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Article

Insight into unprecedented diversity of cyanopeptides in eutrophic ponds using a MS/MS networking approach

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Abstract: Man-made shallow fishponds in the Czech Republic have been facing a high eutrophication since 1950s. Anthropogenic eutrophication and feeding of fish have strongly affected the physico-chemical properties of water and its aquatic community composition leading to harmful algal bloom formation. In our current study, we have characterised the phytoplankton community across three eutrophic ponds to assess the phytoplankton dynamics during the vegetation season. We microscopically identified and quantified 29 cyanobacterial taxa comprised of non-toxigenic and toxigenic species. Further, a detailed cyanopeptides (CNPs) profiling was performed using molecular networking analysis of liquid chromatography tandem mass spectrometry (LC–MS/MS) data coupled with dereplication strategy. This MS networking approach coupled with dereplication on online global natural product social networking (GNPS) web platform led us to putatively identify forty CNPs: fourteen anabaenopeptins, ten microcystins, five cyanopeptolins, six microginins, two cyanobactins, a dipeptide radiosumin, a cyclooctapeptide planktocyclin and epidolastatin 12. We have applied the binary logistic regression to estimate the CNPs producers by correlating the GNPS data with the species abundance. Usage of GNPS web platform has proved as a valuable approach for rapid and simultaneous detection of high number of peptides, and rapidly assessing the risk for harmful bloom.

Keywords: cyanobacteria; cyanopeptides; harmful bloom; liquid chromatography tandem mass spectrometry; Global Natural Product Social networking (GNPS); Dereplication strategy.

Key Contribution: The combination of non-targeted HRMS/MS and GNPS has been proved as a valuable approach for simultaneous, rapid and early detection of bioactive and potentially harmful peptides such as microcystins, anabaenopeptins, microginins, and cyanopeptolins.

1. Introduction

Cyanobacteria are important primary producers in the food chain with high nutritional value [1], tend to proliferate and form dense blooms, scums, and floating mats under favourable environmental conditions [2,3]. Eutrophication and climatic changes have led to increases in the geographical extent, population densities, and duration of cyanobacterial blooms in fresh, brackish, and marine waters [4]. These blooms can be hazardous to humans, animals, and plants due to the production of cyanotoxins apart from disrupting the ecosystem functions such as nutrient cycles, light availability, dissolved oxygen and consequent community reorganization and reduced biodiversity [5]. The most frequently reported cyanotoxins can be classified as cyclic oligopeptides (i.e. microcystins (MCs) and nodularins (NODs)) or alkaloids (i.e. anatoxins and cylindrospermopsin) based on their chemical structures, and as hepatotoxins, neurotoxins, and dermatotoxins based on their mechanism of toxic action in vertebrates [6,7]. The most extensively studied cyanotoxins are cyclic heptapeptides, MCs, produced most often by *Microcystis*, *Planktothrix* and *Dolichospermum* (former *Anabaena*) [4]. NODs, cyclic pentapeptides, are structurally related to MCs, produced mainly by *Nodularia spumigena*. Both, MCs and NODs are hepatotoxins inhibiting serine/threonine protein phosphatase group [8]. To date, about forty cyanobacterial genera have been described as potential cyanotoxins producers [9,10], of which the most common bloom-forming genera include *Microcystis*, *Aphanizomenon*, *Cylindrospermopsis*, *Dolichospermum*, *Nodularia*, *Planktothrix*, *Oscillatoria*, and *Trichodesmium* [4,9]. Moreover, more than six hundred peptides or peptidic metabolites (hereafter "CNPs") are isolated from cyanobacteria [11], many of which are unknown for their toxic potential and are not regularly monitored during the cyanobacterial bloom events. The co-occurrence of CNPs has been reported during the cyanobacterial proliferation events, and the necessity for extending their regular monitoring has been addressed [12-15]. CNPs, such as aeruginosins, microginins, cyanopeptolins, anabaenopeptilides, microviridins, anabaenopeptins , and nostophycins with numerous structural variants are regularly found in the cyanobacterial blooms [13,16-18]. Recent findings suggested that metabolomic profiles consisting of different CNPs affect differently, the cohabiting invertebrates and fish populations [19-21], underlining the need for expansion of the number of regularly monitored and studied CNPs.

Early methods for the detection of toxins were based mostly on animal assays using intraperitoneal or intravenous injections on mice [22-24]. However, recent advancement in the field of fast and accurate methods such as high-performance liquid chromatography connected to tandem mass spectrometry with high resolution mass spectrometry (HPLC-HRMS/MS), and introduction of Global Natural Product Social (GNPS) molecular networking platform has gained considerable attention towards its application in the field of identification of the novel compounds [25]. Further introduction of an in-silico annotation tool (such as Dereplicator+) at GNPS online workflow has revolutionized the detection of known/unknown natural products by comparing experimental MS/MS spectra against chemical structure databases. These tools enable to analyse and curate hundreds to thousands of obtained MS/MS data from analytes within the extract which is almost impossible to analyse manually [26]. Recent application of these tools in the field of annotating metabolites from cyanobacterial bloom led to the discovery of various novel compounds as well as unknown analogues [27-30].

Hence, the current study was focused on three eutrophic shallows ponds in South Bohemia region of Czech Republic and determination of their phytoplankton composition and metabolomic profiles during the vegetation season. The metabolic composition was determined taking the leverage of

GNPS online workflow in silico tools and molecular networking to obtain complete CNP profile of cyanobacterial proliferations of the studied ponds.

2. Results and discussion

The studied ponds have been in use for fish production since the 16th century. During the 20th century natural eutrophication and the intensification of fish production increased, and led to heavy eutrophication of these water bodies at present [31], resulting in intensified cyanobacterial proliferation during the summer months. We have sampled three ponds, KL (Klec), DH (Dehtář) and KV (Kvítkovický), located in South Bohemia of Czech Republic once per month during the whole vegetation season (six months in total), to investigate their phytoplankton and CNPs composition and dynamics. The chemical background data of the studied ponds indicated high concentrations of total nitrogen (TN), total phosphorus (TP) and chlorophyll-a (chl-a) illustrating the hypertrophic status of ponds (Table 1) [32]. All three ponds included in the current study have shown high content of chl-a, the primary and dominant photosynthetic pigment used as a proxy for phytoplankton biomass [33], with lowest concentration in KL-Apr (61.0 µg/L) and highest in KL-Jul (376.1 µg/L). Overall, increase of water temperature and of total nitrogen resulted in higher cyanobacterial proliferation, while chl-a concentrations were correlated with the increase of cyanobacteria and/or diatoms biomass. To study seasonal dynamics of phytoplankton with emphasis on cyanobacterial species composition, we quantified phytoplankton on the species level (where the clear taxonomical identification was possible), and statistically correlated cyanobacterial taxa with detected CNPs.

