Environmental relevancies and variability of cyanobacterial toxins

The aim of the proposed project was to study the role and impact of cyanotoxins and unique cyanobacterial metabolites on aquatic ecosystems by analyzing harmful cyanobacterial blooms in ponds/ lakes and terrestrial habitats. The research complemented with laboratory metabolomic studies, applying different in vitro and in vivo toxicity assays on aquatic organisms naturally affected by cyanotoxins and on model cell/tissue cultures. We aimed to clarify the alterations and variability of unique bioactive peptide metabolites produced by bloom-forming cyanobacterial species.
Regarding the topics focussed in Our proposal were worked up with several experimental study (filed and laboratory as well) and a few review study also.
The results will be summarized in the following sections.

1. Microcystin (MYC) and MYC producer relating results

1.1. Attack of Microcystis aeruginosa bloom on a Ceratophyllum submersum field: Ecotoxicological measurements in real environment with real microcystin exposure

Overproduction of toxic cyanobacteria is a type of harmful algal blooms (HABs). The heptapeptide microcystins (MCs) are one of the most common cyanotoxins. There is increasing research concerning the effects of MCs on growth and physiology of vascular plants, however there is a lack of studies on their direct effects on aquatic macrophytes in the real environment. Here we report the occurrence of a MC producing HAB in Lake Bárdos, Hungary with harmful effects on cytological, histological and biochemical parameters of *Ceratophyllum submersum* (soft hornwort) plants naturally growing at the blooming site. Blue-Green Sinapis Test (BGST) showed high toxicity of HAB samples. Cell-free water samples contained a significant amount of MCs ($7.31 \pm 0.17\ \mu g\ L^{-1}$) while *C. submersum* plants contained $1.01 \pm 0.21\ \mu g\ g\ DW^{-1}$ MCs. Plants showed significant increases of protein content and decreases of anthocyanin content and carotenoid/chlorophyll ratio, indicating physiological stress- as compared to plants from the control (MC free) sampling site of the same water body. Histological and cytological studies showed (i) radial swelling and the abnormal formation of lateral buds at the shoot tip leading to abnormal development; (ii) the fragmentation of nuclei as well as accumulation of phenolics in the nucleus indicating that the HAB induced cell death and stress reactions at the nuclear level. The most relevant effect was the increase of histone H3 phosphorylation in metaphase chromosomes: since MCs are strong inhibitors of protein phosphatases, this alteration is related to the biochemical targets of these toxins. The HAB decreased peroxidase activity, but increased nuclease and protease activities, showing the decreased capacity of plants to face biotic stress and as the cytological changes, the induction of cell death. This study is one of the first to show the complex harmful changes in aquatic plants that co-exist with HABs (fig 1.).

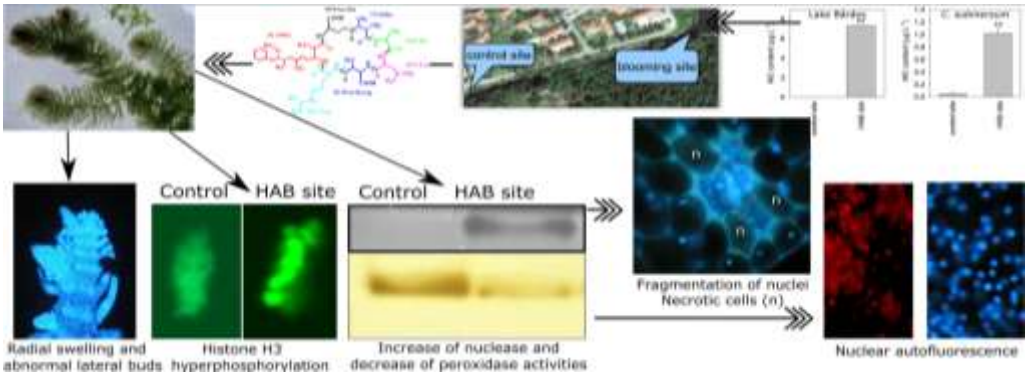

fig 1. Experimental design of 1.1 study

As a conclusion, the MC containing HAB site of Lake Bárdos, Hungary, affected *C. submersum* plants living at this site, at several levels. In this year significant total MC content was observed both in water body and in plants at the HAB site. Decreases of pigment levels (carotenoid-carotenoid/chlorophyll ratio and anthocyanin content) and of pyrogallol peroxidase activity indicated decreased stress defence capacity of the plants. The above parameters, together with the increase of protein level are clear indicators of biotic stress induction. The radial swelling of shoot tips and abnormal lateral bud formation at the shoot apex probably indicated alterations in cytoskeletal organization. This phenomenon, together with chromatin fragmentation and increased autofluorescence of nuclei as well as abnormal secondary cell wall thickenings and increased nuclease/protease activities indicated clearly that MC content of water body could induce cell death in this plant. One of the most relevant alterations observed was hyperphosphorylation of histone H3 in metaphase cells of plants from the HAB site, because this can be related directly to the potent protein phosphatase inhibitory effect of MC. Thus, hyperphosphorylation of histone H3 might be an indicator of the presence of a protein phosphatase inhibitory toxin in a given natural water body. To our knowledge, to date this is one of the first complex studies showing natural effects of cyanobacterial toxins on vascular plants under real environmental conditions.

