School of Doctoral Studies in Biological Sciences
University of South Bohemia in České Budějovice
Faculty of Science

Polyphasic analysis and secondary metabolite patterns in unbranched heterocytous cyanobacteria with different life strategies

Ph.D. Thesis

Mgr. Andreja Kust

Supervisor: RNDr. Jan Mareš, Ph.D.

University of South Bohemia in České Budějovice, Faculty of Science
The Czech Academy of Sciences, Biology Centre, Institute of Hydrobiology

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Annotation

Unbranched heterocytous cyanobacteria exhibit complex filament and colony architectures and variable life strategies from symbionts to free living planktic and non-planktic species. They are counted among microbial groups showing an extensive production of secondary metabolites, resulting in both pharmaceutically important and toxic compounds. The main focus of this thesis is to broaden our knowledge on bioactive secondary metabolite potential in this widespread group of cyanobacteria. An effective combination of methods including whole genome sequencing, bioinformatic analysis, and analytical chemistry techniques are applied to accomplish this task. The discrepancies in distribution of various classes of compounds among ecological groups defined by different life strategies are discussed. Additionally, the thesis endeavours to test multidisciplinary approaches to tackle taxonomic assignments of unresolved unbranched heterocytous cyanobacteria using morphological, phylogenetic and ecophysiological methods, including a meta-analysis of morphological traits.

Declaration [in Czech]

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LIST OF PAPERS AND AUTHOR´S CONTRIBUTIONS

I. Kust A, Kozlíková-Zapomělová E, Mareš J, Řeháková K. 2015: A detailed morphological, phylogenetic and ecophysiological analysis of four benthic *Anabaena* (Nostocales, Cyanobacteria) strains confirms deep heterogeneity within the genus. *Fottea* 15(2): 191-202 **(IF=1.476)**

AK was responsible for performing the experiments, analyses, and data evaluation, she wrote most parts of the manuscript.

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Contents

1. INTRODUCTION

1.1. General Significance of Cyanobacteria

Fossil, geochemical, and molecular evidence has verified cyanobacteria as one of the most ancient lineages of extant organisms, appearing about 3.5 billion years ago (Planavsky et al. 2014, Nutman et al. 2016). They are the only bacterial group/prokaryotes able to perform oxygenic photosynthesis thanks to their complex pigment composition. (Des Marais et al. 2000, Schopf et al. 2000). Cyanobacteria of the early Earth were responsible for the creation of an oxygen-rich atmosphere suitable for aerobic organisms, including plants and animals to live in (Schirrmeister et al. 2015). Cyanobacteria thrive all around the globe due to their ability to colonize all types of habitats and survive extreme conditions (Whitton and Potts 2000, Whitton et al. 2012). The occurrence of cyanobacteria in a broad range of ecological niches relies on maintaining cell function and structure with little energy required for their growth (Gons 1997, Van Liere et al. 1979). Many cyanobacteria are major components of phytoplankton and periphyton communities; being either free-floating organisms in open water or attached to rocks and other substrates.

The ecological significance of cyanobacteria lies in their involvement in global carbon, oxygen, and nitrogen cycles through production of organic compounds utilized by other organisms, and stabilization of sediment and soil (Whitton et al. 2000, Garcia Pichel et al. 2003). Many cyanobacteria from freshwater, marine, and terrestrial habitats are diazotrophs, able to convert dinitrogen gas to two ammonium ions as a result of nitrogenase activity (Fogg et al. 1969, Postgate et al. 1982). The converted nitrogen is later available for utilization in amino acids (proteins and peptides),

nucleic acids, and other cellular parts rich in nitrogen (Zehr et al. 2001, Bothe et al. 2010). Nitrogen fixation by cyanobacteria provides a beneficial resource for symbiotic relationships with non-diazotrophic organisms, thus they are often involved in symbiosis with diverse organisms in which they act as nitrogen suppliers. Nitrogen-fixing cyanobacteria (e.g. *Nostoc* genus) are frequently involved in consortia with liverwort, hornwort, moss, fern, cycads, and angiosperm species (Dodds et al. 1995, Rai et al. 2000). Nitrogen fixation in the often nitrogen-limited marine environment is utilized by both, free-living (Zehr et al. 2007, Bergman et al. 2013) and symbiotic (Thompson et al. 2014, Bar Zeev et al. 2008) diazotrophic cyanobacteria from diverse phylogenetic lineages. Marine cyanobacterial endosymbionts are associated mainly with diatoms and other microalgae. (Momper et al. 2015, Stenegren et al. 2018). The ability to fix atmospheric nitrogen gives nitrogen-fixers a competitive advantage over other microorganisms in environments with nitrogen sources deficiency (Graham et al. 2008, Huisman et al. 2018).

1.2. Cyanobacterial Classification

In the Linnaean classification system, cyanobacteria were described as a group of simple algae. After the recognition of cyanobacteria as prokaryotes, Geitler (1932), inspired by pioneer studies (Thuret 1875, Bornet and Flahaut 1886-1888, and Gomont 1892), established a cyanobacterial taxonomic review and determination manual based solely on the morphology of field-collected specimens of all known cyanobacteria at that time. This system, with several modifications (Elenkin 1938, 1949; Desikachary 1959; Fritsch 1959; Starmach 1966 and others), followed the traditional criteria of botanical taxonomy and has

been utilized by both microbiologists and phycologists. Geitler's monography served as the main identification manual for cyanobacterial taxonomic assignments until the late 20th century. Once classified as prokaryotes, cyanobacteria attracted the attention of bacteriologists who later established the first collections of pure cyanobacterial strains for comprehensive study (Rippka et al. 1979). The recommendations by Rippka et al. (1979) for taxonomic classification of cyanobacteria, based on strains from the Pasteur Culture Collection of Cyanobacteria, have served for a long time as the primary classification source for the Bergey's Manual of Systematic Bacteriology (Castenholz 2001). This classification was still mostly based on morphological features, examined mainly by light microscopy and it recognized five cyanobacterial subsections, I (= Chroococcales), II (= Pleurocapsales), III (= Oscillatoriales), IV (= Nostocales) and V (= Stigonematales) (Castenholz 2001). With the development of electron microscopy and molecular phylogenetic analysis, it became evident that the earlier proposed classification systems would have to pass dramatic changes. A subsequent phylum-wide taxonomic revision of cyanobacteria was carried out by Komárek and Anagnostidis (1999, 2005) and Komárek (2013), it comprised both bacteriological and botanical approaches. While the nomenclature was still based on botanical taxonomic criteria to preserve historical continuity, the taxonomic system partly incorporated bacteriological and molecular information.

Instead of solely morphological characterization, polyphasic (multidisciplinary, consensus) evaluation of taxa has become the basis of the modern revision of cyanobacterial taxonomic classification. The term "polyphasic" had already been introduced in bacterial taxonomy in the 1970s by Colwell, who proposed a combination of genotypic, chemotypic,

and phenotypic information of microorganisms for their unambiguous taxonomic assignments (Colwell et al. 1970, Vandamme et al. 1996). A compilation of work by Anagnostidis and Komárek (1985, 1988), and Komárek and Anagnostidis (1989) introduced the polyphasic (botanical) approach for cyanobacterial taxonomy and since then it has been further conceptualized and applied by scientists all over the world (e.g. Johansen and Casamatta 2005, Castenholz and Norris 2005, Komárek 2010, Osorio–Santos et al. 2014, Dvořák et al. 2015, and many others). This approach has proven to be the best available tool for the most reliable and objective taxonomic designation possible. Although currently based on the monophyletic species concept (Johansen and Casamatta 2005), it requires the synthesis of a detailed description of classical morphological traits, ecology, cell ultrastructure and molecular phylogeny into a congruent picture of a species, defined by both, unique genetic and phenotypic markers (Mareš 2018). The description of new taxa relies on the definition of autapomorphic characters as an integral and obligatory requirement for the revised new taxa (Johansen and Casamatta 2005, Rajaniemi et al. 2005, Řeháková et al. 2007, Komárek 2010). Based on the multidisciplinarity recommendations/polyphasic approach, the latest proposed systematic classification allocates cyanobacteria into eight orders, some of which are still provisional and polyphyletic: Gloeobacterales, Synechococcales, Spirulinales, Chroococcales, Pleurocapsales, Oscillatoriales, Chroococcidiopsidales, and Nostocales (Komárek et al. 2014, Mareš 2018, Fig 1.).

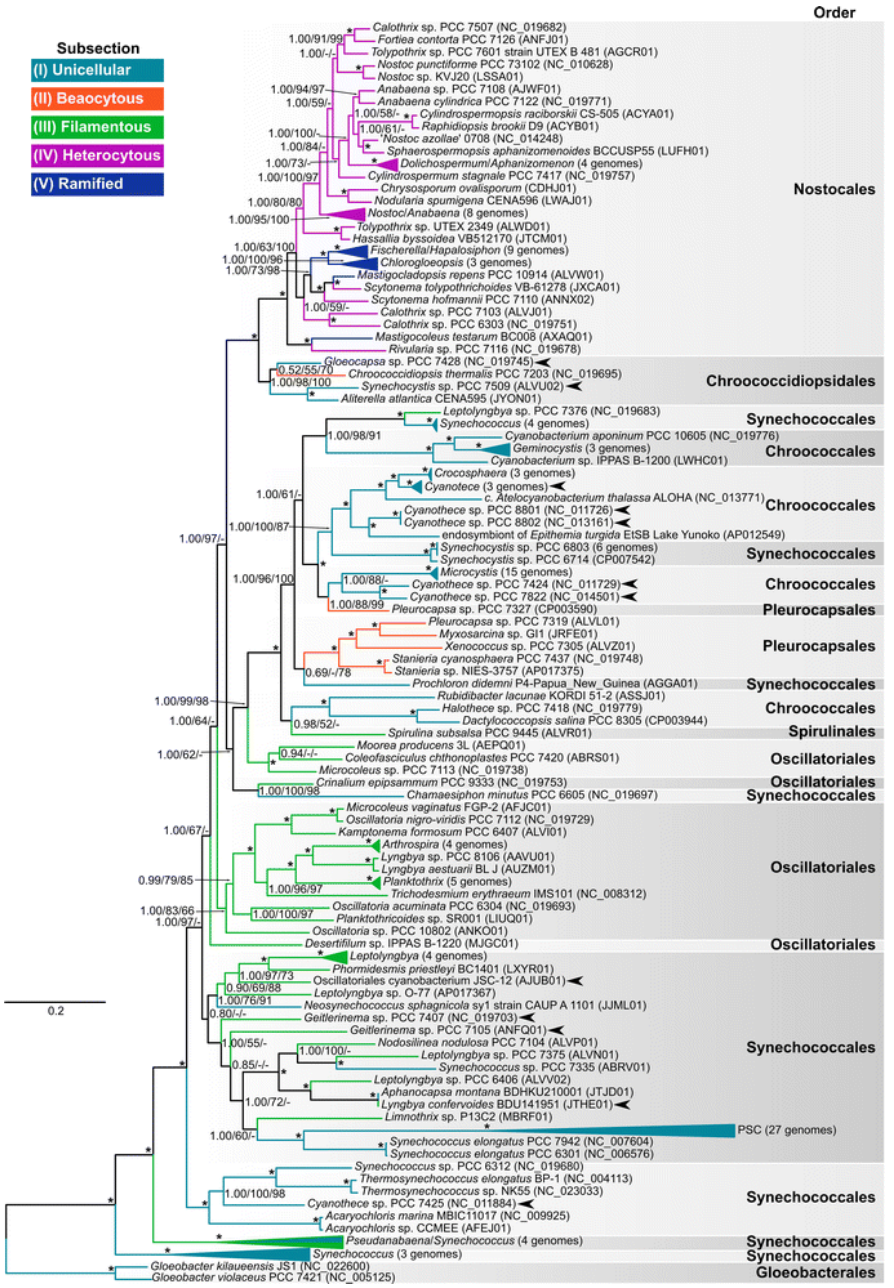

Fig 1. Phylogenetic tree of cyanobacteria (Bayesian Inference), adjusted from Mareš 2018.

1.3. Unbranched Filamentous Heterocytous Cyanobacteria

Members of the order Nostocales (including the main families of unbranched species – Nostocaceae and Aphanizomenonaceae), are defined as diazotrophic cyanobacteria with heterocytes - specialized cells for nitrogen fixation. Heterocytes are pale cells with thickened cell walls, special terminal pores at the site of connection to neighbouring vegetative cells, modified pigment composition, and the absence of an active photosystem II (including the oxygen-evolving complex) (Walsby et al. 1985). This unique set of features ensures anoxic intracellular conditions, providing favourable conditions for the highly oxygen-sensitive enzyme, nitrogenase, which is responsible for nitrogen fixation (Muro Pastor et al. 2012). The thickened cell walls of heterocytes, and in some species, mucilaginous sheath around trichomes, reduces oxygen diffusion into the cells, yet allowing a sufficient influx of N_2 for effective nitrogen fixation (Walsby et al. 1985, Prosperi 1994, Huisman et al. 2018). Another type of specialized cells typical for the non-branching nostocalean cyanobacteria are akinetes, capable of survival under unfavourable conditions and growth resumption when conditions suitable for growth reoccur. The process of akinete development usually includes a decrease in the number of gas vesicles (planktic), size increase, thickened wall, and accumulation of storage granules (Moore et al. 2004). Also akinetes, at least in some cyanobacteria, have an inactivated photosystem II.

A comparison of morphological and molecular features of heterocytous cyanobacteria demonstrated that the development and mutual position of heterocytes and akinetes, together with the presence of aerotopes and variability in cell shapes, are the main morphological features reflecting

evolutionary relationships, and possibly also ecological adaptations (i.e. Rajaniemi et al. 2005a, Zapomělová et al. 2009, 2011, Sant'Anna et al. 2019). The relationships amongst volume, size, shape, and relative position of all three cell types have never been systematically compared between planktic and non-planktic (unbranched) Nostocales. The life strategies of these two ecological groups differ substantially. For instance, while planktic representatives usually develop growth peaks under relatively stable and favourable conditions, non-planktic representatives are often present during the whole year, facing severe fluctuations in temperature, water availability, etc. (Quiblier et al. 2013). As the specialized cells provide prominent metabolic abilities and their development is driven by specific environmental conditions, a life-strategy dependent on a morphological pattern can be expected.

The families Nostocaceae and Aphanizomenonaceae (Komárek et al. 2014) represent unbranched filamentous heterocytous cyanobacteria, with a high diversity of genera. One of the largest traditional genera, *Anabaena*, originally established by Bory in 1822 (Geitler 1932) has recently been divided (Komárek and Zapomělová 2007, 2008, Wacklin et al. 2009, Zapomělová et al. 2009, 2012). Three new planktic genera were erected: *Dolichospermum*, *Sphaerospermopsis*, and *Chrysosporum*, while the non-planktic representatives were distributed among *Anabaena*, *Trichormus*, and *Macrospermum* (Iteman et al. 2002, Gugger et al. 2002, Rajaniemi et al. 2005a, b, Hoffmann et al. 2005, Willame et al. 2006, Komárek 2010, Komárek 2013). The genus *Trichormus* was separated from *Anabaena* based on its apoheterocytic akinete development (Rajaniemi et al. 2005), and *Macrospermum*, usually forming metaphytic mats in tropical regions, is characterized by a subsymmetrical filament structure and a special type

of large akinetes (Komárek 2008). The generic name *Anabaena* was preserved for species without gas vesicles (benthic, periphytic, soil), and at first was considered to be more or less consistent with the modern phylogenetic taxonomic concept. However, recent evidence has demonstrated the polyphyletic nature of the group (Rajaniemi et al. 2005, Halinen et al. 2008, Kozliková-Zapomělová et al. 2016), although all published reports lack a comprehensive connection between the molecular diversity within this group and their morphological diversity observed in nature (Komárek 2005, 2008, Skácelová and Zapomělová 2010, Mareš et al. 2010). One of the oldest genera of heterocytous cyanobacteria is *Nostoc* (Bornet and Flahault 1888), the type genus of the order Nostocales. With complex morphology of morphospecies, often overlapping cell dimensions, macrocolony morphology, and ecology, together with a complicated life cycles, solely morphological classification has virtually been impossible. By applying the polyphasic approach, *Nostoc* has been divided into the "*Nostoc sensu stricto*" cluster, and at least five further different clades: *Mojavia*, *Desmonostoc*, *Aliinostoc*, *Komarekiella*, and *Halotia* (Rajaniemi et al. 2005, Řeháková et al. 2007, Hrouzek et al. 2013, Bagchi et al. 2017, Komárek 2013, Genuário et al. 2015, Hentschke et al. 2017). However, this complex genus still needs to pass revision taking into account polyphasic analysis of a higher number of representatives. *Nodularia (*Mertens ex Bornet and Flahault 1886), another important genus, is a phylogenetically uniform group of planktic, benthic and soil species (Komárek 2012). However, it is a great a challenge to define its members at the species level, even when morphology, biogeography, phylogeny, and secondary metabolite composition are used for identification (Komárek 2013, Řeháková et al. 2014). The genus

Cylindrospermum (Kutz. ex Bornet et Flahault 1886), has been divided into three distinct clades based on distinct morphologies (Johansen et al. 2014). A number of investigated strains exhibited a very high percentage of similarity in ribosomal sequences, however the sequence similarity was in contradiction with high morphological diversity (Johansen et al. 2014). The genus *Wollea* is a traditional genus with type species *Wollea saccata* (Wolle) Bornet et Flahault 1888 (pre–starting–point syn. = *Sphaerozyga saccata* Wolle 1880). Morphologically similar to some of the strains from genus *Anabaena*, the *Wollea* cluster is not well supported by 16S rRNA data, thus further studies including a higher number of representatives and multilocus sequencing are needed for better understanding of this genus (Kozliková-Zapomělová et al. 2016).

Among the representatives described above, morphological characterization in many cases was not sufficient to clearly define the genera and to distinguish them based solely on closely resembling morphological traits. However, with the use of the polyphasic approach employing at least genetic and morphological criteria, a big step forward has been made, and several taxa that are difficult to define have been described. In the future, more comprehensive studies comprising a high number of taxa and multilocus/whole genome sequencing for assessing the phylogenetic relationships (in combination with other criteria) are needed.

1.4. Natural Products Potential of Cyanobacteria

Cyanobacteria are a promising source of novel valuable compounds due to their ability to produce diverse bioactive secondary metabolites with either toxic, or therapeutical potential (Chlipala et al. 2011, Sielaff et al. 2006, Welker and von Döhren 2006). The vast diversity of secondary metabolites

produced by cyanobacteria might be explained by their early appearance in Earth´s history during which they have been adapting to various environmental conditions, developing effective mechanisms and strategies for the purpose of survival and to outcompete other organisms. These secondary metabolites, or natural products, can exhibit various biological activities providing the possibility for application in medicine, agriculture, and biotechnology (Singh et al. 2005). At this time of serious antibiotic resistance and high interest for natural anticancer cures, natural pharmaceutical resources are more than desirable. Despite intensive study, a chemical description of only 10-20 % of cyanobacterial secondary metabolites has been provided, and even less is known about their toxic potential (Hrouzek et al. 2011). Although cyanobacteria comprise a wide range of species, the most prolific sources for natural products are filamentous and colonial cyanobacteria (Méjean and Ploux 2013, Shih et al. 2013). Mining of 89 publicly available genomes evidenced that members of late branching cyanobacteria (Nostocales, Chroococcidiopsidales, Pleurocapsales and Osillatoriales) are richer in putative gene clusters for secondary metabolite production compared with early branching cyanobacteria (Calteau et al. 2014). The deficiency in secondary metabolism in early-branching cyanobacteria is likely connected to their smaller genome size and prevailable primary metabolism (Galica et al. 2017). This is a general trend in all bacteria, because small-sized genomes tend to lack the (usually very large) gene clusters responsible for non-ribosomally synthesized secondary metabolites (Wang et al. 2014).

Members of the cyanobacterial order Nostocales (heterocytous cyanobacteria) are extremely active in terms of general secondary

metabolite production (Rezanka et al. 2006). They are responsible for the production of about a quarter of the total known metabolites produced by filamentous cyanobacteria (Jones et al. 2010). They exhibit diverse chemical structures: peptides, alkaloids, polyketides, lipids, and terpenes (Welker et al. 2006, Yamada et al. 2015, Shah 2017). Cyanotoxins such as microcystins, nodularins, anatoxins, and cylindrospermopsin are often produced by unbranched nostocalean taxa from diverse environments, both free-living and symbiotic (i.e. *Nostoc, Dolichospermum, Aphanizomenon, Cuspidothrix, Cylindrospermopsis, Raphidiopsis*, etc. – Pitois et al. 2018). In addition to common cyanotoxins, they are known to produce other interesting secondary metabolites, as in the case of the first anabaenopeptilide (cyanopeptolin) described from planktic *Dolichospermum* ("*Anabaena"*, Fuji et al. 1996), soil *Cylindrospermum* producing lipopetides-puwainaphycins and muscotoxin (Hrouzek et al. 2012, Tomek et al. 2015), nostopeptolides isolated from terrestrial free living *Nostoc*, and nostocyclopeptides found to be produced by the lichen symbiont *Nostoc* (Golakoti et al. 2000, 2001). All of the peptides from the aeruginosins, microginins, anabaenopeptins, cyanopeptolins, microviridins, and cyclamides classes in cyanobateria have been found in at least one genus from this group (Welker et al. 2006).

Although the role of these various secondary metabolites is generally unknown, some of them have been linked to photoprotection, alteration of the stability and gas permeability of thylakoid membranes, and to protection from predators (reviewed in van Wagoner et al. 2007). Particular secondary metabolites are linked to the last maturation step of heterocytes and with siderophore production, important for nitrogen fixation and iron uptake, respectively (Calteau et al. 2014).

A great deal of attention has been drawn to the potential role of cyanobacterial toxic secondary metabolites – cyanotoxins. Cyanotoxins have been linked to a competitive advantage in microbial communities, as well as to involvement in core physiological functions (Holland et al. 2013). Many studies have tried to find a connection between cyanotoxin production and allelophathy, grazing pressure of zooplankton and protozoans, potential importance in cellular physiology by improving access to nutrients, assisting in photosynthetic efficiency, increasing survival under oxidative stress, homeostasis maintenance, and cell-to-cell signalling (Holland et al. 2013). However, not all secondary metabolites and their variants have a precise natural function. The so called screening hypothesis (Jones and Firn 1991, Firn and Jones 2000) brings up the possibility of an evolutionary benefit for microbes generating a high number of chemically different metabolites at minimum cost, which can serve as a pool of potentially useful compounds sorted by natural selection. It could be compared to the seemingly redundant pool of human antibodies.

1.5. PKS/NRPS Megasynthases

Some of the most important compounds (oligopeptides, polyketides, alkaloids) are synthesized by special types of protein complexes – non-ribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs) and hybrid NRPS/PKSs, accompanied by accessory enzymes (Dittmann et al. 2013) – and proved to be frequent in the majority of cyanobacterial evolutionary clades (Shih et al. 2013, Calteau et al. 2014). These megasynthases are organized in modules consisting of integrated enzymatic domains catalysing individual reaction steps of the

biosynthesis. Prediction of the course of synthesis is often possible once the genes encoding the enzymes responsible for metabolite synthesis are known due to the collinearity rule (Marahiel et al. 1997, Guenzi et al. 1998). The collinearity rule is based on the assumption that the catalytic reaction steps of biosynthesis usually follow the arrangement of enzymatic domains within the NRPS/PKS modules and the order of the corresponding genes in the operon. Additionally, analyses of the binding pockets of the amino-acid activating domains in NRPS elucidated the mechanism of substrate specificity and provided an amino acid contact residue code (Stachelhaus/specificity-conferring code) allowing the prediction of unknown products (Challis et al. 2000). These rules have been recently automatized in software pipelines such as antiSMASH (Weber et al. 2015, Blin et al. 2017). Based on sequence analysis of ketoreductase domains in PKS, it is possible to partly predict the stereochemistry of the final products (Caffrey et al. 2003, Reid et al. 2003).

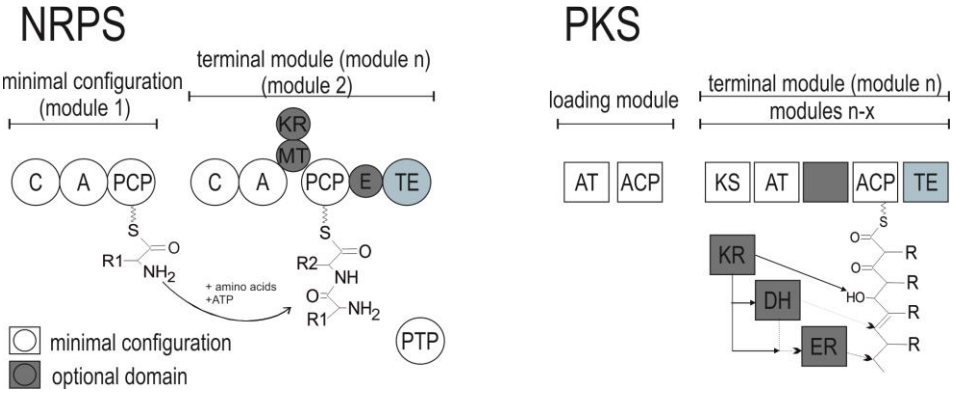

Fig 2. Scheme of main principles of NRPS and PKS megasynthases

1.5.1. Polyketide Synthase (PKS)

Multi-modular polyketide synthases (PKSs) form complex assembly lines for the synthesis of reduced polyketides and their diverse derivates (Piel

2010, Keatinge-Clay et al. 2012). These biosynthetic machineries are responsible for the existence of many compounds utilized as important human medicines (i.e. antibiotics erythromycin, tetracycline, and nystatin, and the antitumor agents epothilone and doxorubicin).

PKSs are one of the largest known proteins, consisting of repeated functional units (modules) which are responsible for chain extension in subsequent steps/cycles. Each module contains a set of different domains performing the chemical transformation of a monomer during its incorporation.

The resulting metabolites are constituted from polyketide chains, starting with a Claisen-type condensation reaction of a ketide to "starter units". Starter units are often acetyl CoA, propionyl CoA or more complex acyl CoAs, biochemically resembling the fatty acid synthases (FASs) (Hertweck et al. 2009, Musiol et al. 2012).

The obligate/essential domains of every PKS are acetyltransferase (AT) the domain responsible for loading the short CoA-bound building blocks to the acyl carrier protein (ACP) domain which, by condensation, covalently binds the starter acyl unit becoming available to keto-synthase (KS) domains responsible for chain elongation (Piel 2010). The usual order of domains is KS-AT-reduction domain loop-ACP, resulting in elongation and additional modification of the polyketide chain, with final transfer of the elongated chain to the downstream module. In addition to the necessary components of the polyketide synthesis apparatus, there are often additional domains, responsible for the existence of a wide diversity of synthesized natural products. Additional modification, performed at PKS by the so called "reduction domain loop" (Caffrey et al. 2003), are usually ketoreduction by ketoreductase (KR), modifying a beta-keto group

to a hydroxyl group, with particular stereospecificity configuration of the hydroxy groups and alkyl subunits (D- or L-). Other frequently performed modifications are dehydration by dehydratase (DH), generating olefinic moieties by elimination of water, and enoyl reduction by enoylreductase (ER) forming double bonds. Regularly, methyltransferase (MT) domains, responsible for methylation of the chain, are part of the synthesis apparatus. PKSs follow the collinearity rule based on which it is possible to predict (at least to some extent) the structure of the final product knowing the genetic background or, the other way around, it is possible to deduce the probable nature of the synthetase and its genes once the structure of the final product is known. The linear multifunctional PKS described above is recognized as PKS type I, however two more types with non-linearly arranged domains exist. PKS II – a dissociable complex of discrete monofunctional enzymes and, PKS III – multifunctional enzymes without AT and ACP with intermediates linked through free coenzyme-A thioesters (Hertweck et al. 2009, Brachman et al. 2007).

1.5.2. Trans-AT

A typical PKS I AT domain is responsible for selecting and loading the acyl CoA unit onto a conserved serine within its active site integrated in modules of the PKS. Integrated AT is referred as “cognate” AT, and it is part of *cis*-AT-PKS type I. However, when the AT domain is not integrated in a PKS module it is a *trans*-acting AT (Fig 3). *Trans*-AT is a single malonyl-CoA specific AT encoded by a separate gene outside of the PKS modules and it interacts with most, or even all PKS modules in the system. The main function of AT in each *cis*-AT-PKS module is substrate loading onto the respective ACP, while in *trans*-AT-PKS, a single separately

encoded AT acylates the entire PKS system. The existence of two types of PKSs was phylogenetically examined, demonstrating likely independent evolution from fatty acid synthesis (FAS) -like systems (Piel 2010).

While the *cis*-AT-PKSs modules usually consist of KS-AT-ACP and additional KR/DH/ER domains, the *trans*-AT PKS systems exhibit rather unique domains with greater module variants (about 50 combinations) (Helfriech and Piel 2016). The most typical domains of the *trans*-AT PKSs are alleged silent KS domains, without histidine residue in an HTGTG motif necessary for decarboxylative condensation, which finally results in nonfunctional KSs incapable of performing the chain elongation. Unlike in the *cis*-AT-PKS, the methylation process can be performed with specific gene cassettes (Chang et al. 2004, Mattheus et al. 2010), and sometimes double or multiple ACP domains can be encoded in a row (Piel et al. 2004, Kampa et al. 2013). *Trans*-acting PKSs deviate from the collinearity rule, which caused delayed understanding of their function (Piel 2010). Given this deviation from collinearity, an alternative model for the product prediction involves phylogenetic comparison of KS domains (Nguyen et al. 2008).

Bacillaene synthetase was the first *trans*-PKS, identified already in 1993 in a sequenced genome of *Bacillus subtilis* (Scotti et al. 1993). Nevertheless, the first reported *trans*-AT PKS system was the pederin synthetase (Piel 2002). After the discovery of pederin many reports of natural products synthesized by *trans*-AT-PKSs have arisen, such as the bioactive, pharmacologically important compounds mupirocin, lankacidin, bryostatin A, and virginiamycin M. Currently there are more than 100 described gene clusters encoding the *trans*-AT-PKSs (Helfriech and Piel 2016)

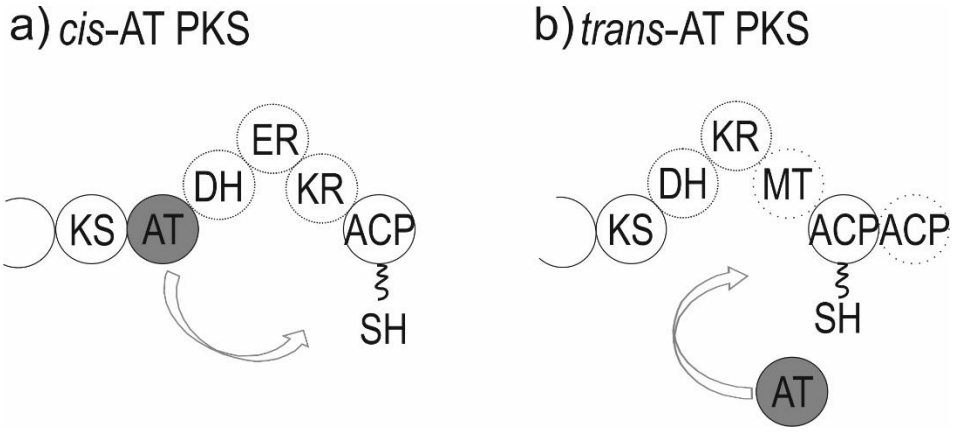

Fig 3. Domain organization of *cis*-AT type I PKS (A) and *trans*-AT type I PKS (B).

Pederin was discovered in the *Paederus* beetle and has been recognized to cause dermatitis in humans (Ueta et al. 1949, Pavan et al. 1953). The pederin family of compounds have a specific *N*-acyl linked tetrahydropyran structure responsible for a number of bioactivities including antitumor activity in nanomolar concentrations (Mosey et al. 2012). With the development of molecular methods and elucidation of the gene cluster responsible for the synthesis of pederin, it has been proven that pederin is actually synthesized by a bacterial symbiont of the *Paederus* beetle (Piel 2002). Later on, symbiotic bacteria of diverse evolutionary origin have been discovered to produce metabolites of the pederin family (Piel 2010). When it comes to cyanobacteria, the first cyanobacterial pederin producer was reported only recently from *Nostoc* in symbiotic relation with lichen *Peltigera membranacea* (Kampa et al. 2013). Since all the pederin members have been identified in symbiotic organisms, it has been hypothesized that these types of compounds are exclusive to

prokaryotic-eukaryotic relationships (Kampa 2013, Wilson et al. 2014, Piel 2004, Fisch et al. 2009, Nakabachi et al. 2013). However, reports of pederin analogues in free-living proteobacterium *Labrenzia* sp. PHM005, (Schleissner et al. 2017) and purportedly free-living cyanobacterium *Nostoc* sp. KVJ10 (Liaimer et al. 2016), have recently shaken the symbiotic theory.

1.5.3. Non-ribosomal Peptide Synthetase (NRPS)

Another type of megasynthases by which natural products are commonly synthesized are NRPSs. Oligopeptides and their derivatives synthesized by NRPS are highly variable due to the possibility of activation and incorporation of non-proteinogenic, as well as D- amino acids, α-hydroxy-, and carboxylic acids. Each NRPS is built from modules with three essential domains (Fig. 2): the adenylation (A) domain, which determines and activates the specific amino acid; the thiolation domain (T), also called peptidyl carrier protein (PCP domain), which carries the intermediate nonribosomal peptide (NRP) and presents it to the catalytic centres; and the condensation (C) domain, catalysing the elongation of the upstream intermediate by the downstream activated aminoacid (AA) through formation of a peptide bond. Similarly to the PKS, in the NRPS, the modules contain additional domains which further modify the synthesized molecule. Usual modifications are oxidation, halogenation, epimerization, and *N*-methylation. The last domain is typically a thioesterase domain (TE) which releases the final product from the NRPS and sometimes performs its cyclization.

The genes encoding NRPS enzymes are conserved and can be predicted by bioinformatics tools. The NRPS systems work on the same collinearity

rule as PKSs, except their building blocks are amino acids. Natural products are also frequently synthesized by hybrid PKS/NRPS. The final product can be further modified by the action of tailoring enzymes (i.e. methyltransferases, aminotransferases, oxygenases, Walsh et al. 2001), increasing the structural diversity of the compound produced non-ribosomally. Natural products of a peptidic nature can be also produced by enzymatic machineries involving conventional ribosomal synthesis, such as pattelamides, microviridins, cyanobactins, and lantipeptides (Schmidt et al. 2005, Arnison et al. 2013).

1.5.4. Discovery of Novel Metabolites

As soon as the gene cluster encoding the production of natural antibiotic erythromycin was elucidated (Weber et al. 1990), the era of genome mining approach started. Genome mining is a generally accepted method for the detection of gene clusters responsible for the synthesis of diverse natural products using analysis of DNA data. With the increased secondary metabolite databases and advanced bioinformatic pipelines, it has become possible not only to detect and identify the gene clusters responsible for the biosynthesis of natural products, but also to predict the course of their biosynthesis. The final structure of novel metabolites is nowadays clarified by a combination of analytical chemistry data and *in vitro* assays, accompanied by genome data mining. There are usually two ways a novel compounds is discovered: either the structure of the compound is characterized, but data about its biosynthetic pathways are missing, or the gene cluster putatively responsible for the synthesis of a compound is known, but the structure is yet to be elucidated (Challis et al. 2008). In the case when the structure is known, sequencing, feeding experiments, gene

knock-out and heterologous gene expression are performed in order to help predict the particular enzymes involved in biosynthesis. Vice versa, when the gene message encoded in the cluster of interest is known, the end product can be predicted and later, if possible, isolated and chemically characterized (Jenke-Kodama and Dittmann 2009).

1.6. Cyanotoxins

Although cyanobacterial species are important primary producers and their general nutritive value is high (Gantar and Svircev 2008), under favourable conditions they proliferate to form cyanobacterial blooms, scums, and floating mats (Schindler 2006, Lampert et al. 2007). Eutrophication and rising global temperatures cause increases in the geographical extent, population densities, and duration of cyanobacterial blooms in fresh, brackish, and marine waters (Cox et al. 2005). They occur in aquatic ecosystems worldwide and can disrupt ecosystem functioning in terms of nitrogen budgets, oxygen and light availability, community reorganization and decreased biodiversity (Robarts et al. 2005, Havens et al. 2008). The biomass levels reached during blooms are extensive and attract a lot of public attention around the globe due to their common toxicity affecting human health, water-based industries, recreation, and wildlife (Hitzfeld et al. 2000, Carmichael et al. 2001, Saqrane and Oudra 2009, Codd et al. 2005, Metcalf et al. 2012, Wilde et al. 2014).

Cyanobacterial species and their ability to produce different types of toxins, known as cyanotoxins, have been intensively studied in the last decades and their course of synthesis, detection methods and possible preventions have been investigated (Sivonen and Jones 1999, Dittman et. al 2013, Meriluoto et al. 2017). Cyanotoxins are a chemically diverse

group of compounds (e.g. alkaloids, cyclic peptides, non-proteinogenic amino acids) with toxic effect on humans, animals, plants and eukaryotic microbes (Metcalf et al. 2012). Based on their toxic effects they are divided into hepatotoxins (e.g. the cyclic peptides microcystins MCs and nodularins NODs), neurotoxins (e.g. the alkaloids anatoxin-a, anatoxin-a (S), paralytic shellfish toxins PSTs or saxitoxins), and cytotoxins (e.g. the alkaloids cylindrospermopsin CYN) (Codd et al. 1999).

The most frequently reported representatives of hepatotoxins are microcystins and nodularins, with microcystins being the most studied cyanotoxins in general (Dittmann and Wiegand 2006, van Apeldoorn et al. 2007). Microcystins are cyclic heptapeptides with several unusual amino acids, including the characteristic tail- shaped amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid (ADDA). Nodularin has a structure similar to microcystin, however missing the most variable amino acids following the dehydro-residue in position 3 in microcystins (Moffitt and Neilan 2004, Rantala et al. 2004, Gehringer et al. 2012). The hepatotoxins inhibit eukaryotic protein phosphatase and can penetrate liver cells via active transport (Runnegar et al. 1995). The natural role of microcystin has recently been related to an increase in cyanobacteria fitness under oxidative stress (Zilliges et al. 2011, Wei et al. 2016, Schuurmans et al. 2018). In aquatic ecosystems, *Microcystis*, *Planktothrix* and *Dolichospermum* are the most common genera responsible for microcystin production, while *n*odularin is usually related to the genus *Nodularia,* with a recent report from *Iningainema pulvinus* gen nov., sp. nov. (McGregor and Sendall, 2017). Both of the hepatotoxins have been reported from symbiotic and non-symbiotic *Nostoc*

(Kaasalainen et al. 2012, Gehringer et al. 2012, Liaimer et al. 2016, Jokela et al. 2017).

Anatoxin-a and its methylated analog homoanatoxin-a, are neurotoxic bicyclic alkaloids with high binding affinity to the nicotinic acetylcholine receptor resulting in an inhibition of the primary function of acetylcholine by blocking neuromuscular activity (Carmichael et al. 1997). Anatoxin was initially named VFDF (Very Fast Death Factor, Gorham et al. 1964) due to the rapid death of animals after intoxication. Its production is reported among a variety of the cyanobacterial genera, from bloom forming *Dolichospermum* to benthic *Phormidium* (Pearson et al. 2016).

Another common cyanotoxin is cylindrospermopsin, a hepatotoxic, nephrotoxic and general cytotoxic alkaloid, an inhibitor of protein synthesis. It was originally isolated from cyanobacterium *Cylindrospermopsis raciborskii*, and since then it has been reported from a diverse number of cyanobacteria from around the globe (reviewed by Pearson et al. 2016). To date, about 40 cyanobacterial genera have been described as potential cyanotoxin producers (Salmaso et al. 2017).

Cyanotoxins can have a real toxic effect on humans, as exemplified by one of the most severe outbreaks of cyanobacterial poisonings, which was reported when more than 50 patients in Brazil died after exposure to hepatotoxins through renal dialysis (Azevedo et al. 2002). Severe acute effects on human health are fortunately not that frequent, however, animal poisoning reports reach higher numbers (i.e. Sivonen and Jones 1999, Metcalf et al. 2012, Meriluoto et al. 2017). These events have commonly been linked to the ingestion of toxins produced by planktic bloom-forming cyanobacteria (Sivonen and Jones 1999).

Besides the planktic environment, toxicity and negative effects on other biota have also been reported from benthic and soil heterocytous cyanobacteria (Izaguirre et al. 2007, Cox et al. 2005, Wood et al. 2012, Quiblier et al. 2013). Benthic cyanobacteria often play the important role of first colonizers in human-impacted areas or newly-emerged (micro) habitats, they can seriously affect revitalization and hamper colonization of these localities by other organisms (Quiblier et al. 2013). They also represent a potential human health risk, as they sometimes overgrow drinking water treatment plants and reservoirs (Hurtado et al. 2008, Izaguirre et al. 2007). In contrast to the most frequent type of planktic blooms, benthic cyanobacteria can a colonize range of environments including oligo- and mesotrophic, seemingly „clean“ waters (Mez et al. 1988, Wood et al. 2012, Whitton et al. 2012). They can detach from the surface, become free floating, and often reach the shore thus putting animals and humans at risk of direct contact. It is assumed that proliferation of benthic cyanobacteria will increase due to climate change and increased nutrients impact (Quiblier et al. 2013).

No systematic toxicological studies have been carried out on freshwater and soil non-planktic filamentous cyanobacteria, although they are repeatedly recognized as toxin producers (e.g. Genuário et al. 2010, Kurmayer et al. 2011). Most of the reports on non gas-vacoulate cyanobacteria and their toxic potential were connected with animal poisonings (Edwards et al. 1992, Carmichael et al. 1997, Mez et al. 1997, Seifert et al. 2007). The studies were rather opportunistic, usually started only after individual cases of poisoning, which creates a large gap in knowledge about the actual extent and frequency of toxin production by non-planktic cyanobacterial representatives.

Early methods for the detection of toxins were based on mostly animal assays using intraperitoneal or intravenous injections on mice (Carmichael 1975, Devlin 1977, Sivonen 1989). Nowadays sensitive physiochemical methods based on chromatography and mass spectrometry have been developed (Kurmayer et al. 2017). The most common cyanotoxins are synthesized by hybrid PKS/NRPS machineries, with characterized gene clusters responsible for their biosynthesis. Since the elucidation of the gene clusters responsible for cyanotoxin synthesis (Tillett et al. 2000, Moffitt and Neilan 2004, Méjean et al. 2009, Shalev-Alon et al. 2002, Mihali et al. 2008), molecular based detecting approaches have become available such as targeted PCR, qPCR and DNA-chip (Kurmayer et al. 2017).

1.7. Life Strategies and Cyanotoxin Potential

Although unbranched heterocytous cyanobacteria, especially the planktic "*Anabaena sensu lato"* (*Dolichospermum*, *Chrysosporum*, *Sphaerospermopsis*) and *Nodularia*, have been reported among the main cyanotoxins producers, their non-planktic counterparts have been understudied. Nevertheless, several case studies have demonstrated that non gas-vacoulate cyanobacteria are capable of producing most of the cyanotoxins found in their planktic relatives, with microcystins and anatoxin being the most common cyanotoxins found in benthic proliferations (Sivonen and Jones 1999, Quiblier et al. 2013).

Non-planktic heterocytous cyanobacteria usually grow attached to surfaces such as rocks or sediments, and they are even able to colonize infertile substrates such as volcanic ash and desert sand (Jaag 1945, Dor and Danin 1996). They often dominate many types of benthic, periphytic and metaphytic mats worldwide (Komárek 2013). Therefore,

understanding their ecological demands is important. It has been demonstrated that they can face extreme conditions, high light irradiation and high temperature, especially in shallow lakes. It is significant to clarify their growth demands, since they often detach from the substrate, becoming free-floating, and reach the shores where they form thick mats along the waterside, influencing water quality. Unlike the planktic representatives which are important components of nuisance blooms, non-planktic nostocacean species have been rather overlooked for broad multidisciplinary research. It has been known that even in a single cyanobacterial population it is possible to find different genotypes of cyanotoxin producing or non-producing strains (Janse et al. 2004, Kurmayer et al. 2004, Sabart et al. 2010). The cyanotoxin composition can also differ between closely related species, belonging to the same genus, but differing in their ecological niche. A good example is the genus *Nostoc*, which contains both symbiotic and free-living isolates (Papaefthimiou et al. 2008). Symbiotic representatives are often nodularin and microcystin producers (Oksanen et al. 2004, Kaasalainen et al. 2009, 2012, Gehringer et al. 2010, Liaimer et al. 2016). However, the pattern of cyanotoxic free-living *Nostoc* isolates is relatively unknown, thus a broad screening study performed on a wide range of strains from different geographical regions could provide valuable clues about the distribution pattern of cyanotoxin potential between symbiotic/free-living representatives.

1.8. Bioactive Cyanobacterial Lipopetides

Lipopeptides are amphiphilic molecules consisting of short linear or cyclic peptides, linked to a fatty acid via ester or amide bonds (or both). The peptidic part of the compound varies in type and number of aminoacids,

while the fatty acid side chains are of different lengths and function which further can be methylated, hydroxylated, halogenated or unsaturated (Mareš et al. 2014, Kang et al. 2012, Hrouzek et al. 2012, Urajová et al. 2016).

More than 70 years ago the first lipopeptide antibiotics applied in clinical use, polymyxins, were isolated form *Bacillus* (Benedict and Langlykke 1947). Since then, a wide range of structural variants of lipopetides have been described, highlighting their rich chemical diversity in nature (Kakinuma et al. 1969, Vestola et al. 2014, Shishido et al. 2015). Most of the known lipopeptides have been isolated form *Bacillus subtillis*, including the first lipopeptide biosynthesis gene cluster of mycosubtilin. Some of these lipopeptides have great pharmaceutical value (Cochrane and Vederas 2016, Velkov et al. 2017, Taylor and Palmer 2016).

Lipopetides from cyanobacteria exhibit a wide range of activities: cytotoxicity (laxaphycins (Frankmolle et al. 1992), hormothamnin A (Gerwick et al. 1992), minutissamides (Kang et al. 2012), pahayokolides (An et al. 2007)), haemolytic activity, antifungal and/or antibacterial activity (Hrouzek et al. 2012, Tomek et al. 2015, Maru et al. 2010, Jokela et al. 2012). Some of the modes of action of cyanobacterial lipopetides have been described. In the case of puwainaphycin, a general cytotoxin, the permeabilization of the membrane causes the influx of calcium ions into the cell which results in cell necrosis (Hrouzek et al. 2012). Another example is anabaenolysin interacting with membranes in a cholesterol-dependent manner with the help of natural cyclodextrins (Shishido et al. 2015). These features render cyanobacterial lipopeptides on one hand promising molecules for biotechnological use, and on the other hand potential environmental toxins.

One of the families of bioactive lipopetides described in cyanobacteria are puwainaphycins/minutissamides (PUW/MIN). These are cyclic β-amino lipopeptides with a ten-membered peptide ring (Gregson et al. 1992, Hrouzek et al. 2012, Kang et al. 2011, 2012). PUWs are synthesized by a hybrid PKS/NRPS accompanied by tailoring enzymes (Mareš et al. 2014). A characteristic feature of PUW synthetase is the fatty acyl-AMP ligase (FAAL) starter unit (Duitman et al. 1999, Mareš et al. 2014). This enzyme was reported to specifically bind and adenylate a FA and pass the activated acyl-adenylate to a downstream phosphopantetheine arm of the PKS acyl carrier protein (ACP) for further processing. All the reported PUW/MIN variants have been isolated from soil strains of unbranched filamentous heterocytous cyanobacteria *Anabaena* and *Cylindrospermum* (Gregson et al. 1992, Kang et al. 2011, 2012, Hrouzek et al. 2012), adding to the importance of these representatives of soil cyanobacteria, often overlooked in natural products surveys. A possible occurrence of puwainaphycin in planktic nostocacean strains of *Sphaerospermopsis* was mentioned by Zapomělová (2009), which however requires further confirmation. Cyanobacterial lipopeptides and their gene clusters in general seem to be markedly more frequent in non-planktic species (Galica et al. 2017), nevertheless systematic surveys dedicated to individual compounds are missing.

