Wissenschaftszentrum Weihenstephan für Ernährung,
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Characterization of cyanobacterial communities of Bavarian lakes based on the *16S rRNA* gene

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Preface

This study aims at contributing to the improvement of future monitoring strategies for freshwater cyanobacteria. Using the example of selected Bavarian lakes, the diversity of cyanobacterial communities was analysed, seasonal variations were studied and changes in community structures due to climate change effects were predicted. The work was accompanied by the development of molecular biology-based methods for the future identification of cyanobacteria.

The introduction of this thesis illustrates the roles of cyanobacteria in aquatic ecosystems and especially in lakes, their taxonomy and genetics, and the different methods used for the identification of this group of organisms. Following a description of the objectives of this thesis, four distinct research topics dealing with methodologies and cyanobacteria in freshwater lakes are presented. Chapters 3 and 5 have been submitted as autonomous research papers in a slightly modified form (following different journal requirements).

The thesis ends with a general discussion on the perspectives in future methods for the detection of cyanobacteria, on specific recommendations for the monitoring of cyanobacteria in their specific habitats and on environmental factors influencing cyanobacteria with predictions on climate change effects.

The new findings on the cyanobacterial communities in Bavarian lakes and the development of new methods as well as the conclusions drawn from the results are finally used to provide concrete advice and recommendations for future monitoring strategies.

Summary

Cyanobacteria are photosynthetic bacteria with a long evolutionary history. The phylum of cyanobacteria is ubiquitous, playing a major role in aquatic ecosystems. Cyanobacteria are a heterogeneous group of organisms. Their heterogeneity appears in their morphology but also in their physiological characteristics.

The main goal of this thesis was to provide concrete advice and recommendations for future monitoring strategies for freshwater cyanobacteria. Molecular biology-based methods, such as PCR-ELISA and quantitative real-time PCR specific for cyanobacterial genera were developed, natural community composition and diversity were monitored, and aquarium experiments were conducted to investigate the impact of climate change on cyanobacterial communities.

In the first study, new methods based on the detection of the *16S rRNA* gene of cyanobacteria were developed. It was possible to establish PCR-ELISA tests and quantitative real-time PCR assays for the detection and quantification of *Limnothrix*, *Microcystis* and *Planktothrix* genera. These methods including a recommended process flow represent powerful tools to support future research and monitoring of cyanobacteria.

The following study focussed on the diversity of cyanobacteria in four selected Bavarian lakes of different trophic status by application of next-generation sequencing technologies. Each lake harboured a specific cyanobacterial community. All studied lakes were dominated by a unique genus, accounting with one exception for more than 50% of the cyanobacterial sequence numbers from the respective sample. In addition to lake-specific differences, cyanobacterial composition was depth-dependent.

The key aspect of the third study was the seasonal monitoring of potentially toxic cyanobacteria by the application of different *16S rRNA* gene detecting methods. Quantitative real-time PCR was applied for the quantification of *Limnothrix* and *Planktothrix* genera in Lake Ammersee and Lake Schliersee. Illumina MiSeq sequencing revealed the natural succession during the different stages of a cyanobacterial bloom in Lake Bergknappweiher. The study proved the applicability of *16S rRNA* gene-based methods for the monitoring of cyanobacteria, which is an important task to extent the knowledge on this group of organisms.

In the fourth study, the potential impacts of global warming on freshwater cyanobacteria in Bavarian lakes were studied by the simulation of increased temperature in aquarium experiments. As a novelty fact, it could be demonstrated that temperature effects on cyanobacterial communities vary depending on the season when temperature increase occurred. As a further result, temperature effects were more severe under nutrient-rich conditions and picocyanobacterial communities were more stable. The results indicate that a

profound knowledge on the current lake-specific cyanobacteria communities is the core basis for predictions on local climate change effects.

In conclusion, the case studies revealed that future cyanobacteria monitoring should be more lake specific. The combination of traditional and molecular biology-based methods is recommended. The study of cyanobacteria deserves more consideration than ever before, as their natural habitats are undergoing changes in consequence of climate change.

Zusammenfassung

Cyanobakterien sind evolutionsgeschichtlich sehr alte, photosynthetisch aktive Bakterien. Der Stamm der Cyanobakterien ist ubiquitär vorhanden und spielt vor allem in aquatischen Ökosystemen eine große Rolle. Cyanobakterien sind eine sehr heterogene Organismengruppe, deren Vielfältigkeit sich sowohl in ihrer Morphologie als auch in ihren physiologischen Eigenschaften äußert.

Das übergeordnete Ziel dieser Arbeit war es, Empfehlungen für zukünftige Monitoringstrategien von Cyanobakterien in Seen zu geben. Dazu wurden kosteneffektive molekularbiologische Methoden zur Detektion und Quantifizierung von Cyanobakterien entwickelt und die natürliche Zusammensetzung von Populationen, deren Diversität und Saisonalität untersucht. Außerdem wurden Aquarienversuche durchgeführt, um den Einfluss des Klimawandels auf diese Organismengruppe zu analysieren.

In der ersten Studie wurden neue Methoden etabliert, die auf dem Nachweis des *16S rRNA* Gens von Cyanobakterien basieren. Es wurden die Methode des PCR-ELISAs, sowie quantitative Echtzeit-PCR Nachweise entwickelt und optimiert. Beide Ansätze ermöglichen die Bestimmung und Quantifizierung der Gattungen *Limnothrix*, *Microcystis* und *Planktothrix*. Auf diese Methoden und die dazu empfohlene Anwendungsempfehlung kann zurückgegriffen werden, um die zukünftige Forschung und das Monitoring von Cyanobakterien zu unterstützen.

In der zweiten Studie wurde die Diversität von Cyanobakterien in vier ausgewählten bayerischen Seen unterschiedlichen Nährstoffgehalts untersucht. Dabei kamen „Next-Generation“ Sequenzierungstechnologien zum Einsatz. Jeder See wies eine eigene charakteristische Cyanobakteriengemeinschaft auf. Alle Seen wurden von einer Hauptgattung dominiert, die mit einer Ausnahme immer mehr als 50% der Sequenzen der jeweiligen Probe aufwies. Zusätzlich zu den See-spezifischen Unterschieden war die Zusammensetzung der Cyanobakterienpopulationen auch tiefenabhängig.

In der dritten Studie wurde das Vorkommen und die Saisonalität potentiell toxischer Cyanobakterien im Jahresverlauf untersucht. Dies geschah unter Anwendung verschiedener Methoden, die das *16S rRNA* Gen detektieren. Mittels quantitativer Echtzeit-PCR wurden die Zellzahlen der Gattungen *Limnothrix* und *Planktothrix* im Ammersee und im Schliersee bestimmt. Die natürliche Sukzession während der verschiedenen Stadien einer Cyanobakterienblüte im Bergknappweiher wurde mittels Illumina MiSeq Sequenzierung aufgedeckt. Die Studie zeigte, dass *16S rRNA* Gen-basierte Methoden für das Monitoring von Cyanobakterien angewendet werden können. In Zukunft muss die Erweiterung des Wissens

über diese Organismengruppe eine immer größere Wichtigkeit erlangen. Dabei können die entwickelten Methoden von großem Nutzen sein.

In der vierten Studie wurden die möglichen Auswirkungen des Klimawandels auf Cyanobakterien in bayerischen Seen untersucht. Dazu wurde in Aquarienversuchen eine Temperaturerhöhung simuliert. Es konnte dabei zum ersten Mal gezeigt werden, dass die Auswirkungen des Klimawandels auf die Cyanobakteriengemeinschaften von der Jahreszeit abhängen, in der die Temperaturerhöhung auftritt. Außerdem konnte gezeigt werden, dass Temperatureffekte vor allem unter nährstoffreichen Bedingungen zu beobachten sind und dass Picocyanobakteriengemeinschaften eine höhere Stabilität aufweisen. Die Ergebnisse zeigen, dass ein tiefergehendes Wissen über die aktuell in Seen vorhandenen Cyanobakteriengemeinschaften eine wichtige Grundlage darstellt, um Aussagen über zukünftige Auswirkungen des Klimawandels treffen zu können.

Zusammenfassend zeigten die einzelnen Studien, dass ein zukünftiges Monitoring von Cyanobakterien See-spezifischer sein muss. Traditionelle und molekularbiologische Methoden sollten dabei kombiniert zur Anwendung kommen. Die Untersuchung von Cyanobakterien ist auf Grund der schwerwiegenden Habitat-Veränderungen, die durch den Klimawandel zu erwarten sind, wichtiger als je zuvor.

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1. Introduction

1.1 The roles of cyanobacteria in aquatic ecosystems

Cyanobacteria form the biggest and most diverse group of photosynthetic bacteria (Schlegel, 1992). Formerly, cyanobacteria have been called "blue-green algae" due to their morphology, their physiological characteristics and their ability to perform photosynthesis, which they have in common with the eukaryotic group of algae (Schlegel, 1992). Although their morphological diversity and size correspond to other microalgae as well as their function in natural biotopes (Palinska & Surosz, 2014), they undoubtedly are classified to the bacteria. Cyanobacteria have a long evolutionary history of about 3.5 billion years. Through the endosymbiotic incorporation with eukaryotes, cyanobacteria became the original source of oxygenic photosynthesis and are responsible for most of the planetary primary production (Palinska & Surosz, 2014). Cyanobacteria have developed various physiological features, such as tolerance to low oxygen conditions, ultraviolet radiation, high temperatures (up to 73 °C) and low pH values (pH < 5.0), adaption to low light, efficient nutrient uptake kinetics and tolerance to desiccation and water stress (Graham & Wilcox, 2000; Whitton & Potts, 2000; Sarma, 2013). They are well adapted to various habitats and can grow in extreme environments (Sarma, 2013). Some cyanobacterial genera are diazotrophic and have the ability to fix elementary nitrogen. Cyanobacteria occur in terrestrial, marine and freshwater ecosystems across all latitudes. Due to their various physiological adaptations and features, cyanobacteria are important factors in earth's nutrient regimes (Schlegel, 1992). On the one hand, cyanobacteria fulfil essential ecological functions and are important contributors to global carbon and nitrogen budgets (Stewart & Falconer, 2008). On the other hand, cyanobacteria often have a negative reputation, as some cyanobacteria form harmful blooms and can produce toxic metabolites, the cyanotoxins.

1.2 Taxonomy of cyanobacteria and genetics

The taxonomy of cyanobacteria has been and still is controversial and disputed. One of the reasons for this controversy is that the prokaryotic cyanobacteria were originally regarded as eukaryotes. Therefore, there are systems which taxonomically treat cyanobacteria as eukaryotes (Lewin, 1976) while others taxonomically treat them as bacteria (Stanier et al., 1978). Historically, morphological features were used to define cyanobacterial taxa (Graham & Wilcox, 2000). During the last decades, the application of molecular methods has substantially changed the knowledge on these organisms. At present, a polyphasic approach - a compromise between bacteriological and botanical approaches combining phenotypic and molecular criteria - is considered as gold standard (Rippka et al., 1979; Komarék & Anagnostidis, 2005;

Komárek, 2010b; Komárek, 2013; Palinska & Surosz, 2014). One of the leading taxonomy specialists recommends a combination of molecular analyses (phylogenetic relationships, diversity of genotypes, diversification progresses and specification), morphological diversity (including variation in nature and in culture), ecological, eco-physiological and biogeographical information, as well as a correct formal designation of taxa that respects the rules of nomenclature (Komárek, 2006).

Cyanobacteria occur in various morphological forms and the group can be divided into five different morphological sections: *Chroococcales* (I), *Pleurocapsales* (II), *Oscillatoriales* (III), *Nostocales* (IV) and *Stigonematales* (V). Sections I and II comprise the unicellular species, sections III-V comprise the multicellular species (Schirrmeister, Antonelli & Bagheri, 2011; Sarma, 2013).

Table 1.1 Orders of Cyanobacteria and their features (adapted after Sarma (2013) according to Castenholz & Waterbury (1989) and Whitton & Potts (2000)).