Table 1. Physico -chemical characteristics of water of investigated lakes during sampling season. Sampling dates, water temperature, pH, conductivity, transparency, dissolved organic carbon (DOC), total nitrogen (TN), total phosphorus (TP), dissolved organic phosphorus (DRP) and chlorophyll-a (Chl-a) during each sampling. KL stands for Klec, DH Dehtář, and KV Kvítkovický

Locality	Sampling date	Temperature	pH	Conductivity	Secchi depth	DOC	TN #200	TP #200	DRP	Chl-a
		(°C)		[µS/cm]	[cm]	[mg/L]	[mg/L]	[mg/L]	[mg/L]	[µg/L]
KL	2018-04-24	19.3		214	50	12.5	1.84	0.18	0.001	61.0
KL	2018-05-15	19.3	6.9	229	90	16.1	1.57	0.18	0.001	67.3
KL	2018-06-19	21.0	8.5	216	40	14.4	2.34	0.13	0.011	106.1
KL	2018-07-17	21.7	9.4	204	20	16.7	4.37	0.31	0.011	376.1
KL	2018-08-14	23.7	8.9	201	25	19.7	6.61	0.37	0.015	270.2
KL	2018-09-11	18.7	9.4	214	20	20.7	7.77	0.39	0.021	351.7
DH	2018-04-26	18.0		330	50	17.8	1.79	0.22	0.001	65.0
DH	2018-05-17	18.9	8.6	338	40	19.6	2.03	0.24	0.031	68.0
DH	2018-06-21	22.7	8.9	337	65	18.9	1.86	0.20	0.009	71.2
DH	2018-07-19	22.1	8.7	338	40	20.0	2.50	0.30	0.026	104.0
DH	2018-08-16	23.5	9.2	329	35	22.3	3.44	0.29	0.013	161.5
DH	2018-09-13	21.0	9.6	323	40	22.0	4.35	0.15	0.014	114.4
KV	2018-04-26	18.8		313	30	15.6	2.22	0.36	0.094	128.0
KV	2018-05-17	17.3	7.7	372	30	19.4	2.63	0.61	0.422	94.3
KV	2018-06-21	21.8	8.3	349	35	19.3	2.03	0.32	0.060	89.0

KV	2018-07-19	20.9	9.0	334	20	17.7	3.45	0.41	0.022	327.7
KV	2018-08-16	22.1	8.6	347	15	22.7	4.20	0.26	0.041	254.7
KV	2018-09-13	19.7	8.6	341	25	22.0	4.87	0.20	0.014	152.4

2.1 Phytoplankton composition and seasonal dynamics

Phytoplankton of the three studied sites were assigned to classes Chlorophyceae, Cyanophyceae, Cryptophyceae, Bacillariophyceae, Euglenophyceae, Dinophyceae, and Zygnematophyceae (Figure S1). During April and May, phytoplankton of all the three studied sites (KL, DH, KV) was dominated by *Chlorophyceae*, while cyanobacterial biomass did not exceed 3 mg/L of total phytoplankton biomass (Table S1). Total cyanobacterial biomass in KL-Apr was 2.2 mg/L from which 90.7% was composed of toxigenic taxa *Cuspidothrix issatschenkoi, Microcystis aeruginosa, Dolichospermum circinale* and *viguieri, Aphanizomenon flos-aquae* and *Planktothrix agardhii.* On the other hand, cyanobacterial taxa in DH-Apr and KV-Apr were composed mainly of picocyanobacteria (84.2% and 88.3%, respectively). During May, low cyanobacterial biomass with dominance of picocyanobacteria was observed in all studied ponds, with exception of 1.9 mg/L (68.1% of cyanobacterial biomass) of toxigenic *Microcystis aeruginosa* in DH-May. Even though, the cyanobacterial biomass was lower during April and May, it still formed an important part of the total phytoplankton biomass in some of the samples, i.e. 17.1%, 6.0%, and 5.9 % in KL-Apr, KV-May and DH-Apr, respectively. The dominance of taxa which have been reported as a CNPs producers was observed in KL-Jun: *Woronichinia naegeliana* 2.6 mg/L (34.7%) and *Microcystis aeruginosa* 2 mg/L (27.5%); in DH-Jun: *Aphanizomenon flos-aquae* 3.3 mg/L (52.8%) and *Dolichospermum circinale* 1.2 mg/L (19.9%), while KV-Jun was dominated by planktic picocyanobacteria 1.5 mg/L (73.7%) and in general had the lowest cyanobacterial biomass (~2mg/L) compared with other two ponds.

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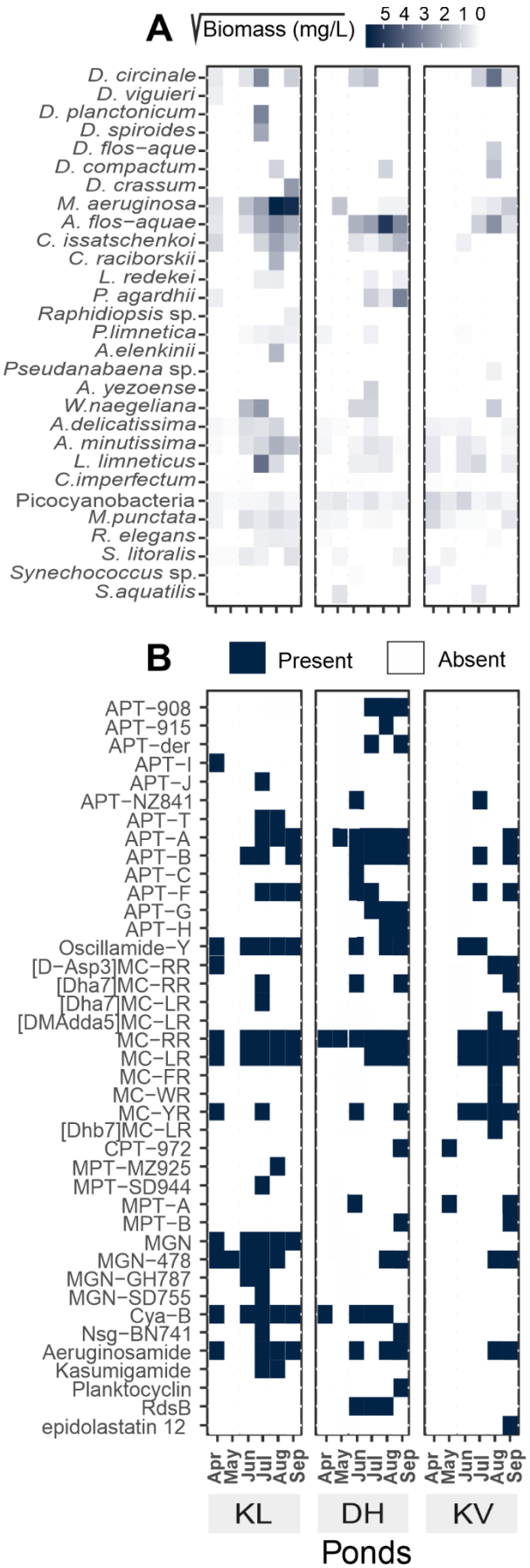

Figure 1. Heat maps showing **A**) the square root of the biomass in mg/L of different cyanobacterial species in all ponds during all sampled months, and **B**) the presence/absence of the different CNPs detected in all ponds during all sampled months. The full names of cyanobacterial species can be found in Figure 7.