1.9. Summary Rationale

Cyanobacteria represent an important group of organisms from many points of view. They exhibit complex morphology, inhabit diverse environments (including extreme ones), have high ecological impact due to excessive proliferation, and are a rich source of diverse secondary

metabolites, either toxic or pharmaceutically important. While free-living planktic representatives have attracted most of the attention and many studies have dealt with these particular groups, non-planktic and symbiotic cyanobacteria have evaded scrutiny. They are indeed represented by diverse taxa, including the prominent group of unbranched Nostocales, but their taxonomy remains often cryptic, and their toxic potential is rather unknown. While genomic data on benthic, soil, and symbiotic representatives is still underrepresented, genome mining of available data has revealed that they have a huge potential for unreported secondary metabolites. Their secondary metabolite pattern seems to differ from their planktic counterparts, mostly in the production of rather uncommon metabolites, representing a potential treasure house of novel compounds with possible pharmaceutical applications. To get a congruent picture of non-planktic and symbiotic unbranched Nostocales, the use of interdisciplinary studies is necessary. Since many aspects characterizing this group are understudied, linking their morphological characters to their ecological demands, and finally to their secondary metabolite production pattern is essential for achieving a congruent picture of these interesting organisms.

2. RESULTS AND DISCUSSION

2.1. The most important outcomes of this thesis

The thesis comprises five studies on various aspects of the unbranched heterocytous cyanobacteria. First of the study was a polyphasic taxonomic study on *Anabaena* strains evidencing polyphyly of the genus, specific growth requirements of its representatives, and discussing the possible links to their life strategies (**Paper I**). This topic was further elaborated in the following study, where we proposed the possible connection between conserved morphological traits and life strategies among number of important unbranched heterocytous genera (**Paper II**). Low occurrence of the most frequently reported cyanotoxins are found in the broad toxicological study on non-planktic unbranched Nostocacales with the discovery of a sole microcystin producing *Nostoc* strain (**Paper III**). The first non-symbiotic planktic cyanobacterial producer of a pederin family compound has been genomically, chemically, and toxicologically characterized (**Paper IV**). Biosynthesis gene clusters of a specific group of lipopetides were elucidated in sequenced genomes of five soil cyanobacterial strains and the probable course of their biosynthesis was proposed. The lipopetides were chemically characterized and their antimicrobial activity was assessed (**Paper V**).

2.2. Paper I. Detailed investigation of non-planktic *Anabaena* spp.

The phylogenetic tree inferred from 16S rRNA gene clustered four studied *Anabaena* strains into separate, relatively little related clades, which was roughly consistent with their morphologies. Studied strains of non–planktic *Anabaena* were resolved as highly polyphyletic, with sometimes

a more distant relationship between themselves than with other genera belonging to nostocacean taxa (i.e. *Trichormus*, *Dolichospermum*, and *Aphanizomenon*). These findings demonstrate the splitting of benthic *Anabaena* spp. into a number of isolated clades (Halinen 2008), and confirm the distinction of planktic *Dolichospermum, Sphaerospermopsis, and Chrysosporum* from the benthic *Anabaena* strains (Zapomělová et al. 2009, 2011, 2012). However, occasional clustering of morphologically defined non–planktic *Anabaena* spp. with planktic *Dolichospermum* strains (Halinen et al. 2008), together with instability of aerotopes during cultivation (Komárek et al. 1993, Laamanen et al. 2001), demonstrated the need for polyphasic investigation on a larger set of populations.

Alongside phylogeny, the morphology of the studied *Anabaena* strains confirmed the considerable morphological and genotypic polymorphy within non-planktic *Anabaena*. Furthermore, the morphology and phylogeny of the studied strains were generally congruent, differing to studies where the observed morphological variability was sometimes not reflected in the genetic relationships based on 16S rRNA sequences (Lyra et al. 2001, Iteman et al. 2002, Gugger and Hoffmann 2004, Řeháková et al. 2014).

On the other hand, it was difficult to establish a correlation between the growth response of the studied strains to various light intensities, temperatures and their morphological or phylogenetic variability. Contradictorily, Miller and Castenholz (2000) showed that various strains of *Synechococcus* isolated from Oregon hot springs grouped into different phylogenetic lineages have different temperature growth optima. Apparently, ecophysiological niche differentiation in cyanobacteria can rapidly evolve in response to the selective pressure of the environment

(Coleman et al. 2006). Hence, the correlation of growth preferences with morphology or phylogenetic position can be expected only among extremely tightly related populations, which was not the case for our strains. Compared with previous studies, our results demonstrated substantial difference in growth demands of related planktic and non-planktic cyanobacteria. Non-planktic *Anabaena* representatives were able to withstand wider fluctuations in temperature and light intensity than their planktic counterparts. This could be explained by their different life strategies, where non-planktic species in contrast to the planktic counterparts occur throughout the year and their microhabitat is not buffered by mixing of large amounts of water. Thus they face more fluctuating and sometimes extreme conditions on a diurnal and annual basis. Although the study itself is based solely on four strains, it summarizes and validates the methodological approaches, and provides the starting point for further studies within this intriguing group of cyanobacteria.

2.3. Paper II. Morphological traits comparison of Nostocaceae

Collected data on the length and width of vegetative and specialized cells, akinetes and heterocytes, from natural samples in the Czech Republic and Argentina, were used to test whether the cell length and width followed a normal distribution, and to compare results among data sets (literature and nature). Cell shape and size of vegetative and specialized cells, and their relative positions in trichomes differ substantially among 59 non-planktic and 47 planktic examined taxa. While heterocyte size and shape were similar across studied taxa, size of vegetative cells and akinetes, and relative position of akinetes to the heterocyte differed significantly.

Morphological similarity of heterocytes across studied taxa could be linked to the best surface to volume ratio minimizing oxygen diffusion hence favouring nitrogen fixation (Lang and Fay et al. 1971, Flores et al. 2006, Walsby 2007). Most akinetes in the studied species were either cylindrical or oval, possibly reflecting akinete evolutionary history where cylindrical shape is present in most ancient akinetes (Tomitani et al. 2006). The different shape of akinetes could be related to formation strategies; differentiation from one, or from several vegetative cells. We found that most species have akinetes developing distant from the heterocyte, particularly in planktic species. It could be assumed that akinete position toward the heterocyte may result in different internal nitrogen stores in the akinete. Additionally, combination with external nutrient availability at germination, and akinete shape may affect population recruitment success (to form blooms or not). Furthermore, smaller vegetative cell size—compared to planktic species—might be an adaptation to tolerate the extreme conditions of temporary habitats, however the reasons for differences in diverse akinetes remain speculative. Although four recently separated genera were used in this study, relevant results should be considered only for well-represented taxa, *Anabaena* and *Dolichospermum*. For further studies and stronger conclusions, a higher number of diverse, both planktic and non-planktic, nostocalean taxa should be investigated.

2.4. Paper III. Large-scale screening of the most common cyanotoxins in non-planktic unbranched Nostocales

Despite of widely reported production of cyanotoxins in non-planktic cyanobacteria, a surprisingly low occurrence of microcystins, nodularin,

cylindrospermopsin, and (homo)anatoxin-a were observed in our study. Among 311 strains of unbranched Nostocales from various environments and geographical locations investigated for cyanotoxins production, a single microcystin producer was detected. Its toxic potential was confirmed by the presence of the mcyE gene from the microcystin synthetase operon. Furthermore, HPLC-ESI-HRMS/MS analysis on the crude extract of microcystin (MC) producer strain revealed the presence of four MC variants. These variants were previously reported from genera *Microcystis, Dolichospermum, and Planktothrix* (Kenichi Harada et al. 1991, Welker et al. 2004, Puddick et al. 2014), and for the first time are reported to be produced by a *Nostoc* representative. MC producer, Nostoc Treb K1/5, clustered inside of the core *Nostoc* lineage which contains both free-living and symbiotic isolates (Papaefthimiou et al. 2008), the latter repeatedly reported as nodularin and microcystin producers (Oksanen et al. 2004, Kaasalainen et al. 2009, 2012, Gehringer et al. 2010). In contrary, reports of free-living MC producing *Nostoc* strains have been relatively scarce and thus far restricted to lineages distant from the core *Nostoc* clade (Bajpai et al. 2009, Genuário et al. 2010, Kurmayer, 2011). Members of the unbranched Nostocales, including all tested strains (267 strains in total with 16S rRNA data available), were resolved in the most derived lineage of heterocytous cyanobacteria, together with representatives of the Tolypothrichaceae family. Among the studied strains, covering a wide range of different linages with toxic and non-toxic representatives, not a single targeted PCR product for anaC, cyrJ, or ndaF were obtained in any of the studied strains. Consistent with that, no actual production of anatoxin-a, cylindrospermopsin or nodularin was detected by HPLC-ESI-HRMS/MS analysis based on the presence of their characteristic

fragments. When considering the relatively high cyanotoxin production in planktic *Dolichospermum* (reviewed in Li et al. 2016), the low occurrence in non-planktic representatives was unexpected. While non-planktic *Anabaena* frequently demonstrated production of a variety of unusual bioactive secondary compounds (Surakka et al. 2005, Oftedal et al. 2012, Jokela et al. 2012, Urajová et al. 2016), only few reports on production of the notoriously known cyanotoxins by *Anabaena* are available (Mohamed et al. 2006, Halinen et al. 2008, Bouma-Gregson et al. 2017). Their obvious scarcity in both our, and previous studies indicates that the composition of bioactive secondary metabolites consistently differs between planktic and non-planktic *Anabaena*-like strains. Similarly, while *Nodularia* planktic blooms are often highly toxic due to the production of nodularin (e.g. Laamanen et al. 2001), benthic and soil representatives have been, up to now, rarely recognized as nodularin producers (Lyra et al. 2005, Řeháková et al. 2014). Although other taxa were limited in the current study, no cyanotoxin producers were identified among them, which was consistent with the general pattern observed in better represented taxa. Our results indicate that the commonly reported cyanotoxins in planktic heterocytous cyanobacteria are almost absent in their non-planktic counterparts. However, based on other studies, non-planktic Nostocaceae are a rich source of unknown secondary metabolites (Voráčová et al. 2017). This situation presents a potential threat that remains uncovered by routine monitoring and deserves further investigation.

2.5. Paper IV. First report of natural product, usually produced by symbiotic organisms, in free-living planktic cyanobacteria

The pederin family includes a number of bioactive compounds isolated from symbiotic organisms of diverse evolutionary origin. They were believed to be specifically associated with prokaryote-eukaryote symbioses as they have been reported exclusively from symbiotic associations (Kampa et al. 2013, Wilson et al. 2014, Piel 2004, Fisch et al. 2009, Nakabachi et al. 2013). Symbiotic theory has been questioned with the discovery of pederin analogues from free-living proteobacterium *Labrenzia* sp. PHM005, (Schleissner et al. 2017) and purportedly free-living cyanobacterium *Nostoc* sp. KVJ10 (Liaimer et al. 2016). The potential of free-living organisms to produce pederins is further corroborated with our discovery of a novel pederin analogue, cusperin, identified for the first time from a non-symbiotic planktic cyanobacterium *Cuspidothrix issatschenkoi* CHARLIE 1. *Cuspidothrix,* a bloom-forming cyanobacterium recognized as a threat for human health (Selwood et al. 2007), belongs to the lineage of typical freshwater planktic heterocytous cyanobacteria, clearly distant from all groups of the typical pederin producers.

Pederins biosynthesis is accomplished by a PKS/NRPS machinery employing an unusual trans-acyltransferase (AT) PKS mechanism (Helfrich & Piel 2016), discovered and elucidated in our strain.

Pederin is linked to beetle-induced dermatitis in humans and pederin family members possess potent antitumor activity caused by selective inhibition of the eukaryotic ribosome (Mosey et al. 2012). However, low bioactivity of cusperin demonstrates that not all natural pederin analogues

exert high cytotoxic activity and thus they do not act generally and exclusively as ribosome poisons.

Our findings contradict the recently suggested restricted occurrence of pederin family members in symbiotic bacteria, and dispute their general role directly associated with symbioses (Kampa et al. 2013). The selective advantage for the production of compound belonging to pederin family by a non-symbiotic organism remains unknown and further studies are needed to decipher its possible ecological significance; especially as cytotoxic activity is weak compared to other pederins.

2.6. Paper V. Characterization of cytotoxic lipopeptides in novel strains of non-planktic cyanobacteria

Our study highlights and explores the extensive structural versatility of cyanobacterial lipopeptides from the puwainaphycin/minutissamides (PUW/MIN) family by introducing previously unknown variants and newly sequenced biosynthetic gene clusters. The strains of PUW/MIN producers from this study were originally isolated from various soil habitats. HPLC-HRMS/MS analysis detected multiple PUW and MIN variants in each of the studied strains. Most of the variations among compounds occurred in the fatty acyl moiety, although the peptidic core differed to some degree as well. Accordingly, bioinformatic analysis identified putative PUW and MIN gene clusters in each of the five newly sequenced strains, indicating a common biosynthetic origin of PUWs and MINs in cyanobacteria.

The biosynthesis of bacterial lipopeptides is typically commenced by FA-activating enzymes (Duitman et al. 1999, Tsuge et al. 2001). In the strains under study three alternative arrangements of the putative fatty acyl-AMP

ligase (FAAL) starter units have been identified. An analogous situation was previously described for the alternative NRPS starter modules in the anabaenopeptin synthetase (Rouhiainen et al. 2010). Each of the arrangements corresponded to a different array of FA side chains detected by HPLC-HRMS/MS, which presumably reflects the range of FA substrates activated during their biosynthesis. Chemical diversity could be generated largely by the presence of two alternative fatty acyl-AMP ligase starter units, one of which exhibits an unusually broad specificity for FA substrates of variable length. The substrate length specificity of the FAAL enzymes in *Mycobacterium tuberculosis* was recently shown to be determined by the size and position of specific amino acid residues protruding into the FA-binding pocket (Goyal et al. 2012). The differences in the peptidic ring could be explained by NRPS modules variability in amino acid adenylation and tailoring domains congruent with the PUW/MIN peptide cores inferred using HPLC-HRMS/MS. Additionally, the diversity of the PUW and MIN variants can be explained by the presence of tailoring enzymes responsible for further oxidation, halogenation and acetylation.

Both PUWs and MINs possess cytotoxic activity against human cells *in vitro* (Hrouzek et al. 2012, Kang et al. 2011, 2012). In the current study, PUW/MIN variants (PUW F and MINs A, C, and D) were tested for antibacterial and antifungal activity. Although none of them exhibited antibacterial effect, PUW F showed antagonistic activity against tested yeast strains. However, as apparent from our and previously published data, PUW/MIN products appear to be effective solely against eukaryotes. This finding is in contrast to the typical antibacterial activity frequently described for many lipopeptides produced by Gram-positive bacteria

(Velkov et al. 2017). Thus, their toxic potential for humans and other animals clearly warrants further investigation, and their possible use as antifungal agents is ripe for exploration.

4. SUMMARY

This thesis outlines diverse approaches used to fill the gaps in knowledge of the important group of unbranched Nostocales cyanobacteria, examining their unresolved taxonomy, ecophysiology and, in particular, secondary metabolites production potential. The first two tasks are addressed by the polyphasic study on four non-planktic *Anabaena* strains with clear phenotypic grouping in agreement with the pylogenetic assignments revealing that the genus is polyphyletic and needs further in-depth studies (**Paper I**). Additionally, the strains have shown rather different growth preferences among studied strains, and when compared with their planktic counterparts (**Paper I**). Moreover, examination of the morphological traits of all described *Anabaena, Dolichospermum, Chrysosporum* and *Sphaerospermopsis* inhabiting planktic and non-planktic environments, demonstrated delimitation of the strains based on the morphological traits and their possible connection with their life style (**Paper II**). Although planktic representatives of unbranched Nostocales are well reported producers of the most common cyantoxins, in the wide toxicological study of non-planktic representatives we have found rare occurrence of the same, represented by a single novel producer of microcystin, harmful hepatotoxin commonly occurred among cyanobacteria (**Paper III**). Study on the anatoxin-producing strain of *Cuspidothrix issatschenkoi* led to the prediction of the course of biosynthesis and structure elucidation of novel moderately cytotoxic pederin compound, the first of its kind produced by non-symbiotic planktic cyanobacteria **(Paper IV**). Finally, after the genome examination of five soil cyanobacterial strains we have elucidated the gene clusters responsible

for biosynthesis of a group of cytotoxic and antifungal lipopetides with further structure elucidation and discussion on the genetic background driving their remarkable structural diversity (**Paper V**).

4. REFERENCES

An TY, Kumar TKS, Wang ML, Liu L, Lay JO, Liyanage R, Berry J, Gantar M, Marks V, Gawley RE, Rein KS (2007) Structures of pahayokolides A and B,cyclic peptides from a *Lyngbya* sp, Journal of Natural Products 70: 730-735.

Anagnostidis K & Komárek J (1985) Modern approach to the classification system of the cyanophytes 1: Introduction. Algological Studies 38/39: 291–302.

Anagnostidis K & Komárek J (1988) Modern approach to the classification system of the cyanophytes 3: Oscillatoriales. Algological Studies 50/53: 327–472.

Arnison PG, Bibb MJ, Bierbaum G, et al. (2013) Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature. Natural Product Reports 30: 108-160.

Azevedo S, Carmichael WW, Jochimsen EM, Rinehart KL, Lau S, Shaw GR & Eaglesham GK (2002) Human intoxication by microcystins during renal dialysis treatment in Caruaru-Brazil. Toxicology 181: 441-446.

Bagchi SN, Dubey N & Singh P (2017) Phylogenetically distant clade of *Nostoc*-like taxa with the description of *Aliinostoc* gen. nov and *Aliinostoc morphoplasticum* sp. nov. International Journal of Systematic and Evolutionary Microbiology 67: 3329-3338.

Bajpai R, Sharma NK, Lawton LA, Edwards C & Rai AK (2009) Microcystin producing cyanobacterium *Nostoc* sp. BHU001 from a pond in India. Toxicon 53: 587-590.

Bar Zeev E, Yogev T, Man-Aharonovich D, Kress N, Herut B, Beja O & Berman-Frank I (2008) Seasonal dynamics of the endosymbiotic, nitrogen-fixing cyanobacterium *Richelia intracellularis* in the eastern Mediterranean Sea. ISME Journal 2: 911-923.

Benedict RG & Langlykke AF (1947) Antibiotic activity of *Bacillus polymyxa*. Journal of Bacteriology 54: 24-25.

Bergman B, Sandh G, Lin S, Larsson J & Carpenter EJ (2013) *Trichodesmium* - a widespread marine cyanobacterium with unusual nitrogen fixation properties. FEMS Microbiology Reviews 37: 286-302.

Blin K, Wolf T, Chevrette MG, et al. (2017) antiSMASH 4.0-improvements in chemistry prediction and gene cluster boundary identification. Nucleic Acids Research 45: W36-W41.

Bornet E & Flahault C (1886-1888). Revision des Nostocacees heterocystees contenues dans les principaux herbiers de France (quatrieme et dernier fragment). Annales des Sciences Naturelles; Botanique Ser. 7: 3:323–81, 4:43–73, 5:51–129, 7:77–262.

Bothe H, Schmitz O, Yates MG & Newton WE (2010) Nitrogen fixation and hydrogen metabolism in cyanobacteria. Microbiology and Molecular Biology Reviews 74: 529-551.

Bouma-Gregson K, Power ME & Bormans M (2017) Rise and fall of toxic benthic freshwater cyanobacteria (*Anabaena* spp.) in the Eel river: Buoyancy and dispersal. Harmful Algae 66: 79-87.

Caffrey P (2003) Conserved amino acid residues correlating with ketoreductase stereospecificity in modular polyketide synthases. Chembiochem 4: 654-657.

Calteau A, Fewer DP, Latifi A, Coursin T, Laurent T, Jokela J, Kerfeld CA, Sivonen K, Piel J & Gugger M (2014) Phylum-wide comparative genomics unravel the diversity of secondary metabolism in Cyanobacteria. BMC Genomics 15: 14.

Carmichael WW (1997) The cyanotoxins. Advances in Botanical Research, 27: 211-256.

Carmichael WW, Azevedo S, An JS, Molica RJR, Jochimsen EM, Lau S, Rinehart KL, Shaw GR & Eaglesham GK (2001) Human fatalities from cyanobacteria: Chemical

and biological evidence for cyanotoxins. Environmental Health Perspectives 109: 663-668.
Carmichael WW, Biggs DF & Gorham PR (1975) Toxicology and pharmacological action of *Anabaena flos-aquae* toxin. Science 187: 542-544.
Castenholz R (2001) General characteristics of cyanobacteria. *In*: Garrity, G. M. [Ed.] Bergey's Manual of Systematic Bacteriology. Springer, New York, pp. 474–78.
Castenholz RW & Norris TB (2005) Revisionary concepts of species in the cyanobacteria and their applications. Algological Studies 117: 53-69
Challis GL (2008) Mining microbial genomes for new natural products and biosynthetic pathways. Microbiology SGM 154: 1555-1569.
Challis GL, Ravel J & Townsend CA (2000) Predictive, structure-based model of amino acid recognition by nonribosomal peptide synthetase adenylation domains. Chemistry & Biology 7: 211-224.
Chang ZX, Sitachitta N, Rossi JV, Roberts MA, Flatt PM, Jia JY, Sherman DH & Gerwick WH (2004) Biosynthetic pathway and gene cluster analysis of curacin A, an antitubulin natural product from the tropical marine cyanobacterium *Lyngbya majuscula*. Journal of Natural Products 67: 1356-1367.
Chlipala GE, Mo SY & Orjala J (2011) Chemodiversity in freshwater and terrestrial cyanobacteria - a source for drug discovery. Current Drug Targets 12: 1654-1673.
Cochrane SA & Vederas JC (2016) Lipopeptides from *Bacillus* and *Paenibacillus* spp.: A gold mine of antibiotic candidates. Medicinal Research Reviews 36: 4-31.
Codd GA, Bell SG, Kaya K, Ward CJ, Beattie KA & Metcalf JS (1999) Cyanobacterial toxins, exposure routes and human health. European Journal of Phycology 34: 405-415.
Codd GA, Morrison LF & Metcalf JS (2005) Cyanobacterial toxins: risk management for health protection. Toxicology and Applied Pharmacology 203: 264-272.
Coleman M, Sullivan MB, Martiny AC, Steglich C, Barry K, DeLong EF & Chisholm SW (2006) Genomic Islands and the Ecology and Evolution of Prochlorococcus. Science 311: 1768-1770.
Colwell RR (1970). Polyphasic taxonomy and the genus *Vibrio*: numerical taxonomy of *Vibrio cholerae*, *Vibrio parahaemolyticus*, and related *Vibrio* species. Journal of Bacteriology 104: 410-433.
Cox PA, Banack SA, Murch SJ, Rasmussen U, Tien G, Bidigare RR, Metcalf JS, Morrison LF, Codd GA & Bergman B (2005) Diverse taxa of cyanobacteria produce beta-N-methylamino-L-alanine, a neurotoxic amino acid. Proceedings of the National Academy of Sciences of the United States of America 102: 9734-9734.
Des Marais DJ (2000) Evolution - When did photosynthesis emerge on Earth? Science 289: 1703-1705.
Desikachary TV (1959) Cyanophyta. Indian Council Agri- cultural Research. Academic Press, New York
Devlin JP, Edwards OE, Gorham PR, Hunter NR, Pike RK & Stavric B (1977) Anatoxin-a, a toxic alkaloid from *Anabaena-flos-aquae* NRC-44H. Canadian Journal of Chemistry-Revue Canadienne De Chimie 55: 1367-1371.
Dittmann E & Wiegand C (2006) Cyanobacterial toxins - occurrence, biosynthesis and impact on human affairs. Molecular Nutrition & Food Research 50: 7-17.
Dittmann E, Fewer DP & Neilan BA (2013) Cyanobacterial toxins: biosynthetic routes and evolutionary roots. Fems Microbiology Reviews 37: 23-43.
Dodds WK, Gudder DA & Mollenhauer D (1995) the ecology of *Nostoc*. Journal of Phycology 31: 2-18.

Dor I, & Danin A (1996) Cyanobacterial desert crusts in the Dead Sea Valley, Israel. Arch. Hydrobiol. Suppl. 117, Algological Studies 83: 197-206

Duitman EH, Hamoen LW, Rembold M, et al. (1999) The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: A multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. Proceedings of the National Academy of Sciences of the United States of America 96: 13294-13299.

Dvorak P, Poulickova A, Hasler P, Belli M, Casamatta DA & Papini A (2015) Species concepts and speciation factors in cyanobacteria, with connection to the problems of diversity and classification. Biodiversity and Conservation 24: 739-757.

Edwards C, Beattie KA, Scrimgeour CM & Codd GA (1992) Identification of anatoxin-a in benthic cyanobacteria (blue-green-algae) and in associated dog poisonings at Loch Insh, Scotland. Toxicon 30: 1165-1175.

Elenkin AA (1936-1949) Monografia algarum cyanophycearum aquidulcium at terrestrium in finibus URSS inventarum [Blue-green algae of the USSR]. Izdatel'stvo AkademiiN auk SSSR, Leningrad.

Firn RD & Jones CG (2000) The evolution of secondary metabolism - a unifying model. Molecular Microbiology 37: 989-994.

Fisch KM, Gurgui C, Heycke N, et al. (2009) Polyketide assembly lines of uncultivated sponge symbionts from structure-based gene targeting. Nature Chemical Biology 5: 494-501.

Flores E, Herrero A, Wolk CP & Maldener I (2006) Is the periplasm continuous in filamentous multicellular Cyanobacteria? Trends in Microbiology 14: 439-443.

Fogg GE, Stewart WDP, Fay P & Walsby AE (1973) The Blue- Green Algae Academic Press, London 459 pp.

Frankmolle WP, Knubel G, Moore RE & Patterson GML (1992) Antifungal cyclic-peptides from the terrestrial blue-green-alga *Anabaena laxa* 2. structures of laxaphycin-a laxaphycin-b, laxaphycin-d and laxaphycin-e. Journal of Antibiotics 45: 1458-1466.

Fritsch FE (1959) The structure and reproduction of the algae, Vol. 1. University Press, Cambridge

Fujii K, Harada KI, Suzuki M, Kondo F, Ikai Y, Oka H, *et al*. (1996) Occurrence of novel cyclic peptides together with microcystins from toxic cyanobacteria, *Anabaena* species. In Harmful and toxic algal blooms. Yasumoto T, Oshima Y, & Fukuyo Y (eds). Paris: Intergovermental Oceanographic Commission of UNESCO, pp. 559–562.

Galica T, Hrouzek P & Mares J (2017) Genome mining reveals high incidence of putative lipopeptide biosynthesis NRPS/PKS clusters containing fatty acyl-AMP ligase genes in biofilm-forming cyanobacteria. Journal of Phycology 53: 985-998

Gantar M & Svircev Z (2008) Microalgae and cyanobacteria: Food for thought. Journal of Phycology 44: 260-268.

Garcia-Pichel F, Belnap J, Neuer S, Schanz F. (2003). Estimates of global cyanobacterial biomass and its distribution. Algological Studies 109: 213–227

Gärtner G, Krienitz L & Schagerl M (eds.) *Süsswasserflora von Mitteleuropa*. Elsevier/Spektrum, Heidelberg, 759pp.

Gehringer MM, Adler L, Roberts AA, Moffitt MC, Mihali TK, Mills TJT, Fieker C & Neilan BA (2012) Nodularin, a cyanobacterial toxin, is synthesized in planta by symbiotic *Nostoc* sp. ISME Journal 6: 1834-1847.

Gehringer MM, Pengelly JJL, Cuddy WS, Fieker C, Forster PI & Neilan BA (2010) Host selection of symbiotic cyanobacteria in 31 species of the Australian cycad genus: *Macrozamia* (Zamiaceae). Molecular Plant-Microbe Interactions 23: 811-822.

Geitler, L. 1932. Cyanobacteria. *In:* Rabenhorst, L. (eds.) *Kryptogamen-Flora. 14. Band.* Akademische Verlagsgesellschaft, Leipzig, 1196 pp.

Genuario DB, Silva-Stenico ME, Welker M, Beraldo Moraes LA & Fiore MF (2010) Characterization of a microcystin and detection of microcystin synthetase genes from a Brazilian isolate of *Nostoc*. Toxicon 55: 846-854.

Genuario DB, Vaz M, Hentschke GS, Sant'Anna CL & Fiore MF (2015) *Halotia* gen. nov., a phylogenetically and physiologically coherent cyanobacterial genus isolated from marine coastal environments. International Journal of Systematic and Evolutionary Microbiology 65: 663-675.

Gerwick WH, Jiang ZD, Agarwal SK & Farmer BT (1992) Total structure of hormothamnin-a, a toxic cyclic undecapeptide from the tropical marine cyanobacterium *Hormothamnion-enteromorphoides*. Tetrahedron 48: 2313-2324.

Golakoti T, Yoshida WY, Chaganty S & Moore RE (2000) Isolation and structures of nostopeptolides A1, A2 and A3 from the cyanobacterium *Nostoc* sp. GSV224. Tetrahedron 56: 9093-9102.

Golakoti T, Yoshida WY, Chaganty S & Moore RE (2001) Isolation and structure determination of nostocyclopeptides A1 and A2 from the terrestrial cyanobacterium *Nostoc* sp. ATCC53789. Journal of Natural Products 64: 54-59.

Gomont M (1892). Monographie des Oscillatoriées (Nostocacées homocystées). Annales des Sciences Naturelles; Botanique Ser**. 7**:15:263–368, 16: 91–264.

Gons HJ (1977) On the light–limited growth of *Scenedesmus protuberans* Fritsch [PhD thesis]. – 120 pp., University of Amsterdam, Amsterdam.

Gorham PR (1964) Toxic Algae. In: Jackson DF (eds) Algae and Man. Springer, Boston, MA

Goyal A, Verma P, Anandhakrishnan M, Gokhale RS & Sankaranarayanan R (2012) Molecular basis of the functional divergence of fatty acyl-amp ligase biosynthetic enzymes of *Mycobacterium tuberculosis*. Journal of Molecular Biology 416: 221-238.

Graham LE, Wilcox LW 2000. Algae. Upper Saddle River. New Jersey, Prentice Hall. 640 p

Gregson JM, Chen JL, Patterson GML & Moore RE (1992) Structures of puwainaphycins A-E. Tetrahedron 48: 3727-3734.

Guenzi E, Galli G, Grgurina I, Pace E, Ferranti P & Grandi G (1998) Coordinate transcription and physical linkage of domains in surfactin synthetase are not essential for proper assembly and activity of the multienzyme complex. Journal of Biological Chemistry 273: 14403-14410.

Gugger MF & Hoffman L (2004) Polyphyly of true branching cyanobacteria (Stigonematales). International Journal of Systematic and Evolutionary Microbiology 54: 349-357.

Gugger M, Lyra C, Henriksen P, Coute A, Humbert JF & Sivonen K (2002) Phylogenetic comparison of the cyanobacterial genera *Anabaena* and *Aphanizomenon*. International Journal of Systematic and Evolutionary Microbiology 52: 1867-1880.

Halinen K, Fewer DP, Sihvonen LM, Lyra C, Eronen E & Sivonen K (2008) Genetic diversity in strains of the genus Anabaena isolated from planktonic and benthic habitats of the Gulf of Finland (Baltic Sea). Fems Microbiology Ecology 64: 199-208.

Havens KE (2008) Cyanobacteria blooms: effects on aquatic ecosystems. In: Hudnell HK (ed) Cyanobacterial harmful algal blooms: state of the science and research needs. Springer Science & Business Media, New York, NY, p 733−747

Helfrich EJN & Piel J (2016) Biosynthesis of polyketides by trans-AT polyketide synthases. Natural Product Reports 33: 231-316.

Hentschke GS, Johansen JR, Pietrasiak N, Rigonato J, Fiore MF & Sant'Anna CL (2017) *Komarekiella atlantica* gen. et sp nov (Nostocaceae, Cyanobacteria): a new subaerial taxon from the Atlantic Rainforest and Kauai, Hawaii. Fottea 17: 178-190.

Hertweck C (2009) The biosynthetic logic of polyketide diversity. Angewandte Chemie-International Edition 48: 4688-4716.

Hitzfeld BC, Hoger SJ & Dietrich DR (2000) Cyanobacterial toxins: Removal during drinking water treatment, and human risk assessment. Environmental Health Perspectives 108: 113-122.

Hoffmann L, Kaštovský J & Komárek J (2005) System of cyanoprokaryotes (cyanobacteria) – state in 2004. Algological Studies 117: 95-115.

Holland A & Kinnear S (2013) Interpreting the possible ecological role(s) of cyanotoxins: compounds for competitive advantage and/or physiological aide? Marine Drugs 11: 2239-2258.

Hrouzek P, Kuzma M, Cerny J, Novak P, Fiser R, Simek P, Lukesova A & Kopecky J (2012) The cyanobacterial cyclic lipopeptides puwainaphycins F/G are inducing necrosis via cell membrane permeabilization and subsequent unusual actin relocalization. Chemical Research in Toxicology 25: 1203-1211.

Hrouzek P, Lukesova A, Mares J & Ventura S (2013) Description of the cyanobacterial genus *Desmonostoc* gen. nov. including *D. muscorum* comb. nov. as a distinct, phylogenetically coherent taxon related to the genus *Nostoc*. Fottea 13: 201-213.

Hrouzek P, Tomek P, Lukesova A, Urban J, Voloshko L, Pushparaj B, Ventura S, Lukavsky J, Stys D & Kopecky J (2011) Cytotoxicity and secondary metabolites production in terrestrial *Nostoc* strains, originating from different climatic/geographic regions and habitats: is their cytotoxicity environmentally dependent? Environmental Toxicology 26: 345-358.

Huisman J, Codd GA, Paerl HW, Ibelings BW, Verspagen JMH & Visser PM (2018) Cyanobacterial blooms. Nature Reviews Microbiology 16: 471-483.

Hurtado I, Aboal M, Zafra E & Campillo D (2008) Significance of microcystin production by benthic communities in water treatment systems of arid zones. Water Research 42: 1245-1253.

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

Izaguirre G, Jungblut A-D & Neilan BA (2007) Benthic cyanobacteria (Oscillatoriaceae) that produce microcystin-LR, isolated from four reservoirs in southern California. Water Research 41: 492-498.

Jaag O (1945) Untersuchungen über die Vegetation und Biologie der Algen des nackten Gesteins in den Alpen, im Jura und im schweizerischen Mittelland. Kryptogamenflora der Schweiz, Band IX, Heft 3. Kommissionsverlag Buchdruckerei Büchler and Co., Bern

Janse I, Kardinaal WEA, Meima M, Fastner J, Visser PM & Zwart G (2004) Toxic and nontoxic microcystis colonies in natural populations can be differentiated on the basis of rRNA gene internal transcribed spacer diversity. Applied and Environmental Microbiology 70: 3979-3987.

Jenke-Kodama H & Dittmann E (2009) Bioinformatic perspectives on NRPS/PKS megasynthases: Advances and challenges. Natural Product Reports 26: 874-883.

Johansen JR, Bohunicka M, Lukesova A, Hrckova K, Vaccarino MA & Chesarino NM (2014) Morphological and molecular characterization within 26 strains of the genus *Cylindrospermum* (Nostocaceae, cyanobacteria), with descriptions of three new species. Journal of Phycology 50: 187-202.

Johansen JR, Casamatta DA (2005) Recognizing cyanobacterial diversity through adoption of a new species paradigm. Algological Studies 117:71-93

Jokela J, Heinila LMP, Shishido TK, Wahlsten M, Fewer DP, Fiore MF, Wang H, Haapaniemi E, Permi P & Sivonen K (2017) Production of high amounts of hepatotoxin nodularin and new protease inhibitors pseudospumigins by the Brazilian benthic *Nostoc* sp. CENA543. Frontiers in Microbiology 8: 14.

Jokela J, Oftedal L, Herfindal L, Permi P, Wahlsten M, Doskeland SO & Sivonen K (2012) anabaenolysins, novel cytolytic lipopeptides from benthic *Anabaena* cyanobacteria. Plos One 7.

Jones AC, Monroe EA, Eisman EB, Gerwick L, Sherman DH & Gerwick WH (2010) The unique mechanistic transformations involved in the biosynthesis of modular natural products from marine cyanobacteria. Natural Product Reports 27: 1048-1065.

Jones CG & Firn RD (1991) On the evolution of plant secondary chemical diversity. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 333: 273-280.

Kaasalainen U, Fewer DP, Jokela J, Wahlsten M, Sivonen K & Rikkinen J (2012) Cyanobacteria produce a high variety of hepatotoxic peptides in lichen symbiosis. Proceedings of the National Academy of Sciences of the United States of America 109: 5886-5891.

Kaasalainen U, Jokela J, Fewer DP, Sivonen K & Rikkinen J (2009) Microcystin production in the tripartite cyanolichen *Peltigera leucophlebia*. Molecular Plant-Microbe Interactions 22: 695-702.

Kakinuma A, Hori M, Isono M, Tamura G & Arima K (1969) Determination of amino acid sequence in surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*. Agricultural and Biological Chemistry 33: 971-972.

Kampa A, Gagunashvili AN, Gulder TAM, Morinaka BI, Daolio C, Godejohann M, Miao VPW, Piel J & Andresson OS (2013) Metagenomic natural product discovery in lichen provides evidence for a family of biosynthetic pathways in diverse symbioses. Proceedings of the National Academy of Sciences of the United States of America 110: E3129-E3137.

Kang HS, Krunic A, Shen Q, Swanson SM & Orjala J (2011) Minutissamides A-D, antiproliferative cyclic decapeptides from the cultured cyanobacterium *Anabaena minutissima*. Journal of Natural Products 74: 1597-1605.

Kang HS, Sturdy M, Krunic A, Kim H, Shen Q, Swanson SM & Orjala J (2012) Minutissamides E-L, antiproliferative cyclic lipodecapeptides from the cultured freshwater cyanobacterium cf. *Anabaena* sp. Bioorganic & Medicinal Chemistry 20: 6134-6143.

Keatinge-Clay AT (2012) The structures of type I polyketide synthases. Natural Product Reports 29: 1050-1073.

Kenichi Harada K, Ogawa K, KimuraY, Murata H, Suzuki M, Thorn PM, Evans WR, Carmichael WW (1991) Microcystins from *Anabaena flos-aquae* NRC 525-17. Chemical Research in Toxicology 4: 535-540.

Komárek J, Hübel M, Hübel H & Šmarda J (1993): The *Nodularia* studies. 2. Taxonomy. Algological Studies 68: 1-25.
Komarek J & Mares J (2012) An update to modern taxonomy (2011) of freshwater planktic heterocytous cyanobacteria. Hydrobiologia 698: 327-351.
Komarek J & Zapomelova E (2007) Planktic morphospecies of the cyanobacterial genus *Anabaena* = subg. *Dolichospermum* - 1. part: coiled types. Fottea 7: 1-31.
Komarek J & Zapomelova E (2008) Planktic morphospecies of the cyanobacterial genus *Anabaena* = subg. *Dolichospermum* - 2. part: straight types. Fottea 8: 1-14.
Komarek J (2005) Studies on the cyanophytes (Cyanobacteria, cyanoprokaryota) of Cuba 11. Freshwater Anabaena species. Preslia 77: 211-234.
Komarek J (2010) Recent changes (2008) in cyanobacteria taxonomy based on a combination of molecular background with phenotype and ecological consequences (genus and species concept). Hydrobiologia 639: 245-259.
Komarek J, Kastovsky J, Mares J & Johansen JR (2014) Taxonomic classification of cyanoprokaryotes (cyanobacterial genera) 2014, using a polyphasic approach. Preslia 86: 295-335.
Komárek J & Anagnostidis K (1989) Modern approach to the classification system of Cyanophytes 4 - Nostocales. Algological Studies/Archiv für Hydrobiologie, Supplement Volumes 56:247–345.
Komárek J & Anagnostidis K (1999) Cyanoprokaryota. 1. Teil: Chroococcales. In: Büdel B, Gärtner G, Krienitz L & Schagerl M (eds.) Süsswasserflora von Mitteleuropa. Spektrum Akademischer Verlag GmbH, Heidelberg, Berlin, pp. 548.
Komárek J & Anagnostidis K (2005) Cyanoprokaryota. 2. Oscillatoriales. In: Büdel, B.,
Gärtner G, Krienitz L & Schagerl M (eds.) Süsswasserflora von Mitteleuropa. Elsevier/Spektrum, Heidelberg, pp. 759.
Komárek, J. 2010. Recent changes (2008) in cyanobacteria taxonomy based on a combination of molecular background with phenotype and ecological consequences (genus and species concept). Hydrobiologia **639**:245–59.
Komárek, J. 2013. Cyanoprokaryota -3. Teil/ 3rd Part: Heterocytous genera. In: Büdel B, Gärtner G, Krienitz L & Schagerl M (eds.) Süsswasserflora von Mitteleuropa Elsevier/Spektrum, Heidelberg, pp. 1130.
Kozlikova-Zapomelova E, Chatchawan T, Kastovsky J & Komarek J (2016) Phylogenetic and taxonomic position of the genus Wollea with the description of Wollea salina sp nov (Cyanobacteria, Nostocales). Fottea 16: 43-55.
Kurmayer R (2011) The toxic cyanobacterium *Nostoc* sp. strain 152 produces highest amounts of microcystin and nostophycin under stress conditions. Journal of Phycology 47: 200-207.
Kurmayer R, Christiansen G, Fastner J & Borner T (2004) Abundance of active and inactive microcystin genotypes in populations of the toxic cyanobacterium *Planktothrix* spp. Environmental Microbiology 6: 831-841.
Kurmayer R, Sivonen K, Wilmotte A, Salmaso N (eds.) 2017. Molecular tools for the detection and quantification of toxigenic cyanobacteria. Wiley 576 pp.
Laamanen MJ, Gugger MF, Lehtimaki JM, Haukka K & Sivonen K (2001) Diversity of toxic and nontoxic *Nodularia* isolates (Cyanobacteria) and filaments from the Baltic Sea. Applied and Environmental Microbiology 67: 4638-4647. Lampert, W. and U. Sommer, 2007. Limnoecology: The ecology of lakes and streams. Oxford University Press, USA., Pages: 324.
Lang N & Fay P (1971) The heterocysts of blue-green algae. II. Details of ultrastructure. Proceedings of the Royal Society of London B 178: 193-203.

Li X, Dreher TW & Li R (2016) An overview of diversity, occurrence, genetics and toxin production of bloom-forming *Dolichospermum* (*Anabaena*) species. Harmful Algae 54: 54-68.

Liaimer A, Jensen JB & Dittmann E (2016) A genetic and chemical perspective on symbiotic recruitment of cyanobacteria of the genus *Nostoc* into the host plant *Blasia pusilla* L. Frontiers in Microbiology 7: 16.

Lyra C, Laamanen M, Lehtimaki JM, Surakka A & Sivonen K (2005) Benthic cyanobacteria of the genus *Nodularia* are non-toxic, without gas vacuoles, able to glide and genetically more diverse than planktonic *Nodularia*. International Journal of Systematic and Evolutionary Microbiology 55: 555-568.

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. International Journal of Systematic and Evolutionary Microbiology 51: 513-526.

Marahiel MA, Stachelhaus T & Mootz HD (1997) Modular peptide synthetases involved in nonribosomal peptide synthesis. Chemical Reviews 97: 2651-2673.

Mares J (2010) *Anabaena fuscovaginata* (Nostocales), a new cyanobacterial species from periphyton of the freshwater alkaline marsh of Everglades, South Florida, USA. Fottea 10: 235-243.

Mares J (2018) Multilocus and SSU rRNA gene phylogenetic analyses of available cyanobacterial genomes, and their relation to the current taxonomic system. Hydrobiologia 811: 19-34.

Mares J, Hajek J, Urajova P, Kopecky J & Hrouzek P (2014) A hybrid non-ribosomal peptide/polyketide synthetase containing fatty-acyl ligase (FAAL) synthesizes the beta-amino fatty acid lipopeptides puwainaphycins in the cyanobacterium *Cylindrospermum alatosporum*. Plos One 9: 11.

Maru N, Ohno O & Uemura D (2010) Lyngbyacyclamides A and B, novel cytotoxic peptides from marine cyanobacteria *Lyngbya* sp. Tetrahedron Letters 51: 6384-6387.

Mattheus W, Gao LJ, Herdewijn P, Landuyt B, Verhaegen J, Masschelein J, Volckaert G & Lavigne R (2010) Isolation and purification of a new kalimantacin/batumin-related polyketide antibiotic and elucidation of its biosynthesis gene cluster. Chemistry & Biology 17: 149-159.

McGregor GB & Sendall BC (2017) *Iningainema pulvinus* gen nov., sp nov (Cyanobacteria, Scytonemataceae) a new nodularin producer from Edgbaston Reserve, north-eastern Australia. Harmful Algae 62: 10-19.

Mejean A & Ploux O (2013) A genomic view of secondary metabolite production in cyanobacteria. Genomics of cyanobacteria, Vol. 65 (Chauvat F & CassierChauvat C, eds.), pp. 189-234. Academic Press Ltd-Elsevier Science Ltd, London.

Mejean A, Mann S, Maldiney T, Vassiliadis G, Lequin O & Ploux O (2009) Evidence that biosynthesis of the neurotoxic alkaloids anatoxin-a and homoanatoxin-a in the cyanobacterium *Oscillatoria* PCC 6506 occurs on a modular polyketide synthase initiated by L-Proline. Journal of the American Chemical Society 131: 7512-75123.

Meriluoto J, Spoof L, Codd G (2017) Handbook of cyanobacterial monitoring and cyanotoxin analysis. Chichester: John Wiley & Sons, 548 pp.

Metcalf JS, Richer R, Cox PA & Codd GA (2012) Cyanotoxins in desert environments may present a risk to human health. Science of the Total Environment 421: 118-123.

Mez K, Beattie KA, Codd GA, Hanselmann K, Hauser B, Naegeli H & Preisig HR (1997) Identification of a microcystin in benthic cyanobacteria linked to cattle deaths on alpine pastures in Switzerland. European Journal of Phycology 32: 111-117.

Mihali TK, Kellmann R, Muenchhoff J, Barrow KD & Neilan BA (2008) Characterization of the gene cluster responsible for cylindrospermopsin biosynthesis. Applied and Environmental Microbiology 74: 716-722.

Miller SR, Castenholz RW (2000) Evolution of thermotolerance in hot spring cyanobacteria of the genus *Synechococcu*s. Applied Environmental Microbiology 66:4222-4229

Moffitt MC & Neilan BA (2004) Characterization of the nodularin synthetase gene cluster and proposed theory of the evolution of cyanobacterial hepatotoxins. Applied and Environmental Microbiology 70: 6353-6362.

Mohamed ZA, El-Sharouny HM & Ali WSM (2006) Microcystin production in benthic mats of cyanobacteria in the Nile River and irrigation canals, Egypt. Toxicon 47: 584-590.

Momper LM, Reese BK, Carvalho G, Lee P & Webb EA (2015) A novel cohabitation between two diazotrophic cyanobacteria in the oligotrophic ocean. ISME Journal 9: 882-893.

Moore D, McGregor GB & Shaw G (2004) Morphological changes during akinete germination in *Cylindrospermopsis raciborskii* (Nostocales, Cyanobacteria). Journal of Phycology 40: 1098-1105.

Mosey RA & Floreancig PE (2012) Isolation, biological activity, synthesis, and medicinal chemistry of the pederin/mycalamide family of natural products. Natural Product Reports 29: 980-995.

Musiol EM & Weber T (2012) Discrete acyltransferases involved in polyketide biosynthesis. Medchemcomm 3: 871-886.

Nakabachi A, Ueoka R, Oshima K, et al. (2013) Defensive bacteriome symbiont with a drastically reduced genome. Current Biology 23: 1478-1484.

Nguyen T, Ishida K, Jenke-Kodama H, Dittmann E, Gurgui C, Hochmuth T, Taudien S, Platzer M, Hertweck C & Piel J (2008) Exploiting the mosaic structure of trans-acyltransferase polyketide synthases for natural product discovery and pathway dissection. Nature Biotechnology 26: 225-233.

Nutman AP, Bennett VC, Friend CRL, Van Kranendonk MJ & Chivas AR (2016) Rapid emergence of life shown by discovery of 3,700-million-year-old microbial structures. Nature 537: 535-548.

Oftedal L, Myhren L, Jokela J, Gausdal G, Sivonen K, Doskeland SO & Herfindal L (2012) The lipopeptide toxins anabaenolysin A and B target biological membranes in a cholesterol-dependent manner. Biochimica Et Biophysica Acta-Biomembranes 1818: 3000-3009.

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. Applied and Environmental Microbiology 70: 5756-5763.

Osorio-Santos K, Pietrasiak N, Bohunická M, Miscoe LH, Kováčik L, Martin MP & Johansen JR (2014) Seven new species of *Oculatella* (Pseudanabaenales, Cyanobacteria): taxonomically recognizing cryptic diversification. European Journal of Phycology 49: 450-70.