Order	Features	Representatives
Chroococcales	unicellular or non-filamentous aggregates of cells; binary division; rarely form akinetes	*Gloeobacter*, *Microcystis*, *Synechococcus*
Pleurocapsales	unicellular or non-filamentous aggregates of cells, internal multiple fissions with production of daughter cells smaller than parent; or by a mixture of multiple fissions and binary fission	*Dermocarpa*, *Pleurocapsa*, *Xenococcus*
Oscillatoriales	binary division in one plane; trichomes do not form heterocysts; akinetes apparently not recorded	*Lyngbya*, *Oscillatoria*, *Phormidium*
Nostocales	binary division in one plane; one or more cells per trichome differentiate into a heterocyst; some produce akinetes	*Aphanizomenon*, *Cylindrospermopsis*, *Dolichospermum*
Stigonematales	binary division in more than one plane, apparently always ability to form heterocysts, some also form akinetes	*Fischeriella*, *Geitleria*, *Stigonema*

The classification of cyanobacteria is still reviewed by means of bacteriological methods as well as traditional botanical methods. The genetic identity and specific ecological roles are explored using molecular tools (Palinska & Surosz, 2014). The analysis of phylogenetic interrelationships among cyanobacteria, which provides insights in the early diversification of

the group, is largely based on analyses of the *16S rRNA* genes (Giovannoni et al., 1988). Other potential genes suitable for molecular investigations of cyanobacteria are the ITS region (Iteman et al., 2000) and *nifH* genes (Stewart et al, 1985; Zehr et al., 2003; Palinska & Surosz, 2014). The unification or division of species, genera and taxa of higher ranks is still done: lately, the classification of different Nostocales genera and *Synechococcus* has been revised (Honda, Yokota & Sugiyama, 1999; Rajaniemi et al., 2005; Wacklin, Hoffmann & Komárek, 2009; Komárek, 2010a; Komárek & Mareš, 2012; Palinska & Surosz, 2014).

1.3 Cyanobacteria in lakes

Cyanobacteria play a major role in the phytoplankton of lakes. Many factors promote their dominance (Hyenstrand, Blomqvist & Pettersson, 1998). Cyanobacteria can have a major impact on human health as well as on aquatic ecosystems and dominance of bloom-forming cyanobacteria is an increasing problem, especially in eutrophic lakes (Downing, Watson & McCauley, 2001).

Cyanobacteria follow the natural phytoplankton succession. The seasonal occurrence of cyanobacteria is dependent on nutrient and light supplies (Paerl, 2008). Other factors such as stratification behaviour and stratification state of the lake also affect the occurrence and predominance of cyanobacteria in general and cyanobacterial genera in detail (Reynolds, Oliver & Walsby, 1987). The impact on the ecosystem, as well as the impact on animal and human health depends on the specific composition of the cyanobacterial community of each lake, as only certain (mostly) bloom-forming cyanobacterial species and strains produce toxins during growth or decay (Downing, Watson & McCauley, 2001). Besides, there are many cyanobacterial genera, species and strains, which do not have toxic metabolites and support the ecological state of the lake by photosynthesis and primary production. Still others increase the available nitrogen compounds in lakes by fixation of elementary nitrogen (Howarth et al., 1988). Due to their often-high abundance in nutrient rich lakes, cyanobacterial communities of eutrophic and hypertrophic lakes are well-studied. Those communities are known for recurrent cyanobacterial blooms (Dokulil & Teubner, 2000). Furthermore, much is known about the occurrence of Microcystin-producing cyanobacteria. Microcystin is a hepatotoxic cyanotoxin. Besides the hepatotoxins, there exist dermatotoxic, neurotoxic and cytotoxic cyanotoxins (Sivonen, 1996). To remediate lake systems and to monitor cyanobacterial incidences, many lakes in Europe are monitored. So far, an exposure to harmful cyanobacteria was expected especially in nutrient rich lakes. In the course of climate change and global warming, changes in cyanobacterial predominance and community structures have

to be expected. Future impacts will vary on global, regional and local scales (Heino & Mykrä, 2008).

1.4 Identification of cyanobacteria

Detection and identification of cyanobacteria is in demand due to their immense role in aquatic environments, their high diversity connected with different characteristics and the connected impact on their environment. A reliable detection and identification is necessary for monitoring purposes, bloom events, early warning, as well as scientific purposes.

Identification of cyanobacteria is traditionally done by microscopy. This method is controversial, as many cyanobacteria, especially picocyanobacteria, can hardly be differentiated by eye. Furthermore, it has been shown that cyanobacteria can vary in their morphology with changing environmental and culture conditions (Rippka et al., 1979; Dor & Hornhoff, 1985; Castenholz & Waterbury, 1989; Palinska et al., 1996; Otsuka et al., 2000; Lyra et al., 2005; Rajaniemi et al., 2005; Palinska & Surosz, 2014).

In recent years, morphological identification of cyanobacteria was complemented by the application of other methods based on spectroscopic or molecular characteristics. This development has been evolved analogously to the application of molecular methods like barcoding for the identification of other groups (Damm, Schierwater & Hadrys, 2010; Schmidt et al., 2015; Belle et al., 2017).

Previous studies showed that the *in vivo* fluorescence method could be used for the detection and quantification of cyanobacteria (Gregor & Maršálek, 2005; Gregor, Maršálek & Šípková, 2007). This method is based on the detection and measurement of phycocyanin, an accessory pigment mainly prevalent in cyanobacteria. *In vivo* fluorescence makes it possible to detect cyanobacteria as a group. Nevertheless, it is not possible to determine the distinct community composition. Furthermore, signals are often false positive when a high number of eukaryotic algae is present (Randolph et al., 2008).

The quantitative mapping of cyanobacterial blooms is more and more frequently done based on hyperspectral measurements (Kutser, 2004; Simis, Peters & Gonz, 2005; Kutser et al., 2006; Randolph et al., 2008). The presence of phycocyanin enables the detection and quantification of the group of cyanobacteria using remote sensing technologies. Only restricted multispectral sensors are able to detect cyanobacteria due to their spectral band configuration (Kutser et al., 2006). In general, results are not reliable, if phycocyanin concentration is low and especially if phycocyanin abundance is low in relation to chlorophyll a (Randolph et al., 2008).

Furthermore, the comparable low spatial resolution is problematic for this field of research (Kutser, 2004). In fact, the spatial coverage and high revisiting times needed for near natural monitoring using multispectral sensors is still insufficient (Kutser et al., 2006). Nevertheless, under optimal conditions, there is a strong relation between phycocyanin concentration and biovolume measurements of cyanobacteria (Randolph et al., 2008).

Another method which has been used for the detection and analysis of cyanobacteria since the 1980s is flow cytometry. Especially in oceanographic research, this technique has been adapted rapidly after the commercial availability of flow cytometers. Nevertheless, the application of flow cytometry in freshwater phytoplankton research is difficult due to the wide variation in the characteristics (size and form) of freshwater phytoplankton assemblages (Cunningham, 1993). Flow cytometry is especially suitable for the detection of picocyanobacterial genera, like *Synechococcus* (Li & Wood, 1988; Olson et al., 1988; Blanchot & Rodier, 1996; Ruber et al., 2018).

Other methods for the identification of cyanobacteria are based on molecular biology. These methods often provide a more detailed analysis of the group of cyanobacteria. Different genes have been used for the detection of cyanobacteria in the past: *16S rRNA* gene, nitrogenase genes (*nifH*) or genes of the internal transcribed spacer (ITS) region. The choice of the appropriate gene for the study is depending on the research question. Due to the high importance and the wide use of the *16S rRNA* gene for the identification of prokaryotes since its publication in the 1980s (Woese, 1987), huge databases are available for the *16S rRNA* gene sequences of bacteria.

Molecular methods for the detection of cyanobacteria comprise polymerase chain reaction (PCR) and a variety of PCR-related methods like quantitative real-time PCR (qPCR), reverse-transcriptase PCR (RT-PCR) and nested PCR (Garcia-Pichel, 2008). These PCR methods are often used for functional analyses (*nifH* genes, encoding enzymes involved in the fixation of atmospheric nitrogen) or toxin genes.

Further molecular tools used for studies on cyanobacteria in the past are genetic markers, fluorescence in situ hybridization, genetic fingerprinting techniques and clone libraries. Clone libraries have formerly been used for the analysis of community structures and diversity in the past. Nowadays, modern next-generation sequencing (NGS) techniques are more up-to-date, provide larger datasets and are therefore about to replace traditional sequencing methods based on clone libraries. Due to the decreasing costs and the more frequent application of NGS technologies, databases on *16S rRNA* gene sequence data increase constantly.

A further possibility to get an insight in cyanobacterial communities is the analysis of cyanotoxins present in the sample. Cyanotoxins are determined using ELISA (Enzyme-Linked-Immuno-Sorbent-Assay) methods, HPLC (High Pressure Liquid Chromatography) or LC-MS/MS (Liquid Chromatography-tandem Mass Spectroscopy (Preußel et al., 2006; Fastner et al., 2007; Brient et al., 2009; Ballot et al., 2010). Nevertheless, toxicity is often determined without further analysis of the cyanobacterial genera or species present. Knowledge on the presence of certain toxins in the water sample can give some restricted information on the cyanobacteria present in the sample. Nevertheless, this information is very superficial and vague as many genera share the same toxins: one example is Microcystin, which can be produced by some strains of *Microcystis*, *Planktothrix* or *Dolichospermum* species. Only further investigation of the cyanotoxin genes could give more precise information on the producer present in the sample.

To sum up, methods based on spectroscopic characteristics enable the detection of cyanobacteria as a group, whereas microscopy and methods based on molecular characteristics allow the identification of genera and species. While molecular methods are sometimes used for scientific research, microscopy – with all its difficulties and uncertainities – is still the method of choice for monitoring purposes. An overview of the methods used for identification and quantification of cyanobacteria is given in Figure 1.1.

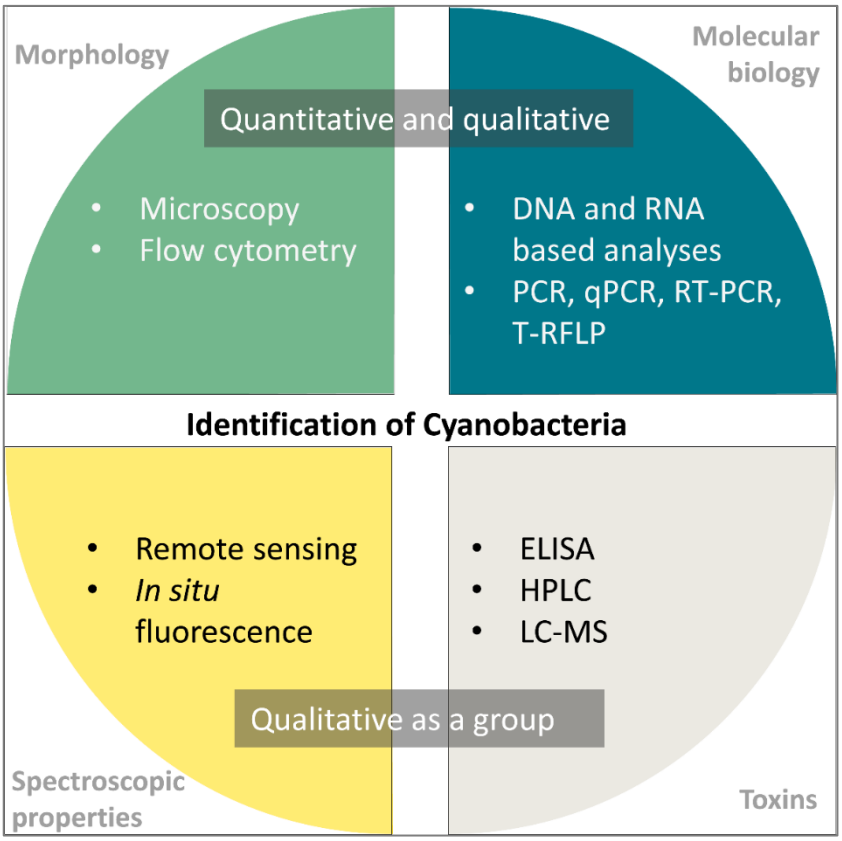

Figure 1.1 Different morphological approaches for the detection and identification of cyanobacteria.

Cyanobacteria are often still treated as a group without any differentiation, and efficient modern monitoring methods based on molecular information are missing. The development of more efficient methodologies is necessary. Due to the high variability within this group of organisms, identification on genus and species or even strain level is very important. To date, often only toxicity is determined, without further analysis of the cyanobacteria present.

Molecular methods based on *16S rRNA* gene information could be a promising approach for future studies or monitoring programmes as they enable the identification of cyanobacteria based on large datasets. For future implementation, a further development of methods in this field is necessary.

1.5 Objectives of the study

Due to the high importance of cyanobacteria in lakes, reliable and effective monitoring strategies are crucial. The knowledge about the composition of the cyanobacterial communities present in lakes is the basis for the analysis of the actual lake status, but it is also the basis for the prediction of future changes and of bloom-formation potential. Due to climate change, changes in community structures (invasive species, toxic strains) can be expected.