1.2. Microcystin-LR, a Cyanobacterial Toxin, Induces DNA Strand Breaks Correlated with Changes in Specific Nuclease and Protease Activities in White Mustard (Sinapis alba) Seedlings

There is increasing evidence for the induction of programmed cell death (PCD) in vascular plants by the cyanobacterial toxin microcystin-LR (MC-LR). Our aim was to detect the occurrence of PCD-related DNA strand breaks and their possible connections to specific nuclease and protease activities. DNA breaks were studied by the deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) method in the photoperiodically grown dicot model of white mustard (*Sinapis alba*). In-gel nuclease and protease activity assays showed changes in the activities of specific isoenzymes during treatments with MC-LR. Strand breaks occurred both in the developing root epidermis and cortex. Several isoenzyme activities were related to these breaks, for example: an increase in the activity of neutral 80–75 kDa, acidic high MW (100–120 kDa) and, most importantly, an increase in the activity of neutral 26–20 kDa nucleases, all of them having single-stranded DNA cleaving (SSP nuclease) activities. Increases in the activities of alkaline proteases in the 61–41 kDa range were also detected and proved to be in relation with MC-LR-induced PCD. This is one of the first pieces of evidence on the correlation of PCD-related DNA strand breaks with specific hydrolase activities in a model dicot treated with a cyanobacterial toxin known to have environmental importance.

In conclusion, the occurrence of MC-LR-induced DNA strand breaks, as revealed by TUNEL labeling in mustard seedlings, can be related to an increase in nuclease (e.g., the neutral 26–20 kDa and 80–75 kDA; the acidic ≥100, 55 and 60 kDa isoenzymes) and protease (e.g., the alkalic 61–51 kDa isoenzymes) activities that altogether lead to cell death. Future research will reveal the functioning of these specific hydrolases in the execution of plant PCD. The present research has an important environmental context as well. It may contribute to a better understanding of the toxicity of MC-LR, a cyanobacterial metabolite with a significant impact on aquatic ecosystems and crop plants.

1.3. Subcellular Alterations Induced by Cyanotoxins in Vascular Plants—A Review

Phytotoxicity of cyanobacterial toxins has been confirmed at the subcellular level with consequences on whole plant physiological parameters and thus growth and productivity. Most of the data are available for two groups of these toxins: microcystins (MCs) and cylindrospermopsins (CYNs). Thus, in this review we present a timely survey of subcellular cyanotoxin effects with the main focus on these two cyanotoxins (fig 2.). We provide comparative insights into how peculiar plant cellular structures are affected. We review structural changes and their physiological consequences induced in the plastid system, peculiar plant cytoskeletal organization and chromatin structure, the plant cell wall, the vacuolar system, and in general, endomembrane structures. The cyanotoxins have characteristic dose-and plant genotype-dependent effects on all these structures. Alterations in chloroplast structure will influence the efficiency of photosynthesis and thus plant productivity. Changing of cell wall composition, disruption of the vacuolar membrane (tonoplast) and cytoskeleton, and alterations of chromatin structure (including DNA strand breaks) can ultimately lead to cell death. Finally, we present an integrated view of subcellular alterations. Knowledge on these changes will certainly contribute to a better understanding of cyanotoxin–plant interactions.

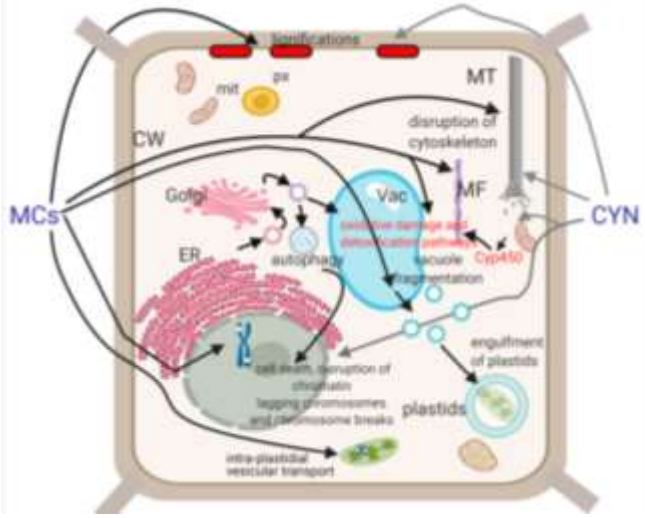

fig 2. Subcellular effects of MYC and CYN

The detrimental effects of both MCs and CYNs on plant cytoskeleton have a distinct importance, since this structure is crucial for the integrated functioning of a eukaryotic cell. Now we know many effects of MCs on MTs and MFs and those of CYNs on MTs in plants. These studies underline that MT and MF disruptions will alter cell division and cell shape, which will ultimately lead to distorted organs and inhibition of elongation growth. One of the most interesting changes are the misorientation of mitotic planes by disruption of normal PPB organization by CYN. Alteration of CMT organization will alter cell shape and elongation growth in MC and CYN treatments.

It is also clear that given the impact of cyanotoxins on aquatic ecosystems and their adverse effects on terrestrial crops (via irrigation with toxin-contaminated water), there is tremendous work to perform in the future for a better understanding of their cellular effects. Here are some directions of study that we consider to be important:

(i)

Endomembrane systems such as the ER are as important as the cytoskeleton for the integrated functioning of the plant cell. There is still a lack of knowledge on the relevant effects of cyanotoxins. For example, do cyanotoxins induce ER stress as related to plant cell death?

(ii)

Although CYN is considered to be a protein synthesis inhibitory toxin in eukaryotes, we still do not know much on its particular molecular targets. Studies on plant cells might be essential in this issue.

(iii)

Most knowledge on the effects of cyanotoxins on plant cells involves MCs and CYNs. What about the other cyanotoxins? Non-MC peptides such as aeruginosins, microginins, etc., are of particular interest because many of them are protein phosphatase inhibitors, proteases or protease inhibitors and as such, they are likely to induce subcellular alterations.

1.4. Microcystin-LR, a cyanobacterial toxin affects root development by changing levels of PIN proteins and auxin response in Arabidopsis roots

Microcystin-LR (MCY-LR) is a heptapeptide toxin produced mainly by freshwater cyanobacteria. It strongly inhibits protein phosphatases PP2A and PP1. Functioning of the PIN family of auxin efflux carriers is crucial for plant ontogenesis and their functions depend on their reversible phosphorylation. We aimed to reveal the adverse effects of MCY-LR on PIN and auxin distribution in Arabidopsis roots and its consequences for root development.

