Cyanobacterial peptides beyond microcystins – A review on co-occurrence, toxicity, and challenges for risk assessment

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Abstract

Cyanobacterial bloom events that produce natural toxins occur in freshwaters across the globe, yet the potential risk of many cyanobacterial metabolites remains mostly unknown. Only microcystins, one class of cyanopeptides, have been studied intensively and the wealth of evidence regarding exposure concentrations and toxicity led to their inclusion in risk management frameworks for water quality. However, cyanobacteria produce an incredible diversity of hundreds of cyanopeptides beyond the class of microcystins. The question arises, whether the other cyanopeptides are in fact of no human and ecological concern or whether these compounds merely received (too) little attention thus far. Current observations suggest that an assessment of their (eco)toxicological risk is indeed relevant: First, other cyanopeptides, including cyanopeptolins and anabaenopeptins, can occur just as frequently and at similar nanomolar concentrations as microcystins in surface waters. Second, cyanopeptolins, anabaenopeptins, aeruginosins and microginins inhibit proteases in the nanomolar range, in contrast to protein phosphatase inhibition by microcystins. Cyanopeptolins, aeruginosins, and aerucyclamide also show toxicity against grazers in the micromolar range comparable to microcystins. The key challenge for a comprehensive risk assessment of cyanopeptides remains their large structural diversity, lack of reference standards, and high analytical requirements for identification and quantification. One way forward would be a prevalence study to identify the priority candidates of tentatively abundant, persistent, and toxic cyanopeptides to make comprehensive risk assessments more manageable.

Keywords: Harmful algal bloom, cyanobacteria, toxin, risk assessment, ecotoxicology, human health

1. Introduction

Our ecosystems and drinking water resources are not only vulnerable towards anthropogenic pollutants but also natural toxins (Bucheli 2014). Among the natural toxins from organisms of various kingdoms, those produced by aquatic organisms, such as cyanobacteria (blue green "algae"), are of particular concern for water resources since these waterborne toxins are released directly into surface waters when the cells die (Bogialli et al. 2017, Flores and Caixach 2015, Saker et al. 2005). Because cyanobacteria produce a diverse mixture of potentially toxic metabolites, there is a need to identify which of these metabolites are of toxicological concern.

Cyanobacteria can grow to extremely high densities forming so-called harmful (algal) blooms within few days or weeks. Such blooms generally occur at warmer temperatures, higher nutrient loads, adequate light penetration into the water and residence time of the water (Schindler 2006, Whitton 2012). Cyanobacterial bloom events have deleterious effects on the ecosystem and on human activities, including decreased biodiversity, higher oxygen consumption due to decaying biomass, restriction of recreational activities, limited usability of water for agriculture, and additional technical measures to provide safe drinking water. While bloom events occur also under pristine conditions and at colder temperatures (Ostermaier and Kurmayer 2010, Trout-Haney et al. 2016), anthropogenic pressure contributes to extended warm periods, and high nutrient loads that favor growth of cyanobacteria (O'Neil et al. 2012). Cyanobacterial blooms occur regularly in numerous lakes (Baumann and Juttner 2008, Gkelis et al. 2015, Kurmayer et al. 2011, Lopes et al. 2012, Mazur-Marzec et al. 2013) and drinking water reservoirs (Ferranti et al. 2013, Grabowska et al. 2014, Jancula et al. 2014, Welker et al. 2006) but our knowledge about the potential risks of the secondary metabolites produced by cyanobacteria remains limited.

Among the bioactive metabolites, cyanobacteria produce low molecular weight toxins, including anatoxin, saxitoxin, and cylindrospermopsin but also non-ribosomal oligopeptides, termed cyanopeptides, which this review will focus on. To date, microcystins are the one class of cyanopeptides that is studied most intensely across scientific disciplines, with more than 300 journal articles published annually on the topic (Figure 1A). The dominance of studies on microcystins may have been catalyzed by the early link of this compound class to human intoxications. In 1996, the death of 52 dialysis patients due to liver failure was traced back to water contaminated with cyanobacteria and the presence of microcystins in a Brazilian hospital (Carmichael et al. 2001, Pouria et al. 1998). These tragic incidents in Brazil triggered the interest in assessing toxicity of microcystins and monitoring environmental concentrations. In addition, intoxication of human has been reported across the globe mostly by recreational exposure through inhalation of aerosols or swallowing contaminated surface water and a comprehensive review on epidemiological research can be found elsewhere (Svircev et al. 2017). Essentially for every country on the globe at least one ISI publication documents the occurrence of microcystin-producing cyanobacteria (Meriluoto et al. 2017, Trout-Haney et al. 2016). Because microcystins are potent liver toxins and can occur at up to micromolar concentrations during cyanobacterial bloom events, several countries include microcystins in their risk management frameworks for water quality (Ibelings et al. 2014). Despite the wealth of publications, even microcystins remain an active field of research, for example to better understand why cyanobacteria produce these complex molecules, how production is regulated, how water treatment can be improved for their effective removal, and which regulatory implementations are needed to protect human and ecosystem health during bloom events (Ibelings et al. 2014).

While microcystins are of ecotoxicological concern, several studies with cyanobacterial extracts observed toxic effects that could not be explained by the presence of microcystins

alone, suggesting that other bioactive metabolites need to be considered in risk assessment as well (Baumann and Juttner 2008, Keil et al. 2002, Le Manach et al. 2016, Smutna et al. 2014, Teneva et al. 2003, Teneva et al. 2005). Recent advancements of analytical techniques enabled researcher to identify hundreds of cyanopeptides beyond the well-known class of microcystins including cyanopeptolins, anabaenopeptins, aerucyclamides, aeruginosines, and microginins with a multitude of structural variants within each class (Welker and von Döhren 2006). However, these cyanopeptides have received little attention thus far regarding their potential human and ecological risk.

This article raises awareness for the diversity of biologically active and potentially toxic cyanopeptides that human and wildlife can be exposed to during cyanobacterial bloom events. Information regarding co-occurrence of cyanopeptides, potential exposure concentrations in surface waters, and toxic effects is synthesized with a particular focus on quantitative data rather than phenomenological observations. Key challenges and research needs towards a risk assessment of cyanopeptide are discussed.

2. Cyanopeptides beyond microcystins

The 1990s present the onset of ever-increasing records of cyanopeptide studies not only for microcystins but also for nodularin, aeruginosins, anabaenopeptins, cyanopeptolins, microginins, microviridins, and aerucyclamides in the 2000s, among others (Figure 1). Over the past decades a legion of other cyanopeptides beyond microcystins has been identified in pure cultures and biomass collected from cyanobacterial bloom events (Beversdorf et al. 2017, Bogialli et al. 2017, Flores and Caixach 2015, Saker et al. 2005, Welker et al. 2004). In the 1960s, some early studies reported the production of aeruginosins from *Microcystis aeruginosa*, a cyanobacterial genus renowned for producing also microcystins (Herbert and Holliman 1964, Holliman 1969, Murakami et al. 1995, Namikoshi and Rinehart 1996).

