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Impact of biotic and abiotic environmental variables on the toxic potential of the cyanobacterium *Microcystis* sp.

Pia Ines Scherer

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Vorsitzender: Prof. Dr. Siegfried Scherer

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1. Prof. Dr. Jürgen Geist
2. Assist. Prof. Andrew D. Millard, Ph.D.
3. Prof. Dr. Wolfgang Liebl

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Preface

This work deals with the toxic potential of the cyanobacterium *Microcystis* sp. First, this thesis briefly introduces cyanobacteria with an emphasis on toxic and bloom forming species and gives background information about the most common cyanotoxin, microcystin. Subsequently, three research chapters are dedicated to individual studies. The first research chapter answers the question whether climate change related abiotic stressors influence toxin gene expression in the toxic cyanobacterium *Microcystis aeruginosa* under laboratory conditions. Similarly, a second corresponding research chapter examines possible effects of biotic stimuli on toxin gene expression in *M. aeruginosa*. A third chapter presents a field monitoring study to assess whether and how biotic and abiotic environmental variables influence toxic cyanobacteria in lakes. Finally, a concluding discussion reviews the relevance of the results for monitoring and risk assessment of harmful algal blooms. By setting those findings in the context of the current literature, open research questions concerning toxic cyanobacteria are identified, and promising avenues for interdisciplinary research avenues are described.

Summary

Cyanobacteria are prokaryotic primary producers and constitute an essential part of the bacterioplankton in many water bodies. However, toxic cyanobacteria such as *Microcystis aeruginosa* are a concern in freshwaters all over the world. *M. aeruginosa* and other toxic species are capable of producing the hepatotoxic secondary metabolite microcystin. Problems caused by the mass occurrence of noxious cyanobacteria may increase in the future due to climate change, which favours cyanobacteria development. Some abiotic environmental variables, such as warm temperatures, and biotic factors, such as grazers, can influence the population growth of cyanobacteria. However, less is known about the effects of those environmental variables on the toxic potential of cyanobacteria.

The main goal of this thesis was to elucidate the influence of abiotic and biotic environmental variables on the toxic potential of the widespread cyanobacterium *Microcystis*. In order to assess how those environmental variables influence toxigenicity, laboratory studies were conducted. In addition, lake monitoring studies were undertaken to disentangle the effects of abiotic and biotic environmental variables on natural bloom toxicity.

In the first laboratory study, the effect of abiotic climate change related factors on the toxigenicity of *M. aeruginosa* was determined. To be able to assess changes in microcystin biosynthesis gene (*mcy*) expression, a novel RT-qPCR assay was developed. It allowed the accurate relative quantification of *mcy* transcripts. The following abiotic environmental variables were chosen for this study: temperature, which was selected because of the expected increase in heat wave frequency and water temperature; perturbation by stirring, which was selected due to the predicted increase in extreme weather events; and increased microcystin concentration, which was selected because of the anticipated upsurge in blooms of toxic cyanobacteria. Results indicate that a 10 °C rise in temperature led to a significant increase in toxigenicity of *M. aeruginosa* but perturbation or increased microcystin-LR concentration (10 µg/l or 60 µg/l) did not.

A corresponding second study assessed the influence of co-occurring microorganisms on the toxic potential of *M. aeruginosa*. Here, as before, *mcy* expression was measured with RT-qPCR. The parameters selected for this laboratory study were: the ubiquitous cyanobacterium *Synechococcus elongatus*; the mixotrophic flagellate *Ochromonas danica*, which is capable of grazing on *M. aeruginosa*; and the virioplankton size fraction from two different lakes, which

contained cyanophages. The picocyanobacterium *S. elongatus* and the virioplankton size fraction from blooming lakes caused increased transcription of *mcy* genes. The mixotrophic flagellate *Ochromonas danica*, however, did not increase *mcy* gene transcription.

Last, a field monitoring study of two recreational lakes was conducted in order to address the question how both abiotic and biotic environmental variables affect the toxic potential of a cyanobacterial bloom under field conditions. Specifically, this study aimed to answer the questions, which environmental factors correlate with bloom toxicity, which bacterial species are responsible for toxicity, and whether *mcy* genes can be used as proxy for bloom toxicity. To perform absolute quantification of *mcy* genes, two quantitative qPCR assays were used. Results from high-throughput sequencing of the 16-S rRNA gene and clone libraries of *mcy* genes indicated that the cyanobacterial community composition was highly dynamic over time and that low abundant *Microcystis* sp. was the main microcystin producer. In addition, bloom toxicity correlated with total phosphorus and *mcy* copy number.

Together these results provide valuable insights into how biotic and abiotic environmental variables influence toxicity and toxic blooms of cyanobacteria. Particularly the facts that some biotic and abiotic environmental factors lead to an increase in toxic potential of the noxious cyanobacterium *M. aeruginosa* independently of growth, and that the main toxin producer in lakes might not be the dominant cyanobacterium species in a bloom, need to be considered in risk assessment and mitigation efforts.

Zusammenfassung

Cyanobakterien spielen als prokaryotische Primärproduzenten eine essenzielle Rolle im Bakterioplankton vieler Gewässer. Allerdings stellen giftige Cyanobakterien wie etwa *Microcystis aeruginosa* ein Problem in Gewässern fast überall auf der Welt dar. Sowohl *M. aeruginosa* als auch andere Arten können den lebertoxischen Sekundärmetabolit Microcystin bilden. Probleme, die vom massenhaften Auftreten giftiger Cyanobakterien herrühren, werden zukünftig auf Grund des Klimawandels noch zunehmen, da dieser Cyanobakterien begünstigt. Einige abiotischen Umweltfaktoren, wie etwa warme Temperaturen, und biotische Umweltfaktoren wie zum Beispiel Protozoen können das Populationswachstum von Cyanobakterien beeinflussen. Jedoch ist wenig darüber bekannt, wie sich diese Umweltfaktoren auf die mögliche Giftigkeit von Cyanobakterien auswirken.

Das übergeordnete Ziel dieser Arbeit war, den Einfluss von abiotischen und biotischen Umweltfaktoren auf das weit verbreitete Cyanobakterium *Microcystis* und sein Potential zur Toxinbildung zu beleuchten. Um einzuschätzen, wie diese Umweltparameter die Toxinbildungsfähigkeit beeinflussen, wurden Laborstudien durchgeführt. Darüber hinaus wurden Monitoringstudien an Seen durchgeführt, um die Einflüsse von Umweltfaktoren auf die Giftigkeit von Cyanobakterienblüten im Freiland zu identifizieren.

In einer ersten Laborstudie wurde der Effekt untersucht, den abiotische Umweltfaktoren, die mit dem Klimawandel in Verbindung gebracht werden, auf die Toxigenität von *M. aeruginosa* haben. Um festzustellen, wie diese Umweltfaktoren die Genexpression von Microcystinbiosynthesegenen (*mcy*) beeinflussen, wurde ein neuer RT-qPCR Test entwickelt. Die in dieser Studie untersuchten abiotischen Umweltfaktoren waren: Temperatur, ausgewählt wegen der erwarteten Zunahme von Hitzewellen und steigenden Wassertemperaturen, Wasserbewegung durch Rühren, welches aufgrund der erwarteten Zunahme von Extremwetterereignissen ausgewählt wurde, und erhöhte Microcystinkonzentration, welche aufgrund der bevorstehenden Häufung von giftigen Cyanobakterienblüten ausgewählt wurde. Die Ergebnisse zeigen, dass eine Temperaturerhöhung von 10 °C die Toxigenität von *M. aeruginosa* mitunter signifikant erhöht, das Rühren oder die erhöhte Microcystinkonzentration jedoch nicht.

In einer zweiten Studie wurde der Einfluss von anderen Mikroorganismen auf das Giftigkeitspotential von *M. aeruginosa* untersucht. Auch hier wurde, wie in der ersten Studie,

die *mcy* Expression mittels RT-qPCR gemessen. Das ubiquitäre und mit *Microcystis* konkurrierende Cyanobakterium *Synechococcus elongatus*, der mixotrophe Flagellat *Ochromonas danica*, welcher ein Fraßfeind von *M. aeruginos*a ist, und die Virioplanktonfraktion aus zwei verschiedenen Seen, welche Cyanophagen enthielt, wurden für diese Laborstudie ausgesucht. Das Picocyanobakterium *S. elongatus* und die Virioplanktonfraktion aus Seen mit Cyanobakterienblüten verursachten eine Erhöhung des toxischen Potentials von *M. aeruginosa*, während sie bei dem mixotrophen Flagellat *O. danica* nicht zu beobachten war.

Zuletzt wurde eine Feldstudie durchgeführt um die Frage zu beantworten, wie abiotische und biotische Umweltparameter das toxische Potential einer Cyanobakterienblüte im Freiland beeinflussen. Im Speziellen war die Zielsetzung dieser Untersuchung die Fragen zu beantworten welche Umweltfaktoren mit der Toxizität von Cyanobakterienblüten korrelieren, welche Spezies für die Toxinbildung verantwortlich sind, und ob *mcy* Kopien als Indikator für Giftigkeit einer Blüte genutzt werden können. Um eine absolute Quantifizierung der Microcystingene vornehmen zu können, wurden zwei quantitative qPCR Protokolle angewendet. Hochdurchsatzsequenzierung und Klonbibliotheken zeigten, dass die Artzusammensetzung der Cyanobakterienpopulation sich äußerst dynamisch in Zeitverlauf veränderte und dass *Microcystis* sp., der nur einen geringen Anteil der gesamten Cyanobakterienpopulation ausmachte, der Haupttoxinproduzent war. Außerdem korrelierte die Giftigkeit der Cyanobakterienblüte mit den Werten für Gesamtphosphat und mit der Zahl der *mcy* Genkopien.

Zusammengenommen erlauben diese Ergebnisse einen wertvollen Einblick in die Auswirkungen von biotischen und abiotischen Umweltfaktoren auf die Giftigkeit von Cyanobakterienblüten. Insbesondere die Tatsachen, dass einige biotische und abiotische Umweltparameter in Laborstudien unabhängig vom Wachstum zu einem Anstieg des Giftbildungspotentials von toxischen Cyanobakterien führen und dass der Toxinproduzent in Seen nicht notwendigerweise der zahlenmäßig dominanten Cyanobakterienart angehört, sind Erkenntnisse, die beim Risikomanagement und der Bekämpfung von giftigen Cyanobakterien bislang nicht berücksichtigt werden.

1 Introduction

1.1 Characteristic traits of cyanobacteria

Cyanobacteria are amongst the oldest organisms on earth. They evolved about 3.5 billion years ago and were the first organisms to perform oxygenic photosynthesis (Graham and Wilcox, 2000). Early cyanobacteria enriched the atmosphere with oxygen up to the current atmospheric oxygen concentration. This great oxygenation event altered earth's community composition in favour of organisms with an aerobic metabolism and enabled the evolution of eukaryotic life forms (Graham and Wilcox, 2000). In addition, cyanobacteria played a crucial role in the evolution of phototrophic eukaryotes through the process of primary endosymbiosis. During primary endosymbiosis an ancient cyanobacterium was taken up as internal symbiont by a heterotrophic cell and became the ancestor of the organelles that are known as chloroplasts today (Graham and Wilcox, 2000; Sagan, 1967).

Some modern day cyanobacteria are capable of fixing atmospheric nitrogen. Diazotrophic cyanobacteria possess heterocysts for the purpose of nitrogen fixation (Sarma, 2012). Heterocysts are specialized terminally differentiated cells, which provide a low oxygen environment required by the nitrogenase enzyme complex that is encoded by the *nif* gene cluster (Sarma, 2012). In contrast to spatial separation of nitrogen fixation and oxygenic photosynthesis by heterocyst formation, some unicellular cyanobacteria realize a temporal separation of both processes. In those cells, nitrogen fixation is restricted to night time when no oxygen is generated (Bergman et al., 1997; Zehr et al., 2001). The ability of some species to fix dinitrogen allows those cyanobacteria to grow under otherwise nitrogen limiting conditions and thus have an advantage over competitors (Whitton and Potts, 2000).

Another major contributor to the success of cyanobacteria is that many bloom forming cyanobacteria species have gas vacuoles (Walsby, 1994; Whitton and Potts, 2000). This gas vacuoles enable cyanobacteria to regulate their buoyancy, and thus avoid sedimentation, move to the place in the water column with optimal light intensity, and gain access to nutrients (Whitton and Potts, 2000). When for example CO_2 becomes limiting in deeper water layers buoyancy regulation allows cyanobacteria to move to the water surface resulting in suddenly appearing surface blooms (Klemer et al., 1996; Walsby and Booker, 1980).

Cyanobacteria have unique abilities to acclimate to the prevailing light conditions on the molecular level. Several mechanisms are known by which cyanobacteria can adjust to changed

light quality and quantity, but the most prominent one is a process called complementary chromatic adaptation which has been studied in the cyanobacteria *Fremyella diplosiphon* and *Calothrix* sp. and which is well understood on the molecular level (Grossman, 2003; Whitton and Potts, 2000). When *Fremyella* for example is grown under green light it accumulates mainly phycoerythrin, which absorbs in the green spectrum, while it accumulates phycocyanin when grown under red light (Whitton and Potts, 2000). Another mechanism to adjust to changes in light quality and quantity is the fine tuning of the ratio between photosystem I and photosystem II (Fujita et al., 1994).

In addition to their extraordinary ability to cope with changing conditions in the water column, cyanobacteria are a rich source of metabolites. Some cyanobacterial products, such as fatty acids, alkanes, alcohols, or isoprene, can be used for the production of renewable energy sources like biofuel (Sarsekeyeva et al., 2015). A wide variety of secondary metabolites are attractive for a range of biotechnological and pharmaceutical applications (Rastogi and Sinha, 2009). Some cyanobacterial secondary metabolites are toxic to humans and animals and are therefore referred to as cyanotoxins. Due to the potential risks associated with them, it is crucial to understand toxin containing cyanobacterial blooms.

1.2 Toxic cyanobacterial blooms

Toxic cyanobacterial blooms have been a major concern for some time (Huisman et al., 2005). An increasing number of bloom incidents are reported, and some of them even gained considerable attention in the media (BBC, 2015; Butler, 2014; N'Diaye, 2017; Orr, 2014). Toxic cyanobacterial blooms cause considerable costs in water treatment and monitoring and are responsible for losses in fisheries and aquaculture and losses in recreational activities and tourism (Dodds et al., 2008). The mass developments of cyanobacteria can potentially have adverse effects on the ecosystem. They supress other biota for example by restricting access to resources such as light (Chorus and Bartram, 1999; Huisman et al., 2005), but the ability of such blooms to produce toxins has received most attention.

1.2.1 Relevant toxic cyanobacteria species

This section focuses on the most relevant toxin producer genera in freshwater lakes with an emphasis on genera that produce the toxin microcystin. Every toxic species, however, encompasses toxic and non-toxic genotypes.

The main planktic microcystin producer genera are *Microcystis*, *Dolichospermum*[1], and *Planktothrix*[2]. The genus *Microcystis* is a well-known microcystin producing freshwater cyanobacterium. It belongs to the order Chroococcales and was first described by Kützing in 1833 (Huisman et al., 2005). This cyanobacterium exhibits a coccoid unicellular shape and can form colonies. *Microcystis* is found in freshwaters all over the world apart from polar regions (Whitton, 2012). *Microcystis* spp. contain gas vacuoles for buoyancy regulation (Whitton, 2012). Typical toxic *Microcystis* species are *M. aeruginosa* or *M. viridis* (Chorus and Bartram, 1999). Species belonging to the commonly found genus *Dolichospermum* have gas vesicles and form filaments that can occur in small clusters. Furthermore, those species form heterocysts, which is why they are capable of fixing dinitrogen. Akinetes, which are specialized cells, allow them to outlast adverse environmental conditions (Sarma, 2012). Members of the genus *Dolichospermum* are also known to produce other toxins besides microcystin, such as anatoxin-a and saxitoxin (Chorus and Bartram, 1999). *Dolichospermum* species that are able to synthesize microcystin are for example *D. flos-aquae* or *D. lemmermanii*. The third important microcystin producing cyanobacterial genus is *Planktothrix*. Members of the genus *Planktothrix* are filamentous cyanobacteria with gas vesicles, but unlike *Dolichospermum* spp., they cannot form heterocysts or akinetes. The most common toxic species of this genus are *P. agardhii* and *P. rubescens.* Both of those species are potential microcystin producers and differ in their pigmentation with the former being green and the latter being red (Kurmayer et al., 2016). Members of the genus *Planktothrix* are able to produce other toxins in addition to microcystins, such as anatoxin and aplysiatoxin (Chorus and Bartram, 1999). In addition to the above mentioned cyanobacterial genera *Microcystis*, *Dolichospermum*, and *Planktothrix*, some non-microcystin producer cyanobacteria are worth mentioning because they produce other cyanotoxins, which might cause problems in freshwater lakes. *Cylindrospermopsis* spp., such as *C. raciborskii*, and *Aphanizomenon* spp., such as *A. flos-aqua* or *A. ovalisporum*, are potentially producing the toxins cylindrospermopsin and saxitoxin, which cause great concern in freshwaters (Chorus and Bartram, 1999; Sarma, 2012).

[1] All members of the genus *Dolichospermum* have until recently been known under the genus name *Anabaena*. The genus *Anabaena* included planktic member with gas vesicles and benthic and soil inhabiting species lacking gas vesicles. The genetic differences revealed by 16-S rRNA gene sequencing between the two *Anabaena* types was sufficiently large to move all planktic species with gas vesicles to the new genus *Dolichospermum* (Wacklin et al., 2009).

[2] This genus was formerly known under the name *Oscillatoria* but forms the new genus *Planktothrix* since 1988 on the basis of its ultrastructural characteristics and 16-S rRNA clustering (Kurmayer et al., 2016).

1.2.2 Risk factors for the development of cyanobacterial blooms

Several abiotic and biotic parameters have been linked to an increased risk of cyanobacterial bloom development. Important abiotic risk factors are: water temperatures above 20 °C, vertical stratification of the water column, and a high nutrient load (Paerl and Otten, 2013). The likelihood of a high nutrient load is increased by catchment areas encompassing agricultural land exposed to fertilizers or manure and the associated runoff (Arbuckle and Downing, 2001; Knoll et al., 2003; Leigh et al., 2010). Other risk factors are alkalinity (Shapiro, 1984) and long water retention times caused by too little flushing (Carvalho et al., 2011; Elliott, 2010). The biology of a water body can also increase the risk of cyanobacterial blooms. The absence of submerse macrophytes competing for resources favours cyanobacteria (Peretyatko et al., 2012). In addition, the absence of large size zooplankton capable to control cyanobacteria by grazing can lead to excessive cyanobacteria growth (Ekvall et al., 2014; Peretyatko et al., 2012). Both, submerse vegetation and large zooplankton are negatively affected by plakti-benthivorous fish, which is why the removal of those fish is used in biomanipulation approaches to reduce the likelihood of cyanobacterial blooms (Peretyatko et al., 2012).

1.2.3 Global change and its consequences for bloom occurrence

Global change can lead to adverse effects for water management by favouring cyanobacterial blooms. This section gives an overview about how global change affects cyanobacteria. Two main contributors to global change have received lots of attention in this context: anthropogenic eutrophication and climate change related rise in mean temperatures (Paerl and Otten, 2013). It has been established that eutrophication of water bodies and climate change are mutually reinforcing each other (Moss et al., 2011). However, other factors linked to or even caused by eutrophication or temperature rise can influence the occurrences of cyanobacterial blooms as well (Figure 1.1).

Mass occurrences of cyanobacteria are often linked to high nutrient loads and eutrophication of water bodies (Chorus and Bartram, 1999; Huisman et al., 2005; Paerl et al., 2001; Paerl et al., 2011; Paerl and Otten, 2013; Whitton and Potts, 2000). The eutrophication of inland waters is a natural process but increased and intense anthropogenic eutrophication has received much attention and due to its drastic impact on ecosystems (Lampert and Sommer, 1993). High total phosphorus (P) levels have been found to lead to cyanobacteria mass development in freshwaters but also high nitrogen (N) input, leading to high N/P ratios can favour harmful algal

blooms especially non-nitrogen fixing species, and it is now accepted that both nutrients must be controlled to reduce cyanobacteria blooms (Paerl et al., 2011; Paerl and Otten, 2013).

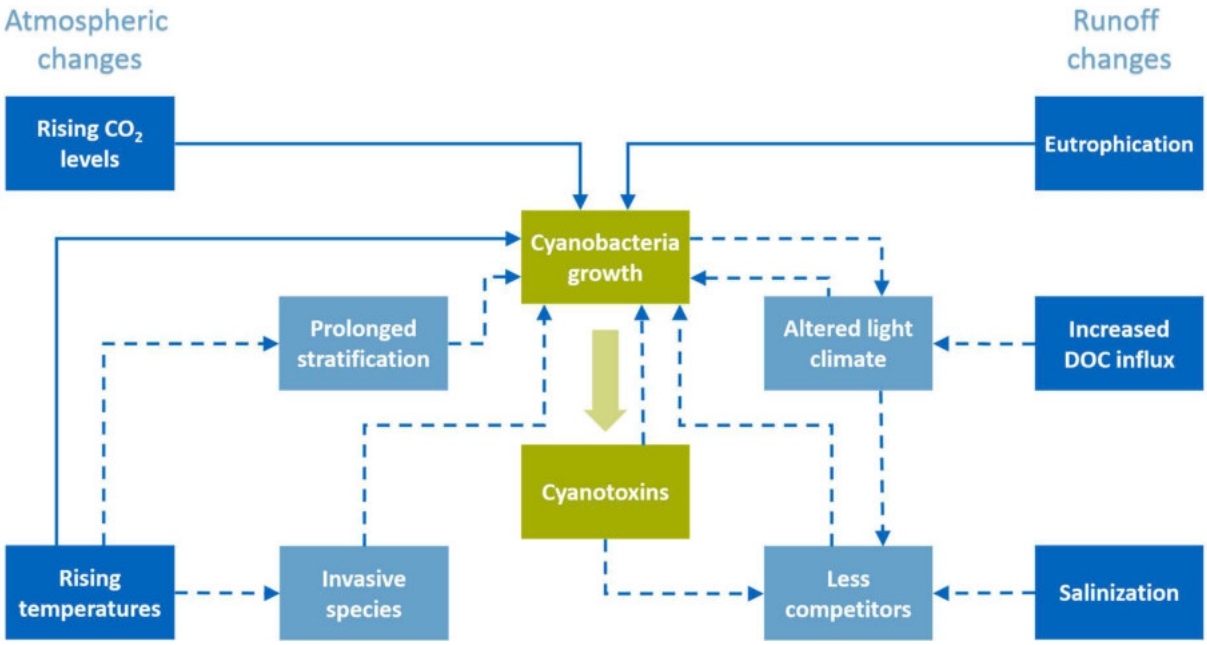

Figure 1.1: Global change affects cyanobacterial growth and consequently the production of cyanotoxins. Direct effect are shown as regular arrows and indirect effects are indicated as dashed arrows.

Rising temperatures favour cyanobacterial blooms in several ways (Paerl and Huisman, 2008; Paerl and Huisman, 2009). Cyanobacteria have generally a higher optimum growth temperature than other phytoplankton species and therefore might be better able to compete in a warmer water bodies or cope with heat waves (Butterwick et al., 2005). Apart from increasing water temperatures, rising temperatures have several consequences that influence cyanobacteria indirectly, the most important being reduced ice cover and prolonged stratification. Historical and current data indicate that the duration of ice cover in inland waters is decreasing and that this trend is likely to continue in the future (IPCC, 2013; Magnuson et al., 2000). In combination with the rising water temperatures, this leads to a longer and more stable stratification of the water column in lakes (IPCC, 2014; Peeters et al., 2007). Stable stratification of the water column generally favours cyanobacteria more than other phytoplankton species due to their ability to regulate buoyancy and therefore move to the most favourable place in the water column (Huber et al., 2012; Paerl et al., 2011). On the other hand, the expected increase in frequency of extreme weather events such as heavy rainfalls, storms, and floods may cause more turbulence in the water column and an altered mixing regime (IPCC, 2014). Turbulent

mixing can promote certain cyanobacterial species, such as potentially toxic *P. agardhii* (Mischke, 2003).

Climate change and associated warmer water temperatures allow invasive cyanobacterial species to expand their geographical range. An example is the toxic and bloom forming cyanobacterium *Cylindrospermopsis raciborskii*, which originated form tropical and sub-tropical regions and is recently found in temperate regions including Germany (Padisák, 1997; Sinha et al., 2012; Wiedner et al., 2007). Long term (Wiedner et al., 2007) and broad scale (Kokociński et al., 2017) environmental monitoring studies, laboratory experiments (Mehnert et al., 2010), modelling approaches (Mehnert et al., 2010; Wiedner et al., 2007), and literature analysis (Sinha et al., 2012; Sukenik et al., 2012) have shown that the expansion of *C. raciborskii*'s geographical distribution is largely caused by climate change. Other potentially toxic invasive cyanobacteria species are *Dolichospermum bergii*, *Aphanizomenon aphanizomenoides*, and *Aphanizomenon ovalisporum* (Sukenik et al., 2012) some of which have been shown to be favoured by climate change due to their higher growth rate at elevated temperatures compared to native species (Mehnert et al., 2010).

Some invertebrates that benefit from climate change favour toxic cyanobacterial blooms as well. Experimental and environmental studies showed that the presence of the invasive zebra mussel *Dreissena polymorpha* increased the abundance of *Microcystis* (Bykova et al., 2006; Fishman et al., 2010; Knoll et al., 2008; Nicholls et al., 2002). *D. polymorpha* selectively expels highly toxic *Microcystis* in their pseudofaeces and thus favours the dominance of this toxic species (Juhel et al., 2006). In addition, the presence of *D. polymorpha* was linked to higher *M. aeruginosa* and *Dolichospermum* spp. biomass and toxin content (Knoll et al., 2008).

Climate change may cause more turbidity in inland waters and thus favour cyanobacteria. The climate change related increase of heavy precipitation events and the associated inflow of dissolved organic carbon (DOC) from the land as well as increased growth of phytoplankton contributes to increasing turbidity of inland waters (IPCC, 2014; Monteith et al., 2007; Sarkkola et al., 2009). Cyanobacteria are able to cope with this change in watercolour due to their various strategies to acclimate to changing light climates. Furthermore, a number of species can handle low light conditions and might even prefer those, such as potentially toxic *Planktothrix s*p. (Kurmayer et al., 2016).

Rising CO_2 levels favour the growth of cyanobacteria. Currently we are experiencing the highest atmospheric partial pressure of CO_2 (pCO_2) in the last 800,000 years. Anthropogenic

activities caused current CO_2 levels, which lie 27 % above the concentration in pre-industrial times (IPCC, 2013; Lüthi et al., 2008). Experimental studies on the toxin producer *Nodularia spumigena* and *Trichodesmium* sp. showed that elevated pCO_2 can increase the growth and nitrogen fixation of diazotrophic cyanobacteria and thus favour potentially toxic cyanobacteria (Barcelos e Ramos et al., 2007; Hutchins et al., 2007; Levitan et al., 2007; Wannicke et al., 2012).

Salinization favours cyanobacteria. Climate change and anthropogenic activities are increasing salinization of inland waters. Climate change leads to higher salinization of lakes by increased evaporation, regionally reduced precipitation, and seawater intrusion as a result of rising sea levels in some areas (IPCC, 2013). Human activities such as de-icing of streets, soil sealing, and water intensive agricultural practices further aggravate the problem of lake salinization (Dugan et al., 2017; Müller and Gächter, 2012), which is currently not adequately addressed (Cañedo-Argüelles et al., 2016). Increasing salinity of freshwaters favours toxic cyanobacterial blooms in several ways. Toxic cyanobacterial genera such as *Microcystis*, *Dolichospermum*, *Cylindrospermopsis*, and *Nodularia* were shown to be more salt tolerant than other phytoplankton species and therefore better equipped to deal with future high salt conditions (Moisander et al., 2002; Tonk et al., 2007). Mazur-Marzec et al. (2005) even found that the toxin concentration in *Nodularia spumigena* positively correlated with salinity.

The climate change related factors presented above as well as ongoing eutrophication of water bodies may not only increase the likelihood of toxic blooms, but also the number of toxic species that are found in temperate regions. The dominance of cyanobacteria may have a positive feedback effect by suppressing competitors such as macrophytes and other phytoplankton (Figure 1.1). All those factors together may lead to increased total toxin content in water reservoirs.

1.2.4 Detection and monitoring of toxic cyanobacterial blooms

From a public health perspective, the detection and monitoring of toxic cyanobacterial blooms are indispensable to avert risks associates with cyanotoxins. Cyanobacterial microscopic cell counts are a popular method to gauge the risk posed by cyanotoxins in recreational waters. The WHO attributes up to 20,000 cyanobacterial cells per mL water to a low probability of adverse health effects. A guideline value of 100,000 cyanobacterial cells per mL indicate a moderate probability of adverse health effects. Cyanobacterial surface scum is always an indicator of high probability of adverse health effects (WHO, 2003). When cyanobacteria dominate the

phytoplankton community, chlorophyll is also a good indicator of potential risks. Similarly to cell counts, the WHO has attributed a guideline value of 50 µg/l chlorophyll-a to a moderate and 5,000 µg/l chlorophyll-a to a high risk level (WHO, 2003). Due to the association of chlorophyll-a content with cyanobacterial biomass, remote sensing approaches detecting chlorophyll-a and possibly other pigments could aid in the detection of cyanobacterial blooms (Anderson, 2009; Huisman et al., 2005). Both, cell count and chlorophyll assessment, have the disadvantage that they measure all cyanobacterial cells including non-toxic ones. While microscopic analysis can at least identify potential toxic species, no statement is possible about actual toxicity. For this reason, monitoring efforts often focus on commonly found toxins such as microcystin.

1.3 The cyanotoxin microcystin

Microcystin is one of the most common and widespread cyanobacterial toxins (Falconer and Humpage, 2005). Poisonings of humans and animals have been reported (Carmichael et al., 2001; Chorus and Bartram, 1999; Van Halderen et al., 1995). Reports of poisoned wildlife, livestock, and pets are more common than fatal incidents with humans. This might partly be explained by the fact that some animals seem to prefer drinking cyanobacterial scums over clear water (Lopez Rodas and Costas, 1999). Due to diffuse symptoms (Weirich and Miller, 2014) and no obligation to report suspected cases of cyanotoxin poisoning to authorities, mild cases might go unrecognized. Microcystin is classed as a hepatotoxin. It is actively taken up into hepatocytes (Runnegar and Falconer, 1982) and inhibits protein phosphatases 1 and 2A (MacKintosh et al., 1990) potentially leading to liver failure (Carmichael et al., 2001). Because of its ability to inhibit protein phosphatases microcystin is also a cell toxin. Chronic exposure to microcystin was linked to liver and colorectal cancer (Chorus and Bartram, 1999; Lun et al., 2002; Svirčev et al., 2009). In addition to being a hepatotoxin, microcystin is also neurotoxic (Hu et al., 2016), and its potential role in neurodegenerative diseases like Alzheimer's disease is still under discussion (Li et al., 2012a; Metcalf and Codd, 2009). Drinking is only one possible exposure route. Exposure during recreational activities such as swimming is relevant, too. Other exposure routes such as irrigation water, food supplements, and renal dialysis have been described (Chorus and Bartram, 1999).

1.3.1 Chemistry and structural variants of microcystin

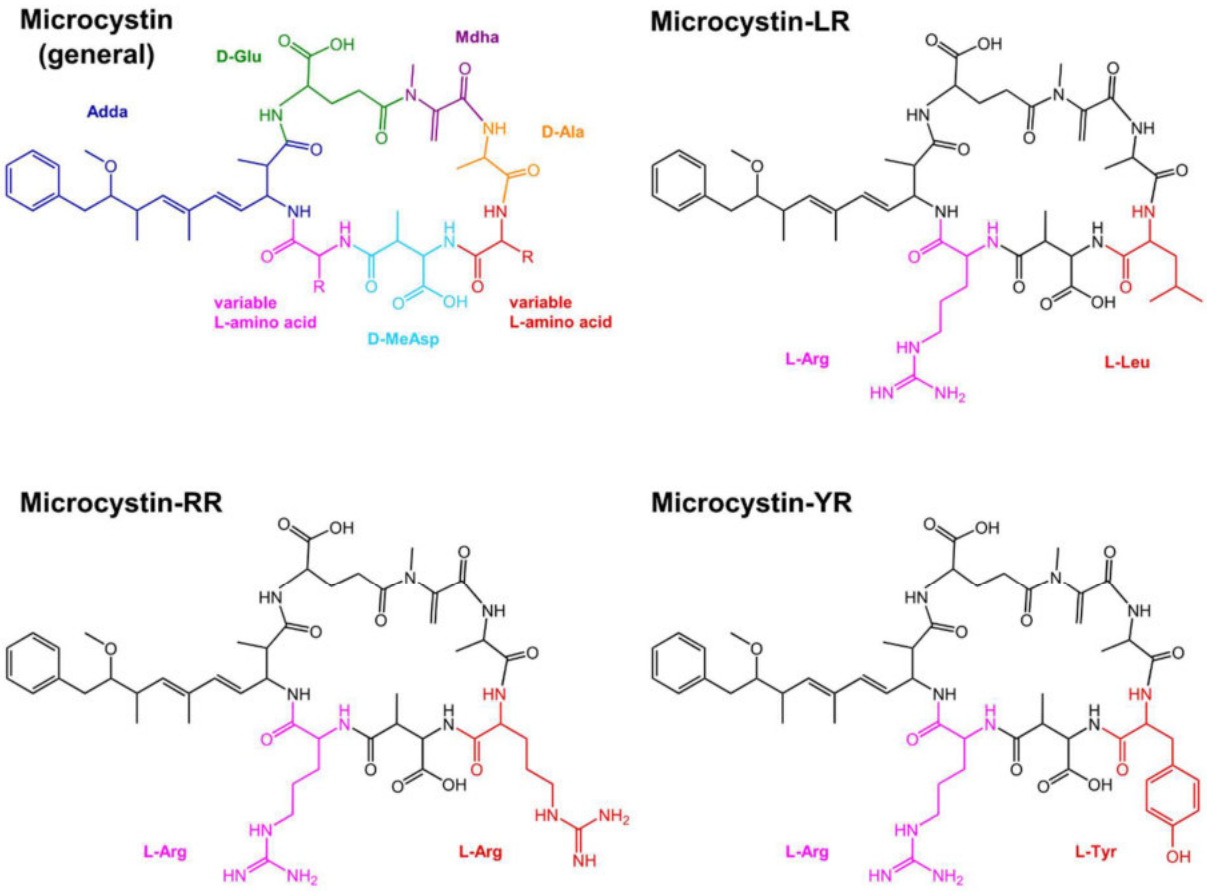

Figure 1.2: Chemical structure of microcystin showing the different amino acids in different colours. The variable amino acids in three commonly found structural variants of microcystin are highlighted in pink and red.

