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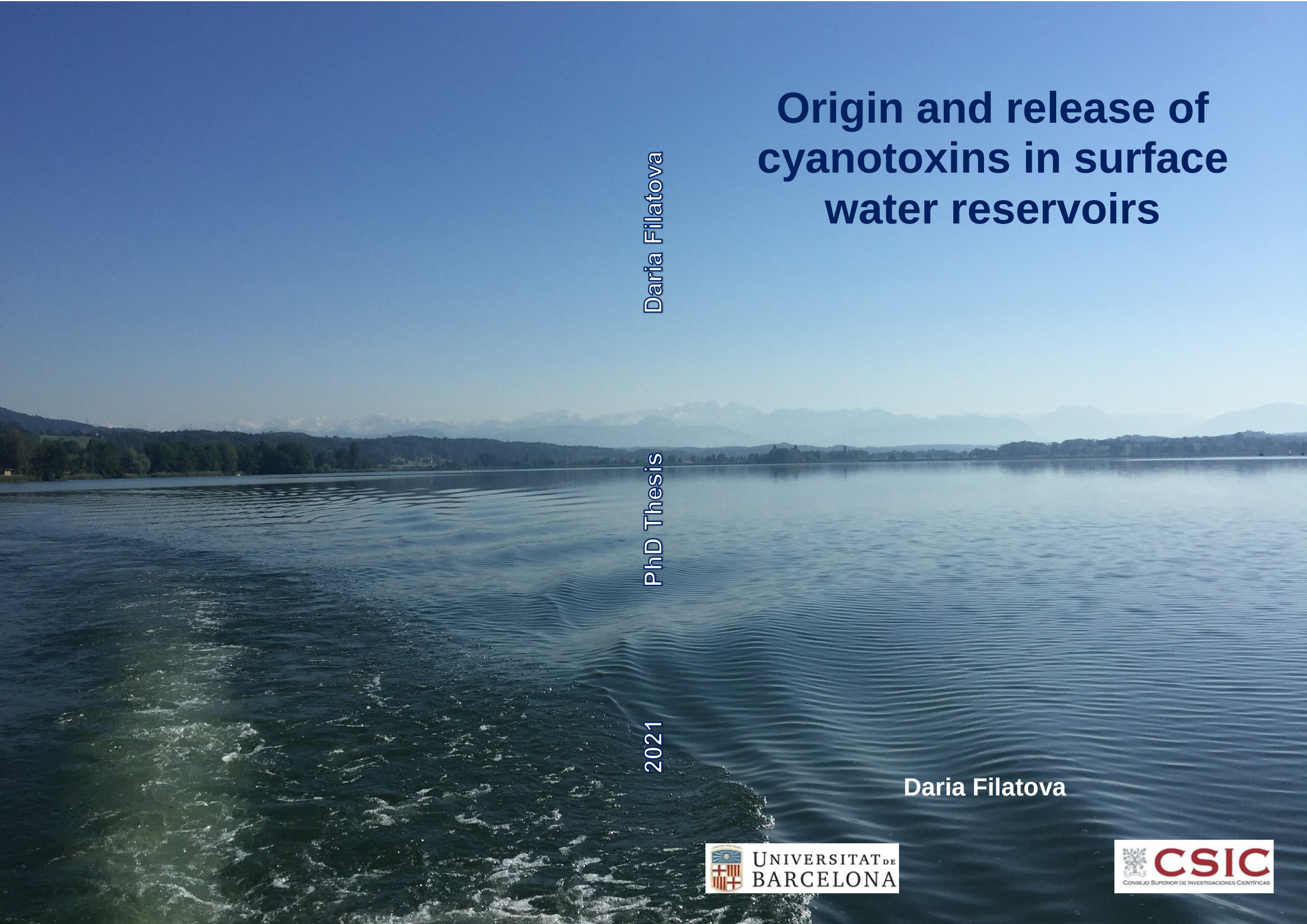
Origin and release of cyanotoxins in surface water reservoirs

Daria Filatova



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PhD Thesis

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Origin and release of cyanotoxins in surface water reservoirs

Memoria de tesis presentada para optar al grado
Doctor por la Universidad de Barcelona a

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Certifican:

Que la presente memoria presentada para optar al título de Doctor, titulada "***Origin and release of cyanotoxins in surface water reservoirs***" ha sido realizada bajo nuestra dirección por la Sra. **Daria Filatova** en el Instituto de Diagnóstico Ambiental y Estudios del Agua (IDAEA), perteneciente al Consejo Superior de Investigaciones Científicas (CSIC), y el departamento de Ingeniería Química y Química Analítica de la Universidad de Barcelona, y que todos los resultados presentados son fruto del trabajo experimental realizado por la mencionada doctoranda.

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Dra. Marinella Farré

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Abbreviations

Ab	Antibody
ACN	Acetonitrile
Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid
AFW	Artificial fresh water
Ag	Antigen
AGC	Automatic gain control
Ahp	3-amino-6-methoxy-2-piperidone
ANA	Anatoxin-a
ANA-s	Anatoxin-a(s)
ATP	Adenosine 5’-triphosphate
CE	Collision energy
Choi	2-carboxy-6-hydroxyoctahydroindole
CyanoHABs	Harmful cyanobacterial blooms
CYN	Cylindrospermopsin
DAD	Diode-array detection
DDA	Data-dependent acquisition
DNA	Deoxyribonucleic acid
DIA	Data-independent acquisition
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisaton
FA	Formic acid
FIA	Flow injection analysis
FWHM	Full width at half maximum
GC-MS	Gas chromatography coupled to mass spectrometry
HESI	Heated electrospray ionisation
HLB	Hydrophilic-lipophilic balance
homoANA	Homoanatoxin-a
Hpla	p-hydroxyphenyl lactic acid
HPLC-MS/MS	High performance liquid chromatography coupled to tandem mass spectrometry
HRMS	High-resolution mass spectrometry

HRMS/MS	High-resolution tandem mass spectrometry
IC_{50}	Half maximal inhibitory concentration
ILOD	Instrumental limit of detection
IMS	Ion-mobility spectrometry
IP	Identification point
LC	Liquid chromatography
LC_{50}	Lethal concentration that kills 50% of tested animals
LC-MS	Liquid chromatography coupled to mass spectrometry
LD_{50}	Lethal dose resulting in 50% deaths
LLE	Liquid-liquid extraction
LOD	Limit of detection
LRMS	Low-resolution mass spectrometry
MC	Microcystin
MLOD	Method limit of detection
MLOQ	Method limit of quantification
MS	Mass spectrometry
NOD	Nodularin
PSI	Photosystem I
PSII	Photosystem II
QIT	Quadrupole ion trap
qPCR	Quantitative polymerase chain reaction
QqQ	Triple quadrupole
RSD	Relative standard deviation
SPE	Solid phase extraction
SPME	Solid phase microextraction
STX	Saxitoxin
ToF	Time-of-flight
UHPLC	Ultra-high performance liquid chromatography
UV	Ultraviolet detection
VAE	Vacuum assisted evaporation
WHO	World Health Organization

Abstract

Cyanobacteria are prokaryotes and components of regular periphyton formation. Overall, there are around 2000 cyanobacterial species that live in various environments. Some cyanobacterial species are known to form blooms, which can cause harmful effects when blooms' intensity is high. Abundant blooms can deplete oxygen causing hypoxic conditions that may result in the death of plants and animals. Another major issue associated with bloom-forming cyanobacteria is the production of bioactive secondary metabolites, some of which are known to be toxic. One of the main routes of human exposure to cyanobacterial toxins occurs through water, both drinking and recreational water use. There are two main drivers that favour cyanobacteria bloom: nutrient over-enrichment and on-going climate change. Nowadays, little quantitative information is available on temporal variations of cyanotoxins, including the European region. Establishing seasonal trends of cyanobacterial toxins will promote the development of effective water management strategies.

Cyanotoxins can be divided into two main groups according to their targeted tissue/organ of toxicity: hepatotoxins and neurotoxins. Main cyanobacterial hepatotoxins are microcystins, nodularins, and cylindrospermopsin, while anatoxin-a, and saxitoxin are the main neurotoxins. Owning to posed toxicological risks by various cyanobacterial metabolites, guidelines values in drinking water have been introduced by several countries. What is more, the update of the WHO guideline has been recently finalized, and now it involves threshold values not only for microcystin variant, but also for cylindrospermopsin, anatoxin-a, and saxitoxins. The assessment of the occurrence and the risks of the exposure to cyanotoxins require robust, straightforward, and sensitive analytical methodologies for their identification and

quantitation in the aquatic environment, and particularly in drinking water reservoirs. Besides, to perform extensive monitoring studies, these methods should be cost-effective and rapid.

Beyond these cyanotoxins, cyanobacteria can produce a variety of other bioactive secondary metabolites, including cyanopeptides. These compounds belong to several classes including cyanopeptolins, anabaenopeptins, aeruginosins, aerucyclamides, and microginins. Some of these compounds are known to be co-produced together with other cyanobacterial toxins. Compounds from these classes have shown acute toxicity in planktonic grazers and are able to inhibit various enzymes. However, there is a knowledge gap in both their occurrence and posed toxicological risks.

In the framework of this thesis, several points were addressed in order to fulfil the current gaps of the research in the area of occurrence of cyanobacterial toxins ant other metabolites in surface water. Literature review on current analytical approaches for analysis of cyanotoxins and their seasonal variations in previously conducted studies in European region was carried out. Main analytical approaches were compared, what provided solid background for analytical method development. Based on available seasonal studies on cyanotoxins in different European climate zones, patterns for continental, Mediterranean, and oceanic climate zones were described.

A method for the assessment of multiclass cyanotoxins in freshwater based on dual solid-phase extraction liquid chromatography coupled with high-resolution mass spectrometry was developed, optimised, and validated. The developed method showed high sensitivity, selectivity, and robustness. The application of an ultra-high pressure liquid chromatography column allowed fast separation, what makes this method more cost-effective.

A targeted method was applied for the analysis of freshwater samples from Spain, Switzerland, and the United Kingdom. Several targeted cyanotoxins were identified and quantified. Additionally, mass spectrometry data acquired in high resolution provided an opportunity of posterior suspect screening, which revealed potential presence of another cyanopeptide – anabaenopeptin.

Additionally, the targeted methodology was expanded for an application of suspect screening for a wide range of cyanopeptides. This method was applied for the analysis of raw drinking water from the United Kingdom. Suspect screening revealed co-occurrence of targeted compounds together with other cyanopeptides. The obtained results are the first to present concentrations of anabaenopeptins, cyanopeptolins, aeruginosins, and microginins, along with microcystins, in the reservoirs of the United Kingdom

Resumen

Las cianobacterias son procariotas y componentes de la formación regular de perifiton. En general, hay alrededor de 2000 especies de cianobacterias que viven en varios entornos. Se sabe que algunas especies de cianobacterias generan episodios de proliferación de toxinas, que pueden causar efectos nocivos cuando la intensidad de dicha proliferación es alta. Las proliferaciones abundantes pueden agotar el oxígeno y causar condiciones hipóxicas que pueden resultar en la muerte de plantas y animales. Otro problema importante asociado con las cianobacterias que conllevan dichas proliferaciones es la producción de metabolitos secundarios bioactivos, algunos de los cuales se sabe que son tóxicos. Una de las principales vías de exposición humana a las toxinas cianobacterianas se produce a través del agua, tanto las de consumo como las de uso recreativo. Hay dos factores principales que favorecen la proliferación de las cianobacterias: el enriquecimiento excesivo de nutrientes y el cambio climático continuo. En la actualidad, se dispone de poca información cuantitativa sobre las variaciones temporales de las cianotoxinas, incluida la región europea. Sin embargo, comprender las tendencias históricas es fundamental, ya que reduce la incertidumbre y proporciona una base sólida para la previsión de dichos episodios. El establecimiento de tendencias estacionales de toxinas cianobacterianas promoverá el desarrollo de estrategias efectivas para la gestión del agua.

Las cianotoxinas se pueden dividir en dos grupos principales según su tejido / órgano de toxicidad objetivo: hepatotoxinas y neurotoxinas. Las principales hepatotoxinas cianobacterianas son microcistinas, nodularinas y cilindrospermopsina, mientras que la anatoxina-a y la saxitoxina son las principales neurotoxinas. Debido a los riesgos toxicológicos que plantean varios metabolitos de las cianobacterias, varios países

han introducido valores de referencia en el agua potable. Es más, la actualización de la guía que la OMS ha finalizado recientemente incluye ahora valores umbral no solo para la variante de microcistina, sino también para cilindrospermopsina, anatoxina-a y saxitoxinas. La evaluación de la presencia y los riesgos de la exposición a las cianotoxinas requieren metodologías analíticas sólidas, sencillas y sensibles para su identificación y cuantificación en el medio acuático y, en particular, en los reservorios de agua potable. Además, para realizar estudios de seguimiento exhaustivos, estos métodos deben ser rentables y rápidos.

Más allá de estas cianotoxinas, las cianobacterias pueden producir una variedad de otros metabolitos secundarios bioactivos, incluidos los cianopéptidos. Estos compuestos pertenecen a varias clases que incluyen cianopeptolinas, anabaenopeptinas, aeruginosinas, aeruciclamidas y microgininas. Se sabe que algunos de estos compuestos se coproducen junto con otras toxinas cianobacterianas. Los compuestos de estas clases han mostrado toxicidad aguda en herbívoros planctónicos y son capaces de inhibir varias enzimas. Sin embargo, existe una laguna de conocimiento tanto con respecto a su aparición como a los riesgos toxicológicos que plantean.

En el marco de esta tesis, se abordaron varios puntos con el fin de cubrir los vacíos actuales de la investigación en el área de la presencia y distribución de toxinas cianobacterianas y otros metabolitos en aguas superficiales. Se llevó a cabo una revisión de la literatura sobre los enfoques analíticos actuales para el análisis de cianotoxinas y sus variaciones estacionales en estudios realizados anteriormente en la región europea. Se compararon los principales enfoques analíticos que proporcionaron una base sólida para el desarrollo de métodos analíticos. Sobre la base de los estudios estacionales disponibles sobre cianotoxinas en diferentes

zonas climáticas europeas, se establecieron patrones para las zonas climáticas continentales, mediterráneas y oceánicas.

Se desarrolló, optimizó y validó un método para la evaluación de cianotoxinas multiclase en agua dulce basado en cromatografía líquida de extracción en fase sólida dual combinada con espectrometría de masas de alta resolución. El método desarrollado una alta sensibilidad, selectividad y robustez. La utilización de una columna de cromatografía líquida de ultra alta presión permitió una separación rápida, lo que hace que este método sea más rentable.

Se aplicó un método dirigido para el análisis de muestras de agua dulce de España, Suiza y Reino Unido. Se identificaron y cuantificaron varias cianotoxinas dirigidas. Además, los datos de espectrometría de masas adquiridos en alta resolución brindaron la oportunidad de realizar una detección posterior de sospechosos, lo que reveló la presencia potencial de otro cianopéptido: la anabaenopeptina.

Además, el método dirigido se amplió para la detección de compuestos sospechosos en relación a una amplia gama de cianopéptidos. Este método se aplicó para el análisis de agua potable del Reino Unido. El cribado de sospechosos reveló la coexistencia de compuestos diana junto con otros cianopéptidos. Los resultados obtenidos son los primeros en presentar concentraciones de anabaenopeptinas, cianopeptolinas, aeruginosinas y microgininas, junto con microcistinas, en los reservorios de agua del Reino Unido.

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Shaded areas highlight the biological data closest to the cyanopeptide data sampled on August 13th, September 3rd, and October 10th 2019.

Figure 26. Isotopic patter of suspected anabaenopeptin B detected in Pasteral reservoir in September sample and simulated isotope.

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Structure of the thesis

This thesis is structured in three chapters: Chapter 1: introduction; Chapter 2: targeted screening for cyanotoxins; and Chapter 3: suspect screening for cyanobacterial metabolites.

- **Chapter 1** is structured in three main parts. The first one, includes an introduction with information about cyanobacteria, cyanotoxins, other cyanobacterial metabolites, and guideline values in drinking water, and it is structured into 3 sections. The first section describes the history of cyanobacteria and their acquired adaptations. Since phylum cyanobacteria counts for more than 2000 species, their classification is also mentioned. Bloom-formed by cyanobacteria is discussed in more detail as harmful effects may occur due to CyanoHABs (harmful cyanobacterial blooms). The second section focus on cyanotoxins (hepatotoxins and neurotoxins) and their structural diversity. Additionally, other bioactive cyanobacterial metabolites are also mentioned as the number of studies on their occurrence and toxicological effects is increasing in the last several years. Lastly, the third section (section 3) summarises information on main guideline values of cyanotoxins in drinking water and its' updates in the last year.

  The second part (section 4) addresses the analysis of cyanotoxins in freshwater. It is the main part of the introductory chapter as development of analytical methodologies for targeted screening of cyanotoxins in freshwater is the main focus of this thesis. Thus, this section covers various techniques and strategies that are applied to date. Firstly, sampling, sample preservation, and sample pre-treatment strategies are described. The chosen techniques would vary depending on the goal of each study. Then, immunochemical and

chemical methods are explained, as they are the most widely used. Among immunochemical methods enzyme-linked immunosorbent assay is the most popular technique. For the chemical analysis, various extraction, pre-concentration, and clean-up techniques are discussed. Nowadays, one of the most popular techniques is solid phase extraction, thus, it is covered in more detail. Additionally, particular attention is brought to techniques based on liquid chromatography coupled to mass spectrometry. The main focus, in this case, is on methods that provide multi-class determination of cyanotoxins. One of the challenges in the analysis of cyanotoxins is confirmation and quantification, thus, current status of reference standards' availability is also explained. Finally, application of high-resolution mass spectrometry for the analysis of cyanotoxins has provided suspect screening of various cyanobacterial metabolites, which are not yet well studied. State-of-the-art approaches for identification and quantification of suspects are also discussed.

The final part of this chapter (section 5) covers levels and seasonal variations of cyanotoxins in European freshwater studies. Nowadays, the information about seasonal variation of cyanobacterial toxins in different regions is scarce. However, understanding historical trends would provide a solid foundation for forecasting of cyanobacterial toxins and blooms, what will promote the development of effective water management strategies. Thus, in this section, information on levels and seasonal variations of cyanotoxins in three different European climatic zones (Mediterranean, humid continental, and oceanic) are summarized. Toxins' variation patters in each region were established and can be used for forecasting and water management. Additionally, relationship

between toxins' concentrations and environmental parameters is discussed in this section.

Main content of this section was published at the beginning of 2020 in the paper: "Analysis, levels and seasonal variation of cyanotoxins in freshwater ecosystems", D. Filatova, M. Picardo, O. Núñez, M. Farré, Trends in Environmental Analytical Chemistry 26 (2020) e00091, and the information was updated in this thesis.

- **Chapter 2** is also structured into three parts. The first one (sections 1 and 2) focus on the development and validation of a targeted screening method for the determination of 10 cyanotoxins in freshwater based on SPE-UHPLC-HRMS/MS. It is worth mentioning that this is the main block of this chapter. The prioritization of targeted compounds was done based on two parameters: 1) the frequency of their detection in European region and 2) the availability of standards. Thus, the list of targeted compound includes 7 MCs (MC-LR, -RR, -YR, -LA, -LW, -LY, -LF), ANA, CYN, and NOD. In the course of this study, no internal or surrogate standards were applied for the determination of cyanotoxins. Since NOD was already reported in freshwater samples, it was added as analyte rather than surrogate standard for MCs. A paper focused on method development and validation was published: "Ultra-Trace Analysis of Cyanotoxins by Liquid Chromatography Coupled to High-Resolution Mass Spectrometry", D. Filatova, O. Núñez, M. Farré, Toxins 12 (2020) 247.

  The second part of this chapter (sections 3 and 4) describes method transfer and modification. In the course of this research project, SPE-UHPLC-HRMS/MS method was transferred to the Department of Environmental Chemistry of the Swiss Federal Institute of Aquatic Science and Technology (Eawag), as a scientific stay was carried out there. The previously developed

targeted method was not only transferred, but also modified. Thus, section 3 of this chapter describes these aspects, and section 4 focus on the methods' performance.

The third part of this chapter (section 5) covers the application of developed and transferred methods for the analysis of freshwater samples from different locations: 1) Ter river basin (Spain); 2) lake Greifensee (Switzerland); and 3) three raw drinking water reservoirs Ingbirchworth, Tophill Low, and Embsay (the United Kingdom). The analysis of samples from Spain was performed at IDAEA-CSIC and UB, and the samples from Switzerland and the United Kingdom were analysed at Eawag. The second part was also done in collaboration with a water company from the United Kingdom - Yorkshire Water. Method application on freshwater samples from the United Kingdom was also published: "Cyanobacteria and their secondary metabolites in three freshwater reservoirs in the United Kingdom", D. Filatova, M.R. Jones, J.A. Haley, O. Núñez, M. Farré, E.M.-L. Janssen, Environmental Sciences Europe 33 (2021) 29.

- **Chapter 3**, the final chapter of this thesis, focus on the suspect screening of other cyanobacterial metabolites. In this case, the main focus was on different cyanopeptides. This part was performed at Eawag in collaboration with Yorkshire Water. As previous chapters, it is also structures into three parts, which in this case correspond to the number of each section. The first section is describing the suspect screening method. It is based on the targeted screening method that was mentioned in the second chapter. For suspect screening, recently-formed database CyanoMetDB was applied.

  The second section focus on identification and confirmation aspects. To process HPLC-MS/MS data files, Compound Discoverer software was

applied. Customized non-targeted work-flow were used for feature detection, grouping, deconvolution, and compound annotation. Since there is a scarcity of reference standards, class-equivalent approach for quantification was used. The final section covers method application. In this case, the samples were provided by our collaborators from Yorkshire Water from three raw drinking water reservoirs.

OBJECTIVES

Nowadays, there is an increasing frequency and intensity of cyanobacterial blooms due to nutrient over-enrichment and on-going climate. This pose risk to aquatic ecosystems and human health. Toxins produced by cyanobacteria is a group of chemically diverse compounds that are hepato- and neurotoxic. Routes of human exposure to cyanotoxins may occur through drinking water and recreational water use. To protect public from exposure, guidelines values in drinking water have been introduced by World Health Organisation for several toxins.

Currently, there is a sacristy of available quantitative information on temporal variations of cyanotoxins, which is needed for effective water management. To study occurrence of cyanotoxins and assess the risk of the exposure in surface (and particularly drinking water) reservoirs, robust, straightforward, and sensitive analytical methods are needed.

Additionally, beyond the regulated cyanotoxins, more than 2000 secondary metabolites from cyanobacteria have been structurally identified to date. They are co-produced with known cyanotoxins by bloom-forming cyanobacteria, however, there occurrence and toxicological risks are not studied well.

Thus, the main objectives of this thesis were:

1. Review literature on seasonal variations of cyanotoxins in different climate zones in Europe and make different patterns to provide information for better forecasting and reservoirs management.
2. Develop, optimise, and validate analytical methodologies for the quantification of targeted multiclass cyanotoxins that occur in freshwater reservoirs for rapid and sensitive analysis.
3. Apply the developed methods for studies of occurrence of targeted toxins in freshwater reservoirs, focusing on drinking water reservoirs.

4. Expand the developed targeted method for the suspect screening of various cyanopeptides and study their co-occurrence with cyanotoxins.

INTRODUCTION

1. Cyanobacteria

Cyanobacteria are prokaryotes and components of regular periphyton formation. They are the only prokaryotic group that perform oxygenic photosynthesis [1]. For their life processes, they require light as a source of energy, water as electron donor and CO_2 as a carbon source [2]. There are two photochemical reaction centres, called Photosystem I (PSI) and PSII, which form the main photosynthetic apparatus in all cyanobacteria. PSII is responsible for water oxidation, then the electrons are passed to PSI through a cytochrome b_6/f complex. Simultaneously, a cross-membrane proton gradient is created, which is used for generation of adenosine 5'-triphosphate (ATP). These two photochemical reaction centres (PSI and PSII) operate in series and generate a biochemical intermediate with a sufficiently low redox potential. This drives the enzymatic reduction of CO_2 to form organic molecules [3]. It is still unclear when did first cyanobacteria-like microbes appear, but cyanobacteria played a major role in the rise of the atmosphere and oceans around 2.4 Gyr ago (also known as the Great Oxidation Event), enabling the oxygenation of oceanic and terrestrial niches and the diversity of complex life [1, 4]. Overall, there are around 2000 cyanobacterial species that live in various environments.

This section will be focused on cyanobacterial adaptations that were developed during their long evolutionary history; their classification including taxonomic scheme and ecological classification; and main aspects of cyanobacterial bloom that some cyanobacterial species are able to form.

1.1. Adaptations

Cyanobacteria species developed a variety of adaptations to different environmental conditions [5]. They are spread globally in different aquatic environments (salty, brackish or fresh waters, in cold and hot springs), and they can also colonise

terrestrial environments such as volcanic ash, desert sand and rocks [6]. Among cyanobacteria adaptations there are presence of pigments, nutrient storage, nitrogen fixation, buoyancy, formation of resting cells known as akinetes, and formation of colonies or filaments. Pigments such as chlorophyll-a, allophycocyanin and phycocyanin harvest light in several parts of the electromagnetic spectrum radiation (green, yellow and orange). Such wide range provides cyanobacteria an advantage in terms of absorbing light for photosynthesis comparing with other phytoplankton species [7]. Phosphorus is commonly stored as polyphosphate and nitrogen as cyanophycin or phycobilin pigments [8]. Irrespective of the fact that N-fixation from N_2 provides a competitive advantage in N-deficient conditions, it is an energetically expensive process. Some species can form akinetes, what ensures their survival during unfavourable conditions such as the lack of light, nutrients scarcity, changes in temperature and desiccation. The akinete outlives in the bottom sediments due to their metabolism and germinate when conditions are favourable. Buoyancy provides the developed bacteria access to well-lit surface waters via the presence of gas vesicles. Gas vesicles are hollow chambers, and have densities ten times lower than that of the water [5, 6, 9].

1.2. Classification

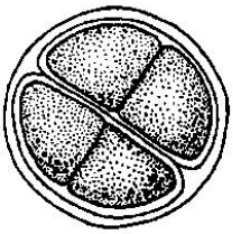
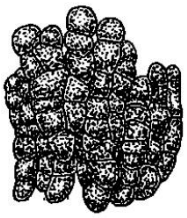
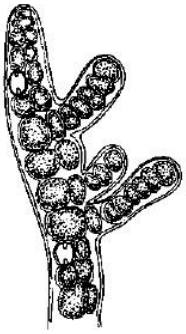
There are several options of cyanobacterial classification. Herein, taxonomic scheme and ecological classification will be mentioned.

1.2.1. Taxonomic scheme

Overall, there are around 2000 cyanobacterial species in 150 genera and 5 orders (Table 1). Focusing on the orders, Chroococcales are unicellular forms that have spherical, ovoid or cylindrical shape. They reproduce by binary fission or budding. The cells may form in irregular colonies. In this case the cells are held together by

the slimy matrix secreted. Pleurocapsales order is characteristic with coccoid cells, which can form aggregates or pseudo-filaments that reproduce by daughter cells (baeocytes or endospores). In this case, a series of successive binary fissions takes place, which converts a single mother cell into many daughter cells. The next three orders, Oscillatoriales, Nostocales and Stigonematales, have filamentous morphology, forming a multicellular structure (Trichome) which consists of a chain of cells, and it can be straight or coiled. In the Oscillatoriales order, species have identical cells and form unseriated and unbranched trichomes. They do not form heterocysts or akinetes. On the other hand, species of both Nostocales and Stigonematales orders form trichomes with heterogeneous cellular composition. In the Nostocles order, filaments divide in only one plane, while in the Stigonematales order filaments divide in more than one plane with true branching and multiseriate forms. Additionally, species of these two orders can form heterocytes [6, 10].

Table 1. *Cyanobacterial classification according to classic botanical taxonomic scheme. Pictures reproduced from Open Access reference [6].*

Order	Representative genera	Illustration
Chroococcales	*Microcystis, Synechocystis, Synechococcus, Aphanocapsa, Aphanothece, Gloeocapsa*	
Pleurocapsales	*Chroococcidiopsis, Pleurocapsa*	
Oscillatoriales	*Oscillatoria (Planktothrix), Lyngbya, Leptolyngbya, Microcoleus, Phormidium*	
Nostocales	*Anabaena, Aphanizomenon, Cylindrospermopsis, Nostoc, Calothrix, Scytonema, Tolypothrix*	
Stigonematales	*Mastigocladus (Fischerella), Stigonema*	

1.2.2. Ecological classification

From the ecological point of view, cyanobacterial species can be divided into three main groups: picocyanobacteria, mat-forming cyanobacteria and bloom-forming cyanobacteria.

Picocyanobacteria have the smallest cells comparing with other cyanobacteria cells (the term *pico* refers to cells smaller than 2 µm, however some genera can exceed the upper limit). Cell size is around one thousandth of a millimetre in diameter. There are two common morphologies: single cells (coccoid, rods) and colonies. Most of the colonies belong to chroococcal cyanobacteria, with a cell size of 0.5 – 3 µm and spherical, ovoid or rod/like cell forms. The cells can be packed loosely or densely, and they can also shape in form of pseudo-filaments or other net-like structures. The most common freshwater colonial picocyanobacteria species belong to the genera *Aphanocapsa*, *Aphanothece*, *Chroococcus*, *Coelosphaerium*, *Cyanobion*, *Cyanodictium*, *Merismopedia*, *Romeria*, *Snowella* and *Tetracercus*. Marine species are the most abundant photosynthetic organisms on Earth, and the most abundant of those are species belonging to *Prochlorococcus* and *Synechococcus* genera. Small cell size can be advantageous for a light capture due to absence of internal shading (‘package effect’) [10-13].

Microbial mats are benthic communities of micro-organisms with high density. They can be encountered in the surfaces of rocks, plants, sand, sediments and other substances. As cyanobacteria are metabolically versatile, mats and films are common in extreme environments such as cryoconites holes on glaciers, in pools on Arctic and Antarctic ice shelves, in saline and hypersaline lakes, and in geothermal springs. The thickness of the mat can vary between millimetres to centimetres. Mat-forming cyanobacteria might give rise to consolidated rocks (laminated structures), which are known as stromatolites (Figure 1). They are dating back to the Precambrian

and are considered to be the first indicators of life on Earth. Cyanobacterial mats are common in wet-land systems, being the most common genera in swamps *Chroococcus*, *Leptolyngbya*, *Lyngbya*, *Phormidium*, *Microcoleus*, *Schizothrix* and *Scytonema* [2, 10].

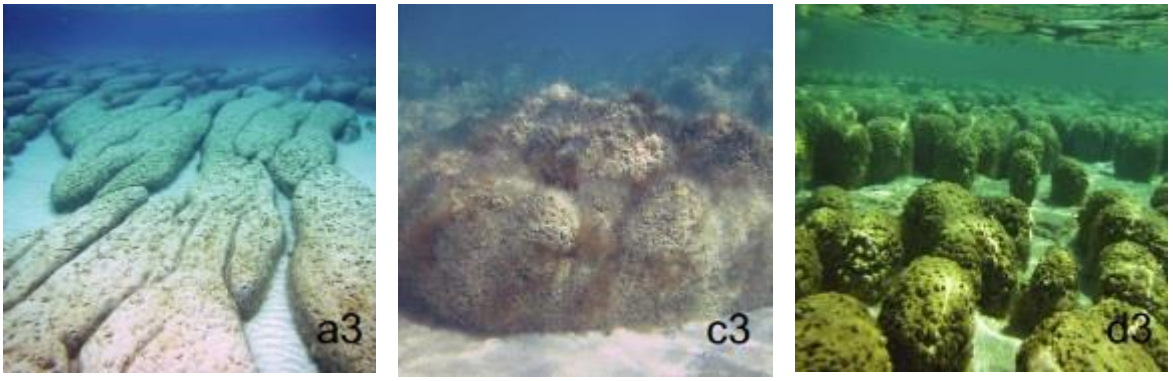


Figure 1. *Stromatolites at Shark Bay in Western Australia. Pictures adopted from Open Access reference [14].*

Bloom-forming species favour warm, stable, nutrient-rich lakes, and they are generally absent in polar and alpine regions. There are several common bloom-forming genera: *Anabaena*, *Aphanizomenon*, *Microcystis*, *Oscillatoria* (*Planktothrix*), *Limnothrix*, *Pseudanabaena* and *Cylindrospermopsis*. In stable water column, the cyanobacterial colonies adjust their position via gas vesicles. Additionally, buoyancy can cause the appearance of surface scums, which are blown by the wind along the shoreline and bays [10, 15]. Bloom-forming cyanobacteria can form harmful blooms (see below), which can be vast events. One example can be seen on Figure 2, which shows algal bloom in the Lake Erie (the United States and Canada) which was captured by the Operational Land Imager on Landsat.

Figure 2. *Algal bloom in Lake Erie, July 28, 2015. Reproduced from NASA Earth Observatory (images by Joshua Stevens, using Landsat data from the United States Geological Survey. Caption by Kathryn Hansen; https://earthobservatory.nasa.gov/images/86327/algae-boom-in-lake-erie)*

1.3. Cyanobacterial bloom

There is no international definition or quantification for what a cyanobacterial bloom is. Jean-François Humbert and Jutta Fastner suggest the following definition:

"*A bloom is an increase of cyanobacterial biomass in a lake (measured, for example, by chlorophyll-a concentration) over a relatively short time (between a few days and 1 or 2 weeks) and is characterized by the dominance (>80%) of only one or a few species within the phytoplankton community. In mesotrophic or less-eutrophic lakes and ponds, biomasses from 30 to 50 µg* L^{-1} *chlorophyll-a correspond to large blooms, whereas in eutrophic and hypereutrophic lakes, biomasses exceeding 300 to 400 µg* L^{-1} *chlorophyll-a can be found*" [15].

When intensity of cyanobacterial blooms is too high, harmful effects may occur [16]. In this case, such blooms are also called CyanoHABs (harmful cyanobacterial

blooms), and this term is frequently used in the literature [17-20]. Abundant blooms can deplete oxygen causing hypoxic conditions that may result in the death of plants and animals [21]. Another major issue associated with bloom-forming cyanobacteria is the production of bioactive secondary metabolites, some of which are identified as toxins. The main routes of human exposure to cyanobacterial toxins may occur through drinking water, recreational water use, and consumption of food in which toxin may have accumulated [22].

In addition to human and animal hazard, cyanobacterial blooms may economically affect several sectors as fisheries, tourism and recreation, and monitoring and management. There is a scarcity of data to evaluate the amount of economic losses in Europe [21]. However, it was reported that freshwater blooms cause economic losses of more than $4 billion annually in the United States alone [16]. In the last decades, growth of the cyanobacterial blooms has been recorded. For instance, Ho et al. [16] have studied long-term trends in intense summertime near-surface phytoplankton blooms by means of high-resolution Landsat 5 satellite imagery for 71 large lakes globally. They found that the bloom intensity increased in 68 % in all lakes. Taranu et al. [23] reported a study which was based on the examination of about 200 years sedimentary records. The results showed a significant increase of cyanobacteria since 1800, and more rapidly during the last decades (since 1945) in north temperate-subarctic lakes. There are two main drivers which favour cyanobacteria bloom: nutrient over-enrichment and on-going climate change [24, 25]. To design efficient management and remediation strategies, an understanding of global patterns, trends and drivers is still necessary [16].