Despite the increasing scientific output across cyanopeptide classes, the publication records are heavily disproportionate as studies on cyanopeptides other than microcystins represent less than 10% of the total scientific output. Particularly, few studies are available within the disciplines of environmental chemistry with less than 100 studies for *other* cyanopeptides together compared to more than 2,000 studies focusing on environmental behavior of microcystins alone. Here, the key challenge for a systematic risk assessment is the large diversity of cyanopeptides. This article will first demonstrate the structural diversity of cyanopeptides before presenting the current understanding of their co-occurrence, exposure concentrations, and quantitative information about toxicity.

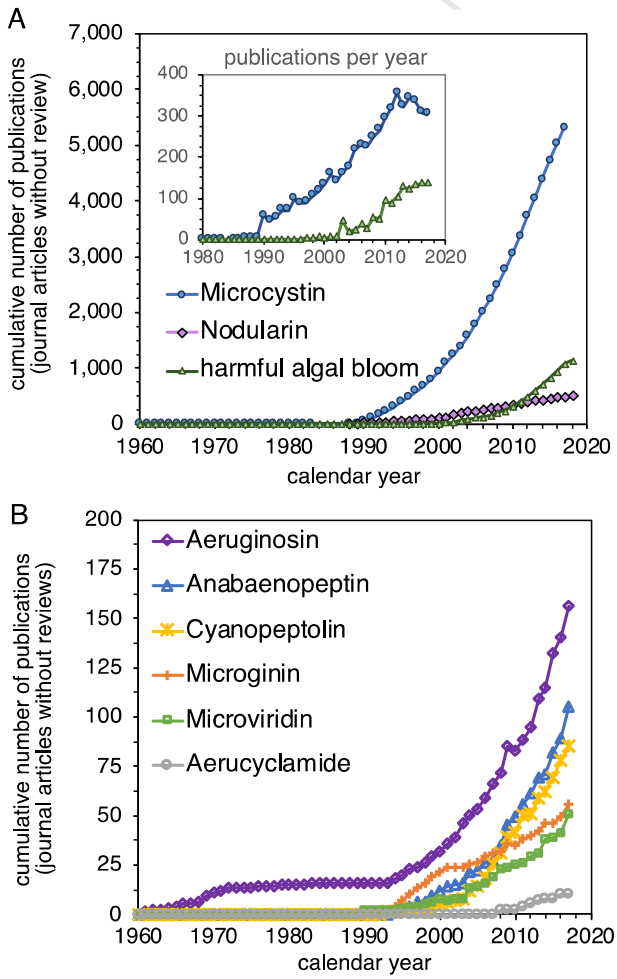

Figure 1. Cumulative number of publications of scientific articles excluding review articles (retrieval from SciFinder 20 June 2018) for the topic of (A) microcystin, nodularin, and harmful algal bloom with annual

publication numbers shown in the insert and for the topics and (B) seven other classes of cyanopeptides in order of the number of publication records. Data is non-exclusive, meaning that one reference may be assigned to multiple categories.

2.1. Structural diversity

Peptides comprise more than 60 percent of the known biological active compounds produced by cyanobacteria (Chlipala et al. 2011). In addition to more than 240 microcystins, a minimum of 500 cyanopeptides ranging from 400-1900 Da have been structurally identified to date and with their variety comes the complexity of the naming system (Meriluoto et al. 2017, Welker and von Döhren 2006). Cyanopeptides consist of cyclic and linear non-ribosomal peptides that can also contain non-proteinogenic residues and posttranslational modifications. Welker and Döhren presented a classification of cyanopeptides based on conserved molecular substructures (Welker and von Döhren 2006). Each class contains a characteristic substructure with a shared biosynthesis pathway while variations of monomers define the different variants within each class. For example, microcystins are heptapeptides with the characteristic Adda moiety (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid) and the different variants result from altering monomers in mainly two positions. The variation is typically expressed by adding the one letter code of the amino acid, so that microcystin-LR refers to the variant with leucine (L) and arginine (R) in positions (2) and (4), respectively. However, the one letter code is limited to the standard amino acids and other modifications require more elaborate suffixes. Nodularins are hexapeptides with a similar structure to microcystins including the Adda moiety. Structural representations of prominent classes beyond microcystins are presented in Figure 2 including the cyclic classes of cyanopeptolins, anabaenopeptins, and (aeru)cyclamides as well as linear peptides classes of aeruginosines and microginins. Each class contains a conserved substructure indicative of this class and variable building blocks. Other than microcystins,

more than two monomeric building blocks can vary in these peptides, resulting in a large combinatorial set of isoforms within each cyanopeptide class. For example, anabaenopeptins carry characteristic ureido bond and several variations in the other five building block is possible (details in Box 1). Among the cyanopeptides beyond microcystins, cyanopeptolins contain the most structurally known compounds (approx. 36%), followed by microginins (approx. 14%), aeruginosins (13%), cryptophycins and anabaenopeptins (approx. 9% each). Other than these cyanopeptide classes, the depsopeptidic crytophycins have mostly been isolated from *Nostoc sp*. and not from a variety of other cyanobacteria (Schwartz et al. 1990). Often several synonyms exist for the same class of cyanopeptides or individual compounds because no standardized naming systems existed early on. For example, the class of cyanopeptolins is also referred to as microcystilides, micropeptins, aeruginopeptins, or oscillapeptins and aeruginosins are also known as microcins or spumigins. In the early literature, the cyanopeptide names are often chosen to refer to the taxon from which the compound had been isolated from and a suffix is added referring to structural properties including characteristic residues, molecular weight, or strain number. Further details of some additional cyanopeptides and their naming systems can be found in comprehensive review on this matter (Chlipala et al. 2011, Van Wagoner et al. 2007, Welker and von Döhren 2006).

179 **BOX 1. Structural characteristics of selected cyanopeptides.**
180

Microcystins are heptapeptides with the characteristic Adda moiety (i.e., 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid) and variable residues in position (2) and (4). **Cyanopeptolins** are hexapeptides with the characteristic Ahp moiety (3-amino-6-methoxy-2-piperidone), and are depsipeptides where a β-lactone ring at the threonine introduces an ester bond and share a mostly conserved linear side chain off position (1). **Anabaenopeptins** are cyclic peptides with a characteristic ureido bond connecting the primary amine of lysine‘s R-group (5) with the carboxyl group of the neighboring amino acid to form an amide bond. **Aerucyclamides** are cyclic hexapeptides characterized by three azole or azoline rings, likely derived from cysteine and threonine modification. **Aeruginosines** are linear tetrapeptides that contain four monomers including the partially substituted Choi moiety (2-carboxy-6-hydroxyoctahydroindole) and Hpla moiety (p-hydroxyphenyl lactic acid) based on tyrosine and often an arginine derivative at the C-terminus. Lastly, **microginins** are linear peptides with four to six amino acids, one of them being the characteristic Ahda moiety (3-amino-2-hydroxydecanoic acid, or -octanoic acid) and predominantly two tyrosine monomers.