Difficulties in the morphological identification of cyanobacteria, the decrease of expertise in this field of research and the need of time- and cost-efficiency require progress.

The overall aim of this study was to provide concrete advice and recommendations for future monitoring strategies. Therefore, molecular biology-based methods to identify cyanobacteria based on their *16S rRNA* genes were developed and these methods were applied and validated in the field.

In order to address this research question, the main objectives of the study were:

1. The development of more advanced and cost-effective molecular biology-based methods for the detection of cyanobacteria.

2. The analysis of the cyanobacterial diversity in selected Bavarian lakes.

3. The seasonal monitoring of potentially toxic cyanobacteria during a normal vegetation period and during a visible surface bloom.

4. The simulation and investigation of the potential impacts of global warming on cyanobacteria in selected Bavarian lakes.

2. Development and comparison of methods for the detection of cyanobacteria

A similar version of this chapter is in preparation: Bauer, F. R., U. Raeder, J. Geist & K. Zwirglmaier. Development and comparison of methods for the detection of cyanobacteria.

2.1 Abstract

In this study, a PCR-ELISA method and a quantitative real-time PCR assay for the detection and quantification of cyanobacteria have been developed, applied on selected cyanobacterial genera, and comparatively discussed. The experimental procedures for the optimization of these two methods are described. Both methods are based on the analysis of the *16S rRNA* gene, a highly conserved region in bacterial genomes. Specific oligonucleotides serve as hybridization probes in the PCR-ELISA and primers in the qPCR. The PCR-ELISA is based on the principle of reverse hybridization. Probe concentrations and hybridization stringency were optimized for the PCR-ELISA test. The optimization of the qPCR assay focused mainly on the design of specific primer pairs and the thermal cycler conditions. A comparison of the developed methods with *16S rRNA* gene-based high-throughput sequencing methods was discussed. Based on the findings of this study, a future extension of the genus range of both methods is possible. Both methods could be used as efficient and easy tools for future monitoring of cyanobacteria.

2.2 Introduction

The identification of cyanobacteria is traditionally done morphologically using microscopy. German state water authorities still identify cyanobacteria in this way. The problem is, that especially small cyanobacteria are hard to identify, and the diverse phylum of cyanobacteria is often only summarized as one group without further specification (Paerl, 1977; Callieri & Stockner, 2000). The interest in cyanobacteria monitoring is rising further, as the importance of cyanobacteria in lakes is likely to increase due to eutrophication and climate change (Paerl & Huisman, 2009; Elliott, 2012; O'Neil et al., 2012). In parallel, morphological identification is difficult and the expertise in this field decreases (Komárek, 2016).

In the last years, the development of molecular biology-based methods has increased in any field of research. With these methods, it is possible to identify organisms based on their genotype. In the field of bacteria research, the *16S rRNA* gene is often used for phylogenetic studies, as it is present in every prokaryotic cell and highly conserved. Next-generation

sequencing (NGS) has opened a new dimension in biodiversity analysis and several high-throughput sequencing methods (e. g. Illumina sequencing) have become commercially available in the last years (Mardis, 2008; Metzker, 2010; Caporaso et al., 2012). Nevertheless, sequencing is not always the method of choice, especially when the interest of analysis is restricted to a certain group of organisms and quick results are needed. Therefore, faster and more specific methods and tests are in demand for future research.

The aim of this study was to develop and compare methods for the identification and quantification of cyanobacterial genera, based on the *16S rRNA* gene. We wanted to develop simple semi-quantitative PCR-ELISA (PCR-based enzyme-linked-immuno-sorbent assay) tests and quantitative real-time PCR (qPCR) assays for the detection and quantification of three important cyanobacterial genera: *Limnothrix*, *Microcystis* and *Planktothrix*. A PCR-ELISA is a combination of molecular biology-based methods (PCR, polymerase chain reaction, and hybridization) and serological methods (ELISA, enzyme-linked-immuno-sorbent-assay). The development of the PCR-ELISA test was based on earlier published methods of DNA-hybridization in microtiter plates (Behr et al., 2000; Schedl et al., 2000) and dot-blot-hybridization (Fuller et al, 2003; Zwirglmaier et al., 2007). Quantitative real-time PCR is a standard method in molecular biology based on the fluorometric detection of the PCR product amplified during the PCR (Heid et al., 1996; Klein, 2002). The experimental approach comprised the general establishment of the PCR-ELISA method and the optimization of the specific steps. The optimization of the qPCR assay focused mainly on the design of specific primer pairs and the optimization of the thermal cycler conditions in consideration of the MIQE guidelines (Bustin et al., 2009).

Here, we report the development of two *16S rRNA* gene-based methods for the detection of cyanobacteria and propose the potential areas of application. We discuss the pros and cons of both methods, also in comparison to Illumina MiSeq sequencing of *16S rRNA* genes.

2.3 Material and Methods

2.3.1 Cyanobacterial culture strains

The cyanobacterial culture strains serving as positive and negative controls in this study were: *Microcystis aeruginosa* (SAG 14.85), *Planktothrix rubescens* (SAG 5.89), *Limnothrix redekei* (SAG 3.89) and *Synechococcus elongatus* (PCC 7942). Strains were obtained from Culture Collection of Algae at Göttingen University (SAG) and from Pasteur Culture collection of Cyanobacteria (PCC).

The DNA of the culture strains was used as template for optimization steps. DNA from these strains was extracted using a phenol/chloroform-based extraction method described earlier (Zwirglmaier et al., 2015). DNA extraction was carried out in 2 ml tubes. To remove culture medium from the cyanobacterial cells, 2 ml of liquid culture were precipitated by centrifugation. Cells in the tubes were lysed in 2 ml DNA lysis buffer (0.25 M TRIS, 25% sterile filtered sucrose) and 20 µl lysozyme (50 mg/ml) at 37 °C. After 30 min, 200 µl SDS (10% w/v) and 15 µl Proteinase K (20 mg/ml) were added and the samples were incubated for another 2 h at 37 °C, followed by 30 min at 50 °C.

After this, 2 ml of phenol/chloroform/isoamylalkohol 25:24:1 were added. Subsequently, the samples were shaken vigorously and then centrifuged 5 min at 3300 × g. The upper aqueous phase was carefully transferred to a new 15 ml tube, 2 ml of chloroform were added, and tubes were shaken and centrifuged again for 5 min at 3300 × g. The upper aqueous phase was transferred to a fresh tube and DNA was precipitated with two volumes of ethanol and 0.1 volumes of 3 M sodium acetate, followed by 45 min centrifugation at 3300 × g and 4 °C. The DNA pellet was washed with 70% ethanol and re-suspended in TE buffer (10 mM TRIS, 1 mM EDTA, pH 8.0). DNA was stored at -80 °C until further use.

2.3.2 Design of oligonucleotide probes

Oligonucleotides used as capture hybridization probes in PCR-ELISA and primers in qPCR are called “probes” in the following. All oligonucleotides used in this study have been ordered via Biomers, Germany. The ARB software package (Ludwig et al., 2004) and an in-house cyanobacteria database, that comprises about 30,000 sequences, were used for the design and the *in silico* evaluation of the probes, when published probes were not available or did not deliver satisfactory results. The oligonucleotides used as capture probes and primers are specified in Tables 2.1 and 2.3.

Table 2.1 Oligonucleotides used for the PCR-ELISA test optimization.

Probe/ Primer	Target organism	Utilisation	Sequence (5' – 3')	Specification	Reference
PlanC	*Planktothrix* sp.	probe	CTGAGAAAAGGGGTTGAC	5'phosphorylated	This study
Limn667	*Limnothrix* sp.	probe	CACCTGGAATTCCTCCTG	5'-phosphorylated	This study
Micr3	*Microcystis* sp.	probe	TCTGCCAGTTTCCACCGCCTTTAGGT	5'-phosphorylated	Rudi et al., 2000
EUB	Eubacteria	probe	GCWGCCWCCCGTAGGWGT	5'-phosphorylated	Amann, 1990
27_f	Eubacteria	forward primer	AGAGTTTGATCMTGGCTCAG	5'-Digoxigenin-labelled or 5'-Biotin-labelled	Weißburg et al., 1991
1492_r	Eubacteria	reverse primer	GGYTACCTTGTTACGACTT	-/-	Weißburg et al., 1991

2.3.3 PCR-ELISA

Hybridization kinetics and stringency depend on primary, secondary and tertiary structure of the probe and the target DNA (Behrens et al., 2003; Zwirglmaier, 2003). Therefore, it was necessary to optimize the hybridization parameters for each PCR-ELISA assay.

2.3.3.1 Preliminary optimizations

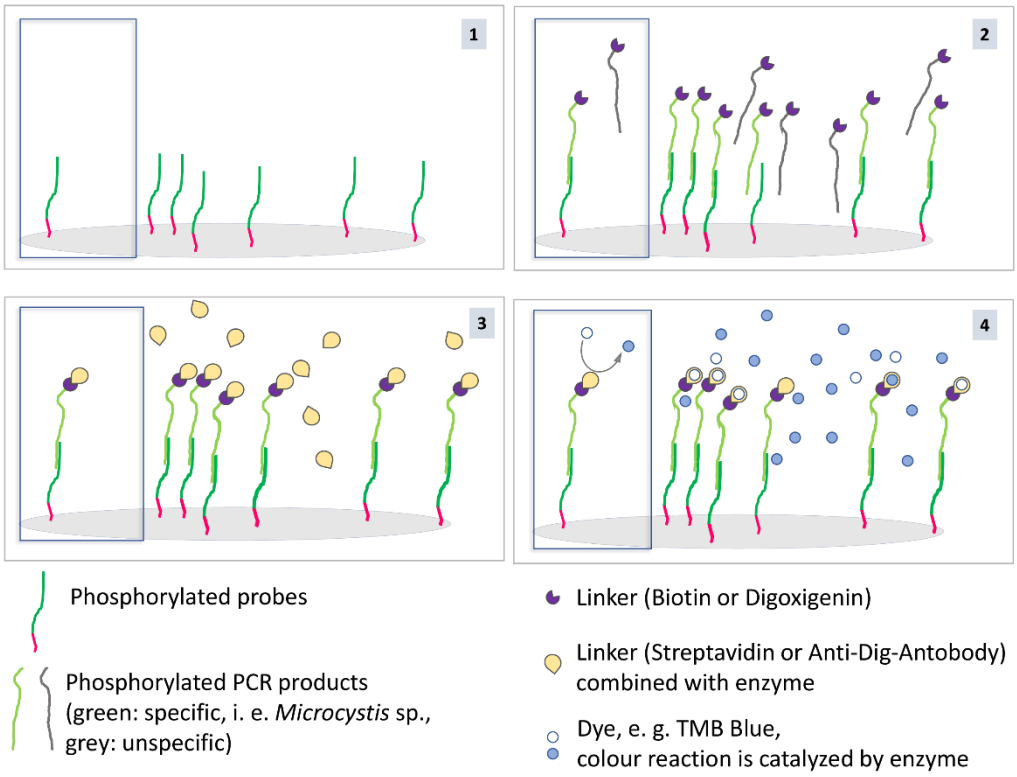

Figure 2.1 PCR-ELISA principle (using the example for detection of *Microcystis* sp.). The individual illustrations (**1-4**) indicate the spots of preliminary optimization. **1** Probe concentration (indirect hybridization). **2** PCR product concentration. **3** Linkage system (Biotin/Streptavidin vs. Digoxigenin/Anti-DIG-antibody). **4** Addition of substrate solution.

For the development of methods like the PCR-ELISAs, many parameters need to be optimized. The general method of the PCR-ELISA is based on the binding of PCR product to a microtiter plate (direct or indirect via probes). These PCR products are equipped with a linker (biotin or digoxigenin). The linker enables (in a later step), the specific binding of an enzyme - which catalyses a colour reaction - to the specific PCR products. The final step in the PCR-ELISA is a colour reaction, which resulting absorption is measured photometrically in a microplate reader.

In the following, a description of the preliminary optimization steps (Fig. 2.1) is given. These steps comprised the testing of direct vs. indirect hybridization of the PCR product to the microtiter plate, the selection of the kind of microtiter plate and the selection of the kind of linker (Biotin vs. Digoxigenin). Before the main optimization steps, the applications of two methodologies were tested:

(i) Direct hybridization (Behr and al., 2000) vs. reverse hybridization (Schedl et al., 2000).
The direct hybridization is based on the principle of binding the targeted PCR products on the hybridization plates. To facilitate the binding, the PCR products need to be phosphorylated at the 5'-end. By contrast, the reverse hybridization is based on the principle of binding pre-phosphorylated probes to the hybridization plate. Due to a better adequacy in the pre-experiments and the possibility to phosphorylate the probes in advance, we used the reverse hybridization for the optimization of the PCR-ELISA assays (data not shown).

