation	*mcyE* PCR*
Trichormus			
T. azollae	BAI/1983	Unknown, 1983	–
T. doliolum	1	Unknown	–
T. variabilis	GREIFSWALD	Unknown, 1992	–
T. variabilis	HINDAK 2001/4	Soil, Dombay valley, Caucasus mountains, Russian Federation, 2001	–

*Determined by PCR with *mcyE* gene-specific primers.
†Strain described previously by Hrouzek *et al.* (2003) (*a*) or Zapomělová (2004) (*b*).

PCR and sequencing. The 16S rRNA gene and ITS region were amplified with primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) (Edwards *et al.*, 1989) and B23S (5′-CTTCGCCTCTGTGTGCCT-AGGT-3′) (Lepère *et al.*, 2000) as described in Gkelis *et al.* (2005). The 16S rRNA gene (1432–1439 bp) was sequenced with internal sequencing primers 16S545R, 16S1092R and 16S979F. The *rbcLX* gene region was amplified and sequenced (782–1003 bp) with primers CX (5′-GGCGCAGGTAAGAAAGGGTTTCGTA-3′) and CW (5′-CGTAGCTTCCGGTGGTATCCACGT-3′) as described by Rudi *et al.* (1998). Despite several trials, amplification of *N. muscorum* strains was not successful and sequences were not obtained. Amplification of the partial *rpoB* gene was performed with primer pair rpoBF (5′-GTAGTTGTARCCNTCCCA-3′) and rpoBR (5′-RCMGCMGACGA-AGAAGACG-3′) or primer pair rpoBanaF (5′-AGCMACMGGTG-ACGTTCC-3′) and rpoBanaR (5′-CNTCCCARGGCATATAGGC-3′), which were designed in this study. For the rpoBF–rpoBR primer pair, amplification was carried out in 50 µl 1× DyNAzyme buffer containing 1·6 U DyNAzyme polymerase (Finnzymes), 0·2 mM each dNTP, 0·2 µM primers and 0·5 µl target DNA. PCR amplification consisted of initial denaturation for 5 min at 94 °C, 30 cycles of amplification: 1 min at 94 °C, 1·5 min at 50 °C and 2 min at 72 °C, and a final elongation for 7 min at 72 °C. For the rpoBanaF–rpoBanaR primer pair, 0·8 U Super Taq Plus polymerase (HT Biotechnology LTA) and 1× Super Taq Plus buffer (HT Biotechnology LTA) replaced DyNAzyme polymerase and buffer, 1 mg BSA ml^{-1} was added and elongation steps of PCR were performed at 68 °C. The *rpoB* fragments of strains 1tu28s8, 1tu33s10, 1tu30s13 and 1tu33s12 were cloned with InsT/Aclone PCR product cloning kit (Fermentas) in order to get high-quality sequences. The *rpoB* gene fragment (520–635 bp) was sequenced with the primers used in the amplification. In addition to studied strains, 13 reference strains, *Anabaena* sp. PCC 7108, 14, 123 and 277; *Anabaena flos-aquae* 202A1, *Anabaena lemmermannii* 66A, *Aphanizomenon* sp. TR183 and 202; *Aphanizomenon flos-aquae* PCC 7905, *Nostoc punctiforme* PCC 72102 and *Nodularia* sp. HEM, HKVV and PCC 7804, were amplified and sequenced. Sequencing of all genes was performed with an Applied Biosystems Big Dye Terminator cycle sequencing kit and 3700 sequencer at Genome Express (Meylan, France) or with an Applied Biosystems PRISM 310 sequencer according to the manufacturer's instructions. The potential microcystin production of strains was based on the detection of the *mcyE* gene in PCR with the specific primers mcyE-F2 and mcyE-R4 as described by Rantala *et al.* (2004).

Phylogenetic analysis. Sequences were aligned in the program ARB (http://www.arb-home.de). The alignment was edited manually and ambiguous bases and hypervariable regions were removed. The highly variable intergenic spacer region between *rbcL* and *rbcX* genes as well as the variable indel region in *rpoB* (positions 322–477 in the alignment) did not allow reliable alignment, and therefore these regions were excluded from the analysis. The *rbcLX* and *rpoB* sequences were studied based on three datasets: one containing all codon positions, one containing only the first and second codon positions of *rbcLX* or *rpoB* genes and a third containing translated amino acid sequences. Only minor differences were found in a comparison of the datasets and the few conflicting nodes had bootstrap support below 65 %. Resolution of clusters and bootstrap values were higher when the third codon positions were included. Therefore, the analysis of *rpoB* and *rbcLX* shown here included all codon positions of the genes. Altogether, 1393 bp of the 16S rRNA gene, 606 bp of *rbcLX* and 451 bp of *rpoB* were used for sequence analysis. Trees based on the 16S rRNA gene, *rbcLX* and *rpoB* were constructed by neighbour-joining (NJ) (Saitou & Nei, 1987) and maximum-parsimony (MP) algorithms in the program PAUP* v10b (Swofford, 2003) and by the maximum-likelihood (ML) algorithm in PHYLIP v3.6 (Felsenstein, 1993). For NJ, the evolutionary model of substitution was evaluated by the program MODELTEST v.3.06 (Posada & Crandall, 1998). The GTR+I+G, TrN+I+G and SYM+I+G evolutionary models of substitution were found to fit the data best for the 16S rRNA gene, *rbcLX* and *rpoB*, respectively. The parameters (base frequencies, rate matrix of substitution types and shape of gamma distribution) were estimated from the data. For NJ and MP analysis, 1000 bootstrap replicates were performed. For ML analysis, only 10 bootstrap replicates were performed due to limited computing power. In addition to these analyses, GTR+G+I evolutionary distances based on the 16S rRNA gene were analysed non-hierarchically with PCA in the program CAP (PISCES). Kishino–Hasegawa (Kishino & Hasegawa, 1989), Templeton (Templeton, 1983) and Winning-Sites tests (Prager & Wilson, 1988) were used to compare the alternative phylogenetic trees based on 16S rRNA gene sequences. The monophyly of planktic *Anabaena*/*Aphanizomenon*, only *Aphanizomenon* and only planktic *Anabaena* sequences as well as subclusters A, F and G were tested as implemented in PAUP* v10b (Swofford, 2003).

RESULTS AND DISCUSSION

Phenotypic analysis of the strains

The 51 strains studied were morphologically heterogeneous. Most of them were planktic *Anabaena* and *Aphanizomenon* strains that were identified as belonging to three *Aphanizomenon* species, *Aphanizomenon flos-aquae* Ralfs ex Born. et Flah., *Aphanizomenon gracile* (Lemm.) Lemm. and *Aphanizomenon issatschenkoi* (Usač) Prošk.-Lavr., and 10 *Anabaena* species: *Anabaena crassa* (Lemm.) Kom.-Legn. et CronB., *Anabaena circinalis* Rabenh. ex Born.

Table 2. Selected morphological characteristics of the studied cyanobacterial strains

The morphology of *Nostoc muscorum*, *N. calcicola*, *N. ellipsosporum* and *N. edaphicum* was described previously by Hrouzek *et al.* (2003).

Strain	Gas vesicles	Shape of terminal cell	Trichome width (μm)*	Akinete			Akinete–heterocyte distance†
				Shape	Width (μm)*	Length (μm)*	
An. cf. *circinalis* var. *macrospora*							
1tu23s3	+	Rounded	5·0 (5·4, 4·4)	Cylindrical with rounded ends	6·9 (7·9, 4·5)	16·1 (21·9, 13·2)	ND
1tu26s10	+	Rounded	4·5 (4·9, 4·0)	Oval	6·3 (7·6, 4·5)	18·6 (26·6, 13·3)	ND
1tu27s5	+	Rounded	4·7 (5·1, 4·1)	Oval	5·9 (7·1, 5·2)	20·4 (24·6, 13·3)	ND
1tu28s13	+	Rounded	3·8 (5·4, 1·7)	Cylindrical	5·5 (8·5, 7·0)	23·0 (37·0, 9·9)	0–1
0tu25s6	+	Rounded	4·9 (5·4, 4·4)	Oval	6·5 (8·2, 5·8)	18·5 (24·3, 12·4)	ND
An. cf. *crassa* 1tu27s7	+	Rounded	6·8 (8·8, 5·0)	Not observed	–	–	–
An. circinalis							
1tu34s5	+	Rounded	8·0 (10·1, 7·8)	Oval	12·3 (14·0, 9·7)	21·0 (30·0,12·0)	0–2
1tu30s11	+	Rounded	8·0 (9·0, 7·4)	Not observed	–	–	–
1tu33s12	+	Rounded	8·2 (9·1, 7·3)	Not observed	–	–	–
An. compacta ANACOM-KOR‡	+	Rounded	4·6 (6·2, 2·9)	Rounded to oval	10·0 (14·8, 5·2)	11·5 (17·3, 5·2)	ND
An. flos-aquae							
1tu31s11	+	Rounded	4·9 (6·3, 4·0)	Rounded to oval	7·7 (8·5, 6·3)	13·9 (15·0, 10·0)	1–2
0tu33s15	+	Hyaline	6·1 (7·6, 3·2)	Oval, slightly curved	8·7 (11·7, 7·1)	17·6 (22·0, 13·5)	ND
0tu33s2a	+	Hyaline	5·6 (6·5, 5·2)	Oval	7·2 (7·9, 6·7)	15·9 (18·6, 11·6)	ND
1tu30s4	+	Hyaline	4·6 (5·3, 4·0)	Not observed	–	–	–
1tu35s12	+	Hyaline	5·1 (5·5, 4·7)	Not observed	–	–	–
An. lemmermannii 1tu32s11	+	Rounded	5·0 (5·0, 3·0)	Oval to cylindrical	6·3 (8·5, 7·0)	11·2 (16·3, 12·0)	ND
An. mucosa 1tu35s5	+	Rounded	9·0 (10·1, 8·2)	Spherical	14·1 (16·2, 12·5)	10·0 (18·0, 13·3)	ND
An. planctonica							
1tu33s10	+	Rounded	5·6 (7·1, 4·1)	Oval to rounded	10·9 (12·8, 9·7)	19·1 (23·5, 13·7)	ND
1tu28s8	+	Rounded	6·2 (6·8, 5·6)	Oval	11·4 (13·7, 10·0)	18·8 (23·7, 15·5)	ND
1tu30s13	+	Rounded	6·3 (11·0, 5·6)	Oval	12·3 (15·0, 9·1)	21·7 (27·0, 13·0)	0–6
1tu33s8	+	Rounded	6·5 (6·9, 5·9)	Not observed	–	–	–
1tu36s8	+	Rounded	5·4 (10·0, 5·2)	Oval to rounded	15·2 (19·1, 12·0)	17·6 (27·6, 12·0)	0–6
An. sigmoidea							
0tu36s7	+	Rounded	4·9 (5·8, 3·8)	Oval	8·5 (9·5, 7·6)	18·1 (20·2, 15·9)	ND
0tu38s4	+	Rounded	4·0 (4·3, 3·8)	Oval, slightly curved	6·0 (6·8, 4·3)	14·1 (15·5, 12·4)	ND
An. smithii 1tu39s8	+	Rounded	9·3 (12·0, 7·2)	Oval to rounded	17·4 (22·4, 13·3)	20·6 (34·2, 15·7)	0–6
Anabaena sp. 0tu37s9	+	Rounded	7·3 (8·4, 6·9)	Not observed	–	–	–
Anabaena sp. 0tu39s7	+	Rounded	7·3 (9·0, 6·5)	Not observed	–	–	–
An. spiroides 1tu39s17	+	Rounded	7·5 (8·4, 6·4)	Oval	11·8 (19·5, 9·5)	18·8 (23·0, 11·5)	ND
Ap. flos-aquae							
1tu29s19	+	Hyaline	4·2 (5·9, 2·9)	Cylindrical	5·9 (8·0, 4·3)	26·6 (39·0, 17·0)	3–12
1tu37s13	+	Hyaline	4·0 (5·4, 2·7)	Cylindrical	7·3 (9·2, 6·1)	62·5 (91·0, 37·0)	10–12
1tu26s2	+	Hyaline	4·5 (5·3, 3·3)	Cylindrical	6·1 (7·9, 4·9)	38·0 (78·2, 7·3)	10–12
Ap. gracile							
Heaney/Camb1986 140 1/1	+	Tapered	4·5 (7·5, 2·5)	Cylindrical	7·2 (12·5, 5·0)	39·5 (95·0, 20·0)	0
1tu26s16	+	Tapered	3·7 (4·4, 2·7)	Cylindrical	5·0 (5·0, 5·0)	11·6 (12·5, 11·3)	2–5
Ap. issatschenkoi 0tu37s7	+	Elongated, pointed	3·1 (4·0, 2·0)	Cylindrical	4·5 (5·5, 3·3)	17·7 (25·5, 10·9)	5–11

Table 2. cont.

Strain	Gas vesicles	Shape of terminal cell	Trichome width (μm)*	Akinete			Akinete–heterocyte distance†
				Shape	Width (μm)*	Length (μm)*	
An. augstumalis SCMIDKE JAHNKE/4a	–	Conical	3·0 (6·1, 1·5)	Oval	7·0 (9·2, 5·3)	18·3 (22·0, 13·0)	0–1
An. cf. *cylindrica* XP6B	–	Rounded	4·4 (5·4, 3·3)	Not observed	–	–	–
An. oscillarioides							
BECID22	–	Rounded	4·2 (5·4, 3·4)	Oval	6·6 (7·9, 5·7)	11·1 (21·0, 11·0)	0
BECID32	–	Rounded	5·5 (2·2, 3·3)	Oval	6·8 (7·9, 5·0)	15·0 (21·0, 11·3)	0
BO HINDAK 1984/43	–	Conical	3·8 (5·2, 2·3)	Not observed	–	–	–
Anabaena sp. 1tu34s7	–	Rounded	2·6 (4·5, 1·2)	Oval	4·5 (6·9, 2·1)	10·2 (15·5, 4·5)	2–10
Anabaena sp. 277	–	Rounded	5·1 (6·6, 3·6)	Not observed	–	–	–
Nostoc sp. 1tu14s8	–	Rounded	3·2 (4·2, 2·3)	Oval	4·1 (5·5, 3·4)	3·2 (4·3, 2·6)	2–10
T. azollae BAI/1983	–	Rounded	4·0 (5·3, 1·4)	Oval	7·5 (7·5, 7·5)	14·5 (15·0, 12·5)	0–14
T. doliolum 1	–	Conical	2·3 (3·0, 1·6)	Lenticular	2·7 (3·7, 1·9)	4·6 (6·5, 3·5)	4–10
T. variabilis							
GREIFSWALD	–	Conical	4·3 (7·7, 2·2)	Oval, slightly compressed in the middle	7·4 (7·4, 7·4)	13·1 (14·8, 12·3)	0–7
HINDAK 2001/4	–	Conical	6·7 (9·6, 2·1)	Oval, slightly compressed in the middle	6·9 (7·4, 5·0)	11·3 (14·8, 8·0)	0–10

*Numbers are means (maximum and minimum values).
†Presented as a number of cells between heterocyte and akinete.
‡Described by Zapomělová (2004).

et Flah., *Anabaena planctonica* Brunnth., *Anabaena mucosa* Kom.-Legn. et Eloranta, *Anabaena spiroides* Kleb., *Anabaena smithii* (Kom.) M. Watan., *Anabaena sigmoidea* Nyg., *Anabaena flos-aquae* [Lyngb.] Bréb. ex Born. et Flah., *Anabaena* cf. *circinalis* var. *macrospora* and *Anabaena lemmermannii* Richt., according to traditional morphological criteria (Geitler, 1932; Desikachary, 1959; Komárek & Anagnostidis, 1989). In addition, nine benthic strains were identified as *Anabaena oscillarioides* Bory ex Born. et Flah., *Anabaena* cf. *cylindrica* Lemm., *Anabaena augstumalis* Scmidle, *Trichormus variabilis* (Born. et Flah.) Kom. et Anag., *Trichormus azollae* (Strasb.) Kom. et Anag., *Trichormus doliolum* (Bharadw.) Kom. et Anag. and *Nostoc* sp. according to traditional morphological criteria. Heterocytes were present in all strains, whereas akinetes were not observed in 12 of the strains studied (Table 2). *Aphanizomenon flos-aquae* strains lost their fascicle-like colony structure during laboratory cultivation. The morphological characteristics of the strains are summarized in Table 2. Other morphological characters measured are available as supplementary material in IJSEM Online. Microphotographs of the selected strains and their important features are shown in Figs 1 and 2.

Aphanizomenon and *Anabaena* strains differed significantly by the mean width of vegetative cells, although the width of vegetative cells of some *Anabaena* (e.g. *Anabaena* cf. *circinalis* var. *macrospora* 1tu28s13) and *Aphanizomenon* strains was overlapping. Generally, the variability in width and length of vegetative cells, heterocytes and akinetes was high (Table 2), which complicates the use of these characters for identification and separation of *Anabaena* and *Aphanizomenon*. These genera were also distinguished by the morphology of the end cells of the trichome which was rounded to oval in planktic *Anabaena* strains and elongated-hyaline to tapered in *Aphanizomenon* strains.

Aphanizomenon strains had more or less straight trichomes, slightly constricted at the cross-walls, and their vegetative cells were from barrel-shaped to cylindrical (Fig. 1). The *Aphanizomenon issatschenkoi* strain was clearly distinguishable from the other *Aphanizomenon* strains by elongated and pointed terminal cells (Table 2; Fig. 1j, k). *Aphanizomenon flos-aquae* strains were characterized by hyaline end cells, cylindrical and long akinetes (up to 91 μm) and a long distance between the heterocytes and akinetes (Table 2; Fig. 1d, e). *Aphanizomenon gracile* trichomes were slightly tapered, but not pointed, and differed from *Aphanizomenon flos-aquae* by the absence of long hyaline end cells and shorter akinetes (Table 2; Fig. 1i, h).

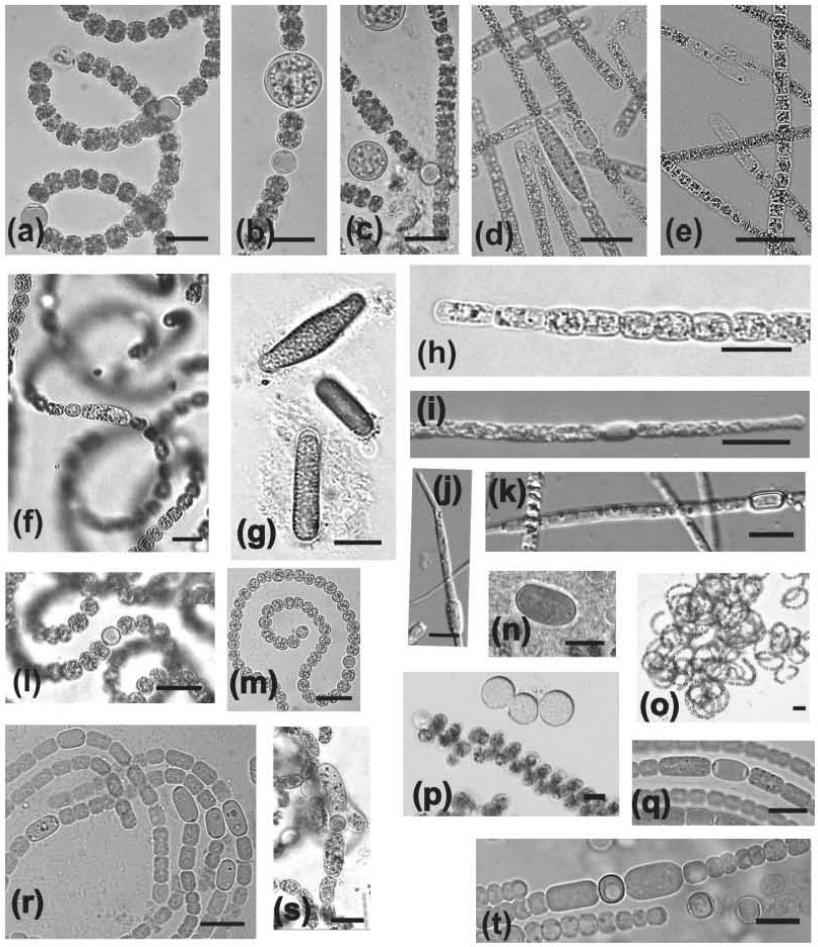


Fig. 1. Microphotographs of some *Anabaena* and *Aphanizomenon* strains in phylogenetic cluster 1, showing important features of the strains. (a) *Anabaena* cf. *crassa* 1tu27S7; (b) *Anabaena planctonica* 1tu30s13; (c) *Anabaena smithii* 1tu39s8; (d) *Aphanizomenon flos-aquae* 1tu26s2; (e) *Aphanizomenon flos-aquae* 1tu37S13; (f) *Anabaena* cf. *circinalis* var. *macrospora* 1tu28s13; (g) akinetes of *Anabaena* cf. *circinalis* var. *macrospora* 1tu23s3; (h) *Aphanizomenon gracile* 1tu26s16; (i) *Aphanizomenon gracile* 1tu26s16; (j) akinete of *Aphanizomenon issatschenkoi* 0tu37s7; (k) *Aphanizomenon issatschenkoi* 0tu37s7; (l) *Anabaena flos-aquae* 1tu33s2a; (m) *Anabaena circinalis* 1tu30s11; (n) akinete of *Anabaena flos-aquae* 1tu33s2a; (o) *Anabaena lemmermannii* 1tu32s11; (p) *Anabaena compacta* ANACOM-KOR; (q) *Anabaena oscillarioides* BECID23; (r) *Anabaena* cf. *cylindrica* XP6B; (s) akinete of *Anabaena lemmermannii* 1tu32s11; (t) *Anabaena oscillarioides* BECID22. Bars, 10 μm.

Anabaena strains can be divided into two groups according to their habitats (Table 2). Planktic species had gas vesicles, which were absent in benthic species. *Anabaena* sp. 1tu34s7, which was isolated from the plankton, did not have gas vesicles and we suspect that it was of benthic origin. In planktic *Anabaena* strains, trichomes varied from coiled to straight (Fig. 1) and two morphological groups were recognized according to the trichome width. Strains of *Anabaena* cf. *crassa*, *Anabaena circinalis*, *Anabaena planctonica*, *Anabaena spiroides*, *Anabaena smithii*, *Anabaena mucosa* and *Anabaena sigmoidea* had significantly ($P<0{\cdot}05$) wider trichomes, heterocytes and akinetes than strains of *Anabaena flos-aquae*, *Anabaena lemmermannii* and *Anabaena* cf. *circinalis* var. *macrospora* (Table 2).

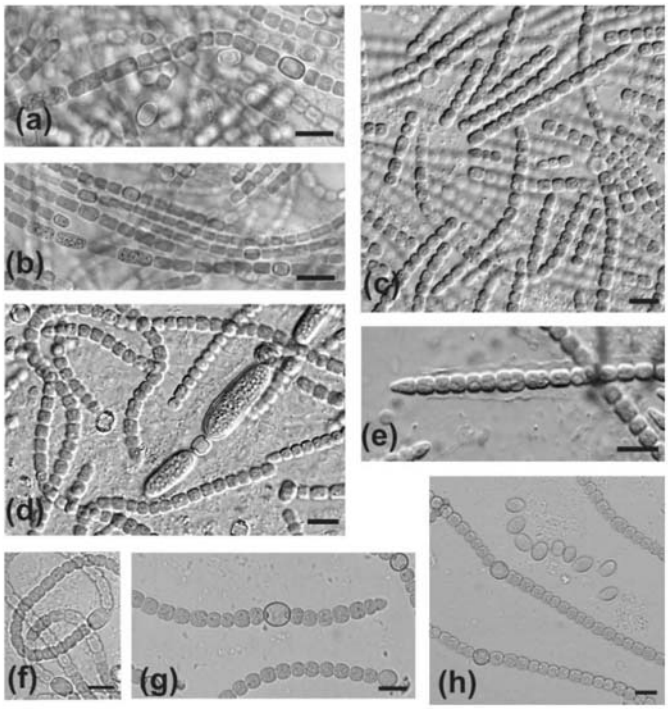


Fig. 2. Microphotographs of some *Anabaena* and *Trichormus* strains in phylogenetic clusters 2–6, showing important features of the strains. (a) *T. variabilis* HINDAK 2001/4; (b) *T. variabilis* GREIFSWALD; (c) *Anabaena* cf. *oscillarioides* BO HINDAK 1984/43; (d) and (e) *Anabaena augstumalis* SCHMIDKE JAHNKE/4a; (f) *T. azollae* BAI/1983; (g) and (h) *T. doliolum* 1. Bars, 10 μm.

However, some transition types with overlapping values were found (e.g. *Anabaena circinalis* and *Anabaena flos-aquae*) (Table 2). Benthic *Anabaena* strains had flexuous trichomes with a diffuse mucilaginous sheath and typical oval morphology of akinetes and terminal heterocytes (Fig. 2).

The genus *Trichormus* was morphologically variable. The special lenticular-shaped akinetes separated *T. doliolum* from all other investigated strains (Fig. 2h). Both strains of *Trichormus variabilis* were morphologically similar, with long wavy filaments and apoheterocytic development of akinetes (Fig. 2a, b). *Trichormus azollae*, a cyanobiont of *Azolla* fern, was morphologically related to the *Nostoc* strains. Similarly to some *Nostoc* strains (*N. muscorum*, *N. ellipsosporum*), it formed long, irregularly coiled trichomes surrounded by a diffuse mucilaginous envelope (Fig. 2f). In addition, the type of akinete development (starting from the middle of the filament between two heterocytes) was similar to that of several *Nostoc* strains. Morphology of the *Nostoc* strains was previously described by Hrouzek *et al.* (2003).

Our results indicated that parameters of akinetes were important taxonomic characters. PCA of all morphological characters showed that most of the total variance is attributable to variance in width and length of akinetes (Fig. 3). Moreover, the shape of the akinetes was quite

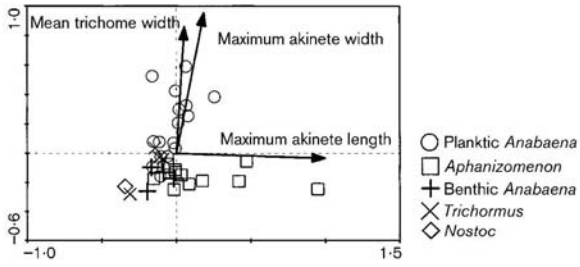


Fig. 3. PCA plot based on the morphological characteristics of studied cyanobacterial strains. The most variable characteristics were length and width of akinetes as well as width of trichomes (shown by the arrows). The first and second principal components accounted for 99% of the total variance.

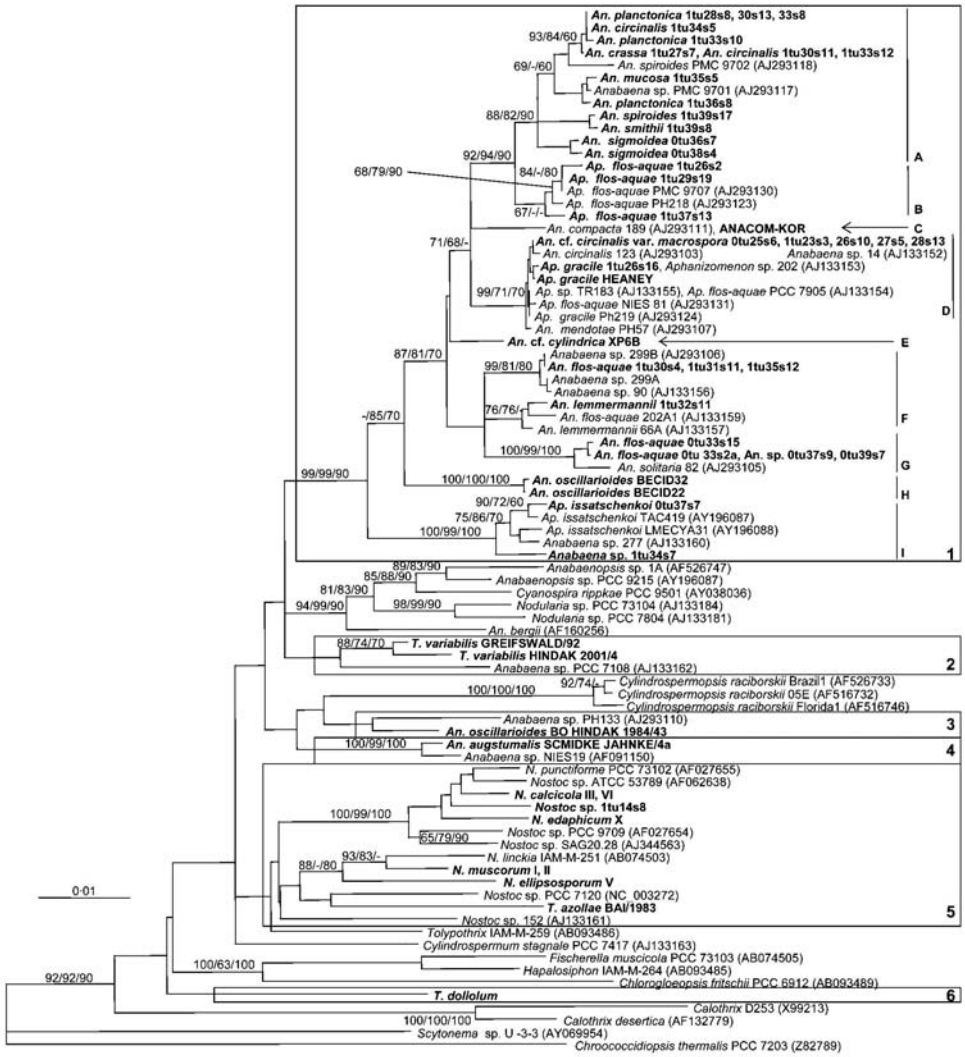


Fig. 4. Neighbour-joining tree based on 16S rRNA gene sequences (1393 bp) showing the clustering of studied *Anabaena* (abbreviated as *An.*), *Aphanizomenon* (*Ap.*), *Trichormus* (*T.*) and *Nostoc* (*N.*) strains (in bold). Numbers near nodes indicate bootstrap values over 65% for NJ, MP and ML analyses.

stable. Previously, Stulp & Stam (1982, 1985) found the position of akinetes, shape of terminal cells and width of vegetative cells to be useful taxonomic characters for *Anabaena*. These morphological features were retained in different light and temperature conditions (Stulp & Stam, 1985) and even brackish water conditions (Stulp & Stam, 1984a).

Genetic relationships of the studied strains

Six clusters were consistently formed in the analysis of 16S rRNA, *rpoB* and *rbcLX* genes: cluster 1 contained all planktic *Anabaena* and *Aphanizomenon* strains as well as five benthic *Anabaena* strains; clusters 2, 3, 4 and 6 contained benthic strains *T. variabilis*, *Anabaena oscillarioides*

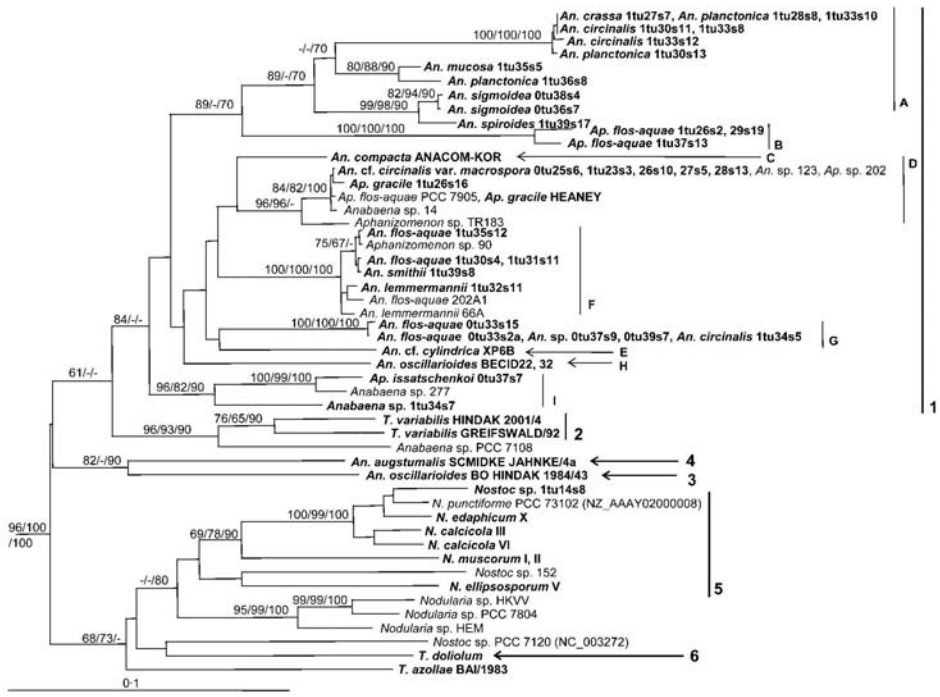


Fig. 5. Neighbour-joining tree based on *rpoB* sequences (451 bp) showing the clustering of studied *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* strains (in bold). Numbers near nodes indicate bootstrap values over 65% for NJ, MP and ML analyses. The outgroup taxa, *Microcystis* sp. 130 and *Planktothrix* NIVA-CYA126, are not shown.

BO HINDAK 2001/4, *Anabaena augstumalis* and *T. doliolum*, respectively; and cluster 5 contained all *Nostoc* strains and *T. azollae*. The tree topologies were similar with all tree-constructing methods, and therefore only the NJ tree is presented for each gene (Figs 4, 5 and 6). The overall topology was in agreement for the 16S rRNA gene, *rbcLX* and *rpoB* trees. However, within closely related *Anabaena* strains, some differences were found: *Anabaena smithii* 1tu39s8 clustered with hepatotoxic *Anabaena* strains in the *rpoB* tree and *Anabaena circinalis* 1tu34s5 with *Anabaena flos-aquae* strains in the *rpoB* and *rbcLX* trees instead of subcluster A (Figs 4, 5 and 6). Otherwise, the few conflicting nodes between gene trees received only low bootstrap support. These were generally lower in *rbcLX* and *rpoB* trees than in the 16S rRNA gene tree, probably because of a smaller number of variable bases. Rudi *et al.* (1998) also found that the topologies of the 16S rRNA gene and *rbcLX* trees were not congruent for genetically closely related *Nostoc* and *Anabaena* strains and stated that this was due to lateral gene transfer between the strains. Nevertheless, in the present study, lateral gene transfer did not seem to play a major role in determining the topologies of gene trees.