Papaefthimiou D, Hrouzek P, Mugnai MA, Lukesova A, Turicchia S, Rasmussen U & Ventura S (2008) Differential patterns of evolution and distribution of the symbiotic behaviour in nostocacean cyanobacteria. International Journal of Systematic and Evolutionary Microbiology 58: 553-564.

Pavan M & Bo G (1953) Pederin, toxic principles obtained in the crystalline state from the beetle *Paederus fuscipes* Curt., Phys. Comp. Oecol. 3: 307-312.

Pearson LA, Dittmann E, Mazmouz R, Ongley SE, D'Agostino PM & Neilan BA (2016) The genetics, biosynthesis and regulation of toxic specialized metabolites of cyanobacteria. Harmful Algae 54: 98-111.

Piel J (2002) A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of *Paederus* beetles. Proceedings of the National Academy of Sciences of the United States of America 99: 14002-14007.

Piel J (2010) Biosynthesis of polyketides by trans-AT polyketide synthases. Natural Product Reports 27: 996-1047.

Piel J, Hui DQ, Wen GP, Butzke D, Platzer M, Fusetani N & Matsunaga S (2004) Antitumor polyketide biosynthesis by an uncultivated bacterial symbiont of the marine sponge *Theonella swinhoei*. Proceedings of the National Academy of Sciences of the United States of America 101: 16222-16227.

Pitois F, Fastner J, Pagotto C & Dechesne M (2018) Multi-toxin occurrences in ten French water resource reservoirs. Toxins 10: 16.

Planavsky NJ, Asael D, Hofmann A, et al. (2014) Evidence for oxygenic photosynthesis half a billiion years before the Great Oxidation Event. Nature Geoscience 7: 283-286.

Postgate JR (1982) The fundamentals of nitrogen fixation. New York, NY: Cambridge University Press.

Prosperi CH (1994) The role of respiration as a protection for nitrogenase activity in the cyanobacterium *Anabaena flos-aquae*. Phyton-International Journal of Experimental Botany 55: 159-162.

Puddick J, Prinsep MR, Wood SA, Kaufononga AFS, Cary SC, Hamilton DP (2014) High levels of structural diversity observed in microcystins from *Microcystis* CAWBG11 and characterization of six new microcystin congeners. Marine Drugs 12: 5372-5395.

Quiblier C, Wood S, Echenique-Subiabre I, Heath M, Villeneuve A & Humbert JF (2013) A review of current knowledge on toxic benthic freshwater cyanobacteria - Ecology, toxin production and risk management. Water Research 47: 5464-5479.

Rai AN, Soderback E & Bergman B (2000) Cyanobacterium-plant symbioses. New Phytologist 147: 449-481.

Rajaniemi P, Hrouzek P, Kastovska K, Willame R, Rantala A, Hoffmann L, Komarek 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.

Rajaniemi P, Komarek J, Willame R, Hrouzek P, Kastovska K, Hoffmann L & Sivonen K (2005a) Taxonomic consequences from the combined molecular and phenotype evaluation of selected *Anabaena* and *Aphanizomenon* strains. Archiv fuer Hydrobiologie Supplement 159: 371-391.

Rantala A, Fewer DP, Hisbergues M, Rouhiainen L, Vaitomaa J, Borner T & Sivonen K (2004) Phylogenetic evidence for the early evolution of microcystin synthesis. Proceedings of the National Academy of Sciences of the United States of America 101: 568-573.

Rehakova K, Johansen JR, Casamatta DA, Xueson 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.

Rehakova K, Mares J, Lukesova A, Zapomelova E, Bernardova K & Hrouzek P (2014) *Nodularia* (Cyanobacteria, Nostocaceae): a phylogenetically uniform genus with variable phenotypes. Phytotaxa 172: 235-246.

Reid R, Piagentini M, Rodriguez E, Ashley G, Viswanathan N, Carney J, Santi DV, Hutchinson CR & McDaniel R (2003) A model of structure and catalysis for ketoreductase domains in modular polyketide synthases. Biochemistry 42: 72-79.

Rezanka T & Dembitsky VM (2006) Metabolites produced by cyanobacteria belonging to several species of the family Nostocaceae. Folia Microbiologica 51: 159-182.

Rippka R, Deruelles J, Waterbury JB, Herdman M & Stanier RY (1979) Generic assignments, strain histories and properties of pure cultures of Cyanobacteria. Journal of General Microbiology 111: 1-61.

Robarts RD, Waiser MJ Arts, MT & Evans MS (2005) Seasonal and diel changes of dissolved oxygen in a hypertrophic prairie lake. Lakes & Reservoirs: Research & Management, 10: 167-177.

Rouhiainen L, Jokela J, Fewer DP, Urmann M & Sivonen K (2010) Two alternative starter modules for the non-ribosomal biosynthesis of specific anabaenopeptin variants in *Anabaena* (Cyanobacteria). Chemistry & Biology 17: 265-273.

Runnegar M, Berndt N, Kong SM, Lee EYC & Zhang LF (1995) *In-vivo* and *in-vitro* binding of microcystin to protein phosphatase-1 and phosphatase-2A. Biochemical and Biophysical Research Communications 216: 162-169.

Sabart M, Pobel D, Briand E, Combourieu B, Salencon MJ, Humbert JF & Latour D (2010) Spatiotemporal variations in microcystin concentrations and in the proportions of microcystin-producing cells in several *Microcystis aeruginosa* populations. Applied and Environmental Microbiology 76: 4750-4759.

Salmaso N, Bernard C, Humbert J, Akçaalan R, Albay M, Ballot A, Catherine A, Fastner J, Häggqvist K, Horecká M, Izydorczyk K, Köker L, Komárek J, Maloufi S, Mankiewicz-Boczek J, Metcalf JS, Quesada A, Quiblier C & Yéprémian C (2017) Basic guide to detection and monitoring of potentially toxic cyanobacteria. In handbook of cyanobacterial monitoring and cyanotoxin analysis (eds J Meriluoto, L Spoof & GA Codd) DOI: 10.1002/978111 9068761.ch6

Sant'Anna CL, Gama WA, Rigonato J, Correa G, Mesquita MCB, Marinho MM (2019) Phylogenetic connection among close genera of Aphanizomenonaceae (Cyanobacteria): *Amphiheterocytum* gen. nov., *Cylindrospermopsis* and *Sphaerospermopsis*, Algal Research 37: 205-214

Saqrane S & Oudra B (2009) CyanoHAB Occurrence and water irrigation cyanotoxin contamination: ecological impacts and potential health risks. Toxins 1: 113-122.

Schindler DW (2006) Recent advances in the understanding and management of eutrophication. Limnology and Oceanography 51: 356-363.

Schirrmeister BE, Gugger M & Donoghue PCJ (2015) Cyanobacteria and the Great Oxidation Event: evidence from genes and fossils. Palaeontology 58: 769-785.

Schleissner C, Canedo LM, Rodriguez P, Crespo C, Zuniga P, Penalver A, de la Calle F & Cuevas C (2017) Bacterial production of a pederin analogue by a free-living marine Alphaproteobacterium. Journal of Natural Products 80: 2170-2173.

Schmidt EW, Nelson JT, Rasko DA, Sudek S, Eisen JA, Haygood MG & Ravel J (2005) Patellamide A and C biosynthesis by a microcin-like pathway in *Prochloron didemni*, the cyanobacterial symbiont of *Lissoclinum patella*. Proceedings of the National Academy of Sciences of the United States of America 102: 7315-7320.

Schopf JW (2000) The fossil record: tracing the roots of the cyanobacterial lineage. In: Whitton BA, Potts M (eds) The ecology of cyanobacteria. Kluwer, Dordrecht, pp 13–35

Schuurmans JM, Brinkmann BW, Makower AK, Dittmann E, Huisman J & Matthijs HCP (2018) Microcystin interferes with defense against high oxidative stress in harmful cyanobacteria. Harmful Algae 78: 47-55.

Scotti C, Piatti M, Cuzzoni A, Perani P, Tognoni A, Grandi G, Galizzi A & Albertini AM (1993) A *Bacillus-subtilis* large ORF coding for a polypeptide highly similar to polyketide synthases. Gene 130: 65-71.

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

Selwood AI, Holland PT, Wood SA, Smith KF & McNabb PS (2007) Production of anatoxin-a and a novel biosynthetic precursor by the cyanobacterium *Aphanizomenon issatschenkoi*. Environmental Science & Technology 41: 506-510.

Shah SAA, Akhter N, Auckloo BN, Khan I, Lu YB, Wang KW, Wu B & Guo YW (2017) Structural diversity, biological properties and applications of natural products from cyanobacteria. A review. Marine Drugs 15: 354.

Shalev-Alon G, Sukenik A, Livnah O, Schwarz R & Kaplan A (2002) A novel gene encoding amidinotransferase in the cylindrospermopsin producing cyanobacterium *Aphanizomenon ovalisporum*. Fems Microbiology Letters 209: 87-91.

Shih PM, Wu D, Latifi A, et al. (2013) Improving the coverage of the cyanobacterial phylum using diversity-driven genome sequencing. Proceedings of the National Academy of Sciences of the United States of America 110: 1053-1058.

Shishido TK, Jokela J, Kolehmainen CT, et al. (2015) Antifungal activity improved by coproduction of cyclodextrins and anabaenolysins in cyanobacteria. Proceedings of the National Academy of Sciences of the United States of America 112: 13669-13674.

Sielaff H, Christiansen G & Schwecke T (2006) Natural products from cyanobacteria: Exploiting a new source for drug discovery. IDrugs 9: 119-127.

Singh S, Kate BN & Banerjee UC (2005) Bioactive compounds from cyanobacteria and microalgae: An overview. Critical Reviews in Biotechnology 25: 73-95.

Sivonen K, Kononen K, Esala AI & Niemela SI (1989) Toxicity and isolation of the cyanobacterium *Nodularia spumigena* from the southern baltic sea in 1986. Hydrobiologia 185: 3-8.

Sivonen K & Jones G (1999) Cyanobacterial toxins. In toxic cyanobacteria in water: A guide to their public health consequences, monitoring, and management. Eds: Chorus I & Bartram J pp. 41-111. E & FN Spoon, London.

Skácelová O & Zapomělová E (2010) Remarks on the occurrence and ecology of several interesting cyanobacterial morphospecies found in South Moravian wetlands. Acta Musei Moraviae, Scientiae biologicae (Brno) 95: 201-221.

Starmach K (1966) Cyanophyta – sinice, Glaucophyta – *glaukofity*. Panstowe Wydawnictwo Naukowe, Warsaw, 806.

Stenegren M, Caputo A, Berg C, Bonnet S & Foster RA (2018) Distribution and drivers of symbiotic and free-living diazotrophic cyanobacteria in the western tropical South Pacific. Biogeosciences 15: 1559-1578.

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. Environmental Toxicology 20: 285-292.

Taylor SD & Palmer M (2016) The action mechanism of daptomycin. Bioorganic & Medicinal Chemistry 24: 6253-6268.

Thompson A, Carter BJ, Turk-Kubo K, Malfatti F, Azam F & Zehr JP (2014) Genetic diversity of the unicellular nitrogen-fixing cyanobacteria UCYN-A and its prymnesiophyte host. Environmental Microbiology 16: 3238-3249.

Thuret G (1875) Essai de classification des Nostochinées. Annales des Sciences Naturelles; Botanique Ser. 6: 372-82.

Tillett D, Dittmann E, Erhard M, von Dohren H, Borner T & Neilan BA (2000) Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system. Chemistry & Biology 7: 753-764.

Tomek P, Hrouzek P, Kuzma M, et al. (2015) Cytotoxic lipopeptide muscotoxin a, isolated from soil cyanobacterium *Desmonostoc muscorum*, permeabilizes phospholipid membranes by reducing their fluidity. Chemical Research in Toxicology 28: 216-224.

Tomitani A, Knoll AH, Cavanaugh CM & Ohno T (2006) The evolutionary diversification of Cyanobacteria: molecular-phylogenetic and paleontological perspectives. Proceedings of the Natural Academy of Science 103: 5442-5447.

Tsuge K, Akiyama T & Shoda M (2001) Cloning, sequencing, and characterization of the iturin A operon. Journal of Bacteriology 183: 6265-6273.

Ueta A (1949) *Kyushu Igaku Zasshi, J. Kurume. Med. College., Kyushu* 249.

Urajova P, Hajek J, Wahlsten M, et al. (2016) A liquid chromatography-mass spectrometric method for the detection of cyclic beta-amino fatty acid lipopeptides. Journal of Chromatography A 1438: 76-83.

van Apeldoorn ME, van Egmond HP, Speijers GJA & Bakker GJI (2007) Toxins of cyanobacteria. Molecular Nutrition & Food Research 51: 7-60.

Van Liere, L. & Mur, L. R. (1979): Chapter 9. Some experiments on the competition between a green alga and a cyanobacterium [PhD Thesis]. – 97 pp., University of Amsterdam.

Van Wagoner RM, Drummond AK & Wright JLC (2007) Biogenetic diversity of cyanobacterial metabolites. Advances in Applied Microbiology, Vol 61 61: 89-217.

Vandamme P, Pot B, Gillis M, De Vos P, Kersters K & Swings J (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiology Reviews 60, 407-438.

Velkov T, Roberts KD & Li J (2017) Rediscovering the octapeptins. Natural Product Reports 34: 295-309.

Vestola J, Shishido TK, Jokela J, Fewer DP, Aitio O, Permi P, Wahlsten M, Wang H, Rouhiainen L & Sivonen K (2014) Hassallidins, antifungal glycolipopeptides, are widespread among cyanobacteria and are the end-product of a nonribosomal pathway. Proceedings of the National Academy of Sciences of the United States of America 111: E1909-E1917.

Voracova K, Paichlova J, Vickova K & Hrouzek P (2017) Screening of cyanobacterial extracts for apoptotic inducers: a combined approach of caspase-3/7 homogeneous assay and time-lapse microscopy. Journal of Applied Phycology 29: 1933-1943.

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

Walsby AE (1985) The permeability of heterocysts to the gases nitrogen and oxygen. Proceedings of the Royal Society Series B-Biological Sciences 226: 366-.

Walsh CT, Chen HW, Keating TA, Hubbard BK, Losey HC, Luo LS, Marshall CG, Miller DA & Patel HM (2001) Tailoring enzymes that modify nonribosomal peptides during

and after chain elongation on NRPS assembly lines. Current Opinion in Chemical Biology 5: 525-534.
Walsby AE (2007) Cyanobacterial heterocysts: terminal pores proposed as sites of gas exchange. Trends in Microbiolgy 15: 340-348.
Wang H, Fewer DP, Holm L, Rouhiainen L & Sivonen K (2014) Atlas of nonribosomal peptide and polyketide biosynthetic pathways reveals common occurrence of nonmodular enzymes. Proceedings of the National Academy of Sciences of the United States of America 111: 9259-9264.
Weber JM, Leung JO, Maine GT, Potenz RHB, Paulus TJ & Dewitt JP (1990) Organization of a cluster of erythromycin genes in *Saccharopolyspora erythraea*. Journal of Bacteriology 172: 2372-2383.
Weber T, Blin K, Duddela S, et al. (2015) antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Research 43: W237-W243.
Wei N, Hu LL, Song LR & Gan NQ (2016) Microcystin-bound protein patterns in different cultures of *Microcystis aeruginosa* and field samples. Toxins 8: 17.
Welker M, Christiansen G, von Döhren H (2004) Diversity of coexisting *Planktothrix* (Cyanobacteria) chemotypes deduced by mass spectral analysis of microystins and other oligopeptides. Archives of Microbiology 182: 288-298.
Welker M & von Dohren H (2006) Cyanobacterial peptides - Nature's own combinatorial biosynthesis. Fems Microbiology Reviews 30: 530-563.
Welker M, Marsalek B, Sejnohova L & von Doehren H (2006) Detection and identification of oligopeptides in *Microcystis* (cyanobacteria) colonies: Toward an understanding of metabolic diversity. Peptides 27: 2090-2103.
Whitton BA, Potts M (2000) Introduction to the cyanobacteria. In: Whitton BA, Potts M (eds) Ecology of cyanobacteria: their diversity in time and space. Kluwer Academic Publishers, Dordrecht, 1–11, 689 pp
Whitton BA, Potts M (2012) Introduction to the cyanobacteria. In: Whitton BA (eds) Ecology of cyanobacteria II: their diversity in time and space Berlin, Springer, 1-11, pp. 760 pp.
Wilde SB, Johansen JR, Wilde HD, Jiang P, Bartelme BA & Haynie RS (2014) *Aetokthonos hydrillicola* gen. et sp. nov.: Epiphytic cyanobacteria on invasive aquatic plants implicated in Avian Vacuolar Myelinopathy. Phytotaxa 181: 243-260.
Willame R, Boutte C, Grubisic S, Wilmotte A, Komarek J & Hoffmann L (2006) Morphological and molecular characterization of planktonic cyanobacteria from Belgium and Luxembourg. Journal of Phycology 42: 1312-1332.
Wilson MC, Mori T, Ruckert C, et al. (2014) An environmental bacterial taxon with a large and distinct metabolic repertoire. Nature 507: 1.
Wood SA, Smith FMJ, Heath MW, Palfroy T, Gaw S, Young RG & Ryan KG (2012) Within-mat variability in anatoxin-a and homoanatoxin-a production among benthic *Phormidium* (cyanobacteria) strains. Toxins 4: 900-912.
Yamada Y, Kuzuyama T, Komatsu M, Shin-ya K, Omura S, Cane DE & Ikeda H (2015) Terpene synthases are widely distributed in bacteria. Proceedings of the National Academy of Sciences of the United States of America 112: 857-862.
Zapomelova E, Hrouzek P, Rezanka T, Jezberova J, Rehakova K, Hisem D & Komarkova J (2011) Polyphasic characterization of *Dolichospermum* spp. and *Sphaerospermopsis* spp. (Nostocales, cyanobacteria): morphology, 16S rRNA gene sequences and fatty acid and secondary metabolite profiles. Journal of Phycology 47: 1152-1163.

Zapomelova E, Jezberova J, Hrouzek P, Hisem D, Rehakova K & Komarkova J (2009) Polyphasic characterization of three strains of *Anabaena reniformis* and *Aphanizomenon aphanizomenoides* (cyanobacteria) and their reclassification to *Sphaerospermum* gen. nov (incl. *Anabaena kisseleviana*)1. Journal of Phycology 45: 1363-1373.

Zapomelova E, Skacelova O, Pumann P, Kopp R & Janecek E (2012) Biogeographically interesting planktonic Nostocales (Cyanobacteria) in the Czech Republic and their polyphasic evaluation resulting in taxonomic revisions of *Anabaena bergii* Ostenfeld 1908 (*Chrysosporum* gen. nov.) and *A. tenericaulis* Nygaard 1949 (*Dolichospermum tenericaule* comb. nova). Hydrobiologia 698: 353-365.

Zehr JP, Bench SR, Mondragon EA, McCarren J & DeLong EF (2007) Low genomic diversity in tropical oceanic N-2-fixing cyanobacteria. Proceedings of the National Academy of Sciences of the United States of America 104: 17807-17812.

Zehr JP and Turner PJ (2001) Nitrogen fixation: Nitrogenase genes and gene expression, p. 271-286. *In* J. H. Paul (ed.), Methods in marine microbiology. Academic Press, San Diego, California

Zilliges Y, Kehr JC, Meissner S, Ishida K, Mikkat S, Hagemann M, Kaplan A, Borner T & Dittmann E (2011) The cyanobacterial hepatotoxin microcystin binds to proteins and increases the fitness of microcystis under oxidative stress conditions. Plos One 6: 11.

5. ATTACHED PUBLICATIONS

Paper I.

Kust A, Kozlíková-Zapomělová E, Mareš J, Řeháková K. 2015: A detailed morphological, phylogenetic and ecophysiological analysis of four benthic *Anabaena* (Nostocales, Cyanobacteria) strains confirms deep heterogeneity within the genus. *Fottea* 15(2): 191-202.

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A detailed morphological, phylogenetic and ecophysiological analysis of four benthic *Anabaena* (Nostocales, Cyanobacteria) strains confirms deep heterogeneity within the genus

Andreja KUST[1,2], Eliška KOZLÍKOVÁ–ZAPOMĚLOVÁ[1,2], Jan MAREŠ[1,2,3], Klára ŘEHÁKOVÁ[1,3]

[1]*Biology Centre of CAS, v.v.i., Institute of Hydrobiology, Na Sádkách 7, CZ–37005 České Budějovice, Czech Republic; Corresponding author e–mail: andreja.kust@gmail.com, tel: +420 387 775 884*
[2]*University of South Bohemia, Faculty of Science, Branišovská 1760, CZ–37005 České Budějovice, Czech Republic;*
[3]*Institute of Botany CAS, v.v.i., Centre for Phycology, Dukelská 135, CZ–37982 Třeboň, Czech Republic*

Abstract: Four benthic *Anabaena* strains isolated from different localities in the Czech Republic were examined from the morphological, phylogenetic, and physiological points of view. The results of combined analysis showed distinct morphological dissimilarity between the studied strains, which were further found to belong to different phylogenetic groups based on the 16S rRNA gene phylogeny. To assess the temperature and light (irradiance) optima of the strains, we exposed them to various combinations of these two parameters. The experiment revealed unexpectedly high temperature and light optima for some of the strains, while others showed optima that were similar to those of previously studied planktic species of related heterocytous genera. Our study is the first of its kind to be applied to benthic *Anabaena* strains. Our results indicate that the benthic *Anabaena* genus is much more complex than previously thought and provide novel insights into the biology and ecology of benthic *Anabaena* species. With the collection of more data, we expect the genus *Anabaena* will be split into several new monophyletic taxa, each covering distinct morpho– and ecotypes.

Key words: *Anabaena*, crossed gradients, ecology, growth optima, morphology, phylogeny, polyphyly

INTRODUCTION

Cyanobacteria are photosynthetic prokaryotes that are considered to be among the first organisms of the early Earth (BROCK 1973; SCHOPF 1996). They are observed worldwide, in all kinds of environments (OREN 2000; STAL 2000; CASTENHOLZ 2001). In aquatic habitats, they represent one of the major groups of organisms in both planktic and benthic communities, and are capable of inhabiting wide temperature and irradiance ranges (CASTENHOLZ 1969, 1973; WARD & CASTENHOLZ 2000). One explanation of their broad distribution is the small amount of energy they require to maintain cell function and structure (VAN LIERE & MUR 1979; GONS 1997).

Methodological improvements and new approaches applied in cyanobacterial studies have recently revealed many new taxa. Numerous traditional genera have been subdivided, usually on the basis of results of molecular analyses (KOMÁREK et al. 2014). After the introduction of new characterization methods based on a polyphasic approach (JOHANSEN & CASAMATTA 2005; KOMÁREK 2010; OSORIO–SANTOS et al. 2014), the traditional genus *Anabaena* established by Bory in 1822 (GEITLER 1932) has recently been divided (KOMÁREK & ZAPOMĚLOVÁ 2007, 2008; WACKLIN et al. 2009; ZAPOMĚLOVÁ et al. 2009, 2012). A large group of planktic taxa with aerotopes (gas vesicles) was reclassified into the new genera *Dolichospermum*, *Sphaerospermopsis*, and *Chrysosporum*, primarily based on the results of 16S rRNA sequencing (ITEMAN et al. 2002; GUGGER et al. 2002; RAJANIEMI et al. 2005a,b; HOFFMANN et al. 2005; WILLAME et al. 2006). Several morphospecies, characterized by the possession of a subsymmetrical filament structure and a special type of large akinete, and forming metaphytic mats in tropical regions, have been included in the newly described genus *Macrospermum* (KOMÁREK 2008). After these revisions, the generic name *Anabaena* was preserved to include the remaining species without gas vesicles (benthic, periphytic, soil), and the

genus is now considered to be more or less consistent. However, up until now, only a few studies have dealt with non–planktic *Anabaena*, whose classification is based solely on morphology (KOMÁREK 2005, 2008; SKÁCELOVÁ & ZAPOMĚLOVÁ 2010; MAREŠ 2010) or on phylogeny (HALINEN et al. 2008). The main weak point with this approach is the inadequate morphological characterization of the majority of non–planktic *Anabaena* strains for which 16S rRNA gene sequences are available. Thus, we have hardly any idea how big the molecular diversity of this cyanobacterial group is and how the morphological diversity of this group observed in nature is related to their evolutionary diversity. Considering this gap in current knowledge, a detailed investigation of benthic *Anabaena* spp. combining morphological and phylogenetic approaches is highly desirable.

Anabaena species are a common component of many types of benthic, periphytic and metaphytic mats worldwide (KOMÁREK 2013), including newly emerging habitats. Therefore, understanding their ecological demands is of exceptional importance. Knowledge about the growth preferences of benthic cyanobacteria in shallow waters is particularly interesting since their living conditions can change dramatically, even during a single day. Unlike planktic cyanobacteria, they face daily extreme fluctuations in environmental factors such as temperature, salinity, and grazing pressure (STAL 2000). The ability to live across a wide range of temperatures is a corollary of their autecological features, i.e., metabolic rate, cell composition, and population differences in growth and temperature optima (DE NICOLA 1996). Several studies, such as those of STULP & STAM (1985) and ZAPOMĚLOVÁ et al. (2008b), have dealt systematically with the growth demands of nostocacean cyanobacteria, but up to now there have been

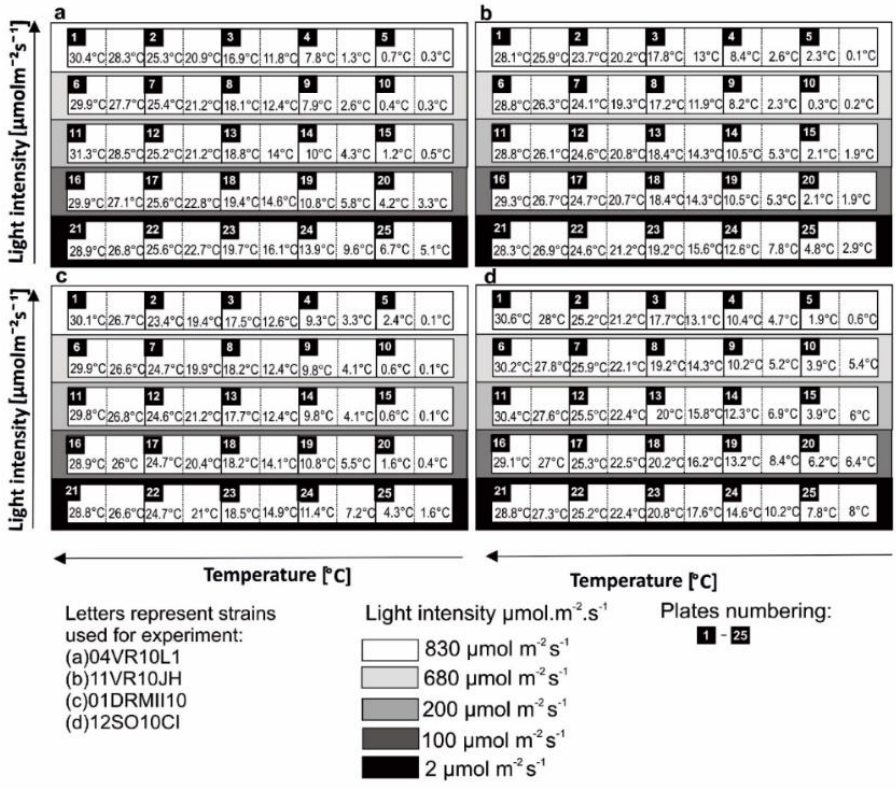

Fig. 1. Design of the crossed–gradient experiment used for the determination of the light and temperature growth optima of the studied *Anabaena* strains. Experimental setups for the four strains studied: (a) 04VR10L1; (b) 11VR10L1; (c) 01DRMII10; (d) 12SO10CI. Gradients of light intensity and temperature are indicated with arrows. The gradient of light intensity is also indicated by shading.

no studies assessing the optimal growth conditions of benthic *Anabaena*. Growth responses to environmental factors are also important because they are fundamental for water management targeting. From this point of view, a full understanding of the ecology of benthic cyanobacteria and their environmental preferences is valuable.

The aim of this study was to examine the morphological traits of four benthic *Anabaena* strains and assess their phylogenetic relationships to each other and to other nostocacean cyanobacteria based on 16S rRNA gene sequences. Another objective was to experimentally evaluate their temperature and light preferences to enable a prediction of their ecological niches and a comparison of these preferences with those of species belonging to related planktic taxa. It is hoped that, by comparing the outputs of these three approaches, correlations/discrepancies in morphological, phylogenetic, and ecophysiological similarities of the studied strains will be uncovered, thereby yielding a more complex picture of the little studied benthic *Anabaena* and providing a starting point for further research.

Materials and Methods

Cyanobacterial Strains. Samples used for this study were collected in 2010 from localities with a water conductivity of cca 600–6000 µS.cm^{-1} and a pH in the range 5.9–7.8 (Table 1), which are probably caused by the high concentration of dissolved minerals released from coal mines in the vicinity, with the exception of SO–CI, which is a natural mineral spring locality. Localities were situated in the northern part of the Czech Republic (Sokolov area). VR–JH is a small lake in a former coal mining pit near Vřesová; VR–L1 is a rainwater puddle in a mine disposal site (on a soil substrate left over after coal mining) near Vřesová; and DR–MII contains drainage water from a coal mine and a SO–CI – Císařský mineral spring in the Soos protected area near Františkovy Lázně. Single trichomes were isolated from the environmental samples using a glass capillary pipette (Zapomělová et al. 2007) and the resulting strains have been maintained in the culture collection of the Biology Centre of AS CR, Institute of Hydrobiology, in WC medium (Guillard & Lorenzen 1972) at 21°C with a light intensity of 50 µmol.m^{-2}.s^{-1} (16:8 L:D cycle). The strains were used for all analyses (examination of morphological traits, phylogenetic analysis, and growth demands) within 1 year after isolation to avoid possible changes induced by long–term cultivation, especially those that result in a loss of important features (Lehtimäki et al. 2000; Gugger et al. 2002b).

Morphological Study. The morphology of the strains was examined using an Olympus BX 51 light microscope equipped with an Olympus DP 70 digital camera. Microphotographs of at least 30 trichomes per strain were taken at a magnification of 400×. Morphometric characterization of the studied *Anabaena* strains was done based on microphotographs using the image analysis software Olympus DP Soft. Lengths and widths of all cell types were measured. Five vegetative cells per trichome were measured in 30 trichomes, 30 heterocytes, and 30 akinetes (if present) in each strain. The positions of akinetes relative to those of heterocytes were determined. Shapes of terminal cells and the length:width ratios of vegetative cells, heterocytes, and akinetes were assessed as additional characters. The identification of strains was done according to classical morphology (Komárek 2013).

Molecular and Phylogenetic Study. The biomasses of the strains were harvested in the exponential phase of growth by repeated centrifugation. Samples were washed by mixing with physiological solution (NaCl solution, concentration 1 g.l^{-1}) to remove mucilaginous substances. The centrifuged biomass samples were stored at – 20 °C until DNA extraction. DNA was extracted using an UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA). The 16S rRNA gene and the adjacent rRNA ITS region was amplified using primers 16S27F (5'–AGAGTTTGATCCTGGCTCAG–3') and 23S30R (5'–CTTCGCCTCTGTGTGCCTAGGT–3') (Taton et al. 2003). Amplification was carried out as follows: an initial denaturation step 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. Primers K6 (5'–GACGGGCCGGTGTGTACA–3'), which is

Table 1. Summary of analyzed strains, including year of isolation, isolation locality, and the parameters of the localities

Strain code	Locality	Environment	Conductivity (µS.m^{-1})	pH	GPS coordinates	Year of isolation
04VR10L1	Vřesová (VR-L1)	black crust	1230	7.1	50°15'51.97"N, 12°43'4.972"E	2010
11VR10JH	Vřesová (VR-JH)	pond	590	7.8	50°15'51.94"N, 12°43'4.973"E	2010
01DRMII10	Sokolov area (DR-MII)	drain	6050	6.7	50°16'18.079"N, 12°45'23.399"E	2010
12SO10CI	Soos Nature Reserve – Císařský spring (SO-CI)	spring	5200	5.9	50°08'51.903"N, 12°24'11.871"E	2010

the reverse complement of Primer 14 described by Wilmotte et al. (1993), K7 (5′–AAGGAGGTGATCCA GCCACA–3′) (FLECHTNER et al. 2002), and 27F (5'AGAGTTTGATCCTGGCTCAG–3') (TATON et al., 2003) were used for PCR product sequencing at the Laboratory of Genomics, Biology Centre of CAS, České Budějovice, Czech Republic. Raw data from the DNA sequencer were analyzed and assembled into final nucleotide sequences using the SeqMan 5.06 (BURLAND 1999) computer program and edited manually to remove unclear bases and the variable ITS region. The DNA sequences from four studied strains were deposited in the NCBI GenBank database under accession numbers KJ679568–571. Additional sequences for phylogenetic analyses were selected from the GenBank online database (http://www.ncbi.nlm.nih.gov). All GenBank sequences included in this study are listed in Supplementary Materials. Sequences were aligned using MAFFT v. 7 (KATOH & STANDLEY 2013) and eventual adjustments of the resulting alignments (deletion of ambiguous sites) were carried out in BioEdit v. 7.0.9.0 (HALL 1999). The alignment was analyzed by Bayesian Inference (BI), Maximum Likelihood (ML), and Neighbor–Joining (NJ) methods. BI trees were constructed using MrBayes 3.2.3 (RONQUIST et al. 2012) and the best ML tree was obtained using RaxML v. 8 (STAMATAKIS 2014); both phylogenies were computed using the CIPRES supercomputing facility (MILLER et al. 2012). NJ tree calculations were conducted in Seaview 4.5.3 (GOUY et al. 2010). The GTR+I+G evolutionary model of substitution used during the ML analysis was obtained for the best fit to the data using jModelTest–2.1.4 (GUINDON & GASCUEL 2003; DARRIBA et al. 2012,). Bayesian analyses were performed using two independent runs, each with four Markov chains that were run for 1 375 000 generations with the default likelihood model (without weighing of bases or base changes) until the average standard deviation of split frequencies was lower than 0.01. For NJ and ML analyses, 1000 bootstrap pseudoreplications with default parameters were performed to evaluate the relative support of branches. The topology of the final phylogenetic tree was derived from that of the BI tree. Trees were rooted using 16S rRNA gene sequences of outgroup non–heterocytous cyanobacteria *Gloeobacter violaceus* PCC 7421, *Trichodesmium erythraeum* IMS 101, *Synechococcus* sp. 1tu21s05, and *Cyanobium* sp. JJ10–3. Trees were edited using FigTree v. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

Crossed–Gradient Experiment. Crossed gradients (KVÍDEROVÁ & LUKAVSKÝ 2001) were used to determine the growth preferences of the four *Anabaena* strains. The experimental table enabled us to establish different combinations of light intensity and temperature. The strains were exposed in sterile culture plates (9×12 cm, 6 wells, 16 ml each) to 50 different combinations (Fig. 1). The temperature gradient was set directly on the table and particular levels of light intensities were achieved by shading. Two replicates of strains were done for each combination of light and temperature. Ranges of temperature and light were modified for each strain according to the results of a pilot experiment (data not shown). The temperature ranged from 0.0 to 31.3 °C, and the range of light intensity was 2–830 $\mu mol.m^{-2}.s^{-1}$. The duration of each experiment was about 2 weeks. The experiments were terminated when the fastest–growing culture (corresponding to the optimal experimental condition) reached the late exponential phase of growth. The late exponential phase was indicated by a slightly yellowish colour of the growing biomass. After the termination of experiments, chlorophyll *a*

Table 2. Morphological characteristics and features of the studied *Anabaena* strains. Dimensions are given as mean values (minimum–maximum). Abbreviations and symbols: L: W, length to width ratio; **A**, akinete; ●, heterocyte; ■, vegetative cells.

	Vegetative cells, n=150					Heterocytes, n=30			Akinetes, n=30			
Strain	**Length (µm)**	**Width (µm)**	**L:W ratio**	**Shape of terminal cells**	**Shape of veg. cells**	**Length (µm)**	**Width (µm)**	**L:W ratio**	**Length (µm)**	**Width (µm)**	**L:W ratio**	**Position**
04VR10L1	4.2 (2.5–7.5)	4.1 (3–5.3)	1 (0.5–1.8)	Pointed arrow like	Isodiametric, cylindrical	6.7 (4.4–9)	5.6 (4.5–5.7)	1.2 (0.7–1.7)	11 (9.6–12.9)	7.6 (5.3–8.9)	1.5 (1.1–2.4)	A●A
11VR10JH	4.7 (3.4–6.2)	6.4 (5.4–7.3)	0.7 (0.5–1)	Spherical rounded	Shorter than wide; barrel shaped	6.4 (5.7.8)	6.6 (5.1–7.8)	1 (0.9–1.1)	less than 30 occurred	less than 30 occurred	less than 30 occurred	A●A
01DRMII10	6.1 (4.1–8.7)	6 (5–7.1)	1 (0.7–1.5)	Cylindrical rounded	Isodiametric, barrel shaped	9.2 (7–12.5)	7.3 (5.6 –8.2)	1.3 (1.1–1.5)	17 (8.9–23.8)	8.8 (7.1–10.8)	2 (1–2.6)	A■■■●
12SO10CI	4.3 (2.7–6.2)	3.5 (2.7–4.6)	1.2 (0.8–1.9)	Cylindrical rounded	Isodiametric, cylindrical	6.6 (4.8–8.4)	4.3 (3.8–5.4)	1.5 (1.2–1.9)	16.8 (12.7–22.5)	6.2 (5.5–7.3)	2.7 (2–3.6)	A●A

Table 3. Summary of temperature and light preferences of the four studied strains. The temperatures and light intensities providing the highest chlorophyll *a* concentration are printed in bold font. The highest chlorophyll *a* concentration is also indicated in bold.

Strain codes	04VR10L1		11VR10JH		01DRMII10		12SO10CI	
Light intensity ($\mu mol.m^{-2}.s^{-1}$)	Temperature (°C)	Highest chl–*a* concentration ($\mu g.l^{-1}$)	Temperature (°C)	Highest chl–*a* concentration ($\mu g.l^{-1}$)	Temperature (°C)	Highest chl–*a* concentration ($\mu g.l^{-1}$)	Temperature (°C)	Highest chl–*a* concentration ($\mu g.l^{-1}$)
830	30.4	5786.97	**28.1**	**3404.35**	**23.4**	**4498.91**	25.2	1513.68
680	25.4	**7200.33**	28.8	2141.29	19.9	3647.11	27.8	1814.63
200	28.5	4540.68	**24.6**	**3400.78**	17.7	3968.41	**25.5**	**2664.65**
100	19.4	1446.92	26.7	671.16	18.2	1196.31	22.5	860.73
2	5.1	417.69	12.6	228.84	14.9	504.08	7.8	328.80

concentrations were determined spectrophotometrically after acetone extraction (Lorenzen 1967) and compared between the individual positions of the crossed gradients. The light and temperature growth preferences of the strains were obtained from a Scatter Chart using R (v.3.1.1; R Development Core Team, 2014).

Results

Morphology

Morphological characters and dimensions of the studied *Anabaena* strains are shown in Table 2. Important morphological features (vegetative cells, terminal cells, heterocytes and akinetes) are further visible in Figure 2. The vegetative cell dimensions and their proportions differed between the different strains, which enabled the recognition of two main morphological types. Strains 04VR10L1, 01DRMII10 and 12SO10CI represent a group with more or less isodiametric vegetative cells, while another morphotype with shorter than wide vegetative cells with a barrel–shape is represented by strain 11VR10JH. Moreover, akinetes were found adjacent to heterocytes in all strains except for 01DRMII10, in which they were situated distant from heterocytes, usually at a distance of 3–10 vegetative cells. Terminal cells in strain 04VR10L1 were pointed, unlike in the other strains where the terminal cells were rounded. The morphological comparisons demonstrated that the strains belong to different morphological groups. Two morphospecies were identified according to currently–used species–defining morphological criteria: strain 12SO10CI was identified as *Anabaena oscillarioides*, and strain 01DRMII10 as *Trichormus variabilis* (syn. *Anabaena variabilis*). Morphological identification of these two strains was in agreement with their phylogenetic position (see below). The remaining two strains (04VR10L1 and 11VR10JH) were impossible to identify because of the insufficient number of observed akinetes, which are crucial for species determination within the *Anabaena/Trichormus* group (Rajaniemi et al. 2005).

Phylogenetic Relationships

Partial 16S rRNA gene sequences (1361 bp) of the four studied *Anabaena* strains were compared with a representative set of sequences available from GenBank. BI, ML and NJ phylogenetic algorithms produced similar topologies; thus only the BI tree is presented here. The bootstrap supports obtained using the ML and NJ methods are given in this tree (Fig. 3). Sequences of non–planktic *Anabaena* were recovered as highly polyphyletic in our phylogeny, forming at least eight separate clades. Each of the four *Anabaena* strains analyzed in this study appeared in one of these different clusters, which was supported by relatively high bootstrap values (Fig. 3). Strains 04VR10L1 and 11VR10JH formed two isolated lineages that were remotely related

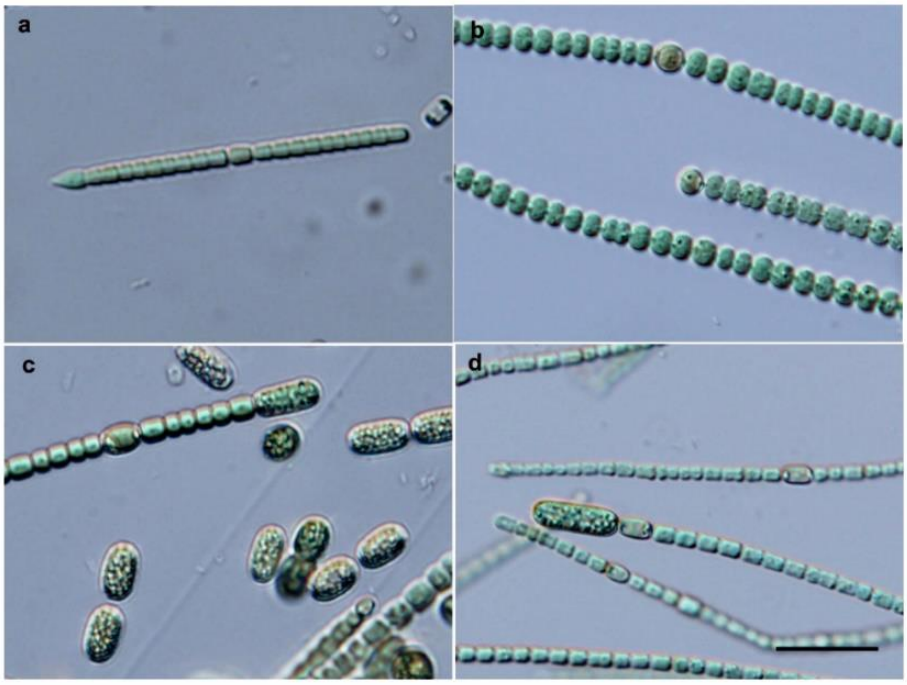

Fig. 2. Microphotographs of the four studied *Anabaena* strains. Strain codes: (a) 04VR10L1; (b) 11VR10JH; (c) 01DRMII10; (d) 12SO10CI. Scale bar 20 µm.

to two different clades of benthic *Anabaena* spp. Strain 12SO10CI grouped tightly in an *Anabaena oscillarioides/Anabaena* sp. cluster, in agreement with its morphological identification (*A. oscillarioides*). Similarly, strain 01DRMII10, identified as *Trichormus variabilis* based on its morphology, clustered together with *Anabaena* sp. and two *Trichormus variabilis* strains with high bootstrap support.

Growth Preferences

Temperature and light growth optima were taken to be those that yielded the highest chlorophyll–*a* concentration. While the temperature and light intensity optima that produced the highest chlorophyll–*a* concentration were evident for three of the strains, strain 11VR10JH gave ambiguous results. The highest chlorophyll *a* concentrations for this strain occurred at 28.1 °C and 830 $\mu mol.m^{-2}.s^{-1}$, both of which are unexpectedly high. The second highest concentration, differing in only ~ 4 $\mu g.l^{-1}$, occurred at 24.6 °C and 200 $\mu mol.m^{-2}.s^{-1}$. The temperature preferences of the other strains were roughly similar, while the light preferences of strains 04VR10L1 and 01DRMII10 were remarkably high and differed from each other (Table 3, Fig. 4). The optimal light preference of strain 12SO10CI (200 $\mu mol\ m^{-2}\ s^{-1}$) was lower than those of the other strains. The results showed that the chlorophyll–*a* concentration was low in all the strains at low irradiance (2 $\mu mol.m^{-2}.s^{-1}$) and across the entire temperature range. At temperatures below 10–15 °C, the chlorophyll–*a* concentration was low in all the strains irrespective of light intensity. In general, the chlorophyll–*a* concentrations of the strains differed. Some of the strains, for example 04VR10L1, accumulated the highest chlorophyll–*a* concentration (over 7000 $\mu g.l^{-1}$) at 630 $\mu mol.m^{-2}.s^{-1}$ and 25.4 °C, while strain 12SO10CI accumulated the lowest concentration (6.78 $\mu g.l^{-1}$) at 830 $\mu mol.m^{-2}.s^{-1}$ and 4.7 °C. Average values of chlorophyll–*a* concentration also differed between the strains (04VR10L1, 1497.29 $\mu g.l^{-1}$; 11VR10JH, 628.36 $\mu g.l^{-1}$; 01DRMII10, 1378.42 $\mu g.l^{-1}$; and 12SO10CI, 635.33 $\mu g.l^{-1}$).

DISCUSSION

The phylogenetic tree inferred from 16S rRNA gene sequences revealed clustering of the four *Anabaena* strains into separate, relatively little related clades that broadly correlated with their morphologies. Members

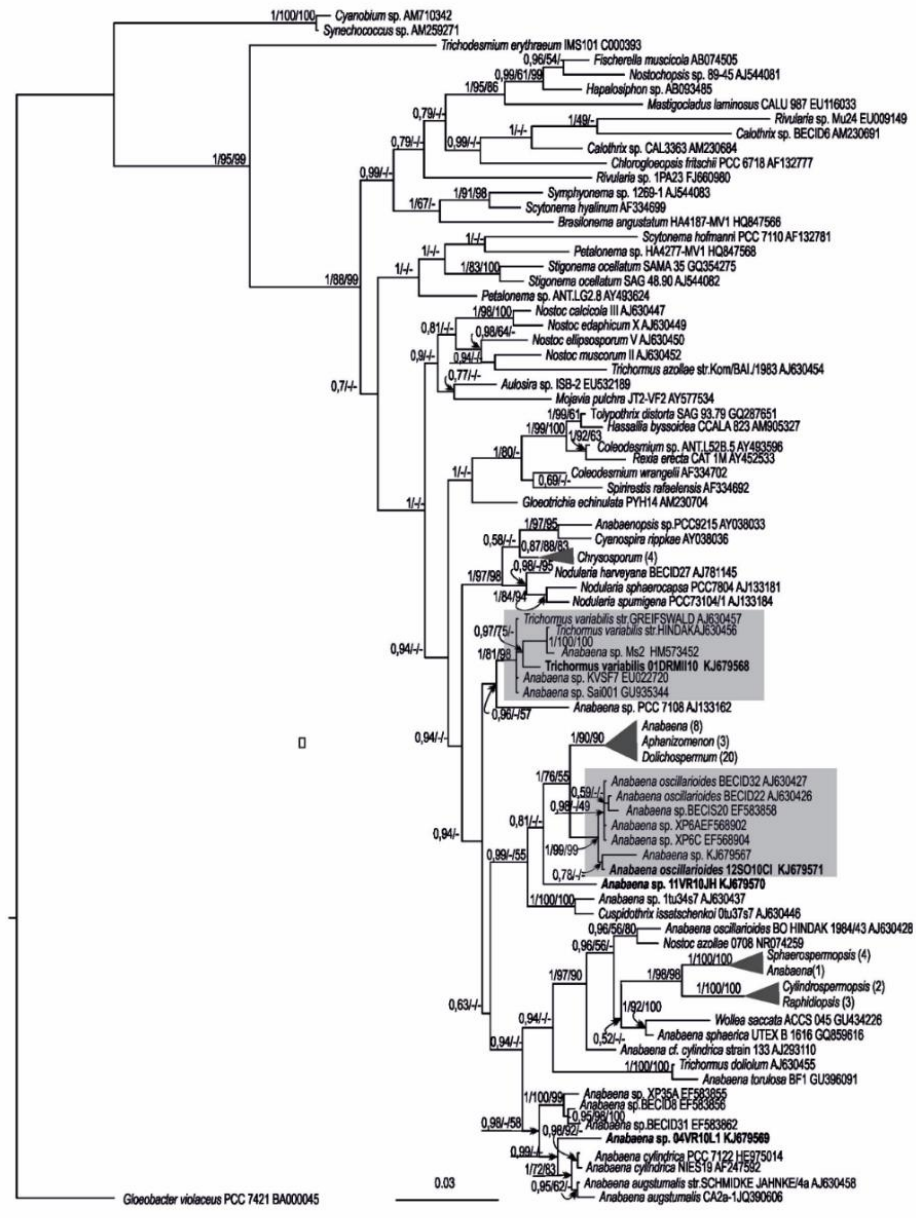

Fig. 3. Bayesian inference (BI) tree based on 16S rRNA data (1361 bp) showing the clustering of *Anabaena* morphospecies without gas vesicles from the Czech Republic (sequences obtained in this work are in bold, clusters containing identified strains are highlighted). Numbers near the nodes indicate branch support values over 50% for BI, Maximum Likelihood (ML), and Neighbor–Joining (NJ) analyses in the following order: BI/ML/NJ. Sequences are labeled with taxon name, strain code, and GenBank accession number. *Synechococcus* sp. 1tu21s05, *Cyanobium* sp. JJ10–3, *Gloeobacter violaceus* PCC 7421, and *Trichodesmium erythraeum* IMS 101 are outgroup taxa.