2. Cyanotoxins and other secondary metabolites

Cyanobacteria produce a variety of bioactive metabolites that can be classified in several ways, such as their targeted tissue/organ of toxicity, their molecular weight, their location in the cell (inside or outside), etc. Some of these secondary metabolites are known to be toxic. The main targeted tissues are liver and nerve; thus, they are called hepatotoxins and neurotoxins. Additionally, there are low molecular weight bioactive compounds and toxins (anatoxins, cylindrospermopsin, and saxitoxin), and high molecular weight cyanopeptides (microcystins). Cyanopeptides include several classes of compounds such as microcystins, nodularin, cyanopeptolins, anabaenopeptins, aeruginosins and microginins [26]. More than 2000 cyanopeptides are structurally identified to date [27]. However, not all of them are classified as bioactive compounds as their toxicity is not sufficiently studied. Cyanobacterial metabolites can be found within bacterial cells (extracellular) or released into the water (intracellular).

In this section, different cyanotoxins are described based on their targeted tissue/organ of toxicity. Additionally, other bioactive cyanopeptides are also mentioned in a separate sub-section.

2.1. Hepatotoxins

Main cyanobacterial hepatotoxins are microcystins, nodularins, and cylindrospermopsin.

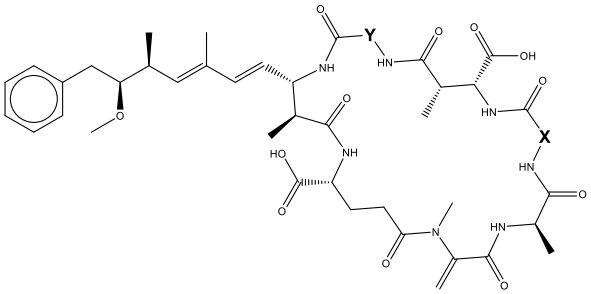
Microcystins (MCs) and nodularins (NODs) are cyclic peptides with hepatotoxic activity containing the β-amino acid Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid) (Figure 3).

The common structure of MCs (heptapeptides - seven amino acids) is cyclo(D-Ala-L-X-D-erythro-methylAsp(iso-linkage)-L-Z-Adda-D-Glu(isolinkage)-N-

methyldehydro-Ala). The main structural difference can be found in the L-amino-acid residues 2 (X) and 4 (Z), which are represented by a two-letter suffix. For example, MC-LR contains leucine (L) in position 2 and arginine (R) in position 4 [6, 28]. Thus, MC-YR is for tyrosine and arginine; -RR is for two arginines; and -LA, -LF, -LY, and -LW are for leucine and alanine, phenylalanine, tyrosine, and tryptophan, respectively. These compounds are produced by multiple cyanobacteria genera, including *Microcystis*, *Anabaena* (now *Dolichospermum* or *Sphaerospermopsis*)*, Oscillatoria (now Planktothrix), Nostoc*, *Oscillatoria*, and *Anabaenopsis*. While more than 300 MC variants have been identified to date [29], few MCs are routinely included in the analysis of surface waters.

NODs are pentapeptides (five amino acids), and their general structure is cyclo(-D-erythro-β-methylAsp(iso-linkage)-L-Y-Adda-D-Glu(iso-linkage)-2-methylamino-2(Z)-dehydrobutyric acid). NODs have generally less structural diversity than MCs [30]. NOD can occur in several variants: two demethylated variants, one with D-Asp instead of D-MeAsp, and the second one with DMAdda instead of Adda. NOD is found in brackish waters, and is synthesized by cyanobacterium *Nodularia spumigena* [31].

MCs



NOD

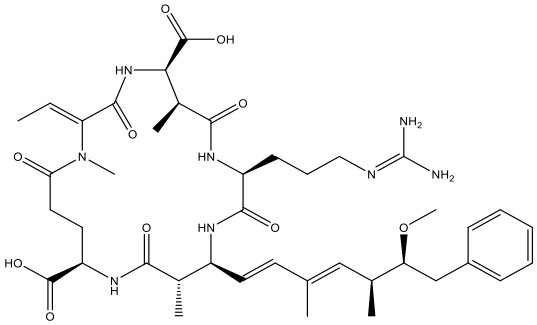


Figure 3. *Chemical structures of MCs and NOD.*

MCs and NODs can cause acute and chronic liver damage. After oral exposure, these cyanotoxins are absorbed in the ileum and they enter hepatocytes and nephrons. They inhibit serine-threonine protein phosphatases 1 and 2A, what causes damage of the cytoskeleton and rearrangement of filamentous actin. As a result, liver cells are disrupted. At high concentration this can lead to acute liver necrosis, intrahepatic hemorrhage, and shock. At low concentrations, these cyanotoxins can lead to a slower onset of liver and kidney failure [32].

The most known case of human poisoning by MCs occurred in Caruaru (Brazil) in 1996. In that case, 100 dialysis patients developed acute liver failure due to usage of inadequately treated water. 52 of these deaths were attributed to cyanotoxins poisoning (now called Caruaru syndrome). Two groups of cyanotoxins were identified in liver tissues: MCs and cylindrospermopsin. However, according to victims' symptoms and pathology using animal studies, it was shown that intravenous

exposure of MCs (MC-YR, -LR and -AR) was the main contributing factor [33]. LD_{50} (lethal dose resulting in 50% deaths) values of MCs depends on exposure route and MCs variant. For example, LD_{50} of MC-LR after oral and intravenous administration to mice were ≥ 5000 µg/kg bw (bodyweight) and 28 µg/kg bw, respectively. Reported LD_{50} values for NOD were between 50 and 70 µg/kg bw [34].

Cylindrospermopsin (CYN) is a tricyclic alkaloid, possessing a guanidine moiety combined with hydroxymethyluracil (Figure 4), which has been demonstrated to be hepatotoxic, cytotoxic, dermatotoxic, and possibly carcinogenic [22]. CYN is highly water-soluble, due to its zwitterionic nature with a positively charged guanidine group and a negatively charged sulphate. Four more CYN variants are known to date: 7-epi-CYN and 7-deoxy-CYN, 7-deoxydesulfo-CYN and 7-deoxydesulfo-12-acetyl-CYN [34]. At first, CYN production was associated exclusively with N_2-fixing filamentous *Cylindrospermopsis raciborskii*, however, the list of potential CYN producing species is expanding and includes *Umezakia*, *Anabaena*, *Aphanizomenon* (now *Chrisosporum*), *Lyngbya* and *Raphidiopsis* generas [22, 31]. *C. raciborskii* is also known to produce saxitoxins. Even though this cyanobacteria was considered to be a tropical/subtropical cyanobacteria, it was also recently found in temperate regions. Such widening occurrence is due to the physiological flexibility of cyanobacteria [35]. Other CYN-producing organisms, *Chrysosporum ovalisporum,* is distributed in tropical, subtropical and Mediterranean areas; *Aphanizomenon gracile* and *Aph. flos-aquae* are the most important CYN producers in Europe [34, 36].

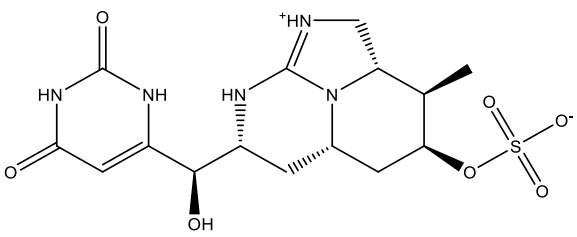


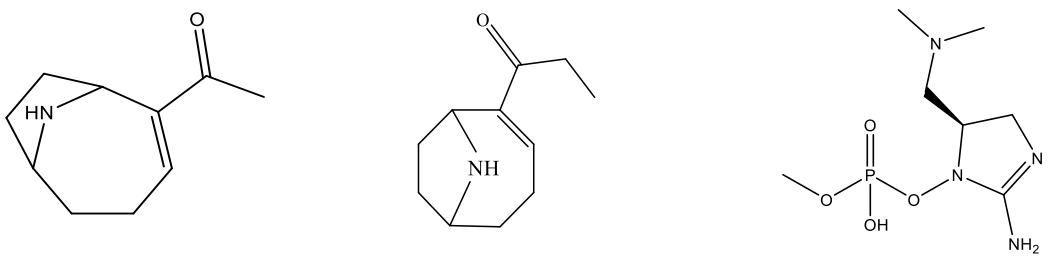
Figure 4. *Chemical structure of CYN.*

There is a scarcity of CYN toxicological studies. Primary mechanism for cytotoxicity is inhibition of protein synthesis, which was demonstrated *in vivo* and *in vitro* [37, 38]. It results in toxicity in liver and kidney. One of the major outbreaks associated with the presence of *C. raciborskii* was reported in Australia in 1979 in Palm Island, northern Queensland. Local drinking water reservoir was treated with copper sulphate to control a dense cyanobacterial bloom. This treatment caused cellular lysis, and thus release of the toxin in water. Consequently, 139 children and 10 adults were hospitalized due to reversible liver and renal damages [34, 39].

2.2. Neurotoxins

There are two most studied cyanobacterial neurotoxins: anatoxins and saxitoxins (another name: paralytic shellfish poisoning toxins).

Anatoxins can be divided into three groups: anatoxin-a (ANA), its structural homologue homoanatoxin-a (homoANA), and the unrelated anatoxin-a(s) (ANA-s) [40]. ANA and homoANA are bicyclic secondary amines (Figure 5). ANA is produced by different species of *Anabaena, Aphanizomenon, Cylindrospermum, Microcystis, Oscillatoria* and *Raphidiopsis* genera. HomoANA is synthesized by some members of *Oscillatoria, Anabaena, Raphidiopsis* and *Phormidium* genera [41]. ANA-s (Figure 5) is an N-hydroxyguanidine methyl phosphate ester, and it is the only natural organophosphate known to date. This toxin was isolated from *Anabaen*a species (*A. flos-aquae*, *A. lemmermannii* and *A. crassa*) [34].



ANA homoANA ANA-s

Figure 5. *Chemical structures of ANA, homoANA, ANA-s.*

ANA can block muscular cells. It operates as a depolarizing neuromuscular blocking agent mimicking acetylcholine. However, its impact on the muscular cells does not stop, because ANA is not degraded by the acetylcholinesterase. Being stimulated, muscular cells are blocked, what causes paralysis. The LD_{50} value of ANA is 200 µg/kg (mouse, intraperitoneal) [42].

Saxitoxins (STXs) are a group of around 30 natural alkaloids, and they can be divided into three main groups: carbamate, sulfamate and decarbamoyl toxins. SXT belongs to carbamate group, and its chemical structure is presented on Figure 6. STXs are produced by marine dinoflagellate genera and by cyanobacteria genera *Cylindrospermopsis*, *Geitlerinema*, *Phormidium*, *Anabeana* and *Lyngbya* [34].

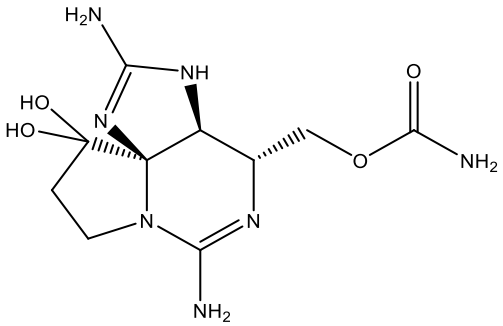


Figure 6. *Chemical structure of STX.*

STX can cause paralytic shellfish poisoning syndrome. The symptoms can be diverse, from a slight tingling or numbness to a complete respiratory paralysis. The degree of toxicity depends on the toxin involved [34]. SXT block sodium conductance in axons, what decreases transmission of electric activity. SXT also block Na^{+}-channels in neuronal cells, what may alter selective membrane permeability, change the ions flux, and thus damage cellular homeostasis.

These toxins may as well block Ca^{+2} and K^{+} channels in cardiac cells by interfering with the opening/closing rhythm of these channels, what can lead to variation in the

flow of ions to the cell. STX is more toxic than ANA, having a LD_{50} value of 10 µg/kg (mouse, intraperitoneal) [34, 42].

2.3. Other cyanopeptides

Beyond these cyanotoxins, cyanobacteria can produce a variety of other bioactive secondary metabolites, including cyanopeptides. These compounds belong to several classes including cyanopeptolins, anabaenopeptins, aeruginosins, aerucyclamides, and microginins. However, these compounds are not well studied yet. Cyanopeptolins, anabaenopeptins and aerucyclamides have cyclic structures, while aeruginosins and microginins are linear peptides. They are co-produced with known cyanotoxins by bloom-forming cyanobacteria [43-47]. Cyanopeptolins are hexapeptides with the characteristic Ahp moiety (3-amino-6-methoxy-2-piperidone). Cyanopeptolins are depsipeptides that contain a β-lactone ring at the threonine, which introduces an ester bond and share a mostly conserved linear side chain off position (Figure 7). Anabaenopeptins contain a five-amino acid ring with a characteristic ureido linkage (Figure 7). Aerucyclamides are cyclic hexapeptides with three azole or azoline rings (Figure 7). As for the linear cyanopeptides, aeruginosines are tetrapeptides with 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 (Figure 7). Microginins have from four to six amino acids, one of them being the characteristic Adda moiety and predominantly two tyrosine monomers (Figure 7) [26, 48].

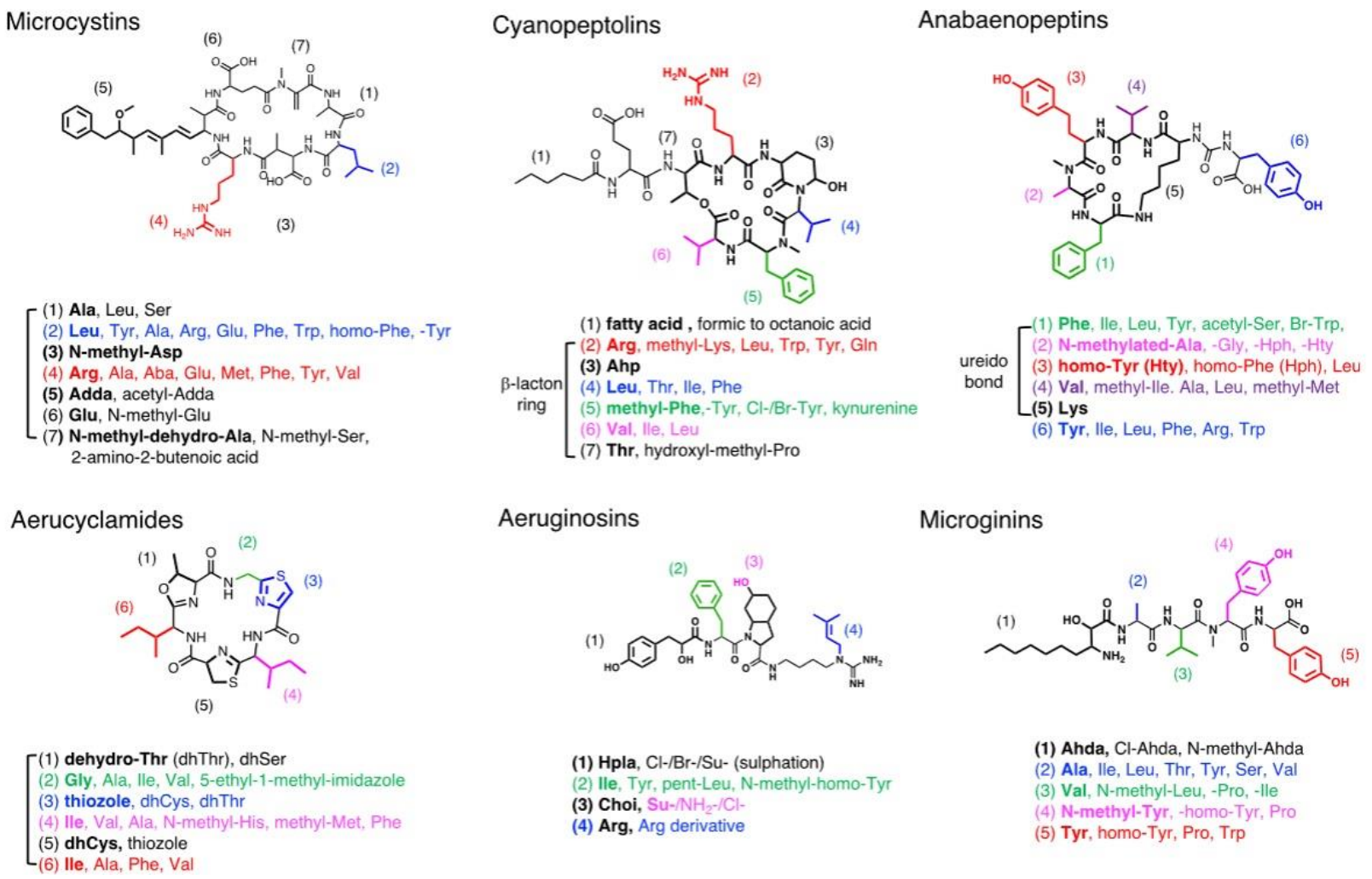


Figure 7. *Representative structures for six cyanopeptide classes. Reproduced from Open Access reference [26].*

Compounds from these classes have shown acute toxicity in planktonic grazers (lethal concentration that kills 50% of tested animals (LC_{50}) in the low mg/L range) and are able to inhibit various enzymes (half maximal inhibitory concentration (IC_{50}) in the low µg/L range) [26]. While their mode of action and toxic potency has not been sufficiently studied yet, their occurrence has been reported in several surface waters in Italy, Spain, Greece, Poland and USA [49-56].

Owning to posed toxicological risks by various cyanobacterial metabolites, guidelines values in drinking water have been introduced by several countries (such as the European Union, the United States of America, Canada, Brazil, Australia, South Africa, China, and Japan) to protect the public from exposure to cyanotoxins [57].

3. Guideline values in drinking water

The risk associated with human health, in particular by drinking contaminated water, promoted different regulations to protect consumers. The World Health Organization (WHO) has previously set a guideline value of 1 µg/L for total (intracellular and extracellular) MC-LR in drinking water [58]. This value is accepted in most of the countries in the world [57]. However, National Centre for Environmental Assessment of the United States of America suggested lowering drinking water guideline value to 0.1 µg/L [59]. As more toxicological studies have been generated, an update of the WHO guideline has been recently finalized, and the value for MC-LR was modified. In the new version not only MC-LR is considered, but a total microcystins' content is introduced. The value for lifetime concentration is 1 µg/L and for short term events is 12 µg/L in drinking water [60]. Additionally, threshold values for CYN, ANA, and STX are now also included, established at 3, 30, and 3 µg/L in drinking water [61-63].

Countries around the world have implemented the WHO guideline values in their internal regulations. Since the new guideline values have been published just

recently, other countries did not adopt the regulations yet (according to the author's knowledge). Table 2 summarises regulated values of cyanotoxins in European countries [64]. For drinking water, several types of regulations are applied, depending on the country. It can be a standard value, (provisional) guideline value, (provisional) maximum value or concentration, and health alert level. Some countries regulate only MC-LR, while others refer to total amount of MCs. Interestingly, not all countries have implemented their own guideline values. For example in Denmark there is no specific regulation as drinking water is from ground water, and if applicable the WHO guideline value is recommended. Similar situation occurs in Germany where only 20% of water originates from surface water. In general, the regulated values are between 1 and 1.5 µg/L across different European countries.

Table 2. *Examples of guideline values or other regulations for managing cyanotoxins in drinking water in European countries [64].*

Country	Cyanotoxin regulated	Value
Czech Republic	Cyanobacteria in raw water	≥2 000 cells/mL
	MC-LR in treated water	1 µg/L
Denmark	WHO guideline values	
France	MCs (total)	1 µg/L
Finland	Potentially toxic cyanobacteria in raw water	>5000 cells/ml o
	MCs (total) in raw water	>1 µg/L
Germany	WHO guideline values	
Italy	WHO guideline values	
Netherlands	WHO guideline values	
Spain	MCs	1 µg/L

The assessment of the occurrence and the risks of the exposure to cyanotoxins require robust, straightforward, and sensitive analytical methodologies for their identification and quantitation in the aquatic environment, and particularly in drinking water reservoirs. What is more, to perform extensive monitoring studies, these methods should be cost-effective and rapid.

4. Analysis of cyanotoxins in freshwater

To date, various techniques and strategies were applied for the analysis of cyanotoxins in freshwater. Nowadays, several approaches are the most frequently used among which enzyme-linked immunosorbent assay (ELISA) assay and liquid chromatography coupled to mass spectrometry (LC-MS) methods, being the second one the most used for identification and quantification of multiclass cyanotoxins. Additionally, prior sample analysis, several sampling, sample preservation and sample pre-treatment strategies are applied depending on the objectives of the analysis.

Thus, in this section, two most applied analytical methodologies will be addressed including immunochemical (especially ELISA) and chemical (especially LC-MS) methods together with sampling, sample preservation, and sample pre-treatment strategies. As LC-MS is the most widely used methodology for determination of multiclass cyanotoxins, and development of such method is one of the objectives of this research, these techniques will be discussed in more detail. Table 3 summarises information on previously published analytical methods which include extraction (where applicable) technique and sorbent used, LC column, MS analyser and ionisation mode, type of cell lysis (where applicable), method recoveries and limits of detection. Additionally, since high-resolution mass spectrometry (HRMS) applications are becoming more favoured, both targeted and suspect approaches will be mentioned, together with availability of reference material and criteria for compounds' identification and confirmation.

Table 3. *Summary of previously published methods for analysis of multiclass cyanotoxins including recoveries and method limits of detection. NE – nor evaluated, extra – extracellular toxins, intra – intracellular toxins.*

Compounds	Technique	Cell lysis	Recoveries, %	MLODs, µg/L	Reference
MCs, microginins, cyanopeptolins, anabaenopeptins	SPE-UHPLC-HRMS/MS SPE: Carboghaph 4 LC: BEH C18 column (2.1 × 100 mm, 1.7 µm), 18 min MS: qToF, +	Freeze-thaw ×1	>85	0.002–0.047	[65, 66]
MCs, ANA, CYN, NOD (and other natural toxins)	SPE-HPLC-HRMS/MS SPE: PGC, PPL, Oasis HLB plus LC: Lichrosphere C18 (2 × 125 mm, 5 µm), 20 min MS: Q Exactive +/-	Sonication	65–94	0.1–1.2	[67]
MCs, CYN, NOD (and domoic acid)	SPE-UHPLS-MS/MS SPE: ENVI-Carb, HLB LC: Acquity HSS T3 (2.1 × 100 mm, 1.8 µm), 9 min MS: QqQ, +	Freeze-thaw ×1, bead beating	35–107	0.0003–0.0056	[68]
MCs, CYN, NOD	SPE-HLPC-DAD-MS/MS SPE: Supelclean 18, ENVI-Carb LC: Chromolith Performance C18 (100 mm × 4.6 mm i.d.) MS: QqQ, +	Sonication	83–90	0.02–0.5	[69]
MCs, ANA, CYN, NOD (and domoicacid, okadaic acid)	SPE-HPLC-MS/MS SPE: Oasis HLB, PGC LC: Atlantis T3 (2.1 × 100 mm, 3 µm), 32 min MS: QqQ, +	-	44–97	0.001–0.007	[70]
MCs, ANA, CYN	online SPE-UHPLC-MS/MS SPE: Hypersil Gold aQ LC: Hypersil Gold (2.1 × 100 mm,1.9 µm), 7 min	Freeze-thaw ×3	91–101	0.01–0.02	[71]

	MS: QqQ, +				
MCs, ANA	online SPE-UHPLC-HRMS/MS SPE: Waters Xbridge C8 2.1 mm × 30 mm × 10 µm LC: Acquity HSS T3 (2.1 × 150 mm, 1.8 µm), 13 MS: qToF, +	Freeze-thaw ×3,	93–105	0.004–0.01	[72]
MCs, CYN, ANA, homo-ANA, anabaenopeptolins	online SPE-UHPLC-HRMS/MS SPE: Hypersil Gold LC: Hypersil Gold C18 column (100 2.1 mm, 1.9 um), 8 min MS: q-Orbitrap, +	Freeze-thaw ×3	NE (based on Fayad 2015)	0.027–0.176	[54]
MCs, ANA, homo-ANA, CYN	DI-UHPLC-HRMS LC: Kinetex® biphenyl (2.1 × 100 mm, 2.6 µm), 20 min MS: qToF, +	Freeze-thaw ×1, bead beating, sonication	extra: 74–110; intra: 84–114	extra: 0.01–0.129; intra: 0.003–0.045	[73]
MCs, ANA, CYN	DI-UHPLC-MS/MS LC: Acquity HSS T3 (2.1 × 100 mm, 1.8 µm), 13 min MS: QqQ, +	Freeze-thaw ×3, sonication	NE	0.1–0.21	[59]
MCs, ANA, CYN, NOD	DI-UHPLC-MS/MS LC: ACQUITY BEH C18 (2.1 × 100 mm, 1.7 µm), 12 min MS: QqQ, +	Freeze-thaw ×3	65–128	0.5 (MLOQ)	[74]
MCs, ANA, CYN	VAE-UHPLC-MS/MS LC: Acquity UPLC HSS T3 (2.1 × 100 mm, 1.8 µm), 12 min MS: QqQ, +	Sonication	NE	2–5	[75]

4.1. Sampling, sample preservation, and sample pre-treatment

Comprehensive monitoring of freshwater reservoirs, which are used for drinking water production, requires extensive sampling comparing and resources in order to cover temporal horizontal and in depth dimensions [76]. To tackle this problem, several approaches were implemented. To assess horizontal distribution of cyanobacteria, remote sensing using satellite data can be applied. However, these tools are rather costly and are not assessable for majority of monitoring programs. Thus, more conventional methods are still widely used [77]. In this section, main aspects of sampling, sample preservation, and sample pre-treatment are addressed. For sampling, glass bottles are widely used. However, plastic bottles (polyethylene or polycarbonate) can be also applied for safety reasons. There are two main types of samples: grab and integrated samples. Grab sampling provides information from a specific spot (such as a site used for drinking water), while integrated sample helps to assess sample from different depths at the same time (for size of cyanobacterial community) [6]. For surface water samples, the water is collected manually (grab sampling) at depth between 0.3 and 1 meter depth [57]. However, for sampling of biomass water can be collected from surface or deeper layers if this is where cyanobacteria are denser. For raw drinking water samples, sampling at deeper layers (where the water intake is usually located) or from the tap at the entrance of the treatment plant can be performed.

For cyanotoxin analysis, samples should be kept in refrigerator and darkness to avoid degradation. However, in this case, storage should be for 24 hours. For long term storage, samples are normally preserved in the freezer at temperatures between -20 and -80 °C. Prior freezing the samples, it is important to consider which type of toxins

are to be analysed (intra-, extracellular, or total amount of toxins). Freeze-thaw of the sample, will break the cell walls of cyanobacteria, and release intracellular toxins. Thus, only total concentration of compounds can then be determined. If the objective of the study is analysis of intracellular and/or extracellular toxins, the water should be first filtered to separate the fractions. Later, both the filters and filtrate can be frozen. For filtering, glass fiber disc with pore sizes of 1.2 µm are normally applied as they retain most cyanobacteria. However, for picoplankton cyanobacteria smaller pore sizes are needed (0.7 µm) [6].

Detailed information on sampling, sample preservation and pre-treatment can be found also in the second edition of “Toxic cyanobacteria in water” which was recently published [78]. Provided information would assist choosing the best strategy for fieldwork, sampling, storage, and pre-treatment.

In order to quantify the total content of cyanotoxins, bacterial cells should be lysed to release intracellular toxins. There are several options that can be applied: sonication, lyophilisation followed by solvent extraction, bead beating, freeze-thaw cycles, and use of lysing solutions. Comparing these five techniques, Kim et al. [79] showed that lyophilisation followed by extraction with 75% methanol or deionised water and bead beating had the highest extraction efficiency for *Microcystis aeruginosa* cells (between 92 and 99%). Thirty minutes of sonication demonstrated a lysis efficiency of 73%. In another study, Pekar et al. [80] showed that three freeze-thaw cycles were the most efficient for MCs in comparison with bead beating and the application of a lysing solution (QuickLyse™). In this case, freeze-thaw conditions differed from the study conducted by Kim at al. However, bead beating and the application of QuickLyse™ are faster and showed reasonable results. Additionally, Loftin et al. [81] did not obtained significant differences between freeze-thaw cycles and QuickLyse™ for the majority of the analysed samples. Table 3 summarises information on

previously published analytical methods including cell lysis approaches. As can be seen, freeze-thaw cycles were the most widely used, especially in triplicate. The second most used method was sonication. All in all, there is not an ideal method, and the choice of the employed cell-lysing procedure will depend on the needs and available labour, time, and equipment resources.

4.2. Immunochemical methods

Immunochemical methods or immunoassays, which are based on biding of antibody (Ab) to an antigen (Ag), have been widely used for quick environmental analysis of both single and multiple compounds. Ab (targeted molecules) are glycoproteins which are involved in immune recognition and defence system of a host. Figure 8 shows the general structure of Ab, which has a Y-shape and consists of two identical ("heavy") polypeptide chains with two identical shorter ("light") chains. On each chain, there are three complementary determining regions, and each six of them on each arm of antibody form an antigen-binding site [82]. Ab are very selective and bind to specific Ag. Ag-Ab interactions have reversible nature and based on electrostatic and Van Der Waals forces and hydrophobic bonds [83].

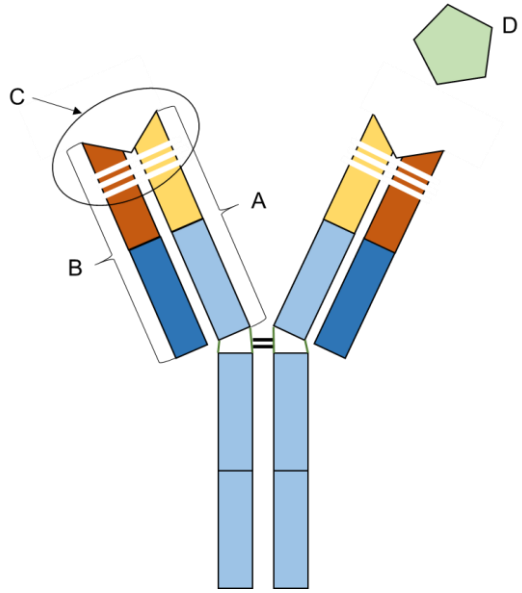


Figure 8. *General structure of an antibody: A – heavy chain, B – light chain, C - complementary determining region, D – antigen.*

Immunoassays has a number of advantages as they are fast, simple and thus cost-effective detection techniques. They provide low sensitivity with minimal sample pre-treatment. Additionally, they can be used in field and designed to operate on-line. However, the main limitations that may occur are poor stability of immunoreagents (both thermal and chemical), cross-reactivity between structurally-related compounds, and matrix effect. Hence, in order to improve both the stability and the specificity of immunoreagents, current development is focused on the application of new materials [84].

Immunochemical methods involve radioimmunoassay, fluoro-immunoassay, chemiluminescence immunoassay, liposome immunoassay, and ELISA. ELISA has been very much employed for analysis of cyanotoxins.

In case of ELISA, an enzyme is attached (conjugated) to the antibody. Enzymes are proteins (like antibodies) that not only bind to specific targets, but also catalyse specific reactions. The substrate is a starting component for an enzyme-catalysed reaction, which can be used for colour, fluorescent or luminescent end product generation. And thus, these signals can be measured by optical and electronic equipment. Moreover, each molecule of enzyme can convert multiple molecules of substrate, what provides sensitive signals [85].

ELISA is in general a plate-based technique, where Ag is immobilised on microplates and then complexed with and Ab which is connected to a reporter enzyme. The reported limits of detection (LODs) using ELISA for cyanotoxins are between 0.02 and 0.1 µg/L [86-88], and 0.1 µg/L for anabaenopeptins [89]. There are several ELISA producers on the market such as Abraxis and Beacon [88].

Another essay format is the antibody-based test strips. They provide a robust, cheap and simple method for initial risk assessment [90]. However, the sensitivity of this type of assays is in general poor in comparison with conventional ELISA, with LODs,

for example, between 1 and 10 µg/L for total MCs [90], which is at the limit for drinking water regulations. During the last decade, ELISA approaches have been widely used for environmental monitoring studies. However, matrix effects and cross-reactivity can lead to overestimation [9, 57, 89, 91]. In case of class antibodies assays, the structurally-related compounds cannot be distinguished, although the affinities are different for the different variants. In case of both NOD and MCs, Adda moiety is used for Ag-Ab reaction, and thus MCs congeners cannot be distinguished, and leads to overestimation. For instance, Birbeck et al. [92] noticed that results obtained with ELISA demonstrate higher MCs concentrations comparing with those obtained by high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). The reason was the cross-reactivity between different MCs variants, as well as the detection of MCs degradation products. Additionally, the calibration curve was non-linear. The ELISA kit for analysis of anabaenopeptins has shown cross-reactivity and overestimation with other cyanopeptides such as MCs, NOD, and cyanopeptolin-a [89]. In another study, Gurbuz et al. [93] observed a false positive result of MCs in fish tissues obtained by ELISA. This could be due to the cross-reactivity of MCs and their detoxification products which formed in animal and plant tissues. These findings demonstrate that results obtained by ELISA should be interpreted carefully. Even though, antibodies are selective, but when the antigen is not compound specific (but class specific), false-positives and cross-reactivity may occur. Thus, despite the fact that ELISA kits are excellent analytical tools for fast screening, obtained results should be confirmed using other methods.

4.3. Chemical methods

Separation techniques such as liquid and gas chromatography are the most widely used for the analysis of cyanobacterial metabolites in freshwater. These techniques

can be coupled with several detectors such as fluorescence and ultraviolet [57]. During the last decade, MS is the most common approach due to the superior sensitivity and selectivity. Gas chromatography coupled to mass spectrometry (GC-MS) is applied for analysis of both low molecular weight (ANA) [94, 95] and higher molecular weight (MCs) [96-100] cyanotoxins. However, the major drawback of these techniques is that a derivatisation step is frequently needed. For instance, in the method developed by Xu et al. [99] for the determination of the total content of MCs, the compounds were firstly oxidized and derivatised using methylchloroformate before their determination by GC.

As cyanotoxins are normally found in aqueous phase and have from medium to high polarity, liquid chromatography is a more convenient option as no derivatisation is required. Different studies were conducted applying LC and diode-array detection (DAD) or ultraviolet detection (UV) [93, 101-104], but identification of cyanobacterial metabolites cannot be confirmed. To enable simultaneous identification and quantification of various compounds, LC-MS approaches are used [9, 54, 57, 66, 105]. However, for the extraction and purification of compounds, sample pre-treatment steps are often employed.

4.3.1. Extraction, pre-concentration, and clean-up

One of the bottle necks of sensitive analysis of cyanobacterial metabolites is sample extraction and clean-up, as these compounds belong to different chemical classes with various polarities, chemical structures, and masses. Nowadays, several techniques such as liquid-liquid extraction (LLE), solid phase microextraction (SPME), solid phase extraction (SPE), and online SPE can be applied. In some cases, extraction and clean-up steps are omitted, and samples are injected directly (after filtering or centrifugation) to the LC-MS system. Table 3, which involves analytical methods used for analysis of multiclass cyanotoxins, summarises sample

pre-treatment techniques used prior to LC-MS analysis. Methods are organised according to sample pre-treatment in the following order: SPE, online SPE, direct injection (DI), and vacuum assist evaporation.