Since hierarchical clustering of 16S rRNA, *rpoB* and *rbcLX* gene sequences did not receive high bootstrap support for all the clusters and subclusters of all gene trees, PCA was performed. All clusters were also found in PCA, confirming the validity of the hierarchical clustering (data not shown). Principal components 1 and 2 together accounted for 84·6% of the total variance in the 16S rRNA distance matrix.

Genetic data did not support the distinction of planktic *Anabaena* and *Aphanizomenon* from benthic *Anabaena* strains as did the morphological data. The benthic *Anabaena* strains BECID22, BECID32, XP6B, 1tu34s7 and 277, which lack visible gas vesicles, were intermixed with planktic *Anabaena* and *Aphanizomenon* strains in 16S rRNA, *rpoB* and *rbcLX* gene trees with high bootstrap support (cluster 1 in Figs 4, 5 and 6). Moreover, a 16S rRNA gene tree with the forced monophyly of planktic *Anabaena*/*Aphanizomenon* strains was significantly worse ($P = 0{\cdot}0010$–$0{\cdot}0013$) than the original tree in all tests performed. Thus, these tests did not support the separation of planktic and benthic *Anabaena*/*Aphanizomenon* strains. This finding contradicts

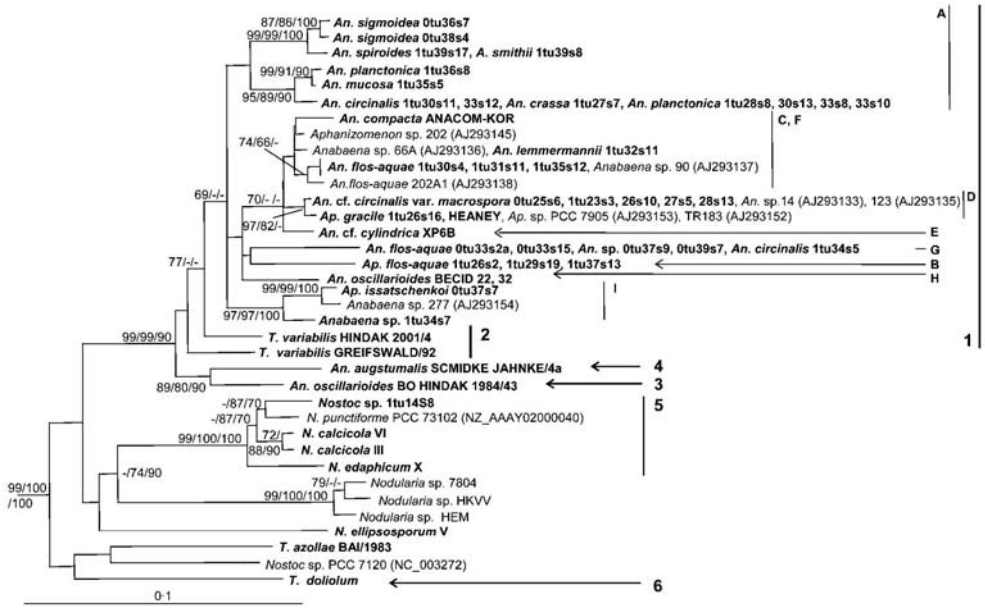


Fig. 6. Neighbour-joining tree based on *rbcLX* sequences (606 bp) showing the clustering of studied *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* strains (in bold). Numbers near nodes indicate bootstrap values over 65 % for NJ, MP and ML analyses. The outgroup taxa, *Microcystis* sp. 130 (Z94894) and *Planktothrix* NIVA-CYA126 (Z94873), are not shown.

Iteman *et al.* (2002), who found a distinct subcluster of planktic heterocytous cyanobacteria with the exception of *Cylindrospermopsis*. Other benthic *Anabaena*/*Trichormus* strains were placed outside cluster 1, which contained all planktic and five benthic *Anabaena* as well as all *Aphanizomenon* strains.

Anabaena and *Aphanizomenon* strains in cluster 1 were genetically heterogeneous (16S rRNA gene sequence similarity >94·8 %) and intermixed in all gene trees (cluster 1 in Figs 4, 5 and 6), confirming the results of earlier studies with other *Anabaena* and *Aphanizomenon* strains (Lyra *et al.*, 2001; Gugger *et al.*, 2002b; Iteman *et al.*, 2002). In addition, the monophyly of *Aphanizomenon* or planktic *Anabaena* was rejected in all statistical tests ($P=0{\cdot}0001$). The monophyly of *Aphanizomenon* strains was proposed in a recent paper of Li *et al.* (2003), but in that study planktic *Anabaena* sequences were not included in the analysis.

Cluster 1, containing all planktic and five benthic *Anabaena* as well as all *Aphanizomenon* strains, was divided into nine subclusters, which mostly received high bootstrap support in all the gene trees (subclusters A–I in Figs 4, 5 and 6). Morphologically, the strains in this cluster were separated from the other strains including benthic strains in clusters 2–4 by absence of terminal heterocysts. The cut-off points 97·5 and 95 % 16S rRNA gene sequence similarity have been suggested for bacterial species and genus definition, respectively (Stackebrandt & Goebel, 1994; Ludwig *et al.*, 1998). According to those definitions, the evolutionary distances of this study suggested that the strains in cluster 1 could be divided into two to three species belonging to a single genus (Table 3). Cluster 1 could also be divided into more than three species (up to nine) according to the

Table 3. Matrix showing *P*-distances based on the 16S rRNA gene (1386 bp) between the subclusters of cluster 1

Subcluster	A	B	C	D	E	F	G	H	I
A	98·6								
B	99·1	99·4							
C	98·3	98·2	100						
D	98·4	98·8	98·5	99·8					
E	98·0	98·0	98·9	98·6	–				
F	97·7	98·0	98·8	98·1	99·2	98·8			
G	96·8	97·0	97·4	97·6	97·9	98·4	99·4		
H	96·5	96·8	97·4	96·9	98·2	98·0	98·0	99·9	
I	95·4	95·7	96·3	96·3	96·6	97·1	96·7	97·0	98·8

subclustering of strains in phylogenetic trees and morphological data presented here, although the evolutionary distances between subclusters were >97·5 %. Most of the phylogenetic subclusters found in all the gene trees were also defined by morphological characters. In addition, the evolutionary distances between the subclusters were discontinuous (Table 3). The subclustering was also reflected in the length and the sequence of the variable indel region of the *rpoB* gene and was confirmed in the PCA of the 16S rRNA gene (data not shown). In addition, the strains in each subcluster shared a similar denaturing-gradient gel electrophoresis (DGGE) pattern of 16S rRNA gene fragments (450 bp) (unpublished results). The studied strains had from one to six different copies of the 16S rRNA gene according to DGGE analysis. These findings further support the subclustering of *Anabaena*/*Aphanizomenon* strains. However, the clustering of the studied *Anabaena* and *Aphanizomenon* strains did not follow the current classification of the genera. In future, DNA–DNA reassociation studies will be able to give more evidence for relationships of studied strains at the species level. Previously, Stam & Stulp (1984b, 1985) found in DNA–DNA reassociation studies that the morphology of *Anabaena* strains was reflected in their genetic relationships. However, our studies indicated that the classification of *Anabaena* strains might be more complicated.

The highly supported subcluster A contained various planktic *Anabaena* strains, *Anabaena planctonica*, *Anabaena crassa*, *Anabaena mucosa*, *Anabaena spiroides*, *Anabaena smithii* and *Anabaena sigmoidea* (Figs 4, 5 and 6), which shared relatively high 16S rRNA gene sequence similarity (>98·6 %). Both irregularly coiled *Anabaena* strains such as *Anabaena circinalis* and straight *Anabaena* species such as *Anabaena planctonica* strains were included in subcluster A, indicating that coiling of the trichome was not useful for classification of *Anabaena* morphotypes. The strains were otherwise morphologically relatively similar to each other and to *Anabaena* strains in subcluster F and G. Nevertheless, a few morphological differences were found between these subclusters. Strains in subcluster A had significantly wider heterocytes, akinetes and trichomes than other *Anabaena* strains in cluster F and G. Thus, the close relationship and shared morphological features suggest that the strains in subcluster A might be related at the species level.

Aphanizomenon flos-aquae strains were included in subcluster B in all the gene trees, although the bootstrap support was low in MP and ML analysis of the 16S rRNA gene (Figs 4, 5 and 6). These strains were closely related and shared identical *rbcLX* gene sequences and highly similar 16S rRNA (>99·4 %) and *rpoB* (>99·8 %) genes. Also, in the study of Gugger *et al.* (2002b) a cluster of mainly *Aphanizomenon flos-aquae* sequences was found in 16S rRNA gene and *rbcLX* trees, but it was not resolved in the ITS tree. The studied *Aphanizomenon flos-aquae* strains shared several morphological features, e.g. hyaline end cells, long akinetes and originally fascicle-like colonies, which separate these strains from the other *Anabaena* and also *Aphanizomenon* strains. The proposed type strain of *Aphanizomenon flos-aquae*, PCC 7905, did not cluster with the *Aphanizomenon flos-aquae* strains of this study. However, Rippka *et al.* (2001b) pointed out that the morphology of the PCC 7905 now in culture does not correspond well with the description of *Aphanizomenon flos-aquae*. The *Aphanizomenon* strains TR183 and 202 in cluster D, described previously as *Aphanizomenon flos-aquae* (Gugger *et al.*, 2002b), are called *Aphanizomenon* sp. in this study, because the original identification of these strains was problematic (Lyra *et al.*, 2001). No morphological description of strain NIES81 was found. *Aphanizomenon flos-aquae* seems to lose its typical colony structure in laboratory cultivation, which might complicate its identification (Gugger *et al.*, 2002b; this study). However, phylogenetic and morphological data from this study suggest that *Aphanizomenon flos-aquae* might form its own species.

Anabaena compacta ANACOM-KOR from the Czech Republic shared an identical 16S rRNA gene sequence with the previously described *Anabaena compacta* strain 189 from Denmark (Gugger *et al.*, 2002b), and these strains formed a distinct cluster, C, in the16S rRNA and *rpoB* trees (Figs 4 and 5). In the *rbcLX* analysis, *Anabaena compacta* ANACOM-KOR was clustered together with hepatotoxic *Anabaena* strains in cluster F (Fig. 6). *Anabaena compacta* was easily identified by its solitary, densely and regularly coiled trichomes (Fig. 1) and thus the morphology was in accordance with genetic data. The dense coiling of *Anabaena compacta* ANACOM-KOR was stable in culture and seems to be characteristic for this species, although the coiling was not a useful feature for the classification of other *Anabaena* species.

Aphanizomenon gracile and *Anabaena* cf. *circinalis* var. *macrospora* strains were closely related and intermixed in all the gene trees (cluster D in Figs 4, 5 and 6; Table 3). The cluster also included neurotoxic *Anabaena* strains described by Gugger *et al.* (2002b) and Lyra *et al.* (2001). This close relationship between neurotoxic *Anabaena* and *Aphanizomenon* strains (PCC 7905, TR183 and 202) had been previously revealed by 16S rRNA-RFLP, the 16S rRNA gene (Lyra *et al.*, 2001; Iteman *et al.*, 2002), *rbcLX* and ITS sequencing (Gugger *et al.*, 2002b), as well as by the analysis of cellular fatty acids (Gugger *et al.*, 2002a). The morphological similarity of *Aphanizomenon gracile* to some *Anabaena* strains has been discussed previously by Komárek & Anagnostidis (1989). These strains grow in solitary trichomes which do not have elongated, hyaline or pointed end cells. Surprisingly, all studied strains (*Anabaena* and *Aphanizomenon*) included in cluster D had very similar morphology and development of akinetes. They all form cylindrical akinetes by fusion of several vegetative cells next to or distant from a heterocyte. This type of akinete development separates it from other subclusters including planktic *Anabaena* strains and could

be used for classification of *Anabaena/Aphanizomenon* species. Previously, the development of akinetes has been found to be a stable character and suggested to have important taxonomic value for *Anabaena* classification (Stulp & Stam, 1982, 1985). The morphological similarities and close evolutionary distances suggest that *Aphanizomenon gracile* and *Anabaena* strains in cluster D might be assignable to a single species.

Benthic *Anabaena cylindrica* XP6B was the only representative of subcluster E (Figs 4, 5 and 6). XP6B was actually closely related to the hepatotoxic strains of subcluster F (16S rRNA sequence similarity >99·2%). Morphologically this strain was separated from strains in subcluster F by a lack of gas vesicles, elliptical vegetative cells and heterocytes as well as presence of diffusive mucilage around trichomes. The benthic strains *Anabaena oscillarioides* BECID22 and BECID32 of cluster 1 formed subcluster H in all the gene trees (Figs 4, 5 and 6) and shared <98·2% 16S rRNA gene sequence similarity with the other subclusters. These BECID strains were morphologically separable from other subclusters by the absence of gas vesicles, cylindrical akinetes (Fig. 1t) and trichomes arranged in fascicle-like formation with diffusive mucilage. More strains closely related to subclusters E and H are needed in order to conclude their morphological similarities and classification. Nevertheless, the close relationship between these benthic *Anabaena* and planktic *Anabaena/Aphanizomenon* strains indicates that gas vesicles are not a useful feature for classification of *Anabaena*.

In all gene trees, subcluster F consists of *Anabaena flos-aquae* and *Anabaena lemmermannii* strains, which were potential microcystin producers according to *mcyE*-PCR. These strains were closely related (16S rRNA gene sequence similarity >98·8%) to each other, but also to non-toxic *Anabaena flos-aquae* strains of cluster G (16S rRNA gene sequence similarity >98·4%) (Fig. 4; Table 3). Cluster F received high bootstrap support only in the *rpoB* analysis (Fig. 5). In the 16S rRNA gene tree, the hepatotoxic strains were divided into two highly supported clusters and in the *rbcLX* analysis also *Aphanizomenon* sp. 202 and *Anabaena compacta* ANACOM-KOR were placed in this cluster (Fig. 6). The close similarity within subcluster F and between it and non-hepatotoxic *Anabaena flos-aquae* strains indicated that the latter might have recently lost their ability to produce microcystins (Rantala *et al.*, 2004). We were not able to find any morphological criteria to distinguish strains from subclusters F and G, which mostly contained strains identified as *Anabaena lemmermannii* and *Anabaena flos-aquae*. These subclusters could be separated only by the phylogenetic analysis and by the potential hepatotoxin production of the strains in subcluster F with the exception of a non-toxic strain, *Anabaena* sp. 299A (this study; Gugger *et al.*, 2002b). Thus, the delineation of morphotypes *Anabaena lemmermannii* and *Anabaena flos-aquae* remains to be confirmed.

Interestingly, planktic *Aphanizomenon issatschenkoi*, 'benthic' *Anabaena* sp. 1tu34s7 and *Anabaena* sp. 277 were grouped together (subcluster I in Figs 4, 5 and 6) and were clearly separated from the other planktic *Anabaena* and *Aphanizomenon* strains in all gene trees as well as in PCA. This cluster shared <96·4% 16S rRNA gene sequence similarity with other clusters/subclusters, indicating that the *Aphanizomenon issatschenkoi* strains, *Anabaena* sp. 277 and 1tu34s7 belong to different species from *Anabaena/Aphanizomenon* strains in cluster 1. The cluster was quite heterogeneous morphologically, containing both benthic and planktic strains. The *Anabaena* strains 1tu34s7 and 277 in this subcluster were isolated from plankton (this study; Lyra *et al.*, 2001) and they might have lost their gas vesicles during cultivation, which has been reported by Rippka *et al.* (2001b) in the case of PCC 7905. However, gas vesicles were not found in 1tu34s7 soon after isolation, and thus at least 1tu34S7 is probably benthic. It is evident that *Aphanizomenon issatschenkoi* strains characterized by solitary trichomes with pointed end cells are both morphologically and genetically clearly separated from the other *Aphanizomenon* strains as well as from typical planktic *Anabaena* strains. The only observed morphological similarity of strains in cluster I was the shape of akinetes, which were cylindrical in *Aphanizomenon issatschenkoi* and *Anabaena* sp. 1tu34s7. Akinetes were not found in *Anabaena* sp. 277.

Trichormus strains, which were separated morphologically from *Anabaena* and *Aphanizomenon* strains by akinete development, were divided into three well-separated clusters, 2, 5 and 6 (Figs 4, 5 and 6), and were not monophyletic. These *Trichormus* strains were found to be morphologically heterogeneous. Two benthic strains with wavy filaments and apoheterocytic akinete formation were assigned as *T. variabilis*. These *T. variabilis* strains GREIFSWALD and HINDAK/2001/4 also shared a high 16S rRNA gene sequence similarity (99·2%) and formed cluster 2, which was loosely grouped with *Anabaena* PCC 7108 (Figs 4 and 5). Also, DNA–DNA reassociation studies of Stulp & Stam (1984b, 1985) with several *Anabaena* species support our results that *Anabaena* (*Trichormus*) *variabilis* strains were clearly separated from other *Anabaena* species. Stulp & Stam (1984b, 1985) found that relative binding values between *Anabaena varibilis* and other *Anabaena* species ranged from 31 to 39%. Interestingly, *T. doliolum*, which was morphologically separated from other *Trichormus* strains by shape of akinete, shared less than 95·3% 16S rRNA gene sequence similarity with any other cyanobacterial sequence and formed cluster 6 (Figs 4, 5 and 6). This indicates that *T. doliolum* might not be related to *Anabaena*, *Trichormus* or *Nostoc* strains at the species or even genus level if the suggested cut-off point for genus definition, 95% 16S rRNA gene sequence similarity, is followed (Ludwig *et al.*, 1998). *T. azollae* BAI/1983 clustered with heterogeneous *Nostoc* strains (Figs 4, 5 and 6) and might actually belong to the genus *Nostoc* rather than to *Trichormus* or *Anabaena*. Similar results were found in analysis of restriction sites in the *nif* region (Meeks *et al.*,

1988). Also, morphological features such as a mucilaginous envelope support the transfer of *T. azollae* to the genus *Nostoc*.

The benthic *Anabaena oscillarioides* BO HINDAK 1984/43 and *Anabaena augstumalis* JAHNKE/4a formed clusters 3 and 4 in the 16S rRNA gene tree (Fig. 4) and these clusters shared 16S rRNA gene sequence similarity <95·5 %. In the *rpoB* and *rbcLX* gene trees, *Anabaena oscillarioides* BO HINDAK 1984/43 and *Anabaena augstumalis* JAHNKE/4a were grouped together, probably because no other closely related sequences of these genes were available (Figs 5 and 6). These *Anabaena* strains were morphologically separated from other *Anabaena* strains in cluster 1 by conical end cells and the presence of terminal heterocytes (Table 2). Among other features, conical end cells were also suggested by Lachance (1981) to separate *Anabaena* and *Nostoc* strains. The benthic *Anabaena*/*Trichormus* clusters 2–4 were grouped with planktic *Anabaena*/*Aphanizomenon* rather than with *Nostoc* strains (Figs 4, 5 and 6). However, the clustering of all *Anabaena*/*Aphanizomenon* and *T. variabilis* received high bootstrap support only in the *rbcLX* tree (Fig. 6). The evolutionary distances between the benthic *Anabaena* strains BO HINDAK 1984/43, JAHNKE/4a and *T. variabilis* as well as *Anabaena*/*Aphanizomenon* strains in cluster 1 were >95·8 %, indicating that these clusters 1–4 are not related at the species level. However, more closely related sequences of benthic *Anabaena* and *Trichormus* are needed before their phylogenetic position and classification can be resolved.

Nostoc strains were separated from genera *Anabaena* and *Trichormus* with the exception of *T. azollae* in all gene trees (Figs 4, 5 and 6) and thus supported the distinction between the genera *Nostoc* and *Anabaena*, as shown previously in studies of 16S rRNA gene sequences (Wilmotte & Herdman, 2001) and the *nifD* gene (Henson *et al.*, 2002). This was opposite to the studies of Tamas *et al.* (2000), which was based on a short fragment of *nifH*. *Nostoc* sequences were heterogeneous (sequence similarity 93·9 % for the 16S rRNA gene) at the bases of all the gene trees. This is in agreement with the DNA–DNA reassociation studies of Lachance (1981). Therefore, the studied *Nostoc* strains may actually represent two different genera. The *Nostoc* strains were clustered together in the 16S rRNA gene tree with low bootstrap support (Figs 4, 5 and 6), and did not form clusters in the *rbcLX* and *rpoB* trees. However, within the *Nostoc* cluster, sequences of *N. calcicola*, *N. edaphicum* and *Nostoc* sp. 1tu14s8 shared high 16S rRNA sequence similarity (97·7 %) and clustered together with high bootstrap values in all the gene trees (Figs 4, 5 and 6). These *Nostoc* strains also shared many common features such as terminal conical heterocytes and narrow, straight hormogonia and were morphologically differentiated from the other studied *Nostoc* strains (Hrouzek *et al.*, 2003; this study). The high sequence and morphological similarity suggest that *N. calcicola*, *N. edaphicum* and *Nostoc* sp. 1tu14s8 could be assigned to a single species. In addition, *N. muscorum* and *N. ellipsosporum* were morphologically (Hrouzek *et al.*, 2003) and genetically more closely related to each other than to the other studied *Nostoc* strains. However, the 16S rRNA gene sequence similarity of these two *Nostoc* strains and *T. azollae* to any other strains was <96·7 %, indicating that these strains are not related to each other or to other *Nostoc* strains at the species level.

Conclusion

This study indicates that planktic *Anabaena*/*Aphanizomenon* and benthic *Anabaena* were not monophyletic, since the planktic *Anabaena* and *Aphanizomenon* as well as five benthic strains in cluster 1 were intermixed. Strains in cluster 1 could be assigned to a single genus according to the genetic data. Cluster 1 could be divided into several (eight or nine) species based on genetic and morphological data, although the 16S rRNA gene sequence similarity between the *Anabaena*/*Aphanizomenon* subclusters was above 97·5 %. Most of these supported phylogenetic *Anabaena*/*Aphanizomenon* subclusters (A–E and H) were found in all the gene trees. Moreover, the strains within the subclusters shared certain morphological features that might be used in classification of the strains at the species level. The subclusters were morphologically separable from each other mainly on the basis of akinete parameters, sometimes in combination with the width of the trichome or potential hepatotoxicity. In contrast, coiling of trichomes, distance between heterocytes or the length of vegetative cells seem not to be useful criteria for separation of the subclusters. *Trichormus* strains were not monophyletic. *T. azollae* might belong to the genus *Nostoc* rather than to *Anabaena*. *T. doliolum* possibly forms a separate genus according to evolutionary distances. *T. variabilis* strains were more closely related to the benthic *Anabaena* strains than *Nostoc* strains, although *T. variabilis* and benthic *Anabaena* strains in clusters 2–4 were genetically relatively heterogeneous. Therefore, more benthic *Anabaena* and *Trichormus* strains need to be studied to confirm their phylogenetic positions. Nevertheless, the shape of end cells and presence of terminal heterocytes were found to be important for discriminating between the benthic *Anabaena* and *T. variabilis* in clusters 2–4 from the *Anabaena* strains in cluster 1. The phylogenetic relationship of the studied strains did not follow the current taxonomic classification of Komárek & Anagnostidis (1989) or that of *Bergey's Manual of Systematic Bacteriology* (Rippka *et al.*, 2001c) and therefore a revision of the taxonomy of these anabaenoid strains is needed.

ACKNOWLEDGEMENTS

This study was funded by the MIDI-CHIP project (EKV2-CT-1999-00026), the Enste Graduate School and the Academy of Finland (grant 201576). We thank Dr Jaana Lehtimäki and Matti Wahlsten for isolating strains XP6B, BECID22 and BECID32, Luydmila Saari for purifying and maintaining the strains during the study and Dr Stefano

Ventura for providing internal 16S rRNA primer sequences. Drs Christina Lyra and David Fewer are thanked for critical reading of the manuscript and helpful comments.

REFERENCES

Desikachary, T. V. (1959). *Cyanophyta part I and II.* New Delhi: Indian Council of Agricultural Research.

Edwards, U., Rogall, T., Blöcker, H., Emde, M. & Böttger, E. C. (1989). Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* **17**, 7843–7853.

Felsenstein, J. (1993). PHYLIP (Phylogeny Inference Package) version 3.6c. Distributed by the author. Department of Genetics, University of Washington, Seattle, USA.

Geitler, L. (1932). Cyanophyceae. In *Kryptogamenflora von Deutschland, Oesterreich und der Schweiz*, vol. XIV. Edited by L. Rabenhorst. Leipzig: Akademische Verlagsgesellschaft (in German).

Gkelis, S., Rajaniemi, P., Vardaka, E., Moustaka-Gouni, M., Lanaras, T. & Sivonen, K. (2005). *Limnothrix redekei* (Van Goor) Meffert (Cyanobacteria) strains from Lake Kastoria, Greece form a separate phylogenetic group. *Microb Ecol* (in press).

Gugger, M. F. & Hoffmann, L. (2004). Polyphyly of the true branching cyanobacteria (Stigonematales). *Int J Syst Evol Microbiol* **54**, 349–357.

Gugger, M., Lyra, C., Suominen, I., Tsitko, I., Humbert, J. F., Salkinoja-Salonen, M. S. & Sivonen, K. (2002a). Cellular fatty acids as chemotaxonomic markers of the genera *Anabaena*, *Aphanizomenon*, *Microcystis*, *Nostoc* and *Planktothrix* (cyanobacteria). *Int J Syst Evol Microbiol* **52**, 1007–1015.

Gugger, M., Lyra, C., Henriksen, P., Couté, A., Humbert, J.-F. & Sivonen, K. (2002b). Phylogenetic comparison of the cyanobacterial genera *Anabaena* and *Aphanizomenon*. *Int J Syst Evol Microbiol* **52**, 1867–1880.

Henson, B. J., Watson, L. E. & Barnum, S. R. (2002). Molecular differentiation of the heterocystous cyanobacteria, *Nostoc* and *Anabaena*, based on complete *nifD* sequences. *Curr Microbiol* **45**, 161–164.

Henson, B. J., Hesselbrock, S. M., Watson, L. E. & Barnum, S. R. (2004). Molecular phylogeny of the heterocystous cyanobacteria (subsections IV and V) based on *nifD*. *Int J Syst Evol Microbiol* **54**, 493–497.

Hönerlager, W., Hahn, D. & Zeyer, J. (1995). Detection of mRNA of *nrpM* in *Bacillus megaterium* ATCC 14581 grown in soil by whole-cell hybridisation. *Arch Microbiol* **163**, 235–241.

Hrouzek, P., Šimek, M. & Komárek, J. (2003). Nitrogenase activity (acetylene reduction activity) and diversity of six soil *Nostoc* strains. *Arch Hydrobiol Suppl* **146**, 87–101.

Iteman, I., Rippka, R., Tandeau de Marsac, N. & Herdman, M. (2002). rDNA analyses of planktonic heterocystous cyanobacteria, including members of the genera *Anabaenopsis* and *Cyanospira*. *Microbiology* **148**, 481–496.

Kishino, H. & Hasegawa, M. (1989). Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data and the branching order in Hominoidea. *J Mol Evol* **29**, 170–179.

Komárek, J. & Anagnostidis, K. (1989). Modern approach to the classification system of Cyanophytes 4 - Nostocales. *Arch Hydrobiol Suppl* **82**, 247–345.

Komárek, J. & Kováčik, L. (1989). Trichome structure of four *Aphanizomenon* taxa (Cyanophyceae) from Czechoslovakia, with notes on the taxonomy and delimitation of the genus. *Plant Syst Evol* **164**, 47–64.

Kotai, J. (1972). *Instructions for preparation of modified nutrient solution Z8 for algae.* Norwegian Institute for Water Research publication B-11/69, pp. 1–5. Oslo: NIWR.

Lachance, M. (1981). Genetic relatedness of heterocystous cyanobacteria by deoxyribonucleic acid-deoxyribonucleic acid reassociation. *Int J Syst Bacteriol* **31**, 139–147.

Lehtimäki, J., Lyra, C., Suomalainen, S., Sundman, P., Rouhiainen, L., Paulin, L., Salkinoja-Salonen, M. & Sivonen, K. (2000). Characterization of *Nodularia* strains, cyanobacteria from brackish waters, by genotypic and phenotypic methods. *Int J Syst Evol Microbiol* **50**, 1043–1053.

Lepère, C., Wilmotte, A. & Meyer, B. (2000). Molecular diversity of *Microcystis* strains (Cyanophyceae, Chroococcales) based on 16S rRNA sequences. *Syst Geogr Plant* **70**, 275–283.

Li, R., Carmichael, W. W. & Pereira, P. (2003). Morphological and 16S rRNA gene evidence for reclassification of the paralytic shellfish toxin producing *Aphanizomenon flos-aquae* LMECYA 31 as *Aphanizomenon issatschenkoi* (Cyanophyceae). *J Phycol* **39**, 814–818.

Ludwig, W., Strunk, O., Klugbauer, S., Weizenegger, M., Neumaier, J., Bachleither, M. & Schleifer, K. H. (1998). Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19**, 554–568.

Lyra, C., Suomalainen, S., Gugger, M., Vezie, C., Sundman, P., Paulin, L. & Sivonen, K. (2001). Molecular characterization of planktic cyanobacteria of *Anabaena*, *Aphanizomenon*, *Microcystis* and *Planktothrix* genera. *Int J Syst Evol Microbiol* **51**, 513–526.

Meeks, J. C., Joseph, C. M. & Haselkorn, R. (1988). Organization of the *nif* genes in cyanobacteria in symbiotic association with *Azolla* and *Anthoceros*. *Arch Microbiol* **150**, 61–71.

Posada, D. & Crandall, K. A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.

Prager, E. M. & Wilson, A. C. (1988). Ancient origin of lactalbumin from lysozyme: analysis of DNA and amino acid sequences. *J Mol Evol* **27**, 326–335.

Rantala, A., Fewer, D. P., Hisbergues, M., Rouhiainen, L., Vaitomaa, J., Börner, T. & Sivonen, K. (2004). Phylogenetic evidence for the early evolution of microcystin synthesis. *Proc Natl Acad Sci U S A* **101**, 568–573.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. & Stanier, R. Y. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J Gen Microbiol* **111**, 1–61.

Rippka, R., Castenholz, R. W. & Herdman, M. (2001a). Subsection IV. (Formerly Nostocales Castenholz 1989b sensu Rippka, Deruelles, Waterbury, Herdman and Stanier 1979). In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, pp. 562–566. Edited by D. R. Boone & R. W. Castenholz. New York: Springer.

Rippka, R., Castenholz, R. W., Iteman, I. & Herdman, M. (2001b). Form-genus I. *Anabaena*. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, pp. 566–568. Edited by D. R. Boone & R. W. Castenholz. New York: Springer.

Rippka, R., Castenholz, R. W., Iteman, I. & Herdman, M. (2001c). Form-genus III. *Aphanizomenon* Morren 1838. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, pp. 569–570. Edited by D. R. Boone & R. W. Castenholz. New York: Springer.

Rudi, K., Skulberg, O. M. & Jakobsen, K. S. (1998). Evolution of cyanobacteria by exchange of genetic material among phyletically related strains. *J Bacteriol* **180**, 3453–3461.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in

the present species definition in Bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Stanier, R. Y., Kunisawa, R., Mandel, M. & Cohen-Bazire, G. (1971). Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriol Rev* **35**, 171–205.

Stulp, B. K. & Stam, W. T. (1982). General morphology and akinete germination of a number of *Anabaena* strains (Cyanophyceae) in culture. *Arch Hydrobiol Suppl* **63**, 35–52.

Stulp, B. K. & Stam, W. T. (1984a). Growth and morphology of *Anabaena* strains (Cyanophyceae, Cyanobacteria) in cultures under different salinities. *Br Phycol J* **19**, 281–286.

Stulp, B. K. & Stam, W. T. (1984b). Genotypic relationships between strains of *Anabaena* (Cyanophyceae) and their correlation with morphological affinities. *Br Phycol J* **19**, 287–301.

Stulp, B. K. & Stam, W. T. (1985). Taxonomy of the genus *Anabaena* (Cyanophyceae) based on morphological and genotypic criteria. *Arch Hydrobiol Suppl* **71**, 257–268.

Swofford, D. L. (2003). PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4b10. Sunderland, MA: Sinauer Associates.

Tamas, I., Svircev, Z. & Andersson, S. G. (2000). Determinative value of a portion of the *nifH* sequence for the genera *Nostoc* and *Anabaena* (cyanobacteria). *Curr Microbiol* **41**, 197–200.

Templeton, A. R. (1983). Phylogenetic interface from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. *Evolution* **37**, 221–224.

ter Braak, C. J. F. & Šmilauer, P. (1998). CANOCO Release 4. Reference manual and user's guide to Canoco for Windows: Software for Canonical Community Ordination. Ithaca, NY: Microcomputer Power.

Turner, S. (1997). Molecular systematics of oxygenic photosynthetic bacteria. *Plant Syst Evol* (Suppl.) **11**, 13–52.

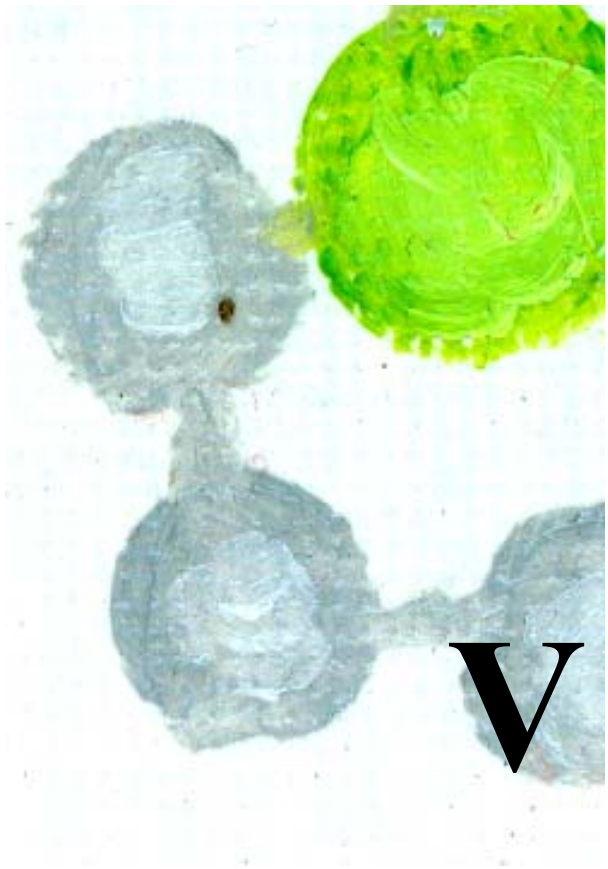
Turner, S., Pryer, K. M., Miao, V. P. W. & Palmer, J. D. (1999). Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *J Eukaryot Microbiol* **46**, 327–338.