In July we detected onset of summer phytoplankton peak (except for DH-Jul), with record of total phytoplankton biomass 104.4 mg/L in KL-Jul. KL-Jul exhibited the highest diversity of cyanobacterial taxa without clear dominance of a single cyanobacterial taxon, while the cyanobacteria from DH-Jul (12.6mg/L) were co-dominated by 5.4 mg/L of *Aphanizomenon floss-aquae*, 2.3 mg/L *Dolichospermum circinale*, and 1.4 mg/L *Planktothrix agardhii*. Unlike the other two ponds, KV-Jul was dominated by Bacillariophyceae with only 3.6 mg/L (9.2%) of total phytoplankton biomass belonging to cyanobacteria, dominated with harmful taxa: 1.2mg/L of *Dolichospermum circinale* and 1.2 mg/L *Aphanizomenon floss-aquae*. During August, all three studied sites exhibited highest cyanobacterial biomass (total phytoplankton was composed of more than 50% cyanobacteria), with dominance of a single or two toxigenic taxa. While toxigenic cyanobacteria formed a major part of KL-Sep phytoplankton, Bacillariophyceae took over cyanobacteria in DH-Sep and KV-Sept, however with still high abundance of toxigenic cyanobacteria (i.e. *Aphanizomenon flos-aquae, Planktothrix agardhii, Microcystis aeruginosa* and *Dolichospermum circinale).* DH and KV showed similar phytoplankton dynamics to the previous studies with an early spring maximum followed by phytoplankton depression, with a final summer peak, while KL had its phytoplankton depression in spring months with a summer maximum (Fig S1). Observed phytoplankton development corresponds to previously reported plankton dynamics in shallow eutrophic ponds [34,35].

2.2 CNPs diversity: Molecular networking

Diverse cyanobacterial communities among studied ponds were reflected in the production of wide array of CNPs. Abundance in CNPs diversity in a given ecosystems could affect any co-existing organisms especially due to their inhibitory and toxic activities [11,18,36,37]. Hypothesis on physiological and ecological relevance of chemotype variability in a single strain, and even higher diversity in natural cyanobacterial bloom population has been proposed to be advantageous for cyanobacterial dominance against other photoautotrophs and protection against grazing zooplankton [38-40]. Applying the online workflow of GNPS for high throughput screening we detected forty CNPs (Table S2.). A molecular network of 87 clusters (Figure 2) was generated using tandem mass spectrometry (MS/MS) spectra data on Global Natural Products Social Molecular Networking (GNPS) online workflow. GNPS algorithm automatically aligned and compared each spectrum against the spectra available in the database and then further grouped them by assigning cosine score (0 to 1).

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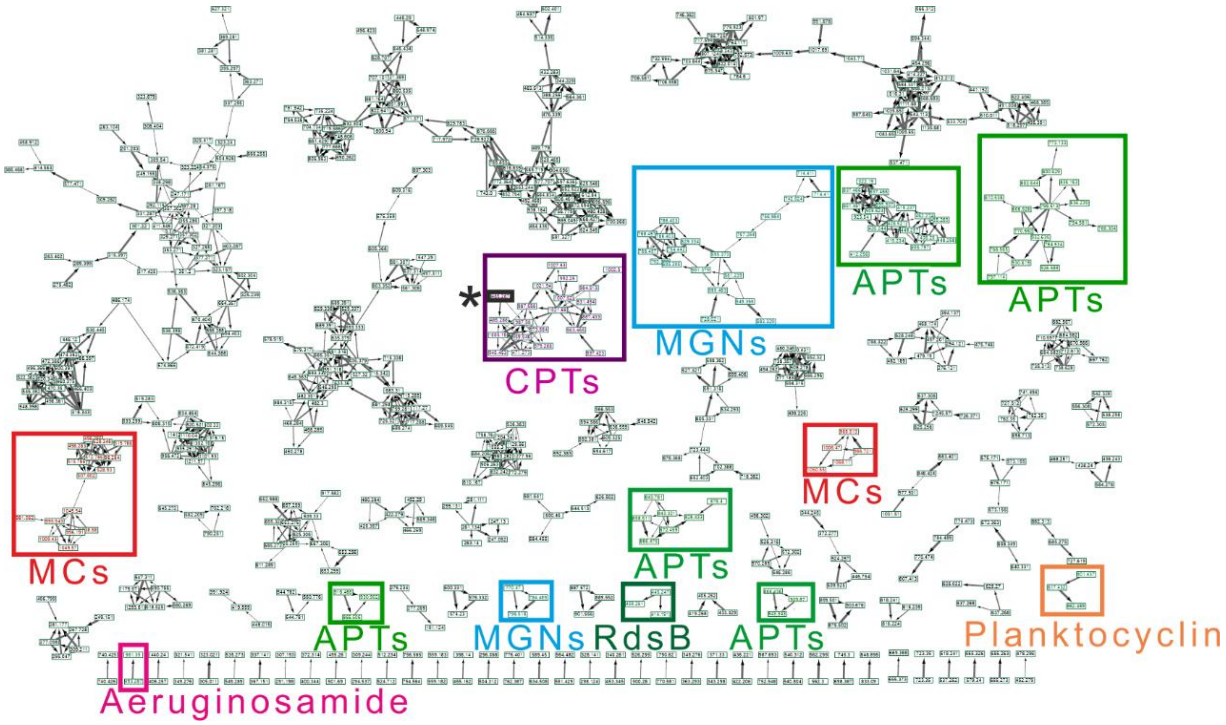

Figure 2. Molecular network generated from MS/MS spectra from all the samples of three ponds using Global Natural Products Social Molecular Networking (GNPS) tool. Analytes were compared with the components from the fragmentation pattern library available from the GNPS server. Only clusters of at least two nodes are represented. APTs: anabaenopeptins, MCs: microcystins, CPTs: cyanopeptolins, MGNs: microginins, RdsB: radiosumin_B, *: epidolastatin 12

Further, the obtained molecular network was annotated using an in-silico tool, dereplicator+. With this tool, it was possible to search all spectra in the GNPS launched molecular network and identify an order of magnitude more natural products than previous dereplication efforts [41]. Eighteen spectrum files were dereplicated generating 26,220 spectrum scans. A total of 568 Peptide-Spectrum Matches (PSMs) were identified with 321 PSMs exhibiting significant score of ≥11.0. Dereplication algorithm enabled us to facilitate the natural product discovery by high-throughput peptide natural product identification among large-scale mass spectrometry-based screening platforms [42]. Different analytes were grouped in the same molecular clusters based on the similarity of their fragmentation patterns, with each cluster being potentially specific to the structure of the chemical families. These un-identified ions that belong to annotated clusters are then considered as potential new analogues of their respective molecular family [43]. Additionally, the availability of HRMS/MS spectral data of known cyanotoxins in GNPS database facilitates the detection process [44-46]. This led to the detection of 14 anabaenopeptins (APTs), ten microcystins (MCs), five cyanopeptolins (CPTs), six microginins (MGNs), two cyanobactins, a dipeptide radiosumin (RdsB), a cyclooctapeptide planktocyclin and epidolastatin 12 from methanolic extract of the biomass (Figure 1B, Table S2). Recently, new MC variants were discovered using MS-based molecular networking approach from the freshwater cyanobacterial harmful bloom at Green Lake, Seattle [28]. Similarly, numerous reports have been published, where molecular networking was employed to track changes in secondary metabolic profiles, including MCs and other peptides [47,48].