Relatively short-term (24 h) MCY-LR treatments decreased the levels of PIN1, PIN2 and PIN7, but not of PIN3 in tips of primary roots. In contrast, levels of PIN1 and PIN2 increased in emergent lateral roots and their levels depended on the type of PIN in lateral root primordia. DR5:GFP reporter activity showed that the cyanotoxin-induced decrease of auxin levels/responses in tips of main roots in parallel to PIN levels. Those alterations did not affect gravitropic response of roots. However, MCY-LR complemented the altered gravitropic response of *crk5-1* mutants, defective in a protein kinase with essential role in the correct membrane localization of PIN2. For MCY-LR treated Col-0 plants, the number of lateral root primordia but not of emergent laterals increased and lateral root primordia showed early development. In conclusion, inhibition of protein phosphatase activities changed PIN and auxin levels, thus altered root development. Previous data on aquatic plants naturally co-occurring with the cyanotoxin showed similar alterations of root development. Thus, our results on the model plant Arabidopsis give a mechanistic explanation of MCY-LR phytotoxicity in aquatic ecosystems.

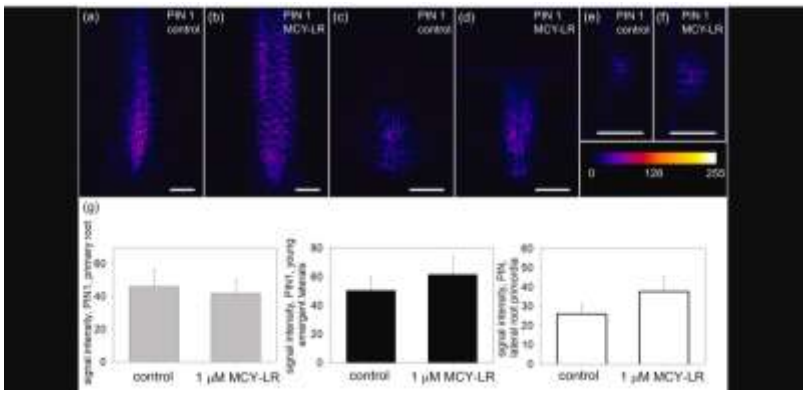

fig 3. Short-term MYC treatments induce altered levels of PINs

This study shows that even relatively short-term cyanotoxin treatments induce inhibition of root elongation and promote initiation of lateral root development in Arabidopsis-related to altered levels of PINs and in consequence, auxins (fig. 3). Thus, the model presented here suggests that developmental alterations induced by the cyanotoxin in natural plant communities originate from alterations of local auxin concentrations in roots.

To conclude, MCY-LR induces alterations of root development in the model plant Arabidopsis and this is correlated to changes in the levels of PIN auxin efflux carriers and of local auxin levels in roots. These changes are related directly to the protein phosphatase inhibitory effects of the cyanotoxin at 24 h treatments. The present study is the first one to show such phytotoxic effects of a cyanotoxin. It contributes to a better understanding of phytotoxicity of a well-known cyanotoxin both under laboratory conditions and the real environment.

2. Cylindrospermopsin (CYN) and CYN producer relating results.

2.1. Cellular effects of cylindrospermopsin (cyanobacterial alkaloid toxin) and its potential medical consequences

Cylindrospermopsin (CYN) is a tricyclic guanidino alkaloid toxin produced by several cyanobacterial genera. It alters cellular functioning in eukaryotes, including animal and plant organisms. Over the past decades, more and more evidence shows its potential hazardous effects on animal and human health. In this review, we give a critical survey and interpretation of data currently available on its biochemical and consequently, cellular effects. CYN is considered to be a cytotoxin. Several reports suggest that it is a potent inhibitor of eukaryotic protein synthesis, though the exact mechanisms are not completely understood. Here we show that the biochemical changes induced by CYN are complex, possibly involving multiple modes of action (fig. 4). Glutathione metabolism and pyrimidine nucleotide synthesis is affected besides the proposed protein synthesis inhibition. Biochemical alterations lead to the following cellular/subcellular alterations both in animals and plants: (i) changes in cell division rates due to perturbations in chromatin and cytoskeleton; (ii) perturbations of structure and functioning of endomembranes including endoplasmic reticulum; (iii) general metabolic alterations leading to genotoxicity and programmed cell death/apoptosis. The underlying mechanisms and possible health consequences are discussed.

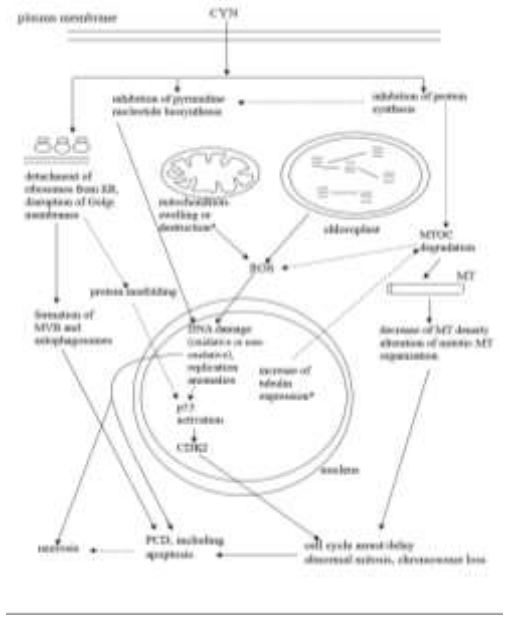

fig 4. CYN alters cellular functioning in eukaryotes

2.2. Cylindrospermopsin induces biochemical changes leading to programmed cell death in plants

In the present study we provide cytological and biochemical evidence that the cyanotoxin cylindrospermopsin (CYN) induces programmed cell death (PCD) symptoms in two model vascular plants: the dicot white mustard (Sinapis alba) and the monocot common reed (Phragmites australis). Cytological data include chromatin fragmentation and the increase of the ratio of TUNEL-positive cells in roots, the latter being detected in both model systems studied. The strongest biochemical evidence is the elevation of the activity of several singlestranded DNA preferring nucleases-among them enzymes active at both acidic and alkaline conditions and are probably directly related to DNA breaks occurring at the initial stages of plant PCD: 80 kDa nucleases and a 26 kDa nuclease, both having dual (single-