Wilmotte, A. (1994). Molecular evolution and taxonomy of the cyanobacteria. In *The Molecular Biology of Cyanobacteria*, pp. 1–25. Edited by D. A. Bryant. Dordrecht: Kluwer Academic.

Wilmotte, A. & Herdman, M. (2001). Phylogenetic relationships among the cyanobacteria based on 16S rRNA sequences. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, pp. 487–493. Edited by D. R. Boone & R. W. Castenholz. New York: Springer.

Zapomělová, E. (2004). *Morfologická variabilita a růst vybraných kmenů sinic rodu Anabaena a Aphanizomenon v závislosti na podmínkách prostředí* [*Morphological variability and growth of chosen cyanobacterial strains of genera Anabaena and Aphanizomenon in the dependence on environmental conditions*]. MSc thesis, University of South Bohemia, Czech Republic (in Czech).

Zehnder in Staub, R. (1961). Ernährungphysiologish-autökologische Untersuchung an der planktonischen Blaualge *Oscillatoria rubescens* DC. *Schweiz Z Hydrol* **23**, 82–198 (in German).



Zapomělová E., Hrouzek P., Řezanka T., Jezberová J., Řeháková K., Hisem D. & Komárková J.:

Are fatty acid profiles and secondary metabolites good chemotaxonomic markers of genetic and morphological clusters of Dolichospermum spp. and Sphaerospermopsis spp. (Nostocales, Cyanobacteria)?

Manuscript

Are fatty acid profiles and secondary metabolites good chemotaxonomic markers of genetic and morphological clusters of *Dolichospermum* spp. and *Sphaerospermopsis* spp. (Nostocales, Cyanobacteria)?

Eliška Zapomělová[a,*], Pavel Hrouzek[b,c,d], Tomáš Řezanka[e], Jitka Jezberová[a], Klára Řeháková[a,f], Daniel Hisem[b,d] and Jaroslava Komárková[a,f]

[a]*Biology Centre of AS CR, Institute of Hydrobiology, Na Sádkách 7, CZ-37005 České Budějovice, Czech Republic.*
[b]*Institute of Microbiology, AS CR, Department of Autotrophic Microorganisms, Opatovický mlýn, CZ-379 81 Třeboň, Czech Republic*
[c]*Institute of Physical Biology, Zámek 136, CZ-37333 Nové Hrady, Czech Republic*
[d]*University of South Bohemia, Faculty of Science, Branišovská 31, CZ-37005 České Budějovice, Czech Republic*
[e]*Institute of Microbiology, AS CR, Vídeňská 1083, CZ-14220 Prague, Czech Republic*
[f]*Institute of Botany, AS CR, Dukelská 135, CZ-37982 Třeboň, Czech Republic*
** Corresponding author. Tel.: +420 387 775 886; fax: +420 385 310 248. E-mail address: eliska.zapomelova@seznam.cz.*

ABSTRACT
The genera *Dolichospermum* (Ralfs ex Bornet et Flahault) Wacklin et al. 2009 and *Sphaerospermopsis* Zapomělová et al. 2010 represent highly diversified group of planktonic cyanobacteria that have been recently separated from the traditional genus *Anabaena* Bory ex Bornet et Flahault 1888. In this study, morphological diversity, phylogeny of 16S rRNA gene, production of fatty acids and secondary metabolite profiles were compared among 33 strains of 14 morphospecies isolated from the Czech Republic. Clustering of the strains based on 16S rRNA gene sequences corresponded to wider groups of species in terms of morphology. On the contrary, the overall secondary metabolite and fatty acid profiles were neither correlated to each other, nor to 16S rRNA phylogeny and morphology of the strains suggesting that these compounds are not good chemotaxonomic tools for the cyanobacterial genera studied. Nevertheless, a minor part of the detected secondary metabolites (19% of all compounds) were present solely in the closest relatives and can be thus considered as autapomorphic features.

Keywords: *Anabaena, Dolichospermum, Sphaerospermopsis,* 16S rRNA, fatty acids, secondary metabolites, taxonomy.

Abbreviations: *D., Dolichospermum; S., Sphaerospermopsis;* FAs, fatty acids; GC, gas chromatography; HPLC-MS, High Performance Liquid Chromatography Mass Spectrometry; MS, Mass Spectrometry; MSD, mass selective detector; *m/z,* mass divided by charge; NJ, neighbour joining; RT, retention time.

Introduction

Taxonomy of organisms in general has been traditionally based on morphological characteristics. However, progress and subsequent routine use of molecular and analytical methods have brought new insight into the taxonomic classification.
Modern taxonomy implies mainly molecular but also biochemical markers to describe and delimit new taxa. These data are especially useful in groups of organisms where morphological characters are insufficient, e.g. in case of cryptic species, organisms with simple morphology etc. Congruency between the metabolite production and molecular or morphological features has been demonstrated several times in higher plants (Aitzetmuller, 1993; Grayer et al., 1999; Wink, 2003; Mongrand et al., 2005), fungi (Frisvad et al., 2008) and several groups of eukaryotic algae (Marshal et al., 2002; de Carvalho and Roque, 2004; Falshaw and Furneaux, 2009). The description of chemosyndromes is a routine method to define relationships among some lichens' taxa and these chemosyndromes were found well correlated with molecular data (Søchting 2002).
In prokaryotes, their extremely simple morphology lead to the need of alternative traits such as biochemical markers (fatty acids, secondary metabolites or enzymes) to characterize taxonomic units, especially on generic level (e.g. Skerratt et al., 1991; Romano et al., 2000; Gugger et al., 2002b). Chemotaxonomy including fatty acid analysis has been suggested as one of the obligatory criteria for characterization of new bacterial taxa (Kämpfer et al., 2003).
The genera *Dolichospermum* (Ralfs ex Bornet et Flahault) Wacklin et al. 2009 and *Sphaerospermopsis* Zapomělová et al. 2010 are groups of planktonic cyanobacteria that have been recently separated from the traditional nostocacean genus *Anabaena* (Bory ex Bornet et Flahault) sensu lato (Wacklin et al., 2009; Zapomělová et al., 2009, 2010a). These cyanobacteria are distributed worldwide, colonizing planktonic habitats and often forming heavy water blooms. They are responsible for the production of secondary metabolites representing human and animal health risks, and malodorous substances worsening the quality of water (Izaguirre et al., 1981; Hayes and Burch, 1989; Chorus and Bartram, 1999).
Traditional taxonomy within the genus *Anabaena* s. l. was based on morphometric parameters (Komárek and Zapomělová, 2007, 2008). However, many previous studies consistently reported on morphological, ecological and genetic heterogeneity within the genus *Anabaena,* especially the pronounced differences between planktonic and benthic representatives (Rajaniemi et al. 2005a, b; Halinen et al. 2008). The main molecular cluster of planktonic *Anabaena* morphospecies, as was presented by Rajaniemi et al. (2005a, b), was therefore reclassified into the new genus *Dolichospermum* (Wacklin et al., 2009). The generic name *Anabaena* has been retained for benthic morphospecies. Moreover, some other morphospecies exist that have been traditionally classified as *Anabaena* but form evidently separate genetic clusters, e.g. *Sphaerospermopsis* erected by Zapomělová et al. (2009, 2010a).
It is evident from this recapitulation that the traditional genus *Anabaena* is very complicated and has to be studied and revised from many points of view to produce good and practical taxonomic system (polyphasic approach; Castenholz, 1992).
Besides morphology and molecular biology, another useful tool for improving the classification may be chemotaxonomy. Most studies of *Anabaena* chemotaxonomy have focused on chemotaxonomic value of fatty acids (FAs), i.e. primary metabolites (Li and Watanabe, 2001; Gugger et al., 2002b; Li and Watanabe, 2004). Their conclusions are rather inconsistent. Fatty acid composition of *Anabaena* strains with straight trichomes (Li and Watanabe, 2001) matched neither with their morphology nor with the phylogenetic relationships among the same species reported by Rajaniemi et al. (2005a, b). On the contrary, fatty acid profiles of *Anabaena* strains with coiled trichomes (Li and Watanabe,

2004) corresponded almost exactly to the recently published phylogenetic analyses (Gugger et al., 2002a; Rajaniemi et al., 2005a, b). Gugger et al. (2002b) demonstrated that fatty acid composition of planktonic *Anabaena (Dolichospermum)* strains agreed with their toxicity, i.e. secondary metabolite production. Halinen et al. (2008) and Stüken et al. (2009) referred a correlation of toxin production (microcystin and cylindrospermopsin, respectively) and phylogenetic affiliation of *Anabaena (Anabaena / Dolichospermum / Sphaerospermopsis)* strains.
Nevertheless, the interconnection of toxicology, fatty acid analysis, molecular approach and morphological description is missing although it could bring useful taxonomic conclusions. Li and Watanabe (2001, 2004) did not evaluate secondary metabolite production and phylogenetic relationships of their *Anabaena* strains and, on the contrary, most of the strains by Gugger et al. (2005b) were identified simply as *Anabaena* sp.
Consequently, we compared morphologies, 16S rRNA gene phylogeny, fatty acid production, and secondary metabolite profiles among 33 selected strains of *Dolichospermum* and *Sphaerospermopsis* from the Czech Republic, representing a wide spectrum of various morphospecies. We examined the ability of morphological and chemical characteristics to establish species relationships among the taxa studied and discussed these relationships with a phylogeny constructed from 16S rRNA gene sequences.

Materials and Methods

Sampling, strain isolation and cultivation: Samples of water blooms were collected in years 2004–2008 from various fishponds and reservoirs in the Czech Republic using 20 μm mesh plankton net. Morphology of fresh material was evaluated immediately as described below. Single trichomes were isolated from the phytoplankton samples as described by Zapomělová et al. (2007). Clonal cultures were grown in WC medium (Guillard and Lorenzen, 1972) at 21 °C and the light intensity of 70 $\mu mol.m^{-2}.s^{-1}$ (16:8 L:D cycle). 33 strains representing 14 different morphospecies of the genera *Dolichospermum* and *Sphaerospermopsis* were studied (Table 1).

Morphometry: Microphotographs of at least 30 fresh trichomes per each natural population were taken with a digital camera (Olympus DP 70, magnification 400x). As a population we mean a set of trichomes of the same morphospecies observed in a natural sample collected from a certain locality at a certain time. Dimensions of all cell types were measured (five vegetative cells per trichome measured in 30 trichomes and as many heterocytes and akinetes as were possible to find in each sample). Length:width ratios of vegetative cells, heterocytes and akinetes were computed to roughly characterize the cell shapes. All size measurements were performed using image analysis (Olympus DP Soft).

Statistical evaluation of morphological data: Basic statistical characteristics such as mean values, 25% and 75% percentiles and extreme values were computed for the morphological features of the field populations. To describe the variability within all morphological data, principal component analysis (PCA) was carried out in the program CANOCO (Ter Braak and Šmilauer, 1998), where the mean values, 25% and 75% percentiles and extreme values of all morphological features were the input data. The data were centred and standardized. CanoDraw software (Šmilauer, 1992) was used for construction of the PCA plot.

Table 1. Cyanobacterial strains used in this study and the summary of the analyses performed (*D., Dolichospermum*; *S., Sphaerospermopsis*; FA, fatty acid composition; SM, secondary metabolite content; 16S rRNA, sequencing of 16S rRNA gene; morph., analysis of morphological characteristics). First two numbers of the strain code indicate the year of isolation (all strains isolated in 2000 or later).

Taxa	Strain code	Locality of isolation	FA	SM	16S rRNA	Morph.
Anabaena sp. Bory ex Born. et Flah.	08-05	Máchovo fishpond, Czech Republic	+		+	+
D. affine (Lemm.) Wacklin et al.	04-44	Svět fishpond, Czech Republic	+	+	+	+
D. affine(Lemm.) Wacklin et al.	05-03	Staňkovský fishpond, Czech Republic	+		+	+
D. flos-aquae (Bréb. ex Born. et Flah.) Wacklin et al.	04-10	Byňovský fishpond, Czech Republic	+		+	+
D. flos-aquae (Bréb. ex Born. et Flah.) Wacklin et al.	04-40	Skalka reservoir, Czech Republic	+	+	+	+
D. flos-aquae (Bréb. ex Born. et Flah.) Wacklin et al.	04-57	Vajgar fishpond, Czech Republic		+	+	+
D. circinale (Rabenh. ex Born. et Flah.) Wacklin et al. x *D. crassum* (Lemm.) Wacklin et al.	04-22	Husinec reservoir, Czech Republic		+	+	+
D. circinale (Rabenh. ex Born. et Flah.) Wacklin et al. x *D. crassum* (Lemm.) Wacklin et al.	04-26	Jesenice reservoir, Czech Republic		+	+	+
D. circinale (Rabenh. ex Born. et Flah.) Wacklin et al. x *D. crassum* (Lemm.) Wacklin et al.	04-28	Hodějovický fishpond, Czech Republic	+	+	+	+
D. circinale (Rabenh. ex Born. et Flah.) Wacklin et al. x *D. crassum* (Lemm.) Wacklin et al.	04-59	Valcha fishpond, Czech Republic		+	+	+
D. compactum (Nygaard) Wacklin et al.	04-17	Dubnenský fishpond, Czech Republic		+	+	+
D. compactum (Nygaard) Wacklin et al.	06-02	Pěšák fishpond, Czech Republic	+		+	+
D. compactum (Nygaard) Wacklin et al.	06-03	Svět fishpond, Czech Republic	+		+	+
D. curvum (Hill) Wacklin et al.	04-19	Hejtman fishpond, Czech Republic	+	+	+	+
D. lemmermannii (Richter in Lemm.) Wacklin et al.	04-24	Husinec reservoir, Czech Republic	+	+	+	+
D. lemmermannii (Richter in Lemm.) Wacklin et al.	04-38	Senecký fishpond, Czech Republic		+	+	+
D. lemmermannii (Richter in Lemm.) Wacklin et al.	04-42	Svět fishpond, Czech Republic		+	+	+
D. mendotae (Trelease) Wacklin et al. x *D. sigmoideum* (Nygaard) Wacklin et al.	04-06	Březová reservoir, Czech Republic		+	+	+
D. mendotae (Trelease) Wacklin et al. x *D. sigmoideum* (Nygaard) Wacklin et al.	04-11	Černiš fishpond, Czech Republic		+	+	+
D. mendotae (Trelease) Wacklin et al. x *D. sigmoideum* (Nygaard) Wacklin et al.	04-33	Orlík reservoir, Czech Republic	+	+	+	+
D. mendotae (Trelease) Wacklin et al. x *D. sigmoideum* (Nygaard) Wacklin et al.	04-45	Svět fishpond, Czech Republic	+	+	+	+
D. mendotae (Trelease) Wacklin et al. x *D. sigmoideum* (Nygaard) Wacklin et al.	05-01	Lipno reservoir, Czech Republic	+		+	+
D. mucosum (Kom-Legn. et Eloranta) Wacklin et al.	06-04	Horák fishpond, Czech Republic	+		+	+
D. mucosum (Kom-Legn. et Eloranta) Wacklin et al.	08-03	Mařka fishpond, Czech Republic	+		+	+
D. planctonicum (Brunnthaler) Wacklin et al.	00-05	Nechranice reservoir, Czech Republic	+		+	+
D. planctonicum (Brunnthaler) Wacklin et al.	03-02	Lipno reservoir, Czech Republic	+		+	+
D. smithii (Komárek) Wacklin et al.	05-05	Dubnenský fishpond, Czech Republic	+		+	+
D. smithii (Komárek) Wacklin et al.	08-02	Mařka fishpond, Czech Republic	+		+	+
D. spiroides (Bréb. ex Born. et Flah.) Wacklin et al.	04-51	Svět fishpond, Czech Republic		+	+	+
D. viguieri (Denis et Frémy) Wacklin et al.	08-04	Mařka fispond, Czech Republic	+		+	+
S. aphanizomenoides (Forti) Zapomělová et al.	04-43	Svět fishpond, Czech Republic	+		+	+
S. reniformis (Lemm.) Zapomělová et al.	06-01	Pěšák fishpond, Czech Republic	+	+	+	+
S. reniformis (Lemm.) Zapomělová et al.	07-01	Vyšehrad fishpond, Czech Republic	+		+	+

16S rDNA sequencing: The biomass of the strains was harvested in the exponential phase of growth by repeated centrifugation, during which the trichomes were washed several times by NaCl solution (concentration 1 $g.L^{-1}$) to remove or reduce mucilaginous substances. The biomass samples were stored at –20 °C until DNA extractions. DNA was extracted using UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA). The 16S rRNA gene and ITS region were amplified with primers 16S27F and 23S30R (Taton et al., 2003). Amplification was carried out as follows: one cycle of 5 min at 94 °C; 10 cycles of 45 s at 94 °C, 45 s at 57 °C, and 2 min at 72 °C; 25 cycles of 45 s at 94 °C, 45 s at 54 °C, and 2 min at 72 °C; and a final elongation step of 7 min at 72 °C. PCR products were cleaned using NucleoSpin ExtractII kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). PCR products were then used as templates for sequencing with primers 16S27F, 23S30R (Taton et al., 2003), primer cAlaR (Wilmotte et al., 1994), primers WAW1486R and Primer 14 (Wilmotte et al., 1993), and primer CYA781F(a) (Nübel et al., 1997). The sequencing was performed at a commercial facility (Laboratory of Genomics, Biology Centre of AS CR, České Budějovice, Czech Republic).

Phylogenetic analyses: Sequences were aligned in the program ARB (http://www.arb-home.de) and the alignment was edited manually. For the phylogenetic analysis, trees were built with the Neighbour joining method (NJ) (Saitou and Nei, 1987) for that 500 bootstrap replicates were performed. Nucleotide sequences were deposited at GenBank under the accession numbers AM940218, AM940219, FM161348–FM161350, FM242083–FM242088, and FN691905–FN691926.

Fatty acid profiles: A Hewlett Packard 6890 (series II) gas chromatograph modified for glass-capillary work and a HP-GC mass selective detector (5973B MSD) were used. Fatty acid methyl esters were prepared according to Metcalfe and Wang (1981) and analyzed by GC on capillary column, i.e. RTX 1701, 30 m, 0.32 mm, 0.25 mm film (Restek, USA). The GC oven was programmed for 40 ºC per 2 min, increase 2 ºC min^{-1} to 300 ºC, 20 min at 300 ºC. The injector temperature was kept at 180 ºC. The flow rate of the carrier gas (helium) was 0.5 $mL.min^{-1}$. The MS detector operated at 200 ºC, ionization energy was 70 eV. The scan range was from 30 to 700 *m/z* at 0.9 scan per second. Solvent delay was 9 min. Fatty acid methyl esters were identified using mass spectral libraries search (Wiley 7th, and NIST-98).
Statistical package Statistica 9.0 for Windows (Stat Soft, Inc, Tulsa, OK) was used for evaluation of similarities among fatty acid profiles of the strains studied (cluster analysis – Euclidean distance, single linkage).

Secondary metabolite content: extract preparation and high performance liquid chomatography with connection to mass spetrometry (HPLC-MS) analysis
Lyophilized biomass (approx. 40 mg) was disintegrated by grinding and extracted in 2 mL of 70% methanol (MeOH) in microtubes for 30 min. The microtubes were centrifuged at 3170 g at 4 °C for 15 min. Supernatant was concentrated ten times using a rotary vacuous drier. HPLC-MS analysis was performed in order to determine the content of secondary metabolites. The extracts were analyzed on a reversed phase column (Zorbax XBD C8, 4.6 x 150 mm, 5 μm) using MeOH / H_2O gradient with the flow rate of 0.6 $mL.min^{-1}$. For a more effective ionization 0.1% formic acid was added into the eluents. The extract composition was analyzed by the HP 1100 Agilent mass spectrometer HP 100 MSD SL-Ion trap in positive mode. The settings were selected to cover the mass range between 50–2000 Da, and the ion trap was targeted to molecular masses near 900 Da. Automatic fragmentation of the most intensive peak was applied. The molecular ions were determined based on the presence of sodium and potassium adducts and based on the distribution of isotopologues. Only

metabolites found in at least two of the strains studied were taken into consideration. Photosynthetic pigments, identified based on absorption spectra, were eliminated from the analysis. The similarities of whole secondary metabolite profiles among the strains studied were visualized by cluster analysis (Euclidean distance, complete linkage) computed by Statistica for Windows (Stat Soft, Inc, Tulsa, OK).

2. Results and discussion

The present study provides a unique chemotaxonomic dataset of the genera *Dolichospermum* and *Sphaerospermopsis*. These data are comparable among all of the strains studied, since they were grown, harvested and analyzed in the same time under identical conditions. This is extremely important because both fatty acid composition and secondary metabolite production were previously shown to be influenced by environmental conditions and growth phase (Sivonen, 1996; Rapala and Sivonen, 1998; Dembitsky et al., 2003, 2005; Temina et al., 2007), which hampers the overall comparison of the results obtained in the past by different authors (Li and Watanabe, 2001; Gugger et al., 2002b; Li and Watanabe, 2004). Morphology of our strains has been described in detail (Appendix 1–3), which, together with the information on genotypic relationships among the strains, is a prerequisite of reliable interpretation of the results.

3.1. Morphological characteristics

Principal Component Analysis (PCA) was employed to compare morphometric parameters of 33 natural populations representing 12 different morphospecies of the genus *Dolichospermum* and 2 morphospecies of the genus *Sphaerospermopsis.* The analysis has demonstrated a continuum of morphological variability within this cyanobacterial group where no distinctly delimited groups of populations representing traditional species can be recognized (Fig. 1).

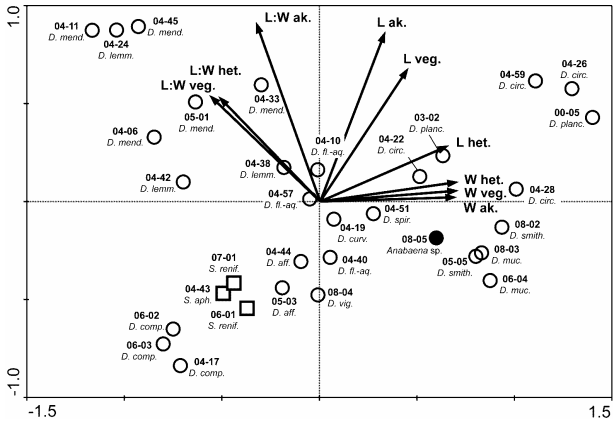


Fig. 1. PCA diagram based on the morphological characteristics of the studied cyanobacterial populations from the field conditions. Each strain is symbolized by plain circle (*Dolichospermum*), full circle (*Anabaena* sp.) or square (*Sphaerospermopsis*). The most variable morphological characteristics are shown by the arrows (veg., vegetative cells; het., heterocytes; ak., akinetes; L, length; W, width; L:W, length:width ratio). The first and the second canonical axes explain together 74.9% of the total variance. *D. aff., Dolichospermum affine; D. circ., D. circinale; D. curv., D. curvum; D. dan, D. danicum; D.*

fl.-aq., D. flos-aquae; D. lemm., D. lemmermannii; D. mend., D. mendotae; D. muc., D. mucosum; D. planc., D. planctonicum; D. smith., D. smithii; D. spir., D. spiroides; D. vig., D. viguieri; S. aph., Sphaerospermopsis aphanizomenoides; S. renif., S. reniformis. The strain codes are explained in Table 1.

The exceptions are *D. compactum* and *Sphaerospermopsis* morphospecies *(S. aphanizomenoides* and *S. reniformis*), which were morphologically clearly defined by the shape and position of the akinetes and the morphology of trichome coiling. This is consistent with former study on morphological diversity among natural populations of various *Dolichospermum* morphospecies (Zapomělová et al., 2007). For general morphologies of all of the strains studied see the microphotographs in Appendix 1–3.
In several cases, wider groups of morphospecies were only recognizable while the borderlines between single morphospecies were unclear (*D. circinale* & *D. crassum,* and *D. mendotae* & *D. sigmoideum).* Laboratory experiments by Zapomělová (2008) and Zapomělová et al. (2008, 2010b) aimed at morphological plasticity of selected *Dolichospermum* strains supported the existence of wider complexes of morphospecies within this cyanobacterial group. The authors demonstrated that single strains under manipulated experimental conditions (temperature, light, concentration of N and P) were able to span the variability of both the species *D. circinale* and *D. crassum,* or *D. mendotae* and *D. sigmoideum* respectively, as they were originally described.
The herein presented results indicated that additional morphological criteria (i.e. shape of akinetes, arrangement of heterocytes and akinetes, filament aggregation, occurrence and pattern of trichome coiling) have to be used besides morphometry for reliable identification of other than *D. compactum* or *Sphaerospermopsis* morphospecies.

3.2. Molecular characteristics and phylogenetic analyses

Sequencing of 16S rRNA gene was performed in pure clonal strains isolated from the above-mentioned natural populations of *Dolichospermum* spp. and *Sphaerospermopsis* spp. Two neighbor-joining trees were constructed; the first one for the strains where fatty acids were analyzed (Fig. 2) and the second one for the strains where HPLC-MS analysis of secondary metabolites was performed (Fig. 3). Both of the trees displayed consistent clustering of the strains, which was in a good agreement with their morphospecies affiliations.
The genera *Dolichospermum* and *Sphaerospermopsis* were located in two distinctly separated clusters A and B, highly supported (Fig. 2–3), which was one of the main reasons why the genus *Sphaerospermopsis* was established (Zapomělová et al., 2009, 2010a). The cluster A comprised four distinct subclusters A1–A4 of significant bootstrap supports and an outlying strain *D. curvum* 04-19. The strain 04-19 displayed evident morphological differences in comparison to other *Dolichospermum* strains, i.e. specific trichome coiling and clumps of trichomes in distinct mucilaginous envelope. It was identified as *D. curvum,* since its morphlogy resembled morphological features of the strain *Anabaena curva* Ana Ao presented by Li et al. (2000). Particularly the general morphology of trichome coiling of our strain 04-19 and the accumulation of its filaments in thick mucilage were highly similar to the strain Ana Ao. On the contrary, the shape of akinetes, which was also identical in both strains, did not agree with the original description of *A. curva* (Hill, 1976). According to the original description, the akinetes of *A. curva* should be markedly curved and elongated, while both our strain 04-19 and the strain Ana Ao by Li et al. (2000) exhibited kidney-shaped akinetes.

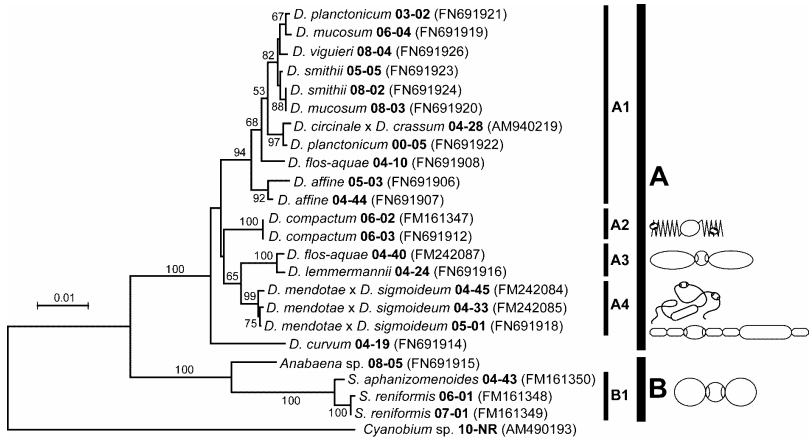


Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences (1419 bp) showing the clustering of cyanobacterial strains from that fatty acid profiles were analyzed. Numbers near nodes indicate bootstrap values over 50%. Drawings demonstrate morphological features that are typical and common for the strains of single genetic clusters. *D., Dolichospermum, S., Sphaerospermopsis.*

The subcluster A1 showed the highest genotypic and morphological diversity. It comprised wide range of morphospecies: *D. planctonicum, D. mucosum, D. viguieri, D. smithii, D. flos-aquae, D. affine* and strains from morphological complex of *D. circinale* x *D. crassum*. Common morphological features of all these strains were relatively low length:width ratios of all cell types (vegetative cells, heterocytes, akinetes) and their relatively large dimensions (Fig. 1).

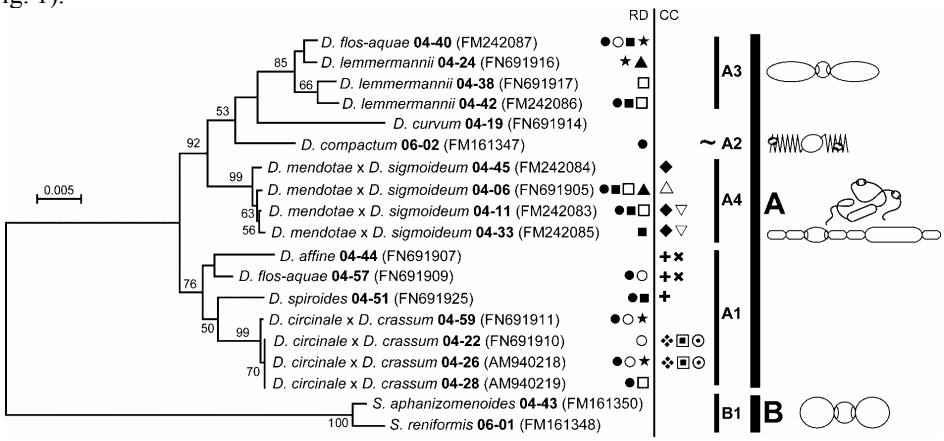


Fig. 3. Neighbour-joining tree based on 16S rRNA gene sequences (1392 bp) showing the clustering of cyanobacterial strains from that secondary metabolites were analyzed. Symbols represent the most frequent randomly distributed compounds (RD) and compounds exhibiting correlation with 16S rRNA phylogeny (CC): ● (*m/z* 685.2, RT 11.6 min), ○ (*m/z* 715.3, RT 21.6 min), ■ (*m/z* 701.3, RT 11.2 min), □ (*m/z* 256.3, RT 21.7 min), ★(*m/z* 569.4, RT 20.4 min), ▲ anabaenopeptin B (*m/z* 837.4, RT 13.1 min), ✚ (*m/z* 727.4, RT 12.6 min), ✖ (*m/z* 534.4, RT 12.2 min), ❖ (*m/z* 714.4, RT 7.2 min), ▣ (*m/z* 756.3, RT 9.6 min), ⊙ (*m/z* 731.3, RT 19.4 min), ◆ unknown variant of anabaenopeptin (*m/z* 842.4,

RT 20.1 min), △ anabaenopeptin A (*m/z* 844.4, RT 17.1 min) , ▽anabaenopeptin D (*m/z* 828.5, RT 19.6 min). Numbers near nodes indicate bootstrap values over 50%. Drawings demonstrate morphological features that are typical and common for the strains of single genetic clusters.

Clustering of these morphospecies together is consistent with phylogenetic analyses based on 16S rRNA gene sequences performed by Rajaniemi et al. (2005a) on strains from Finland. The uniform subcluster A2 consisted of the strains of *D. compactum,* which agreed exactly with the findings by Rajaniemi et al. (2005a, 2005b). These strains were clearly characterized by tight regular coiling of their trichomes and widely ovoid akinetes with low length:width ratios, distant from heterocytes. For the strains of the subcluster A3 formation of ovoid akinetes next to heterocytes was typical. This akinete position was stable in all strains of *D. lemmermannii* and was only rarely disrupted in the strain *D. flos-aquae* 04-40. Though *D. lemmermannii* is known as highly diversified taxon from morphological point of view (Komárková, 1988; Zapomělová et al., 2007), the arrangement of akinetes is invariable and was shown to be a good indicator of its phylogenetic affiliation vs. *D. mendotae* and *D. sigmoideum* morphospecies that occur in different molecular cluster (Zapomělová et al., 2010b).
The subcluster A4 contained strains from morphological complex of *D. mendotae* x *D. sigmoideum.* Typical morphological characteristics of these strains are the shapes of vegetative cells and akinetes (high length:width ratios; Fig. 1) and the position of akinetes distant from heterocytes.
The cluster B contained a highly supported subcluster B1 (*Sphaerospermopsis* strains with nearly-spherical akinetes adjacent to heterocytes as the autapomorphic feature) and an outlying strain *Anabaena* sp. 08-05, preliminarily identified as *D.* cf. *danicum* according to its morphology. Phylogenetic affiliation based on 16S rRNA gene sequence, nevertheless, indicated, that the strain 08-05 cannot be classified as a morphospecies of *Dolichospermum;* and neither of *Sphaerospermopsis,* from which it is separated with 100% bootstrap support. This strain probably represents a specific group of *Anabaena*-like cyanobacteria on generic level that has not yet been described. Further studies are highly required for the definite identification of this group corresponding to a potential new genus. It is questionable whether our strain 08-05 actually represented *D. danicum.* The width of its filaments, their mucilaginous envelope, and the dimensions and shapes of the akinetes corresponded with the original description of *D. danicum* (Komárková-Legnerová and Eloranta, 1992). On the contrary, the trichomes of our strain 08-05 were distinctly narrowed towards the ends and the terminal vegetative cells were elongated, especially in the original natural population (Appendix 2 C). And the shape of vegetative cells in general was also not typical for true *D. danicum.* However, 16S rRNA gene sequences of any *D. danicum* strains are not available either in literature or in GenBank. Sequence of 16S rRNA gene of the strain 08-05 cannot be therefore compared with the sequences of this taxon.

3.3. Fatty acid profiles

As seen from Table 2, the cyanobacteria produced large amounts of linoleic and linolenic acids, whose content in some strains exceeded 50% of total FA. To our knowledge such high content has not yet been described in the genus *Dolichospermum* and closely related cyanobacteria (cf. Li and Watanabe, 2001, 2004; Gugger et al., 2002b). This can be explained most likely by geographic differences at the site of collection and hence by different growth conditions (temperature, sunshine, amount of inorganic substances in the water, etc.; see Temina et al., 2007). Noteworthy is also the content of two positional isomers of hexadecenoic acid, which were found in previous analyses (Li and Watanabe,

2001, 2004; Gugger et al., 2002b). Overall, the FA of our cyanobacterial strains, with the exception of the features caused by ecological and geographical conditions, do not differ from FA described so far.