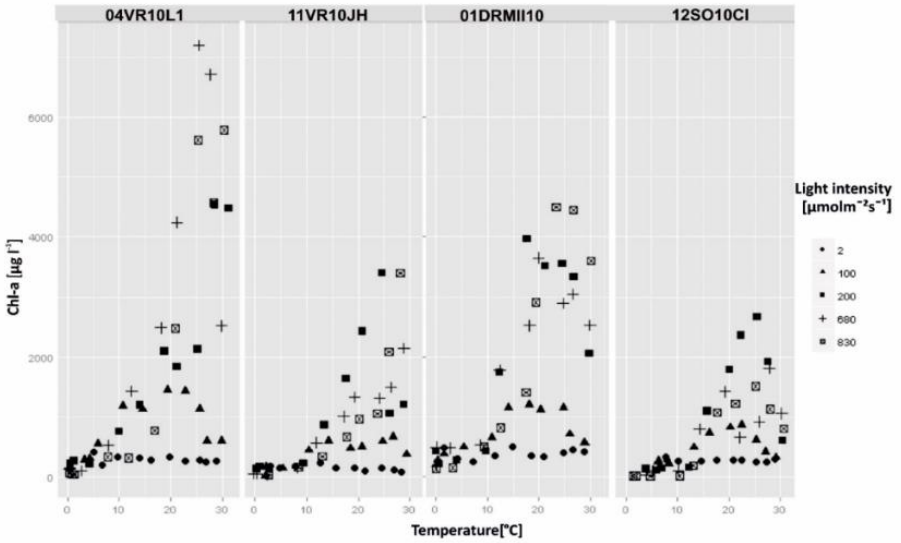

Fig. 4. Scatter Charts describing chlorophyll–*a* concentrations [µg.l^{-1}] of the strains as a function of temperature and light. Identical inocula of each strain were exposed to various combinations of temperature and light intensity. The biomass was harvested in the exponential phase of growth corresponding to that of the fastest–growing culture, and chlorophyll–*a* concentrations were determined.

of non–planktic *Anabaena* were resolved as highly polyphyletic, with sometimes a more distant relationship between themselves than with other species of other Nostocacean genera (for example, *Trichormus*, *Dolichospermum*, and *Aphanizomenon*). These findings are in accordance with those of HALINEN et al. (2008), who also identified several relatively unrelated phylogenetic clusters of benthic *Anabaena* spp. *Trichormus,* belonged for a long time to the genus *Anabaena* and only recently was separated from this genus on the basis of its apoheterocytic akinete development (RAJANIEMI et al. 2005). Some of the *Anabaena* strains (01DRMII10 and related *Anabaena* sp.) in our phylogeny formed a tight cluster together with members of the genus *Trichormus* (Fig. 3), namely, with a couple of strains identified as its type species *T. variabilis* (former *Anabaena variabilis*). Based on the high bootstrap support, and the fact that the morphology of 01DRMII10 matched that of *T. variabilis*, an affiliation of these strains with true *Trichormus* should be considered. However, there is no clear morphological description available for the other *Anabena* sp. in this cluster, and further polyphasic examinations will be required before any decision can be made. Strains of other species assigned to *Trichormus* (*T. azollae* and *T. doliolum*) clustered in completely different positions in the tree, once again emphasizing the polyphyly of the genus (RAJANIEMI et al. 2005; HROUZEK et al. 2013), which is in urgent need of a comprehensive taxonomic revision.

Strain 12SO10CI was identified as *Anabaena oscillarioides* based on its morphology and source habitat. This conclusion was further supported by its position in a cluster of several strains designated as *A. oscillariorides* and *Anabaena* sp. Because *A. oscillarioides* is the type species of *Anabaena*, this lineage may be a candidate for the definition of "true" *Anabaena*. Nevertheless, as demonstrated earlier (RAJANIEMI et al. 2005; KOZHEVNIKOV & KOZHEVNIKOVA 2011), putative *A. oscillarioides* can be found at least in one more, relatively distant lineage (see also Fig. 3, strain BO HINDAK 1984/43). There are currently not as many sequences available for this genus as there are for planktic taxa, and, moreover, the strains are usually poorly defined. One of the reasons for this lies in the morphological plasticity of *Anabaena* and their frequent lack of morphological features that are critical for identification during the different parts of its life cycle or when it is grown under culture conditions. This was also the case for two strains in our study that could be identified solely as *Anabaena* sp. because they lacked akinetes.

The results of this study further support the distinction of planktic *Dolichospermum, Sphaerospermopsis, and Chrysosporum* (ZAPOMĚLOVÁ et al. 2009, 2011, 2012) from benthic *Anabaena* strains. On the other hand, HALINEN et al. (2008) showed that some non–gas–vacuolate *Anabaena* spp. are intermixed with planktic *Dolichospermum* strains. The occurrence of aerotopes was demonstrated to be an unstable feature

of species in some related cyanobacterial taxa, especially after long–term cultivation (Komárek et al. 1993; Laamanen et al. 2001). Thus, detailed molecular and morphological studies involving a larger set of populations are still required to test this hypothesis.

Alongside phylogeny, the morphology of the studied *Anabaena* strains confirmed the considerable morphological and genotypic polymorphy within benthic *Anabaena*. However, the morphology and phylogeny of the studied strains were generally congruent, which is in contrast to studies where the observed morphological variability is sometimes not reflected in the genetic relationships based on 16S rRNA sequences (Lyra et al., 2001; Iteman et al. 2002; Gugger & Hoffmann 2004; Řeháková et al. 2014). On the other hand, it was difficult to find a correlation between the growth response of the studied strains to various light intensities and temperatures and their morphological or phylogenetic variability. Strain 01DRMII10 was clearly distinct from other strains based on its morphology and phylogeny, whereas it was not different from other strains with respect to its growth optima. Strain 12SO-10CI had growth preferences that were the most different from those of the other studied strains, while the results of strain 11VR10JH were ambiguous, similar to both 01DRMII10 and 04VR10L1. In contrast to our results, Miller & Castenholz (2000) showed that strains of *Synechococcus* isolated from Oregon hot springs that grouped into different phylogenetic lineages have different temperature growth optima. Apparently, ecophysiological niche differentiation in cyanobacteria can rapidly evolve in response to the selective pressure of the environment (Coleman et al. 2006). Hence, the correlation of growth optima with morphology or phylogenetic position can be expected only in extremely tightly related populations, which was not the case for our strains.

Since benthic or periphytic species often have to face severe fluctuations in temperature and light intensity (Stal 2000), we expected their growth optima or tolerances to be lower or higher than those of their planktic counterparts. Indeed, some of the studied strains showed remarkably high temperature and light preferences. Interestingly, strains showing preference for a higher light intensity (01DRMII10 and 04VR10L1) were isolated from localities with shallow water, with high exposure to sun irradiance. Strains preferring higher light intensities had also higher chlorophyll *a* concentrations (an approximation of biomass), which is in agreement with the greater photosynthetic yield expected from an increased energy input. We did not observe any link between the conductivity or the water pH of the localities and morphology or phylogenetic clustering.

Our strains survived over the whole experimental temperature range (0.0–31.3 °C), and at light intensities 2–830 $\mu mol.m^{-2}.s^{-1}$. Most of the studied strains (04VR10L1, 11VR10JH, and 01DRMII10) showed similar light intensity optima, which were relatively close to those of previously studied planktic species of *Dolichospermum* (Zapomělová et al. 2008a). The *Dolichospermum* strains exhibited an ability to grow over a wide temperature range (10–28 °C), but some of them did not survive extremely low or high light intensities (20 $\mu mol.m^{-2}.s^{-1}$, 750 $\mu mol.m^{-2}.s^{-1}$) (Zapomělová et al. 2008a). Although cyanobacteria generally require little energy to maintain cell function and structure (Gons 1997; Van Liere & Mur 1979), the low light intensities employed were apparently insufficient to provide the minimal amount of energy necessary for the growth of *Dolichospermum* strains studied by Zapomělová et al. (2008a). Their death at high light intensities could be explained by oxidative stress and photoinhibition (Van Liere & Mur 1980; Herman & D'Ari 1998; Muller 2000). In general, planktic strains seem to be adapted to a more homogenous and stable environment without the need for mechanisms to protect against abrupt changes in environmental conditions, whereas the benthic strains investigated in this study presumably possess protective mechanisms that allow them to grow over a wide range of temperatures and light intensities. Such mechanisms possibly involve increased carotenoid production to protect cells from photoinhibition (Paerl et al. 1983) or efficient cyclic electron flow (Marathe 2012). Considering that the four strains used in this study were collected from different sampling sites, their morphological differences, clustering in different phylogenetic lineages, and variable growth preferences are not very surprising. Previously, Rapala & Sivonen (1998) referred to strain–specific differences in growth rates of various planktic *Anabaen*a strains (recently reclassified to *Dolichospermum*) exposed to different temperature and light conditions. Similarly, Zapomělová et al. (2008b) described the markedly different and non–overlapping temperature and light optima of different strains in the species complex of *Dolichospermum circinale* / *D. crassum*.

Our study contributes to the current knowledge on non–gasvaculate *Anabaena* mostly by combining the morphological, phylogenetic and eco–physiological approaches in attempt at obtaining a congruent picture of the studied strains. Although the study itself is based solely on four strains, it summarizes and validates the methodological approaches, and provides the starting point for further studies within this intriguing group of cyanobacteria. The main weak point of previous phylogenetic studies of non–planktonic *Anabaena*–like cyanobacteria was the missing analysis of morphology. Furthermore, growth preferences of benthic *Anabaena* species have not been experimentally assessed before. Some of the strains included in the current study exhibited much higher light intensity preferences compared to their planktonic counterparts, raising further questions related to their biology and ecology.

Our study has once again stressed the importance of combining different approaches to reach reliable conclusions about individual cyanobacterial species and populations, which in turn should result in improvements in the taxonomy of cyanobacteria and better resolution of their life history. The phylogenetic heterogeneity (polyphyly) of the studied strains indicates that benthic and periphytic *Anabaena* will certainly have to be divided into several genera. Further investigation is necessary to reveal the whole range of the diversity of this cyanobacterial group and to create a reliable taxonomic classification. Taken together with the results of previous studies, our results show that the growth demands of related planktic and benthic cyanobacteria differ substantially from each other. This can be explained by their different life strategies, resulting from the different properties of their habitats. Planktic species usually live in relatively stable environments, whereas benthic species inhabit environments with extremely fluctuating conditions (light intensity, temperature, water and nutrient availability, grazing pressure etc.). Moreover, benthic or periphytic mats can occur during the whole year, whereas those of planktic species of cyanobacteria predominantly occur during the warmer season. The results of this study indicate that benthic *Anabaena* species can tolerate wider fluctuations in temperature and light intensity than their planktic counterparts. Further investigation of the physiological and biochemical aspects of these growth preferences is desirable.

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REFERENCES

BROCK, T.D. (1973): Evolutionary and ecological aspects of the cyanophytes. – In: CARR, N. G. & WHITTON, B. A. (eds): The Biology of the Blue–Green Algae. – pp. 487–500. Blackwell Scientific Publications, Oxford.

BURLAND, T.G. (1999): DNASTAR's Lasergene Sequence Analysis Software. – Methods in Molecular Biology 132: 71–91.

CASTENHOLZ, R.W. (1969): Thermophilic blue–green algae and the thermal environment. – Bacteriology Reviews 33: 476–504.

CASTENHOLZ, R.W. (2001): General characteristics of the cyanobacteria. – In: CASTENHOLZ, R. W. & BOONE, D. R. (eds): Bergey's Manual of Systematic Bacteriology:1. – pp. 474–487, 2nd ed., Springer – Verlag, New York.

CASTENHOLZ, R.W. (1973): Ecology of blue–green algae in hot springs. – In: CARR, N.G. & WHITTON B.A. (eds): The Biology of Blue–Green Algae. – pp. 379–414. Blackwell Scientific Publications, Oxford.

COLEMAN, M.; SULLIVAN, M.B.; MARTINY, A.C.; STEGLICH C.; BARRY, K.; DELONG, E.F. & CHISHOLM, S.W. (2006): Genomic Islands and the Ecology and Evolution of *Prochlorococcus*. – Science 311: 1768–1770.

DARRIBA, D.; TABOADA, G.L.; DOALLO, R. & POSADA, D. (2012): jModelTest 2: more models, new heuristics and parallel computing. – Nature Methods 9: 772.

DENICOLA, D.M. (1996): Periphyton responses to temperature at different ecological levels. – In: STEVENSON, R.J.; BOTHWELL, M.L. & LOWE, R.L. (eds): Algal ecology: freshwater benthic ecosystems. Academic Press. – pp. 149–181, San Diego, California.

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.

GEITLER, L. (1932): Cyanophyceae. – In: RABENHORST, L. (eds): Kryptogamen–Flora von Deutschland, Österreich und der Schweiz. – pp. 673–1056, Akademische Verlagsgesellschaft, Leipzig, Germany.

GONS, H.J. (1977): On the light–limited growth of *Scenedesmus protuberans* Fritsch [PhD Thesis]. – 120 pp., University of Amsterdam, Amsterdam.

GOUY, M.; GUINDON, S. & GASCUEL, O. (2010): SeaView version 4 : a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. – Molecular Biology and Evolution 27: 221–224.

GUGGER, M. F. & HOFFMAN, L. (2004): Polyphyly of true branching cyanobacteria (Stigonematales). – International Journal of Systematic and Evolutionary Microbiology 54: 349–357.

GUGGER, M.; LYRA, C.; HENRIKSEN, P.; COUTÉ, A.; HUMBERT, J.F. & SIVONEN, K. (2002): Phylogenetic comparison of the cyanobacterial genera *Anabaena* and *Aphanizomenon*. – International Journal of Systematic and Evolutionary Microbiology 52: 1–14.

GUILLARD, R.R.L.; LORENZEN, C.J. (1972): Yellow–green algae with chlorophyllide c. – Journal of *Phycology* 8: 10–14.

GUINDON, S. & GASCUEL, O. (2003). A simple, fast and accurate method to estimate large phylogenies by maximum–likelihood". – Systematic Biology 52: 696–704.

HALINEN, K.; FEWER, D.P.; SIVONEN, L.M.; LYRA, C.; ERONEN, E. & SIVONEN, K. (2008): Genetic diversity in strains of the genus *Anabaena* isolated from planktonic and benthic habitats of the Gulf of Finland (Baltic Sea). – FEMS Microbiology Ecology 64: 199–208.

HALL, T.A. (1999): BioEdit: a user–friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. – Nucleic Acids Symposium Series 41: 95–98.

HERMAN, C. & D' ARI, R. (1998): Proteolysisand Chaperones: The Destruction/Reconstruction Dilemma. – Current Opinion in Microbiology 1: 204–09.

HOFFMAN, L.; KOMÁREK, J. & KAŠTOVSKÝ, J. (2005): System of cyanoprokaryotes (cyanobacteria) – state in 2004. – Algological Studies 117: 95 –115.

HROUZEK, P.; LUKEŠOVÁ, A.; MAREŠ, J. & VENTURA, S. (2013): Description of the cyanobacterial genus *Desmonostoc* gen. nov. including *D. muscorum* comb. nov. as a distinct, phylogenetically coherent taxon

and Evoutionaryl Microbiology 51: 513–526.

Gugger, M.; Lenoir, S.; Berger, C.; Ledreux, A.; Druart, J.C.; Humbert, J.F.; Guette, C. & Bernard, C. (2005): First report in a river in France of the benthic cyanobacterium *Phormidium favosum* producing anatoxin–a associated with dog neurotoxicosis. – Toxicon 45: 919–928.

Marathe, A. (2012): Cyclic electron transfer pathways in *Synechococcus* sp. PCC 7002 cyanobacteria during photosynthesis at high light intensity [PhD. Thesis]. – 133 pp., University of Wisconsin.

Mareš, J. (2010): *Anabaena fuscovaginata* (Nostocales), a new cyanobacterial species from periphyton of the freshwater alkaline marsh of Everglades, South Florida, USA. – Fottea 10: 235–243.

Miller, M.A.; Pfeiffer, W. & Schwartz, T. (2012): The CIPRES science gateway: enabling high impact science for phylogenetics researchers with limited resources. – In: Proceedings of the 1st Conference of the Extreme Science and Engineering Discovery Environment: Bridging from the eXtreme to the campus and beyond. – pp. 1– 8, ACM, Chicago, Illinois. http://doi.acm.org/10.1145/2335755.2335836

Miller, S.R. & Castenholz, R.W. (1998): Effects of visible light and UV radiation on photosynthesis in a population of a hot spring cyanobacterium, a *Synechococcus* sp., subjected to high–temperature stress. – Applied Environmental Microbiology 64: 3893–3899.

Muller, F. (2000): The nature and mechanism of superoxide production by the electron transport chain: its relevance to aging. Journal of the American Aging Association 23: 227–253.

Oren, A. (2000): Salts and brines. – In: Whitton, B. A. & Potts, M. (eds): The ecology of cyanobacteria. Their diversity in time and space. Kluwer Academic Publishers: 281–319. Dordrecht, The Netherlands.

Osorio–Santos, K.; Pietrasiak, N.; Bohunická, M.; Miscoe, L.H.; Kováčik, Ľ.; Martin, M.P. & Johansen, J.R. (2014): Seven new species of *Oculatella* (Pseudanabaenales, Cyanobacteria). – European Journal of Phycology 49: 450–470.

Paerl, H.W.; Tucker, J. & Bland, P.T. (1983): Carotenoid enhancement and its role in maintaining blue green (*Microcystis aeruginosa*) surface blooms.– Oceanography 28: 847–857.

R Core Team (2014): R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3–900051–07–0.

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). – International Journal of Systematic and Evolutionary Microbiology 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. – Archiv für Hydrobiologie – Algological Studied 117: 371–391.

Rapala, J. & Sivonen, K. (1998): Assessment of environmental conditions that favour hepatotoxic and neurotoxic *Anabaena* spp. strains cultured under light limitation at different temperatures. – Microbial Ecology 36: 181–192.

Řeháková, K.; Mareš, J.; Lukešová, A.; Zapomělová, E.; Bernardová, K. & Hrouzek, P. (2014): *Nodularia* (Cyanobacteria, Nostocaceae): a phylogenetically uniform genus with variable phenotypes. – Phytotaxa 172: 235–246.

Ronquist, F.; Teslenko, M.; van der Mark, P.; Ayres, D.L.; Darling, A.; Höhna, S.; Larget, B.; Liu, L.; Suchard, M.A. & Huelsenbeck, J.P. (2012): MRBAYES 3.2: Efficient Bayesian phylogenetic inference and model selection across a large model space. – Systematic Biology 61: 539–542. Published online 2012 February 22. doi: 10.1093/sysbio/sys029

Schopf, J.W. (1996): Cyanobacteria. Pioneers of the early Earth. – In: Prasad, A.K.S.K.; Nienow, J.A. & Rao, V.N.R. (eds): Contributions in Phycology. – pp. 13–32. Nova Hedwigia, Beiheft 112, Cramer, J., Berlin.

Skácelová, O. & Zapomělová, E. (2010): Remarks on the occurrence and ecology of several interesting cyanobacterial morphospecies found in South Moravian wetlands. – Acta Musei Moraviae, Scientiae biologicae 95: 201–221.

Stal, L.J. (2000): Cyanobacterial mats and stromatolites. – In: Whitton, B.A. & M. Potts (eds): The Ecology of Cyanobacteria. – pp. 61–120, Kluwer, Dordrecht.

Stamatakis, A. (2014): "RAxML Version 8: A tool for Phylogenetic Analysis and Post–Analysis of Large Phylogenies". – Bioinformatics. open access link: http://bioinformatics.oxfordjournals.org/content/early/2014/01/21/bioinformatics.btu033.abstract?keytype=ref&ijkey=VTEqgUJYCDcf0kP

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

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. – Applied Environmental Microbiology 69: 5157–5169.

Van Liere, L. & Mur, L. R. (1979): Chapter 9. Some experiments on the competition between a green alga and a cyanobacterium [PhD Thesis]. – 97 pp., University of Amsterdam.

Van Liere, L. & Mur, L.R. (1980). Occurrence of *Oscillatoria agardhii* and some related species, a survey. – Developments in Hydrobiology 2: 67–77.

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

Ward, D.M. & Castenholz, R.W. (2000): Cyanobacteria in geothermal habitats. – In: Potts, M. & Witton, B. A. (eds): Ecology of cyanobacteria: their diversity in time and space. – pp. 37–59, 669, Kluwer Academic Publishers, Dordrecht.

Willame, R.; Boutte, C.; Grubisic, S.; Wilmotte, A.; Komarek, J. & Hoffmann, L. (2006): Morphological and molecular characterization of planktonic cyanobacteria from Belgium and Luxembourg. – Journal of Phycology 42: 1312–1332.

Wilmotte A.; Van der Auwera, G. & De Wachter, R. (1993): Structure of the 16S ribosomal RNA of the thermophilic cyanobacterium Chlorogloeopsis HTF

related to the genus *Nostoc*. – Fottea 13: 201–213.

ITEMAN, I.; RIPPKA, R.; MARSAC, N.T. & HERDMAN, M. (2002): rDNA analyses of planktonic heterocystous cyanobacteria, including members of genera *Anabaenopsis* and *Cyanospira*. – Microbiology 148: 481–496.

JOHANSEN, J.R. & CASAMATTA, D.A. (2005): Recognizing cyanobacterial diversity through adoption of a new species paradigm. – Archiv für Hydrobiologie–Algological Studies. Suppl. 117: 71–93.

KATOH, K. & STANDLEY, D. M. (2013): MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. – Molecular Biology and Evolution 30: 772–80.

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.

KOMÁREK, J. (2005): Studies on the cyanophytes (Cyanobacteria, cyanoprokaryota) of Cuba 11. Freshwater *Anabaena* species. – Preslia 77: 211–234.

KOMÁREK, J. (2008): The cyanobacterial genus *Macrospermum*. – Fottea 8: 79–86.

KOMÁREK, J. (2010): Recent changes (2008) in cyanobacterial taxonomy based on a combination of molecular background with phenotype and ecological consequences (genus and species concept). – Hydrobiologia 639: 245–259.

KOMÁREK, J. (2013): Cyanoprokaryota –3. Teil/ 3rd Part: Heterocytous genera. –In: BÜDEL, B.; GÄRTNER, G.; KRIENITZ, L. & SCHAGERL, M. (eds.): Süsswasserflora von Mitteleuropa. – 1130 pp. Elsevier/Spektrum, Heidelberg.

KOMÁREK, J.; HÜBEL, M.; HÜBEL, H. & ŠMARDA, J. (1993): The *Nodularia* studies. 2. Taxonomy. –Algological Studies 68: 1–25.

KOMÁREK, J.; KAŠTOVSKÝ, J.; MAREŠ, J. & JOHANSEN, J.R. (2014): Taxonomic classification of cyanoprokaryotes (cyanobacterial genera) 2014 according to the polyphasic approach. – Preslia 86: 295–335.

KOZHEVNIKOV, I.V. & KOZHEVNIKOVA, N.A. (2011): Phylogenetic and morphological evaluation of *Wollea saccata* (Nostocales, Cyanobacteria) isolated from the Yenissei River basin (Eastern Siberia, Russia). – Fottea 11:99–106.

KVÍDEROVÁ, J. & LUKAVSKÝ, J. (2001): A new unit for crossed gradients of temperature and light. – Beiheift Nova Hedwigia 123: 541–550.

LAAMANEN, M.J.; GUGGER, M.F.; LEHTIMÄKI, J.M.; HAUKKA, K. & SIVONEN K. (2001): Diversity of toxic and nontoxic *Nodularia* isolates (Cyanobacteria) and filaments from the Baltic Sea. – Applied and Environmental Microbiology 67: 4638–4647.

LORENZEN, C.J. (1967): Determination of chlorophyll and phaeo-pigments: Spectrophotometric equation. – Limnology and Oceanography 12: 343–346.

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. – International Journal of Systematic ('Mastigocladus laminosus HTF') strain PCC7518, and phylogenetic analysis. – FEBS Microbiology Letters 317: 96–100.

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*). – Journal of Phycology 45: 1363–1373.

ZAPOMĚLOVÁ, E.; HISEM, D.; ŘEHÁKOVÁ, K.; HROUZEK, P.; JEZBEROVÁ, J.; KOMÁRKOVÁ, J.; KORELUSOVÁ, J. & ZNACHOR, P. (2008a): Experimental comparison of phenotypical plasticity and growth demands of two strains from the *Anabaena circinalis / A. crassa complex* (cyanobacteria). – Journal of Plankton Research 30: 1257–1270.

ZAPOMĚLOVÁ, E.; HROUZEK, P.; ŘEHÁKOVÁ, K.; ŠABACKÁ, M.; STIBAL, M.; CAISOVÁ, L.; KOMÁRKOVÁ, J. & LUKEŠOVÁ, A. (2008b): Morphological variability in selected heterocystous cyanobacterial strains as a response to varied temperature, light intensity and medium composition. – Folia Microbiologica 53: 333–341.

ZAPOMĚLOVÁ, E.; HROUZEK, P.; ŘEZANKA, T.; JEZBEROVÁ, J.; ŘEHÁKOVÁ, K.; HISEM, D. & KOMÁRKOVÁ, J. (2011): Polyphasic characterization of *Dolichospermum* spp. and *Sphaerospermopsis* spp. (Nostocales, Cyanobacteria): morphology, 16S rRNA gene sequences and fatty acid and secondary metabolite profiles. – Journal of Phycology 47: 1152–1163.

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. – Cryptogamie/Algologie 28: 353–371.

ZAPOMĚLOVÁ, E.; SKÁCELOVÁ, O.; PUMANN, P.; KOPP, R.; JANEČEK, E. (2012): Biogeographically interesting planktonic Nostocales (Cyanobacteria) in the Czech Republic and their polyphasic evaluation resulting in taxonomic revisions of *Anabaena bergii* Ostenfeld 1908 (*Chrysosporum* gen. nov.) and *A. tenericaulis* Nygaard 1949 (*Dolichospermum tenericaule* comb. nova). – Hydrobiologia 698: 353–365.

Supplementary material

the following supplementary material is available for this article:

Table S1.Summary of GenBank sequences used in this study.

This material is available as part of the online article (http://fottea.czechphycology.cz/contents)

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PHYTOPLANKTON & SPATIAL GRADIENTS

Morphological traits in nitrogen fixing heterocytous cyanobacteria: possible links between morphology and eco-physiology

Paula de Tezanos Pinto · Andreja Kust · Melina Devercelli · Eliška Kozlíková-Zapomělová

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Abstract Heterocytous cyanobacteria are able to fix nitrogen (in heterocytes) and to produce dormant cells (akinetes). Heterocyte and akinete shape, size, and relative position have taxonomical relevance and possibly ecological value too. We collected—from literature and nature—and compared morphological data on vegetative cells, heterocytes, and akinetes across four genera taxonomically separated from *Anabaena*. In average, heterocyte size doubled that of vegetative cells—probably because of extra cell wall deposition. Heterocyte morphology was remarkably similar across genera, both in size and shape (spherical). The latter may decrease oxygen diffusion from adjoining vegetative cells. Akinetes were huge (one order of magnitude bigger) compared to vegetative cells, probably because of its massive genome replication, extra deposition of wall layers, allocation of storage and number of vegetative cells fused during akinete differentiation. Akinete shape was mostly cylindrical, or oval, but rarely spherical. In line with molecular data, we found morphological differences between *Anabaena* (non-aerotopated, soil or benthic) and *Dolichospermum* (aerotopated, planktonic), including vegetative cell size, and akinete size, shape, and relative position to the heterocyte. Differences may relate to adaptations to their contrasting environments (benthic versus planktic). Further research is needed to generalize our results to other heterocytous genera.

Keywords Traits · Heterocyte · Akinete · Shape · Size · Phytoplankton

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P. de Tezanos Pinto (✉)
Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, C1428EHA, Buenos Aires Argentina
IEGEBA (CONICET-UBA), Buenos Aires, Argentina
e-mail: paulatezanos@ege.fcen.uba.ar

A. Kust · E. Kozlíková-Zapomělová
Biology Centre, AS CR, v.v.i, Institute of Hydrobiology, Na Sádkách 7, České Budějovice, Czech Republic

A. Kust
Faculty of Science, University of South Bohemia in České Budějovice, Branišovská 31, České Budějovice, Czech Republic

M. Devercelli
Instituto Nacional de Limnología (CONICET-UNL), Santa Fe, Argentina

Introduction

Many heterocytous nitrogen fixing cyanobacteria cause blooms in water bodies around the globe occasioning severe ecological, economical, and social problems. This ecological group have a wide suite of

traits that provide fitness in a range of environmental settings, including the ability to fix nitrogen (in heterocytes) and to produce dormant cells (akinetes). Both heterocytes and akinetes differentiate from vegetative cells—in response to environmental triggers—and undergo major morphological and functional changes. The shape and size of akinetes, as well as the relative position of heterocytes and akinetes have taxonomical relevance (Komárek, 2013) and we believe that they have an ecological value too.

Exploring the morphological traits in heterocytous nitrogen fixing cyanobacteria will allow us to explore its trait distributions and trait–trait relations (e.g., positive relations, trade-offs). It also sets the basis to assess the links between morphological and physiological traits (e.g., how cell size relates to growth rate or light acquisition traits). Indeed, ecological traits are used in phytoplankton studies to explain and predict species distribution along environmental gradients (Litchman et al., 2010; Schwaderer et al., 2011; Salmaso et al., 2014). Both size and shape are important traits to study phytoplankton distribution (Reynolds et al., 2002; Salmaso & Padisák, 2007; Padisák et al., 2009; Kruk et al., 2010). Morphological traits impact on phytoplankton reproduction, resource acquisition, and grazer resistance capabilities (Litchman & Klausmeier, 2008) and are relatively simple to measure (Arnold, 1983; Hodgson, 1999).

The heterocyte is a good example of how a morphological trait relates to a function, in this case the fixation of atmospheric nitrogen. The nitrogenase enzyme, responsible of the reduction of N_2 to NH_3, NH_4 or any organic nitrogen compound (Jaffe, 1992), has an extreme sensitivity to oxygen (Gallon, 1992). Within the heterocyte, oxygen concentration is extremely low (e.g., 0.6 µm in *Anabaena*, Tomitani et al., 2006), hence ensuring the functioning of the nitrogenase. When a vegetative cell differentiates into a heterocyte, major morphological (and physiological) changes occur, which ensure low oxygen availability within mature heterocytes, including the loss of photosystem II (hence lacking the ability to perform oxygenic photosynthesis and fixing carbon dioxide), the addition of several wall layers (which decrease oxygen diffusion from the environment), increased oxidase activity (Wolk et al., 1994), and the development of nodule poles (rich in cyanophycin) in the points of contact with vegetative cells (which decrease molecular flux, Mullineaux et al., 2008). These changes are reflected in heterocyte morphology: they are pale colored (as they lack one photosystem), have thick walls and polar nodule, hence it is very easy to identify heterocytes in the microscope.

Moreover the timing of the evolution of the heterocyte as a trait innovation—about 2.5 billion years ago—coincided with the oxygenation of the planet Earth (Tomitani et al., 2006) when high oxygen levels hindered nitrogen fixation. The capability to differentiate heterocytes allowed Nostocales to be able to fix nitrogen at daytime, in aerobic environments. This monophyletic clade (e.g., Rajaniemi et al., 2005a, b) has dramatically diversified compared to other non-heterocytous nitrogen fixing cyanobacteria, underscoring the fitness provided by the trait of fixing nitrogen within the heterocyte. Nevertheless, we lack knowledge on whether morphological traits such as shape and size vary across heterocytous cyanobacteria, or if these traits may relate to heterocyte functioning.

The akinete allows the survival of populations through dormancy in periods which hinder growth or cause mortality (Kaplan-Levy et al., 2010). These resting cells appeared in the evolution after heterocytes; the most ancient akinetes date from 2.1 to 1.5 billion years ago (Tomitani et al., 2006) and only a subgroup of Nostocales have this trait. These specialized cells may develop from one vegetative cell (Clark & Jensen, 1969; Cmiech et al., 1984) or from the fusion of several neighboring cells (Komárek, 1975; Hindák, 1999, 2008). Akinete differentiation, such as heterocyte differentiation, involves major morphological and physiological modifications. These include: the formation of a multilayered cell wall, the loss of gas vesicles (Komárek, 2013), the allocation of storage in the form of granules of cyanophycin and glycogen (Simon, 1987), massive multiplication of genome (Sukenik et al., 2011), and loss of inorganic polyphosphate bodies (Sukenik et al., 2009). Akinetes can have different shapes (mostly cylindrical, spherical, or oval) and relative positions to the heterocyte (adjacent or distant). These traits are species-specific and are used in taxonomical classification (Komárek, 2013). There is lack of a quantification of the frequency distribution of akinete morphological traits—shape, size, and relative position toward the heterocyte- and of the possible causes leading to morphological differences in this dormant cell.

The genus *Anabaena* Bory 1822, considered a consistent genus for a long time, has recently been divided into several new genera—planktic *Dolichospermum*, *Sphaerospermopsis*, and *Chrysosporum,* and non-aerotopated *Anabaena* (Komárek & Zapomělová, 2007, 2008; Wacklin et al., 2009; Zapomělová et al., 2009, 2012)—based primarily on the 16S rRNA gene sequencing (Iteman et al., 2002; Gugger et al., 2002; Rajaniemi et al., 2005a, b; Hoffman et al., 2005). It would be interesting to test if differences in morphological traits across genera support the molecular findings.

In this investigation, we aim to build a solid knowledge on morphological traits within heterocytous cyanobacteria. For this we collected, synthesized and compared morphological data of different cell types (vegetative cells, heterocytes, and akinetes) in a wide range of heterocytous species within the recently separated genera *Anabaena*, using information from literature and from natural populations. We assessed trait distributions as well as trait–trait relationships and tried to find links between morphology and function within this relevant ecological group.

Materials and methods

Data collected from the literature

We collected, from the third volume of "Cyanoprokaryotes" (Komárek, 2013), data on length, width, and shape of vegetative cells, heterocytes, and akinetes for all species within the non-aerotopated genus *Anabaena*, and the planktic genera *Dolichospermum*, *Sphaerospermopsis,* and *Chrysosporum*. As mentioned in the Introduction, these four genera used to belong to the traditional genus *Anabaena* but were separated according to modern phenotypic and molecular criteria (Iteman et al., 2002; Gugger et al., 2002; Rajaniemi et al., 2005, b; Hoffman et al., 2005; Komárek & Zapomělová, 2007, 2008; Wacklin et al., 2009; Zapomělová et al., 2009, 2012, 2013).

For each species and cell type, we computed the average between the minimum and maximum length and width, assuming a normal distribution of these traits, based on the argument of Kerkhoff & Enquist (2009) that most traits have a normal distribution. We validated this approach by assessing the same cell dimensions in natural samples and testing if these followed a normal distribution (see Data collected from natural populations).

Next, we calculated cell size (volume) following Hillebrand et al. (1999). We also collected information about the relative position of the akinete to the heterocyte (e.g., distant or adjacent) from Komárek (2013).

Data collected from natural populations

We collected data on length and width of vegetative and specialized cells from natural samples collected in the Czech Republic and Argentina (Table 1). For each species we: (a) took microphotographs of at least 30 trichomes under 400× magnification using a digital camera (Olympus DP70 and AxioCam 5S Carl Zeiss) attached to a light microscope (Olympus BX51 and Nikon TS100 Eclipse) and (b) measured five vegetative cells per trichome in 30 trichomes, and as much akinetes and heterocytes as possible. Only mature akinetes were measured (those which possessed fully developed thickened cell wall) as recommended by Komárek (1996). We used this information to test whether cell length and width followed a normal distribution (Jarque–Bera test) and to compare results among data sets (literature and nature).

Statistical analyses

For the data collected from literature we:

(i) assessed the following trait–trait relationships: length to width (of each cell type), vegetative to akinete size, and vegetative to heterocyte size. In each case, we tested which curve fitted best the data (linear, logarithm, power, or exponential) based on the significance of ANOVA test and the coefficient of determination (R^2).

(ii) compared length to width relationships between cell types (vegetative cell versus heterocyte, vegetative cell versus akinete, and heterocyte versus akinete) using ANCOVA test after meeting the assumptions. Also, for each cell type (vegetative, heterocyte, akinete) we compared the length to width relationship across data sets (e.g., vegetative cell length to width relationship from literature data versus those obtained

Table 1 Geographical position and environment where the natural samples were obtained

Species	Geographical position and environment
Anabaena sp.	Sokolov area, Czech Republic
Anabaena sp.	50.1503689 N, 12.4016761E, Soos Natural Reserve, (spring), Františkovy Lázně, Czech Republic
Anabaena sp.	50.1500939 N, 12.4044656E, Soos Natural Reserve, (mud surface), Františkovy Lázně, Czech Republic
Anabaena sp.	50.2384222 N, 12.7046586E, (pool after coil mining), Vřesová, Czech Republic
Anabaena sp.	50.2384222 N, 12.7046586E, (temporary puddle), Vřesová, Czech Republic
Anabaena sp.	50.2384222 N, 12.7046586E, (pool after coil mining), Vřesová, Czech Republic
Anabaena sp.	50.2714039 N, 12.7563286E, Smolnická (mine disposal site), Chodov, Czech Republic
Anabaena sp.	50.1484439 N, 12.4030494E, Soos Natural Reserve, Císařský (spring), Františkovy Lázně, Czech Republic
Anabaena sp.	50.1484439 N, 12.4030494E, Soos Natural Reserve, Císařský (spring) Františkovy Lázně, Czech Republic
Anabaena sp.	50.1500939 N, 12.4044656E, Soos Natural Reserve, (mud surface), Františkovy Lázně, Czech Republic
Anabaena sp.	50.1503689 N, 12.4016761E, Soos Natural Reserve, (spring), Františkovy Lázně, Czech Republic
Anabaena sp.	50.1503689 N, 12.4016761E, Soos Natural Reserve, (spring), Františkovy Lázně, Czech Republic
Dolichospermum lemmermannii	48.9989158 N, 14.7704314E, Svět (fishpond), Třeboň, Czech Republic
Dolichospermum flos-aquae	49.0313686 N, 14.4398547E, Naděje (fishpond), Bavorovice, České Budějovice, Czech Republic
Dolichospermum sigmoideum	50.1975339 N, 12.8572225E, Březová (reservoir), Karlovy Vary, Czech Republic
Dolichospermum sigmoideum	49.0039836 N, 14.4347478E, Černiš (fishpond), České Budějovice, Czech Republic
Dolichospermum lemmermannii	49.0382619 N, 13.9928483E, Husinec (reservoir), Prachatice, Czech Republic
Dolichospermum sigmoideum	49.0707172 N, 14.7009086E, Koclířov (fishpond), Lomnice nad Lužnicí, Czech Republic
Dolichospermum sigmoideum	49.0105144 N, 14.3056583E, Dehtář (fishpond), Dehtáře, Czech Republic
Dolichospermum lemmermannii	49.7845892 N, 13.3931064E, Senecký (fishpond), Plzeň, Czech Republic
Dolichospermum sp.	Czech Republic
Dolichospermum tenericaule	50.5766417 N, 14.6476078E, Máchovo (fishpond), Doksy, Czech Republic
Sphaerospermopsis aphanizomenoides	48.9989158 N, 14.7704314E, Svět (fishpond), Třeboň, Czech Republic
Sphaerospermopsis reniformis	48.7819850 N, 16.7836761E, Hlohovecký (fishpond), Lednice, Czech Republic
Sphaerospermopsis aphanizomenoides	34°10′-34°17′S—58°48-58°53′W (shallow lake) Grande, Buenos Aires, Argentina
Sphaerospermopsis torques reginae	34°10′-34°17′S—58°48-58°53′W (shallow lake) Grande, Buenos Aires, Argentina
Sphaerospermopsis aphanizomenoides	34°21′11.44″S—62°13′2.50″W (shallow lake) La Picasa, Santa Fe, Argentina
Sphaerospermopsis aphanizomenoides	34°21′11.44″S—62°13′2.50″W (shallow lake) La Picasa, Santa Fe, Argentina
Sphaerospermopsis reniformis	49.1246686 N, 14.7404336E, Pěšák (fishpond), Lomnice nad Lužnicí, Czech Republic
Sphaerospermopsis aphanizomenoides	50.0428369 N, 14.5311786E, Hostivař (reservoir), Prague, Czech Republic
Chrysosporum bergii	Očko sandpit, Czech Republic

from nature). Finally, we compared size trait relationships (vegetative cell to heterocyte, and vegetative cell to akinete) between literature and natural data sets.

(iii) tested, for each cell type (vegetative cell, heterocyte, and akinete), if there existed significant differences in size among *Anabaena*, *Dolichospermum*, *Sphaerospermopsis,* and *Chrysosporum*. For this, we run one-way ANOVA tests (all variables were transformed to logarithm to meet the test assumptions). When significant differences were found, we used Hochberg GT2 post hoc test, which is suitable for comparing unbalanced treatments.

(iv) Examined the frequency distributions of akinete relative position to the heterocyte in the genera with high number of species (*Anabaena* and *Dolichospermum*) running a Chi square test.

All analyses were performed with SPSS 17 Software, and Past 3 Software (Hammer et al., 2001).

Results

The four genera analyzed in the literature dataset encompassed 106 species. Most of these belonged to the non-aerotopated genus *Anabaena* (59 species), while the remaining (47 species) were planktic (aerotopated, allowing buoyancy regulation). In the latter group, most species belonged to *Dolichospermum* (38) while few belonged to *Sphaerospermopsis* (5) and *Chrysosporum* (4).

For natural populations, we measured cell dimensions in 866 individuals across 31 species (12 of *Anabaena*, 10 of *Dolichospermum*, 8 of *Sphaerospermopsis,* and 1 of *Chrysosporum*, Table 1). The total number of normality tests run was 186: 2 cell dimensions (length and width) *3 cell types (vegetative, heterocyte, and akinete)*31 species. The majority of traits studied (92%) had a normal distribution ($P > 0.05$) on linear scale. This suggests that the approach used in this study (calculation of average cell length and width from the minimum and maximum values informed in the literature) is adequate for estimating—and comparing—cell size across different genera and cell types.

We found significant ($P < 0.05$, $n = 106$) and positive relationship between cell width and length, for each cell type; in all cases the power curve was the best fit for the data (Fig. 1a–c). The coefficient of determination (R^2) and the slope of the regression models were much higher in vegetative cells and heterocytes than in akinetes (Fig. 1a–c). In line with the previous information, the vegetative cell and the heterocyte showed a similar length to width ratio (close to the unit and with low standard deviation: 1.1 ± 0.3 in vegetative cell and 1.2 ± 0.4 in heterocyte, $n = 106$ for each cell type). For the akinete, however, its length more than doubled its width, and showed higher variability (2.5 ± 1.4, $n = 106$). We found similar slopes (ANCOVA, $P > 0.05$, $n = 106$) for the length to width relationships between cell types (vegetative to heterocyte, vegetative to akinete, and heterocyte to akinete). For each cell type (vegetative, heterocyte, and akinete) the length to width relationship was similar (ANCOVA $P > 0.05$) between literature ($n = 106$) and nature ($n = 31$) data sets (e.g., akinete length to width relationship obtained from literature data compared to that obtained in natural data).

Vegetative cell size ranged from 2.5 to 1022.1 μm^3 (mean: 160.5 μm^3); it was significantly smaller in *Anabaena* than in *Dolichospermum* ($P < 0.001$) (Fig. 2a). Vegetative cell shape was mostly cylindrical (71.4% in *Anabaena*, 39.5% in *Dolichospermum*, 60% in *Sphaerospermopsis* and 100% in *Chrysosporum*) or spherical (25.4% in *Anabaena*, 47.4% in *Dolichospermum* and 40% in *Sphaerospermopsis*); very few were oval (3.4% in *Anabaena* and 13.2% in *Dolichospermum*). Heterocyte size was similar across genera ($P > 0.05$) (Fig. 2b) and ranged between 8.2 to 950.3 μm^3 (mean: 228 μm^3); the most frequent shape was spherical (68%). Akinete size ranged from 54 to 12477 μm^3 (mean: 1823 μm^3) and was significantly smaller in *Anabaena* than in *Dolichospermum* ($P = 0.032$) (Fig. 2c). In most species, akinetes were cylindrical (50%) or oval (39%); few species had akinetes with spherical shape (11%). Within *Sphaerospermopsis,* all species have spherical akinetes and within *Chrysosporum* all species have oval shape. In *Dolichospermum* and *Anabaena* species have akinetes with cylindrical, oval, or spherical shape.

Regarding the relative position of the akinete to the heterocyte, most species showed either distant (60%)

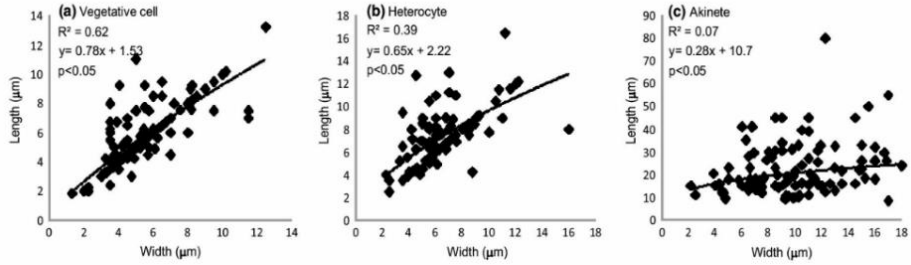

Fig. 1 Length to width relationship in **a** vegetative cell, **b** heterocyte and **c** akinete, $n = 106$ species. The coefficient of determination (R^2) slope, constant, and significance of each curve fit are included in the graphs

or adjacent (30%) position; very few species (9%) differentiate akinetes in both positions (Fig. 3, $n = 106$). In *Anabaena,* there was a similar frequency of species with akinetes distant (50%) or adjacent (37%) to the heterocyte, while in *Dolichospermum* most species (81%) formed akinetes in a distant position to the heterocyte (Fig. 3). The difference in the frequency distribution of akinete position between *Anabaena* and *Dolichospermum* was statistically significant ($\lambda_1 = 8.08$, $P = 0.04$). In all species within *Sphaerospermopsis* ($n = 5$), the akinete develops adjacent to the heterocyte. The opposite happened in *Chrysosporum*, where the akinete develops distant from the heterocyte in 3 out of all 4 species. In *C. ovalisporum* akinetes usually develop distant to the heterocyte, but sometimes may occur in an adjacent position.

Vegetative cell size was significantly and positively related to heterocyte size; the best curve fit was linear ($P < 0.001$, $n = 106$) (Fig. 4a). This pattern was similar (ANCOVA $P > 0.05$) between the literature and the natural population data sets. The ratio of heterocyte to vegetative cell size, in average was two (mean: 2.1, range 0.3–10.6, $n = 106$).

The relationship between vegetative cell and akinete size showed a saturating trend around 4000 µm^3; the best curve fit was power ($P < 0.001$, $R^2 = 0.49$, $n = 106$) (Fig. 4b). This pattern differed from the natural populations (ANCOVA, $P = 0.026$). Both data sets showed positive relationships; however data obtained from nature showed a more limited distribution compared to the range of values obtained in the literature data. This is not surprising as the number of species surveyed from the literature was much higher than those compiled from nature. The ratio of the akinete to the vegetative cell size was in average 9.4 (range 1–55, $n = 106$).

Discussion

We found striking differences in morphological traits among the heterocytous cyanobacteria studied here. Across the four genera analyzed, we found remarkable morphological similarity for the heterocyte, both in terms of size and shape. Conversely, vegetative cell size, as well as akinete size, shape, and relative position to the heterocyte differed between genera.