Among above mentioned techniques, SPE is the most widely used due to its versatility and lower solvent use (in comparison with LLE). SPE itself is an extraction and clean-up technique, but it is often followed by evaporation, what leads to pre-concentration. However, usually matrix clean-up is not 100% effective, and higher pre-concentration leads to higher matrix effect. Thus, pre-concentration level should be considered during method development. Nowadays, there is a high range of available SPE stationary phases on the market. It is also possible to connect SPE cartridges sequentially or to combine several sorbents in one cartridge, what allows to retain analytes of various structures and polarities in one run. The most common stationary phases for the extraction of cyanotoxins are octadecyl silica (C_{18}) and copolymeric sorbents: Oasis HLB, Oasis MAX, BondElut C18, HyperSep Hypercarb porous graphitic carbon (PGC), and ImmunoSep [57]. In fact, HLB (hydrophilic-lipophilic balance) is the most common sorbent for analysis of water samples. It is widely applied for targeted, suspect and non-targeted approaches (discussed below) for broad chemical enrichment [106, 107]. In some studies, a combination of cartridges with different polarities was used, because the selectivity of only one sorbent could not ensure high recoveries for compounds of different chemical classes [67, 70]. For instance, Zervou et al. [70] used dual SPE based on Oasis HLB and HyperSep Hypercarb PGC to extract multi-class cyanotoxins dissolved in water. For efficient recovery of ANA from HyperSep Hypercarb PGC, the amine group of ANA was neutralised (pH >10.5) what ensured adsorption to the stationary phase. Additionally, mixed-bed multi-layered in-house SPE cartridges were applied for the analysis of cyanotoxins. For example, Picardo et al. [67] applied the combination of

three stationary phases including HLB plus, porous graphitized carbon and Bond-Elut PPL for suspect screening of natural toxins (including cyanotoxins) in surface and drinking water. Overall for suspect and non-targeted screening of organic contaminants in water, SPE cartridge with combination of five sorbents was developed and optimized for analysis of more than 400 compounds (mostly pesticides and pharmaceuticals) [108]. In this case, cartridge consisted of Oasis HLB, Strata-X-AW, Strata-X-CW, IsoluteENV+, and Supelclean ENVI-Carb. Such cartridge demonstrates how versatile SPE can be. Another variation of SPE that was used for analysis of cyanotoxins is an on-line SPE approach. The main advantage of the method is that is an automated one, which provides higher sample throughput. One of the disadvantages is that setting up the method can be challenging and expensive, however, for routine analysis the price will be cheaper than with off-line SPE. Several sorbents were used, among which Hypersil Gold aQ with C_{18} selectivity and Xbridge C_8 for analysis of multiclass cyanotoxins [71, 72]. Obtained limits of detection are comparable or higher than those obtained by off-line SPE. For instance, in two separate studies similar LC-MS configuration was applied for the analysis of multiclass cyanotoxins [72, 109]. In both cases, the MS analyser was a hybrid quadrupole time-of-flight instrument coupled with reversed phase LC. Obtained method limits of detection were between 0.001 and 0.01 µg/L for the method with off-line SPE [109], and between 0.004 and 0.01 µg/L for method with on-line-SPE [72] (see Table 3). Thus, SPE and on-line SPE are approaches that should be applied when the matrix effect are expected to be higher (freshwater samples) what allows to reach lower limits of detection. Additionally, versatility of available stationary phases and their possible combination provides extraction of compounds of various chemical classes and polarities.

Additionally to the described SPE approaches, solid-phase microextraction, monolith-based SPE and immunoaffinity extraction were applied for analysis of cyanotoxins [110, 111], however, these techniques are not widely used.

Alternatively to SPE approaches, samples can be directly injected to LC-MS systems. The main advantage of this approach lays in fast and easy sample handling. The only sample manipulation that has to be done prior the injection is filtration or centrifugation in order to prevent both LC and MS system contamination and breakage. However, as samples are not pre-concentrated, higher limits of detection are expected. The way to tackle with the issue of sensitivity is to employ a larger volume injection. For example, 100 µL of sample can be injected [74] instead of 20 or 10 µL. In this case, method limits of quantification of cyanotoxins in freshwater were at 0.5 µg/L level (see Table 3), which was lower comparing to the methods using SPE or on-line SPE. Another limitation is connected with matrix effect and possible ion suppression as there is no clean-up step included. In this case, larger injection can cause higher matrix effect, what should be considered during method optimisation. Hence, direct injection is more recommended for less complex matrices (drinking water), or when higher limits of detection are acceptable.

Another technique that was applied for multiclass cyanotoxins pre-concentration is vacuum assisted evaporation (VAE). For example, this technique was applied by Rodriguez et al, and 10 mL (neither pre-concentration factor nor reconstituted volume were given) of fresh and brackish water samples [112]. It is challenging to pre-concentrate higher volumes of freshwater samples due to accumulation of matrix effect (discussed above) without application of clean-up step (for example SPE). This leads to higher limits of detection, such as 2-5 µg/L (see Table 3). However, this method can be applied for cleaner matrices such as drinking water (for cyanotoxins' analysis) and groundwater (for other organic contaminants). To the author's

knowledge, VAE was not yet applied for analysis of multiclass cyanotoxins in drinking water, but it was successfully applied for targeted and suspect screening of wide range (in terms of molecular masses and polarities) of organic contaminants in groundwater [113], and it was possible to pre-concentrate the sample up to 150 times. Additionally, recoveries obtained by VAE were comparable to those obtained by SPE with five sorbents (this cartridge is mentioned above) in nanopure, wastewater influent and effluent for analysis of more than 500 organic contaminants [114]. To sum up similarly to direct injection, VAE can be also used for analysis of cyanotoxins in freshwater, but only higher limits of detection can be achieved. Even though VAE is a pre-concentration method, due to accumulation of matrix effect pre-concentration factor is usually low. However, this method was successfully applied for analysis of various organic contaminants in less complex matrices (such as ground and nanopure water), and thus can have a high potential for analysis of multiclass cyanotoxins in drinking water.

4.3.2. Liquid chromatography coupled to mass spectrometry

LC-MS/MS and LC-HRMS have been widely used as these techniques provide qualitative, quantitative, and structural information. In this sub-section, methods based on LC-MS for analysis of multiclass cyanotoxins will be discussed (Table 3). Both targeted and suspect screening approaches will be addressed. In separate sub-sections, current status about availability of reference standards and the assessment of cyanotoxins and criteria for their confirmation and identification will be also commented.

For separation, both HPLC and ultra-high performance liquid chromatography columns (UHPLC) are applied (see Table 3). Reversed phase C18 columns are the most frequently employed. As expected, UHPLC columns provide faster separation (down to 7 minutes), and lower method limits of detection (MLODs). Due to these

lower MLODs, it was often in combination with DI. UHPLC columns were also combined with SPE, in this case, MLODs were the lowest reaching ng/L range [68] even with HRMS [65, 66].

Both low-resolution mass spectrometry (LRMS) and HRMS were applied for analysis of cyanotoxins. For LRMS, triple quadrupoles were usually employed. Among high-resolution mass analysers, Q-Exactive Orbitrap and quadrupole time-of-flight were proposed. As the ionisation source, electrospray ionisation (ESI) is usually applied, as it allows the analysis of compounds within a wide polarity range

LC-MS enables application of both targeted and suspect screening approaches for the analysis of cyanotoxins. The main requirement for targeted approaches is the use of reference standards for confirmation and quantification purposes. Thus, retention time and MS/MS fragmentation can be employed as confirmation criteria. For targeted analysis, both LRMS and HRMS can be employed. The working horses of targeted approaches are instruments with triple quadrupole (QqQ) and to a lesser extent quadrupole ion trap (QIT) technologies [115] operated in tandem.

In contrast to targeted analysis, suspect screening does not require reference standards for confirmation purpose, although unequivocal identification will be granted only when reference standards are available. The suspect screening approach involves: introduction of a suspect list, exact mass filtering, isotope pattern filtering (matching of measured and theoretical isotope patterns), retention time filtering (matching of measured retention time with predicted one), matching of MS/MS fragmentation. As a result, a list of likely present suspect compounds will be obtained [115]. For reliable identification of suspects (increased selectivity against the matrix background and for correct formula assignment), the use of high-resolution mass spectrometry is mandatory [115]. The most common HRMS analysers are Orbitrap and time-of-flight (ToF) mass spectrometers [9, 66, 67, 105]. For example,

Roy-Lachapelle et al.[54] employed a Q Exactive Orbitrap with data-independent acquisition (DIA) mode for the suspect screening of cyanopeptides. This approach enabled the confirmation and quantification of cyanopeptides, for which reference standards were available, and the semi-quantification of suspects, for which reference standards were not available. Another advantage of HRMS is that the generated data can be posteriorly re-analysed, what can contribute to effective risk assessment strategies and distinguishing historical trends.

One of the challenges in the determination of cyanotoxins is the scarcity of reference standards, which prevents quantification of wide ranges of cyanotoxins and complicates confirmation of suspects. For this reason, a common approach is to use MC-LR for the quantification of other toxins, for which reference standards or bioreagents are not available. For instance, Natumi and Janssen [44] applied MC-LR equivalent units and class-specific equivalent for estimation of cyanopeptides levels. Class-specific approach considers different compound structures and functional groups, and thus provides more specific estimation of concentrations.

4.3.3. Reference standards

Even though more than 2000 cyanopeptides [27, 29] and overall cyanotoxins were identified, reference materials are still scarce. Overall, reference standards for most studied MCs (MC-LR, -RR, -YR, -LA, -LF, -LY, -LW, [D-Asp^3]MC-LR, [D-Asp^3]MC-RR), NOD, ANA, CYN, and several other bioreagents are available. Additional problem is the use of internal standards for analysis of cyanotoxins. The price of these standards is also high.

Ideally, an isotope dilution method using isotopically-labelled cyanobacterial metabolites would be a perfect approach for correction of SPE recoveries and assessment of matrix effect which might appears during ionization applying ESI. These compounds are even scarcer and more expensive, and were not available

until recently. Nevertheless, several recent studies applied isotopically-labelled standards that showed acceptable results. For example, Haddad et al. [116] demonstrated that internal standard corrections improved matrix effect. Absolute matrix effect was between -22 to -77% for MCs and NOD, and it became between -4.2 to +5.4% with the use of internal standards. For ANA, CYN and STX absolute values were in the range of -44 and -50%, and became between -16 and +10% employing an internal standard. However, such standards are not usually appropriate for routine monitoring due to its cost.

Taking into account the above mentioned drawback of internal standards' usage, possible alternatives can be applied. The appointed compounds should follow several criteria [117]:

- Retention time should be close to that of the target toxin;
- It should have the same ionization mode in ESI as the target;
- It should have similar matrix effects as the target toxins;
- It was not detected in freshwater samples.

In this case such compounds are also called surrogate standards. Several surrogates were applied instead of labelled cyanotoxins. For example, acetaminophen-d_4 demonstrated recoveries between 95 and 106%, and was suggested for ANA and homoANA determination [117]. In the same study, L-phenylalanine-d_5 as an internal standard for both ANA and homoANA was also tested. It demonstrated overestimated results, and thus, it is not recommended. In case of CYN, two studies applied 1,9-diaminononane and controversial results were obtained. In one case, SPE efficiency was in the range of 83–94% [69], while in the other case much poorer efficiency was observed (0.14%) [117]. However, the applied analytical techniques were not identical, what could have caused the variation. One of the most used internal

standards for MCs is in fact NOD [54, 72] due to its similar structure. However, NOD was found in freshwater in *Nodularia* specie [118], what makes it less attractive as internal standard [71]. All in all, scarcity of analytical standards contributes to the challenge of the analysis of cyanotoxins.

4.3.4. Criteria for the confirmation and identification of compounds

Development of HRMS provided new opportunities for the analysis of cyanotoxins and allowed better assignment of confidence levels for detection and prioritization of new bioactive compounds. Once these bioactive compounds are released into the environment, different biotransformation reactions may take place.

Recently, the photochemical fate of a wide range of cyanopeptides from *Microcystis aeruginosa* and *Planktothrix rubescens*, and *Dolichospermum flos-aquae*, focusing on the half-lives during sunlight and transformation kinetics of various compounds was studied [119]. However, many of these metabolites, their transformation products and their toxicity and persistence are still unknown, thus, analytical tools for their structural characterisation are still needed. In this section, confidence levels' criteria for assessment of targeted and suspect compounds is addressed.

In order to provide concise and accurate comparison between reported studies, several strategies were established. The European Community has implemented Council Directive 96/23/EC of 29 April 1996 [120] on measures to monitor certain substances and residues. It describes monitoring of two groups of substances: substances having anabolic effect and unauthorized substances (group A); veterinary drugs and contaminants (group B). Group A involves compounds such as stilbenes, antithyroid agents, and steroids, while group B involves antibacterial substances, veterinary drugs, environmental contaminants and other substances. In 2002, Commission Decision 2002/657/EC was published introducing the system of

identification points [121]. For confirmation of substances from group B by LC-MS methods, at least 3 identification points (IP) are needed. The relationship between the employed mass spectrometric technique and the IPs gained is:

- LRMS – 1 IP;
- LR-MS^n precursor ion – 1 IP;
- LR-MS^n transition product – 1.5 IPs;
- HRMS – 2 IPs;
- HR-MS^n precursor ion – 2IPs;
- HR-MS^n transition product – 2.5 IPs.

Application of HRMS provides more IPs due to its high selectivity, sensitivity, and quantitation within high linear dynamic ranges. However, there is variation in levels of confidence of compounds' identification for suspect (and non-targeted: not mentioned here) approaches. Schymanski et al. [122] have suggested a methodology to unify confidence levels, which covers new possibilities provided by HRMS. Here is the summary of identification levels and minimal required data:

- Level 1: Confirmed structure by reference standard – requires MS, MS/MS, retention time, reference standard;
- Level 2: Probable structure
    a) by library spectrum match – requires MS, MS/MS, library MS/MS;
    b) by diagnostic evidence – requires MS, MS/MS, experimental data;
- Level 3: Tentative candidates (structure, substituent, class) – requires MS, MS/MS, experimental data;
- Level 4: Unequivocal molecular formula – requires MS isotope/adduct;
- Level 5: Exact mass of interest – requires MS.

This approach was implemented in various environmental studies [67, 123-126]. Recently, it has been expanded, by adding criteria for ion-mobility spectrometry (IMS) coupled with MS (based on collision cross section value) [127]. To the author's knowledge, IMS-MS was not yet applied either for suspect or non-targeted screening of cyanobacterial metabolites in freshwater.

Other approaches such as quantitative polymerase chain reaction (qPCR), phosphatase inhibition assay, and electrochemical biosensors are also used for analysis of cyanotoxins in freshwater, however, as they are less employed, they are not discussed in this thesis.

5. Cyanotoxins' levels and seasonal variation in European freshwater reservoirs

Nowadays, there are many studies about the presence of cyanotoxins in the environment, and in the development of analytical methods and approaches to assess them at low concentrations (ng/L). Nevertheless, little quantitative information is available on temporal variations, including the European region. Understanding historical trends is crucial as it decreases uncertainty and provides a solid foundation for forecasting. Establishing seasonal trends of cyanobacterial toxins will promote the development of effective water management strategies for resource distribution and establishing objectives for different seasons and climate zones [128].

This section is based on a review paper that was published earlier [9]. Herein, several aspects will be addressed, among which: temporal variations of cyanotoxins and their levels during seasonal studies; relationship between environmental parameters, cyanotoxins' concentrations and cyanobacterial biomass; patterns and peaking periods for three main European climate zones (Mediterranean, humid continental, and oceanic).

5.1. Levels of cyanotoxins in seasonal studies

Before to start describing seasonal patterns, it is worth to highlight the levels of cyanotoxins in different freshwater reservoirs. Table 4 summarises the detected groups of cyanotoxins, the maximum levels, and the months of the peak season in European freshwater reservoirs. The sections are already divided according to different climate zones, which will be described below. Results are grouped according to the European climate zones. It should be commented that due to the variety of analytical techniques and sampling strategies applied, in some cases the described results are difficult to compare. Nevertheless, the available literature provides an overview of the occurrence and the most abundant groups of cyanotoxins in European bloom events. As can be seen in Table 4, MCs is the most detected group of cyanotoxins. In contrast, other cyanotoxin groups such as CYN [129, 130], ANA [129, 131-136], and STX [129, 131, 137] are identified only in some studies. Even in the studies that focus in the analysis of different groups of toxins, MCs are often the predominant ones. ELISA is often the method of choice for the determination of MCs due to its sensitivity, simple sample manipulation, and short analysis time in comparison to LC-MS based methodologies [129, 131, 137-142]. However, the immunoassays present the handicap that are not selective enough towards different cyanotoxins species, as previously addressed.

Table 4. *Reported levels of cyanotoxins in surface freshwater during seasonal sampling campaigns.*

Country	Sampling point	Sampling period	Toxins	Max levels, µg/L	Peaking period	Reference
Mediterranean						
Italy	Lake Vico	February 2009 – December 2010	MCs	2009: 3.4 2010: 5.205	2009: May and October 2010: February and November	[143]
Italy	Lake Alto Flumendosa	October 2011 – May 2013	MCs	100	May, October	[144]
Italy	15 reservoirs in Sicily	August 2016 – July 2017	MCs	0.3	August	[138]
Spain	Reservoirs Ojos and Cenajo	October 2000 – September 2001	MCs	Ojos: 0.17 Cenajo: 0.085	Ojos: spring, summer Cenajo: summer, autumn	[139]
Spain	Reservoir Rosarito	June – October 2013	MCs, ANA, STXs	MCs: 18.6; ANA 2.1; STXs 0.12	MCs and ANA: September STXs: July	[131]
Portugal	Reservoirs Alvito, Enxoé, Odivelas and Roxo	May – December2005, April – July 2006	MCs	Alvito: 2.58 Enxoé: 0.63 Odivelas: 0.5 Roxo: 7.2	Alvito: July, September Enxoé: April Odivelas: July, September Roxo: September	[140]
Portugal	Reservoirs Alqueva and Beliche	February, April, June, July, September, November 2011	MCs	0.776	September	[112]
Portugal	River Tâmega, Reservoir Torrão,	April – September 2017	MCs ANA	MCs: 18.8 ANA: 6.8	MC: August ANA: June	[129]

	Lakes of Porto City, Lagoons Mira and Vela		SXT CYN	SXT: 4.3 CYN: 0.1	SXT: August	
Greece	Lake Pamvotis	October 2007, June 2008, Desember 2008	MCs	Water: 0.0034 Scum: 0.0036	October	[141]
Greece	Lake Pamvotis	January 2008 – February 2009	MCs, STXs	MCs: 19 STXs: 2.1	March, September	[137]
Greece	Lake Marathonas	July 2007 – December 2010	MCs	0.717	February, September – October	[145]
Turkey	Lake Egirdir	April – December 2013	MCs	20.5	April, August	[93]
Turkey	Lake Sapanca	September 2012 – October 2013	MCs	1.522	March	[146]
			Continental			
Italy	Lakes Occhito, Pusiano, Lerdo, Garda	April 2009 – December 2012	MCs	Occhio: 7.5 Pusiano: 4.6 Ledro: 1.15 Garda: 0.26	Occhio: April Pusiano and Ledro: November Garda: August	[147]
Italy	Lake Garda	September 2008 – September 2013	MCs	0.23	September	[148]
Italy	Lake Garda	February 2014 – October 2015	ANA	2.2	May	[132]
Italy, Switzerland	Lakes Garda, Iseo, Como	Grada: September 2009 – December 2016; Iseo: February	MCs	Grada: 0.23 Iseo: 0.43	Grada: August, September Iseo: May, June	[133]

		–December 2016; Como: April – December 2016;	ANA	Como: 0.053 Grada: 2.7 Iseo: 1.3 Como: 0.53	Como: July – August Grada: June Iseo: May, June Como: June – August	
Poland	Lakes Mytycze and Tomaszne	May – September 2010 and 2011	MCs	Mytycze: 30.68 Tomaszne: 23.62	Mytycze: mid-August – September Tomaszne: July – mid-August	[149]
Poland	Reservoir of dam Zemborzycki	May – September 2005 –2011	MCs, ANA	MCs: 22.2 ANA: 14.4	August, September	[134]
Poland	Lake Lubosinskie	July 2006 – March 2008	MCs	71.2	October	[150]
Poland	Lakes Niegocin, Piłwąg and Rekąty	July – September 2007	MCs	0.03	September	[151]
Czech Republic	94 water reservoirs	July – September 2004	MCs	37	August, September	[142]
Germany	Lakes Langer See and Melangsee	June – September 2004, April-October 2005	CYN	Langer See: 1.8 Melangsee: 0.5	June and September	[130]
Germany	Lakes Klostersee, Bergknappweiher	Klostersee: May – October 2015, Bergknappweiher: August – October	MCs	Klostersee: 5.0 Bergknappweiher: 6.7	Klostersee: October Bergknappweiher: September	[152]
Russia	Lakes Suzdal and Sestroretskij Razliv	June – October 2010, June – September	MCs, ANA	MCs: 41.37 ANA: 0.54	August – September	[135]

		2011, May – September 2012				
			Oceanic			
France	Lake Aydat	September – October, 2011 – 2013	MCs, ANA	MCs: 0.077 ANA <LOD	September	[136]
France	Reservoir Pen Mur	May 2016 – April 2018	MCs	60	June, September	[153]
England	Lakes Longham	May 2016 - May 2017	MCs	7.1	September	[154]
Spain	Reservoir Trasona	January 2006 – December 2010	Cyanotoxins (predicted)	>7000 (predicted)	October	[155]

The most frequently detected MCs are MC-RR, -LR, -YR and the demethylated forms of MC-RR and -LR [93, 112, 133, 139, 144-148, 150, 155, 156]. For instance, it was shown that in the Lake Vico, the most abundant MC variant was the demethylated form of MC-RR representing more than 95 % of the total MCs content [143]. Overall, 8 MCs species were analysed. In another study, which included the analysis of water samples from Lakes Occhito, Pusiano, Lerdo and Garda, the demethylated forms of both MC-RR and -LR were the dominating ones among 8 MCs monitored in the analysis [147, 148]. MC-YR, RR, and LR were also present but in a lower concentration. In a following study by the same research group, which involved analysis of samples from 4 deep subalpine lakes (Garda, Iseo, Como, and Lugano), demethylated MC-RR was again the dominating compound [133]. Other studies have also showed that MC-LR, -RR and -YR were the prevalent toxins in freshwater reservoirs [139, 145, 147, 153]. However, there is a grate variability in abundances even within the same climate zone. For instance, MC-LR ranged between 2.6 and 74%, MC-RR between 3 and 75 %, and MC-YR between 1 and 53% of total amount of cyanotoxins in the Mediterranean Lakes Ojos and Cenajo. MC-RR variant dominated in Lake Ojos during autumn and in Lake Cenajo during spring, which was probably attributed to the dominating species of *Oscillatoria, Lyngbya* and *Phormidium* [139]. Levels of cyanotoxins' concentrations within the season and variation in their composition could be associated with fluctuation of environmental parameters and bacterial biomass.

5.2. Relationship between cyanotoxins' concentrations, environmental parameters and biomass

Different environmental parameters such as weather conditions [137, 157], pH [137], light intensity [130], and nutrient availability [86, 130, 137, 158-161] have shown to influence phytoplankton structure and distribution, as well as levels of cyanotoxins

[162]. Recently, a European Multi Lake Survey studied the effects of temperature and nutrients on the variability of cyanotoxins at a continental scale [163]. The obtained results demonstrated that both direct and indirect effects of temperature have a high influence on cyanotoxins' distribution and the toxic potential of the lakes [154]. In a study focusing on the effect of temperature alone (without confounding influence of nutrients' variation), Walls et al. [157] showed that the amount of intracellular MCs released by *Planktothrix agardhii* rises significantly at temperatures that are above the optimal growing ones. Thus, intracellular toxins reached maximal values when biomass and cell density were decreased. This study shows that elevated temperatures lead to higher cyanotoxins' concentrations. Different cyanobacteria have different optima growth temperatures [137, 157], however, in general, they are higher in comparison with optimum growth temperature of most algae. H. Paerl [17] reported (Figure 9) that the optimal temperature for cyanobacteria is higher than 25 °C (arriving at circa 33 °C), what overlaps with the optimum one for chlorophytes (27-32.8 °C) but clearly differs from with the one for dinoflagellates (17-27 °C) and diatoms (17-22 °C). For one of toxin-producing stains (*Microcystis flos-aquae* AUTH 1410), it was shown that a 5 °C temperature increase (between 25 and 30 °C) resulted in a 25% higher growth rate [137]. Such pattern is suggesting that cyanobacteria may outcompete eukaryotic algae in warmer temperature, and it may lead to loss of biodiversity.

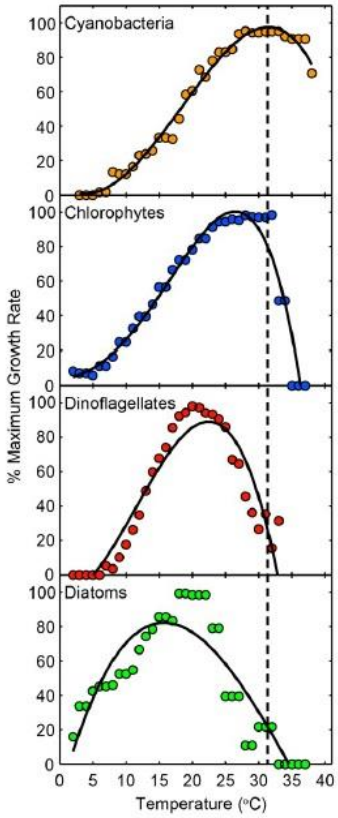


Figure 9. *Temperature-growth dependence among four different taxonomic groups. Reproduced from Open Access reference [17].*

Nutrients over-enrichment could have a synergic interaction with elevated water temperatures and increase cyanobacterial growth. The relationship between cyanotoxins' levels, biomass and the environmental parameters were fluctuating among lake studies. Most frequently, cyanotoxins' levels were positively correlated with water temperature, total nitrogen, total phosphorus, and pH [130, 137, 158, 159, 161, 164]. Paerl [17] summarized major anthropogenic and environmental drivers, ecosystem responses and effects on harmful cyanobacterial blooms (CyanoHABs) in a scheme (Figure 10). Most of the ecosystem responses contribute to the proliferation of CyanoHABs, except for changes in flushing rate and nutrient ratio. These parameters can also lower the bloom. For example, higher amount of precipitation will dilute water, and thus decreases the bloom density. In another review, which focused on factors affecting MCs production, Dai et al. [165] showed

that light intensity, temperature, nitrogen, and phosphorus are the main physical and chemical drivers for cyanobacterial proliferation and MCs production. For instance, in a study centred in the Lake Pamvotis, Gkelis et al. [137] found that MCs levels were positively correlated with temperature and nutrient concentrations. Overall, there is a scarcity of seasonal studies that involve nutrients measurement [68, 92, 93, 140], what tangles an elicitation of patterns that would facilitate prediction and regulation of cyanobacterial blooms and produced toxins.

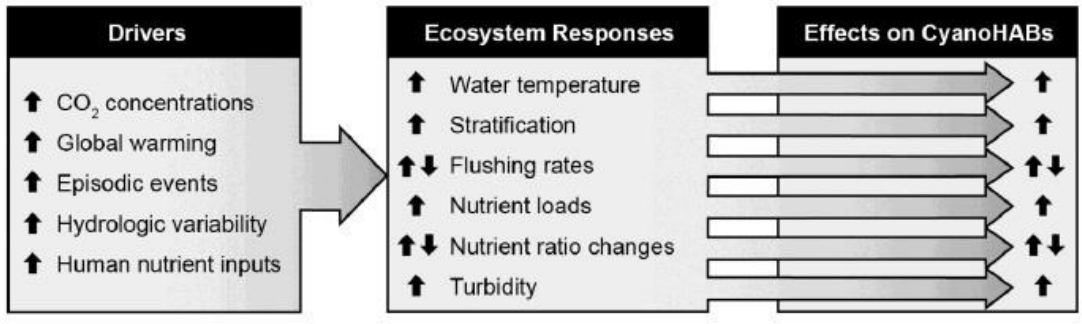


Figure 10. *Relationship between major anthropogenic and environmental drivers, ecosystem change, and potential influence on CyanoHAB. Reproduced from Open Access reference [17].*

Another factor that has positive correlation with cyanotoxins' concentrations is cyanobacterial biomass [86, 137, 158-161, 166]. For example, dominating MCs (MC-LF > -LY > -LA > -LR) in reservoir of Zemborzycki dam were positively correlating with biomass of toxin-producing *Anabaena planctonica*, *Anabaena affinis* and *Microcystis spp*. [134]. However, in some cases, no [164, 167] or negative [154] correlation was observed. Several authors highlighted that cyanotoxins' levels cannot be always directly connected with the total number of bacterial cells; also, a variation in the ratio of toxic/non-toxic genotype should be considered [137, 166, 167]. Manganelli et al. [167] mentioned another two parameters that should be taken into account for the determination of toxins' variation: a) shifts in toxins' production rate and b) potential utilisation of toxin inside the cell. Additionally, the occurrence of degrader heterotrophic groups of bacteria may lead to decline of MCs levels.

Nowadays, there is a scarcity of a deep understanding on which factors influence the production of cyanotoxins. One of the contributing factors is data heterogeneity from the field studies. To tackle this problem and build a robust tool for both monitoring and prediction of seasonal patterns of cyanotoxins' variation, several steps could be implemented. First of all, standardisation of sampling, sample treatment and analysis are needed. This will assure comparable results between the studies. Next, implementation of multidisciplinary studies could be helpful. It is necessary to include measurement of environmental parameters, and cyanobacterial abundance together with cyanotoxins' measurements. Obtained data will be helpful to determine drivers of both cyanotoxins' and biomass growth. Such combined strategy will contribute to effective lake management and consequently minimization of human health hazard [9].

5.3. Seasonal variations of cyanotoxins in different climate zones of Europe

Since cyanobacterial blooms are spread globally across different climate zones, distinguishing toxins' variations patterns in different climatic conditions is needed. This will contribute to implement effective water monitoring strategies. To assess this problem, the Köppen-Geiger climate classification used by Peel et al. [168] could be applied. In Europe, three main climatic zones can be distinguished: cold, arid, and temperate, representing a land area of 44.4, 36.3, and 17.0%, respectively [168]. These zones can be classified as Mediterranean, humid continental, and oceanic (Atlantic) (Figure 11).

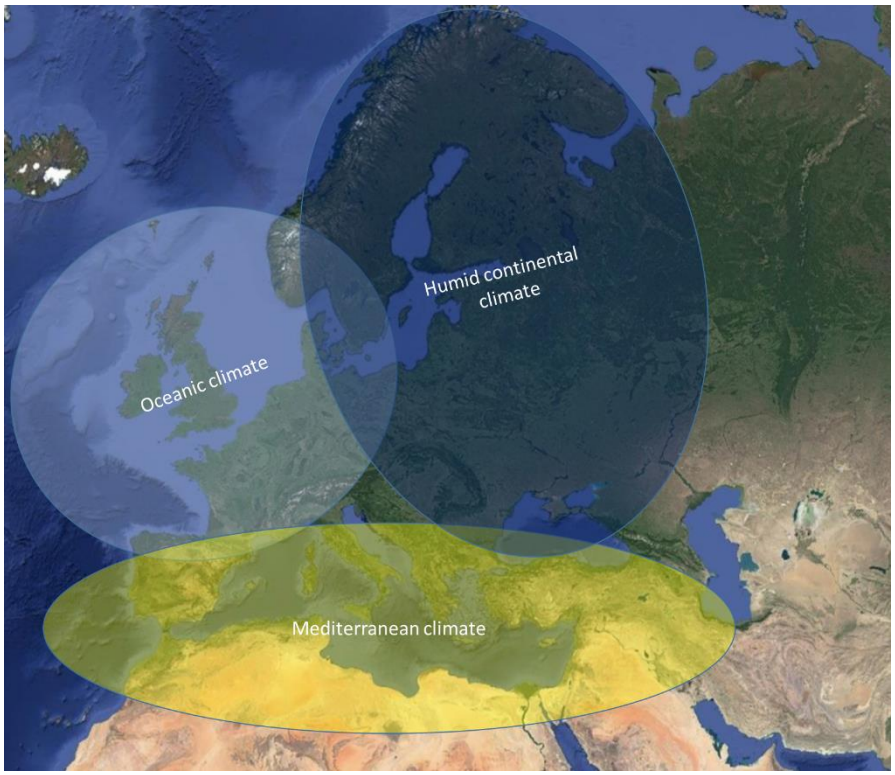


Figure 11. *Main European climate zones.*

The Mediterranean climate zone, which is located in the sub-region around the Mediterranean sea, contains several climate types according to Köppen-Geiger map (see detailed map in [168]), among them Csa, Csb, and BSk. Csa stand for temperate climate with dry and hot summers. In this case, temperature of the hottest month is ≥ 22 °C and is between 0 and 18 °C for the coldest month. Precipitations of the driest month in summer are below 40 mm, and are below 120 mm for the wettest month in winter. Csb type (temperate climate with dry and warm summer) has the same precipitation criteria as Csa. The difference is in the temperature criteria, which is above 10 °C at least 4 months per year. BSk is the arid steppe cold type. It has mean annual temperatures of less than 18 °C, and the mean precipitation can be estimated according to the following criteria MAP ≥ $5 \times P_{threshold}$, where MAP is the mean annual precipitation, and $P_{threshold}$ = varies following the next rules: if 70% of MAP is in winter then $P_{threshold} = 2 \times MAT$, if 70% of MAP is in summer then $P_{threshold} = 2 \times MAT + 28$, otherwise $P_{threshold} = 2 \times MAT + 14$ [168]. As an example, the graphs of temperature

and precipitation variation during the year for two Mediterranean cities, Rome and Athens, are presented in Figure 12.

Humid continental zone consists of two climatic types Dfb and Dfc. Dfb is cold climate without dry season with warm summer. In this case, the temperature of the coldest month is bellow or equals 0 °C, and it is above 10 °C at least 4 months per year. Precipitation of driest month in summer is more than 40 mm, and wettest month in summer has 10 times higher amount of precipitations than driest winter month [168]. Dfc type means cold climate without dry season with cold summer. This type follows the same precipitation criteria, and temperature of the coldest month is bellow or equals 0 °C, and it is above 10 °C during the hottest month. Munich and Trento were taken as representative cities of humid continental climate zone, and their temperature and precipitation variations around the year are shown in Figure 12.

The Atlantic or oceanic climate zone manly consist of a Cfb climate type, which is temperate climate without dry season and with warm summer. For this type, the temperature during the hottest month is above 10 °C, and it maintains for at least 4 months per year. As for the coldest month, the temperature is between 0 and 18 °C. Precipitation criteria is the same as for Sfb and Dfc: for the driest month in summer it is more than 40 mm, and wettest month in summer has 10 times higher amount of precipitations than driest winter month [168]. Temperature and precipitation variation graphs of two cities, Gijon and Amsterdam, within the oceanic climate, are shown in the Figure 12.