Table 2. Fatty acid compositions (%) of different *Dolichospermum* and *Sphaerospermopsis* strains

Taxa	Strain code	14:0	15:0	16:2	16:1a	16:1 b	16:0	17:0a	17:0 b	18:3	18:2	18:1	18:0	19:0
Anabaena sp.	08-05	0.8	0.0	2.9	4.2	2.8	31.9	0.1	0.3	41.9	9.5	0.8	4.7	0.1
D. affinis	04-44	2.1	0.7	5.2	4.5	5.1	25.6	0.0	0.1	43.5	11.1	1.1	0.9	0.1
D. affinis	05-03	5.8	0.0	5.8	2.9	3.9	17.5	0.1	0.1	34.1	26.4	0.8	2.6	0.0
D. circinalis x *D. crassum*	04-28	2.2	1.4	4.1	8.0	4.8	22.4	0.0	0.1	41.8	13.3	0.1	1.8	0.0
D. compactum	06-02	0.7	0.1	7.4	8.3	3.8	20.2	0.0	0.0	38.2	20.1	0.5	0.7	0.0
D. compactum	06-03	0.8	0.0	7.6	7.6	4.6	8.2	0.1	0.1	51.3	18.7	0.2	0.8	0.0
D. curvum	04-19	2.2	0.3	3.0	1.3	3.5	26.9	0.0	0.1	51.6	7.1	1.6	2.3	0.1
D. flos-aquae	04-10	0.7	0.1	7.1	5.2	2.4	21.9	0.0	0.1	40.4	19.4	0.2	2.4	0.1
D. flos-aquae	04-40	6.1	0.3	3.2	0.2	5.0	29.4	0.0	0.0	47.9	4.9	1.2	1.6	0.2
D. lemmermannii	04-24	5.0	2.0	2.5	0.2	5.2	24.8	0.0	0.1	50.8	3.9	2.4	3.0	0.1
D. lemmermannii	04-42	2.2	0.3	3.4	2.1	3.0	24.2	0.0	0.1	55.1	7.4	0.6	1.5	0.1
D. mendotae x *D. sigmoideum*	04-11	0.7	0.5	6.6	3.6	4.9	21.7	0.0	0.1	46.4	14.2	0.4	0.8	0.1
D. mendotae x *D. sigmoideum*	04-33	0.9	1.6	4.0	3.7	4.9	25.7	0.0	0.0	48.1	9.6	0.8	0.6	0.1
D. mendotae x *D. sigmoideum*	04-45	1.0	0.3	6.2	4.0	4.6	8.6	0.0	0.2	56.8	16.5	0.6	1.1	0.1
D. mendotae x *D. sigmoideum*	05-01	0.7	0.1	7.1	5.2	4.5	15.0	0.0	0.1	50.3	16.1	0.1	0.8	0.0
D. mucosum	06-04	0.5	0.5	4.6	2.1	7.8	28.2	0.4	0.2	36.7	12.9	4.2	1.8	0.1
D. mucosum	08-03	0.7	0.0	5.5	2.7	2.9	31.4	0.1	0.1	36.9	17.2	0.6	1.8	0.1
D. planctonicum	00-05	1.7	0.2	5.3	5.4	3.9	25.7	0.1	0.1	42.5	12.6	0.9	1.5	0.1
D. planctonicum	03-02	1.0	0.2	0.1	0.7	21.0	30.2	0.7	1.3	27.5	6.8	4.6	5.5	0.4
D. smithii	05-05	1.3	0.1	0.0	0.3	12.0	53.6	0.2	0.7	7.0	1.8	3.7	18.9	0.4
D. smithii	08-02	0.8	0.0	5.7	3.6	3.2	28.5	0.1	0.1	38.9	17.2	0.2	1.6	0.1
D. viguieri	08-04	0.3	0.2	0.1	0.0	19.3	28.2	0.2	0.4	44.0	3.8	1.8	1.6	0.1
S. aphanizomenoides	04-43	1.5	2.5	0.2	0.2	23.9	37.5	0.0	0.2	19.2	1.9	9.8	3.0	0.1
S. reniformis	06-01	2.7	0.2	0.1	0.2	19.8	52.9	0.0	0.3	8.1	1.8	8.8	4.8	0.3
S. reniformis	07-01	1.5	0.8	0.1	0.3	15.6	27.4	0.0	0.1	41.2	6.1	5.2	1.6	0.1
Cyanobium sp.	10-NR	10.7	0.0	0.0	36.2	39.8	8.2	0.0	0.9	0.2	0.0	2.9	1.1	0.0

The complete linkage cluster analysis of fatty acid profiles resulted in four clusters of strains (Fig. 4), which displayed low correlation with morphospecies affiliation of the strains and their phylogenetic clustering based on 16S rRNA gene. Studies of Li and Watanabe (2001, 2004) indicated that planktonic *Anabaena* (currently *Dolichospermum)* strains can be divided into two groups differing in fatty acid production, i.e. Type 2A producing 16:2 and 16:3, and Type 2B where no 16:2 and 16:3 were found. The Type 2B in the studies by Li and Watanabe (2001, 2004) comprised morphospecies *D. planctonicum, D. danicum, D. affine, S. kisseleviana,* and strains *Anabaena eucompacta* and *A. oumiana*. Taxonomic status of the two latter morphospecies *(A. eucompacta, A. oumiana)* has not yet been satisfactorily clarified but they both form spherical akinetes adjacent to heterocytes, suggesting that they may belong more likely to the genus *Sphaerospermopsis* than to *Dolichospermum* (Zapomělová et al., 2009). Contrary to the above-mentioned studies, Gugger et al. (2002b) found neither 16:2 nor 16:3 in their *Anabaena* strains.

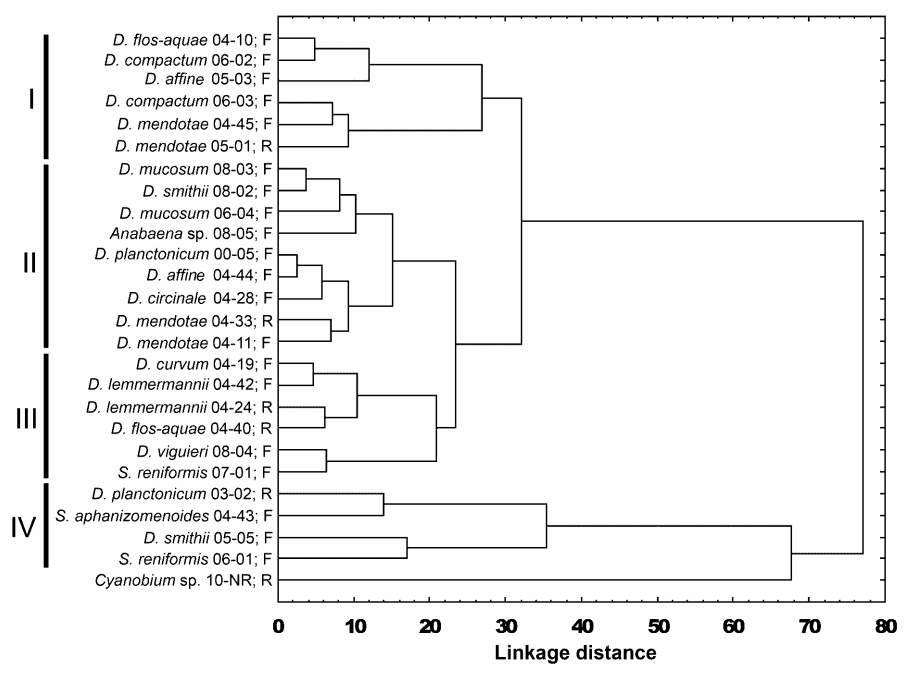


Fig. 4. Complete linkage cluster analysis dendrogram showing the similarities (Euclidean distances) of fatty acid profiles among planktonic *Anabaena* sp., *Dolichospermum* spp. and *Sphaerospermopsis* spp. strains of various morphospecies. The capitals indicate the type of localities from where the strains were isolated: F, fishpond, R, reservoir.

We only detected production of 16:2, while 16:3 was not detected in any of our *Dolichospermum* and *Sphaerospermopsis* strains in amouts higher than 0.1% of total fatty acid methyl esters. Very low or no production of 16:2, corresponding to Type 2B, was detected in all of our *Sphaerospermopsis* strains (04-43, *S. aphanizomenoides*; 06-01, *S. reniformis;* 07-01, *S. reniformis)* and in one of the *D. planctonicum* strains (03-02), which is in a good agreement with the results of Li and Watanabe (2001, 2004). On the contrary, the strains 05-05 *(D. smithii),* and 08-04 *(D. viguieri)* also belonged to Type 2B, while Li and Watanabe (2001, 2004) referred these morphotypes belonging to Type 2A. Besides the fatty acid 16:2, production of the 18:1 appeared to be characteristic for *Sphaerospermopsis*. All of the three *Sphaerospermopsis* strains studied displayed relatively higher concentration of 18:1 compared to other strains analyzed (Table 2).

2.4. Secondary metabolite contents

For the purposes of the present study we selected an approach that considered each compound of certain molecular mass (expressed as *m/z* value) at certain retention time (RT) as one biochemical marker. The main reason was an enormous diversity of cyanobacterial secondary metabolites (e.g. Welker and von Dohren, 2006; Van Wagoner et al., 2007). Most of their structural variants have been probably not yet described and remain unknown (Welker et al., 2006; Hrouzek et al., 2010). Similar approach was successfully applied to analysis of whole bacterial cells spectra by means of MALDI-TOF MS (Holland et al., 1996).

Extract analysis of the 19 cyanobacterial strains studied revealed 170 compounds, 26 of which were present at least in two strains. Distribution of compounds that were common for two or more strains was compared with reconstructed 16S rRNA gene phylogeny in order to find possible correlations. The complete linkage cluster analysis of total metabolite content resulted in four clusters of chemotypes (Fig. 5), which displayed low correlation with morphospecies affiliation of the strains, their phylogenetic clustering based on 16S rRNA gene and also with the clustering based on fatty acid profiles. The strain *S. reniformis* 06-01 was clearly separate from all other strains in the complete linkage cluster analysis, which is in a good agreement with results of phylogenetic analysis and was also reported previously (Zapomělová et al., 2009).

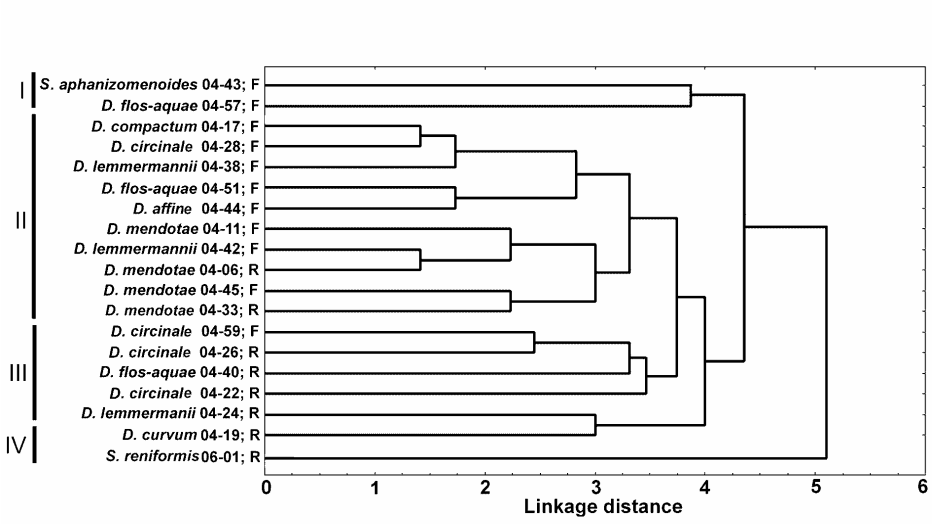


Fig. 5. Complete linkage cluster analysis dendrogram showing the similarities (Euclidean distances) of MS spectra of secondary metabolites among planktonic *Dolichospermum* spp. and *Sphaerospermopsis* spp. strains of various morphospecies. The capitals indicate the type of localities from where the strains were isolated: F, fishpond, R, reservoir.

The pattern of the compound distribution was further categorized into three groups:

1. Particular compound is randomly dispersed across the genotypic clusters based on 16S rRNA gene sequences.
2. The compound occurs in several strains of a monophyletic genotypic cluster but not in all strains of the cluster.
3. The compound is present in the closest relatives and nowhere else in the phylogenetic tree, and can be considered as autoapomorphy.

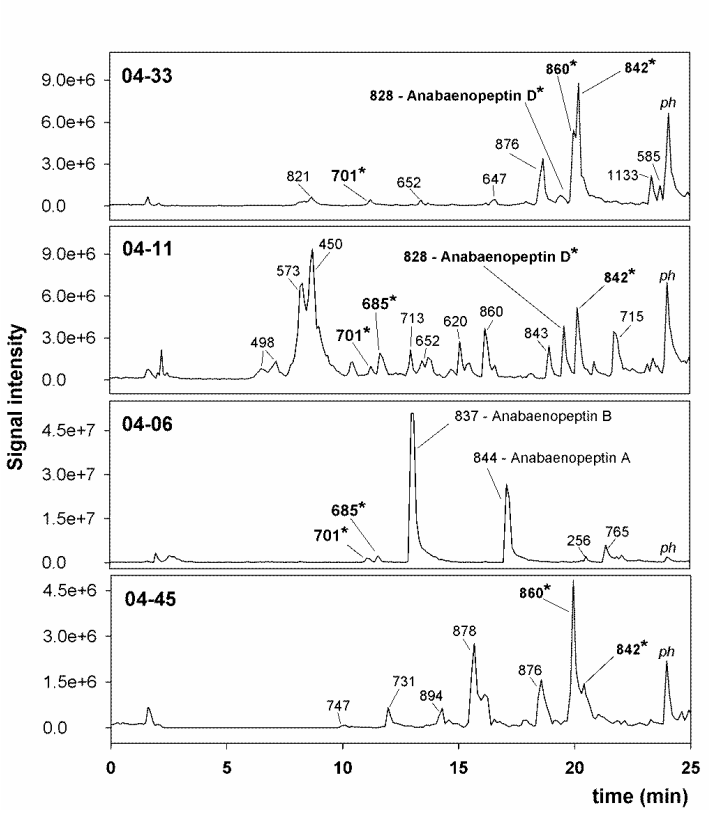


Fig. 6. Secondary metabolite profiles of *Dolichospermum* strains of cluster A4 (base peak HPLC-MS chromatograms). Production of different anabaenopeptin conformers and probably unknown anabaenopeptin variants was found in all members of the cluster A4, which supports clustering of this group. Compounds which were detected in more than one strain of cluster A4 are in bold and marked by asterisk. *ph,* peak corresponding to phtalate contamination.

Most of the compounds (67%) fell into the first category and their random distribution across 16S rRNA gene phylogeny is demonstrated on the most frequently occurring compounds (Fig. 3). Detailed example of this random pattern is given using the compound of *m/z* 685.2 and RT 11.6 min (Fig. 3, full circles), which was spread both among all subclusters of *Dolichospermum* and also within these subclusters (Fig. 6–7).

Only 14% of the detected compounds fell into the second group and were produced by some of strains within a monophyletic cluster.

Production of unknown compound of *m/z* 842.4 and RT 20.1 min was recorded exclusively in three strains of *D. mendotae* x *D.sigmoideum* complex (04-33, 04-11, 04-45), tightly clustering in subcluster A4; however, in fourth member of this group (04-06) this compound was missing (Fig. 6). In all members of this cluster, production of cyclic peptides anabaenopeptines was recorded based on molecular weight, UV-spectra and retention behavior, following the methodology by Fujii et al. (2002). Strain 04-06 was found to contain anabaenopeptin A and B, while strains 04-33 and 04-11 were proved to synthesize anabaenopeptin D. Thus it is highly probable that the above-mentioned compound produced by 04-33, 04-11 and 04-45 is an unknown variant of anabaenopeptin. This suggestion is

highly supported by similarities of this compound with other anabaenopeptins in MS3 spectrum (data not shown). Thus, the strains of the phylogenetic cluster A4 can be characterized by anabaenopeptin production. Within the strains studied, production of anabaenopeptin B was also recorded in *D. lemmermannii* 04-24 located in cluster A3 (Fig. 3 and 7) and anabaneopeptin production cannot be therefore used as an exclusive biochemical marker for cluster A4. However, it definitely supports the grouping of cluster A4.

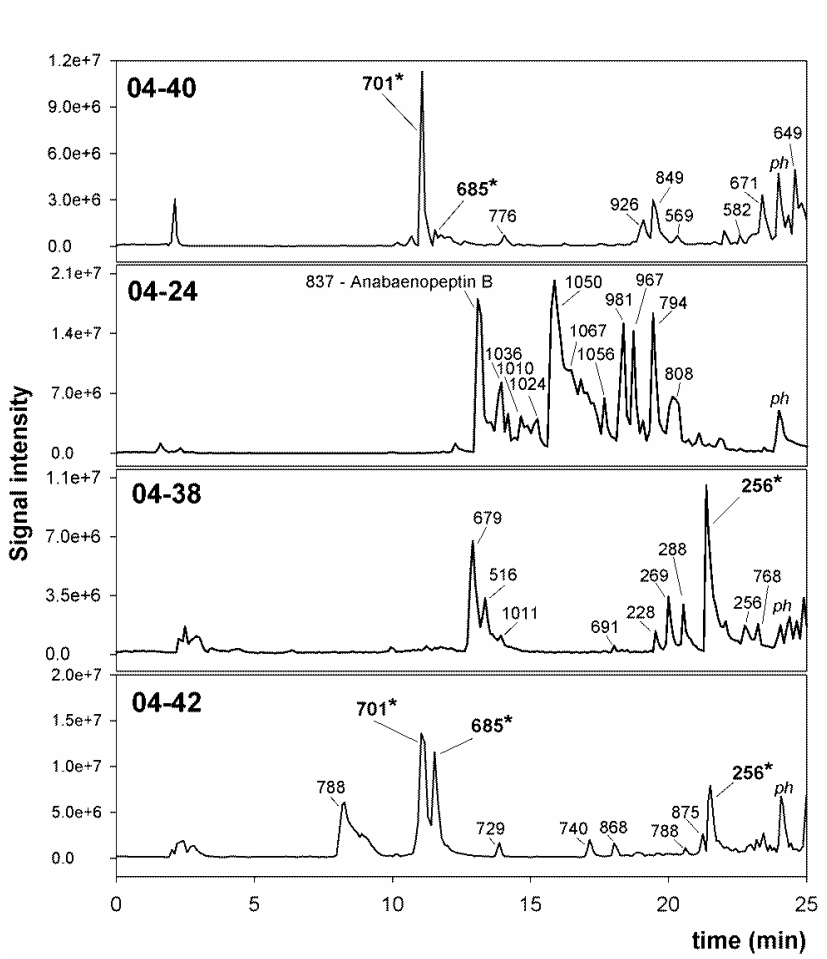


Fig. 7. Secondary metabolite profiles of *Dolichospermum* strains of cluster A3 (base peak HPLC-MS chromatograms). None of observed compounds was found to be characteristic for this cluster. Particular compounds are characterized by their *m/z* values. Compounds that were detected in more than one strain of cluster A3 are in bold and marked by asterisk. Production of cyclic peptide anabaenopeptin B was recorded in *D. lemmermanni* 04-24. *ph,* peak corresponding to phtalate contamination.

Tight clustering of *D. circinale* x *D. crassum* strains 04-22 and 04-26 was absolutely confirmed by production of three compounds (*m/z* 714.4, RT 7.2 min; *m/z* 756.3, RT 9.6 min; *m/z* 731.3, RT 19.4 min) that were only detected in these two strains and were not produced by any other strains studied. Nevertheless, these compounds were missing in the

third *D. circinale* x *D. crassum* strain (04-28) that exhibited 100% 16S rRNA gene sequence similarity with previous two isolates.
Finally, 5 compounds (19% of the compounds found in at least two strains) fell into the third category and were present in closely related strains (strictly monophyletic group) and nowhere else in the phylogenetic tree. Thus, they can be considered as autapomorphic characters with a good potential for taxonomic purposes. In both strains of *S. reniformis,* molecular ion of *m/z* 1235.7 and RT 19.8 min corresponding to puwainaphycin A was found (Zapomělová et al., 2009). Production of this compound was not proved in any *Dolichspermum* strains and thus this result is in congruence with morphological and molecular data.
Strains *D. affine* 04-44 and *D. flos-aque* 04-57, which clustered within subcluster A1, were both found to produce two compounds (*m/z* 534.4, RT 12.2 min; *m/z* 727.4, RT 12.6 min). Also their relation to *D. spiroides* 04-51 was confirmed by production of the compound of *m/z* 727.4 in this strain. Production of anabaenopeptin D (*m/z* 828, RT 19.6 min) and unidentified compound of *m/z* 652.3 and RT 13.7 min by *D. mendotae* x *D. sigmoideum* strains 04-33 and 04-11 was in a good agreement with their tight clustering within the cluster A4.

3.5. General discussion, conclusions

Majority of the detected secondary metabolites and fatty acids exhibited random pattern compared with each other and with the 16S rRNA gene phylogeny of the strains studied. Total secondary metabolite content and fatty acid profiles in general therefore appear not to be reliable chemotaxonomic tools for the cyanobacterial genera studied. As was suggested by Thacker and Paul (2004), who found similarly low consistency between 16S rRNA gene phylogeny and chemical traits in cyanobacterial genera *Lyngbya* and *Symploca*, divergence in chemosynthetic genes may not be reflected in 16S rRNA gene sequences if the ribosomal sequences are relatively more conserved. Earlier studies demonstrated differences in toxin production among genetically similar strains and vice versa (Lyra et al., 2001; Baker et al., 2002; Gugger et al., 2002b). The random distribution of microcystin synthesis genes across the phylogenetic spectrum of cyanobacteria was suggested to be the result of ancient origin of the synthetic pathway rather than the result of lateral gene transfer (Rantala et al., 2004). However, the evidence of lateral gene transfer for cyanobactin synthetic genes has been recently referred (Leikoski et al., 2009) and probably it will play more important role then it was assumed. These facts are strong arguments that cyanobacterial peptides are not good chemotaxonomic markers, which is in agreement with our data. Moreover, the synthetic pathways are probably rapidly evolving systems, since cyanobacterial strains of almost identical 16S rDNA can display markedly different metabolite profiles (Zapomělová et al., 2008). This is supported by our observation that 144 of the total 170 compounds were produced by only one of the strains studied. Little is known about the evolution of genes of fatty acid biosynthesis in prokaryotic autotrophs. Nevertheless, the effect of lateral gene transfer cannot be excluded, since some indications of lateral transfer of genes of fatty acid biosynthesis have been reported from other prokaryotes, e.g. *Mycobacterium tuberculosis* (Kinsella et al., 2003).
On the other hand, we demonstrated that certain correlations between particular compound production and 16S rRNA gene phylogeny exist. In all suchlike cases the *Dolichospermum* strains were isolated from distinct localities and it is therefore highly probable that they represented different clones. Secondary metabolite synthetic apparatus is supposedly rapidly evolving; and the correlations found despite this can be therefore considered as a support of taxonomic classification at certain level.

Acknowledgement

The authors are particularly grateful to Prof. Jiří Komárek for valuable comments and discussions during the work. Many thanks also belong to Marie Kupková, Martina Vožechová and Jindra Bučková for the reliable technical assistance. We thank the Třeboň Fishery, Inc. and the Woods and Fishponds of the Town of České Budějovice for permission for sampling. This study was largely supported by the GA ASCR (project No. KJB600960703). Partial financial assistance was provided by Grant Agency of the Czech Republic (projects No. P504/10/1501 and 206/09/0309), GA ASCR (No. AV0Z60170517) and by *Institutional Research Concepts* AV0Z5020910.

References

Aitzetmuller, K., 1993. Capillary GLC fatty-acid fingerprints of seeds lipids – a tool in plant chemotaxonomy. HRC – Journal of High Resolution Chromatography 16, 488–490.

Baker, J.A., Entsch, B. , Neilan, B.A., McKay, D.B., 2002. Monitoring changing toxigenicity of a cyanobacterial bloom by molecular methods. Appl. Environ. Microbiol. 68, 6070–6076.

de Carvalho, L.R., Roque, N.F., 2004. Correlations between primary and secondary metabolites in Ceramiales (Rhodophyta). Biochem. Syst. Ecol. 32, 337–342.

Castenholz, R. W., 1992. Species usage, concept, and evolution in the cyanobacteria (blue-green algae). J. Phycol. 28, 737–745.

Chorus, I., Bartram, J., 1999. Toxic cyanobacteria in water. E. & F. N. Spon, London and New York.

Dembitsky, V.M., Rezanka, T., 2005. Metabolites produced by nitrogen-fixing Nostoc species. Folia Microbiol. 50, 363–391.

Dembitsky, V.M., Rezankova, H., Rezanka, T., Hanus, L.O., 2003. Variability of the fatty acids of the marine green algae belonging to the genus *Codium*. Biochem. Syst. Ecol. 31, 1125–1145.

Falshaw, R., Furneaux, R.H., 2009. Chemotaxonomy of New Zealand red algae in the family *Gigartinaceae* (Rhodophyta) based on galactan structures from the tetrasporophyte life-stage. Carbohydr. Res. 344, 210–216.

Frisvad, J.C., Andersen, B.,Thrane, U., 2008. The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. Mycol. Res. 112, 231–240.

Fujii, K., Sivonen, K., Nakano, T., Harada K., 2002. Structural elucidation of cyanobacterial peptides encoded by peptide synthetase gene in *Anabaena* species. Tetrahedron 58, 6863–6871.

Guillard, R.R.L., Lorenzen, C.J., 1972. Yellow-green algae with chlorophyllide c. J. Phycol. 8, 10–14.

Grayer, R.J., Chase, M.W., Simmonds, M.S.J., 1999. A comparison between chemical and molecular characters for the determination of phylogenetic relationships among plant families: An appreciation of Hegnauer's "Chemotaxonomie der Pflanzen". Biochem. Syst. Ecol. 27, 369–393.

Gugger, M., Lyra, C., Henriksen, P., Couté, A., Humbert, J.-F., Sivonen, K., 2002a. Phylogenetic comparison of cyanobacterial genera *Anabaena* and *Aphanizomenon*. Int. J. Syst. Evol. Micr. 52, 1–14.

Gugger, M., Lyra, C., Suominen, I., Tsitko, I., Humbert, J.-F., Salkinoja-Salonen, M.S., Sivonen, K., 2002b. Cellular fatty acids as chemotaxonomic markers of the genera *Anabaena, Aphanizomenon, Microcystis, Nostoc* and *Planktothrix* (Cyanobacteria). Int. J. Syst. Evol. Micr. 52, 1007–1015.

Halinen, K., Fewer, D.P., Sihvonen, L.M., Lyra, C., Eronen, E., Sivonen, K., 2008. Genetic diversity in strains of the genus Anabaena isolated form planktonic and benthic habitats of the Gulf of Finland (Baltic Sea). FEMS Microbiol. Ecol. 64, 199–208.

Hayes, K.P., Burch, M.D., 1989. Odorous compounds associated with algal blooms in south Australia waters. Wat. Res. 23, 115–121.

Hill, H., 1976. A new species of *Anabaena* (Cyanophyta, Nostocaceae) from a Minnesota lake. I. Phycologia 15, 61–64.

Holland, R.D., Wilkes, J.G., Rafii, F., Sutherland, J.B., Persons, C.C., Voorhees, K.J., Lay, J.O., 1996. Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom.10, 1227–1232.

Hrouzek, P., Tomek, P., Lukešová, A., Urban J., Voloshko, L., Pushparaj, B., Lukavský, J., Štys, D., Kopecký, J., 2010. Cytotoxicity and secondary metabolites production in terrestrial *Nostoc* strains originating from different climatic / geographic regions and habitats: Is their cytotoxicity environmentaly dependent? Environ Tox. (DOI: 10.1002/tox.20561).

Izaguirre, G., Hwang, C.J., Krasner, S.W., Mcguire, M.J., 1981. Geosmin and 2-methylisoborneol from cyanobacteria in three water supply systems. Appl. Environ. Microbiol. 43, 708–714.

Kämpfer, P., Buczolits, S., Albrecht, A., Busse, H.-J., Stackebrandt, E., 2003. Towards a standardized format for the description of a novel species (of an established genus): Ochrobactrum gallinifaecis sp. nov. Int. J. Syst. Evol. Microbiol. 53, 893–896.

Kinsella, R.J., Fitzpatrick, D.A., Creevey, C.J., McInerney, J.O., 2003. Fatty acid biosynthesis in *Mycobacterium tuberculosis:* Lateral gene transfer, adaptive evolution, and gene duplication. Proc. Natl. Acad. Sci. USA 100, 10320–10325.

Komárek, J., Zapomělová, E., 2007. Planktic morphospecies of the cyanobacterial genus *Anabaena* = subg. *Dolichospermum* – 1. part: coiled types. Fottea 7, 1–31.

Komárek, J., Zapomělová, E., 2008. Planktic morphospecies of the cyanobacterial genus *Anabaena* = subg. *Dolichospermum* – 2. part: straight types. Fottea 8, 1–14.

Komárková, J., 1988. Morphological variation in natural populations of *Anabaena lemmermannii* in respect to existence of *Anabaena utermoehlii*. Arch. Hydrobiol. Suppl. 80: 93–108.

Komárková-Legnerová, J., Eloranta, P., 1992. Planktic blue-green algae (Cyanophyta) from Central Finland (Jyväskylä region) with special reference to the genus *Anabaena*. Algol. Stud. 67: 103–133.

Leikoski, N., Fewer, D.P., Sivonen, K., 2009. Widespread Occurrence and Lateral Transfer of the Cyanobactin Biosynthesis Gene Cluster in Cyanobacteria. Appl. Environ Microbiol. 75, 853–857.

Li, R., Watanabe, M., Watanabe, M. M., 2000. Taxonomic studies of planktic species of *Anabaena* based on morphological characteristics in cultured strains. Hydrobiologia 438, 117–138.

Li, R., Watanabe, M. M., 2001. Fatty acid profiles and their chemotaxonomy in planktonic species of *Anabaena* (Cyanobacteria) with straight trichomes. Phytochemistry 57, 727–731.

Li, R, Watanabe, M. M., 2004. Fatty acid composition of planktonic species of *Anabaena* (Cyanobacteria) with coiled trichomes exhibited a significant taxonomic value. Curr. Microbiol. 49, 376–380.

Lyra, C., Suomalainen, S., Gugger, M., Vezie, C., Sundman, P., Paulin, L., Sivonen, K., 2001. Molecular characterization of planktic Cyanobacteria of *Anabaena, Aphanizomenon, Microcystis,* and *Planktothrix* genera. Int. J. Syst. Evol. Microbiol. 51, 513–526.

Marshall, J.A, Nichols, P.D., Hallegraeff, G.M., 2002. Chemotaxonomic survey o f sterols and fatty acids in six marine raphidophyte algae. J. Appl. Phycol. 14, 255–265.

Mongrand, S., Badoc, A., Patouille, B., Lacomblez, C., Chavent, M., Bessoule, J.J., 2005. Chemotaxonomy of the *Rubiaceae* family based on leaf fatty acid composition. Phytochemistry 66, 549–559.

Nübel, U., Garcia-Pichel, F., Muyzer, G., 1997. PCR primers to amplify 16S rRNA genes from cyanobacteria. Appl. Env. Microbiol. 63, 3327–3332.

Rajaniemi, P., Hrouzek, P., Kaštovská, K., Willame, R., Rantala, A., Hoffmann, L., Komárek, J., Sivonen, K., 2005a. Phylogenetic and morphological evaluation of the genera *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* (Nostocales, Cyanobacteria). Int. J. Syst. Evol. Micr. 55, 11–26.

Rajaniemi, P., Komárek, J.,Willame, R., Hrouzek, P., Kaštovská, K., Hoffmann, L., Sivonen, K., 2005b. Taxonomic consequences from the combined molecular and phenotype evaluation of selected *Anabaena* and *Aphanizomenon* strains. Algol. Stud. **117:**371–391.

Rantala, A., Fewer, D.P., Hisbergues, M., Rouhiainen, L., Vaitomaa, J., Börner, T., Sivonen, K., 2004. Phylogenetic evidence for the early evolution of microcystin synthesis. Proc. Natl. Acad. Sci. USA 101, 568–573.

Rapala, J., Sivonen, K., 1998. Assessment of Environmental Conditions That Favor Hepatotoxic and Neurotoxic *Anabaena* spp. Strains Cultured under Light Limitation at Different Temperatures. Microb. Ecol. 36, 181–192.

Romano, I., Bellitti, M.R., Nicolaus, B., Lama, L., Manca, M.C., Pagnotta, E., Gambacorta, A., 2000. Lipid profile: a useful chemotaxonomic marker for classification of a new cyanobacterium in *Spirulina* genus. Phytochemistry 54, 289–294.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Sivonen, K., 1996. Cyanobacterial toxins and toxin production. Phycologia 35,12–24.

Skerratt J.H., Nichols P.D., Mancuso C.A. 1991. The phospoholipid ester-linked fatty-acid composition of the members of the family *Halomonadaceae* and the genus *Flavobacterium* – a chemotaxonomic guide. Syst. Appl. Microbiol. 14, 8–13.

Šmilauer, P., 1992. *CANODRAW users guide v. 3.0.* Microcomputer Power, Ithaca, New York.

Søchting, U., Frödén, P., 2002. Chemosyndromes in the lichen genus *Teloschistes* (Teloschistaceae, Lecanorales). *Mycol. Prog.* 1, 257–266.

Stüken, A., Campbell, R.J., Quesada, A., Sukenik, A., Dadheech, P.K., Wiedner, C., 2009. Genetic and morphologic characterization of four putative cylindrospermopsin producing species of the cyanobacterial genera *Anabaena* and *Aphanizomenon.* J. Plank. Res. 31, 465–480.

Taton, A., Grubisic, S., Brambilla, E., De Wit, R., Wilmotte, A., 2003. Cyanobacterial diversity in natural and artificial microbial mats of Lake Fryxell (McMurdo dry valleys, Antarctica): A morphological and molecular approach. Appl. Env. Microbiol. 69, 5157–5169.