The trait–trait analysis show that heterocyte size increased proportionally to vegetative cell size. Because the heterocyte develops from a single vegetative cell (Komárek, 2013), the linear relationship found between these cell types seems related to the fact that heterocytes differentiate from one vegetative cell. Heterocytes were about two times bigger than vegetative cells. We hypothesize that this may be related to the extra cell wall deposition that develops during heterocyte differentiation. As heterocytes differentiate from vegetative cells, we were expecting heterocytes to be smaller in *Anabaena* than in *Dolichospermum*; however, we found that heterocyte size was similar (around 200 µm^3, in average) across genera. We also found that the majority of heterocytes were spherical. Though we still lack evidence to explain the causes leading to the striking similar morphology in heterocytes across the genera analyzed here, we speculate that it might be linked to surface to volume relationships minimizing oxygen diffusion

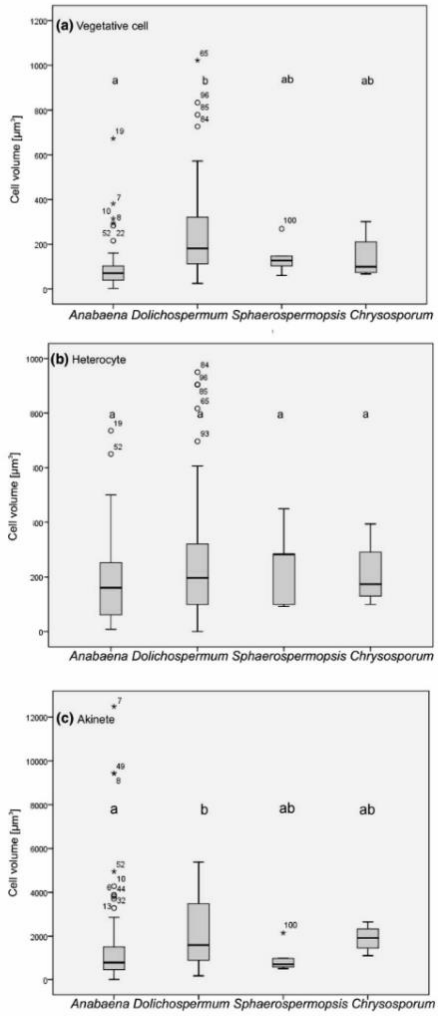

Fig. 2 Comparison of cell size (volume) across the 4 genera analyzed (*Anabaena*, *Dolichospermum*, *Sphaerospermopsis*, and *Chrysosporum*) for the different cell types across 106 species: **a** vegetative cell, **b** heterocyte, and **c** akinete. *Lowercase letters* "*a*" and "*b*" indicate significant differences ($P < 0.05$); *letters* "*ab*" indicate similarities ($P > 0.05$) among genera

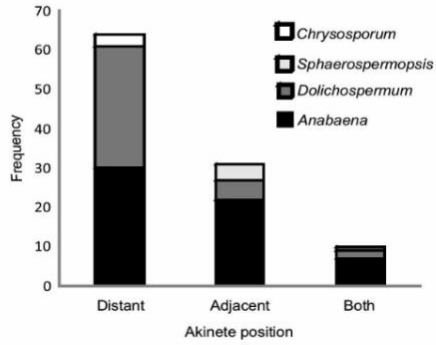

Fig. 3 Frequency distribution of akinete position (*adjacent*, *distant*, or *both*) in each of the 4 genera analyzed ($n = 106$)

(hence favoring nitrogen fixation). Indeed, several authors suggest that the narrow "neck" at the heterocyte-vegetative cell interface reduces the area of contact among cells (Lang & Fay, 1971, Flores et al., 2006; Walsby, 2007). In average, there are about 50 microplasmodesmata at the heterocyte-vegetative cell interface compared to the 200–300 at vegetative–vegetative cell interfaces (Giddings & Staehelin, 1981).

Fay (1969) and others later (Nichols & Adams, 1982; Herdman, 1987, 1988; Sukenik et al., 2013) acknowledged that akinetes are much bigger than the vegetative cells. In line with this evidence, we found that average akinete size was one order of magnitude bigger than average vegetative cell size. The huge size of mature akinetes may be linked to the massive genome replication (Sukenik et al., 2011), extra deposition of wall layers and allocation of storage (Komárek, 2013). We further propose that akinete size may be also linked to the number of vegetative cells fused to form an akinete. For example, akinete differentiation in *Anabaena augstumalis* occurs after the fusion of several neighbor cells (Hindák, 1999). However, our understanding of the number of vegetative cells involved in forming an akinete is very poor, and we still do not know if it occurs in a species-specific way or if it responds similarly within a genus. Finally, the trait–trait relationship between vegetative cell and akinete size showed a saturating trend,

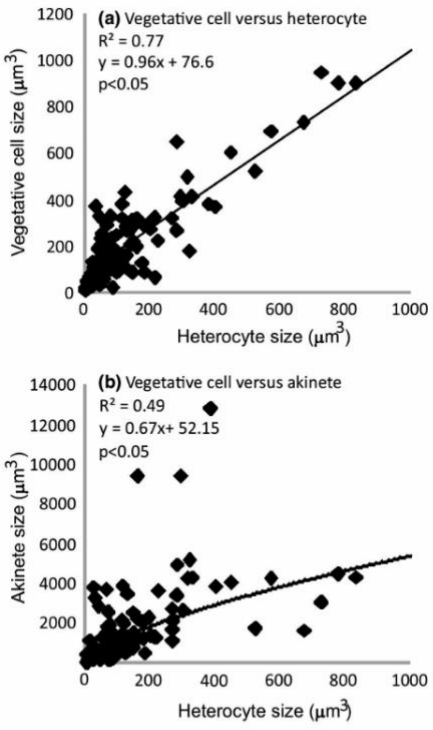

Fig. 4 Trait-trait relationship between cell types: *a* vegetative cell and heterocyte and *b* vegetative cell and akinete ($n = 106$). The coefficient of determination (R^2) slope and constant of each curve fit are included in the graphs

indicating that akinete size increases with vegetative cell size up to a threshold after which akinetes seem to reach its maximum size. Conversely, heterocyte size (as mentioned before) increases proportionally with vegetative cell size.

Most akinetes in the species that we studied were either cylindrical or oval. This may reflect akinete evolutionary history; most ancient akinetes were cylindrical (Tomitani et al., 2006, p. 5444, Fig. 2). Only 12 species (5 benthic and 7 planktonic) out of the 106 species here analyzed had spherical akinetes, including 5 species belonging to *Sphaerospermopsis* (in this genus akinetes exclusively develop adjacent to the heterocyte). We are still unable to explain why differences in akinete shape exist, but we believe it must have an ecological meaning (if shape was driven by chance, we would expect similar proportions of all three akinete shapes). Plausible explanations are that the shape is related to akinete formation strategies, that is, if akinetes differentiate from one or from several vegetative cells. Moreover, though there is consensus that akinete function is related to dormancy and reproduction, current germination experiments that we are undertaking (Paula de Tezanos Pinto Daniela Gangi, Andreja Kust & Eliška Kozlíková-Zapomělová, unpublished data) suggest that shape may be linked to different germination strategies: either if the endospore divides within the akinete cell wall or after breaking the akinete wall. We believe that this may result in different needs of external nutrient supply at germination, though more studies are needed.

We found that most species have akinetes developing distant from the heterocyte, particularly in planktonic species. We speculate that akinete position toward the heterocyte may result in different internal nitrogen stores in the akinete. This, in combination with external nutrient availability at germination, and akinete shape (proxy of germination strategies?), may affect population recruitment success (to form blooms or not). Future studies will allow testing our ideas.

Morphological trait differences among recently taxonomically separated genera

We found several morphological differences between the genera *Anabaena* (non-aerotopated) and *Dolichospermum* (planktonic, aerotopated). These groups have recently been taxonomically separated based on molecular criteria (Iteman et al., 2002; Gugger et al., 2002; Rajaniemi et al., 2005a, b; Hoffman et al., 2005; Komárek & Zapomělová, 2007, 2008; Wacklin et al., 2009; Zapomělová et al., 2009, 2012, 2013). The morphological differences that we identified include: (a) smaller size of vegetative cells and akinetes in *Anabaena* compared to *Dolichospermum*, (b) prevalence of cylindrical akinete shape in *Anabaena* compared to dominance of oval akinete shape in *Dolichospermum*, and (c) similar proportion of species which develop akinetes distant and adjacent to the heterocyte in *Anabaena*, while most species within *Dolichospermum* develop akinetes in a distant position from the heterocyte. We suppose that these morphological differences may be linked to the contrasting

habitats where these genera thrive (benthic or soil in *Anabaena* and planktic in *Dolichospermum*). For example, *Anabaena* species tolerate extreme temperatures and irradiances (Kust et al., 2015), probably because they inhabit sites prone to desiccation. We think that their smaller vegetative cell size—compared to planktonic species—might be an adaptation to tolerate the extreme conditions of temporary habitats. We are still unable to hypothesize why akinete shape and position differ among these groups.

For the other two planktonic genera analysed in this study—*Sphaerospermopsis* and *Chrysosporum*—cell morphology (vegetative, heterocyte, and akinete) were similar to both planktonic *Dolichospermum* and benthic *Anabaena*. This finding seems controversial, as we would expect only to find similarities among planktonic species. Nevertheless because in *Sphaerospermopsis* and *Chrysosporum* diversity is low (≤ 5 species) compared to *Dolichospermum* and *Anabaena* (38 and 59, respectively), results are probably affected by the power of the test, i.e., the ability of the statistical test to find differences when these exist (even if the one-way ANOVA test and the comparisons used are robust to compare means in treatments with unbalanced data).

Concluding remarks

We found remarkable morphological differences among the three cell types and genera analyzed. Though we tried to find mechanistic explanations for the observed morphological differences, we need more research to test if the patterns found across four genera hold for other heterocytous cyanobacteria species, particularly within genera which recurrently form harmful algal bloos (e.g., *Aphanizomenon*, *Anabaenopsis,* and *Cylindrospermopsis*).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Arnold, S. J., 1983. Morphology, performance and fitness. American Zoologist 23: 347–361.

Clark, R. L. & T. E. Jensen, 1969. Ultrastructure of akinete development in a blue green alga. Cylindrospermum sp. Cytologia 34: 439–448.

Cmiech, H. A., G. F. Leedale & C. S. Reynolds, 1984. Morphological and ultrastructural variability of planktonic Cyanophyceae in relation to seasonal periodicity. I. *Gleotrichia echinulata*: vegetative cells, polarity, heterocysts, akinetes. British Phycological. Journal 19: 259–275.

Fay, P., 1969. Cell differentiation and pigment composition in *Anabaena cylindrica*. Archives of Microbiology 67: 62–70.

Flores, E., A. Herrero, C. P. Wolk & I. Maldener, 2006. Is the periplasm continuous in filamentous multicellular Cyanobacteria? Trends in Microbiology 14: 439–443.

Gallon, J. R., 1992. Reconciling the incompatible: N_2 fixation and oxygen. New Phytologist 122: 571–609.

Giddings, T. H. & L. A. Staehelin, 1981. Observation of microplasmodesmata in both heterocyst-forming and non-heterocyst forming filamentous cyanobacteria by freeze-fracture electron microscopy. Archives of Microbiology 129: 295–298.

Gugger, M., C. Lyra, P. Henriksen, A. Couté, J. F. Humbert & K. Sivonen, 2002. Phylogenetic comparison of the cyanobacterial genera *Anabaena* and *Aphanizomenon*. International Journal of Systematic and Evolutionary Microbiology 52: 1–14.

Hammer, Ø., D. A. T. Harper & P. D. Ryan, 2001. PAST: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4: p. 9. http://palaeo-electronica.org/2001_1/past/issue1_01.htm.

Hindák, F., 1999. Akinete development in *Anabaena augstumalis* Schmidle (Cyanophyta/Cyanobacteria) by fusion of several pro-akinetes. Algological Studies 94: 147–161.

Hindák, F., 2008. Colour Atlas of Cyanophytes. Academy of Sciences, Veda Bratislava: 253.

Hodgson, J. G. E. A., 1999. Allocating C-S-R plant functional types: a soft approach to a hard problem. Oikos 85: 282–294.

Hoffman, L., J. Komárek & J. Kaštovský, 2005. System of cyanoprokaryotes (cyanobacteria)—state in 2004. Algological Studies 117: 95–115.

Herdman, M., 1987. Akinetes: structure and function. In Fay, P. & C. van Baalen (eds.), The Cyanobacteria. Elsevier, Amsterdam: 227–250.

Herdman, M., 1988. Cellular differentiation: akinetes. Methods Enzymology 167: 222–232.

Hillebrand, H., C.-D. Durselen, D. Kirshtel, U. Pollingher & T. Zohary, 1999. Biovolume calculation for pelagic and benthic microalgae. Journal of Phycology 35: 403–424.

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

Jaffe, D. A., 1992. The nitrogen cycle. In Butcher, S. S., R. J. Charlson, G. H. Orians & G. V. Wolfe (eds.), Global Biogeochemical Cycles. Academic Press, London: 265–284.

Kaplan-Levy, R. N., O. Hadas, M. L. Summers, J. Rucker & A. Sukenik, 2010. Akinetes: dormant cells of Cyanobacteria. In Lubzens, E., et al. (eds.), Dormancy and Resistance in Harsh Environments, Topics in Current Genetics 21. Springer, Heidelberg: 5–27.

Kerkhoff, A. J. & B. J. Enquist, 2009. Multiplicative by nature: why logarithmic transformation is necessary in allometry. Journal of Theoretical Biology 257: 519–521.

Komárek, J., 1975. Blaualgen aus dem Naturschutzgebiet "Řežabinec" bei Ražice. Nova Hedwigia 26: 601–643.

Komárek, J., 1996. Towards a combined approach for the taxonomy and species delimitation of picoplanktic cyanoprokaryotes. Algological Studies/Archiv für Hydrobiologie 83: 377–401.

Komárek, J., 2013. *Cyanoprokaryota* 3. *Teil/3rd Part:* Heterocytous genera. In Büdel, B., G. Gärtner, L. Krienitz & M. Schagerl (eds.), Süwasserflora von Mitteleuropa/Freshwater flora of Central Europe 19/3. Springer Spektrum, Berlin, Heidelberg: 1130.

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

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

Kruk, C., V. L. M. Huszar, E. Peeters, S. Bonilla, L. Costa, M. Lurling, C. S. Reynolds & M. Scheffer, 2010. A morphological classification capturing functional variation in phytoplankton. Freshwater Biology 55: 614–627.

Kust A, E. Kozlíková–Zapomělová, J. Mareš1 K. Řeháko, 2015. A detailed morphological, phylogenetic and ecophysiological analysis of four benthic *Anabaena* (Nostocales, Cyanobacteria) strains confirms deep heterogeneity within the genus. Fottea, Olomouc 15: 113–122.

Lang, N. & P. Fay, 1971. The heterocysts of blue-green algae. II. Details of ultrastructure. Proceedings of the Royal Society of London B 178: 193–203.

Litchman, E. & C. A. Klausmeier, 2008. Trait-based community ecology of phytoplankton. The Annual Review of Ecology, Evolution, and Systematics 39: 615–639.

Litchman, E., P. de Tezanos Pinto, C. Klausmeier, M. Thomas & K. Yoshiyama, 2010. Linking traits to species diversity and community structure in phytoplankton. Hydrobiologia 653: 15–28.

Mullineaux, C. W., V. Mariscal, A. Nenninger, H. Khanum, A. Herrero, E. Flores & D. G. Adams, 2008. Mechanism of intracellular molecular Exchange in heterocyt-forming cyanobacteria. The EMBO Journal 27: 1299–1308.

Nichols, J. M. & D. G. Adams, 1982. Akinetes. In Carr, N. G. & B. A. Whitton (eds.), The Biology of Cyanobacteria. Blackwell, Oxford: 387–412.

Padisák, J., L. Crossetti & L. Naselli-Flores, 2009. Use and misuse in the application of the phytoplankton functional classification: a critical review with updates. Hydrobiologia 621: 1–19.

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

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

Reynolds, C. S., V. L. M. Huszar, C. Kruk, L. Naselli-Flores & S. Melo, 2002. Towards a functional classification of the freshwater phytoplankton. Journal of Plankton Research 24: 417–428.

Salmaso, N. & J. Padisák, 2007. Morpho-functional groups and phytoplankton development in two deep lakes (Lake Garda, Italy and Lake Stechlin, Germany). Hydrobiologia 578: 97–112.

Salmaso, N., L. Naselli-Flores & J. Padisák, 2014. Functional classifications and their application in phytoplankton ecology. Freshwater Biology 60: 603–619.

Schwaderer, A., K. Yoshiyama, P. de Tezanos Pinto, N. G. Swenson, C. A. Klausmeier & E. Litchman, 2011. Eco-evolutionary differences in light utilization traits help explain phytoplankton distribution patterns. Limnology and Oceanography 56: 589–598.

Simon, R. D., 1987. Inclusion bodies in the cyanobacteria: cyanophycin, polyphospate and poyhedral bodies. In Fay, P. & C. van Baalen (eds.), The Cyanobacteria: current Research. Elsevier/North Holland Biomedical Press, Amsterdam: 199–226.

Sukenik, A., S. Stojkovic, N. Malinsky-Rushansky, Y. Viner-Motzini & J. Beardall, 2009. Fluorescence approaches reveal variations in cellular composition during formation of akinetes in the cyanobacterium *Aphanizomenon ovalisporum*. European Journal of Phycology 44: 309–317.

Sukenik, A., R. N. Kaplan-Levy, J. M. Welch & A. F. Post, 2011. Massive multiplication of genome and ribosomes in dormant cells (akinetes) of *Aphanizomenon ovalisporum* (Cyanobacteria). The International Society for Microbial Ecology Journal 6: 670–679.

Sukenik, A., R. N. Kaplan-Levy & Y. Viner-Mozzini, 2013. Potassium deficiency triggers the development of dormant cells (akinetes) in *Aphanizomenon ovalisoporum* (Nostocales, Cyanoprokaryota). Journal of Phycology 49: 580–587.

Tomitani, A., A. H. Knoll, C. M. Cavanaugh & T. Ohno, 2006. The evolutionary diversification of Cyanobacteria: molecular-phylogenetic and paleontological perspectives. Proceedings of the Natural Academy of Science 103: 5442–5447.

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

Walsby, A. E., 2007. Cyanobacterial heterocysts: terminal pores proposed as sites of gas exchange. Trends in Microbiolgy 15: 340–348.

Wolk, C. P., A. Ernst & J. Elhai, 1994. Heterocyst metabolism and development. In Bryant, D. A. (ed.), Molecular Biology of Cyanobacteria. Advances in Photosynthesis and Respiration, Vol. 1. Springer, Dordrecht: 769–823.

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

Zapomělová, E., O. Skácelová, P. Pumann, R. Kopp & E. Janeček, 2012. Biogeographically interesting planktonic Nostocales (Cyanobacteria) in the Czech Republic and their polyphasic evaluation resulting in taxonomic revisions of *Anabaena bergii* Ostenfeld 1908 (*Chrysosporum* gen. nov.) and *A. tenericaulis* Nygaard 1949 (*Dolichospermum tenericaule* comb. nova). Hydrobiologia 698: 353–365.

Zapomělová, E., J. Mareš, A. Lukešová, O. Skácelová, K. Řeháková & A. Kust, 2013. Extensive polyphyly of non-gas-vacuolate *Anabaena* species (*Nostocales*, *Cyanobacteria*)—a challenge for modern cyanobacterial taxonomy. In: Book of abstracts, 19th Symposium of the International Society for Cyanophyte Research, Cleveland, Ohio, 51 pp.

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A new microcystin producing *Nostoc* strain discovered in broad toxicological screening of non-planktic Nostocaceae (cyanobacteria)

Andreja Kust[a,b,c], Petra Urajová[b], Pavel Hrouzek[b,c], Dai Long Vu[b], Kateřina Čapková[a], Lenka Štenclová[a,c], Klára Řeháková[a], Eliška Kozlíková-Zapomělová[a], Olga Lepšová-Skácelová[c], Alena Lukešová[d], Jan Mareš[a,b,c,*]

[a] The Czech Academy of Sciences, Biology Centre, Institute of Hydrobiology, Na Sádkách 7, CZ-37005, České Budějovice, Czech Republic
[b] Centre Algatech, Institute of Microbiology, Czech Academy of Sciences, Opatovický mlýn, CZ-379 81, Třeboň, Czech Republic
[c] Faculty of Science, University of South Bohemia, Branišovská 1760, CZ-37005, České Budějovice, Czech Republic
[d] Institute of Soil Biology, The Czech Academy of Sciences, Biology Centre, Na Sádkách 7, CZ-37005, České Budějovice, Czech Republic

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ABSTRACT

Benthic cyanobacteria recognized as producers of natural products, including cyanotoxins, have been neglected for systematic toxicological studies. Thus, we have performed a broad study investigating cyanotoxin potential of 311 non-planktic nostocacean representatives combining molecular and chemical analyses. Out of these, a single strain *Nostoc* sp. Treb K1/5, was identified as a new microcystin producer. Microcystins [Asp^3]MC-YR, [Asp^3]MC-FR, [Asp^3]MC-HtyR and Ala-Leu/Ile-Asp-Arg-Adda-Glu-Mdha are reported for the first time from the genus *Nostoc*. All the studied strains were also analyzed for the occurrence of nodularins, cylindrospermopsin and (homo)anatoxin-a, yet no novel producer has been discovered. Our findings indicate rare occurrence of the common cyanotoxins in non-planktic nostocaceae which is in contrast with frequent reports of cyanotoxin producers among phylogenetically closely related planktic cyanobacteria.

1. Introduction

Production of all known major cyanotoxin types has been found in non-planktic cyanobacteria: hepatotoxic microcystins, nodularins, cylindrospermopsins, and the neurotoxic, anatoxin-a, and homoanatoxin-a (e.g., Gugger et al., 2005; Izaguirre et al., 2007; Wood et al., 2007, 2010a,b; Řeháková et al., 2014; Bohunická et al., 2015). However, previous studies were performed on a restricted number of strains, resulting in an incomplete understanding of the actual extent of cyanotoxin production.

The cyanobacterial order Nostocales (heterocytous cyanobacteria) and the family Nostocaceae are extremely active in terms of general secondary metabolite production. They are responsible for production of 26% of the total known metabolites produced by filamentous cyanobacteria (Jones et al., 2010). However, no systematic toxicological studies have been carried out on any non-planktic Nostocacean representatives, despite the fact that particular strains have been repeatedly recognized as cyanotoxin producers (e.g., Genuário et al., 2010; Kurmayer, 2011).

For example, while a number of planktic strains of the nostocacean genera *Nodularia* and *Dolichospermum* (previously *Anabaena*) (Laamanen et al., 2001; Li et al., 2016) are well known cyanotoxins producers, their benthic counterparts have evaded scrutiny. Systematic data on cyanotoxin production are also missing for genera frequently occurring in soil and benthic habitats, such as *Nostoc, Trichormus*, and *Cylindrospermum*. An exception to this was provided by a broad study performed on *Nostoc* lichen cyanobionts (Kaasalainen et al., 2012), which reported high incidence of microcystin biosynthetic genes and microcystins production pointing out the toxic potential of *Nostoc*.

The aim of this study was therefore to systematically investigate non-planktic Nostocaceae and their genetic potential and actual production of several frequently reported cyanotoxins using a combination of molecular and chemical methods.

2. Materials and methods

2.1. Strains under study

Soil and benthic samples were collected from different habitats across a wide range of localities on different continents (for details see Table S1). Monoclonal strains representing eight genera (*Anabaena, Cylindrospermum, Desmonostoc, Mojavia, Nodularia, Nostoc, Trichormus,*

* Corresponding author. The Czech Academy of Sciences, Biology Centre, Institute of Hydrobiology, Na Sádkách 7, CZ-37005, České Budějovice, Czech Republic.
E-mail address: jan.mares@centrum.cz (J. Mareš).

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and *Wollea*) were isolated and maintained in the culture collection of the Institute of Hydrobiology and Institute of Soil Biology, Biology Centre of the Czech Academy of Sciences (CAS), and Centre Algatech, Institute of Microbiology of the CAS. Strains were grown in liquid and agar-solidified (1.5% agar) WC or BG11 media (Guillard and Lorenzen, 1972; Rippka et al., 1979), depending on growth preference. Conditions in the culture collection room were set at 21 °C with a light intensity of 50 μmol $m^{-2}s^{-1}$ (16:8 light/dark cycle). Strain identification was performed based on morphological characteristics (Komárek, 2013) and phylogenetic position in the 16S rRNA tree, where available. A list of the strains and their origin is shown in Table S1.

2.2. Phylogenetic study

The biomasses of the strains were harvested in Eppendorf microcentrifuge tubes (2 mL) by centrifugation and dried at room temperature for 48 h in silica gel, and subsequently pulverized in a Retsch MM200 (Retsch GmbH, Haan, Germany) laboratory mill with wolfram carbide beads (3 min, 30 s^{-1}). Total genomic DNA was isolated following a modified xanthogenate-SDS (Sodium Dodecyl Sulfate) buffer extraction protocol with the addition of 3% PVPP (Polyvinyl Polypyrrolidone) and PEG (Polyethylene Glycol)–$MgCl_2$ precipitation (Yilmaz et al., 2009). The quality of isolated DNA was checked on a 1.5% agarose gel. PCR (polymerase chain reaction) amplification of the 16S rRNA gene was performed using Plain PP Master Mix (Top Bio, Czech Republic) with primer 1 and primer 2 (Table 1), as described by Boyer et al. (2001). Amplification was carried out as follows: an initial denaturation step of 5 min at 94 °C, followed by 38 cycles of 45 s at 94 °C, 45 s at 55 °C, and 2 min at 72 °C, followed by a final elongation step of 10 min at 72 °C. Alternatively, a PCR protocol using the primers 16S27F and 23S30R (Taton et al., 2003, Table 1) included an initial denaturation step 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 10 min at 72 °C. PCR products were sequenced at SEQme s.r.o. (Dobříš, Czech Republic) using the primers listed in Table 1. The SeqMan 5.06 (Burland, 1999) computer program was used for analysis and assembly of the sequences. Out of 267 studied strains used for phylogenetic analyses, 192 were newly sequenced at the time of publication, covering a wide range of phylogenetic clusters across all the studied genera. The sequences of the 16S rRNA gene of strains were deposited in the NCBI GenBank database under the accession numbers KX424390–KX424499, KX442795, and MG596677-MG596761. Additional 177 sequences for phylogenetic analyses were selected from the GenBank online database (http://www.ncbi.nlm.nih.gov) including published 16S rRNA available sequences of Nostocacean taxa previously proven by other authors to contain the synthetase genes for microcystin, nodularin, cylindrospermopsin, or anatoxin-a, and representative strains of the major lineages of Nostocales. MAFFT v. 7 (Katoh and Standley, 2013) was used for sequence alignment. Ambiguous positions and the variable ITS regions were removed prior to phylogenetic analysis. A Maximum Likelihood phylogenetic tree was obtained using RaxML v. 8 (Stamatakis, 2014) and was calculated by the CIPRES supercomputing facility (Miller et al., 2015); 1000 bootstrap pseudo-replications were performed to evaluate the relative support of branches. The GTR + I + G evolutionary substitution model was selected using the AIC criterion in jModelTest-2.1.6 (Guindon and Gascuel, 2003; Darriba et al., 2012). The tree was edited using FigTree v. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

2.3. Toxic potential and toxin production

Toxic potential of the tested strains was examined by PCR for microcystin (*mcy*E), nodularin (*nda*F), cylindrospermopsin (*cyr*J), and anatoxin-a (*ana*C) synthetase genes using published protocols and primers (Table 1). DNA samples isolated from known toxin-producing strains were used as positive controls. The PCR product obtained in *Nostoc* sp. Treb K1/5 was sequenced commercially at SEQme s.r.o. (Dobříš, Czech Republic) using the same primers and the sequence was deposited in GenBank under accession number KX495361. A BLAST search was performed to confirm the result and identify the most similar *mcy*E sequence in other cyanobacteria.

All strains were simultaneously analyzed for actual toxin production using high-performance liquid chromatography connected to high resolution mass spectrometer with electrospray ionization (HPLC-ESI-HRMS/MS). After centrifugation and lyophilization, 10–20 mg biomass samples were extracted in Eppendorf tubes (500 μL) using sea sand and a pestle; 100–150 μL of 70% MeOH was used for microcystin and nodularin extraction, 100–150 μL of 80% acetonitrile solution acidified with 0.1% formic acid was used for extraction of anatoxin-a and cylindrospermopsin (Dell'Aversano et al., 2004). To detect microcystins, 70% methanol extracts were analyzed on a Dionex UltiMate 3000 UHPLC+ (Thermo Scientific, Sunnyvale, CA, USA) equipped with a diode-array detector (range 220–650 nm). Separation of compounds was performed on reversed-phase C18 column (Phenomenex Kinetex, 150 × 4.6 mm, 2.6 μm, Torrance, CA, USA) using H_2O (A) and acetonitrile (B) (both containing 0.1% HCOOH) as a mobile phase at a flow rate of 0.5 mL min^{-1}. The linear gradient was as follows: A/B 85/15 (0 min), 85/15 (in 1 min), 0/100 (in 19 min), 0/100 (in 25 min), and 85/15 (in 30 min). The acetonitrile extracts were analyzed on the same HPLC-ESI-HRMS/MS system, using a TSK gel Amide-80 column (Tosoh Bioscience, 250 × 4.6 mm, 5 μm, Stuttgart, Germany) with isocratic elution with an A/B ratio of 21/79 and a flow rate of 0.5 mL min^{-1}.

Table 1
Primers used for PCR and sequencing.

Target gene	Primer pair	Sequence (5′-3′)	Reference
*ana*C (anatoxin-a)	anaxgenF	ATG GTC AGA GGT TTT ACA AG	Rantala-Ylinen et al., 2011
	anaxgenR	CGA CTC TTA ATC ATG CGA TC	
*cyr*J (cylindrospermopsin)	cyrJF	TTC TCT CCT TTC CCT ATC TCT TTA TC	Mazmouz et al., 2010
	cyrJR	TGC TAC GGT GCT GTA CCA AGG GGC	
*mcy*E (microcystin)	mcyEF2	GAA ATT TGT GTA GAA GGT GC	Vaitomaa et al., 2003
	mcyER4	AAT TCT AAA GCC CAA AGA CG	Rantala et al., 2004
*nda*F (nodularin)	ndaF8452	GTG ATT GAA TTT CTT GGT CG	Koskenniemi et al., 2007
	ndaF8640	GGA AAT TTC TAT GTC TGA CTC AG	
16S rRNA	primer1	CTC TGT GTG CCT AGG TAT CC	Boyer et al., 2001
	primer2	GGG GAA TTT TCC GCA ATG GG	
	16S27F	AGA GTT TGA TCC TGG CTC AG	Taton et al., 2003
	23S30R	CTT CGC CTC TGT GTG CCT AGG T	
	CYA781F(a)	AAT GGG ATT AGA TAC CCC AGT AGT C	Nübel et al., 1997
	K6	GAC GGG CCG GTG TGT ACA	Wilmotte et al., 1993
	K8	AAG GAG GTG ATC CAG CCA CA	Flechtner et al., 2002
	16S781R(a)[b]	GAC TAC TGG GGT ATC TAA TCC CAT T	Taton et al., 2003
	16S1494R	TAC GGC TAC CTT GTT ACG AC	Taton et al., 2003

Table 2
Characteristic MS/MS fragments of the studied cyanotoxins.

Compound	Characteristic Fragment Ions	Reference
Anatoxin-a	166.1226 $[M+H]^+$, 149.0961 $[M\text{-}NH_3+H]^+$, 131.0855 $[M\text{-}NH_3\text{-}H_2O+H]^+$	Furey et al., 2003
Homoanatoxin-a	180.1383 $[M+H]^+$, 163.1117 $[M\text{-}NH_3+H]^+$, 145.1012 $[M\text{-}NH_3+H]^+$	Furey et al., 2003
11-carboxylanatoxin-a	210.1125 $[M+H]^+$,166.1226 $[M\text{-}CO_2+H]^+$, 133.0648 $[M\text{-}H_2O+C_2H_2O\text{-}NH_2+H]^+$	Selwood et al., 2007
Cylindrospermopsin	416.1246 $[M+H]^+$, 336.1671 $[M+SO_3+H]^+$, 274.0860 $[M\text{-}C_5H_8N_2O_3+H]^+$, 194.1317 $[M\text{-}C_5H_8N_2O_3\text{-}SO_3+H]^+$	Guzmán-Guillén et al., 2012
Nodularin	135.0808 Adda side chain	Mazur-Marzec et al., 2006
Adda-MCYST	135.0808 Adda side chain	Genuário et al., 2010
ADMAdda-MCYST	265.16 ADMAdda - $[CH_3CO_2H - NH+H]^+$	Yuan et al., 1998
DMAAdda-MCYST	163.11 [DMAdda + H]$^+$	Puddick et al., 2015

The HPLC was connected to an Impact HD high resolution mass spectrometer (Bruker, Billerica, Massachusetts, USA) with electrospray ionization. The analyses were performed in positive mode. The following settings were used: drying temperature, 200 °C; drying gas flow, 12 L min^{-1}; nebulizer gas pressure, 3 bars; capillary voltage, 3.8 kV; end plate offset, 500 V. Spectra were collected in the range *m/z* 20–2000 with a spectra rate of 2 Hz. The automated fragmentation was used in the method with the precursor ion selection 20–2000 *m/z* for microcystins/nodularins and 50–1000 *m/z* for anatoxin/cylindrospermopsin with the isolation window width of 1Da. The ramping of the collision energy was based on the molecular mass: 20 eV at 400 *m/z* and 70 eV at 1000 *m/z* for microcystins/nodularins and 20 eV at 400 *m/z* and 70 eV at 1000 *m/z* for anatoxin/cylindrospermopsin analysis. The mass spectrometer was calibrated with sodium formate clusters at the beginning of each analysis. Hexakis (1H,1H,2H-perfluoroethoxy) phosphazene (LockMass Tuning Mix ES-TOF *m/z* 622.02896, 97%) was purchased from ABCR GmbH & Co., KG, Germany.

The detection of anatoxin, homoanatoxin, 11-carboxylanatoxin and cylindrospermopsin was based on the presence of their molecular ion and their characteristic fragments referred in literature (Table 2).

To cover production of described microcystins/nodularins as well as their abundant undescribed variants we screened for characteristic Adda (3*S*-amino-9*S*-methoxy-2*S*,6,8*S*-trimethyl-10-phenyldeca-4*E*,6*E*-dienoic acid) fragments together with fragments derived from modified Adda variants (ADMAdda and DMAdda (Puddick et al., 2015, Table 2)). The methods were optimized using analytical standards – microcystin-LR (Enzo Life Sciences, Farmingdale, NY, USA), anatoxin-a (Enzo Life Sciences), nodularin (Fluka Analytical, Sigma Aldrich) and cylindrospermopsin (Enzo Life Sciences). *Nostoc* sp. 152 (Sivonen et al., 1992) was used as an internal standard for detection of Adda, ADMAdda and DMAdda containing microcystin variants (Table S2, Fig. S1). The limits of detection (LOD) for particular toxins were calculated as LOD = 3sd/slope (where sd stands for standard deviation of peak area of the toxin) based on the measurement of peak areas of the toxin standards in matrix (cyanobacterial extract of concentration 100 mg mL^{-1} of 70% MeOH). The resulting LODs were: 6.6 ng.mL^{-1}for nodularin, 5.8 ng mL^{-1} for anatoxin, and 3.1 ng mL^{-1} for microcystin-LR. For cylindrospermopsin the LOD was 2.8 ng mL^{-1} as referred previously (Bohunická et al., 2015). The Skyline software (v.3.5) was used to inspect data for particular transitions (Table 2) in case of anatoxin-a, homoanatoxin-a, 11-carboxylanatoxin and cylindrospermopsin and the presence of currently described microcystin and nodularin variants (Table S3) using mass tolerance of 0.04 *m/z*. For the undescribed microcystin and nodularin variants the Bruker Data Analysis software was used to plot the extracted ion chromatograms of Adda characteristic ions (Table 2) in MS/MS spectra with the tolerance of 0.02 *m/z*. The detected MS/MS spectra were manually inspected for the molecular weights of the parent ion and its molecular formula, the exact mass and the relative intensity of the characteristic Adda fragment ions. All hits showing Adda fragment ions relative intensity below 1% were excluded from the further analysis. The peptide sequences of microcystins found in *Nostoc* sp. Treb K1/5 were inferred from the MS/MS spectra (54eV) of the parent ion. To distinguish between *N*-methyl dehydroalanine (Mdha) or dehydrobutyrine (Dhb) in the position 7 mercaptoethanol derivatization of crude extracts and microcystin-LR (MC-LR) standards for 48 h (30 °C, 99% 2-Mercaptoethanol, Fluka Biochemika) was used as described previously (Miles et al., 2012, 2013). Reaction mixtures were analyzed by HPLC-ESI-HRMS/MS method as described above.

3. Results

A set of 311 strains of Nostocaceae collected from different environments and geographical locations (Table S1) have been investigated for their cyanotoxin production. Genetic potential to synthesize cyanotoxins and the actual toxin production was tested using specific PCR protocols and HPLC-ESI-HRMS/MS, respectively. To determine their taxonomic identity and asses their overall genetic diversity (phylogenetic span), phylogenetic analysis of sequenced strains (267) was performed.

3.1. Presence of known cyanotoxins

We detected a single microcystin producer (Treb K1/5) among the 311 non-planktic strains. Based on its morphology (Komárek, 2013) and the 16S rRNA (KX424473) it was identified as *Nostoc*. Its toxic potential was confirmed by the presence of the *mcy*E gene from the microcystin synthetase operon. A 757 bp region of the *mcy*E gene exhibited 93.3% nucleotide sequence identity to the *mcy*E gene of *Nostoc* sp. 152 KC699835 (the closest BLAST hit) confirming the identity of this gene. However, these two strains were not closely related in the 16S rRNA tree (Fig. 1, Fig. S2).

HPLC-ESI-HRMS/MS analysis revealed four microcystin variants detected in the strain extract, exhibiting clear loss of the non-modified Adda fragment (Fig. 2) with protonated molecules at *m/z* 1031.5218, 981.5405, 1015.5248, 1045.5314 (Table 3).

Based on the MS/MS data interpretation and their comparison with MS/MS of the MC-LR standard (cyclo-(D-Ala^1-L-Leu^2-D-MeAsp^3-L-Arg^4-Adda^5-D-Glu^6-Mdha^7)), it was possible to determine three of the four detected variants. Product ions corresponding to the presence of the molecule fragment Glu^6-R^7-Ala^1 were obtained for all detected variants as well as for the MC-LR standard (Table S4). To distinguish between isobaric amino acids (Mdha/Dhb) occurring in microcystins in position R^7, additional step of β-mercaptoethanol derivatization was performed (Miles et al., 2013). In all variants, the reaction led to mass shift of 78, corresponding to reaction of Mdha with mercaptoethanol and its presence in position R^7 (Fig. S3). The position R^3 was found to be occupied by Asp instead of Me-Asp present in MC-LR. This was proved by the difference of 14 in the corresponding Arg-R^3 fragment (Table S4). Based on the presence of fragments Arg-R^3-R^2-Ala and Arg-R^3-R^2 and the immonium ions, amino acid in position R^2 was identified as Phe for *m/z* 1015.5248, Tyr for 1031.5218 and HtyR in case of 1045.5314. In 981.5405, this position was occupied by either Ile or Leu. Based on this structural data we were able to assign microcystin variants 1015.5248, 1031.5218, and 1045.5314 as [Asp^3]MC-FR, [Asp^3]MC-YR and [Asp^3]MC-HtyR, respectively (Table 3, Fig. 2). The MS/MS data for the fourth variant (*m/z* at 981.5405) were insufficient to fully determine its

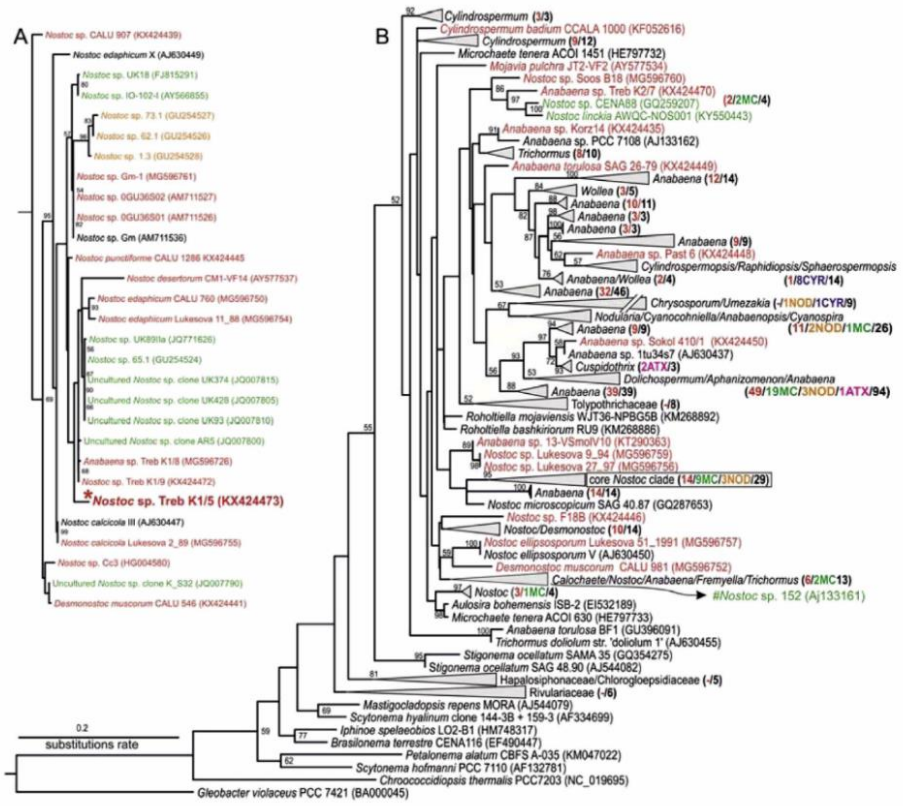


Fig. 1. Phylogenetic tree (Maximum Likelihood) of Nostocales inferred from 16S rRNA gene sequences. (A) Uncollapsed core *Nostoc* clade with strain *Nostoc* sp. Treb K1/5, new microcystin producer from this study (red, bold, asterisk). (B) Collapsed tree of all the strains used for phylogentic analysis, with indication of *Nostoc* sp. 152. The tree is rooted with *Gloeobacter violaceus* PCC 7421. Bootstrap values (1000 pseudo-replications) > 50% are given near the nodes. Strains screened for toxic potential (PCR) and toxin production (HPLC-ESI-HRMS/MS) are in red with indication of number of studied strains included in collapsed clades (first, red value in brackets). The following colors and abbreviations indicate the positions and number of strains with known toxin synthetase genes (taken from the NCBI nucleotide collection): green and MC – microcystin, orange and NOD – nodularin, blue and CYR – cylindrospermopsin, pink and ATX – anatoxin-a. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

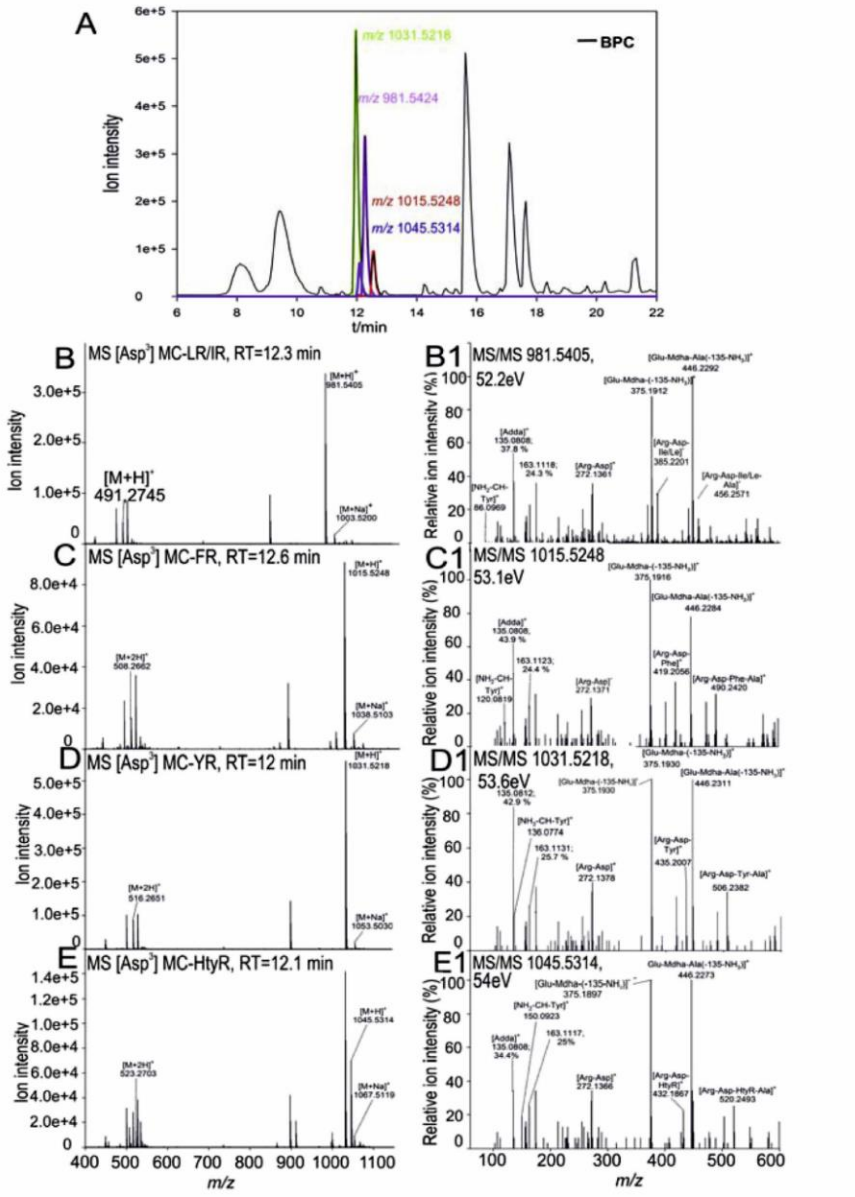
structure, since Leu/Ile was impossible to distinguish using our method.

None of the 311 studied strains was positive in the production of microcystins containing modified Adda variants. Since we included characteristic fragments of all known Adda structural variants and our methods were successful in detecting of these variants in the microcystin producing reference strain *Nostoc* sp. 152 (see Supplementary Fig. S1), we can conclude that, except for *Nostoc* sp. Treb K1/5, there was no other microcystin producer.

Potential producers of anatoxin-a and its analogues, cylindrospermopsin, and nodularin were also studied by PCR amplification of the corresponding synthetase genes. No targeted PCR products for *ana*C, *cyr*J, or *nda*F were obtained in any of the studied strains. Consistent with that, no actual production of anatoxin-a, cylindrospermopsin or nodularin was detected by HPLC-ESI-HRMS/MS analysis based on the presence of their characteristic fragments (Table 2).

3.2. Phylogenetic analysis

Members of the Nostocaceae family, including all tested strains (267 strains in total with 16S rRNA data available), were resolved in the most derived lineage of heterocytous cyanobacteria, together with representatives of the Tolypothrichaceae family (Fig. 1B). Although the majority of strains analyzed in this study belonged to the morphologically defined genus *Anabaena* (224 strains), they were extremely polyphyletic, clustering into more than ten different clades. Similarly, *Nostoc* (38 strains) appeared in four different clusters, although they were more closely related to each other than the clusters of *Anabaena*. Tested strains of several other genera were monophyletic (*Desmonostoc* – 5 strains, *Nodularia* – 13 strains, *Trichormus* – 8 strains), while others



(caption on next page)

Fig. 2. Production of microcystin variants by the strain *Nostoc* sp. Treb K1/5, as evidenced by HPLC-ESI-HRMS/MS analysis. Chromatographic peaks of protonated molecules corresponding to particular microcystin variants are shown as an extracted ion chromatogram (A). All protonated molecules, double charged molecular ions and sodium adducts are presented in MS spectra (B, C, D, E). B1, C1, D1, E1- Adda fragment ions and the most important fragments of the microcystin variants in their MS/MS spectra. Retention time (RT) and the collision energy is given in the left upper corner. BPC - base beak chromatogram, EIC – extracted ion chromatogram, 981.5405- [Asp^3]MC-LR/IR, 1015.5248 - [Asp^3]MC-FR, 1031.5218 -[Asp^3]MC-YR and, 1045.5314 - [Asp^3]MC-HtyR.

were restricted to a few related clades (*Cylindrospermum* – 14 strains, *Wollea* – 5 strains). A single strain was tested for *Mojavia*. Two strains (Treb K2-9 and Past 3) could not be identified at the genus level based on either morphology or phylogenetic position. The strains analyzed for toxic potential and toxin production covered most of the nostocacean evolutionary lineages. Many were also recovered inside, or sister to clades containing strains known to possess toxin synthetase genes, such as *Dolichospermum* and several *Nostoc* lineages (microcystin, nodularin, and anatoxin-a genes), *Nodularia* (nodularin), *Cylindrospermopsis/Raphidiopsis* and *Chrysosporum/Umezakia* (cylindrospermopsin), and *Cuspidothrix* (anatoxin-a) (Fig. 1B and Fig. S2). The single microcystin-producing strain discovered in our study, *Nostoc* sp. Treb K1/5, clustered inside the core *Nostoc* clade. This clade contained also other strains producing microcystins and nodularins, mostly *Nostoc* lichen cyanobionts (Fig. 1A).

