From genes to blooms

Diversity in microcystin phenotypes and mcy biosynthesis genes in the cyanobacterium Microcystis

Johansson, Emma

2023

Document Version:
Publisher's PDF, also known as Version of record

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Diversity in microcystin phenotypes and *mcy* biosynthesis genes in the cyanobacterium *Microcystis*

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LUND UNIVERSITY

DOCTORAL DISSERTATION

By due permission of the Faculty of Science, Lund University, Sweden.
To be defended at the Blue Hall, Ecology Building, Sölvegatan 37, Lund.
Thursday 15th of June 2023 at 09.00.

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Title and subtitle: From genes to blooms. Diversity in microcystin phenotypes and *mcy* biosynthesis genes in the cyanobacterium *Microcystis*

Abstract: Cyanobacterial blooms are increasing in occurrence and frequency world-wide, mainly due to eutrophication and increased water temperatures. In freshwater, *Microcystis* is one of the most common bloom-forming genera, renowned for producing the toxin microcystin which is harmful to humans and other mammals. The ability of *Microcystis* strains to produce microcystins is largely due to the presence or absence of genes encoding microcystin biosynthesis (i.e. the *mcy* gene cluster), and the toxicity of *Microcystis* blooms is therefore dependent on the presence of toxin producing genotypes in the population. Numerous laboratory studies and field studies have aimed at explaining what environmental factors drive toxic blooms, however, with various results. Moreover, there is limited understanding of what the eco-physiological role of microcystin might be, and what factors influence microcystin concentration in the water. The aims of this thesis were to examine 1) the phenotypic and genotypic composition and variation in *Microcystis*, with regards to microcystin-production, 2) the association between *mcy* genotypes and the observed microcystin phenotypes, and 3) what environmental conditions favour microcystin-producing *Microcystis* during blooms, and are associated to microcystin concentrations in lake systems.

In **paper I**, *Microcystis botrys* strains were isolated and cultured from a single bloom, and their microcystin-profiles were analysed with mass spectrometry. I could show that the microcystin-producing *M. botrys* subpopulation contained multiple microcystin-phenotypes, and that the phenotypic diversity varied on a temporal scale. Not only were the proportions of microcystin-producing strains higher during early and late summer, but the microcystin-profiles were more diverse, and the number of microcystin variants produced by individual strains were higher. In **paper II**, I performed whole genome sequencing of the strains analysed in the first study. Thereby, I could characterise the variation within the *mcy* gene cluster, encoding for microcystin biosynthesis, and show how the composition of *mcy* genotypes relates to the observed phenotypes. One main finding was that both microcystin-producing and non-producing strains consist of several genotypes, that either possess the full *mcy* gene cluster, or partial operons. Based on the results of **paper II**, in **paper III** I developed a population-tailored marker for quantitative polymerase chain reaction (qPCR) targeting the *mcyJ* gene in the *mcy* gene cluster. I was thereby able to detect and quantify the abundance of toxigenic cells in natural populations of *Microcystis* spp., sampled from two Swedish lakes. I also sampled and analysed several environmental variables to determine which factors favour toxigenic vs non-toxigenic strains. The results confirmed the temporal succession of microcystin-producing and non-producing phenotypes observed in **paper I**. I could also show that toxigenic *Microcystis* spp. were associated with high concentrations of inorganic nitrogen, whereas microcystin concentrations were associated with soluble reactive phosphorus.

To conclude, my results show that there is inherent phenotypic and genotypic variation in *Microcystis*: the studied subpopulations contain multiple microcystin-phenotypes, as well as *mcy* genotypes. The presence of several *mcy* genotypes, found in both microcystin-producing and non-producing phenotypes, indicate that microcystin production might not be attributed to the presence or absence of *mcy* genes alone. Furthermore, toxic *Microcystis* blooms are likely driven by nutrient availability in the studied systems, which confirms that management strategies for bloom mitigation should focus on both phosphorus and nitrogen reduction.

Key words: *Microcystis*, cyanobacteria, microcystin, cyanotoxin, cyanobacterial harmful blooms, CHAB, qPCR, whole genome sequencing, *mcy*, secondary metabolite, nutrients

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language:** English
ISSN and key title		**ISBN:** 978-91-8039-664-6 (print) 978-91-8039-663-9 (electronic)
Recipient's notes	**Number of pages:**	Price
	Security classification	

Signature Date 2023-05-11

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Cover by Jonas Johansson van Doorn

Photo from lake Vombsjön (back cover) by Emma Johansson

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Department of Biology

ISBN: 978-91-8039-664-6 (print)
978-91-8039-663-9 (electronic)

Printed in Sweden by E-husets Tryckeri
Lund 2023

Jag kunde gjort allting tusen gånger bättre,
men nu gjorde jag inte det.
(Errol Norstedt)

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II. Johansson, E., Manoharan, L., Gollnisch, R., Mazur-Marzec, H., Tromas, N., Pérez-Carrascal, O., Legrand, C., Säll, T., Ahrén, D., Rengefors, K. High Diversity of *mcy* Genotypes within a Population of the Microcystin-producing Cyanobacterium *Microcystis* spp. *Manuscript*.

III. Johansson, E., Rabow, S., Legrand, C., Säll, T., Rengefors, K. Dissolved Nitrogen Favours Toxigenic *Microcystis* spp. In Natural Systems. *Manuscript*.

Author contributions

I. KR, AG, and CL conceived the study. KR, AG, CL, and CB designed the sampling. Field work and isolation of colonies was carried out by CB. HM-M performed mass spectrometry analysis. EJ analysed the data with support from TS. EJ, KR, TS, CL, and AG discussed the results. EJ wrote the manuscript, with comments from KR and TS. All co-authors read and edited the manuscript. AG passed away before the manuscript was finalized.

II. EJ and KR designed the study. EJ performed molecular work. EJ analysed the data with valuable input from LM, NT, OP-C, and DA. EJ wrote the manuscript, with comments from KR, LM, NT, OP-C and DA. All co-authors read and edited the manuscript.

III. EJ, KR, and CL conceived the study. EJ and KR planned the sampling. EJ performed sampling and data collection. EJ and SR designed primers for quantitative polymerase chain reaction. SR optimised the qPCR protocol. EJ performed laboratory work. EJ analysed the data, with input from TS and KR. EJ wrote the manuscript with contribution from KR, SR and TS. All co-authors read and edited the manuscript.

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Abbreviations and definitions

Adda	3-amino-9methoxy-2, 6,8-trimethyl-10-phenyl-deca-4,6-dienoicacid. Amino acid group that is unique for microcystins and the closely related nodularin.
Batch culture	Technique for growing/cultivating microorganisms in enclosed systems, with a limited amount of nutrients and trace metals. Opposite to continuous cultures, in which microorganisms are grown with a continuous supply of nutrients. In this thesis work, I have only cultured *Microcystis* cells in batch cultures.
Bloom	Rapid proliferation of cyanobacteria or eukaryotic microalgae to high densities and biomasses, often as a result of nutrient (mainly phosphorus and nitrogen) enrichment.
Cyanobacteria	Prokaryotic organisms that are capable of performing oxygenic photosynthesis. Represent the earliest known form of life! Often referred to as blue-green algae, but are bacteria, not algae.
CHAB	Cyanobacterial harmful bloom. Mass proliferation of cyanobacteria, in such densities that the biomass cause negative effects on ecosystems and/or society. Most often used to refer to blooms of cyanobacteria that produce metabolites that are toxic to other organisms.
d-MeAsp	D-erythro methylaspartic acid, one of the amino acids in the microcystin-LR molecule.
De-methylation	Chemical reaction in which a methyl-group (CH_3) is removed from a molecule. A process that contribute to the multitude of microcystin variants detected.
ELISA	Method to detect and measure e.g. cyanotoxins in samples.
Eutrophic	Nutrient-rich aquatic environments. One definition is that the phosphorus concentrations range between 30-100 µg L^{-1}. The high nutrient concentrations support high primary production by plants and phytoplankton, and the

	decomposition of biomass often cause oxygen depletion in sediments.
Genotype	The genetic makeup of an organism. The genotype describes the type of gene variants (alleles) present at a location (locus) in the genome.
Hepatotoxin	Substance with toxic effects, mainly on cells in the mammalian liver.
Hypereutrophic	Aquatic environments with phosphorus concentrations above 100 µg L^{-1}, i.e. "extra eutrophic".
mcy/Mcy	Microcystin genes, and the corresponding amino acid product. For example, *mcyA* encodes biosynthesis of the NRPS McyA in the microcystin synthetase complex.
Mdha	N-methyldehydroalanine, one of the amino acids in the microcystin-LR molecule.
Metagenomics	The study of all genomes (i.e. from various organisms) present in a sample. Often used to study microbial communities in various environments, and to identify and characterise microbes that are difficult to isolate and cultivate in the laboratory.
Methylation	Chemical reaction in which a methyl group (CH_3) is added to a molecule. A process that contribute to the multitude of microcystin variants detected.
Microcystins	A family of secondary metabolites produced by cyanobacteria, e.g. *Microcystis* and *Planktothrix*. Consist of seven amino acids (i.e. is a heptapeptide) and mainly targets liver cells in mammals (i.e. is a hepatotoxin).
Morphospecies	Species classification based on morphological characters. Can be defined as a group of organisms that are morphologically different from related species. For example, species within *Microcystis* are determined based on colony morphology. However, based on sequence similarities rather than morphology, several studies have shown that *Microcystis* species are not coherent.
NRP	Non-ribosomal peptide(-s), a class of peptide secondary metabolites that are produced non-ribosomally (!) by prokaryotes and fungi. NRPs are synthesised on NRPS and PKS enzyme complexes (see below).

NRPSs	Non-ribosomal peptide synthases. NRPS are modular enzymes on which peptides are synthesised from a variety of amino acid substrates.
PC	Phycocyanin. In cyanobacteria, PC is an accessory pigment to chlorophyll. The pigment molecule absorbs orange and red light, and therefore gives cyanobacteria their characteristic blue-green colour. In this thesis and in **paper III**, PC is also referred to as the phycocyanin operon, i.e. the genes coding for synthesis of phycocyanin molecules.
PCA	Principal Component Analysis, a dimensionality-reduction method used to explain variance and co-variance in large datasets containing multiple observations. PCA increase the interpretability of data, while minimising information loss.
PCR	Polymerase Chain Reaction, a method to amplify and detect DNA-sequences. To put it simple: PCR is a method to obtain multiple (in the magnitude of 10^3 up to 10^6) copies of a specific DNA-sequence (targeted by primers) from a sample which contains a mixture of DNA molecules. In this thesis work, quantitative PCR (qPCR) was used to target specific genes in the *Microcystis* spp. *mcy* gene operon as well as the PC operon. Thereby, it was possible to detect and quantify possible microcystin-producers in relation to the whole *Microcystis* spp. population.
Phenotype	The observable characteristics of an organism. Most often referred to as the detectable expression of the genotype, however the phenotype is also influenced by environment.
Phytoplankton	Microscopic, free-floating algae that live in aquatic environments. The term is derived from the Greek *phyto* (plant) and *plankton* (made to wander, or drift). They are photosynthetic organisms, however some can also get energy by consumption of e.g. other organisms (i.e. they are mixotrophic).
PKS	Polyketides. Secondary metabolites produced by prokaryotes, fungi, plants, and animals. They are building blocks for a variety of natural products, and constitute a group of structurally diverse products with a broad range of biological activities and pharmaceutical (e.g. antibiotic, antifungal, or cytostatic) properties.

PKSs	Polyketide synthases. A family of large, multifunctional, modular enzymes or enzyme complexes, on which polyketides and non-ribosomal peptides are synthesised.
Recombination	Exchange/"reshuffling" of gene variants on the chromosome, or between chromosomes. The term is also used to describe the transfer of genetic material between bacterial cells, creating genetic variation. Such processes are, for example, involved in the build-up of antibiotic-resistance in bacteria.
Redfield ratio	The atomic ratio of carbon, nitrogen, and phosphorus (C:N:P 106:16:1). First described by the American oceanographer Alfred C. Redfield in 1934, who discovered that the C:N:P ratio are relatively constant in marine plankton, and that they correspond to the ratio of these chemical elements in oceans.
Secondary metabolites	Organic compounds that are not directly involved in cellular metabolism, or organismal growth, development, or reproduction. Caffeine and nicotine are examples of secondary metabolites produced by plants, and botulinum ("botox") is a metabolite produced by a bacteria.
SRP	Soluble reactive phosphorus. Measure of orthophosphate, the form of soluble, inorganic phosphorus that can be directly taken up by phytoplankton and plants.
Stoichiometry	The "measure of elements": the term is derived from the Greek *stoikhein* (element) and *metron* (measure). In ecology and biology, stoichiometry can be defined as the relationship and balance of energy and chemical elements in ecological systems.
Strain	A genetic variant of an organism. In microbiology, a strain can be defined as a group of organisms that belong to the same species, but have certain genetic characteristics that are not found in other members of the species.

Abstract

Cyanobacterial blooms are increasing in occurrence and frequency world-wide, mainly due to eutrophication and increased water temperatures. In freshwater, *Microcystis* is one of the most common bloom-forming genera, renowned for producing the toxin microcystin which is harmful to humans and other mammals. The ability of *Microcystis* strains to produce microcystins is largely due to the presence or absence of genes encoding microcystin biosynthesis (i.e. the *mcy* gene cluster), and the toxicity of *Microcystis* blooms is therefore dependent on the presence of toxin producing genotypes in the population. Numerous laboratory studies and field studies have aimed at explaining what environmental factors drive toxic blooms, however, with various results. Moreover, there is limited understanding of what the eco-physiological role of microcystin might be, and what factors influence microcystin concentration in the water. The aims of this thesis were to examine 1) the phenotypic and genotypic composition and variation in *Microcystis*, with regards to microcystin-production, 2) the association between *mcy* genotypes and the observed microcystin phenotypes, and 3) what environmental conditions favour microcystin-producing *Microcystis* during blooms, and are associated to microcystin concentrations in lake systems.

In **paper I**, *Microcystis botrys* strains were isolated and cultured from a single bloom, and their microcystin-profiles were analysed with mass spectrometry. I could show that the microcystin-producing *M. botrys* subpopulation contained multiple microcystin-phenotypes, and that the phenotypic diversity varied on a temporal scale. Not only were the proportions of microcystin-producing strains higher during early and late summer, but the microcystin-profiles were more diverse, and the number of microcystin variants produced by individual strains were higher. In **paper II**, I performed whole genome sequencing of the strains analysed in the first study. Thereby, I could characterise the variation within the *mcy* gene cluster, encoding for microcystin biosynthesis, and show how the composition of *mcy* genotypes relates to the observed phenotypes. One main finding was that both microcystin-producing and non-producing strains consist of several genotypes, that either possess the full *mcy* gene cluster, or partial operons. Based on the results of **paper II**, in **paper III** I developed a population-tailored marker for quantitative polymerase chain reaction (qPCR) targeting the *mcyJ* gene in the *mcy* gene cluster. I was thereby able to detect and quantify the abundance of toxigenic cells in natural populations of *Microcystis* spp., sampled from two Swedish lakes. I also sampled and analysed several

environmental variables to determine which factors favour toxigenic vs non-toxigenic strains. The results confirmed the temporal succession of microcystin-producing and non-producing phenotypes observed in **paper I**. I could also show that toxigenic *Microcystis* spp. were associated with high concentrations of inorganic nitrogen, whereas microcystin concentrations were associated with soluble reactive phosphorus.

To conclude, my results show that there is inherent phenotypic and genotypic variation in *Microcystis*: the studied subpopulations contain multiple microcystin-phenotypes, as well as *mcy* genotypes. The presence of several *mcy* genotypes, found in both microcystin-producing and non-producing phenotypes, indicate that microcystin production might not be attributed to the presence or absence of *mcy* genes alone. Furthermore, toxic *Microcystis* blooms are likely driven by nutrient availability in the studied systems, which confirms that management strategies for bloom mitigation should focus on both phosphorus and nitrogen reduction.