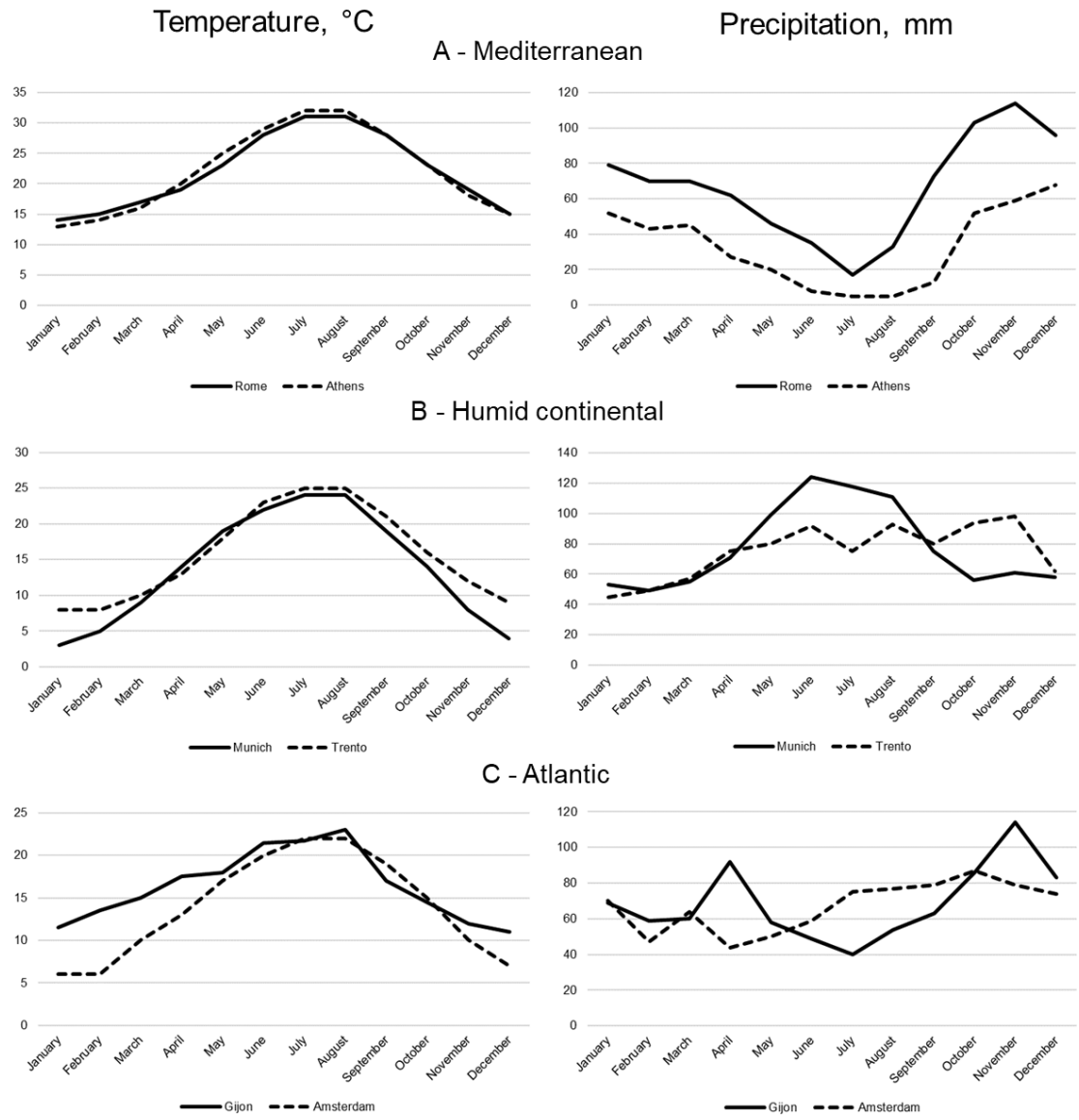


Figure 12. *Average temperature and precipitation variations for cities in A - Mediterranean, B - Humid continental, C - Atlantic climate zones.*

Considering that each climate zone has characteristic variations in temperature and precipitation, dynamic of cyanobacterial growth and levels of released cyanotoxins are expected to follow different patterns.

Even though levels of cyanotoxins are frequently evaluated, there is still a scarcity of data to establish the seasonal variations. In some studies, it was impossible to observe the toxins' changes during a whole year due to the short sampling periods, which were covering only several months [92, 129, 130, 136, 142, 169]. In such cases, studies were focused on a blooming period, which was usually not enough to

determine when the toxins' levels start to grow. To distinguish the peaking seasons in different European climate zones, collected data from various sampling campaigns is presented in Figure 13. Data were normalised to have a maximum level at 1.

Figure 13-A demonstrates cyanotoxins' seasonal variations in four different Mediterranean countries (Italy, Greece, Turkey, and Portugal) [68, 93, 137, 140, 167]. In the graph chart, two peaking periods can be observed. The first one was between March and May and the second one between August and October. However, seasonal variation of the reservoir Alvito does not fit the first peaking sector. This can be due to the sampling period that started only in April [140]. These patterns also align with peaking months for shorter sampling campaigns. For example, maximal cyanotoxins levels were reached between August and October in other fresh water reservoirs in Spain, Greece, Italy, and Portugal [129, 140, 144, 145, 170].

Figure 13-B illustrated the variation of cyanotoxins' concentrations during a year in humid continental climate [130, 132-134, 150, 166] . As in the Mediterranean zone, two peaking areas can be distinguished. The first one was in May and June, and the second one was within August and September. In the case of the humid continental zone, peaking months of the studies with shorter sampling periods match this pattern. The second peaking period is aligned with the trend in reservoir of Zemborzycki dam in Poland [134], where August and October were usually the peaking months for MCs and ANA [9]. Moreover, higher levels of cyanobacterial toxins were reached during August and September in the lakes in Poland (Mytycze [149], Niegocin, Piłwąg, Rekąty [151], and Germany (Bergknappweiher [152]).

In Figure 13-C, normalised variations of cyanotoxins in the oceanic zone are shown [153-155]. Here, only one peaking season between September and October could be observed. However, the amount of seasonal studies in this zone is lower comparing with the other regions.

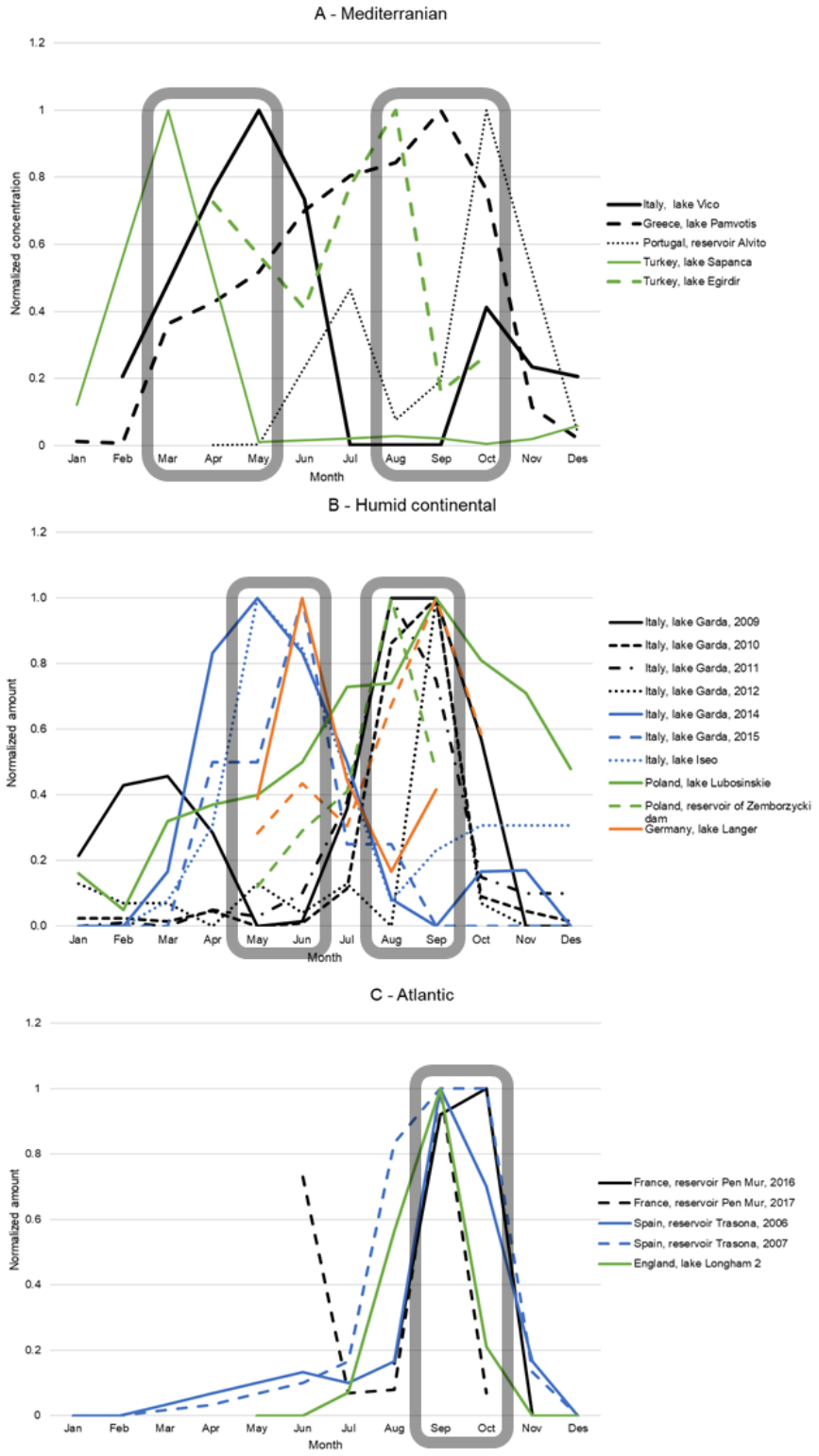


Figure 13. *Seasonal variations of cyanotoxins in A - Mediterranean, B - Humid continental, C - Atlantic climate zones in European region. Grey frames show the peaking periods.*

Considering all three graphs together, several features can be observed. In the Mediterranean zone the blooms are more persistent along the year compared with the other two climate zones. In total 6 peaking months were observed, while the continental and oceanic zones have 4 and 2 peaking months, respectively. However, such differences were expected due to the weather conditions. In the Mediterranean zone, summers are hotter and drier, favouring cyanobacterial proliferation. Shorter peaking seasons in continental climate can be described by higher precipitation and lower temperatures, preventing bloom development. Moreover, in the Alpine region, frequent rainfalls contribute to higher turbidity and dilution. In oceanic climate zone, the absence of the first peak can be explained not only by scarcity of available data, but also due to characteristic weather conditions. A slow growth of cyanotoxins' levels until July could be attributed to the slow slope of the temperature growth, which is reduced in comparison with other regions (Figure 12). Additionally, higher number of rains during spring and summer seasons leads to lower concentrations of cyanotoxins. Nevertheless, the presence of cyanobacterial toxins was, for instance, reported in England in spring (April and May) [171].

All things considered, the obtained seasonal variation patterns in the three European climate zones suggest that climate conditions (such as temperature and precipitation regime) are the main drivers of cyanotoxins' variation.

TARGETED SCREENING

As was mentioned in the previous chapter, in the recent years there is an increase of cyanobacterial blooms due to nutrient over-enrichment and on-going climate changes. Bloom-forming cyanobacteria pose risk to ecosystems and human health as they are known to produce bioactive secondary metabolites, some of which are identified as toxins. The main routes of human exposure to cyanobacterial toxins may occur through drinking water and recreational water use. The assessment of the occurrence and the risks of the exposure to cyanotoxins require robust, straightforward, and sensitive analytical methodologies for their identification and quantitation in the aquatic environment, and particularly in drinking water reservoirs. Sensitivity of the method, can be achieved by applying sample pre-concentration and clean-up. As these compounds belong to different chemical classes (with various polarities, chemical structures, and masses), the choice of pre-concentration and clean-up techniques together with their optimisation is one of the critical points of their analysis. One of the most versatile techniques that showed good performance in terms of recoveries and achieved low LODs is SPE. It provides a wide choice of sorbents that allows to cover various polarities and structures of cyanotoxins, and removes (to some extend) matrices interferences at the same time. Further evaporation, may allow even higher sensitivity.

Cyanotoxins' structural diversity creates an obstacle not only for sample pre-treatment, but also for its further analysis. Additionally, cyanotoxins classes can be also very diverse. For example, more than 300 MCs variants have been identified to date. These compounds are still structurally similar, as the main structural difference can be found in the L-amino-acid residues. This fact complicates differentiation between variants. For example, ELISA method for assessment of MCs is widely used and can provide LODs in the same range as other techniques (LC-MS for example). However, it is not compound-specific, but class-specific, as Adda moiety is used for

Ag-Ab reaction. As was described in the previous chapter, both MCs and NOD have Adda moiety, what makes differentiation between congeners impossible. The application of LC-MS techniques helps to tackle this problem, as it allowed simultaneous analysis of compounds of various structures, polarities, and masses. This is one of the most widely used techniques nowadays.

New WHO guideline that now includes not only MC-LR but other compounds, not only highlights the need of multiclass analytical strategies but also that more knowledge about cyanotoxins' toxicity and occurrence need to be generated. In addition to the toxins mentioned in the guidelines, cyanobacteria are known to produce other bioactive metabolites, while their toxicity in not well studied yet, they are known to co-occur with known cyanotoxins [43-47]. Thus, analytical methods that would enable assessment of compounds beyond the well-studied ones and posterior suspect screening are of great importance. HRMS would provide such opportunity.

All things considered, the goal of this chapter is to provide robust, straightforward, selective, and sensitive analytical methodologies for the identification and quantitation of multiclass cyanotoxins enabling posterior suspect screening in the aquatic environment, and particularly in drinking water reservoirs. Thus, the developed methods are based on SPE-LC-ESI-HRMS, which was then applied for the analysis of freshwater samples from reservoirs in different European climatic zones during the bloom period. Selected water reservoirs are used for recreational activities and production of drinking water.

This chapter covers method development and validation based on dual SPE-UHPLC-ESI-HRMS/MS (which was published [172]), its transfer to Eawag during the scientific stay, and its application for the analysis of freshwater samples from Spain, Switzerland, and the United Kingdom.

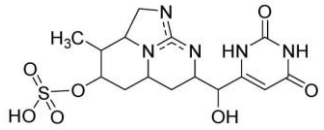
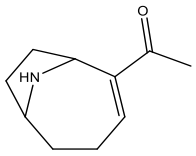
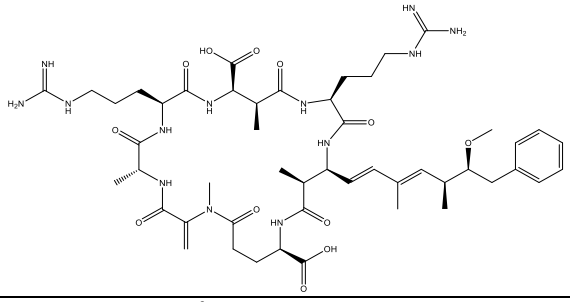
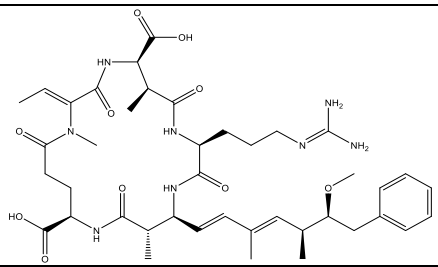
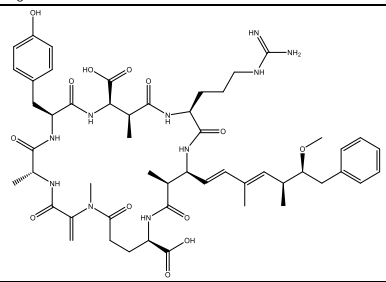
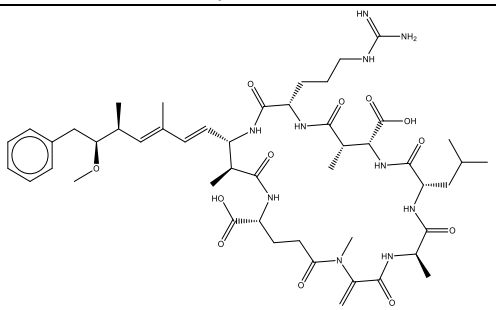
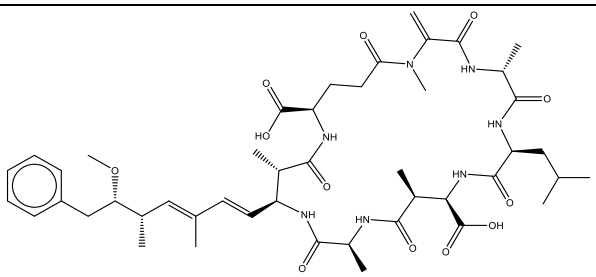
1. Method development and validation

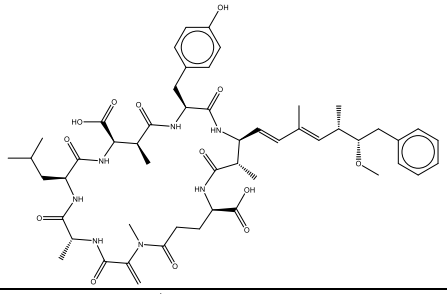
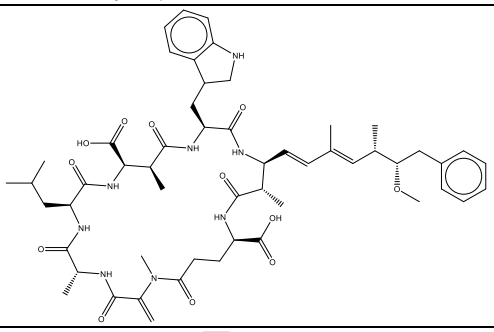
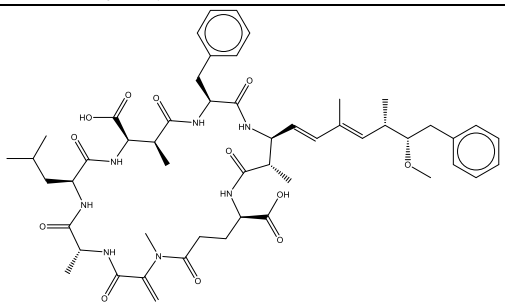
The proposed targeted screening method was developed applying several techniques. The prioritization of targeted compounds was done based on two parameters: 1) the frequency of their detection in European region; and 2) the availability of standards.

Recently, Svirčev et al. [24] described global occurrence of cyanotoxins. Among 341 studies, MCs were the most detected group (in 198 studies). In another recent study, Namsaraev et al. discussed the occurrence of cyanoHABs in various climate zones in Russia. Considering seasonal studies presented in the Table 4, MCs were detected across three studied climate zones. Depending of the type of freshwater sample, concentrations of MCs were varying between ng/L levels (dissolved in water) to 100 µg/L (freshwater bloom) (Table 4). Considering large scale of MCs' occurrence, they were the main group of targeted compounds. ANA was also detected in different European countries including Spain, Portugal, France, Italy, Switzerland, Poland, and Russia (Table 4). Detected concentrations were reaching 14.4 µg/L. Even though, concentration levels of ANA were lower in comparison with MCs, it was still detected in many freshwater samples and, thus, added to the list of targeted compounds. CYN was also reported in many countries all over Europe including Germany, Hungary, Italy, Spain, Finland, Czech Republic, and France [22]. Its concentrations were reaching 1.8 µg/L. Detected CYN concentrations were lower comparing with ANA and MCs, however its wide occurrence was the reason to add the compound to the targeted screening. NOD was found less extensively in freshwater, and they are more frequently found in brackish water [173]. But taking into account structural similarity to MCs (what would help to evaluate selectivity of the method and LC separation when similar classes are analysed simultaneously), the presence in freshwater in *Nodularia* specie [118], and availability of standard,

NOD was added to the list of targeted compounds. In this study, NOD was not considered as an option for a surrogate standard. Thus, the list of targeted compounds selected in this study includes 7 MCs (MC-LR, -RR, -YR, -LA, -LW, -LY, -LF), CYN, ANA, and NOD. Structure of each targeted compound is shown in Table 5. In the course of this study, no internal or surrogate standards were applied for the determination of cyanotoxins. Internal standards were not applied as they were either not available for some compounds, or too expensive. Available surrogate standards (that was mentioned in Introduction section 4.3.3.) were not applied as previously reported results were either ambiguous or not satisfactory.

Table 5. *Structures and elemental compositions of targeted compounds.*

Cyanotoxin	Elemental Composition	Structure
CYN	$C_{15}H_{21}N_5O_7S$	
ANA	$C_{10}H_{15}NO$	
MC-RR	$C_{49}H_{75}N_{13}O_{12}$	
NOD	$C_{41}H_{60}N_8O_{10}$	
MC-YR	$C_{52}H_{72}N_{10}O_{13}$	
MC-LR	$C_{49}H_{74}N_{10}O_{12}$	
MC-LA	$C_{46}H_{67}N_7O_{12}$	

MC-LY	$C_{52}H_{71}N_7O_{13}$	
MC-LW	$C_{54}H_{72}N_8O_{12}$	
MC-LF	$C_{52}H_{71}N_7O_{12}$	

The goal was to assess the concentrations of total toxins: both extracellular and intracellular. As was mentioned in Introduction (section 4.3.1), there are several methods to disrupt cyanobacterial cells and release the intracellular toxins. In studies carried out by Kim et al. [79], sonication for 30 minutes provides 73% of lysis efficiency. Event though, lyophilisation followed by extraction with 75% methanol showed higher efficiency (between 92 and 99%), sonication was chosen in this study, because it is easier and faster approach with satisfactory efficiency. Therefore, samples were sonicated for 30 min at a power of 200 W and a frequency of 60 Hz.

As was mentioned in the previous section, targeted screening method will be based on dual SPE-UHPLC-HRMS/MS. Two different sorbents were applied in order to retain cyanotoxins of various polarities. For the LC part, reversed phase HPLC and UHPLC columns were evaluated. UHPLC column are known to be more efficient in

comparison with HPLC columns due to higher number of theoretical plates. They provide faster separation and lower LODs, what is very beneficial for quick and sensitive analysis. Thus. The final method is applying UHPLC column. For the HRMS part, a Q-Exactive Orbitrap (ThermoFisher Scientific) instrument was used. It allows resolution up to 140,000 full width at half maximum (FWHM) (at *m/z* 200). For data acquisition, two modes were applied: full scan at high resolution for quantification and fragmentation mode in lower resolution for confirmation. As for ionisation, a heated electrospray ionisation (HESI) source was used, as it allows the analysis on compounds within a wide polarity range. In this thesis, positive ionisation mode was applied.

The sequence of method development steps was the following: optimisation of ionisation for each toxins applying HESI, optimisation of LC applying HPLC column, method transfer from HPLC to UHPLC column, and adjustment of HESI parameters. After the method was developed, it was validated.

1.1. Development and optimization of instrumental method

First step in method development was to ensure determination of the compounds in the MS analyser. For this, ionisation of the targeted toxins was studied. As previously commented, detection and determination of the targeted compounds was performed with a Q-Exactive Orbitrap mass spectrometer equipped with a HESI source. To determine optimal mass spectrometer conditions for the determination of the 10 targeted cyanotoxins, standards were directly infused into the ESI source in both positive and negative modes. For each compound, the observed *m/z* values of the corresponding ion was compared to the theoretical *m/z* that was calculated by Xcalibur 2.1 software (ThermoFisher Sientific).

Table 6 summarises information about theoretical and experimental *m/z* values obtained in both ionization modes together with the mass errors. Calculated values were below 2 ppm (except for CYN and MC-LF in negative ionization mode, which were −2.17 ppm and −2.03 ppm, respectively). This represents high instrumental selectivity provided by HRMS. ANA was not detected in the negative ionisation mode. This is due to the fact that ANA is a bicyclic secondary amine, and having a basic –NH group means that proton will not be easily lost.

Further, the ionization of the targeted toxins was studied by flow injection analysis (FIA) using isocratic mobile phase composed of acidified with formic acid (FA) (0.1%) water/acetonitrile (ACN), (50/50, v/v) at a flow rate of 0.07 mL/min. The injected standards were at 0.75 mg/L for MC-LY, -LW, -LF, and 1 mg/mL for the other seven targeted compounds. Capillary temperature (275, 325, and 375 °C), heater temperature (225, 275, and 325 °C), which were changed pairwise with increase of 50 °C each time, spray voltage (3 kV, 3.5 kV, 4 kV), and S-lens RF levels (60% and 70%) were evaluated. These tests were performed in both positive and negative ionization modes. Finally, the optimal parameters for both positive and negative mode to obtain, as a compromise, the highest signal for most of the targeted toxins, were the following: sheath gas at 10 a.u.; sweep gas at 0 a.u.; auxiliary gas at 5 a.u.; capillary temperature at 320 °C; HESI probe temperature at 275 °C; electrospray voltage at 3.5 kV; S-lens RF level at 60%. In the end, positive ionisation mode was chosen, as ANA was not detected in the negative one.

Table 6. *The most abundant m/z values for both positive and negative ionization modes.*

Compound	Neutral mass (theoretical)	Ion specie in positive mode	*m/z* in positive mode (theoretical)	Mass error in positive mode (ppm)	Ion specie in negative mode	*m/z* in negative mode (theoretical)	Mass error in negative mode (ppm)
CYN	415.1156	$[M+H]^+$	416.1234	−0.48	$[M-H]^-$	414.1089	−2.17
ANA	165.1148	$[M+H]^+$	166.1226	1.81	$[M-H]^-$	164.1081	N/D
MC-RR	1037.5664 518.7824	$[M+2H]^{2+}$	519.7902	0.96	$[M-H]^-$	1036.5585	0.09
NOD	824.4438	$[M+H]^+$	825.4505	1.57	$[M-H]^-$	823.4360	−1.21
MC-YR	1044.5286 522.2635	$[M+H]^{2+}$	523.2713	1.72	$[M-2H]^{2-}$	521.2567	−1.73
MC-LR	994.5488 497.2738	$[M+H]^{2+}$	498.2817	1.61	$[M-H]^-$	993.5415	−0.80
MC-LA	909.4848	$[M+H]^+$	910.4920	1.31	$[M-H]^-$	908.4775	−1.32
MC-LY	1001.5110	$[M+H]^+$	1002.5183	−0.59	$[M-H]^-$	1000.5037	−1.09
MC-LW	1024.5270	$[M+H]^+$	1025.5342	1.56	$[M-H]^-$	1023.5197	−1.07
MC-LF	985.5233 492.7575	$[M+H]^+$	986.5223	0.71	$[M-2H]^{2-}$	491.7508	−2.03

Once the ionization parameters were optimized, the next step was to optimise the chromatographic separation. As a first step, the optimization of the separation conditions for the 10 selected cyanotoxins was performed on a C_{18} reversed-phase HPLC column LichtoCART® (2 × 125 mm, 5 µm particle diameter). The analysis was performed using an Accela LC instrument (ThermoFisher Scientific). Gradient elution was performed with water (solvent A) and acetonitrile or methanol (solvent B) both with 0.1% formic acid at a constant flow rate of 0.2 mL/min with the following gradient program: 10/10/90/90/10/10% B at 0/3/11/16/18/25 min, respectively. Firstly, the effect of the organic modifier in the mobile phase was studied. Thus, methanol and acetonitrile both acidified with 0.1% FA were evaluated and several chromatographic parameters were compared. Table 7 presents the evaluated chromatographic parameters using different mobile phase compositions. Evaluated parameters were the following: retention time, retention factor, tailing factor, selectivity, resolution, and peak width at the peak base. As can be seen by retention time and retention factor, CYN and ANA were the first compounds to elute. This is expected, as polar compounds elute the first while applying reversed reversed-phase columns. The tailing factor for ANA and MC-RR was lower using ACN. Better resolution for NOD, MC-LA, and -LW was observed when ACN was used. In addition, chromatographic peaks of MC-RR, -YR, -LR, and NOD were narrower when applying ACN. This factor also may contribute to lower LODs. Overall, ACN provided slight improvements in comparison to methanol, thus it was chosen as organic modifier of mobile phase.

Table 7. *Chromatographic parameters using different mobile phase compositions.*

Compound	Solvent A	Retention time, min	Retention factor, K	Tailing factor, T_f	Selectivity, $\alpha_{(a,a+1)}$	Resolution, R_s	Peak width (base), W_b
CYN	ACN	1.68	0.39	0.73	0.98	0.03	0.18
	MeOH	1.73	0.40	0.7	1.02	0.03	0.18
ANA	ACN	1.67	0.38	1.14	13.76	13.81	0.5
	MeOH	1.74	0.40	2.13	17.78	19.74	0.47
MC-RR	ACN	7.54	5.23	1.28	1.06	1.21	0.35
	MeOH	10.13	7.17	1.43	1.06	1.51	0.38
NOD	ACN	7.92	5.55	1.24	1.03	0.75	0.28
	MeOH	10.68	7.61	1.08	1.00	0.06	0.35
MC-YR	ACN	8.1	5.69	1.31	1.01	0.21	0.2
	MeOH	10.7	7.63	0.93	1.02	0.48	0.29
MC-LR	ACN	8.15	5.74	1.47	1.31	9.06	0.27
	MeOH	10.85	7.75	1.3	1.14	5.00	0.33
MC-LA	ACN	10.28	7.50	0.7	1.01	0.37	0.2
	MeOH	12.15	8.80	0.7	1.00	0.15	0.19
MC-LY	ACN	10.36	7.56	0.88	1.10	3.36	0.23
	MeOH	12.12	8.77	0.95	1.03	1.65	0.2
MC-LW	ACN	11.3	8.34	0.83	1.04	1.08	0.33
	MeOH	12.45	9.04	1.22	1.02	0.93	0.2
MC-LF	ACN	11.75	8.71	1.08			0.5
	MeOH	12.65	9.20	1.66			0.23

Conditions for chromatographic separation: gradient elution was performed with water (solvent A) and acetonitrile or methanol (solvent B) both with 0.1% formic acid at a constant flow rate of 0.2 mL min^{-1} with the following gradient program: 10/10/90/90/10/10% B at 0/3/11/16/18/25 min. The injection volume was 20 µ L.

Once the separation of the 10 targeted toxins was established, their signal detection was evaluated. For that purpose, the amount of FA in the mobile phase was optimized, in order to improve their ionization and detection. The tested concentrations were between 0.05 and 1% of FA. The normalized signal (to 1 as maximum) for the studied mycotoxins using different FA contents in the mobile phase is shown in Figure 14. As can be seen, the lower is the amount of FA, the better is the ionisation. Thus, 0.05% of FA provided the highest signal intensities for most of the compounds (except for MC-RR and -LA) comparing with higher concentrations of FA. Hence, 0.05% FA was selected, as a compromise, for the determination of the 10 targeted cyanotoxins.

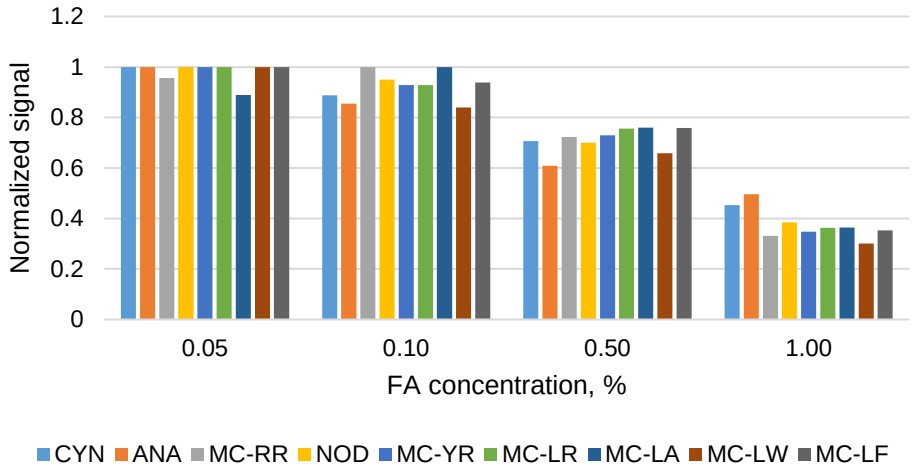


Figure 14. *Optimization of FA concentration in the mobile phase: ACN (solvent A), H_2O (solvent B).*

The current method provides separation within 25 min. In order to increase the throughput of the method, what would make it more appropriate for routine analysis, it was decided to decrease the analysis time. To reduce chromatographic analysis time, the method was transferred to a C_{18} reversed-phase UHPLC column Hibar® (2.1 × 150 mm, 2 µm particle diameter). Application of UHPLC column will also decrease LOD due to narrower peaks. UHPLC column with smaller particle sizes

were not tested as they could be blocked, what was demonstrated in another study for analysis of lake water samples during the blooming period [174].

As the UHPLC column was applied, the flow rate was increased from 0.2 to 0.3 mL/min, and the separation time was shortened to 10 min. In order to evaluate the performance of both HPLC and UHPLC columns, several chromatographic parameters such as retention time, retention factor, tailing factor, selectivity, resolution, and peak width were compared. Obtained results are summarized in Table 8. Similarly to HPLC column, polar compounds eluted first. Tailing factor and selectivity of HPLC and UHPLC columns were very similar. Resolution and peak width parameters were better when UHPLC column was applied, as expected. Since UHPLC column provides better resolution, narrower peaks, and the analysis time is 2.5 times lower, Hibar® UHPLC column was then selected for the final proposed method. The extracted ion chromatograms for the 10 targeted cyanotoxins at concentrations of 5 µg/L are shown in Figure 15. Gradient elution was performed with water (solvent A) and acetonitrile (solvent B) both with 0.05% formic acid at a constant flow rate of 0.3 mL/min with the following gradient program: 10/10/90/90/10/10% B at 0/1/5/8/8.5/10 min, respectively. The injection volume was 20 µL using a 20 µL stainless sample loop.

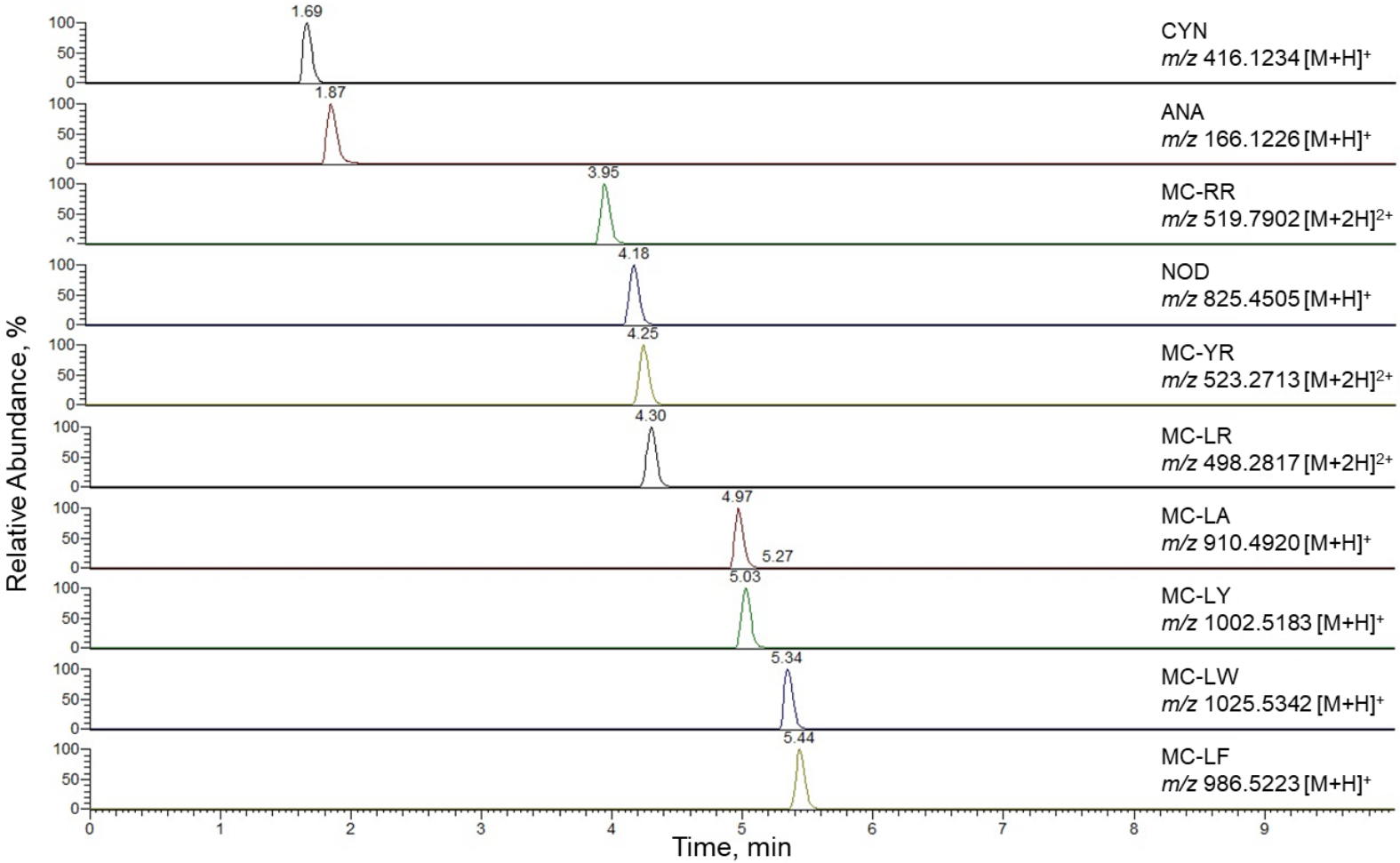


Figure 15. *Extracted ion chromatograms for the 10 targeted cyanotoxins at 5 µg/L.*

It is worth mentioning that some co-elutions were observed (between NOD, MC-YR, and -LR; -LA and -LY; and -LW and -LF) applying UHPLC column. As was mentioned before, ESI was applied for ionisation of the compounds. When ESI is applied and co-elutions occur, enhancement and suppression effects may also take place and this will affect the sensitivity of the proposed methodologies. Thus, since co-elutions were observed, these effects were studied and neither ion enhancement nor ion suppression effects were observed. Therefore, the observed co-elutions did not represent a problem on the proposed method as all co-eluted compounds have different *m/z* value and they can be perfectly distinguished by the high selectivity provided by the HRMS instrument, without any effect on their ionization signal as no ion-suppression nor ion-enhancement was observed.