4. Discussion

Despite widely reported production of cyanotoxins in non-planktic cyanobacteria a surprisingly low occurrence of microcystins, nodularin, cylindrospermopsin, and (homo)anatoxin-a was observed in our study. Among the 311 tested strains, only one, *Nostoc* sp. Treb K1/5, showed the presence of *mcy*E gene, which is involved in the synthesis of microcystins. Three out of four microcystin variants produced by this strain were unambiguously identified as [Asp^3]MC-FR, [Asp^3]MC-YR and [Asp^3]MC-HtyR using HPLC-ESI-HRMS/MS methods and employing thiol derivatization to distinguish the amino-acid at position 7 (Miles et al., 2012). These microcystin variants were previously reported only in planktic cyanobacteria of genera *Microcystis*, *Dolichospermum*, and *Planktothrix* (Kenichi Harada et al., 1991; Welker et al., 2004, Puddick et al., 2014). Our study described these variants production for the first time from *Nostoc* strain. Some *Nostoc* strains were previously shown to produce microcystins including the frequently occurring variants such as MCYST-LR and WR (Bajpai et al., 2009; Oudra et al., 2009; Genuário et al., 2010) and microcystins bearing acetylation on ADDA moiety (Sivonen et al., 1992; Oksanen et al., 2004; Kaasalainen et al., 2012).

Our study included in total 46 strains of *Nostoc* and the recently split genera *Desmonostoc* and *Mojavia* (Řeháková et al., 2007; Hrouzek et al., 2013), covering at least six different lineages across the phylogenetic tree (Fig. 1B and Fig. S2). The new microcystin producer *Nostoc* sp. Treb K1/5 clustered inside the core *Nostoc* lineage (Fig. 1B), which contains both symbiotic and free-living isolates (Papaefthimiou et al., 2008). *Nostoc* cyanobionts from this lineage were repeatedly reported to produce microcystins and nodularins (Oksanen et al., 2004; Kaasalainen et al., 2009, 2012; Gehringer et al., 2010). The most comprehensive of these reports demonstrated high frequency of microcystin producers among lichen cyanobionts, with approximately 12% of 803 tested strains exhibiting the ability to produce microcystins (Kaasalainen et al., 2012). In contrary, reports of free-living MCYST producing *Nostoc* strains have been relatively scarce and thus far restricted to lineages distant from the core *Nostoc* clade (Bajpai et al., 2009; Genuário et al., 2010, Kurmayer, 2011).

Besides *Nostoc*, our study included a broad array of *Anabaena*-like morphotypes (223 strains) that were spread over more than ten unrelated clades based on phylogenetic analysis (Fig. 1B and Fig. S2). Our results indicate the existence of a number of cryptic genera within the non-planktic *Anabaena*, which is in accordance with analogous reports of morphologically cryptic cyanobacterial taxa generated by serial convergence events (Dvořák et al., 2015; Shalygin et al., 2017). However, none of the multiple cryptic lineages within the *Anabaena*-complex yielded positive hits in the presented toxin screening. This is intriguing considering that the closely allied planktic *Anabaena*-like genera, especially *Dolichospermum* (Fig. 1B and Fig. S2), are recognized worldwide as producers of microcystins, anatoxin, and cylindrospermopsin (reviewed in Li et al., 2016). In the past, screening studies of non-planktic *Anabaena* frequently demonstrated production of a variety of unusual bioactive secondary compounds (Surakka et al., 2005; Oftedal et al., 2012; Jokela et al., 2012; Urajová et al., 2016), while only few reports on production of the notoriously known cyanotoxins by *Anabaena* are available (Mohamed et al., 2006; Halinen et al., 2008; Bouma-Gregson et al., 2017). Their obvious scarcity in both our study and previous studies indicates that the composition of bioactive secondary metabolites consistently differs between planktic and non-planktic *Anabaena*-like strains.

A similar pattern was observed in planktic and non-planktic *Nodularia* species. While *Nodularia* planktic blooms are often highly toxic due to the production of nodularin (e.g., Laamanen et al., 2001), benthic and soil representatives have been, up to now, rarely recognized as nodularin producers (Řeháková et al., 2014). Among the tested strains, 13 soil and benthic *Nodularia* isolates were examined, with no positive results.

To our knowledge, no systematic study has examined toxic potential and cyanotoxin production in other common benthic and soil taxa such as *Cylindrospermum*, *Trichormus*, and *Wollea*. Although the number of tested strains of these genera was limited in the current study, no

Table 3
Microcystin variants detected in *Nostoc* sp. Treb K1/5 showing their *m/z*, molecular formula, retention time (RT) and percentage of Adda and modified Adda fragments.

Compound	MS		MS/MS (Adda fragments)			Reference
m/z (error-ppm)	Molecular Formula (predicted amino acid sequence)	RT (min)	*m/z* 163.11	*m/z* 135.08	*m/z* 265.15	
1031.5218 (-4.5)	[Asp^3]MC-YR (Ala-Tyr-Asp-Arg-Adda-Glu-Mdha)	12	163.1131; **25.7%**	135.0812; **42.9%**	265.1610; **11.4%**	Niedermeyer et al., 2014
981.5405 (-0.6)	$C_{48}H_{72}N_{10}O_{12}$ (Ala-Leu/Ile-Asp-Arg-Adda-Glu-Mdha)	12.3	163.1118; **24.3%**	135.0808; **37.8%**	265.1589; **8.1%**	
1015.5248 (-0.1)	[Asp^3]MC-FR (Ala-Phe-Asp-Arg-Adda-Mdha)	12.6	163.1123; **24.4%**	135.0808; **43.9%**	265.1540; **4.9%**	Chen et al., 2006
1045.5314 (-3.8)	[Asp^3]MC-HtyR (Ala-hTyr-Asp-Arg-Adda-Glu- Mdha)	12.1	163.1117; **25%**	135.0808; **34.4%**	265.1593; **9.4%**	Miles et al., 2013

All the percentages describe relative intensity.

cyanotoxin producers were identified, which was consistent with the general pattern observed in better represented taxa.

Our results indicate that the commonly reported cyanotoxins in planktic heterocytous cyanobacteria are almost absent in their non-planktic counterparts. However, based on other studies, non-planktic Nostocaceae are a rich source of unknown secondary metabolites. This situation presents a potential threat that remains uncovered by routine monitoring and deserves further investigation.

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References

Bajpai, R., Sharma, N.K., Lawton, L.A., Edwards, C., Rai, A.K., 2009. Microcystin producing cyanobacterium *Nostoc* sp. BHU001 from a pond in India. Toxicon 53, 587–590.

Bohunická, M., Mareš, J., Hrouzek, P., Urajová, P., Lukeš, M., Šmarda, J., Komárek, J., Gaysina, L.A., Strunecký, O., 2015. A combined morphological, ultrastructural, molecular and biochemical study of the peculiar family Gomontiellaceae (Oscillatoriales) reveals a new cylindrospermopsin-producing clade of cyanobacteria. J. Phycol. 51, 1040–1054.

Bouma-Gregson, K., Power, M.E., Bormans, M., 2017. Rise and fall of toxic benthic freshwater cyanobacteria (*Anabaena* spp.) in the Eel river: Buoyancy and dispersal. Harmful Algae 66, 79–87.

Boyer, S.R., Flechtner, V.R., Johansen, J.R., 2001. Is the 16S–23S rRNA internal transcribed spacer region a good tool for use in molecular systematics and population genetics? A case study in cyanobacteria. Mol. Biol. Evol. 18, 1057–1069.

Burland, T.G., 1999. DNASTAR's Lasergene sequence analysis software. Method Mol. Biol. 132, 71–91.

Chen, Y.M., Lee, T.H., Lee, S.J., Huang, H.B., Huang, R., Chou, H.N., 2006. Comparison of protein phosphatase inhibition activities and mouse toxicities of microcystins. Toxicon 47, 742–746.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9 772–772.

Dell'Aversano, C., Eaglesham, G.K., Quilliam, M.A., 2004. Analysis of cyanobacterial toxins by hydrophilic interaction liquid chromatography-mass spectrometry. J. Chromatogr. A 1028, 155–164.

Dvořák, P., Casamatta, D.A., Hašler, P., Poulíčková, A., 2015. Putative extremely long evolutionary stasis in bacteria might be explained by serial convergence. Proc. Natl. Acad. Sci. U. S. A. 112 E2559-E2559.

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

Furey, A., Crowley, J., Lehane, M., James, K.J., 2003. Liquid chromatography with electrospray ion-trap mass spectrometry for the determination of anatoxins in cyanobacteria and drinking water. Rapid Commun. Mass Spectrom. 17, 583–588.

Gehringer, M.M., Pengelly, J.J.L., Cuddy, W.S., Fieker, C., Forster, P.I., Neilan, B.A., 2010. Host selection of symbiotic cyanobacteria in 31 species of the Australian cycad Genus: *Macrozamia* (Zamiaceae). Mol. Plant-Microbe Interact. 23, 811–822.

Genuário, D.B., Silva-Stenico, M.E., Welker, M., Beraldo Moraes, L.A., Fiore, M.F., 2010. Characterization of a microcystin and detection of microcystin synthetase genes from a Brazilian isolate of *Nostoc*. Toxicon 55, 846–854.

Gugger, M., Lenoir, S., Berger, C., Ledreux, A., Druart, J.C., Humbert, J.F., Guette, C., Bernard, C., 2005. First report in a river in France of the benthic cyanobacterium *Phormidium favosum* producing anatoxin-a associated with dog neurotoxicosis. Toxicon 45, 919–928.

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

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.

Guzmán-Guillén, R., Prieto, A.I., González, A.G., Soria-Díaz, M.E., Cameán, A.M., 2012. Cylindrospermopsin determination in water by LC-MS/MS: optimization and validation of the method and application to real samples. Environ. Toxicol. Chem. 31, 2233–2238.

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

Hrouzek, P., Lukešová, A., Mareš, J., Ventura, S., 2013. Description of the cyanobacterial genus *Desmonostoc* gen. nov. including *D. muscorum* comb. nov. as a distinct, phylogenetically coherent taxon related to the genus *Nostoc*. Fottea 13, 201–213.

Izaguirre, G., Jungblut, A.D., Neilan, B.A., 2007. Benthic cyanobacteria (Oscillatoriaceae) that produce microcystin-LR, isolated from four reservoirs in southern California. Water Res. 41, 492–498.

Jokela, J., Oftedal, L., Herfindal, L., Permi, P., Wahlsten, M., Doskeland, S.O., Sivonen, K., 2012. Anabaenolysins, novel cytolytic lipopeptides from benthic *Anabaena* cyanobacteria. PLOS One 7, e41222.

Jones, A.C., Monroe, E.A., Eisman, E.B., Gerwick, L., Sherman, D.H., Gerwick, W.H., 2010. The unique mechanistic transformations involved in the biosynthesis of modular natural products from marine cyanobacteria. Nat. Prod. Rep. 27, 1048–1065.

Kaasalainen, U., Jokela, J., Fewer, D.P., Sivonen, K., Rikkinen, J., 2009. Microcystin production in the tripartite cyanolichen *Peltigera leucophlebia*. Mol. Plant-Microbe Interact. 22, 695–702.

Kaasalainen, U., Fewer, D.P., Jokela, J., Wahlsten, M., Sivonen, K., Rikkinen, J., 2012. Cyanobacteria produce a high variety of hepatotoxic peptides in lichen symbiosis. Proc. Natl. Acad. Sci. U. S. A. 109, 5886–5891.

Katoh, K., Standley, D.M., 2013. MAFFT Multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780.

Kenichi Harada, K., Ogawa, K., Kimura, Y., Murata, H., Suzuki, M., Thorn, P.M., Evans, W.R., Carmichael, W.W., 1991. Microcystins from *Anabaena flos-aquae* NRC 525-17. Chem. Res. Toxicol. 4, 535–540.

Komárek, J., 2013. Cyanoprokaryota. 3. Heterocytous genera. In: Büdel, B., Gärtner, G., Krienitz, L., Schagerl, M. (Eds.), Süsswasserflora von Mitteleuropa/Freshwater flora of Central Europe. Springer Spektrum, pp. 1130.

Koskenniemi, K., Lyra, C., Rajaniemi-Wacklin, P., Jokela, J., Sivonen, K., 2007. Quantitative real-time PCR detection of toxic *Nodularia* cyanobacteria in the Baltic sea. Appl. Environ. Microbiol. 73, 2173–2179.

Kurmayer, R., 2011. The toxic cyanobacterium *Nostoc* sp. strain 152 produces highest amounts of microcystin and nostophycin under stress conditions. J. Phycol. 47, 200–207.

Laamanen, M.J., Gugger, M.F., Lehtimaki, J.M., Haukka, K., Sivonen, K., 2001. Diversity of toxic and nontoxic *Nodularia* isolates (cyanobacteria) and filaments from the baltic sea. Appl. Environ. Microbiol. 67, 4638–4647.

Li, X., Dreher, T.W., Li, R., 2016. An overview of diversity, occurrence, genetics and toxin production of bloom-forming *Dolichospermum* (*Anabaena*) species. Harmful Algae 54, 54–68.

Mazmouz, R., Chapuis-Hugon, F., Mann, S., Pichon, V., Méjean, A., Ploux, O., 2010. Biosynthesis of cylindrospermopsin and 7-epicylindrospermopsin in *Oscillatoria* sp. strain PCC 6506: identification of the *cyr* gene cluster and toxin analysis. Appl. Environ. Microbiol. 76, 4943–4949.

Mazur-Marzec, H., Meriluoto, J., Pliński, M., Szafranek, J., 2006. Characterization of nodularin variants in *Nodularia spumigena* from the Baltic Sea using liquid chromatography/mass spectrometry/mass spectrometry. Rapid Commun. Mass Spectrom. 20, 2023–2032.

Miles, C.O., Sandvik, M., Nonga, H.E., Rundberget, T., Wilkins, A.L., Rise, F., Ballot, A., 2012. Thiol derivatization for LC-MS identification of microcystins in complex matrices. Environ. Sci. Technol. 46, 8937–8944.

Miles, C.O., Sandvik, M., Haande, S., Nonga, H.E., Ballot, A., 2013. Lc-ms analysis with thiol derivatization to differentiate Dhb(7) - from Mdha(7) -microcystins: analysis of cyanobacterial blooms, *Planktothrix* cultures and European crayfish from lake Steinsfjorden, *Norway*. Environ. Sci. Technol. 47, 4080–4087.

Miller, M.A., Schwartz, T., Pickett, B.E., He, S., Klem, E.B., Scheuermann, R.H., Passarotti, M., Kaufman, S., O'Leary, M.A., 2015. A RESTful API for access to phylogenetic tools via the CIPRES science gateway. Evol. Bioinf. 11, 43–48.

Mohamed, Z.A., El-Sharouny, H.M., Ali, W.S.M., 2006. Microcystin production in benthic mats of cyanobacteria in the Nile River and irrigation canals, Egypt. Toxicon 47, 584–590.

Niedermeyer, T.H.J., Daily, A., Swiatecka-Hagenbruch, M., Moscow, J.A., 2014. Selectivity and potency of microcystin congeners against OATP1B1 and OATP1B3 expressing cancer cells. PLoS One 9, e91476.

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

Oftedal, L., Myhren, L., Jokela, J., Gausdal, G., Sivonen, K., Doskeland, S.O., Herfindal, L., 2012. The lipopeptide toxins anabaenolysin A and B target biological membranes in a cholesterol-dependent manner. Biochim. Biophys. Acta, Biomembr. 818, 3000–3009.

Oksanen, I., Jokela, J., Fewer, D.P., 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.

Oudra, B., Dadi-El Andaloussi, M., Vasconcelos, V.M., 2009. Identification and quantification of microcystins from a *Nostoc muscorum* bloom occurring in Oukaïmeden River

(High-Atlas mountains of Marrakech, Morocco). Environ. Monit. Assess. 149, 437–444.
Papaefthimiou, D., Hrouzek, P., Mugnai, M.A., Lukešová, A., Turicchia, S., Rasmussen, U., Ventura, S., 2008. Differential patterns of evolution and distribution of the symbiotic behaviour in nostocacean cyanobacteria. Int. J. Syst. Evol. Microbiol. 58, 553–564.
Puddick, J., Prinsep, M.R., Wood, S.A., Kaufononga, A.F.S., Cary, S.C., Hamilton, D.P., 2014. High levels of structural diversity observed in microcystins from *Microcystis* CAWBG11 and characterization of six new microcystin congeners. Mar. Drugs 12, 5372–5395.
Puddick, J., Prinsep, M.R., Wood, S.A., Cary, S.C., Hamilton, D.P., Holland, P.T., 2015. Further characterization of glycine-containing microcystins from the McMurdo dry valleys of Antarctica. Toxins 7, 493–515.
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.
Rantala-Ylinen, A., Känä, S., Wang, H., Rouhiainen, L., Wahlsten, M., Rizzi, E., Berg, K., Gugger, M., Sivonen, K., 2011. Anatoxin-a synthetase gene cluster of the cyanobacterium *Anabaena* sp. strain 37 and molecular methods to detect potential producers. Appl. Environ. Microbiol. 77, 7271–7278.
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.
Selwood, A.I., Holland, P.T., Wood, S.A., Smith, K.F., McNabb, P.S., 2007. Production of anatoxin-a and a novel biosynthetic precursor by the cyanobacterium *Aphanizomenon issatschenkoi*. Environ. Sci. Technol. 41, 506–510.
Shalygin, S., Shalygina, R., Johansen, J.R., Pietrasiak, N., Gomez, E.B., Bohunická, M., Mareš, J., Sheil, C.A., 2017. *Cyanomargarita* gen. nov. (Nostocales, Cyanobacteria): convergent evolution in a cryptic genus. J. Phycol. 53, 762–777.
Sivonen, K., Namikoshi, M., Evans, W.R., Fardig, M., Carmichael, W.W., Rinehart, K.L., 1992. Three new microcystins, cyclic heptapeptide hepatotoxins, from *Nostoc* sp. strain 152. Chem. Res. Toxicol. 5, 464–469.
Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312–1313.
Surakka, A., Sihvonen, L.M., Lehtimaki, J.M., 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.
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, 5157–5169.
Urajová, P., Hájek, J., Wahlsten, M., Jokela, J., Galica, T., Fewer, D.P., Kust, A., Zapomělová-Kozlíková, E., Delawská, K., Sivonen, K., Kopecký, J., Hrouzek, P., 2016. A liquid chromatography-mass spectrometric method for the detection of cyclic beta-amino fatty acid lipopeptides. J. Chromatogr. A 1438, 76–83.
Vaitomaa, J., Rantala, A., Halinen, K., Rouhiainen, L., Tallberg, P., Mokelke, L., Sivonen, K., 2003. Quantitative real-time PCR for determination of microcystin synthetase E copy numbers for *Microcystis* and *Anabaena* in lakes. Appl. Environ. Microbiol. 69, 7289–7297.
Welker, M., Christiansen, G., von Döhren, H., 2004. Diversity of coexisting *Planktothrix* (Cyanobacteria) chemotypes deduced by mass spectral analysis of microystins and other oligopeptides. Arch. Microbiol. 182, 288–298.
Wilmotte, A., Van der Auwera, G., Dewachter, R., 1993. Structure of the 16S ribosomal-RNA of the thermophilic cyanobacterium *Chlorogloeopsis* HTF (*Mastigocladus laminosus* HTF) strain PCC 7518, and phylogenetic analysis. FEBS Lett. 317, 96–100.
Wood, S.A., Selwood, A.I., Rueckert, A., Holland, P.T., Milne, J.R., Smith, K.F., Smits, B., Watts, L.F., Cary, C.S., 2007. First report of homoanatoxin-a and associated dog neurotoxicosis in New Zealand. Toxicon 50, 292–301.
Wood, S.A., Heath, M.W., Holland, P.T., Munday, R., McGregor, G.B., Ryan, K.G., 2010a. Identification of a benthic microcystin-producing filamentous cyanobacterium (Oscillatoriales) associated with a dog poisoning in New Zealand. Toxicon 55, 897–903.
Wood, S.A., Heath, M.W., Kuhajek, J., Ryan, K.G., 2010b. Fine-scale spatial variability in anatoxin-a and homoanatoxin-a concentrations in benthic cyanobacterial mats: implication for monitoring and management. J. Appl. Microbiol. 109, 2011–2018.
Yilmaz, M., Phlips, E.J., Tillett, D., 2009. Improved methods for the isolation of cyanobacterial DNA from environmental samples. J. Phycol. 45, 517–521.
Yuan, M., Namikoshi, M., Otsuki, A., Sivonen, K., 1998. Effect of amino acid side-chain on fragmentation of cyclic peptide ions: differences of ESI-MS/CID mass spectra of toxic heptapeptide microcystins containing ADMAdda instead of Adda. Eur. J. Mass Spectrom. 4, 287–298.
Řeháková, K., Johansen, J.R., Casamatta, D.A., Xueson, 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.
Řeháková, K., Mareš, J., Lukešová, A., Zapomělová, E., Bernardová, K., Hrouzek, P., 2014. *Nodularia* (Cyanobacteria, Nostocaceae): a phylogenetically uniform genus with variable phenotypes. Phytotaxa 172, 235–246.

Paper IV.

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Letters

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Discovery of a Pederin Family Compound in a Nonsymbiotic Bloom-Forming Cyanobacterium

Andreja Kust,[▽,†,‡,§] Jan Mareš,[▽,†,‡,§] Jouni Jokela,[∥] Petra Urajová,[†] Jan Hájek,[†,§] Kumar Saurav,[†] Kateřina Voráčová,[†] David P. Fewer,[∥] Esa Haapaniemi,[⊥] Perttu Permi,[⊥,#] Klára Řeháková,[‡] Kaarina Sivonen,[∥] and Pavel Hrouzek*[,†,§]

[†]Institute of Microbiology CAS, Center Algatech, Třeboň, Czech Republic

[‡]The Czech Academy of Sciences, Biology Centre, Institute of Hydrobiology, České Budějovice, Czech Republic

[§]University of South Bohemia, Faculty of Science, České Budějovice, Czech Republic

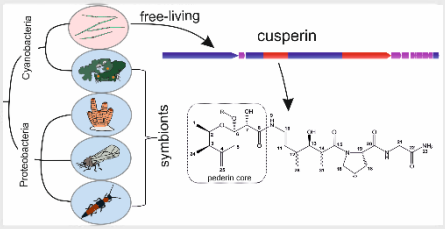
[∥]Department of Microbiology, University of Helsinki, FI-00014 Helsinki, Finland

[⊥]Department of Chemistry, University of Jyväskylä, FI-40010 Jyväskylä, Finland

[#]Department of Biology and Environmental Science, Nanoscience Center, University of Jyväskylä, FI-40010 Jyväskylä, Finland

Supporting Information

ABSTRACT: The pederin family includes a number of bioactive compounds isolated from symbiotic organisms of diverse evolutionary origin. Pederin is linked to beetle-induced dermatitis in humans, and pederin family members possess potent antitumor activity caused by selective inhibition of the eukaryotic ribosome. Their biosynthesis is accomplished by a polyketide/nonribosomal peptide synthetase machinery employing an unusual *trans*-acyltransferase mechanism. Here, we report a novel pederin type compound, cusperin, from the free-living cyanobacterium *Cuspidothrix issatschenkoi* (earlier *Aphanizomenon*). The chemical structure of cusperin is similar to that of nosperin recently isolated from the lichen cyanobiont *Nostoc* sharing the tehrahydropyran moiety and major part of the linear backbone. However, the cusperin molecule is extended by a glycine residue and lacks one hydroxyl substituent. Pederins were previously thought to be exclusive to symbiotic relationships. However, *C. issatschenkoi* is a nonsymbiotic planktonic organism and a frequent component of toxic water blooms. Cusperin is devoid of the cytotoxic activity reported for other pederin family members. Hence, our findings raise questions about the role of pederin analogues in cyanobacteria and broaden the knowledge of ecological distribution of this group of polyketides.



Pederin is a toxin containing two tetrahydropyran cores that causes dermatitis.[1] Pederin was first reported from the beetle *Paederus fuscipes* and widely believed to be produced by eukaryotes.[2,3] However, the discovery of the pederin biosynthetic gene cluster demonstrated that bacterial symbionts of insects, marine invertebrates, and lichens are the true producers of these compounds.[4] Biosynthesis of pederin and its homologues is accomplished by hybrid polyketide/nonribosomal peptide synthases (PKS/NRPS) employing an unusual *trans*-acyltransferase (AT) PKS mechanism,[5] in which the PKS enzymatic modules utilize a common AT encoded in a separate gene. The biosynthesis of all pederin family compounds seems to share the first several steps forming the typical tetrahydropyran moiety attached to an acyl chain with a glycine residue.[6]

Metagenomic sequencing of symbiotic consortia has recently uncovered a large and highly specific repertoire of natural products, particularly in bacterial symbionts of marine sponges.[7,8] Pederins are currently also believed to be specifically associated with prokaryote−eukaryote symbioses as they have been reported exclusively from symbiotic associations.[6,7,9−11] However, a recent discovery of a pederin analogue in a purportedly free-living strains of cyanobacterium *Nostoc* sp. KVJ10[12] and proteobacterium *Labrenzia* sp. PHM005[13] isolated from the soil arond the *Nostoc* hosting moss Blasia and marine sediment, respectively, challenged this hypothesis.

The potential of free-living organisms to produce pederins is further corroborated with our report of a novel pederin analogue, cusperin, identified for the first time in a nonsymbiotic cyanobacterium, *Cuspidothrix issatschenkoi* (Usačev) Rajaniemi *et al. Cuspidothrix* (earlier *Aphanizomenon*) is a frequent bloom-forming species in freshwater bodies,[14] which belongs to the lineage of typical freshwater planktonic heterocytous cyanobacteria, clearly distant from all groups of the typical pederin producers (Figure 1).

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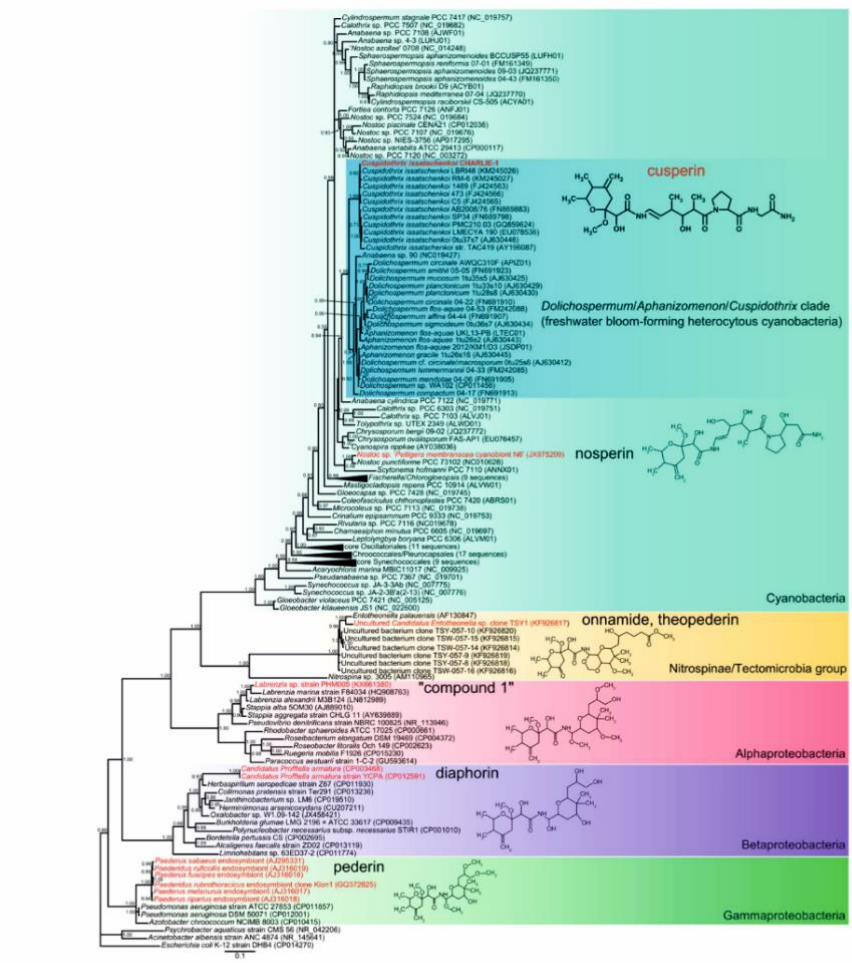

Figure 1. Phylogenetic position of *Cuspidothrix issatschenkoi* CHARLIE-1 among the producers of pederin family compounds. The unrooted tree was inferred from 16S rRNA gene data using Bayesian Inference (BI). BI posterior probabilities are indicated near nodes. The major bacterial lineages containing producers of pederin analogues are color-shaded. The strains producing pederin family compounds are printed in red; representative structures of the products are shown for each group.

Inspection of a draft genome of *C. issatschenkoi* strain CHARLIE-1 led to the identification of a previously unreported PKS/NRPS gene cluster, exhibiting >70% similarity with the nosperin biosynthetic gene cluster.[6] The core region of this biosynthetic gene cluster is almost identical to that reported for pederin compounds (Figure 2a, Supporting Information Tables 1 and 2). The putative biosynthetic gene cluster (~54 kb) consisted of 11 protein coding ORFs: two genes encoding multidomain PKS/NRPS proteins, eight accessory genes, and a single gene encoding a peculiar AT in the *trans* position (Figure 2a). Further evidence suggesting the presence of a *trans*-AT PKS system in the cusperin synthetase is the absence of AT domains in the deduced PKS proteins (Figure 2b). Detailed analysis of the individual components of the gene cluster are provided in the Supporting Information.

Analysis of methanol extracts of *C. issatschenkoi* CHARLIE-1 led to the identification of two putative products of the PKS/NRPS gene cluster. Their chemical structures were elucidated using a combination of high-performance liquid chromatography connected to high resolution mass spectrometer with

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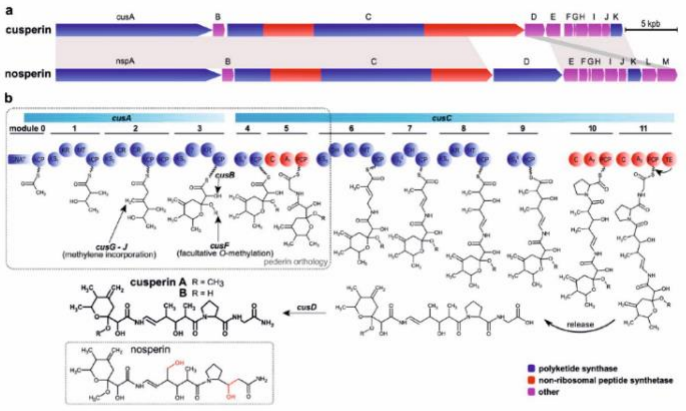

Figure 2. Predicted biosynthetic pathway of cusperin. (a) Gene arrangement of the proposed cusperin biosynthetic gene cluster and comparison to nosperin biosynthetic gene cluster. Homologous regions are indicated by gray shading. (b) Proposed biosynthesis of cusperin A and B is colinear with the arrangement of modules in CusA and CusC. Part of the pathway orthologous to pederin, onnamides, theopederins, and diaphorin is indicated by a dashed box. The structure of nosperin is shown for comparison; differences from cusperin A are highlighted in red color. A, adenylation domain; ACP, acyl carrier protein; C, condensation domain; CR, crotonase (enoyl-CoA hydratase); DH, dehydratase; GNAT, GCN5-related N-acetyltransferase; MT, methyltransferase; NRPS, nonribosomal peptide synthetase; KR, ketoreductase; KS, ketosynthase; PCP, peptidyl carrier protein; PKS, polyketide synthase; TE, thioesterase.

electrospray ionization (HPLC-ESI-HRMS/MS) and nuclear magnetic resonance (NMR) spectroscopy (Figures 3 and 4 Supporting Information Figures 1−6, Supporting Information Tables 3 and 4). A prominent chromatographic peak was obtained by HPLC-MS analysis of *Cuspidothrix* extract

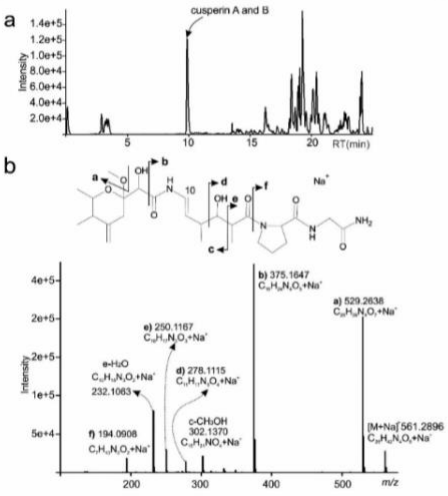

Figure 3. Structural analysis of cusperin A. (a) HPLC-HRMS/MS base peak chromatogram of *Cuspidothrix* crude extract. (b) Fragmentation pattern and HRMS/MS spectrum of cusperin A.

containing a compound detected at m/z = 561.2896 $[M + Na]^+$ with a calculated elemental composition of $C_{26}H_{42}N_4O_8+Na^+$ (Δ 0.2 ppm) corresponding to cusperin A (Figure 3). Its MS/MS exhibited attributes analogous to those reported for nosperin, *e.g.*, the loss of methanol (producing ion a) and the loss of a whole substituted tetrahydropyran moiety (producing ion b) identical to those of nosperin[6] (Figure 3b).

Data from the analysis of ^1H, $^1H-^1H$ NOESY, ^{13}C HSQC, ^{13}C HMBC, and ^{15}N HSQC spectra (Supporting Information Figures 1−5) of cusperin showed high similarity to nosperin NMR data,[6] which proved that their general structure was the same (Supporting Information Table 4). Compared to nosperin, cusperin lacked C-28 hydroxyl. C-20 hydroxyl was changed to carbonyl, and next to it there was an extra amide group (C-21-NH). Due to the lack of the hydroxyl, C-28 showed a methyl signal (δC 19.8, δH 1.00), and this difference affected the nearby shift values (Supporting Information Table 4). Four carbonyl signals were found (Supporting Information Figure 3), one more than in nosperin as one hydroxyl of nosperin is replaced with carbonyl in cusperin. The cusperin NH-9 signal (d, δH 8.40) matched with nosperin (d, δH 8.57). The extra C-21-NH signal (dd, δH 7.11) of cusperin was broad, but the signals' dd structure could be seen, which was characteristic for glycine in peptide structures. ^{15}N-HSQC spectrum showed the δH 7.11 proton connection to a nitrogen atom (Supporting Information Figure 4). The most important ^{13}C-HMBC and $^1H-^1H$ NOESY correlations are shown in Supporting Information Figure 4.

The MS/MS analysis further detected the presence of a second cusperin variant with m/z at 547.2740 $[M + Na]^+$ and calculated elemental composition of $C_{25}H_{40}N_4O_8+Na^+$ (Δ −0.3 ppm). While cusperin A provided ions corresponding to $[M-CH_2OH+H]^+$ in both MS and MS/MS (m/z 507. 2812, Δ 0.3 ppm), cusperin B provided only a $[M-H_2O+H]^+$ fragment (Supporting Information Figure 6). This corresponds to the

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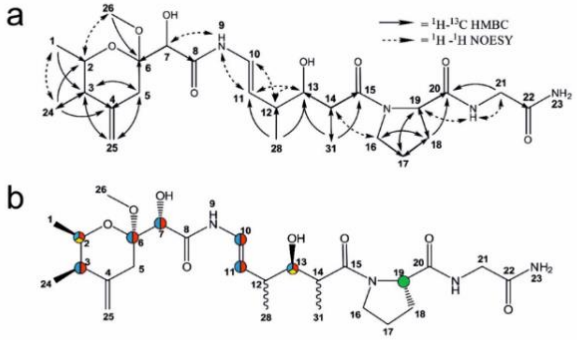

Figure 4. NMR analysis and stereochemical assignment of cusperin A. (a) ^{13}C HMBC (solid arrows) and ^{1}H−^{1}H NOESY (dashed arrows) correlations of cusperin. (b) Stereochemical arrangement of cusperin chiral centers based on NMR and bioinformatic analysis. Blue, domain organization identical to nosperin, supporting a similar arrangement of chiral centers; red, NMR coupling constants and shifts; yellow, ketoreductases (KRs) predicted specificity based on a specific sequence motif (the presence of a conserved Asp residue); green, nonribosomal peptide synthase (NRPS) domain prediction (absence of an epimerase domain).

losses of a methoxy and a hydroxy group from the molecular ion of cusperin A and B, respectively. On the basis of the combination of MS and NMR data, we concluded that cusperin A presents the methylacetal variant (bearing methoxy group on C-6), and cusperin B presents the hemiacetal variant (bearing hydroxy group on C-6). Purification (see Supporting Information Table 5) yielded 0.3 mg of cusperin A and B in a molar ratio of 70:30 (Supporting Information Figure 6).

The origin of the structural differences between cusperin and nosperin as well as the occurrence of cusperin A and B variants were explained by bioinformatic prediction of cusperin biosynthesis (Figure 2b). First, the presence of a terminal glycine in cusperin instead of a reduced biketide was consistent with an additional NRPS module encoded in *cusC* containing an A domain with a predicted substrate specificity for glycine (Supporting Information Table 2). The second difference observed was the lack of the hydroxyl on C-12 in cusperin, explained by the lack of cytochrome-450 like protein responsible for the hydroxylation step in nosperin.[6] Four nitrogen atoms were predicted in the cusperin scaffold based on the presence of three NRPS modules each incorporating a single amino acid and an asparagine synthetase responsible for the final transamination step (Figure 2b). The number of nitrogen atoms was further confirmed by ^{15}N isotopic substitution (m/z at 565.2685, Δ −0.9 ppm). The predicted product of *cusF* was an *O*-methyltransferase, likely involved in facultative *O*-methylation of the hydroxy group at C6, which results in formation of the observed cusperin methylacetal/hemiacetal variants (cusperin A and B). The individual predicted biosynthetic steps are explained in detail in Supporting Information.

The cusperin gene cluster exhibited high similarity in the arrangement of the first five PKS/NRPS modules to those observed in several pederin members (pederin, theopederins, mycalamides, onnamides), which allowed us to infer the absolute configuration at chiral carbons C2, C3, C6, and C7 (Figure 4b). In addition, the arrangement of the first 10 modules was consistent with the nosperin gene cluster, further suggesting the configuration of C10, C11, and C13 are identical to nosperin. Comparison of the NMR coupling constants and shift values (Supporting Information Table 4) further indicates that the stereochemistry is likely identical between these two compounds (Figure 4b). Bioinformatics analysis of the specific sequence motifs of the KR domains has again confirmed D−OH configuration at C2 and C13 based on the presence of a conserved aspartate residue recently demonstrated to play a key role in stereospecificity of the KR domain[15−17] (Supporting Information Table 6). Finally, the lack of the epimerization domain in the NRPS module 10 supported the incorporation of an L-Pro residue as per bioinformatics analysis of pathway A domains. Equivalent to nosperin, C12 and C14 remained without stereochemical assignments.

The strong cytotoxic activity of pederin family members was found due to their specific binding to the large ribosomal subunit.[18] Recent structure−activity studies defined the key features of pederin analogues involved in their action.[19] First, the presence of alkoxy or hydroxy substitution at C10 facilitates the molecule rotation and subsequent binding, while the lack of any substituent at this position leads to diminished activity.[19] Additionally, the presence of a methoxy group at C13 is favored over the hydroxyl group. In comparison to other natural pederin analogues, cusperin A and B exhibited only weak cytotoxic activity (Supporting Information Table 7). The cusperin molecule features both traits, which do not favor the interaction with ribosome as C10 is not functionalized and C13 bears the less efficient −OH group.

The bioactivity of cusperin demonstrates that not all natural pederin analogues exert high cytotoxic activity, and thus they do not act generally and exclusively as ribosome poisons. Interestingly, high production levels of cusperin A and B were recorded in the *Cuspidothrix* culture (up to 2.5 mg/g of dry weight). With regard to the high content and easy isolation protocol, discovery of cusperin opens up an opportunity to address the ecological role of this type of compound.

Cuspidothrix, a bloom-forming cyanobacterium which has the potential to produce compounds that are a threat to human health,[20] has been characterized as the first free-living cyanobacterium producing a pederin-type compound, cusperin.

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Our findings contradict the recently suggested restricted occurrence of pederin family members in symbiotic bacteria and dispute their general role directly associated with specific symbioses between eukaryotes and bacteria.[6] Cusperin production may however play a role in other microbe−microbe relationships occurring in freshwater blooms that are currently not well characterized. The selective advantage for the production of a pederin family compound by a nonsymbiotic organism remains unknown, and further studies are needed to decipher its possible ecological significance, especially as cytotoxic activity is weak compared to other pederins.

METHODS

Cultivation of the Strain. *C. issatschenkoi* CHARLIE-1 was isolated from a water bloom sample collected from fishpond Papež near Dobříš, Czech Republic. A monoclonal culture was established from a single filament and maintained in the culture collection of the Biology Centre of CAS, Institute of Hydrobiology in 250 mL Erlenmeyer flasks with WC medium[21] at 21 °C and a light intensity of 50 μmol m^{-2} s^{-1} (16:8 L/D cycle). For the isolation procedure, the batch culture of the strain was grown in 10 L Erlenmeyer flasks using BG-11 medium bubbled with 1.5% CO_2 enriched air at 25 °C with an illumination of 100 μmol m^{-2} s^{-1}. The biomass for isolation of ^{15}N isotopically substituted cusperin was cultivated under the same cultivation conditions in BG-11 medium supplemented with $Na^{15}NO_3$ (Sigma-Aldrich, Cat. No. 364606), and the culture was reinoculated twice, each time after 20 days of cultivation period. Strain identification was performed based on morphological characters[22] and position in a phylogenetic tree based on the 16S rRNA gene.

Phylogenetic Analysis. The 16S rRNA gene sequences of pederin family compound producers available through NCBI and the cusperin producer *C. issatschenkoi* CHARLIE-1 were aligned with a set of closest BLAST hits, representatives of the corresponding bacterial phyla, and a representative set of cyanobacterial sequences. A phylogenetic tree was constructed using the Bayesian inference. See the Supporting Information for details.

Genomic and Bioinformatic Analysis. Single filaments of *C. issatschenkoi* CHARLIE-1 were isolated using the glass capillary technique and prepared for whole genome sequencing as described previously.[23] The filaments were utilized as a template for whole-genome amplification by multiple-displacement amplification (MDA) using a Repli-G Mini Kit (Qiagen). Sixteen MDA products that passed the quality check were pooled and sent for commercial *de novo* genome sequencing (EMBL Genomics Core Facility, Heidelberg, Germany) using an Illumina MiSeq Pair-End library with 250 bp reads, 350 bp average insert length, and 1.4 Gbp data yield. The data from Illumina were assembled using CLC Bio Genomics Workbench v. 10.5 (Qiagen). The genomic assembly is available under NCBI accession number PGEM00000000.1, and the cusperin biosynthetic gene cluster is available under accession number MG518226.

Genomic scaffolds were investigated using BLASTp in the Geneious Pro R10 (Biomatters) software package to identify putative PKS/NRPS gene clusters, employing cyanobacterial A domains and KS domains as queries. In target genomic regions, open reading frames were predicted (Glimmer 3).[24] The deduced proteins were functionally annotated (BLASTp and CDD searches), and in the putative PKS/NRPS proteins individual enzymatic domains were identified (antiSMASH 4.0).[25] The substrate specificities of KS domains in the *trans*-AT PKS modules were predicted based on similarity to the closest functionally characterized hits in the BLASTp analysis (Supporting Information Table 2), which were unequivocally represented by KS domains from the nosperin pathway (analyzed by phylogenetics in a previous study[9]). The predicted specificity of NRPS adenylation domains was inferred using a combination of tools implemented in antiSMASH 4.0 (Stachelhaus code, NRPS Predictor3, pHMM search). A biosynthetic scheme was constructed based on the functional annotation of the deduced PKS/NRPS domains and accessory enzymes, and by comparing to known biosynthesis pathways of pederin-family compounds. Stereochemical assignment of cusperin was assessed based on NMR data, specific KR sequence motifs,[15−17] and overall arangement of NRPS/PKS modules.[15]

HPLC-ESI-HRMS. The crude extract of *C. issatschenkoi* CHARLIE-1 was analyzed using a high-performance liquid chromatograph (Dionex UltiMate 3000 UHPLC+ (Thermo Scientific, Sunnyvale, CA, USA)) connected to a Bruker Impact HD high resolution mass spectrometer (Bruker, Billerica, Massachusetts, USA) with electrospray ionization in positive mode. Separation was performed on an RP-C18 column (Phenomenex Kinetex, 2.6 μm; 4.6 × 150, Torrance, CA, USA) using H_2O (A) and acetonitrile (B) (both containing 0.1% HCOOH) as a mobile phase at a flow rate of 0.5 mL min^{-1}. The linear gradient was as follows: A/B 85/15 (0 min), 85/15 (in 1 min), 0/100 (in 19 min), 0/100 (in 25 min), and 85/15 (in 30 min). The following settings of the mass spectrometer were used: drying temperature, 200 °C; drying gas flow, 12 L min^{-1}; nebulizer gas pressure, 3 bar; capillary voltage, 3.8 kV; end plate offset, 500 V. Spectra were collected in the range m/z 20−2000 with a spectra rate of 2 Hz. Automated fragmentation was used in the method with a precursor ion selection of 20−2000 m/z for an isolation window width of 1 Da. The ramping of the collision energy was based on the molecular mass: 20 eV at 400 m/z and 70 eV at 1000 m/z for analysis. The mass spectrometer was calibrated with sodium formate clusters at the beginning of each analysis. Further, the lock mass calibration was applied during the analysis using hexakis (1*H*,1*H*,2*H*-perfluoroethoxy) phosphazene (ES-TOF m/z 622.02896, 97%) purchased from ABCR GmbH & Co., KG, Germany. Quantification of cusperin A and B in the *C. issatschenkoi* CHARLIE-1 extract was performed using the method described above with purified compounds as external standards (concentrations 2.5, 5, 10, 25, 50, and 100 μg mL^{-1} were used to establish the calibration curve). See the Supporting Information for details.

Extraction, Purification, and Quantification. Freeze-dried biomass of *C. issatschenkoi* CHARLIE-1 was extracted twice with 400 mL methanol/water (40/60, v/v) using bath sonicator, resulting supernatants were combined and partially evaporated on rotary evaporator and remaining solvent was diluted by distilled water so that the final MeOH concentration did not exceed 5%. Further the sample was loaded on DCS 18 SPE cartridge and retained compounds were eluted with MeOH. Eluent was evaporated until dryness and reconstituted in 10 mL of MeOH prior the high-performance liquid chromatography (HPLC) purification. The compounds were purified using three consecutive preparative HPLC separation steps (for detailed information on the purification procedure see the Supporting Information).

Nuclear Magnetic Resonance Spectroscopy. All NMR spectra were collected using a Bruker Avance III HD 800 MHz NMR spectrometer, equipped with a cryogenically cooled TCI ^1H, ^{13}C, ^{15}N triple resonance probehead. Data were collected at 25 °C. For the assignment of cusperin ^1H, ^{13}C, and ^{15}N resonances, a ^1H preset experiment along with two-dimensional total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY) experiments as well as heteronuclear single quantum coherence (^{13}C HSQC and ^{15}N HSQC) and heteronuclear multiple bond correlation (^{13}C HMBC) experiments were employed. For details of the NMR measurement, please see the Supporting Information.