Anabaenopeptins (APTs)

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Anabaenopeptins are a highly diverse family of cyclic hexapeptides firstly described from *Anabaena flos-aqae* NRC 525-17 [49]. They exhibit diverse biological activities; however studies are mostly focused on their serine protease and chymotrypsin inhibiting activity [50]. They are *N*-methylated and contain a conserved ureido linkage connecting the side-chain amino acid residue to the D-lys [11]. In the current study, we detected the presence of 14 APTs variants; APT-908, APT-915, APT-I, APT-J, APT-NZ841, APT-T, APT-A, APT-B, APT-C, APT-F, APT-G, APT-H, Oscillamide-Y, and one defined as APT-derivative using dereplication strategy (Figure 1B, Table S2). Oscillamide-Y (a serine protease inhibitor, isolated from *Planktothrix agardhii* NIES -610; [50] was detected in ten samples, including KL-Apr with low cyanobacterial abundance (Figure 1). Apt-A and -B were detected nine times, while Apt-F seven times. Apt-A and -B inhibit carboxypeptidase A and protein phosphatase 1 with varying potency, but no inhibition against chymotrypsin, trypsin and thrombin was reported [51]. Detected APTs formed five clusters (Figure 2) corresponding to ions presenting a match with the mass of previously described variants, to compounds that very likely correspond to potentially new analogues (Figure 3). Some of the compounds formed single nodes and were removed from the networking. However, we report here their putative presence based on dereplication. The recent increased reports of APTs co-occurrence with MCs raise the attention to this class of CNPs since their impact on the cohabiting aquatic organisms remains unclear, and their ecological role is uncertain [47].

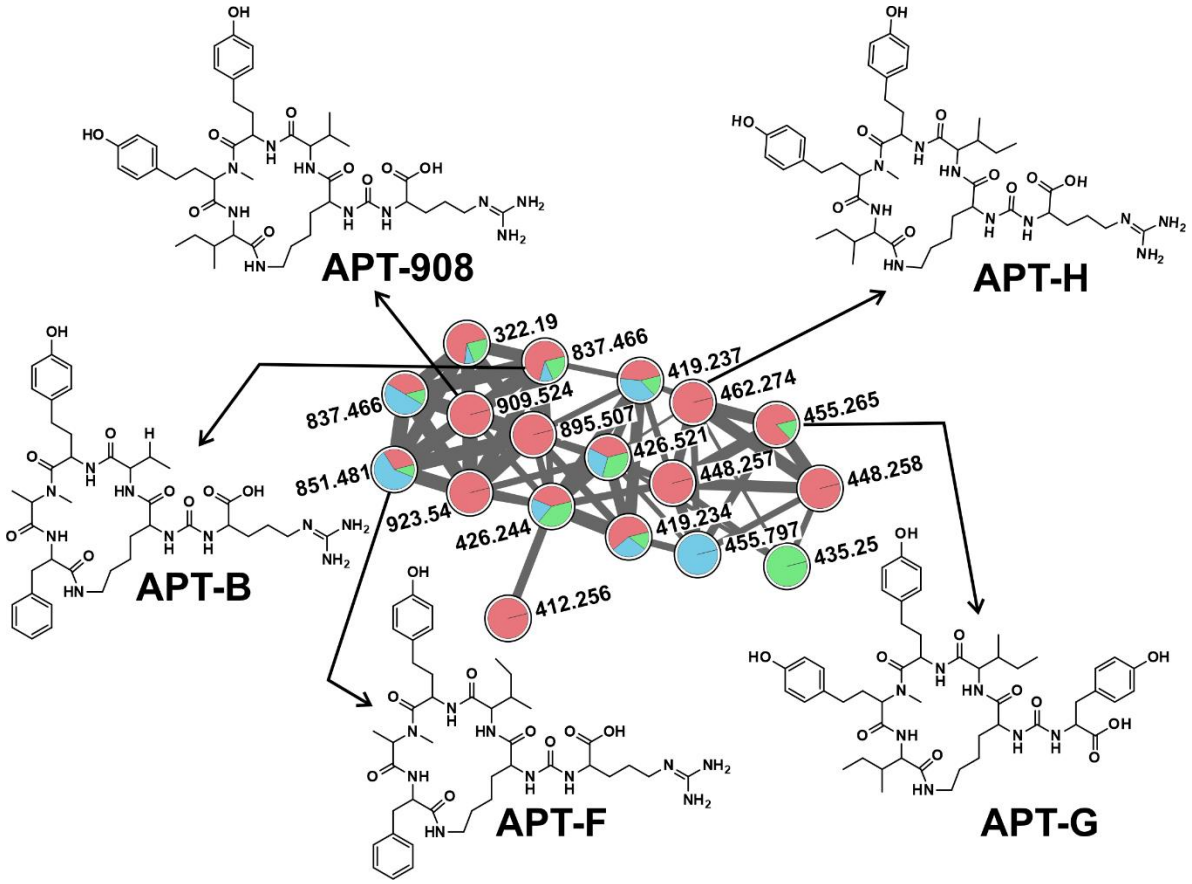

Figure 3. Anabaenopeptin (APT) cluster formed by the GNPS analysis based on the MS/MS fragmentation spectra obtained from all the three sampling sites (Red: DH, blue: KL, green: KV). APTs congener chemical structures detected in this respective cluster are depicted here, whose fragmentation patterns are available in library of the GNPS server. Note that $(M+H)^+$ and $(M+2H)^{2+}$ ions are grouped together in the molecular cluster.

Microcystins (MCs)

Microcystins are cyclic heptapeptides produced, among others, by *Microcystis*, *Anabaena*/*Dolichospermum*, *Nodularia* and *Oscillatoria*. While the liver is the primary target of MCs, MCs are also a skin, eye, throat irritant and immunomodulating agents [52,53]. Microcystin-LR was the first identified cyanotoxin and is the most studied. The WHO has established a provisional guideline value of 1 ug/L for microcystin-LR in drinking water [54].

We were able to detect putatively ten microcystin congeners; MC-LR, MC-RR, MC-FR, MC-WR, MC-YR, [D-Asp3]MC-RR, [Dha7]MC-RR, [Dha7]MC-LR, [DMAdda5]MC-LR and [Dhb7]MC-LR (Figure 1B) using dereplication and molecular networking (Figure 2). Potentially new analogues were also observed in these clusters showing distinct but similar fragmentation patterns to those of other known variants spectra present in GNPS library (Figure 4). MC-RR and MC-LR have been detected in all studied ponds, MC-RR was detected in 15 samples, while MC-LR in 12. However, the presence of other congeners was more scattered, with highest diversity in KV-Aug (eight MC variants).