Popular science summary

Cyanobacteria are ancient prokaryotes that are found in a multitude of different habitats world-wide. Their ability to perform photosynthesis (to use sunlight, water, and carbon dioxide to produce oxygen and sugar-molecules) makes them important primary producers, forming the base of aquatic food webs together with eukaryotic phytoplankton and algae. During optimal growth conditions in aquatic ecosystems, cyanobacterial populations often increase rapidly, forming dense blooms. While bloom-formation is not a new phenomenon, during the past ~100-150 years they have been increasing in occurrence, frequency, and duration. This has mainly been explained by increased nutrient inputs from e.g. agriculture and wastewater, but also by increased water temperatures and changes in precipitation patterns. Cyanobacterial blooms can have negative effects on aquatic ecosystems, for example by impeding light availability for other organisms, by depleting oxygen in water upon degradation, or by outcompeting other phytoplankton for nutrients. The preference and ability of zooplankton to graze on cyanobacteria is also restricted, limiting the transfer of energy, and therefore growth, of other organisms in aquatic habitats. Most importantly, cyanobacteria are able to produce an array of secondary metabolites, which are compounds that often have harmful effects on other organisms. Cyanobacterial blooms can therefore degrade water quality and negatively impact the production of clean and safe drinking water, and have emerged as significant concern for societies world-wide. Understanding the causes for expansion of cyanobacterial blooms is therefore of great importance.

In freshwater systems, species within the genus *Microcystis* are among the most common bloom-forming cyanobacteria. They are known to produce secondary metabolites named microcystins, that have harmful effects on aquatic organisms, and are known to be toxic for mammals. Why *Microcystis* and other cyanobacteria produce microcystins is not known. Several studies have aimed at explaining the role of microcystin production, however with various results. Furthermore, *Microcystis* strains ("individuals") are variable in terms of microcystin production: not all strains are able to produce microcystin, and those that do can produce multiple variants of the microcystin molecule. Despite extensive research focussing on *Microcystis* and microcystin production, what environmental factors influence the dynamics (the variation in space and time) of toxic and non-toxic strains within a bloom are also not fully understood.

In this thesis work, I have examined the variation genes and traits (microcystin-production) in local populations of the species *Microcystis botrys*, with regards to microcystin production. I have also investigated how environment influences the prevalence of microcystin-producing and non-microcystins producing *Microcystis*, and on microcystin concentrations, in two sampled lakes located in southern Sweden, that are known for cyanobacterial blooms

Using chemical analytical methods, I could show that the microcystin-producing subpopulation consisted of several unique phenotypes (or "microcystin profiles"). I could also show that the diversity of these phenotypes varied throughout the sampling period. For example, strains that produced many microcystins were more common during the early sampling (in June), whereas the highest proportion of microcystin-producing strains occurred during late sampling (in August). Based on these results, I then wanted to examine if there was variation also in the genes that codes for microcystin synthesis (the *mcy* genes). To answer this question, I sequenced the full genomes of a subset of the *Microcystis botrys* strains that were analysed in the first project. I found that there was considerable diversity in *mcy* genes, not only among microcystin-producing strains, but also among those that did not produce microcystins. Most interestingly, I discovered that non-microcystin producing strains did not lack all *mcy* genes, but specific genes within the *mcy* gene cluster. This is the first time such variation is described within *Microcystis* populations from Sweden.

In the third project of my thesis, my aim was to investigate which environmental factors were most related to the prevalence of toxic *Microcystis* and to microcystin concentrations in the two study-systems. To do this, I sampled and analysed the phytoplankton community, as well as a multitude of environmental (both biotic and abiotic) parameters in the studied lakes during a two-year period. I also developed a method to detect and quantify toxic versus non-toxic *Microcystis* strains from lake water samples. My main findings were that toxic *Microcystis* spp. had a positive association with high concentrations of inorganic nitrogen, whereas microcystin concentrations were positively associated with inorganic phosphorus.

To conclude, my results show that there is a lot of diversity in *Microcystis*, both in mcy genes and in microcystin-profiles. These results suggest that microcystin production might not be caused only by the presence or absence of *mcy* genes. Furthermore, toxic *Microcystis* blooms are likely driven by nutrient availability in the studied systems, which suggest that management actions should focus on the dual reduction of phosphorus and nitrogen, in order to mitigate toxic *Microcystis* blooms.

Populärvetenskaplig sammanfattning

Cyanobakterier är urgamla prokaryoter, som finns i en mängd olika livsmiljöer över hela världen. Deras förmåga att utföra fotosyntes (det vill säga att använda solljus, vatten och koldioxid för att producera syre- och kolhydrater) gör dem till viktiga primära producenter, och tillsammans med andra växtplankton utgör de basen för akvatiska näringsvävar.

Vid optimala tillväxtförhållandena kan populationer av cyanobakterier snabbt öka i biomassa, och bilda täta blomningar. Dessa kallas ofta för algblomningar, även om cyanobakterier inte är egentliga alger, utan just bakterier. Algblomningar är inte ett nytt fenomen, men har under de senaste cirka 100-150 åren ökat både i förekomst och varaktighet. Detta beror till stor del på ökad näringstillförsel från t.ex. jordbruk och avlopp, men också av ökade vattentemperaturer och förändringar i nederbörd och klimat. Täta blomningar av cyanobakterier kan ha negativa effekter på akvatiska ekosystem, till exempel genom att ljustillgången minskar för andra organismer, på grund av minskad syretillgången i vatten då höga biomassor bryts ned, eller genom att konkurrera med andra växtplankton om näringsämnen. Många djurplankton kan inte, eller vill inte, äta cyanobakterier, och detta kan begränsa energiöverföringen från primärproduktion till högre trofiska nivåer. Framför allt kan många arter av cyanobakterier producera olika typer av sekundär-metaboliter, som ofta kan vara skadliga för andrar organismer. Blomningar av cyanobakterier kan därför leda till försämrad vattenkvalitet, vilket kan hota tillgången till dricksvatten. Blomningar av cyanobakterier är alltså problematiska, för ekosystem och också för samhällen världen över. Att förstå varför cyanobakterieblomningar generellt, och toxiska blomningar specifikt, ökar är därmed av stor vikt.

Arter inom släktet *Microcystis* är bland de vanligaste blommande cyanobakterierna i sötvatten. De är kända för att producera mikrocystiner, som är sekundärmetaboliter med skadliga effekter på vattenlevande organismer. Mikrocystiner har också toxiska effekter på däggdjur, exempelvis har kraftiga *Microcystis*-blomningar kopplats till dödsfall av husdjur och boskap, och lever- och tjocktarmscancer har kopplats till mikrocystin-förekomst i dricksvatten. Man vet dock inte varför *Microcystis* (och andra cyanobakterier) producerar mikrocystiner. Flertalet studier har genomförts, med syfte att kunna förklara vilken roll mikrocystin kan ha för cellen, dock med varierande resultat och slutsatser. *Microcystis*-stammar ("individer") är dessutom variabla när det gäller mikrocystin-produktion: alla stammar kan inte syntetisera mikrocystin, och de som kan producera dessa metaboliter samexisterar med stammar

som inte kan göra det. Dessutom kan mikrocystin-producerande stammar syntetisera flera olika varianter av mikrocystiner. Trots omfattande forskning är det heller inte helt klarlagt vilka miljöfaktorer som påverkar dynamiken (det vill säga variationen i tid och rum) av mikrocystin-producerande och icke mikrocystin-producerande stammar av *Microcystis* under en blomning.

I mitt avhandlingsarbete har jag undersökt den fenotypiska och genotypiska variationen i lokala populationer av arten *Microcystis botrys*. Stammar av *Microcystis* är kapabla till att producera även andra sekundära metaboliter, men jag har i mitt arbete valt att fokusera på enbart mikrocystiner. Detta på grund av att de ofta är starkt toxiska, som jag beskrev i stycket ovan. Jag har också undersökt vilka miljöfaktorer som påverkar förekomsten av mikrocystin-producerande och icke mikrocystin-producerande stammar, samt mikrocystin-koncentrationer, i två skånska sjöar.

Med hjälp av kemiska analysmetoder kunde jag visa att den mikrocystin-producerande subpopulationen bestod av flera unika fenotyper ("mikrocystin-profiler"). Jag kunde också visa att mångfalden av dessa fenotyper varierade under provtagningsperioden. Till exempel var stammar som producerade många mikrocystiner vanligare under den tidiga provtagningen (i juni), medan den högsta andelen mikrocystin-producerande stammar förekom under den senare provtagningen (i augusti). Baserat på dessa resultat ville jag sedan undersöka om det finns variation även inom de gener som kodar för mikrocystinsyntes (*mcy*-generna). Jag sekvenserade därför hela genomen (det vill säga hela det genetiska materialet) av en del av de *M. botrys*-stammar jag analyserade i mitt första projekt. Jag kunde bland annat visa att stammar som inte producerade mikrocystin inte saknade alla gener i det genkluster som kodar för mikrocystin-syntes. Jag kunde också visa att det finns en stor variation i de gener som kodar för mikrocystin-syntes, både bland stammar som producerade mikrocystin och bland de som inte gjorde det. Det är första gången en sådan variation beskrivs i *Microcystis*-populationer från svenska sjöar.

I mitt tredje projekt undersökte jag vilka miljöfaktorer som är mest relaterade till förekomsten av mikrocystin-bildande *Microcystis*, samt till mikrocystin-koncentrationer, i två skånska sjöar. Under sommarmånaderna år 2018 och 2019 genomförde jag min fältprovtagning, och analyserade en mängd miljöparametrar (både biotiska och abiotiska) i sjöarna. Inom projektet utvecklade jag en metod för att kunna upptäcka och kvantifiera mikrocystin-producerande och icke microcystin-producerande *Microcystis*. Jag kunde visa att proportionerna av mikrocystin-producerande *Microcystis* var högre då halterna av inorganiskt kväve var höga, i början av sommaren (juni och tidiga juli), medan mikrocystin-koncentrationerna var högre i slutet av sommaren (augusti/september), vilket sammanföll med högre halter av inorganiskt fosfor.

Sammanfattningsvis visar mina resultat att det finns en hel del fenotypisk och genotypisk variation i *Microcystis*, och att mikrocystin-produktion inte enbart beror på om alla gener inom *mcy*-genkomplexet finns. Dessutom påverkas förekomsten av giftiga *Microcystis*-blomningar sannolikt av tillgången på näringsämnen i sjöar. För att minska riskerna att *Microcystis*-blomningar ska uppkomma, bör man inom vattenförvaltningen fokusera på att samtidigt reducera både fosfor och kväve.

Preface

Cyanobacteria are ancient microorganisms, with an evolutionary history dating back more than three billion years (Schirrmeister et al., 2015). Due to their photosynthetic capacity, they were largely responsible for the oxygenation of the Earth's atmosphere during the Proterozoic, 2.5-2.3 billion years ago (Schirrmeister et al., 2015, Hamilton et al., 2016), and thus have had major impacts on the biosphere. Their long evolutionary history has enabled cyanobacteria to adapt to changes in geochemistry and climate. To quote Hans Paerl (2018): "they have 'seen it all' with regard to major geochemical changes that the earth has experienced". This likely contributes to their diversity in terms of e.g. ecology, morphology, and life strategies. For example, they occur in freshwater, marine and brackish environments. They can grow attached to hard substrates, forming benthic mats or biofilms, live in moist soils, or live in symbiosis with fungi, forming lichens (Glibert et al., 2016). They have strategies to tolerate extreme conditions (such as temperature, salinity, desiccation, solar radiation), and are therefore able to survive and proliferate in various extreme environments e.g. hot springs or Antarctic lakes (Raven et al., 2005, Codd et al., 2017). Some are even found growing in the hollow structures of polar bear fur (Raven et al., 2005). They can be very small, such as the marine, single-celled *Prochlorococcus* sp. which is about 0.6 µm in size, or form large filaments or colonies, that are visible to the naked eye. Due to their photosynthetic capacity, the ability of several genera to fix atmospheric nitrogen, as well as other traits (that will be presented in subsequent parts of the thesis), cyanobacteria are primary colonisers in many habitats. When resources are abundant, cyanobacteria can store nutrients to use for growth during resource-limited conditions. I like to think of cyanobacteria as "the Rolling Stones of phytoplankton". Here's why: they are ancient (no need to explain that further), they survive against all odds (*sensu* Keith Richards falling from a palm tree, hit his head, and still be alive), they can manage on limited resources and then BAM before you know it, success! (Sold out venues world-wide vs. suddenly appearing massive blooms, also world-wide.) Above all, cyanobacteria are sturdy and stubborn as nothing I ever encountered (except for my daughter).

When I started my PhD-studies, one of the first aims was to do whole genome sequencing using PacBio-technology. To briefly inform the un-initiated reader: to do this kind of sequencing you need to extract and purify large DNA-fragments in rather high quantities. How difficult can it be? Turns out it is very difficult! I spent about a year growing *Microcystis* and trying out different extraction protocols. I

grinded cells with pestles, I vortexed them with metal balls, I poured liquid nitrogen on them. I tried cleaning protocols (that mainly resulted in high losses of DNA, but hey at least the remaining fragments were large and of good quality!). I grew and harvested so much *Microcystis* biomass! I found a DNA-extraction method that worked out alright, ran a number of agarose gels, and then FINALLY (figure 1):

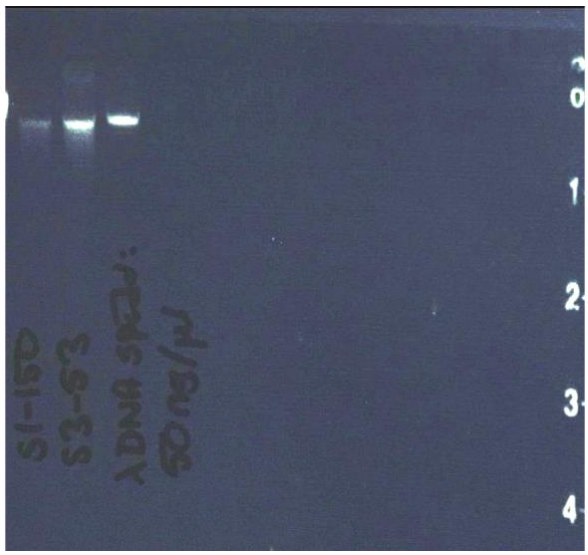

Figure 1. Photo of an agarose gel, from when I finally got a "good enough-sized" DNA-fragment (size ca 40.000 bp) from *Microcystis botrys*. One of the best moments in my PhD studies! Photo: E. Johansson.

It is now almost seven (!) years since I started my PhD-studies, and during this time I have sort of developed a love-hate relationship to these little creatures. It has at times been super-fun, sometimes super-frustrating. Writing **paper II** took ages (sorry Karin!), due to me re-analysing my results fifty-eleven times, because genes were there when I thought they would not, and likewise absent when I had expected them to be present. I have been confident in my results, questioned them, questioned myself, and then gotten confident in the results again. Now that I am summing up the work I have done, trying to put my research in a broader context, and reading through some of the scientific papers that have been published about *Microcystis* (and that is an immense amount!), all I can think about is how stubborn my little cyanos are, not following predictions or expectations. Instead, they have definitely proven to me how much (unexpected) variation and diversity they contain, and how much we still do not know about them.

Introduction and background

> "About two Leagues from this Town there lyes an Inland-Sea called Berkelse-Sea, whose bottom in many places is very moorish. This water is in Winter very clear, but about beginning or in the midst of Summer it grows withish, and there are then small green clouds permeating it … Passing lately over this Sea at a time, when it blew a fresh gale of wind, and observing the water as above-described, I took up some of it in a Glass-vessel, which having view'd the next day, I found moving in it several Earthy particles, and some green streaks, spirally ranged, after the manner of the Copper or Tinworms, used by Distillers to cool their distilled waters; and the whole compass of each of these streaks was about the thickness of a man-hair on his head. Other particles had but the beginning of the said streak; all consisting of small green gobuls interspersed."
>
> Antonie van Leeuwenhoek, 1674

Cyanobacteria are important primary producers, and are key players e.g. for global carbon, oxygen, and nitrogen cycling. In aquatic environments (mainly brackish and freshwater), mass occurrences (blooms) (figure 2) of cyanobacteria have become an increasing problem. These can have negative impacts on ecosystems (e.g. by affecting the abundance and diversity of other species in a system), as well as for society and public health (e.g. by decreasing water quality) (Huisman et al., 2018). Several bloom-forming cyanobacteria, including *Microcystis*, the key player of this thesis, are able to produce secondary metabolites that have toxic effects on organisms and are harmful to aquatic environments (Carmichael, 1994). Hence, these metabolites are referred to as cyanotoxins, and blooms of toxin-producing cyanobacteria are referred to as cyanobacterial harmful blooms (*abbr*. CHABs). In freshwater systems, CHABs are often dominated by the genus *Microcystis*. Most species within the genus are known to produce microcystins, metabolites that have toxic effects mainly on the liver. However, not all strains ("individuals") are capable of synthesising these metabolites. Natural populations are polymorphic, and consist of both microcystin-producing and non-microcystin producing cells. The environmental and/or biological factors that contribute to toxic *Microcystis* blooms is yet not established (Wilhelm et al., 2020). In addition, the eco-physiological function(-s) of the microcystins, and what factors are regulating microcystin-production, are not fully understood.