As the HPLC column was substituted with an UHPLC column for a faster separation, ionization parameters were readjusted as the mobile phase flow rate was increased from 0.2 mL/min to 0.3 mL/min. All parameters were slightly increased. Thus, the optimal source HESI parameters were set as follows: spray voltage of +4 kV, sheath gas, auxiliary gas and sweep gas at 35, 17, and 1 a.u. (arbitrary units), respectively, the heater temperature at 300 °C, the capillary temperature at 350 °C, and S-lens RF level at 60%.

Table 8. *Chromatographic parameters of LichtoCART® HPLC and Hibar® UHPLC columns.*

Compound	Solvent A	Retention time, min	Retention factor, K	Tailing factor, T_f	Selectivity, $\alpha_{(a,a+1)}$	Resolution, R_s	Peak width (base), W_b
CYN	HPLC	1.68	0.39	0.73	0.98	0.03	0.18
	UHPLC	1.69	0.72	1.04	1.25	1.89	0.10
ANA	HPLC	1.67	0.38	1.14	13.76	13.81	0.5
	UHPLC	1.87	0.91	1.13	3.34	18.09	0.09
MC-RR	HPLC	7.54	5.23	1.28	1.06	1.21	0.35
	UHPLC	3.95	3.03	1.1	1.08	2.00	0.14
NOD	HPLC	7.92	5.55	1.24	1.03	0.75	0.28
	UHPLC	4.18	3.27	1.05	1.02	0.74	0.09
MC-YR	HPLC	8.1	5.69	1.31	1.01	0.21	0.2
	UHPLC	4.25	3.34	1.04	1.02	0.51	0.10
MC-LR	HPLC	8.15	5.74	1.47	1.31	9.06	0.27
	UHPLC	4.3	3.39	1.1	1.20	5.00	0.10
MC-LA	HPLC	10.28	7.50	0.7	1.01	0.37	0.2
	UHPLC	4.97	4.07	1.02	1.02	0.41	0.17
MC-LY	HPLC	10.36	7.56	0.88	1.10	3.36	0.23
	UHPLC	5.03	4.13	1.1	1.08	2.14	0.12
MC-LW	HPLC	11.3	8.34	0.83	1.04	1.08	0.33
	UHPLC	5.34	4.45	1.1	1.02	0.57	0.17
MC-LF	HPLC	11.75	8.71	1.08			0.5
	UHPLC	5.44	4.55	1.11			0.18

Conditions for chromatographic separation for UHPLC coulunm: gradient elution was performed with water (solvent A) and acetonitrile (solvent B) both with 0.05% formic acid at a constant flow rate of 0.3 mL min^{-1} with the following gradient program: 10/10/90/90/10/10% B at 0/1/5/8/8.5/10 min. The injection volume was 20 µL.

After the optimisation of the chromatographic separation and the ionisation of the targeted cyanotoxins, MS acquisition parameters were optimised. Full scan at high resolution was used for quantification and identification, while fragmentation was run in parallel and it was used for confirmation purposes.

Full-scan MS data were acquired between 90 and 1100 *m/z* with a nominal resolving power of 70,000 FWHM (at *m/z* 200), with automated gain control of 1e6, and a maximal injection time of 100 ms with 1 ppm mass accuracy. In order to increase the number of identification points (see section 4.3.4. in the Introduction), fragmentation studies were also performed. Some fragments were also observed in the full scan spectra due to in-source fragmentation. However, these fragments were not considered as method transfer (to Eawag) was planned in advance. Even though the same ionisation source was used in both cases, the in-source fragmentation varies between equipment meaning that same in-source fragmentation cannot be guaranteed between different instruments. Thus, fragmentation was run in parallel to full scan, in order to generate product ions for confirmation of the compounds.

For quantification, the most abundant ions in full scan HRMS mode were chosen (see Table 9). For MCs, single-charged $[M + H]^+$ ion were mostly produced. However, for MC-RR, -LR, and -YR both single- $[M + H]^+$ and double-charged $[M + 2H]^{2+}$ specie occurred. Arginine-containing MCs are known to produce ions at the guanidine group in the arginine (Arg) residue [175]. Thus, in case of MC-RR, which contains two arginine residues, abundance of double-charged ions was significantly higher than that of single charged once. Similarly, to MC-RR, both MC-LR and MC-YR had also double charged ions with high abundances. In this case, protonation occurs on both the methoxy residue of the Adda side chain and arginine residue [176]. However, single-charged ions were still dominating. The main transition for MCs was attributed to $PhCH_2CH(OCH_3)$ or with the loss of $PhCH_2CH(OCH_2)$. The ion at *m/z* 135 is a

fragment from the α-cleavage of the methoxy group of the Adda residue, and it agrees with previously reported studies [177, 178]. NOD formed single-charged ion $[M + H]^+$ due to the protonation of Arg, and the most abundant fragment was again *m/z* 135 due to the protonation of the methoxy group of the Adda residue [179]. The precursor ion for CYN was also single-charged $[M + H]^+$ at *m/z* 825. The most intense fragment was m/z 336 due to the loss of SO_3 [180]. ANA with $[M + H]^+$ precursor ion at m/z 166 provided the most abundant product ion at m/z 149, corresponding to the loss of the amine NH_3 [181].

For the optimization of fragmentation, parallel reaction monitoring mode (PRM) was applied. This mode is generally used for short inclusion lists, as scan speed is not high enough for longer inclusion lists in the same time window. For example, PRM was previously applied to obtain the MS/MS spectra of five cyanotoxins [54]. However, for the application of this method to the analysis of 10 compounds, PRM mode is too slow and will not provide enough (6-10) scans per fragmentation peak. As the first step of fragmentation optimization, collision energies (CEs) for each toxin had to be established. Thus, CE between 10 and 60 eV with an increase of 5 eV were studied. Later, CE was optimized more precisely (changing only ± 2 eV around the chosen value) for the selection of the optimal product ions for confirmation. Three parameters were considered: retention time, and abundances of both product and precursor ions.

Optimal CEs, and selected precursors and product ions are summarised in Table 9. Observed fragmentation is in accordance with previously-reported works [70, 145, 177, 179-188].

Table 9. *Details on the optimized HRMS parameters for 10 targeted cyanotoxins.*

Toxin	Retention time (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	CE (eV)
CYN	1.73	416.1241 $[M + H]^+$	336.1664 $[M + H - SO_3]^+$	30
ANA	1.75	166.1229 $[M + H]^+$	149.0959 $[M - NH_3 + H]^+$	35
MC-RR	4.66	519.7902 $[M + 2H]^{2+}$	135.0803 $[C_9H_{11}O]^+$	30
NOD	4.89	825.4518 $[M + H]^+$	135.0803 $[C_9H_{11}O]^+$	32
MC-YR	4.97	1045.5353 $[M + H]^+$	135.0803 $[C_9H_{11}O]^+$	30
MC-LR	5.03	995.5560 $[M + H]^+$	135.0803 $[C_9H_{11}O]^+$	30
MC-LA	5.78	910.4904 $[M + H]^+$	776.4176 $[M + H - C_9H_{10}O]^+$	10
MC-LY	5.86	1002.5177 $[M + H]^+$	868.4444 $[M + H - C_9H_{10}O]^+$	10
MC-LW	6.23	1025.5334 $[M + H]^+$	891.4594 $[M + H - C_9H_{10}O]^+$	10
MC-LF	6.33	986.5253 $[M + H]^+$	852.4490 $[M + H - C_9H_{10}O]^+$	10

After optimization of fragmentation by PRM, the fragmentation mode had to be changed, as for fragmentation of higher (than 5) number of compounds in the same method, PRM mode was not fast enough. For larger number of compounds there are two ways to record fragmentation spectra while full scan is also acquired: data dependent acquisition (DDA) and data independent acquisition (DIA). In the case of DDA, mass lists of targets/suspects (inclusion list) has to be specified in the method. Thus, when the specified mass is detected in full scan, respective fragmentation will be triggered. The precursor ion is isolated in the quadrupole, fragmented in the collision cell, and then the fragments are measured in the Orbitrap analyser. Distinct relation between precursor and fragment provides compound specific fragmentation spectra. On the other hand, DIA does not provide a compound specific fragmentation spectra as it is not triggered by single mass. Herein, precursor ion package, which is defined by a selected *m/z* window, is fragmented and all fragments are measured at the same time. DDA is normally used in two cases, for specific compounds or when the fragmentation spectra of compounds are not known. However, when the suspect lists are long, DDA mode can be slow as it is limited by the number of cycles (top-n – top n most intense ions). DIA is used when fragmentation of the compounds is

known or when the suspect list is longer. Data generated during DIA is complex and can be difficult to interpret in case of co-eluting compounds. In order to simplify data evaluation, different width of *m/z* windows are applied [105]. As was earlier mentioned (section 4.3.2. of Introduction) Roy-Lachapelle et al. [105] used DIA for suspect screening of MCs and anabaenopeptins (in-house database consisted of 660,960 MCS and 61,152 anabaenopeptins). The most optimal isolation window width was 50 *m/z* and there were 22 of them in the scan range of 300 – 1400 *m/z*.

In the developed method in this thesis for targeted screening of 10 cyanotoxins, DDA was applied. The ThermoFisher Scientific software for methods establishment this mode is named as ddMS2, thus ddMS2 top-3 (top 3 most intense ions) was applied. Since UHPLC column was employed, to ensure the maximal amount of scans in ddMS2 mode, the 'pick others' function was switched off. The final conditions for MS data acquisition were the following: full-scan mode at a resolving power of 70,000 FWHM (*m/z* 200), AGC target of 1e6, and maximal injection time of 100 ms with 1 ppm mass accuracy. The ddMS2 (top3) mode was acquired at a resolving power of 17,000 FWHM, AGC target of 1e5, and maximal injection time of 50 ms with optimal CE for each compound (Table 9). The precursor ion in the full-scan was used for quantification, and the most abundant fragment from ddMS2 mode was used for confirmation. The positive identification of target compounds was carried out by comparing the retention times in the samples and standards in matrix-matched in artificial fresh water (AFW) with a maximum tolerance of ±2%. To ensure selectivity of the obtained data, the mass tolerance was set at ±5 ppm for the extracted *m/z* values from acquisition.

1.1.1. Validation of instrumental parameters

For the evaluation of the instrumental performance of the method, the following quality parameters were assessed: instrumental limits of detection (ILODs), linearity,

inter-day and intra-day precision, and the obtained results are summarised in Table 10.

For the determination of ILODs, a standard solution of the 10 selected cyanotoxins was prepared at an initial concentration of 50 µg/L. The ILODs were determined by progressive dilution with an injection volume of 20 µL. Obtained ILODs were between 0.02 pg and 1.5 pg on the column. It is not always possible to compare ILODs with other reported methods, because usually only method LODs are reported. However, when direct injection is applied and injection volume is given, it is possible to compare. Obtained ILODs are in the same concentration level as the ones calculated from the reported method LODs (MLODs) and the injection volume for MCs and NOD (between 0.04 and 3 pg on the column) [189] and for ANA and CYN (between 0.3 and 1.4 pg on the column) [117]. However, these are two separate methods based on UHPLC-MS/MS for low-molecular weight cyanotoxins (ANA, CYN) and higher molecular weight MCs and NOD, while method developed in this thesis involves all these classes of compounds.

The linearity of the method was established by analysing mixtures of the 10 targeted cyanotoxins at 16 different concentrations in the range of 1–50 µg/L. The Pearson's correlation coefficient (R^2) and the slopes of the calibration curves in solvent were determined. Since the calibration range was high, two linear ranges were distinguished for each compound. Good linearity was obtained, with R^2 values below 0.9928. This method can be applied in wide range of concentrations, what is of an advantage, as cyanotoxins were reported at various concentrations levels (Introduction, Table 4).

For evaluation of the inter-day precision, the average percentage of the relative standard deviation (RSD%) of standard solutions at 10 µg/L concentration. Nine replicates were injected for intra-day precision and three replicates were injected for

inter-day (three days) precision evaluation. Obtained results were below 10% and 20% (except for 2 compounds) for intra- and inter-day precision, respectively, what demonstrates satisfactory precision.

Table 10. *Instrumental quality parameters of the developed targeted method.*

Compound	ILOD, pg	Linearity range µg/L, R^2	Precision, RSD%	
			Intra-day	Inter-day
CYN	0.5	0.025–0.5, 0.9992 1–50, 0.9998	5.2	2
ANA	0.2	0.01–0.25, 0.998 0.5–50, 0.9998	2.1	22.6
MC-RR	0.02	0.001–0.5, 0.9992 1–25, 0.9997	1.6	17.9
NOD	0.5	0.025–0.25, 0.999 0.5–25, 0.9996	1.5	17.3
MC-YR	1	0.05–0.1, 0.9928 0.25–50, 0.9943	2	22.4
MC-LR	1	0.05–0.25, 0.998 0.5–50, 0.9992	2.5	23.2
MC-LA	1	0.05–0.25, 0.9943 0.5–50, 0.9971	2.7	17.7
MC-LY	0.76	0.038–0.75, 0.9995 2–38, 0.9993	5	18.7
MC-LW	1.5	0.075–0.75, 0.9986 2–38, 0.9997	8.8	14.1
MC-LF	0.76	0.038–0.75, 0.9994 2–38, 0.9994	7.5	13.2

1.2. Development and optimization of sample pre-treatment

As commented at the beginning of this chapter, a sample pre-treatment method based on SPE was optimised. To achieve the effective extraction of all the targeted cyanotoxins, two cartridges were preselected and tested: Oasis HLB (500 mg, 6cc, Waters Corporation) and Supelclean™ ENVI-Carb™ (500 mg, 6cc, Supelco). These two cartridges were chosen as selectivity of this stationary phases have demonstrated satisfactory recoveries of cyanotoxins (Introduction section 4.3.1. and Table 3). Oasis HLB is a hydrophilic-lipophilic-balanced, reversed-phase sorbent,

which is composed of a copolymer of the hydrophilic N-vinylpyrrolidone and the lipophilic divinylbenzene. It provides high retention capacity for analytes within a wide range of polarities. Supelclean™ ENVI-Carb™ is a reversed-phase sorbent which is made of graphitized non-porous carbon. The surface of the carbon is comprised of hexagonal ring structures, which are interconnected and layered into graphitic sheets. Its non-porous nature provides fast processing as the analyte adsorption does not require their dispersion into the pores. For the extraction optimization, an AFW fortified with 75 ng/L of MC-LY, -LW, and -LF, and with 100 ng/L for the rest of the selected toxins, was used.

It is worth mentioning that the selectivity of only one cartridge was not enough to retain all ten selected cyanotoxins as they belong to different chemical classes and have different polarities. At first trials – evaluation of the elution step, it was observed that Oasis HLB retained MCs and NOD effectively, while ENVI-Carb™ retained better CYN. ANA was retained better on the Oasis HLB cartridge, although the recoveries were below 21%. During the evaluation of the elution step, loading was carried out at neutral pH, and the effect of the solvent, the temperature, and the pH on the elution step was studied. In several previously published studies, trifluoroacetic acid was used for pH adjustment at different SPE steps [190-192]. However, trifluoroacetic acid is known to strongly supress the electrospray signals of many compounds, especially when working in negative ionization mode, which would clearly reduce the method sensitivity. Thus, in this study, formic acid (FA) was employed. The studied elution conditions were: 10 mL of methanol (MeOH) at room temperature (25 °C), 5 mL of methanol at 50 °C, 5 mL of acetonitrile (ACN), 5 mL of methanol acidified with FA (0.5%), and 5 mL of methanol basified with NH_4OH (0.1%). The recoveries obtained during the evaluation of this step are summarized in Table 11. As can be seen, MCs were eluted more efficiently from the Oasis HLB sorbent with methanol,

either when using 10 mL at 25 °C or 5 mL at 50 °C. Even though, MC-LW and MC-LF were slightly better eluted from Oasis HLB with basified methanol. Further elution with 10 mL of heated methanol resulted in 1–9% better recoveries for cyanotoxins. Dimitrakopoulos et al. [190] has described the highest recoveries of ANA using PGC cartridges in comparison to Oasis HLB and silica based ENVI C_{18} at basified loading conditions. As ANA is a very polar alkaloid with a basic secondary amine group (pK_a of 9.6), neutralization of the amine group is needed to achieve higher retention via adsorption on the reversed-phase cartridge. Thus, water sample should be basified during the loading step [190]. As for CYN, it was better eluted from the ENVI-Carb™ with acidified methanol, which was expected as CYN has shown higher recoveries at graphitized carbon cartridges with acidified elution solvents before [70, 193]. However, obtained recoveries were below 23% and required further optimization. Hence, the next step of SPE optimization was focused on the retention and elution of ANA and CYN applying the ENVI-Carb™ cartridge.

Table 11. *SPE optimization of elution conditions for Oasis HLB and Supelclean™ ENVI-Carb™ (±standard deviation) in triplicate.*

Compound	Conditions				
	10 mL MeOH	**5 mL Heated MeOH**	**5 mL ACN**	**5 mL MeOH with 0.5% FA**	**5 mL MeOH with 0.1% NH_4OH**
			Oasis HLB		
CYN	< 3	< 3	< 3	< 3	< 3
ANA	6.9 ± 1.9	10.2 ± 1.3	5.0 ± 0.4	20.2 ± 5.0	13.6 ± 3.6
MC-RR	55.0 ± 4.9	56.3 ± 0.7	5.5 ± 0.1	46.3 ± 2.2	37.9 ± 1.9
MC-YR	49.4 ± 3.6	46.4 ± 0.7	< 3	27.1 ± 0.9	35.6 ± 3.3
MC-LR	47.4 ± 4.0	44.3 ± 0.9	< 3	27.1 ± 0.6	33.2 ± 2.2
MC-LA	57.5 ± 3.7	58.5 ± 2.3	8.9 ± 0.4	32.8 ± 0.8	51.9 ± 2.1
MC-LW	13.6 ± 1.3	29.9 ± 10.2	< 1	< 1	36.3 ± 5.2
MC-LF	51.1 ± 3.5	63.1 ± 3.8	< 3	9.9 ± 2.0	63.6 ± 2.5
			Supelclean™ ENVI-Carb™		
CYN	4.0 ± 0.6	< 3	< 3	22.6 ± 3.7	< 3
ANA	< 1	< 1	< 1	< 1	< 1
MC-RR	< 1	< 1	< 1	< 1	< 1
MC-YR	< 1	< 1	< 1	< 1	< 1
MC-LR	< 1	< 1	< 1	< 1	< 1
MC-LA	< 1	< 1	< 1	< 1	< 1
MC-LW	< 1	< 1	< 1	< 1	< 1
MC-LF	< 1	< 1	< 1	< 1	< 1

In order to optimize the recoveries from the ENVI-Carb™ cartridge for both CYN and ANA, a mass balance experiment was performed. For this step, 250 mL of water at neutral pH spiked with 1 µg/L of ANA and CYN were passed through the cartridge. Water was collected and analysed. For this experiment water was spiked at a higher concentration (1 µg/L, while it was 100 and 75 ng/L in previous optimisation steps) to assure detection in both not pre-concentrated and percolated water. As expected, ANA was not retained at neutral pH and passed through, while CYN was entirely retained. In the collected water ANA had the same concentration level as before passing through cartridge, while CYN was not detected in the collected water. Next, optimization of loading step was carried out. Different pH sample loading conditions (neutral, and with 0.1% and 0.01% of ammonium hydroxide (NH_4OH)) were tested. Back-flush elution with 10 mL of heated methanol with 0.5% of FA was applied. Back-flush was used for better elution of CYN since it was highly retained in the cartridge. Obtained results demonstrated improvement on both CYN and ANA recoveries of up to 68% and 46% respectively, with basified (0.1% of NH_4OH) loading. Loading with 0.01% of NH4OH and neutral pH recovered 60% and 51% of CYN, and 40% and 2% of ANA, respectively.

Finally, it was also observed that better recoveries for all targeted mycotoxins were achieved by increasing the amount of elution solvent up to 20 mL of methanol at 50 °C for the Oasis HLB sorbent and 20 mL of methanol at 50 °C with 0.5% of FA for the ENVI-Carb™ sorbent. This allowed to recover up to 2.87% more analytes: 2.4% for CYN, 2.65% for ANA, 1.75% for MC-RR, 2.87% for MC-YR, 0.56% for MC-LR, 0.72% for MC-LA, 0.91% for MC-LF. In Table 12, the recoveries for each cartridge are presented. Overall, recoveries obtained by Oasis HLB were between 66.6 and 80.6 % (at 20 ng/L) for 6 MCs and NOD respectively. ANA was retained in both

cartridges with total recoveries of 87.8% (at 20 ng/L). Retention of CYN was obtained exclusively by ENVI-Carb™ with final effectiveness of 87.2% (at 20 ng/L).

Table 12. *Mean recoveries of Oasis HLB and ENVI-Carb™ at three concentration levels (±standard deviation) in triplicate.*

Compound	Concentration, ng/L					
	Oasis HLB			Supelclean™ ENVI-Carb™		
	2	10	20	2	10	20
CYN	<3	<3	<3	53.4 ± 5.5	52.2 ± 2.1	87.2 ± 8.6
ANA	46.8 ± 6.7	25.6 ± 2.4	34.2 ± 1.9	34.8 ± 1.0	44.6 ± 1.8	53.0 ± 0.6
MC-RR	72.2 ± 7.3	62.8 ± 5.8	66.6 ± 7.5	<1	<1	<1
NOD	81.1 ± 5.1	66.1 ± 2.0	82.1 ± 2.4	<1	<1	<1
MC-YR	71.6 ± 9.0	73.6 ± 12.4	70.6 ± 6.7	<1	<1	<1
MC-LR	57.7 ± 9.7	70.3 ± 9.6	80.4 ± 8.5	<1	<1	<1
MC-LA	82.8 ± 4.8	70.0 ± 7.1	80.0 ± 2.8	<1	<1	<1
MC-LY	84.3 ± 4.9 [a]	65.0 ± 5.7 [b]	80.6 ± 5.9 [c]	<1	<1	<1
MC-LW	9.2 ± 2.2 [a]	32.3 ± 4.1 [b]	48.7 ± 7.2 [c]	<1	<1	<1
MC-LF	63.9 ± 7.1 [a]	66.4 ± 12.0 [b]	70.2 ± 4.7 [c]	<1	<1	<1

[a] Concentration level 1.5 ng/L. [b] Concentration level 7.5 ng/L. [c] Concentration level 15 ng/L.

The final sample pre-treatment involves cyanotoxins extraction and pre-concentration applying ultrasonication and two sequential SPE procedures. To disrupt cells and release the intracellular toxins, 300 mL of each freshwater sample was sonicated in an ultrasonic bath (30 min, 200 W, 60 Hz). Then, the samples were centrifuged for 7 min at 3219.84 g. After this process, a 250 mL supernatant aliquot was collected and subjected to two sequential SPE procedures based, respectively, on Oasis HLB (500 mg, 6cc, Waters Corporation, Milford, MA, USA) and Supelclean™ ENVI-Carb™ (500 mg, 6cc, Supelco, Sigma-Aldrich, St. Louis, MO, USA) cartridges. In the first step of SPE, the HLB cartridges were used. They were conditioned with methanol and equilibrated with water (10 mL each). Then, 250 mL of the supernatant was loaded at 1 mL/min, and the elution was accomplished using 20 mL of methanol at 50 °C. The percolated sample is then collected, basified up to 0.1% ammonia, and then transferred to an ENVI-Carb™ cartridge, previously pre-conditioned with methanol and equilibrated with water containing 0.1% ammonium hydroxide (10 mL each). After loading at 1 mL/min under vacuum, elution was carried

out by back-flushing the cartridge with 20 mL of methanol at 50 °C containing 0.5% formic acid. Thus, the loading was sequential, and the extraction step was performed separately for each cartridge. Both Oasis HLB and ENVI-Carb™ extracts were then combined, dried in a Turbovap (Biotage) not entirely, but until the drop under a gentle stream of gaseous nitrogen at 25 °C, and re-dissolved in 500 µL acetonitrile:water (1:9, v/v) and stored at -20 °C until analysis.

1.2.1. Method validation

After method development, method validation was performed. In this case several quality parameters were evaluated including method limits of detection (MLODs) and quantification (MLOQs), matrix effect, and recoveries. Evaluated parameters are summarised in Table 13.

Table 13. *Quality parameters of the developed targeted method.*

Compound	MLOD, pg/L	MLOQ, pg/L	Mean recoveries, %			Matrix effect, %
			2 ng/L	10 ng/L	20 ng/L	
CYN	100	300	53.4	52.2	87.2	−59
ANA	20	60	81.6	70.2	87.8	17
MC-RR	4	12	72.2	62.8	66.6	−11
NOD	100	300	81.1	66.1	82.1	−35
MC-YR	100	300	71.6	73.6	70.6	−24
MC-LR	100	300	57.7	70.3	80.4	−26
MC-LA	100	300	82.8	70	80	−23
MC-LY	75	225	84.3 [a]	65.0 [b]	80.6 [c]	15
MC-LW	150	450	9.2 [a]	32.3 [b]	48.7 [c]	46
MC-LF	75	225	63.9 [a]	66.4 [b]	70.2 [c]	35

[a] Concentration level 1.5 ng/L. [b] Concentration level 7.5 ng/L. [c] Concentration level 15 ng/L

The MLODS and MLOQs were based on matrix-matched (in fortified AFW) calibration curve points. MLOD of each analyte was defined as the lowest concentration for which the peak area was, at least, three times the signal-to-noise, while the MQLs were established as the lowest concentrations which fulfilled the criteria: signal-to-noise ratio, at least, 10; relative standard deviation of three replicates, below 19%;

Gaussian peak shapes; less than 3 ppm of exact mass error; and molecular isotopic pattern accomplishing the standard ratio. MLODs and MLOQs for the targeted compounds ranged between 4–150 pg/L and 12–450 pg/L, respectively (Table 13). To author's knowledge, these are the lowest reported MLODs for the determination of cyanotoxins of different chemical classes (Table 3 in Introduction).

The recoveries of the developed method were evaluated comparing responses of compounds in extracted samples with that of extracts of matrix spiked with standards post extraction.

$$Recovery = \frac{A_{pre}}{A_{post}} \times 100\%,$$

where $\mathrm{A_{pre}}$ is the measured peak area of the matrix blank spiked before SPE, while $\mathrm{A_{post}}$ is the measured peak area of the matrix blank spiked after SPE in the reconstitution step.

The recoveries were assessed at three concentration levels (2, 10, and 20 ng/L for CYN, ANA, MC-RR, -YR, -LR, -LA, NOD; 1.5, 7.5, 15 ng/L for MC-LY, -LW, -LF). Experimental blanks were also analysed in all batch of samples. The mean recoveries at the lowest, medium, and highest concentration levels ranged between 53.4 – 84.3%, 52.2 – 73.6%, and 66.6 – 87.3%, respectively, for nine compounds. Recoveries of MC-LW were lower and in agreement with the results obtained in previous studies [70]. As can be seen in the Table 13, the recovery values were often different for different concentration levels. Overall, it is difficult to compare recovery values with other methods directly, because recoveries at different concentration levels were evaluated in different studies. Since different recoveries were recorded at different concentration levels for some compounds in this thesis, recoveries between different studies cannot be compared directly. For example, recoveries were evaluated at 100 ng/L level in one of the studies with application of two sorbents [70],

while herein the maximal spiking level was 20 ng/L. Additionally, in methods where NOD was used as a surrogate standard [54, 59, 72], relative recoveries are mentioned. If surrogate is applied the relative recoveries are quantified as:

$$Relative\ recovery = \frac{Recovery_{MC}}{Recovery_{NOD}} \times 100\%.$$

In this case, relative recoveries are higher than the absolute ones. For example, if in this work NOD would be applied as a surrogate, recoveries of MC-LR at 20 ng/L would be 97.9% instead of 80.4%. Thus, it is another complication for comparison of recoveries across different methods. Overall, recoveries were comparable with other methods were more than one sorbent was applied [67-70].

The matrix effect was evaluated in order to determine a possible signal enhancement or ion suppression that could appear during the ionization process in liquid phase due to presence of interferences in natural waters. To evaluate the matrix effects, measured peak areas of compounds in experimental blank (applying AFW) and in pure solvent were compared. The following equation was applied:

$$Matrix\ effect = \frac{A_{AFW}}{A_{solvent}} \times 100\%,$$

where $\mathrm{A_{AFW}}$ is the measured peak area of the compound in the experimental blank, while $\mathrm{A_{solvent}}$ is the measured peak area of the compound in solvent. Both positive and negative matrix effects were obtained, which means that both ion enhancement and suppression were observed. The values ranged between -59 to 46% at concentration of 20 ng/L. To tackle this issue of the matrix effects, matrix-matched calibration curves were used for the quantification of targeted compounds in real samples.

This method was applied for analysis of surface water samples from Spain and will be discussed in the section 3.2.1.

2. SPE-UHPLC-HRMS/MS method transfer and modification at Eawag

As was mentioned before, part of the research of this thesis was done during a scientific stay at Eawag (Switzerland). Thus, the developed method was transferred according to the instrumentation available there. This resulted in the proposal of two methods in this thesis for the targeted screening of cyanotoxins. The main difference between these two methods is that one of them is focused on cyanopeptides (mainly MCs) and enables suspect screening for other groups of cyanopeptides. In the second method, several extra targets were added, as reference standards for these compounds were available at Eawag. In this chapter, only the targeted approach of this method will be commented. The suspect screening approach will be addressed in the next chapter.

In the course of the method transfer several parts were changed. One of such is the LC column. UHPLC column had to be exchanged back to an HPLC column as stainless steel capillaries were not available to connect autosampler with the LC system. When UHPLC column is applied, naturally, higher pressure occurs. Thus the pressure can reach up to 600 bar, depending on the method. When HPLC columns are applied, the pressure can reach about 300 bar. In case of lower pressure, peek capillaries are sufficient to resist the pressure generated in the system. However, for higher pressure only stainless steel capillaries must be used. Thus, in order to avoid this problem and be able to perform the analysis of toxins, an HPLC column was used instead of an UHPLC column.

Another thing that was changed is the MS analyser. Instead of a Q-Exactive instrument, Q-Exactive Plus was applied. Comparing with Q-Exactive, the Exactive-Plus allows resolution up to 240,000 FWHM (at 200 *m/z*). However, we did not

change the resolution. However, since a different instrument was used, collision energies were slightly adjusted for the targeted screening.

Regarding the method that enables suspect screening, scan range was also changed, as the main focus was on the analysis of cyanopeptides. Thus, lower *m/z* values were not relevant, and would just prolong data analysis time during the suspect screening. In this case, a different approach for fragmentation was applied. Since the method's inclusion list consisted of more than 1000 ions, and CE for each compound was not possible to apply, stepped fragmentation was applied for all suspects. Stepped fragmentation is normally applied for analysing mixtures of compounds, when a variation of CE is required. During this process, several normalised CE ("steps") are applied.

Even though some instrumental parameters were changed, sample pre-treatment (sonication and dual SPE) of cyanotoxins remained the same, and thus, it is not commented in this section.

In the next sections, the employment of both targeted methods applied at Eawag, for cyanotoxins and for cyanopeptides, respectively, are covered in more detail.

2.1. Targeted screening of cyanotoxins (full scan data acquired from 90 to 1100 *m/z*)

After performing dual SPE, evaporation and re-constitution, extracted cyanotoxins were injected into an HPLC system (Ulti-mate3000, Dionex, ThermoFisher Scientific) using a CTC PAL autosampler fitted with a 20 µL stainless sample loop; the injection volume was 20 µL. For targeted screening of the 10 cyanotoxins, chromatographic separation was carried out on an Atlantis T3® column (3 × 150 mm, 3 µm particle diameter) fitted with pre-column (VanGuard® Cartridge) and in-line filter (BGB®). This column was chosen because it showed good performance for various compounds in targeted and suspect screening. The mobile phase consisted of nanopure water

(solvent A) and acetonitrile (solvent B) both acidified with FA (0.05%) as in the original method. Several gradients were tested for the optimisation of separation, and the final gradient profile was the following: 0/5/55/75/95/95/5% B at 0/1/10/20.5/24/26/26.1 min, respectively; and column was re-equilibrated for 3.4 min under the initial conditions. The flow rate was 0.3 mL/min. Since the flow rate was the same as the one employed in the previous UHPLC method developed using a Hibar® column, HESI parameters were only slightly corrected based on the response of each toxin. The optimal conditions were the following: +4 kV spray voltage, 325 °C capillary temperature, 35 a. u. sheath gas, 17 a.u. auxiliary gas, 1 a.u. spare gas, 275 °C probe heater temperature. In this case, the scan rage was the same as in the previously developed UHPLC-HRMS/MS method. Full-scan MS data were acquired between 90 to 1100 *m/z* with a resolving power of 70,000 FWHM (at 200 *m/z*), AGC target of 1e6, and maximal injection time of 100 ms with 1 ppm mass accuracy. For fragmentation, ddMS2 mode was employed at a resolving power of 17,000 FWHM, AGC target of 1e5, and maximal injection time of 50 ms. Fragmentation acquisition was optimized for target compounds due to method transfer. Table 14 summarises standard analytical information including retention time, precursor ions, product ions and collision energies. For majority of the compounds the precursor ions were the same. However, for MC-LR and -YR double-charged ions were more abundant than single-charged ones. As was mentioned before for these two compounds, protonation occurs on both the methoxy residue of the Adda side chain and arginine residue [176]. Obtained optimal CE values were different. Both variation in the precursor ions and CE values can be explained by the fact that different instrumentation is employed.