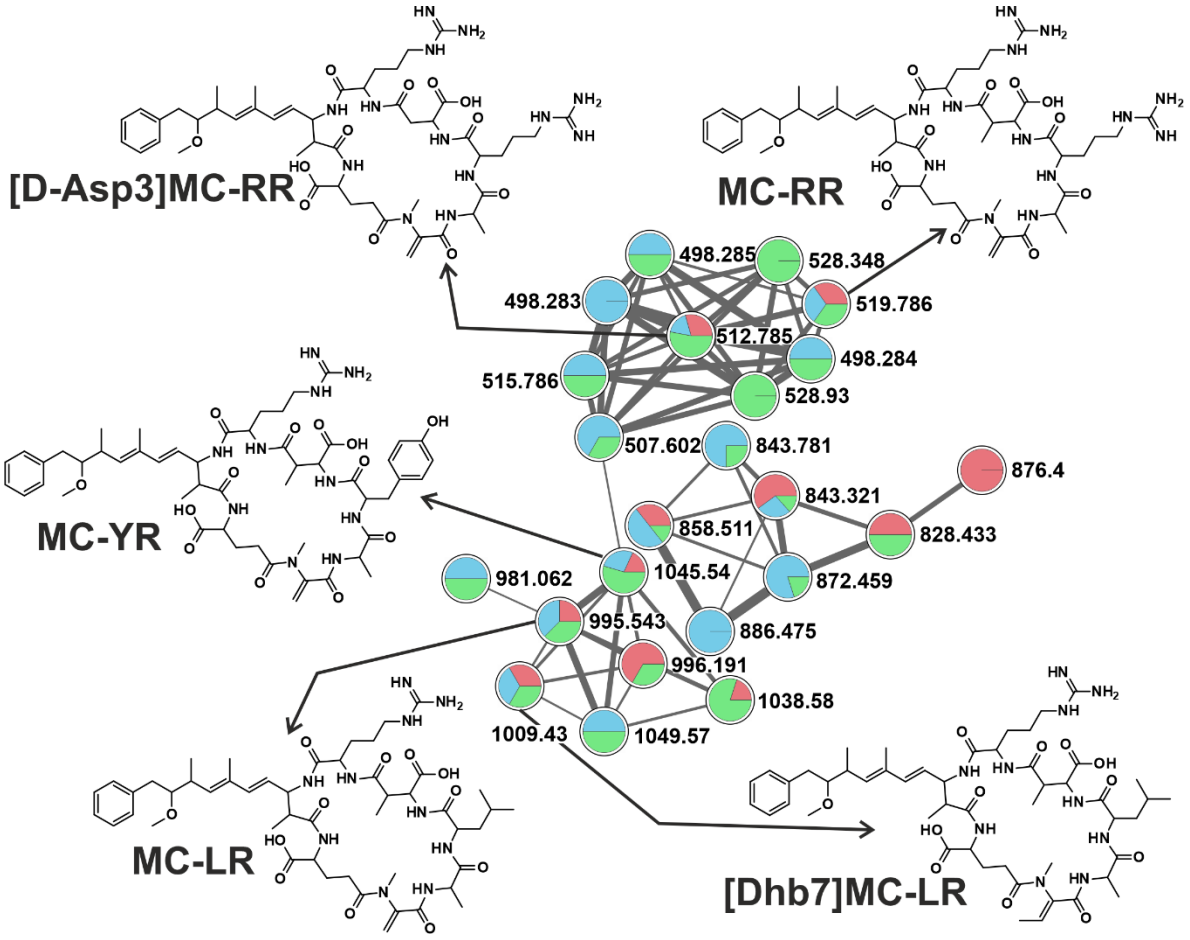

Figure 4. A microcystin (MC) cluster formed by the GNPS analysis based on the MS/MS fragmentation spectra obtained from all the three sampling sites (Red: DH, blue: KL, green: KV). MCs congener chemical structures detected in this respective cluster is depicted here, whose fragmentation patterns are available in library of the GNPS server. Note that $(M+H)^+$ and $(M+2H)^{2+}$ ions are grouped together in the molecular cluster.

Cyanopeptolins (CPTs)

CPTs are diverse class of cyclic depsipetides previously isolated from *Microcystis* sp. PCC 7806 [55], composed of six amino acid residue ring structure, a conserved 3-amino-6-hydroxy-2- piperidone (AHP) residue and a variable length side chain [55,56]. We detected five CPTs variants (micropeptin

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(MPT) -MZ925, -SD944, -A, -B, and CPT 972 (Figure 1B) exclusively from summer samples) with high abundance of cyanobacteria in the phytoplankton community (total nine hits). Two micropeptins and a epidolastatin12 formed a cluster together with compounds that very likely correspond to potentially new analogues (Figure 5). The general activities reported from CPTs are protease inhibitory, fungicidal, cytotoxic, and antitumor activities [57], and recent elucidation of molecular basis of Ahp - cyclodepsipeptides has opened new possibilities for customizing them as serine protease-specific inhibitors [56].

Another peptide, epidolastatin 12, detected in the current study formed a cluster together with CPTs (Figure 5). Dolastatins were originally reported from mollusk *Dolabella auricularia* [58], however their structural variants were found to be produced by axenic cyanobacteria implying the possibility that even the first reported dolastatin is produced by cyanobacteria [59,60]. It has been reported as an epimer of dolastatin 12 isolated form marine *Lyngbya majuscula/Schizothrix calcicola* cyanobacterial assemblages [59]. Detection of dolastatins epimer in a single sample (KV-Sep) is to our knowledge, the first report of dolastatin variant detected from freshwater source.

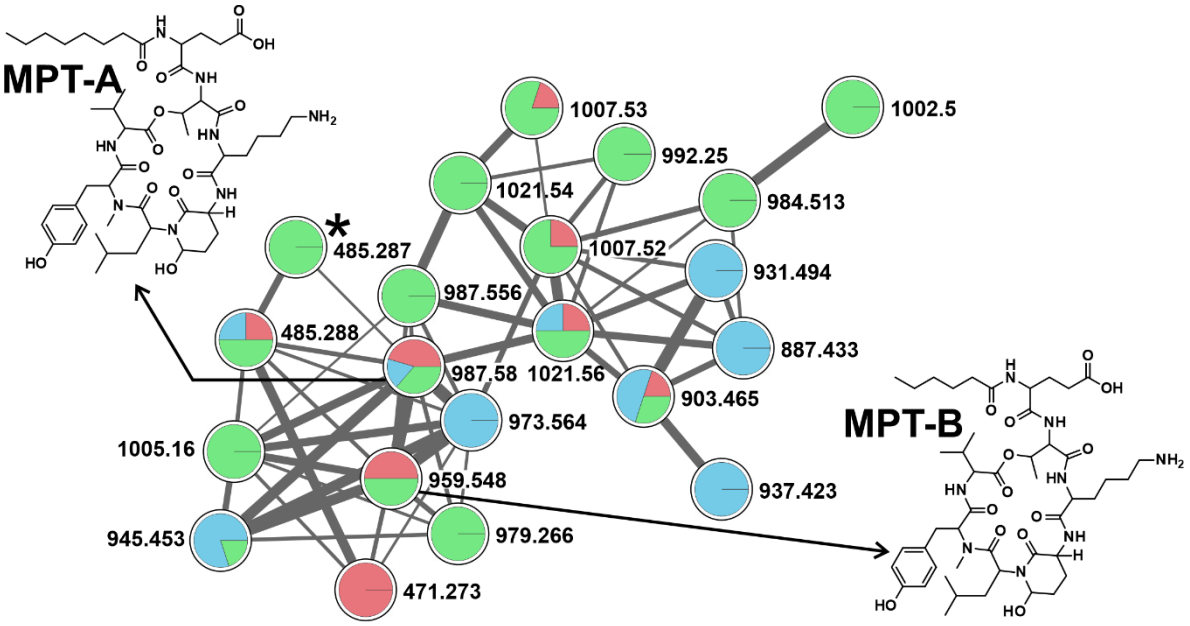

Figure 5. Cyanopeptolins (CPTs) cluster formed by the GNPS analysis based on the MS/MS fragmentation spectra obtained from all the three sampling sites (Red: DH, blue: KL, green: KV). CPTs congener chemical structures (MPT: micropeptin) detected in this respective cluster is depicted here together with epidolastatin 12 (*), whose fragmentation patterns are available in library of the GNPS server. Note that $(M+H)^+$ and $(M+2H)^{2+}$ ions are grouped together in the molecular cluster.