The term microcystin refers to a family of cyanobacterial secondary metabolites that are synthesized non-ribosomally. Microcystins are cyclic heptapeptides and contain unusual and non-proteinogenic amino acids, such as Adda and D-amino acids (Botes et al., 1984; Botes et al., 1985). The general structure of microcystins is cyclo(-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-) where X and Z are variable L amino acids, D-MeAsp is D-erythro-β-methylaspartic acid, Adda is 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4.6-dienoic acid, and Mdha is N-methyldehydro-alanine (Figure 1.2) (Botes et al., 1984; Botes et al., 1985). Over 90 different structural variants of microcystin are known (Neilan et al., 2008; Welker and von Döhren, 2006). Most structural variants of microcystin differ in their two L-amino acids or the presence of methyl groups on D-erythro-β-methylaspartic acid (D-MeAsp) and N-methyldehydroalanine (Mdha) (Welker and von Döhren, 2006). The variable amino acids are typically indicated by single-letter amino acid code, e.g. Microcystin-LR comes with Leucine

(L) and Arginine (R) at the variable positions (Figure 1.2). Microcystin-LR is by far the best studied of all microcystin isoforms and appears to be one of the most widespread structural variants, albeit this might be due to potential bias in detection methods. Toxicity of microcystin is highly dependent on the isoform, but only for microcystin-LR enough toxicological data is available for the Wold Health Organization to propose a provisional save guideline value of 1 µg/L for drinking water (WHO, 1998). Most countries that regulate microcystin, have adopted this guideline for their legislation (Burch, 2008).

The question, why different isoforms of microcystin exist remains unclear from a functional perspective and has only partly been answered from a biosynthesis point of view. Mikalsen et al. (2003) found that the synthesis of certain isoforms (microcystin-LR and microcystins-RR) correlates with genetic variations in the adenylation domain of the *mcyB* gene. Moreover, the amino acid sequence is more variable in the enzymes that incorporate the variable L-amino acids than in the remaining microcystin biosynthesis enzymes (Rouhiainen et al., 2004). This indicates that the enzyme binding pockets responsible for incorporating the variable L-amino acids have a relaxed substrate specificity allowing the incorporation of various amino acids. Therefore, the resulting isoform could be a direct result of the substrate available during biosynthesis of microcystin. However, microcystin producing cyanobacterial strains typically produce several structural variants of microcystin (Puddick et al., 2014; Sivonen and Jones, 1999), and in strains that produce more than one isoform, the ratio between the isoforms can vary in response to environmental factors such as light (Tonk et al., 2005).

1.3.2 The biological function of microcystin

The function of microcystin is still very much under discussion. The reason why in closely related cyanobacteria toxic and non-toxic strains co-exist in natural environments remains an enigma, especially when considering the energetic expense for microcystin synthesis (Briand et al., 2008; Kardinaal et al., 2007; Sabart et al., 2010). Comparing microcystin producing and non-producing stains (Alexova et al., 2011b) or knocking out a microcystin synthesis gene (Dittmann et al., 1997), did not yield a single and simple answer to this question. Several possible functions for microcystin have been proposed. The toxic properties of microcystin that attracted initial attention led scientists to hypothesize that cyanobacteria produce this substance to poison and thus deter natural enemies such as grazers (Kurmayer and Jüttner, 1999; Pineda-Mendoza et al., 2014). This hypothesis is somehow supported by the fact that the growth, survival, and development of cladocerans is affected by microcystin (Dao et al., 2010; DeMott

et al., 1991; Fulton and Paerl, 1987b; Herrera et al., 2015; Ortiz-Rodríguez et al., 2012; Wiegand et al., 2002). However, the early evolution of the microcystin biosynthesis gene cluster, which even predates the earliest metazoans, rules out the deterrence of metazoan grazers as microcystin's primary function (Rantala et al., 2004). Whether microcystin acts as a defence mechanism against unicellular grazers, is less clear, but experimental studies with mixotrophic flagellates argue against it (Wilken et al., 2010).

Apart from a grazer deterrent, microcystin was proposed to act against intracellular parasites, such as fungi or bacteriophages. The predominantly intracellular localization of microcystin supports this supposition (Park et al., 1998; Young et al., 2005). In addition, experimental data about *Planktothrix* sp. that was able to combat intracellular parasitic fungi by producing microcystin and other secondary metabolites supported this hypothesis (Rohrlack et al., 2013).

Microcystin was proposed to play a role in the acquisition of micronutrients such as iron due to its binding capacity to certain metal ions (Klein et al., 2013; Utkilen and Gjølme, 1995). The ability of microcystin to act as an iron chelator is supported by experiments that showed that cells increased their microcystin content when iron was limiting (Lukač and Aegerter, 1993; Wang et al., 2016; Yeung et al., 2016). Moreover, cyanobacteria producing microcystin take up iron more efficiently than non-toxin producers (Utkilen and Gjølme, 1995). In addition, transcription of microcystin biosynthesis genes is linked to iron metabolism by means of the ferric uptake regulator Fur, which is present in *M. aeruginosa* and binds to promotor regions of the microcystin gene cluster (Martin-Luna et al., 2006a; Martin-Luna et al., 2006b).

Microcystin was believed to play a role in intercellular signalling between cyanobacterial cells of a population (Phelan and Downing, 2014; Schatz et al., 2007). Not only the active export out of the cell, but also the passive release of large amounts of microcystin after cell death might alert neighbouring cells to potential danger (Schatz et al., 2007). Furthermore, a role of microcystin in sensing and possibly regulating cell density was discussed (Wood et al., 2011). In addition, allopathic interactions of microcystin were observed (Hu et al., 2004; Pflugmacher, 2002; Phelan and Downing, 2014).

More recent studies pointed towards the intracellular function of microcystin (Makower et al., 2015). Especially its role in coping with oxidative stress became the focus of attention. Microcystin is upregulated during high light when the cyanobacterial cell encounters stressful conditions (Zilliges et al., 2011). In addition, microcystins binds to important enzymes of the

energy metabolism such as RubisCo and stabilizes them during times of oxidative stress (Meissner et al., 2013; Zilliges et al., 2011).

1.3.3 Genetic basis of microcystin synthesis

The microcystin biosynthesis gene cluster is the genetic basis of microcystin biosynthesis. It encodes for the proteins of a multi-enzyme complex that is responsible for the non-ribosomal synthesis of microcystin. The structure of the gene cluster and most of the reactions necessary for microcystin biosynthesis have been determined (Tillett et al., 2000). The gene cluster spans approximately 55 kb and consists of about 10 open reading frames, termed *mcyA-mcyJ* (Figure 1.3). Those open reading frames code for polyketide synthases, peptide synthetases, chimeric enzymes containing both domains, or enzymes with a peptide tailoring function (Christiansen et al., 2003; Nishizawa et al., 2000; Tillett et al., 2000). The order and orientation of the open reading frames within the gene cluster is different in the three main microcystin producer genera: *Microcystis* spp. contain two operons, which are transcribed in opposite directions. One operon comprising the genes *mcyA-mcyC* and the other the genes *mcyD-J* (Figure 1.3) (Nishizawa et al., 1999; Nishizawa et al., 2000; Tillett et al., 2000). *Dolichospermum* spp. contain the same genes in a slightly different order (Rouhiainen et al., 2004). *Planktothrix* spp. lack *mcyF* and *mcyI* but possesses the gene *mcyT* instead (Christiansen et al., 2003). Insertional mutagenesis of some of the genes in the microcystin biosynthesis cluster showed that disruption of one gene (*mcyA*, *mcyB*, *mcyD,* or *mcyE*) disables microcystin production and that only a single cluster is responsible for the production of all structural microcystin variants (Dittmann et al., 1997; Nishizawa et al., 1999; Nishizawa et al., 2000; Tillett et al., 2000).

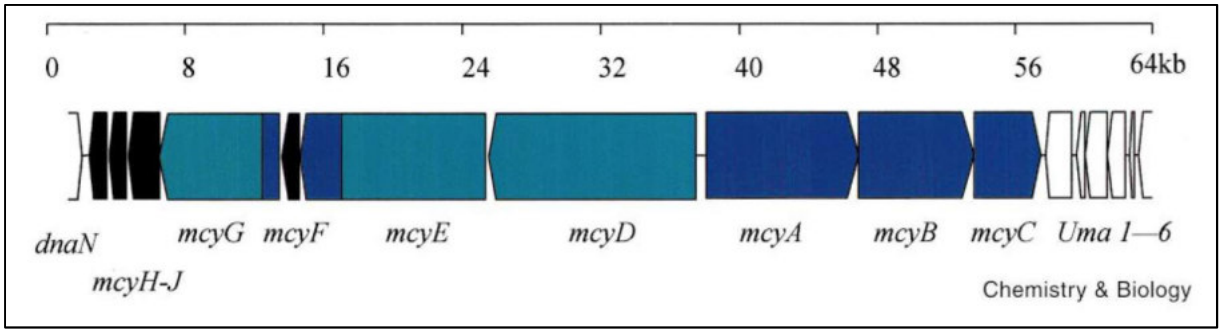

Figure 1.3: Microcystin biosynthesis gene cluster in Microcystis indicating the direction of transcription and relative size of open reading frames. Genes encoding polyketide synthase are indicated in light blue and gene encoding peptide synthetase are indicated in dark blue. Genes of putative microcystin tailoring function are in black and neighbouring genes not belonging to the microcystin synthesis gene cluster are white (Reprinted from Chemistry & Biology, (Tillett et al., 2000) with permission from Elsevier).

1.3.4 Regulation of microcystin synthesis at the genetic level

Several lines of evidence suggest that microcystin is not expressed constitutively but subject to regulation (Wood et al., 2011). More specifically, regulation takes place at the transcriptional level. The transcriptional regulation of microcystin synthesis has mainly been studied on *M. aeruginosa*, but the regulatory elements for toxin genes have been described for other cyanobacteria as well (Jones et al., 2009). Regulator proteins and alternative transcription start points direct the transcriptional regulation of gene products involved in microcystin biosynthesis.

So far, two regulator proteins, which are associated with the microcystin biosynthesis gene cluster, have been found in *Microcystis*: Fur and NtcA. The genomes of *M. aeruginosa* and *Dolichospermum* sp. harbour *furA* genes (Hernández et al., 2002; Martin-Luna et al., 2006a). Fur is a protein which acts as repressor with iron as co-repressor (Litwin and Calderwood, 1993). Fur from *Microcystis* binds to the intercistronic promotor region between the two operons upstream of *mcyA* and *mcyD* (*mcyA/D* promotor region) (Martin-Luna et al., 2006b) and thus regulates the transcription of the microcystin biosynthesis genes in response to the iron concentration (Alexova et al., 2011a). The second regulator for microcystin biosynthesis genes is NtcA. NtcA is a regulator protein, which regulates the transcription of genes of the nitrogen metabolism, photosynthesis, and reaction to stress in cyanobacteria (Frías et al., 1993; Herrero et al., 2001; Kuniyoshi et al., 2011). In *M. aeruginosa* NtcA binds to the *mcyA/D* promotor region (Ginn et al., 2010; Kuniyoshi et al., 2011) and acts as a repressor of microcystin gene transcription similar to Fur (Kuniyoshi et al., 2011).

The *mcyA/D* promotor region contains several alternate transcription start points (Kaebernick et al., 2002; Sevilla et al., 2008). A total of four different transcription start points were identified for *mcyA* (Kaebernick et al., 2002; Sevilla et al., 2008), and eight different transcription start points were identified for *mcyD* (Kaebernick et al., 2002; Sevilla et al., 2008). Some of those alternative transcription start points are associated with the transcriptional regulation of the microcystin biosynthesis genes in response to changing light condition (Kaebernick et al., 2002). Apart from the promotors in the *mcyA/D* promotor region, additional intercistronic promotors were identified within the microcystin biosynthesis gene cluster (Kaebernick et al., 2002).

1.3.5 Methods and problems in microcystin detection

The following section summarizes the most relevant methods for detection of microcystin and presents advantages and disadvantages for each method.

Traditionally, mouse bioassays were used to gauge toxicity of cyanobacterial samples and cultures (Douma et al., 2010; Masango et al., 2008; Sivonen et al., 1989). An intraperitoneal injection of toxin extract was administered to mice and toxic response was monitored to deduce the class of toxin. This assay is able to distinguish between cyanobacterial hepatotoxins, which cause symptoms of liver damage and death within four hours, and neurotoxins, which lead to death within 15 min accompanied by neuromuscular symptoms (Nicholson and Burch, 2001). Results of the mouse bioassay can only be described qualitatively and not quantified. The assay is not suitable for the monitoring of water samples due to the large number of animals required and the low sensitivity and specificity of the method (Kaushik and Balasubramanian, 2013; Sangolkar et al., 2006).

Due to issues with animal use and practicability bioassays utilizing invertebrate organisms were developed. In most cases, crustaceans are used. One of the most commonly used assays is the brine shrimp assay which uses *Artemia salina* to determine the percent mortality of *A. salina* larvae after 24 hours (Kiviranta et al., 1991). Test kits using the fairy shrimp *Thamnocephalus platyurus* (Törökné, 1999; Törökné et al., 2000) and *Daphnia* sp. (Kyselková and Maršálek, 2000) were successfully used for cyanobacterial toxins. The disadvantage of these assay is the low sensitivity and specificity (Campbell et al., 1994; Lahti et al., 1995) as the invertebrates also react to other substances than microcystin such as cylindrospermopsin (Metcalf et al., 2002).

Protein phosphatase inhibition assays utilize the capacity of microcystin to inhibit protein phosphatase (mostly protein phosphatase 2A) (Heresztyn and Nicholson, 2001; Lambert et al., 1994; Wong et al., 1999). This detection method is sensitive and suitable for quantitative analysis, but due to the extensive laboratory equipment needed (e.g. for radiolabelling), it is not commonly used in routine monitoring (Kaushik and Balasubramanian, 2013). In addition, protein phosphatase inhibition assays show a differential sensitivity to the different microcystins (Mountfort et al., 2005).

Immunological assays are currently amongst the most widely used methods to detect microcystin, especially commercially available enzyme-linked immunosorbent assays (ELISAs). The performance of microcystin ELISA assays is largely dependent on the antibody

utilized. Polyclonal antibodies in most commercial kits have variable affinities to different microcystin isoforms so that more than one structural variant of microcystin is recognized. However, this cross reactivity is not 100% and usually does not extend to all microcystin isoforms. In addition, a degree of cross reactivity with other substances must be considered with those assays (Merel et al., 2013). Apart from ELISAs, other antibody-based test were developed, most notably lateral flow dipstick assays (Melnik et al., 2017; Pyo et al., 2006; Tippkotter et al., 2009). Like ELISA assays, their specificity is very much dependent on the antibody used and sensitivities do vary. Quantification of the microcystin concentration is not possible with dipstick tests. The potential advantage over all other detection method is the extremely short turnaround time and the possibility for use on site without laboratory equipment or trained personnel.

Mass spectroscopy and chromatography are sensitive and specific methods for the detection of microcystins. Detection of toxins by mass spectroscopy coupled with either gas or liquid chromatography for separation (GC-MS, LC-MS) is a frequently used technique for the detection and quantification of microcystin. This method is, like high-pressure liquid chromatography (HPLC), dependent on the availability of purified microcystin standards. The small range of commercially available standards limits analysis with GC/LC-MS and HPLC (McElhiney and Lawton, 2005). Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectroscopy is a rapid and sensitive method that can be used on very small sample volumes, but it is not quantitative and thus not suitable for effective monitoring of microcystin concentrations in water samples (Kaushik and Balasubramanian, 2013).

Nucleic acid based molecular methods such as PCR, qPCR, and microarrays detect the presence of microcystin biosynthesis genes not the microcystin itself. Moreover, RT-qPCR can quantitatively assess the microcystin biosynthesis gene transcription. Those methods overcome some of the disadvantages of the methods mentioned above (Sangolkar et al., 2006). They can be very sensitive, and are independent of available toxin standards. Those methods do not discriminate between microcystin isoforms but account for all structural variants of microcystin. While PCR is not quantitative, qPCR and microarrays give quantitative results, but those quantitative results cannot be translated into toxin concentration (Kaushik and Balasubramanian, 2013).

2 Objectives

Previous research established that toxic cyanobacteria blooms are going to continue to present a problem in the future and are likely going to appear even more frequently than in the past due to global change. The toxic cyanobacterium *Microcystis* is of particular interest because it is a well-known toxin producer commonly found in waterbodies almost all over the world, and it often forms toxic cyanobacterial blooms. Furthermore, it was shown that the expression of microcystin genes is subject to transcriptional regulation in *Microcystis* and other cyanobacteria.

Traditionally, research focused on the impact of abiotic environmental drivers on the growth of *Microcystis*. Such approaches, however, failed to address the impact such factors might have at the level of toxin gene expression. Little is known about how climate change related factors influence the toxic potential of *Microcystis* at the transcriptional level. Even less is known about the role of co-occurring microorganisms on the toxic potential of cyanobacteria like *Microcystis*. To increase knowledge about possible future changes in toxic potential of cyanobacteria, this work focused on gene expression in laboratory experiments and the study of toxin content and relative abundance of toxic cyanobacteria in field studies.

The primary objectives of this thesis were:

i. To assess any effect of climate change related abiotic factors on the toxic potential of *Microcystis aeruginosa* under laboratory conditions.

   In order to assess transcriptional changes in the expression of microcystin biosynthesis genes, an assay to do this accurately was developed for this research. The climate change related factors under investigation were: increased temperature, changed perturbation patterns, and increased microcystin content. This study tested the hypothesis whether those factors influence toxic potential by affecting microcystin gene transcription.

ii. To elucidate possible effects of biotic environmental factors, in particular co-occurring microorganisms, on the toxic potential of *M. aeruginosa* under laboratory conditions.

   Transcriptional changes of microcystin biosynthesis genes were used to determine possible effects of the following co-occurring organisms: cyanobacteria, mixotrophic flagellates, and the virioplankton size fraction containing cyanophages. This research

aimed to answer whether and how co-occurring microorganisms affect microcystin gene transcription.

iii. To determine the influence of abiotic and biotic environmental variables on the cyanobacterial community composition in the field, in particular on relative abundance and toxicity of the toxin producer *Microcystis* sp.

In order to do this, high throughput sequencing, *mcy* gene copy quantification, toxin measurements, and phylogenetic analyses were performed on samples from two Bavarian recreational lakes. In addition, this project aimed to answer the questions whether the microcystin concentrations and the number of toxic genotypes correlate and whether the main microcystin producer can be identified in the natural community. Moreover, the correlation of biotic and abiotic environmental variables with toxicity and relative abundance of toxigenic cyanobacteria was explored.

3 Influence of temperature, mixing, and addition of microcystin-LR on microcystin gene expression in *Microcystis aeruginosa*

A similar version of this chapter was published: Scherer PI, Raeder U, Geist J, Zwirglmaier K. 2017. Influence of temperature, mixing, and addition of microcystin-LR on microcystin gene expression in *Microcystis aeruginosa*. MicrobiologyOpen 6(1):e00393.

3.1 Abstract

Cyanobacteria, such as the toxin producer *Microcystis aeruginosa*, are predicted to be favoured by global warming both directly, through elevated water temperatures, and indirectly, through factors such as prolonged stratification of waterbodies. *M. aeruginosa* is able to produce the hepatotoxin microcystin, which causes great concern in freshwater management worldwide. However, little is known about the expression of microcystin synthesis genes in response to climate change-related factors. In this study, a new RT-qPCR assay employing four reference genes (*GAPDH*, *gltA, rpoC1* and *rpoD*) was developed to assess the expression of two target genes (the microcystin synthesis genes *mcyB* and *mcyD*). This assay was used to investigate changes in *mcyB* and *mcyD* expression in response to selected environmental factors associated with global warming. A 10 °C rise in temperature significantly increased *mcyB* expression, but not *mcyD* expression. Neither mixing nor the addition of microcystin-LR (10 µg/l or 60 µg/l) significantly altered *mcyB* and *mcyD* expression. The expression levels of *mcyB* and *mcyD* were correlated but not identical.

3.2 Introduction

Microcystis aeruginosa is a cyanobacterium found in waterbodies worldwide (Chorus and Bartram, 1999; Moreira et al., 2014; Van Gremberghe et al., 2011). Many strains of *M. aeruginosa* produce the toxin microcystin, which is harmful to humans, livestock and aquatic animals. Microcystin is actively taken up by hepatocytes and is, therefore, classified as a liver toxin (Runnegar and Falconer, 1982). Toxic *Microcystis* blooms have been associated with human and livestock fatalities or disease (Carmichael et al., 2001; Van Halderen et al., 1995), and microcystin-producing cyanobacterial blooms regularly pose a threat to the safety of the water supply (Hudnell, 2010).

Toxic cyanobacterial blooms are expected to occur more often in the future because of global warming (Paerl and Huisman, 2008; Paerl and Huisman, 2009; Paerl and Paul, 2012). Direct effects, such as rising temperatures, and indirect effects, such as intensified stratification, favour cyanobacterial blooms, that might be toxic in some cases. Increased water temperature is expected to give cyanobacteria a selective advantage over competing phytoplankton because of the high optimal growth temperature of studied cyanobacterial species (Elliott et al., 2006; Jöhnk et al., 2008). Also, some findings suggest that toxic *Microcystis* genotypes are favoured over non-toxic ones at warmer temperatures (Davis et al., 2009). However, the link between temperature and toxin production remains largely unknown (El-Shehawy et al., 2012). Consequently, increased temperature is one of the stressors related to climate change that we chose to investigate as a possible trigger of increased *M. aeruginosa* toxigenicity. Another change expected to be brought about by global warming is a shift in waterbody perturbation patterns. Model-based analyses forecast increased water column stability and a warmer epilimnion (Wahl and Peeters, 2014), as well as an earlier seasonal stratification of waterbodies (Peeters et al., 2007). These effects will be caused, in part, by a shorter annual duration of ice cover (Magnuson et al., 2000). These developments are expected to favour cyanobacterial blooms (Jöhnk et al., 2008) because many cyanobacteria, such as *Microcystis*, are able to thrive under those conditions by actively regulating buoyancy and finding their optimal depth in the water column (Huisman et al., 2004; Walsby et al., 1997). On the contrary, intensified vertical mixing of the epilimnion caused by more frequent wind stress and extreme weather events, such as storms and floods, is expected for temperate regions (Gastineau and Soden, 2009; Helbling et al., 2015). Because the effect of such profound environmental changes on cyanobacterial toxicity or toxigenicity is unknown, we selected the process of mixing as another environmental stressor to investigate with regard to *M. aeruginosa* toxigenicity. Other factors, such as rising carbon dioxide levels (Wannicke et al., 2012) and increased eutrophication (Huisman et al., 2005) of waterbodies, have also been linked to the enhanced growth of cyanobacteria. Taken together, these factors might not only enhance the growth of toxic cyanobacteria but also increase toxin concentrations in the environment. Thus, we investigated microcystin at different concentrations as a third environmental stressor that is expected to influence *M. aeruginosa* toxigenicity.

Microcystin, a toxin commonly associated with toxic cyanobacterial blooms, is a cyclic heptapeptide that is produced non-ribosomally at a multienzyme complex. The genes necessary for the synthesis of microcystin are organized in a gene cluster with two bi-directionally

transcribed operons (*mcyA-C* and *mcyD-J*) (Neilan et al., 1999; Tillett et al., 2000). Not all strains of *M. aeruginosa* have the genetic capability to produce microcystin. Unfortunately, toxic and non-toxic genotypes cannot be distinguished by means of microscopy (Komárek, 1991). Based on knowledge about the genetic basis of microcystin synthesis, PCR-based assays have been developed to distinguish toxic from non-toxic strains at the molecular level (Al-Tebrineh et al., 2012; Fortin et al., 2010; Hautala et al., 2013; Hisbergues et al., 2003; Kurmayer and Kutzenberger, 2003; Ostermaier and Kurmayer, 2010; Pimentel and Giani, 2014; Rantala et al., 2006). The detection and quantification of toxicity genes are of special interest because the direct detection of microcystin has certain limitations and drawbacks. More than 90 isoforms of microcystin, which differ in their two L-amino acids or the presence of methyl groups on D-erythro-β-methylaspartic acid (D-MeAsp) and N-methyldehydroalanine (Mdha), are known (Neilan et al., 2008; Welker and von Döhren, 2006). Commonly used enzyme-linked immunosorbent assays (ELISAs) usually measure only one of the microcystin isoforms (microcystin-LR) and have a certain degree of cross reactivity with other isoforms (Kaushik and Balasubramanian, 2013; Sangolkar et al., 2006). Other methods, such as high pressure liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS), can distinguish several isoforms, but are also more expensive and labour-intensive. Another limitation of these methods is the fact that methanol extracts are usually measured; therefore, protein-bound microcystin is not detected (Meissner et al., 2013).

The genes encoding the proteins required to synthesize the secondary metabolite microcystin are not constitutively expressed (Wood et al., 2011). Therefore, expression of the genes belonging to the microcystin gene cluster has been of interest for some time. Several approaches have been employed to study gene expression in *M. aeruginosa* in general and the expression of toxicity genes in particular. Microarrays, for example, have led to a deeper understanding of the systemic effects of microcystin on cells (Makower et al., 2015). Methods such as the RNase protection assay (Kaebernick et al., 2000) or competitive reverse-transcriptase polymerase chain reaction (RT-PCR) (Kim et al., 2005) have been used to quantify *mcyB* and *mcyD* transcripts in the past. Reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) has been used to understand the impact of nutritional and other factors on the expression of microcystin genes. For instance, the influence of micro- and macronutrients on *mcyD* expression was investigated (Kuniyoshi et al., 2013; Pimentel and Giani, 2014; Sevilla et al., 2008; Sevilla et al., 2010), and the effect of high light stress as well as co-occurring

cyanobacteria and cell concentration on *mcyE* expression was studied (Ngwa et al., 2014; Tran et al., 2013; Wood et al., 2011).

Most of the previous RT-qPCR studies, while making valuable contributions to our understanding of the microcystin synthesis gene expression dynamics of *M. aeruginosa*, normalized gene expression to a single reference gene; this single reference gene was mostly the 16S rRNA gene (Kuniyoshi et al., 2013; Pimentel and Giani, 2014; Sevilla et al., 2008). Although it is possible to use a single stably expressed gene as a reference, some scientists have found that the 16S rRNA gene is not a suitable reference gene (Radonić et al., 2004; Tran et al., 2013). Other studies have relied on a panel of several reference genes to average out small fluctuations in reference gene expression (Tran et al., 2013; Zhao et al., 2011). To our knowledge, there is no RT-qPCR assay for the microcystin synthesis genes of *M. aeruginosa* that employs a whole reference gene panel to assess the expression of one or more target genes. This led us to develop a novel RT-qPCR assay with a panel of four reference genes (*GAPDH*, *gltA, rpoC1* and *rpoD*) that can be used to assess the expression of two microcystin synthesis genes (*mcyB* and *mcyD*).

In summary, the aims of this study were i) to design laboratory experiments suitable to test the hypothesis that those stressors increase microcystin gene expression and ii) to determine the fold change in *mcyB* and *mcyD* gene expression in response to elevated temperature, mixing regimes and treatment with microcystin-LR.

3.3 Material and methods

3.3.1 Experimental design

Cyanobacterial cultures were grown to early exponential phase under laboratory conditions and then divided into two cohorts. One cohort continued growth under the original conditions and acted as a control, whereas the other was treated with a stressor. Three different stressors were tested: temperature rise, mixing and the addition of microcystin-LR. To investigate the effects of elevated temperature, cultures grown at 20 °C were shifted to 30 °C. To investigate the effect of mixing, liquid cultures were stirred in such a way that a vortex in the liquid was just about not forming (4 cm stir bar, 150 rpm). This mimics, to a degree, the perturbation of cyanobacteria in waterbodies subjected to mixing events such as storms or floods. To test whether increased levels of extracellular microcystin-LR have an effect, two different concentrations of microcystin-LR were added to batch cultures. Both concentrations tested (10 µg/l and 60 µg/l) are well above the safe level (1 µg/l) for drinking water (WHO, 2011) and within a range that

can be realistically found in surface waters (Backer et al., 2008; Backer et al., 2010; Lee et al., 2015). Three independent biological replicates were analysed for each experimental condition. RNA was extracted from cultures that were harvested 72 h after stressor application. Expression of the target genes *mcyB* and *mcyD* was assessed using RT-qPCR with two technical replicates. Each experiment was repeated a minimum of two times.

3.3.2 Strains and cultivation techniques

M. aeruginosa strain SAG14.85, which is known to produce microcystin (Lyra et al., 2001), was obtained from the Culture Collection of Algae at Göttingen University in Germany (SAG). *M. aeruginosa* cells were grown in batch cultures in 300 mL Erlenmeyer flasks made of borosilicate glass. Cultures were grown in 200-mL volumes of BG-11 medium (Rippka et al., 1979) supplemented with 0.5 mM ammonium chloride under a light-dark regime of 14 h light and 10 h dark. MASTER TL5 HO 39W/865 1SL fluorescent light tubes emitting cool daylight (Phillips, Amsterdam, Netherlands) were used as light sources. The light intensity was 130 µmol s^{-1} m^{-2} for both control and treated cultures (measured with a PAR sensor, LI-COR, Lincoln, USA). Cultivation temperatures of 20 °C and 30 °C were used during the temperature rise experiments. The intermediate temperature of 25 °C was used during the mixing and microcystin addition experiments for both control and treated cultures. Cyanobacterial growth was monitored by measuring the optical density at 730 nm (OD_{730}) using a spectrophotometer (model 150-20, HITACHI, Chiyoda, Japan), and cultures were regularly checked for contamination under a microscope (LEICA DM R, Leica Microsystems, Wetzlar, Germany).

3.3.3 Harvesting and RNA extraction

Harvesting was performed as follows: Twenty millilitre culture samples were filtered through a 0.2 µm pore-size cellulose nitrate filter (Sartorius, Göttingen, Germany), and then the filters, which retained the cyanobacteria, were frozen immediately at −80 °C. Storage at −80 °C did not exceed 14 days.

RNA extraction was performed as described by Penn et al. (2014), with minor modifications. In short, filters were cut, placed into 1.5 mL reaction tubes and vortexed with 1 mL lysozyme solution (15 mg/mL). After incubation for 10 min at 37 °C, 2 g of 1.8–2.0-mm ceramic beads (Sigmund Linder, Warmensteinach, Germany) were added, and the mixture was beaten for 10 min using a Mikro-dismembrator II (Braun, Melsungen, Germany), submerging the tubes in ice every 2 min for 30 s. The resulting liquid was transferred to a fresh tube and subjected to centrifugation at 6,200 ×*g* at 4 °C for 3 min. The pellet was resuspended in 1 mL TRIsure

(Bioline, Luckenwalde, Germany) and incubated for 5 min at 20 °C. Addition of 0.2 mL chloroform and mixing was followed by 8 min of incubation and centrifugation at 13,800 ×*g* for 15 min. The upper phase was transferred into a fresh tube, and the chloroform wash was repeated. Addition of 1 µl of 15 µg/µl glycogen and 0.5 mL of isopropanol was followed by overnight incubation at −80 °C. Subsequently, the samples were defrosted, mixed and centrifuged at 18,800 ×*g* for 30 min. The resulting pellet was washed with ethanol, dried and resuspended in DEPC-treated water.

The isolated RNA was cleaned up with the RNA Clean & Concentrator kit (Zymo Research, Irvine, USA) and stored at −80 °C. Total RNA was quantified and checked for purity using a NanoVue Plus spectrophotometer (GE healthcare, Little Chalfont, UK), and its integrity was checked on a 1% agarose gel stained with GelRed (Biotium, Hayward, USA).