Cytotoxicity Testing. In order to test the antiproliferative and cytotoxic activity usually reported for pederin family members, cusperin bioactivity was tested on human cervical cancer cells (HeLa). For cell maintenance and plating before the experiment, see the Supporting Information. Serial dilutions (in the range from 0.5−20 μM) of cusperin A and B variants were added as technical triplicates, so the concentration of the vehicle (MeOH) did not exceed 1%. The viability was determined after 72 h of exposure. To assess cell viability, we used three end-point methods: the ATP content was measured using the CellTiter-Glo Luminescent Viability Assay (Promega, G7570), MTT assay, and determination of cell counts (384-well plates) of treated and control cells at 72 h by optical microscopy. The output data represent results of three independent experiments. For a detailed method description, see the Supporting Information.

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■ ASSOCIATED CONTENT

Supporting Information

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Additional details on methods and results concerning the phylogenetic analysis, genomic and bioinformatic analysis, HPLC-MS/MS, NMR and cytotoxicity tests; Supporting Figures 1−6; and Supporting Tables 1−7 (PDF)

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■ AUTHOR INFORMATION

Corresponding Author

*Tel.: +420-384-340-470. Fax: +420-384-340-415. E-mail: hrouzekp@gmail.com.

ORCID

Andreja Kust: 0000-0003-4016-6454
Jouni Jokela: 0000-0001-5096-3575
David P. Fewer: 0000-0003-3978-4845
Kaarina Sivonen: 0000-0002-2904-0458
Pavel Hrouzek: 0000-0002-2061-0266

Author Contributions

[▽]These authors contributed equally to this work

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Frank, J.-H., and Kanamitsu, K. (1987) *Paederus*, sensu lato (Coleoptera: Staphylinidae): natural history and medical importance. *J. Med. Entomol. 24*, 155−191.

(2) Ueta, A. (1949) *J. Kurume. Med. College, Kyushu*, 2489.

(3) Pavan, M., and Bo, G. (1953) Pederin, toxic principles obtained in the crystalline state from the beetle *Paederus fuscipes* Curt. *Physiol. Comp. Oecol. 3*, 307−312.

(4) Piel, J. (2002) A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of *Paederus* beetles. *Proc. Natl. Acad. Sci. U. S. A. 99*, 14002−14007.

(5) Helfrich, E.-J.-N., and Piel, J. (2016) Biosynthesis of polyketides by trans-AT polyketide synthases. *Nat. Prod. Rep. 33*, 231−316.

(6) Kampa, A., Gagunashvili, A.-N., Gulder, T.-A.-M., Morinaka, B. I., Daolio, C., Godejohann, M., Miao, V.-P.-W., Piel, J., and Andresson, O.-S. (2013) Metagenomic natural product discovery in lichen provides evidence for a family of biosynthetic pathways in diverse symbioses. *Proc. Natl. Acad. Sci. U. S. A. 110*, E3129−E3137.

(7) Wilson, M.-C., Mori, T., Ruckert, C., Uria, A.-R., Helf, M.-J., Takada, K., Gernert, C., Steffens, U.-A.-E., Heycke, N., Schmitt, S., Rinke, C., Helfrich, E.-J.-N., Brachmann, A.- O., Gurgui, C., Wakimoto, T., Kracht, M., Crusemann, M., Hentschel, U., Abe, I., Matsunaga, S., Kalinowski, J., Takeyama, H., and Piel, J. (2014) An environmental bacterial taxon with a large and distinct metabolic repertoire. *Nature 506*, 58−62.

(8) Freeman, M.-F., Vagstad, A.-L., and Piel, J. (2016) Polytheonamide biosynthesis showcasing the metabolic potential of sponge-associated uncultivated *'Entotheonella'* bacteria. *Curr. Opin. Chem. Biol. 31*, 8−14.

(9) Piel, J., Hui, D.-Q., Wen, G.-P., Butzke, D., Platzer, M., Fusetani, N., and Matsunaga, S. (2004) Antitumor polyketide biosynthesis by an uncultivated bacterial symbiont of the marine sponge *Theonella swinhoei*. *Proc. Natl. Acad. Sci. U. S. A. 101*, 16222−16227.

(10) Fisch, K.-M., Gurgui, C., Heycke, N., van der Sar, S.-A., Anderson, S.-A., Webb, V.-L., Taudien, S., Platzer, M., Rubio, B.-K., Robinson, S.-J., Crews, P., and Piel, J. (2009) Polyketide assembly lines of uncultivated sponge symbionts from structure-based gene targeting. *Nat. Chem. Biol. 5*, 494−501.

(11) Nakabachi, A., Ueoka, R., Oshima, K., Teta, R., Mangoni, A., Gurgui, M., Oldham, N.-J., van Echten-Deckert, G., Okamura, K., Yamamoto, K., Inoue, H., Ohkuma, M., Hongoh, Y., Miyagishima, S., Hattori, M., Piel, J., and Fukatsu, T. (2013) Defensive bacteriome symbiont with a drastically reduced genome. *Curr. Biol. 23*, 1478−1484.

(12) Liaimer, A., Jensen, J.-B., and Dittmann, E. (2016) A genetic and chemical perspective on symbiotic recruitment of cyanobacteria of the genus Nostoc into the host plant Blasia pusilla L. *Front. Microbiol. 7*, 1693.

(13) Schleissner, C., Canedo, L.-M., Rodriguez, P., Crespo, C., Zuniga, P., Penalver, A., de la Calle, F., and Cuevas, C. (2017) Bacterial production of a pederin analogue by a free-living marine Alphaproteobacterium. *J. Nat. Prod. 80*, 2170−2173.

(14) Kaštovský, J., Hauer, T., Mareš, J., Krautová, M., Bešta, T., Komárek, J., Desortová, B., Heteša, J., Hindáková, A., Houk, V., Janeček, E., Kopp, R., Marvan, P., Pumann, P., Skácelová, O., and Zapomělová, E. (2010) A review of the alien and expansive species of freshwater cyanobacteria and algae in the Czech Republic. *Biol. Invasions 12*, 3599−3625.

(15) Ueoka, R., Uria, A.-R., Reiter, S., Mori, T., Karbaum, P., Peters, E.-E., Helfrich, E.-J.-N., Morinaka, B.-I., Gugger, M., Takeyama, H., Matsunaga, S., and Piel, J. (2015) Metabolic and evolutionary origin of actin-binding polyketides from diverse organisms. *Nat. Chem. Biol. 11*, 705−712.

(16) Caffrey, P. (2003) Conserved amino acid residues correlating with ketoreductase stereospecificity in modular polyketide synthases. *ChemBioChem 4*, 654−657.

(17) Reid, R., Piagentini, M., Rodriguez, E., Ashley, G., Viswanathan, N., Carney, J., Santi, D. - V., Hutchinson, C. - R., and McDaniel, R. (2003) A model of structure and catalysis for ketoreductase domains in modular polyketide synthases. *Biochemistry 42*, 72−79.

(18) Mosey, R.-A., and Floreancig, P.-E. (2012) Isolation, biological activity, synthesis, and medicinal chemistry of the pederin/mycalamide family of natural products. *Nat. Prod. Rep. 29*, 980−995.

(19) Wan, S.-Y., Wu, F.-H., Rech, J.-C., Green, M.-E., Balachandran, R., Horne, W.-S., Day, B.-W., and Floreancig, P.-E. (2011) Total synthesis and biological evaluation of pederin, psymberin, and highly potent analogs. *J. Am. Chem. Soc. 133*, 16668−16679.

(20) Selwood, A.-I., Holland, P.-T., Wood, S.-A., Smith, K.-F., and McNabb, P.-S. (2007) Production of anatoxin-a and a novel biosynthetic precursor by the cyanobacterium *Aphanizomenon issatschenkoi*. *Environ. Sci. Technol. 41*, 506−510.

(21) Guillard, R. L., and Lorenzen, C. J. (1972) Yellow-green algae with chlorophyllide c. *J. Phycol. 8*, 10−14.

(22) Komárek, J. (2013) Cyanoprokaryota. 3. Heterocytous Genera, in *Süswasserflora von Mitteleuropa/Freshwater Flora of Central Europe* (Büdel, B., Gärtner, G., Krienitz, L., and Schagerl, M., Eds), pp 1130, Springer Spektrum.

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ACS Chem. Biol. 2018, 13, 1123−1129

(23) Mareš, J., Hájek, J., Urajová, P., Kopecký, J., and Hrouzek, P. (2014) A hybrid non-ribosomal peptide/polyketide synthetase containing fatty-acyl ligase (FAAL) synthesizes the beta-amino fatty acid lipopeptides puwainaphycins in the cyanobacterium *Cylindrospermum alatosporum*. *PLoS One* 9, e111904.
(24) Delcher, A.-L., Bratke, K.-A., Powers, E.-C., and Salzberg, S.-L. (2007) Identifying bacterial genes and endosymbiont DNA with Glimmer. *Bioinformatics* 23, 673−679.
(25) Blin, K., Wolf, T., Chevrette, M. G., Lu, X.-W., Schwalen, C. J., Kautsar, S. A., Suarez Duran, H. G., de los Santos, E. L. C., Kim, H. U., Nave, M., Dickschat, J. S., Mitchell, D. A., Shelest, E., Breitling, R., Takano, E., Lee, S. Y., Weber, T., and Medema, M. H. (2017) antiSMASH 4.0-improvements in chemistry prediction and gene cluster boundary identification. *Nucleic Acids Res.* 45, W36−W41.

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Paper V.

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Structural diversity of cytotoxic puwainaphycin and minutissamide lipopeptides is generated by a common biosynthetic pathway employing two alternative starter modules

Jan Mareš,[1,2,3] Jan Hájek,[2,3] Petra Urajová,[2] Andreja Kust,[1,2,3] Jouni Jokela,[4] Kumar Saurav,[2] Tomáš Galica,[2,3] Kateřina Čapková,[1] Antti Mattila,[4] Esa Haapaniemi,[5,6] Perttu Permi,[5] Ivar Mysterud,[7] Olav M. Skulberg,[8] Jan Karlsen,[9] David P. Fewer,[4] Kaarina Sivonen,[4] Hanne Hjorth Tønnesen,[9] Pavel Hrouzek[2,3#]

[1]The Czech Academy of Sciences, Biology Centre, Institute of Hydrobiology, České Budějovice, Czech Republic
[2]The Czech Academy of Sciences, Institute of Microbiology, Center Algatech, Třeboň, Czech Republic
[3]University of South Bohemia, Faculty of Science, České Budějovice, Czech Republic
[4]Department of Microbiology, Helsinki, Finland
[5]Department of Chemistry, University of Jyväskylä, Finland
[6]Department of Biological and Environmental Science, Nanoscience Center, University of Jyväskylä, Finland
[7]Department of Biosciences, University of Oslo, Oslo, Norway
[8]Norwegian Institute for Water Research (NIVA), Oslo, Norway
[9]School of Pharmacy, University of Oslo, Oslo, Norway

Running title: Cyanobacterial lipopeptide biosynthesis

#Address correspondence to Pavel Hrouzek, hrouzekp@gmail.com

Abstract: Puwainaphycins (PUWs) and minutissamides (MINs) are structurally analogous cyclic lipopeptides possessing cytotoxic activity. Both types of compound exhibit high structural variability, particularly in the fatty acid (FA) moiety. Although biosynthetic gene cluster for several PUW variants has been proposed in a cyanobacterial strain, the genetic background for MINs remains unexplored. Herein, we report PUW/MIN biosynthetic gene clusters and structural variants from six cyanobacterial strains. Comparison of biosynthetic gene clusters indicates a common origin of the PUW/MIN hybrid nonribosomal peptide synthetase and polyketide synthase. Surprisingly, the biosynthetic gene clusters encode two alternative biosynthetic starter modules, and analysis of structural variants suggests that initiation by each of the starter modules results in lipopeptides of differing length and FA substitution. Among additional modifications of the FA chain, chlorination of minutissamide D was explained by the presence of a putative halogenase gene in the PUW/MIN gene cluster of *Anabaena minutissima* UTEX B 1613. We detected PUW variants bearing an acetyl substitution in *Symplocastrum muelleri* NIVA-CYA 644, consistent with an *O*-acetyltransferase gene in its biosynthetic gene cluster. The major lipopeptide variants did not exhibit any significant antibacterial activity, and only the PUW F variant was moderately active against yeast, consistent with previously published data suggesting that PUW/MIN interact preferentially with eukaryotic plasma membranes.

Importance: Herein, deciphered the most important biosynthetic traits of a prominent group of bioactive lipopeptides. We reveal evidence for initiation of biosynthesis by two alternative starter units hardwired directly

in the same gene cluster, eventually resulting in the production of a remarkable range of lipopeptide variants. We identified several unusual tailoring genes potentially involved in modifying the fatty acid chain. Careful characterization of these biosynthetic gene clusters and their diverse products could provide important insight into lipopeptide biosynthesis in prokaryotes. Some of the identified variants exhibit cytotoxic and antifungal properties, and some are associated with a toxigenic biofilm-forming strain. The findings may prove valuable to researchers in the fields of natural product discovery and toxicology.

Introduction

Bacterial lipopeptides are a prominent group of secondary metabolites with pharmaceutical potential as antibacterial, antifungal, anticancer, and antiviral agents (1). Compounds such as fengycin, the iturin family antibiotics, octapeptins, and daptomycin are important pharmaceutical leads, the latter of which is already in clinical use (1–3). Their biological activity is the result of an amphipathic molecular structure that allows micellar interaction within the cell membranes of target organisms (4).

Lipopeptides are widespread in cyanobacteria and possess cytotoxic and antifungal activities (5-8). Puwainaphycins (PUWs) and minutissamides (MINs) are lipopeptides featuring a β-amino fatty acid and a 10-membered peptide ring (5, 9–11). Both classes exhibit considerable structural variability in terms of length and functionalization of the fatty acyl (FA) side chain attached to the stable peptide core (10–14). Only minor discrepancies in length and substitution of the FA chain separate these two

types of lipopeptides. A wide array of bioactivities has been reported for these compounds. PUW C is a cardioactive compound (15) as demonstrated by positive inotropic activity in mouse atria, while PUW F/G exhibit cytotoxicity against human cells *in vitro* through cell membrane permeabilization (5). MINs A–L exhibited antiproliferative effects when tested against human cancer cell lines over a concentration range similar to PUWs (10, 11). The overall structural similarity suggests that PUWs and MINs share a similar biosynthetic origin. However, the biosynthetic mechanisms generating the conspicuous chemical variability remain unknown.

PUWs are synthesized by a hybrid polyketide/non-ribosomal peptide synthetase (PKS/NRPS) accompanied by tailoring enzymes (12). A characteristic feature of the PUW synthetase is the fatty acyl-AMP ligase (FAAL) starter unit (12). This enzyme specifically binds and adenylates FAs and passes the activated acyl-adenylate to a downstream phosphopantetheine arm of the PKS acyl carrier protein (ACP) for further processing (12). The whole process bears resemblance to the biosynthesis of iturin-family lipopeptides (16–19) as well as small lipopeptide-like cyanobacterial metabolites such as hectochlorin (20), hapalosin (21), and jamaicamide (22), as discussed previously (23). Bacterial FAAL enzymes originate from basal cell metabolism, and likely evolved from fatty acyl-CoA ligases (FACLs) following a specific insertion that hampered subsequent ligation to CoASH (24) or altered the catalytic conformation (25). FAAL enzymes play an important role in the assembly of other metabolites including olefins (26) and unusual lipids (27) in addition to lipopeptide synthesis. The exact substrate-binding mechanism employed by FAALs was demonstrated experimentally in *Mycobacterium*

tuberculosis using several homologous FAAL enzymes and FA substrates as models (28). The substrate specificity of these enzymes corresponds to the structure of the substrate-binding pocket (25, 28), although it overlaps among homologs.

Herein, we combined recently developed bioinformatics and highperformance liquid chromatography combined with high resolution tandem mass spectrometry (HPLC-HRMS/MS) approaches (13, 23) to identify biosynthesis gene clusters for PUWs/MINs in five new cyanobacterial strains and characterized the chemical variability of their products. We discuss the specific structural properties of the identified lipopeptide variants and compare the predicted functions of synthetase enzymes.

Results and Discussion

Structural variability vs. common biosynthetic origin of PUWs and MINs

In the present study, we collected all known PUW/MIN producers (except for *Anabaena* sp. UIC10035). The strains were originally isolated from various soil habitats (Table 1). HPLC-HRMS/MS analysis detected multiple PUW and MIN variants in each of the strains studied (Fig. 1), ranging from 13 to 26 in strains 3 and 1, respectively (Table S1).

The MS/MS data acquired for crude extracts were used to create a molecular network (Fig. 2), analysis of which demonstrated that *Cylindrospermum* strains 1–3 and *Anabaena* strains 4 and 5 formed a single group with MIN A as the only variant common to all the strains (Fig. 2a). All major structural variants of these strains shared the common peptide sequence FA^{1}-Val^{2}-Dhb^{3}-Asn^{4}-Dhb^{5}-Asn^{6}-Ala^{7}-Thr^{8}-*N*MeAsn^{9}-

Pro[10] (Fig. 3), described previously for PUW F and MIN A (5, 10). The pattern of variant production was almost identical in *Cylindrospermum* strains 2 and 3, which in addition to MIN A contained PUW F (Fig. 1, Table S1). By contrast, *Anabaena* strains 4 and 5 produced MIN C and D in addition to the major variant MIN A (Fig. 1). The peptide core of the molecule was different in *Symplocastrum muelleri* strain 6 (Fig. 3), forming a separate group in the molecular network (Fig. 2b), with the general peptide sequence FA[1]-Val[2]-Dhb[3]-Thr[4]-Thr/Val[5]-Gln[6]-Ala[7]-*O*Me-Thr[8]-*N*MeAsn[9]-Pro[10] (Fig. 3), identical to PUW A–D and MIN I, K, L isolated previously from *Anabaena* sp. (9, 11).

Strain No.	Strain	Isolated by	Date	Locality	Reference
1	*Cylindrospermum alatosporum* CCALA 988	A. Lukešová	1989	Canada, Manitoba, Riding Mountain National Park, soil	Johansen *et al.* 2014[55]
2	*Cylindrospermum moravicum* CCALA 993	A. Lukešová	2008	Czech Republic, South Moravia, Moravian Karst, Amaterska Cave, cave sediment	Johansen *et al.* 2014[55]
3	*Cylindrospermum alatosporum* CCALA 994	A. Lukešová	2011	Czech Republic, Moravian Karst, earthworms collected from soil above Amaterska Cave, earthworm casings	Johansen *et al.* 2014[55]
4	*Anabaena* sp. UHCC-0399	M. Wahlsten	N/A	Finland, Jurmo, Southwestern Archipelago National Park, copepods	Tamrakar 2016[56]
5	*Anabaena minutissima* UTEX B1613	T. Kantz	1967	South Texas, USA, soil	Kantz & Bold 1969[57]
6	*Symplocastrum muelleri* NIVA-CYA 644	O.M. Skulberg	2009	Norway; Møre og Romsdal county; Halsa municipality, western slope of Slettfjellet mountain in semiterrestrial alpine habitat, biofilm on turf in ombrotrophic blanket bog	Skulberg *et al.* 2012[58]

Table 1. Strains analyzed for PUW/MIN production

The peptide core of the variants included in the network differed to some degree, but most variation was detected in the FA moiety (Fig. 4) when crude extracts were analyzed for the presence of characteristic FA immonium fragments (13).

Accordingly, bioinformatic analysis identified putative PUW and MIN gene clusters in each of the five newly sequenced strains (Fig. 5, Table 2). Based on BLASTp, CDD, and AntiSMASH searches, these gene clusters exhibited synteny and functional homology with the previously characterized *puw* biosynthesis gene cluster in strain 1 (12) (Fig. 5). Therefore, our results strongly indicate a common biosynthetic origin of PUWs and MINs in cyanobacteria.

Variability in the peptide core

A common set of NRPS genes (*puwA, puwE–H*; Fig. 5) encoding a sequence of nine amino acid-incorporating modules (Fig. 6) was detected in all analyzed strains. Individual NRPS modules displayed variability in amino acid adenylation and tailoring domains that was generally congruent with the PUW/MIN peptide cores inferred using HPLC-HRMS/MS (Fig. 3). The two major observed types of peptide cores (represented by PUW A and PUW F, respectively) differed in the amino acids at positions 4 (Thr→Asn), 5 (Thr→Dhb), 6 (Gln→Asn), 7 (Ala→Gly) and 8 (Thr→*O*Me-Thr), as shown in Fig. 3 and Table S1. This was reflected in the predicted substrates of the corresponding A-domains, and by the presence of an *O*-methyltransferase domain in PuwH of *S. muelleri* strain 6, which is responsible for the methoxylation of Thr^8 (Fig. 6, Table S2). In contrast to the variability observed at the previously noted amino acid

positions, the two positions adjacent to both sides to the modified fatty acid (*N*MeAsn^{9}-Pro^{10}-(FA^{1})-Val^{2}-Dhb^{3}) are conserved in all known PUW/MIN variants described here and previously (5, 9, 13–15) (Fig. 3, Table S1). Accordingly, no functional variation in A-domains corresponding to these positions was observed within the deduced PuwA, PuwE, and PuwF proteins (Table S2). This is interesting because these four hydrophobic amino acids surround the FA moiety, which is likely responsible for the membrane disruption effect suggested previously (5). Thus, we hypothesize that such an arrangement could further support hydrophobic interactions with the lipid layer of the plasma membrane. For some of the other positions, minor variants were observed involving substitution of amino acids similar in structure and hydrophobicity, including Asn-Gln at position 4, Thr-Val at position 5, Ala-Gly at position 7, and Thr-Ser at

position 8 (Fig. 3, Table S1), indicative of probable substrate promiscuity in their respective adenylation domains (29).

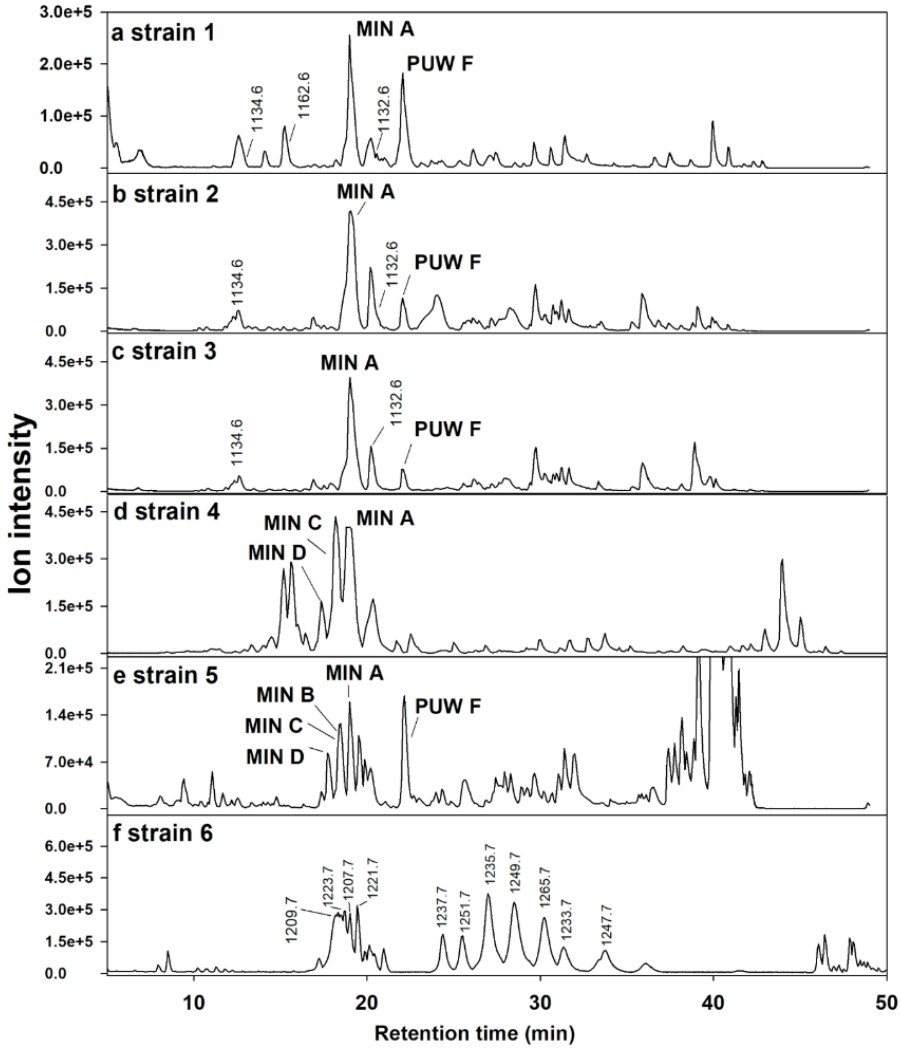


Fig. 1. HPLC-HRMS/MS analysis of crude extracts from the investigated strains. Major puwainaphycin (PUW) and minutissamide (MIN) variants are highlighted. For variants without complete structural information, only *m/z* values are shown.

The A6-domains in strains 4 and 5 activated Ala as a major substrate, and Gly to a lesser extent, even though *in silico* analysis predicted Gly as their main substrate (Table S2).

In strain 6, Gly was incorporated, in agreement with the predicted substrate specificity. An epimerase domain was present in each of the sixth NRPS modules of the pathways (Fig. 6), indicating probable formation of a D-amino acid enantiomer at position 7 of the peptide core. Indeed, the presence of D-Ala was previously confirmed in PUW F (5) and MIN A-H (10, 11), and D-Gly was identified in MIN I-L (10, 11).

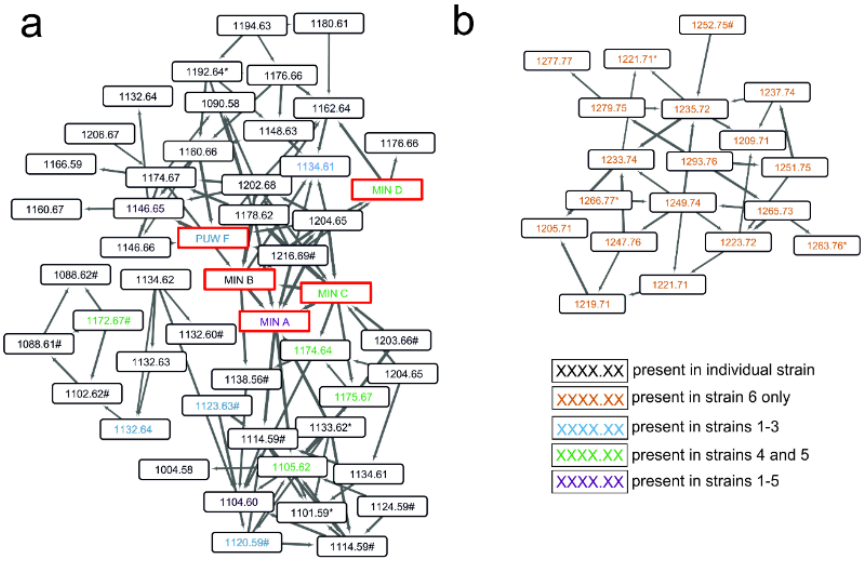


Fig. 2. Molecular network created using the Global Natural Products Social Molecular Networking (GNPS) web platform. Two separate networks were obtained during GNPS analysis; (a) a group containing *Cylindrospermum* strains 1–3 and *Anabaena* strains 4–5, and (b) a group containing only variants detected in *Symplocastrum muelleri* strain 6. The separate groups differ mainly in the peptide core of the molecule. For

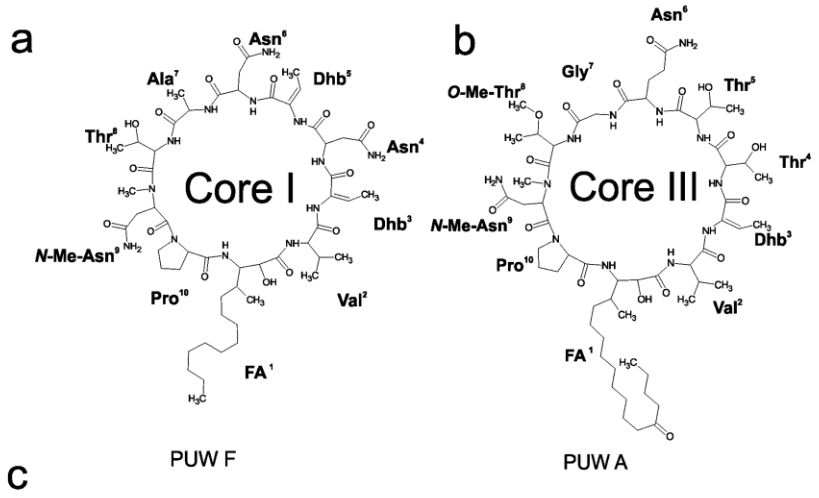
variants without complete structural information, only *m/z* values are shown. (*) refers to compounds present in trace amounts and (#) refers to compounds for which MS/MS data failed to resolve the structural information.

In two cases, the adenylation domains A3 (PuwF) and A6 (PuwG) are capable of incorporating significantly different amino acids such as Asn^4-Thr^4 and Ala^7-Ser^7, respectively (Fig. 6). This degree of substrate promiscuity is relatively uncommon. Activation of two divergent amino acids (Arg/Tyr) by a single adenylation domain, based on point mutations in just three codons, was previously demonstrated in the anabaenopeptin synthetase from the cyanobacterium *Planktothrix agardhii* (30). The substrate exchange of Ala vs. Ser was previously reported from fungal class IV adenylate-forming reductases that contain A-domains homologous to NRPS enzymes (31). The last synthetase enzyme in the pathway (PuwA) is equipped with a terminal thioesterase domain (Fig. 6), which presumably catalyzes cleavage of the final product and formation of the cycle via a peptide bond between the terminal prolyl and the β-amino group of the FA chain, as previously suggested (12).

Two hypothetical starter units and their substrate range

The biosynthesis of bacterial lipopeptides is typically commenced by FA-activating enzymes (16, 18). Initiation of the biosythesis of PUW/MIN is performed by a FAAL enzyme (12) and allows a much broader array of activated substrates than the relatively conserved oligopeptide core (13) (Fig. 4). We identified three alternative arrangements of the putative FAAL starter units (Fig. 5 and 6), each corresponding to a different array of FA side chains detected by HPLC-HRMS/MS, which presumably

reflects the range of FA substrates activated during their biosynthesis (Fig. 4). *Cylindrospermum* sp. strains 1–3 possess the Type I putative starter unit consisting of a standalone FAAL enzyme PuwC and a separate ACP PuwD (Fig. 5, Table 2). By contrast, the biosynthetic gene cluster of *S. muelleri* strain 6 contains the Type II putative starter unit (PuwI) consisting of a FAAL fused to an ACP (Fig. 5, Table 2). *Anabaena* spp. strains 4 and 5 combine both Type I and Type II putative starter units in their biosynthetic gene clusters (Fig. 5, Table 2).



c

	AA_1	AA_2	AA_3	AA_4	AA_5	AA_6	AA_7	AA_8	AA_9	AA_{10}	known compounds	variants found in strains 1-6
Core I	FA	Val	Dhb	Asn	Dhb	Asn	Ala	Thr	*N*-Me-Asn	Pro	MIN A, MIN B, MIN C, MIN D, PUW F	**MIN A, MIN B, MIN C, MIN D, PUW F,** Comp. 1,3,10,11,12,14,16,19,20, 25,26,28,30,31,37,45,51
Core II	FA	Val	Dhb	Gln	Dhb	Asn	Ala	Thr	*N*-Me-Asn	Pro	PUW G	**PUW G,** Comp. 2,6,13,15,17,27, 29,32
Core III	FA	Val	Dhb	Thr	Thr	Gln	Gly	*O*-Me-Thr	*N*-Me-Asn	Pro	PUW A, PUW C, PUW E, MIN I, MIN K	Comp. 38,46,50,52,53,56,58
Core IV	FA	Val	Dhb	Thr	Thr	Gln	Ala	*O*-Me-Thr	*N*-Me-Asn	Pro	MIN E, MIN G	Comp. 39,47,53,57,59
Core V	FA	Val	Dhb	Thr	Val	Gln	Gly	*O*-Me-Thr	*N*-Me-Asn	Pro	PUW B, PUW D, MIN L	Comp. 40,48,54,60
Core VI	FA	Val	Dhb	Thr	Val	Gln	Ala	*O*-Me-Thr	*N*-Me-Asn	Pro	MIN H	Comp. 41,49,55,61
Core VII	FA	Val	Dhb	Asn	Dhb	Asn	Gly	Thr	*N*-Me-Asn	Pro		Comp. 7,36,44
Core VIII	FA	Val	Dhb	Asn	Dhb	Asn	Ser	Thr	*N*-Me-Asn	Pro		Comp. 8,23,35
Core IX	FA	Val	Dhb	Asn	Dhb	Asn	Ala	Ser	*N*-Me-Asn	Pro		Comp. 9,18,24
Core X	FA	Val	Dhb	Thr	Dhb	Asn	Ala	Thr	*N*-Me-Asn	Pro		Comp. 5,34,43

Fig. 3. Structural variability of the peptide core of PUW/MIN variants. Examples of structural variants PUW F (a) and PUW A (b) with designated aminoacid positions representing the two major peptide cores. (c) Table

summarizing all types of the PUW/MIN peptide core found in known compounds reported in literature and compounds (Comp.) detected in studied strains. Columns shaded in grey highlight the conserved aminoacid positions.

Although the functions and substrate ranges of these hypothetical starter units requires further confirmation by gene manipulation experiments, they are supported by the patterns of lipopeptide variants detected by HPLC-HRMS/MS (Fig. 4, Table S1). In *Cylindrospermum* strains 1–3 that exclusively contain the Type I starter unit, the PUW/MIN products exhibited an almost continuous FA distribution between C_{10}–C_{15} (up to C_{17} in negligible trace amounts; Fig. 4). In *S. muelleri* strain 6, the presence of the Type II loading module resulted in production of PUW/MIN variants with discrete FA lengths of C_{16} and C_{18}. Strains containing both Type I and Type II starter units (*Anabaena* strains 4 and 5) produced two sets of PUW/MIN products with no overlap (C_{12}–$C_{14\text{-}15}$ for the Type I pathway, and C_{16} for the Type II pathway), but exhibited a slightly shifted length distribution (Fig. 4).

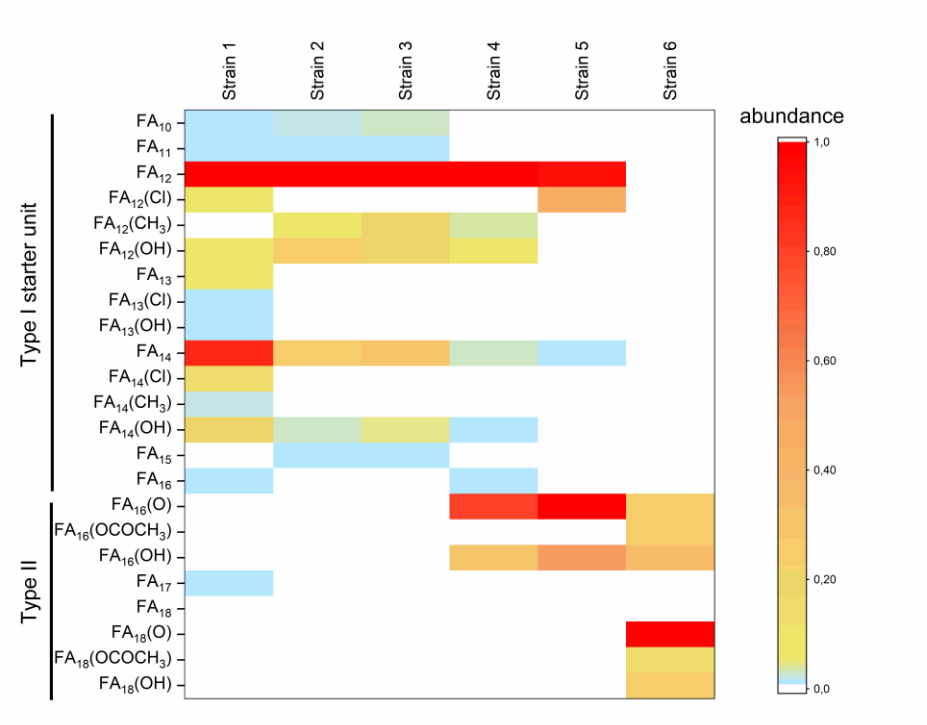


Fig. 4. Structural variability of the FA moiety of PUW/MIN variants. The relative proportion of variants with differences in FA length and substitution (y-axis) is depicted using a color scale (z-axis). For comparison, the peak area of a given variant was normalized against the peak area of the major variant present in the strain (MIN A for strains 1–5, and *m/z* 1235.7 for strain 6).

Based on these results, it seems plausible that PuwC/D and PuwI represent two alternative FAAL starter modules capable of initiating PUW/MIN biosynthesis (Figs. 5, 6). An analogous situation was previously described for the alternative NRPS starter modules in the anabaenopeptin synthetase (32).

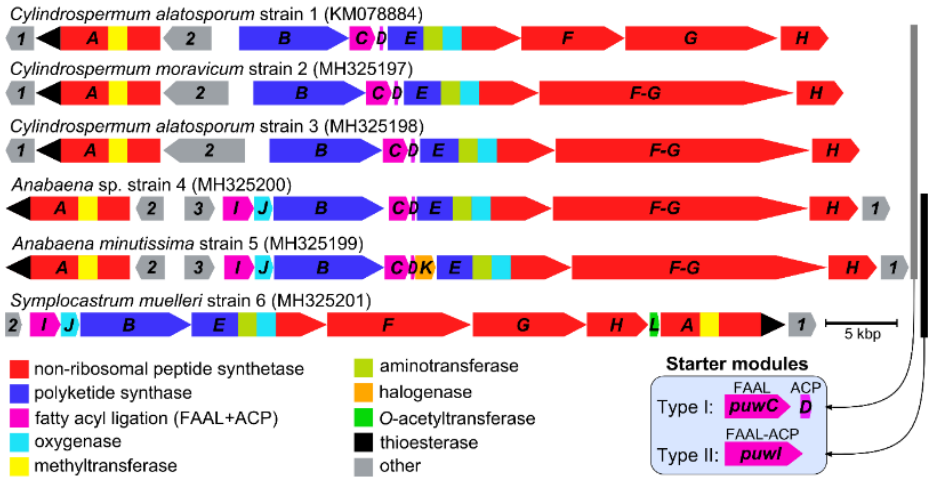


Fig. 5. Structure of the *puw* gene cluster in the six investigated cyanobacterial strains. Gene arrangement and functional annotation of *puwA–L* genes and selected PKS/NRPS tailoring domains is indicated by colored arrows. The distribution of the two observed types of putative starter modules (shaded boxes) is indicated by bars.

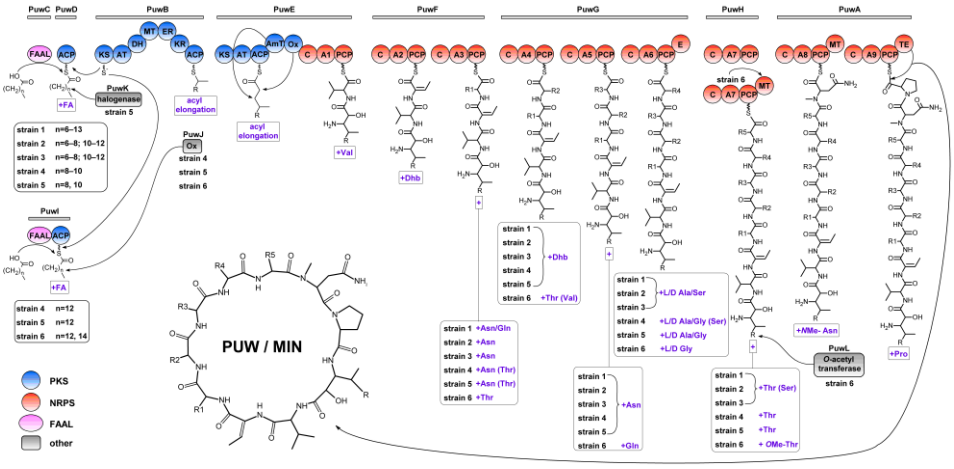


Fig. 6. Schematic view of the proposed biosynthesis assembly line of puwainaphycins and minutissamides. Variable amino acid positions and the ranges of fatty acyl lengths incorporated by the two putative alternative

starter units are listed for individual strains. A, adenylation domain; ACP, acyl carrier protein; AmT, aminotransferase; AT, acyltransferase; C, condensation domain; DH, dehydratase; E, epimerase; ER, enoylreductase; FAAL, fatty acyl-AMP ligase; MT, methyltransferase; NRPS, non-ribosomal peptide synthetase; KR, ketoreductase; KS, ketosynthetase; Ox, monooxygenase; PCP, peptidyl carrier protein; PKS, polyketide synthetase; TE, thioesterase

In the FA residue of the lipopeptide, proximal carbons in the linear aliphatic chain are incorporated into the nascent product by PKS enzymes (12). The PKS domains of PuwB and PuwE (Fig. 6, Table 2) catalyze two elongation steps. Therefore, the fatty acid is expected to be extended by four carbons. The substrate length specificity of the FAAL enzymes in *Mycobacterium tuberculosis* was recently shown to be determined by the size and position of specific amino acid residues protruding into the FA-binding pocket (28).

Experimental replacement of Gly or Ile by a larger Trp residue i n the upper and middle parts of the pocket blocked the binding of the original C_{12} substrate, but shorter chains (C_2 or C_{10}, respectively) were still activated (28). Experimental data on FAAL substrate specificity in cyanobacteria are currently lacking. Alignment of amino acid residues from all putative PuwC and PuwI proteins demonstrates overall homology (Fig. S2a), including the positions corresponding to the FA-binding pocket, as previously shown in *Mycobacterium* (28) (Fig. S2b). Experimental evidence such as *in vitro* activity assays and crystallization of protein-ligand complexes is required to explain the variable substrate specificity of PuwC vs. PuwI.

Table 2. Deduced proteins encoded by the *puw* gene cluster in six cyanobacterial strains, including length and functional annotation. ACP, acyl carrier protein; FAAL, fatty acyl-AMP ligase; PKS, polyketide synthase; NRPS, non-ribosomal peptide synthetase.

Protein		Strain No.						Predicted Function
		1	2	3	4	5	6	
ORF1	Length (aa)	659	664	664	643	643	647	ABC transporter
PuwA		2870	2870	2870	2854	2854	2866	NRPS
ORF2		1116	1499	1875	643	670	376	patatin-like phospholipase
ORF3		-	-	-	696	696	-	dynamin family protein
PuwI		-	-	-	709	702	711	FAAL, ACP
PuwJ		-	-	-	427	427	529	cytochrome-like protein
PuwB		2534	2592	2592	2549	2537	2555	hybrid PKS/NRPS, aminotransferase, oxygenase
PuwC		597	590	590	597	589	-	FAAL
PuwD		101	104	96	93	92	-	ACP
PuwK		-	-	-	-	465	-	halogenase
PuwE		3077	3121	3121	3099	3112	3113	NRPS

Also, we cannot exclude the possibility that the FA substrate length range is partially determined by the pool of free FAs available to the FAAL enzyme. Indeed, this possibility is supported by observations of *Cylindrospermum* strains 1–3, which share highly conserved PuwC proteins (Fig. S2a) with identical residues in the predicted FA-binding pockets (Fig. S2b), but display slightly different ranges and ratios of incorporated FAs in the PUW/MIN variants produced (Fig. 1, 4).

FA tailoring reactions: oxidation, halogenation, and acetylation

Intriguingly, all products originating from biosynthesis initiated by the Type II starter unit (variants with a C_{16} and C_{18} FA tail in *Anabaena* strains 4–5 and *S. muelleri* strain 6) include substitution of a hydroxy- or oxo-moiety (Fig. 6). For minutissamides C and D, this substitution takes place on the third carbon from the FA terminus (C_{14}), as described previously (10), and this position was confirmed by NMR in variants produced by *Anabaena sp.* strain 4 in our study (Table S3, Figs. S3–6). In agreement with this hydroxy- and oxo-substitution, the respective gene clusters each encode PuwJ, a putative cytochrome P450-like oxidase (Table 2), immediately downstream of the gene encoding the Type II starter module. We therefore hypothesize that the PuwJ enzyme is responsible for hydroxylation of FA residues activated by PuwI (Fig. 6). However, the formation of the keto variant remains unexplained by our data.

Another gene, the putative halogenase *puwK*, was associated with the Type II starter module in *Anabaena* sp. strain 5 (Table 2). Although no conserved enzymatic domain was detected in the deduced protein, it shares similarity with proteins postulated to be involved in halogenation of cyanobacterial chlorinated acyl amides known as columbamides (33), and *N*-oxygenases similar to *p*-aminobenzoate *N*-oxygenase AurF (34–36). The possible functional designation of this enzyme as a halogenase is further supported by the fact that the ω-chlorinated product MIN B, originally described in strain *Anabaena* sp. strain 5 (10), was also detected in this study (Table S1) as one of the major variants, while no MIN B or any other chlorinated PUW/MIN products were detected in *Anabaena* sp. strain 4 (Fig. 1). *Anabaena* sp. strains 4 and 5 share identical organization across the entire gene cluster, and lack of the putative halogenase gene

puwK is the only difference between these two clusters in terms of presence of genes (Fig. 5).

In *Cylindrospermum* sp. strains 1–3 that exclusively possess the Type I starter unit, the presence of minor amounts of hydroxylated and chlorinated variants (Fig. 4) suggests the involvement of another biosynthetic mechanism unexplained by the current data. This ambiguity warrants experimental research such as gene knock-out experiments to confirm the proposed functions of *puwJ* and *puwK*.

Finally, the gene cluster identified in *S. muelleri* strain 6 was the only one containing gene *puwL*. The deduced product of this gene shares 53.4% similarity with the *O*-acetyltransferase McyL (Table 2) involved in acetylation of the aliphatic chain of microcystin in cyanobacteria (37). Additionally, this gene is similar to chloramphenicol and streptogramin A *O*-acetyltransferases that serve as antibiotic resistance agents in various bacteria (38). The functional annotation of PuwL as a putative *O*-acetyltransferase is consistent with the detection of *O*-acetylated lipopeptide variants in *S. muelleri* strain 6 (Table 3, Fig. 7).

Five PUW variants (*m/z* 1265.7338, 1279.7496, 1277.7695, 1291.7870 and 1293.7654) yielding high-energy fragments, proving the presence of an acetyl group bonded to the FA moiety, have been detected. In the *m/z* 1279.8 and 1293.8 peaks, the high-energy fragment ion at *m/z* 312 corresponds to the FA immonium ion bearing an acetyl group, and fragment ion at *m/z* 439 corresponds to the prolyl-FA-acetyl fragment. The subsequent loss of an acetyl group resulted in the presence of ions at *m/z* 252 and 379, respectively (Table 3, Fig. 7). Similarly, analysis of the *m/z* 1265.7 peak revealed analogous fragments at *m/z* 284/411 and 351/224 (Table 3).