Microginins (MGNs)

Microginins are linear pentapetides originally isolated from *Microcystic aeruginosa* NIES-100 as an angiotensin-converting enzyme inhibitor [61]. We detected six MGNs variants (MGN, MGN-478, -GH787, -SD755, cyanostatin B (Cya-B), nostoginin BN741 (NSG-BN741)) throughout the sampling season (Figure 1B). In KL-Jul, the most cyanobacterial diverse sampling point (16 taxa, Figure 1), we detected six MGNs variants matching with the library spectra match. One of the cluster comprising of three variants is depicted in Figure 6. Similarly, to MCs and APTs, MGNs were detected also in samples with low cyanobacterial biomass (KL-Apr, -May, DH-Apr). The most frequently detected variants were MGN-478 and Cya-B, both detected in nine samples. The biological activity among

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MGN variants also vary widely, for example, Cya-B being reported as an aminopeptidase M inhibitor [50], whereas MGN-478 have not exhibited any protease inhibitory activity [62].

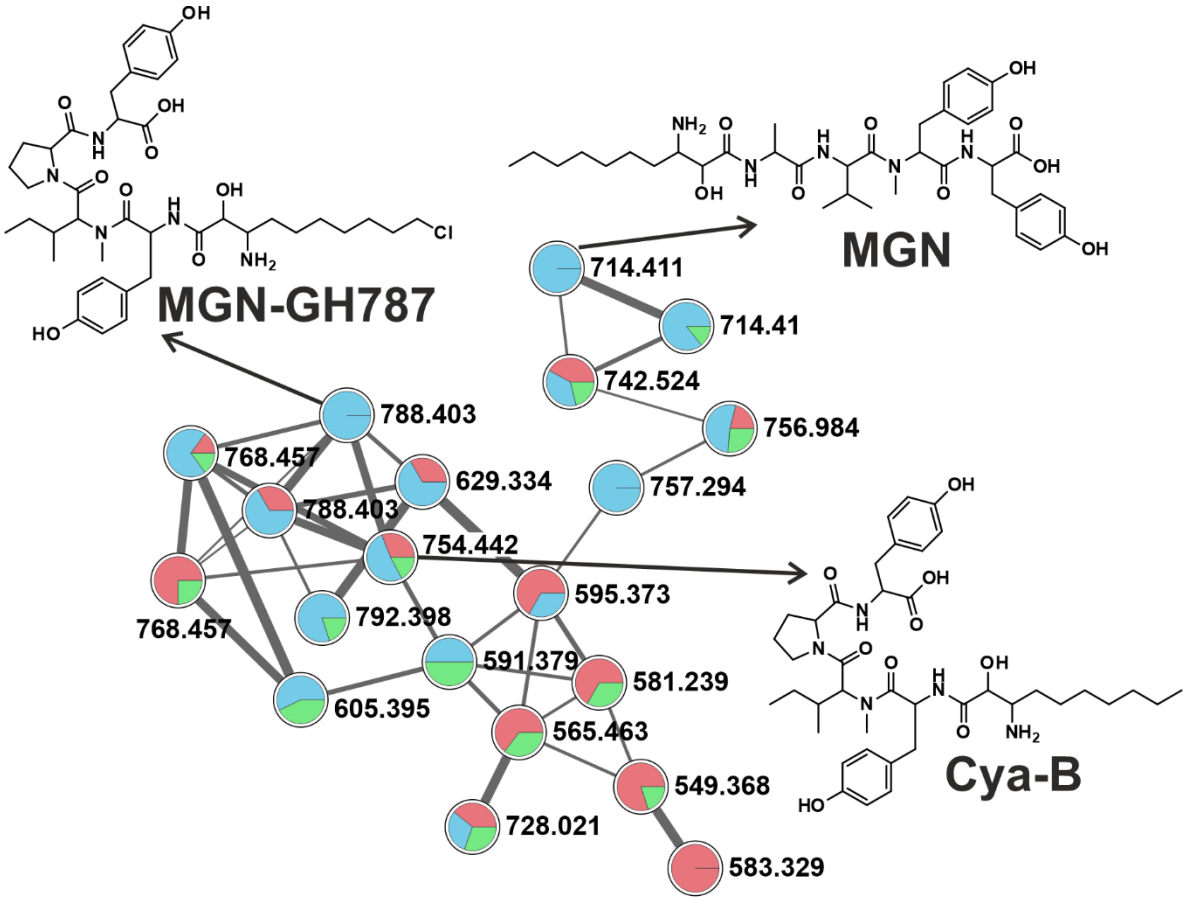

Figure 6. Microginin (MGN) compounds clustered together by the GNPS analysis based on the MS/MS fragmentation spectra obtained from all the three sampling sites (Red: DH, blue: KL, green: KV). Selective known MGNs congener chemical structures detected in this respective cluster is depicted here, whose fragmentation patterns are available in library of the GNPS server. Note that $(M+H)^+$ and $(M+2H)^{2+}$ ions are grouped together in the molecular cluster.

2.3 CNPs composition and seasonal dynamics

Cyanobacterial blooms are formed by diverse coexisting cyanobacterial species resulting in the production of wide array of CNPs altering the natural habitat with their toxic activities [11,18,36,37,63]. Recent studies have reported the co-production of diverse CNPs with MCs [14], suggesting high chemotype variability in a single strain and even higher in natural cyanobacterial blooms [64,65].

The obtained array of CNPs (MCS, APTs, CPTs, MGNs) in our study in general corresponds to the expected chemical composition of cyanobacterial blooms dominated by commonly reported toxigenic planktic taxa such as *Microcystis*, *Dolichospermum*, *Aphanizomenon*, and *Planktothrix* (Table S1) [14,51,66]. However, amino-protease inhibitor Nsg-BN741 was previously reported only from periphytic and terrestrial heterocytous cyanobacteria *Nostoc* [67], thus it was surprising to find it in the planktic environment (ponds KL and DH). Despite of the absence of *Plectonema radiosum* and *Planktothrix rubescens* in the phytoplankton in DH pond, we have detected the presence RdsB and planktocyclin [68,69].

In the ponds we detected also two cyanobactins, unlike other CNPs reported here, produced ribosomally [70]. Cyanobactin kasumigamide, a ribosomal tetrapeptide isolated originally from

Microcystis aeruginosa [62], occurred only in one pond (KL-Jul and -Aug), sampling points which showed the highest cyanobacterial diversity. On the other hand, second cyanobactin, aeruginosamide, reported originally from *Microcystis*, [71], occurred in all three ponds in range of sampling points with different abundances of *Microcystis aeruginosa* co-occurring with diverse cyanotaxa (Figure 1).

As mentioned above, spring samples of all three ponds were dominated by Chlorophyceae with low cyanobacteria abundance. While no CNPs were detected in KV-Apr, we detected nine CNPs in KL-Apr where the common toxigenic taxa were present, and two CNPs in DH-Apr without presence of common CNPs producers. Although detection of the CNPs already in samples with low cyanobacterial biomass has been reported previously [72], they are often neglected for detailed monitoring. Detection of diverse CNPs in our samples further addresses a need for detailed monitoring of water bodies even in samples with low cyanobacterial abundance.