Table 3.1: Criteria for reference gene primer

Tested parameter	**Pass Criteria**
Specificity *in silico*	BLAST search does not identify an alternate biding site in cyanobacterial genome
Specificity *in vitro*	Single band of expected size on agarose gel Melting curve examination /single melting peak No false positive results
Similar product quantity as target genes	C_q values similar to C_q of target genes Baseline must be present
Annealing temperature	Can be used at same temperature as the target genes
Stable expression	M values < 0.5 (geNorm)
Efficiency of reaction	Between 90 and 110%
R^2 value of reaction	> 0.980

3.3.4 Primer design and testing

Several possible reference genes were evaluated in this study to assemble a suitable reference gene panel. Criteria for selecting reference gene primer pairs are listed in Table 3.1. These potential reference genes included the 16S rRNA gene and the genes encoding glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), citrate synthase (*gltA*), DNA gyrase subunit B (*gyrB*), DNA-directed RNA polymerase subunit gamma (*rpoC1*) and RNA polymerase sigma factor RpoD (*rpoD*). Primers targeting *GAPDH, gltA*, *gyrB* and *rpoD* of *M. aeruginosa* NIES843 (Gen

Bank AP009552.1) were designed using Primer3 software (Rozen and Skaletsky, 2000). Both primers designed in this study and primers obtained from the literature (Table 3.2) were checked for specificity using the BLAST algorithm (Altschul et al., 1990), and no homology to unwanted targets was found. Melting curves and agarose gel electrophoresis were used to ensure the specificity of the reaction under the chosen conditions (see Figure 3.1). The efficiency of qPCR reactions and the R^2 values of standard curves were determined for all primers included in the assay (see Table 3.3 and Figure 3.2). Oligonucleotides were synthesized by biomers.net (Ulm, Germany).

The suitability of a gene to serve as reference gene under a certain experimental condition was verified using geNorm software (Vandesompele et al., 2002).

3.3.5 RT-qPCR

Prior to reverse transcription, the RNA samples were freed from residual genomic DNA using DNase I digestion according to the manufacturer's instructions (Thermo Fisher, Waltham, USA).

Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, USA) according to the manufacturer's instructions. A no-reverse-transcriptase control reaction, which acted as a negative control for reverse transcription, was performed for every sample by omitting reverse transcriptase from the reaction. cDNA was stored at -20 °C until analysis commenced.

The qPCR analyses were performed using a BioRad CFX 96 cycler, Sso Advanced universal SYBR Green supermix (BioRad, Hercules, USA) and primers at 0.2 µM in a 20-µl reaction. Primer pairs for the target genes *mcyD* and *mcyB* were RmcyDF/RmcyDR (Sevilla et al., 2008) and mcyB30F/mcyB108R (Kurmayer and Kutzenberger, 2003), respectively. Primer pairs for the reference genes *rpoC*, *gltA*, *rpoD* and *GADPH* were rpoC1F/rpoC1R (Alexova et al., 2011b), gltA429F/gltA528R, rpoD230F/rpoD354R and GAPDH727F/GAPDH825R, respectively (Table 3.2). A 1-µl volume of cDNA (50 ng) was used as template. The cycling conditions were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 64.7 °C for 1 min and finally a melt curve of 95 °C for 10 min and then a ramp from 65 °C to 95 °C in 0.5 °C increments. Two technical replicates of each cDNA sample were analysed using qPCR. A single no-reverse-transcriptase qPCR reaction was performed for each sample in order to detect possible contamination of the RNA with genomic DNA, and a no-template control was performed, in duplicate, and for every target to check for contaminants.

Table 3.2: Information about genes and primers in this study

Target gene	**Primer name**	**Sequence 5‘ to 3‘**	**Source**
mcyB peptide synthetase	mcyB30F mcyB108R	CCTACCGAGCGCTTGGG GAAAATCCCCTAAAGATTCCTGAGT	(Kurmayer and Kutzenberger, 2003)
mcyD polyketide synthase	RmcyDF RmcyDR	ACCCGGAACGGTCATAAATTGG CGGCTAATCTCTCCAAAACATTGC	(Sevilla et al., 2008)
16S-rRNA 16S ribosomal RNA	16S-For 16S-Rev	TGCGTAGAGATTGGGAAGAACATC GCTTTCGTCCCTGAGTGTCA	(Sevilla et al., 2008)
GAPDH glyceraldehyde-3-phosphate dehydrogenase	GAPDH727F GAPDH825R	GTTTCGGCGGTGGATTTAACC ACCTTTCATCGGACCTTCG	this study
gltA citrate synthase	gltA429F gltA528R	AGGTAATCATCCCATTCAGCCC AACTTTCGCCGCTAAATCCG	this study
gyrB DNA gyrase subunit B	gyrB1041F gyrB1124R	AGTCCGGGGTATTGTTGATTCC ATAATCGTGTCGGCTACTTGGG	this study
rpoC1 DNA-directed RNA polymerase subunit gamma	rpoC1F rpoC1R	CCTCAGCGAAGATCAATGGT CCGTTTTTGCCCCTTACTTT	(Alexova et al., 2011b)
rpoD RNA polymerase sigma factor RpoD	rpoD230F rpoD354R	GCAGGATTCGGTTATTGAGAGC CTGTTTTCCCCATTCAGCATCG	this study

Table 3.3: Efficiency and R^2 values of qPCR reactions and amplicon size for target and reference genes

Primer name	Target gene	Efficiency (%)	R^2	Amplicon length (bp)
mcyB30F mcyB108R	*mcyB* peptide synthetase	97.9	0.998	97
RmcyDF RmcyDR	*mcyD* polyketide synthase	93.5	0.995	80
GAPDH727F GAPDH825R	*GAPDH* glyceraldehyde-3-phosphate dehydrogenase	101.4	0.996	99
gltA429F gltA528R	*gltA* citrate synthase	97.4	0.998	100
rpoC1F rpoC1R	*rpoC1* DNA-directed RNA polymerase subunit gamma	93.9	0.998	181
rpoD230F rpoD354R	*rpoD* RNA polymerase sigma factor RpoD	94.8	0.998	125

Only technical replicates with a difference of no more than 0.5 cycles were included in the subsequent analysis. Results were only included in the analysis if the difference between the C_q value of the sample and that of the no-reverse-transcriptase control or the no-template control (NTC) was five or larger. C_q values of NTCs were either not defined (as signal did not cross the threshold until cycle 40) or ≥ 31.

Data analysis was performed according to the $\Delta\Delta C_q$ method (Pfaffl, 2001) with BioRad CFX Manager software.

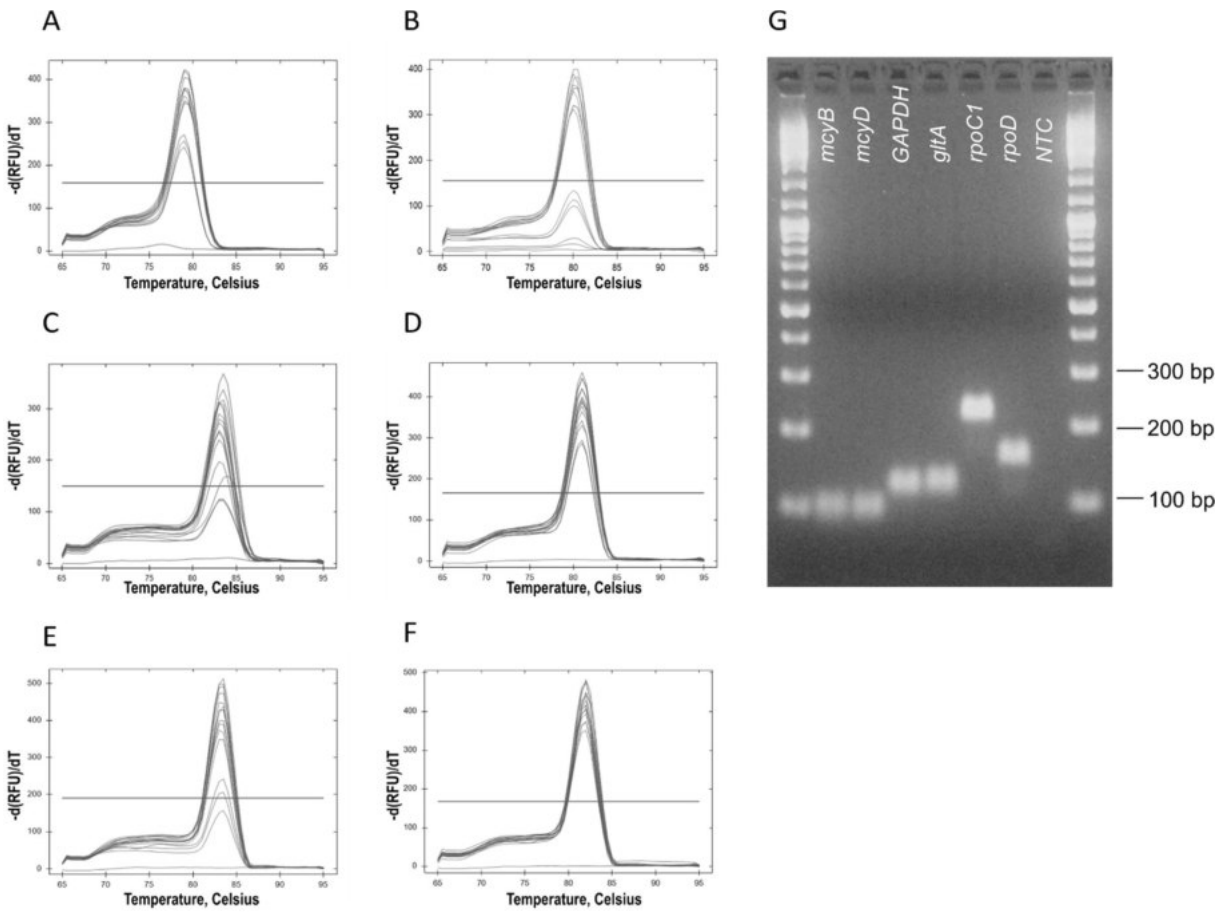

Figure 3.1: Primer specificity for reference and target genes. Melting curves profiles of target and reference gene PCR products. Genes: (A) mcyB, (B) mcyD, (C) GAPDH, (D) gltA, (E) rpoC1, and (F) rpoD. (G) qPCR products on 3 % agarose gel.

3.3.6 Statistical analysis

The fold change values were examined to compare the relative normalized expression of target genes between control and stressor treatments. Statistical analyses were carried out using PAST v3.10 software (Hammer et al., 2001). The fold change values were tested for normal distribution using the Shapiro-Wilk test. Subsequently, the normally distributed data was analysed using a two-sample t-test with the level of significance set at $p \leq 0.05$. Pearson's r and linear regression were used to assess the correlation of ΔC_q values.

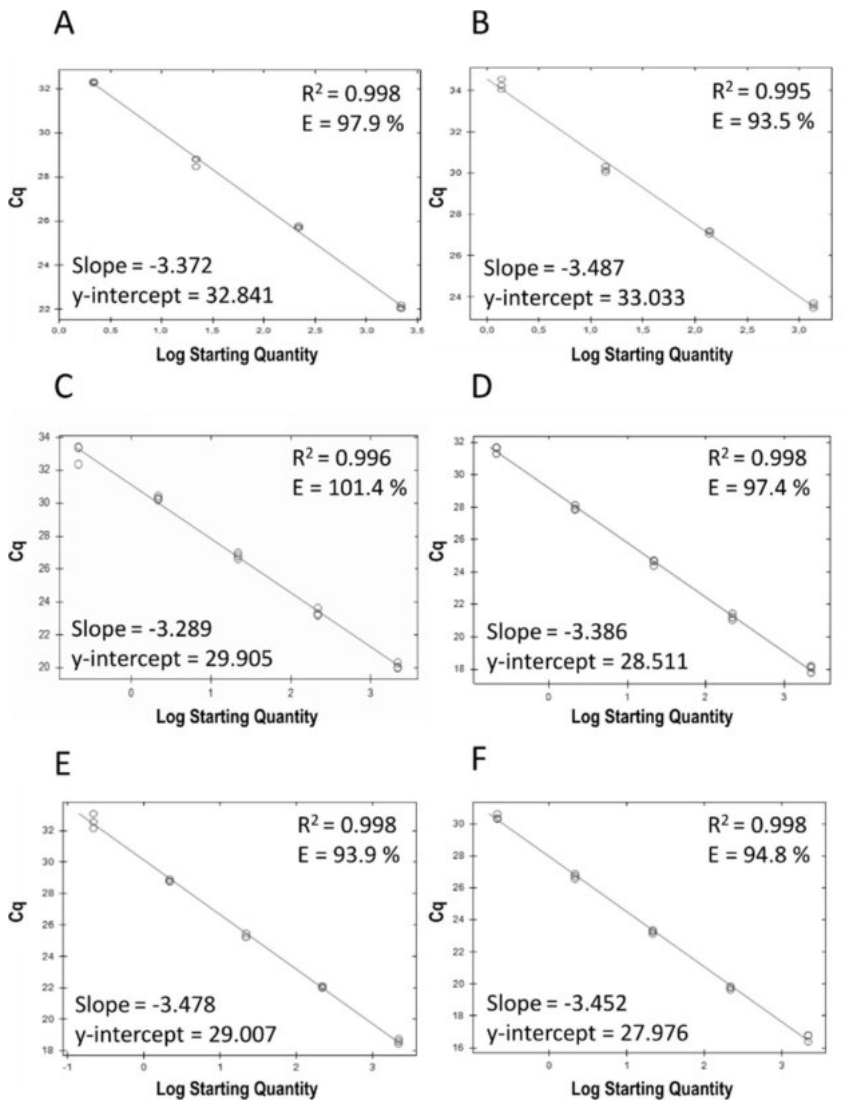

Figure 3.2: Standard curves of target and reference gene qPCR products. Genes: (A) mcyB*, (B)* mcyD*, (C)* GAPDH*, (D)* gltA*, (E)* rpoC1*, and (F)* rpoD*.*

3.4 Results

3.4.1 Reference and target genes

Of the six reference genes evaluated, only *GAPDH*, *gltA*, *rpoC1* and *rpoD* were found to be suitable. The corresponding amplification efficiencies were 101.4%, 97.4%, 93.9% and 94.8%, respectively (Table 3.3). R^2 values for the selected reference gene reactions were found to be 0.996, 0.998, 0.998 and 0.998, respectively (Table 3.3). Analysis of the melting curves and agarose gel electrophoresis data demonstrated that the RT-qPCR reactions were specific (Figure 3.1). Two of the candidate reference genes tested were disregarded because of a false positive signal (*gyrB*) (data not shown) or low C_q values (*16S rRNA*) (Figure 3.3). The selected reference genes (*GAPDH*, *gltA, rpoC1* and *rpoD*) were included in the reference gene panel for this study,

and the suitability of every reference gene for each experimental condition could be confirmed using geNorm (M values <0.5).

The two targets (*mcyB* and *mcyD*) amplified with efficiencies of 97.9% and 93.5% (Table 3.3), respectively. The R^2 values for the target gene reactions were 0.998 and 0.995 (Table 3.3), respectively. The specificity of the target gene reactions was verified by evaluation of the melting curves and agarose gel electrophoresis data (Figure 3.1).

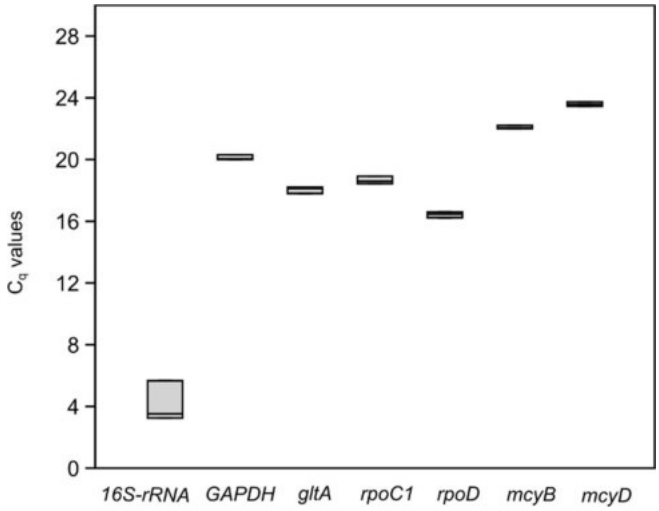

Figure 3.3: C_q values of reference and target gene candidates (n=4) obtained during assay optimisation.

3.4.2 **Response to elevated temperature**

Elevated temperature was the only stressor tested that induced a significant increase in expression of one of the microcystin genes, *mcyB*. Relative quantification of the *mcyB* and *mcyD* transcript levels was performed for both temperature conditions. The control condition of 20 °C was used as the calibrator (Figure 3.4A). For the *mcyB* transcripts, the mean fold change values from control cultures were found to be significantly higher than those of the cultures subjected to elevated temperature (30 °C) (mean [standard error of the mean] = 1.00 [.13] versus 1.72 [.12]; t = 3.57, $p \leq 0.05$). This indicates that the transcription of the *mcyB* gene was significantly upregulated at the warmer temperature. For the *mcyD* transcripts, however, the mean fold change values from control cultures were not significantly different from those of the cultures subjected to 30 °C treatment (1.00 [.15] versus 1.33 [.09]; t = 0.50, $p = 0.63$). This shows that the expression of *mcyB,* but not that of *mcyD*, was significantly upregulated when cultures were exposed to elevated temperature.

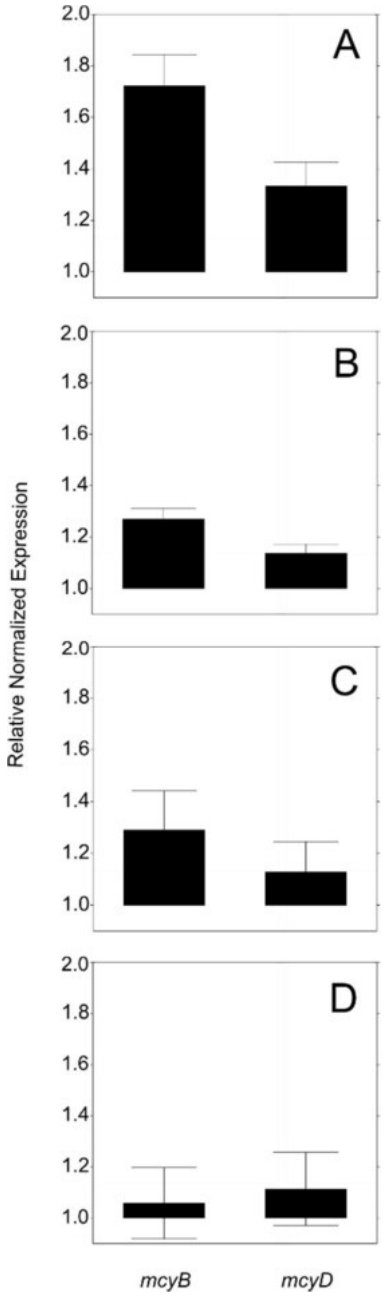

Figure 3.4: Gene expression in response to abiotic environmental variables. Relative quantification of mcyB and mcyD gene expression. Error bars indicate standard error of the mean. (A) Elevated temperature. mcyB expression (left) was elevated 1.72-fold when cultures were exposed to 30 °C compared with mcyB expression in control cultures grown at 20 °C. mcyD expression (right) was elevated 1.33-fold when cultures were exposed to 30 °C compared with mcyD expression in control cultures grown at 20 °C. (B) Mixing. mcyB expression (left) was elevated 1.27-fold when cultures were mixed compared with non-mixed cultures. mcyD expression (right) was elevated 1.14-fold when cultures were mixed compared with mcyB expression in non-mixed cultures. (C) Low amount of microcystin-LR. mcyB gene expression (left) was elevated 1.29-fold when 10 µg/l microcystin-LR was added to the culture. mcyD gene expression (right) was elevated 1.13-fold when 10 µg/l microcystin-LR was added to the cultures. (D) High amount of microcystin-LR. mcyB gene expression (left) was elevated 1.06-fold when 60 µg/l microcystin-LR was added to the culture. mcyD gene expression (right) was elevated 1.11-fold when 60 µg/l microcystin-LR was added to the culture.

Nonetheless, there was a weak positive correlation between the ΔC_q values of *mcyB* and *mcyD* transcripts (Pearson's r = 0.41) under elevated temperature conditions. The mean normalized *mcyD* expression level was found to be 77.46% of the mean normalized *mcyB* expression level. The difference between the two was not statistically significant (t = 2.10; $p = 0.06$).

3.4.3 Effect of mixing

Mixing did not significantly alter microcystin gene expression. Relative quantification of *mcyB* and *mcyD* transcript levels was performed for both conditions, mixed and non-mixed. The non-mixed control was used as a calibrator (Figure 3.4B). For the *mcyB* transcripts, the mean fold change values from control cultures were not significantly different from those of the cultures subjected to mixing (1.00 [.03] versus 1.27 [.04]; t = 1.72, $p = 0.12$). Similarly, for the *mcyD* transcripts, the mean fold change values from the control cultures were not significantly different from those of the cultures subjected to stirring (1.00 [.04] versus 1.14 [0.04]; t = 0.92, $p = 0.38$).

The ΔC_q values for the *mcyB* and *mcyD* transcripts under stirring conditions correlated strongly (Pearson's r = 0.97). The mean normalized *mcyD* expression was 89.46% of the mean normalized *mcyB* expression, but the difference was not significant, (t = 1.04; $p = 0.33$).

3.4.4 Effects of the microcystin-LR concentration

The addition of microcystin-LR did not significantly alter microcystin gene expression. When investigating the effect of adding 10 µg/l microcystin-LR on *mcyB* transcription, we compared the mean fold change values from control cultures with those of the cultures treated with an additional 10 µg/l microcystin-LR. The untreated control was used as calibrator (Figure 3.4C). The difference in gene expression was not significant (1.00 [.12] versus 1.29 [.15]; t = 1.78; $p = 0.09$). Similarly, for the *mcyD* transcripts, the mean fold change values of the control and microcystin-LR-treated cultures were not significantly different (1.00 [.11] versus 1.13 [.12]; t = 0.51, $p = 0.62$) (Figure 3.4C).

When investigating the effect of adding 60 µg/l microcystin-LR on *mcyB* transcription, we compared the mean fold change values from control cultures with those from cultures exposed to 60 µg/l microcystin-LR and found they did not differ significantly (1.00 [.09] versus 1.06 [0.14]; t = 0.24, $p = 0.82$). The untreated control was used as calibrator (Figure 3.4D). The same was true for the *mcyD* transcripts, where the mean fold change values from control cultures

were not significantly different than those from cultures exposed to 60 µg/l microcystin-LR (1.00 [.08] versus 1.11 [.14]; t = 0.36, $p = 0.72$) (Figure 3.4D).

There was a strong positive correlation between the mean ΔC_q values for *mcyB* and *mcyD* transcripts when low (Pearson's r = 0.76) or high (Pearson's r = 0.97) concentrations of microcystin-LR were added. For the low microcystin concentration, the mean normalized *mcyD* expression was found to be 87.37% of the mean normalized *mcyB* expression, and the difference was not statistically significant (t = 0.51; $p = 0.62$). For the high microcystin concentration, the mean normalized *mcyD* expression was found to be 105.30% of the mean normalized *mcyB* expression, but the difference was not statistically significantly (t = 0.22; $p = 0.83$).

3.5 Discussion

Our findings support the hypothesis that elevated temperature leads to increased gene expression of at least one microcystin synthesis gene (*mcyB*). The other hypotheses, presuming a change in microcystin gene expression in response to mixing or additional microcystin, could not be confirmed in this study.

Our results suggest that an increase in microcystin production could possibly be expected with elevated temperatures because we demonstrated that increased temperature led to an upregulation of at least one microcystin synthesis gene. The expression of *mcyB* was significantly upregulated, whereas that of *mcyD* did not change significantly. This shows that a rise in temperature of 10 °C does have an effect on the expression of a microcystin synthesis gene (Figure 3.4A), increasing our understanding of the answer to the question of how gene expression in *M. aeruginosa* reacts to elevated temperature. Our results, however, are contradictory to some findings that describe a decrease in *mcyB* transcripts by competitive RT-PCR when comparing 20 °C to 30 °C (Kim et al., 2005).

Even though some studies suggest otherwise (Helbling et al., 2015), rising global temperatures are thought to favour blooms of toxic algae such as *Microcystis* by affecting several important factors (Paerl and Huisman, 2008; Paerl and Huisman, 2009; Paerl and Paul, 2012). First, many potentially toxic cyanobacterial species have a growth advantage at warmer temperatures (Davis et al., 2009; Elliott et al., 2006; Jöhnk et al., 2008). Second, invasive species from tropical regions, such as the toxic cyanobacterium *Cylindrospermopsis raciborskii*, are able to spread to temperate zones (Wiedner et al., 2007). In this study, we now add a third aspect that needs to be considered in the context of rising global temperatures. Because climate change is

believed to promote mass occurrences of cyanobacteria, even a small increase in toxin gene expression (Figure 3.4A) could amplify the risks posed by toxic cyanobacteria considerably. Even though mixing did not lead to increased microcystin gene expression in this study, additional effects on aquatic ecosystems can be expected due to the effects of climate change on the perturbation patterns of waterbodies. On the one hand, increased vertical mixing of the epilimnion caused by more frequent wind stress has been predicted (Gastineau and Soden, 2009; Helbling et al., 2015). On the other hand, model-based analyses predicted not only an increased water column stability and a warmer epilimnion (Wahl and Peeters, 2014), but also an earlier stratification of waterbodies (Peeters et al., 2007) in response to rising temperatures. Historical trends, furthermore, provide evidence for a shorter annual duration of ice cover of lakes and rivers (Magnuson et al., 2000), a phenomenon connected to earlier onset of phytoplankton blooms (Peeters et al., 2007). Cyanobacteria are well adapted to thrive under those conditions (Huber et al., 2012). The elevated cyanobacteria growth will result in higher risks associated with cyanotoxins because elevated temperatures might simultaneously increase the transcription of microcystin genes.

Relevant studies of gene expression in *Microcystis* are often conducted in bioreactors where the cultures are agitated by stirring or a gas mixing system (Makower et al., 2015). This is why learning how mixing affects microcystin gene expression in *M. aeruginosa* is not only interesting from an ecological point of view but also important for assessing the transferability of laboratory-based results to the field. To our knowledge, this is the first time the effect of mixing on microcystin synthesis gene expression has been explored. Like mixing, the addition of microcystin-LR could not be shown to impact microcystin gene expression. These findings are in agreement with results from Makower et al. (2015), where the authors found a non-significant upregulation of *mcyA* gene when adding 50 µg/l microcystin-LR.

The reason why the cell upregulates microcystin synthesis with rising temperature remains unclear. Recent studies suggest that microcystin might have an intracellular function in coping with oxidative stress (Makower et al., 2015). Zilliges et al. (2011) showed that microcystin stabilizes critical proteins such as RubisCO as well as enzymes of the Calvin cycle by binding covalently to their cysteine residues during the oxidative stress conditions caused by high light or H_2O_2. Although elevated temperature has not been studied in conjunction with the protective function of microcystin, it is easy to envision that the protein-modulating function of microcystin is beneficial at higher temperatures and in conjunction with high light conditions,

like in this study. Such a mechanism could also explain the selective advantage of toxic *Microcystis* genotypes over non-toxic ones at elevated temperatures (Davis et al., 2009).

In this study, we introduced a novel RT-qPCR assay for the relative quantification of microcystin gene expression in *M. aeruginosa*. We established a new reference gene panel consisting of four reference genes: *GAPDH*, *gltA*, *rpoC1* and *rpoD*. To the best of our knowledge, this is the first time this unique combination of reference genes was used to investigate gene expression in *Microcystis*. A reference gene panel should be preferred over a single reference gene because it is more resilient to small fluctuations in expression stability. Ideally, reference genes should have a range of expression similar to that of the target genes. Therefore, the 16S rRNA gene, which was also considered as a reference gene in this study, was found not to be suitable due to its high expression levels (Figure 3.3). This is in agreement with other studies that assessed this gene as a candidate reference gene (Tran et al., 2013). In addition, extremely low C_q values lead to technical difficulties with baseline correction of the amplification curves (data not shown). On the other hand, using the 16S rRNA gene as reference gene worked for others (Kuniyoshi et al., 2013; Ngwa et al., 2014; Pimentel and Giani, 2014; Sevilla et al., 2008). It is good practice to assess the suitability of a reference gene for each new experimental condition or treatment. All four reference genes presented here were found to be suitable reference genes, i.e., they were stably expressed under the conditions tested in this study. Therefore, the reference gene panel presented here is a valuable starting point for finding suitable reference genes for future experimental studies. By offering four reference genes to choose from, there is an increased chance of identifying at least one stable reference gene for other experimental conditions.

Two different target genes, *mcyB* and *mcyD,* were used in this assay. Both are suitable target genes for several reasons. First, they are both essential for the synthesis of microcystin (Dittmann et al., 1997; Nishizawa et al., 2000; Tillett et al., 2000). Second, because they are located on two bi-directionally transcribed operons of the microcystin gene cluster, they represent both of these operons (Tillett et al., 2000). Finally, primer sequences for these genes are available from previous studies (Kurmayer and Kutzenberger, 2003; Sevilla et al., 2008). Although the primers for *mcyD* had been used for gene expression studies before (Kuniyoshi et al., 2013; Pimentel and Giani, 2014; Sevilla et al., 2008; Sevilla et al., 2010), those for *mcyB* had only been used for absolute quantification in qPCR studies (Kurmayer and Kutzenberger, 2003). To the best of our knowledge, the qPCR assay introduced here is the first to target both of these genes at the same time. To measure two target genes from the microcystin gene cluster

gives an idea of the risks associated with certain environmental stressors, potentially even before microcystin is produced. Although most studies target only one gene from the microcystin gene cluster, the two target genes we choose here represent not only the two operons of the microcystin gene cluster, but also target representative non-ribosomal peptide synthase (*mcyB*) and polyketide synthase (*mcyD*) genes.

Both genes were found to be suitable target genes, and their expression correlated well in most experiments. This means that they are largely co-expressed. The co-expression of *mcyB* and *mcyD* is explained by the fact that both genes encode proteins of a common multienzyme complex that performs the non-ribosomal synthesis of microcystin. It is notable that the relative normalized expressions of *mcyB* and *mcyD* differed consistently in our experiments. This is an interesting observation even though the differences were not significant, drawing attention to the fact that the mechanisms regulating microcystin production are still largely unknown. The target genes are on different polycistronically transcribed operons and are, therefore, potentially subjected to differential regulation. The microcystin synthesis genes may be regulated by light-dependent alternate transcription start points like those found upstream of *mcyA* and *mcyD*, or their regulation may be governed by intercistronic promoters (Kaebernick et al., 2002). In addition, putative binding boxes for the ferric uptake regulator Fur (Martin-Luna et al., 2006b) and the global nitrogen regulator NtcA (Ginn et al., 2010) in the microcystin gene cluster suggest transcriptional regulation in response to nutrients. These regulatory mechanisms imply regulation of the microcystin genes on the transcript level, emphasizing the relevance of transcriptional studies.

Although no microcystin measurements were performed in this study, it has been shown that expression of microcystin synthesis genes correlates with levels of microcystin in some studies (Kuniyoshi et al., 2013; Pimentel and Giani, 2014; Sevilla et al., 2008), while other studies could not show this connection (Kaebernick et al., 2000; Ngwa et al., 2014; Sipari et al., 2010; Wood et al., 2011). Microcystin measurements can also be misleading because the lag phase between transcription and actual microcystin formation remains unknown and because protein-bound microcystin might be missed (Zilliges et al., 2011). Although other research on the effects of elevated temperature have mostly concentrated on the presence of potentially toxigenic cyanobacteria (Davis et al., 2009; Dziallas and Grossart, 2011) or the amount of microcystin itself (Dziallas and Grossart, 2011; Kleinteich et al., 2012; Sivonen, 1990; van der Westhuizen and Eloff, 1985), our research bridges the gap between the two by focusing on gene expression.

3.6 Conclusion

For the first time, an RT-qPCR assay was used to explore three environmental factors connected to climate change. The results revealed that increased temperature leads to the upregulation of the microcystin synthesis gene *mcyB*, whereas mixing and the addition of microcystin-LR does not cause increased expression of *mcyB* or *mcyD*. This led us to conclude that future rising temperatures may increase the risks associated with toxic cyanobacteria by influencing microcystin gene expression, whereas changed perturbation patterns and increased microcystin levels might not increase the risks. To make even more substantiated predictions about the effect of climate change on microcystin synthesis gene expression, other factors connected to and caused by global warming need to be explored in future studies.

4 Influence of cyanobacteria, mixotrophic flagellates, and virioplankton size fraction on transcription of microcystin synthesis genes in the toxic cyanobacterium *Microcystis aeruginosa*

A similar version of this chapter was published: Scherer PI, Absmeier C, Urban M, Raeder U, Geist J, Zwirglmaier K. 2017. Influence of cyanobacteria, mixotrophic flagellates, and virioplankton size fraction on transcription of microcystin synthesis genes in the toxic cyanobacterium *Microcystis aeruginosa*. MicrobiologyOpen: e538.