and double-stranded nucleic acid) specificity. Moreover, the total protease activity and in particular, a 53–56 kDa alkaline protease activity increases. This protease could be inhibited by PMSF, thus regarded as serine protease. Serine proteases are detected in all organs of Brassicaceae (Arabidopsis) having importance in differentiation of specialized plant tissue through PCD, in protein degradation/processing during early germination and defense mechanisms induced by a variety of biotic and abiotic stresses. However, knowledge of the physiological roles of these proteases and nucleases in PCD still needs further research. It is concluded that CYN treatment induces chromatin fragmentation and PCD in plant cells by activating specific nucleases and proteases. CYN is proposed to be a suitable molecule to study the mechanism of plant apoptosis.

As a conclusion, we provide the first clear evidence that CYN, an environmentally relevant cyanobacterial toxin induces PCD in plants, mainly in roots. DNA fragmentation typical to PCD is related to increases in the levels and concerted effects of well defined sspDNases and proteases. CYN induced DNA strand breaks were previously related exclusively to ROS in animals, humans and partially in plants. Now we show for the first time that CYN induced nuclease and protease activation plays a pivotal role in the triggering of plant PCD.

2.3. Carotenoid glycoside isolated and identified from cyanobacterium Cylindrospermopsis raciborskii

The freshwater cyanobacterium *Cylindrospermopsis raciborskii w*as investigated for carotenoid composition. Besides β-carotene, echinenone and (9/9′*Z*)-echinenone a carotenoid glycoside was found to be the main component. This compound was isolated and subsequently acetylated for structural elucidation. The acetyl derivative was fully characterized by UV–vis, ECD, NMR and HRMS techniques. The detailed ^{1}H and ^{13}C NMR chemical shift assignment of the major carotenoid supported the unequivocal identification of (2′*S*)-2-hydroxymyxol 2′-α-L-fucoside. A heavy and rather unusual *C. raciborskii* bloom was detected early November in the Eastern part of Hungary (Fancsika pond), resulting strong discoloration of the water (fig 5.). Instead of the well-known cyanobacterial pale green-bluish discoloration, the water turned to orange color which suggested uncommon pigments or uncommon pigment concentration in the water. Nostoxanthin, caloxanthin, and zeaxanthin were absent, whereas the major carotenoid in cyanobacterium *Cylindrospermopsis raciborskii* was identified as (2*R*,3*R*,2′*S*)-2-hydroxymyxol 2′-α-L-fucoside on the basis of UV/Vis, ECD, NMR and mass spectra of its acetylated derivative. Its semi-systematic name is (2*R*,3*R*,2′*S*)-2′-(α-L-fucopyranosyloxy)-3′,4′-didehydro-1′,2′-dihydro-β,ψ-carotene-2,3,1′-triol.

fig 5. *C. raciborskii* strain and its bloom

Although the same compound was previously found as one of the carotenoid component in the thermophilic cyanobacterial species *Thermosynechococcus elongatus* , this glycoside is the major carotenoid in *C. raciborskii*. Our results suggest that the biomass of this cyanobacterial species would be an economically justified natural source for the production and purification of this unique compound for the industrial approaches. In addition the presence of this hydrophilic antioxidant pigment suggests another explanation why this cyanobacterial species could spread and adapt efficiently and can built huge biomass in different aquatic habitats.

2.4. Potential role of the cellular matrix of Aphanizomenon strains in the effects of cylindrospermopsin—an experimental study

Some literature data suggest that one of the possible roles of the cyanotoxin cylindrospermopsin (CYN) is forcing other phytoplankton species in the environment to produce alkaline phosphatase, which enables the cyanobacterium to take up the enzymatically liberated phosphate. In this study, cultures of a planktonic green alga, Scenedesmus obtusus (Chlorophyta, Sphaeropleales), were treated with CYN producer Aphanizomenon (Cyanobacteria, Nostocales) crude extract (C+), with non-CYN producer Aphanizomenon crude extract (C−), and with non-CYN producer Aphanizomenon crude extract supplemented with CYN (C−+C). The results showed that C+ treatment induced both acidic and alkaline phosphatases of the studied cosmopolitan green alga, which otherwise was neither sensitive to the relatively high CYN concentration, nor to phosphate limitation. In cases of C− and C−+C treatments, these phenomena were not observed. Several studies suggest that additional compounds may support CYN action. The results presented here suggest in a more direct way that other components present in the cellular matrix of the producer organism itself are involved in the effects of CYN, activation of phosphatases (not only alkaline ones) among them. These other components are absent in C− crude extract or cannot actively contribute to the effects of exogenously added CYN. In this study, effects of crude extracts of phenotypically closely related Aphanizomenon strains were introduced on growth, phosphate uptake, and phosphatase activities of a green alga S. obtusus. Responses of the green alga in phosphate-limited circumstances were also investigated. Our results show that the Scenedesmus strain is sensitive neither to the relatively high concentration of CYN nor to phosphate limitation. Nonetheless, alkaline phosphatase activity of algal cells was significantly higher during C+ treatments; therefore, the concept that CYN is forcing other phytoplankton species in the environment to produce alkaline phosphatase was confirmed also in the case of an insensitive species. Acidic phosphatase activity also increased during C+ treatments. The lack of growth inhibition and phosphatase induction in C− treatments strongly support the role of CYN in these phenomena. At the same time, the lack of growth inhibition and the weaker effects on phosphatases in the case of C−+C treatments highlight the possible role of synergistic metabolites in originally CYN-containing crude extract. Our results also suggest that these metabolites together with CYN contribute to external phosphate uptake, since in the lack of the living CYN producer (Aphanizomenon), the phosphate uptake of the treated green alga increased. Currently, it is unknown how these theoretical additional compounds may support CYN action. To assess whether they directly contribute to the effects of CYN or rather they influence CYN bioavailability requires further studies. The results presented here suggest that CYN, together with other molecules of its producer, could affect significantly even non-sensitive phytoplankton species, thus could affect the processes in algal assemblages.