In following month, all three ponds were dominated mainly by picocyanobacteria, where we found one CNPs in KL-May, and two in DH-May. The increase in cyanobacterial abundance during later sampling season was reflected by increase in CNPs. Increase in the diversity of common toxigenic cyanobacterial taxa in June resulted in higher number of detected CNPs (Figure 1). Particularly, KL-Jun and DH-Jun exhibited higher cyanobacterial biomass and diversity of common toxigenic taxa compared with previous samples, thus in these sampling points we detected eight and 13 CNPs, respectively. KV-Jun was less abundant in cyanobacteria, resulting in detection of only four CNPs.

The highest diversity of the CNPs was detected during the cyanobacterial proliferation in the months of July, August and September (Fig 1). The most prolific sample in both, cyanobacterial and CNP diversity, was KL-Jul. This sampling point was characterised by presence of 16 cyanotaxa and detection of 20 CNPs, referring that co-occurrence of several toxigenic taxa would result in higher metabolic diversity [14,15].

As mentioned above, *Aphanizomenon flos-aquae* was the most abundant species in DH-Jul where we detected nine CNPs, while KV-Jul was unlike the other ponds dominated by Bacillariophyceae, and had the lowest cyanobacterial biomass, accordingly, we detected lower CNP diversity. Generally, KV pond exhibited lower cyanobacterial and CNP diversity in comparison with the other two studied ponds.

All ponds showed increased cyanobacterial abundance and dominance of a single taxon in August. *Microcystis aeruginosa*, reported diverse CNPs producer [73,74] was the most abundant cyanobacterial taxon in KL-Aug (55%), where we detected 12 diverse CNPs. 13 CNPs have been detected in DH-Aug dominated by *Aphanizomenon flos-aquae* (90%), while *Dolichospermum* dominated KV-Aug (50%) showed presence of 12 CNPs, with highest detected diversity of MCs congeners (eight) among all samples. Both of dominating taxa have been demonstrated as rich secondary metabolite producers [75,76]. KL-Sept had the highest abundance of *Microcystis aeruginosa* (65% of cyanobacterial biomass) with presence of 11 more taxa resulting in the detection of nine CNPs. Known for their high CNPs potential, *Aphanizomenon flos-aquae* and *Planktothrix agardhii* [75,76] were the most abundant species in DH-Sep where 17 CNPs were found. KV-Sep showed a lower diversity of cyanobacteria when compared with the previous sampling point (KV-Aug), and the other two ponds. Dominated with *Microcystis*, KV-Sep exhibited the highest CNPs diversity (13) when compared with previous sampling points of the same pond.

Among all the samples, four samples (DH-May, KL-Aug, KL-Sep, KV-Sep), were dominated mainly by *Microcystis aeruginosa* where we detected the presence of different variants of CNPs found in

another samples not dominated by *Microcystis aeruginosa*; only MPT-MZ925 and epidolastatin 12 were detected exclusively in KL-Aug and KV-Sep, respectively.

Since high cyanobacterial and CNPs diversity co-occurred throughout the sampling campaign, a binary logistic regression was performed in order to correlate specific cyanobacterial taxa with individual CNP occurrence. A number of common toxigenic cyanobacterial taxa have exhibited strong correlation with some of the CNPs, on the other hand we also observed previously unreported associations (Figure 7). Presence of several reported APTs have been significantly correlated with a certain cyanobacterial taxon previously reported as a producer [51]. APT-915 and APT-H have been associated with *Aphanizomenon flos-aquae;* APT-der with *Planktothrix agardhii*, and APT-I with *Dolichospermum viguieri*. Three APTs showed correlation with two taxa, oscillamide Y with *Cuspidothrix issatschenkoi* and *Limnococcus limneticus*, while APT-908 and APT-G has been correlated with *Planktothrix agardhii* and *Aphanizomenon flos-aquae*. Other APTs were correlated with three or more taxa, or not correlated to any.

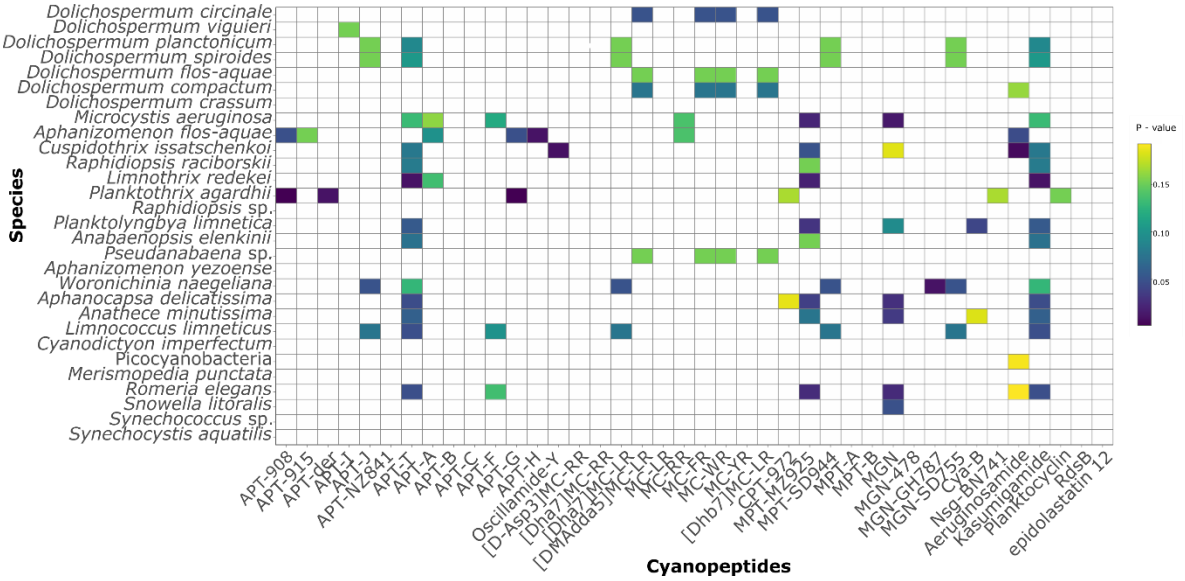

Figure 7. Binary logistic regression of cyanobacterial taxa with distinct CNP production. The full names of CNPs are in Table S2.

Six out of 10 MCs have been correlated with two or more taxa; four MCs (MC-FR,-WR, [DMAdda5]MC-LR and [Dhb7]MC-LR) detected only in one sample, KV-Aug, shown the same correlation pattern with four cyanobacteria (*Dolichospermum circinale*, *Dolichospermum flos-aquae*, *Dolichospermum compactum*, and *Pseudanabaena sp.*). Similarly, three CPTs have shown correlation with more than one taxon, including not previously reported producers (*Aphanocapsa delicatissima*, *Anathece minutissima*, *Romeria elegans).* MGN-GH787 was correlated with a single taxon, *Woronichinia naegeliana,* previously reported as MGNs producing species [77]. Nsg-BN741, previously reported from *Nostoc*, exhibited correlation with *Planktothrix agardhii,* while MGN and MGN-SD755 have shown a correlation with seven and four taxa, respectively. Furthermore, Cya-B showed association not only to *Planktolynbya limnetica*, but as well as to *Anathece minutissima* (Figure 7), often reported in cyanobacterial blooms, but never directly associated with CNPs production so far [64,78]. Aeruginosamide was correlated with five cyanobacteria, while planktocyclin, previously reported from *Planktothirx rubescens* exhibited correlation with *Planktothrix agardhii*. APT-T and kasumigamide

were both detected only in KL-Jul and KL-August and were significantly correlated to the same thirteen cyanotaxa, including non-toxigenic taxa. While *Limnococcus limneticus* (formerly *Chroococcus limneticus*) is generally not considered as toxigenic, it has been associated to MGN-SD755, APT-I, -T, -F, [Dha7]MC-LR, Mpt-SD944, MGN-SD755 and kasumigamide. Similarly, picocyanobacterial taxa have been exhibiting correlation with several CNPs. Picocyanobacteria have been in general considered as cyanobacteria with low secondary metabolite potential, although their correlation with MCs production has been repeatedly reported since the eighties [79-81].