Figure 2. Satellite image showing the phytoplankton bloom in lake Vombsjön, on 2019-07-26. Image based on Copernicus Sentinel-2 data [2019-07-26]. Retrieved from Creodias [2023-05-02], processed by the European Space Agency (ESA).

Descriptions of cyanobacterial blooms can be found as far back as during the 12th century (Codd, 1996), and toxic blooms linked to the death of both wild and domesticated animals have been reported since at least the mid 1800s (Hilborn and Beasley, 2015). However, the occurrence of CHABs have increased globally during the past ~200 years, and more rapidly since the 1950's (Taranu et al., 2015, Ho et al., 2019). This have mainly been linked to combined effects of anthropogenic nutrient pollution (i.e. eutrophication) and global climate change (Elser et al., 2009, Moss, 2012, Paerl, 2018). For society, it is crucial to understand the factors driving CHAB formation, in order to be able to manage and mitigate CHABs. In addition, knowledge on cyanobacterial traits, physiology, and genomics are important to be able to predict their response to future changes in land use and climate.

Cyanobacterial harmful blooms

Cyanobacteria are primary producers that, together with other phytoplankton, form the base of the food-web in aquatic systems. As other photosynthetic organisms, they use light energy, carbon dioxide (CO_2), water (H_2O), and macronutrients to synthesise organic matter (i.e. to grow), producing oxygen as a by-product. Hence, nutrient and light availability are the main factors affecting population growth (Harke et al., 2016). In addition, biological and physiological variables such as thermal stratification, predation, and competition impact the dynamics of cyanobacterial populations.

When environmental conditions are favourable, cyanobacterial blooms often appear rapidly. This is due partly to increases in abundance and biomass, but also by upward migration of populations present in the water column or in the benthos (Reynolds, 1984, Brunberg and Blomqvist, 2003). In temperate areas, bloom-formation is mainly initiated during summer, when the water temperature is >20°C and the water column has stratified thermally. Dense blooms can have severe impacts on environment, economy, on ecosystem function, and on human health (Bullerjahn et al., 2016, Huisman et al., 2018). The senescence and degradation of large bloom biomass can cause anoxia/hypoxia in aquatic systems, which can cause e.g. fish-kills, or affect the internal nutrient cycling and enhance eutrophication processes in aquatic systems (see e.g. Paerl and Huisman, 2008). In addition, the ability of several cyanobacterial taxa to produce secondary metabolites with toxic effects on animals (including humans) (Konst et al., 1965, Campos and Vasconcelos, 2010) makes cyanobacterial blooms of increasing growing concern for public health and for environment.

Cyanobacterial traits associated with bloom development

Cyanobacteria possess numerous traits making them effective competitors for light and nutrients, which enhances their potential to proliferate and outcompete eukaryotic phytoplankton (Huisman et al., 2018). Many genera, including *Microcystis*, can regulate their buoyancy and are thereby able to migrate vertically in the water column, increasing their access to light and shading other, non-buoyant, phytoplankton (Walsby, 1994, Jöhnk et al., 2008). Increased water temperatures increase cyanobacterial growth rates (Jöhnk et al., 2008, Paerl and Huisman, 2008), but also enhances thermal stratification, thereby causing decreased mixing of the water column which further favours buoyant cyanobacteria (Jöhnk et al., 2008). Increased turbidity due to high cell numbers, or shading caused by surface-blooms, can further impede light availability for macrophytes and other phytoplankton (Huisman et al., 2018). During photosynthesis, dense blooms can reduce concentrations of dissolved CO_2, causing pH to increase. The equilibrium of inorganic carbon is thereby shifted to bicarbonate and carbonate (Verspagen et al.,

2014) ($CO_2 + H_20 \leftrightarrows H_2CO_3 \leftrightarrows H^+ + HCO_3^- \leftrightarrows CO_3^{2-} + 2H^+$). Cyanobacteria have evolved effective CO_2-concentration mechanisms (Sandrini et al., 2016, Visser et al., 2016), enabling them to adjust carbon fixation when the availability of inorganic carbon declines (Sandrini et al., 2016). Furthermore, morphological traits such as size or colony formation decrease the preference and ability of zooplankton to graze on cyanobacteria, thereby reducing predation pressure (Brooks and Dodson, 1965, Wilson et al., 2006, Gobler et al., 2007). Cyanobacteria are of low food quality for zooplankton, and selective feeders can reject cyanobacterial cells (Müller-Navarra et al., 2000, Wilson et al., 2006). In addition, many cyanobacterial genera are able to produce secondary metabolites, of which several has toxic effects on a diversity of aquatic organisms (Christoffersen, 1996, Catherine et al., 2017). It has therefore been suggested that these compounds might function as grazer deterrents (Hairston et al., 2001, Ger et al., 2016).

Environmental drivers of toxic cyanobacterial blooms

The occurrence, as well as societal awareness, of CHABs have increased during the past decades (O'Neil et al., 2012, Harke et al., 2016). The increase of CHABs are mainly caused by eutrophication due to anthropogenic nutrient pollution from e.g. agricultural run-off or from wastewater, but also to the global increase of atmospheric CO_2, changes in hydrology, and to increased water temperatures (van de Waal et al., 2010, Ho and Michalak, 2019, Zepernick et al., 2023). Climate change scenarios predict that CHABs will continue to increase in frequency and duration, due to alterations in e.g. precipitation patterns, nutrient loading, and temperatures (Paerl and Huisman, 2009, O'Neil et al., 2012, Visser et al., 2016).

Much research has aimed at finding relationships between various environmental parameters and the occurrence of toxic blooms and/or cyanotoxin-production. For example, studies have been performed to assess the individual or combined effects of iron (Utkilen and Gjolme, 1995), light (Utkilen and Gjolme, 1992, Wiedner et al., 2003, Leblanc Renaud et al., 2011, Chaffin et al., 2018), nutrients (Orr and Jones, 1998, Yoshida et al., 2007, Dolman et al., 2012, Gobler et al., 2016, Suominen et al., 2017, Chaffin et al., 2018), CO_2 (Van de Waal et al., 2011, Sandrini et al., 2016), and temperature (Lürling et al., 2017, Mantzouki et al., 2018) on cyanobacterial growth and/or microcystin production/content. The results of such studies have provided a framework of what conditions might initiate toxic blooms, or favours toxin-producers. However, with regards to what factors are most related to the prevalence of toxin-producing cyanobacteria and microcystin content in lake water, results are inconclusive.

The study organism: *Microcystis* spp.

In freshwater systems, CHABs are often dominated by the genus *Microcystis* (Kützing ex Lemmermann 1907) (figure 3). In temperate areas, *Microcystis* cells overwinters in the benthos and migrates to the epilimnion during summer, forming surface blooms and scums (Brunberg and Blomqvist, 2002, Brunberg and Blomqvist, 2003). The genus comprises approximately 25 species (Cronberg and Annadotter, 2006, Komárek and Anagnostidis, 2008), most of which are known to produce microcystins and other metabolites (Bishop et al., 1959, Humbert and Fastner, 2017).

Several incidents of human and animal intoxication linked to toxic *Microcystis* blooms have been reported (Cronberg and Annadotter, 2006), and long-term exposure to microcystin has been associated to e.g. liver cancer (Nishiwaki-Matsushima et al., 1992). Drinking water guidelines for microcystin have been developed by the World Health Organization (WHO), who propose a threshold concentration of 1 µg L^{-1} for life-time drinking water (corresponding to a tolerable daily intake level of 0.04 µg per kg bodyweight), 12 µg L^{-1} for short-term drinking water, and 24 µg L^{-1} in recreational water (Chorus and Welker, 2021).

The ability of *Microcystis* to produce toxic metabolites have been known since the late 1950's (Bishop et al., 1959). However, not all *Microcystis* strains are able to synthesise microcystins. The co-existence of microcystin-producing (often referred to as "toxic", or "toxigenic") and non-microcystin producing ("non-toxic", or "non-toxigenic") *Microcystis* strains in natural populations have been observed in several studies (e.g. Via-Ordorika et al. (2004), Sabart et al. (2010), Yancey et al. (2022)). To understand what environmental conditions act as drivers on toxic blooms, many field studies have focused on measuring the relative abundance of toxic and non-toxic strains during bloom succession. The often observed temporal succession of toxic and non-toxic strains suggest that these phenotypes are favoured by different environmental conditions. Thus, natural *Microcystis* populations likely consist of ecologically distinct strains with specific adaptations to the various environmental conditions experienced during bloom succession.

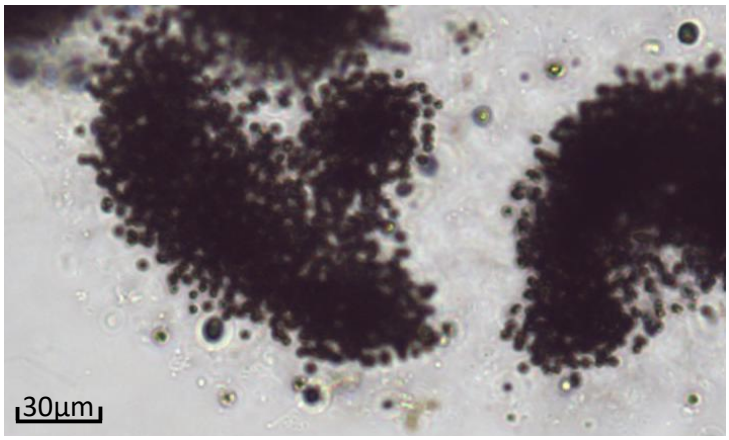

Figure 3. *Microcystis botrys* colony, 200x magnification. Species within the genus are single-celled, but form colonies by binary fission within a mucilage sheet (Komárek and Anagnostidis, 2008). Photo by Emma Johansson.

Cyanobacterial toxins

Cyanobacteria, as many other prokaryotes, are known to produce a large variety of secondary metabolites. These are often non-ribosomal peptides, of which many have toxic effects on other organisms (Konst et al., 1965, Christoffersen, 1996), and are thus referred to as cyanotoxins. The diversity of secondary metabolites produced by prokaryotes is immense, but for the majority, the biological function remains largely unknown (Carlson et al., 2017). It has been suggested that cyanobacterial secondary metabolites are involved in protection against predators and/or pathogens, that they protect against oxidative stress, that they might function as metal-transporting agents, or as signalling molecules (allelochemicals) (Gokulan et al. (2014), Omidi et al. (2017), see also Jones et al. (2021)).

Cyanotoxins can be categorized based on their chemical structure (cyclic peptides, e.g. microcystin and nodularin, heterocyclic compounds such as anatoxin-a, saxitoxin, and lipopolysaccharides), but also on their biological effect. Hepatotoxins, such as microcystin, inhibit protein phosphatases 1 and 2A in liver-cells, thereby causing e.g. cell damage and liver haemorrhage (Fontanillo and Kohn, 2018). Neurotoxins affect the functioning of the neuro-muscular system, cytotoxins inhibits serine proteases and protein synthesis, dermatoxins cause dermatitis, and endotoxins can cause irritation, allergic reactions, or gastroenteritis (Harke et al., 2016, Omidi et al., 2017, Meriluoto et al., 2017). The various cyanotoxins are produced by several cyanobacterial genera. Microcystins are typically produced by freshwater genera such as *Microcystis*, *Planktothrix*, *Dolichospermum* (*Anabaena*), and *Fischerella* (Catherine et al., 2017, Heck et al., 2018), but have also been detected e.g. in lichen-symbiotic cyanobacteria (Oksanen et al., 2004). Furthermore, some species are able

to produce several toxins simultaneously. *Microcystis* is known to produce not only microcystins, but also other cyanotoxins with toxic effects as described above, such as aeruginosins, cyanopeptolines, and BMAA (Meriluoto et al., 2017, Pérez-Carrascal et al., 2019). Thus, a cyanobacterial bloom can contain several different cyanotoxins. However, even though several cyanobacterial species produce microcystins, not all strains of a species are able to synthesize them (Chorus and Welker, 2021) and studies have shown that microcystin-producing and non-microcystin producing strains co-exist in natural populations.

Microcystin

Most research on cyanotoxins has focussed on the microcystins. This is mainly because they are the most frequently produced cyanotoxins, because of their high toxic potential, and also because of the high concentrations often reported during bloom events. While microcystin concentrations in pelagic waters often are in the range of 0-10 µg L^{-1}, concentrations as high as 2.8 mg L^{-1} has been reported (Catherine et al., 2017).

Microcystin was first isolated from *Microcystis aeruginosa*, hence the name, and was originally named as "the fast death factor" (Bishop et al., 1959). Microcystins are cyclic heptapeptides, i.e. consist of seven amino acids in a circular structure. The general structure of the microcystin molecule MC-LR is described as cyclo D-Ala^{1}-X^{2}-D-MeAsp^{3}-Z^{4}-Adda^{5}-D-Glu^{6}-Mdha^{7} (Carmichael et al., 1988, Nishizawa et al., 1999), where X and Z are variable L-amino acids on position two and four in the molecule, and Adda is an unique amino acid, characteristic for microcystin and the closely related pentapeptide nodularin (Rinehart et al., 1988, Catherine et al., 2017) (figure 4). (For definitions on the other amino acids in MC-LR, see the section "Abbreviations and definitions".) To date, more than 300 structural variants (congeners) have been described (Jones et al., 2021). The large variety is mainly attributed to the variant amino acids on position 2 and 4, but there can also be variations on the amino acids on other positions, such as presence or absence of methyl groups, or amino acid substitutions (Carmichael et al., 1988, Welker et al., 2004a). Microcystin-producing cyanobacteria can concurrently produce multiple microcystin congeners. Studies have shown that *Microcystis* strains are able to produce a range from one to more than 25 microcystin variants simultaneously (Tonk et al., 2009, Agha et al., 2012, Drugă et al., 2013, Puddick et al., 2014, Johansson et al., 2019), with a median number of 4-5 microcystins (Puddick et al., 2014).

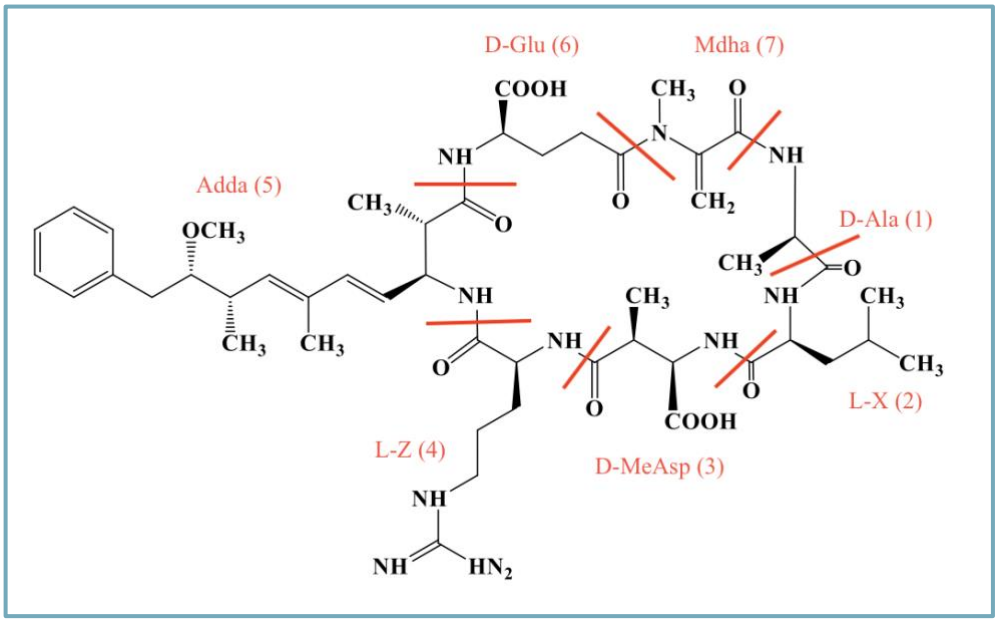

Figure 4. General structure of the microcystin molecule (MC-LR). Amino acids constituents are given in red text, number in brackets show their position in the microcystin molecule. Red lines shows delimitations between amino acids. Modified from Nishizawa et al. (1999), and Tillett et al. (2000).