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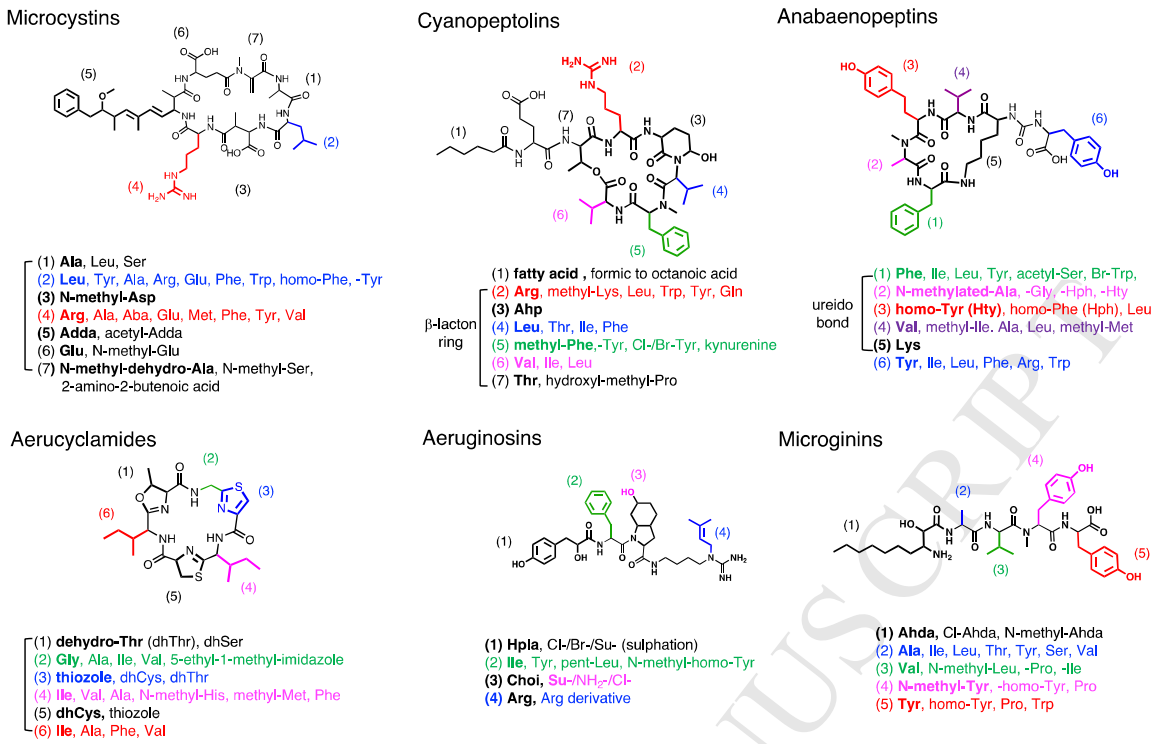


Figure 2. Schematic of representatives for six cyanopeptide classes: Microcystin-LR, Cyanopeptolin A, Anabaenopeptin A, Aerucyclamide A, Aeruginosin KB 676, and Microginin 713. Colored areas in the structure represent variable parts that can be substituted and examples of alternative building blocks are presented below each structure in the respective legend. The stereochemistry is not considered in these representations and annotations were adopted from Welker and Döhren (2006).

3. Occurrence of cyanopeptides during bloom events

The chemical diversity of cyanopeptides is large, but how common are these compounds in surface waters dominated by cyanobacteria? While the abundance of cyanobacteria can be monitored successfully, following cyanobacterial peptides is more challenging because of the variety of potential target compounds. Few studies focused on the presence of cyanopeptides other than microcystins but those that did search further, discovered that microcystins *never* occurred alone. While the ecological benefit for cyanobacteria to produce these complex and metabolically expensive cyanopeptides has not been completely resolved, not even for microcystins, evidence is presented below on their abundance and bioactivity.

200

201 **3.1 Ability of cyanopeptide production**

202 The likelihood of cyanopeptides to occur in the environment depends on the presence of
203 cyanobacteria that carry the genes for toxin production and that these genes are actually
204 expressed. The exposure concentrations of cyanopeptides depend on the abundance of those
205 toxin producers and the cyanpeptide production dynamics as well as the degradation and fate
206 processes of toxins in surface waters.

207 Genotype analysis can reveal gene clusters that are indicative of the metabolic synthesis
208 pathways for cyanopeptides (Kurmayer et al. 2015, Kurmayer et al. 2011). Cyanopeptide
209 biosynthesis involves multi-enzyme complexes of non-ribosomal peptide synthetases and
210 polyketide synthases that can be conserved across cyanobacterial genomes (Kurmayer et al.
211 2015, Kurmayer et al. 2011, Neilan et al. 2013). For example, the biosynthetic cluster code
212 and highly conserved multidomain proteins required to produce microcystins have been
213 identified in the genus of *Microcystis*, *Planktothrix, Nostoc*, and *Dolichospermum*
214 (*Anabaena*) (Christiansen et al. 2003, Kosol et al. 2009, Kurmayer and Christiansen 2009,
215 Merel et al. 2013, Rantala et al. 2004, Rouhiainen et al. 2004, Zikova et al. 2010). Within one
216 species the genes may not always be expressed and also non-producing strains exists that lack
217 the ability to produce microcystins altogether (Bogialli et al. 2017, Briand et al. 2016, Davis
218 et al. 2009, Kurmayer and Christiansen 2009, Kurmayer et al. 2004, Welker and von Döhren
219 2006). At the same time, one cyanobacterial species can harbor biosynthesis pathways for
220 several cyanopeptides beyond microcystins (Kurmayer and Christiansen 2009, Neilan et al.
221 2013, Shishido et al. 2017). For example, the biosynthesis genes for microcystins,
222 anabaenopeptins, aeruginosins, microginins, microviridins, and cyanopeptolins were
223 identified in the common cyanobacteria *Planktothrix rubescens* and *P. agardhii* as well as in
224 *Microcystis sp*. from a bloom event in San Francisco Bay (Kurmayer et al. 2016, Otten et al.