4.1 Abstract

Toxic cyanobacteria such as *Microcystis aeruginosa* are a worldwide concern in freshwater reservoirs. Problems associated with their mass occurrence are predicted to increase in the future due to global warming. The hepatotoxic secondary metabolite microcystin is of particular concern in this context. This study aimed to determine whether co-occurring microorganisms influence the expression of microcystin biosynthesis genes. To this end, we performed co-cultivation experiments and measured *mcyB* and *mcyD* transcripts in *M. aeruginosa* using RT-qPCR. We utilized representatives from three different plankton groups: the picocyanobacterium *Synechococcus elongatus*, the unicellular flagellate grazer *Ochromonas danica*, and virioplankton from two different lakes. The presence of *S. elongatus* significantly increased *mcyB* and *mcyD* transcription in *M. aeruginosa*. Co-cultivation with the mixotrophic chrysophyte *O. danica* did not increase the transcription of *mcyB* and *mcyD*; in fact, *mcyD* transcripts decreased significantly. The virioplankton size fraction of environmental water samples induced a significant increase in *mcyB* and *mcyD* transcription when obtained from lakes with cyanobacterial blooms. Our results show that co-occurring microorganisms influence the expression of microcystin biosynthesis genes in *M. aeruginosa*.

4.2 Introduction

Many strains of *Microcystis aeruginosa* and other cyanobacteria produce the toxic secondary metabolite microcystin, which inhibits protein phosphatases in eukaryotes (MacKintosh et al., 1990) and is thus toxic to humans and animals. Therefore, microcystin is a growing concern in

freshwater management worldwide (Moreira et al., 2014; Sivonen and Jones, 1999; Van Gremberghe et al., 2011).

The adverse effects of microcystin on aquatic organisms have been studied intensely. Particular attention has been given to mussels (Juhel et al., 2006), fish (Hou et al., 2016; Liu et al., 2015; Xie et al., 2015), members of the zooplankton community such as cladocerans (Dao et al., 2010; DeMott et al., 1991; Fulton and Paerl, 1987b; Herrera et al., 2015; Ortiz-Rodríguez et al., 2012; Wiegand et al., 2002), and aquatic macrophytes (Mitrovic et al., 2005; Pflugmacher, 2002; Pflugmacher, 2004; Yin et al., 2005). However, much less is known about the inverse relationship, i.e., the effect that co-occurring organisms might have on microcystin producers such as *Microcystis* sp. One of the few studies to address this question found an increase in McyB protein in *Microcystis* sp. caused by the presence of the dinoflagellate *Peridinium gatunense* (Vardi et al., 2002). The little data available on *M. aeruginosa* toxin gene expression are limited to co-cultivation with *Daphnia magna,* a metazoan grazer, which was observed to cause overexpression of *mcyA* in *M. aeruginosa* (Pineda-Mendoza et al., 2014). In addition, colony formation as a response to grazing danger has been observed but is not restricted to toxic strains (Fulton and Paerl, 1987a; Yang et al., 2006).

The fate of *M. aeruginosa* cells in the environment is affected by co-occurring microorganisms in several ways: Other phototrophs compete with *M. aeruginosa* for similar resources. At the same time, *M. aeruginosa* may be subject to and cause of interactions by means of secondary metabolites. Furthermore, *M. aeruginosa* cells are grazed on by zooplankton or utilized as a host by intracellular parasites such as bacteriophages, which can cause lysis of the *M. aeruginosa* cell.

In aquatic environments, several strains and species of cyanobacteria generally co-occur (Al-Tebrineh et al., 2012; Glowacka et al., 2011; Via-Ordorika et al., 2004; Zwart et al., 2005), and studies suggest that microcystin producers may have an advantage in the competition for micronutrients such as iron (Lukač and Aegerter, 1993; Utkilen and Gjølme, 1995). Interaction of cyanobacteria with other members of the microbial community by means of secondary metabolites has been described and is an ongoing and expanding field of study (Kaplan et al., 2012; Kaplan et al., 2016). The picocyanobacterium *Synechococcus elongatus* is of particular interest in this context because it is almost ubiquitously distributed (Stockner et al., 2002). The effect of *Synechococcus* on *Microcystis* gene expression is of ecological relevance since those cyanobacteria do not only co-occur (Fortin et al., 2015; Kolmonen et al., 2004; Magana-Arachchi et al., 2011; Ouellette et al., 2006; Zwart et al., 2005) but also can constitute the two

most dominant cyanobacteria species in the phytoplankton community (Berry et al., 2016; Feng et al., 2016; Teneva et al., 2010; Ye et al., 2011).

Cyanobacteria serve as prey for unicellular zooplankton. For instance, protists such as the mixotrophic flagellate *Ochromonas* sp. have been shown to feed on *Microcystis* sp. in the laboratory (Wilken et al., 2010). Furthermore, environmental studies have revealed that *Ochromonas* spp. co-occur with *Microcystis* spp. in natural habitats (Van Donk et al., 2009) and that *Ochromonas* spp. are among the most widespread and abundant bacterivores in aquatic environments (Arndt et al., 2000). For these reasons Wilken et al. (2014) evaluated *Ochromonas* as a tool to control *Microcystis* blooms.

Besides being grazed on by protists, lysis by bacteriophages is a major mortality factor for cyanobacteria (Proctor and Fuhrman, 1990). Viruses are the most abundant biological entities in both marine and freshwater ecosystems (Bergh et al., 1989; Wommack and Colwell, 2000). Laboratory and field studies suggest that phages regulate bloom dynamics of *Microcystis* sp. (Manage et al., 1999; Tucker and Pollard, 2005; Yoshida et al., 2008). Cyanophages that infect *M. aeruginosa* have been isolated in earlier studies and have been discussed as a tool for biological control of toxic cyanobacteria (Phlips et al., 1990; Yoshida et al., 2006).

The biological function of microcystin is still under investigation and while its role in the survival of the *Microcystis* cell is not yet fully understood, there have been several foci of research over the years: Microcystin, a peptide toxic to many animals, was suspected to act as a defence against grazers such as *Daphnia* (Kurmayer and Jüttner, 1999; Pineda-Mendoza et al., 2014). Other studies, however, provide phylogenetic evidence (Rantala et al., 2004) as well as proteomic and physiological data (Zilliges et al., 2011) that suggest that a defence against metazoan grazers is not microcystin's primary function. Nevertheless, those grazers might have an effect on the expression of microcystin biosynthesis genes (Pineda-Mendoza et al., 2014). In addition, some doubt has been cast on microcystin as a primary defence mechanism against unicellular grazers such as mixotrophic flagellates (Wilken et al., 2010), albeit the situation is less clear here. Instead, more recent studies have focused increasingly on the intracellular and regulatory functions of microcystin within *Microcystis* cells (Makower et al., 2015; Meissner et al., 2013; Zilliges et al., 2011).

However, the discoveries that microcystin has multiple intracellular effects and that defence against metazoan grazers is not its primary role, do not rule out the possibility that microcystin regulation might be affected by secondary metabolites of other microbial community members

(viruses, protists, or other cyanobacteria). This is suggested by observations that toxins may increase in the presence of other microorganisms (Vardi et al., 2002). For this reason, it is of the utmost importance to understand how interaction between members of the microsphere and toxigenic cyanobacteria affects their expression of toxicity genes. In addition, any biological agent considered for use in the control of harmful algal blooms should first be scrutinized for its potential effects on toxin gene expression to prevent exacerbation of the problem.

The effect of co-occurring microorganisms on toxin gene expression in *Microcystis* sp. is not well understood. The aim of this study was to determine whether transcription of the microcystin biosynthesis genes *mcyB* and *mcyD* is altered by the presence of different types of microorganisms. We co-cultivated *M. aeruginosa* with representative protists, cyanobacteria, or the virioplankton size fraction of environmental water samples from two different lakes and assessed the transcription of *M. aeruginosa* microcystin biosynthesis genes.

4.3 Material and methods

4.3.1 Sampling and preparation of environmental samples

Water samples for experiments with the virioplankton size fraction were collected from Lake Klostersee and Lake Bergknappweiher, two Bavarian lakes frequently presenting with cyanobacterial blooms. Lake Klostersee (GPS coordinates 48.08, 11.96) is a polytrophic artificial lake with a maximum water depth of 2.5 m, located 545 m above sea level in Ebersberg, Germany (Gesundheitsamt Landratsamt Ebersberg, 2014). Lake Bergknappweiher (GPS coordinates 47.85, 11.23) is a small meso-eutrophic lake with a maximum water depth of 2.5 m, located 617 m above sea level and about 50 km south-west of Munich, Germany (Teubner et al., 2004). Water samples (4 L) were taken on 25 Jun 2015 from Lake Klostersee and on 27 Jul 2015 and 27 Aug 2015 from Lake Bergknappweiher. Samples were collected from surface water near the shore, and bacterioplankton and zooplankton were removed by filtering the water through a 0.2-µm pore-size cellulose nitrate filter (Sartorius, Göttingen, Germany). The filtrate containing the virioplankton and other particles smaller than 0.2 µm as well as dissolved organic matter is referred to as 0.2 µm filtrate in this study. Subsequently, cross-flow filtration was performed to concentrate the particles present in the 0.2 µm filtrate (Vivaflow50, 10,000 MWCO, Sartorius, Göttingen, Germany). We obtained 100 mL cross-flow filtrate from each litre of 0.2 µm filtrate. This ten-fold concentration of particles larger than 10 kDa is referred to as particle concentrate. The particle concentrate contains all substances present in the 0.2 µm filtrate and is enriched in particles such as viral particles. For

some experiments 0.2 µm filtrate was inactivated by means of autoclaving (121°C, 20 min) and is referred to as autoclaved filtrate.

4.3.2 Strains and cultivation techniques

The microcystin-producing cyanobacterium *M. aeruginosa* SAG14.85 and the mixotrophic flagellate *Ochromonas danica* SAG933-7 were obtained from the Culture Collection of Algae at Göttingen University in Germany (SAG). *Ochromonas* was selected for being a phytoplankton grazer. The picocyanobacterium *S. elongatus* PCC7942 was obtained from the Pasteur Culture Collection of Cyanobacteria at the Institut Pasteur in Paris, France (PCC). *Synechococcus* was selected due to its almost ubiquitous distribution and its ability to compete for similar resources as *Microcystis* (Stockner et al., 2002).

All three strains were cultured at 25 °C and under a light-dark regime of 14 h light and 10 h dark. Cool daylight with an intensity of 100 µmol/s/m^2 was provided by MASTER TL5 HO 39W/865 1SL fluorescent light tubes (Phillips, Amsterdam, Netherlands). Cultures were regularly checked for contamination via microscopy (LEICA DM R, Leica Microsystems, Wetzlar, Germany).

M. aeruginosa and *S. elongatus* cells were grown separately for 10±2 days in 100 mL volumes of BG-11 medium (Rippka et al., 1979) supplemented with 0.5 mM ammonium chloride in 300 mL borosilicate Erlenmeyer flasks. Both strains grew unicellular and were pre-cultured under the same experimental conditions. Optical density at 730 nm (OD_{730}) was measured with a spectrophotometer (model 150-20, HITACHI, Chiyoda, Japan) to monitor cyanobacterial growth. Growth rates for *M. aeruginosa* and *S. elongatus* without treatment were 0.15 d^{-1} and 0.13 d^{-1}, respectively. Spent medium from *S. elongatus* cell cultures was obtained by filtering cultures through a 0.2-µm pore-size cellulose nitrate filter (Sartorius, Göttingen, Germany) to remove any cells from the medium.

O. danica cells were grown in 50 mL Ochromonas medium (Röderer, 1986) in 100 mL borosilicate Erlenmeyer flasks. *O. danica* was grown for five days to a concentration of $1.5\pm0.5\times10^6$ cells/mL. *O. danica* growth was monitored by counting cells immobilized with 1 % (v/v) glycerine in a Neubauer improved counting chamber (Paul-Marienfeld GmbH & Co. KG, Lauda Königshofen, Germany) under a microscope (LEICA DM R, Leica Microsystems, Wetzlar, Germany). *O. danica* cells grew unicellular and were pre-cultured under the same conditions.

For each experimental condition, three replicates of *M. aeruginosa* culture were grown to the early exponential phase (OD_{730} of 0.5±0.1). At this point, equal volumes of either control medium, autoclaved filtrate, 0.2 µm filtrate, particle concentrate, *S. elongatus* (grown to OD_{730} of 0.15±0.01), or *S. elongatus* spent medium were added, or *O. danica* was added at the desired concentrations (10^3 or 10^4 cells/mL) to the *Microcystis* culture and incubated for 48 hours before harvesting.

4.3.3 Plaque assay

Plaque assay was performed as described by (Millard, 2009) with modifications to accommodate bacteriophages from a freshwater environment. In short, concentrated log phase *M. aeruginosa* cells were mixed with 0.2 µm filtrate and grown on 1% (w/v) agar plates with 0.4% (w/v) top-agar. Plates were prepared with washed agar-agar (Carl Roth, Karlsruhe, Germany) and BG-11 medium (Rippka et al., 1979) supplemented with 0.5 mM ammonium chloride.

4.3.4 Humic acid treatment

To mimic the effect of the humic acids present in Lake Bergknappweiher water under laboratory conditions, artificial humic acid extract (HuminFit, Dohse Aquaristik GmbH & Co. KG, Grafschaft, Germany) was used to obtain a medium of visual similar colour to the Lake Bergknappweiher water. To this end, 80 µL artificial humic acid extract was added to 200 mL of *M. aeruginosa* culture (four times the manufacturer's recommended concentration). To test a 2.5-times higher humic acid concentration, 200 µL artificial humic acid extract (ten times the manufacturer's recommended concentration) was added to 200 mL of *M. aeruginosa* culture.

4.3.5 Harvesting and RNA extraction

Harvesting of cell cultures and RNA extraction were carried out as described previously (Scherer et al., 2017). Briefly, 20 mL liquid culture was filtered through a 0.2-µm pore-size cellulose nitrate filter (Sartorius Göttingen, Germany). RNA from the filter was extracted as described in Penn et al. (2014), cleaned using the RNA Clean & Concentrator kit (Zymo Research, Irvine, USA), and quantified and quality-checked using a NanoVue Plus spectrophotometer (GE healthcare, Little Chalfont, UK) and 1 % agarose gel stained with GelRed (Biotium, Hayward, USA), respectively.

4.3.6 RT-qPCR

Relative quantification of transcripts by reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) and subsequent data analyses were performed as described previously (Scherer et al., 2017). Two target genes, *mcyB* and *mcyD*, representatives of the microcystin synthetase gene cluster, and four reference genes (*rpoC1*, *gltA*, *rpoD* and *GAPDH*) were used in this assay. Suitability of the four reference genes as reference gene panel for each experimental condition was confirmed with geNorm (M-values < 0.5). For primers and cycling conditions refer to Scherer et al. (2017). Normalized expression was determined as described in (Vandesompele et al., 2002). In order to determine relative normalized expression, expression level of the control samples is set to one.

4.3.7 Statistical analysis

To test for statistically significant changes in gene expression, fold-change values were tested for normal distribution (Shapiro–Wilk Test). Subsequently, the data were analysed using one-way ANOVA with the level of significance defined as $p \leq 0.05$. If applicable, Tukey's post hoc test was performed. All statistical analyses were performed using PAST v3.10 software (Hammer et al., 2001).

4.4 Results and discussion

4.4.1 Response to cyanobacteria

Co-cultivation of *M. aeruginosa* with the picocyanobacterium *S. elongatus* or spent medium from *S. elongatus* cultures resulted in increased transcription of microcystin biosynthesis genes in *M. aeruginosa* (Figure 4.1). Transcription of *mcyB* significantly increased in the presence of spent medium (*p*, 0.003) or cells of *S. elongatus* (*p*, 0.001) compared to the control treatment. For *mcyD* transcripts, a significant increase was found for cultures exposed to *S. elongatus* cells (*p*, 0.025) but not cultures exposed to mere spent medium of *S. elongatus* cells (*p*, 0.064). These results indicate that microcystin biosynthesis genes in *M. aeruginosa* might be upregulated by a mechanism involving the direct presence of *S. elongatus* or, to a lesser extent, the presence of soluble compounds released into the medium by this cyanobacterium. Close communication of cyanobacteria with other members of the biosphere has been observed in several previous studies (Briand et al., 2016; Kaplan et al., 2012; Kaplan et al., 2016). One possible means of interaction between co-occurring microorganisms is through small molecules. *S. elongatus*, for instance, has been shown to release a large variety of such small molecules into its surroundings

(Fiore et al., 2015). However, little is known about how these molecules influence the expression of toxicity genes. The identification and characterization of the secondary metabolites or other chemicals that mediate the interaction between *S. elongatus* and *M. aeruginosa* are avenues for future research.

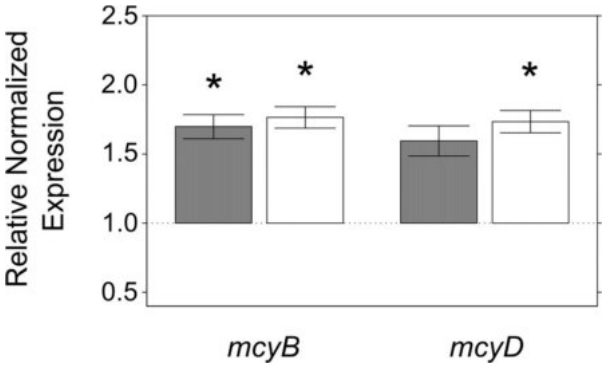

*Figure 4.1: Gene expression in response to S. elongatus. Relative normalized mcyB (left) and mcyD (right) mRNA expression in M. aeruginosa. Error bars show standard error of the mean. * statistically significant difference from control treatment, $p \leq 0.05$. Co-cultivation of M. aeruginosa with cell-free spent medium from S. elongatus culture (grey) or S. elongatus cells in spent medium (white) for 48 hrs. The control treatment, BG-11 medium, was used for normalization.*

A possible allelopathic role of microcystin against *S. elongatus* would explain the observed upregulation of *mcyB* and *mcyD* in the presence of *S. elongatus*. Negative allelopathic effects of microcystin on picocyanobacteria have been described previously by Phelan and Downing (2014) and Hu et al. (2004). In our study, however, growth curves failed to reveal any effect of spent medium from *M. aeruginosa* cultures on the growth of *S. elongatus* cultures (data not shown).

4.4.2 Response to mixotrophic flagellates

Co-cultivation of *M. aeruginosa* with *O. danica* did not increase the transcription levels of microcystin biosynthesis genes. In the contrary, *mcyD* transcription significantly decreased in the presence of *O. danica* (Figure 4.2). For *mcyD* transcripts, the mean fold-change values decreased significantly for the cultures with 10^4 cells/mL *O. danica* (p, 0.049) but not for 10^3 cells/mL *O. danica* (p, 0.078). Transcription of *mcyB* was also decreased, but the difference was not significant (Appendix, Table 11.1). These results show that the eukaryotic grazer *O. danica* does not induce the upregulation of microcystin biosynthesis genes but does even cause downregulation of relevant genes at the concentrations tested here. Therefore, the hypothesis that microcystin constitutes an inducible defence mechanism against unicellular grazers was

not confirmed. Our results rather support the findings of others who found no evidence that microcystin acts as a defence against mixotrophic flagellates (Wilken et al., 2010). The possible role of microcystin as an infochemical has been discussed (Schatz et al., 2007), and using microcystin as an intercellular signal in the event of grazing pressure could potentially benefit the *M. aeruginosa* population. In light of this discussion, our findings are all the more surprising. However, we cannot exclude the possibility that another molecule serves such a signalling function.

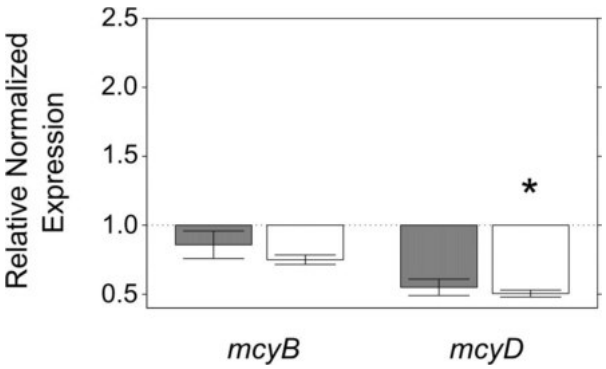

*Figure 4.2: Gene expression in response to O. danica. Relative normalized mcyB (left) and mcyD (right) mRNA expression in M. aeruginosa. Error bars show standard error of the mean. * statistically significant difference from control treatment, $p \leq 0.05$. M. aeruginosa co-cultivated with 10^3 cells/mL (grey) or 10^4 cells/mL (white) O. danica for 48 hrs. The control treatment, Ochromonas medium, was used for normalization. O. danica concentrations chosen were 10^3 or 10^4 cells/mL, as used in previous studies (Guo and Song, 2010; Van Donk et al., 2009).*

In our study, *O. danica* did react to the presence of *M. aeruginosa* with a behavioural change like the one described by Pfandl et al. (2004). When no prey *M. aeruginosa* was present, the chrysophyte remained highly motile, moving around rapidly and frequently.[3] In the presence of *M. aeruginosa* cells, however, *O. danica* was mostly attached to the substratum and created a current with its flagella to capture prey bacteria.[3] The pivotal assumption that *O. danica* SAG933-7 does prey on *M. aeruginosa* SAG14.85 was verified by microscopic observation of engulfed *M. aeruginosa* cells (Figure 4.3), which is in agreement with previous studies (Van Donk et al., 2009; Wilken et al., 2010).

[3] Micrographic videos of this behaviour can be accessed in the supporting information section at http://onlinelibrary.wiley.com/doi/10.1002/mbo3.538/abstract.

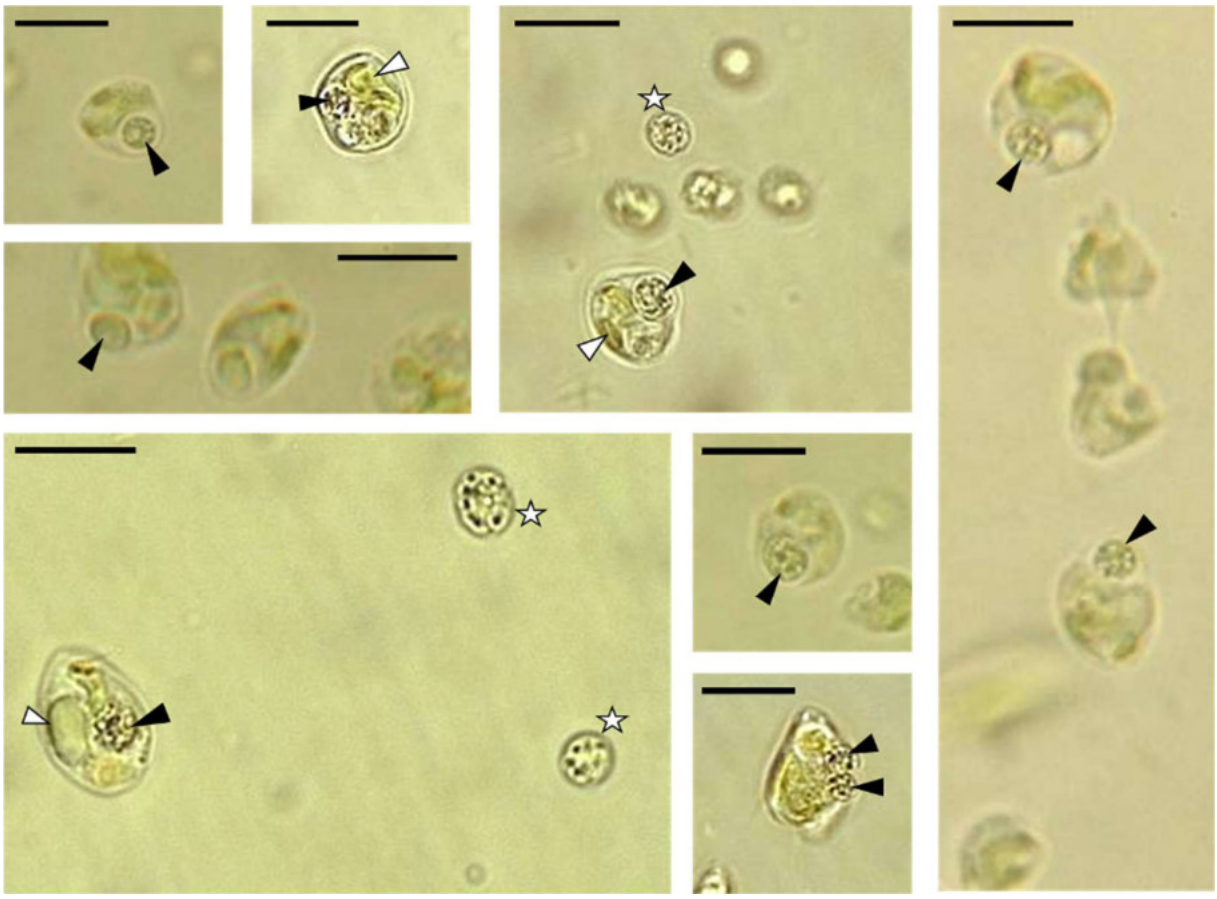

Figure 4.3: *O. danica feeding on M. aeruginosa. Black arrowheads indicate engulfed M. aeruginosa cell within O. danica cell. White arrowheads indicate clearly distinguishable O. danica chloroplast. Stars indicate M. aeruginosa cells that are not engulfed. Bar = 10 µm.*

Flagellates are thought to be responsible for more bacterial mortality than phages (Bettarel et al., 2003). This, and the fact that we did not observe an increase in toxin biosynthesis gene transcription in the presence of *O. danica*, suggest that the use of hetero- or mixotrophic flagellates is an interesting avenue to explore in the pursuit of controlling toxic cyanobacterial blooms.

4.4.3 Effects of the virioplankton size fraction

4.4.3.1 Effects of the virioplankton size fraction from Lake Bergknappweiher before and during algal bloom

Cultivation with 0.2 µm filtrate or particle concentrate from non-blooming Lake Bergknappweiher did not increase the transcription of *mcyB* or *mcyD* significantly (Figure 4.4A and Appendix, Table 11.1).

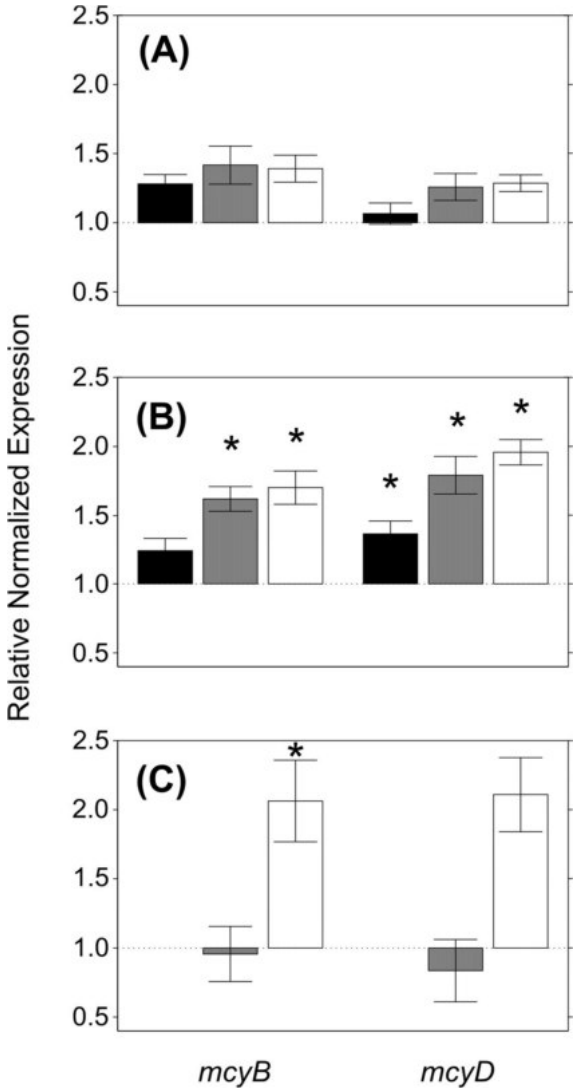

*Figure 4.4: Gene expression in response to virioplankton size fraction. Relative normalized mcyB (left) and mcyD (right) mRNA expression in M. aeruginosa. Error bars show standard error of the mean. * statistically significant difference from control treatment, $p \leq 0.05$. The control treatment, BG-11 medium, was used for normalization. (A+B) Co-cultivation of M. aeruginosa with autoclaved filtrate (black), 0.2 µm filtrate (grey), and particle concentrate (white) from Lake Bergknappweiher sampled before (A) and during (B) a cyanobacteria bloom for 48 hrs. (C) Co-cultivation of M. aeruginosa with 0.2 µm filtrate (grey) and particle concentrate (white) from Lake Klostersee sampled during a cyanobacteria bloom for 48 hrs.*

However, 0.2 µm filtrate and particle concentrate from Lake Bergknappweiher sampled during an algal bloom did cause significant upregulation of microcystin biosynthesis genes in *M. aeruginosa* (Figure 4.4B). The cyanobacterial bloom was characterized by macroscopically visible phytoplankton aggregates on the water surface and a mass development of *Microcystis*-like and *Dolichospermum*-like cells. The *mcyB* mean fold-change values of *M. aeruginosa* cultures exposed to 0.2 µm filtrate (*p*, 0.003) or particle concentrate (*p*, 0.002) differed significantly from that of controls. However, there was no significant change in cultures

exposed to autoclaved filtrate (*p*, 0.240). Similarly, a statistically significant increase in *mcyD* transcripts of *M. aeruginosa* cultures exposed to 0.2 µm filtrate ($p < 0.001$) or particle concentrate ($p < 0.001$) was observed. For *mcyD*, even autoclaved filtrate caused a statistically significant rise in transcription (*p*, 0.020). These results show that *mcyB* and *mcyD* mRNA increased in *M. aeruginosa* co-cultivated with the virioplankton size fraction from Lake Bergknappweiher water taken during an algal bloom. The different results for 0.2 µm filtrate from blooming and non-blooming water likely reflect higher concentrations of the effective components in the bloom samples (Figure 4.5 A+B).

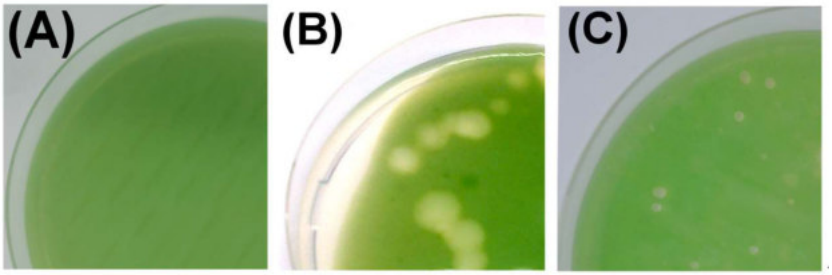

Figure 4.5: Plaque assay with M. aeruginosa lawn. Water samples were collected on (A) 27 Jul 2015 from Lake Bergknappweiher when no cyanobacteria bloom was present, (B) 27 Aug 2015 from Lake Bergknappweiher with a cyanobacteria bloom present, and (C) on 25 Jun 2015 from Lake Klostersee with cyanobacteria bloom present. (B+C) Plaques on cyanobacteria lawn indicate presence of bacteriophages capable of infecting and lysing M. aeruginosa.

The presence of humic acid in Lake Bergknappweiher water was taken into account in a separate experiment. The change in watercolour due to the presence of humic acid, at two different concentrations, did not alter the transcription of microcystin biosynthesis genes significantly (Figure 4.6). The results show that the humic acid preparation tested in this study does not influence the transcription of selected toxicity genes and suggest that the observed changes in gene transcription after treatment with 0.2 µm filtrate and particle concentrate are not caused by humic acid in the water. Whether the chemical composition of the humic acid in Lake Bergknappweiher water influenced results, is less clear and needs to be subject of further study.

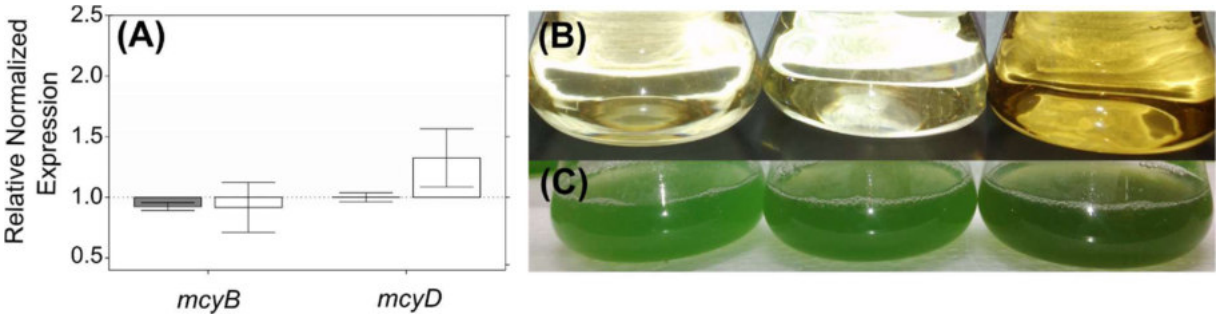

Figure 4.6: Gene expression in response to humic acid. (A) Relative normalized mcyB (left) and mcyD (right) expression in M. aeruginosa. Error bars show standard error of the mean. Co-cultivation of M. aeruginosa with humic acid preparation similar to that from Lake Bergknappweiher (grey) or a 2.5-times more concentrated humic acid preparation (white). The control treatment, BG-11 medium, was used for normalization. Neither the humic acid preparation similar to the one in Lake Bergknappweiher nor the 2.5-times more concentrated humic acid preparation caused a significant mean fold-change in mcyB (p, 0.455) and mcyD (p, 0.058) expression compared to the control treatment. (B) From left to right: BG-11, humic acid preparation similar to that from Lake Bergknappweiher, and a 2.5-times more concentrated humic acid preparation. (C) From left to right: M. aeruginosa cultures mixed with BG-11, humic acid preparation similar to that from Lake Bergknappweiher, and a 2.5-times more concentrated humic acid preparation.