Mode of toxicity

Microcystins mainly affect cells in the liver of mammals, hence their classification as hepatotoxins. If ingested, microcystins are actively transported into cells by organic anion transport proteins (OATPs, a family of membrane transporters) (Chorus and Welker, 2021). These are expressed particularly in liver cells, but also in other tissues and the intestinal tract. When inside a cell, microcystins inhibit protein phosphatases (mainly PP1 and PP2A), a class of enzymes that are involved in several regulatory pathways (Fontanillo and Kohn, 2018). The inhibition is caused by high affinity binding of the microcystin molecule to these enzymes. Inhibition of protein phosphatases decrease protein dephosphorylation, and thereby phosphorylated proteins accumulate in the cell. This ultimately cause alterations in cell metabolism, destabilisation of the cytoskeletal structure, and cell shape, followed by apoptosis and necrosis of the liver cell, which in turn cause intrahepatic haemorrhage. Microcystin has also been associated to gastroenteritis (Cronberg and Annadotter, 2006), and linked to oxidative stress in cells, which can trigger apoptotic processes (Fontanillo and Kohn, 2018). The different congeners varies in toxicity from more or less non-toxic to highly toxic (e.g. [(6Z)-Adda^{5}]MC-LR with a LD_{50} exceeding 1200 µg kg^{-1}, to MC-LR with a LD_{50} of 50 µg kg^{-1}) (Rinehart et al., 1994).

The *mcy* gene cluster and microcystin biosynthesis

Microcystins are synthesised non-ribosomally by a large multi-enzyme complex, encoded for by the microcystin synthetase (*mcy*) gene cluster. The *mcy* genes were first identified and sequenced in strains of *M. aeruginosa* (Meissner et al., 1996, Dittmann et al., 1997, Nishizawa et al., 1999, Nishizawa et al., 2000, Tillett et al., 2000), but have also been characterized in *Planktothrix* sp. (Christiansen et al., 2003, Rounge et al., 2009), *Dolichospermum* sp. (Rouhiainen et al., 2004), and *Fischerella* sp. (Heck et al., 2018). In *Microcystis*, the *mcy* gene cluster consists of ten genes (*mcyA-J*) located on two bidirectionally translated operons, with a total size of approximately 55 kbp (figure 5). These encode non-ribosomal peptide synthetases (NRPS, *mcyABC*), polyketide synthases (PKS, *mcyD*), hybrid NRPS/PKS (*mcyE* and *mcyG*), an ABC-transporter (*mcyH*), and tailoring enzymes (*mcyFIJ*) (Tillett et al., 2000, Kaebernick et al., 2002). It has been shown that the *mcy* gene cluster differ slightly between genera (see e.g. Méjean and Ploux, 2013). *Dolichospermum/Anabaena* sp. strain 90 and *P. agardhii* strain CYA-128/1 both have the *mcyABC* genes in a similar arrangement as in *Microcystis*, but the remaining genes are differently arranged. In addition, *P. agardhii* CYA-128/1 lack *mcyF* and *mcyI*, but have the additional gene *mcyT*. It has been suggested that this is the result of horizontal gene transfers, further supported by the presence of transposase genes in proximity to the *mcy* gene clusters in *Microcystis* and *Dolichospermum/Anabaena* (Rantala et al., 2004)

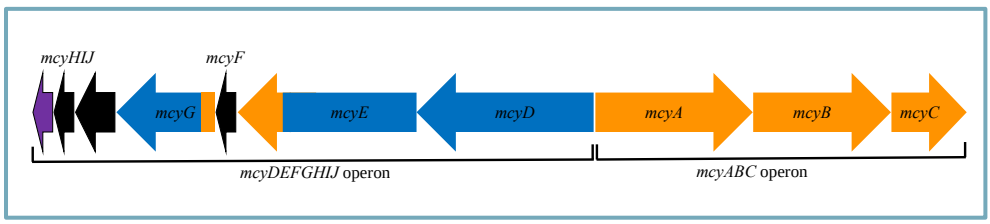

Figure 5. The microcystin (*mcy*) gene cluster in *Microcystis aeruginosa*. The genes are located on two operons, with a total size of circa 55 kbp. Genes coding for non-ribosomal peptide synthetases (NRPS) are indicated in orange, polyketide synthases in blue, tailoring enzymes in black, and ABC-transporters in purple. Modified from Tillett et al. (2000).

Microcystin synthetase complexes are composed of PKS modules, responsible for Adda-formation, and seven NRPS modules, on which peptide assembly is performed in a "step-by-step"-fashion (figure 6). Each single module is responsible for the activation, thioester formation, condensation, and incorporation of an amino acid in the growing peptide chain (Pearson et al., 2016, Baunach et al., 2021). The biosynthesis of microcystin starts by the assembly of the amino acid Adda, which is characteristic for microcystins and nodularins (Rinehart et al., 1988). Adda assembly is initiated by activation and loading of phenylacetate on McyG (see e.g. Méjean and Ploux, 2013). The first product in the Adda-assembly is thereafter methylated by the

tailoring enzyme McyJ (an O-methyltransferase, (Christiansen et al., 2003)), and further transferred to, extended, and modified by the PKS modules of McyG, McyD and McyE (Tillett et al., 2000, Méjean and Ploux, 2013). The remaining amino acids in the microcystin molecule are incorporated by the NRPS modules of McyE and McyABC, and modified by the tailoring enzymes McyF and McyI.

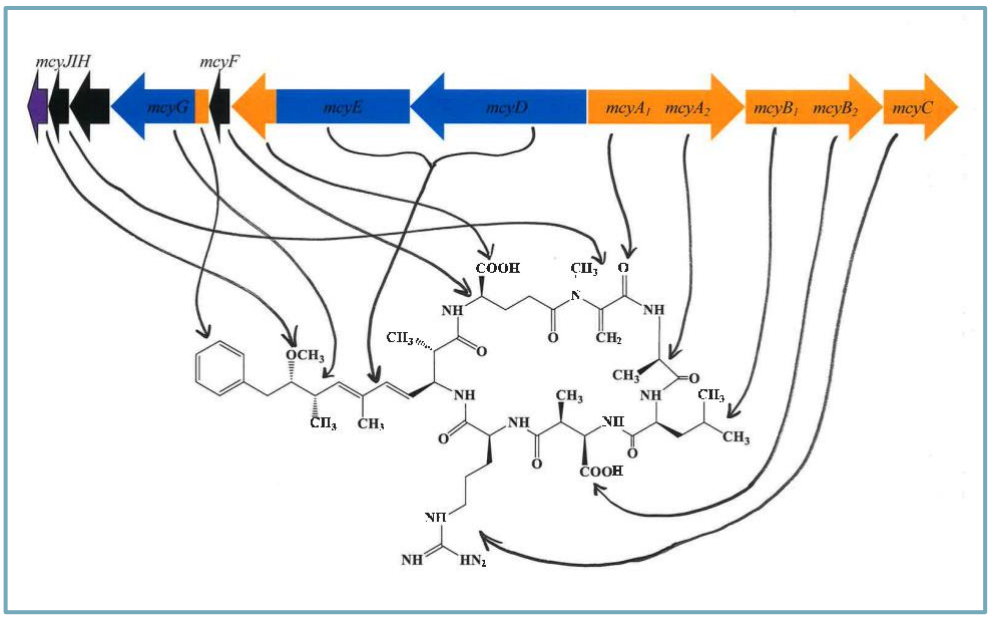

Figure 6. Simplified biosynthetic model of the MC-LR molecule. The Adda moiety is first assembled on by the PKSs/NRPSs encoded for by *mcyDEGHJ*. The remaining amino acids are mainly incorporated by NRPSs encoded for by *mcyABC*. Intermediate products are modified by enzymes encoded for by *mcyF* and *mcyI*.

The ability of cyanobacterial strains to produce microcystins have generally been contributed to the presence and functionality of all genes within the *mcy* gene cluster. Several studies have shown that non-microcystin producing cyanobacterial strains lack either the full *mcy* gene cluster, or some of the *mcy* genes (Nishizawa et al., 1999, Rantala et al., 2004, Schatz et al., 2005, Nishizawa et al., 2007, Christiansen et al., 2008, Yamaguchi et al., 2015). However, there are also observations of cyanobacterial strains in which the full *mcy* gene cluster is present, that do not produce detectable amounts of microcystins (including *Microcystis* (Nishizawa et al., 1999, Kaebernick et al., 2001, Mikalsen et al., 2003, Nishizawa et al., 2007), *Planktothrix* (Kurmayer et al., 2004), and *Dolichospermum* (Fewer et al., 2011)). Microcystin-deficient mutant strains have been generated by insertional mutagenesis causing inactivation of *mcy* genes (e.g. *mcyB* (Dittmann et al., 1997) and *mcyH* (Pearson et al., 2004)). These mutants can produce other metabolites, but not microcystins, and these strains are therefore useful for studying the potential function

of microcystins. In contrast to the mutant strains where *mcy* genes have been inactivated, it has also been shown that partial deletion of *mcyA* in *Dolichospermum* (Fewer et al., 2008) and *mcyJ* in *Planktothrix* (Christiansen et al., 2003) did not reduce microcystin biosynthesis, but instead altered the production to other microcystin variants. More recent studies (including **paper II** in this thesis) have shown that natural *Microcystis* populations consist of co-existing toxic and non-toxic strains, in which the *mcy* gene cluster is either fully present, partial, or absent (Yancey et al., 2022, see also Pérez-Carrascal et al., 2019). Altogether, there is variability in the *mcy* gene cluster, but the effects on microcystin synthesis is not fully resolved.

Potential roles of microcystin

Most research on microcystins have focused on their effect on other organisms, but their potential physiological and/or ecological function for the *Microcystis* cell is to a large extent unresolved (Omidi et al., 2017, Huisman et al., 2018). Several potential functions have been suggested. Here, I have chosen to bring up those that are most relevant in context of my research.

It has been suggested microcystins might function as a grazer deterrent (Hansson et al., 2007, Ger et al., 2016) or have a protective role against parasites and pathogens (Rohrlack et al., 2013, Urrutia-Cordero et al., 2013). However, a transcriptome study showed that *mcy* gene expression was largely unaffected in *Microcystis* that were exposed to grazing (Harke et al., 2017). Furthermore, the evolution of the *mcy* genes pre-dates the occurrence of metazoans (Rantala et al., 2004). This suggest that, even though there might be an effect of microcystins on predators, grazing deterrence was not the primary function of microcystins. The long evolutionary history of secondary metabolites, such as microcystins, instead imply that they could be involved in cellular functions, such as siderophoric trace metal scavenging and iron chelation, protein binding, or photosynthesis (Rantala et al., 2004, Holland and Kinnear, 2013, Pearson et al., 2016). Laboratory studies have shown that microcystin production rate is affected by irradiance (Utkilen and Gjolme, 1992), and microcystin production has also been linked to chlorophyll a content, growth rates, and photosynthetic activity (Leblanc Renaud et al., 2011, Phelan and Downing, 2011, Meissner et al., 2015). High light irradiances cause oxidative stress in photosynthetic cells, and it has been suggested that microcystins might have a protective function, by binding to photosynthetic proteins that are sensitive for oxidative damage (Dziallas and Grossart, 2011, Zilliges et al., 2011). Moreover, microcystins have been shown to affect *Microcystis* colony formation (Gan et al., 2012). This might have effects on colony buoyancy and subsequently on *Microcystis* ability to e.g. compete for light and CO_2 at lake surface, but colony size can also aid in escaping grazers, that cannot ingest large *Microcystis* aggregates.

To sum up, despite the large amount of research conducted, there are still many things about *Microcystis* and microcystins that are unknown. For example, why do cyanobacteria do produce microcystins and other metabolites? What is the ecological or physiological function? If microcystin-production is beneficial – why do not all strains synthesise these compounds? Some earlier studies have shown that there are variation in the *mcy* genes, which has been corroborated by more recent research based on e.g. metagenomic or genome sequencing. The effects on microcystin synthesis is, however, not resolved. What factors (environment, genes, other traits than microcystin-production? A combination?) influences the temporal and spatial distribution of toxic and non-toxic *Microcystis* strains in natural environments is not fully understood Furthermore, studies aiming to explain how toxic and non-toxic *Microcystis* are favoured by various environmental factors, often vary in their results. It would probably take a whole bunch of PhD-students to answer just a tiny fraction of all possible questions. In my thesis work, I have aimed to at least contribute to a small part of the "general *Microcystis*-knowledge", by addressing the research questions specified on the next page.

Objectives of the thesis

In my thesis, I investigated phenotypic and genotypic variation in the freshwater cyanobacterium *Microcystis* sp., with emphasis on the ability of individual strains to produce the hepatotoxin microcystin. I have aimed to provide critical understanding of 1) the temporal dynamics and variation of microcystin-producing and non-microcystin producing phenotypes, 2) how the underlying *mcy* genotypes might explain the observed phenotypes, 3) identify environmental factors that favours microcystin-producing *Microcystis* over non-producing phenotypes, and are associated to microcystin concentrations measured in natural systems.

Specifically, the following objectives were addressed in the chapters of my thesis:

- Examine the temporal dynamics of microcystin-producing and non-microcystin producing phenotypes in natural *Microcystis* populations (**paper I, paper III**).
- Investigate variation in microcystin-profiles in microcystin-producing phenotypes (**paper I**).
- Based on the results from **paper I**, investigate the variation within the *mcy* gene cluster and how the *mcy* genotypes relate to microcystin phenotypes (**paper II**).
- Based on the results from **paper II**, develop a population-specific quantitative PCR method for the detection of potentially microcystin-producing *Microcystis* (**paper III**).
- Investigate the impact of environmental factors on 1) the proportions of *Microcystis* strains with the *mcyJ* genotype, and 2) on microcystin concentrations in lake water (**paper III**).

Summary of methods

In this chapter, I describe some of the key methods used in this thesis. I cannot assume that all readers of this thesis have knowledge of e.g. methods in limnology or population genetics, and therefore I aim at giving a broad overview of the methods and analyses conducted. Detailed descriptions of methods are presented in the original publications/manuscripts (**paper I-III**).

Sampling

Study systems

In two of the studies (**paper I** and **paper II**), I used monoclonal cultures of the morphospecies *Microcystis botrys* (Teiling 1942), originating from lake Vombsjön (55°41'44.0"N, 13°35'41.0"E). Lake Vombsjön (figure 7) is located in the Kävlingeån catchment, in Lund, Eslöv, and Sjöbo municipalities (Scania, southern Sweden). The lake provides several municipalities, including Lund and Malmö, with drinking water via Sydvatten AB/the Vomb drinking water plant (Sydvatten, 2021). Vombsjön is an hypertrophic lake, surrounded mainly by agricultural land (Alström et al., 2017). The ecological and chemical status is considered poor (Länsstyrelserna, the County Administrative Board Skåne), partly due to heavy nutrient loading through run-off from the surrounding land (Alström et al., 2017, Li et al., 2018). Heavy phytoplankton blooms dominated by cyanobacteria has been observed since the 1940's, and *Microcystis* is the most common genus observed within blooms (Alström et al., 2017).

In the third study (**paper III**), I conducted a field study in lake Vombsjön and in the eastern basin of lake Ringsjön (E. Ringsjön). Ringsjön is located in the Rönne å catchment, in Hörby and Höör municipalities, Scania. Similar to Vombsjön, E. Ringsjön is a hypertrophic lake, surrounded by agricultural land and forest. Historically, phytoplankton blooms used to be dominated by diatoms. Since the 1970's, the lake has experienced an increase in phytoplankton biomass, and summer blooms have been dominated mainly by *Microcystis* sp. (Länsstyrelserna).

These two study systems were chosen based on the following:

- They provide many consumers with drinking water, and are important for recreation and fisheries.
- Environmental monitoring have been conducted since the 1960's (Ekologgruppen, 2012, Alström et al., 2017). Therefore, it would be possible to compare my results to earlier observations, and understand the results in a "local context".
- During the past 50-80 years, both lakes have experienced an increase in cyanobacterial blooms dominated by *Microcystis* (Ekologgruppen, 2012, Alström et al., 2017).

Figure 7. Map showing the location of lakes Ringsjön (top, indicated in blue) and Vombsjön (bottom, indicated in brown), located in southern Sweden. Scale bar shows 2 km. Modified from Lantmäteriet, map of Sweden from https://pixabay.com.