Temina, M., Rezankova, H., Rezanka, T., Dembitsky, V.M., 2007. Diversity of the fatty acids of the Nostoc species and their statistical analysis. Microbiol. Res. 162, 308–321.

Ter Braak, C.J.F., Šmilauer, P., 1998. *CANOCO reference manual.* Microcomputer Power, Ithaca, New York.

Thacker, R.W., Paul, V. J., 2004. Morphological, Chemnical, and Genetic Diversity of Tropical Marine Cyanobacteria *Lynbya* spp. and *Symploca* spp. (Oscillatoriales). Appl. Env. Microbiol. 70, 3305–3312.

Van Wagoner, R.M., Drummond, A.K., 2007. Biogenetic diversity of cyanobacterial metabolites. Adv. Appl. Microbiol. 61, 89–217.

Wacklin, P., Hoffmann, L., Komárek, J., 2009. Nomenclatural validation fo the genetically revised cyanobacterial genus *Dolichospermum* (Ralfs ex Bornet et Flahault) comb. Nova. Fottea 9, 59–64.

Welker, M., Maršálek, B., Šejnohová, L., von Dohren, H., 2006. Detection and identification of oligopeptides in *Microcystis* (cyanobacteria) colonies: Toward an understanding of metabolic diversity. Peptides 27, 2090–2103.

Welker, M., von Dohren, H., 2006. Cyanobacterial peptides – Nature's own combinatorial biosynthesis. Fems Microbiol. Rev. 30, 530–563.

Wilmotte, A., Van der Auwera, G., De Wachter, R., 1993. Structure of the 16S ribosomal RNA of the thermophilic cyanobacterium *Chlorogloeopsis* HTF (*'Mastigocladus laminosus* HTF') strain PCC7518, and phylogenetic analysis. FEBS Microbiol. Lett. 317, 6–100.

Wilmotte, A., Neefs, J. M., De Wachter, R., 1994. Evolutionary affiliation of the marine nitrogen-fixing cyanobacterium *Trichodesmium* sp strain NIBB 1067, derived by 16S ribosomal RNA sequence analysis. Microbiology 140, 2159–2164.

Wink, M., 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry 64, 3–19.

Zapomělová, E., Řeháková, K., Znachor, P., Komárková, J., 2007. Morphological diversity of coiled planktonic types of the genus *Anabaena* (cyanobacteria) in natural populations – taxonomic consequences. Crypt. Algol. 28, 353–371.

Zapomělová, E., 2008. *Anabaena* – Phenotypic and genotypic diversity of planktonic strains in fishponds and reservoirs of the Czech Republic. Ph.D. Dissertation. University of South Bohemia, Faculty of Science, České Budějovice, Czech Republic, 125 pp.

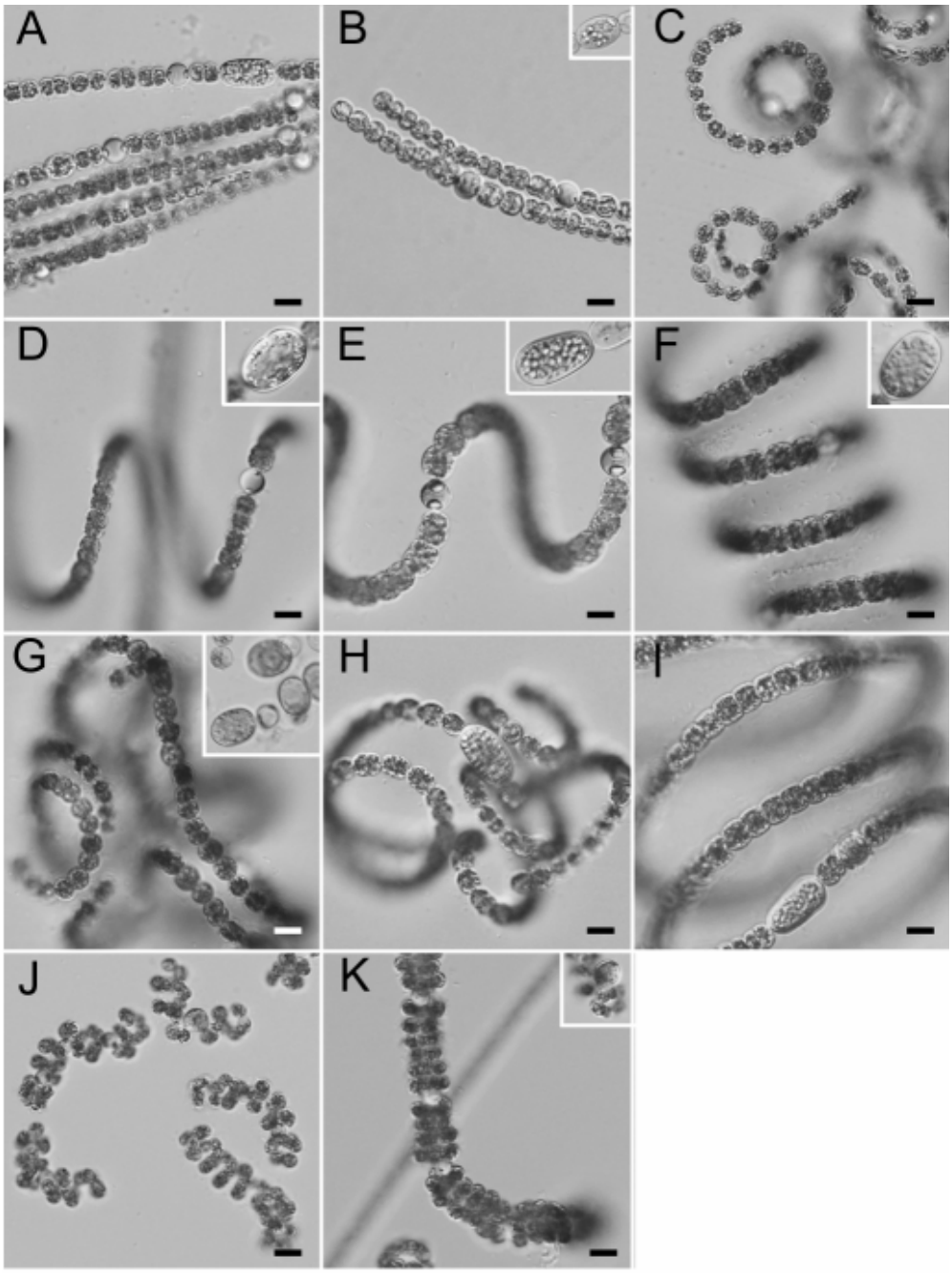
Zapomělová, E., Hisem, D., Řeháková, K., Hrouzek, P., Jezberová, J., Komárková, J., Korelusová, J., Znachor, P., 2008. Experimental comparison of phenotypical plasticity and growth demands of two strains from the *Anabaena circinalis / A. crassa* complex (cyanobacteria). J. Plank. Res. 30, 1257–1269.

Zapomělová, E., Jezberová, J., Hrouzek, P., Hisem, D., Řeháková, K., Komárková, J., 2009. Polyphasic characterization of three strains of *Anabaena reniformis* and *Aphanizomenon aphanizomenoides* (cyanobacteria) and their re-classification to *Sphaerospermum* gen. nov. (incl. *Anabaena kisseleviana).* J. Phycol. 45, 1363–1373.

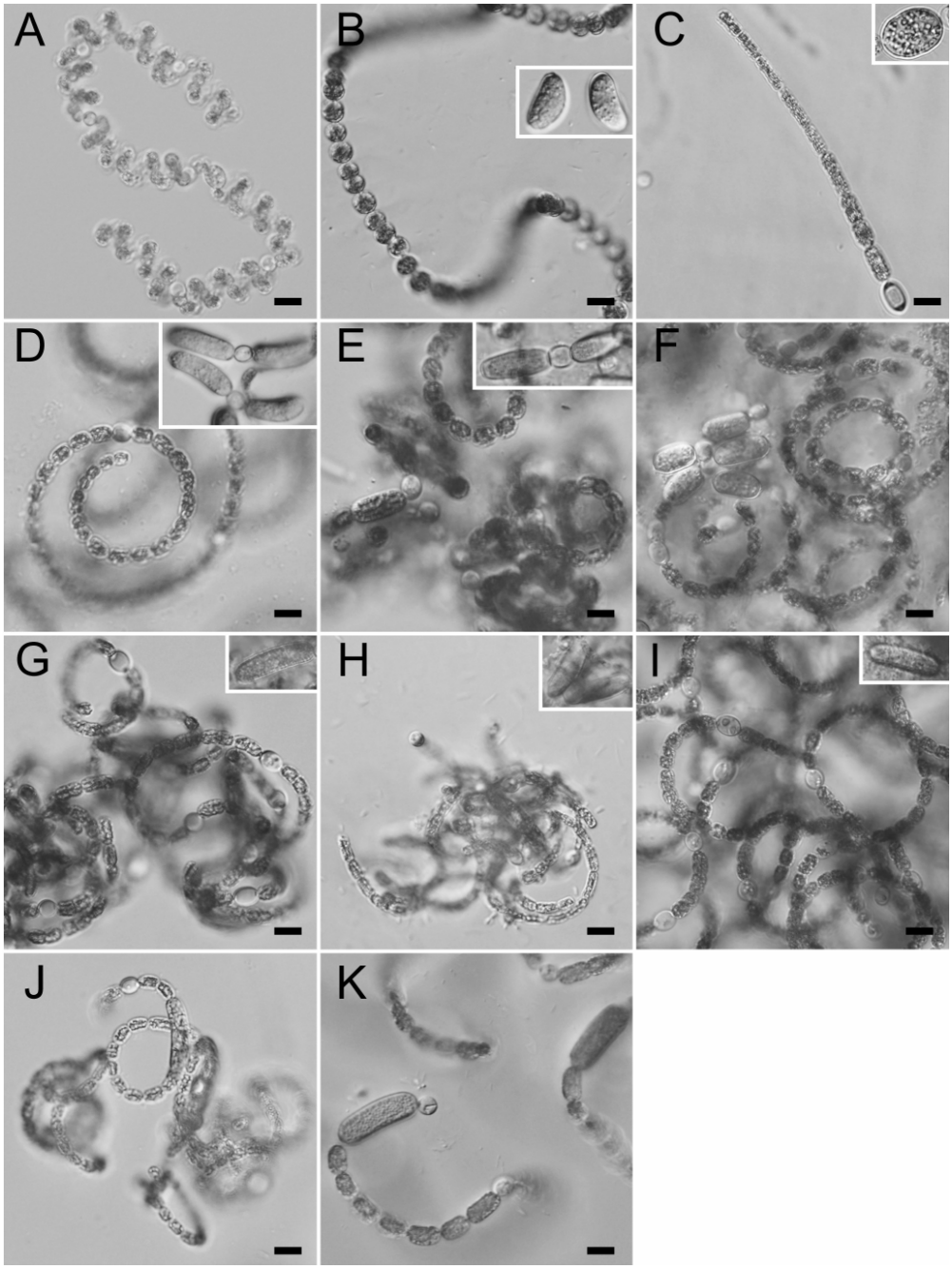
Zapomělová, E., Jezberová, J., Hrouzek, P., Hisem, D., Řeháková, K., Komárková, J., 2010a. Polyphasic characterization of three strains of *Anabaena reniformis* and *Aphanizomenon aphanizomenoides* (cyanobacteria) and their re-classification to *Sphaerospermum* gen. nov. (incl. *Anabaena kisseleviana).* Nomenclatural Note. J. Phycol., in press.

Zapomělová, E., Řeháková, K., Jezberová, J., Komárková, J., 2010b. Polyphasic characterization of eight planktonic *Anabaena* strains (cyanobacteria) with reference to the variability of 61 *Anabaena* populations observed in the field. Hydrobiologia 639, 99–113.

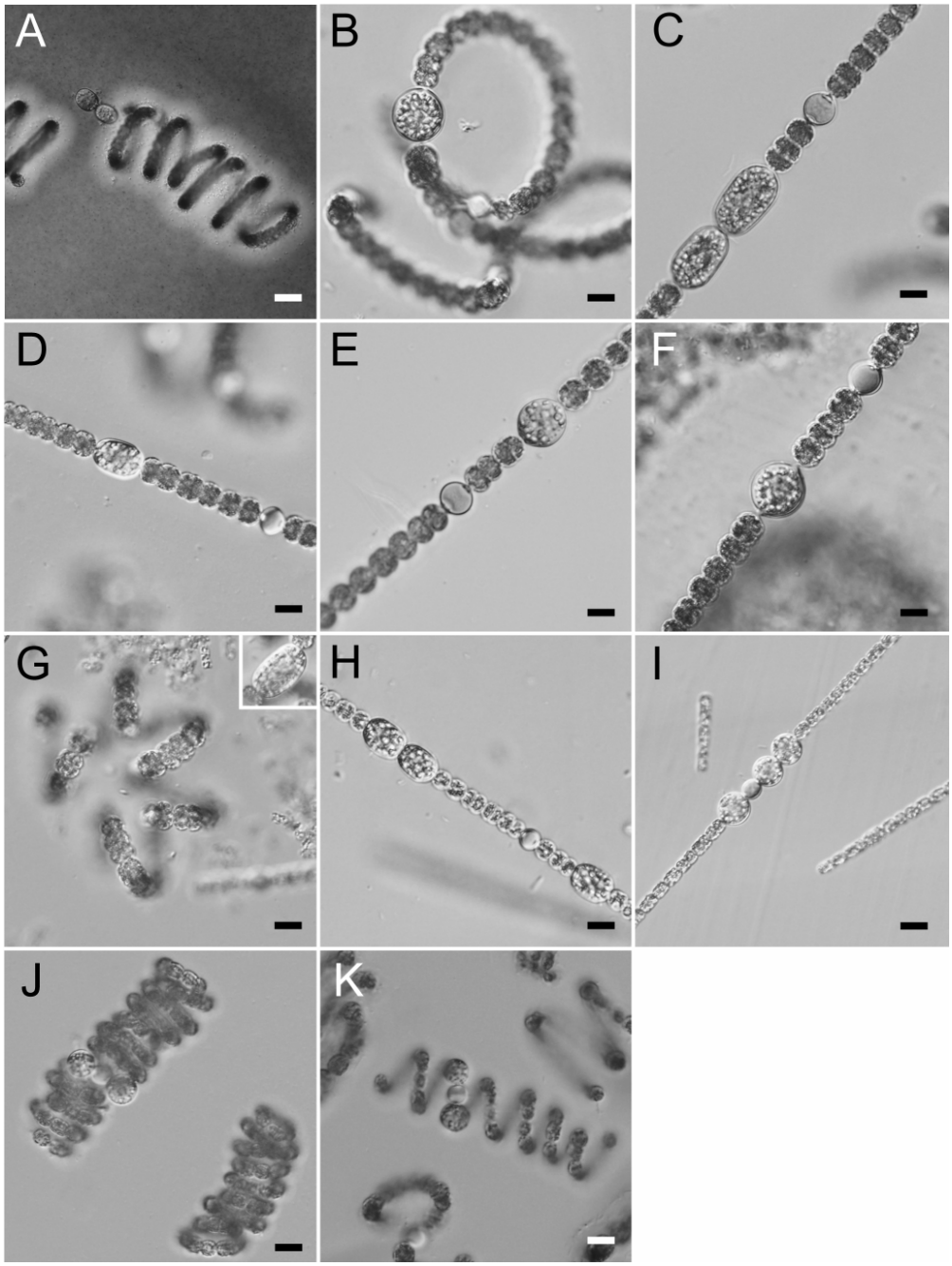
Appendixes



Appendix 1: Microphotographs of cyanobacterial strains studied. (A) *D. affine* 04-44, (B) *D. affine* 05-03, (C) *D. flos-aquae* 04-10, (D) *D. circinale* x *D. crassum* 04-22, (E) *D. circinale* x *D. crassum* 04-26, (F) *D. circinale* x *D. crassum* 04-28, (G) *D. flos-aquae* 04-40, (H) *D. flos-aquae* 04-57, (I) *D. circinale* x *D. crassum* 04-59, (J) *D. compactum* 04-17, (K) *D. compactum* 06-02. Scale bars represent 10 μm.



Appendix 2: Microphotographs of cyanobacterial strains studied. (A) *D. compactum* 06-03, (B) *D. curvum* 04-19, (C) *Anabaena* sp. 08-05, (D) *D. lemmermannii* 04-24, (E) *D. lemmermannii* 04-38, (F) *D. lemmermannii* 04-42, (G) *D. sigmoideum* x *D. mendotae* 04-06, (H) *D. sigmoideum* x *D. mendotae* 04-11, (I) *D. sigmoideum* x *D. mendotae* 04-33, (J) *D. sigmoideum* x *D. mendotae* 04-45, (K) *D. sigmoideum* x *D. mendotae* 05-01. Scale bars represent 10 μm.



Appendix 3: Microphotographs of cyanobacterial strains studied. (A) *D. mucosum* 06-04, (B) *D. mucosum* 08-03, (C) *D. planctonicum* 00-05, (D) *D. planctonicum* 03-02, (E) *D. smithii* 05-05, (F) *D. smithii* 08-02, (G) *D. spiroides* 04-51, (H) *D. viguieri* 08-04, (I) *S. aphanizomenoides* 04-43, (J) *S. reniformis* 06-01, (K) *S. reniformis* 07-01. Scale bars represent 20 μm (A) or 10 μm (B–K).

Appendix 4: Secondary metabolites detected by HPLC-MS analysis in extracts of the *Dolichospermum* and *Sphaerospermopsis* strains studied. *m/z* values and retention times (RT) are given for each compound.

m/z	RT (min)	04-06	04-11	04-19	04-22	04-24	04-26	04-28	04-33	04-38	04-40	04-42	04-44	04-45	04-51	04-57	04-59	06-02
714.4	7.2				1		1											
756.3	9.6				1		1											
909.1	9.9												1		1			
701.3	11.2	1	1						1		1	1						
453.6	11.3					1		1		1								
685.2	11.6	1	1				1	1			1	1			1	1	1	1
823.3	11.9			1		1												
543.4	12.2												1			1		
1022.2	12.5			1		1												
938.3	12.6										1			1				
727.4	12.6												1		1	1		
837.5	13.1	1				1												
652.3	13.7		1						1									
1036.8	13.8			1		1												
587.4	17.5				1						1							
794.5	19.4					1												1
731.3	19.4				1		1											
828.4	19.6		1						1									
842.4	20.3		1						1					1				
569.4	20.4					1	1				1						1	
571.3	21.0					1										1	1	
715.3	21.6				1		1				1					1	1	
256.3	21.7	1	1					1		1		1						
671.4	23.1										1					1	1	
813.5	23.4						1		1									
768.5	23.4			1						1					1			

Zapomělová E., Jezberová J., **Hrouzek P.,** Hisem D., Řeháková K. & Komárková J. 2009.

Polyphasic characterization of three strains of *Anabaena reniformis* and *Aphanizomenon aphanizomenoides* (Cyanobacteria) and their reclassification to *Sphaerospermum* gen. nov. (incl. *Anabaena kisseleviana*.

Journal of Phycology 45: 1363-1373.

J. Phycol. **45**, 1363–1373 (2009)

DOI: 10.1111/j.1529-8817.2009.00758.x

POLYPHASIC CHARACTERIZATION OF THREE STRAINS OF *ANABAENA RENIFORMIS* AND *APHANIZOMENON APHANIZOMENOIDES* (CYANOBACTERIA) AND THEIR RECLASSIFICATION TO *SPHAEROSPERMUM* GEN. NOV. (INCL. *ANABAENA KISSELEVIANA*)[1]

Eliška Zapomělová[2]

University of South Bohemia, Faculty of Science, Branišovská 31, CZ-37005 České Budějovice, Czech Republic
Biology Centre of AS CR, Institute of Hydrobiology, Na Sádkách 7, CZ-37005 České Budějovice, Czech Republic

Jitka Jezberová

Biology Centre of AS CR, Institute of Hydrobiology, Na Sádkách 7, CZ-37005 České Budějovice, Czech Republic

Pavel Hrouzek, Daniel Hisem

University of South Bohemia, Faculty of Science, Branišovská 31, CZ-37005 České Budějovice, Czech Republic
Institute of Microbiology, AS CR, Department of Autotrophic Microorganisms, Opatovický mlýn, CZ-379 81 Třeboň, Czech Republic
Institute of Physical Biology, Zámek 136, CZ-37333, Nové Hrady, Czech Republic

Klára Řeháková

Biology Centre of AS CR, Institute of Hydrobiology, Na Sádkách 7, CZ-37005 České Budějovice, Czech Republic
Institute of Botany, ASCR, Dukelská 135, CZ-37982 Třeboň, Czech Republic

and Jaroslava Komárková

University of South Bohemia, Faculty of Science, Branišovská 31, CZ-37005 České Budějovice, Czech Republic
Biology Centre of AS CR, Institute of Hydrobiology, Na Sádkách 7, CZ-37005 České Budějovice, Czech Republic
Institute of Botany, AS CR, Dukelská 135, CZ-37982 Třeboň, Czech Republic

Occurrences of rare cyanobacteria *Anabaena reniformis* Lemmerm. and *Aphanizomenon aphanizomenoides* (Forti) Horecká et Komárek were recently detected at several localities in the Czech Republic. Two monoclonal strains of *An. reniformis* and one strain of *Aph. aphanizomenoides* were isolated from distant localities and different sampling years. They were characterized by a combination of morphological, genetic, and biochemical approaches. For the first time, partial 16S rRNA gene sequences were obtained for these morphospecies. Based on this gene, all of these strains clustered separately from other planktonic *Anabaena* and *Aphanizomenon* strains. They appeared in a cluster with *Cylindrospermopsis* Seenaya et Subba Raju and *Raphidiopsis* F. E. Fritsch et M. F. Rich, clustered closely together with two *An. kisseleviana* Elenkin strains available from GenBank. A new generic entity was defined (*Sphaerospermum* gen. nov., with the type species *S. reniforme*, based on the traditional species *An. reniformis*). These results contribute significantly to the knowledge base about genetic heterogeneity among planktonic *Anabaena*–like and *Aphanizomenon*–like morphospecies. Accordingly, the subgenus *Dolichospermum*, previously proposed for the group of planktonic *Anabaena*, should be revaluated. Secondary metabolite profiles of the *An. reniformis* and *Aph. aphanizomenoides* strains differed considerably from 17 other planktonic *Anabaena* strains of eight morphospecies isolated from Czech water bodies. Production of puwainaphycin A was found in both of the *An. reniformis* strains. Despite the relatively short phylogenetic distance from *Cylidrospermopsis*, the production of cylindrospermopsin was not detected in any of our strains.

Key index words: **16S rRNA gene; *Anabaena*; cyanobacteria; morphology; new genus; phytoplankton; secondary metabolites; taxonomy**

Abbreviations: **HPLC–MS, high-performance liquid chromatography–mass spectrometry; m/z, mass divided by charge; MP, maximum parsimony; NJ, neighbor joining; OTU, operational taxonomic unit; RT, retention time**

An. reniformis has only been reported a few times from isolated localities worldwide (Cronberg and Annadotter 2006, Komárek and Zapomělová 2007) and is considered to be very rare in the Czech

[1]Received 13 June 2008. Accepted 12 June 2009.
[2]Author for correspondence: e-mail eliska.zapomelova@seznam.cz.

Republic (three findings to date; Komárek 1996, Keršner 1997). It is mainly characterized by the shape and position of the akinetes (spherical, on one or both sides adjacent to the heterocytes). Other coiled morphospecies of identical akinete shape and position, and of similar cell sizes, have been described from Japan (*An. eucompacta* R. Li et M. M. Watan. [Li and Watanabe 1999] and *An. oumiana* M. Watan. [Watanabe 1996]) and also from Cuba (*An. torques-reginae* Komárek [Komárek 1984]). In addition, highly similar morphospecies with straight trichomes were described as *An. kisseleviana* Elenkin from Siberia (Elenkin 1938) and *An. austro-africana* Cronberg et Komárek (Cronberg and Komárek 2004).

Aph. aphanizomenoides shows identical shape and arrangement of akinetes as the above-mentioned *Anabaena* morphospecies. The cyanobacterium was originally described as *Anabaena aphanizomenoides* Forti from a lake in Anatolia (Geitler 1932), and later reclassified to the genus *Aphanizomenon* (Horecká and Komárek 1979). The synonymic name is *Aphanizomenon sphaericum* Kisselev. However, its taxonomic status remains unclear, since it displays morphological characteristics typical for both *Anabaena* (constricted cell walls, isodiametrical akinetes) and *Aphanizomenon* (tapered and elongated terminal cells), and its genotypic features have not yet been analyzed.

Cluster analysis of fatty-acid composition (Li and Watanabe 2004) has shown that *An. eucompacta* and *An. oumiana* group together in a distant cluster, separately from other planktonic *Anabaena* strains. This finding suggests that not only morphological but also some other features are common for similar *Anabaena* strains, discriminating them from other planktonic *Anabaena* morphospecies. Nevertheless, no study focusing on their genotypic characteristics has yet been published to confirm this segregation.

The main objective of the present study was to characterize *An. reniformis* and *Aph. aphanizomenoides* strains, using the polyphasic approach. Morphometry, 16S rRNA gene sequence, and secondary metabolite content were analyzed. New evidence on the occurrence and distribution of *An. reniformis* and *Aph. aphanizomenoides* in the Czech Republic is reported.

MATERIALS AND METHODS

Sampling. The sampling area, South Bohemia, is in the southwest of the Czech Republic, with a long tradition of fish farming. Two main fishpond systems are situated there, the first in the Třeboň Basin and another in the České Budějovice Basin. Most fishponds are intensively farmed, overstocked, and fertilized shallow water bodies where the temporary stratification is often disrupted by wind. Therefore, nutrients are easily available for the phytoplankton, and as a consequence, cyanobacterial blooms of varying intensity and species composition develop at the localities (Pechar et al. 2002). Phytoplankton samples of 100 fishponds from both the Třeboň Basin (60 localities) and České Budějovice Basin (40 localities) were repeatedly collected during the vegetation seasons 2004–2007 (from April to October) using a 20 μm mesh plankton net (Sefar, 9425 Thal, Switzerland). Species composition of the water blooms was evaluated, from fresh samples, using an Olympus BX 50 light microscope (Olympus Czech Group, Prague, Czech Republic).

Cultivation. Single trichomes were isolated from the samples using a glass capillary, and from these, clonal strains were grown. The trichomes were transferred repeatedly from a drop of sterile culture medium WC (Guillard and Lorenzen 1972) to another one, until all of the other organisms had been excluded. One strain of *Aph. aphanizomenoides* (strain 04-43, Svět fishpond, 2004) and two *An. reniformis* strains (strain 06-01, Pěšák fishpond, 2006; strain 07-01, Vyšehrad fishpond, 2007) were isolated. In addition, a strain of *An. compacta* (strain 06-02, Pěšák fishpond, 2006) was isolated from the same phytoplankton community as one of the two *An. reniformis* strains and was used as the reference strain in the phylogenetic analysis. Clonal nonaxenic cultures were grown in WC medium (Guillard and Lorenzen 1972) at 21°C and 70 μmol photon $\cdot m^{-2} \cdot s^{-1}$ light intensity (16:8 light:dark [L:D] cycle). The strains are maintained in the culture collection at the Biology Centre of the AS CR, Institute of Hydrobiology (České Budějovice, Czech Republic). The strain 06-01 was deposited in two official culture collections: (1) Culture Collection of Algal Laboratory (CCALA), Institute of Botany, Academy of Sciences of the Czech Republic, Centre of Phycology, Dukelská 135, CZ-37982 Třeboň, Czech Republic (accession no. CCALA862); and (2) Culture Collection of Algae (SAG), Albrecht-von-Haller Institute, the University of Göttingen, Nikolausberger Weg 18, 37073 Göttingen, Germany (accession no. SAG 2284).

Statistical evaluation of morphological data. Morphology of both *An. reniformis* and *Aph. aphanizomenoides* was analyzed on living materials immediately after sampling. Microphotographs of at least 30 nonfixed trichomes per population were taken using a digital camera (Olympus DP 70, magnification 400×). Size measurements were performed using image analysis (Olympus DP Soft). The basic statistical parameters (mean values, 25% and 75% percentiles, and extreme values) of all morphometric characteristics were computed for the original populations of *An. reniformis* and *Aph. aphanizomenoides* strains using the Graph Pad Prism software (version 4.0 for Windows; San Diego, CA, USA; http://www.graphpad.com). Measurements of morphometric parameters were carried out as follows: length and width of five vegetative cells per trichome in 30 trichomes (i.e., 150 cells in total), length and width of 30 heterocytes, length and width of as many as possible akinetes, length and width of as many as possible terminal cells, 150 trichome coil diameters and distances between coils.

Phylogenetic study. The biomass was harvested in the exponential phase of growth by repeated centrifugation (centrifuge Z 233 MK-2; Hermle Labortechnik GmbH, Wehingen, Germany), during which the trichomes were washed several times by physiological solution (NaCl solution, concentration $1 g \cdot L^{-1}$) to remove mucilaginous substances. The biomass samples were stored at −20°C until DNA extraction. DNA was extracted using the UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA). The 16S rRNA gene and ITS region were amplified using primers 16S27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 23S30R (5′-CTTCGCCTCTGTGTGCCTAGGT-3′) (Taton et al. 2003). Amplification was carried out as follows: one cycle of 5 min at 94°C; 10 cycles of 45 s at 94°C, 45 s at 57°C, and 2 min at 72°C; 25 cycles of 45 s at 94°C, 45 s at 54°C, and 2 min at 72°C; followed by a final elongation step of 7 min at 72°C. The PCR product was used as a template for sequencing with primers 16S27F (Taton et al. 2003) CYA781F(a) (5′-AATGGGATTAGA-

TACCCCAGTAGTC-3′) (Nübel et al. 1997), K6 (5′-GACGGGCCGGTGTGTACA-3′), which is reverse complement of Primer14 of Wilmotte et al. (1993), and K8 (5′-AAGGAGGTGATCCAGCCACA-3′) (Flechtner et al. 2002). Sequences were aligned in the ARB program (http://www.arb-home.de). The alignment was edited manually, and ambiguous bases were removed. For the phylogenetic analysis, trees were built with the neighbor-joining (NJ) method (Saitou and Nei 1987) and also the maximum-parsimony (MP) algorithms in the Phylip program (Felsenstein 2004). Five hundred bootstrap replicates were performed for both NJ and MP analyses. Nucleotide sequences were deposited at GenBank under accession numbers FM161347–FM161350.

The list of those strains whose 16S rRNA gene sequences were used for the construction of the phylogenetic trees in this study is available in Table S1 (see the supplementary material). Only those sequences of the genera *Anabaena* and *Aphanizomenon* whose morphologies were clearly and reliably described (Rajaniemi et al. 2005a,b) were chosen from GenBank. The strains of *An. kisseleviana, Raphidiopsis,* and *Cylindrospermopsis* were chosen based on the BLAST (the closest matches). Morphologies of the *An. kisseleviana* strains were described by Li et al. (2000); polyphasic characterization of the *Raphidiopsis* strains was given by Li et al. (2008).

Secondary metabolite content—extract preparation and high-performance liquid chromatography–mass spectrometry (HPLC–MS). The lyophilized biomass (~40 mg) was disintegrated by grinding and extracted in 2 mL of 70% methanol (MeOH) in microtubes for 30 min. Microtubes were centrifuged at 4,500 rpm (3,400*g*) for 15 min at 4°C. The supernatant was concentrated 10 times at 37°C using rotary vacuous drier ROTAVAPOR B-840 and WATERBATH R-114 (BÜCHI, Flawil Switzerland). Extracts were analyzed on a reversed phase column (Zorbax XBD C8, 46 × 150 mm, 5 μm; Agilent Technologies Inc., Santa Clara, CA, USA) using a MeOH/H_2O gradient with a flow rate of 0.6 mL · min^{-1}. For more effective ionization, 0.1% formic acid was added in the eluents. Extract composition was analyzed by an HP 1100 Agilent mass spectrometer, with an HP 100 MSD SL-Ion trap (Agilent Technologies Inc.) in positive mode. These settings were selected to cover the mass range 50–2,000, and the ion trap was targeted to molecular masses near 900. Automatic fragmentation of the most intensive peak was applied. The molecular ions were determined, based both on the presence of sodium and potassium adducts and on the distribution of isotopologues. Two alternative extraction protocols were performed for the evaluation of cylindrospermopsin content: 20 mg of lyophilized biomass was extracted using 90% MeOH, according to Törökné et al. (2004), and the same amount was extracted using 5% acetic acid as suggested by Kikuchi et al. (2007). Simultaneously, the samples of the cultivation medium were taken for analysis. The extracts were injected directly into the HP 1100 Agilent mass spectrometer, with an HP 100 MSD SL-Ion trap targeted at molecular ions near 400; the positive and negative mode was changed manually during the direct infusion.

RESULTS AND DISCUSSION

Occurrence of An. reniformis *and* Aph. aphanizomenoides *in South Bohemia. An. reniformis* has been newly observed at nine localities, and *Aph. aphanizomenoides* at 14 localities in the Třeboň Basin (Tables S2 and S3 in the supplementary material), while they were not detected in the České Budějovice Basin.

Only one previous observation of *An. reniformis* was reported by Keršner (1997) from a rather distant region of Southern Moravia, Czech Republic. The foregoing paper mentioned two former findings of *An. reniformis* in the Czech Republic, without further specification. In September 2009, it has been newly found in fishponds Hlohovecký (48°46′59.388″ N, 16°47′22.347″ E) and Mlýnský (48°46′38.566″ N, 16°47′36.7″ E) in Southern Moravia (E. Zapomělová and O. Skácelová, unpublished data). Scanty records exist on the occurrence of *An. reniformis* around the world. It appears to be sparsely distributed, at very distant localities: Germany, Ukraine, Japan, Cuba, and Africa (Lemmermann 1898, Aptekar in Elenkin 1938, Watanabe et al. 2004, Komárek 2005, Cronberg and Annadotter 2006, Komárek and Zapomělová 2007). However, Cronberg and Annadotter (2006) remarked that it might well have been overlooked.