2017). Overall, much less genetic information, for example regarding indicative syntheses gene clusters or distribution of non-/producing strains is available for cyanopeptides beyond microcystins. Cyanobacteria often co-occur during bloom events and their genotypes suggest that they are *able* to produce different cyanopeptides but not whether these metabolites are actually produced.

3.2 Production during bloom events.

Chemical analysis verifies that cyanobacteria can indeed use their genetic ability effectively to biosynthesize cyanopeptides during bloom events. A variety of cyanopeptides was frequently detected in lakes, rivers, estuaries, and reservoirs affected by cyanobacteria across the globe including studies in Italy, Greece, Poland, Portugal, Israel, Switzerland, and Finland (Ferranti et al. 2013, Gkelis et al. 2015, Grabowska et al. 2014, Lifshits and Carmeli 2012, Lopes et al. 2012, Rohrlack et al. 2009). Additional studies inspected field isolates, which constitute cyanobacterial cultures isolated from environmental samples and regrown under laboratory conditions. All 26 cyanobacterial isolates from a drinking water reservoir in Portugal produced a range of aeruginosines, anabaenopeptins, and microcystins (Saker et al. 2005). Similar field isolates originating from a Czech reservoir showed up to 90 co-occurring cyanopeptides, from Brazilian surface waters up to 38 cyanopeptides, and nearly 60 cyanopeptides from Baltic Sea isolates (Mazur-Marzec et al. 2016, Sanz et al. 2015, Welker et al. 2006). Advanced analytical techniques further revealed the diverse cyanopetidome on the cellular scale realized by analyzing single bacterial colonies and cyanobacterial filaments with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Harustiakova and Welker 2017, Welker et al. 2004).

3.3 Exposure concentrations in surface waters

Quantification of cyanopeptides is a key prerequisite to characterize the potential exposure concentrations in surface waters where cyanobacteria proliferate. Most studies, however, only report qualitative information such as frequencies of detection of cyanopeptides, as mentioned above. To date, only few studies quantified absolute concentrations of cyanopeptides beyond microcystins (Figure 3). A recent study quantified three cyanopeptolins, one microginins, two anabaenopeptins, and five microcystins in samples from six eutrophic lakes in the United States (Beversdorf et al. 2017). Another study assessed the aqueous concentrations of five anabaenopeptins, one cyanopeptolin and ten microcystins in European lakes using analytical standards isolated from biomass extracts. Here, all cyanopeptides were detected frequently with 87% for microcystins, 90% for a cyanopeptolin, and 100% for anabaenopeptins (Chorus et al. 2006). Data in Figure 3 show that concentrations were in the μg/L range (i.e., nanomolar) and microcystins were not always dominating, particularly in the European lakes. The study on European Lakes further showed that cyanopeptide production from one cyanobacterial species can vary between different lakes and only correlates with biovolume of cyanobacteria for some cyanopeptides (Chorus et al. 2006).

Furthermore, a recent study of the U.S. Great Lakes Basin demonstrates that the total load of cyanopeptolins and anabaenopeptins entering drinking water treatment plants can be comparable to microcystins and also correlated with abundance of cyanobacteria (cell count, chlorophyll-a) and organic carbon concentrations (UV absorbance, turbidity) (Beversdorf et al. 2018).

Such quantitative assessments are rare because of the lack of standard reference materials for most cyanopeptides that would allow to convert a signal from analytical instruments into the absolute mass of a cyanopeptide present in the sample. Currently, reference standards of 12 microcystins (MC-LR, -RR, -YR, -LA, -LF, -LW, -WR, -D-Asp3-LR, D-Asp3-RR, HtyR,

HilR) are commercially available and partially included in standardized analytical methods (ISO 20179:2005, US EPA Method 544), which can reach sensitivities in the low nanomolar range (e.g., by liquid chromatography coupled to mass spectrometry). The limited availability of reference standards restricts comprehensive quantification of cyanopeptide concentrations considering that more than 240 microcystin isoforms and many more additional cyanopeptides have been identified (Meriluoto et al. 2017). Organic synthesis was achieved for some linear aeruginosines that also allowed to selectively test the effect of subtle changes of functional moieties, i.e., chloride and sulfate removal (Scherer et al. 2016, Scherer and Gademann 2017). However, organic synthesis is currently not feasible for the structurally more complex cyanopeptides particularly considering the stereochemistry of these molecules. Most commercially available cyanopeptide materials stem from isolation efforts of cyanobacterial biomass, called bioreagents, which do not strictly qualify as reference material because of their lower purity and even certified reference materials come in varying purity and require batch-to-batch verification of the absolute concentration. Without reference standards for each compound of interest, one common alternative is to report semi-quantitative concentrations relative to an available standard: microcystin-LR.

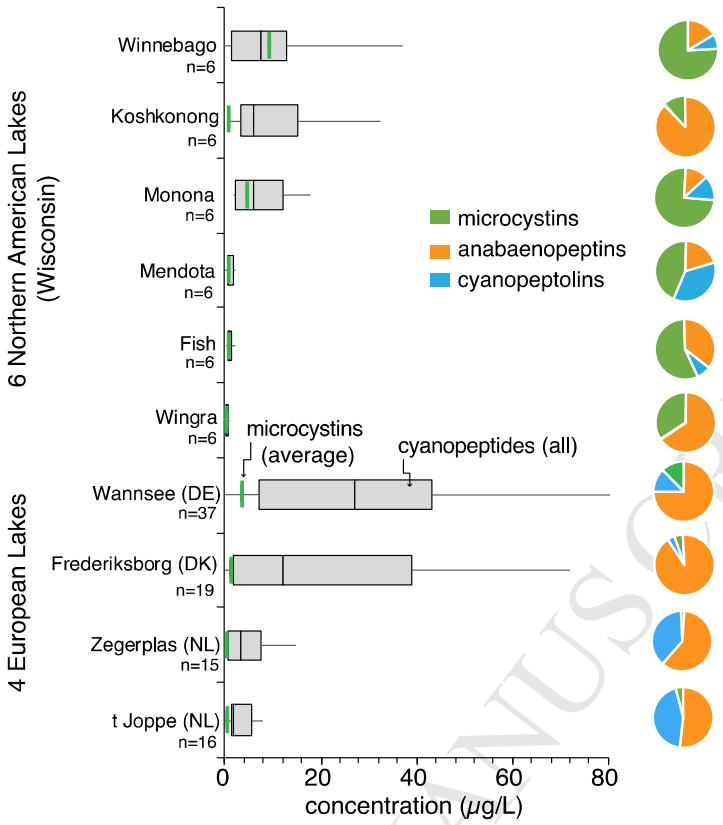


Figure 3. Cyanopeptide concentrations (µg/L) for six eutrophic lakes in the United States (top) and four European lakes (bottom) (Beversdorf et al. 2017, Chorus et al. 2006). The boxplot shows data of total cyanopeptides including 4 microcystins (MC-LR, -YR, -LA, -RR), 2 anabaenopeptins (Anabaenopeptin-B, -F) and 2 cyanopeptolins (Cyanopeptolin-1007, -1041) for the U.S. lakes and 10 microcystins (MC-RR, -YR, -LR, -Asp3-RR, -Dha7-RR, -Asp3, -Dhb7-RR, -Asp3-HTyr, 2 unidentified MCs), 5 anabaenopeptins (Anabaenopeptin A,-B, -F, Aeruginosinamide, Oscillamide Y) and 1 cyanopeptolin (Cl-Cyanopeptolin W) for the European lakes. The black line within the boxplot indicates the median value, whiskers indicate 25th and 75th percentiles. For comparison, the average values for total microcystins are shown as green lines within the boxplot. The pie charts present the relative distribution of concentrations for total microcystins (green), anabaenopeptins (orange), and cyanopeptolins (blue) and the sample number (n) is indicated for each lake.