Microcystis bloom samples and cultures

In the summer 2014, the cyanobacterial bloom in lake Vombsjön was sampled during five occasions (see table 1 in **paper I**). Plankton biomass was screened by microscopy and individual *M. botrys* colonies were isolated. The isolates were maintained in climate rooms under controlled conditions. After established growth, the isolated, clonal *M. botrys* strains were grown as batch cultures, and were transferred to new, fresh growth medium every three to four weeks. Since *M. botrys* cultures grew in non-axenic conditions, they were continuously monitored (by microscope) for growth and survival, and to detect contaminating organisms. In total 843 colonies were isolated, and of the initial isolates, 15% (130 out of 843 strains) survived and their microcystin content could be analysed (**paper I**).

Microcystis samples for qPCR

Environmental samples for quantification of toxic and non-toxic *Microcystis* with qPCR, and microcystin analysis with ELISA (**paper III**) were collected in lake Vombsjön and from the eastern basin of lake Ringsjön during June-September 2018 and June-October 2019. Lake water was filtered on polycarbonate filters, thereby collecting cells from the whole plankton community. The qPCR method (see description below) then allowed for detection and quantification of *Microcystis* cells specifically: one primer-pair was used to target a part within the phycocyanin gene operon, thereby quantifying the whole *Microcystis* spp. population, and three primer pairs were used to target the *mcyB*, *mcyF*, and *mcyJ* genes within the *mcy* gene operon. This allowed me to quantify the toxigenic subpopulation to the whole *Microcystis* population.

Environmental variables

Nutrients

Nutrients are chemical substances, mainly nitrogen and phosphorus, required for organismal growth. When a nutrient is necessary for growth, but present in smaller quantities than needed, they are referred to as limiting nutrients. Changes in concentrations and ratios of limiting nutrients can have effects on lake ecosystems.

In freshwater systems, the growth of phytoplankton and aquatic plants are most affected by phosphorus availability. Phosphorus occurs in two forms: dissolved phosphorus (PO_4, or SRP, phosphorus that can be used by organisms), and particulate phosphorus (that is incorporated in particles, organisms et c.). The total phosphorus (totP) include both dissolved and particulate phosphorus, and is used to estimate the amount of phosphorus in a system. By measuring SRP and totP, it is possible to estimate the productivity of lake systems.

In addition to phosphorus, nitrogen is also necessary for the growth of primary producers. Nitrogen are present in various forms, both organic and inorganic. The organic forms are present in living organisms, e.g. in proteins, whereas inorganic forms are not incorporated in biologic material, but are mineral based. These forms include, for example, nitrate (NO_3^-), nitrite (NO_2^-), and ammonia (NH_4^+). It is the inorganic forms that can be utilised by aquatic primary producers. The total nitrogen (totN) include all various forms of nitrogen in a water sample.

The ratio of nitrogen to phosphorus (the N:P quota) is used to determine which of these two nutrients are limiting productivity in a system. Based on the Redfield ratio, systems with a N:P quota <16 are considered being limited by nitrogen availability, whereas a quota >16 is limited by phosphorus

Chlorophyll a

Chlorophyll is the green pigment in plants, that absorbs light to provide energy for photosynthesis. Since (almost) all phytoplankton contain this pigment, chlorophyll a-concentrations are commonly used as estimates of algal biomass in water samples.

pH

The pH of a solution is defined as the logarithmic hydrogen (H^+) ion activity, which is approximately equal to the H^+ concentration in solutions. In natural waters, pH usually range between 4.0 to 9.0 (mostly between 6.0 to 8.0). Deviations (decreases or increases) from this range can stress aquatic organisms, reducing fitness and survival. Furthermore, the pH might reflect biological activity (i.e. primary production) and changes in the natural chemistry of aquatic systems.

Dissolved oxygen

Dissolved oxygen (DO) is an estimate of how much oxygen is dissolved in water bodies, thereby available for aquatic organisms. In aquatic systems, photosynthesis is a major source of oxygen. Since the photosynthetic process is dependent on light, oxygen is mainly produced during daytime. All living organisms also consume oxygen (they respire). Too high respiration rates (e.g. by bacterial degradation of organic material) can cause anoxia in water bodies. By measuring dissolved oxygen throughout the water column, it is possible to determine the quality and "health" of a system.

Temperature

The temperature in the water column can be measured in order to determine thermal stratification (the thermocline) in lakes. As a general rule, the warmer, upper layer of the water column do not mix with the cooler, deeper layer. Temperature differences in stratified lakes can affect the amount of oxygen that is available for living organisms.

Light attenuation and water colour

Solar radiation is critical for primary production, both in aquatic and terrestrial systems: the radiant energy is used to build e.g. carbohydrates from CO_2 and water during photosynthesis. By measuring the light at different depths, it is possible to determine the depth of the photic zone (the part of the water column where photosynthesis can occur). These measurements are used to calculate the light attenuation coefficient (k_D), which is the depth at which light is no longer able to penetrate the water column. Another method to measure the depth of light penetration is to use a Secchi disk, which is lowered into the water. The depth at which the disk "disappears" (the Secchi depth) can be used to estimate light attenuation.

Water colour is a measure of dissolved organic material (such as humus, peat, or decaying organic matter) and metallic ions (iron and manganese) in lake water. These substances enter lake systems e.g. via surface water runoff from the surroundings, by precipitation (rain or snow), or from decomposing plant material within the lake. The colour of lake water can also indicate that there is presence of suspended particles, plankton, or bacteria. The depth of the euphotic zone is mainly determined by the amount of resuspended and dissolved substances. Measures of water colour and light can thus be used to understand primary production in lakes.

Detection and quantification of microcystins

LC/MS-MS

To investigate the temporal dynamics of microcystin-phenotypes, identify the specific microcystin variants present, and to analyse the phenotype diversity in microcystin-producing strains, cultured cyanobacterial cells were harvested and analysed with liquid chromatography tandem mass spectrometry (LC/MS-MS) (**paper I**). Liquid chromatography (LC) is a method used to separate and identify the components of a mixture. Mass spectrometry (MS) is a method used to measure the mass-to-charge (*m/z*) ratio of ions. Tandem mass spectrometry (MS/MS) is a technique where two mass analysers are coupled together, which increase the abilities to detect and identify fragments that have very similar *m/z* ratios. By coupling LC to MS/MS, the capabilities of the separate techniques are synergistically enhanced, making it possible to determine the amount and elements of a certain compound also in complex, or highly volatile or unstable samples.

Enzyme-Linked Immunosorbent Assay (ELISA)

In the work included in this thesis, I have also used indirect competitive ELISA to assay the microcystin content in lake water (**paper III**). The ELISA methods are immunoassays, used for the detection and quantification of e.g. antibodies, proteins, et c. in biological samples. The indirect competitive ELISA measures the concentrations of antigens in a sample by spectrophotometry. In short, the technique is based on the competitive binding of labelled antigens (reference antigens) and antigens present in the sample (sample antigens) to antibodies. The analysis is performed in multi-well plates, onto which standards (solutions with known microcystin concentrations) and samples are loaded. Reference antigens and antibodies are added to each sample. Depending on the amount of sample antigen present, more or less of the reference antigen can bind to antibodies. A colour solution is added, and the absorbance is analysed by spectrophotometry at 450 nm. Based on the absorbance values of the included standards, a standard curve is constructed, which in turn is used for evaluating the microcystin concentrations in samples. The ELISA method is generally considered being sensitive, and is recommended e.g. by the United States Environmental Protection Agency (EPA) for the monitoring of microcystins and other cyanotoxins in drinking water (Holsinger et al., 2015).

Molecular methods

DNA sequencing and *mcy* gene analysis

In the first project of this thesis (**paper I**), I showed that there was variation in microcystin phenotypes in the sampled *M. botrys* population. Therefore, in **paper II** I wanted to investigate how *mcy* genotypes correspond to the microcystin phenotypes (characterised as microcystin-producers or non-microcystin producers). I originally intended to examine the genotypes of all 130 *M. botrys* strains that were analysed in the first project, but discovered that many of the toxic strains that were still maintained as laboratory cultures did no longer produce detectable amounts of microcystin. The subset of strains in **paper II** were harvested for microcystin-analysis and for DNA-extraction at the same time, ensuring that the microcystin-phenotypes and *mcy* genotypes are linked.

Whole genome sequencing (WGS) is the analysis of an organism's entire genome, i.e. the determination of all nucleotides in the organism's DNA. The workflow (figure 8) starts with DNA extraction, a process that I will not explain further here. During sequencing, DNA is first sheared into smaller fragments, and each fragment is sequenced to a given length (in **paper II**, the length was 150 nucleotides). The quality of the resultant sequence *reads* is then evaluated, and if necessary, low

quality sequences are removed. Sequences are then *assembled*: the short reads are puzzled back together into longer, continuous sequences (*contigs*), that in turn are joined together in longer sequences, *scaffolds*. It is important that there is enough overlap between sequence reads at each position in the genome, otherwise there is risk of mis-assembly (i.e. that reads are puzzled together incorrectly). This requires sufficient sequence *coverage* (or read depth, the number of times each individual nucleotide has been sequenced).

After genome assembly, genomes are usually *annotated*. To simplify, genome annotation is the process where coding regions, as well as RNAs, CRISPR loci, insertion sequences, and other mobile elements in a genome is identified, and their function predicted. The annotation process thus infers the structure and function of assembled sequences. In the process, nucleotide sequences are first translated into protein sequences. This is because protein sequences are better conserved than their corresponding nucleotide sequences (a change in a nucleotide triplet (a codon) do not necessarily translate to a change in amino acid). The approach used in this thesis work is based on comparisons: the structural annotation is determined by comparing open reading frames (ORFs, a portion of DNA sequence between a start and stop codon) to protein libraries available in various curated databases, or to characterized reference genomes.

In **paper II**, my approach to detect the presence or absence of *mcy* genes in the *M. botrys* genomes was based om similarity searches. A database consisting of Mcy sequences (i.e. translated protein sequences from *mcy* nucleotide sequences) from multiple *Microcystis* spp. genomes was created, to which I could compare the annotated genomes via BLAST (Basic Local Alignment Search Tool). Hence, I was able to evaluate the presence or absence, as well as potential ambiguities, in the annotated *mcy* genes in *M. botrys*.

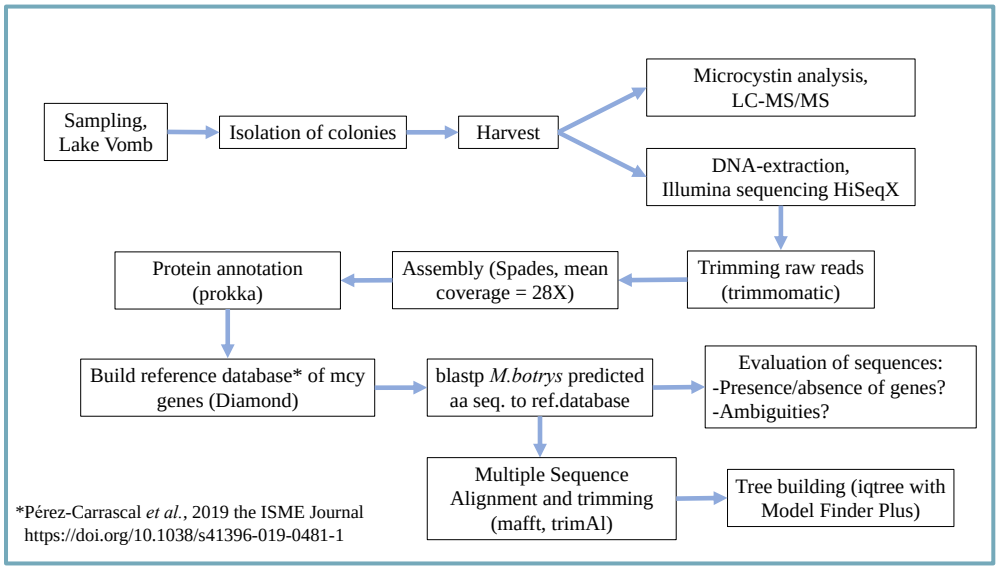

Figure 8. Graphic summary of the bioinformatic methods used in **paper II**.

Quantitative Polymerase Chain Reaction (qPCR)

Polymerase Chain Reaction (PCR) is a technique used to detect and amplify specific pieces of DNA. In contrast to the conventional PCR, the qPCR method also allows for quantification of the amplified DNA in samples, i.e. enables the determination of relative or absolute amounts of the target DNA ("the DNA of interest"). In order to quantify DNA, the amplified DNA is first labelled (usually with fluorescent dyes, for the work in **paper III**, I used SYBR green). The amount of fluorescence detected during the amplification process is proportional to the amount of amplified target DNA. To be able to quantify the amount of target DNA, standard samples (with known amounts of DNA) are included during each qPCR analysis. Sample fluorescence is monitored during the PCR process, and the information is used to create amplification curves. By comparing the fluorescence of standards and samples, it is possible to calculate the number of gene copies, or estimate the amounts of DNA present in samples.

Main findings

Dynamics and variation of microcystin phenotypes in natural *Microcystis* populations

In **paper I**, I investigated the temporal dynamics of toxic and non-toxic producing phenotypes in a natural population of *Microcystis botrys*. I also aimed at getting a more comprehensive understanding of the phenotypic diversity in microcystin-producing strains. To achieve this goal, the phytoplankton community in lake Vombsjön was sampled during five occasions in the summer of 2014. In lake Vombsjön, *M. botrys* is the most common species within the genus. Therefore, sampling of the species rather than the whole *Microcystis* community was performed. Individual *M. botrys* colonies were isolated, and the microcystin content in established strains analysed with liquid chromatography tandem mass spectrometry (LC/MS-MS). In **paper III**, I further investigated how the proportions of microcystin-producing and non-producing *Microcystis* phenotypes change during the course of a bloom. Bi-weekly sampling was performed in lakes Vombsjön and E. Ringsjön for two consecutive years (June-September in 2018, and June-October in 2019). To determine the proportions of microcystin-producing and non-microcystin producing *Microcystis* spp., a population-specific primer for qPCR was developed, targeting the *mcyJ* gene in the *mcy* gene cluster. The qPCR method used here do not allow to distinguish *M. botrys* from the remaining morphospecies within the genus, and therefore I chose to target the whole *Microcystis* population.

My results showed that 1) microcystin-producing and non-microcystin producing phenotypes co-exist in the studied lakes, 2) the proportions of microcystin-producing and non-microcystin producing phenotypes in the sampled *Microcystis* populations varied temporally during the sampling periods, and 3) the proportions of microcystin-producing phenotypes were higher in early (June) and late (end of August/early September) summer (figure 9). This had not previously been described in *M. botrys*, nor in other lakes in Sweden (**paper I**). The co-existence and temporal succession of microcystin-producing and non-producing phenotypes have been shown in *Microcystis*, as well as in other microcystin-producing cyanobacterial genera (e.g. Welker et al. (2004b), Briand et al. (2008)). In **paper I** and **paper III**, I could show that microcystin-producing phenotypes are favoured during both early and late bloom phase in the studied lakes. These results are similar to the findings by Briand et al. (2009). In contrast, several studies have shown that microcystin-

producing phenotypes tend to dominate during the early and peak bloom phases, and non-microcystin producing phenotypes during the end of blooms (Welker et al., 2007, Briand et al., 2008, Gobler et al., 2016, Chaffin et al., 2018). This might indicate that different strains are ecologically adapted to different environmental conditions, as suggested by Dick et al. (2021). The seasonal succession of toxigenic and non-toxigenic *Microcystis* in our study systems could thus represent ecotypes that are adapted to the variable environmental conditions experienced during bloom succession.