(ii) Microplates.
Several microplates are available for the binding of oligonucleotides. We carried out preliminary tests on the suitability of MaxiSorp (Thermo Fisher Scientific, USA), Nucleolink (Thermo Fisher Scientific, USA) and Corning (Corning, USA) microplates. As also described in Behr et al. (2000), MaxiSorp plates, which are normally used for the binding of protein, are quite good for the hybridization of nucleotides. As these plates gave the best price to quality ratio, we used them for the further optimizations (data not shown).

(iii) Linkage of PCR product and enzyme.
The PCR-ELISA tests were conducted using Biotin and Streptavidin as well as Digoxigenin and Anti-Digoxigenin-antibodies for the linkage of PCR product and enzyme (horseradish peroxidase HRP). As there were high interactions between Biotin and PCR products, final optimization steps were conducted using Digoxigenin and Anti-Digoxigenin-antibody.
The PCR-ELISA assays were optimized for different probes. The target genus provided the DNA for the positive control. The DNAs of two related genera served as negative controls. For the use in PCR-ELISA, a 10-Thymin-linker was attached to the 5' end of the probe.

2.3.3.2 *In vitro amplification and labelling of the targeted DNA*

The *16S rRNA* gene was amplified from genomic DNA of the target organisms[1] by PCR, using the following temperature profile: Three minutes at 94 °C for denaturation, followed by 30 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30s) and elongation (72 °C, 60 s) and final incubation at 72 °C for 5 minutes. For simultaneous labelling, Dioxigenin-labelled primers were supplied (Table 2.1). The primer pair used for the amplification is specific for bacteria and has been described and recommended by Klindworth et al. (2012). The components of the mastermix are shown in Table 2.2.

Table 2.2 Components of standard PCR assay.

Component	Volume for each reaction [µl]
10x Dream Taq Buffer	5
dNTP Mix (10 mM; Roth, Germany)	3
10 mM Primer (reverse)	1
10 mM Primer (forward)	1
DNA template	1
Dream Taq (5U/µl; Thermo Fisher Scientific, USA)	0.5
BSA	2
ddH_2O	36.5
Total volume	50

PCR products were purified using MSP Spin PCRapace (Stratec Molecular, Germany) according to manufacturer's recommendations and finally analysed by gel electrophoresis.

2.3.3.3 *Coating*

5'-phosphorylated oligonucleotides were immobilized on MaxiSorp plates (flat bottom, clear) using a similar protocol as Schedl et al. (2000). Probe concentrations of 0, 5, 10, 20, 30 and 50 ng/well were tested. The probes were added to each well in 100 µl coupling buffer (10mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, EDC). The plates were incubated overnight (up to 15 h) at 37 °C in a thermomix incubator. Afterwards, the wells were washed for 5 min at 37 °C three times using 200 µl washing solution (0.4 N NaOH, 0.25%

[1] During the optimization, the DNA of pure cyanobacteria cultures was used as positive or negative control. During the application of the method, DNA extracted from environmental samples must be used at this step.

Tween20) per well. Final washing was done using 200 µl TRIS-washing solution (100 mM TRIS-HCl, 150 mM NaCL, 0.1% Tween20, pH 7.5) per well, again for 5 min at 37 °C.

2.3.3.4 Hybridization and washing

Hundred microliters of prehybridization buffer (5xSSC, 1% Blocking, 0.1% n-Laurylsarcosine, 0.02% SDS; Blocking = Albumin Fraction V, Roth, Germany) were added to each well and the microplates were incubated at 37 °C for 30 min. During the incubation, DIG-labelled target DNA (90 ng/well) was denatured at 95 °C for 10 min and then immediately chilled on ice to avoid renaturation. Afterwards, 100 µl of hybridization buffer (5xSSC, 1% Blocking, 0.1% n-Larylsarcosine, 0.02% SDS + X% formamide) and 90 ng PCR products were added to each well. Then, the plate was incubated for 120 min at 37 °C, followed by three washing steps (three times, 10 min, 37 °C) with TMA-washing solution (3 M TMA, 50 mM TRIS-HCl, 2 mM EDTA, 0.1% SDS + Y% formamide). For the stringency of the hybridization, various formamide concentrations in hybridization and TMA-washing solution (X%/Y%) were tested: 5%/10%, 20%/25%, 30%/35%, 50%/55%. Afterwards, the plate was rinsed with 200 µl PBS (130 mM NaCl, 10 mM Na2HPO4, pH 7.2) per well.

2.3.3.5 Detection of hybrids

Anti-Digoxigenin-Antibody (HRP) solution (Abcam, UK) was diluted 1:20,000 by adding Blocking-PBS (1% Blocking, 1xPBS). To avoid unspecific hybridization of the antibody, 100 µl of Blocking-PBS without enzyme were added to each well and incubated for 15 min at room temperature in the dark. Afterwards, 100 µl of antibody solution were added to each well. The plate was incubated for 30 min at room temperature in the dark. Three washing steps followed the binding of the antibody with TRIS washing solution (200 µl, 10 min, and room temperature). For colorimetric detection, 100 µl of 1-StepTM Ultra TMB-ELISA substrate solution (Thermo Scientific, USA) were added to each well. Colour development was stopped after about 10 min (depending on the reaction speed) with 100 µl 5% H_2SO_4. The absorption was measured at 450 nm (reference measurement 620 nm) with a multiplate reader (Tecan, Switzerland).

2.3.3.6 Measurement of environmental samples

To determine the relative hybridization of environmental samples, the PCR-ELISA was conducted in parallel with a specific probe (e. g. specific for *Microcystis* sp.) and a probe specific for bacteria. The relative hybridization is calculated according to the equation published earlier by Fuller et al. (2003):

$$\textbf{\% relative hybridization} = \left[\left(\frac{Hs}{He}\right) \times \left(\frac{Ss}{Se}\right)^{(-1)}\right] \times 100$$

, where H_s and H_e represent hybridization of the environmental DNA of the specific and eubacteria probes, respectively, and S_s and S_e are the slopes of the specific and eubacterial probe binding curves. These curves were determined by hybridization of a dilution series of homogenous control DNAs.

In each plate, several NTCs (no template controls, without PCR product) need to be conducted. The hybridization values of the NTCs are needed to normalize the sample values.

2.3.4 Quantitative real-time PCR

The qPCR assays were conducted to quantify cell numbers by the determination of *16s rRNA* gene copies. The developed probes for *Limnothrix* sp., *Microcystis* sp. and *Planktothrix* sp. were used as reverse primers for the qPCR assays. Adequate forward primers were designed accordingly to represent the optimal length of the amplicon of ~ 100 bp (Table 2.3). The number of gene copies per cell must be known to determine the number of cells. *Microcystis* sp. has two gene copies (Schirrmeister et al., 2012) per cell, whereas *Planktothrix* sp. has four gene copies per cell (Savitcheva et al., 2011). For *Limnothrix* sp., the gene copy number of the *16S rRNA* gene is not known. Therefore, we calculated with three gene copy numbers per cell, which is the average value for cyanobacteria (Savitcheva et al., 2011).

Table 2.3 Oligonucleotides used as primer pairs for the optimization of the qPCR assays.

Target organism	Primer	Sequence (5'-3')	Amplicon length [bp]	Reference
***Microcystis* sp.**	Q_Micr3_f Q_Micr3_r	CGGTACTTGAGGAATCAGCCTCG TCTGCCAGTTTCCACCGCCTTTAGGT	122	This study Rudi et al., 2000
***Limnothrix* sp.**	Q_Limn667_f Q_Limn667_r	GGATGCAAGCGTTATCCG CACCTGGAATTCCTCCTG	112	This study
***Planktothrix* sp.**	Q_PlanC_f Q_PlanC_r	CTGAGAGGATGATCAGCC CTGAGAAAAGGGGTTGAC	117	This study

2.3.4.1 Optimization

To optimize the PCR conditions, a magnesium chloride gradient and an annealing temperature gradient were tested for each primer pair to determine optimal reaction conditions. At first, the adequacy of the primer pairs was tested in PCR reactions in a T100 thermal cycler (Biorad, Germany). Therefore, a standard PCR mastermix (Table 2.2) and program were used. Afterwards, temperature gradients that are more precise were conducted in a real-time PCR cycler. All qPCR reactions were done in the CFX Connect cycler (Biorad, Germany). For the mastermix, the SSO Advanced Universal SYBR Green Super Mix (Biorad, Germany) was used (Table 2.4). Cycling conditions for these reactions were as follows: 3 min, 95 °C; 39 cycles of 15 s, 95 °C and 30 s determined annealing temperature for the primer pair.

Table 2.4 Components of the qPCR mastermix.

Component	Volume for each reaction [µl]
SSO Advanced Universal SYBR Green Super Mix (2x)	5
10 mM Primer (forward)	0.2
10 mM Primer (reverse)	0.2
DNA template	1
Reaction water (ddH_2O)	3.6
Total volume	10

2.3.4.2 Measurements of environmental samples

Measurements should be conducted at optimal conditions for each primer pair (Table 2.6). Detection limits need to be considered and values below these must be discarded.

2.4 Results

The aim of the study was to develop and optimize PCR-ELISA and qPCR assays for the quantification of cyanobacteria genera. The following data summarize results from the optimization of different experimental parameters.

2.4.1 PCR-ELISA

The first part of the graphs shows results based on the application of Biotin and Streptavidin (B/S) and the second part shows results based on the application of Digoxigenin and Anti-Digoxigenin-Antibody (D/A) for the linkage of PCR product and enzyme.

Unless specified otherwise, the following standard conditions were used for preliminary optimization steps:

Table 2.5 Standard conditions for preliminary optimizations of the PCR-ELISA method.

Probe concentration	20 pmol/well
PCR product concentration	90 pmol/well
Formamide in hybridization buffer/TMA washing solution	5%/0%

2.4.1.1 Optimal probe concentration (B/S)

The optimal probe concentration is the concentration when the plateau-reaching curve is in the linear phase.

The optimal probe concentrations for PlanC (Fig. 2.2 A) and Limn667 (Fig. 2.2 B) are about 10 pmol/well. The results indicate unspecific bindings between probe and PCR products of the negative controls.

The optimal probe concentration of the EUB probe is about 20 pmol/well (Fig 2.2 C). The binding affinity of the EUB probe to the PCR products of the positive controls is different

depending on the genus. The binding affinity of EUB to *Limnothrix*-DNA is the highest, the binding affinity of the EUB probe to *Planktothrix*-DNA the lowest.