4. Toxicity of cyanopeptides

Considering that other cyanopeptides indeed occur at similar concentrations as microcystins during cyanobacterial bloom events, there is a need to evaluate their potential

306 (eco)toxicological risk. In the following, first those studies are highlighted that assessed the
307 (sub-)lethal effects for these cyanopeptides. Then, studies are presented that focused on
308 microcystins alone but observation suggest that additional compounds might be present in the
309 cyanobacterial extracts that contributed to toxic effects.
310
311 **4.1 (Sub-)lethal effects**
312 One common effect observed for cyanopeptides is the inhibition of enzymes. Particularly,
313 inhibition of proteases has been frequently reported for cyanopeptolins, anabaenopeptins,
314 aeruginosins, and microginins and inhibitory potencies (IC_{50} values) down to the low
315 nanomolar range, comparable to concentrations observed during bloom events (Figure 4). For
316 cyanopeptolins, anabaenopeptins, and aeruginosins inhibition of human serine proteases
317 involved in blood coagulation was observed, i.e., Factor VIa/VIIa, thrombin, plasmin, and
318 kallikrein (Bonjouklian et al. 1996, Gademann et al. 2010, Hanessian et al. 2006, Kodani et
319 al. 1999, Kohler et al. 2014, Schreuder et al. 2016) and inhibition of pancreatic and leucocyte
320 elastases for cyanopeptolins and anabaenopaptins (Bubik et al. 2008, Sedmak et al. 2008).
321 Further tests with proteases showed inhibition of carboxypeptidases A+B and leucine
322 aminopeptidase involved in food metabolism for cyanopeptolins, anabaenopeptins, and
323 microginins (Ishida et al. 2000a, Ishida et al. 1998, Ishida et al. 1997, Itou et al. 1999, Kodani
324 et al. 1999, Murakami et al. 2000, Walther et al. 2009), inhibition of angiotensin-converting
325 enzymes involved in blood pressure regulation for microginins (Kodani et al. 1999, Okino et
326 al. 1993), and inhibition of trypsin and chymotrypsin activity for cyanopeptolins and
327 aeruginosins (Banker and Carmeli 1999, Bister et al. 2004, Bonjouklian et al. 1996,
328 Gademann et al. 2010, Hanessian et al. 2006, Kodani et al. 1999, Kohler et al. 2014, Raveh
329 and Carmeli 2009, Reshef and Carmeli 2001, Sano and Kaya 1995, Von Elert et al. 2005).
330 Microginins were also observed to inhibit *Mycobacterium tuberculosis* protein tyrosine

phosphatase (Muller et al. 2006), and aerucyclamides showed activity against leukemia cells and human parasites (Ishida et al. 2000b, Portmann et al. 2008b). Cyanopeptides also show antimicrobial, antifungal and antimycobacterial activity as recently reviewed elsewhere (Swain et al. 2017).

Another common observation is the toxicity against the grazing crustacean *Thamnocephalus platyurus* reported for microcystins, cyanopeptolins, aeruginosins, microginin, and aerucyclamides with LC_{50} values in the low micromolar range (mg/L) *(Blom et al. 2003, Blom et al. 2001, Bober and Bialczyk 2017, Gademann et al. 2010, Kohler et al. 2014, Portmann et al. 2008a, Portmann et al. 2008b).* While the observed concentrations in lake water were one order of magnitude lower (Figure 3), much higher exposure concentration must be expected for organisms that feed directly on cyanobacterial cells by ingestion of biomass and also when human swallow contaminated water or inhale cells with aerosols. The hypothesis that cyanobacteria produce these complex molecules to defend themselves against predators is in line with observed toxicity against grazers and a promising avenue to further explore in future mechanistic studies. Cyanopeptides have been detected in tissue of exposed fish, frogs, snails, and mussels but the toxicokinetics of internal distribution and bioaccumulation is not completely understood (Gkelis et al. 2006).

4.2 Toxicity of microcystins and beyond

Microcystins are hepatotoxins that can enter liver cells by hitchhiking a specific membrane transporter, i.e., organic anionic transporting polypeptide (Fischer et al. 2005). Once in the liver cell, microcystins suppress enzyme activities by binding to protein phosphatases (PP1 and PP2), which causes dysregulation, formation of reactive oxygen species, and eventually liver failure (Ding et al. 2000, Honkanen et al. 1990, Konst et al. 1965, Mackintosh et al. 1990). The toxicity depends critically on the microcystin variant, the

exposure route, and the target organisms. For example, the lethal dose to kill 50% of mice (acute LD_{50}) ranges between 5,000-10,000 μg/kg for oral and 50 μg/kg for intraperitoneal administration (Kuiper-Goodman et al. 1999). Epidemiological studies indicate human health effects and potential cancer development from acute and chronic exposure to cyanopeptides as recently reviewed (Liu et al. 2017, Svircev et al. 2017). Some toxicological studies on microcystins offer clues concerning the potential toxicity of other cyanopeptides. These studies did not include other cyanopeptides specifically but hint towards their toxicity when (a) the observed toxic effects exceeded the toxicity expected from microcystins alone or (b) the symptoms could not be associated with microcystins. One example is the case of human exposure to cyanotoxins by recreational activities. Here, the concurrence of illness was correlated with cyanobacterial cell number but vomiting, diarrhea, and flu-symptoms and could not directly be associated with the presence of known liver toxins (microcystins) or neurotoxins (e.g., saxitoxin, anatoxin) (Berg et al. 2011, Pilotto et al. 1997). Other studies compared microcystin-producing strains (wild-type) with cyanobacterial strains that specifically cannot produce microcystins (knock-out mutants). For example, while only the microcystin producing strain had lethal effects on crustaceans (*Daphnia geleata, D. magna*), also the non-producing strain caused decreased ingestion rates, increased enzyme production involved in biotransformation, and altered reproductive processes, suggesting that compounds other than microcystins also affect aquatic organisms (Dao et al. 2013, Hulot et al. 2012, Rohrlack et al. 1999). Additional studies frequently observed that extracts from the microcystin knock-out mutants also caused adverse effects on aquatic organisms and cell lines (Baumann and Juttner 2008, Best et al. 2001, Dao et al. 2013, Fastner et al. 2003, Hulot et al. 2012, Keil et al. 2002, Liang et al. 2017, Smutna et al. 2014). For example, the formation of oedema and bent tails in zebrafish larvae and dysregulation in fish were observed also upon exposure to extracts of non-microcystin-producing cyanobacteria (Keil et

al. 2002, Le Manach et al. 2016). Information on the toxicity of some additional cyanopeptides including cryptophycins, tolytoxin, scytophycins, and microviridins can be found elsewhere (Chlipala et al. 2011).