3. Results relating cyanobacterial peptides

3.1. Microcystis chemotype diversity in the alimentary tract of bigheaded carp

Most cyanobacterial organisms included in the genus *Microcystis* can produce a wide repertoire of secondary metabolites. In the mid-2010s, summer cyanobacterial blooms of *Microcystis* sp. occurred regularly in Lake Balaton (fig 6.).

fig 6. Microcystis sp local bloom at lake Balaton

During this period, we investigated how the alimentary tract of filter-feeding bigheaded carps could deliver different chemotypes of viable cyanobacteria with specific peptide patterns. Twenty-five *Microcystis* strains were isolated from pelagic plankton samples (14 samples) and the hindguts of bigheaded carp (11 samples), and three bloom samples were collected from the scums of cyanobacterial blooms. An LC-MS/MS-based untargeted approach was used to analyze peptide patterns, which identified 36 anabaenopeptin, 17 microginin, and 13 microcystin variants. Heat map clustering visualization was used to compare the identified chemotypes (fig 7.). A lack of separation was observed in peptide patterns of *Microcystis* that originated from hindguts, water samples, and bloom-samples. Except for 13 peptides, all other congeners were detected from the viable and cultivated chemotypes of bigheaded carp. This finding suggests that the alimentary tract of bigheaded carps is not simply an extreme habitat, but may also supply the cyanobacterial strains that represent the pelagic chemotypes.

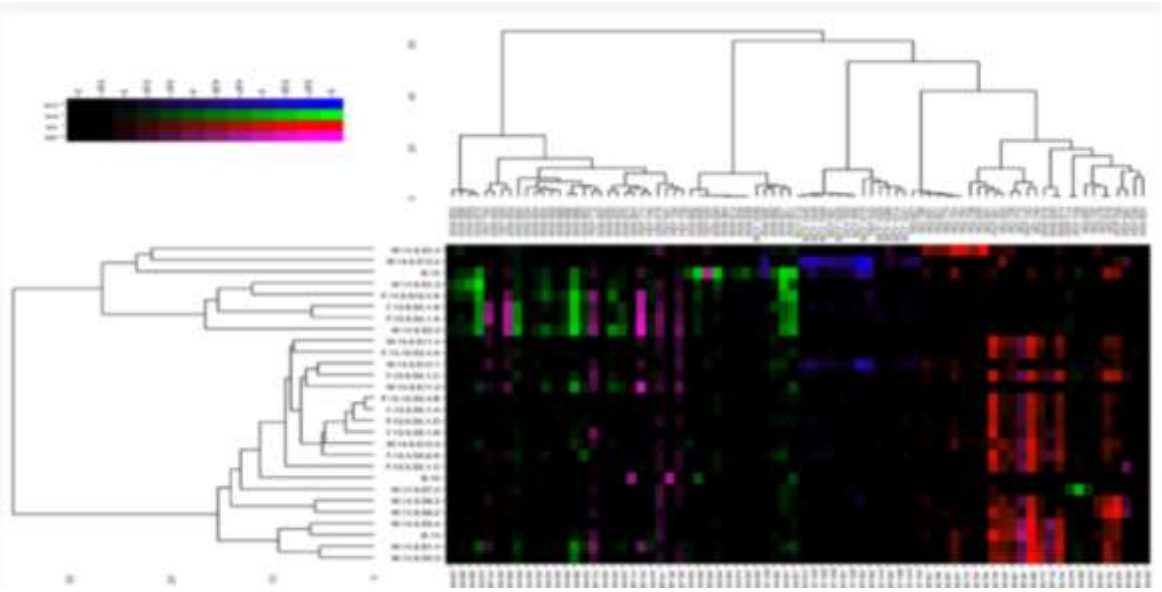
fig 7. Heat map clustering of Microcystis chemotypes

While the traditional approaches of toxin and/or bioactive metabolite research of cyanobacteria have mainly focused on individual peptides, exploring their effects or biosynthesis, our chemotyping study with non-targeted analysis investigated the occurrence of various peptides in *Microcystis* strains that originated from bloom, pelagic plankton samples, and from the gut of a notorious invasive fish species. Except for 13 peptides, all other congeners were detected from viable and cultivated chemotypes originating from bigheaded carps (fig 8).

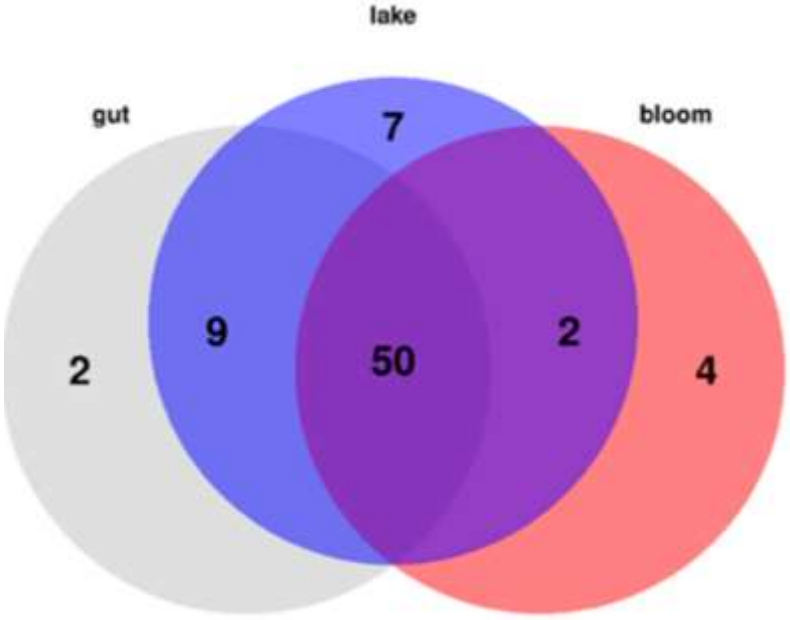

fig 8. Venn diagram showing the presence/absence of identified peptide natural products in the dataset containing the gut, bloom, and lake samples.