Jakubowska [78] suggested more in-depth toxicological studies on picocyanobacteria in general since CNPs have been already detected in bloom samples with high picocyanobacterial abundance. Only several recent studies investigated picocyanobacterial capability for bioactive secondary metabolites production such as hepatotoxins, BMAA, LPS, and other bioactive metabolites, i.e. bacteriocins [82]. However, the direct proofs of picocyanobacteria toxicity are still scarce and authors tend to consider them as non-toxic.

3. Conclusions

Our non-targeted CNPs detection survey of three ponds used for fish farming in Czech Republic raises concerns on the high presence of potentially harmful CNPs. In this study, we detected a range of harmful MCs variants, and other potential harmful CNPs during the whole sampling season and especially (but not exclusively) in samples where cyanobacterial proliferation occurred. There is no study which investigates high range CNPs profile of fish farming ponds regularly used in fish market for human consumption, and their potential (eco)toxicological risk has not been investigated in sufficient detail [14]. Furthermore, detected CNPs (i.e. APT, CPT, MGN) have been reported as a co-product along with the MCs, thus the possible synergistic effect of several compounds produced has been addressed. We introduce a rapid and efficient monitoring approach combining the GPNS approach and binary logistic regression for the detection of wide range of CNPs even in the samples with low cyanobacterial biomass, which could help understanding the early development and dynamics of CNPs production in aquaculture ponds.

4. Material and methods

4.1. Study sites and sampling

Three nutrient rich shallow eutrophic ponds were sampled monthly, to cover the growth season, from April until September 2018. The investigated ponds are used for fish production in the Czech Republic: KL (Klec 49.090N, 14.767E, max. depth 2 m, area 0.64 km^2), DH (Dehtář) 49.006N, 14.294E, max. depth 4 m, area 2.28 km^2), KV (Kvítkovický 48.963N, 14.337E max. depth 3 m, area 0.24 km^2). During each sampling point, temperature, pH, conductivity, and transparency were measured. (Table 1) Water samples for plankton and background physico-chemical analysis were collected as described previously [32]. Briefly, horizontally integrated mixed water samples from surface water were collected from seven different points with van Dorn sampler (length of 1 m, 6.4 L volume). Chlorophyll a (Chl a) was determined spectrophotometrically after the extraction of samples collected on GF/C filters as described elsewhere [83] (Table 1). Subsample (3-5 L) was taken for chemical analysis, and 100 mL was preserved with Lugol's solution for phytoplankton analysis. For the CNPs analysis, surface water samples were repeatedly collected with the plankton net (20 μm mesh) until obtaining dense biomass, refrigerated on the way, transferred to the lab, and kept at -80°C until the analysis.

4.2. Phytoplankton analysis

Biomass of individual phytoplankton taxa was determined in Lugol preserved samples using Utermöhl's sedimentation method [84] and the inverted microscope (Olympus IMT2). Abundance of each taxon was multiplied by their respective biovolume calculated from mean cell dimensions using an approximation to geometrical solids [85]. For the taxonomic determination of cyanobacteria, the taxonomic keys by Komárek and Anagnostidis were used [86-88].

4.3. Crude extracts preparation and HPLC-MS/MS analysis

Crude extracts were prepared following the pre-established protocol [46,89]. Briefly, freeze-dried biomass of collected pond samples (~20 mg) were grinded (with the sea sand) and extracted three times with 75% MeOH in water followed by bath sonication. Extracts were evaporated under vacuum using a rotary vacuum evaporator (Heidolph, Germany) and dissolved with DMSO to get a final concentration of 4 mg/mL prior to analysis. Thermo Scientific DionexUltiMate 3000 UHPLC (Thermo Scientific) equipped with a diode array detector (DAD) and high-resolution mass spectrometry with electrospray ionization source (ESI-HRMS; Impact HD Mass Spectrometer, Bruker) was used for analysis of the crude extracts. HPLC separation was performed on reversed phase Kinetex Phenomenex C_{18} column (150 × 4.6 mm, 2.6 µm) with H_2O/acetonitrile containing 0.1 % HCOOH as a mobile phase. Flow rate during analysis was 0.6 mL/min. The gradient was as follows: H_2O/MeOH 85/15 (0 min), 85/15 (in 1 min), 0/100 (in 20 min), 0/100 (in 25 min) and 85/15 (in 30 min). Mass spectrometer settings were as follows: dry temperature 200 °C; drying gas flow 12 L/min; nebulizer 3 bar; capillary voltage 4500 V; endplate offset 500 V. The spectra were collected in the range 20-2000 *m*/*z* with spectra rate 4 Hz. A ramp was set with collision induced dissociation from 20 to 60 eV on successive *m*/*z* 200-1200. Data was collected by an initial precursor ion survey scan, followed by product ion generation from precursor ions selected in small isolation windows (≈4 Da wide). Calibration was performed using LockMass 622 as internal calibration solution and CH_3COONa clusters at the beginning of each analysis.

4.4. Molecular networking

The raw data files obtained from HPLC-HRMS/MS analysis were converted to mzXML format using MSConvert from the ProteoWizard suite (http://proteowizard.sourceforge.net/tools.shtml) [90]. A molecular network was created using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu). The data was filtered by removing all MS/MS fragment ions within +/- 17 Da of the precursor m/z. MS/MS spectra were window filtered by choosing only the top 6 fragment ions in the +/- 50Da window throughout the spectrum. The precursor ion mass tolerance was set to 2 Da and a MS/MS fragment ion tolerance of 0.1 Da. A network was then created where edges were filtered to have a cosine score above 0.65 and more than 6 matched peaks. Further, edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.65 and at least 4 matched peaks. Further, the network was annotated using dereplicator+ to putatively identify the structural details of the compounds present. For annotation using dereplication+, precursor Ion Mass Tolerance of 0.1 Da, fragment ion mass tolerance of 0.01 Da, max charge of 2, Min Score to Consider a PSM as 8.25 and fragmentation mode applied as general_6_1_6.

4.5. Statistical analysis

Statistical analyses were performed in R v. 3.6.1. [91]. The association of specific cyanobacterial species abundance (continuous variable) with distinct cyanotoxin presence/absence (nominal variable) was evaluated using a binary logistic regression in R ('*glm*' function from 'stats' package) [92]. An asymptotic chi-square statistic based on the deviance was used to assess the goodness-of-fit of each model. P-values were adjusted in order to reduce the number of false positives using the Benjamini-Hochberg procedure [93] with a FDR threshold of 0.2. Heatmaps were generated using the 'heatmaply' function in R ('heatmaply' package). The R code for the entire analysis is available in Supplementary Data.

4.6. Data deposition

The mass spectrometry data was deposited on MassIVE public repository (MSV000085840). The molecular networking job can be publicly accessed at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=c2034223333641b3a06a72b40d27b2e4.

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