4.4.3.2 Effects of the virioplankton size fraction from Lake Klostersee

Particle concentrate but not 0.2 µm filtrate from Lake Klostersee sampled during an algal bloom increased the transcription of microcystin biosynthesis genes (Figure 4.4C). The cyanobacterial bloom was characterized macroscopically by visible phytoplankton aggregates on the water surface and a mass development of *Microcystis*-like cells. Mean *mcyB* fold-change values of *M. aeruginosa* cultures exposed to particle concentrate differed significantly from those exposed to 0.2 µm filtrate and control cultures (*p*, 0.031). Mean *mcyD* fold-change values in cultures exposed to particle concentrate differed significantly from those exposed to 0.2 µm filtrate (*p*, 0.049) but not control cultures (*p*, 0.064). These results show that microcystin synthetase gene transcription in *M. aeruginosa* increased when the cyanobacterium came into contact with small molecules and concentrated particles from water taken during an algal bloom in Lake Klostersee.

4.4.3.3 Effective components in the virioplankton size fraction

There are several possible explanations for the observed effects caused by the virioplankton size fraction from environmental samples. One potential explanation is the presence of cyanophages in the environmental samples. We could demonstrate the presence of plaque forming units capable to infect *M. aeruginosa* in 0.2 µm filtrate from algal blooms (Figure 4.5B+C), and we found no such plaque forming units in the non-bloom water sample from Lake

Bergknappweiher (Figure 4.5A). This presence of cyanophages is also reflected in a transcript upregulation when co-cultivating *M. aeruginosa* with the virioplankton size fraction from bloom samples (Figure 4.4B+C). A further cue suggesting cyanophages as causative agents, is the fact that the particle concentrate caused a higher increase in gene transcription than the 0.2 µm filtrate (Figure 4.4B+C). Viral particles are concentrated in the process of obtaining the particle concentrate unlike other small soluble compounds. Co-occurring cyanophages might cause increased microcystin expression as defence against parasites. As microcystin is mostly located inside the cell (Park et al., 1998; Young et al., 2005), a defence function against intracellular pathogens such as bacteriophages is plausible. Microcystin and other secondary metabolites have been shown to reduce the virulence of parasitic fungi to the cyanobacterium *Planktothrix* (Rohrlack et al., 2013). The observation that autoclaved filtrate increased *mcyD* transcription in some cases (Figure 4.4B), seems to contradict this proposition. However, inactivated virus particles have been shown to cause infection-independent transcriptional responses in the host cell (Huipao et al., 2017).

In addition, infochemicals or secondary metabolites produced by competing species may account for the observed effects on gene transcription, especially when considering the virioplankton size fraction from Lake Bergknappweiher. Such molecules are most likely present in larger amounts during algal blooms, which explains the difference in effects caused by the bloom and non-bloom virioplankton size fraction (Figure 4.4A+B). Additionally, the fact that autoclaved filtrate from an algal bloom caused an increase in *mcyD* transcripts (Figure 4.4B) points towards small heat stable metabolites as causative agent rather than heat sensitive bacteriophages. This explanation is strongly supported by the results reported here for the co-cultivation of *M. aeruginosa* with spent medium from *S. elongatus* where soluble compounds caused an upregulation of *mcyB* transcripts (Figure 4.1).

Regardless of whether cyanophages or metabolites from other bacteria or a combination of both cause the observed effects on toxin gene transcription, our results show that those mechanisms are probably relevant not only for laboratory but also field conditions.

4.5 Conclusion

For the first time, the effect of a set of different microorganisms and the virioplankton size fraction from environmental water samples on *M. aeruginosa* was determined in a gene expression study. This study reveals the evident effects that co-occurring microorganisms and the virioplankton size fraction have on the transcription of the microcystin biosynthesis genes

mcyB and *mcyD* under laboratory conditions. While the virioplankton size fraction or the picocyanobacterium *S. elongatus* resulted in increased *mcyB* and *mcyD* transcription, the presence of the mixotrophic gazer *O. danica* resulted in decreased or unchanged transcription levels of those genes. Based on our observations, we do not deem cyanobacteria suitable tools for the biological control of toxic cyanobacterial algal blooms. Our findings highlight the complexity of interspecies interactions on the molecular level, and more research is needed to identify the key biological drivers in the expression of microcystin biosynthesis genes and microcystin synthesis. This study can serve as a starting point for future considerations in research and bioremediation.

5 Temporal dynamics of the microbial community composition with a focus on toxic cyanobacteria and toxin presence during harmful algal blooms in two South German lakes

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5.1 Abstract

Bacterioplankton plays an essential role in aquatic ecosystems, and cyanobacteria are an influential part of the microbiome in many water bodies. In freshwaters used for recreational activities or drinking water, toxic cyanobacteria cause concerns due to the risk of intoxication with cyanotoxins, such as microcystins. In this study, we aimed to unmask relationships between toxicity, cyanobacterial community composition, and environmental factors. At the same time, we assessed the correlation of a genetic marker with microcystin concentration and aimed to identify the main microcystin producer. We used Illumina MiSeq sequencing to study the bacterioplankton in two recreational lakes in South Germany. We quantified a microcystin biosynthesis gene (*mcyB*) using qPCR and linked this information with microcystin concentration to assess toxicity. Microcystin biosynthesis gene (*mcyE*)-clone libraries were used to determine the origin of microcystin biosynthesis genes. Bloom toxicity did not alter the bacterial community composition, which was highly dynamic at the lowest taxonomic level for some phyla such as Cyanobacteria. At the OTU level, we found distinctly different degrees of temporal variation between major bacteria phyla. Cyanobacteria and Bacteroidetes showed drastic temporal changes in their community compositions, while the composition of Actinobacteria remained rather stable in both lakes. The bacterial community composition of Alpha- and Beta-proteobacteria remained stable over time in Lake Klostersee, but it showed temporal variations in Lake Bergknappweiher. The presence of potential microcystin degraders and potential algicidal bacteria amongst prevalent Bacteroidetes and Alphaproteobacteria implied a role of those co-occurring heterotrophic bacteria in cyanobacterial bloom dynamics.

Comparison of both lakes studied revealed a large shared microbiome, which was shaped towards the lake specific community composition by environmental factors. Microcystin variants detected were microcystin-LR, -RR, and -YR. The maximum microcystin concentrations measured was 6.7 µg/L, a value still acceptable for recreational waters but not drinking water. Microcystin concentration correlated positively with total phosphorus and *mcyB* copy number. We identified low abundant *Microcystis* sp. as the only microcystin producer in both lakes. Therefore, risk assessment efforts need to take into account the fact that non-dominant species may cause toxicity of the blooms observed.

5.2 Introduction

Cyanobacteria are of special interest in aquatic microbiology. They occur alongside other prokaryotes in aquatic environments and the extent of the interspecies interaction with those bacteria is only just emerging (Lee et al., 2016; Li et al., 2011; Li et al., 2012b; Parulekar et al., 2017). In particular, cyanobacteria capable of producing the hepatotoxic secondary metabolite microcystin cause problems in drinking and recreational water reservoirs (He et al., 2016; Hudnell, 2010; Roegner et al., 2014). For this reason, identification of potentially microcystin-producing species by microscopy and microcystin analyses is commonly used to assess risks associated with toxic cyanobacteria (Chorus and Bartram, 1999; Gaget et al., 2017; Kaushik and Balasubramanian, 2013; Sangolkar et al., 2006).

Deducing the toxicity of an algal bloom based on the cyanobacterial community composition can be difficult because microcystins are potentially produced by different species (Chorus and Bartram, 1999). To address this problem, quantitative polymerase chain reaction (qPCR) assays have been introduced to quantify toxic genotypes (Al-Tebrineh et al., 2012; Martins et al., 2011; Ostermaier and Kurmayer, 2010).

When assessing the microbial diversity in water bodies, culture-independent techniques are commonly used. Molecular approaches outperform microscopy when estimating the diversity of heterotrophic prokaryotes and are increasingly applied to evaluate cyanobacterial community composition. The most widespread molecular methods for the study of aquatic microbiomes are denaturing gradient gel electrophoresis (DGGE) (Li et al., 2012b; Shi et al., 2011), terminal restriction fragment length polymorphism (T-RFLP) (Chen et al., 2010; Li et al., 2011), Sanger sequencing of clone libraries (Cai and Kong, 2013; Chen et al., 2010; Li et al., 2012b), and more recently high-throughput sequencing (Berry et al., 2017; Fortin et al., 2015; Kurilkina et al., 2016; Lee et al., 2016; Zwirglmaier et al., 2015).

So far only a few studies have combined high-throughput sequencing-based assessment of cyanobacterial community composition with toxic genotype quantification by qPCR (Fortin et al., 2015; Lee et al., 2016), and even fewer included microcystin measurements (Fortin et al., 2015). These research efforts focused on water bodies in North America (Berry et al., 2017; Fortin et al., 2015; Lee et al., 2016), and Norwegian lakes (Parulekar et al., 2017).

This study aimed to explore possible correlations between microcystin occurrence and factors such as environmental parameters, cyanobacterial community composition, or genetic markers. Specifically, we hypothesized a positive correlation between microcystin synthesis gene copy number and microcystin concentration. Furthermore, we aimed to identify the main microcystin producer in Lake Klostersee and Lake Bergknappweiher.

Here we present the first assessment of the bacteria and cyanobacteria community in two South German lakes during toxic cyanobacterial blooms with Illumina sequencing of the 16S-rRNA gene. Gene quantification by qPCR, phylogenetic identification of the main microcystin producer through sequencing of clone libraries of one microcystin synthesis gene region, and two different microcystin measurement methods underpin the information about community composition.

5.3 Material and methods

5.3.1 Sampling

5.3.1.1 Sampling area

Both lakes used in this study are known for recurrent cyanobacterial blooms and used for recreational purposes (Figure 5.1). Lake Klostersee (GPS coordinates: 48.08, 11.96) is an artificial eutrophic lake with a maximum water depth of 2.5 m (Gesundheitsamt Landratsamt Ebersberg, 2014; Teubner et al., 2004). It is located 545 m above sea level in Ebersberg, Germany, ca 20 km south-east of Munich. Lake Klostersee was sampled in a biweekly rhythm from May to October 2015. Lake Bergknappweiher (GPS coordinates: 47.85, 11.23) is a small eutrophic and dystrophic lake with a maximum water depth of 2.5 m (Teubner et al., 2004). This lake is located 617 m above sea level about 30 km south-west of Munich, Germany. Lake Bergknappweiher was sampled in a weekly rhythm from August to October 2015.

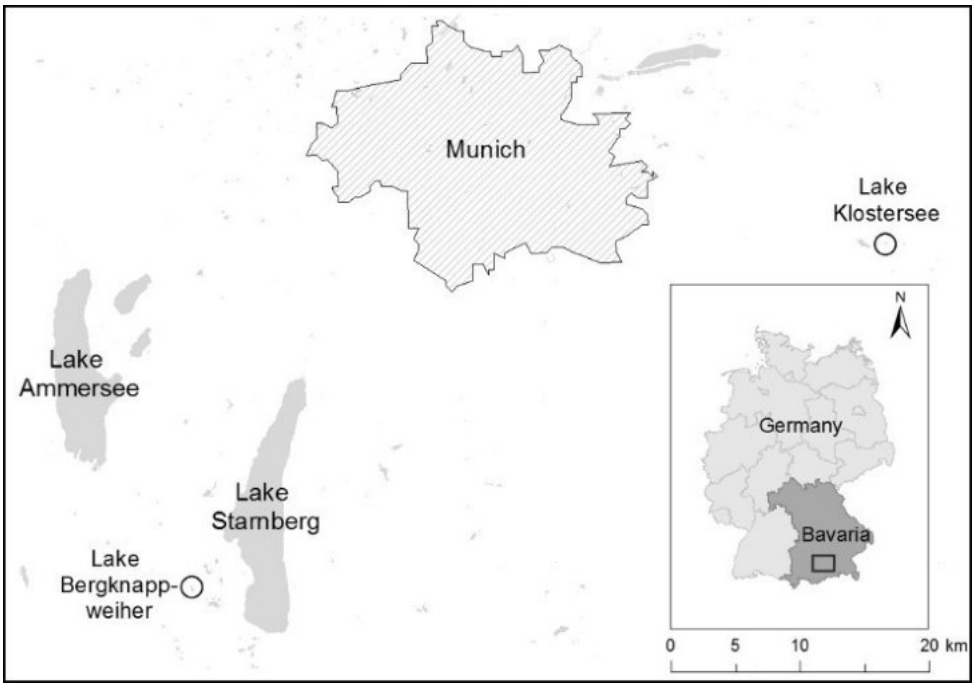

Figure 5.1: Geographic location of Lake Klostersee and Lake Bergknappweiher.

5.3.1.2 Sampling for DNA, water chemistry, and microcystin analysis

Surface water samples (1 L) were taken from one near shore sampling point at Lake Klostersee and Lake Bergknappweiher using a 2 L beaker on a pole. From the water sample, 0.5 L was dedicated for DNA analysis, 100 mL was dedicated for microcystin measurements, and 400 mL was dedicated for water chemistry measurements. Water samples were chilled on ice and transported to the laboratory within 2 hrs, where they were processed immediately (DNA, water chemistry) or frozen at -20 °C until sample preparation and extraction or measurement commenced.

5.3.2 Water chemistry

Ammonium was measured using an ion chromatograph (Dionex ICS-1100, Thermo Scientific, Waltham, US) and analysed using the Chromeleon Software Version 7.2.1.5833 (Thermo Scientific, Waltham, US). Phosphorus and NO_3-nitrogen were measured spectrophotometrically (model 150-20; HITACHI, Chiyoda, Japan). Total phosphorus (TP) was measured according to the German standard methods for the examination of water, wastewater, and sludge (DEV, 2013). NO_3-nitrogen was measured according to Navone (1964). The quotient of inorganic nitrogen and phosphorus ($N_{inorg.}$/P) was calculated from measured ammonium, NO_3-nitrogen, and TP.

5.3.3 Water physics

The following physical water parameters were determined using a multi-parameter probe Multi 350i (WTW, Weilheim, Germany): pH, temperature, oxygen concentration, and conductivity. Secchi depth was measured to the nearest 5 cm using a Secchi disk.

5.3.4 Microcystin measurements with ELISA

5.3.4.1 Sample preparation and extraction

Sample preparation was performed according to the manufacturer's instructions of a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Beacon Analytical Systems, Portland, US). Duplicate water samples (5 mL each) were subjected to three freeze-thaw cycles to lyse cells and release microcystins into solution (Zaffiro et al., 2016). This was followed by filtration of the 5 mL water sample using an Acrodisc LC 25 mm syringe filter, 0.45 µm PVDF (Sigma-Aldrich, St. Louis, US). The filtrate was stored at 4-8 °C for a maximum of 12 hrs.

5.3.4.2 Measurement

Total microcystin (particulate and dissolved microcystin measured together) was measured immunologically in duplicates using a commercially available ELISA kit (Beacon Analytical Systems, Portland, US). Samples (50 µL) were subjected to the microcystin ELISA kit according to manufacturer's instructions. The limit of detection was 1 µg/L and the upper limit of quantification was 20 µg/L.

5.3.5 Microcystin measurement with HPLC

5.3.5.1 Sample preparation and extraction

Water samples (90 mL) were subjected to three freeze-thaw cycles, and after centrifugation at 20,000 ×*g* for 10 min samples were filtered through a 47 mm diameter glass fibre filter with 1.2 µm pore size (Whatman, Maidstone, UK). Microcystins and nodularin in the filtrate were concentrated by solid phase extraction using Oasis MAX 6 cc cartridges (150 mg, 60 µm particle size, Waters Corporation, Milfort, US). The filtrate from water samples was applied after conditioning the cartridge with 4 mL methanol and 6 mL water. The cartridge was washed with a 4 mL mixture of methanol-water (5:1, v/v) and microcystin was eluted from the cartridge with 4 mL methanol containing 2 % formic acid. The resulting eluent was evaporated by a gentle stream of nitrogen at 40 °C and the residue was re-dissolved in 500 µL methanol-water (5:1, v/v). Immediately before chromatographic analysis, the solution was membrane filtered using Acrodisc LC 25 mm syringe filters, 0.45 µm PVDF (Sigma-Aldrich, St. Louis, US).

5.3.5.2 Measurement

Individual total microcystins and nodularin (particulate and dissolved microcystins and nodularin measured together) were measured together by high-performance liquid chromatography equipped with photodiode array detector (HPLC-PDA). According to Spoof et al. (2010), a sub-3 µm particle-based reversed-phase column was used for chromatography and the mobile phase gradient was optimized for separation of twelve microcystins and nodularin-R (Figure 5.2). HPLC was performed with a Summit® HPLC system equipped with a photo diode array detector (Dionex, Germany). Individual microcystins and nodularin were separated on a 3.0 mm i.d. × 100 mm Kinetex® C18 column (100 Å pore size, 2.6 mm particle size) equipped with a 4 mm i.d. C18 securityguard™ cartridge (Phenomenex, Torrance, USA). The column temperature was set at 40 °C. Solvent A consisted of 0.5 mL trifluoroacetic acid and 250 mL methanol adjusted to a final volume of 1,000 mL with water, and solvent B was acetonitrile with 0.05 % trifluoroacetic acid. The gradient elution program started with 20 % B and increased to 40 % B in 13 min, then to 63 % B in 5 min and subsequently to 100 % B in 3 min at a flow rate of 0.5 mL/min. The injection volume was 20 µL and microcystins (MC) were detected at 238 nm. [D-Asp3]-MC-RR, MC-RR, MC-YR, MC-HtyR, MC-LR, [D-Asp3]-MC-LR, MC-WR, MC-HilR, MC-LA, MC-LY, MC-LW, MC-LF and nodularin-R were obtained from Enzo Life Sciences (Germany) and solutions in methanol-water (5:1, v/v) (0.5-2.5 µg/mL) were used for identification and quantification. The recovery was determined by spiking tap water with microcystins (2.5 µg/L). Recoveries were between 64 ± 3 % for MC-WR and 123 ± 4 % for nodularin-R (mean ± standard deviation, n = 5). The limit of detection for each microcystin variant was 1 µg/L.

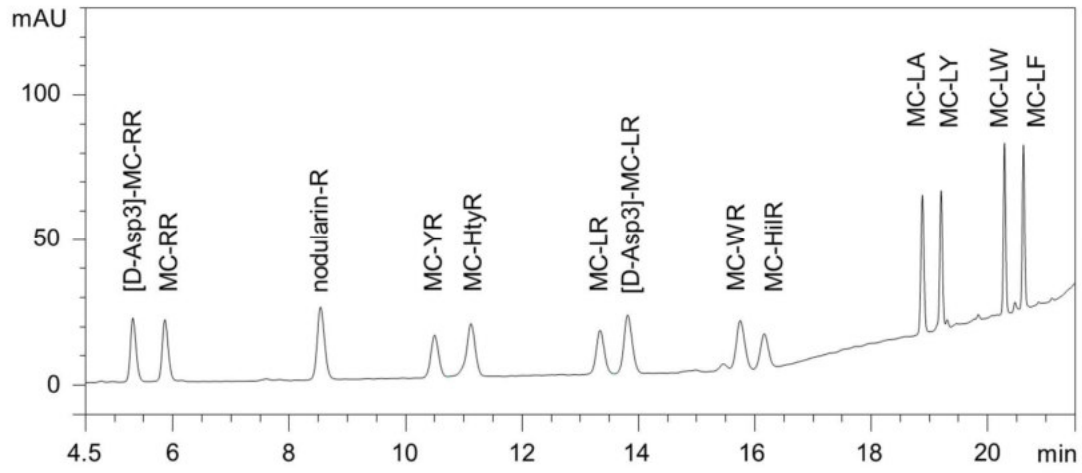

Figure 5.2: Separation of microcystins and nodularin-R by HPLC-PDA (standard solution, 2.5 µg/mL of each toxin, detection at 238 nm).

5.3.6 DNA isolation

A volume of 0.25-0.5 L of lake water was filtered through a 0.2 µm cellulose nitrate filter (Sartorius, Göttingen, Germany) to concentrate cellular organisms on the filter. Filters were stored at -20 °C prior to DNA isolation. DNA isolation was performed using a phenol-chloroform based method, which was modified from Fuller et al. (2003). Briefly, 2 mL of lysis buffer (0.75 M sucrose, 0.4 M NaCl, 50 mM Tris, 20 mM EDTA, pH 9.0) and 20 µL lysozyme (50 mg/mL) were added to the filter and incubated while rotating for 30 min at 37 °C, followed by heating to 55 °C for 10 min. After addition of 2 mL phenol/chloroform/isoamylalcohol (25:24:1 v/v/v) samples were spun at 3,310 ×*g* for 5 min at 4 °C. Subsequently, the upper phase was separated and mixed with 2 mL of chloroform and spun at 4,000 ×*g* for 5 min at 4 °C. DNA was then precipitated with two volumes of ethanol and 0.1 volume of sodium-acetate 3 M, pH 5.2 for a minimum of 1 h at -20 °C. The samples were centrifuged for 45 min at 4,000 ×*g* at 4 °C, and the resulting pellet was washed with 0.5 mL 80 % ethanol. DNA was re-suspended in 50-100 µL Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and quantified with a NanoVue Plus spectrophotometer (GE Healthcare, Little Chalfont, UK). The 260 nm/280 nm ratios of DNA samples ranged from 1.68 to 2.11. Isolated DNA was aliquoted and stored at -20 °C until further analysis.

5.3.7 qPCR

To quantify the *mcyB* gene copies of the genus *Microcystis* and the *mcyE* gene copies of the genus *Dolichospermum*, quantitative polymerase chain reaction (qPCR) was performed. *Planktothrix mcy* genes were not quantified because of the absence of this genus from our Illumina Miseq sequencing data set.

5.3.7.1 *Microcystis mcyB* quantification

For targeting the *mcyB* gene of *Microcystis* spp., the primers 30F and 108R and a 5'-FAM labelled hydrolysis probe was used (Table 5.1) (Kurmayer and Kutzenberger, 2003). Cycling conditions for this reaction were as follows: 10 min, 95 °C; 45 cycles of 15 sec, 95 °C and 1 min 60 °C. A seven-point standard curve was created using five-fold serial dilutions of genomic DNA from *Microcystis aeruginosa* SAG14.85. Calculations were made assuming a genome size for *M. aeruginosa* of 5,842,795 bp as published for *M. aeruginosa* NIES-843 (NC_010296.1) and a single *mcyB* copy per genome. The qPCR amplification and analysis were performed with a magnetic induction cycler and the micPCRv2.4.0 software (Bio Molecular Systems, Sydney, Australia). We used 25-µL reactions with 0.9 µM primers,

0.25 µM hydrolysis probe, and Taqman Master Mix (Bioron, Ludwigshafen, Germany). All standard curve dilutions and samples were analysed in triplicates. All environmental DNA samples were diluted one in twenty in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Raw data from qPCR runs had to fulfil the following criteria before gene copy quantification commenced: Samples had to be in the dynamic range of the standard curve. The C_q values of triplicates had to be no more than 0.5 cycles apart.

5.3.7.2 *Dolichospermum mcyE* quantification

For targeting the *mcyE* gene of *Dolichospermum* spp., the primers 611F and 737R were used (Table 5.1) (Sipari et al., 2010). Cycling conditions for this reaction were as follows: 3 min, 98 °C; 15 sec, 95 °C; 50 sec, 64.7 °C; 40 cycles, followed by a melting curve analysis of 95 °C for 10 min and subsequently a ramp from 65 °C to 95 °C in 0.5 °C increments. A six-point standard curve was created using five-fold serial dilutions of genomic DNA from *Dolichospermum lemmermannii* NIVA-CYA270/1. Calculations were made assuming a genome size of 5,305,670 bp as published for *Dolichospermum sp.* 90 (NC_019427.1), and a single *mcyE* copy per genome. The qPCR amplification and analysis were performed with a BioRad CFX96 cycler and the BioRad CFX Manager software (BioRad, Hercules, USA). We used 20-µL reactions with 0.2 µM primers and SsoAdvanced™ Universal SYBR® Green Supermix (BioRad, Hercules, USA). The qPCR assay was optimized for annealing and extension temperature and time, initial denaturation temperature and time, and primer concentration. All standard curve dilutions and samples were analysed in triplicates. All environmental DNA samples were diluted one in twenty in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Raw data from qPCR runs had to fulfil the following criteria before gene copy quantification commenced: Samples had to be in the dynamic range of the standard curve. The C_q values of triplicates had to be no more than 0.5 cycles apart. Melting curve peaks of samples had to show a single peak at the same temperature and of the same shape as the melting curve peaks of the standard curve dilutions.

Table 5.1: Information about primers and probes used in this study. Overhanging adapter of primers are shown in bold.

Target gene	Primer name	Sequence 5' to 3'	Use	Approx. product size (bp)	Source
mcyE (*Microcystis*, *Dolichospermum*, and *Planktothrix*)	mcyE-F2 mcyE-R4	GAAATTTGTGTAGAAGGTGC AATTCTAAAGCCCAAAGACG	clone library	809	(Rantala et al., 2006)
mcyB (*Microcystis*)	30F 108R *mcyB* probe	CCTACCGAGCGCTTGGG GAAAATCCCCTAAAGATTCCTGAGT CACCAAAGAAACACCCGAATCTGAGAGG	qPCR	102	(Kurmayer and Kutzenberger, 2003)
mcyE (*Dolichospermum*)	611F 737R	CTAGAGTAGTCACTCACGTC GGTTCTTGATAGTTAGATTGAGC	qPCR	148	(Sipari et al., 2010)
16S-rRNA	Bakt_341F Bakt_805R	**TCGTCGGCAGCGTCAGATGTGTATAAG** **AGACAG**CCTACGGGNGGCWGCAG **GTCTCGTGGGCTCGGAGATGTGTATAA** **GAGACAG**GACTACHVGGGTATCTAATCC	Illumina amplicon PCR	498	Modified from (Herlemann et al., 2011)

5.3.8 Clone library and Sanger sequencing

A clone library of the environmental ca. 809 bp *mcyE* gene fragment was prepared as follows. The initial PCR reaction was performed with the primer pair *mcy*E-F2/*mcy*E-R4 described in Rantala et al. (2006) (see Table 5.1). A 50-µL reaction volume was used with 2.5 U DreamTaq DNA polymerase and DreamTaq buffer (10x) (Fisher Scientific, Schwerte, Germany), 0.75 mM $MgCl_2$, 0.2 µM of each primer, 0.15 mM dNTP (Fisher Scientific, Schwerte, Germany), and 0.1 mg/mL BSA (Fisher Scientific, Schwerte, Germany). PCR was performed using a BioRad T100 thermal cycler and the following program: 5 min, 95 °C; 15 sec, 95 °C; 30 sec, 56 °C; 50 sec, 72 °C; 35 cycles. The presence and correct size of PCR products were confirmed on a 1.8 % agarose gel. The clone library was prepared using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, US) according to the manufacturer's instructions. Sanger sequencing services were provided by LGC Genomics (Berlin, Germany). Sequences were quality checked, trimmed, and aligned. Individual and consensus sequences were added to a neighbour-joining tree of published *mcyE* sequences using Geneious version 7.1.5 (Kearse et al., 2012). Sequence data were submitted to GenBank (accessions numbers: MF947220 - MF947378).

5.3.9 Illumina sequencing

Material for Illumina sequencing was obtained as described above in the sections on sampling and DNA isolation. An Illumina MiSeq library was prepared according to the Illumina 16S Metagenomic Sequencing Library Preparation guide (Part # 15044223 Rev. B). In short, a metagenomics-sequencing library was prepared using universal bacterial primers with an overhanging adapter targeting the V3 and V4 region of the bacterial 16S-rRNA gene (see Table 5.1). The cycling conditions of the amplicon PCR were: 3 min, 95 °C; 30 sec, 95 °C; 30 sec, 55 °C; 30 sec, 72 °C; 25 cycles; 5 min, 72 °C. PCR products were purified with Agencourt® AMPure® CP (Beckman Coulter, Krefeld, Germany) magnetic beads according to the manufacturer's instructions. Index PCR was performed as per the dual indexing principle in the Nextera XT Index Kit (Illumina, San Diego, US) and the following PCR conditions: 3 min, 95 °C; 30 sec, 95 °C; 30 sec, 55 °C; 30 sec, 72 °C; 8 cycles; 5 min, 72 °C. For both PCR reactions, the proof reading polymerase Accuzyme Taq (Bioline, Cambridge, UK) was used in a 50-µL reaction volume, and the presence and correct size of PCR products was confirmed on a 1.8 % agarose gel. Index PCR products were purified with Agencourt® AMPure® CP (Beckman Coulter, Krefeld, Germany) magnetic beads according to the manufacturer's instructions. Samples were quantified using Qubit (Thermo Fisher Scientific, Waltham, US), pooled, and normalized to a 4 nM concentration. Sequencing was performed bi-directionally using the

Illumina MiSeq v3 2x300 paired-end sequencing. Sequence analysis was performed with Usearch version 7 (Edgar, 2010). Sequences were trimmed to 400 bp length and low-quality reads were removed by setting the truncqual parameter to 3. De-replication of files and the removal of singletons were carried out. Operational taxonomic unit (OTU) clustering was carried out at the level of 97 % similarity and chimeras were removed afterwards. OTU numbering was done with python. OTUs were classified using greengenes (DeSantis et al., 2006) via the SINA aligner and classification tool (www.arb-silva.de) (Pruesse et al., 2007). The sequence data was not normalized to avoid loss of information (McMurdie and Holmes, 2014). For rarefaction curves and sequence numbers see Figure 5.3 and Table 5.2. The sequence data were submitted to the European Nucleotide Archive (PRJEB21009). The universal bacteria primers used in this study also amplified the chloroplast 16S-rRNA gene sequence of phototrophic eukaryotes due to the cyanobacterial origin of chloroplasts (Sagan, 1967; Schwartz and Dayhoff, 1978). The number of eukaryotic chloroplast reads could not be directly linked to eukaryotic algal relative abundance because eukaryotic algae have a varying number of chloroplasts and chloroplasts have a varying number of genome copies. We identified chloroplasts down to the lowest taxonomic level possible, which was at the taxonomic order level. A classification down to lower taxonomic level was only possible in a few cases (data not shown).

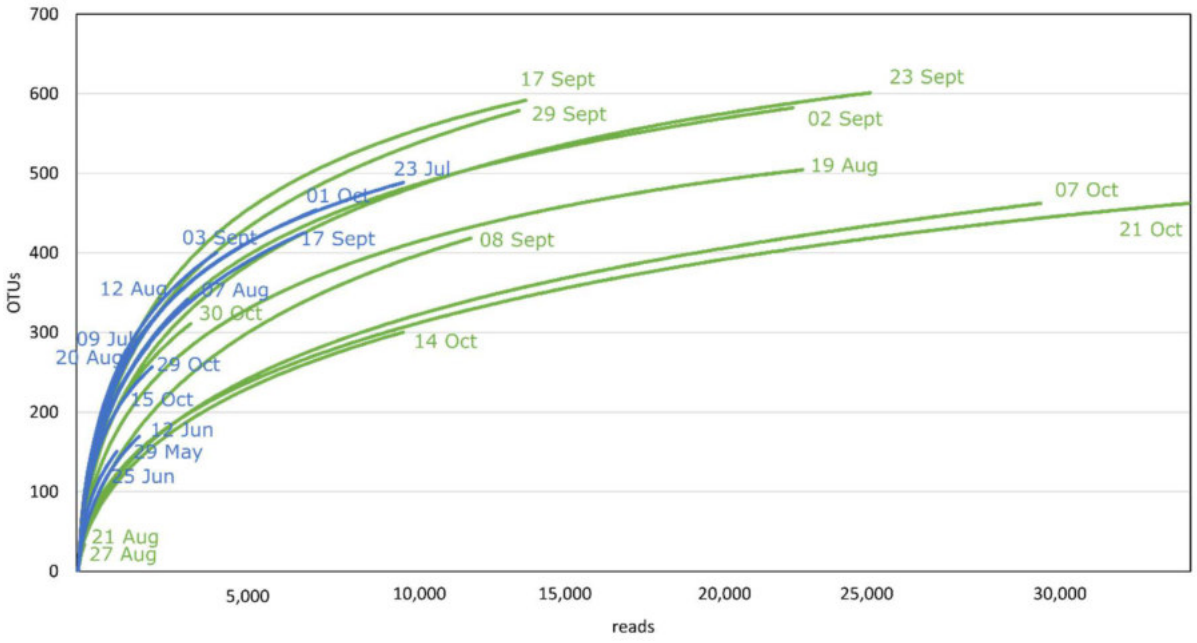

Figure 5.3: Rarefaction curves for each Illumina sequencing sample. Curves and sampling dates of Lake Klostersee samples in blue and Lake Bergknappweiher in green.