This finding suggests that the alimentary tract of bigheaded carps is not only a special habitat, but also a supplier for strains that represent the pelagic chemotypes and can initiate blooms in the waterbody. This potentially malicious feature can come from the ability of this fish species to filter plankton efficiently, but a few organisms such as the peptide-producing mucilaginous enveloped cyanobacterial species *M. flos-aquae* are digested improperly or not at all in the digestive system. In addition, several studies have noted that the toxicity of cyanobacteria remained unaffected or even increased after defecation. It is not easy to calculate the retention of viable *Microcystis* cells in the gut. Although it is worth raising the opportunity that bigheaded carps carrying cyanobacterial chemotypes in their guts from one habitat can invade new areas, and that the viable cyanobacterial cells may be released by defecation from fish.

In our chemotyping study, *Microcystis* strains isolated from the invasive non-native bigheaded carps and their peptide patterns were compared to pelagic and bloom material strains. Our results draw attention to the fact that bigheaded carps not only carry and spread viable, mucilaginous envelope-covered *Microcystis* cells from their alimentary tracts, but harmful cyanobacterial strains can also be found among them according to the chemotypes.

3.2. The cyanobacterial oligopeptides microginins induce DNA damage in the human hepatocellular carcinoma (HepG2) cell line

Microginins (MGs) are bioactive metabolites mainly produced by *Microcystis spp.*, (Cyanobacteria) commonly found in eutrophic environments. In this study, the cytotoxic and genotoxic activities of four MG congeners (MG FR3, MG GH787, cyanostatin B, MGL 402) and a well characterized cyanobacterial extract B-14-01 containing these metabolites were evaluated in the human hepatocellular carcinoma (HepG2) cell line. The cytotoxicity was measured with the MTT assay, while genotoxicity was studied with the comet, γH2AX and cytokinesis block (CBMN) micronucleus assays. The viability of cells after 24 h was significantly affected only by the extract, whereas after 72 h a concentration dependent decrease in cell proliferation was observed for the extract and tested microginins, with MGL 402 being the most potent and MG FR3 the least potent congener. The extract and all tested congeners induced DNA strand breaks after 4 and 24 h exposure. The most potent was the extract, which induced concentration and time dependent increase in DNA damage at concentrations ≥0.01 μg mL^{-1}. Among microginins the most potent was MGL 402 (increase in DNA strand breaks at ≥ 0.01 μg mL^{-1}) and MG FR3 was the least potent (increase in DNA strand breaks at ≥ 1 μg mL^{-1}). However, no induction of DNA double strand breaks was observed after 24 and 72-h exposure to the cyanobacterial extract or MGs. Induction of genomic instability was observed in cells exposed to MG GH787, cyanostatin B and the extract B-14-01. This study is the first to provide the evidence that microginins exert genotoxic activity (fig 9.).

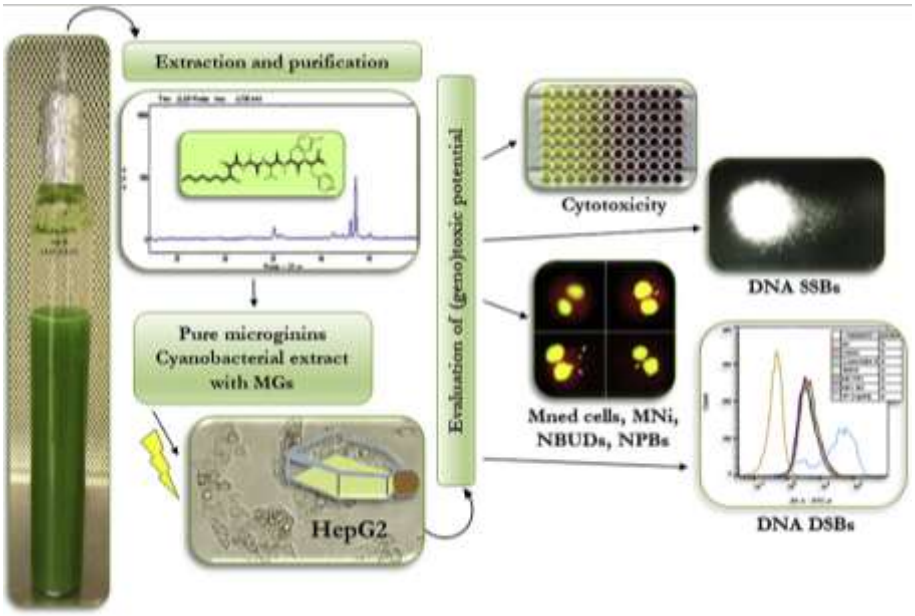

fig 9. experimental design of 3.2 study

The results from the present study are the first that demonstrated genotoxic potential of MGs indicating that MGs may represent hazard for aquatic environment and humans that has not been recognized before. In humans, exposure to genotoxic compounds is associated with development of several chronic diseases including cancer, whereas in aquatic environment reproduction of aquatic organisms. This is particularly important as MGs are produced by a common *Microcystis* species that are ubiquitously present in the aquatic environment. Therefore, further studies are needed to explore the mechanisms of the genotoxic activity of MGs as well as studies of their occurrence in the aquatic environment.