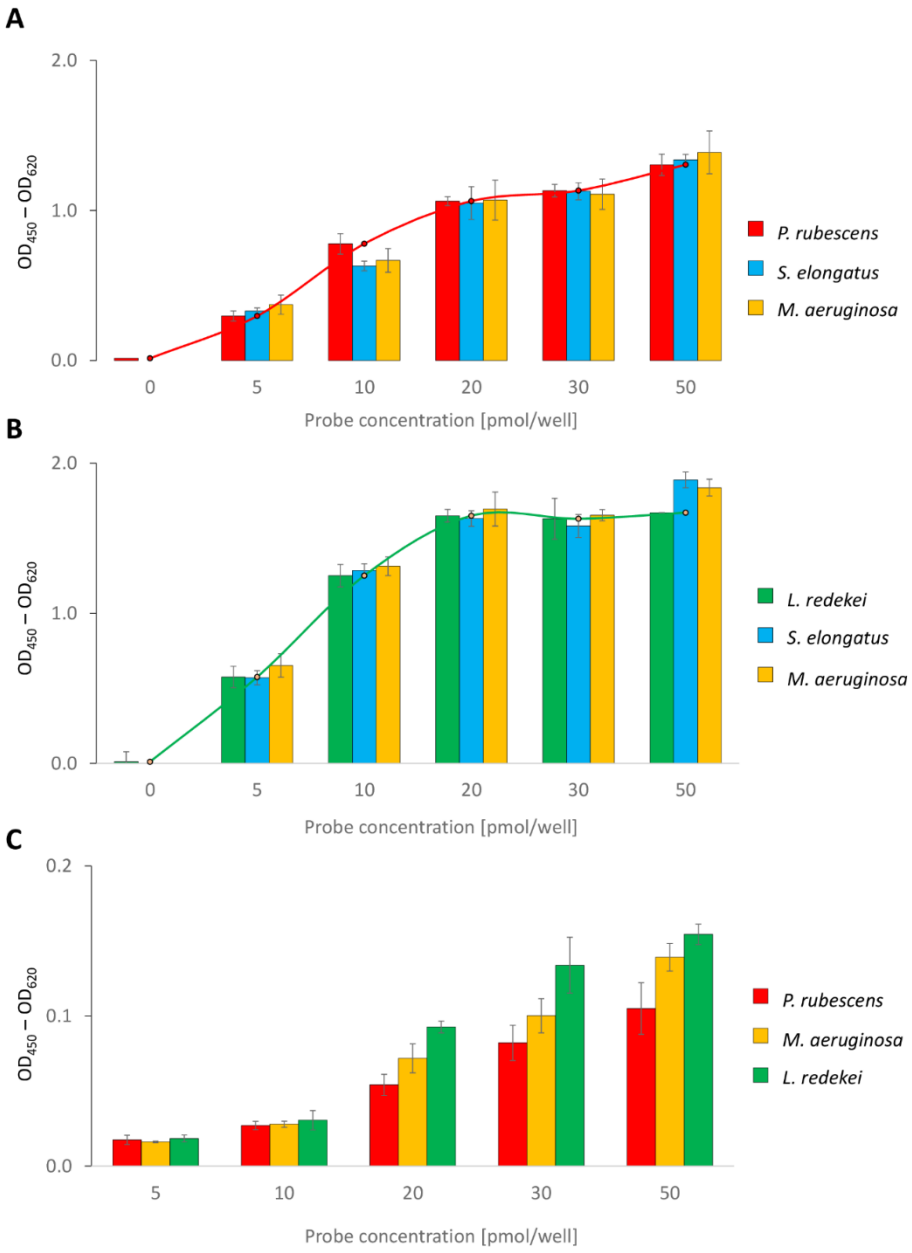

Figure 2.2 Optimization of different probe concentrations. Data is presented in bar charts. Lines are added in A/B to clarify the slopes. **A** Probe: PlanC, positive control: *Planktothrix rubescens*, negative controls: *Synechococcus elongatus* and *Microcystis aeruginosa*. **B** Probe: Limn667, positive control: *Limnothrix redekei*, negative controls: *Synechococcus elongatus* and *Microcystis aeruginosa*. **C** Probe: EUB, positive controls: *Planktothrix rubescens*, *Microcystis aeruginosa* and *Limnothrix redekei*.

2.4.1.2 Optimization of PCR product concentration (B/S)

The optimization of the PCR product concentrations did not show any differences between the different PCR product concentrations (Fig. 2.3). For both positive controls and unspecific binding to negative controls, all tested concentrations resulted in similar absorption values. Even wells without PCR product gave comparable high signals.

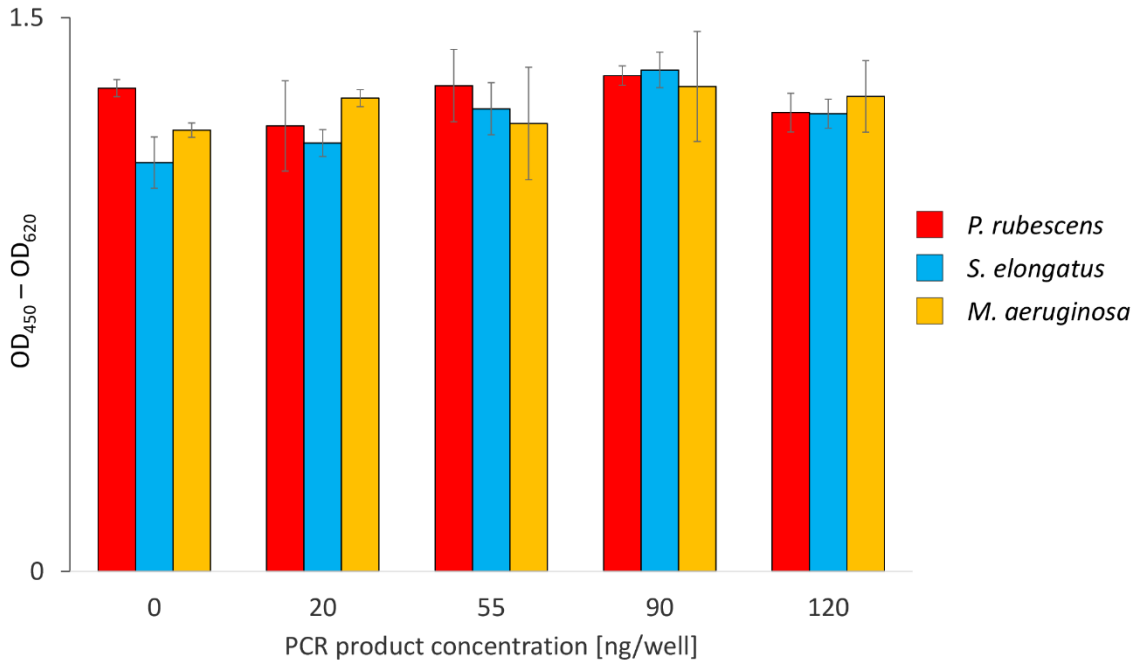

Figure 2.3 Optimization of PCR product concentration (PlanC). Positive control: *Planktothrix rubescens*, negative controls: *Synechococcus elongatus* and *Microcystis aeruginosa*.

2.4.1.3 Optimization of hybridization stringency (D/A)

The optimizations of hybridization stringency were conducted using 90 ng PCR product per well and the optimized probe concentrations: EUB 20 pmol/well, Limn667 10 pmol/well, Micr3 20 pmol/well and PlanC 10 pmol/well. The optimization of the hybridization stringency (optimization of formamide concentrations) was done with the approach based on Digoxigenin and Anti-DIG-antibody. A normalization of the background noise with the NTC was necessary. Binding specificity increases with addition of formamide (Gorkin et al., 2010). Formamide builds hydrogen bonds with the bases of the DNA, and the DNA bases cannot bind to each other any longer. Different formamide concentrations were tested in the hybridization buffer and the TMA washing solution, to determine the optimal concentrations for hybridization stringency

(Fig. 2.4). The optimal formamide concentration is the concentration with the highest amplitude between the absorption of the positive control and the absorptions of the negative controls.

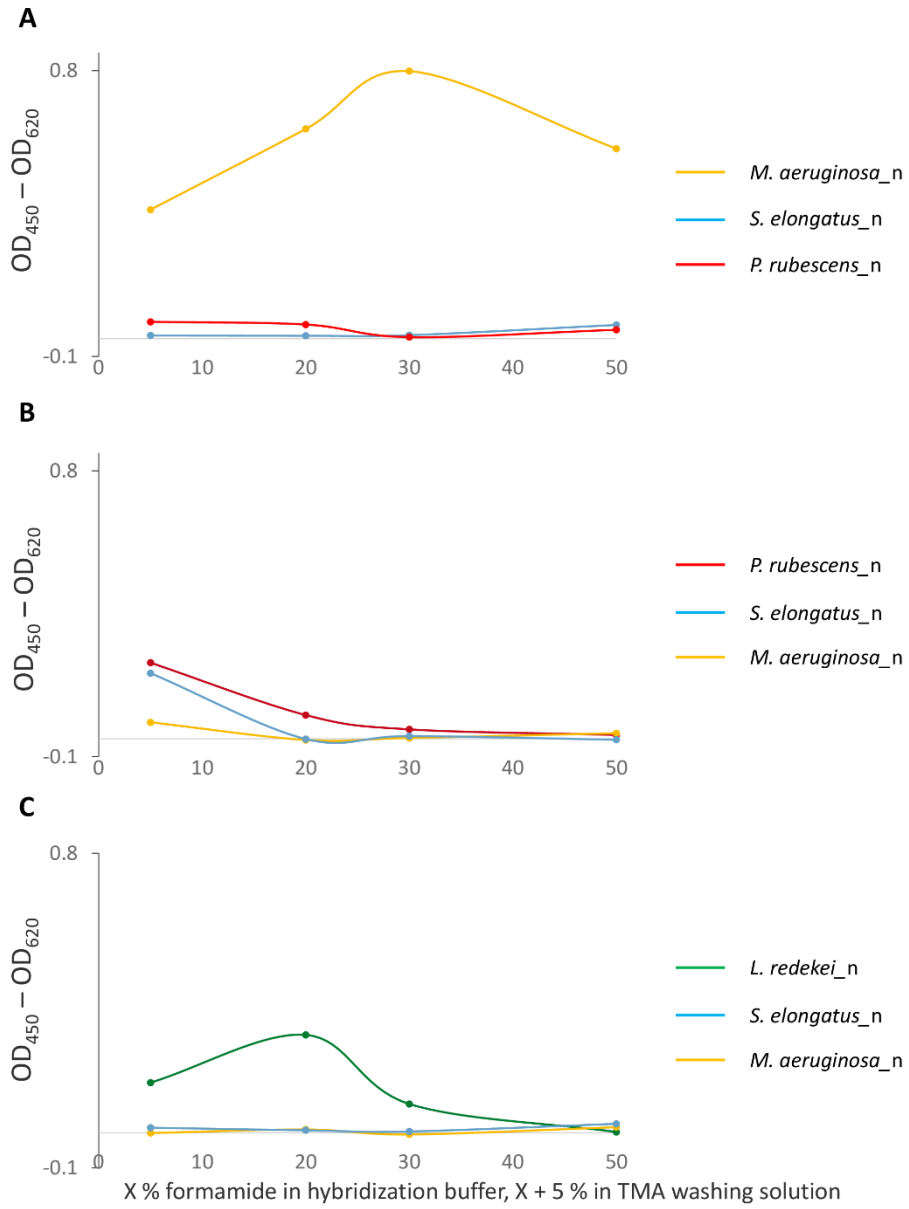

Figure 2.4 Optimization of formamide concentration in hybridization buffer and TMA washing solution. **A** probe: Micr3, positive control: *Microcystis aeruginosa*, negative controls: *Synechococcus elongatus* and *Planktothrix rubescens*; normalized values. **B** Probe: PlanC, positive control: *Planktothrix rubescens*, negative controls: *Synechococcus elongatus* and *Microcystis aeruginosa*; normalized values. **C** Probe: Limn667, positive control: *Limnothrix redekei*, negative controls: *Synechococcus elongatus* and *Microcystis aeruginosa*; normalized values.

The optimal formamide concentration for Micr3 is 30% in the hybridization buffer and 35% in the TMA washing solution. Testing PlanC, absorbance decreased with increasing formamide concentration and specificity increased. The highest specificity for PlanC was achieved with 20% formamide in hybridization buffer and 25% formamide in TMA washing solution. The absorbance of the positive control testing the Limn667 probe had a maximum using 20% formamide in hybridization buffer and 25% formamide in TMA washing solution. Negative controls were low, independent on the formamide concentrations. The optimal formamide concentrations for the Limn667 probe are therefore the concentrations of the maximum.

2.4.2 Quantitative real-time PCR

The qPCR assays were developed for the quantification of three cyanobacterial genera: *Limnothrix*, *Microcystis*, and *Planktothrix*.

Annealing temperature gradients for each primer pair were first analysed in simple PCR (Fig. 2.5). The addition of further magnesium chloride to the mastermix was not necessary.