This is the first report on the occurrence of *Aph. aphanizomenoides* in the Czech Republic in almost 30 years, since it was reported by Horecká and Komárek (1979) from Central Moravia. Currently, it has also been found in some other regions of the Czech Republic (R. Kopp and P. Pumann, unpublished data; Table S4 in the supplementary material). Although *Aph. aphanizomenoides* has predominantly been described from the tropical and subtropical regions, within the last few years, it seems to have expanded to the temperate zones of Central Europe, such as Hungary (Padisák and Kovács 1997), Slovakia (Hindák 2000), Poland (Stefaniak and Kokociński 2005), and northeast Germany (Stüken et al. 2006).

Reliable identification of *An. reniformis* and *Aph. aphanizomenoides* is only possible when akinetes are present. Thus, their occurrence could be more frequent than has so far been reported.

Morphology of An. reniformis. The populations of *An. reniformis* were easily identifiable by the position of akinetes relative to heterocytes. Besides similar morphometric parameters, they also shared some other phenotypical features: dark-green-colored to brownish vegetative cells containing gas vesicles, undifferentiated terminal cells, and distinct mucilaginous sheaths (Figs. 1–2). Despite a larger coiling diameter in all *An. reniformis* populations, differentiation from *An. compacta* (Nygaard) B. Hickel might be disputed in cases of the akinete absence. Apparent dissimilarities, mainly in the shape (length:width ratio) of vegetative cells and in the tightness of trichome coiling, were observed between the *An. reniformis* populations from both the Pěšák fishpond, 2006, and Vyšehrad fishpond, 2007 (Figs. 1–2; Table 1). This finding is in good agreement with the molecular data, since 16S rRNA gene sequences of the strains isolated from those populations were not identical (see below).

Under culture conditions, some morphometric characteristics of *An. reniformis* strains were modified

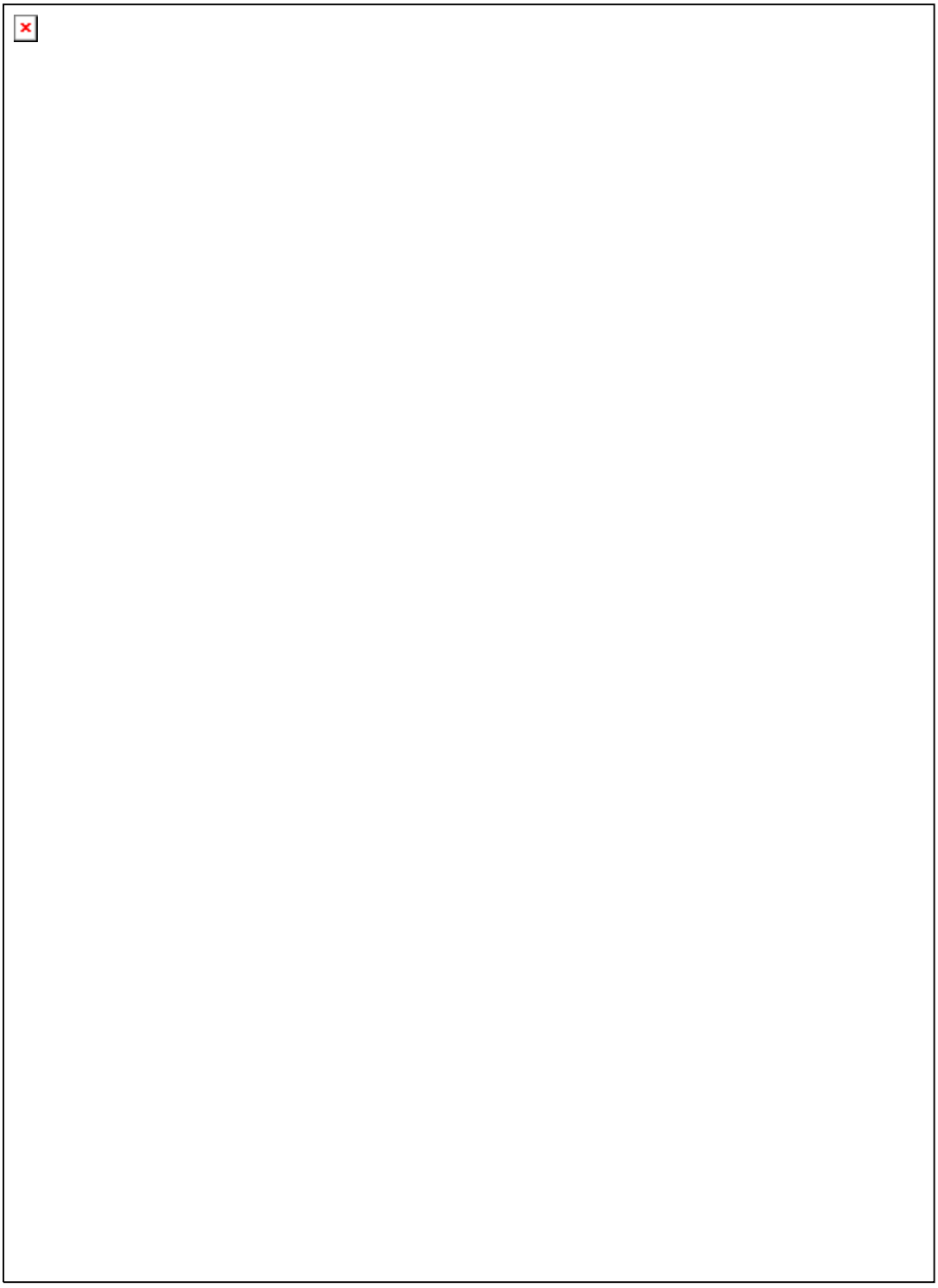


TABLE 1. Morphological characteristics of the populations of *Anabaena reniformis* and *Aphanizomenon aphanizomenoides* observed in field conditions (A, akinetes; ●, heterocytes; ■■, vegetative cells). Values are (minimum) 25% percentile-**mean value**-75% percentile (maximum).

Strain code Morphospecies	06-01 *Anabaena reniformis*	07-01 *Anabaena reniformis*	04-43 *Aphanizomenon aphanizomenoides*
Vegetative cells			
Length [μm]	(4.1) 4.7-**5.3**-5.8 (7.7)	(3.9) 5.3-**6.1**-6.8 (9.5)	(3.3) 4.5-**5.5**-6.5 (11.2)
Width [μm]	(4.3) 5.0-**5.2**-5.5 (6.0)	(3.9) 4.6-**4.8**-5.0 (5.6)	(3.1) 4.1-**4.4**-4.7 (7.2)
lw ratio	(0.7) 0.9-**1.0**-1.2 (1.4)	(0.8) 1.1-**1.3**-1.4 (2.1)	(0.7) 1.0-**1.3**-1.5 (2.3)
Heterocytes			
Length [μm]	(5.8) 6.4-**6.9**-7.4 (7.6)	(6.0) 6.6-**7.0**-7.4 (7.9)	(5.2) 5.7-**6.3**-7.0 (8.5)
Width [μm]	(6.3) 6.6-**7.1**-7.4 (8.2)	(5.4) 6.0-**6.5**-7.0 (7.6)	(4.8) 5.1-**5.9**-6.8 (7.1)
lw ratio	(0.87) 0.92-**0.96**-1.00 (1.11)	(0.9) 1.0-**1.1**-1.2 (1.3)	(0.9) 1.0-**1.1**-1.2 (1.3)
Akinetes			
Length [μm]	(8.5) 9.5-**10.0**-10.3 (12.3)	(8.7) 9.0-**9.5**-10.0 (10.5)	(7.5) 9.2-**10.0**-10.9 (11.4)
Width [μm]	(8.0) 9.1-**9.5**-10.0 (10.5)	(9.0) 9.2-**9.4**-9.6 (9.8)	(8.9) 9.8-**10.3**-11.1 (11.4)
lw ratio	(0.9) 1.0-**1.1**-1.1 (1.2)	(0.9) 0.96-**1.01**-1.07 (1.11)	(0.8) 0.9-**1.0**-1.1 (1.2)
Position	A●A or A●■■■	A●A or A●■■■	A●A or A●■■■ or AA●A
Trichome coiling			
Diameter [μm]	(12.0) 15.5-**16.9**-18.6 (22.3)	(12.3) 15.4-**17.3**-18.8 (27.3)	Straight trichomes
Distance [μm]	(3.5) 5.9-**6.6**-7.5 (11.9)	(4.3) 7.6-**10.5**-12.9 (21.9)	Straight trichomes
diam:dist ratio	(1.3) 2.3-**2.6**-2.9 (5.7)	(0.8) 1.3-**1.8**-2.4 (4.2)	Straight trichomes
Regularity	Regular	Regular	Straight trichomes
Terminal cells			
Length [μm]	Undifferentiated	Undifferentiated	(10.7) 11.1-**13.6**-16.7 (16.9)
Width [μm]	Undifferentiated	Undifferentiated	(2.5) 2.6-**3.1**-3.6 (3.9)
lw ratio	Undifferentiated	Undifferentiated	(3.2) 3.3-**4.5**-5.9 (6.8)

Bohemia shared not only similar mophometric parameters but also some other phenotypic features: dark-green-colored to brownish vegetative cells containing gas vesicles and elongated, tapered but unpointed terminal cells (Fig. 3). Formation of two or more akinetes at one side of a heterocyte sometimes occurred, both from the field and in cultures. Mucilaginous substances were not detected, using India-ink staining. Most of the akinetes retained their characteristic arrangement in culture; however, akinetes remote from heterocytes were also observed several times (Fig. 3, g–h). In culture, the terminal cell morphology was not as clearly differentiated as under field conditions, but terminal cells were always detectable on at least some trichomes.

Phylogenetic analysis. The two *An. reniformis* strains (06-01, 07-01) and the strain of *Aph. aphanizomenoides* (04-43) appeared in a common cluster, closely together with two *An. kisseleviana* strains (NIES74, TAC34) published by Li et al. in GenBank (Fig. 4, Table 2). All of these strains were placed close to *Raphidiopsis* Fritsch et F. Rich and *Cylindrospermopsis raciborskii* (Wołosz.) Seenaya et Subba Raju. Their cluster was distinct from the big cluster of other planktonic *Anabaena* and *Aphanizomenon* strains presented by Rajaniemi et al. (2005a,b), which also

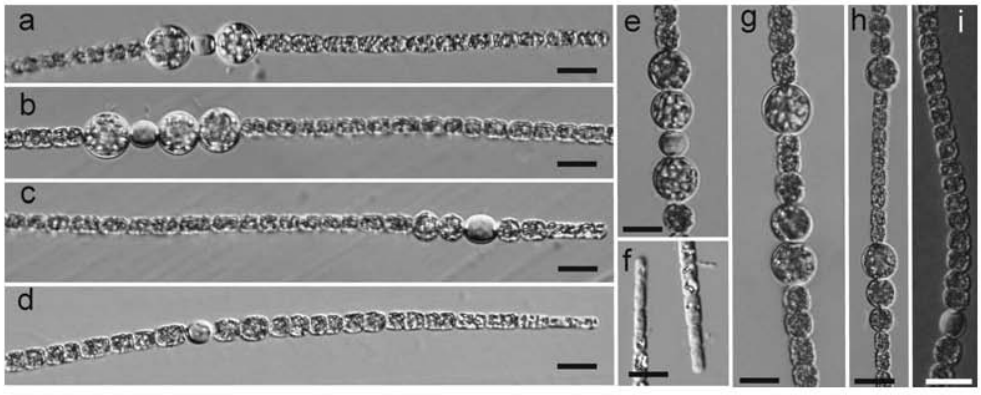


FIG. 3. *Aphanizomenon aphanizomenoides* from the Svět fishpond in original phytoplankton sample (a–d) and in culture (e–i). Absence of mucilage is demonstrated using India ink (i). Scale bars, 10 μm.

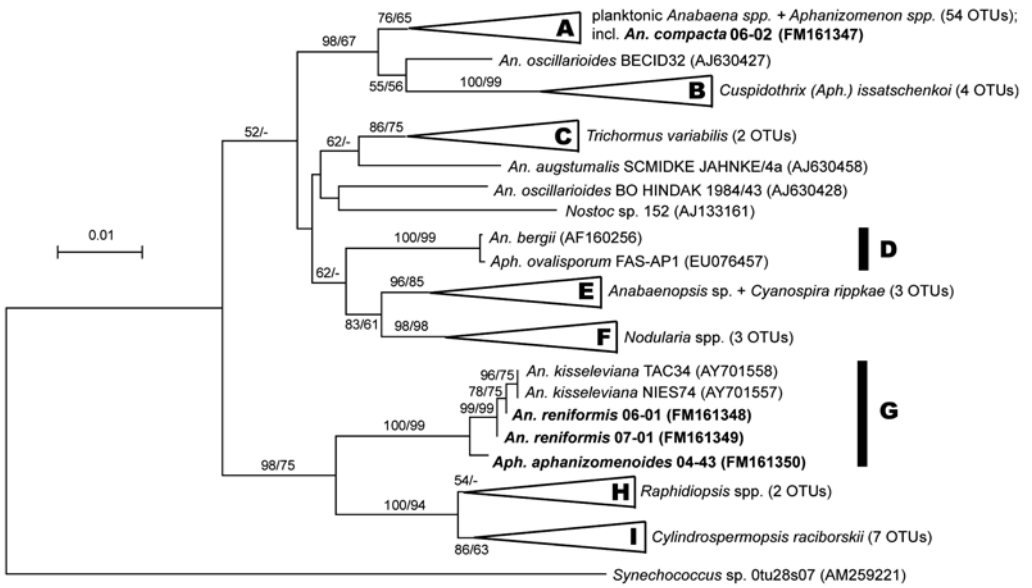


FIG. 4. Neighbor-joining tree based on 16S rRNA gene sequences (1,318 bp) showing the clustering of *Anabaena reniformis* strains (strain codes 06-01 and 07-01), *Aphanizomenon aphanizomenoides* (04-43), and *Anabaena compacta* strain (06-02). The studied strains are in bold; numbers near nodes indicate bootstrap values >50% for the neighbor-joining and maximum-parsimony analyses.

included the strain of *A. compacta* (06-02). NJ and MP phylogenetic algorithms produced trees with a very similar topology, and therefore only the NJ tree is presented (Fig. 4).

These results are an important contribution to the knowledge on genotypic heterogeneity within the genera *Anabaena* and *Aphanizomenon*, since up to the present, the group of planktonic *Anabaena* has appeared to be monophyletic and intermixed with *Aphanizomenon* strains (Gugger et al. 2002, Rajaniemi et al. 2005a,b, Willame et al. 2006), except for *An. bergii* and *Aph. ovalisporum*. Our results do not support the previous suggestion that all planktonic *Anabaena* and *Aphanizomenon* strains could be

TABLE 2. Matrix showing P distances (%), based on the 16S rRNA gene (1,318 bp). All positions containing alignment gaps and missing data were only eliminated in pair-wise sequence comparison.

	Strain	Cl.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	***An. reniformis* 06-01**	G																
2	***An. reniformis* 07-01**	G	99.9															
3	***Aph. aphanizomenoides* 04-43**	G	99.4	99.5														
4	*An. kisseleviana* TAC34	G	99.9	99.8	99.3													
5	*An. kisseleviana* NIES74	G	99.9	99.8	99.3	100.0												
6	***An. compacta* 06-02**	A	93.3	93.4	93.5	93.2	93.2											
7	*An. lemmermannii* 1tu32s11	A	93.2	93.3	93.1	92.9	92.9	98.0										
8	*Aph. flos-aquae* 1tu29s19	A	93.5	93.6	93.7	93.4	93.4	98.1	98.2									
9	*Aph. gracile* 1tu26s16	A	93.3	93.4	93.2	93.1	93.1	98.2	98.9	98.8								
10	*Cuspidothrix (Aph.) issatschenkoi* 0tu37s7	B	93.8	93.9	94.0	93.5	93.5	96.4	96.2	95.8	96.2							
11	*Trichormus variabilis* HINDAK2001/4	C	95.0	95.0	95.2	94.9	94.9	95.9	95.3	95.6	95.6	96.0						
12	*An. bergii* (AF160256)	D	94.1	94.2	94.2	94.1	94.1	95.4	95.1	95.6	95.2	94.9	96.2					
13	*Aph. ovalisporum* FAS-AP1	D	94.0	94.1	94.1	94.1	94.1	95.3	95.0	95.5	95.1	94.9	96.2	99.9				
14	*Anabaenopsis* sp. PCC9215	E	93.7	93.7	93.9	93.7	93.7	95.3	95.6	95.3	95.0	94.9	95.7	96.9	96.9			
15	*Nodularia* sp. PCC73104/1	F	93.3	93.3	93.5	93.3	93.3	95.3	95.2	95.0	95.0	94.5	96.2	96.8	96.7	96.9		
16	*Raphidiopsis mediterranea* HB2	H	96.2	96.3	96.6	96.0	96.0	93.2	93.6	93.6	93.6	93.3	93.8	93.3	93.4	93.9	92.5	
17	*Cylindrospermopsis raciborskii* LMECYA132	I	95.9	96.0	96.4	95.8	95.8	92.8	93.2	93.3	93.1	93.0	93.7	93.4	93.5	93.8	92.4	99.3

Cl., cluster; letters correspond to the numbering of clusters in Figure 4; the studied strains are in bold. *An.*, *Anabaena*; *Aph.*, *Aphanizomenon*.

assigned to a single genus (Rajaniemi et al. 2005a). Evolutionary distances between the strains of the herein presented cluster G (*An. reniformis*, *Aph. aphanizomenoides*, *An. kisseleviana*) and other planktonic *Anabaena* and *Aphanizomenon* strains (Table 2) suggested that these strains should be classified into a separate genus (see below). Moreover, the subgeneric name *Dolichospermum* (Ralfs ex Bornet et Flahault) Komárek et Anagn. 1989, proposed by Rajaniemi et al. (2005b) for the planktonic *Anabaena* group excl. *Aphanizomenon*, should be revaluated.

All of the strains from the cluster G exhibited spherical akinetes adjacent to heterocytes, suggesting that this could be a good indicator of phylogenetic affiliations of similar *Anabaena* and *Aphanizomenon* morphospecies. Morphologies of both *An. kisseleviana* strains (TAC34 and NIES74) were thoroughly described by Li et al. (2000), and the shape and position of their akinetes were identical to the herein presented strains. The separate status of similar morphospecies is also supported by the results of Li and Watanabe (2004) on fatty-acid composition of *An. eucompacta* and *An. oumiana*. Their profiles considerably differed from other planktonic *Anabaena* strains studied. However, 16S rRNA gene sequences of these taxa have not yet been analyzed as well as the sequences of other *Anabaena* morphospecies with spherical akinetes adjacent to heterocytes, such as *An. torques-reginae* and *An. austro-africana*. Phylogenetic analyses of these morphospecies are to be performed to confirm their affiliation to the newly discovered cluster G. The subgenus *Dolichospermum* should then be limited to those planktonic morphospecies with oval, cylindrical, or reniform akinetes and/or akinetes remote from heterocytes. This delimitation would be in agreement with nomenclatorical rules of the Botanical Code, since both *An. reniformis* and *Aph. aphanizomenoides* as well as other similar morphospecies (*An. austro-africana*, *An. eucompacta*, *An. kisseleviana*, *An. oumiana*, and *An. torques-reginae*) were described later than *Dolichospermum*; thus the subgeneric name does not encompass these morphospecies, in any case.

The strains of *An. lemmermannii* P. Richt. appeared in the separated cluster A, together with other planktonic *Anabaena* and *Aphanizomenon* strains (Fig. 4), although the arrangement of their akinetes was identical to the cluster G. The main difference as compared with the cluster G was the shape of akinetes, which were kidney shaped in *An. lemmermannii*. Some other *Anabaena* and *Aphanizomenon* morphospecies can be found, whose akinetes are adjacent to heterocytes but considerably elongated (ovoid or cylindrical), such as *An. orientalis* S. C. Dixit, *An. iyengarii* Bharadwaja (Komárek 2005), and *Aph. capricorni* Cronberg et Komárek (Cronberg and Komárek 2004). From these morphospecies, 16S rRNA gene sequences are only available for several *An. iyengarii* strains in GenBank. All of these sequences are too short (up to 757 bp) to be included in our phylogenetic analyses, which are based on 1,318 bp. Nevertheless, the BLAST search for the closest matches did not detect any of the strains from the cluster G to be significantly similar to *An. iyengarii* sequences.

The classification of *Aph. aphanizomenoides* into the genera *Aphanizomenon* or *Anabaena* has been discussed frequently, since it displays morphological characteristics of both genera (Hindák 2000). A similar situation exists also with some other taxa, such as *An. bergii* Ostenf., *An. minderi* Hub.-Pest., *An. recta* Geitler et Ruttner, *Aph. ovalisporum* Forti, and *Aph. gracile* (Lemmerm.) Lemmerm. (Hindák 2000). The phylogenetic analyses presented have shown that the 16S rRNA gene sequence of *Aph. aphanizomenoides* differs markedly from *An. bergii*, *Aph. ovalisporum*, and *Aph. gracile* (Fig. 4). *Aph. gracile* clustered with the majority of planktonic *Anabaena* and *Aphanizomenon* in the cluster A, as previously shown by Rajaniemi et al. (2005a,b). *An. bergii* and *Aph. ovalisporum* appeared together in the cluster D, which was separated both from the big planktonic *Anabaena–Aphanizomenon* cluster A and from the cluster G, herein presented. Morphologies of both *An. minderi* (Hindák 2000) and *An. recta* (Cronberg and Komárek 2004) are highly similar to *An. bergii* and *Aph. ovalisporum* (shape of vegetative cells, conical terminal cells, shape of akinetes and their position apart from heterocytes), but their 16S rRNA genes have not yet been sequenced to elucidate their phylogenetic affiliations.

The high 16S rRNA gene similarity of *An. reniformis*, *An. kisseleviana*, and *Aph. aphanizomenoides* supports the previously reported indications that the occurrence of trichome coiling does not reflect the phylogeny of planktonic *Anabaena* (Rajaniemi et al. 2005b). *Anabaena* strains with coiled and straight trichomes, but of similar morphometric characteristics, often appear in the same clusters in phylogenetic trees, based on 16S rRNA gene sequences (Beltran and Neilan 2000, Rajaniemi et al. 2005a,b).

Production of secondary metabolites. To expand the strain characterization into biochemical properties, production of secondary metabolites was determined in *An. reniformis* 06-01, 07-01; *Aph. aphanizomenoides* 04-43; and *An. compacta* 06-02 using HPLC–MS analysis. The results were compared with secondary metabolite production of 17 other planktonic *Anabaena* strains.

The secondary metabolite content of the *An. reniformis* and *Aph. aphanizomenoides* strains differed from all other planktonic *Anabaena* strains analyzed. Based on the 16S rRNA gene sequences (unpublished data), all of the other strains corresponded to cluster A herein (Fig. 4) and to cluster 1 of Rajaniemi et al. (2005a). None of the compounds found in MS spectra of *An. reniformis* and *Aph. aphanizomenoides* strains was produced by any of the other 17 *Anabaena* strains (Table 3). Two

TABLE 3. Matrix showing numbers of identical secondary metabolites (molecular masses) that were recognized in planktonic *Anabaena* (*An.*) and *Aphanizomenon* (*Aph.*) strains from the Czech Republic; the studied strains are in bold.

	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	***An. reniformis* 06-01**																				
2	***An. reniformis* 07-01**	1																			
3	***Aph. aphanizomenoides* 04-43**	0	0																		
4	***An. compacta* 06-02**	0	0	0																	
5	*An. circinalis* 04-59	0	0	0	1																
6	*An. flos-aquae* 04-57	0	0	0	1	4															
7	*An. mendotae* 04-11	0	0	0	1	1	1														
8	*An. circinalis* 04-26	0	0	0	1	2	2	2													
9	*An. mendotae* 04-45	0	0	0	0	0	0	0	0												
10	*An. lemmermannii* 04-33	0	0	0	0	0	0	2	1	0											
11	*An. spiroides* 04-51	0	0	0	1	1	2	2	1	1	0										
12	*An. flos-aquae* 04-40a	0	0	0	1	3	1	2	2	1	1	0									
13	*An. circinalis* 04-22	0	0	0	0	1	0	0	4	0	0	1	2								
14	*An. affinis* 04-44	0	0	0	0	0	2	0	0	0	0	2	0	0							
15	*An. lemmermannii* 04-24	0	0	0	1	1	0	0	0	0	0	0	0	0	0						
16	*An. lemmermannii* 04-42	0	0	0	1	1	2	4	1	0	1	1	2	0	0	0					
17	*An.* cf. *curva* 04-19	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0				
18	*An. compacta* 04-17	0	0	0	1	0	0	1	0	0	0	0	0	0	0	3	1	0			
19	*An. circinalis* 04-28	0	0	0	1	1	0	2	1	0	0	1	1	0	0	1	2	0	2		
20	*An. lemmermannii* 04-38	0	0	0	0	0	1	2	0	0	0	1	0	0	0	1	2	1	2	1	
21	*An. mendotae* 04-06	0	0	0	1	1	0	3	1	0	1	1	2	0	0	1	3	0	1	0	1

compounds (MW = 684 and 643) recognized in the strain *An. compacta* 06-02 were also detected in other planktonic *Anabaena* strains from the Czech Republic, which was in a good agreement with clustering of the strain 06-02 based on 16S rRNA gene sequence (cluster A, Fig. 4). The compound MW = 684 was measured in *An. circinalis* 04-26, 04-28, 04-59; *An. flos-aquae* 04-40, 04-57; *An. mendotae* 04-11; *An. lemmermannii* 04-42; and *An. spiroides* 04-51. The compound of MW = 643 was recognized in *An. compacta* 04-17 and *An. mendotae* 04-06. Although the random distribution of toxin or protein production throughout a range of distantly related cyanobacterial groups has been repeatedly demonstrated (Palinska et al. 1996, Bolch et al. 1999, Rantala et al. 2004), the pronounced difference of secondary metabolite profiles of *An. reniformis* and *Aph. aphanizomenoides* strains supports their outlying position in the phylogeny of *Anabaena*–like cyanobacteria.

TABLE 4. Main molecular ions $[M+H]^+$ found in crude extracts of the studied strains. *An.*, *Anabaena*; *Aph.*, *Aphanizomenon*.

Strain	Main molecular ions $[M+H]^+$
An. reniformis 06-01	751.3, 576.8, 714.4, 847.2, 833.3, 1109.4, 861.4, 875.2, 590.5, 648.3, 882.4, 634.4, 866.6, 1235.7 (puwainaphycin A), 643.4.
An. reniformis 07-01	415.1, 1235.7 (puwainaphycin A)
Aph. aphanizomenoides 04-43	486.4, 500.3, 453.6, 351.3, 556.4, 540.4, 568.4, 540.4, 568.4, 421.4, 621.4, 639.3, 643.4
An. compacta 06-02	685.5, 794.7, 415.1

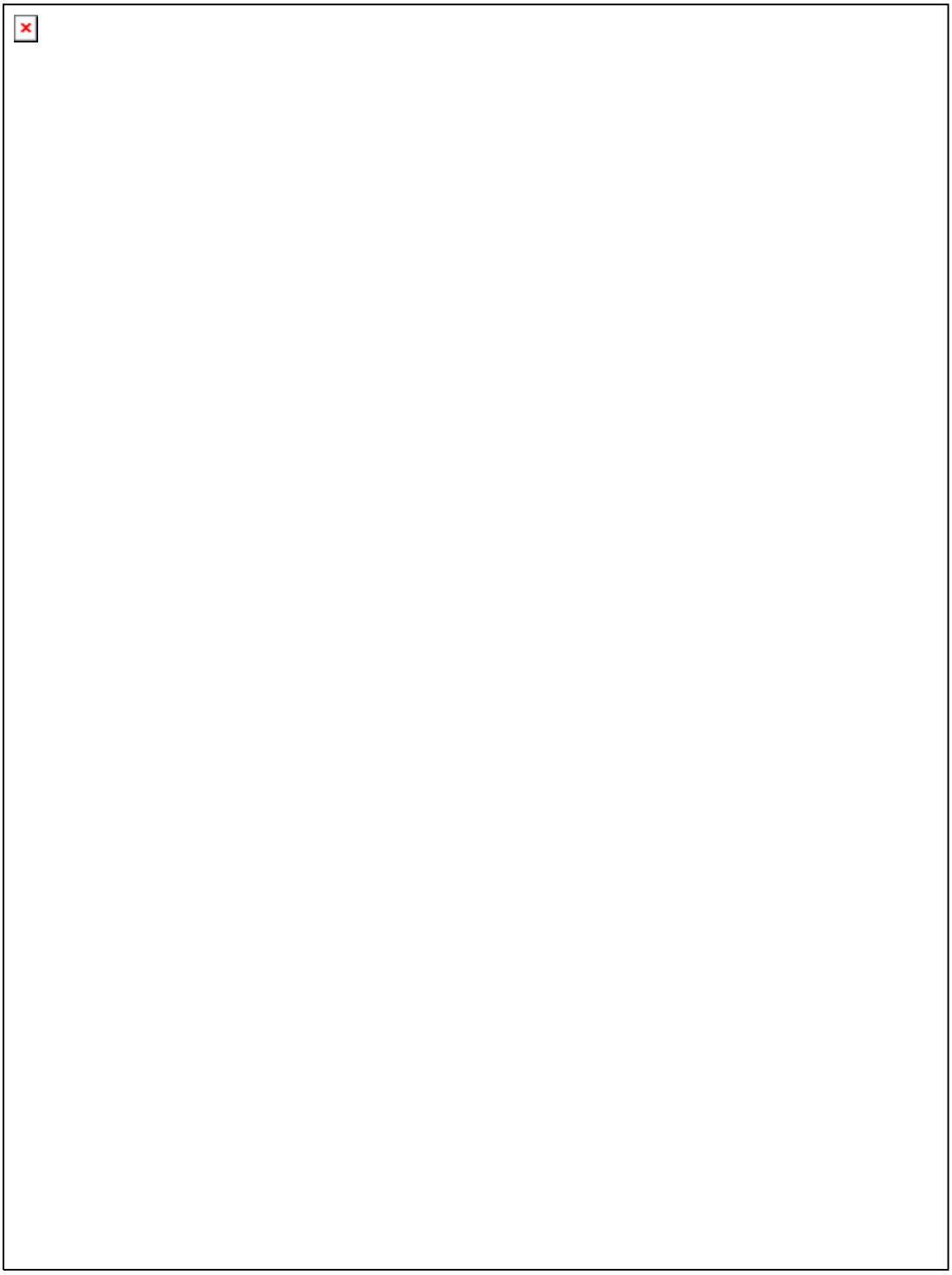
The molecular ions found in the HPLC–MS spectra of the strains under study are presented in Table 4. With the exception of cyclic lipopeptide puwainaphycin A, occurring in extracts of *An. reniformis* 06-01 and 07-01 (1235.7 $[M+H]^+$ and RT = 19.8′ and 20.1′ for the strain 06-01 and 07-01, respectively; Fig. 5), no other molecular ion corresponded to a known compound. Presence of this compound within extracts of both *An. reniformis* strains confirms their close affinity. Production of puwainaphycin was previously reported from terrestrial cyanobacterium *Anabaena* sp. BQ-16-1 (Gregson et al. 1992). However, this strain has not yet been characterized by any available gene sequence.

We were not able to detect the molecular ion corresponding to the cytotoxic alkaloid cylindrospermopsin (416 in MS^+ and 414 in MS^-) within the extracts or the cultivation media of our *An. reniformis* and *Aph. aphanizomenoides* strains. The ability to synthesize cylidrospermopsin has been previously reported from *C. raciborskii* and *Raphidiopsis curvata* F. E. Fritsch et M. F. Rich (Li et al. 2001, Fergusson and Saint 2003), which could be considered as relatively closely related strains. In addition to this, cylindrospermopsin has also been found in *An. bergii*, *Aph. ovalisporum*, *Aph. flos-aquae*, *Lyngbya wollei* (Farl.) Farl., and *Umezakia natans* M. Watanabe (Saker and Neilan 2001, Preussel et al. 2006, Seifert et al. 2007).

The validation of the new genus according to the botanical nomenclatoric rules.

Sphaerospermum gen. nov.

Diagnosis: Trichomata solitaria, libere natantia, brevia vel longa, circinata vel recta, plus minuusque constricta ad septa, cum vel sine vaginis mucosis, ad apices attenuata vel non-attenuata. Cellulae vacuolis



Cronberg, G. & Annadotter, H. 2006. *Manual on Aquatic Cyanobacteria. A Photo Guide and a Synopsis of Their Toxicology.* International Society for the Study of Harmful Algae and the UNESCO, Copenhagen, Denmark, 106 pp.

Cronberg, G. & Komárek, J. 2004. Some nostocacean cyanoprokaryotes from lentic habitats of eastern and southern Africa. *Nova Hedwigia* 78:71–106.

Elenkin, A. A. 1938. *Monographia algarum cyanophycearum aquidulcium et terrestrium in finibus URSS inventarum. (Sinezelenyje vodorosli SSSR).* Izd. AN SSSR, Moskva, Leningrad, 1908 pp.

Felsenstein, J. 2004. *PHYLIP (Phylogeny Inference Package) Version 3.6.* Distributed by the author, Department of Genome Sciences, University of Washington, Seattle (http://evolution.genetics.washington.edu/phylip.html).

Fergusson, K. M. & Saint, C. P. 2003. Multiplex PCR assay for *Cylindrospermopsis raciborskii* and cylindrospermopsin-producing cyanobacteria. *Environ. Toxicol.* 18:120–5.

Flechtner, V. R., Boyer, S. L. & Johansen, J. R. 2002. *Spirirestis rafaelensis* gen. et sp. nov. (Cyanophyceae), a new cyanobacterial genus from arid soils. *Nova Hedwigia* 74:1–24.

Forti, A. 1912. Diagnoses Myxophycearum novarum. *Atti Acad. Agric. Sci. Lett. Ser. 4* 12:122–7.

Geitler, L. 1932. *Cyanophyceae,* 2nd ed. Rabenhorst's Kryptogamen-Flora von Deutschland, Österreich und der Schweiz. Reprint 1985, Koeltz Scientific Books, Koenigstein, Germany, 1196 pp.

Gregson, J. M., Chen, J. L., Patterson, G. L. M. & Moore, R. E. 1992. Structures of puwainaphycin A-E. *Tetrahedron* 48:3727–34.