3.3. A new aspect of grassland vegetation dynamics: cyanobacterium colonies affect establishment success of plants

Cyanobacteria may have considerable effects on community functioning, mostly because they produce various metabolites that adversely affect other organisms. Here we synthesized existing knowledge about the effects of toxic cyanobacteria on the germination and growth of terrestrial plants. We also aimed to test the chemical effects of a *Nostoc* (Cyanobacteria) extract on the germination and growth of species of alkali habitats to investigate whether cyanobacteria can alter community structure and diversity via affecting the establishment success of plants.
Cyanobacterium colonies from the Hortobágy National Park, east Hungary; indoor experiments at the University of Debrecen, Hungary.
To review the effects of toxic cyanobacteria on terrestrial plants, we conducted a literature search. To test these effects on native plants, field-collected *Nostoc* colonies were used to prepare a cell-free water extract, and treatments (watering with *Nostoc* extract and watering with tap water) were tested on 3 × 100 seeds of nine alkali grassland species. After 5 wk, seedling number, seedling length and fresh and dry weights were measured.
We collected data on the effects of cyanobacteria on 27 species, but they were mostly focused on crops irrigated with cyanobacteria-containing water, not on floras native to natural ecosystems. In the germination experiment species identity and treatment had a significant effect on almost all variables, but their interaction only affected germination rate and fresh weight. Fresh weight decreased significantly only in the invasive *Hordeum jubatum*, but germination rate decreased significantly in five species.
Based on our findings, terrestrial cyanobacterium colonies can affect the establishment success of grassland plants, through which they may be important in determining which species can be incorporated into the community. Thus, cyanobacteria might play an important role in shaping diversity, species composition and the structure of natural plant communities.
In conclusion, the potential effects of cyanobacterium colonies on grassland vegetation and their role in vegetation dynamics have been largely overlooked so far. Although many other factors must be kept in mind when assessing the effects of cyanobacteria, our results draw attention to the fact that cyanobacteria should be considered not just in aquatic ecosystems, but also in terrestrial ecosystems where they occur. Our results suggest that by altering crucial measures of plant performance *Nostoc* colonies might play an important role in shaping the diversity, species composition and structure of natural plant communities.

3.4. Chemotyping of terrestrial Nostoc-like isolates from alkali grassland areas by non-targeted peptide analysis

The *Nostoc* genus is a well-known heterocytous, filamentous cyanobacterium which can be found all over the world. The size of terrestrial and/or freshwater colonies can be microscopic and macroscopic as well. In addition, *Nostoc* species are one of the most common photosynthetic cyanobacterial partners in symbiotic interactions.

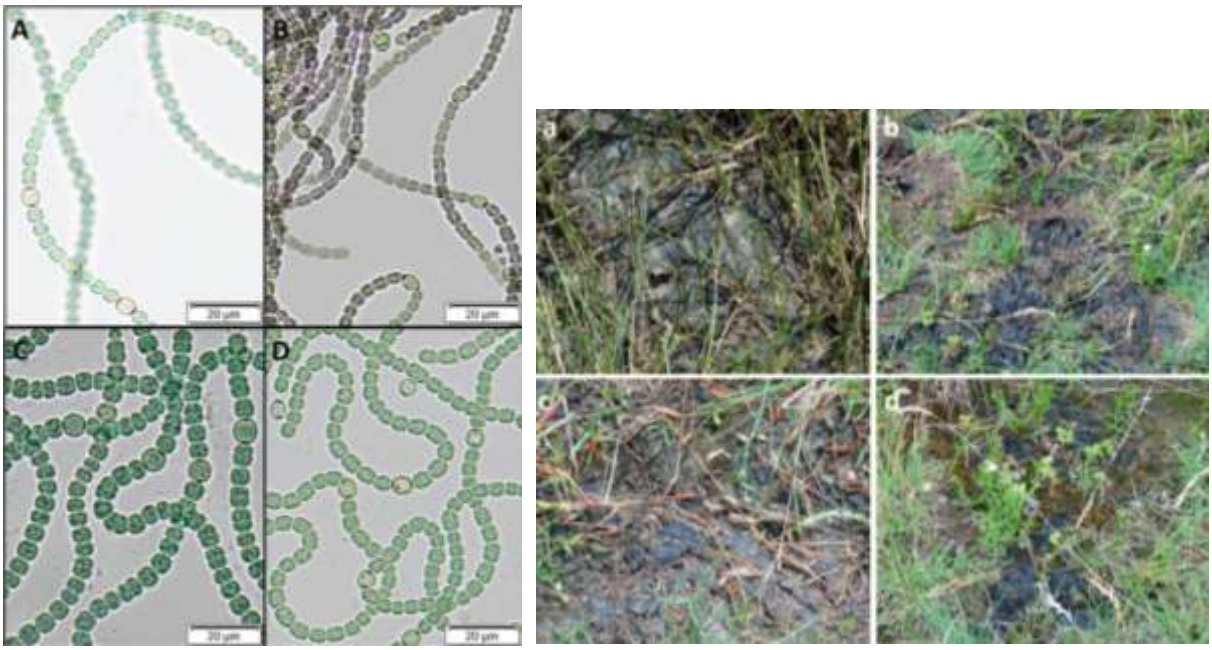

fig 10. Terrestrial Nostoc-like cyanobacterial strains and colonies

Terrestrial cyanobacterial colonies were collected and isolated (fig 10) in this study from various alkali grassland habitats (Great Hungarian Plain). Altogether 133 colonies were isolated from the 65 collected samples. The peptide patterns of the *Nostoc*-like strains were examined using HPLC-ESI-MS/MS and 41 peptides were identified from 45 isolated *Nostoc*-like strains; these compounds belonged to 4 different peptide classes (fig 11). Twelve nostoginin/microginin, 16 anabaenopeptin, 12 banyaside/suomilide variants were identified. 37% of our isolated *Nostoc*-like strains produced some of the peptide metabolites we tested. These strains showed distinct chemotypes according to their peptide patterns, and can be divided into 4 groups based on their metabolisms. Strains either contained: (1) nostoginins/microginins, (2) anabaenopeptins, (3) anabaenopeptins and banyasides or (4) banyasides as major compounds. Banyasides were present in many of our strains and showed very high intensity in some cases. A number of previously unknown banyaside variants have been identified.