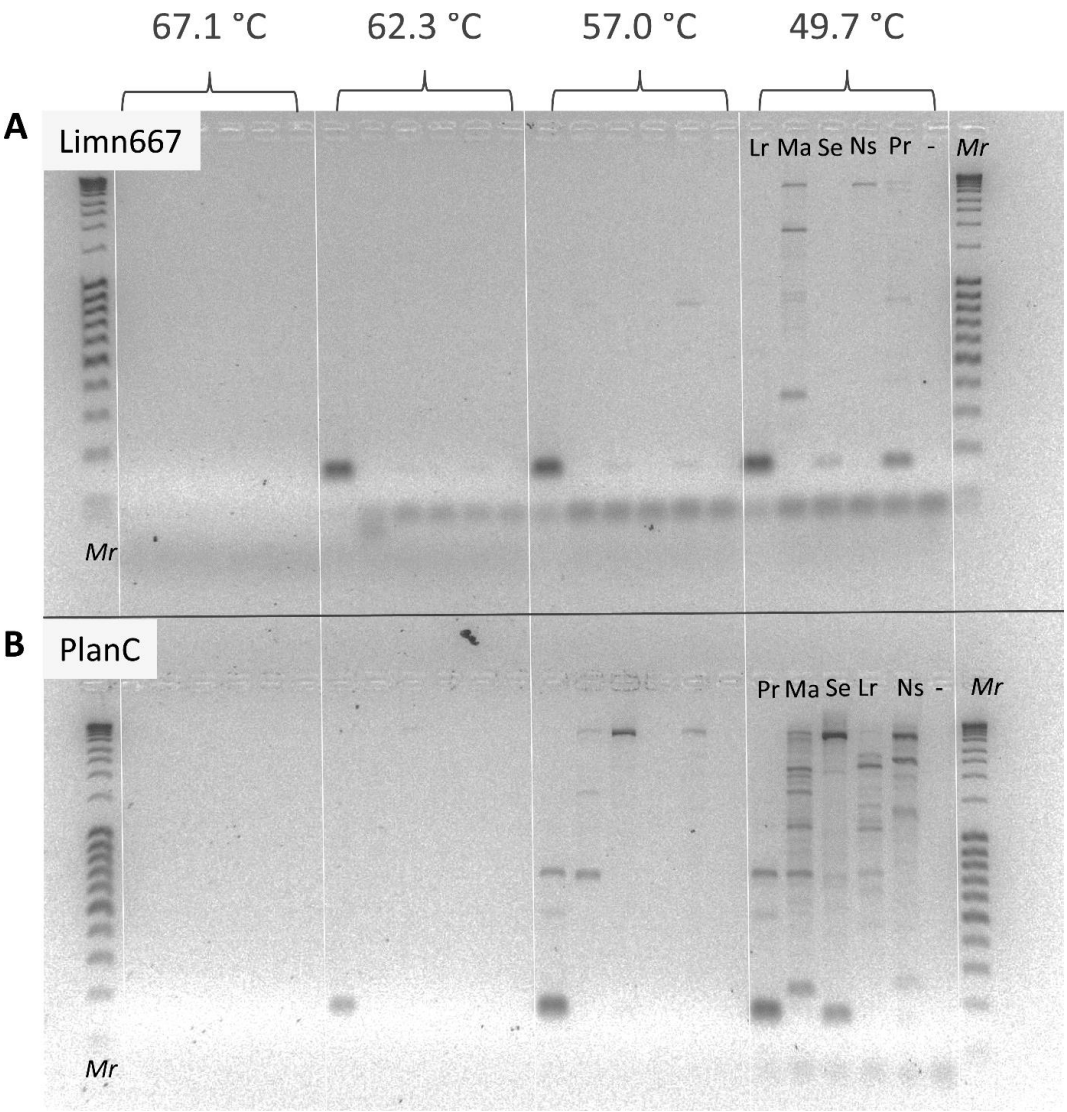

Figure 2.5 Gel electrophoresis of the annealing temperature gradients (PCR). **A** Limn667 primer pair, **B** PlanC primer pair. Positive and negative controls were distributed as shown in the right column.

Well 1: positive control, wells 2-5: negative controls, well 6: NTC (without template).

Lr: *Limnothrix redekei*, Pr: *Planktothrix rubescens*, Ma: *Microcystis aeruginosa*, Se: *Synechococcus elongatus*, Ns: *Nostoc* sp., -: NTC, Mr: MassRuler DNA ladder mix (Thermo Scientific, USA).

Afterwards, a more precise determination of annealing temperature was conducted during qPCR. For the detection of amplicons, a SYBR green based assay was applied. Resulting optimal annealing temperatures are shown in Table 2.6.

Table 2.6 Optimal annealing temperatures and detection limits of the primer pairs used in qPCR.

Target organism	Primer	Sequence (5'-3')	Determined annealing temperature	Detection limit	Reference
***Microcystis* sp.**	Micr3_f	CGGTACTTGAGGAATCAGCCTCG	67.0	10^5	This study
	Micr3_r	TCTGCCAGTTTCCACCGCCTTTAGGT			Rudi et al., 2000
***Limnothrix* sp.**	Limn667_f	GGATGCAAGCGTTATCCG	65.0	10^4	This study
	Limn667_r	CACCTGGAATTCCTCCTG			
***Planktothrix* sp.**	PlanC_f	CTGAGAGGATGATCAGCC	64.5	10^6	This study
	PlanC_r	CTGAGAAAAGGGGTTGAC			

To avoid false positive results, a detection limit had to be determined for all primer pairs for the absolute quantification of the *16S rRNA* gene. The detection limit for each primer pair was defined as the lowest standard concentration above which no false positive signals of the negative controls had been detected. To prove that the appropriate section of the DNA has been amplified, melting curves were conducted and melting peaks were compared.

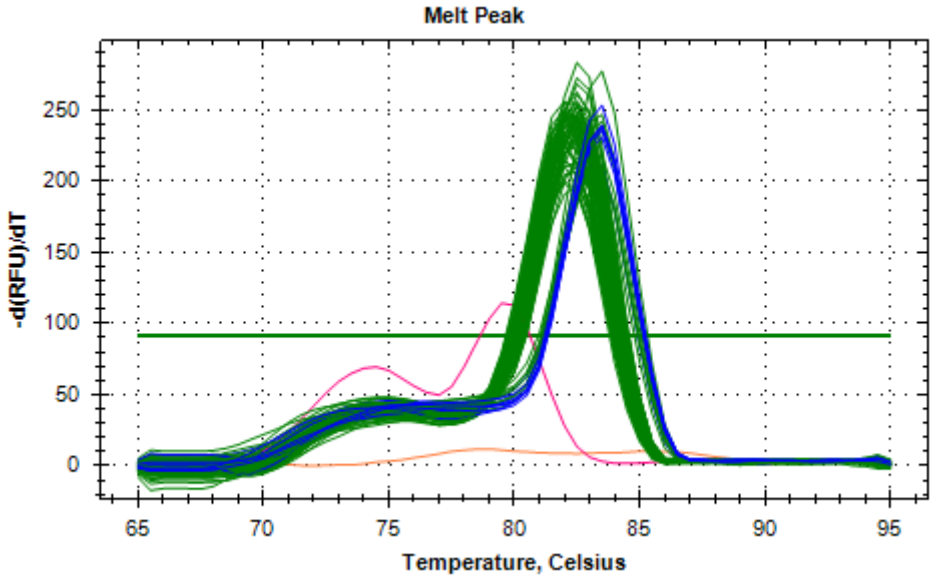

Figure 2.6 Melting curves of amplicons amplified with Limn667 primer pair. Blue: standard dilutions of the positive control (*L. redekei*), green: environmental samples, pink: negative control (*S. elongatus*) and orange: no template control (NTC).

The melting peaks of the environmental samples varied within a small deviation from the melting peaks of the culture standards. The amplicons of the negative controls had a melting peak clearly distinguishable from the positive controls (Figure 2.6).

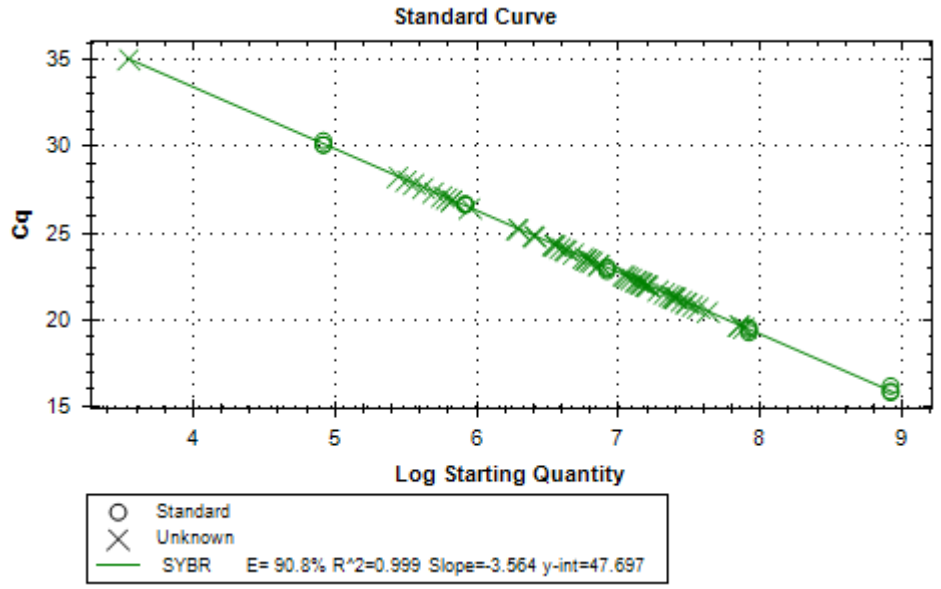

Figure 2.7 Exemplary standard curve of Limn667 primer pair based on five standard dilutions. Efficiency of the assay is 90.8% with a R^2 of 0.999.

The amplification efficiencies were 90.8% (R^2 = 0.999) for Limn667 primer pair, 105.2% (R^2 = 0.993) for Micr3 primer pair, and 97.1% (R^2 = 0.999) for PlanC primer pair. For each assay, at least five standard solutions were used for the calculation of slopes and efficiencies (Fig. 2.7).

2.5 Discussion

2.5.1 Development of methods

Here, we report the development and the optimization of two methods for the detection and quantification of cyanobacterial genera. In future, both methods can be applied in the monitoring of cyanobacteria to pursue different quantification levels. There is the possibility to extend the future genus spectrum for both assays.

The PCR-ELISA provides a method for the semi-quantitative determination of cyanobacteria. The results are relative abundances of one genus compared to the whole bacterioplankton or to all cyanobacteria, depending on the primer pair used for the amplification of the *16S rRNA* gene fragment. Differences in abundances of a certain genus compared across different samples are easy to detect.

The use of Nunc MaxiSorp microplates is cost efficient and very reliable. The binding affinity of phosphorylated probes to this plate kind, which is developed for proteins, is lower compared to the binding affinity to NucleoLink microplates, developed for DNA. Nevertheless, Nunc

MaxiSorp plates provide a good hardware for the PCR-ELISA, as has already been described in Schedl et al. (2000).

Optimal probe concentrations were dependent on the sequence structure of the probes and had to be determined for each probe. For the probes tested, the optimal probe concentrations ranged between 10 and 20 pmol/well. This finding reflects the recommendation of Schedl and al. (2000), who stated that optimal probe concentrations usually vary between 0.1 and 50 µmol/well.

During the optimization experiments on the PCR product concentrations, we found that the usage of biotin and streptavidin as linker between PCR product and enzyme is not applicable. The results indicated that streptavidin was binding non-specifically to the oligonucleotide probes and thus provided unspecific signalling. One possible explanation would be that the probes were contaminated with biotin due to production processes. Nevertheless, this contamination was excluded by the manufacturer (Biomers, Germany). We have not found any scientific references reporting unspecific bindings between streptavidin and oligonucleotides. This phenomenon has been discussed in various scientific forums concerning the unspecific binding of streptavidin magnetic beads to non-biotinylated DNA (http://www.researchgate.net, http://www.chemicalforums.com; visited on 21th January 2015).

The major problem of the unspecific binding due to the usage of biotin and streptavidin could be eliminated by the usage of digoxigenin and Anti-DIG-antibody instead. This approach is also used by default in molecular biology to label DNA fragments (Sigma Aldrich, Technical Documents, DIG labelling methods). Furthermore, an NTC (no template control) must be conducted within each plate to deduct background noise. After these preliminary optimizations it was possible to determine the optimal formamide concentrations. A final stringency optimization was done. In general, the relative abundances provided by the established PCR-ELISA are overestimated, but a comparison of tendencies between different samples is possible.

The qPCR assay provides the absolute quantification of cyanobacteria cells of three genera. The PCR conditions of the developed primer pairs could be optimized. The primers based on the *16S rRNA* gene are specific but only within a certain range. Low cell numbers cannot be detected, because of the high danger of false positive results. Therefore, a detection limit had to be stated for each primer pair. For the detection, a SYBR green based dye was used, which provides a reliable alternative to more expensive TaqMan protocols (Vilalta, Whitlow & Martin, 2002).