Gugger, M., Lyra, C., Henriksen, P., Couté, A., Humbert, J.-F. & Sivonen, K. 2002. Phylogenetic comparison of the cyanobacterial genera *Anabaena* and *Aphanizomenon*. *Int. J. Syst. Evol. Microb.* 52:1867–80.

Guillard, R. R. L. & Lorenzen, C. J. 1972. Yellow-green algae with chlorophyllide c. *J. Phycol.* 8:10–4.

Hindák, F. 2000. Morphological variation of four planktic nostocalean cyanophytes – members of the genus *Aphanizomenon* or *Anabaena*? *Hydrobiologia* 438:107–16.

Horecká, M. & Komárek, J. 1979. Taxonomic position of three planktonic blue-green algae from the genera *Aphanizomenon* and *Cylindrospermopsis*. *Preslia* 51:289–312.

Keršner, V. 1997. Two rare planktic cyanophytes from Southern Moravia (Czech Republic). *Biologia* 52:485–8.

Kikuchi, S., Kubo, T. & Kaya, K. 2007. Cylindrospermopsin determination using 2-[4-(2-hydroxyetyl)-1-piperazinyl] ethanesulfonic acid (HEPES) as internal standard. *Anal. Chim. Acta* 583:124–7.

Komárek, J. 1984. Sobre las cyanofíceas de Cuba: (3) Especies planctónicas que forman florecimientos de las aguas. *Acta Bot. Cubana* 19:1–33.

Komárek, J. 1996. Klíč k určování vodních květů sinic v České republice [A key for determination of water-bloom-forming cyanobacteria in the Czech Republic]. *In* Maršálek, B., Keršner, V. & Marvan, P. [Eds.] *Vodní květy sinic [Cyanobacterial water blooms].* Nadatio flos-aquae, Brno, pp. 22–85 (in Czech).

Komárek, J. 2005. Studies on the cyanophytes (Cyanobacteria, Cyanoprokaryota) of Cuba, 11. Freshwater *Anabaena* species. *Preslia* 77:211–34.

Komárek, J. & Zapomělová, E. 2007. Planktic morphospecies of the cyanobacterial genus *Anabaena* = subg. *Dolichospermum* – 1. part: coiled types. *Fottea* 7:1–31.

Lemmermann, E. 1898. Beiträge zur Kenntniss der Planktonalgen. *Bot. Zentralbl.* 76:150–6.

Li, R. H., Carmichael, W. W., Brittain, S., Eaglesham, G. K., Shaw, G. R., Liu, Y. D. & Watanabe, M. M. 2001. First report of the cyanotoxins cylindrospermopsin and deoxycylindrospermopsin from *Raphidiopsis curvata* (Cyanobacteria). *J. Phycol.* 37:1121–6.

Li, R. & Watanabe, M. M. 1999. *Anabaena eucompacta* sp. nov. (Nostocales, Cyanobacteria), a new planktonic species with tightly spiraled filaments from Japan. *Bull. Natl. Sci. Mus. Tokyo Ser. B* 25:89–94.

Li, R. & Watanabe, M. M. 2004. Fatty acid composition of planktonic species of *Anabaena* (cyanobacteria) with coiled trichomes exhibited a significant taxonomic value. *Curr. Microbiol.* 49:376–80.

Li, R., Watanabe, M. & Watanabe, M. M. 2000. Taxonomic studies of planktic species of *Anabaena* based on morphological characteristics in cultured strains. *Hydrobiologia* 438:117–38.

Li, R., Wilhelm, S. W., Carmichael, W. W. & Watanabe, M. M. 2008. Polyphasic characterization of water bloom forming *Raphidiopsis* species (cyanobacteria) from central China. *Harmful Algae* 7:146–53.

Nübel, U., Garcia-Pichel, F. & Muyzer, G. 1997. PCR primers to amplify 16S rRNA genes from cyanobacteria. *Appl. Environ. Microbiol.* 63:3327–32.

Padisák, J. & Kovács, A. 1997. *Anabaena compacta* (Nygaard) Hickel – Új kékalga faj a Balaton üledékében és planktonjában [*Anabaena compacta* (Nygaard) Hickel – a new blue-green algal species in the sediments and plankton of the lake Balaton] (in Hungarian with English summary). *Hidrológiai Közlöny* 77:29–32.

Palinska, K. A., Liesack, W., Rhiel, E. & Krumbein, W. E. 1996. Phenotype variability of identical genotypes: the need for a combined approach in cyanobacterial taxonomy demonstrated on *Merismopedia*-like isolates. *Arch. Microbiol.* 166: 224–33.

Pechar, L., Přikryl, I. & Faina, R. 2002. Hydrobiological evaluation of Třeboň fishponds since the end of the nineteenth century. *In* Květ, J., Jeník, J. & Soukupová, L. [Eds.] *Freshwater Wetlands and Their Sustainable Future.* UNESCO and The Parthenon Publishing Group, Paris, pp. 31–61.

Preussel, K., Stuken, A., Wiedner, C., Chorus, I. & Fastner, J. 2006. First report on cylindrospermopsin producing *Aphanizomenon flos-aquae* (Cyanobacteria) isolated from two German lakes. *Toxicon* 47:156–62.

Rajaniemi, P., Hrouzek, P., Kaštovská, K., Willame, R., Rantala, A., Hoffmann, L., Komárek, J. & Sivonen, K. 2005a. Phylogenetic and morphological evaluation of the genera *Anabaena, Aphanizomenon, Trichormus* and *Nostoc* (Nostocales, Cyanobacteria). *Int. J. Syst. Evol. Microbiol.* 55:11–26.

Rajaniemi, P., Komárek, J., Willame, R., Hrouzek, P., Kaštovská, K., Hoffmann, L. & Sivonen, K. 2005b. Taxonomic consequences from the combined molecular and phenotype evaluation of selected *Anabaena* and *Aphanizomenon* strains. *Algol. Stud.* 117:371–91.

Rantala, A., Fewer, D. P., Hisbergues, M., Rouhianen, L., Vaitomaa, J., Börner, T. & Sivonen, K. 2004. Phylogenetic evidence for the early evolution of microcystin synthesis. *Proc. Natl. Acad. Sci. U. S. A.* 101:568–73.

Saitou, N. & Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406–25.

Saker, M. L. & Neilan, B. A. 2001. Varied diazotrophies, morphologies, and toxicities of genetically similar isolates of *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyceae) from Northern Australia. *Appl. Environ. Microb.* 67:1839–45.

Seifert, M., McGregor, G., Eaglesham, G., Wickramasinghe, W. & Shaw, G. 2007. First evidence for the production of cylindrospermopsin and deoxy-cylindrospermopsin by the freshwater benthic cyanobacterium, *Lyngbya wollei* (Farlow ex Gornont) Speziale and Dyck. *Harmful Algae* 6:73–80.

Stefaniak, K. & Kokociński, M. 2005. Occurrence of invasive cyanobacteria species in polimictic lakes of the Wielkopolska region (Western Poland). *Ocean. Hydrob. Stud.* 34(Suppl. 3):137–48.

Stüken, A., Rücker, J., Endrulat, T., Preussel, K., Hemm, M., Nixdorf, B., Karsten, U. & Wiedner, C. 2006. Distribution of three alien cyanobacterial species (Nostocales) in northeast Germany: *Cylindrospermopsis raciborskii, Anabaena bergii* and *Aphanizomenon aphanizomenoides*. *Phycologia* 45:696–703.

Taton, A., Grubisic, S., Brambilla, E., De Wit, R. & Wilmotte, A. 2003. Cyanobacterial diversity in natural and artificial microbial mats of Lake Fryxell (McMurdo dry valleys, Antarctica): a morphological and molecular approach. *Appl. Environ. Microbiol.* 69:5157–69.

Törökné, A., Asztalos, M., Bákiné, M., Bickel, H., Borbély, G., Carmeli, S., Codd, G. A., et al. 2004. Interlaboratory comparison trial on cylidrospermopsin measurement. *Anal. Biochem.* 332:280–4.

Watanabe, M. 1996. Studies on planktonic blue-green algae 6. Bloom-forming species in Lake Biwa (Japan) in the summer of 1994. *Bull. Natl. Sci. Mus., Tokyo Ser. B* 22:1–10.

Watanabe, M., Niiyama, Y. & Tuji, A. 2004. Studies on planktonic blue-green algae. 10. Classification of planktonic *Anabaena* with coiled trichomes maintained in the National Science Museum, Tokyo. *Bull. Natl. Sci. Mus. Tokyo Ser. B* 30:135–49.

Willame, R., Boutte, C., Grubisic, S., Wilmotte, A., Komárek, J. & Hoffmann, L. 2006. Morphological and molecular characterization of planktonic cyanobacteria from Belgium and Luxembourg. *J. Phycol.* 42:1312–32.

Wilmotte, A., Van der Auwera, G. & De Wachter, R. 1993. Structure of the 16S ribosomal RNA of the thermophilic cyanobacterium *Chlorogloeopsis* HTF (*'Mastigocladus laminosus* HTF') strain PCC7518, and phylogenetic analysis. *FEBS Lett.* 317:96–100.

Zapomělová, E., Řeháková, K., Znachor, P. & Komárková, J. 2007. Morphological diversity of coiled planktonic types of the genus *Anabaena* (cyanobacteria) in natural populations – taxonomic consequences. *Cryptogam. Algol.* 28:353–71.

Supplementary Material

The following supplementary material is available for this article:

Table S1. Cyanobacterial strains whose 16S rRNA gene sequences were used in this study for the construction of neighbor-joining and maximum-parsimony phylogenetic trees. The studied strains are in bold. *An.*, *Anabaena*; *Aph.*, *Aphanizomenon*.

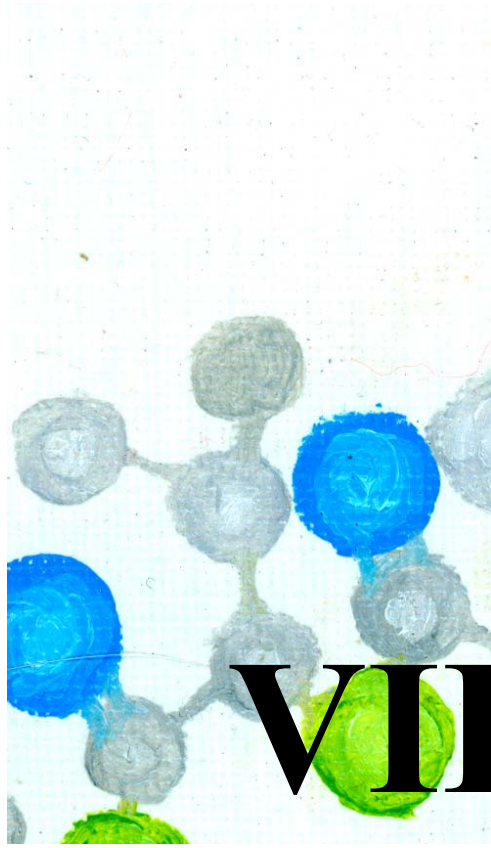
Table S2. Occurrence of *Anabaena reniformis* during the sampling period 2004–2007 in South Bohemia and its relative abundances (+, rare; ++, sporadic; +++, abundant; +++D, dominant; –, not observed).

Table S3. Occurrence of *Aphanizomenon aphanizomenoides* during the sampling period 2004–2007 in South Bohemia and its relative abundances (+, rare; ++, sporadic; +++, abundant; +++D, dominant; –, not observed).

Table S4. Summary of the localities in the Czech Republic where *Aphanizomenon aphanizomenoides* has been recently found by other authors (unpublished data).

This material is available as part of the online article.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.



Zapomělová E., Hisem D., Řeháková K., **Hrouzek P.,** Jezberová J., Komárková J., Korelusová J. & Znachor P. 2008.

Experimental comparison of phenotypical plasticity and growth demands of two strains from the *Anabaena circinalis*/*A. crassa* complex (cyanobacteria).

Journal of Plankton Research 30 (11): 1257-1269.

Experimental comparison of phenotypical plasticity and growth demands of two strains from the *Anabaena circinalis/A. crassa* complex (cyanobacteria)

ELIŠKA ZAPOMĚLOVÁ[1,2]*, DANIEL HISEM[1,3], KLÁRA ŘEHÁKOVÁ[2,4], PAVEL HROUZEK[1,3,5], JITKA JEZBEROVÁ[2], JAROSLAVA KOMÁRKOVÁ[1,2], JANA KORELUSOVÁ[1,2] AND PETR ZNACHOR[1,2]

[1]UNIVERSITY OF SOUTH BOHEMIA, FACULTY OF SCIENCE, BRANIŠOVSKÁ 31, CZ-37005 ČESKÉ BUDĚJOVICE, CZECH REPUBLIC, [2]BIOLOGY CENTRE OF AS CR, INSTITUTE OF HYDROBIOLOGY, NA SÁDKÁCH 7, CZ-37005 ČESKÉ BUDĚJOVICE, CZECH REPUBLIC, [3]DEPARTMENT OF AUTOTROPHIC MICROORGANISMS, INSTITUTE OF MICROBIOLOGY, AS CR, OPATOVICKÝ MLÝN, CZ-379 81, TŘEBOŇ, CZECH REPUBLIC, [4]INSTITUTE OF BOTANY, AS CR, DUKELSKÁ 135, CZ-37982 TŘEBOŇ, CZECH REPUBLIC AND [5]INSTITUTE OF PHYSICAL BIOLOGY, ZÁMEK 136, CZ-37333 NOVÉ HRADY, CZECH REPUBLIC

*CORRESPONDING AUTHOR: eliska.zapomelova@seznam.cz

Received April 25, 2008; accepted in principle July 18, 2008; accepted for publication July 31, 2008; published online August 2, 2008

Corresponding editor: William Li

Two cyanobacterial strains were isolated in 2004 from different localities in the Czech Republic. Field morphology of the strain 04-26 (Jesenice reservoir) matched with the species description of Anabaena crassa (Lemm.) Kom.-Legn. et Cronb. 1992, whereas the strain 04-28 (Hodějovický fishpond) was identified as A. circinalis Rabenh. ex Born. et Flah. 1888. Both these strains, exposed to various experimental conditions (temperature, light intensity, nitrogen and phosphorus concentration), displayed highly similar morphologies and spanned the morphological variability of both of the above-mentioned species. Significant relationships between environmental conditions (temperature, phosphorus) and morphological characteristics (vegetative cell and heterocyte dimensions, trichome coiling parameters) have been recorded for the first time within the genus Anabaena. The strains studied differed in their temperature and light growth optima and in secondary metabolite contents. However, both were identical (100% similarity) in their 16S rRNA gene sequence and showed 99.9–100% similarity to the published 16S rRNA sequences of A. circinalis strains from northern Europe.

INTRODUCTION

The planktonic cyanobacterium *Anabaena circinalis* Rabenh. ex Born. et Flah. 1888 is a common morphospecies that has been reported from all continents and its blooms are a major worldwide problem due to the production of a wide range of toxins (Beltran and Neilan, 2000). The knowledge of its ecology, morphological variability, toxicity and phylogenetic status is therefore of particular interest.

A similar morphospecies, *A. crassa* (Lemm.) Kom.-Legn. et Cronb. 1992, was described from Sweden (Komárková-Legnerová and Cronberg, 1992) and is common in the temperate zone worldwide (Komárek, 1996; Cronberg and Annadotter, 2006). The trichome width and the diameters of trichome coils are regarded as the main distinguishing criteria between *A. crassa* and *A. circinalis* (Komárek, 1958; Komárková-Legnerová and Cronberg, 1992; Komárková-Legnerová and Eloranta, 1992; Komárek and Zapomělová, 2007). However, morphological comparison of 13 *A. circinalis* and *A. crassa* populations from the Czech Republic has demonstrated

continuous transitions of both the trichome widths and the coil diameters (Zapomělová *et al.*, 2007).

Phylogenetic comparison of several strains of *A. circinalis* and *A. crassa* was published by Rajaniemi (Rajaniemi *et al.*, 2005a, b). The results were consistent with the above-mentioned morphological study (Zapomělová *et al.*, 2007), since both the morphospecies clustered closely together, based on 16S rRNA gene, *rpoB* and *rbcLX* sequences.

Ecological demands of the cyanobacteria from the *A. circinalis/A. crassa* complex, the range of their morphological variability under varying growth conditions or secondary metabolite production have not been compared so far. Consequently, the present study focuses on morphological plasticity of two strains of this cyanobacterial complex under varied conditions of temperature, light intensity and nitrogen or phosphorus concentration. The temperature and light growth optima and secondary metabolite content of these strains have been compared. In addition to morphological evaluation, the studied strains have also been characterized by partial sequences of the 16S rRNA gene.

METHOD

Sampling, isolation and cultivation

Samples of blooms were collected in August and September 2004 from two localities (Jesenice reservoir, Hodějovický fishpond) using a 20 μm mesh plankton net. Jesenice (50°5′1.88″N, 12°28′29.71″E) is a deep dimictic mesotrophic reservoir in the west of the Czech Republic. It is situated on the river Odrava and serves for recreational purposes. Hodějovický fishpond (48°56′36.63″N, 14°29′35.88″E) is a small and shallow polymictic eutrophic pond on an unnamed brook in the south of the Czech Republic. Morphology of fresh material was evaluated immediately as described below. Single trichomes were isolated from the phytoplankton samples as described by Zapomělová (Zapomělová *et al.*, 2007). Clonal cultures were grown in WC medium (Guillard and Lorenzen, 1972) at 21°C and a light intensity of 70 μmol m $^{-2}$ s $^{-1}$ (16:8 L:D cycle). The strain isolated from Jesenice reservoir was named 04-26 and the strain from Hodějovický fishpond 04-28.

Crossed gradients of light and temperature

Crossed gradients (Kvíderová and Lukavský, 2001) were used to test the effect of light and temperature on the cyanobacterial morphology and to determine the growth optima. For the morphological experiments, the strains were exposed in sterile culture plates (9 × 12 cm, 12 wells, 6.5 mL each) to nine different combinations of light and temperature in crossed gradients (Fig. 1a) for 10 days. The temperature ranged from 10°C to 28°C and the range of light intensity, provided by sodium-vapour lamps, was 20–750 μmol m $^{-2}$ s $^{-1}$. To estimate the temperature and light growth optima of the strains, a modified design of the cross-gradient experiments was used (Fig. 1b). Identical volumes of stirred dense batch culture were inoculated into sterile culture plates containing fresh WC medium (9 × 12 cm, 6 wells, 16 mL each). The plates were then exposed to 25 positions of the cross-table. The temperature range was 6–34°C and the light intensity 20–750 μmol m $^{-2}$ s $^{-1}$. The experiments were terminated in the exponential phase of growth of

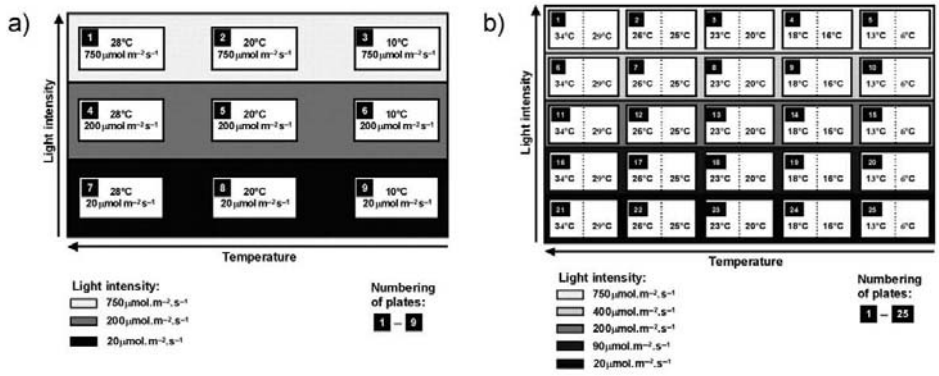


Fig. 1. Design of the cross-gradient experiments: (**a**) evaluation of morphological variability of the strains in relation to light and temperature and (**b**) determination of light and temperature growth optima of the strains. Gradients of light intensity and temperature are indicated with arrows.

the fastest growing cultures. Chlorophyll *a* concentrations were determined spectrophotometrically after acetone extraction (Lorenzen, 1967) and compared among the positions of the crossed gradients.

Concentration series of nitrogen and phosphorus

Modified types of WC medium containing different concentrations of nitrogen (N) and phosphorus (P) (Table I) were used to test the effect of nutrients on cyanobacterial morphology. The concentration series were designed with respect to N and P concentrations commonly occurring in fishponds and reservoirs of the Czech Republic (Znachor *et al.*, 2006). In order to force the strains to deplete their intracellular nutrient reserves, cyanobacterial biomass was incubated in a modified WC medium without N and P ("starving medium", Table I) for 7 days prior to the experiment. Equimolar concentrations of KCl were added to the "starving medium", $WC_{0\times P}$ $WC_{0.001\times P}$ and $WC_{0.1\times P}$ in order to retain the original K^+ concentration.

Morphometry

Microphotographs of at least 30 fresh trichomes from each field population or for each experimental treatment were taken with a digital camera (Olympus DP 70, magnification ×400). Dimensions of all cell types were measured (five vegetative cells per trichome measured in 30 trichomes and as many heterocytes and akinetes as it was possible to find in each sample). Length:width ratios of vegetative cells, heterocytes and akinetes were computed to roughly characterize the cell shapes. Trichome coil diameters and distances between neighbouring coils were measured. Diameter:distance ratios were calculated to characterize the tightness of trichome coiling. All size measurements were performed using image analysis (Olympus DP Soft).

Table I: Modifications of WC medium used in the experiments

Medium type	P (μmol L^{-1})	N (μmol L^{-1})
$WC_{0\times P}$	0	5.7×10^{2}
$WC_{0.001\times P}$	2.6×10^{-2}	5.7×10^{2}
$WC_{0.1\times P}$	2.6×10^{0}	5.7×10^{2}
$WC_{1\times P}$=$WC_{1\times N}$=WC	2.6×10^{1}	5.7×10^{2}
$WC_{10\times P}$	2.6×10^{2}	5.7×10^{2}
$WC_{0\times N}$	2.6×10^{1}	0
$WC_{0.001\times N}$	2.6×10^{1}	5.7×10^{-1}
$WC_{0.1\times N}$	2.6×10^{1}	5.7×10^{1}
$WC_{10\times N}$	2.6×10^{1}	5.7×10^{3}
"Starving medium"	0	0

Secondary metabolite content: extract preparation and HPLC-MS analysis

Lyophilized biomass (~40 mg) was disintegrated by grinding and extracted in 2 mL of 70% methanol (MeOH) in microtubes for 30 min. The microtubes were centrifuged at 3170 g at 4°C for 15 min. Supernatant was concentrated 10 times in a rotary vacuum drier. HPLC-MS analysis was performed in order to determine the content of secondary metabolites. The extracts were analysed on a reversed phase column (Zorbax XBD C8, 46 × 150 mm, 5 μm) using a MeOH/H_2O gradient with a flow rate of 0.6 mL min^{-1}. For a more effective ionization, 0.1% formic acid was added to the eluents. The extract composition was analysed with an HP 1100 Agilent mass spectrometer HP 100 MSD SL-Ion trap in positive mode. The settings were selected to cover the mass range between 50 and 2000, and the ion trap was targeted to molecular masses near 900. Automatic fragmentation of the most intensive peak was applied. The molecular ions were determined based on the presence of sodium and potassium adducts and on the distribution of isotopologues.

Phylogenetic study

The biomass was harvested in the exponential phase of growth by repeated centrifugation, during which the trichomes were washed several times with physiological solution (NaCl solution, concentration 1 g L^{-1}) to remove mucilaginous substances. The biomass samples were stored at −20°C until DNA extraction. DNA was extracted using UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). The 16S rRNA gene and ITS region were amplified with primers 16S27F and 23S30R (Taton *et al.*, 2003). Amplification was carried out as follows: 1 cycle of 5 min at 94°C; 10 cycles of 45 s at 94°C, 45 s at 57°C and 2 min at 72°C; 25 cycles of 45 s at 94°C, 45 s at 54°C and 2 min at 72°C and a final elongation step of 7 min at 72°C. PCR product was used as a template for sequencing with primers 16S27F, 23S30R (Taton *et al.*, 2003), primer cAlaR (Wilmotte *et al.*, 1994) and primer CYA781F(a) (Nübel *et al.*, 1997). Sequences were aligned in the program ARB (http://www.arb-home.de). The alignment was edited manually and ambiguous bases were removed. For the phylogenetic analysis, trees were built with the neighbour joining (NJ) method (Saitou and Nei, 1987) and the maximum parsimony (MP) algorithm in the Phylip program (Felsenstein, 2004). Five hundred bootstrap replicates were performed both for NJ and MP analyses.

Nucleotide sequences have been deposited at Gen Bank under the accession numbers AM940218 and AM940219. Besides the strains whose accession numbers are given in the phylogenetic tree, the following sequences were used for the construction of the phylogenetic trees: AJ293126, AJ630441, AJ293127, AY701569, AJ293109, AJ630418, AJ293111, AJ133154, AJ293131, AJ293124, AJ133155, AJ630408, AJ630410, AJ630412, AJ630409, AJ133151, AJ293103, AJ293104, AJ133159, AJ630424, AJ630422, AJ13156, AJ293113, AJ293106, AY196088, AJ293108, AY196087, AJ630446, AJ630457, AJ630456, AJ630458, AJ630428, AF160256, AY038033, AF516747, AY038036, AJ781144, AJ133184, AJ133181, AY701557, AY701558, AY763116, AY763117, EU076459, AF067819, AF516724, AJ582102, AY699989, EU078547, EU076458, EU078548.

Statistical analysis

The effect of light, temperature, phosphorus and nitrogen on the morphometric characteristics was tested by redundancy analysis (RDA) with forward selection. A Monte-Carlo permutation test was used for calculation of *P*-values.The data were centred and standardized. These statistical analyses were performed using the program CANOCO (Ter Braak and Šmilauer, 1998) and ordination diagrams were created using CanoDraw software (Šmilauer, 1992). Basic statistical characteristics such as average values, 25 and 75% percentiles and extreme values were computed for each morphological feature. Box–whisker plots were created by the GraphPad Prism program (GraphPad Software, San Diego California USA, www.graphpad.com). Surface plots demonstrating the light and temperature growth optima were created using the program Statistica (Anonymous, 1996).

RESULTS

Morphological plasticity under varied growth conditions

The experiments demonstrated the range of morphological characteristics of the strains 04-26 and 04-28, which was markedly greater than their morphological variability observed under field conditions (Table II). The strains displayed highly similar morphologies during the experiments, although their width of trichomes, dimensions of akinetes and trichome coiling characteristics differed in the field.

Significant effects of temperature and phosphorus concentration on the morphologies of the strains studied were confirmed by RDAs, while the effects of light intensity and nitrogen concentration were insignificant (Table III). The strongest response to varied environmental factors was observed in vegetative cells, whose dimensions were significantly influenced by the phosphorus concentration in both the strains and by the temperature in the strain 04-26 (Table III). On the contrary, no significant effect of the experimental factors on the akinete dimensions and shape was found in both the strains.

The dimensions of vegetative cells and heterocytes were largest at 28°C, especially in the strain 04-26, where the effect of the temperature was stronger (Fig. 2). However,

Table II: Summary of morphological features of the strains observed during the experiments and in the field

	Strain 04-26		Strain 04-28	
Morphological characteristics	Experiments	Field	Experiments	Field
Vegetative cells				
Length (μm)	6.0–12.0	8.2–10.6	5.5–17.0	6.4–8.6
Width (μm)	8.5–13.5	11.2–12.2	7.0–13.0	10.0–11.0
Length:width ratio	0.7–1.2	0.7–0.9	0.6–2.1	0.6–0.8
Heterocytes				
Length (μm)	7.5–13.0	10.6–12.5	8.5–12.6	10.2–12.0
Width (μm)	8.5–13.0	11.2–12.2	9.0–13.0	10.6–11.8
Length:width ratio	0.9–1.1	0.9–1.0	0.9–1.2	0.9–1.0
Akinetes				
Length (μm)	20.0–30.0	24.0–29.0	18.0–29.0	17.0–25.0
Width (μm)	12.5–18.5	16.5–18.5	13.0–18.5	12.8–15.2
Length:width ratio	1.3–1.9	1.5–1.6	1.4–2.0	1.3–1.7
Trichome coiling				
Coil diameter (μm)	35.0–62.0	45.0–56.0	15.0–110.0	40.0–52.0
Coil distance (μm)	20.0–57.0	38.0–45.0	20.0–130.0	25.0–40.0
Diameter:distance ratio	0.9–1.9	0.8–1.4	0.2–2.8	1.0–1.8

Values between 25 and 75% percentiles are shown and the outlying values were omitted.

Table III: Morphological characteristics for which a significant effect of at least one environmental factor was demonstrated by RDA

Morphological criterion	Strain	Factor	Variability explained by the model (%)	Variability explained by the factor (%)	*F*-value	*P*-value
Vegetative cell morphology (length, width, length: width ratio)	04-26	Temperature	52.5	44.8	**4.871**	**0.0260**
		Light		7.7	0.806	0.4540
		Phosphorus	80.1	75.4	**21.469**	**0.0080**
		Nitrogen		4.7	1.425	0.2740
	04-28	Temperature	32.9	26.5	2.524	0.1000
		Light		7.4	0.559	0.5940
		Phosphorus	75.9	70.9	**17.073**	**0.0260**
		Nitrogen		5.0	1.242	0.3280
Heterocyte morphology (length, width, length: width ratio)	04-26	Temperature	85.4	82.8	**28.985**	**0.0020**
		Light		2.6	0.876	0.4920
		Phosphorus	31.9	29.5	2.929	0.0640
		Nitrogen		1.0	0.070	0.9560
	04-28	Temperature	33.8	32.5	2.884	0.0720
		Light		1.6	0.100	0.8680
		Phosphorus	50.6	30.7	3.097	0.0840
		Nitrogen		19.9	1.739	0.1860
Trichome coiling (coil diameters, distances of adjacent coils, coil: distance ratio)	04-26	Temperature	38.9	35.4	**3.294**	**0.0480**
		Light		3.5	0.283	0.7680
		Phosphorus	16.2	13.7	1.112	0.3700
		Nitrogen		2.2	0.154	0.8760
	04-28	Temperature	60.8	42.0	5.076	0.0500
		Light		18.8	2.876	0.1240
		Phosphorus	20.7	11.0	0.867	0.3440
		Nitrogen		8.8	0.675	0.4120

Significant effects are given in bold.

the shape of these cells did not change throughout the temperature gradient since their length:width ratios remained constant. The vegetative cell width of both the strains reached 12–13 μm at 28°C.

On the contrary, the thinnest vegetative cells (8–9 μm) were observed in the lowest phosphorus concentrations ($WC_{0\times P}$ $WC_{0.001\times P}$) and their width increased with increasing P (Fig. 3). Together with reduced width of vegetative cells at low P concentrations, their length increased. Therefore, length:width ratios of vegetative cells were higher at lower P concentrations, i.e. the cells were obviously elongated.

Gas vesicles tended to accumulate in vegetative cells more at lower light intensities and at higher nutrient concentrations, especially P (Fig. 4).

Temperature and phosphorus concentration also affected trichome morphology. A clear decrease in coil diameter and coil distance was observed at the lowest temperature (Fig. 5), although the effect of temperature was slightly below the 5% significance level for the strain 04-26 and at the 5% level of significance for the strain 04-28 (Table III). The effect of P concentration on trichome coiling parameters was not supported by RDA (Table III). Nevertheless, the intervals of 25% and 75% percentiles of coil diameters and distances in higher P concentrations ($WC_{0.1\times P}$ $WC_{1\times P}$ $WC_{10\times P}$) did not overlap those at lower P concentrations (Fig. 4). Together with increasing distances of adjacent trichome coils at low P concentrations ($WC_{0\times P}$ $WC_{0.001\times P}$), trichome coil diameters of the strain 04-28 decreased until almost straightened trichomes (Fig. 4). At higher P concentrations, the trichomes were coiled regularly, forming more or less tight spirals. After inoculation to fresh WC medium, the straightened trichomes from $WC_{0\times P}$ and $WC_{0.001\times P}$ media were able to grow into a culture of regularly coiled trichomes.

Temperature and light growth optima

Both the light and the temperature optima of the strains differed markedly and did not overlap in their ranges. The strain 04-26 displayed the temperature growth optimum between 17.5°C and 22.5°C and the light optimum 220–360 μmol m^{-2} s^{-1}, whereas the temperature growth optimum of the strain 04-28 was 22–28°C and the light optimum was 100–210 μmol m^{-2} s^{-1} (Fig. 6).

Secondary metabolite content

Secondary metabolite contents were determined in the strains studied. Only one compound was produced by both the strains 04-26 and 04-28. The identity of this compound (MW = 684, $m/z = 685$ $[M+H]^+$) is unknown.

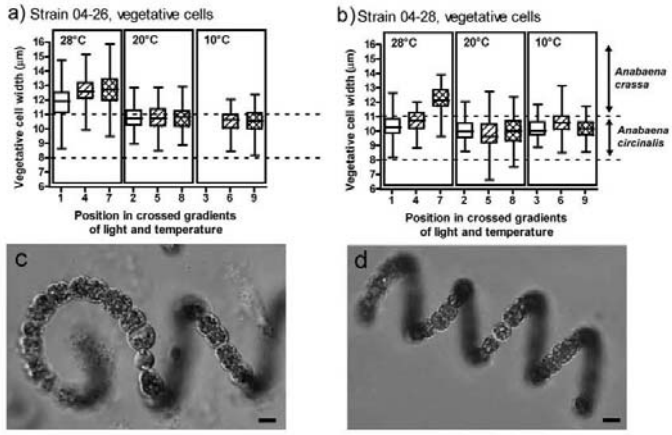


Fig. 2. Vegetative cell width of the strain 04-26 (**a**, **c**, **d**) and 04-28 (**b**) in various positions of crossed gradients of light and temperature. Light intensity is symbolized by shading (750 µmol m^{-2} s^{-1}, plain boxes; 200 µmol m^{-2} s^{-1}, simple shading; 20 µmol m^{-2} s^{-1}, double shading). Whiskers represent the minimal and maximal values, boxes symbolize the 25 and 75% percentiles and lines inside the boxes show the mean values. Ranges of values for the morphospecies *Anabaena circinalis* and *A. crassa* after Komárek (Komárek, 1996) are indicated. Microphotographs show differences in vegetative cell width of the strain 04-26 grown at 28°C (**a**) and 10°C (**b**). Scale bars represent 10 µm.