Table 14. *Standard analytical information including retention time, precursor ions, product ions, and collision energies for 10 targeted compounds applying Atlantis T3® column.*

Toxin	Retention time (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	CE (eV)
CYN	5.30	416.1241 $[M + H]^+$	336.1664 $[M + H - SO_3]^+$	25
ANA	5.50	166.1229 $[M + H]^+$	149.0959 $[M - NH_3 + H]^+$	10
MC-RR	9.54	519.7902 $[M + 2H]^{2+}$	135.0803 $[C_9H_{11}O]^+$	27
NOD	10.42	825.4518 $[M + H]^+$	135.0803 $[C_9H_{11}O]^+$	50
MC-YR	10.86	523.2712 $[M + 2H]^{2+}$	135.0803 $[C_9H_{11}O]^+$	10
MC-LR	10.93	498.2819 $[M + 2H]^{2+}$	135.0803 $[C_9H_{11}O]^+$	10
MC-LA	17.29	910.4904 $[M + H]^+$	776.4176 $[M + H - C_9H_{10}O]^+$	20
MC-LY	17.64	1002.5177 $[M + H]^+$	868.4444 $[M + H - C_9H_{10}O]^+$	20
MC-LW	20.54	1025.5334 $[M + H]^+$	891.4594 $[M + H - C_9H_{10}O]^+$	20
MC-LF	21.58	986.5253 $[M + H]^+$	852.4490 $[M + H - C_9H_{10}O]^+$	20

2.1.1. Method performance

Since the sample pre-treatment was the same as in the original method, same recoveries were applied.

Several instrumental parameters were evaluated to assess the performance of the method. Full validation was not carried out due to the fact that equipment was similar to the one at IDAEA-CSIC and UB. Thus, instrumental LODs, LOQs, and linearity were evaluated. Here, calibrants in mobile phase (initial conditions) were prepared in the range of 0.05 and 50 µg/L. Linear regression models of the calibration curves were determined. LOQ and LOD were calculated from the regression models as three or ten times, respectively, the standard deviation of the response, divided by the slope parameter. Table 15 summarises LODs. LOQs and linearity for the reference standards in mobile phase (nanopure water). LOD and LOQ ranged in the high ng/L to low µg/L range. These levels were lower than the ones achieved with UHPLC column, but low enough for assessment of targeted compounds. Linearity was also determined from linear regression models as the squared Pearson index (R^2), obtaining good linearity values (R^2 lower than 0.99).

Table 15. *LODs, LOQs and linearity of 10 targeted cyanotoxins in nanopure water applying Atlantis T3® column.*

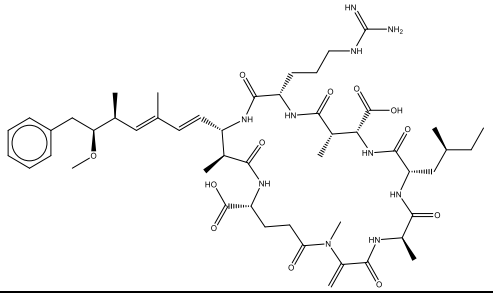
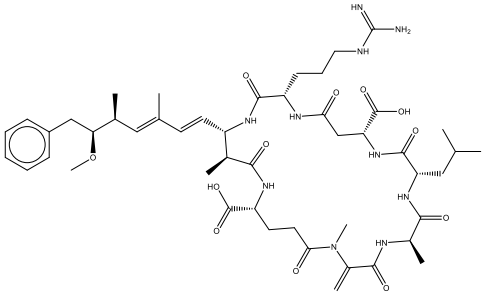
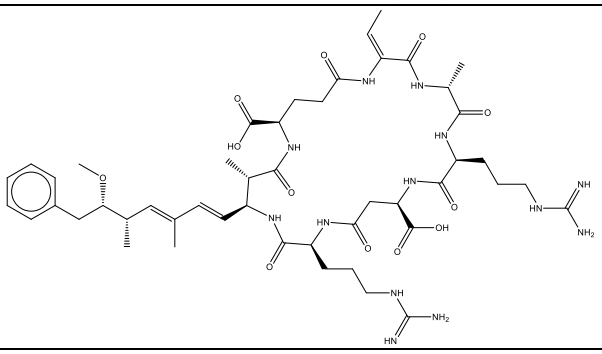
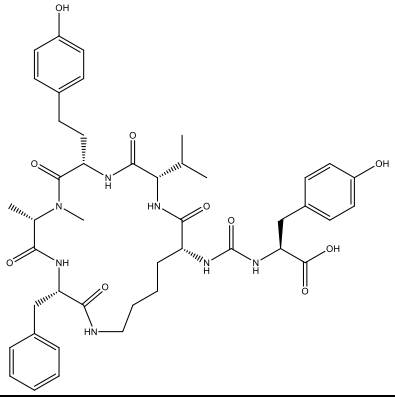
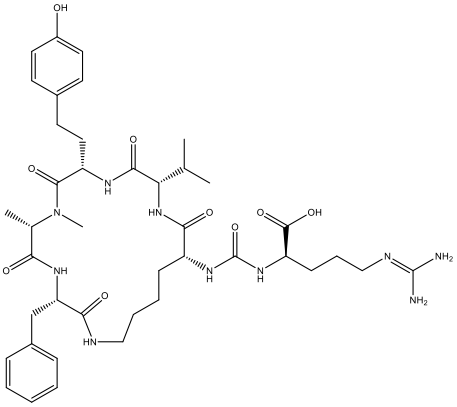
Toxin	LOD (µg/L)	LOQ (µg/L)	Linearity, R^2
CYN	0.02	0.05	0.9997
ANA	0.08	0.23	0.999
MC-RR	0.02	0.08	0.9994
NOD	0.34	1.02	0.9952
MC-YR	0.02	0.06	0.9999
MC-LR	0.03	0.09	0.9991
MC-LA	0.3	0.91	0.9962
MC-LY	0.41	1.26	0.9982
MC-LW	0.37	1.12	0.9942
MC-LF	0.18	0.55	0.9986

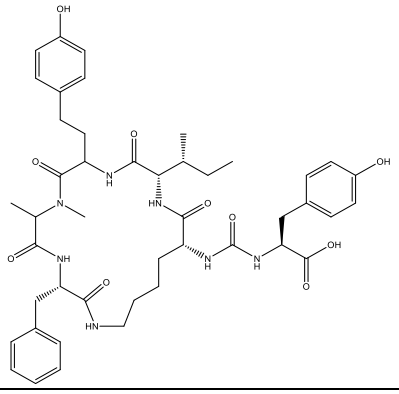
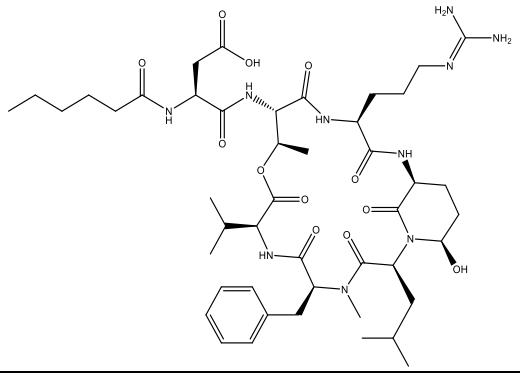
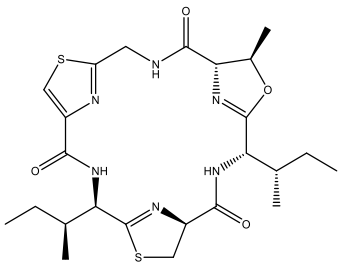
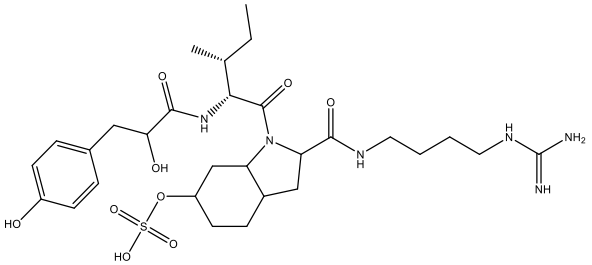
This method was applied for analysis of freshwater samples from Switzerland, and obtained results discussed in section 3.2.2 of this chapter.

2.2. Targeted screening of cyanopeptides (full scan data acquired from 450 to 1350 m/z)

As was mentioned before, a different method was employed for the analysis of cyanopeptides, which also included suspect screening for other cyanopeptides (the suspect screening method will be addressed more in depth in the next chapter – Suspect Screening). Several compounds were added to the targeted screening because cyanopeptides reference standards (additionally to the original 10 targeted cyanotoxins) were also available. The added compounds were MC-HilR, [D-Asp^3]MC-LR, [D-Asp^3,(E)-Dhb^7]MC-RR, anabaenopeptin A, anabaenopeptin B, oscillamide Y, cyanopeptolin A, aerucyclamide A, and aeruginosin 98B. Their structures and elemental compositions are summarised in Table 16. Thus, overall, targeted screening included 17 cyanopeptides (MC-LR, -RR, -YR, -LA, -LF, -LY, -LW, NOD were from the original targeted method.

Table 16. *Structures and elemental compositions of cyanopeptides that were added to the targeted method.*

Cyanopeptide	Elemental composition	Structure
MC-HilR	$C_{50}H_{76}N_{10}O_{12}$	
[D-Asp^3]MC-LR	$C_{48}H_{72}N_{10}O_{12}$	
[D-Asp^3,(E)-Dhb^7]MC-RR	$C_{48}H_{73}N_{13}O_{12}$	
Anabaenopeptin A	$C_{44}H_{57}N_7O_{10}$	
Anabaenopeptin B	$C_{41}H_{60}N_{10}O_9$	

Oscillamide Y	$C_{45}H_{59}N_7O_{10}$	
Cyanopeptolin A	$C_{46}H_{72}N_{10}O_{12}$	
Aerucyclamide A	$C_{24}H_{34}N_6O_4S_2$	
Aeruginosin 98B	$C_{29}H_{46}N_6O_9S$	

Similarly as in the transferred method mentioned above, after performing dual SPE, evaporation and re-constitution, extracted cyanotoxins were injected into an HPLC system (Ulti-mate3000, Dionex, ThermoFisher Scientific) using a CTC PAL autosampler fitted with a 20 µL stainless sample loop; the injection volume was 20 µL. Chromatographic separation was performed on a Kinetex® column (2.1 × 100 mm, 2.6 µm particle diameter). fitted with a SecurityGuard C18 guard cartridge and in-line filter (aluminium frit, 0.7 µm pore diameter) at 40 °C. The mobile phase

consisted of nanopure water (solvent A) and methanol (solvent B) both acidified with FA (0.05%), which were used to generate the following binary gradient elution profile: 0/20/50/70/100/100/20/20% B at 0/1.5/5.5/21.4/21.5/26/26.1/30 min, respectively, at a flow rate of 0.255 mL/min. The applied LC method (column, gradient, temperature) was based on the method for suspect screening for cyanopeptides in freshwater, which was previously developed within Dr. Janssen's research team. Since this method was developed on different instrument and different instrumental configuration (on-line SPE-HPLC-HRMS/MS), elution gradient was slightly adjusted according to the pump system applied in the instrumentation used for the current method.

Since the flow rate was lower than those applied for the two previously commented methods in this thesis (applying Hibar® and Atlantis T3® columns) HESI voltage was adjusted accordingly, and the optimal parameters were the following: +3.5 kV spray voltage, 325 °C capillary temperature, 35 a.u. sheath gas, 17 a.u. auxiliary gas, 1 a.u. spare gas, and 275 °C probe heater temperature.

As was mentioned before, several changes in MS parameters were introduced. The scan range was changed because the focus was on cyanopeptides only, which have higher molecular weights in comparison to ANA and CYN. Full-scan MS data were acquired from 450 to 1350 *m/z* at 70,000 FWHM (at 200 *m/z*) resolution with AGC of 5e5, 100 ms maximum ion injection time, 1 microscan, and 60% S-lens RF setting. For analytes detection, a data-dependent top-n MS2 acquisition procedure was again used. Data-dependent MS2 scans were triggered for the top-3 most-intense ions (with intensity > 2e4) from the preceding full scan. The applied parameters were the following: profile acquisition mode, 17,500 FWHM resolution, 1 *m/z* isolation window (0 *m/z* offset), 1 microscan, 5e4 AGC target, 70 ms maximum ion injection time, 5 s dynamic exclusion, 'True' for 'pick others'. As was mentioned before, in this method

CE was not optimized for each compound. Instead, stepped normalized collision energies of 15, 30, and 45% was applied. The scan range for MS2 events was dynamically adjusted (by the instrument) based on the target ion's *m/z* value. In total, the suspect screening method included 1219 cyanopeptides (as previously commented, more details about the suspect screening method will be addressed in Chapter 3).

2.2.1. Method performance

It is worth mentioning that recoveries for newly added compounds were not evaluated, even though the same sample pre-treatment (sonication and dual SPE extraction) was performed. The reason is that the original 8 cyanopeptides were still the prime objective of this thesis. However, the availability of the standards at Dr. Janssen's research team could not be ignored, as it provided an opportunity to analyse and confirm other compounds. Additionally, new compounds were enabling class-equivalent quantification of other cyanopeptides (new classes such as anabaeneopeptins, cyanopeptolins, aerucyclamide, and aeruginosin were added). One more reason for not evaluating recoveries of additional cyanopeptides was the scarcity of time during the scientific stay. Hence, since in this method, recoveries were evaluated only for 8 cyanopeptides, and both types of cyanopeptides (with and without evaluated recoveries) were detected in analysed fresh water samples, recoveries were not applied. This was done to provide homogeneous data - to maintain relative proportion of targeted cyanopeptides for comparison of concentrations.

To process HPLC–MS/MS data files, Compound Discoverer version 3.1.0.305 was applied for targeted screening. In this case, peak integration was done automatically by the software, and the data extraction parameters are explained in the next chapter (Suspect Screening) as the same procedure was applied.

Similarly to the first transferred method, only several instrumental parameters were evaluated to assess the performance of the method. For each compound instrumental LOD, LOQ, and linearity were evaluated. In this case, to support cyanopeptide quantification, linear regression models of the calibration curves were determined. Matrix-matched calibrants were prepared in the range 5–125 µg/L for samples from three water reservoir from the United Kingdom, using water derived from the samples as calibrant matrix. Also calibrants in the mobile phase were prepared in the range 0.5–500 µg/L. To prepare the calibrants, reference standards of 10 microcystins and nodularin as well as 6 bioreagents of additional cyanopeptides were employed. In Table 17, the dominant precursor ions and the limits of detection and quantification in nanopure water and lake matrices (Ingbirchworth, Tophill Low, and Embsay) are listed**.** LODs and LOQs were calculated from the regression models as three or ten times, respectively, the standard deviation of the response, divided by the slope parameter. For nanopure water, the LODs were between 0.02 and 1.01 µg/L, what is low enough for this part of work. It can be seen, that LODs applying matrix-matched calibrants were higher than those obtained in nanopure water. This can be attributed to the matrix effect. Additionally, t-test statistics was carried out in order to evaluate whether the difference of application calibrants in nanopure water and in matrix was significantly different or not ($p > 0.05$). It was significantly different, for majority of compounds in three different matrices. Thus, matrix-matched calibration was applied for quantification.

Table 17. *Standard analytical information including LODs and LOQs in µg/L for the reference standards and bioreagents in nanopure water and lake water from three reservoirs from the United Kingdom.*

Cyanopeptide	Dominant precursor	Nanopure		Ingbirchworth		Tophill Low		Embsay	
		LOD (µg/L)	LOQ (µg/L)	LOD (µg/L)	LOQ (µg/L)	LOD (µg/L)	LOQ (µg/L)	LOD (µg/L)	LOQ (µg/L)
MC-LR	$[M+H]^+$	0.23	0.71	1.32	3.99	2.92	8.84	0.74	2.26
MC-RR	$[M+2H]^{2+}$	0.31	0.94	1.40	4.24	1.45	4.38	0.58	1.76
MC-YR	$[M+2H]^{2+}$	0.23	0.71	1.31	3.98	0.95	2.88	1.64	4.97
MC-LA	$[M+H]^+$	0.30	0.89	1.26	3.82	2.02	6.13	1.11	3.38
MC-LF	$[M+H]^+$	0.27	0.83	1.66	5.04	1.44	4.35	1.74	5.28
MC-LY	$[M+H]^+$	0.24	0.72	1.40	4.23	1.68	5.08	1.97	5.97
MC-LW	$[M+H]^+$	0.27	0.83	1.76	5.34	1.37	4.16	1.43	4.33
MC-HilR	$[M+H]^+$	0.24	0.71	0.88	2.66	1.82	5.50	0.93	2.83
$[D\text{-}Asp^3]$MC-LR	$[M+H]^+$	0.27	0.81	0.70	2.11	1.09	3.31	0.62	1.89
$[D\text{-}Asp^3,(E)\text{-}Dhb^7]$MC-RR	$[M+2H]^{2+}$	0.05	0.15	1.25	3.80	0.67	2.02	n.a.	n.a.
NOD	$[M+H]^+$	0.25	0.76	1.06	3.23	2.09	6.34	0.47	1.43
Anabaenopeptin A	$[M+H]^+$	0.25	0.75	11.96	36.26	1.63	4.94	1.06	3.21
Anabaenopeptin B	$[M+H]^+$	0.29	0.88	4.43	13.43	1.21	3.67	0.56	1.70
Oscillamide Y	$[M+H]^+$	0.27	0.81	3.31	10.02	1.76	5.34	1.80	5.47
Cyanopeptolin A	$[M+H]^+$	1.01	3.06	2.18	6.62	1.66	5.04	2.35	7.11
Aerucyclamide A	$[M+H]^+$	0.24	0.74	2.17	6.58	0.92	2.78	3.19	9.66
Aeruginosin 98B	$[M+H]^+$	0.35	1.05	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

n.a. = not analysed

Linearity was also determined from linear regression models as the squared Pearson index (R^2), obtaining again very good results (R^2 below 0.99, except for cyanopeptolin A). Obtained linearities are summarised in Table 18.

Table 18. *Linearity for the reference standards and bioreagents in nanopure water and lake water from three reservoirs from the United Kingdom.*

Cyanopeptide	Linearity, R^2			
	Nanopure	Ingbirchworth	Tophill Low	Embsay
MC-LR	0.9960	0.9999	0.9998	0.9999
MC-RR	0.9939	0.9999	0.9993	0.9999
MC-YR	0.9959	0.9998	0.9999	0.9996
MC-LA	0.9937	0.9999	0.9997	0.9999
MC-LF	0.9944	0.9998	0.9998	0.9997
MC-LY	0.9958	0.9998	0.9996	0.9995
MC-LW	0.9945	0.9997	0.9998	0.9998
MC-HilR	0.9958	0.9999	0.9995	0.9999
[D-Asp^3]MC-LR	0.9947	0.9999	0.9998	0.9999
[D-Asp^3,(E)-Dhb^7]MC-RR	0.9954	0.9966	0.999	n.a.
NOD	0.9953	0.9999	0.9996	0.9999
Anabaenopeptin A	0.9955	0.9875	0.9996	0.9998
Anabaenopeptin B	0.9938	0.9983	0.9998	0.9999
Oscillamide Y	0.9947	0.999	0.9997	0.9997
Cyanopeptolin A	0.9292	0.9996	0.9998	0.9995
Aerucyclamide A	0.9956	0.9996	0.9999	0.9991
Aeruginosin 98B	0.9912	n.a.	n.a.	n.a.

n.a. = not analysed

This method was applied for analysis of freshwater samples from the United Kingdom and it is described in section 3.2.3 of this chapter.

3. Method application

In this section, three method application studies are discussed. Targeted methods were applied for analysis of freshwater samples from 3 countries: Spain, Switzerland, and the United Kingdom. In case of Spain, surface water samples were collected from three water reservoirs. In Switzerland, samples from both surface water and from the depth of 3 meters were collected from Greifensee Lake. Regarding the

samples from the United Kingdom, they were collected at the entrance of drinking water treatment plants from three locations.

3.1. Study sites, sample collection, and storage

Targeted methods were applied for the analysis of fresh water samples from 3 countries: Spain, Switzerland, and the United Kingdom. Figure 16 summarises the sampling periods. The first method based on UHPLC-HRMS/MS was applied for the determination of 10 cyanotoxins in the analysis of freshwater from Spain from three water reservoirs from Ter river basin. The second method – targeted screening (full scan data acquired from 90 to 1100 *m/z*) – was applied for the determination of 10 cyanotoxins in the analysis of freshwater samples from Switzerland from Lake Greifensee, and the analysis of samples from the United Kingdom for ANA and CYN. Cyanopeptides in freshwater samples from the United Kingdom were assessed by the third method where full scan data was acquired from 90 to 1100 *m/z*. As was mentioned before, several other cyanopeptides were added. Freshwater samples from the United Kingdom were raw drinking water samples from Ingbirchworth, Tophill Low, and Embsay reservoirs.

Analysis of samples from Switzerland and the United Kingdom was performed at Eawag in Dr. Janssen's team during the scientific stay. The last part (the samples from the United Kingdom) was also done in collaboration with water company Yorkshire Water. John Haley of Yorkshire Water is one of the consortium members of European Training Network NaToxAq.

Details on each sampling sight, sample collection, measured parameters, and storage are mentioned bellow.

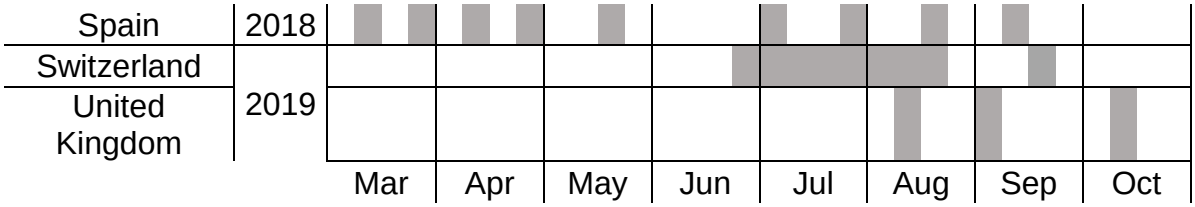


***Figure 16**. Sampling periods.*

3.1.1. Study sites in Spain

Samples from three water reservoirs of Ter river basin were collected. The Ter River originates in the Catalan Pyrenees, and falls into the Mediterranean Sea. In total, its length is 167 km. Ter River contains a system of reservoirs of Sau-Susqueda-Pasteral, and it serves urban water supply (being the main water supply systems for the metropolitan area of cities such as Barcelona and Girona), irrigation and production of hydroelectric energy. Before the reservoirs, Ter river is impacted by several industries (metallurgic, pulp mill, textile and tannery industries) [55]. Additionally, Sau and Susqueda reservoirs are used for recreational activities.

Sau (41°58′5″N 2°24′47″E) is the first of the three reservoirs. Created dam enables storage capacity of 216 hm^3, the water covers former town of Sant Romà de Sau. The second water reservoir is Susqueda (41°58′45″N 2°31′38″E) with a storage capacity of 233 hm^3. Pasteral (41°59′3.95″N 2°36′4.28″E) is the last water reservoir, and its storage capacity is 153 hm^3. Susqueda and Sau are the largest reservoirs of the system, however, the water catchment is located in Pasteral [55].

The dam of Sau water reservoir (60 m height) has a spillway, and thus bottom side drains allow water to pass to Susqueda. The half-bottom drain (located at the same heights as the other two but in the centre of the dam) is equipped with Howell Bunger valves that allow oxygenation [55].

Twenty-seven grab surface water samples were collected at depth of 0-20 cm at each water reservoir between March and September 2018 (Figure 16). The exact location

of sampling points is given above. Sampling period was expected to cover the months of both algal bloom peaks according to the literature review of seasonal variations of cyanotoxins in Mediterranean climate zone (Introduction section 5.3).

The samples were collected in 2 L amber glass bottles. The pH, temperature, pO_2, and conductivity were measured on-site and summarized in Table 19. Samples were transported at 4 °C and then were frozen at -40 °C until the analysis. Samples were analysed in triplicate (technical), except for Samples from Susqueda on 28.03.2018, 24.04.2018, and Pasteral on 24.04.2018. These samples were analysed in duplicate.

Table 19. *Chemical physical parameters of the sampling points in Ter river basin.*

Reservoir	Date	Temperature, °C	Conductivity	pH	Oxygen, mg/L	Oxygen, %
Pasteral	28.03.2018	11.1	312.4	9.3	20.91	190.3
	13.04.2018	16	302.2	9.2	7.6	69.8
	25.05.2018	18	348.4	8.1	9.6	107.1
	17.07.2018	18.5	302	8.4	8.4	77.2
	24.08.2018	22.3	256	8.4	5.6	51.5
	20.09.2018	20.3	286	8.2	5	45.9
Susqueda	28.03.2018	9	306.3	8.9	8.2	75.3
	13.04.2018	7.2	423	8.5	6.2	57
	25.05.2018	13.5	451	7.6	7	64.3
	17.07.2018	17.2	394	7.8	8.3	76.3
	24.08.2018	20.5	384.2	8.2	9.3	85.4
	20.09.2018	21.7	426	8.2	10.9	100.2
Sau	28.03.2018	6.1	321.2	8.9	13.6	125
	13.04.2018	8.2	345.8	8.4	7.3	67.1
	25.05.2018	20.8	332.9	8.1	8.29	92.7
	17.07.2018	19.2	306.2	8.1	7.6	69.8
	24.08.2018	21.6	278.1	8.6	5.3	48.7
	20.09.2018	20.4	326	8.2	6.3	57.9

3.1.2. Study site in Switzerland

Greifensee (47°21′N 8°41′E) is a prealpine lake with a surface area of 8.45 km^2 and a maximum depth of 32 m [194]. The main supply river is the Aabach River. The lakeside of Greifensee is under UNESCO protection. This lake was chosen for sampling because Eawag in-house sampling campaign was already running there,

and thus it was easier to join and contribute to the running research as Dr. Janssen's team is focused on cyanopeptides, and does not study low molecular weight cyanobacterial metabolites such as ANA and CYN. Moreover, several departments of Eawag also study this site. In this case, sampling was done in collaboration with researches of Aquatic Ecology Department. Group supervised by Dr. Francesco Pomati studies phytoplankton and lake-ecosystem using innovative approaches such as monitoring lake station Aquaprobe (more information can be found at www.eawag.ch within Aquatic Ecology Department projects). This lake station is equipped with plankton camera that allows plankton microbes' imaging in their natural environment (www.aquascope.eawag.ch). And cyanobacterial community and its secondary metabolites were monitored and studied in collaboration with Dr. Janssen's group.

Sampling was performed at two depths levels: surface (0-20 cm) and at the depth of three meters where the plankton camera is located. This would provide an opportunity to compare cyanotoxins and cyanobacterial dynamics, and compare levels of cyanotoxins in different water layers. Thus, eight surface water and samples from the three-meter depth were collected weekly between June and August 2019, samples from September were collected only at three-meter depth (Figure 16) next to the monitoring station. Samples were collected in glass bottles and transported to the laboratory in coolers, in the dark, where the samples were kept in the fridge, in the dark for 18-20 hours and processed the next day. Then the extracts were kept frozen and evaporated and re-constituted before the analysis by LC-MS.

3.1.3. Study sites in the United Kingdom

As was mentioned above, sampled water reservoirs were Tophill Low (53°54′59.2416" N, 000°22′20.4024" W), Ingbirchworth (53°32′59.3808" N, 001°40′40.9800" W), and Embsay (53°59′13.9344" N, 002°00′11.1492" W). Tophill

Low consists of 2 water reservoirs D and O (due to their shape) with volumes, maximal depth, and surface area of 900 million L (ML) and 773 ML, 3.8 m and 6.1 m, and 0.239 km^2 and 0.141 km^2, respectively. Each of these reservoirs provides 45 ML per day (ML/d) of drinking water. The Ingbirchworth reservoir is the biggest reservoir, its volume is1370 ML, maximal depth of 18.5 m, and a surface area of 0.235 km^2. Embsay has a volume of 797 ML, maximal depth of 15 m, and a surface area of 0.11 km^2. Both Ingbrichworth and Embsay provide 20 ML/d of drinking water each.

The primary purpose of all three reservoirs is to serve as drinking water sources, however, they are also used for recreational activities, including bird watching, game bird shooting, and sailing. At Tophill Low, water from the River Hull is pumped into both storage reservoirs, which are operated in series, and water is abstracted for treatment after about 30 days retention time via subsurface draw-offs at around 4 m depth. Ingbirchworth reservoir receives water from the Blackwater Dike, which enters the treatment plant under gravity via a draw-off tower at a depth of around 14 m. Embsay reservoir receives water from both Lowburn Gill and Moor Beck, and here water again enters the treatment plant by gravity via a draw-off tower at a depth of around 12 m.

Samples were collected at the inlets to the respective water treatment facilities operated by Yorkshire Water on August 13th, September 3rd, and October 10th 2019 (Figure 16); in each case travel time of the raw water storage at the reservoir to the treatment plant is only a few minutes, thus, the samples were representing water from the raw water storage.

For biological analyses, 1 L water samples were kept at 4–8 °C in the dark. Taxonomic analysis by microscopy was performed on the following day after sampling and chlorophyll-a concentrations were assessed by filtration and solvent extraction followed by spectrophotometric measurement. Additionally, total

ammonium, nitrate, and total phosphate were also spectrophotometrically measured. These data were provided by Yorkshire Water and mentioned in suspect screening section.

For cyanotoxin and cyanopeptide analysis, samples were collected in green polyethylene terephthalate bottles and transported to the laboratory in coolers, in the dark, where the samples were frozen and shipped to Eawag. The samples were stored frozen until the analysis.

3.2. Occurrence of targeted compounds in surface water reservoirs

3.2.1. Samples from Spain

In twenty-seven samples form Sau-Susqueda-Pasteral system of reservoirs, only MC-RR was detected among the 10 targeted cyanotoxins. Overall, MC-RR was detected in 22% of samples at ng/L level. Table 20 summarises information on MC-RR determined concentrations. The toxin was most frequently found in Susqueda reservoir, with maximal concentration of 1.4 ng/L in March. In Sau and Pasteral, cyanopeptide MC-RR was detected only once in September at 1 ng/L and in April at 1.2 ng/L levels respectively. MC-LR was detected at the levels below LODs and thus not reported.

Table 20. *Concentration of MC-RR in ng/L (±standard deviation) in a system of reservoirs of Sau-Susqueda-Pasteral.*

Date	Sau	Susqueda	Pasteral
28.03.2018	n.d.	1.36±0.02	n.d.
24.04.2018	n.d.	1.19±0.02	1.19±0.05
23.08.2018	n.d.	1.34±0.08	n.d.
12.09.2018	1.00±0.06	1.3±0.1	n.d.

Comparing obtained data with the other seasonal studies in Mediterranean area (Table 4), most of the studies showed higher concentration levels at µg/L range. However, in a seasonal study in Greece in water sample from lake Pamvotis, same

concentration range of MCs was detected, reaching 3.4 ng/L [141]. Similarly to the Mediterranean seasonal variation pattern that has two peaking periods (March – May and August – October), highest concentrations of MC-RR in Susqueda reservoir were recorded in March and August – September, while the sampling period was between March and September (Figure 16). Since only several samples had targeted cyanotoxin, it is not enough information to evaluate seasonal variation pattern in comparison with the one obtained during literature review (Figure 13) for Mediterranean climate zone. More seasonal studies have to be carried out in surface water reservoirs of Mediterranean climate zone, in order to evaluate and enrich obtained pattern.

Low concentrations (ng/L range) of MCs in the reservoir system of Sau-Susqueda-Pasteral were also detected in the recently published study [55]. The sampling campaign was carried out three years earlier – in 2015. Thus, results obtained in this research project are in accordance with previous studies. Additionally, in the study carried out by Flores and Caixach [55], high (up to mg/L range) levels of anabaenopeptins were recorded in samples from Sau water reservoir. Taking into account this findings and the fact that full scan data was recorded at high resolution, retrospective suspect screening for several anabaenopeptins was carried out, and discussed in the next chapter.

3.2.2. Samples from Switzerland

Regarding the analysis of targeted toxins in 19 samples from Greifensee Lake, no targeted toxins were detected above limits of detection. MC-LA was found in the last two samples of the sampling campaign (from the 21st of August and from the 17th of September), however, the number of scan for peak was not enough to identify it as a peak and to quantify the amount of the toxin. Within Dr. Janssen's team sampling campaign was longer than the 17th of September, and higher concentrations of MC-

LA were recorded. Overall, the goal of the study that was carried out within Dr. Janssen's team was to study occurrence of a wider range of cyanopeptides in Greifensee, and thus suspect and targeted screening for wider range of cyanopeptides was carried out. Obtained results demonstrated the presence of other cyanopeptides. However, this data is not discussed in this thesis, and it is being prepared for publication.

Taking into consideration that targeted compounds were found starting from the end of August, and was recorded at higher concentration later in the season by our collaborators at Eawag, it is recommended to have longer sampling campaign at Greifensee Lake in order to improve research of occurrence of targeted cyanotoxins.

3.2.3. Samples from the United Kingdom

Analysis of cyanotoxin and cyanopeptide profile of raw drinking water collected from three freshwater reservoirs in the United Kingdom showed presence of 8 targeted compounds: 7 cyanopeptides and ANA.

Targeted compounds were found in Ingbirchworth and Tophill Low reservoirs, and no targeted cyanobacterial metabolites were found in Embsay reservoir. Figure 17 summarises the total concentration and number of identified cyanobacterial metabolites (cyanopeptides and ANA) recorded for both reservoirs and month. Overall, samples from Ingbirchworth reservoir had the highest concentration of cyanobacterial secondary metabolites reaching 3.6±0.4 µg/L in August, 36.2±1.2 µg/L in September, 0.27±0.01 µg/L in October. Overall, all cyanopeptides showed the same concentration pattern, with lower concentrations occurring in August and October, and the highest concentration in September in Ingbirchworth samples. Only one ANA was not following this pattern with overall lower concentrations in the ng/L range (26 ng/L in August, 12 ng/L in September, and 6 ng/L in October). Cyanopeptides in the Tophill Low reservoir were detected only in September,

reaching 90.8±2.3 ng/L. Total concentrations of cyanobacterial metabolites are within the range of concentrations that were detected in Oceanic climate during seasonal studies (up to 60 µg/L) (Table 4). September and October were peaking months for cyanotoxins' concentrations during seasonal sampling campaigns, what aligns with maximal concentrations detected in this thesis. However, in the current study only 3 sampling dates were chosen, and in order to improve the understanding of seasonal variation of cyanotoxins in these reservoirs, a more detailed temporal resolution is desired, for example with weekly frequency.

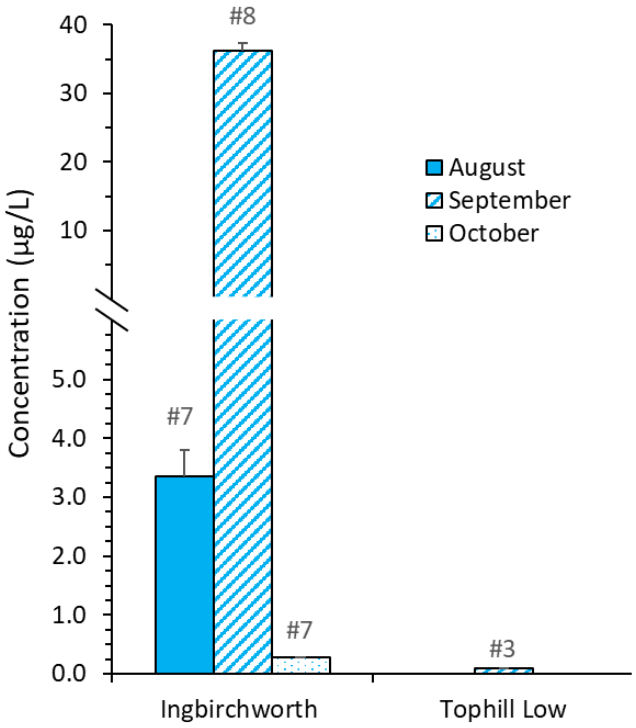


Figure 17. *Concentrations (µg/L) of cyanobacterial metabolites of two UK water reservoirs sampled in August, September, and October 2019. # at the top of each bar denotes the number of individual compounds identified. Error bars represent the standard deviation of triplicate analysis except for duplicates in September at Ingbirchworth.*

As for the diversity of cyanopeptides, compounds belonging to two classes were identified: MCs and anabaenopeptins. Figure 18 shows the concentrations of individual metabolites recorded in Ingbirchworth reservoir samples in September,

when the highest concentrations and greatest number of cyanobacterial metabolites were observed. Anabaenopeptins was the domination class representing the 72% of total number of identified compounds. Among the anabaenopeptins, the dominating compounds were anabaenopeptin B (12±2 µg/L), anabaenopeptin A (9.3±0.7 µg/L), and oscillamide Y (1.5±0.6 µg/L). Among the detected microcystins, the most abundant were MC-RR, [Dha^{7}]MC-LR, and MC-LR. The maximum MC-LR concentration recorded in Ingbirchworth reservoir was 1.8±0.2 µg/L, while the maximum total microcystin concentration summing all variants was 13±1 µg/L. Both of these values are below the provisional recreational water guideline value of 24 µg/L for MC-LR [60].