2.5.2 Comparison of 16S rRNA gene-based methods

The developed methods, the PCR-ELISA and the qPCR, both have advantages and disadvantages. A further often applied method for the determination of bacterial communities is the sequencing of the *16S rRNA* gene using modern sequencing technologies like Illumina. The applied methods differ in the type of quantification. The Illumina MiSeq and the PCR-ELISA test provide relative abundances, whereas the qPCR assay provides absolute abundances. Depending on the pursued target, the methods have differing suitability. The sequencing of environmental samples provides an overview over the whole bacterioplankton, while the PCR-ELISA and the qPCR assay enable the observation of a specific genus. The application of the qPCR assays provides exact results, whereas the use of PCR-ELISA tests results in the detection of trends. In some cases, PCR-ELISA is less prone to error. Aquatic environmental samples are often contaminated with humic acids and other inhibitory substances, leading to failure of PCR (Rossen et al., 1992; Opel, Chung & McCord, 2009; Matheson et al., 2010). In most cases, this effect can be eliminated by the dilution of the samples. A dilution of samples to a high extent risks the false negative determination of samples, due to the proximity to the detection limit. The effects of inhibition are negligible in PCR-ELISA and Illumina MiSeq sequencing. Detection limits for these two methods are unknown. The sequencing provides many advantages. However, in contrast to the sequencing, PCR-ELISA and qPCR are both methods suitable for the regular monitoring of water bodies.

Table 2.7 Comparison of high-throughput sequencing, PCR-ELISA and qPCR.

	High-throughput sequencing	**PCR-ELISA**	**qPCR**
Quantification	Relative	Relative	Absolute
Target organism	All bacteria (at once)	Specific genus	Specific genus
Applicability	Description of bacterial community, rather qualitative	Trends detectable, semi-quantitative	Exact cell numbers detectable, quantitative
Efficiency	High efficiency, many data, little effort	Medium efficiency	Medium efficiency
Cost	Approx. 2000 €/run (i.e. Illumina MiSeq)	Approx. 8 €/sample	Approx. 5 €/sample
Reliability of results	High reliability (prerequisite: good primers and enough sequences)	Good buffering against inhibition	High reliability (without inhibition), standard curve's coefficient of determination
Error susceptibility	Less prone to error	Less prone to error	Prone to error when samples are inhibited
Limits	Not precisely definable	Not precisely definable	Detection limit due to high similarity between *16S rRNA* genes
Laboratory requirements	Thermocycler, gel electrophoresis, centrifuge (samples go out for sequencing)	Thermocycler, gel electrophoresis, centrifuge, microplate reader	Real-time thermocycler

2.6 Conclusion

The two developed methods of PCR-ELISA and qPCR represent powerful tools to support future research and monitoring on cyanobacteria. The final decision about the usage of the two methods depends on the target of the study. The recommended process flow is the application of the PCR-ELISA test for regular monitoring and a further analysis by qPCR to specify selected results.

3. Cyanobacterial diversity in four Bavarian lakes of different trophic status

A similar version of this chapter is submitted: Bauer, F. R., A. D. Millard, U. Raeder, J. Geist & K. Zwirglmaier, 2018. Cyanobacterial diversity in four Bavarian lakes of different trophic status.

3.1 Abstract

The nutrient level of lake ecosystems is an important factor influencing species composition and diversity. Much is known on cyanobacteria in eutrophic lakes, whereas their distribution and indicator function in lakes of different trophic status is rarely known. This study characterized cyanobacterial communities in four well-studied Bavarian lakes of differing trophic status to identify individual characteristics of cyanobacterial communities. Cyanobacterial diversity was analysed at different seasons and depths by Illumina MiSeq sequencing of the *16S rRNA* gene. Community structure of cyanobacteria varied greatly between the lakes. Each of the studied lakes was characterized by an individual cyanobacterial genus, which contributed > 50% of the total cyanobacteria population of the respective lake, independent of the sampling date. A vertical stratification of the cyanobacterial community was revealed, indicating that *Planktothrix* sp. and *Synechococcus* sp. share the same ecological niche, and become competitors during lake thermal mixing. The findings indicate at first sight, that Cyanobacteria as a group are not very effective for lake trophic classification. Nevertheless, the occurrence of specific cyanobacterial species, and their monitoring with modern next-generation sequencing technologies, may have the potential to be used as an indicator for trophic or other environmental parameters influencing a lake.

3.2 Introduction

Trophic status is an important factor determining ecological processes and food webs in lake ecosystems, as it affects taxon composition of all groups of lake organisms. Earlier studies have shown that different water bodies harbour microorganisms that are specific for their environmental conditions (Zwart et al., 2002) and that nutrient resources are a main factor controlling prokaryotic community composition (Jardillier et al., 2005). Increasing phosphor availability, which is one of the key factors determining lake trophic status, induces shifts in the lake trophic structure (Reynolds, 1998; Vollenweider, 1971). Numerous species have been identified as indicators for the trophic status of lakes, comprising zooplankton (Gannon &

Stemberger, 1978; Gulati, 1983), macrozoobenthos (Wiederholm, 1980), macrophytes (Melzer, 1999), benthic diatoms (Dokulil, Schmidt & Kofler, 1997; Seele et al., 2000; Kitner & Poulícková, 2003) and phytoplankton (Reynolds, 1980; Salmaso et al., 2006). Cyanobacteria are crucial components of many aquatic ecosystems as primary producers. Some species have been identified as bioindicators for monitoring running waters (Rott et al., 1997; Rott et al., 1999; Gutowski, Foerster & Schaumburg, 2004; Rott et al., 2006; Schneider & Lindstrøm, 2011; Rott & Schneider, 2014; Mateo et al., 2015). The situation in lakes is different. Although cyanobacteria occur in a wide range of trophic statuses, indicator species among them have to date mainly been identified for eutrophic lakes (Schaumburg et al., 2005). Furthermore, cyanobacteria have for a long time generally been considered as one big group indicating eutrophic and problematic conditions (Mateo et al., 2015). The reasons for that might be that in the past, much of the work concentrated on cyanobacteria typical for eutrophic lakes. Therefore, cyanobacterial genera like *Microcystis* are well known concerning their requirements and distribution. However, especially smaller species have often been put together and treated as a single group.

The presence of certain species of cyanobacteria can have several ecological implications for the lake, as well as for the ecosystem services derived from lakes. Cyanobacteria play a key role in primary production, resource cycling and food webs (Whitton & Potts, 2000). The widely distributed picocyanobacterial cells of the genus *Synechococcus* do not form blooms and most strains are generally benign. Bloom-forming species such as *Microcystis aeruginosa* and *Planktothrix rubenscens* can produce potent toxins (e. g. Microcystins) that present a human health threat and cause severe ecological and economic problems (Carmichael et al., 2001; Pflugmacher, 2002; Zurawell et al., 2005; Funari & Testai, 2008). Thus, the ecological importance of cyanobacteria in lakes is not limited to the production of toxins and it is critical to differentiate distinct genera, species or strains.

Cyanobacteria are commonly detected with traditional methods such as microscopy, which requires experts in the field of taxonomy. Furthermore, it has been shown, that there is a high variability in the comparability of taxonomic species identification even done by experts so that intercalibrations of freshwater phytoplankton analyses to avoid variations have been in demand (Vuorio, Lepistö & Holopainen, 2007).

Studies on biological communities in connection with nutrient loading are important for the characterization of the biology of lakes and form the basis for potential future lake management (Dokulil & Teubner, 2000; Downing, Watson & McCauley, 2001; Gulati & Van Donk, 2002; Jeppesen et al., 2005; Smith & Schindler, 2009; Hering et al., 2013). In the last

decades, morpho-functional groups of phytoplankton have been described and refined (Reynolds et al., 2002; Padisák, Crossetti & Naselli-Flores, 2009). The classification based on morphological and functional characteristics has the potential to overcome problems related to possible differences of taxonomic accuracy and identification (Salmaso & Padisák, 2007). It has been shown that this approach is useful for a large proportion of phytoplankton, but for the group of cyanobacteria similar morphologies often have different ecological adaptions (Padisák, Crossetti & Naselli-Flores, 2009).

Different genera and species of picocyanobacteria – whose identification is at the limits of light microscopy - might be suitable indicators for a lower nutrient status. This hypothesis is supported by the restricted occurrence of certain picocyanobacteria such as *Prochlorococcus* sp. to oligotrophic conditions in marine ecosystems (Partensky, Blanchot & Vaulot, 1999; Zwirglmaier et al., 2007), but needs to be verified for freshwater habitats. Rajaneesh et al. (2015) suggested that picoplankton might be a useful indicator for trophic status in the Cochin backwaters (India), a chain of brackish lagoons and lakes.

In the last years, molecular methods, such as next-generation sequencing (NGS) have become increasingly popular. They provide high throughput data collection and have the potential to revolutionize surveys of microorgansism diversity in many fields of research (Shendure & Ji, 2008; Medinger et al., 2010). Next-generation sequencing methods will further extend the application of DNA-based methods for routine biomonitoring applications, like indices for trophic status (Domaizon et al., 2013; Mateo et al., 2015; Salmaso et al., 2016). Sequencing of cyanobacterial genomes has the potential to start where microscopy and identification based on morphological characteristics end. The genomic approach might reveal many other cyanobacterial species, which could be used not only for lake trophic classification but also for a deeper understanding of biodiversity and ecological function of cyanobacteria.

Here, we present high-throughput NGS data on lake microbial diversity with a focus on cyanobacterial diversity. Four lakes in Southern Germany were selected to represent a trophic gradient.

Eutrophic Lake Bergknappweiher has a documented record of recurrent visible surface algal blooms (Teubner et al., 2004). Mesotrophic Lake Ammersee is known for mass occurrences of the potentially toxic *Planktothrix rubescens* (Ernst, Hitzfeld & Dietrich, 2001), and oligo-mesotrophic Lake Schliersee continually harbours high biomasses of potentially toxic *Limnothrix redekei* (Schaumburg et al., 2005). Less is known about cyanobacterial communities in oligo-mesotrophic Lake Ostersee, but previous studies have indicated a high abundance of *Synechococcus* sp. (Zwirglmaier et al., 2015).

The data collected was used to get a first molecular insight into the links between trophic status and the diversity of cyanobacteria in the selected lakes. To consider spatial and temporal changes in the cyanobacteria distribution, different seasons and water depths were sampled. The two main objectives of this study were (i) to characterize cyanobacterial communities in these four lakes using NGS, and (ii) to associate lake trophic status with the occurrence of characteristic genera. Specifically, we hypothesized that (i) a higher trophic status would result in a higher dominance of cyanobacteria, (ii) lakes of different trophic status would harbour different genera of cyanobacteria, and (iii) that distinct depth-patterns of cyanobacteria can be found. This snapshot of the cyanobacterial communities based on a limited number of samples is intended to form the foundation for future, more detailed studies on the identification of cyanobacterial indicator species through NGS.

3.3 Materials and Methods

3.3.1 Study sites

The study was conducted in four lakes of different trophic status in Upper Bavaria, Germany: the oligo-mesotrophic lakes Ostersee and Schliersee, the mesotrophic Lake Ammersee, and the eutrophic Lake Bergknappweiher. Lakes Ammersee and Schliersee are both glacial finger lakes and Lake Ostersee was formed by a dead ice field during the last ice age (Grimminger, 1982). Lake Bergknappweiher is a small lake originating from a peat pit. All the lakes are used as bathing lakes. Lake Ostersee is situated in a nature conservation area. Lakes Ammersee and Schliersee are touristically used lakes. While villages surround Lake Ammersee, Lake Schliersee is mostly surrounded by agricultural land. Lake Bergknappweiher is surrounded by agricultural land and forests. Figure 3.1 illustrates the location of the studied lakes and the sampling sites. Information on the lakes' parameters and surroundings are given in Table 3.1.

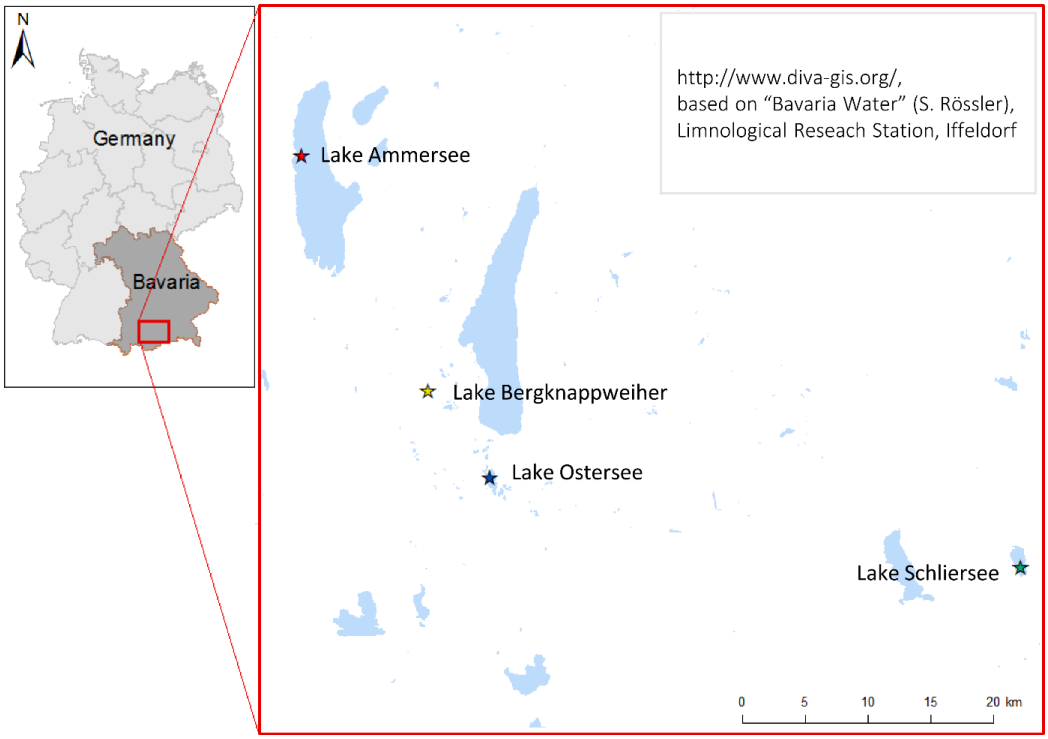

Figure 3.1 Map of the study area. The map shows the location of the four sampled Bavarian lakes. The stars illustrate the sampling points.