In this study, we demonstrated the peptide metabolite-producing ability of terrestrial nitrogen-fixing cyanobacteria from different sites of the special alkaline habitats of Hungary. The isolated *Nostoc*-like strains could be classified into different chemotype groups based on their metabolic pattern (fig 12). A total of 41 peptide-type metabolites were identified which belonged to 4 different peptide families.

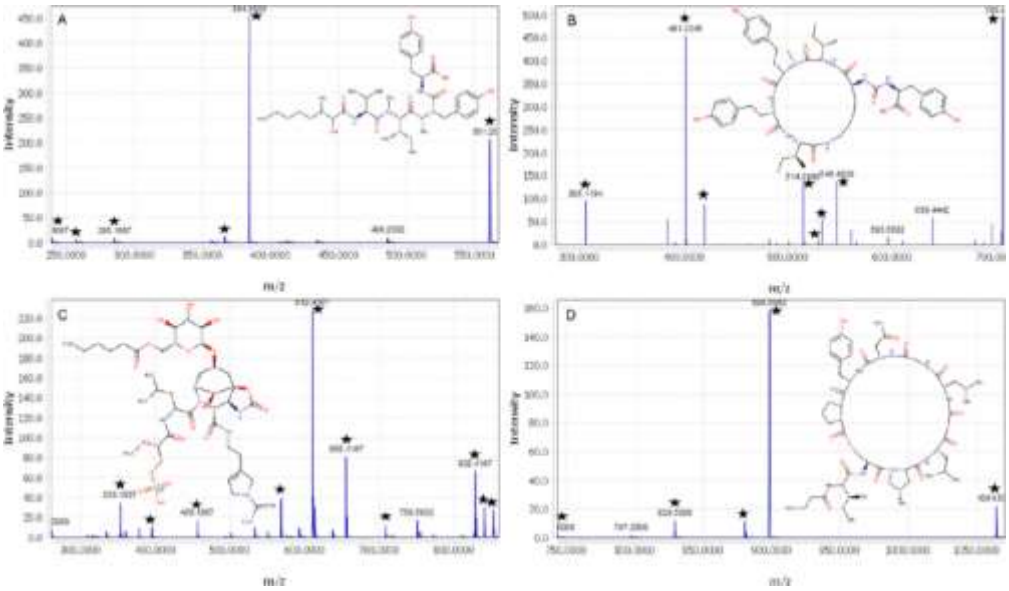

fig 11. Identified cyanopeptide examples belonged to 4 different peptide classes

None of the well-studied cyanotoxins (e.g. MCYs) were found, and many previously unknown biologically active peptide metabolites were identified. Several analyses, in which cyanobacterial strains collected from environmental or bloom/mats samples were targeted, showed negative results if focusing on "just" the well-known toxin families such as like MCYs, nodularins, anatoxins, saxitoxins and β-N-methylamino-L-alanine variants. However other studies emphasized that field and laboratory bioactivity/toxicity cannot be explained (just) with the mentioned cyanotoxins and encourage to get to know new agents.

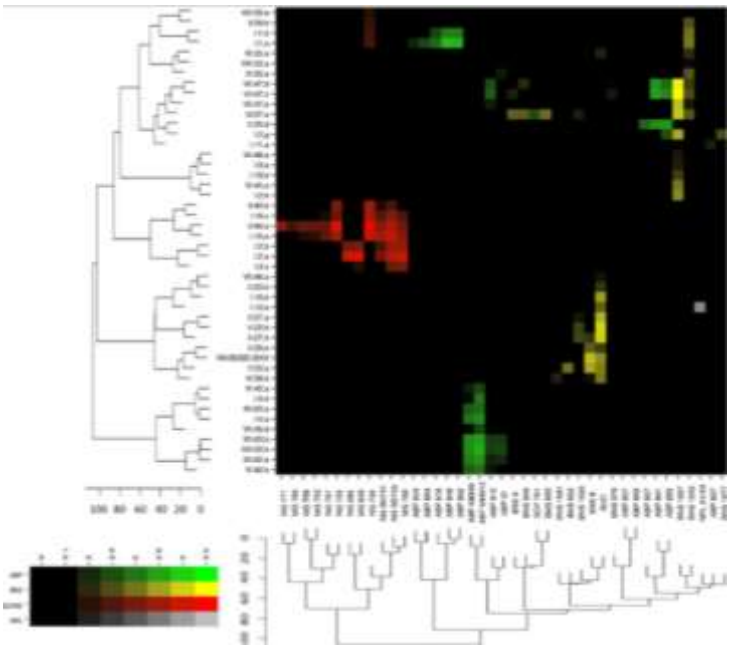

fig 12. Heat map clustering of Nostoc-like chemotypes

The exact role of these lesser-known or unknown metabolites remains to be clarified, and they can be significant from the medical point of view also, as they may be important in the development of potential pharmaceutically active ingredients. Our results also draw attention to the use of untargeted test

methods. Among our achievements, we highlighted the significance of banyaside producers within the *Nostocaceae* family which were represented in high number in our investigated area and moreover appeared as a prominent chemotype. These glycopeptides which are structurally similar to aeruginosins and found to be protease inhibitors are much more water-soluble than several other peptides because of the sugar moiety. On the basis of the occurrence and the intensity, this peptide family needs more research and further investigation in the future.

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