Table 5.2: Number of Illumina sequences after quality control.

Lake	Sampling time point	Bacteria sequences	Cyanobacteria sequences	Chloroplast sequences (% of total)
Lake Klostersee	29 May 15	1,255	381	54
	12 Jun 15	1,976	529	70
	25 Jun 15	432	56	80
	09 Jul 15	2,235	154	119
	23 Jul 15	9,071	1,392	1,167
	07 Aug 15	3,322	230	227
	12 Aug 15	2,182	245	222
	20 Aug 15	1,597	362	54
	03 Sept 15	4,211	450	220
	17 Sept 15	5,721	782	1,446
	01 Oct 15	6,775	356	813
	15 Oct 15	883	219	207
	29 Oct 15	1,853	346	592
Lake Bergknappweiher	19 Aug 15	21,975	8,151	648
	21 Aug 15	351	259	1
	27 Aug 15	36	15	2
	02 Sept 15	21,523	9,495	798
	08 Sept 15	12,300	8,333	36
	17 Sept 15	12,681	6,066	1,364
	23 Sept 15	23,377	12,801	1,404
	29 Sept 15	12,656	5,238	1,161
	07 Oct 15	28,402	15,809	1,650
	14 Oct 15	9,125	5,349	1,119
	21 Oct 15	29,945	14,453	4,722
	30 Oct 15	2,517	338	1,177

5.3.10 Statistical analysis

To assess coverage in the amplicon datasets, rarefaction curves were plotted. Pearson's correlation coefficient was utilized to shed light on correlations between *mcyB* gene abundance, microcystin concentrations, environmental factors, bacterial communities, and cyanobacterial taxa. All statistical analyses were done with Past v3.06 (Hammer et al., 2001).

5.4 Results

5.4.1 Microbial community composition

5.4.1.1 Total bacterial community composition (BCC) at the phylum level

The overall composition of bacterial phyla in Lake Klostersee was rather stable over the sampling period. The most abundant phyla throughout the sampling period were Proteobacteria, Actinobacteria, and Cyanobacteria (Figure 5.4A). The sample from 12 Jun 2015 with high abundance of Verrucomicrobia and the sample from 29 May 2015 with high abundances of Bacteroidetes formed exceptions.

Like in Lake Klostersee, in Lake Bergknappweiher the overall composition of bacterial phyla was relatively stable over the sampling period (Figure 5.4B). Exceptions were the samples from 21 Aug and 30 Oct 2015 with high and low relative abundances of Cyanobacteria, respectively. The single most abundant phylum in Lake Bergknappweiher throughout the sampling period was Cyanobacteria with an exception at the end of the sampling period on 30 Oct 2015 when Bacteroidetes and Proteobacteria were more abundant than Cyanobacteria. The second most abundant phylum in this lake was Proteobacteria.

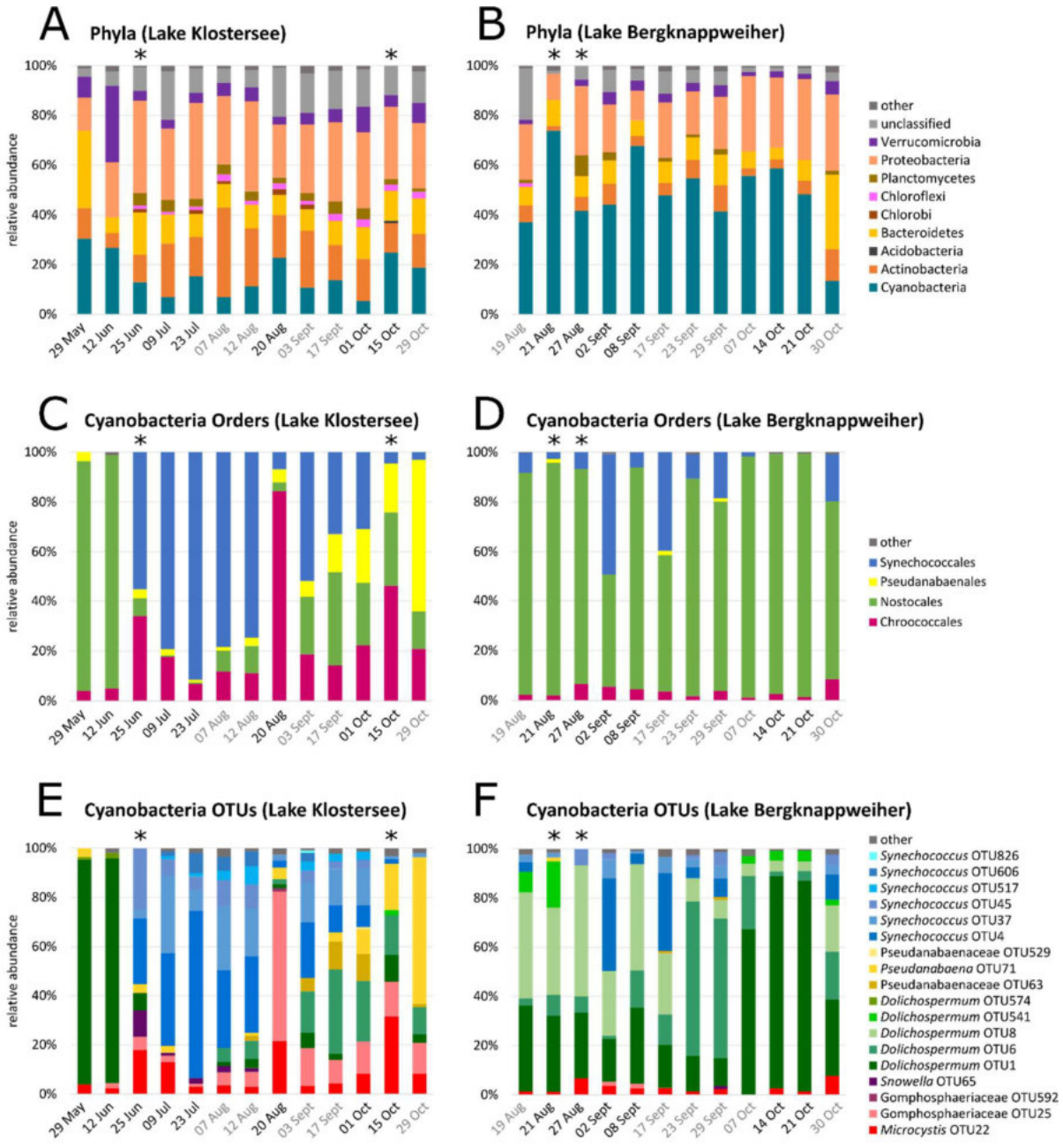

Figure 5.4: Bacterioplankton community composition in Lake Klostersee (left) and Lake Bergknappweiher (right) (relative abundances < 1 % shown as "other"). Dates in black indicate VSBs. Dates in grey indicate absence of VSBs. Asterisks indicate < 1,000 sequences. (A+B): Percent relative abundances of bacterial taxa at the phylum level. (C+D): Percent relative abundances of cyanobacterial taxa at the order level. (E+F): Percent relative abundances of cyanobacterial taxa at the level of OTUs (97 % sequence identity).

5.4.1.2 Cyanobacterial community composition

The community composition of Cyanobacteria in Lake Klostersee changed considerably over the sampling period (Figure 5.4C+E). We observed a major shift from Nostocales, which were dominated by a single *Dolichospermum* OTU, at the beginning of the sampling period to a dominance of Synechococcales, which were represented by up to six *Synechococcus* OTUs, from late June to mid-August. A sudden peak of Chroococcales followed this on 20 Aug 2015. After that, we observed a more balanced distribution of the main cyanobacterial orders with ever decreasing relative abundances of Synechococcales and increasing relative abundances of Pseudanabaenales.

In Lake Bergknappweiher, Cyanobacteria made up the largest fraction of the total bacterial community. Mean relative abundance of Cyanobacteria in Lake Bergknappweiher was more than three times higher than in Lake Klostersee. The community composition of Cyanobacteria in Lake Bergknappweiher changed during the sampling period, in particular at the OTU level (Figure 5.4D+F). Nostocales, which were represented by up to five *Dolichospermum* OTUs, were very dominant throughout the entire sampling period. In mid-October, a single *Dolichospermum* OTU dominated the Nostocales population. Peaks in omnipresent Synechococcales were evident beginning- and mid-September. *Microcystis* was present in every sample.

5.4.1.3 Community composition of main heterotrophic phyla

OTU level analysis revealed that Actinobacteria in Lake Klostersee and Lake Bergknappweiher displayed a similar community composition, which remained stable over the sampling period (Figure 5.5A+B). Three members of the order Actinomycetales (OTU9, 20, and 13) and one Acidimicrobiales (OTU12) dominated the Actinobacteria community. All four OTUs showed a significant negative correlation with the cyanobacterial genus *Dolichospermum* and a positive correlation with the genus *Synechococcus* (Appendix, Table 11.2).

In contrast to this, the many members of the highly diverse phylum Bacteroidetes showed a highly dynamic community composition (Figure 5.5C+D). OTU23, a member of the family Saprospiraceae correlated positively with *Microcystis* and negatively with the genus *Dolichospermum* (Appendix, Table 11.2).

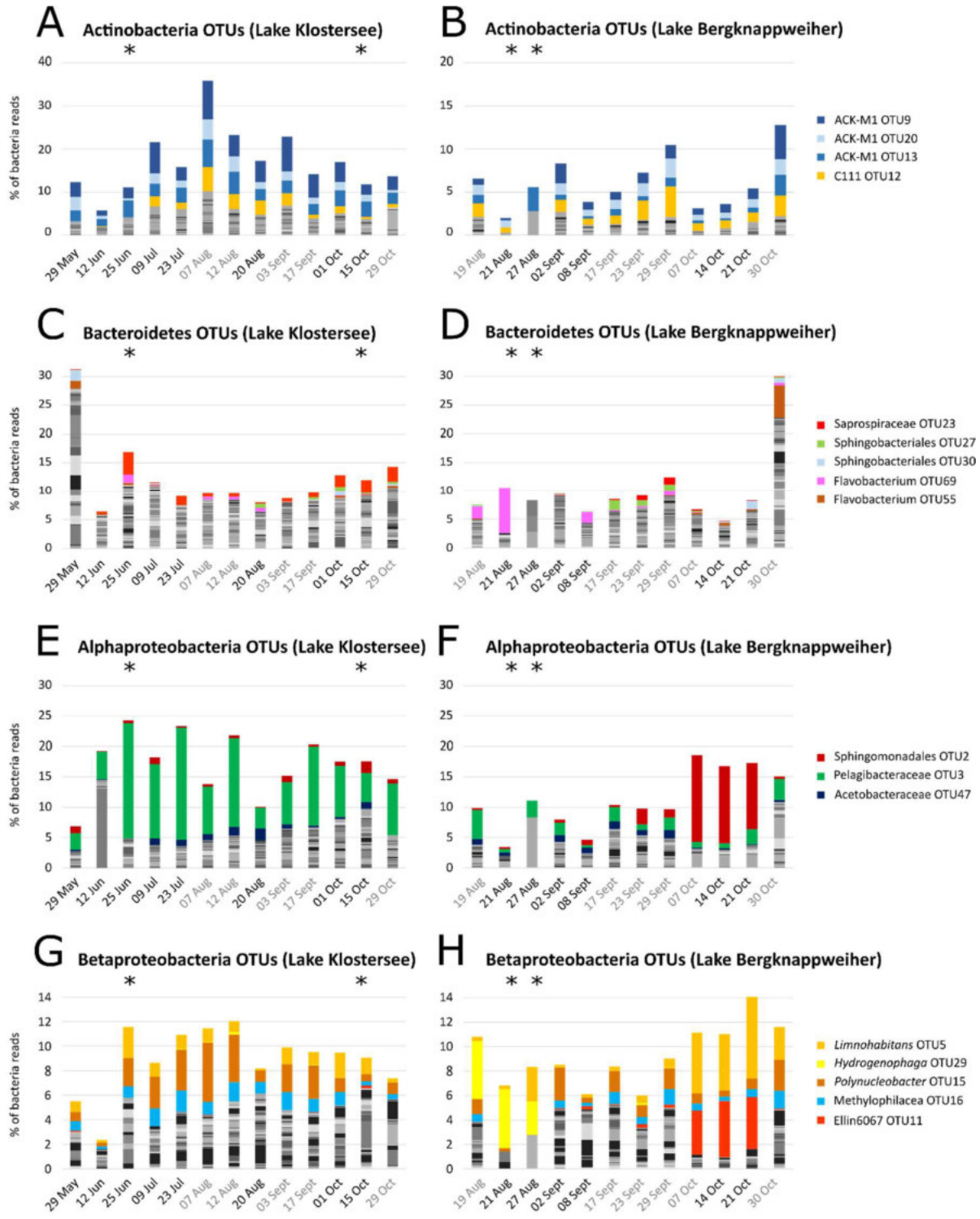

Figure 5.5: Non-cyanobacteria community composition in Lake Klostersee (left) and Lake Bergknappweiher (right) at the OTU level (97 % sequence identity). Dates in black indicate VSBs. Dates in grey indicate absence of VSBs. (A+B): Actinobacteria (C+D): Bacteroidetes. (E+F): Alphaproteobacteria (G+H): Betaproteobacteria (note different y-axis).

Proteobacteria showed distinctively different patterns in the two lakes. In Lake Klostersee, Alpha- and Betaproteobacteria formed communities, which were stable over the sampling period (Figure 5.5E+G). By contrast, in Lake Bergknappweiher those classes showed considerable temporal variability in their community composition (Figure 5.5F+H). The dominant OTU in the Alphaproteobacteria community was a member of the order Sphingomonadales, OTU2, and a member of the family Pelagibacteraceae, OTU3. The first correlated positively with *Dolichospermum* (especially OTU1 in Lake Bergknappweiher) and the latter correlated positively with *Synechococcus* and negatively with *Dolichospermum* (Appendix, Table 11.2). The dominant Betaproteobacteria were three different OTUs from the family Comamonadaceae (the genus *Limnohabitans*, *Hydrogenophaga*, and *Polynucleobacter*) and a member of the family Methylophilaceae and the order Ellin60607 (almost absent in Lake Klostersee). *Limnohabitans* OTU5 and Ellin60607 OTU11 correlated positively with *Dolichospermum* OTU1 (Appendix, Table 11.2). None of the dominant non-cyanobacterial OTUs in either phylum correlated positively with microcystin concentration.

5.4.1.4 Chloroplast community composition

In the samples from Lake Klostersee, chloroplasts from Cryptophyta, Stramenopiles, and Haptophyceae dominated. Noteworthy abundances of Euglenozoa occurred only on 03 Sept 2015 (Figure 5.6A). The percentage of reads identified as being from chloroplasts showed a weak negative correlation with the relative abundance of Cyanobacteria in Lake Klostersee ($r = -0.20$) (Figure 5.6C). In samples from Lake Bergknappweiher, chloroplasts from Cryptophyta and Stramenopiles dominated most of the time with some Euglenozoa and Haptophyceae (Figure 5.6B). In Lake Bergknappweiher the percentage of reads identified as chloroplast showed a strong negative correlation with the relative abundances of Cyanobacteria ($r = -0.80$) (Figure 5.6D).

In total, the relative abundance of Cyanobacteria and percentage of chloroplast reads were negatively correlated (Appendix, Table 11.3). The different chloroplast orders showed distinctive correlations with environmental parameters and cyanobacterial orders (Appendix, Table 11.3). None of the chloroplast orders showed any correlation with microcystin concentration or *mcyB* abundance.

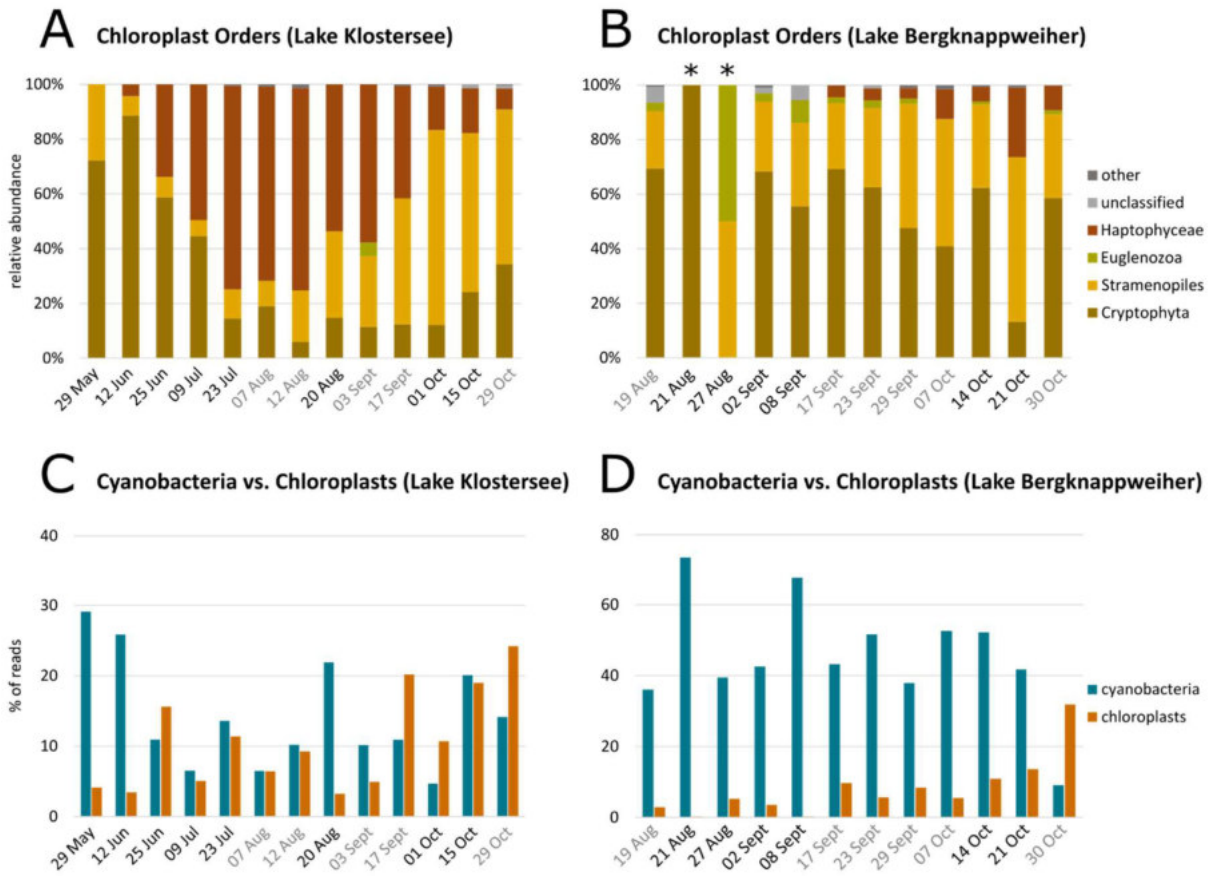

Figure 5.6: Chloroplast community composition and percent sequence reads in Lake Klostersee (left) and Lake Bergknappweiher (right). (A+B): Relative abundance of chloroplast sequences at the order level (relative abundances < 1 % shown as "other", asterisks mark samples with low sequence number). Dates in black indicate VSBs. Dates in grey indicate absence of VSBs. (C+D): Relative abundance of cyanobacteria vs. chloroplast percent sequence reads (note different y-axis).

5.4.1.5 Comparison of Lake Bergknappweiher and Lake Klostersee BCC

A total of 1,076 different OTUs were identified in this study. Of those OTUs 60 OTUs were chloroplast sequences and excluded from the bacterioplankton analysis. We identified 795 different bacterial OTUs in Lake Klostersee out of which approximately one quarter (22.77 %) were unique to this lake and not present in Lake Bergknappweiher. In Lake Bergknappweiher, we identified 835 different bacterial OTUs out of which approximately one quarter (26.47 %) were unique to this lake. The OTUs unique to the lakes were rare and together made up only a small fraction of the microbiome in terms of relative abundance (4.43 % in Lake Klostersee and 2.73 % in Lake Bergknappweiher). A common set of 614 OTUs was present in both lakes (see Figure 5.7A). This means that the lakes shared approximately three quarters of bacterial taxa

(73.53 % of Lake Bergknappweiher and 77.23 % of Lake Klostersee). Those common OTUs contributed to over 95 % of the microbiome in terms of relative abundance.

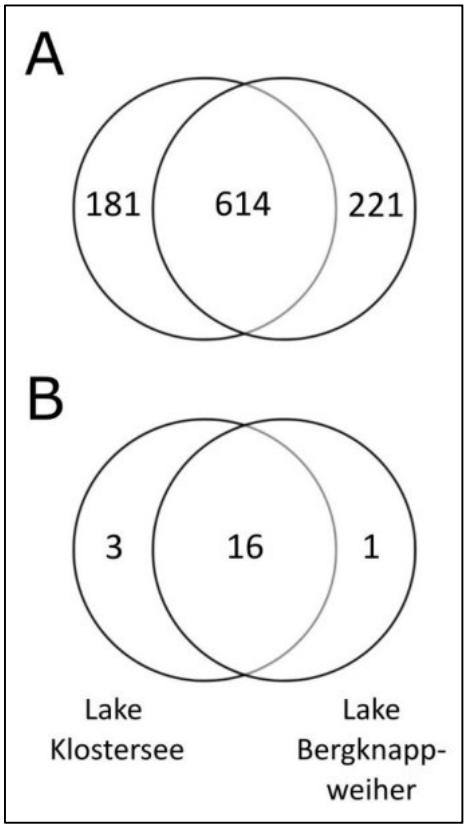

Figure 5.7: Unique and shared OTUs. (A) Number of unique and shared bacterial OTUs between Lake Bergknappweiher and Lake Klostersee. (B) Number of unique and shared cyanobacterial OTUs between Lake Bergknappweiher and Lake Klostersee. Chloroplasts sequences were excluded from both analyses. Circles not drawn to proportion.

In this study, 20 different OTUs belonged to the phylum Cyanobacteria. In Lake Bergknappweiher, 17 different cyanobacterial OTUs were present out of which only one was unique to this lake. In Lake Klostersee, we found 19 different cyanobacterial OTUs out of which only three were unique to this lake. Hence, a common set of 16 cyanobacterial OTUs was found in both lakes (see Figure 5.7B). Thus, the lakes shared the majority of cyanobacterial OTUs (for Lake Klostersee 84.21 % and for Lake Bergknappweiher 94.12 %).

One of the most abundant cyanobacterial OTUs was *Dolichospermum* (OTU1). It was the main contributor to visible surface blooms in both the spring sampling points in Lake Klostersee and the autumn sampling points in Lake Bergknappweiher (Figure 5.4E+F). During those times, it accounted for over 85 % of the whole cyanobacterial population. A BLAST search revealed *Dolichospermum flos-aquae* (accession number KY327796.1, identity 100 %) as the closest

match for *Dolichospermum* OTU1. *Dolichospermum flos-aquae* has previously been found to dominate in Lake Bergknappweiher (Teubner et al., 2004).

5.4.2 Microcystin-producing cyanobacteria

5.4.2.1 Toxigenicity

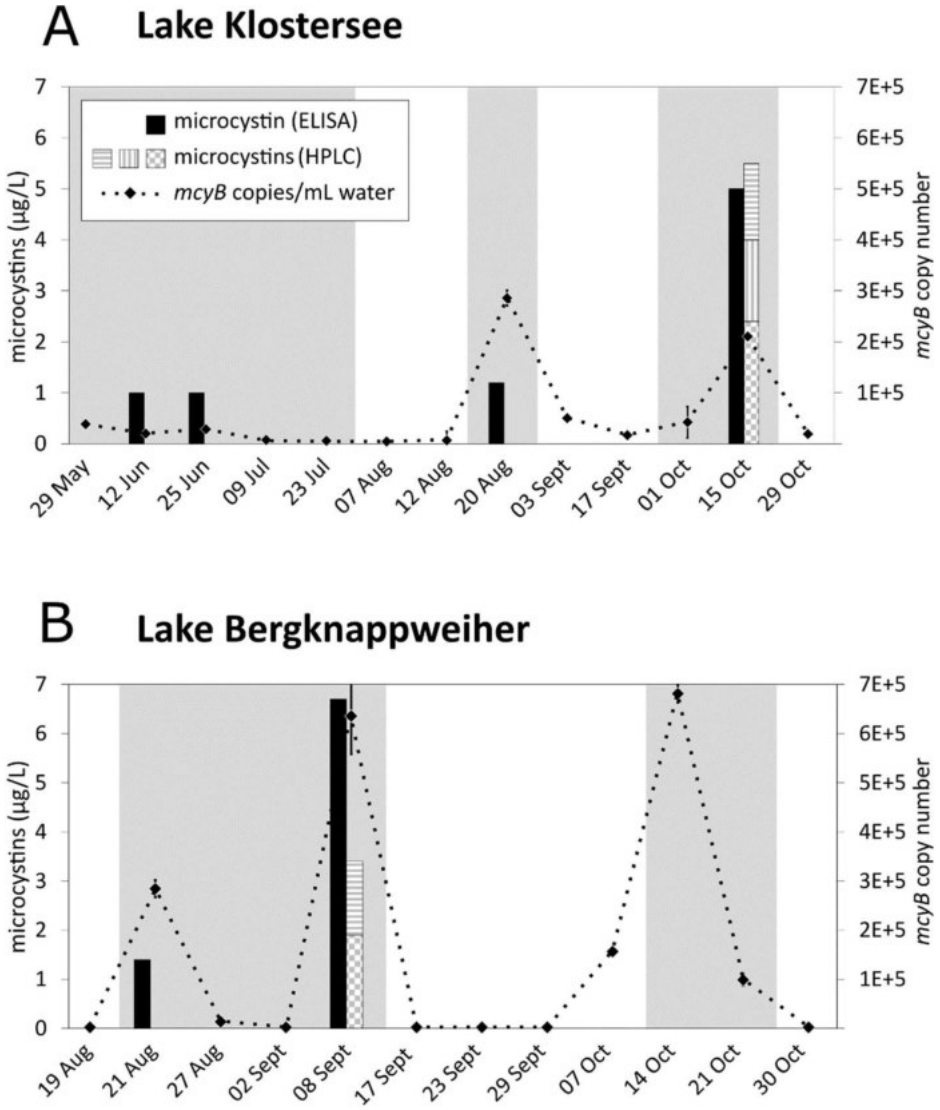

Figure 5.8: Microcystins and mcyB copy number over the bloom period 2015 in (A) Lake Klostersee and (B) Lake Bergknappweiher. Grey background indicates visible surface blooms. Microcystins (HPLC): Horizontal grey stripes indicate microcystin-LR, vertical grey stripes indicate microcystin-YR, and grey check indicates microcystin-RR.

To gauge bloom toxigenicity, the microcystin biosynthesis genes *mcyB* and *mcyE* were assessed. The limit of detection (LOD) for *Microcystis mcyB* was 6.12×10^2 copies/mL water. For *mcyB* gene copy numbers see Figure 5.8 and Table 5.3. The qPCR reaction detecting *mcyE* was specific for *Dolichospermum* (Appendix, Figure 11.1). The LOD was 3.38×10^3 copies/mL

water. All environmental samples gave a negative result for *Dolichospermum mcyE* (Table 5.3). In both lakes, peaks in *mcyB* copy number coincided with visible surface blooms (VSBs) (Figure 5.8).

Table 5.3: qPCR results for each sampling date.

Lake	Sampling time point	*Microcystis mcyB* (copies/mL)	*Microcystis mcyB error of the mean*	*Dolichospermum mcyE* (copies/mL)
Lake Klostersee	29 May 15	44,200	5,600	< LOD
	12 Jun 15	22,800	200	< LOD
	25 Jun 15	32,600	1,700	< LOD
	09 Jul 15	8,400	1,400	< LOD
	23 Jul 15	6,300	500	< LOD
	07 Aug 15	4,600	600	< LOD
	12 Aug 15	7,300	300	< LOD
	20 Aug 15	330,000	20,300	< LOD
	03 Sept 15	57,700	17,600	< LOD
	17 Sept 15	19,000	2,300	< LOD
	01 Oct 15	48,500	3,100	< LOD
	15 Oct 15	243,200	35,500	< LOD
	29 Oct 15	21,700	800	< LOD
Lake Bergknappweiher	19 Aug 15	< LOD	ND	< LOD
	21 Aug 15	270,000	16,800	< LOD
	27 Aug 15	10,700	ND	< LOD
	02 Sept 15	< LOD	ND	< LOD
	08 Sept 15	606,400	74,500	< LOD
	17 Sept 15	< LOD	ND	< LOD
	23 Sept 15	< LOD	ND	< LOD
	29 Sept 15	< LOD	ND	< LOD
	07 Oct 15	147,500	1,800	< LOD
	14 Oct 15	649,900	17,800	< LOD
	21 Oct 15	92,200	11,400	< LOD
	30 Oct 15	< LOD	ND	< LOD

5.4.2.2 Diversity and origin of *mcyE* genes

We assessed the diversity and taxonomic origin of the microcystin biosynthesis gene *mcyE* for one sampling point in Lake Klostersee and two sampling points in Lake Bergknappweiher. In the Lake Klostersee sample from 29 May 2015 we identified 82 highly similar partial *mcyE* sequences with a pairwise percent identity of 98.8 %. In the Lake Bergknappweiher samples from 08 Sept and 14 Oct 2015 a total of 61 and 16 highly similar partial *mcyE* sequences were identified with a pairwise percent identity of 98.3 % and 98.5 %, respectively. All of the partial *mcyE* sequences and their consensus sequences clustered with *mcyE* sequences from *Microcystis* sp. in a neighbour-joining tree with published *mcyE* sequences (Figure 5.9). None of the sequences clustered with *mcyE* sequences from other genera such as *Dolichospermum* or *Planktothrix*, indicating that all *mcyE* sequences detected originated from *Microcystis* sp.

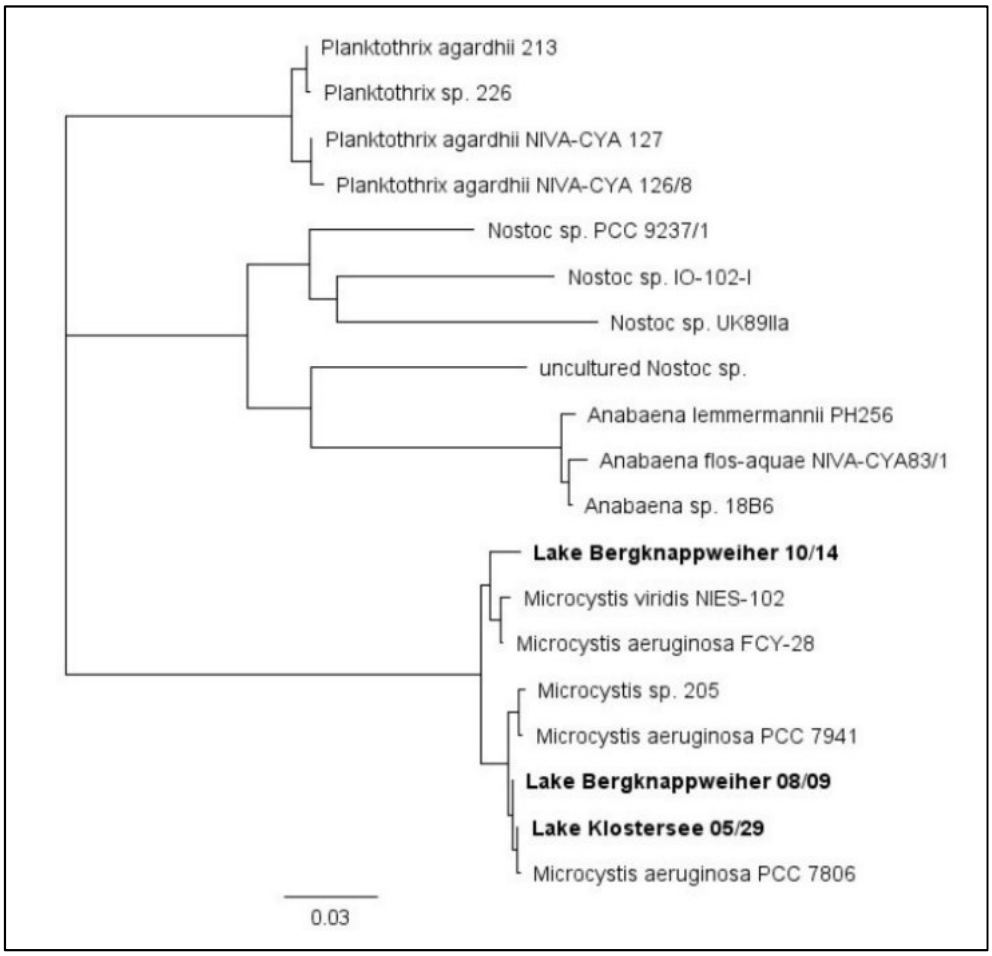

Figure 5.9: Neighbour-joining tree based on published partial and full mcyE sequences and mcyE consensus sequences obtained from clone libraries in this study (in bold).

5.4.3 Physico-chemical parameters

For microcystin concentrations in both lakes see Figure 5.8 and Appendix Table 11.6. For further physico-chemical water parameters see Appendix Table 11.6.