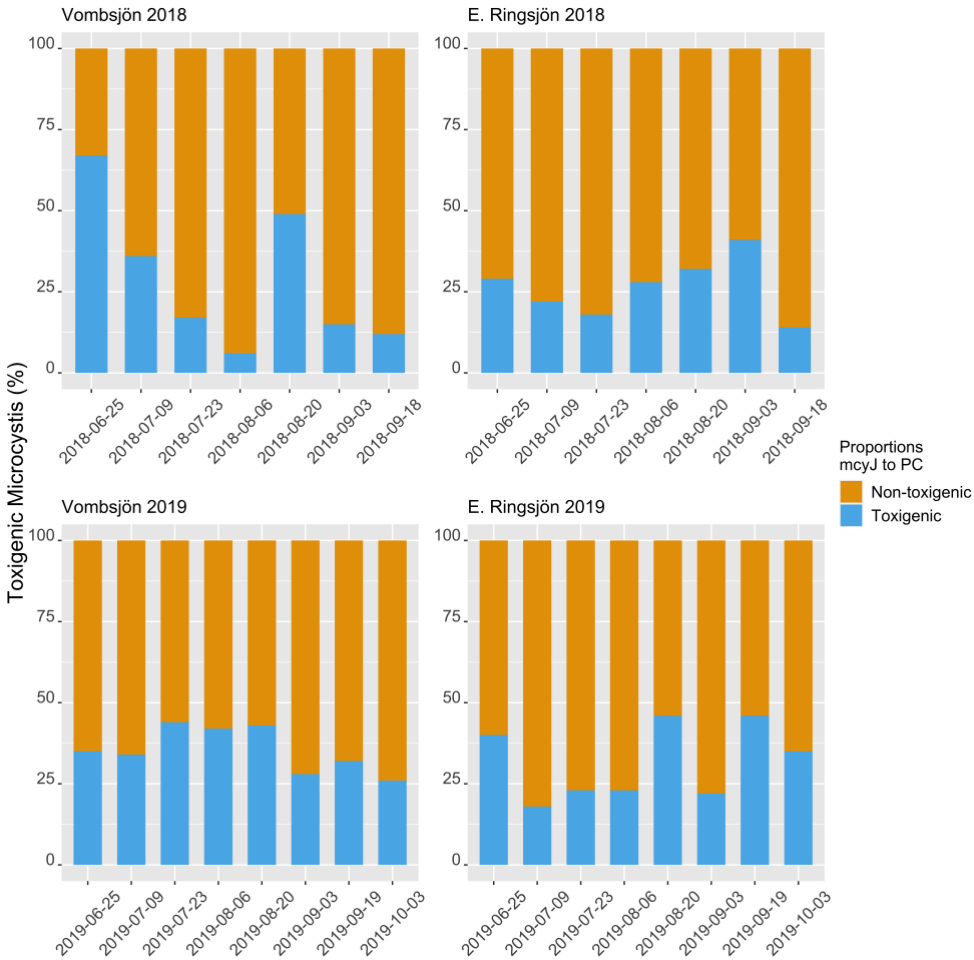

Figure 9. Proportions of microcystin-producing (toxigenic, blue bars) and non-microcystin producing (non-toxigenic, orange bars) *Microcystis* spp. in lakes Vombsjön and East Ringsjön 2018 (top) and 2019 (bottom).

The use of two different approaches (screening of individual strains to assess actual microcystin-production in **paper I**, vs. qPCR to assess the abundance of *Microcystis* spp. cells with and without the *mcyJ* gene in environmental samples in **paper III**) rendered similar results. By isolating individual colonies and analysing their microcystin-profiles with LC-MS/MS, we could show that the proportion of microcystin-producing phenotypes ranged between 8-52% in lake Vombsjön in 2014. Using qPCR, proportions ranged between 5.5-67% in Vombsjön 2018, 25-44% in Vombsjön 2019, 14-41% in E. Ringsjön 2018, and 18-46% in E. Ringsjön 2019. The fact that similar proportions and patterns were observed, support the reliability of the qPCR method. However, it is also important to acknowledge that the culturing-method might be biased. The survival and performance of isolated colonies might differ, and thus the strains that survived until analysis might not be a true representative of the sampled population. Similarly, there is evidence that the use of qPCR in order to assess toxic and non-toxic *Microcystis* might over- or underestimate the abundances of toxic strains, depending on which *mcy* gene is targeted (Beversdorf et al., 2015, Zuo et al., 2018). This might be because *Microcystis* cells that are unable to synthetise microcystins can contain partial *mcy* gene clusters, thereby giving positive results in qPCR assays (Yancey et al., 2022). To obtain more reliable results, it would be preferable to target more than one *mcy* gene, as done in **paper III**.

While much research on microcystin-production in cyanobacteria focuses on whether a strain is able to produce microcystins or not, it is also important to acknowledge that a single strain can produce several microcystin congeners (structural variants) simultaneously. To date, more than 300 microcystin congeners have been identified (Jones et al., 2021), which vary in toxicity from more or less non-toxic to highly toxic (Rinehart et al., 1994). In **paper I**, I identified the specific microcystin variants produced by individual strains, and could thus characterize the microcystin profiles, or chemotypes, present in the *M. botrys* population. Among the analysed strains, 28.5% were microcystin-producers and found to produce a range from one up to twelve microcystins simultaneously, with a median number of 4.9 microcystins (table 1). To put these results in context, a literature review by (Puddick et al., 2014) concluded that *Microcystis* produce a median number of four to five microcystins, however larger numbers has been observed (Fewer et al., 2007). In the population sampled from Vombsjön in 2014, I could identify 26 unique microcystin-profiles/chemotypes, producing a total number of 18 different microcystin variants. With one exception, all strains that only produced one microcystin congener produced the MC-RR variant. These chemotypes were present during almost the whole sampling period, from July to September. The remaining strains displayed unique chemotypes, that were only sampled once during the bloom. This indicates that *Microcystis* with different microcystin profiles might be favoured during certain environmental conditions.

Table 1. Microcystin-producing and non-producing *Microcystis botrys* in lake Vombsjön, sampled in 2014. Columns show the number of non-microcystin producing strains, and microcystin-producing strains, as revealed by LC-MS/MS.

Sampling date (2014)	Number of analysed *M. botrys* colonies	Percentage microcystin-producers	Microcystins produced (range, median)
June 30	25	20	3-12, 12
July 14	25	8	1, 1
August 3	27	15	1-4, 1
August 25	30	45	1-9, 2
September 8	23	52	1-11, 7

In addition to the temporal variation in proportions of microcystin-phenotypes discovered in **paper I**, I also showed that strains isolated during early (June) and late (September) summer produced a higher number of microcystin variants, compared to strains isolated during mid-summer (July-August) (table 1). Furthermore, in **paper III** I showed that that microcystin concentrations, in general, increased during the course of a bloom (figure 10). This is most likely related to *Microcystis* total biomass, rather than an increase in per cell microcystin production. With the method used in **paper I**, I could not get information on the total production of the microcystin variants (i.e. microcystin content per biomass). This information would have been useful and could have added to the assessment of temporal variation in chemotypes present in a bloom.

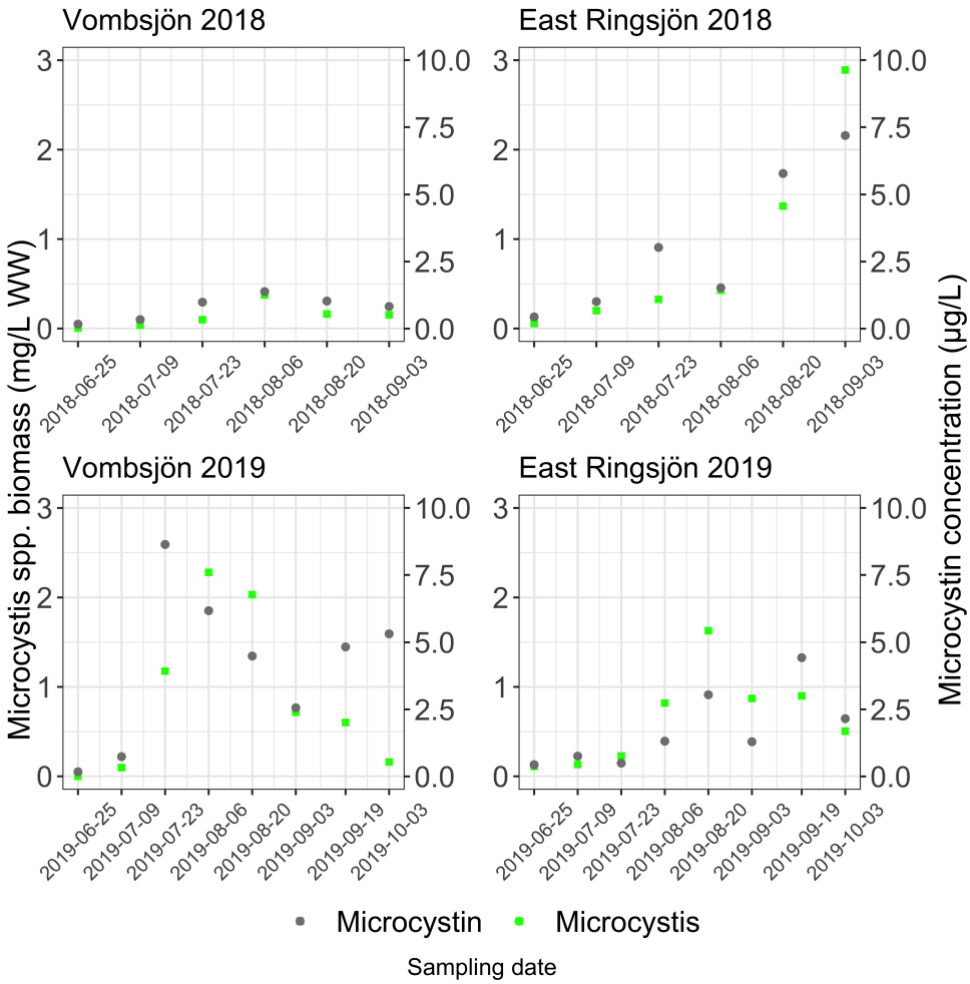

Figure 10. *Microcystis* spp. biomass (µg L^{-1} wet weight (WW), left y-axis) and microcystin concentrations (µg L^{-1}, right y-axis) for each sample date in Vombsjön and East Ringsjön 2018 (top), and Vombsjön and E. Ringsjön 2019 (bottom). Biomasses are based on microscopy counts.

Effects of environmental variables on the prevalence of toxic *Microcystis* in natural systems

In **paper III**, I aimed to identify what environmental factors favours microcystin-producing *Microcystis* over non-producing phenotypes in the studied systems. I also wanted to examine how the environment might influence microcystin concentrations in lake water, with the aim to better understand the role of microcystin. To achieve this, I used quantitative PCR as a tool for detection and quantification of microcystin-producing and non-microcystin producing *Microcystis* cells in lake water samples. Based on the results from **paper II**, two population-specific primers targeting the *mcyF* and *mcyJ* genes were developed. In addition, I used an already published primer pair targeting the *mcyB* gene (Kurmayer et al., 2003), so that the results of our population-specific primers could be compared and evaluated to a marker that has been used previously. A primer pair targeting the PC-IGS operon (Kurmayer et al., 2003) was used to obtained estimates of the total *Microcystis* population. The results from qPCR using the three primers targeting *mcy* genes gave slightly dissimilar estimates of gene copy numbers. However, a regression analysis showed that there was a strong relationship between *mcy* genes (see methods section, **paper III**). Linear regression was also performed on *mcy*-genes to *Microcystis* biomass (*mcyB* to biomass: $R^2 = 0.2$, $p = 0.016$; *mcyF* to biomass: $R^2 = 0.19$, $p = 0.045$; *mcyJ* to biomass: $R^2 = 0.32$, $p = 0.0019$), and *mcy* genes to microcystin-concentration (see methods section**, paper III**). I therefore concluded that the use of *mcyJ* was most suitable in the specific systems. The environmental variables included in the study were chosed based on their proposed influences on toxic vs. non-toxic *Microcystis*, and on microcystin production (see Methods section).

Using principal component analysis (PCA) (figure 11) and multivariate analysis of covariance (MANCOVA), I could show that among the environmental variables sampled (see supplementary table ST2 in **paper III**), the ratios of microcystin-producing phenotypes to non-producers showed a positive association to NO_3^- + NO_2^- concentrations (from here on NO_X), and a negative relation to pH. Microcystin concentrations were positively related to SRP concentrations, and negatively associated with copepod biomass.

My observations show that NO_X-concentrations, as well as totN were highest during the early sampling period (June), and started to decline from early to mid-July. SRP and totP were low in June-July, and started to increase from approximately late July/early August. Phosphorus concentrations were highest in September and declined towards the end of the sampling periods (as indicated in figure 12, see also figure 5 and figure 6 in **paper III** for details). The association of NO_X and the proportions of microcystin-phenotypes suggest that nitrogen-availability might be a potential driver, favouring microcystin-producing strains over non-producers (see e.g. Yancey et al. (2022)). Microcystin is a nitrogen-rich metabolite (Harke and

Gobler, 2013, Wagner et al., 2021), and the result of several studies have shown that microcystin production increase when nitrogen is replete, whilst nitrogen depletion cause microcystin-synthesis to decrease (Harke and Gobler, 2013, Holland and Kinnear, 2013, Van de Waal et al., 2014, Gobler et al., 2016, Newell et al., 2019, Brandenburg et al., 2020, Duan et al., 2021). This suggest that microcystin-producing strains have higher nitrogen requirements than non-microcystin producers. The decrease in microcystin production during N-limitation could potentially be explained by a higher loss than benefit when N-availability is constrained: the (energetic) cost of microcystin production might be higher than the possible benefit for the cell. Considering the general trend that microcystin production is favoured during conditions when N is available in excess, it would have been reasonable to find a positive association of either NO_X or total nitrogen (totN) to microcystin-concentrations, as well as to the proportions of microcystin-phenotypes. Instead, there was a positive relationship of the concentrations of SRP and microcystin concentrations. On the one hand, this suggest that microcystin-production is favoured when P is in excess. This is in support of the results of a meta-analysis by Van de Waal et al. (2014), in which it was shown that P-limitation were linked to decreased microcystin production. The authors of this study suggested that this could partly be due to the ability of cyanobacteria to shunt excess N in cyanophycin (a nitrogen-storage molecule) when N is replete, preventing N to be accumulated in microcystin molecules. To be a bit speculative: if too high microcystin-concentrations within the cell could be a cause of damage, allocating N into cyanophycin rather than microcystin would be an adequate strategy to avoid cellular "self-harm". On the other hand, high phosphorus is also related to phytoplankton population growth. It is therefore possible that the link between microcystin and SRP in fact might be an artefact, and that the microcystin-concentrations observed are the results from *Microcystis* spp. population growth (figure 10) rather than the SRP concentrations *per se*.

The negative relationship of pH on the proportions of microcystin-producing strains was unexpected. Studies have linked microcystin production to chlorophyll a content, growth rates, and photosynthetic activity (Leblanc Renaud et al., 2011, Phelan and Downing, 2011, Meissner et al., 2015). A recent study based on metagenome read mapping-analysis showed a positive relationship of pH and *Microcystis* strains containing the full *mcy* cluster, and a negative relationship to *Microcystis* strains without *mcy* genes (Yancey et al., 2022), and suggested that genotype succession during the bloom might be influenced by photosynthetic rates and subsequent alterations in pH. Increased carbon fixation by photosynthetic cells would also cause pH to increase. Furthermore, high photosynthetic rates would potentially increase the formation of reactive oxygen species (ROS) in cells. Since one of the suggested potential functions of microcystin is that it protects the cell from ROS that are formed during photosynthesis (Zilliges et al., 2011), I had expected to find a positive association of both microcystin concentrations and the proportions of toxic *Microcystis* to pH. The results of **paper III** instead showed that the proportions

of toxigenic strains decreased with increasing pH, and that there were no association to microcystin concentrations.