Low fragmentation energy (60eV)	X=Ala, Y=Thr, FA=C_{16}			X=Gly, Y=Thr, FA=C_{18}			X=Ala, Y=Thr, FA=C_{18}			X=Gly, Y=Val, FA=C_{18}			X=Ala, Y=Val, FA=C_{18}		
	m/z	Δ(ppm)	Sum formula	*m/z*	Δ(ppm)	Sum formula	*m/z*	Δ(ppm)	Sum formula	*m/z*	Δ(ppm)	Sum formula	*m/z*	Δ(ppm)	Sum formula
$[M]^+$	**1265.7338**	**+0.7**	**$C_{68}H_{101}N_{13}O_{18}$**	**1279.7496**	**+0.9**	**$C_{69}H_{103}N_{13}O_{18}$**	**1293.7654**	**+0.8**	**$C_{70}H_{105}N_{13}O_{18}$**	**1277.7695**	**+1.6**	**$C_{70}H_{105}N_{13}O_{17}$**	**1291.7870**	**+0.1**	**$C_{71}H_{107}N_{13}O_{17}$**
$[M\text{-}CH_3OH]^+$	1233.7170	-6.6	$C_{68}H_{97}N_{13}O_{17}$	1247.7194	+4.1	$C_{69}H_{99}N_{13}O_{17}$	1261.7494	-7.3	$C_{70}H_{101}N_{13}O_{17}$	low int.		$C_{69}H_{101}N_{13}O_{16}$	low int.		$C_{70}H_{103}N_{13}O_{16}$
$[M\text{-}CH_3OH\text{-}NMeAsn]^+$	1105.6558	-4.9	$C_{63}H_{89}N_{10}O_{15}$	1119.6619	+3.7	$C_{64}H_{91}N_{10}O_{15}$	1133.681	+0.6	$C_{65}H_{93}N_{10}O_{15}$	1117.6924	-5.0	$C_{65}H_{93}N_{10}O_{14}$	1131.7307	-25.0	$C_{66}H_{95}N_{10}O_{14}$
$[M\text{-}CH_3OH\text{-}NMeAsn\text{-}dhb]^+$	1022.6180	-4.7	$C_{49}H_{84}N_9O_{14}$	1036.6365	-7.3	$C_{50}H_{86}N_9O_{14}$	1050.6478	-3.1	$C_{51}H_{88}N_9O_{14}$	1134.6603	-10.3	$C_{51}H_{88}N_9O_{13}$	1048.6671	-1.7	$C_{52}H_{90}N_9O_{13}$
$[M\text{-}CH_3OH\text{-}NMeAsn\text{-}dhb\text{-}X]^+$	951.5785	-2.5	$C_{46}H_{81}N_8O_{13}$	979.589	+18.8	$C_{48}H_{85}N_8O_{13}$	979.6041	+3.4	$C_{48}H_{85}N_8O_{13}$	977.6481	-20.5	$C_{49}H_{85}N_8O_{12}$	977.6518	-24.1	$C_{50}H_{87}N_8O_{12}$
$[M\text{-}CH_3OH\text{-}NMeAsn\text{-}dhb\text{-}X\text{-}Gln]^+$	823.5253	-9.4	$C_{41}H_{71}N_8O_{11}$	851.5473	+1.8	$C_{43}H_{75}N_8O_{11}$	851.5478	+1.2	$C_{43}H_{75}N_8O_{11}$	849.5838	-16.7	$C_{44}H_{75}N_8O_{10}$	849.5589	+12.5	$C_{44}H_{77}N_8O_{10}$
$[M\text{-}CH_3OH\text{-}NMeAsn\text{-}dhb\text{-}X\text{-}Gln\text{-}Y]^+$	722.4729	-4.2	$C_{37}H_{64}N_7O_9$	750.5005	+0.9	$C_{39}H_{68}N_7O_9$	750.5147	-18.1	$C_{39}H_{68}N_7O_9$	low int.		$C_{40}H_{70}N_7O_9$	low int.		$C_{41}H_{72}N_7O_9$
$[M\text{-}CH_3OH\text{-}NMeAsn\text{-}dhb\text{-}X\text{-}Gln\text{-}Y\text{-}Thr]^+$	621.4223	-0.2	$C_{33}H_{57}N_6O_7$	649.4526	+1.4	$C_{35}H_{61}N_6O_7$	649.4539	-0.6	$C_{35}H_{61}N_6O_7$	649.465	-17.8	$C_{36}H_{61}N_6O_7$	649.4483	+8.0	$C_{35}H_{61}N_6O_7$
High fragmentation energy (100eV)															
Fragment 1	411.3208	+2.2	$C_{23}H_{43}N_4O_4$	439.3559	-6.5	$C_{25}H_{47}N_4O_4$	439.3556	-5.8	$C_{25}H_{47}N_4O_4$	439.3556	-5.8	$C_{25}H_{47}N_4O_4$	439.3508	+5.1	$C_{25}H_{47}N_4O_4$
Fragment 1 - $C_2H_4O_2$	351.3006	+0.0	$C_{21}H_{39}N_4O_2$	379.3334	-4.0	$C_{23}H_{43}N_4O_2$	379.3329	-2.6	$C_{23}H_{43}N_4O_2$	379.3328	-2.4	$C_{23}H_{43}N_4O_2$	379.3360	-10.8	$C_{23}H_{43}N_4O_2$
Fragment 2	284.2583	+0.4	$C_{17}H_{34}NO_2$	312.2919	-6.9	$C_{19}H_{38}NO_2$	312.2892	+1.6	$C_{19}H_{38}NO_2$	low int.		$C_{19}H_{38}NO_2$	low int.		$C_{19}H_{38}NO_2$
Fragment 2 - $C_2H_4O_2$	224.2367	+2.6	$C_{15}H_{30}N$	252.2686	0.0	$C_{17}H_{34}N$	252.2687	-0.5	$C_{17}H_{34}N$	252.2684	+0.7	$C_{17}H_{34}N$	252.2677	+3.5	$C_{17}H_{34}N$

Table 3. Fragmentation of PUW variants from *Symplocastrum muelleri* strain 6 bearing acetyl substitutions on the FA moiety revealed by high energy (100 eV) fragmentation, and amino acid composition deduced by fragmentation at 60 eV.

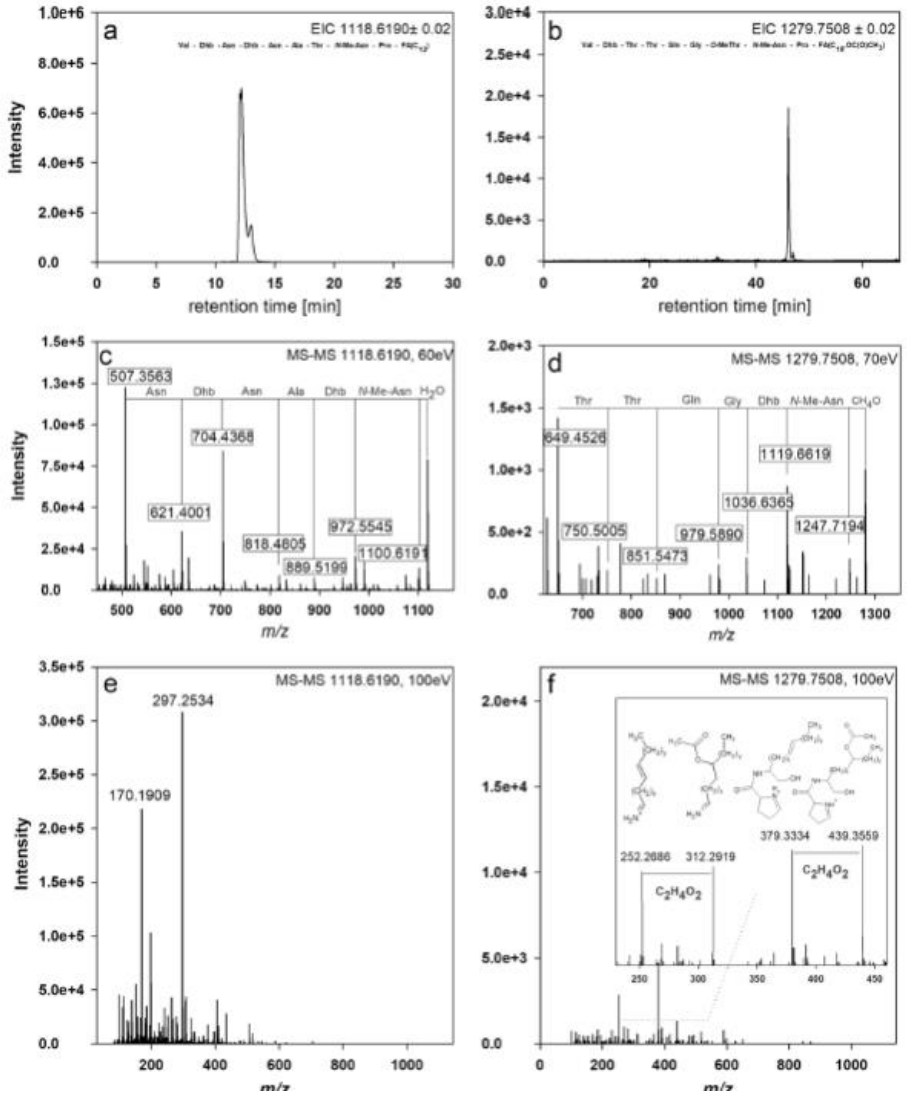


Fig. 7 MS-MS fragmentation of MIN A (a, c, e) and the PUW variant at m/z 1,279 bearing an acetyl substitution of the fatty acid chain (b, d, f). (a, b) Base peak chromatograms. (c, d) Fragmentation of the protonated molecule at low fragmentation energy, yielding b series of ions, corresponding to the losses of particular amino acid residues. (e, f)

Fragmentation of the protonated molecule at high energy (100 eV), yielding fragments characteristic for the β -amino fatty acid**.**

Antimicrobial activity

Both PUWs and MINs possess cytotoxic activity against human cells *in vitro* (5, 10, 11). In the current study, we demonstrated that the major PUW/MIN variants (PUW F and MINs A, C, and D) did not exert antibacterial effects against either Gram-positive or Gram-negative bacteria using a panel of 13 selected strains (Table 4).

PUW F was the only tested variant manifesting antagonistic activity against two yeast strains utilized in our experiment, namely *Candida albicans* HAMBI 261 and *Saccharomyces cerevisiae* HAMBI 1164, with inhibition zones of 14 and 18 mm, respectively, and minimum inhibitory concentration (MIC) values of 6.3 µg mL^{-1} (5.5 µM; Fig. 8).

Table 4. Bacterial and yeast strains used for antimicrobial testing of PUW F and MIN A, C, and D. HAMBI, culture collection of University of Helsinki, Faculty of Agriculture and Forestry, Department of Microbiology.

Test organisms (HAMBI nr.)	**Media[a]**	**Incubation temp. (°C)**	**Incubation time (h)**	**Gram strain reaction (+\-)**
Pseudomonas sp. (2796)	TGY	28	24	-
Micrococcus luteus (2688)	TGY	28	24	+
Bacillus subtilis (251)	TGY	28	24	+
Pseudomonas aeruginosa (25)	TGY	37	24	-

Escherichia coli (396)	TGY	37	24	-
Bacillus cereus (1881)	TSA	28	24	+
Burkholderia cepacia (2487)	TSA	37	24	-
Staphylococcus aureus (11)	TSA	37	24	+
Xanthomonas campestris (104)	NA	28	24	-
Burkholderia pseudomallei (33)	NA	37	24	-
Salmonella typhi (1306)	NA	37	24	-
Arthrobacter globiformis (1863)	NA	28	24	-
Kocuria varians (40)	NA	28	24	+
Candida albicans (261)	YM agar	37	24	yeast
Cryptococcus albidus (264)	YM agar	28	24	yeast
Saccharomyces cerevisiae (1164)	YM agar	28	24	yeast

[a]The composition of all media was obtained from the American Type Culture Collection (ATCC).

TGY, tryptone glucose yeast; TSA, tryptic soy agar; NA, Nutrient agar; YM agar, yeast malt agar.

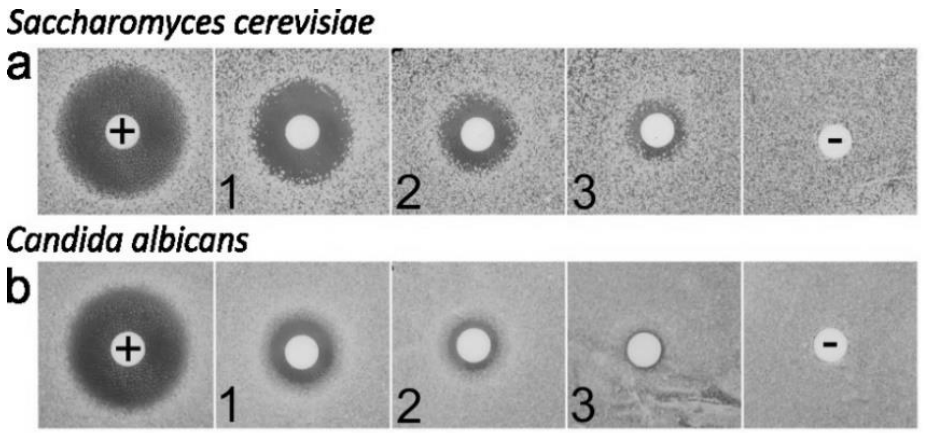


Fig. 8. Antifungal activity of PUW F against yeast strains (a) *Saccharomyces cerevisiae* HAMBI 1164 and (b) *Candida albicans* HAMBI 261. Discs were treated with a concentration range from 25.2 µg mL^{-1} to 0.0394 µg/mL to determine the minimum inhibitory concentration (MIC). Numbers represent concentrations: (1) = 25.2 µg mL^{-1}; (2) = 12.6 µg mL^{-1}; (3) = 6.3 µg mL^{-1}; (+) = positive control (10 µg of nystatin). (-) = negative control (10 µL of methanol).

No antifungal activity was recorded for the MIN C and D variants, and only weak inhibition of the two yeast strains was recorded for MIN A (Fig. S7). PUW F differs only slightly from MIN A by a -CH_2-CH_3 extension of the FA moiety, indicating that the FA length affects bioactivity. Furthermore, the lack of bioactivity for MIN C and MIN D suggests that hydroxy- and oxo- substitution also compromises antifungal efficacy. As previously demonstrated, cytotoxicity is due to membrane permeabilization activity accompanied by calcium flux into the cytoplasm (5), consistent with the membrane effects documented for other bacterial lipopeptides (4). However, as apparent from our data (Fig. 8), PUW/MIN products appear to be effective solely against eukaryotes (thus far tested only on human and yeast cells). This finding is in contrast to the typical antibacterial activity frequently described for many lipopeptides produced by Gram-positive bacteria (4). Analogously, the cyanobacterial lipopeptides anabanenolysin A and hassalidins preferentially interact with cholesterol-containing membranes, hence their predisposition for activity against eukaryotic cells (6, 8).

Distribution of PUWs and MINs in cyanobacteria

PUWs and MINs form one of the most frequently reported groups of lipopeptides in cyanobacteria, and have been isolated from heterocytous cyanobacteria, particularly members of the genera *Anabaena* and *Cylindrospermum* that inhabit soil (5, 9–11). Only a single study has mentioned the probable occurrence of puwainaphycins in a planktonic cyanobacterium (*Sphaerospermopsis*) (39). Our current comprehensive analysis of these lipopeptides and their biosynthetic genes further supports the hypothesis that lipopeptides occur predominantly in non-planktic biofilm-forming cyanobacteria (23). In this context, it is worth mentioning that *S. muelleri* strain 6 was isolated from a wetland bog in alpine mountains in coastal Norway (40). This strain is a toxigenic member of a biofilm microbiome and suspected to play a role in the development of severe hemolytic Alveld disease among outfield grazing sheep (41, 42). Biomass harvested from pure cultures of this strain exhibited strong cytotoxic activity toward primary rat hepatocytes (43, 44), which indicates the production of secondary metabolites with cytotoxic properties. Thus, the possible toxic potential of cyanobacterial lipopeptides such as PUWs and MINs in the environment warrants further attention.

Conclusions

Our study highlights and explores the extensive structural versatility of cyanobacterial lipopeptides from the PUW/MIN family by introducing previously unknown variants and newly sequenced biosynthetic gene clusters. Intriguingly, all variants are synthesized by a relatively conserved PKS/NRPS machinery with a common genetic origin. We hypothesize that chemical diversity is generated largely by the presence of two alternative

fatty acyl-AMP ligase starter units, one of which exhibits an unusually broad specificity for FAsubstrates of variable length. Additionally, putative halogenase and *O*-acetyltransferase genes were present in some gene clusters. This knowledge provides novel insight into the genetic background underpinning the biosynthesis of bacterial lipopeptides. The proposed biosynthetic mechanisms allow the studied microbes to generate a large pool of products that can be readily expanded by introducing relatively small genetic changes. This is consistent with the so-called 'Screening Hypothesis' (45, 46), which predicts an evolutionary benefit for organisms producing a large chemical diversity of secondary metabolites at minimum cost. Accessory antimicrobial tests on bacteria and yeasts, together with previously published results, suggest a specific toxic effect of PUWs against eukaryotic cells. Thus, their toxic potential for humans and other animals clearly warrants further investigation, and their possible use as antifungal agents is ripe for exploration.

Materials and Methods

Cultivation of cyanobacterial strains

Six cyanobacterial strains were included in the present study: *Cylindrospermum moravicum* CCALA 993 (strain 1), *Cylindrospermum alatosporum* CCALA 994 (strain 2), *Cylindrospermum alatosporum* CCALA 988 (strain 3), *Anabaena sp.* UHCC-0399 (previously *Anabaena* sp. SMIX 1; strain 4), *Anabaena minutissima* UTEX B 1613 (strain 5), and *Symplocastrum muelleri* NIVA-CYA 644 (strain 6). The origins of the strains are listed in Table 1. For chemical analysis, strains 1–5 were cultivated in BG-11 media (47) in glass columns (300 mL) bubbled with air enriched in 1.5% CO_2 at a temperature of 28°C and constant

illumination of 100 µmol photons m^{-2} s^{-1}. Strain 6 was maintained in culture using a custom liquid medium obtained by mixing 200 mL of Z8 medium (48), 800 mL distilled water, 30 mL soil extract, and common vitamin pre-mix (according to SAG – Sammlung von Algenkulturen der Universität Göttingen, but without biotin). Cultivation was performed in 100–200 mL Erlenmeyer flasks at 20°C with a 16:8 light:dark photoperiod under static conditions. Cultures were kept at low irradiance (4 µmol m^{-2} s^{-1} PHAR generated using RGB LED strips). Cells were harvested by centrifugation (3125 × *g*), stored at -80°C, and subsequently lyophilized. Strain 4 was cultivated at a larger scale for purification of major lipopeptide variants in a 10 L tubular photobioreactor under the above-mentioned conditions in BG-11 medium.

Molecular and bioinformatic analyses

Single filaments of strains 2, 3, 5, and 6 were isolated for whole-genome amplification (WGA) and subsequent preparation of a whole-genome sequencing (WGS) library, as described previously (12). Briefly, the glass capillary technique was used to isolate filaments excluding minor bacterial contaminants. A set of 20 filaments from each strain was then used as a template for WGA. Multiple displacement amplification (MDA) using a Repli-g Mini Kit (Qiagen, Hilden, Germany) was followed by PCR and sequencing to monitor the cyanobacterial 16S rRNA gene using primers 16S387F and 16S1494R (49). Positive samples (7–10 MDA products yielding clear 16S rRNA gene sequences of the respective genera) were then pooled to create a template for WGS. DNA samples were sent for commercial *de novo* genome sequencing (EMBL Genomics Core Facility, Heidelberg, Germany) using the Illumina MiSeq platform (Illumina, San

Diego, CA, USA) with a ~350 bp average insert length Pair-End library and 250 bp reads (~1.4 Gbp data yield per strain). Raw data from *de novo* WGS were assembled using CLC Bio Genomics Workbench v. 7.5 (CLC Bio, Aarhus, Denmark). Genomic DNA was isolated from strain 4 as previously described (37) and the quality was assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and an Agilent TapeStation (Agilent Technologies, Santa Clara, CA, USA). High-molecular-weight DNA was used to construct an Illumina TruSeq PCR Free 350 bp library and sequenced using an Illumina HiSeq 2500 platform with a paired-end 100 cycles run. Genome data (1Gb for each strain) were first checked using SPAdes version 3.7.1 (51) for read correction and removal of erroneous reads, and then assembled using Newbler version 3.0 (454 Life Sciences, Branford, CT, USA). Genomic scaffolds were loaded into Geneious Pro R10 (Biomatters; available from http://www.geneious.com) and investigated for FAAL and NRPS genes using BLASTp searches to identify putative lipopeptide synthetase gene clusters (23). FAAL and NRPS adenylation domains (A-domains) from the single known PUW gene cluster (strain 1; KM078884) were used as queries since homologous gene clusters were expected. Contigs yielding high similarity hits (E-value $<10^{-20}$) were then analyzed using the Glimmer 3 (50) algorithm to discover putative open-reading frames (ORFs). Functional annotation of ORFs was conducted by applying a combination of BLASTp/CDD searches against the NCBI database, and using the antiSMASH 4.0 secondary metabolite gene cluster annotation pipeline (52, 53). Pairwise sequence identities and the presence of conserved residues in homologous putative proteins encoded in the gene clusters were assessed using Geneious Pro software based on amino acid alignment

(MAFFT plugin, default parameters). Minor assembly gaps were identified in the genomic scaffolds of all investigated strains, either directly after pair-end read assembly, or based on mapping to the reference gene cluster from *C. alatosporum* CCALA 988. Gaps in PUW/MIN gene clusters were closed by PCR, and subsequent Sanger sequencing of PCR products was performed using custom primer annealing to regions adjacent to the assembly gaps.

Extraction and analysis of PUWs/MINs

To obtain comparable results, each strain was extracted using an identical ratio of lyophilized biomass (200 mg) to extraction solvent (10 mL of 70% MeOH, v/v). Extracts were evaporated using a rotary vacuum evaporator at 35°C and concentrated to 1 mL of 70% MeOH. The methanolic extracts were analyzed using a Thermo Scientific Dionex UltiMate 3000 UHPLC+ instrument equipped with a diode-array detector connected to a Bruker Impact HD (Bruker, Billerica, MA, USA) high-resolution mass spectrometer with electrospray ionization. Separation of extracts was performed on a reversed-phase Phenomenex Kinetex C18 column (150 × 4.6 mm, 2.6 µm) using H_2O (A)/acetonitrile (B) containing 0.1% HCOOH as a mobile phase, at a flow rate of 0.6 mL min^{-1}. The gradient was as follows: A/B 85/15 (0 min), 85/15 (over 1 min), 0/100 (over 20 min), 0/100 (over 25 min), and 85/15 (over 30 min). For better resolution of minor PUW variants, another analytical method with a longer gradient (67 min) adopted from our previous study (13) was applied. The peptide sequence was reconstructed from the *b* ion series obtained after opening of the ring between the proline and *N*-methylasparagine residues, followed by the sequential loss of water and all the amino acids with exception of the last

residue (Pro). The number of carbons in the FA moiety in PUW/MIN variants containing nonsubstituted and hydroxy-/chloro-substituted FA was determined using a method described previously by our team (13). Characteristic FA immonium fragments in oxo-substituted PUW/MIN variants were identified by employing this method to crude extracts of *Anabaena* strain 5 containing the oxo-substituted MIN D variant (10). Since a stable, prominent, and characteristic FA immonium fragment with the sum formula $C_{15}H_{30}NO^+$ was obtained for MIN D (Fig. S1), analogous fragments with general formula $C_xH_{2x}NO^+$ were used to identify oxo-substituted components in unknown PUW/MIN variants from other investigated strains.

Molecular networking

A molecular network was created using the Global Natural Products Social Molecular Networking (GNPS) online workflow (54). Data were filtered by removing all MS/MS peaks within +/- 17 Da of the precursor *m/z*. MS/MS spectra were window-filtered by choosing only the top six peaks in the +/- 50 Da window throughout the spectrum. Data were then clustered with MS-Cluster with a parent mass tolerance of 0.1 Da and a MS/MS fragment ion tolerance of 0.025 Da to create consensus spectra. Additionally, consensus spectra comprised of fewer than two spectra were discarded. A network was then created in which edges were filtered using a cosine score above 0.75 and more than three matched peaks. Additional edges between pairs of nodes were retained in the network only when both nodes were included in each other's respective top 10 most similar nodes. Spectra in the network were then searched against the GNPS spectral libraries, and library spectra were filtered in the same manner as the input

data. All matches obtained between network spectra and library spectra were retained only when the score was above 0.7 and at least four peaks matched. Analog searching was performed against the library with a maximum mass shift of 200 Da.

Purification of MINs from Anabaena sp. strain 4 and its NMR analysis

Freeze-dried biomass of strain 4 (10 g) was extracted with 70% MeOH (500 mL). The extract was evaporated using a rotary vacuum evaporator to reduce the MeOH content, and the sample was subsequently diluted with distilled water to reach a final MeOH concentration >5%. The diluted extract was pre-purified using a Supelco C18 SPE cartridge (10 g, 60 mL) pre-equilibrated with 60 mL of MeOH and 120 mL of H_2O. After loading, retained components were eluted with 60 mL of pure MeOH, concentrated to dryness, and resuspended in 10 mL of pure MeOH. MINs A, C, and D were purified in two HPLC purification steps. The first step was performed on a preparative chromatographic system (Agilent 1260 Infinity series) equipped with a multi-wavelength detector and automatic fraction collector. A preparative Reprosil 100 C18 column (252 × 25 mm) was employed for separation at a flow rate of 10 mL min^{-1} using the following gradient of MeOH containing 0.1% HCOOH (A) and 10% MeOH containing 0.1% HCOOH (B): 0 min (100% B), 6 min (100% B), 15 min (43% B), 43 min (12% B), 45 min (0% B), 58 min (0% B), 60 min (100% B), and 64 min (100% B). Fractions were collected using an automatic fraction collector at 1 min intervals, and fractions were analyzed for MIN A, C, and D using the method described above. Fractions containing MIN A, C, and D were collected in separate vials and concentrated using a rotary evaporator.The second purification step was performed on a semi-

preparative HPLC (Agilent 1100 Infinity series) using a Reprosil 100 Phenyl column (250 × 8 mm) with (A) acetonitrile containing 0.1% HCOOH and (B) water containing 0.1% HCOOH using the following gradient: 0 min (60% B), 2 min (60% B), 6 min (50% B), 28 min (18% B), 30 min (0% B), 30 min (0% B), 32 min (0% B), 31 min (60% B), and 36 min (60% B). The flow rate was 1 mL min^{-1} throughout, fractions were collected manually, and the purity was analyzed using the HPLC-HRMS method described above. NMR spectra of minutissamides were measured in dimethyl sulfoxide (DMSO)-*d6* at 30°C. All NMR spectra were collected using a Bruker Avance III 500 MHz NMR spectrometer, equipped with a 5 mm ∅ BBI probehead with actively shielded z-gradient.

Antibacterial and antifungal assays

The antimicrobial activity of four major variants (PUW F, and MINs A, C, and D) was tested against 13 bacterial and two yeast strains (Table 4) using disc diffusion assays (8) in three independent experiments with kanamycin/nystatin and MeOH as positive and negative controls, respectively. Antifungal activity of PUW F was further evaluated by determining the MIC against *Candida albicans* (HAMBI 261) and *Saccharomyces cerevisiae* (HAMBI 1164) as described previously (8). PUW F was isolated from *Cylindrospermum* strain 1 according to a protocol described previously (5), and isolation of MIN A, C, and D was performed as described above. The variants produced by *S. muelleri* strain 6 were impossible to isolate due to the slow growth of the cyanobacterium, resulting in low biomass yields during the study period.

Accession numbers for the newly sequenced complete putative biosynthetic gene clusters uploaded to the NCBI GenBank database are MH325197-MH325201.

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References

1. Cochrane SA, Vederas JC. 2016. Lipopeptides from *Bacillus* and *Paenibacillus* spp.: A gold mine of antibiotic candidates. Med Res Rev 36:4–31.

2. Taylor SD, Palmer M. 2016. The action mechanism of daptomycin. Bioorg Med Chem 24:6253–6268.

3. Velkov T, Roberts KD, Li J. 2017. Rediscovering the octapeptins. Nat Prod Rep 34:295–309.

4. Ines M, Dhouha G. 2015. Lipopeptide surfactants: Production, recovery and pore forming capacity. Peptides 71:100–112.

5. Hrouzek P, Kuzma M, Černý J, Novák P, Fišer R, Šimek P, Lukešová A, Kopecký J. 2012. The cyanobacterial cyclic lipopeptides puwainaphycins F/G are inducing necrosis via cell membrane permeabilization and subsequent unusual actin relocalization. Chem Res Toxicol 25:1203–1211.

6. Oftedal L, Myhren L, Jokela J, Gausdal G, Sivonen K, Doskeland SO, Herfindal L. 2012. The lipopeptide toxins anabaenolysin A and B target biological membranes in a cholesterol–dependent manner. Biochim Biophys Acta-Biomembr 1818:3000–3009.

7. Tomek P, Hrouzek P, Kuzma M, Sýkora J, Fišer R, Černý J, Novák P, Bártová S, Šimek P, Hof M, Kavan D, Kopecký J. 2015. Cytotoxic lipopeptide muscotoxin A, isolated from soil cyanobacterium *Desmonostoc muscorum*, permeabilizes phospholipid membranes by reducing their fluidity. Chem Res Toxicol 28:216–224.

8. Vestola J, Shishido TK, Jokela J, Fewer DP, Aitio O, Permi P, Wahlsten M, Wang H, Rouhiainen L, Sivonen K. 2014. Hassallidins, antifungal glycolipopeptides, are widespread among cyanobacteria and are the end-product of a nonribosomal pathway. P Natl Acad Sci USA 111:E1909–E1917.

9. Gregson JM, Chen JL, Patterson GML, Moore RE. 1992. Structures of puwainaphycins A-E. Tetrahedron 48:3727–3734.

10. Kang HS, Krunic A, Shen Q, Swanson SM, Orjala J. 2011. Minutissamides A-D, antiproliferative cyclic decapeptides from the cultured cyanobacterium *Anabaena minutissima*. J Nat Prod 74:1597–1605.

11. Kang HS, Sturdy M, Krunic A, Kim H, Shen Q, Swanson SM, Orjala J. 2012. Minutissamides E-L, antiproliferative cyclic lipodecapeptides from the cultured freshwater cyanobacterium cf. *Anabaena* sp. Bioorg Med Chem 20:6134–6143.

12. Mareš J, Hájek J, Urajová P, Kopecký J, Hrouzek P. 2014. A hybrid non-ribosomal peptide/polyketide synthetase containing fatty-acyl ligase (FAAL) synthesizes the beta-amino fatty acid lipopeptides puwainaphycins in the cyanobacterium *Cylindrospermum alatosporum*. Plos One 9: e111904.

13. Urajová P, Hájek J, Wahlsten M, Jokela J, Galica T, Fewer DP, Kust A, Zapomělová-Kozlíková E, Delawská K, Sivonen K, Kopecký J, Hrouzek P. 2016. A liquid chromatography-mass spectrometric method for the detection of cyclic beta-amino fatty acid lipopeptides. J Chromatogr A 1438:76–83.

14. Cheel J, Urajová P, Hajek J, Hrouzek P, Kuzma M, Bouju E, Faure K, Kopecký J. 2017. Separation of cyclic lipopeptide puwainaphycins from cyanobacteria by countercurrent chromatography combined with polymeric resins and HPLC. Anal Bioanal Chem 409:917–930.

15. Moore RE, Bornemann V, Niemczura WP, Gregson JM, Chen JL, Norton TR, Patterson GML, Helms GL. 1989. Puwainaphycin C, a cardioactive cyclic peptide from the blue-green alga *Anabaena* BQ-16-1. Use of two dimensional carbon-13-carbon-13 and carbon-13-nitrogen-15 correlation spectroscopy in sequencing the amino acid units. J Am Chem Soc 111:6128–6132.

16. Duitman EH, Hamoen LW, Rembold M, Venema G, Seitz H, Saenger W, Bernhard F, Reinhardt R, Schmidt M, Ullrich C, Stein T, Leenders F, Vater J. 1999. The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: A multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. P Nat Acad Sci USA 96:13294–13299.

17. Tsuge K, Akiyama T, Shoda M. 2001. Cloning, sequencing, and characterization of the iturin A operon. J Bacteriol 183:6265–6273.

18. Koumoutsi A, Chen XH, Henne A, Liesegang H, Hitzeroth G, Franke P, Vater J, Borriss R. 2004. Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. J Bacteriol 186:1084–1096.

19. Sood S, Steinmetz H, Beims H, Mohr KI, Stadler M, Djukic M, von der Ohe W, Steinert M, Daniel R, Muller R. 2014. Paenilarvins: Iturin family lipopeptides from the honey bee pathogen *Paenibacillus larvae*. ChemBioChem 15:1947–1955.

20. Ramaswamy AV, Sorrels CM, Gerwick WH. 2007. Cloning and biochemical characterization of the hectochlorin biosynthetic gene cluster from the marine cyanobacterium *Lyngbya majuscula*. J Nat Prod 70:1977–1986.

21. Micallef ML, D'Agostino PM, Sharma D, Viswanathan R, Moffitt MC. 2015. Genome mining for natural product biosynthetic gene clusters in the Subsection V cyanobacteria. Bmc Genomics 16:669.

22. Edwards DJ, Marquez BL, Nogle LM, McPhail K, Goeger DE, Roberts MA, Gerwick WH. 2004. Structure and biosynthesis of the jamaicamides, new mixed polyketide-peptide neurotoxins from the marine cyanobacterium *Lyngbya majuscula*. Chem Biol 11:817–833.

23. Galica T, Hrouzek P, Mareš J. 2017. Genome mining reveals high incidence of putative lipopeptide biosynthesis NRPS/PKS clusters containing fatty acyl-AMP ligase genes in biofilm-forming cyanobacteria. J Phycol 53:985–998.

24. Arora P, Goyal A, Natarajan VT, Rajakumara E, Verma P, Gupta R, Yousuf M, Trivedi OA, Mohanty D, Tyagi A, Sankaranarayanan R, Gokhale RS. 2009. Mechanistic and functional insights into fatty acid activation in *Mycobacterium tuberculosis*. Nat Chem Biol 5:166–173.

25. Liu Z, Ioerger TR, Wang F, Sacchettini JC. 2013. Structures of *Mycobacterium tuberculosis* FadD10 protein reveal a new type of adenylate-forming enzyme. J Biol Chem 288:18473–18483.

26. Coates RC, Podell S, Korobeynikov A, Lapidus A, Pevzner P, Sherman DH, Allen EE, Gerwick L, Gerwick WH. 2014. Characterization of cyanobacterial hydrocarbon composition and distribution of biosynthetic pathways. Plos One 9:e85140.

27. Mohanty D, Sankaranarayanan R, Gokhale RS. 2011. Fatty acyl-AMP ligases and polyketide synthases are unique enzymes of lipid biosynthetic machinery in *Mycobacterium tuberculosis*. Tuberculosis 91:448–455.

28. Goyal A, Verma P, Anandhakrishnan M, Gokhale RS, Sankaranarayanan R. 2012. Molecular basis of the functional divergence of fatty acyl-AMP ligase biosynthetic enzymes of *Mycobacterium tuberculosis*. J Mol Biol 416:221–238.

29. Villiers BRM, Hollfelder F. 2009. Mapping the limits of substrate specificity of the adenylation domain of TycA. ChemBioChem 10:671–682.

30. Christiansen G, Philmus B, Hemscheidt T, Kurmayer R. 2011. Genetic variation of adenylation domains of the anabaenopeptin synthesis operon and evolution of substrate promiscuity. J Bacteriol 193:3822–3831.

31. Brandenburger E, Braga D, Kombrink A, Lackner G, Gressler J, Künzler M, Hoffmeister D. 2018. Multi-genome analysis identifies functional and phylogenetic diversity of basidiomycete adenylate-forming reductases. Fungal Genet Biol 112:55–63.

32. Rouhiainen L, Jokela J, Fewer DP, Urmann M, Sivonen K. 2010 Two alternative starter modules for the non-ribosomal biosynthesis of specific anabaenopeptin variants in *Anabaena* (Cyanobacteria). Chem Biol 17:265–273.

33. Kleigrewe K, Almaliti J, Tian IY, Kinnel RB, Korobeynikov A, Monroe EA, Duggan BM, Di Marzo V, Sherman DH, Dorrestein PC, Gerwick L, Gerwick WH. 2015. Combining mass spectrometric metabolic profiling with genomic analysis: A powerful approach for discovering natural products from cyanobacteria. J Nat Prod 78:1671–1682.

34. Voráčová K, Hájek J, Mareš J, Urajová P, Kuzma M, Cheel J, Villunger A, Kapuscik A, Bally M, Novák P, Kabeláč M, Krumschnabel G, Lukeš M, Voloshko L, Kopecký J, Hrouzek P. 2017. The cyanobacterial metabolite nocuolin A is a natural oxadiazine that triggers apoptosis in human cancer cells. Plos One 12:e0172850.

35. He J, Hertweck C. 2004. Biosynthetic origin of the rare nitroaryl moiety of the polyketide antibiotic aureothin: Involvement of an unprecedented N-oxygenase. J Am Chem Soc 126:3694–3695.

36. Choi YS, Zhang HJ, Brunzelle JS, Nair SK, Zhao HM. 2008. In vitro reconstitution and crystal structure of p-aminobenzoate N-oxygenase (AurF) involved in aureothin biosynthesis. P Natl Acad Sci USA 105:6858-6863.

37. Fewer DP, Wahlsten M, Osterholm J, Jokela J, Rouhiainen L, Kaasalainen U, Rikkinen J, Sivonen K. 2013. The genetic basis for O-Acetylation of the microcystin toxin in cyanobacteria. Chem Biol 20:861–869.

38. Murray IA, Shaw WV. 1997. O-acetyltransferases for chloramphenicol and other natural products. Antimicrob Agents Chemother 41:1–6.

39. 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*). J Phycol 45:1363–1373.

40. Skulberg OM, Mysterud I, Karlsen J, Tønnesen HH, Laane CMM, Schumacher T. 2012. Alveld research per annum 2012: Searchlight on cyanobacteria we have minor knowledge of. (In Norwegian). Biolog 30: 32–41.

41. Tønnesen HH, Mysterud I, Karlsen J, Skulberg OM, Laane CMM., Schumacher T. 2013. Identification of singlet oxygen photosensitizers in lambs drinking water in an alveld risk area in West Norway. J Photochem Photobiol B 119: 37–45.

42. Hegge AB, Mysterud I, Karlsen J, Skulberg OM, Laane CMM, Schumacher T, Tønnesen HH. 2013. Impaired secondary oxidant deactivation capacity and enhanced oxidative stress in serum from alveld affected lambs. J Photochem Photobiol B 126: 126–134.

43. Heinze R. 1996. A biotest for hepatotoxins using primary rat hepatocytes. Phycologia 35 (Supplement), 89–93.

44. Skulberg, OM. 1996. Toxins produced by cyanophytes in Norwegian inland waters – health and environment. – In Chemical data as a basis of geomedical investigations. (J. Låg, J. Ed), pp. 197–216, The Norwegian Academy of Science and Letters.

45. Jones CG, Firn RD. 1991. On the evolution of plant secondary chemical diversity. Phil Trans R Soc Lond B 333:273–280.

46. Firn RD, Jones CG. The evolution of secondary metabolism - a unifying model. Mol Microbiol 37: 989–994.

47. Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111: 1–61.

48. Skulberg R, Skulberg OM. 1990. Research with algal cultures – NIVA's Culture Collection of Algae. *Norwegian Institute for Water Research*, 1–32. ISBN: 82–577–1743–6.

49. 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 69:5157–5169.

50. Delcher AL, Bratke KA, Powers EC, Salzberg SL. 2007. Identifying bacterial genes and endosymbiont DNA with Glimmer. Bioinformatics 23:673–679.

51. Bankevich A, Nurk S, Antipov D, Gurevich A, Dvorkin M, Kulikov AS, Lesin V, Nikolenko S, Pham S, Prjibelski A, Pyshkin A, Sirotkin A, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455–497.

52. Weber T, Blin K, Duddela S, Krug D, Kim HU, Bruccoleri R, Lee SY, Fischbach MA, Muller R, Wohlleben W, Breitling R, Takano E, Medema MH. 2015. antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Res 43:W237–W243.

53. Blin K, Wolf T, Chevrette MG, Lu XW, Schwalen CJ, Kautsar SA, Duran HGS, Santos E, Kim HU, Nave M, Dickschat JS, Mitchell DA, Shelest E, Breitling R, Takano E, Lee SY, Weber T, Medema MH. 2017. antiSMASH 4.0-improvements in chemistry prediction and gene cluster boundary identification. Nucleic Acids Res 45:W36–W41.

54. Wang MX, Carver JJ, Phelan VV, Sanchez LM, Garg N, Peng Y, Nguyen DD, Watrous J, Kapono CA, Luzzatto-Knaan T, Porto C, Bouslimani A, Melnik AV, Meehan MJ, Liu WT, Criisemann M, Boudreau PD, Esquenazi E, Sandoval-Calderon M, Kersten RD, Pace LA, Quinn RA, Duncan KR, Hsu CC, Floros DJ, Gavilan RG, Kleigrewe K, Northen T, Dutton RJ, Parrot D, Carlson EE, Aigle B, Michelsen CF, Jelsbak L, Sohlenkamp C, Pevzner P, Edlund A, McLean J, Piel J, Murphy BT, Gerwick L, Liaw CC, Yang YL, Humpf HU, Maansson M, Keyzers RA, Sims AC, Johnson AR, Sidebottom AM, Sedio BE, *et al.* 2016. Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. Nat Biotechnol 34:828–837.

55. Johansen JR, Bohunická M, Lukešová A, Hrčková K, Vaccarino MA, Chesarino NM. 2014. Morphological and molecular characterization within 26 strains of the genus *Cylindrospermum* (Nostocaceae, Cyanobacteria) with descriptions of three new species. J. Phycol. 50:187-202.

56. Tamrakar A. 2016. Isolation of benthic cyanobacteria and screening of bioactivities and natural products from culture collection strains. Master Thesis. University of Helsinki, Faculty of Agriculture and Forestry, Department of Food and Environmental Sciences.

57. Kantz T., Bold HC. 1969. Phycological studies IX. Morphological and taxonomic investigations of *Nostoc* and *Anabaena* in culture. Publication no. 6924. University of Texas, Austin.

58. Skulberg OM, Mysterud I, Karlsen J, Třnnesen HH, Laane CMM, Schumacher T. 2012. Alveld research per annum 2012: Searchlight on cyanobacteria we have minor knowledge of. (In Norwegian). Biolog 30: 32-41.

8. CURRICULUM VITAE

Name: Andreja Kust
Nationality: Croatian
Date of Birth: 06.04.1988
Email: andreja.kust@gmail.com

Research experience and Education

2016 - Until now: Ph.D student at the Institute of Microbiology Czech Academy of Sciences, Center Algatech, Třeboň, Czech Republic. Team: Pavel Hrouzek
July 2013 - Until now: PhD student of Faculty of Science, University of South Bohemia
April 2013 - Until now: Ph.D. student at the Czech Academy of Sciences, Biology Centre, Institute of Hydrobiology, České Budějovice, Czech Republic. Team: Jan Mareš
2010 - 2013: Master of Science, Graduate Programme in Biology and Chemistry Education, University J. J. Strossmayer in Osijek, Croatia
2007 - 2010: Bachelor (Baccalaurea) of Biology, University J. J. Strossmayer in Osijek, Croatia

Abroad Internships

5.10-1.12.2017 and 1.10.2018-15.12.2018: LIED UMR CNRS 8236, University Paris Diderot, France. Team: Annick Mejean
June 2017: University of Helsinki, Finland. Team: Kaarina Sivonen
March 2014: Faculty of Exact and Natural Sciences, University of Buenos Aires, Argentina. Team: Paula de Tezanos Pinto
March 2012 - July 2012 Erasmus study stay at Czech Academy of Sciences, Biology Centre, Institute of Hydrobiology, České Budějovice, Czech Republic

Teaching experience

2014 and 2015: teaching assistant during courses of Microbiology practicals at Faculty of Science, University of South Bohemia
Workshop leader during the International conference FBFW2017 České Budějovice, Czech Republic.

Grants and fellowships

Awarded by IBERA Grant Program BC 2018, and French Government Scholarship for international stay abroad at LIED UMR CNRS 8236, University Paris Diderot, France. Team: Annick Méjean

National Grants as Collaborator

2016-2018: Project of Czech Science Foundation GA CR No. 16-09381S (Bioactive cyanobacterial lipopeptides: genome mining, detection, and structure-activity relationships), team member

2014-2016: Czech Science Foundation GA CR No. 14-18067S (Toxic potential, evolution of toxin synthesis, and factors driving anatoxin-a production in benthic and soil nostocacean cyanobacteria), team member

2010-2012: Project of Czech Science Foundation GA CR No. P504/10/1501 (Taxonomic revision of the genera *Anabaena* and *Aphanizomenon* (cyanobacteria) based on complex phenotypic and molecular approach), team member

Publications

Achieved results: 4 published articles in international peer-reviewed journals

Citations: h- index=2, citations=11 (Listing out publications 2013 onwards)

Kust A, Kozlíková-Zapomělová E, Mareš J, Řeháková K. 2015: A detailed morphological, phylogenetic and ecophysiological analysis of four benthic Anabaena (Nostocales, Cyanobacteria) strains confirms deep heterogeneity within the genus. Fottea 15(2): 191-202 **(IF=1.350)**

de Tezanos Pinto P, **Kust A,** Devercelli M, Kozlíková – Zapomělová E. 2015: Morphological traits in nitrogen fixing heterocytous cyanobacteria: possible links between morphology and eco-physiology. Hydrobiologia. Volume 764 (1): 271-281 **(IF=2.056)**

Urajová P, Hájek J, Wahlsten M, Galica T, Fewer D.P, Jokela J, **Kust A**, Zapomělová-Kozlíková E, Delawská K, Sivonen K, Kopecký J, Hrouzek P. 2016: A liquid chromatography–mass spectrometric method for the detection of cyclic β-amino fatty acid lipopeptides. Journal of Chromatography A. DOI: 10.1016/j.chroma.2016.02.013 **(IF=4.169)**

Kust A, Mareš J, Jokela J, Urajová P, Hájek J, Saurav K, Voráčová K, Fewer D. P, Haapaniemi E, Permi P, Řeháková K, Sivonen K, Hrouzek P. 2018: Discovery of a pederin family compound in a non-symbiotic bloom-forming cyanobacterium. *ACS Chemical Biology* **13**: 1123-1129. **(IF=4.592)**

Kust A, Urajová P, Hrouzek P, Long Vu D, Čapková K, Štenclová L, Řeháková K, Kozlíková-Zapomělová E, Lepšová-Skácelová O, Lukešová A, Mareš J. 2018: A new microcystin producing Nostoc strain discovered in broad toxicological screening of non-planktic Nostocaceae (Cyanobacteria). *Toxicon* 150: 66-73. **(IF=2.352)**

Mareš J, Hájek J, Urajová P, **Kust A,** Jokela J, Saurav K, Galica T, Čapková K, Mattila A, Haapaniemi E, Permi P, Mysterud I, Skulberg OM, Karlsen J Fewer DP, Sivonen K, Tonnesen HH Hrouzek P. 2018: Alternative biosynthetic starter units enhance the structural diversity of cyanobacterial

lipopeptides**.** *Applied and Environmental Microbiology*, DOI: 10.1128/AEM.02675-18 (**IF=3.807)**

COMMUNICATIONS

Member of organizing committee and workshop leader during the International conference FBFW2017 České Budějovice, Czech Republic.

Conference on Aquatic Microbial Ecology SAME-15, Zagreb, Croatia, 2017 (volunteering)

Oral presentations

Toxic potential and factors driving toxin production by nostocacean cyanobacteria

PhD conference of Department of Ecosystem Biology University of South Bohemia, České Budějovice, Czech Republic, 2018 (first price for the oral presentation)

Genetic background, biosynthesis, and structural variability of puwainaphycins and minutissamides

University of Helsinki, Finland, 2017

An in-detail morphological, phylogenetic and ecophysiological analysis of four benthic *Anabaena* strains (Nostocales, Cyanobacteria) confirms deep heterogeneity within the genus.

FBFW Fresh Blood for Fresh Water, 15 -17 May 2015, Mondsee, Austria

Benthic *Anabaena* - polyphyletic group of extremophiles or not?

Universidad de Buenos Aires, Buenos Aires, Argentina, 2014 and Instituto Nacional de Limnologia (CONICET-UNL), Santa Fe, Argentina, 2014

Rock 'n' Soil, Argentinian Anabaena Favorite Home, University of South Bohemia, České Budějovice, Czech Republic, 2014

Posters

Cytotoxic cyanobacterial lipopeptides puwainaphycins and minutissamides: from outsiders towards widespread potential toxins

a) Cyano2018, 3rd Early Reasercher Symposium on Cyanobacteria, 12.-14. of September 2018 in Freiburg, Germany

b) ICHA2018, The 18th international conference on harmful algae 21-26 October 2018, Nantes, France

First record of a putative pederin-type gene cluster and its product in a free-living cyanobacterium Cuspidothrix issatschenkoi.

a) International Summer School on Natural Products (ISSNP), 4-7 July 2017. Naples, Italy

b) 16th International Symposium on Microbial Ecology (ISME), 21- 26 August 2016. Montreal, Canada

Occurrence of the most common toxins vs. cytotoxicity in non-gasvacuolate heterocytous cyanobacteria

Second EMBO Conference on Aquatic Microbial Ecology: SAME-14, 23-28 August 2015 Uppsala, Sweden

Teaching experience
2014 and 2015. Microbiology practicals at University of South Bohemia. 60 hours

Research interest:
Morphological, molecular and ecophysiological analysis of cyanobacteria. Broad research experience in toxic potential, evolution of toxin synthesis, and factors driving toxins production in cyanobacteria. Techniques of isolation and cultivation of cyanobacteria and algae, quantification of secondary metabolites production by RT-qPCR; detection of secondary metabolites by PCR and HPLC/MS, genome analyses.

andreja.kust@gmail.com

Polyphasic analysis and secondary metabolite patterns in unbranched heterocytous cyanobacteria with different life strategies
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University of South Bohemia in České Budějovice
Faculty of Science
Branišovská 1760
CZ-37005 České Budějovice, Czech Republic

Phone: +420 387 776 201
www.prf.jcu.cz, e-mail: sekret-fpr@prf.jcu.cz