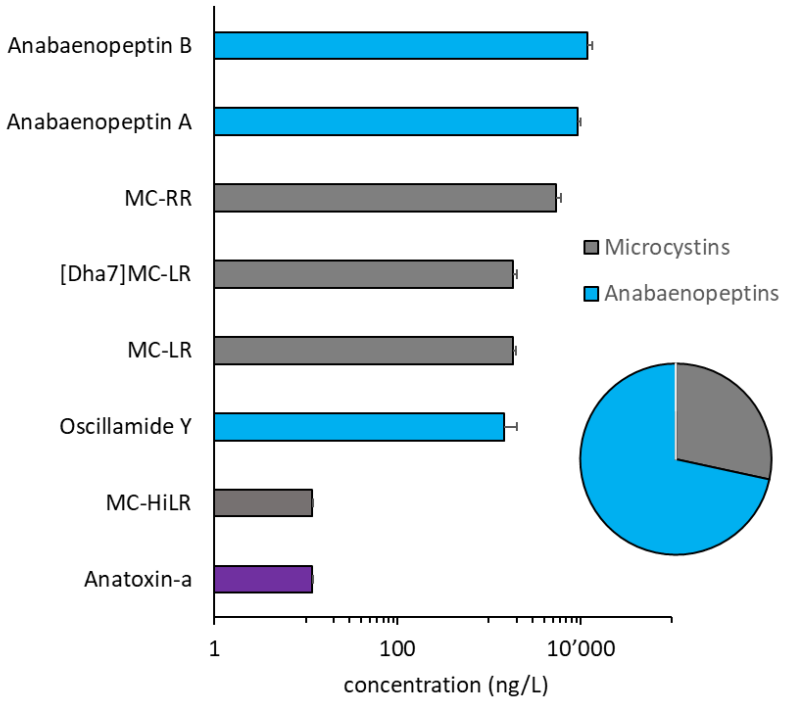
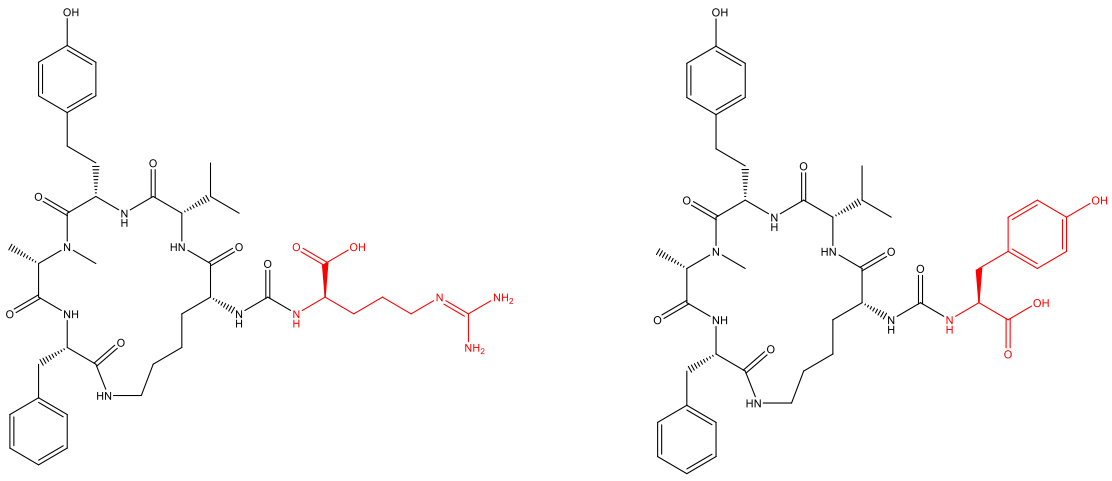


Figure 18. *Concentrations (ng/L) of individual cyanobacterial metabolites detected in September 2019 samples from Ingbirchworth reservoir. The relative proportion of major metabolite classes is presented in the inset pie chart. Error bars represent the standard deviation of triplicate samples.*

Notably, anabaenopeptins appeared at higher concentrations than MCs. The risks posed by anabaenopeptins is still unclear. They are not classified as cyanotoxins, yet do have inhibitory effects on enzymes (for this reason they are called bioactive compounds). Similarly to some MC congeners, anabaenopeptins have demonstrated to inhibit protein phosphatases, albeit with lower potency [195]. Additionally, anabaenopeptins were identified as potent inhibitors of carboxypeptidases and the concentrations recorded here (1.5-12 µg/L individual and >22 µg/L total), exceed the IC_{50} values for anabaenopeptin B (1 µg/L) by ten-fold (IC_{50} of 371 µg/L for anabaenopeptin A) [196]. The only difference between these two anabaenopeptins is the C-terminal amino acid outside of the cyclic structure, where anabaenopeptin B has an arginine while anabaenopeptin A has a tyrosine moiety (Figure 19).



Anabaenopeptin B Anabaenopeptin A

Figure 19. *Structures of anabaenopeptin B and anabaenopeptin A*

Table 21 summarises information about concentrations of individual cyanopeptide for the September sample from Tophill Low reservoir when, analogous to Ingbirchworth, the peak of concentrations was observed. In case of Tophill Low, only in September sample cyanobacterial metabolites were identified, which belong to the same class –

anabaenopeptins. Similarly to Ingbirchworth reservoir, anabaenopeptins were the dominating class (72% of total concentration of cyanobacterial metabolites). Compared to Ingbirchworth (23±2 µg/L) the anabaenopeptin concentrations were two orders of magnitude lower at Tophill Low, and bellow the range reported to be have inhibitory effects, as discussed above.

Table 21. *Concentrations of detected anabaenopeptins in Tophill Low September sample.*

Compound	Concentration, ng/L
Anabaenopeptin A	48.1±2.1
Anabaenopeptin B	23.3±0.6
Oscillamide Y	19.3±0.6

Overall, different results were obtained from three different sampling countries. Spanish and Swiss samples showed low (ng/L) or below LODs levels, while samples from the UK showed higher concentrations of cyanobacterial metabolites. When considering only the 10 targeted cyanotoxins which were included in all three targeted methods, concentrations in the samples from the United Kingdom were reaching 5.4±0.7 µg/L for individual MC. Total concentrations of MCs did not exceed WHO guideline level of relatively low probability of adverse health effects in recreational water.

While comparing diversity cyanobacterial metabolites from the three different locations, one common trend was observed: MCs co-occur with other cyanopeptides, in these cases with anabaenopeptins. Even though targeted for anabaenopeptins in the samples from Spain and Switzerland was not carried out in this thesis, presence of other cyanopeptides was recorded in the other studies of the same locations. What is more, in the samples from the United Kingdom, anabaenopeptins were the domination class in both reservoirs where targeted compounds were determined.

SUSPECT SCREENING

As was mentioned in the Introduction, cyanobacteria can produce a variety of other bioactive secondary metabolites, and more than 2000 secondary metabolites from cyanobacteria have been structurally identified to date [27]. These compounds belong to several classes including cyanopeptolins, anabaenopeptins, aeruginosins, aerucyclamides, and microginins. However, these cyanopeptides are not well studied yet, and compounds from these classes have shown acute toxicity in planktonic grazers and inhibition of various enzymes [26].

The goal of this part of the thesis was to study cyanopeptides beyond MCs in freshwater samples, their occurrence and concentration levels. Additionally, co-occurrence of targeted compounds and suspects is also discussed.

This method was applied for the analysis of freshwater samples from 3 reservoirs from the United Kingdom. Method application is described in section 3.1 of this chapter. For suspect screening, recently-formed database CyanoMetDB was applied. Additionally, after discussing occurrence of suspect cyanopeptides, seasonal trends of chlorophyll-a and cell counts in two reservoirs is discussed. The data on both of chlorophyll-a and cell counts was provided by Yorkshire Water.

As was mentioned in previous chapter, MS data obtained during targeted screening for cyanotoxins in Ter river basin were used for retrospective screening for several anabaenopeptins. It was done due to the reported results in recently published work by Flores and Caixach [55] , where authors observed presence of anabaenopeptins. Thus, it was decided to do a retrospective analysis to check if the same compounds could be found.

1. Suspect screening of cyanopeptides as part of targeted screening method (full scan data acquired from 450 to 1350 m/z)

As was mentioned in the previous chapter, suspect screening was integrated in the targeted screening method proposed at Eawag, and most of the instrumental parameters are mentioned in the previous chapter. In total, the inclusion list for the suspect screening consisted of 1219 cyanopeptides, including 160 microcystins, 177 cyanopeptolins, 73 anabaenopeptins, 65 cyclamides, 78 microginins, 79 aeruginosins, and 587 other compounds, accounting for structural isomers within the mass window of 450–1350 *m/z*.

1.1. Identification and quantification

In the case of the suspect screening method, identification and quantification of the compounds cannot be done in the same way as for the targeted screening one. The main reason is the absence of reference standards. When reference standard of a suspect is available on the market, after identification, reference standard will be purchased, included in the method, and the suspect will be confirmed. However, this is not possible for most of the cyanopeptides, as reference standards of these compounds are not available. Thus, class-equivalent approach for quantification was used and will be described later in this section.

As was mentioned in previous chapter, HPLC–MS/MS data files were processed applying Compound Discoverer version 3.1.0.305 for suspect screening. Peaks were integrated directly from Compound Discoverer software. Customized non-targeted work-flow based on CyanoMetDB (v01), a database of known cyanopeptides [27] were used for feature detection (extracting mass-chromatographic peaks), grouping (grouping of features with correlated retention time pro-files), deconvolution (assigning adduct and isotope annotations to grouped features), and compound annotation. Compound annotations were assigned based on one or more of the

following requirements: match between a) an experimental MS2 spectrum and one or more mzCloud library spectrum/spectra; b) an experimental MS2 spectrum and mzVault library spectrum/spectra, c) base ion (i.e. $[M + H]^{+}$ or $[M + 2H]^{+2}$) mass and one or more entries in the mass list, i.e., metabolites listed in CyanoMetDB or; d) predicted elemental composition and one or more formulae in the mass list. More details about parameters of the applied workflow can be found in Table 22, where parameters for each node can be found. During the next step, the features (i.e., mass-chromatographic peaks of dominant adducts + H, + Na,+ NH_4) associated with each annotation were checked to ensure Gaussian-like peak integration and an isotopic 'S-fit' greater than 50%. This step was performed manually. Where no peak was identified, e.g., in the blank samples, the 'Fill gaps' workflow node was activated, retaining filled peak areas associated with 'filled by re-detected peak' and 'filled by matching ion' flags. Overall, there are four "Filled gaps" flags additionally including 'filled by simulated peak' and 'filled by spectrum noise'. Figure 20 illustrates all four options for anabaenopeptin B and MC-LW. As can be seen, the first two flags integrate real peak, however, the last two flags do not. What is more, the magnitude of "not real" peaks can be as high as of the real ones. Such example can be seen on a) and c) parts of Figure 20. These wrong assignments of the peaks, created high RSD of the measurements. Thus only two flags were considered ('filled by re-detected peak' and 'filled by matching ion'), a custom Python script was used to extract data from the CD results file using SQL queries (script was provided by collaborator from Eawag Dr. Martin R. Jones).

Table 22. *Applied workflow parameters for suspect screening of cyanopeptides.*

Workflow node	Category	Processing parameter name	Processing parameter value
Select Spectra	General Settings	Precursor Selection	Use MS(n - 1) Precursor
		Peak Picking Algorithm	PD
		Use Caching	TRUE
		Use Isotope Pattern in Precursor Reevaluation	TRUE
		Provide Profile Spectra	Automatic
		Store Chromatograms	FALSE
	Spectrum Properties Filter	Min. Precursor Mass	450 Da
		Max. Precursor Mass	1350 Da
		Total Intensity Threshold	0
		Minimum Peak Count	1
	Scan Event Filters	Mass Analyzer	(Not specified)
		MS Order	Any
		Activation Type	(Not specified)
		Min. Collision Energy	0
		Max. Collision Energy	1000
		Scan Type	Any
		Polarity Mode	(Not specified)
	Peak Filters	S/N Threshold (FT-only)	3
	Replacements for Unrecognized Properties	Unrecognized Charge Replacements	1
		Unrecognized Mass Analyzer Replacements	FTMS
		Unrecognized MS Order Replacements	MS2
		Unrecognized Activation Type Replacements	HCD
		Unrecognized Polarity Replacements	+
		Unrecognized MS Resolution@200 Replacements	70000
		Unrecognized MSn Resolution@200 Replacements	17500
	Precursor Pattern Extraction	Precursor Clipping Range Before	2.5 Da
		Precursor Clipping Range After	5.5 Da
Fill Gaps	General Settings	Mass Tolerance	5 ppm
		S/N Threshold	3
		Use Real Peak Detection	TRUE
Mark Background Compounds		Hide Background	TRUE
Group Compounds	Compound Consolidation	Mass Tolerance	5 ppm
		RT Tolerance [min]	0.2
	Fragment Data Selection	Preferred Ions	$[2M+H]^{+1}$; $[M+2H]^{+2}$; $[M+H]^{+1}$
Calculate Mass Defect	Mass Defect	Fractional Mass	FALSE
		Standard Mass Defect	FALSE
		Relative Mass Defect	FALSE
		Kendrick Mass Defect	TRUE

		Nominal Mass Rounding	Floor
	Kendrick Formula	Formula 1	C20 H29 N O2
Predict Compositions	Prediction Settings	Mass Tolerance	5 ppm
		Min. Element Counts	C H
		Max. Element Counts	C150 H250 Cl4 N25 O50 P3 S5
		Min. RDBE	0
		Max. RDBE	40
		Min. H/C	0.1
		Max. H/C	3.5
		Max. # Candidates	25
		Max. # Internal Candidates	500
	Pattern Matching	Intensity Tolerance [%]	30
		Intensity Threshold [%]	0.1
		S/N Threshold	3
		Min. Spectral Fit [%]	30
		Min. Pattern Cov. [%]	80
		Use Dynamic Recalibration	FALSE
	Fragments Matching	Use Fragments Matching	TRUE
		Mass Tolerance	5 ppm
		S/N Threshold	3
Assign Compound Annotations	General Settings	Mass Tolerance	5 ppm
	Data Sources	Data Source #1	mzCloud Search
		Data Source #2	mzVault Search
		Data Source #3	MassList Search
		Data Source #4	Predicted Compositions
	Scoring Rules	Use mzLogic	TRUE
		Use Spectral Distance	TRUE
		SFit Threshold	20
		SFit Range	20
Generate Molecular Networks (beta)	Spectral Similarity	Use Full MSn Tree	TRUE
		Match Mass Shift	TRUE
		Match Transformations	FALSE
		Variate Transformations	FALSE
		S/N Threshold	3
		Mass Tolerance	2.5 mmu
		Min. Fragment m/z	50
	Transformations	Max. # Phase II	1
		Max. # All Steps	3
	Applied View Filters	Require Transformation	FALSE
		Require MSn	TRUE
		Min. MSn Score	50
		Min. MSn Coverage	70
		Min. Fragments	3

	Applied Thresholds	Require Transformation	FALSE
		Require MSn	FALSE
		Min. MSn Score	20
		Min. MSn Coverage	20
		Min. Fragments	0
Search mzCloud	General Settings	Compound Classes	All
		Precursor Mass Tolerance	10 ppm
		FT Fragment Mass Tolerance	10 ppm
		IT Fragment Mass Tolerance	0.4 Da
		Library	Autoprocessed; Reference
		Post Processing	Recalibrated
		Max. # Results	10
		Annotate Matching Fragments	TRUE
	DDA Search	Identity Search	HighChem HighRes
		Match Activation Type	FALSE
		Match Activation Energy	Any
		Activation Energy Tolerance	20
		Apply Intensity Threshold	TRUE
		Similarity Search	Confidence Forward
		Match Factor Threshold	60
	DIA Search	Use DIA Scans for Search	FALSE
		Max. Isolation Width [Da]	500
		Match Activation Type	FALSE
		Match Activation Energy	Any
		Activation Energy Tolerance	100
		Apply Intensity Threshold	TRUE
		Match Factor Threshold	20
		Identity Search	Cosine
Search mzVault	Search Settings	mzVault Library	Internal databases of Eawag: CyanoMetDB, MassBank
		Compound Classes	All
		Match Ion Activation Type	FALSE
		Match Ion Activation Energy	Any
		Ion Activation Energy Tolerance	20
		Match Ionization Method	FALSE
		Apply Intensity Threshold	FALSE
		Remove Precursor Ion	FALSE
		Precursor Mass Tolerance	10 ppm
		FT Fragment Mass Tolerance	10 ppm
		IT Fragment Mass Tolerance	0.4 Da
		Match Analyzer Type	FALSE
		Search Algorithm	HighChem HighRes
		Match Factor Threshold	50
		Max. # Results	10

Suspect screening

		Use Retention Time	FALSE
		RT Tolerance [min]	2
Search Mass Lists		Mass Lists	CyanoMetDB_massList.massList
		Mass Tolerance	5 ppm
		Use Retention Time	FALSE
		RT Tolerance [min]	0.5
Apply mzLogic		FT Fragment Mass Tolerance	10 ppm
		IT Fragment Mass Tolerance	0.4 Da
		Max. # Compounds	0
		Max. # mzCloud Similarity Results to consider per Compound	10
		Match Factor Threshold	30
		Forward Similarity Search	Confidence Forward
		Reverse Similarity Search	Confidence Reverse
		Search algorithm	Confidence
Apply Spectral Distance	Pattern Matching	Mass Tolerance	5 ppm
		Intensity Tolerance [%]	30
		Intensity Threshold [%]	0.1
		S/N Threshold	3
		Use Dynamic Recalibration	TRUE
Detect Compounds	General Settings	Mass Tolerance [ppm]	5 ppm
		Intensity Tolerance [%]	30
		S/N Threshold	3
		Min. Peak Intensity	500000
		Ions	$[M+2H]^{+2}$; $[M+3H]^{+3}$; $[M+H]^{+1}$; $[M+K]^{+1}$; $[M+Na]^{+1}$; $[M+NH4]^{+1}$ [a]
		Base Ions	$[M+2H]^{+2}$; $[M+H]^{+1}$; $[M+NH4]^{+1}$
		Min. Element Counts	C H
		Max. Element Counts	C150 H250 Br3 Cl4 F6 K2 N25 Na2 O50 P3 S5
	Peak Detection	Filter Peaks	TRUE
		Max. Peak Width [min]	1
		Remove Singlets	TRUE
		Min. # Scans per Peak	3
		Min. # Isotopes	1
Merge Features	Peak Consolidation	Mass Tolerance	5 ppm
		RT Tolerance [min]	0.2

[a] Additional ions: $[2M+H]^{+1}$; $[2M+K]^{+1}$; $[2M+Na]^{+1}$; $[2M+NH4]^{+1}$; $[M+H+K]^{+2}$; $[M+H+MeOH]^{+1}$; $[M+H+Na]^{+2}$; $[M+H+NH4]^{+2}$; $[M+H-H2O]^{+1}$; $[M+H-NH3]^{+1}$

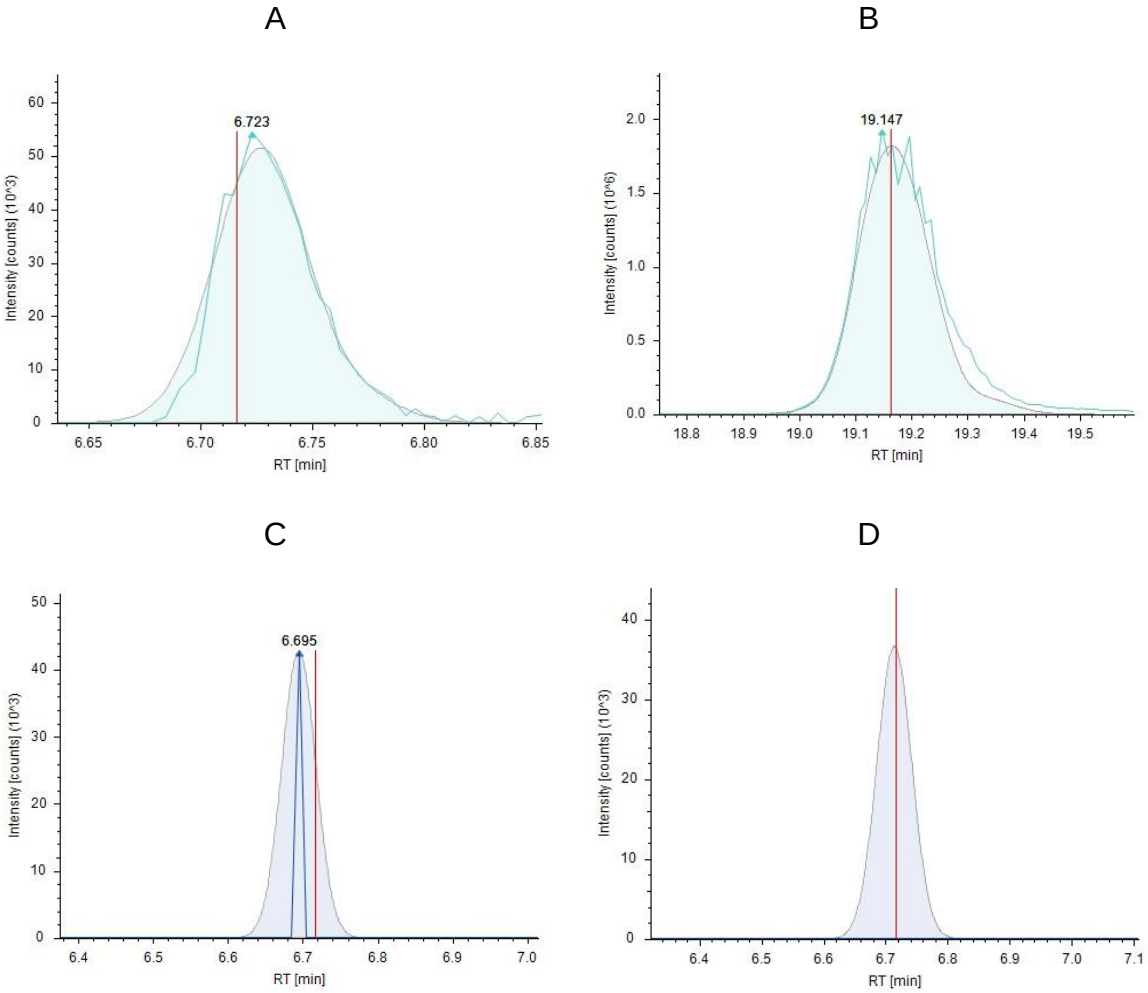


Figure 20. *Selected ion chromatograms of ions plotted in Compound Discoverer with activated 'Fill gaps' flags A - by re-detected peak of anabaenopeptins B $[M+Na]^+$ ion specie in standard solution at concentration of 0.005 mg/L 5; B - by matching ion of MC-LW $[M+H+Na]^{+2}$ ion specie in standard solution at concentration of 0.5 mg/L; C - by simulated peak of anabaenopeptins B $[M+H]^+$ ion specie in lake sample (Tophill Low reservoir, August 2019); D - by spectrum noise of anabaenopeptins B $[M+Na]^+$ ion specie in lake sample (Tophill Low reservoir, September 2019). Solid line shows point-to-point peak plotting; Gaussian-smoothened peak is a peak generated by 'Fill gaps'.*

For confirmation and identification of the compounds, the confidence level scheme for mass spectrometry outlined by Schymanski et al. [122] (Introduction section 4.3.4.) was used. Only those cyanopeptides that could be identified by one of the following criteria would be reported: a compound was defined as a tentative candidate (Level 3) based on exact mass (< 5 ppm mass error), accurate isotopic pattern, and evidence from fragmentation data (which was evaluated manually); a cyanopeptide was defined as a probable structure by diagnostic evidence (Level 2b)

based on indicative fragmentation information supporting the connectivity of the building blocks of the peptide; and a compound was defined as a confirmed structure (Level 1) when these parameters and the retention time were in agreement with available reference standards or bioreagents.

Since standard reference materials were not available for most cyanopeptides, class-equivalent approach for quantification was used. This approach was according to previous work that was carried out at Eawag within another NaToxAq PhD project [44] (also mentioned in Introduction section 4.3.2.) with slight modifications. In this case, quantification of cyanopeptides is achieved using the regression models of the structurally most similar reference standard or bioreagent assigned for each compound. If no class equivalent could be assigned, the calibration parameters of MC-LR could be used for quantification. Herein, recoveries were again taken as 100% in order to maintain relative proportion of suspected cyanopeptides for comparison of concentrations.

2. Retrospective suspect screening for 5 anabaenopeptins

As was mentioned above, recently published data showed that MCs were not found or only found at low ng/L levels while five suspected anabaenopeptins were found at µg/L or mg/L levels in samples obtained from Sau water reservoir during extensive cyanobacterial bloom event in 2015 [55]. Thus, full scan MS data obtained during Ter river basin sampling campaign was retrospectively screened to evaluate the presence of five anabaenopeptins. The suspects were: Anabaenopeptin A, B, C, and F variants, and oscillamide Y. More compounds were not considered due to the lack of time. This part of the research was not initially planned, however, it was decided to perform it as the obtained results would contribute to the knowledge of the occurrence and dynamics of cyanobacterial metabolites in important water reservoir

of the area and provide additional information for drinking water management for water quality assurance strategy.

2.1. Identification and quantification

In order to assess identification and quantification of suspects, HPLC–MS/MS data files were processed applying Qual Browser node of Xcalibur software (ThermoFisher Scientific). Peaks were integrated directly from Xcalibur software. The identification of suspects was performed according to their experimental exact *m/z*. For confirmation and identification of the compounds, the confidence level scheme for mass spectrometry outlined by Schymanski et al. [122] (Introduction section 4.3.4.) was used Since only exact mass of interest was considered, only the confidence level 5 could be achieved. In this case, only $[M+H]^+$ ion specie were considered, with a maximal mass tolerance of 5 ppm. Table 23 summarises information for each ion.

Table 23. *Details on 5 suspect for retrospective screening.*

Suspect	Molecular formula (neutral)	Monoisotopic mass $[M+H]^+$
Anabaenopeptin A	$C_{44}H_{57}N_7O_{10}$	844.4240
Anabaenopeptin B	$C_{41}H_{60}N_{10}O_9$	837.4617
Anabaenopeptin C	$C_{41}H_{60}N_8O_9$	809.4556
Anabaenopeptin F	$C_{42}H_{62}N_{10}O_9$	851.4774
Oscillamide Y	$C_{45}H_{59}N_7O_{10}$	858.4396

As was mentioned earlier in this chapter, absence of reference standards is a limitation factor for suspect screening of cyanotoxins. In this case, MC-LR was used for quantification of anabaenopeptins. This approach is less effective for concentrations' estimations in comparison to class-equivalent approach. Nevertheless, application of MC-LR also has an advantage in this particular case, as it allows better comparison between results obtained in this study and those obtained in the previously published study by Flores and Caixach [55]. Besides, ESI-HRMS

instrumental set-up (ESI source and Q-Exactive Orbitrap MS analyser) was similar with some variations in parameters. Table 24 summarises both methods: the one developed and validated in this thesis and the one employed by Flores et al. , which was developed and evaluated elsewhere [197]. As can be seen, although similar methods were employed, some differences need to be commented. In the method developed in this thesis, sensitivity was of high priority, thus, dual SPE and UHPLC column were applied, while the other method aimed for faster sample treatment approach. The common objective of these methods is to provide reliable identification and quantification of cyanobacterial metabolites (list of targeted compounds differs) by HRMS. One of the main differences in MS data acquisition lays in fragmentation mode. As was previously explained, in the developed targeted screening method developed in this thesis $ddMS^2$ acquisition mode was employed and it was only focused on 10 targeted cyanotoxins, while all-ion fragmentation (AIF) mode was applied in the work by Flores et al. In this case. no precursor preselection is needed, and all ions in the scan range monitored will be fragmented, however it is possible to use different time segments to apply different fragmentation parameters (such as different collision energies). More details on the method developed by Flores et al. can be found elsewhere [197].

Table 24. *Parameters of the method developed and validated in this thesis and the method applied in previous study by Flores and Caixach [55] and evaluated elsewhere [197].*

Parameter	Values	
	This thesis	**Previous study**
Type of metabolites	total (intra- and extracellular together)	intra- and extracellular separately
Extraction and pre-concentration	dual SPE, pre-concentration factor around 500	no, filtered samples were injected directly
Injection volume, µL	20	10 (for extracellular); 95 (for intracellular)
LC column	C_{18} Hibar® (150 × 2.1 mm, 2µm)	C_{18} Phenomenex Luna (150 × 2.0 mm, 5 µm).
Total method duration	12 min: 10 min separation, 3 min re-equilibration	65 min: 55 min separation, 10 min re-equilibration
Ionisation source	HESI +	HESI, +/-
Full scan *m/z* range	90-1100	60-1200
Full scan resolution (*m/z* 200, FWHM)	70,000	50,000
Fragmentation	$ddMS^2$ for 10 targets	AIF

3. Occurrence of suspected cyanopeptides in surface water reservoirs

In this section, obtained results from suspect screening for cyanopeptides in the samples from the United Kingdom and from posterior suspect screening for several anabaenopeptins in the samples form Ter river basin are discussed.

3.1. Samples from the United Kingdom

Water samples from three reservoirs from the United Kingdom was analysed for the presence of suspected cyanopeptides. The analysed samples represent water-soluble concentrations after partial liberation of intracellular compounds (cell lysis during sonication). Sampling points are described in the previous chapter as well as the sample treatment and LC-MS method.

Overall, 20 cyanobacterial metabolites were revealed through suspect screening, with level of confidence of ‘probable structures’ based on the interpretation of the fragmentation spectra. Table 25 summarises information on the detected cyanopeptides: dominant precursor and its m/z, and respective reference standard or bioreagent used for quantification by class equivalent. For majority of the compounds $[M+H]^+$ was the dominant precursor. Quantification equivalent was assigned due to structural similarity of molecular structures between suspects and available reference standards of bioreagents. Thus, for classes where only one standard or bioreagent were available, it was used for all suspects within the class. For example, for all four aeruginosins (variants NLO1, 850, 822, 98A) identified aeruginosin 98B was used. In contrast, for MCs where more than one reference standard was available, the most structurally related equivalent was used. For those compounds where no class equivalent was available, MC-LR was used.

Table 25. *Cyanopeptides detected in lake samples and the respective reference standard or bioreagent used for quantification by class equivalent.*

Cyanopeptide	Molecular formula	Dominant precursor	*m/z* of precursor	Quantification equivalent
MC-RR 1024 Group[a]	$C_{48}H_{73}N_{13}O_{12}$	$[M+2H]^{2+}$	512.78	[D-Asp^3,(E)-Dhb^7]MC-RR
MC-VF	$C_{51}H_{69}N_7O_{12}$	$[M+H]^+$	972.51	MC-LF
MC-FL	$C_{52}H_{71}N_7O_{12}$	$[M+H]^+$	986.52	MC-LF
[D-Asp^3,(E)-Dhb^7]MC-HtyHty	$C_{56}H_{71}N_7O_{14}$	$[M+H]^+$	1066.51	MC-YR
Anabaenopeptilide 202A	$C_{51}H_{71}N_9O_{15}$	$[M+H]^+$	1050.51	Cyanopeptolin A
Anabaenopeptin D	$C_{44}H_{57}N_7O_9$	$[M+H]^+$	828.43	Anabaenopeptin A
Anabaenopeptin NZ841	$C_{45}H_{59}N_7O_9$	$[M+H]^+$	842.44	Anabaenopeptin A
Aeruginosin NOL1	$C_{26}H_{40}N_6O_6$	$[M+H]^+$	533.31	Aeruginosin 98B
Aeruginosin 850	$C_{41}H_{66}N_6O_{13}$	$[M+H]^+$	851.48	Aeruginosin 98B
Aeruginosin 822	$C_{39}H_{62}N_6O_{13}$	$[M+H]^+$	823.44	Aeruginosin 98B
Aeruginosin 98A	$C_{29}H_{45}ClN_6O_9S$	$[M+H]^+$	689.27	Aeruginosin 98B
Cyanopeptolin CP992	$C_{49}H_{69}N_9O_{13}$	$[M+H]^+$	992.51	Cyanopeptolin A
Microginin 767	$C_{41}H_{61}N_5O_9$	$[M+H]^+$	768.45	MC-LR
Microginin KR604	$C_{32}H_{52}N_4O_7$	$[M+H]^+$	605.39	MC-LR
Aeruginosamide	$C_{30}H_{48}N_4O_4S$	$[M+H]^+$	561.35	MC-LR
Bacteriohopanetetrol	$C_{41}H_{73}NO_8$	$[M+H]^+$	708.54	MC-LR
Veraguamide G	$C_{37}H_{62}N_4O_8$	$[M+NH_4]^+$	708.49	MC-LR
Micropeptin LH1062	$C_{53}H_{78}N_{10}O_{13}$	$[M+H]^+$	1063.58	MC-LR
Nostosin B	$C_{22}H_{37}N_5O_5$	$[M+H]^+$	452.29	MC-LR
Almiramide G	$C_{36}H_{64}N_6O_6$	$[M+H]^+$	677.50	MC-LR

[a] Microcystin-RR isomeric group 1024 include: [Dha7]MC-RR, [Gly1,D-Asp3,Dhb7]MC-Rhar, [DMAdda5]MC-RR, [D-Asp3]MC-RR, [D-Asp3,(E)-Dhb7]MC-RR

Figure 21 summarises the total concentration and number of identified cyanopeptides recorded for each individual reservoir and month. Similarly, as in the targeted screening, samples from Ingbirchworth reservoir had the highest concentration of cyanobacterial secondary metabolites, reaching 1.8±0.1 µg/L in August, 29±1 µg/L in September, and 0.81±0.05 µg/L in October. Overall, 9 out of 13 suspects showed the same concentration pattern, with lower concentrations in August and October, and the highest concentration in September in Ingbirchworth samples. Among the compounds that did not follow this pattern were aeruginosin NOL1, aeruginosin 98A, cyanopeptolins CP992, and microginin KR604.8. Samples from Tophill Low reservoir also had the highest concentration of suspect cyanopeptides during September, reaching 2.31±0.08 µg/L. In August and October, concentrations were below 0.2 µg/L for this reservoir. Only traces of suspects were tentatively identified in samples from the Embsay reservoir with comparable concentrations across the sampling months (0.09±0.02 µg/L).