All the other compounds identified in extracts of the strains 04-26 and 04-28 differed in their retention times and MS^2/MS^3 fragmentation spectra (data not shown).

Eight compounds were clearly identified from the mass spectra of strain 04-26. Molecular weights of these compounds were 845, 713, 887, 910, 755, 684, 812 and 1132. No ion corresponding to the loss of an amino acid was found, excluding the possibility that this compound was a peptide.

Five principal compounds were identified in the extract of strain 04-28. The molecular ions and related sodium and potassium adducts confirmed the presence of compounds with molecular weights 452, 684, 786, 870 and 854. None of these compounds were identified as a known structure. Analysis of the MS^2 spectra of molecular ions 786, 870 and 854 revealed one intensive peak corresponding to loss of water and/or CO_2. Further fragmentation of this ion led to the creation of fragments

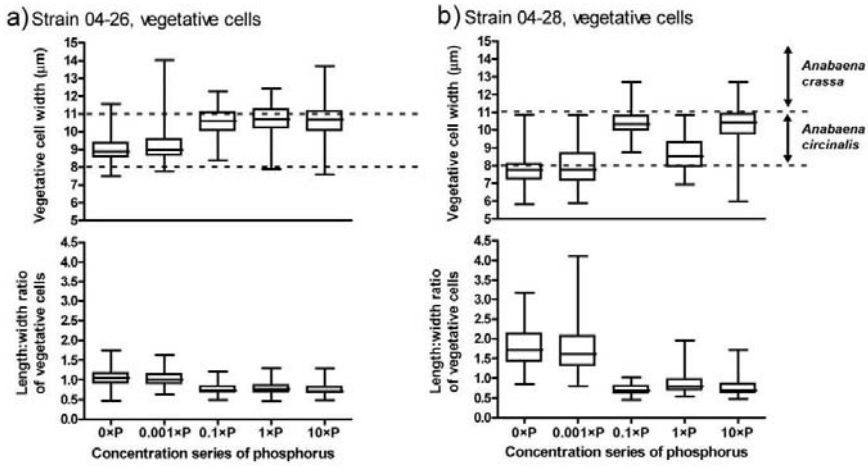


Fig. 3. Vegetative cell morphometric characteristics of the strain 04-26 (**a**) and 04-28 (**b**) under various phosphorus concentrations. Whiskers represent the minimal and maximal values, boxes symbolize the 25 and 75% percentiles and lines inside the boxes show the mean values. Ranges of values for the morphospecies *Anabaena circinalis* and *A. crassa* after Komárek (Komárek, 1996) are indicated.

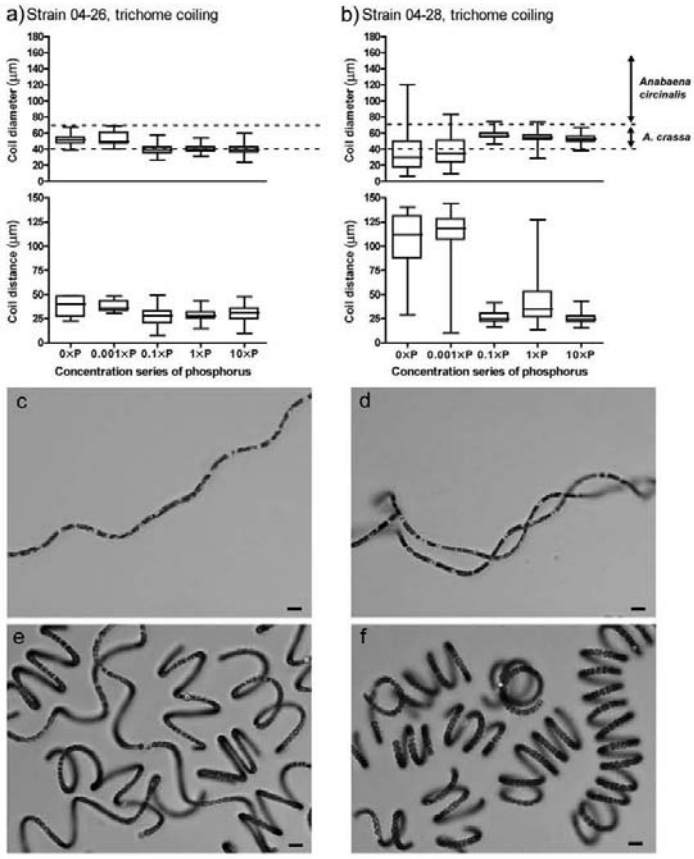


Fig. 4. Parameters of trichome coiling of the strain 04-26 (**a**) and 04-28 (**b f**) under various phosphorus concentrations. Whiskers represent the minimal and maximal values, boxes symbolize the 25 and 75% percentiles and lines inside the boxes show the mean values. Ranges of values for the morphospecies *Anabaena circinalis* and *A. crassa* after Komárek (Komárek, 1996) are indicated. Microphotographs demonstrate the variability in trichome coiling of the strain 04-28 under different phosphorus concentrations: (**c**) 0 µmol L^{-1} ($WC_{0\times P}$); (**d**) 2.6×10^{-2} µmol L^{-1} ($WC_{0.001\times P}$); (**e**) 2.6×10^{1} µmol L^{-1} ($WC_{1\times P}$); (**f**) 2.6×10^{2} µmol L^{-1} ($WC_{10\times P}$). Scale bars represent 20 µm.

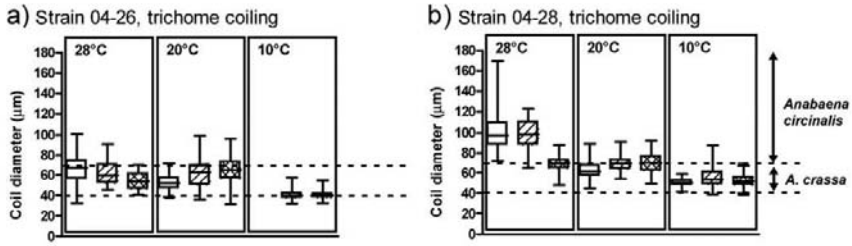


Fig. 5. Parameters of trichome coiling of the strain 04-26 (**a**) and 04-28 (**b**) in various positions of crossed gradients of light and temperature. Light intensity: 750 µmol m^{-2} s^{-1}, plain boxes; 200 µmol m^{-2} s^{-1}, simple shading; 20 µmol m^{-2} s^{-1}, double shading. Whiskers represent the minimal and maximal values, boxes symbolize the 25 and 75% percentiles and lines inside the boxes show the mean values. Ranges of values for the morphospecies *Anabaena circinalis* and *A. crassa* after Komárek (Komárek, 1996) are indicated.

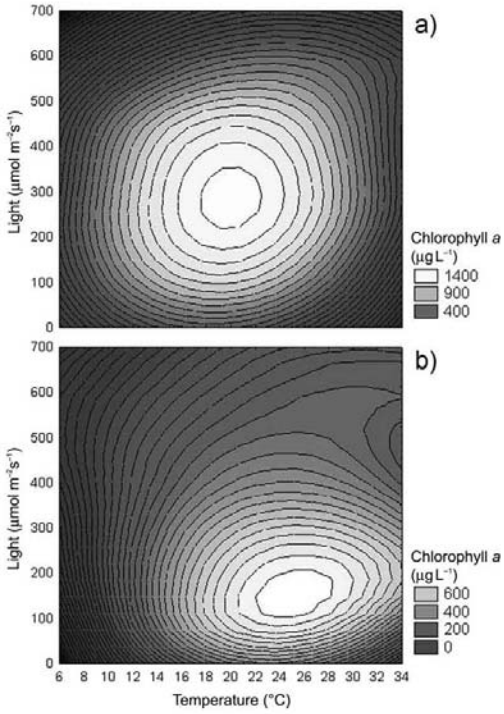


Fig. 6. Contour plots describing chlorophyll *a* concentrations ($\mu g\ L^{-1}$) of the strains as a function of temperature and light: (**a**) strain 04-26, (**b**) strain 04-28. Identical inocula of each strain were exposed to various combinations of temperature and light intensity. The biomass was harvested in exponential phase of growth of the fastest growing cultures.

that corresponded to amino acid loss. Fragmentation of the compound of MW 786 resulted in the loss of Gly, Ala, Val and Asn. Fragmentation of the compound of MW 870 resulted in the loss of Ala, Val, Lys/Gln and Met. Fragmentation of the compound of MW 854 resulted in the loss of Ala, Leu/Ile, Asp and Lys/Gln.

16s rRNA gene structure

Partial 16S rRNA sequences (1212 bp) of the strains 04-26 and 04-28 were compared with sequences of planktonic *Anabaena* strains available in GenBank. NJ and MP phylogenetic algorithms produced similar topologies, and therefore only the NJ tree is presented (Fig. 7). Both the strains exhibited 100% sequence identity and appeared in a common cluster together with *A. circinalis*, *A. crassa* and *A. planctonica* strains from lake Tuusulanjärvi, Finland (cluster A). Sequences of the 16S rRNA genes of all these strains were almost identical (similarities higher than 99.8%).

DISCUSSION

Morphology

Morphological plasticity under varied experimental conditions was described for the first time in the cyanobacterial species complex *Anabaena circinalis/A. crassa*. In most cases, the responses of morphological characteristics to varied conditions (temperature, light, nitrogen, phosphorus) were similar in both the strains, including *F*- and *P*-values of RDAs and percentages of the variability explained. This indicates that the results may be generalized to the whole group of similar planktonic *Anabaena* morphotypes, corresponding to the "large" species of Rajaniemi (Rajaniemi *et al.*, 2005b). Saker and Neilan (Saker and Neilan, 2001) found a similar strong consistency in morphological responses of seven *Cylindrospermopsis raciborskii* isolates to different nitrogen sources. The present study is the first report of significant relationships between *Anabaena* morphology and environmental parameters, since the only previous study dealing with effects of growth conditions (light and temperature) on *Anabaena* strains referred to the stability of *Anabaena* morphology (Stulp and Stam, 1985).

Our experiments demonstrated that both the strains studied covered the range of variability of both *A. circinalis* and *A. crassa*, as they were originally described. The strains displayed the range of trichome width of 8.5–13.5 μm (strain 04-26) and 7.0–13.0 μm (strain 04-28). The widest trichomes (12–13 μm) were observed at the highest experimental temperature (28°C), the thinnest trichomes (8–9 μm) at the lowest concentrations of phosphorus (WC_{0xP}, $WC_{0.001xP}$). Diameters of trichome coils of strain 04-26 were 35–62 μm, whereas strain 04-28 had a wider range (15–110 μm). These results suggest that no reliable morphological criterion exists for distinguishing *A. circinalis* and *A. crassa*. This agrees with the suggestion made by Zapomělová *et al.* (Zapomělová *et al.*, 2007), who observed continuous variability of trichome widths and coil diameters of 13 *A. circinalis* and *A. crassa* populations in the Czech Republic. Consequently, a revision of these two *Anabaena* species is required, using a combination of molecular and morphological methods applied to different strains of these cyanobacteria.

An important finding of our study is the stability of akinete morphometry under varied experimental conditions. Thus, the dimensions and the shape of akinetes appear to be reliable criteria for identification of these *Anabaena* morphospecies, discriminating them from

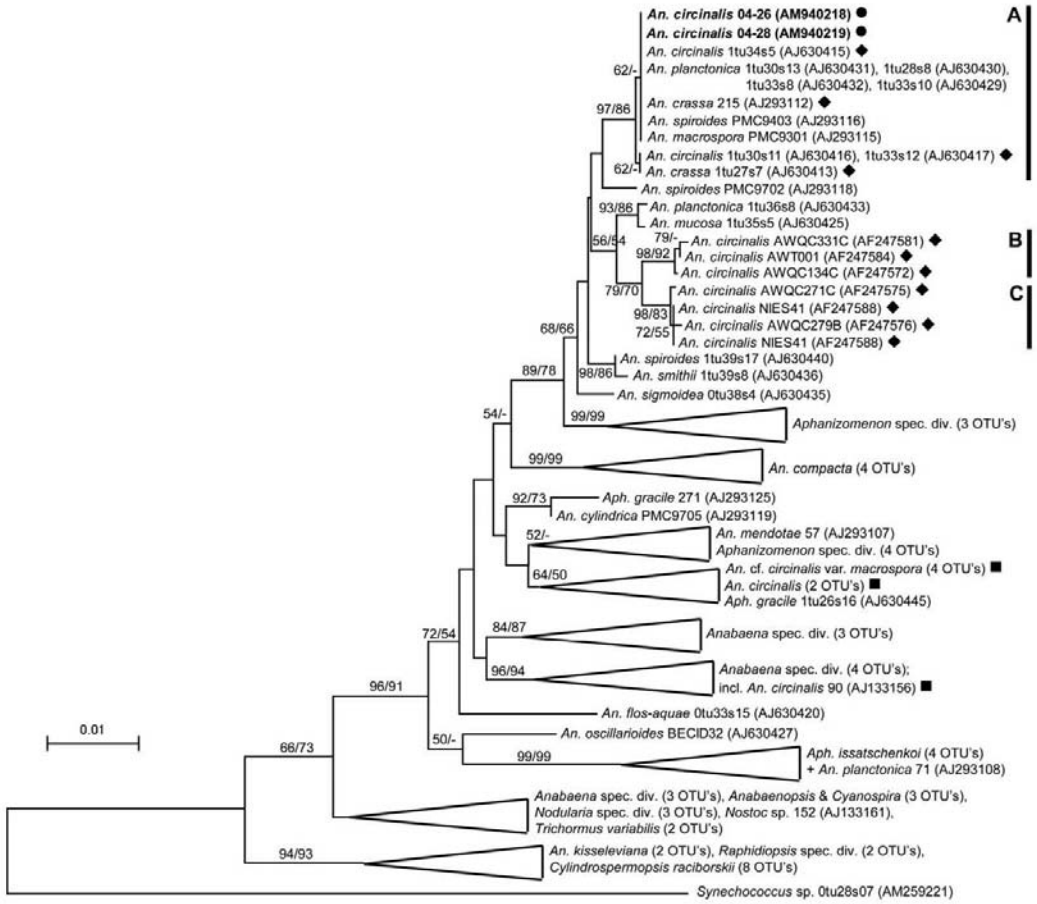


Fig. 7. NJ tree based on 16S rRNA gene sequences (1212 bp) showing the clustering of studied strains 04-26 and 04-28 (highlighted) with *A. circinalis* and *A. crassa* strains from Finland (cluster A). Numbers near the nodes indicate the bootstrap values over 50% for NJ and MP analyses. Abbreviations and symbols: *An.*, *Anabaena*; *Aph.*, *Aphanizomenon*; incl., including; OTU, operational taxonomic unit; spec. div., different species; filled circle, studied *Anabaena circinalis* strains; filled diamond, *A. circinalis* and *A. crassa* strains published by other authors; filled square, misidentifications of *A. circinalis* in other studies.

morphospecies with similar trichome width, such as *A. mucosa* Komárková et Eloranta 1992 and *A. ucrainica* (Schkorb.) M. Watanabe 1996.

Our study has demonstrated a high plasticity of trichome coil diameters and distances between adjacent coils under varied experimental conditions. An analogous conclusion has been derived from the study of Zapomělová *et al.* (Zapomělová *et al.*, in press), who observed modifications in trichome coiling of *A. spiroides* related to the composition of the culture medium. Numerous reports on trichome straightening of *Anabaena* strains in cultures have been published (Booker and Walsby, 1979; Hickel, 1982; Zapomělová, 2004; Zapomělová *et al.*, in press), indicating the instability of *Anabaena* trichome coiling in general. Recently published analyses of 16S rRNA, *rpoB* and *rbcLX* sequences have shown that trichome coiling does not reflect phylogeny, since *Anabaena* strains with coiled and straight trichomes, but with similar morphometric characteristics, often appear in the same clusters in phylogenetic trees (Beltran and Neilan, 2000; Rajaniemi *et al.*, 2005a, b).

Temperature

Our experiments confirmed temperature as an important factor influencing growth and morphology of planktonic

Anabaena. A significant effect of temperature was found on morphometry of vegetative cells and heterocytes and on trichome coiling parameters. Previously published papers have emphasized the importance of persistent thermal stratification for the occurrence of blooms of *A. circinalis* (Mitrovic *et al.*, 2003; Westwood and Ganf, 2004a, b). It is controversial whether this is due to enhanced metabolic rates at higher temperatures (Robarts and Zohary, 1987) or more likely the result of the physical and chemical status of the water column (Mitrovic *et al.*, 2003). Accumulation of metabolic products as a result of enhanced metabolic rates may be a potential explanation of the vegetative cell and heterocyte enlargement that we observed in our strains at 28°C.

The temperature optima of our strains differed one from another but both of them corresponded more or less to the optimum of *A. circinalis* (20–25°C) reported by Tsujimura and Okubo (Tsujimura and Okubo, 2003). Inter-strain variability can probably be expected in temperature and light preferences, since Rapala and Sivonen (Rapala and Sivonen, 1998) referred to strain-specific differences in growth rates of various *Anabaena* strains as a function of temperature and light. On the contrary, Stulp and Stam (Stulp and Stam, 1985) demonstrated similar growth response to temperature and light of various representatives of one and the same morphological group of *Anabaena*.

The growth optimum of strain 04-28 was slightly higher than the water temperature commonly observed in Czech water bodies during the summer, which is 21–24°C (Znachor *et al.*, 2006). A similar finding was published from the Baltic Sea where the water temperature fluctuates around 15°C in summer, whereas the optimum growth temperature of a *Nodularia* strain isolated from these waters was 25–28°C (Lehtimäki *et al.*, 1997). One explanation for these discrepancies may be the adaptation to higher temperature during cultivation. This, however, seems unlikely in the case of our strain since both the strains studied were cultured at 21°C, which is lower than the temperature optimum determined for strain 04-28.

Light

A significant effect of light on morphological features of our strains was not observed, although physiological consequences (photoinhibition) could be expected from the high light intensities reached in the crossed gradients (Wyman and Fay, 1987). Similarly, the lowest light intensities of the crossed gradients can be considered limiting for cyanobacterial growth (Wyman and Fay, 1987). Our findings agree with the results of Stulp and Stam (Stulp and Stam, 1985) who also did not observe any effect of light on morphology of *Anabaena* strains.

A decrease in gas vesicle content was observed in our strains with rising light intensity and with decreasing P concentration, which is consistent with previous findings (Reynolds and Walsby, 1975; Konopka *et al.*, 1987; Brookes and Ganf, 2001).

The light optima of our strains were narrow, falling at the higher end of the range of light intensities determined optimal for the growth of *A. circinalis* (Westwood and Ganf, 2004b).

Nutrients (phosphorus, nitrogen)

Phosphorus concentration was the main factor influencing the morphology of vegetative cells, and consequently, trichome coiling characteristics were modified in the strains studied. Significant differences in the trichome width were previously observed between P-limited and non-limited populations of *C. raciborskii*. The thinner form from P-limited conditions was able to grow into normal-sized trichomes under favourable culture conditions (Komárková *et al.*, 1999), which agrees with our results.

The phosphorus-dependent trichome straightening of the strain 04-28, which was observed in our experiments, is not consistent with the ecological context. Trichomes should tend to sink to lower parts of the water column when P is limited in the euphotic zone, whereas trichome straightening would counteract this (Booker and Walsby, 1979; Padisák *et al.*, 2003). It seems to be more likely a geometrical consequence rather than an ecological adaptation. The cells of the strain 04-28 became longer at the lowest P concentrations, supposedly because of the lack of sources necessary for cell division. Then the increased cell length affected the trichome shape.

The insignificant effect of low nitrogen concentrations can be easily explained by the N_2-fixing ability of *Anabaena* (heterocytes). However, our study also demonstrated that the high N concentrations achievable in field had no effect on *Anabaena* morphology. This contrasts with the results of Saker and Neilan (Saker and Neilan, 2001) who found significant response of morphological variability of *C. raciborskii* strains to different sources and concentrations of N.

Secondary metabolite content

Despite high morphological and 16S rRNA gene sequence similarities, we found a very low similarity in the production of secondary metabolites between the two strains studied. This finding is consistent with the

published results on discrepancies between phylogenetic features and protein or toxin production (Palinska *et al.*, 1996; Bolch *et al.*, 1999; Iteman *et al.*, 2002). The random distribution of peptide synthetase genes across the phylogentic spectrum was demonstrated by Rantala *et al.* (Rantala *et al.*, 2004) in the case of microcystin synthesis. The authors suggested that the ability to synthesize different peptides is scattered across the phylogenetic spectrum because of extinction of the ancient synthetic pathway in different evolution lineages. We have shown that even cyanobacteria within one lineage can produce markedly different types of metabolites. This can refer to fast changes in regulation and pattern of non-ribosomal synthetic pathways, the pathways which cyanobacterial peptides are synthesized by (Welker and von Döhren, 2006). Secondary metabolite production can vary considerably in response to growth conditions (Rapala *et al.*, 1993, 1997) or to the growth phase (Negri *et al.*, 1997). However, the effect of the above-mentioned factors seems to be limited in this case, since both of our strains were isolated almost at the same time, kept under identical culture conditions and harvested in exponential phase of growth.

Phylogenetic relationships based on 16s rRNA gene structure

Our strains displayed very high 16S rDNA sequence similarities with all Finnish *A. circinalis* and *A. crassa* strains (marked with diamonds in Fig. 7) corresponding to subcluster A of Rajaniemi *et al.* (Rajaniemi *et al.*, 2005a, b). On the other hand, Australian strains of *A. circinalis* (Beltran and Neilan, 2000), which were located separately from the European cluster, were divided into two distinct branches of higher heterogeneity (clusters B and C). The strains designated as *A. circinalis* var. *macrospora* by Rajaniemi *et al.* (Rajaniemi *et al.*, 2005a, b) or as *A. circinalis* in other studies (Lyra *et al.*, 2001; Gugger *et al.*, 2002) were placed into clusters considerably distant from all other *A. circinalis* strains in the present phylogenetic tree (marked with squares in Fig. 7). Morphology of the strains of *A. circinalis* var. *macrospora* evidently differed from the "true" *A. circinalis* strains described in the same paper (Rajaniemi *et al.*, 2005a) and also from our strains 04-26 and 04-28. Phenotypic characteristics of the strains *A. circinalis* studied by Lyra *et al.* (Lyra *et al.*, 2001) and Gugger *et al.* (Gugger *et al.*, 2002) were not published in detail but presumably, these strains belonged to *Anabaena* morphotypes with thinner trichomes since they appeared near *A. circinalis* var. *macrospora* or even separately.

FUNDING

Financial assistance for this study was provided by the GA ASCR (Project Nos KJB600960703, AV0Z60170517) and by Grant Agency of the Czech Republic (Project No. 206/06/0462).

ACKNOWLEDGEMENTS

We want to express our thanks to Marie Kupková, Martina Vožechová and Jindra Bučková for valuable technical help and Dr Valerie Flechtner for language correction.

REFERENCES

Anonymous (1996) *Statistica for Windows [Computer program manual].* Statsoft, Tulsa, OK.

Beltran, E. C. and Neilan, B. A. (2000) Geographical segregation of the neurotoxin-producing cyanobacterium *Anabaena circinalis*. *Appl. Environ. Microb.*, **66**, 4468 4474.

Bolch, C. J. S., Orr, P. T., Jones, G. J. *et al.* (1999) Genetic, morphological, and toxicological variation among globally distributed strains of *Nodularia* (cyanobacteria). *J. Phycol.*, **35**, 339 355.

Booker, M. J. and Walsby, A. E. (1979) The relative form resistance of straight and helical blue green algal filaments. *Bt. Phycol. J.*, **14**, 141 150.

Brookes, J. D. and Ganf, G. G. (2001) Variations in the buoyancy response of *Microcystis aeruginosa* to nitrogen, phosphorus and light. *J. Plankton Res.*, **23**, 1399 1411.

Cronberg, G. and Annadotter, H. (2006) *Manual on Aquatic Cyanobacteria. A Photo Guide and a Synopsis of their Toxicology.* International Society for the Study of Harmful Algae and the UNESCO.

Felsenstein, J. (2004) *PHYLIP (Phylogeny Inference Package) version 3.6. 2004.* Computer program, distributed by the author. Department of Genome Sciences, University of Washington, Seattle.

Gugger, M., Lyra, C., Henriksen, P. *et al.* (2002) Phylogenetic comparison of the cyanobacterial genera *Anabaena* and *Aphanizomenon*. *Int. J. Syst. Evol. Microb.*, **52**, 1867 1880.

Guillard, R. R. and Lorenzen, C. J. (1972) Yellow green algae with chlorophyllide c. *J. Phycol.*, **8**, 10 14.

Hickel, B. (1982) A helical bloom-forming *Anabaena*-like blue green alga (Cyanophyta) from hypertrophic lakes. *Arch. Hydrobiol.*, **95**, 115 124.

Iteman, I., Rippka, R., de Marsac, N. T. *et al.* (2002) rDNA analyses of planktonic heterocystous cyanobacteria, including members of the genera *Anabaenopsis* and *Cyanospira*. *Microbiology*, **148**, 481 496.

Komárek, J. (1958) Die taxonomische Revision der planktischen Blaualgen der Tschechoslowakei. In Komárek, J. and Ettl, H. (eds), *Algologische Studien*. Verlag der Tschechoslowakischen Akademie der Wissenschaften, Prag, pp. 10 206.

Komárek, J. (1996) Klíč k určování vodních květů sinic v České republice [A key for determination of water-bloom-forming cyanobacteria in the Czech Republic]. In Maršálek, B., Keršner, V. and Marvan, P.

(eds), *Vodní květy sinic [Cyanobacterial water blooms]*. Nadatio flos-aquae, Brno (in Czech), pp. 22 85.

Komárek, J. and Zapomělová, E. (2007) Planktic morphospecies of the cyanobacterial genus *Anabaena*=subg. *Dolichospermum* 1. part: coiled types. *Fottea*, **7**, 1 31.

Komárková-Legnerová, J. and Cronberg, G. (1992) New and recombined filamentous Cyanophytes from lakes in South Scania, Sweden. *Algol. Studies*, **67**, 21 31.

Komárková-Legnerová, J. and Eloranta, P. (1992) Planktic blue-green algae (Cyanophyta) from Central Finland (Jyväskylä region) with special reference to the genus *Anabaena*. *Algol. Studies*, **67**, 103 133.

Komárková, J., Laudares-Silva, R. and Senna, P. A. C. (1999) Extreme morphology of *Cylindrospermopsis raciborskii* (Nostocales, Cyanobacteria) in the Lagoa do Petri, a freshwater coastal lagoon, Santa Catarina, Brazil. *Algol. Studies*, **94**, 207 222.

Konopka, A., Kromkamp, J. C. and Mur, L. R. (1987) Regulation of gas vesicle content and buoyancy in light- and phosphate-limited cultures of *Aphanizomenon flos-aquae* (Cyanophyta). *J. Phycol.*, **23**, 70 78.

Kviderová, J. and Lukavský, J. (2001) A new unit for crossed gradients of temperature and light. Beiheft algae and extreme environment. *Nova Hedwigia*, **123**, 541 550.

Lehtimäki, J., Moisander, P., Sivonen, K. *et al.* (1997) Growth, nitrogen fixation and nodularin production by two Baltic Sea cyanobacteria. *Appl. Environ. Microb.*, **63**, 1647 1656.

Lorenzen, C. J. (1967) Determination of chlorophyll and phaeopigments: Spectrophotometric equation. *Limnol. Oceangr.*, **12**, 343 346.

Lyra, C., Suomalainen, S., Gugger, M. *et al.* (2001) Molecular characterization of planktic cyanobacteria of *Anabaena, Aphanizomenon, Microcystis* and *Planktothrix* genera. *Int. J. Syst. Evol. Microb.*, **51**, 513 526.

Mitrovic, S. M., Oliver, R. L., Rees, C. *et al.* (2003) Critical flow velocities for the growth and dominance of *Anabaena circinalis* in some turbid freshwater rivers. *Freshwater Biol.*, **48**, 164 174.

Negri, A. P., Jones, G. J., Blackburn, S. I. *et al.* (1997) Effect of culture and bloom development and of sample storage on paralytic shellfish poisons in the cyanobacterium *Anabaena circinalis*. *J. Phycol.*, **33**, 26 35.

Nübel, U., Garcia-Pichel, F. and Muyzer, G. (1997) PCR primers to amplify 16S rRNA genes from cyanobacteria. *Appl. Environ. Microbiol.*, **63**, 3327 3332.

Padisák, J., Soroczki-Pinter, E. and Rezner, Z. (2003) Sinking properties of some phytoplankton shapes and the relation of form resistance to morphological diversity of plankton an experimental study. *Hydrobiologia*, **500**, 243 257.

Palinska, K. A., Liesack, W., Rhiel, E. *et al.* (1996) Phenotype variability of identical genotypes: the need for a combined approach in cyanobacterial taxonomy demonstrated on *Merismopedia*-like isolates. *Arch. Microbiol.*, **166**, 224 233.

Rajaniemi, P., Hrouzek, P., Kaštovská, K. *et al.* (2005a) Phylogenetic and morphological evaluation of the genera *Anabaena, Aphanizomenon, Trichormus* and *Nostoc* (Nostocales, Cyanobacteria). *Int. J. Syst. Evol. Microb.*, **55**, 11 26.

Rajaniemi, P., Komárek, J., Willame, R. *et al.* (2005b) Taxonomic consequences from the combined molecular and phenotype evaluation of selected *Anabaena* and *Aphanizomenon* strains. *Algol. Studies*, **117**, 371 391.

Rantala, A., Fewer, D. P., Hisbergues, M. *et al.* (2004) Phylogenetic evidence for the early evolution of microcystin synthesis. *Proc. Natl Acad Sci*, **101**, 568 573.

Rapala, J. and Sivonen, K. (1998) Assessment of environmental conditions that favour hepatotoxic and neurotoxic *Anabaena* spp. strains cultured under light limitation at different temperatures. *Microb. Ecol.*, **36**, 181 192.

Rapala, J., Sivonen, K., Luukkainen, R. *et al.* (1993) Anatoxin-a concentrations in *Anabaena* and *Aphanizomenon* under different environmental conditions and comparison of growth by toxic and notn-toxic *Anabaena* strains a laboratory study. *J. Appl. Phycol.*, **5**, 581 591.

Rapala, J., Sivonen, K., Lyra, C. *et al.* (1997) Variation of microcystins, cyanobacterial hepatotoxins, in *Anabaena* sp. as a function of growth stimuli. *Appl. Environ. Microbiol.*, **63**, 2206 2212.

Reynolds, C. S. and Walsby, A. E. (1975) Water-blooms. *Biol. Rev.*, **50**, 437 481.

Robarts, R. D. and Zohary, T. (1987) Temperature effects on photosynthetic capacity, respiration, and growth rates of bloom-forming cyanobacteria. *New Zealand J. Freshwater Res.*, **21**, 391 399.

Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**, 406 425.

Saker, M. L. and Neilan, B. A. (2001) Varied diazotrophies, morphologies, and toxicities of genetically similar isolates of *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyceae) from Northern Australia. *Appl. Environ. Microb.*, **67**, 1839 1845.

Šmilauer, P. (1992) *CANODRAW Users Guide v. 3.0.* Microcomputer Power, Ithaca, New York.

Stulp, B. K. and Stam, W. T. (1985) Taxonomy of the genus *Anabaena* (Cyanophyceae) based on morphological and genotypic criteria. *Arch. Hydrobiol. Suppl.*, **71**, 257 268. (*Algol. Studies*, **38/39**).

Taton, A., Grubisic, S., Brambilla, E. *et al.* (2003) Cyanobacterial diversity in natural and artificial microbial mats of Lake Fryxell (McMurdo dry valleys, Antarctica): a morphological and molecular approach. *Appl. Environ. Microbiol.*, **69**, 5157 5169.

Ter Braak, C. J. F. and Šmilauer, P. (1998) *CANOCO Reference Manual.* Microcomputer Power, Ithaca, New York.

Tsujimura, S. and Okubo, T. (2003) Development of *Anabaena* blooms in a small reservoir with dense sediment akinete population, with special reference to temperature and irradiance. *J. Plankton Res.*, **25**, 1059 1067.

Welker, M. and von Döhren, H. (2006) Cyanobacterial peptides nature's own combinatorial biosynthesis. *FEMS Microbiol. Rev.*, **30**, 530 563.

Westwood, K. J. and Ganf, G. G. (2004a) Effect of cell flotation on growth of *Anabaena circinalis* under diurnally stratified conditions. *J. Plankton Res.*, **26**, 1183 1197.

Westwood, K. J. and Ganf, G. G. (2004b) Effect of mixing patterns and light dose on growth of *Anabaena circinalis* in a turbid, lowland river. *River Res. Appl.*, **20**, 115 126.

Wilmotte, A., Neefs, J. M. and De Wachter, R. (1994) Evolutionary affiliation of the marine nitrogen-fixing cyanobacterium *Trichodesmium* sp strain NIBB 1067, derived by 16S ribosomal RNA sequence analysis. *Microbiology*, **140**, 2159 2164.

Wyman, M. and Fay, P. (1987) Acclimation to the natural light climate. In Fay, P. and Van Baalen, C. (eds), *The Cyanobacteria*. Elsevier Science Publishers B. V., (Biomedical Division), pp. 347 376.

Znachor, P., Jurczak, T., Komárková, J. *et al.* (2006) Summer changes of cyanobacterial bloom composition and microcystin

concentration in eutrophic Czech reservoirs. *Environ. Toxicol.*, **21**, 236 243.

Zapomělová, E. (2004) Morfologická variabilita a růst vybraných kmenů sinic rodu Anabaena a Aphanizomenon v závislosti na podmínkách prostředí [Morphological variability and growth of chosen cyanobacterial strains of genera Anabaena and Aphanizomenon in the dependence on environmental conditions]. MSc thesis. University of South Bohemia, Czech Republic (in Czech).

Zapomělová, E., Řeháková, K., Znachor, P. *et al.* (2007) Morphological diversity of coiled planktonic types of the genus *Anabaena* (cyanobacteria) in natural populations taxonomic consequences. *Cryptogamie Algol.*, **28**, 353 371.

Zapomělová, E., Hrouzek, P., Řeháková, K. *et al.* (2008) Morphological variability in selected heterocystous cyanobacterial strains as a response to varied temperature, light intensity and medium composition. *Folia Microbiologica*, **53**, 333 341.