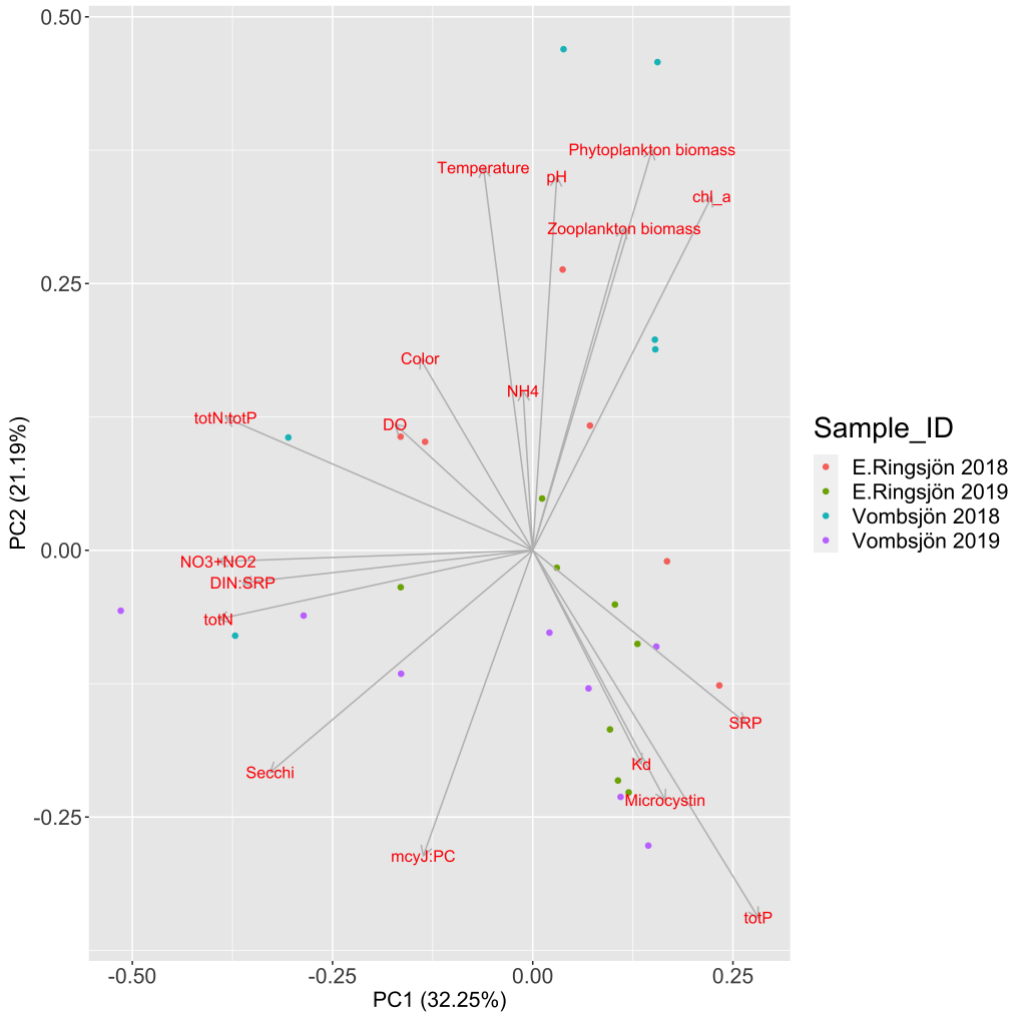

Figure 11. Principal component plot showing the associations between sampled environmental variables, microcystin concentrations, and the proportions of microcystin-producing and non-microcystin producing *Microcystis* spp. (denoted as *mcyJ*:PC). Sampling was conducted in Vombsjön and E. Ringsjön in 2018 and 2019. Dots represent the coordinates for each sample from E. Ringsjön 2018 (red), E. Ringsjön 2019 (green), Vombsjön 2018 (blue), and Vombsjön 2019 (purple).

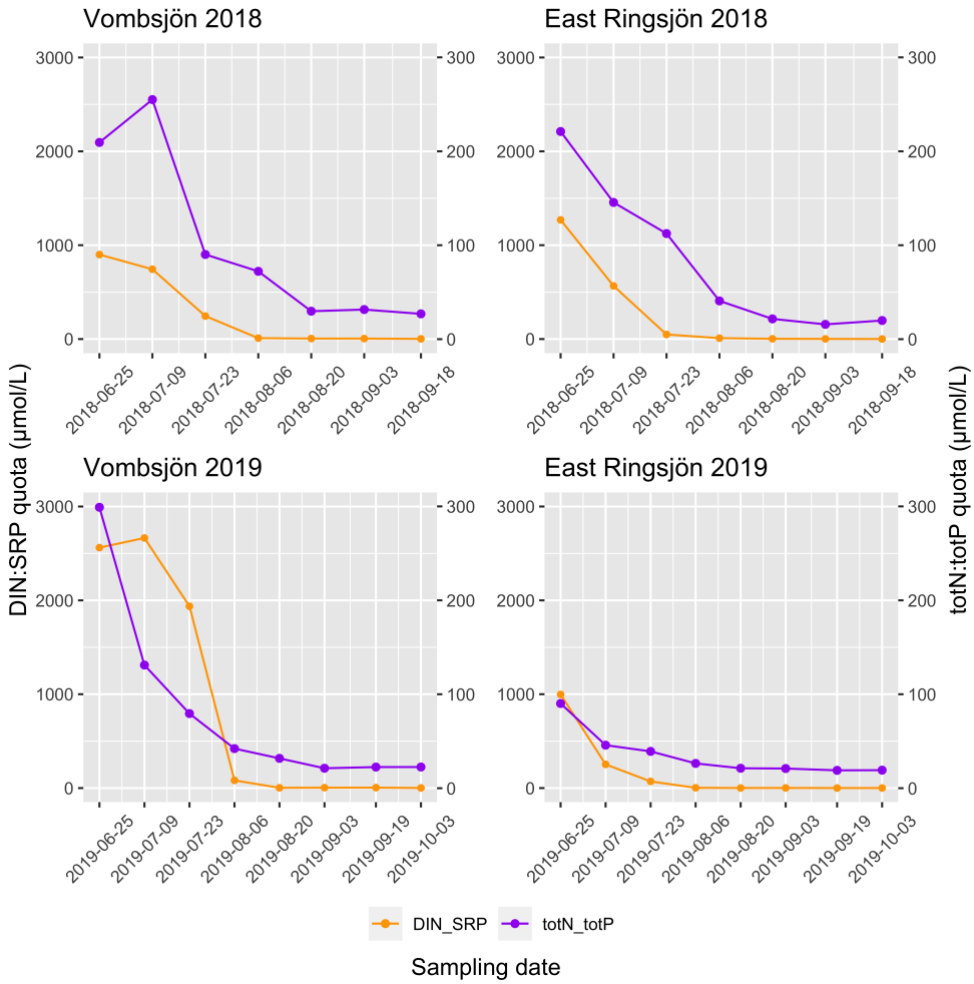

Figure 12. Quotas (µmol L^{-1}) of inorganic nitrogen (nitrate, nitrite, and ammonia) to soluble reactive phosphorus (DIN:SRP, left y-axis), and total nitrogen to total phosphorus (totN:totP, right y-axis). DIN:SRP is shown in orange, and totN:totP in purple. The figure shows the quotas per sampling date, for each lake and year.

Several studies have shown that microcystins might protect cyanobacteria against grazers, parasites, and pathogens (Hansson et al., 2007, Rohrlack et al., 2013, Ger et al., 2016, Urrutia-Cordero et al., 2016). However, since the evolution of the *mcy* genes pre-dates the occurrence of metazoans (Rantala et al., 2004), this hypothesis has been questioned. In **paper III**, I found a significant negative association of copepod biomass and microcystin concentration. In addition, the PCA showed a negative relationship between the proportion of toxic *Microcystis* cells and copepod biomass, but the results from MANCOVA showed that this relationship was not statistically significant. It could thus be argued that the negative association between microcystin concentrations and copepod biomass support the hypothesis that

microcystins might have anti-grazer functions. However, it is also possible that the negative relation between copepods and microcystins might be due to other traits than microcystin production, which have a role in predator avoidance. For example, it has been shown that genes associated to other traits than microcystin production, such as colony formation, are upregulated in *Microcystis* exposed to grazers (Harke et al., 2017). Furthermore, cyanobacteria constitute poor food sources for zooplankton (Müller-Navarra et al., 2000). That copepod biomass was lower when microcystin concentrations were higher might be simply a correlation, but not necessarily an effect of microcystin production in the lakes: correlations are not causations!

mcy genotype variation in *Microcystis* spp.

In the second study (**paper II**), I analysed the *mcy* genotypes on a subset of the microcystin-producing and non-producing phenotypes analysed in **paper I**. My aim was to investigate how the *mcy* genotypes correlate to the previously observed microcystin phenotypes. To be able to examine the full *mcy* gene cluster in *M. botrys* strains, I performed *de novo* high throughput whole genome sequencing, followed by functional annotation of the assembled genomes, and sequence similarity comparisons of the annotated *M. botrys* sequences to a custom-built database. Noteworthy is that strains were categorised as either microcystin-producing or non-producing, i.e. I did not consider what specific, or how many, microcystin variants were produced. Based on sequence alignment length and sequence similarity, *mcy* genes were categorised as present, absent, or as ambiguous.

The results showed that the studied *M. botrys* population contained multiple *mcy* genotypes. Based on the phenotypes from **paper I**, and the categorisation of *mcy* genes described above, *mcy* genotypes were classified into four groups (table 2). Groups 1 and 2 were non-microcystin producing phenotypes, and groups 3 and 4 were microcystin-producing phenotypes. Groups 1 and 3 consisted of strains where most or all *mcy* genes were detected, irrespective of phenotype. Multiple *mcy* genes were absent in strains within group 2 and 4. I did not detect any genotypes in which the full *mcy* gene cluster was absent, but rather that the *mcy* gene cluster was incomplete. Typically, the genes *mcyF*, *mcyG*, and *mcyJ* were completely lacking, whereas sequences corresponding to the other *mcy* genes were variable in terms of sequence similarity and sequence alignment lengths. The presence of partial genotypes are corroborated by the findings of Yancey et al. (2022), who showed that the *Microcystis* population in western Lake Erie consist of genotypes with the full, partial, or absent *mcy* gene cluster.

Table 2 Table showing phenotypes and genotypes of *Microcystis botrys* strains analysed in **paper I** and **paper II**. Microcystin-production was determined with LC-MS/MS (**paper I**), and is denoted with ✓ = non-microcystin producing strain, and ✖ = microcystin-producing strain. The number of microcystins produced are given in the third column. *mcy* genotypes were analysed in **paper II**, and are characterised as full or partial. Simplified from table 2 in **paper II**.

Strain ID	Microcystin-producing	Number of microcystins	Genotype
S5-4	✖		Full *mcy* gene cluster
S5-16	✖		Partial, lack *mcyFGJ*
S5-18	✖		Partial, lack *mcyFGJ*
S5-48	✖		Partial, lack *mcyFGJ*
S5-99	✖		Partial, lack *mcyFGJ*
S5-135	✖		Partial, lack *mcyFGJ*
S5-137	✖		Partial, lack *mcyFGJ*
S5-139	✖		Partial, lack *mcyFGJ*
S5-21	✓	7	Lack *mcyE*
S5-23	✓	8	Full, *mcyBC* ambiguous
S5-34	✓	8	Full *mcy* gene cluster
S5-73	✓	11	Full, *mcyBC* ambiguous
S5-74	✓	5	Full, *mcyBC* ambiguous
S5-79	✓	6	Full *mcy* gene cluster
S5-93	✓	4	Full *mcy* gene cluster
S5-110	✓	7	Full *mcy* gene cluster
S5-144	✓	9	Full *mcy* gene cluster
S5-152	✓	1	Partial, lack *mcyFGJ*
S5-163	✓	1	Partial, lack *mcyFGJ*

To further explore sequence similarities within the observed *mcy* genotypes, I performed multiple sequence alignment (MSA) of the translated *M. botrys mcy* sequences and a subset of the *Microcystis* spp. sequences included in the reference database. Based on the MSA, a phylogenetic *mcy* tree was built (figure 13). All *M. botrys* strains were subdivided within one of the main clades, except for strain S5-4 (group 1). Strains did not cluster together completely, forming a separate *M. botrys* clade. Instead, some of the strains within group 3 clustered together with the reference strains. All strains within group 2 and 4 did however form a separate group.

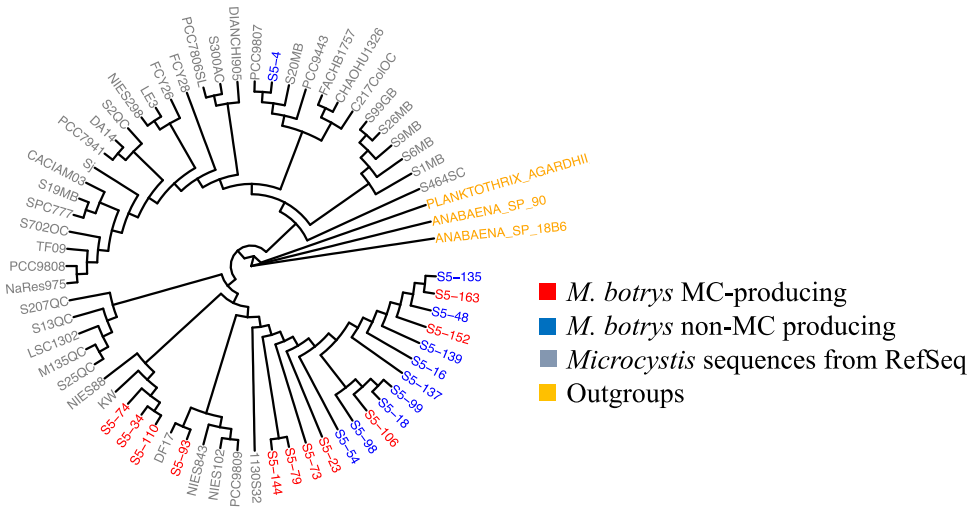

Figure 13. Simplified phylogenetic tree, based on *mcy* sequences. The tree is representative, and branches do not indicate divergence times. Strain S5-106 in this figure were not included in the analysis in **paper II**. Microcystin-producing *M. botrys* strains are shown in red, and non-microcystin producers in blue. *Microcystis* spp. *mcy* sequences obtained from RefSeq are shown in grey, and outgroups (*Planktothrix agardhii* and *Anabaena* sp.) in orange.

Among both toxic and non-toxic genotypes, I found possible recombination of domains within the *mcyA*, *mcyB*, and *mcyC* genes. Although the interpretations I have done of these results are somewhat speculative, they do support earlier studies in which recombination events between adenylation domains of *mcyB* and *mcyC* have been detected (Mikalsen et al., 2003, Kurmayer et al., 2005, Fewer et al., 2007, Tooming-Klunderud et al., 2008). Furthermore, one of the analysed strains did not produce microcystins, but nevertheless contained the full *mcy* gene cluster. This has been observed previously in *Microcystis* (Kaebernick et al., 2001, Nishizawa et al., 2007), *Planktothrix* (Kurmayer et al., 2004), and *Dolichospermum* (Fewer et al., 2011). The variation in *mcy* genes suggest that microcystin synthesis is not only due to the presence or absence of *mcy* genes, but could be subject to e.g. point mutations, or be disrupted by transposable elements.

Molecular tools such as PCR, qPCR, and amplicon sequencing, have been used extensively in order to e.g. detect *Microcystis* in environmental samples, to sequence genes of interest, or for phylogenetic analysis. While much early work aimed at quantifying and characterising the genotypes of strains in natural populations, the increased availability of (curated) *Microcystis* genome sequences, in combination with high-throughput sequencing methods, have allowed for more extensive analysis of *Microcystis* genomes.

Conclusions and future perspectives

During the past years of research studies, I have touched upon several aspects of *Microcystis* biology and ecology that might be important for understanding toxic *Microcystis* blooms, at least for lake systems in southern Sweden. I have demonstrated that the freshwater cyanobacterium *Microcystis* spp. are diverse in *mcy* genotypes and microcystin phenotypes. I have also shown that, in the studied systems, there is an association of certain environmental conditions to the proportion of toxigenic *Microcystis*, as well as to microcystin content in lake water. The presence of several co-occurring *mcy* genotypes, as well as the diversity in microcystin-phenotypes, corroborates previous research, where it has been found that natural *Microcystis* populations are shifting dynamically during the bloom period, and that these shifts might be influenced by environmental conditions. Finally, I developed a population-tailored marker to use for qPCR, targeting the *mcyJ* gene in the *mcy* gene operon, thereby allowing to detect and estimate the proportions of toxigenic *Microcystis* in the two studied lake systems.

When I first started my PhD-studies, I can honestly say that I did not fully understand how extensive the field of CHAB research is. Considering the effects of CHABs on environment, ecosystem function, economy (for example, the estimated economic costs of CHAB effects in the U.S. exceed $2 billion per year, see Burford et al. (2020)), and human health, it might not be surprising that so many studies have examined different aspects of *Microcystis* physiology, biology and ecology. Researchers have aimed at identifying and new microcystin congeners and describe their structure, to investigate how microcystins are synthesised, to do genome sequencing of *Microcystis* strains, to identify temporal and spatial variation in genotypes, to identify key drivers that favour toxic or non-toxic strains (in the laboratory and in field), to elucidate the function of microcystin… just to name a few "fields of study". For me, it has sometimes been overwhelming when trying to get an overview of the research that has been published, and synthesise the results of previous studies. Even more, when I have analysed my own results, trying to understand them, and put them into perspective, it has at times been rather frustrating that so many studies have reached different results and conclusions. How can I fit in my results in a broader context, when there is no apparent scientific consensus? Why is there so much variation? How can this be explained?