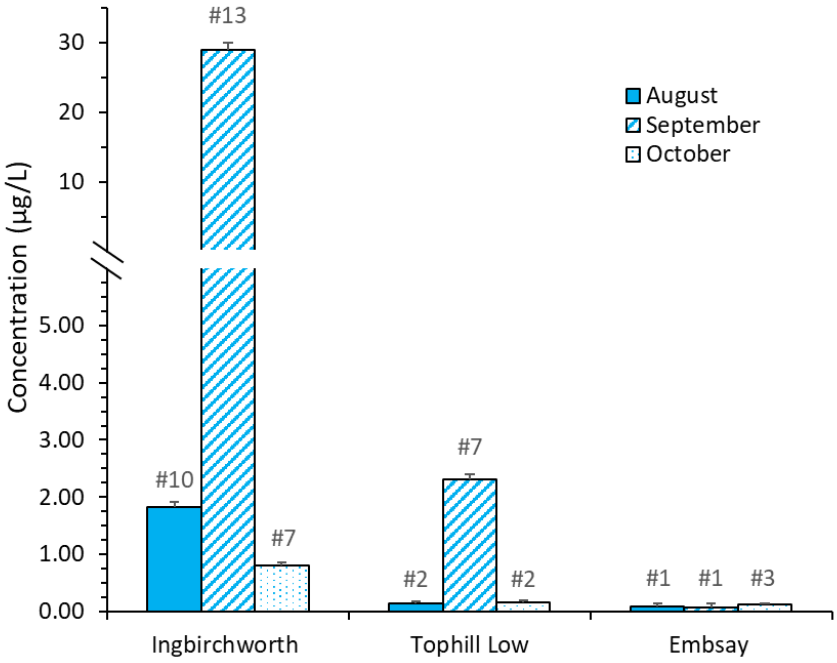


Figure 21. *Concentrations (µg/L) of cyanobacterial metabolites of three UK water reservoirs sampled in August, September, and October 2019. # at the top of each bar denotes the number of individual compounds identified. Error bars represent the standard deviation of triplicate analysis except for duplicates in September at Ingbirchworth.*

Regarding the diversity of suspects, compounds belonging to different cyanopeptide classes were identified, including microcystins, anabaenopeptins, aeruginosins, cyanopeptolins, and microginins. Figure 22 shows the concentrations of individual suspect cyanopeptide recorded in Ingbirchworth reservoir samples in September, when the highest concentrations and greatest number of cyanopeptides were observed. Cyanopeptolins and MCs were the two dominating classes of cyanopeptides representing the 74% and 23% of total number of identified compounds, respectively.

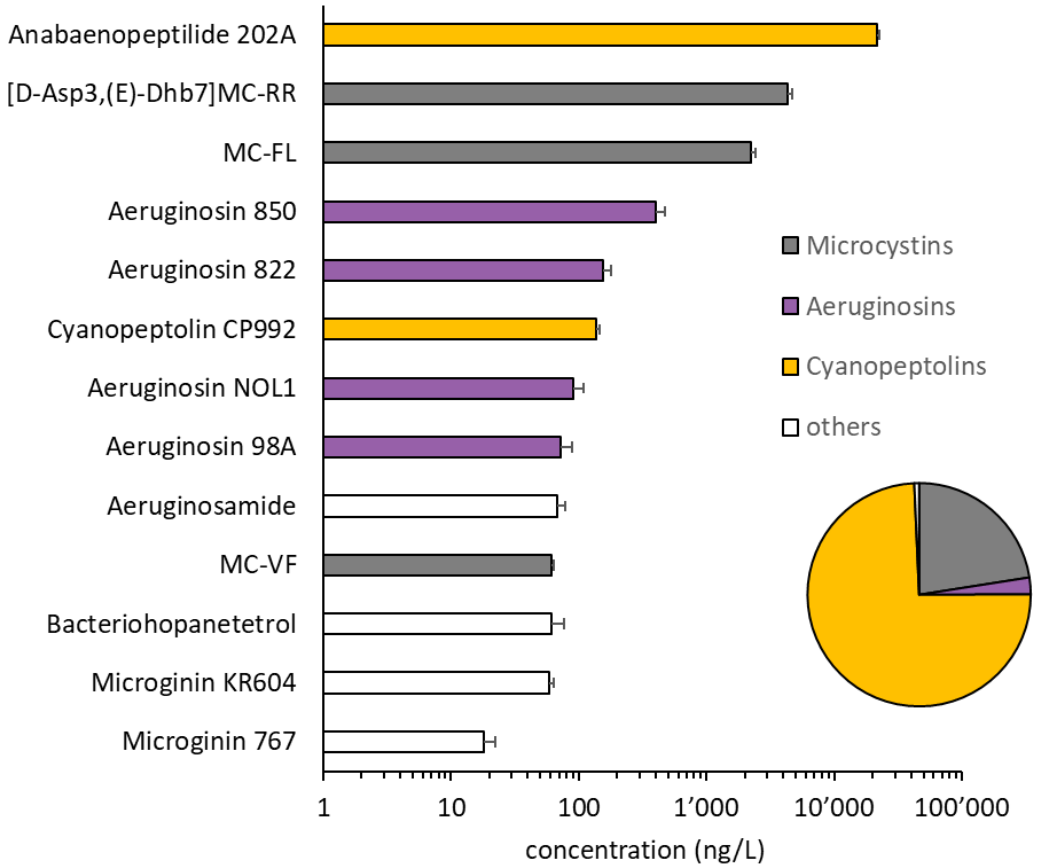
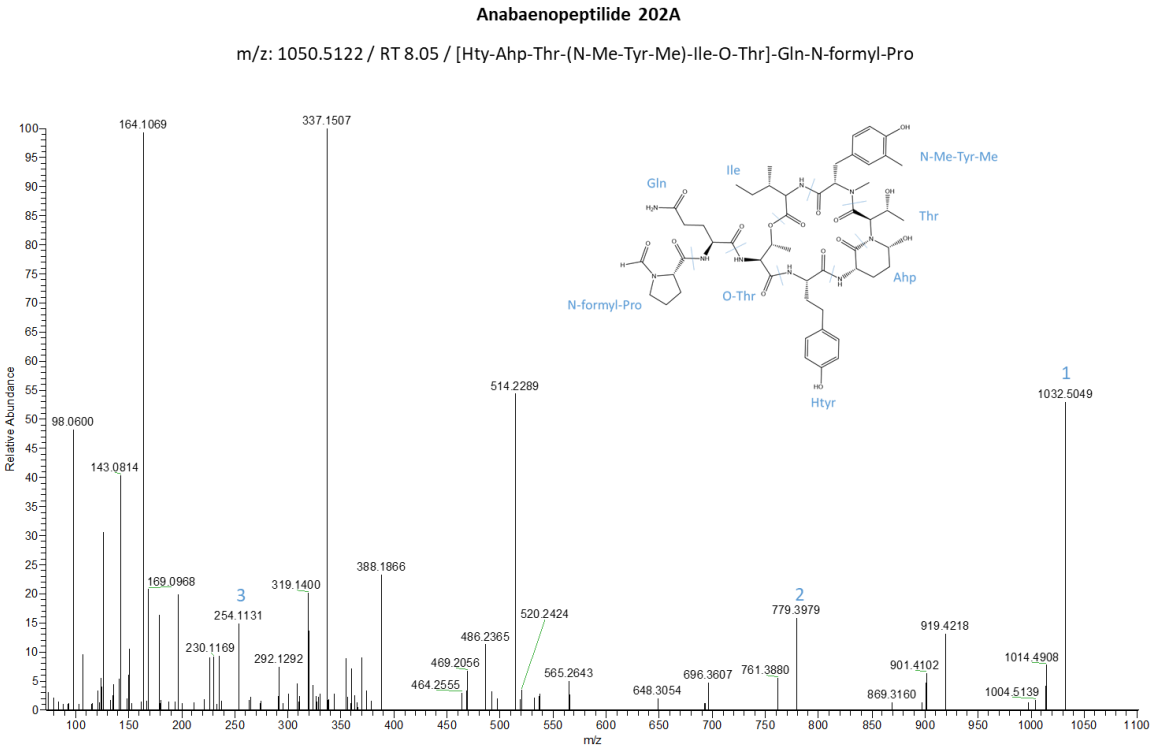


Figure 22. *Concentrations (ng/L) of individual suspects detected in September 2019 samples from Ingbirchworth reservoir. The relative proportion of major cyanopeptide classes is presented in the inset pie chart. Error bars represent the standard deviation of triplicate samples.*

The cyanopeptolin anabaenopeptilide 202A (fragmentation spectra is shown on the Figure 23) was the most abundant compound with 22±2 µg/L. This compound can be produced by genera *Anabaena* and while toxicological studies have not been reported for this cyanopeptolin, other variants are known to inhibit proteases involved in metabolism and blood coagulation [26, 198].



Fragment number	*m/z*	Fragment
1	1032.50	$[M\text{-}H_2O+H]^+$
2	779.40	$[(O\text{-}Thr\text{-}Hty\text{-}Ahp\text{-}Thr\text{-}(N\text{-}Me\text{-}Tyr\text{-}Me)\text{-}Ile)\text{-}H_2O]^+$
3	254.11	$[CO\text{-}Gln\text{-}N\text{-}formyl\text{-}Pro]^+$

Figure 23. *Fragmentation spectrum of Anabaenopeptilide 202A at HCD 15, 30, 45% stepped normalised collision energy. Precursor m/z, retention time (RT) and the building block string are noted at the top. The flat structure is shown with annotated building blocks and sites of fragmentation. The table specifies the m/z value and building block fragments that support the identification of this compound.*

Figure 24 shows the concentration of individual suspects for the September sample from Tophill Low reservoir, when, analogous to Ingbirchworth, the highest concentrations and greatest number of cyanopeptides were observed. Anabaenopeptins accounted for 84% or 1.95±0.05 µg/L of the total concentration, with anabaenopeptin D and anabaenopeptin NZ842 being the most abundant of the annotated anabaenopeptins. Total amount of anabaenopeptins is in the range reported to have inhibitory effects, as discussed above in the section of targeted

screening. Aeruginosins accounted for 9% or 0.20±0.06 µg/L at Tophill Low. Until now, aeruginosins have only been shown to induce toxic effects at high (mg/L) concentrations for *Thamnocephalus platyurus* (LC_{50} values of 10-41 mg/L) [199]. Aeruginosins also inhibit human serine proteases involved in blood coagulation at IC_{50} values ranging from 4-93 µg/L [200, 201].

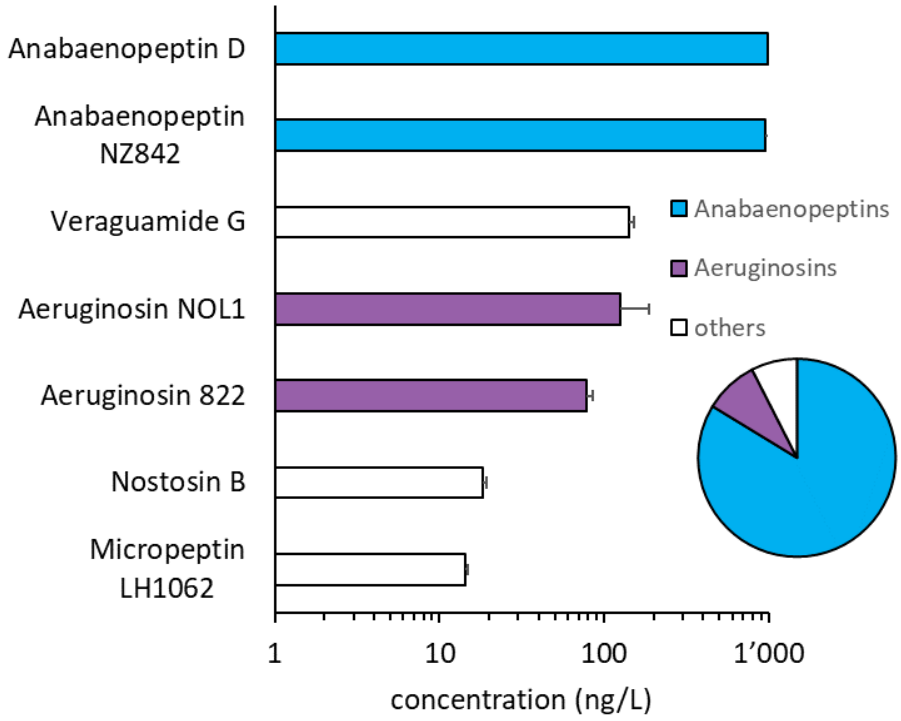


Figure 24. *Concentrations (ng/L) of individual cyanobacterial metabolites detected in September 2019 samples from Tophill Low reservoir. The relative proportions of major metabolite classes is present in the inset pie chart. Compounds were identified by suspect screening and quantified as class-equivalents. Error bars represent the standard deviation of triplicate samples.*

3.1.1. Seasonal trends of chlorophyll-a and cyanobacterial cell counts

Cyanobacteria are known to be present in the reservoirs analysed herein, which is supported by the detection and identification of cyanobacterial metabolites (both targeted and suspect) discussed above. In this section, concentrations of total cyanobacterial peptides (targeted and suspect) are addressed. In the recent years, Ingbirchworth reservoir has experienced periphyton blooms, which may be related to increase of farming activity in the catchment area. Water quality and cyanobacteria

are monitored by authorities in order to manage possible taste and odour development and to prevent issues related to cyanotoxins. Figure 25 summarises both cyanobacterial cell counts and the chlorophyll-a concentrations recorded at Ingbirchworth and Tophill Low reservoirs, between July and October 2019.

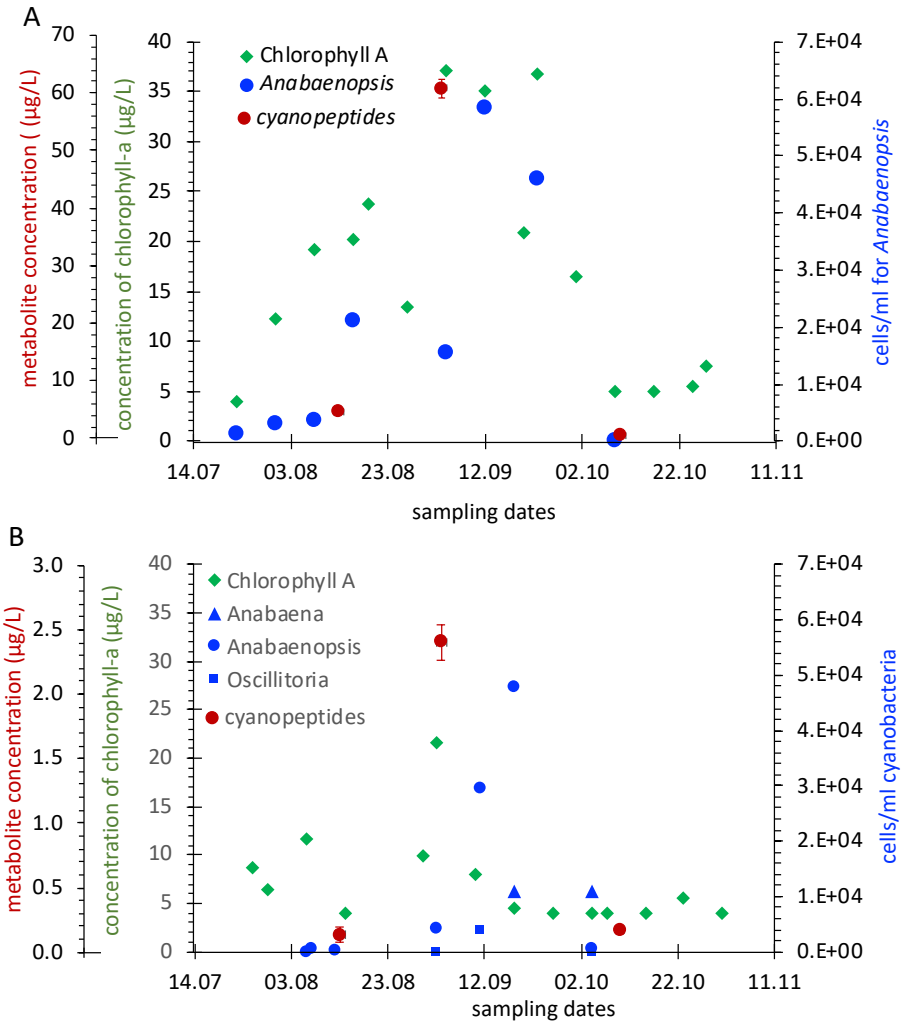


Figure 25. *Chlorophyll-a concentrations and cyanobacteria cell counts (secondary y-axis) measured in A - Ingbirchworth and B - Tophill Low reservoir samples. Total cyanopeptide concentrations are plotted for comparison (additional y-axis on the left). Shaded areas highlight the biological data closest to the cyanopeptide data sampled on August 13th, September 3rd, and October 10th 2019.*

And tables 26 and 27 provide information on chlorophyll-a, total ammonium, nitrate, total phosphate, and temperature measured in Ingbirchworth and Tophill Low

reservoirs samples respectively in 2019. In both reservoirs, the maximum chlorophyll-a concentrations of 37 µg/L and 22 µg/L, respectively, coincided with the maximum concentration of cyanopeptides, which occurred during September 2019. These values exceed the WHO guideline level for relatively-low probability of adverse health effects, which is currently defined as 10 µg/L chlorophyll-a [202]. For Ingbirchworth and Tophill Low reservoirs, this was the case most of the year, for 9 and 6 months in 2019, respectively. While a general trend of chlorophyll-a concentration with cyanobacterial abundance is expected [203-205], an absolute relationship across water bodies cannot be inferred.

Table 26. *Chlorophyll-a, total ammonium, nitrate, total phosphate, and temperature measured in Ingbirchworth reservoir samples in 2019.*

Date	Chlorophyll-a, µg/l	Ammonium total, mg/l	Nitrate, mg/l	Phosphate total, mg/l	Temperature, °C
02.01.2019	12			0.03	3.7
10.01.2019	7			0.03	2.6
18.01.2019	13	0.026	4.40	0.03	2.5
21.01.2019	15			0.03	1.7
29.01.2019	19			0.03	0.9
06.02.2019	28			0.03	0.7
15.02.2019	33	0.008	4.29	0.03	1.8
22.02.2019	23			0.03	3.3
25.02.2019	15			0.02	3.4
05.03.2019	13			0.02	3.8
13.03.2019	5			0.03	3.0
21.03.2019	19	0.008	4.59	0.04	4.6
29.03.2019	17			0.03	4.8
01.04.2019	11			0.02	5.2
09.04.2019	9			0.02	7.6
17.04.2019	6	0.024	4.18	0.02	8
25.04.2019	5			0.03	8.2
03.05.2019	6			0.04	9.8
06.05.2019	6			0.02	10.3
14.05.2019	7	0.013	2.91	0.02	9.6
22.05.2019	4			0.02	9.7
30.05.2019	4			0.02	14.1
07.06.2019	4			0.01	14.3
10.06.2019	4			0.03	13.6
18.06.2019	4	0.004	2.44	0.03	12.5
26.06.2019	6			0.04	12.3
04.07.2019	5			0.02	14.7
12.07.2019	6			0.03	14.3
15.07.2019	7	0.047	1.97	0.03	14.8
23.07.2019	4			0.04	16.9
31.07.2019	12			0.08	16.4
08.08.2019	19			0.07	16.7
16.08.2019	20			0.04	16.3
19.08.2019	24	0.026	1.69	0.04	16.3
27.08.2019	14			0.04	15.9
04.09.2019	37			0.03	15.5
12.09.2019	35			0.03	14.5
20.09.2019	21	0.020	0.99	0.03	14.2
23.09.2019	37			0.03	14.3
01.10.2019	17			0.13	13.3
09.10.2019	5			0.06	12.4
17.10.2019	5	0.040	2.37	0.07	11.1
25.10.2019	6			0.05	10.1
28.10.2019	8			0.12	9.5
05.11.2019	4			0.07	8.3
13.11.2019	4			0.07	7
21.11.2019	4	0.040	3.05	0.06	5.8
29.11.2019	4			0.06	6.1
02.12.2019	4			0.06	
10.12.2019	4			0.06	

Table 27. *Chlorophyll-a, total ammonium, nitrate, total phosphate, and temperature measured in Tophill Low reservoir samples in 2019.*

Date	Chlorophyll-a, µg/l	Ammonium total, mg/l	Nitrate, mg/l	Phosphate total, mg/l	Temperature, °C
08.01.2019		0.047	11.50	0.13	
16.01.2019	11			0.13	
24.01.2019	74	0.004	10.37	0.12	
01.02.2019	76			0.08	
04.02.2019	59	0.005	10.30	0.07	
12.02.2019	35			0.07	
20.02.2019	21	0.043	9.62	0.08	
28.02.2019	13			0.07	
08.03.2019	12	0.055	9.37	0.09	
11.03.2019	17			0.07	
19.03.2019	29	0.014	8.65	0.05	
27.03.2019	46			0.07	
04.04.2019	32	0.066	9.13	0.05	
01.05.2019	4	0.176	7.36	0.10	
09.05.2019	4			0.06	
17.05.2019	16	0.093	7.95	0.07	
20.05.2019	12			0.06	
28.05.2019	9	0.055	7.64	0.03	
05.06.2019				0.03	
13.06.2019	10	0.123	6.91	0.03	
21.06.2019	7			0.04	
24.06.2019	9	0.148	6.33	0.04	
02.07.2019	4			0.10	
10.07.2019	4	0.187	5.47		19.6
18.07.2019	4			0.05	20.6
26.07.2019	9	0.089	4.86	0.05	20.7
29.07.2019	7			0.06	21.6
06.08.2019	12	0.156	4.97	0.05	21.3
14.08.2019	4			0.10	18.9
30.08.2019	10			0.06	18.8
02.09.2019	22	0.038	5.06	0.05	18.8
10.09.2019	8			0.06	16.5
18.09.2019	5	0.023	5.06	0.03	15.9
26.09.2019	4			0.04	16
04.10.2019	4	0.065	4.92	0.04	14.4
07.10.2019	4			0.05	13.8
15.10.2019	4	0.020	5.31	0.04	12.3
23.10.2019	6			0.05	11.3
31.10.2019	4	0.040	6.51	0.05	9.4
08.11.2019	4			0.04	8.8
11.11.2019	4	0.031	7.75	0.06	8.1
19.11.2019	4			0.05	6.8
05.12.2019	4			0.05	
13.12.2019	4	0.037	9.53	0.05	
16.12.2019	5			0.06	
24.12.2019	4	0.031	9.47	0.07	
25.12.2019		0.003	9.76		
26.12.2019		0.037	9.67		
28.12.2019		0.047	9.92		
29.12.2019		0.037	9.71		

Based on the evaluated samples, the maximal cyanopeptide concentration at Ingbirchworth was almost 30-fold higher compared to Tophill Low, while the peak of chlorophyll-a concentrations was more comparable. The cyanobacterial cell counts peaked in both reservoirs about two weeks after the peak of cyanopeptide concentration, reaching $6x10^4$ cells/mL with *Anabaenopsis* being the most abundant genus. Similar to the chlorophyll-a concentrations, the relationship between absolute cyanopeptide concentrations and cyanobacterial cell count differs between two water reservoirs. Less information is available about secondary metabolites produced by *Anabaenopsis*, in comparison with other bloom-forming cyanobacteria, such as *Microcystis*, *Dolichospermum/Anabaena,* and *Oscillatoria/Planktothrix*, though it has been reported that *Anabaenopsis* produce microcystins [206, 207]. At Tophill Low, *Dolichospermum/Anabaena* ($1.1x10^4$ cells/mL) and *Oscillatoria/Planktothrix* ($0.4x10^4$ cells/mL) also contributed to the cyanobacterial abundance in September. While at Ingbirchworth, only a low co-abundance of *Microcystis* ($0.09x10^4$ cells/mL) and *Snowella* ($0.05x10^4$ cells/mL) was detected together with the dominant *Anabaenopsis* ($5.8x10^4$ cells/mL). Even though, *Anabaenopsis* was the major cyanobacterial genus at both reservoirs, the cyanopeptide profiles of Ingbirchworth and Tophill Low reservoirs were not identical. Both had high concentrations of anabaenopeptin A, anabaenopeptin B, oscillamide Y, and aeruginosin 822. However, only Ingbirchworth samples were rich in the cyanopeptolin (anabaenopeptilide 202A) as well as different microcystins. Tophill Low samples contained no detectable microcystins, but did contain additional anabaenopeptins and aeruginosins. This discrepancy suggests that other cyanobacterial species may have contributed to the identified cyanopeptide profiles, and/or that the production dynamics of metabolites of *Anabaenopsis* varies between reservoirs. No cyanobacteria were identified in Embsay reservoir, which agrees with detected herein cyanotoxins at only trace

levels. Also, no correlation of total ammonium, nitrate, total phosphate, and temperature with cyanobacterial abundance or toxin concentrations was observed in Ingbirchworth or Tophill Low reservoirs (data in Tables 26, 27).

In conclusion of this section, recently published paper can be directly cited:

"To the best of our knowledge, this is the first time that anabaenopeptins, cyanopeptolins, aeruginosins and microginins have been quantified in waters at the United Kingdom along with microcystins and anatoxin-a. Globally, there is a knowledge gap regarding entry of these cyanopeptides into drinking water treatment plants and the effectiveness of their abatement during water treatment, along with cyanobacterial cells and other known toxins. A better understanding of those cyanopeptides that are abundant in drinking water reservoirs would help to guide monitoring strategies. Further, abundant cyanopeptides should be prioritized to study their abatement during water treatment.

Herein, we successfully selected three sampling dates that captured a summer peak of toxin concentrations at the intake to the drinking water treatment plants of two reservoirs. However, to improve the understanding of seasonal variation of cyanotoxins in these reservoirs, a more detailed temporal resolution is desired, for example with weekly frequency. As cell count and particularly toxin analysis are resource intensive, monitoring can switch to increase sampling and analysis for cyanobacterial toxins once the WHO guidelines value of 10 µg/L is reached. For Ingbirchworth and Tophill Low reservoirs, this was the case most of the year, for 9 and 6 months in 2019, respectively (Additional file 1: Tables S3, S4). With limited resources, increased toxin analysis may be at least considered during the summer/fall peak period" [56]:

3.2. Samples from Spain

Since recent study reported presence of several anabaenopeptins in Sau reservoir [55], retrospective analysis for 5 anabaenopeptins in samples analysed in this thesis was carried out. Posterior suspect screening revealed presence of anabaenopeptin B in two samples from Pasteral. Table 28 summarises information about detected concentrations, and average mass error. Additionally, isotope simulation in Xcalibur softwater (ThermoFisher Scientific) was carried out. In this case, chemical formula, adduct, and the most abundant ion specie were set. For anabaenopeptins B these parameters were the following: $C_{41}H_{60}N_{10}O_9$, H adduct, and most abundant ion 1. Isotopic patter of suspect compound and simulated isotope are shown in the Figure 27.

Table 28. *Results of the posterior suspect screening analysis of five anabaenopeptins: estimated concentration of anabaenopeptin B with relative standard deviation of technical replicates and average mass error in Pasteral water reservoir. Samples of 26.07.2018 were analysed in duplicate.*

Date	Estimated concentration, ng/L	Mass error, ppm
26.07.2018	2.6±0.2	-0.4
12.09.2018	4.57±0.07	0.3

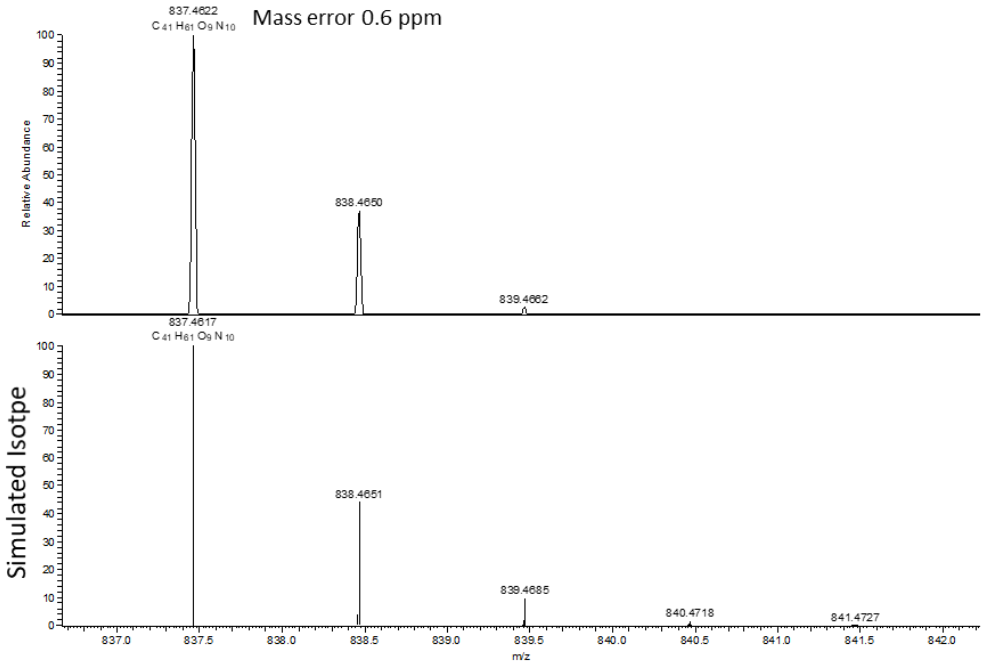


Figure 26. *Isotopic patter of suspected anabaenopeptin B detected in Pasteral reservoir in September sample and simulated isotope.*

Estimated concentrations of anabaenopeptin B were at 2.6±0.2 and 4.57±0.07 ng/L levels, which is higher than concentrations of MC-RR detected during targeted screening that reached 1.40±0.02 ng/L, however, the range is the same (ng/L). As was mentioned before, accurate mass and isotopic pattern were taken into account to confirm the compound. Mass error of identified suspect was below 0.5 ppm, what is within mass tolerance values (which is 5 ppm for HRMS). As for the isotopic pattern, match of the first three isotopes can be seen on the Figure 27. Thus, anabaenopeptin B is confirmed at the confidence level 4 according to the confidence level scheme for mass spectrometry outlined by Schymanski et al. [122] (Introduction section 4.3.4.)

Regarding comparison of results obtained in the current study and the ones reported in the previous study by Flores and Caixach [55], trace levels of anabaenopeptins were reported in the September 2015 samples from Susqueda and Pasteral in the previous study, while the highest concentrations were detected in the samples from

Sau at µg/L and mg/L. In contrast, in the current study, no suspects were found in the samples from Sau and Susqueda, and identified anabaenopeptin in Pasteral samples was at lower levels of ng/L. These differences could be due to different sample types. For example, maximal concentrations of anabaenopeptins were reported in scums, while surface water was analysed in this study. Another reason could be that after bloom episode in 2015 risk management and assessment plan was implemented by Agència Catalana de l'Aigua, Aigües Ter Llobregat, and scientific experts [55]. Thus, lower levels of suspects could be a consequence of the performed actions to prevent cyanobacterial blooms and associated with them the low presence of cyanobacterial metabolites even 3 years after the risk management actions. Additionally, the weather during the sampling campaign in 2018 was especially rainy and with temperatures colder than usual in that region of Spain, what created less favourable conditions for proliferation of cyanobacterial bloom.

CONCLUSIONS

Conclusions

According to the aims that were set in the beginning of my theses and were tackled throughout the thesis, several conclusions can be reported:

1. The seasonal variation patterns of cyanotoxins for three European climate zones were described based on the available literature. In Mediterranean and continental climate zones, two peaking periods were distinguished. As for oceanic climate zone, only one peaking period was observed. However, the amount of seasonal studies in this zone was lower comparing with the other regions. Obtained results were published [9].
2. A multi-residue method has been developed and validated for the analysis of 10 targeted cyanotoxins in freshwater. For sample clean up and pre-concentration, SPE was applied. In order to cover compounds of various polarities and classes, two cartridges were employed (HLB Oasis and Supelclean™ ENVI-Carb™). The developed method showed high sensitivity, selectivity, and robustness. The application of an UHPLC column allowed fast separation, what makes this method more cost-effective. The developed method can be proposed for both environmental and food analysis due to the number of confirmation criteria such as HRMS, and MS/MS ions. Data acquired in full scan at high-resolution can be used for posterior suspect screening of other natural toxins and cyanotoxins. The developed method was published [172].
3. Application of targeted methods on freshwater samples from drinking water reservoirs from Spain demonstrated the presence of microcystin-RR at low ng/L levels, however posterior suspect screening of mass spectrometry data acquired in high-resolution revealed potential presence of another cyanopeptide – anabaenopeptin. In freshwater samples from Switzerland traces of microcystin-LA were found, however, the concentrations were bellow

limits of detection. In raw drinking water from three freshwater reservoirs in the United Kingdom, 8 targeted compounds were identified and quantified with reference standard or bioreagents. The highest concentration of total cyanobacterial metabolites was reaching 36.2±1.2 µg/L.

4. Targeted methodology was expanded for an application of suspect screening for a wide range of cyanopeptides. This method was applied for the analysis of raw drinking water from the United Kingdom. Suspect screening revealed co-occurrence of targeted compounds together with other cyanopeptides. The obtained results are the first to present concentrations of anabaenopeptins, cyanopeptolins, aeruginosins, and microginins, along with microcystins, in the reservoirs of the United Kingdom. Results of targeted and suspect screening for the samples from the United Kingdom were published [56]

FUTURE PERSPECTIVES

After conducting four years of research in the field of analysis and occurrence of cyanotoxins in freshwater, I could observe several gaps in in the analysis of cyanobacterial metabolites that require fulfilment. Herein, I will address two of them that in my opinion can be solved with recent developments in analytical approaches. One of such gaps is the need of faster, selective and high-throughput analytical tools for screening of cyanotoxins what would help to identify early presence of compounds in comparison to SPE-LC-HRMS based methods. Even though, immunochemical methods can partially fulfil these needs, currently they are not selective enough to differentiate between different variants of microcystins. However, recently developed coated blade spray (CBD) [208] extraction technique launched on the market by Restek (catalogue number 23248 at www.restek.com) could be a possible solution. CBD is a solid-phase microextraction-based technology that can be coupled directly to HRMS; more details can be found elsewhere [208, 209]. In this case, pre-concentration and clean-up is fast and easy in comparison with SPE, and its direct coupling to MS provides higher selectivity comparing with immunoassays. Such combination could be a powerful analytical tool for fast and selective analysis of cyanotoxins. However, to support this hypothesis, method development and application have to be performed. In the course of this PhD thesis with support from Dr. Elisabeth Janssen and her team, I have initiated collaboration with Restek in order to perform preliminary trials of CBS application. However, due to the delays caused by COVID-19, material transfer agreement between Restek and Eawag was not finalised, and thus, this work was not carried out.

Another gap that recent analytical developments can assist solving is complex identification of suspects. Cyanobacterial metabolites are a large group of compounds that is not well studied yet. Scarcity of standards contributes greatly to this challenge as not many fragmentation libraries can be generated and applied for

compounds' confirmation. However, application of ion mobility separation (IMS) coupled to HRMS can contribute in solving this issue as it provides extra parameter (collision cross-section value) for the identification of suspects. More details on IMS can be found elsewhere [210]. Recently published study adopted existing confidence levels for LC-HRMS analysis for IMS-HRMS analysis, and provided examples of the potential of IMS-HRMS for environmental analysis and additional value of collision cross-section parameter for screening strategies [127]. Since collision cross-section is not affected by the matrix or chromatographic separation, it might help to distinguish structurally related cyanopeptides better. To support this hypothesis, experimental work has to be carried out.

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