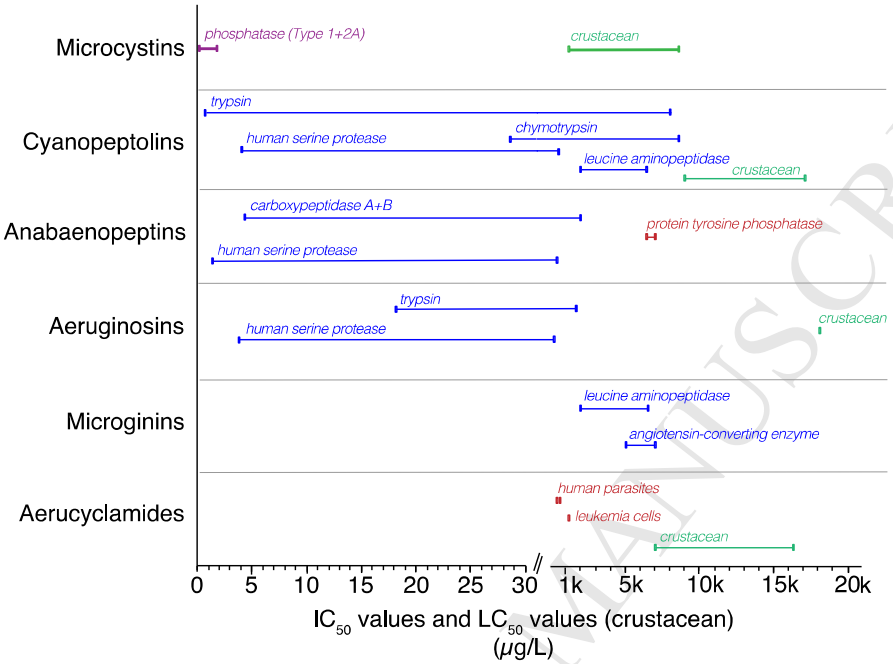


Figure 4. Ranges of inhibitory concentrations of enzymes, IC_{50} values (µg/L) for the different cyanopeptide classes including proteases (blue) (Banker and Carmeli 1999, Bister et al. 2004, Bonjouklian et al. 1996, Gademann et al. 2010, Hanessian et al. 2006, Ishida et al. 1998, Ishida et al. 1997, Itou et al. 1999, Kodani et al. 1999, Kohler et al. 2014, Murakami et al. 2000, Okino et al. 1993, Raveh and Carmeli 2009, Reshef and Carmeli 2001, Sano and Kaya 1995, Schreuder et al. 2016, Von Elert et al. 2005, Walther et al. 2009), phosphatases (purple) (An and Carmichael 1994, Honkanen et al. 1990) and others (red) (Ishida et al. 2000b, Muller et al. 2006, Portmann et al. 2008b) and the lethal concentrations, LC_{50} values (μg/L) for the grazing crustacean *Thamnocephalus platyurus* (green) (Blom et al. 2001, Bober and Bialczyk 2017, Gademann and Portmann 2008, Kohler et al. 2014, Portmann et al. 2008a, Portmann et al. 2008b, Scherer et al. 2016). Note break in x-axis, hereafter 1k represents 1,000 μg/L.

5. Implications for risk assessment

The key challenge for future risk assessment of cyanobacterial peptides is the need to cover a large chemical and biological space. Implications and research needs for analytical methods, assessment of abundant and persistent peptides, as well as toxic peptides to prioritize relevant cyanotoxins are discussed in the following (Figure 5).

5.1 Analytical Methods

Hundreds of cyanopetides have been structurally identified and the lack of reference standard materials and comprehensive databases of all cyanopeptides are the key challenges for standardized and sensitive analytical detection. High-resolution tandem mass spectrometry (HRMS/MS) has become more widely available in the scientific research institutions, regulatory laboratories, and even at larger water treatment utilities. While HRMS/MS analysis alone is not sufficient for structurally elucidating newly identified compounds, it can be directed to specifically detect and differentiate structurally known cyanopeptides. So-called suspects screening with HRMS/MS allows to identify cyanopeptides without available reference materials based on the exact mass (*m/z*), presence of one or more charge states (z=1, 2 etc.), expected isotope pattern and common adducts (e.g., +H, +Na, $+NH_4$) as well as secondary fragmentation (MS2) following procedures established for small organic pollutants and their transformation products (Krauss et al. 2010, Schymanski et al. 2014). Those cyanopeptide that are structurally known comprise the suspect list in this scenario. The high resolving power and mass spectral accuracy is essential for a reliable assignment of exact mass of a cyanopeptide. Other than for small organic molecules, most cyanopeptides are large molecules and automated chemical formula assignment is often not reliable for identification. To increase the confidence of the identification, the secondary fragmentation pattern can be matched with in-silico predictions

and literature information. Because we also face isobaric compounds one needs to obtain fragmentation spectra that are unambiguously identifying a single cyanopeptide, e.g., due to unique fragments. For reliable and faster cyanopeptide identification, a public spectral library should be built and maintain by collaborative efforts of the scientific community. The use of extracts of pure cyanobacterial laboratory cultures may serve as qualitative reference materials for the identification of cyanopeptide in field samples. For example, when the dominating cyanobacterial species in a water body are known, the field samples can be compared to the extract of the respective cyanobacterial species from laboratory culture. This approach would allow to compare not only the spectral fragmentation but also the retention behaviour during chromatographic separation (retention time match). With such diagnostic evidence, cyanopeptides can be identified as the "probable structure" even without reference standards available (Level 2 after Schymanski et al. 2014).