I think that there are several plausible explanations for this incongruence. In the previous part of this thesis summary, I presented and discussed the results I have

obtained. In this section of my thesis, I will instead take the opportunity to highlight some questions that I believe are important to consider in future research.

Research and management are too microcystin-centred?

The harmful effects of microcystin on ecology and society have led to a rather microcystin-centred focus of cyanobacterial research (not only with regards to *Microcystis*, but also other genera that produce microcystins, such as *Planktothrix* and *Dolichospermum*). Strains are usually defined as toxic or non-toxic, based on their ability to synthesise microcystins. However, as shown e.g. in **paper I**, *Microcystis* are able to produce a suite of different microcystin variants, that differ in toxicity. Furthermore, cyanobacteria are able to produce several secondary metabolites, as shown by e.g. Pérez-Carrascal et al. (2019), and Chen et al. (2021). There is little knowledge about the function of other metabolites produced by cyanobacteria, but, as for microcystins, it has been suggested that they might be involved in e.g. cell signalling, grazing deterrence, or have intracellular functions (reviewed in Dick et al. (2021)). However, in most studies, characterisation of other metabolites is usually not performed. From a societal perspective, the effects of toxic (i.e. microcystin-producing) blooms might be severe and acute. Access to reliable methods for detection of microcystins is of great importance for management, and there is definitely a need to be able to predict the occurrence of CHABs. However, I believe that if we are to understand the function of microcystins and how microcystin affect the performance of *Microcystis*, scientists also need to consider the production and potential functions of other metabolites (see the review by Agha and Quesada (2014) and also the work by Welker et al. (2004b, 2006)).

Should we consider cyanobacteria as functional groups, rather than focus on their toxic/non-toxic properties?

As already mentioned in previous chapters, CHABs are increasing globally. This has mainly been explained by increasing lake water temperatures, in combination with alterations in trophic status caused by anthropogenic nutrient pollution (Chorus et al., 2021). However, the increase of CHABs have been reported from a variety of freshwater systems, regardless of exposure to anthropogenic disturbances, trophic statuses, or thermal regime (Bramburger et al., 2022, Reinl et al., 2023). This suggests that cyanobacteria are able to thrive throughout a variety of environmental conditions. One plausible explanation to this might be that cyanobacterial communities might contain considerable functional diversity (see Bramburger et al. (2022)). Freshwater systems vary in their chemical and physical properties, due to lake morphometry, hydrography, trophic status, and the surrounding catchment. They are also subject to different influences from anthropogenic activities. Such variation affects the dynamics of the phytoplankton community in a system. Cyanobacteria possess several traits that make them competitive in comparison to eukaryotic phytoplankton, but strains of the same species might also differ in their traits and properties. Such varieties have, for example, been shown in the

manuscripts included in this thesis, where I could show that *Microcystis* spp. are diverse in both phenotypes (**paper I**) and genotypes (**paper II**). Such polymorphisms can influence the functional variation in a population.

In my perspective, the long evolutionary history of cyanobacteria and their potential of genomic divergence might explain their prevalence in various (sometimes extreme) environments (also discussed by Dick et al. (2021)). This could also explain why cyanobacteria are able to adapt and proliferate during rapidly changing environmental conditions. Cyanobacterial communities consisting of species or strains with different genomic and physiological properties, might also be more resilient to environmental perturbation. It has been suggested that ecological niche and habitat might influence the diversity in cyanobacterial functional traits (Prabha et al., 2016). High genomic variation could also increase the adaptive potential, and the structure of cyanobacterial genomes might facilitate rapid adaptations to changing environmental conditions (Biller et al., 2014, Kashtan et al., 2014, Alvarenga et al., 2017, Berube et al., 2019). Cyanobacterial genomes consist of a stable set of core genes, that are conserved, and accessory genes, that are more plastic (Biller et al., 2014, Meyer et al., 2017, Cai et al., 2023). Genome diversification has been contributed to changes (such as genome re-arrangements, transposable insertion elements, gene/function gain by horizontal gene transfer, and gene loss) in the accessory genome (Prabha et al., 2016). While species within *Microcystis* are characterised based on colony morphology (morphospecies), more recent research based on (pan-)genome analyses, suggest that most species within the genus are not coherent (see e.g. Pérez-Carrascal et al. (2019), Cai et al. (2023)). In my second study (however not part of **paper II**), I wanted to know how the *M. botrys mcy* genotypes are distributed within a phylogenetic tree consisting of Mcy sequences (i.e. amino acid sequences translated from the *mcy* nucleotide sequences) from several *Microcystis* spp. genomes (figure 13). My results indicate that the *M. botrys* morphospecies might not be a monophyletic clade, which is corroborated by the results of Pérez-Carrascal et al. (2019), and Cai et al. (2023). To further confirm my observations, it would be useful to include other genes than *mcy* sequences in phylogenetic analysis. For example, Cai et al. (2023) based their analysis on both core- and pan-genes, however they did not include *M. botrys* genome sequences in their study.

Some thoughts about study design.

Many studies, including **paper III**, aim to find relationships of environmental variables and the prevalence of toxic cyanobacteria, and/or toxin production (including microcystin). Such variables often include nutrients, light, temperature, competition, or predation. However, most experimental studies often investigate the effect of one or a few variables on microcystin-producing or non-microcystin producing strains, and the effects are often evaluated using only one or a few strains of each phenotype (see e.g. Utkilen and Gjolme (1995), Van de Waal et al. (2011),

Zilliges et al. (2011), Suominen et al. (2017)). The microcystin-producing *M. aeruginosa* reference strain PCC7806 and its non-microcystin producing mutant are commonly used in laboratory studies. The results (e.g. growth and performance of the toxigenic and non-toxigenic strain) are often generalised, and assumed to be valid for all toxigenic/non-toxigenic strains. However, my results (**paper I** and **paper II**) showed that there was high phenotypic and genotypic variation in *M. botrys*, and that both toxigenic and non-toxigenic strains consisted of several genotypes. This suggest that generalisations should not be based on microcystin-production alone. Furthermore, field studies often correlate environmental factors to the prevalence of toxic strains or microcystin concentrations, but have mostly not accounted for interactions of abiotic and biotic factors, possibly with synergic or antagonistic effects (see e.g. Ho et al. (2019), Buley et al. (2022)). Natural systems are complex, and organisms inhabiting these are subject to multiple environmental stressors simultaneously, such as competition for resources (e.g. nutrients or light), predation, and changes in temperature or pH. In addition, plankton blooms (including CHABs) are always made up by more than one species (Reynolds, 1984), and several genotypes of the same taxa can co-exist. During the course of a bloom, there can be rapid shifts both in species composition, in the proportions of toxic and non-toxic phenotypes, and in toxin production (**paper I** and **paper III**, see also e.g. Hotto et al. (2008), Briand et al. (2009), Rinta-Kanto et al. (2009), Pobel et al. (2012), and Yu et al. (2014)). Altogether, this can complicate our understanding of how changes in environmental conditions might affect the frequency and toxicity of CHABs.

How should we manage and mitigate CHABs?

One of my thesis aims was to investigate how environmental factors might favour toxic *Microcystis* in freshwater systems, and how microcystin concentrations might be affected. Primary production is limited by nutrient availability and stoichiometry (Sterner and Elser, 2002, Elser et al., 2007), and traditionally, phosphorus is considered to be the main limiting nutrient in freshwater systems (based on the results by e.g. Schindler, 1974). I therefore expected to find a link between nitrogen and phosphorus, and *Microcystis* bloom toxicity. In fact, the results of **paper III** show that the availability of NO_x as well as SRP were associated to the proportions of toxic *Microcystis* cells, and to microcystin concentrations in lakes. *Microcystis* biomass determined from microscope counts, as well as the abundances of toxic and non-toxic *Microcystis* estimated from qPCR, were associated to microcystin concentrations in the studied lakes. The environmental variables associated to microcystin concentrations could therefore be related to factors that are associated to increased population growth, rather than to increased microcystin production in the population.

Despite the somewhat reluctant reasoning above, I do not doubt that nutrients are key for mitigating cyanobacterial blooms. However, during my PhD studies I have learned that the relationship between nutrients and CHABs are more complex than I

had previously realised. For example, as phosphorus loading has increased in many freshwater systems (thereby decreasing N:P ratios), phytoplankton community composition have become more dominated by cyanobacteria (see the review by Gobler et al., 2016) and it has for long been assumed that cyanobacterial growth is controlled by phosphorus. With regards to *Microcystis*, recent research has shown that *Microcystis* are capable of growing also during low phosphorus conditions (Harke et al., 2012). Many studies have also shown that nitrogen is a key factor influencing *Microcystis* blooms, and *Microcystis* toxicity (Orr and Jones, 1998, Vezie et al., 2002, Kosten et al., 2012). In addition, studies aiming at synthesising current knowledge highlight the inconclusiveness of much previous research. For example, Buley et al. (2022) found that phosphorus rather than nitrogen was positively correlated to microcystins, and the results of Brandenburg et al. (2020) nicely illustrated how the conclusions of several studies are, in fact, affected by strain variation. While the paradigm has long been to reduce phosphorus inputs, recent research has raised the need of dual P and N reduction, in order to mitigate CHABs (Gobler et al., 2016, Chorus et al., 2021). The results of **paper III** do support such management strategies. Regardless of what the impact of nutrients is (i.e. if they favour toxic *Microcystis*, or *Microcystis* in general), I think it is important that management strategies related to the mitigation of *Microcystis* blooms need to consider both nitrogen and phosphorus dynamics in the affected lake systems.

Conclusions

In the introduction of this thesis, I explained that the sometimes contradictory results of many field and laboratory studies makes it difficult to pinpoint particular conditions that drive CHABs and microcystin production in freshwater systems. While I do believe that the results of the field study (**paper III**) will add on to the general framework of what conditions might affect the prevalence of toxic *Microcystis* blooms, I think it is important to consider the uniqueness of each system. Much of my work has focussed on the variation in microcystin-profiles (phenotypes) (**paper I)** and *mcy* genotypes (**paper II)** of *Microcystis*, but I think the results also highlight some aspects to why predicting the response of cyanobacteria to various environmental stressors is cumbersome. Furthermore, the results of **paper II** shows that *Microcystis* populations are heterogeneous in terms of *mcy* genotypes. The presence of multiple *mcy* genotypes among both microcystin-producing and non-producing phenotypes suggest that the ability of strains to synthesise microcystins might not be due only to the presence or absence of the *mcy* gene cluster. This might have implications when choosing methods for monitoring the development and prevalence of toxic blooms. With regards to management, the use of qPCR as an approximation of microcystins in lake water is useful. However, the variation in *mcy* genotypes detected (see also Yancey et al., 2022) highlights the importance to target appropriate *mcy* genes for each system.

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Acknowledgments

In August 2016, I moved from Gothenburg (the best coast!) to Skåne, to start my doctoral studies at Lund University. Even though I had never worked with prokaryotes previously, I was excited to finally begin my own project. During the past seven years, there has been a few ups and un-countable downs. Summing up the past years work, there are a whole bunch of people that I would like to thank. This thesis would not have been possible without your support and encouragement.

First of all, there are three people that I owe so much, and whom without I do not think I would have been able to finish this thesis. **Kasra**, tack för att du såg och förstod, när jag inte själv fattade att jag var både utbränd och deprimerad. **Jannike** utmanade mig på så många sätt, och gav mig verktyg att hantera och förekomma stressen. **Jonathan**, MCT kändes flummigt och jag var rätt tveksam till en början, men jösses vilka framsteg jag nått det senaste året! Jag nöjer mig med ”good enough”. Och tack för Kierkegaard! Jag vet att ni alla bara gjorde ert jobb, men för mig gjorde det skillnad!

A whole bunch of supervisors and advisors has pushed me throughout the past seven years. **Karin**, the number of times I have been able to keep a deadline can be counted on both hands, but you have always been understanding. Perhaps a bit more than I deserved. Thank you for your patience Thank you also for tirelessly correcting were/was and are/is. **Torbjörn**, during our meetings we have often ended up talking about other things than *Microcystis* (such as Turing’s Enigma, swimming schools in Ringsjön, or PhD studies “back in the days”). Thanks for all enjoyable conversations, I have really appreciated them! **Dag** and **Catherine**, thank you so much for all your encouragement, and for helping me believe I could do this. **Eric**, the best mentor I could ever have wished for! **Anders**, thank you for being an engaged and supportive examiner. **Lokesh**, not an official advisor, but without your guidance I could not have managed paper II. I learned so much from you! And finally, **Anna**! I wish you were still here with us. Your geist and never-ending enthusiasm is deeply missed!

I am very happy that I ended up at the **Aquatic Ecology Unit**, being surrounded by so many nice and friendly colleagues! I have really enjoyed lunches, fikas, small-talks, unit days et c. with all of you! I would especially like to thank **Anders N.** for help with statistics, and **Christer** and **Lars-Anders** for having their door open whenever I needed to talk. Lasse, nu kan jag uppskatta att plogfårorna bakom mig inte är raka. De vindlar sig hit och dit, och de tog mig framåt!

Marie, the friendliest person I know! Always with a happy smile, even when times were tough. You have the ability to make people feel welcome, no matter what position they have and for how long they are going to stay at the unit. **Mia**, you have been such an asset for the group! I wish you all the best with the new path in your life (but I will miss you!). I am so grateful to both of you, for all your help with lab work, cultures, and of course for the fika!

Thank you also to my fellow PhD students and post docs (past and present) that have been part in making the Aquatic Unit such a friendly and creative environment! **Gustaf**, the best office mate I could have wished for! You are great to talk to, and to be silent with. The office has felt empty since you and your dinosaurs left! **Anna** and **Marcus**, without the both of you I do not know how I would have managed the past months! Thank you! **Sandra** – you are awesome! I still hope that my failures can help you feel that there is hope. **Qinyang**, great walks! Great job! You are welcome for all the candy. **Franca**, thank you for your encouragement and pep-talk when I needed it! **Kevin** and Gustaf, cheers for the greatest playlist of all times! **Nan**, you are such a kind and caring person! I wish you and your family all the best for the future! **Caroline**, thank you for being such a good friend. Thanks **Pablo** for letting me whine about my low scientific confidence, and for introducing me to crossfit! Thank you also to **Raphael**, **Hannah**, **Àngels**, **Jana**, **Cai**, and everyone else in the KER group, and to **Huan**, **Simon**, **Yongcui**, **Martin**, **Jerker**, **Alex**, **Varpu**, **Marja**, **Johanna**, **Harmony**, and everyone else. Thanks also **Sylvie** and **Giuseppe** for being such good friends. Without all you, my time here would have been less enjoyable!

A special thanks to **Olga** and **Nico**! I am so glad for our collaboration, and for the meetings crossing three time-zones. I learned so much from both of you!

I am also lucky to have amazing friends and family, who has helped me to focus on the more important aspects of life. I met **Julian**, **Eva**, and **Marie** through GENECO, and you have become such important persons in my life! **Eva-Lotta**, **Swantje** och **Angelica**, det är så fint att veta att ni alltid finns! **Marie** & **Anders**, **Martin**, **Daniel**, **Adam**, **Kniven** & **Klara**, **Ragnar** & **Johanna, Jo** & **Tania**, ni är så jävla bra! **Nellie** och **Jenny**, den bästa sortens vänskap är när man ses med typ två års mellanrum, men ändå alltid kan fortsätta från där man avslutade! Mina tass-tastiska vänner **Emelie** och **Niclas**, vilken mjau-trolig tur att våra avkommor började på samma förskola så att vi kunde bli kompisar! Tack till alla **Duvetorparna** för att jag fått vara en del av er familj!

Till hela min **familj**: jag är så obeskrivligt tacksam för all support under de här åren, och speciellt nu på sluttampen av avhandlingsarbetet! Ni är bäst och jag älskar er! **Mårten**, tack för att du pallade med mig i fjorton år. Jag hade nog inte gjort det. **Elise**, min älskade unge. Du är det absolut bästa jag någonsin har gjort! Jag hoppas att jag nu kan bli en mer närvarande (och mindre stressad) mamma, att jag lyckats lära dig att värdesätta vikten av vila, och att du en dag kommer titta igenom den här boken och känna dig lite stolt över din gamla morsa.