In both lakes microcystin measurements by ELISA and HPLC correlated strongly (Pearson's r (r) = 0.89) and showed a positive correlation with *mcyB* copy number (Appendix, Table 11.4). All three parameters were positively correlated with TP and negatively with $N_{inorg.}$/P (Appendix, Table 11.4). The correlations of microcystins with NO_3-nitrogen, however, were different for both lakes. In Lake Klostersee, microcystin concentrations were negatively correlated with NO_3-nitrogen (ELISA r = -0.47, HPLC r = -0.42) while in Lake Bergknappweiher they were positively correlated with NO_3-nitrogen (ELISA r = 0.51, HPLC r = 0.41). In both lakes, actual microcystin measurements coincided with visible surface blooms (VSBs). However, not all VSBs were characterized by detectable microcystin concentrations (Figure 5.8 and Appendix, Table 11.6).

Correlation analysis revealed a clear two-way division between cyanobacterial genera. The relative abundance of OTUs belonging to the genus *Dolichospermum* showed a very strong negative correlation with NO_3-nitrogen and a negative correlation with TP (Appendix, Table 11.5). All other genera correlated positively with NO_3-nitrogen and showed positive or no correlation with TP (Appendix, Table 11.5). *Microcystis* showed a positive correlation with TP and NO_3-nitrogen (Appendix, Table 11.5).

5.5 Discussion

In this study, we identified environmental parameters that correlated with microcystin occurrence. Of the environmental parameters recorded in this study, N_{inorg}/P and TP showed a correlation with microcystin occurrence. In line with our hypothesis, we showed that *mcyB* gene copy number correlated positively with microcystin concentration. We identified *Microcystis* sp. as the sole microcystins producer. *Microcystis* sp. was not very abundant and never dominated the cyanobacterial community. Furthermore, we showed that the BCC was relatively stable at the phylum level throughout the sampling period, but temporal changes in the community composition at the OTU level were evident. Our study adds to the few previous studies that combine various community aspects with toxicity (Berry et al., 2017; Fortin et al., 2015; Parulekar et al., 2017) and differs from previous work where either *Microcystis* was dominating the Cyanobacteria community (Fortin et al., 2015) or only the phylum Cyanobacteria was resolved down to OTU level (Fortin et al., 2015; Parulekar et al., 2017).

5.5.1 Microbial community composition

5.5.1.1 Phylum level vs. OTU level distribution

The appearance of a VSB did not coincide with massive changes in BCC at the phylum level, which was mostly stable throughout the sampling period. This partly contradicts other studies, which have found a low resistance but high resilience of the BCC towards cyanobacterial blooms (Li et al., 2012b) or temporal changes in phylum level distribution (Parulekar et al., 2017). However, we observed considerable temporal changes in bacterial community composition at the level of order and even more at the OTU taxonomic level (Figure 5.4 and Figure 5.5). These data show that it is important to evaluate the BCC at the OTU level in addition to the phylum or order level because most of the temporal variation in bacterial community composition occurred at the OTU level.

5.5.1.2 Community composition of main heterotrophic phyla

Interestingly, major bacterial phyla exhibited not only highly different levels of diversity but also different degrees of temporal variation in community composition (see Figure 5.5). In our study, the community of Actinobacteria was stable throughout the sampling period and similar between the lakes. In contrast to our findings, Berry et al. (2017) described an Actinobacteria community that was highly dynamic over time and showed a strong correlation to bloom dynamics. This link to cyanobacteria dynamics might also explain our contrasting observation of a relatively stable Actinobacteria community. The changes in total cyanobacteria relative abundance over time observed in our study were much smaller than the ones observed by Berry et al. (2017). In our study, the cyanobacterial community composition was associated with some species-specific correlations with non-cyanobacterial taxa. However, we observed less bloom-induced changes in the bacterial community composition than others did (Berry et al., 2017; Woodhouse et al., 2016). Most notably the frequently reported bloom related changes in Actinobacteria community composition were not observed here. Possible explanations are low bloom intensity or low abundance of the microcystin producer *Microcystis*. The main Actinobacteria OTUs (Figure 5.5) belong to the most common taxa in freshwater. This phylum has been shown to be more successful under lower nutrient conditions (Newton et al., 2011), which is in line with our results that show a two times higher relative abundance of Actinobacteria in the lake with the lower nutrient load (Lake Klostersee) compared to the lake with the higher nutrient load (Lake Bergknappweiher).

The drastic temporal variations in bacterial community composition within the highly diverse phylum Bacteroidetes has previously been described in the context of toxic cyanobacteria

blooms (Woodhouse et al., 2016). The link between Bacteroidetes and Cyanobacteria is also evident in our study. The three most abundant OTUs of the phylum Bacteroidetes were members of the order Sphingobacteriales (OTU23, OTU27, and OTU30) (see Figure 5.5C+D). Members of this taxon, such as Saprospiraceae, are known for their ability to degrade toxins and other cyanobacterial secondary metabolites (Li et al., 2017; McIlroy and Nielsen, 2014). Moreover, certain Sphingobacteriales lyse cyanobacteria by means of algicidal metabolites, and members of the Saprospiraceae are known to even prey on cyanobacteria (Lewin, 1997). Thus, the positive correlation of Saprospiraceae OTU23 with the toxin producer *Microcystis* emphasizes the close interaction between members of the toxin producing cyanobacteria and heterotrophic bacteria (Appendix, Table 11.2).

The different patterns of Alpha- and Betaproteobacteria in the two lakes studied could be explained by the fact that environmental parameters in Lake Bergknappweiher, such as nutrients and pH fluctuated more than in Lake Klostersee (Appendix, Table 11.6). The peculiar and constant community composition of both Alpha- and Betaproteobacteria in Lake Bergknappweiher between 07 Oct and 21 Oct 2015 (Figure 5.5F+H) coincided with the overwhelming dominance of *Dolichospermum* OTU1 (Figure 5.4F). One of the most abundant Alphaproteobacteria during this time was a member of the order Sphingomonadales (OTU2) (see Figure 5.5F). Several members of this taxon are able to degrade microcystins (Li et al., 2017), and the high relative abundance of OTU2 between 07 Oct and 21 Oct 2015 might hint toward the presence of a similar secondary metabolite possibly produced by *Dolichospermum* or the presence of a yet undetectable microcystin isoform. This would explain the peak in *mcyB* gene copy concentration (qPCR data) that lacks a concurrent peak in microcystin concentration (ELISA and HPLC data) during this period (Figure 5.8). One of the dominant Betaproteobacteria belonged to the genus *Limnohabitans*. *Limnohabitans* can utilize algal derived substrates possibly produced by *Dolichospermum* (Šimek et al., 2011). The fact that high relative abundances of Sphingomonadales and *Limnohabitans* were not observed in Lake Klostersee, while the same *Dolichospermum* dominated the cyanobacterial community (29 May and 12 Jun 2015), can be explained by the overall lower relative abundance of cyanobacteria in Lake Klostersee.

The data from our study complement other studies that have looked at the cyanobacterial bloom associated BCC (Berry et al., 2017; Lee et al., 2016; Shi et al., 2011; Wu et al., 2007). This additional information from aquatic habitats in other geographic locations is important to distinguish patterns in BCC that are influenced by environmental factors (Bacteroidetes and

partly Proteobacteria in our study) from those that are influenced by disturbances such as cyanobacterial blooms (Actinobacteria in Berry et al. (2017)). This is a prerequisite to understanding the dynamics in bloom associated bacterial community composition and better gauge environmental implications of toxic cyanobacterial blooms. The sampling period in this study spanned a single season in two lakes. Nevertheless, our results show that the high temporal resolution, which was higher than in many studies and which was called for by Parulekar et al. (2017), was necessary for tracing BCC dynamics at the OTU level at least for some phyla.

5.5.1.3 Diversification within the dominant cyanobacterial genera

Analysis at the lower taxonomic levels revealed that the cyanobacterial community composition was highly dynamic throughout the sampling period. Nevertheless, a single genus was able to dominate the cyanobacterial community for several months. In Lake Klostersee, this was *Synechococcus* and in Lake Bergknappweiher *Dolichospermum* (Figure 5.4E+F). Analysis of community composition at the OTU level showed that several different OTUs contributed to the high relative abundance of the dominant genus, but the dominance of any single OTU never lasted longer than about two weeks. Top-down control by cyanophages, grazers or algicidal bacteria may prevent any one OTU from becoming dominant over an extended period. Any dominant species might be decimated according to the kill-the-winner hypothesis allowing competitors to thrive (Thingstad and Lignell, 1997). This idea is supported by findings presented by Yoshida et al. (2006) who showed that cyanophages are largely strain specific when infecting their host. Thus, a top down control by viruses can stimulate host diversity (Thingstad and Lignell, 1997; Weinbauer and Rassoulzadegan, 2004; Zwart et al., 2005). Correspondingly, selective feeding on even closely related cyanobacteria strains by grazers has been shown (Zwirglmaier et al., 2009), which might prevent one OTU from becoming dominant and further promote diversification within the dominant cyanobacterial genus. In addition, different algicidal bacteria are capable of lysing cyanobacteria in a species-specific manner (Rashidan and Bird, 2001; Redhead and Wright, 1978). Those algicidal bacteria can shape the cyanobacterial community composition and lead to the decline of algal blooms (Rashidan and Bird, 2001). The filamentous cyanobacterium *Dolichospermum* has been shown to be particularly sensitive to lysis by co-occurring algicidal bacteria (Rashidan and Bird, 2001; Redhead and Wright, 1978). Algicidal bacteria thus might have caused the drop in *Dolichospermum* relative abundance in Lake Klostersee after the 12 Jun 2015 (Figure 5.4E). This conclusion is supported by the fact that the Illumina Miseq sequencing data set contained

bacteria that have previously been shown to have algicidal activity, such as members of the order Sphingobacteriales and the family Saprospirace (Lewin, 1997) (Figure 5.5) and the genera *Pseudomonas* sp. and *Microbacterium* sp. (Chen et al., 2012; Redhead and Wright, 1978). The fact that the potential algicidal bacteria Sphingobacteriales and Saprospiracea (OTU23, 27, and 30) are amongst the most abundant Bacteroidetes (Figure 5.5C+D) hints towards a potential role in regulation of the bacterial community composition. In addition, less abundant Alpha- and Gammaproteobacteria from the family Rhodobacteraceae, Aeromonadaceae, and Enterobacteriacea were identified in this study, and members of those families have been associated with algicidal activity against cyanobacteria (Chen et al., 2012; Liao et al., 2014; Redhead and Wright, 1978; Yang et al., 2013).

5.5.1.4 Chloroplasts

The negative correlation of cyanobacteria and chloroplast reads we observed on a temporal scale (Figure 5.6C+D) has been noted before (Zwirglmaier et al., 2015), albeit in a spatial distribution. This contrasting distribution might be due to the occupation of a somewhat similar ecological niche by eukaryotic algae and cyanobacteria and competition for similar resources. We found a negative correlation of chloroplast reads with water temperature ($r = -0.54$). Our results support the findings of others who found that eukaryotic phytoplankton is better adapted to lower temperatures than cyanobacteria (Butterwick et al., 2005; de Senerpont Domis et al., 2007; Jöhnk et al., 2008).

5.5.1.5 Comparison of Lake Bergknappweiher and Lake Klostersee BCC

Both lakes sampled displayed a distinct bacterial community composition at the phylum level (Figure 5.4A+B) albeit sharing a majority of OTUs (Figure 5.7). OTUs unique to each lake contributed less than 5 % to the BCC in terms of relative abundance. This means that the difference in relative abundance of taxa, rather than their presence or absence accounted for the dissimilarity in BCC of both lakes, a conclusion similar to the one drawn by Staley et al. (2013). Regardless of their low relative abundances, the taxa unique to a lake might still be important because rare taxa were proposed to play an important role in shaping of future communities (Sogin et al., 2006).

The relative close geographic proximity of the lakes studied here is not sufficient to explain the large common microbiome on the OTU level. Even adjacent and interconnected lakes were found to share only a much smaller fraction of their microbiome, and environmental conditions were suggested as drivers for differential prokaryotic community composition (Zwirglmaier et al., 2015).

The most striking difference in the bacterial community composition between Lake Klostersee and Lake Bergknappweiher is the higher abundance of cyanobacteria in the latter. OTU level analysis revealed that those cyanobacteria are for the most part *Dolichospermum* species. This genus is diazotrophic and was the only cyanobacterial genus, which correlated negatively with NO_3-nitrogen (Appendix, Table 11.5). It is thus less prone to suffer from nitrogen limitation and therefore can take advantage of the high TP concentrations in Lake Bergknappweiher compared to Lake Klostersee. This shows that environmental conditions shape differences in lakes' community composition.

5.5.1.6 Taxonomic classification

In this study, we used the 16S-rRNA gene for taxonomic classification of bacteria. The 16S-rRNA gene is a suitable marker for identification of prokaryotes at the species level and has been widely used for investigation of cyanobacterial diversity (Foster et al., 2009; Kirkwood et al., 2008; Kormas et al., 2010; Lymperopoulou et al., 2011; Ruber et al., 2016; Zwirglmaier et al., 2015). It should be noted that some bacteria carry more than one copy of the 16S-rRNA gene (Pei et al., 2010). Schirrmeister et al. (2012) found *Nostoc* spp. and *Dolichospermum variabilis* to carry four 16S-RNA gene copies while *M. aeruginosa* NIES-843 only carried two. The number of 16S-rRNA gene copies cannot necessarily be inferred from the genus: for example, different members of the genus *Synechococcus* were found to carry either one or two copies of the 16S-rRNA gene in the same study (Schirrmeister et al., 2012). Nevertheless, Parulekar et al. (2017) showed that *Dolichospermum flos-aquae* was not overestimated by high-throughput sequencing compared to microscopic analysis.

5.5.2 Microcystin-producing cyanobacteria

5.5.2.1 Correlations with microcystin occurrence

Microcystis was present in almost every sample from Lake Klostersee and Lake Bergknappweiher and its highest relative abundances on 25 Jun, 20 Aug, and 15 Oct 2015 coincided with microcystin detections (Figure 5.8A). The highest relative abundances of *Microcystis* in Lake Bergknappweiher on 27 Aug and 30 Oct 2015 did not coincide with positive microcystin results (Figure 5.8B). A strong positive correlation of relative abundance of *Microcystis* sp. (based on the 16S rRNA gene) with microcystin concentration was found in Lake Klostersee (ELISA $r = 0.70$, HPLC $r = 0.68$) but not in Lake Bergknappweiher. There are several possible explanations for these observations apart from the relative nature of the Illumina sequence data. First, a shift in the ratio of *mcyB* genotypes and *mcyB* gene free genotypes during the sampling period might have occurred. Genotypes containing *mcyB* and

mcyB gene free members of the same species not only co-occur in natural environments but also vary in their contribution to the total population (Briand et al., 2008; Kardinaal et al., 2007; Martins et al., 2011; Sabart et al., 2010). Second, microcystin biosynthesis genes may not be expressed constitutively, which still awaits independent confirmation (Wood et al., 2011). Regulation of microcystin biosynthesis genes at the transcriptional level has been described in a laboratory study (Pimentel and Giani, 2014; Scherer et al., 2017; Sevilla et al., 2008) and in the environment (Penn et al., 2014). A third explanation is the possible presence of *mcyB* genotypes with microcystin biosynthesis gene clusters that are inactivated by mutations. Such non-functional gene clusters have been described in environmental studies (Christiansen et al., 2006; Christiansen et al., 2008; Ostermaier and Kurmayer, 2009).

For analysis of total microcystin in surface water samples extracellular as well as intracellular microcystins have to be considered. Extraction of intracellular microcystins from the cyanobacteria is thus a critical step for the quantitative analysis of microcystin. Microcystin levels detected in this study were far below the action level of 30 µg/L recommended by the German Federal Environment Agency for recreational water (Bundesgesundheitsblatt, 2015). Four samples exhibited a microcystin level above the limit of detection of the ELISA (1.0 µg/L to 1.4 µg/L). In these samples, specific microcystins were not detected by HPLC, probably because they were below the limit of detection of the HPLC method. Higher microcystin levels detected by ELISA in two samples (5.0 µg/L and 6.7 µg/L) were confirmed by HPLC (5.5 µg/L and 3.4 µg/L). Deviations observed for results obtained by ELISA and HPLC are due to different principles of detection and possibly differential affinities of the ELISA antibodies to various microcystin isoforms. Other potentials and limitations of different methods for microcystin analysis have been reported (Kaushik and Balasubramanian, 2013; Meriluoto et al., 2017; Sangolkar et al., 2006; Schmidt et al., 2014).

5.5.2.2 Low abundant microcystin producer

In this study, we identified the microcystin-producing cyanobacterium without the need for time-consuming isolation and cultivation. *Microcystis* sp. was present in much lower abundances than *Dolichospermum* spp. in both lakes (Figure 5.4E+F). *Planktothrix* spp. were absent in both lakes. We found no evidence that any of the *Dolichospermum* OTUs identified by high-throughput sequencing carried the genes for microcystin biosynthesis. Data from qPCR as well as data from clone libraries suggested that in both lakes *Microcystis* sp. was the only microcystin producer present. Those findings are in line with other studies, which found that the dominant microcystin producer is not necessarily the dominant cyanobacterial species in a

water body (Dadheech et al., 2014). Similar to our study, Lee et al. (2015) identified low abundant *Microcystis* sp. as the sole microcystin producer. More awareness about the possibility of low abundant microcystin producers is required to avoid underestimating toxicity risks in blooms that predominantly contain non-toxic species.

5.5.3 Physico-chemical parameters

The positive correlation of TP with microcystin concentration and microcystin synthesis gene copy number observed in this study has previously been reported (Amer et al., 2009; Rinta-Kanto et al., 2009). A negative correlation between the copy number of a microcystin biosynthesis gene and NO_3-nitrogen like the one we discovered in Lake Klostersee was shown before (Rinta-Kanto et al., 2009). On the other hand, the positive correlation of those factors as observed in Lake Bergknappweiher was also demonstrated in another study (Yoshida et al., 2007). Our results and previous studies, therefore, suggest that the influence of NO_3-nitrogen may vary in different water bodies depending on the limiting factor in a particular lake.

We found a positive correlation between *mcyB* copy number (based on qPCR results) and microcystin concentration, which is in agreement with previous work (Al-Tebrineh et al., 2012; Martins et al., 2011; Ostermaier and Kurmayer, 2010). Whether the *mcy* copy number quantified by qPCR can be used as a proxy for microcystin occurrence or not, is still under discussion (Pacheco et al., 2016). The results presented in this study do not settle the matter definitively but indicate it might be possible.

5.6 Conclusion

For the first time, high-throughput sequencing was used to reveal differential temporal variations in the microbial community composition during toxic cyanobacterial blooms in two lakes in South Germany. A focused analysis revealed that the microcystin producing species never dominated the bloom. In addition, *mcyB* gene copy number correlated positively with microcystin concentration. These findings are relevant for risk assessment and therefore need to be the subject of further research. The collective results presented in this study will aid in future risk assessment for recreational waters and help to put microcystin measurements into greater ecological perspective.

6 Overall discussion and outlook

This thesis explored the influence of abiotic and biotic environmental variables on the toxic potential of cyanobacteria using laboratory and field approaches. Growth independent effects of those variables were assessed in laboratory gene expression studies. Results showed that climate change associated abiotic variables can increase toxin gene transcription. Response was dependent on the tested variable and most pronounced for increased temperature. Two of the three biotic environmental variables tested increased toxin gene expression. The relationships of abiotic and biotic factors with cyanobacterial bloom toxicity were addressed in field monitoring studies. Field studies identified abiotic (TP, *mcyB* copy number, $N_{inorg.}$/P, NO_3-nitrogen) and biotic factors (*Microcystis* sp.) that correlated with toxicity and identified *Microcystis* sp. as the cyanobacterium responsible for toxin production. This combination of findings has important implications for current monitoring practices, but more importantly for the development of future monitoring tools and future research perspectives.

6.1 The impact of abiotic environmental factors on toxic cyanobacteria

Results reported in this thesis indicate that some environmental factors can influence the expression of toxicity genes and correlate with cyanobacterial bloom toxicity. The current section discusses the overall findings of this thesis in the context of two environmental factors: total phosphorus and rising temperature. In addition, this section offers links and possible research perspectives on environmental factors that were not directly assessed in this thesis and are less well studied with regard to their effect on cyanobacteria. Those are iron concentration and light climate.

6.1.1 The effect of temperature on toxic cyanobacteria

Water temperature was not only monitored in the field studies but also manipulated in the laboratory experiments. This thesis expands on the current body of knowledge by for the first time providing information from laboratory experiments about a possible increase of toxigenicity brought about by higher temperatures. Under field conditions, however, no positive correlation between water temperature and bloom toxicity was found. Nevertheless, the negative correlation between water temperature and chloroplast sequence reads observed in the field study suggests that eukaryotic phytoplankton might be less competitive than cyanobacteria at higher temperatures (Appendix, Table 11.3). Overall, our findings from laboratory and field

studies did not only confirm the conclusions of others who found cyanobacteria might be favoured disproportionally by climate change but also expand on current knowledge by reporting a transcriptional response to elevated temperatures. Temperature is not only an important factor for the cyanobacteria population, but is also predicted to remain highly relevant or even gain importance in the future due to climate change (1.2.3 Global change and its consequences for bloom occurrence). As mentioned previously, other studies found that the growth of cyanobacteria is favoured by climate change and associated warmer water temperatures (Paerl and Huisman, 2008; Paerl and Huisman, 2009). This is supported by data on rather high growth temperature optima of cyanobacteria species in comparison to other phytoplankton species (Butterwick et al., 2005; Elliott et al., 2006; Lürling et al., 2013). Regarding the bloom toxicity this field study is in agreement with other field studies who found no correlation between microcystin concentration and water temperature (Kotak et al., 1995); but it contradicts others who associated higher temperatures with increased toxicity (Kleinteich et al., 2012; van der Westhuizen and Eloff, 1985). A possible explanation for those discrepancies might be the prevalence of different species and strains of cyanobacteria in different water bodies. Different species might react differently to changes in environmental conditions such as temperature. This can for example be inferred from the fact that different cyanobacteria isolates have distinctly dissimilar optimal growth temperatures (Lürling et al., 2013).

6.1.2 The impact of TP on toxic cyanobacteria

The field study during this thesis found a positive correlation between phosphate concentration and bloom toxicity, and thus establishing a link between those two parameters (Appendix, Table 11.4). However, phosphate concentration did not show a positive correlation with the relative abundance of *Microcystis,* which was the toxic cyanobacterium in the bloom. This might be explained by mere increase of total cyanobacteria cells number during high phosphate concentration. Limnologists have conducted extensive research in order to understand the relationship between abiotic environmental factors and the growth of bloom-forming cyanobacteria, and a high phosphate concentration is traditionally associated with increased cyanobacterial growth (Whitton and Potts, 2000). Furthermore, bloom toxicity has been correlated to high phosphate concentration (Kotak et al., 1995).

6.1.3 The micronutrient iron is connected to cyanobacterial toxicity

Laboratory experiments presented in this thesis show that some biotic factors increased toxigenicity of *M. aeruginosa*, while others did not. *M. aeruginosa* increased the expression of microcystin biosynthesis genes in the presence of the cyanobacterium *S. elongatus* and in the presence of *S. elongatus* spent medium. Microcystin biosynthesis genes were not upregulated in the presence of the mixotrophic flagellate *O. danica*, but they were upregulated in the presence of virioplankton size fractions. Humic acid on the other hand did not show any significant effect on microcystin biosynthesis gene expression.

Micronutrient-related effects could possibly explain the aforementioned observations made in this thesis. The concentration of micronutrients is one of the abiotic environmental factors that affect growth and toxicity of cyanobacteria and that has received only little attention. Especially iron is of interest in this context because of strong links between microcystin and iron acquisition. Microcystin producers have been shown to take up iron more efficiently than non–toxin-producers (Utkilen and Gjølme, 1995), and the microcystin content increases under iron limitation (Wang et al., 2016; Yeung et al., 2016). These observations support the proposed function of microcystin as an iron chelator (Utkilen and Gjølme, 1995). In addition, transcription of microcystin biosynthesis genes was linked to iron metabolism by means of the ferric uptake regulator Fur, which is present in *M. aeruginosa* and binds to promotor regions of the microcystin gene cluster (Martin-Luna et al., 2006a; Martin-Luna et al., 2006b).

Securing micronutrients such as iron is a task that is even more crucial for *Microcystis* in the presence of competing cyanobacteria like *S. elongatus*; thus, its presence might trigger increased expression of microcystin biosynthesis genes in *Microcystis* (4.4.1 Response to cyanobacteria). Moreover, soluble factors from *S. elongatus* such as siderophores might withhold iron from *Microcystis*, thereby increasing the need for microcystin as an iron chelator and possibly increasing *mcyB* and *mcyD* expression.

This hypothesis could also explain the lack in *mcyB* and *mcyD* upregulation in the presence of *O. danica* (4.4.2 Response to mixotrophic flagellates). As a grazer, *O. danica* can acquire iron from its prey and thus is not competing with *M. aeruginosa* for dissolved iron. Therefore, *Microcystis* did not increase its synthesis of iron-chelating microcystin.

Upregulation of microcystin biosynthesis genes in the presence of phages from the virioplankton size fraction might be connected to iron metabolism as well (4.4.3 Effects of the virioplankton size fraction). First, recent studies have demonstrated that phages bind iron

(Bartual et al., 2010; Browning et al., 2012; Penner et al., 2016; Yamashita et al., 2011); phage-bound iron composes a significant fraction of the total organically complexed dissolved iron in the oceans (Bonnain et al., 2016). Furthermore, certain phages released by the human pathogen *Pseudomonas aeruginosa* have been shown to inhibit competing *Aspergillus fumigatus* by sequestering iron (Penner et al., 2016). While this phenomenon has been demonstrated only in clinical isolates thus far, this mechanism might also occur in aquatic ecosystems, where micronutrients are often scarcer than within a human host. In such a situation, again, it could be beneficial for *Microcystis* to overexpress genes for the synthesis of iron-chelating microcystin to compensate for iron withdrawn by phages. Second, phage infection in cyanobacteria can influence photosynthesis, a process that is dependent on iron for pigment synthesis and electron transport (Kosakowska et al., 2004). Cyanophages have been found to carry photosynthesis genes, which are most likely used to boost photosynthesis if needed, as in providing protection from photoinhibition (Alperovitch-Lavy et al., 2011; Bailey et al., 2004; Sandaa et al., 2008; Sharon et al., 2009). Photosynthesis can be a source of oxidative stress; microcystin has been shown to bind to enzymes crucial to energy metabolism, thereby stabilizing them during times of oxidative stress (Zilliges et al., 2011). For this reason, microcystin production might be upregulated in the event of cyanophage infection to cope with the increased oxidative stress.

Increased iron bioavailability caused by humic substances has been found to favour *M. aeruginosa* growth (Kosakowska et al., 2007), but opposite effects also have been reported because humic substances can complex iron, rendering it unavailable for cyanobacteria (Imai et al., 1999; Sun et al., 2005). In this thesis, no significant effect of humic acid on the expression of microcystin biosynthesis genes was found (4.4.3.1 Effects of the virioplankton size fraction from Lake Bergknappweiher before and during algal bloom). This suggests that either these antithetical effects are cancelling each other out or the change in iron availability does not influence microcystin synthetase gene expression under the given conditions.

In this thesis, the direct effect of iron concentration on toxigenicity of *M. aeruginosa* was not tested, because only a limited number of environmental factors could be assessed. However, when analysing the results, the possible links to the micronutrient iron sparked interest and should receive more attention in the future when investigating the topic. As other studies are limited to laboratory-based approaches (Alexova et al., 2011a; Martin-Luna et al., 2006b; Sevilla et al., 2008), the role of micronutrient limitation in the toxicity of blooms under field conditions would be another interesting avenue to explore in future studies.

6.1.4 The link between light conditions and cyanobacterial toxicity

In this thesis, no increase in toxigenicity by stirring *M. aeruginosa* was found (3.4.3 Effect of mixing). However, it has been suggested that under environmental conditions the drastic changes in light climate in a moving water column might constitute much more stress for the cyanobacterial cells than the movement itself (Ingrid Chorus, personal communication). This and the fact that climate change might cause changing light conditions either by increased influx of DOC, increased phytoplankton growth, or changed perturbation patterns in water bodies, calls for future work focussing on the response of toxic cyanobacteria to changing underwater light conditions. Even though the effects of light quality or light stress on toxic bacteria has been determined in some studies (Kaebernick et al., 2000; Zilliges et al., 2011), this has not been performed in controlled simulated light fields resembling natural underwater light conditions so far. Such experiments have only recently been made possible by the development of the ENVILAB, a novel automated spectrometric bioreactor setup with a tunable multispectral light source that is able to simulate underwater light fields with a spectral composition similar to natural aquatic environments (Göritz et al., 2017). The development and realisation of the ENVILAB was part of another doctoral thesis within the framework of the IGSSE's (TUM International Graduate School of Science and Engineering) Focus Area Water. The ENVILAB is an ideal setup for both physiological studies and studies of inherent optical properties of phytoplankton, and therefore, is an excellent platform for future interdisciplinary research. In the context of changing light conditions, elucidating the link between toxin metabolism and complimentary chromatic adaptation in cyanobacteria (CCA) might be a promising avenue for research. Many different cyanobacteria are able to perform CCA, among them toxin producing genera (Tandeau de Marsac, 1977). Toxin biosynthesis and CCA might be connected at the molecular level. First, microcystin has been shown to bind to phycobiliproteins in cyanobacteria under certain conditions (Zilliges et al., 2011). Second, the expression of genes responsible for the biosynthesis of cyanobacterial toxins is connected to the expression of genes involved in the CCA by common regulatory elements, suggesting co-regulation of these genes (Jones et al., 2009). And third, changes in light quantity (Jähnichen et al., 2011; Jodłowska and Latała, 2010; Wiedner et al., 2003) but more importantly in light quality (Kaebernick et al., 2000) have been shown to result in changes in microcystin content or gene transcription in toxic cyanobacteria species. Future research should be undertaken to investigate whether the link between toxicity and CCA and the associated changes in optical properties could be ultimately harnessed for a better detection of toxic cyanobacteria blooms by remote sensing methods.

6.2 The impact of biotic factors on toxic cyanobacteria

Field and laboratory studies in the course of this work confirmed the crucial role of co-occurring microorganisms in the bacterioplankton community of toxic blooms. It was shown in this thesis that biotic environmental factors such as co-occurring microorganisms can influence the expression of toxicity genes under laboratory conditions. One of the co-occurring organisms that particularly stood out in the laboratory and field studies was the picocyanobacterium *Synechococcus* sp. Recent research on interspecies communication and signalling suggests that toxic cyanobacteria are connected to co-occurring microorganisms by means of secondary metabolites (Briand et al., 2016; Kaplan et al., 2012; Kaplan et al., 2016; Schatz et al., 2007; Vardi et al., 2002), a notion supported by results from this thesis (4.4.1 Response to cyanobacteria). The second biotic factor able to increase microcystin biosynthesis gene expression, are the cyanophages. The potential of cyanophages, to control cyanobacterial blooms, has widely been recognized (Brussaard, 2004), and cyanophages have even been proposed as a biological algicide (Phlips et al., 1990; Safferman and Morris, 1964; Yoshida et al., 2006). However, cyanophages have not been used as a control measure in the field so far, but other aquatic organisms have been used for combating cyanobacterial blooms. An example is the practice of biomanipulation (Peretyatko et al., 2012). A third interesting factor in field studies were the heterotrophic bacteria population. Heterotrophic bacteria have been found to be associated with toxic cyanobacteria (Berry et al., 2017; Shi et al., 2011; Woodhouse et al., 2016). Their potential effects on cyanobacteria toxicity, however, is not well understood and their role in the bloom dynamics of toxic cyanobacterial is only just emerging.

6.2.1 *Synechococcus* an underestimated factor in toxic blooms

In this thesis, it was for the first time demonstrated that the picocyanobacterium *S. elongatus* increases toxin gene expression in the toxic cyanobacterium *M. aeruginosa*. This shows that the importance of picocyanobacteria such as *Synechococcus* species might play a much more crucial role in toxic cyanobacterial blooms than previously thought. This conclusion was even strengthened by our field data from two lakes with cyanobacteria blooms where unexpectedly high relative abundances of *Synechococcus* spp. were found. Despite this, no positive correlation between toxicity and *Synechococcus* relative abundance was evident. Still, our findings suggest that the picocyanobacterium *Synechococcus* does not only occur in high abundances in eutrophic and cyanobacteria bloom presenting lakes but also that it may even influence the toxicity of other toxic cyanobacteria such as *Microcystis* sp. under some circumstances.

Contrary to bloom-forming cyanobacterial genera associated with high nutrient conditions (Chorus and Bartram, 1999; Paerl and Otten, 2013), picocyanobacteria, such as *Synechococcus*, can thrive in oligotrophic conditions (Sherr et al., 2005). However, *Synechoccoccus* is not exclusively found under low nutrient conditions. Cells belonging to the marine *Synechococcus* clade II have been detected in meso-eutrophic marine environments (Zwirglmaier et al., 2007, 2008). Whether there is also a high-nutrient-adapted *Synechococcus* clade in freshwater systems is still under investigation. Several *Synechococcus* OTUs were found in most samples of the two eutrophic lakes examined in this study. *Synechococcus* even dominated the cyanobacterial community composition in half of the Lake Klostersee samples. Therefore, our findings support the notion of a high nutrient adapted *Synechococcus* clade and emphasize its importance in freshwater. Our results therefore challenge the widely accepted view that picocyanobacteria do not play a significant role in eutrophic inland water bodies. Previous studies on cyanobacterial community composition may have overlooked *Synechococcus* in microscopic analysis due to its small size and low sedimentation speed or due to sampling bias (Eiler et al., 2013; Parulekar et al., 2017). Therefore, more research must be conducted on this important and ubiquitous cyanobacterium to elucidate its role in meso- and eutrophic freshwater bodies and in bloom toxicity.