While identification of cyanopeptide is possible with conclusive HRMS/MS data, the quantification without certified reference standards presents another critical challenge. Analytical chemistry widely relies on semi-quantitative exposure concentrations thus far, for example by microcystin-LR-equivalent concentrations for all cyanopeptides. One avenue to explore can be a class-specific semi-quantitative equivalent approach that uses representative reference material of one abundant and potentially toxic cyanopeptides of each class. However, even among microcystin variants, the analytical response can vary by a multitude, due to different response factors in mass spectrometry, which leads to higher uncertainties when relying on equivalent concentrations. Because variants of a cyanopeptide class vary only in a limited number of moieties, a structure-based prediction of response factors for variants of a cyanopeptide class would be one possible avenue to explore. In the case of electronspray ionization mass spectrometry (ESI-MS), commonly employed to analyse cyanopeptides, the response factor comprises how well a structural moiety can be ionized,

447 which depends on instrumental parameters (source settings, mobile phase composition, pH
448 etc.), on physico-chemical properties of the analyte, and matrix-specific interferences. For
449 example, the ionization of microcystins in ESI-MS depends largely on the presence of basic,
450 ionizable arginine moieties (Yuan et al. 1999) but can also be greatly affected by polarity,
451 and vaporability of the target compounds (Kiontke et al. 2016). In silico tools have been used
452 in proteomic research to prioritize the best ionizable, i.e., best observable proteolytic peptides
453 for target proteins based on the primary sequence of linear peptides (Matsuda et al. 2017).
454 However, such prediction becomes more complex with the stereochemistry of cyanopeptides
455 particularly for cyclic structures (Wang and Cole 1994) and in presence of sample matrix
456 from biomass extracts, e.g., ion suppression (Kankaanpaeae et al. 2009, Karlsson et al. 2005).
457 While these quantification methods may be sufficient to screen for general presence of
458 cyanopeptide and tentative abundance, reference standard materials of those cyanopeptide of
459 toxicological concern are ultimately required.
460
461 **5.2 Abundant and persistent peptides**
462 To make future risk assessment of cyanopeptides more manageable, one approach is to
463 assess the number of tentatively relevant compounds based on exposure concentrations by
464 prioritizing those compounds that are produced in considerable amounts by common
465 cyanobacteria. Peptide profiles can be assessed and curated for common strains in
466 comprehensive archives and compared across geographic and temporal trends. When the
467 major cyanobacterial strains in a bloom event are identified, one can explore whether the
468 expected cyanopeptides can be predicted based on archived peptide profiles from laboratory
469 cultures and field isolates. While current literature suggests that only a smaller subset of
470 cyanopeptides is expected at an individual site, this may still comprise dozens of compounds.

Among the abundant cyanopeptides, those that are persistent could be further prioritized for risk assessment. Here, both persistence in environmental and engineered systems is of interest. Environmental fate studies include biodegradability as well as mass transfer, complexation, abiotic hydrolysis, and photochemical transformation processes. Microcystins are susceptible to microbial degradation and photochemical transformation but can still persist in water for several days or weeks (de la Cruz et al. 2011, Edwards et al. 2008, Sharma et al. 2012, Walker 2014). While open questions regarding the behavior of microcystins remain, essentially no information currently exists on the fate of the other cyanopeptides.

The relatively long half-life of microcystins in surface waters suggest that cyanopeptides can reach water treatment plants influents when blooms occur in freshwater reservoirs. Consequently, the engineered processes need to be inspected for their ability to remove cyanopeptides by conventional treatment including sand and activated carbon filtration as well as advanced oxidation processes including UV/H_2O_2, ozonation, and membrane filtration. All treatment options need to consider that intracellular toxins can be released by lysing the bacterial cells. For example, ozonation can effectively oxidize microcystins but also lyses cells and thus does not always result in sufficient total removal of the toxin (Hoeger et al. 2005). Since microcystins have been frequently detected even in finished drinking waters (Carmichael 2000, Hoeger et al. 2005, Miller et al. 2017) avoiding the intake of contaminated water and developing adoptive treatment strategies during bloom events needs to be considered for impacted source waters. Overall, the fate and transformation behavior of cyanopeptides will define the exposure concentrations after release from the bacterial cells that need to be considered for risk assessment.

5.3 Toxic peptides

In addition to chemical analysis, effect-based tests are needed in the process of prioritizing cyanopeptides. First, the most abundant and persistent compounds might not be the most toxic. Second, not all cyanopeptides might be structurally known and would be missed in a suspect screening by HRMS/MS as described above. In addition, toxicological analysis is promising to investigate mixture toxicities of co-occurring cyanopeptides.

To identify and quantify the main toxicological effects of one cyanopeptide, one can focus on a targeted approach with a specific hypothesis for the toxic mode of action or effect-based analysis by screening various endpoints. The hypothesis-driven approach is challenged by the lack of information about potential target sites of these cyanopeptides. We currently lack a clear understanding of internal distribution and internal concentrations of the cyanopeptides. In the case of microcystins, they do not only cause liver toxicity but have also been reported to induce neurological symptoms in exposed patients (Hu et al. 2016). Also, the organic anion transporting polypeptides (OATP), which mediate the cellular uptake of microcystins, are not only expressed in the liver but also at the blood brain barrier (Fischer et al. 2005). A better understanding of the exposure routes can further guide which tissue will be in contact with cyanopeptides, for example via aerosol inhalation and swallowing contaminated water. The current literature demonstrates that cyanopeptides inhibit different target enzymes compared to microcystins, which needs to be considered when selecting toxicity tests. Hence, not only liver, but also the brain, gill/lung and gut are among the plausible target organs to investigate.

In addition to the challenge that we are dealing with hundreds of cyanopeptides, also many possible toxic modes of action need to be considered. High-throughput toxicity screening can be a powerful approach to sieve through thousands of potential endpoints. High-throughput toxicity screening uses *in vitro* toxicity assays and is being used extensively for testing the effects of chemicals and their mixtures in risk assessment and drug discovery (Szymanski et

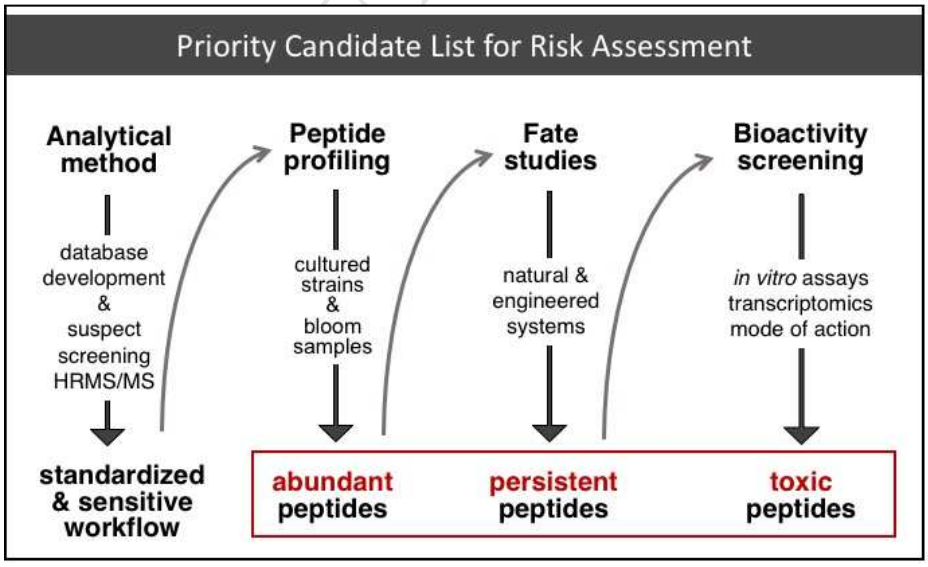
al. 2012). Cellular assays that measure defined toxic endpoints in automated and miniaturized systems present an opportunity to overcome the bottle neck of extremely low throughput in conventional in vivo tests. However, toxicity can not only be toxin-specific but also cell-specific, individual cellular models can not cover all possible toxic mechanisms, processes that rely on cellular communication, and control systems throughout the organisms (Astashkina et al. 2012). Transcriptomic analysis offers another holistic approach to predict potential adverse effects and molecular mechanisms considering the whole organism. For example, a reduced transcriptome atlas of the zebrafish was investigated for 74 chemicals including microcystin-LR and cyanopeptolin-1020 revealing their effects on early neuronal development (Zhang and Zhao 2018). The transcriptomics approach can help characterize and quantify unanticipated toxic effects but the relationship between gene expression and toxic mechanisms still needs to better understood and annotating the function of genes of organisms with ecological relevance is still limited (Schirmer et al. 2010). In the future, advanced data processing and mechanistic understanding, and reduced costs may enable the wider use of transcriptomic screening for the risk assessment of cyanopeptides.

These screening approaches can also be used for crude cyanobacterial extracts without identifying each cyanopeptide first. The chemical demand of several mg for comprehensive toxicity tests are high compared to chemical analysis where even a few μg can be sufficient. Hence, bioactive screening of complex extracts can be attractive to delineate potential toxic sample early on. When an effect is observed, fractionation of extracts allows to further curtail the origin of a toxic effect but only isolation efforts of a single compound can yield clarity about toxic potencies of one specific cyanopeptide. The risk assessment for cyanotoxins can learn from development in drug discovery that uses high-throughput screening in combination with *in silico* modelling for toxicity but also for adsorption, distribution, metabolism, and excretion (van de Waterbeemd and Gifford 2003). *In silico* models rely on

546 molecular modelling of the potential interaction of compound with a target site, e.g., an
547 enzyme, as well as data modelling including structure-activity relationships. Once a target
548 site has been identified for one cyanopeptide, the molecular mechanism of toxicity and
549 quantitative structure activity relationships can be further explored for other variants in the
550 cyanopeptide class. For example, the binding affinities to carboxypeptidase has been recently
551 modelled for a range of anabaenopeptins (Walther et al. 2009). Also, the development of
552 antibodies should be explored in the future to allow for quantification of total cyanopeptide
553 of one class analogous to the enzyme-linked immunosorbent assay (ELISA) existing for
554 microcystins.

555 The combined approach of chemical and effect-based screening aims for an informed
556 decision about the tentatively abundant, persistent, and toxic cyanopeptides to make a
557 comprehensive risk assessment more manageable. In the process of prioritizing cyanopeptide
558 for risk assessment, additional factors may be decisive, for example the value of a field site
559 harbouring particularly sensitive organisms, impacts on natural resource utilization and water
560 treatment options, or recreational values, which represent different personal and public
561 interests, priorities for human and ecological health.

562



563
564

565 **Figure 5. Strategy for a prevalence studies to reduce the large number of theoretically known**
566 **cyanopeptides to be a priority candidate list for risk assessment of tentatively abundant, persistent, and**
567 **toxic compounds.** One prerequisite is a state-of-the art analytical method for sensitive and standardized
568 identification of cyanopeptides including comprehensive, open-access databases for all structurally known
569 cyanopeptides (i.e., suspects) and spectral libraries for suspect screening by high-resolution mass spectrometry
570 (HRMS/MS). The abundant peptide can be identified by peptide profiling of common cyanobacterial strains
571 from laboratory cultures and in-situ bloom samples. Fate studies in will point to the most persistent peptides in
572 environmental and engineered systems (e.g. drinking water treatment). The bioactivity screening including *in*
573 *vitro* assays and transcriptomics can inform about the mode of action across different compounds or compound
574 classes from fractionated extracts containing mixtures of toxins and isolated compounds. The potentially
575 abundant, persistent, and toxic cyanopeptides are the priority candidates for future risk assessments.

576

577 ## 6. Conclusions

578 The current state of the literature supports that cyanopeptides are bioactive and produced
579 in legions at comparable frequency as microcystins in surface waters that are impacted by
580 cyanobacteria. Consequently, risk assessment of cyanopeptides beyond microcystins and
581 their mixtures is necessary. The few studies that quantified cyanopeptides beyond
582 microcystins find that they can reach comparable concentrations and complementary studies
583 are desired to verify these phenomena for different surface waters, dominating cyanobacterial
584 species, and bloom dynamics. Also, additional toxicity studies are needed that target human
585 and ecosystem health with relevant exposure scenarios. The key challenge is the need to
586 cover a large chemical and biological space for risk assessment without essential reference
587 standards for the hundreds of known cyanopeptides. To move forward from here, advanced
588 analytical methods to identify and quantify cyanopeptides need to be used to determine toxin
589 profiles in field samples and laboratory strains, to assess their fate and transformation
590 processes, and to screen for their potential bioactivity. Such prevalence study could reveal the
591 priority candidates among cyanopeptides and their mixtures for which a comprehensive risk

assessment will be more manageable (Figure 5). The combined approach of chemical and effect-based screening aims for an informed decision about the tentatively abundant, persistent, and toxic cyanopeptides to make a comprehensive risk assessment more manageable. In the process of prioritizing cyanopeptides for risk assessment, additional factors may be decisive, for example the value of a field site harbouring particularly sensitive organisms, impacts on natural resource utilization and water treatment options, or recreational values, which represent different personal and public interests, priorities for human and ecological health.

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Highlights

- Lack of studies on cyanopeptides beyond microcystins especially in environmental sciences
- Occurrence of cyanopeptides comparable in frequency and concentration to microcystins
- Cyanopeptides inhibit various proteases in the nanomolar range (IC50)
- Key challenge for risk assessment is the structural diversity of cyanopeptides
- Identify priority candidates of potentially abundant, persistent, and toxic cyanopeptides

Who is abundant + persistent + toxic ?