6.2.2 Bacteriophages in cyanobacterial blooms

Both the results from laboratory experiments as well as from field studies hint towards cyanophages playing a pivotal role in toxic cyanobacterial blooms. This thesis showed that in the lakes sampled during the field studies, cyanophages were present and some of them were able to infect and lyse the toxic cyanobacterium *M. aeruginosa* (Figure 4.5). Those cyanophages might influence toxicity gene expression (5.5.1.3 Diversification within the dominant cyanobacterial genera). In addition, OTU-level analysis of the cyanobacteria community in both lakes revealed some hints that cyanophages might drive diversification in the cyanobacterial community composition. This is important from a water management perspective because it might severely impact bloom dynamics, and an apparently homogenous bloom might consist of several different species.

6.2.3 The unknown role of heterotrophic bacteria in toxic blooms

In the field studies conducted in this thesis, selected OTUs such as OTU23 (Saprospiraceae), OTU5 (*Limnohabitans*), or OTU11 (Ellin60607) were found to correlate positively with potentially toxic cyanobacterial species (Appendix, Table 11.2). However, those correlations

and interesting temporal patterns in the heterotrophic bacterial community composition could not be directly tied to bloom toxicity. Nevertheless, by analysing how the community of heterotrophic bacteria interplays with the cyanobacterial composition, this work attempts to close a knowledge gap. The effect of heterotrophic bacteria on bloom toxicity or their connection to bloom dynamics is even less well understood than the interactions between different cyanobacteria. Despite recent research successes mainly made possible by the advances of culture independent techniques, there are not many clear patterns emerging. This is partly because most studies did not perform an in-depth analysis beyond phylum level in the bloom (Fortin et al., 2015; Parulekar et al., 2017). Others who found a large part of the heterotrophic bacteria community to change along bloom dynamics (Berry et al., 2016; Li et al., 2012b) contradict results from this thesis. For this reason, more research on interactions between bloom forming cyanobacteria and co-occurring heterotrophic bacteria is still needed.

6.3 Implications for water monitoring

Some of the results presented in this thesis may have serious implications for water monitoring considerations. The following section addresses these issues and indicates open research questions in this context.

6.3.1 The need for identification of the toxin producer cyanobacterium

One unanticipated finding in the field studies presented here was that the microcystin producer cyanobacterium was not the dominant cyanobacterium in the toxic blooms. While such an observation has been reported before (Dadheech et al., 2014; Lee et al., 2015), it cannot be made when the cyanobacterial community composition is analysed by microscopy. Wrong conclusions might be drawn especially when other potentially toxic cyanobacteria are present in high abundance, such as *Dolichospermum* spp. in our case (Figure 5.4).

The fact that low abundant species may be the primary toxin producers in a community, has potential implications for the development and use of remote sensing techniques that are proposed for the monitoring of potentially affected inland waters. Remote sensing with the help of satellites, planes, or unmanned aerial vehicles (UAVs) has the potential to offer a cost-effective alternative to traditional sampling based monitoring approaches. However, when the toxic species is not the dominant one, remote sensing approaches face the challenge to discriminate between different species of cyanobacteria based on their optical properties. This challenge must be addressed early on in the development process. Consequently, systematic studies on cyanobacteria optical properties in mixed cultures need to be conducted under

laboratory conditions. An ideal tool to do this is the ENVILAB setup because it allows for the controlled growth of phytoplankton and the characterisation of optical properties, such as attenuation, absorption, and fluorescence (Göritz et al., 2017).

To know the genus, or better yet, the exact species of the toxin producing cyanobacterium in a lake is useful for several reasons. First, different species of cyanobacteria might have a very different ecology, and therefore might be associated with different degrees of actual risk. An example would be the toxic cyanobacteria *Planktothrix rubescens* and *Planktothrix agardhii*. *P. rubscens* is usually associated with a lower risk than *P. agardhii* in recreational waters, because it is mostly located in the metalimnion of deep stratified lakes (Kurmayer et al., 2016). Second, different species and genera of cyanobacteria might be sensitive to different means of mitigation. For example, *Planktothrix* and other *Oscillatoria*-like cyanobacteria are more sensitive to water exchange but less sensitive to grazing by zooplankton (Kurmayer et al., 2016). Another example is the differential sensitivity of various species to manipulations in lake nutrient content. While diazotrophic cyanobacteria are usually less sensitive to nitrogen scarcity than non-diazotrophic species, the often practiced reduction of phosphorus inputs may favour non-nitrogen fixing cyanobacteria such as *Microcystis* spp. over Nostocales (Paerl and Otten, 2013; Sarma, 2012). However, a shift in community composition from diazotrophic *Dolichospermum* spp. to non-diazotrophic *Microcystis* sp., in Lake Bergknappweiher for example, could lead to higher microcystin concentrations because *Microcystis* sp. was the microcystin producer in this lake. It is not evident from the current literature whether a low abundant toxin producer is a frequent phenomenon or just an exception. The fact that this phenomenon was observed in both lakes studied prompts further studies to answer this question, ideally by conducting a systematic survey of a larger number of water bodies.

In this thesis, a culture-independent approach to identify the toxin producing cyanobacterium was used. While this was faster and more accurate than approaches that rely on strain isolation, culturing, and subsequent toxin measurements, it still involved the combination of results from several techniques such as high-throughput sequencing, clone libraries, and phylogenetic analysis. For future analysis, the development of a simpler technique to identify the toxin producer ideally at the species level in a lake is required. This would allow a more accurate assessment of the risks posed and would support more targeted mitigation efforts that are tailored to the specific cyanobacterium.

6.3.2 The potential of qPCR as a monitoring tool

One of the objectives of the field study in this thesis was to determine whether genetic markers determined by qPCR could be used as an alternative approach in bloom toxicity assessment instead of direct microcystin measurement. The results of this study indicate that qPCR measurements are sensitive enough and could be a reasonably good qualitative proxy for bloom toxicity. This findings are consistent with others studies (Al-Tebrineh et al., 2012; Martins et al., 2011; Ostermaier and Kurmayer, 2010) but disagree with others who found gene copy number assessed by qPCR not to be a good toxicity proxy (Beversdorf et al., 2015). Due to conflicting results from several studies, a recent review on the subject concluded that qPCR currently should be used as a supporting tool in combination with other methods for risk assessment (Pacheco et al., 2016). The results from the field study presented in this thesis support this notion because one sampling date with a high *mcyB* copy number determined by qPCR lacked measurable microcystin concentrations (Figure 5.8B).

Using qPCR for bloom toxicity assessment has several advantages over measurement of the toxin. On the one hand, it can give valuable information about the toxin producing genus of cyanobacterium if genus specific primers or probes are used. On the other hand, this technique is independent of toxin isoforms and suitable available standard solutions. This second aspect is certainly currently relevant for microcystins, but its implications might even be more significant when considering other cyanotoxins. Those toxins and their possibly existing isoforms are less well understood and standards and assays for routine measurement are not readily available for all of them. It is much easier and quicker to adapt the qPCR assay for the detection of additional toxin genes, than, for example, developing and optimizing new immunological tests. The occurrence of non-microcystin cyanotoxins in freshwater bodies is just being uncovered and not routinely assessed, yet. Nevertheless, qPCR-based methods are most likely one of the most effective ways to do so in upcoming studies.

6.4 Outlook

Building on the results presented and methods developed in this thesis, three main prospects should be considered in future research:

i. The study of further abiotic factors

   In this work, the molecular tools for the assessment of cyanobacterial toxin biosynthesis gene expression were established (3 Influence of temperature, mixing, and addition of

microcystin-LR on microcystin gene expression in *Microcystis aeruginosa*). At the same time a spectrometric bioreactor setup was developed in a related project (Göritz et al., 2017). Both advances provide ideal prerequisites to investigate the effects of additional and so far neglected environmental parameters on toxic cyanobacteria. One such factor could be the underwater light field.

ii. The in depth analysis of interactions with biotic factors

Field and laboratory studies in this work showed that certain biota such as picocyanobacteria might affect toxic algal blooms. However, to date little is known about the nature of the interactions between them and toxin producing cyanobacteria.

iii. Improving the identification of toxic cyanobacteria

It was demonstrated in this thesis that pinpointing a bloom's toxin producer cyanobacterium is not trivial. This shows the need for developing tools for not only detecting harmful algal blooms but also reliably identifying the toxic species. Such tools could encompass molecular methods and/or remote sensing methods and ideally should be rapid, cost-effective, and easy to use.

7 Conclusion

This study set out to explore the effects of abiotic and biotic environmental variables on the toxic potential of cyanobacteria. The findings of this research strengthen the idea that temperature increase in the context of climate change might increase risks associated with toxic cyanobacteria blooms. This research also expands our knowledge about the interactions between toxic cyanobacteria and other microorganism and highlights the considerable temporal variation in cyanobacterial communities. To put it all in a nutshell, this thesis highlights the complexity of interspecies interactions at the molecular level. It supports and expands current knowledge on the influence of abiotic factors on cyanobacteria. Further studies therefore can build on techniques and findings established in this thesis to address other decisive factors for bloom toxicity.

8 Publication list

8.1 Peer-reviewed publication included in this thesis

Scherer PI, Raeder U, Geist J, Zwirglmaier K. 2017. Influence of temperature, mixing, and addition of microcystin-LR on microcystin gene expression in *Microcystis aeruginosa*. MicrobiologyOpen 6(1):e00393.

Scherer PI, Absmeier C, Urban M, Raeder U, Geist J, Zwirglmaier K. 2017. Influence of cyanobacteria, mixotrophic flagellates, and virioplankton size fraction on transcription of microcystin synthesis genes in the toxic cyanobacterium *Microcystis aeruginosa*. MicrobiologyOpen; e538.

Scherer PI, Millard AD, Miller A, Schoen R, Raeder U, Geist J, Zwirglmaier K. Temporal dynamics of the microbial community composition with a focus on toxic cyanobacteria and toxin presence during harmful algal blooms in two South German lakes. Frontiers in Microbiology (accepted).

8.2 Selected oral contributions related to this thesis

Scherer PI, Göritz A. Identification and monitoring of toxic cyanobacteria. FA Water Workshop, Garching, Germany, February 2015

Scherer PI, Geist J, Zwirglmaier K. How toxigenic cyanobacterium *Microcystis aeruginosa* responds to its environment on the gene expression level. International Symposium on Phototrophic Prokaryotes, Tübingen, Germany, July 2015

Scherer PI, Göritz A, Gege P, Ruber J, Geist J, Bamler R, Zwirglmaier K. Identification and monitoring of toxic cyanobacteria. FA Water Midterm Meeting, Freising, Germany, April 2016

Scherer PI, Göritz A, Gege P, Raeder U. Influence of biotic and abiotic factors on the toxicity of microcystin producing cyanobacteria. Universität Göttingen, Germany, May 2016 (invited)

Scherer PI, Göritz A. Von der Mikrobiologie zur Fernerkundung – Cyanobakterien in Inlandgewässern. Umweltbundesamt, Dessau-Roßlau, Germany, May 2017 (invited)

8.3 Selected poster presentations related to this thesis

Scherer PI, Geist J, Zwirglmaier K. Phylogenetic analysis of genes involved in microcystin biosynthesis and their diversity in Lake Ammersee, Bavaria. 9th European Workshop on the Molecular Biology of Cyanobacteria, Texel, Netherlands, September 2014

Ruber J, Scherer PI, Kar A, Göritz A, Zwirglmaier K, Geist J, Gege P, Bamler R. IMOTOX Identification and monitoring of toxic cyanobacteria. 8th IGSSE Forum, Burghausen, Germany, July 2014

Scherer PI, Geist J, Zwirglmaier K. Gene expression studies on toxicity genes of *Microcystis aeruginosa* in response to environmental stressors. Fresh Blood for Fresh Water, Mondsee, Austria, April 2015

Ruber J, Scherer PI, Kar A, Göritz A, Zwirglmaier K, Geist J, Gege P, Bamler R. IMOTOX Identification and monitoring of toxic cyanobacteria. 9th IGSSE Forum, Burghausen, Germany, July 2015 (Vodafone poster award)

Ruber J, Scherer PI, Kar A, Göritz A, Zwirglmaier K, Geist J, Gege P, Bamler R. IMOTOX Identification and monitoring of toxic cyanobacteria. 10th IGSSE Forum, Burghausen, Germany, June 2016

Ruber J, Scherer PI, Göritz A, Riedel S, Zwirglmaier K, Geist J, Gege P, Bamler R. IMOTOX Identifizierung und Monitoring giftiger Cyanobakterien. Raitenhaslach opening exhibition, Burghausen, Germany, June 2016

Ruber J, Scherer PI, Göritz A, Riedel S, Zwirglmaier K, Raeder U, Geist J, Gege P, Bamler R. IMOTOX, quo vadis? Identification and monitoring of toxic cyanobacteria (Water 01). 11th IGSSE Forum, Burghausen, Germany, May 2017

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11 Appendix

11.1 Statistics results ANOVA

Table 11.1: ANOVA results. ANOVA: df: degrees of freedom, F: F-value, p: p-value; Tukey's post hoc test: SEM: standard error of the mean, ND: not determined, upper number indicating result of statistic test for mcyB, lower number indicating result of statistic test for mcyD.

		ANOVA				Tukey's post hoc test			
		df between groups	df within groups	*F*	*p*		SEM	Mean fold change	Tukey's post hoc *p*
Cyanobacteria	*mcyB*	2	15	12.68	<0.001	control	0.08	1.00	
	mcyD	2	15	5.02	0.021		0.07	1.00	
						spent medium	0.09	1.70	0.003
							0.11	1.59	0.064
						cells	0.08	1.76	0.001
							0.08	1.73	0.025
Mixotrophic flagellates	*mcyB*	2	15	1.20	0.329	control	0.22	1.00	
	mcyD	2	14	4.40	0.033		0.15	1.00	
						10^3 cells/mL	0.10	0.86	ND
							0.06	0.55	0.078
						10^4 cells/mL	0.03	0.75	ND
							0.02	0.50	0.049
Lake Bergknappweiher virioplankton size fraction (no bloom)	*mcyB*	3	8	4.61	0.037	control	0.07	1.00	
	mcyD	3	8	3.02	0.094		0.06	1.00	
						autoclaved phages	0.07	1.28	0.572
							0.08	1.07	ND
						env. phages	0.14	1.42	0.052
							0.10	1.26	ND
						phage concentrate	0.10	1.39	0.062
							0.06	1.29	ND
Lake Bergknappweiher virioplankton size fraction (algal bloom)	*mcyB*	3	8	16.25	<0.001	control	0.10	1.00	
	mcyD	3	8	41.48	<0.001		0.09	1.00	
						autoclaved phages	0.10	1.24	0.240
							0.09	1.37	0.020
						env. phages	0.09	1.62	0.003
							0.14	1.79	<0.001

		ANOVA				Tukey's post hoc test		
		df between groups	df within groups	*F*	*p*		SEM	Mean fold change	Tukey's post hoc *p*
						phage	0.12	1.70	0.002
						concentrate	0.09	1.96	<0.001
Lake Klostersee virioplankton size fraction (algal bloom)	*mcyB*	2	7	8.25	0.026	control	0.13	1.00	
	mcyD	2	7	5.85	0.049		0.10	1.00	
						env. phages	0.20	0.96	0.998
							0.23	0.84	0.967
						phage concentrate	0.30	2.06	0.031
							0.27	2.11	0.064
Humic acid preparation	*mcyB*	2	7	0.93	0.455	control	0.05	1.00	
	mcyD	2	7	5.29	0.058		0.06	1.00	
						humic acid 1x	0.03	0.92	ND
							0.04	1.00	ND
						humic acid 2.5x	0.21	0.92	ND
							0.24	1.32	ND

11.2 Specificity of *Dolichospermum mcyE* primer

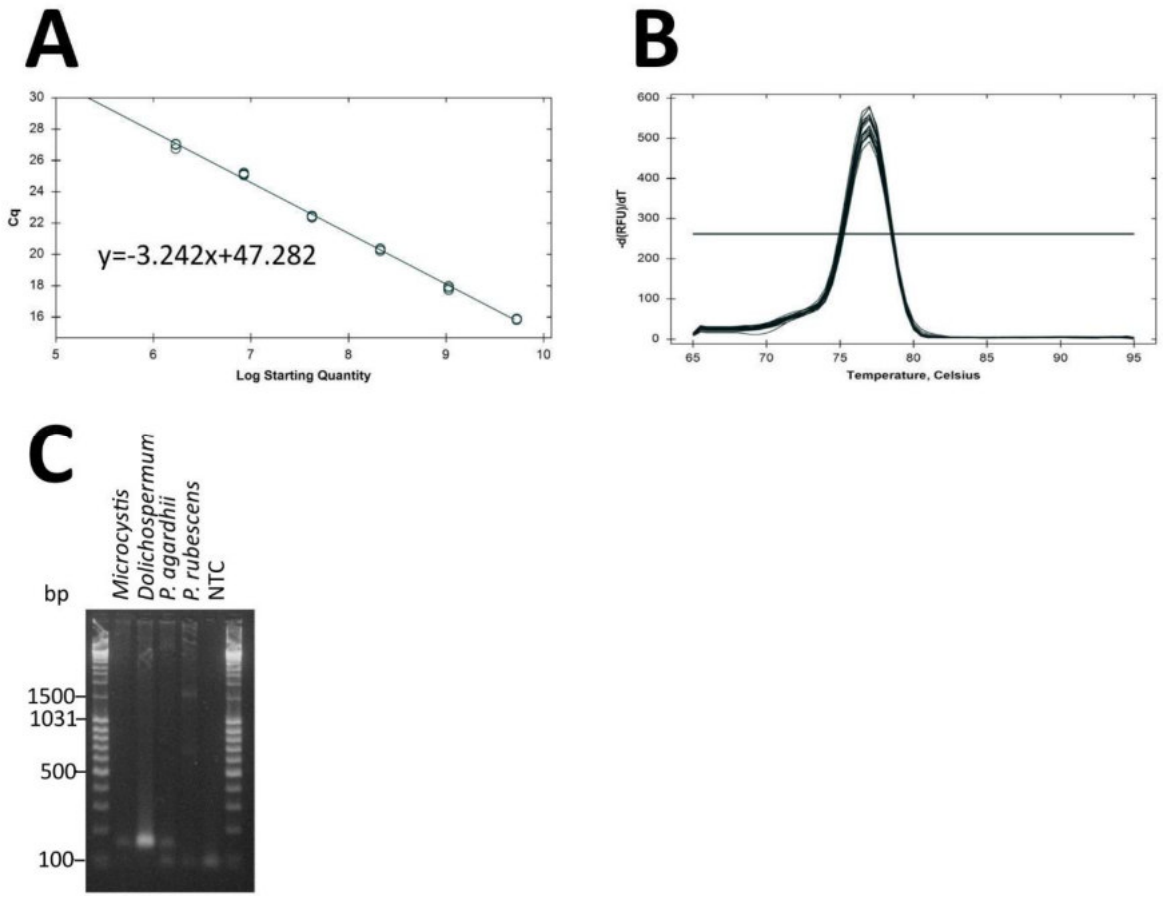

Figure 11.1: qPCR assay for the detection of Dolichospermum mcyE. (A+B) Standard curve. (A) Samples were measured in triplicates. Efficiency of standard curve is 103.4 % indicating near perfect doubling of PCR product in each cycle. R^2 value of standard curve is 0.998 indicating good linearity and low variability between technical replicates. Standard curve covers a dynamic range from $1.69x10^3$ to $5.28x10^6$ copies/reaction. (B) Each dilution of standard curve shows a single symmetrical melting curve peak indicating a single product. (H) qPCR products of specificity test on 2 % agarose gel. From left to right: MassRuler DNA Ladder Mix (Thermo Scientific, Waltham, US), MassRuler DNA Ladder Mix (Thermo Scientific, Waltham, US). Significant product of the right size is only present for D. lemmermanni as template.

11.3 Correlation analysis

Table 11.2: Correlation of heterotrophic bacteria with cyanobacteria. Pearson's correlation coefficient and p-value in brackets. Results with correlation coefficients > 0.3 or < -0.3 and p-values < 0.05 are shown in bold indicating a significant correlation.

Common non-cyanobacteria	Microcystis	Dolicho-spermum	Dolicho-spermum OTU1	Synecho-coccus
Actinomycetales ACK-M1 OTU9	-0.06 (0.78)	**-0.73 (3.44E-5)**	**-0.49 (0.01)**	**0.64 (5.58E-4)**
Actinomycetales ACK-M1 OTU20	-0.03 (0.88)	**-0.41 (0.04)**	-0.26 (0.21)	**0.54 (0.01)**
Actinomycetales ACK-M1 OTU13	0.39 (0.34)	**-0.75 (1.35E-5)**	**-0.50 (0.01)**	**0.63 (8.24E-4)**
Acidimicrobiales OTU12	-0.06 (0.78)	**-0.43 (0.03)**	**-0.46 (0.02)**	**0.49 (0.01)**
Saprospiraceae OTU23	**0.51 (0.01)**	**-0.53 (0.01)**	**-0.43 (0.03)**	0.21 (0.32)
Sphingomonadales OTU2	-0.21 (0.32)	**0.44 (0.03)**	**0.60 (0.001)**	-0.34 (0.09)
Pelagibacteraceae OTU3	0.24 (0.26)	**-0.76 (1.07E-5)**	**-0.51 (0.01)**	**0.74 (2.08E-5**)
***Limnohabitans* OTU5**	-0.09 (0.68)	0.31 (0.13)	**0.49 (0.01)**	-0.21 (0.32)
Ellin60607 OTU11	-0.23 (0.26)	**0.46 (0.02)**	**0.64 (5.9E-4)**	-0.35 (0.09)

Table 11.3: Correlation of chloroplasts with cyanobacteria and environmental factors. Pearson's correlation coefficient and p-value in brackets. Results with correlation coefficients > 0.3 or < -0.3 and p-values < 0.05 are shown in bold indicating a significant correlation.

Environmental parameters	Chloroplast reads (total)	Crypto-phyta	Strameno-piles	Euglenozoa	Hapto-phyceae
Total phosphorus	-0.41 (0.05)	-0.25 (0.26)	-0.26 (0.24)	-0.02 (0.96)	**-0.42 (0.05)**
$N_{inorg.}$/P	0.17 (0.46)	0.31 (0.17)	0.24 (0.28)	-0.08 (0.72)	**0.53 (0.01)**
pH	-0.07 (0.74)	-0.14 (0.52)	-0.02 (0.93)	**0.53 (0.01)**	-0.12 (0.59)
Water temperature	**-0.54 (0.01)**	**-0.52 (0.01)**	**-0.69 (2.21E-4)**	0.29 (0.18)	0.30 (0.16)
Secchi depth	0.13 (0.53)	-0.04 (0.86)	0.02 (0.94)	-0.14 (0.50)	**0.49 (0.02)**
Conductivity	0.13 (0.55)	-0.07 (0.75)	0.09 (0.68)	-0.25 (0.24)	**0.51 (0.01)**
Oxygen	0.13 (0.56)	-0.01 (0.97)	0.14 (0.51)	**0.49 (0.02)**	0.04 (0.86)
NO_3-nitrogen	-0.24 (0.26)	0.06 (0.78)	-0.21 (0.34)	0.24 (0.26)	**-0.64 (9.46E-4)**
Ammonium	0.41 (0.05)	0.22 (0.31)	**0.54 (0.01)**	-0.12 (0.57)	0.11 (0.61)
Cyanobacteria (total)	**-0.49 (0.01)**	-0.21 (0.32)	-0.31 (0.14)	0.10 (0.65)	**-0.64 (6.05E-4)**
Chroococcales	-0.05 (0.82)	-0.09 (0.65)	0.05 (0.80)	-0.01 (0.97)	0.002 (0.99)
Nostocales	**-0.46 (0.02)**	-0.21 (0.32)	-0.30 (0.14)	0.11 (0.59)	**-0.65 (4.30E-4)**
Pseudana-baenales	**0.47 (0.02)**	0.22 (0.29)	**0.70 (9.10E-05)**	-0.10 (0.63)	0.07 (0.76)
Synecho-coccales	-0.15 (0.46)	-0.04 (0.86)	-0.33 (0.11)	-0.08 (0.71)	0.13 (0.53)

Environmental parameters	Chloroplast reads (total)	Crypto-phyta	Stramenopiles	Euglenozoa	Haptophyceae
mcyB	-0.20 (0.33)	-0.11 (0.61)	-0.09 (0.68)	-0.11 (0.61)	-0.26 (0.21)
Microcystin (ELISA)	-0.12 (0.57)	-0.12 (0.57)	0.02 (0.94)	-0.09 (0.69)	-0.14 (0.50)
Microcystin (HPLC)	0.07 (0.74)	-0.05 (0.83)	0.25 (0.25)	-0.06 (0.77)	-0.06 (0.80)

Table 11.4: Correlation of water quality parameters. Pearson's correlation coefficient and p-value in brackets. Results with correlation coefficients > 0.3 or < -0.3 and p-values < 0.05 are shown in bold indicating a significant correlation.

Toxicity parameters	*mcyB*	Total phos-phorus	$N_{inorg.}/P$	pH	Water tempe-rature	Secchi depth	Con-ductivity	Oxygen	NO_3-nitrogen	Ammo-nium
mcyB		**0.56 (0.01)**	**-0.61 (0.003)**	-0.23 (0.28)	-0.32 (0.13)	-0.31 (0.14)	-0.26 (0.21)	-0.14 (0.50)	0.31 (0.15)	-0.22 (0.32)
Microcystin (ELISA)	**0.61 (1.53E-3)**	**0.59 (0.003)**	**-0.63 (0.002)**	-0.25 (0.25)	-0.19 (0.40)	-0.12 (0.59)	-0.01 (0.96)	-0.16 (0.46)	0.08 (0.71)	0.04 (0.84)
Microcystin (HPLC)	**0.44 (0.04)**	0.35 (0.11)	**-0.49 (0.02)**	-0.21 (0.34)	-0.28 (0.19)	-0.03 (0.89)	0.05 (0.84)	-0.20 (0.37)	-0.04 (0.84)	0.16 (0.46)

Table 11.5: Correlation of cyanobacteria with environmental factors. Pearson's correlation coefficient and p-value in brackets. Results with correlation coefficients > 0.3 or < -0.3 and p-values < 0.05 are shown in bold indicating a significant correlation.

Environmental parameters	Microcystis	Dolicho-spermum	Chroococcales OTU594	Gomphos-phaeriaceae	Snowella OTU65	Pseudana-baenaceae	Pseudana-baena OTU71	Synecho-coccus
Total phosphorus	**0.44 (0.03)**	**-0.45 (0.03)**	0.30 (0.16)	0.07 (0.74)	**0.86 (1.48E-7)**	-0.06 (0.80)	-0.05 (0.81)	**0.43 (0.04)**
$N_{inorg.}$/P	-0.39 (0.07)	0.35 (0.11)	-0.29 (0.19)	-0.19 (0.40)	**-0.54 (0.01)**	-0.04 (0.86)	0.19 (0.41)	-0.32 (0.14)
pH	**0.52 (0.01)**	**-0.45 (0.03)**	**0.44 (0.03)**	0.35 (0.09)	0.11 (0.58)	0.11 (0.60)	-0.002 (0.99)	0.26 (0.22)
Water temperature	-0.33 (0.11)	0.16 (0.44)	-0.09 (0.67)	-0.31 (0.14)	0.16 (0.45)	-0.39 (0.06)	**-0.48 (0.02)**	0.24 (0.26)
Secchi depth	-0.32 (0.13)	**0.65 (6.56E-4)**	-0.27 (0.21)	-0.27 (0.20)	**-0.46 (0.02)**	-0.35 (0.10)	-0.17 (0.43)	**-0.47 (0.02)**
Conductivity	**-0.51 (0.01)**	**0.91 (1.19E-9)**	**-0.50 (0.01)**	**-0.50 (0.01)**	-0.35 (0.09)	**-0.44 (0.03)**	-0.32 (0.13)	**-0.59 (0.003)**
Oxygen	**0.47 (0.02)**	-0.09 (0.68)	**0.40 (0.06)**	0.15 (0.49)	0.01 (0.98)	0.002 (0.99)	0.12 (0.58)	-0.12 (0.58)
NO_3-nitrogen	**0.44 (0.04)**	**-0.93 (1.68E-10)**	**0.50 (0.01)**	0.38 (0.07)	**0.48 (0.02)**	0.34 (0.12)	0.28 (0.20)	**0.69 (2.76E-4)**
Ammonium	-0.21 (0.34)	**0.43 (0.04)**	-0.25 (0.25)	-0.26 (0.23)	-0.20 (0.35)	-0.26 (0.23)	-0.14 (0.52)	-0.27 (0.22)

11.4 Water parameters in field studies

Table 11.6: Chemical and physical water parameters for each sampling date, ND = value not determined, LOD = limit of detection.

Lake	Sampling time point	Micro-cystin ELISA (µg/L)	Micro-cystin HPLC (µg/L)	Visible surface bloom	Total phos-phorus (µg/L)	$N_{inorg.}$/P (stoi-chiome-tric)	pH	Water Tempe-rature (°C)	Secchi depth (m)	Con-ductivity (µS/cm²)	Oxygen (mg/L)	NO_3-nitrogen (mg/L)	Ammoni-um (mg/L)
Lake Kloster-see	29 May 15	ND	ND	yes	ND	ND	8.09	19.0	1.00	431	9.16	ND	ND
	12 Jun 15	1.0	< LOD[b]	yes	80	12	8.36	21.3	0.75	423	10.52	0.29	0.19
	25 Jun 15	1.0	< LOD	yes	57	18	8.40	19.5	0.75	415	12.22	0.35	0.15
	09 Jul 15	< LOD[a]	< LOD	yes	60	22	8.06	24.3	0.80	414	6.71	0.42	0.23
	23 Jul 15	< LOD	< LOD	yes	39	28	8.07	26.1	1.50	405	7.33	0.36	0.16
	07 Aug 15	< LOD	< LOD	no	47	21	8.52	24.9	0.75	399	10.13	0.30	0.17
	12 Aug 15	< LOD	< LOD	no	43	20	8.22	25.8	0.75	390	9.46	0.40	0.00
	20 Aug 15	1.2	< LOD	yes	160	5	7.85	21.6	0.75	398	ND	0.39	0.00
	03 Sept 15	< LOD	< LOD	no	62	11	7.89	21.7	0.60	380	6.97	0.32	0.00
	17 Sept 15	< LOD	< LOD	no	52	18	8.67	18.5	0.40	377	12.33	0.34	0.12
	01 Oct 15	< LOD	< LOD	yes	67	18	8.32	13.6	0.75	392	9.83	0.37	0.23
	15 Oct 15	5.0	5.5	yes	230	5	8.16	11.4	0.70	396	8.48	0.29	0.24
	29 Oct 15	< LOD	< LOD	no	68	25	7.95	10.1	0.90	410	8.05	0.40	0.48

Lake	Sampling time point	Micro-cystin ELISA (µg/L)	Micro-cystin HPLC (µg/L)	Visible surface bloom	Total phos-phorus (µg/L)	$N_{inorg.}$/P (stoi-chiome-tric)	pH	Water Tempe-rature (°C)	Secchi depth (m)	Con-ductivity (µS/cm²)	Oxygen (mg/L)	NO_3-nitrogen (mg/L)	Ammoni-um (mg/L)
Lake Berg-knapp-weiher	19 Aug 15	< LOD	ND	no	282	ND	ND	ND	ND	ND	ND	ND	ND
	21 Aug 15	1.4	< LOD	yes	1,034	2	8.51	20.7	0.15	257	10.78	1.08	0.06
	27 Aug 15	< LOD	< LOD	yes	181	13	9.13	25.1	0.45	243	14.85	1.00	0.06
	02 Sept 15	< LOD	< LOD	yes	155	16	8.20	21.9	0.50	256	5.20	1.09	0.06
	08 Sept 15	6.7	3.4	yes	834	3	8.08	15.4	0.40	255	7.79	1.07	0.06
	17 Sept 15	< LOD	< LOD	no	162	13	8.54	18.2	0.40	253	8.82	0.92	0.06
	23 Sept 15	< LOD	< LOD	no	204	10	8.70	15.7	0.50	253	10.21	0.83	0.05
	29 Sept 15	< LOD	< LOD	no	132	15	8.49	14.8	0.40	253	11.80	0.85	0.07
	07 Oct 15	< LOD	< LOD	no	110	17	8.57	14.2	0.40	256	9.57	0.83	0.05
	14 Oct 15	< LOD	< LOD	yes	174	12	8.27	10.5	0.40	259	9.04	0.91	0.05
	21 Oct 15	< LOD	< LOD	yes	ND	ND	8.70	10.2	0.60	259	12.98	0.83	0.05
	30 Oct 15	< LOD	< LOD	no	94	22	8.15	9.7	0.50	270	9.81	0.87	0.06