Table 3.1 Characteristics of the studied lakes. Hydrophysical and hydrochemical parameters were determined each sampling day. Hydrochemical parameters were measured in triplicates. M, May; S, September; A, Lake Ammersee; B, Lake Bergknappweiher; O, Lake Ostersee; S, Lake Schliersee; SF, surface; SC, Secchi depth; 10M, 10 metres depth; n. d., not detected. (The table is continued on the next page)

	Lake Ammersee	**Lake Bergknappweiher**	**Lake Ostersee**	**Lake Schliersee**
Latitude and longitude	48.009595, 11.117341	47.852775, 11.238334	47.790296, 11.298131	47.726726, 11.860286
Altitude [m above sea level]	531	629	588	788
Surface area [ha]	4660	5	118	220
Max. depth [m]	81.1	2.5	29.5	40.5
Surroundings	Area of high tourism load, mixture of villages and unspoiled nature, forest	Agricultural land, forest	Nature conservation area, agricultural land, forest	Area of high tourism load, agricultural land, next to a busy street
Trophic status	mesotrophic	eutrophic	oligo-mesotrophic	oligo-mesotrophic

Sample IDs	**AM**	**AS**	**A-SF**	**A-SC**	**A-10M**	**BM**	**BS**	**OM**	**OS**	**SM**	**SS**
Time of sampling	06th May 2013	12th Sept 2013	26th Aug 2014	26th Aug 2014	26th Aug 2014	07th May 2013	10th Sept 2013	07th May 2013	10th Sept 2013	08th May 2013	11th Sept 2013
Depth	0 – 20 m	0 – 20 m	0.2 m	3 m	10 m	0 – 2 m	0 – 2 m	0 - 20 m	0 – 20 m	0 – 20 m	0 – 20 m
TP [µg/l]	15	15	n. d.	n. d.	n. d.	129	227	12	12	n. d.	13
NO_3^--N [µg/l]	1,400	1,000	n. d.	n. d.	n. d.	800	100	1,700	800	800	200
NH_4^+-N [µg/l]	20	40	n. d.	n. d.	n. d.	10	60	20	30	10	10
Secchi depth [m]	7.1	4.0	-	3.0	-	0.6	0.5	1.5	2.5	2.9	1.4

3.3.2 Physical and chemical measurements

Water temperature, dissolved oxygen, oxygen saturation, pH and conductivity were concurrently measured *in situ* at each sampling day with an electronic multi-parameter probe (MPP 930 IDS, WTW, Germany). Water transparency was determined with a Secchi disk (Hydrobios, Germany) from the shaded side of the boat. For hydrochemical analyses, 0.5 L samples were taken in spring (May 2013) and in late summer (September 2013) every metre from 0-10 m and every two metres from 10-20 m. Water samples were collected from 0, 1 and 2 m depths at the shallow Lake Bergknappweiher (Z_{max} = 2.5 m). This is the standard method for sampling phytoplankton used by the Bavarian State Water Authority (pers. comm., Schaumburg, 2013). Trophic classification was done according to Melzer & Schneider (2001); Mischke & Nixdorf (2008) and OECD (Organization for Economic Cooperation and Development) based on the total phosphorus content.

Sampled water was filtered through a 0.2 µm pore size membrane filter (Whatman/GE Healthcare, UK) prior to measurement of nitrate-nitrogen (NO_3^--N) and ammonium- nitrogen (NH_4^+-N) concentrations. Total phosphorus (TP) concentration was calculated from unfiltered water samples. Values of TP and NH_4^+-N were determined following established methods by the German chemists' association GdCH (2013), NO_3^--N values were determined using the method described by Navone (1964).

3.3.3 Molecular analyses

As seasonal-induced changes are important structuring forces for lake bacterial communities, with the most dramatic changes expected in summer (Yannarell et al., 2003), depth integrated water samples from the euphotic zones were taken in spring and autumn. Depth integrated samples for molecular analyses were collected in spring (May 2013) and in autumn (September 2013) from all four lakes. During the sampling period in May and September 2013, all four lakes were sampled within three days. At these times, the deep lakes (Ammersee, Ostersee, and Schliersee) were stratified. An aliquot of water was sampled every metre from the surface down to 20 m, and equal volumes of each aliquot were combined in a 1 L bottle. In the case of Lake Bergknappweiher, the depth integrated sample was derived from the upper two metres. As *Planktothrix rubescens* accumulates regularly in the metalimnion of Lake Ammersee (Ernst et al., 2009), we also analysed a depth profile for this lake to obtain more discrete information on the depth distribution of cyanobacteria. The one litre samples of August 2014 from Lake Ammersee were taken from the surface, the Secchi depth (3.0 m), and 10 m depth. Long-term data of Lake Ammersee have shown that during stratification the

highest abundance of *P. rubescens* is in the metalimnic layer (Ernst et al., 2009). Immediately after sampling, 0.5 L of the sample were filtered through a 47-mm diameter 0.2 µm pore size cellulose nitrate membrane filter (Whatman/GE Healthcare, UK). The filters were stored at -20 °C until DNA was extracted using a phenol-chloroform-based protocol previously described by Zwirglmaier et al. (2015).

Samples were sequenced bidirectionally using Illumina MiSeq v3 2x300 paired end sequencing. Samples were prepared according to the manufacturer's recommendations ("Illumina 16S Metagenomic Sequencing Library Preparation", Illumina 2013). Polymerase chain reaction (PCR) primers used for the first PCR step were S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 (Table 3.2), which cover the *16S rRNA* gene variable regions V3-V4. The amplicon length was about 440 base pairs.

Table 3.2 Sequences of the polymerase chain reaction primers used for the preparation of the amplicon library (Illumina overhang adapter in italic).

Primer indication	Sequence (5'→ 3')	Reference
S-D-Bact-0341-b-S-17	*TCGTCGGCAGCGTCAGATGTGTATAAGAGACA*G CCTACGGGNGGCWGCAG	Klindworth et al., 2012
S-D-Bact-0785-a-A-21	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGAC*AG GACTACHVGGGTATCTAATCC	Klindworth et al., 2012

The hypervariable regions V3 or V4 combined with a paired-end sequence configuration are recommended as an effective study design (Mizrahi-Man, Davenport & Gilad, 2013) and the selected primers have been identified as the most promising bacterial primer pair in a study evaluating the overall coverage and phylum spectrum of 512 primer pairs (Klindworth et al., 2012). The conditions for PCR amplification were 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and final extension for 5 min at 72 °C using Accuzyme DNA Polymerase (Bioline, UK). To validate the correct size of the PCR products, they were separated by agarose gel electrophoresis and compared to a standard size marker. The PCR products were purified using AMPure XP beads (Beckman Coulter, USA) according to the manufacturer's protocol. The dual indices and the Illumina sequencing adapters were attached during the index PCR step. These PCR conditions were 95 °C for 3 min, 8 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and final extension for 5 min at 72 °C. The indexed PCR products were checked on an agarose gel prior to purification with AMPureXP beads. DNA was quantified

using Qubit (Thermo Fisher Scientific, USA). The samples were pooled and normalized to a 4 nM concentration and were sequenced at University of Warwick, UK.

The Illumina MiSeq sequence data were analysed with USEARCH v7.0 and associated python scripts (Edgar, 2010). Quality control of the sequences was carried out within USEARCH. In a first step, the forward and reverse reads of each sequence pair were combined. Low quality reads were removed. The values of the filter parameters were adjusted (fastq_trunclen=400, fastq_maxee=0.5). Chimeric sequences were detected and discarded with the UCHIME algorithm (Edgar et al., 2011) within USEARCH. Afterwards, operational taxonomic units (OTUs) were clustered at 97% sequence identity and subsequently classified with SINA online using greengenes (DeSantis et al., 2006). After quality control, the sample depth was normalized to 30,000 sequences per sample using the bioinformatics software QIIME (Caporaso et al., 2010), which allows the analysis of high-throughput community sequencing data. Normalization to a sample size of 30,032 sequences per sample (minimum original sample size) was done to remove sampling depth heterogeneity. Chloroplast sequences were excluded from all analyses, as they represent sequences derived from chloroplasts of eukaryotic organisms. Rarefaction curves are shown in the supplementary data (Supplementary Fig. 11.1). Sequences have been submitted to GenBank (accession PRJNA295806).

3.3.4 Statistical analysis

All statistical analyses were carried out using the PAST software (Hammer, Harper & Ryan, 2001). Non-metric, multidimensional scaling (NMDS) analyses based on Bray Curtis distance of abundance data was computed to visualize potential differences between the lakes and seasonal shifts within one lake. The OTUs primarily responsible for the observed cyanobacteria differences were assessed with similarity percentage (SIMPER).

3.4 Results

3.4.1 Physical and chemical characteristics

Depth profiles of the measured physical parameters (Supplementary Figs. 11.2 – 11.4) indicated the onset of the stratification in May and a pronounced stratified water column in September 2013. Only the shallow Lake Bergknappweiher was mixed during summer. The greatest epilimnion extension was found in Lake Ammersee (8.0 m), followed by Lake Schliersee (6.0 m) and Lake Ostersee (4.0 m). The metalimnion of Lake Schliersee and Lake Ostersee comprised about 6.0 m, the metalimnion of Lake Ammersee about 10.0 m. The depth

integrated samples taken in May and September 2013 spanned over the entire epi- and metalimnion, and parts of the hypolimnion of the lakes. The samples taken in August 2014 from the surface and the Secchi depth of Lake Ammersee originated from the epilimnion, the sample taken from 10.0 m depth from the metalimnion.

The lakes varied in their water transparencies, as measured by Secchi depths (Table 3.1). Lake Bergknappweiher had the smallest Secchi depth, never exceeding 0.6 m, whilst the Secchi depths of the other lakes always exceeded 1.5 m.

Lakes Ostersee, Schliersee, and Ammersee were similar in their TP concentrations (Table 3.1; Supplementary Figs. 11.5 and 11.6) and classified as either oligo-mesotrophic (Lakes Ostersee and Schliersee) or mesotrophic (Lake Ammersee). Lake Bergknappweiher had a considerably higher TP concentration. Lake Bergknappweiher was classified as eutrophic lake. Both, Lakes Ammersee and Ostersee showed similar values of NO_3^--N and NH_4^+-N concentrations in May and in September 2013. Lakes Bergknappweiher and Schliersee also had similar nitrogen concentrations (NO_3^--N and NH_4^+-N), which were lower than in the other two lakes. Due to the good oxygenation of the lake water columns (Supplementary Figs. 11.2 – 11.4), NO_3^--N always exceeded NH_4^+-N concentrations, although Lake Bergknappweiher exhibited a distinctly higher NH_4^+-N concentration in late summer (Table 3.1).

3.4.2 Cyanobacterial composition

The most abundant bacterial phyla in all samples were Proteobacteria (particularly Alpha- and Betaproteobacteria), Actinobacteria, Bacteroidetes, Cyanobacteria and Verrucomicrobia (Fig. 3.2). The proportions of Cyanobacteria sequences in relation to all sequences varied between 5% (Lake Bergknappweiher, September 2013; Lake Ammersee surface and 10 m depth, August 2014) and 20% (Lake Schliersee, May 2013). In Lake Schliersee, Cyanobacteria were high abundant (20% of all sequences) but Proteobacteria were still the dominant bacterial phylum (26% of all sequences) in May 2013.

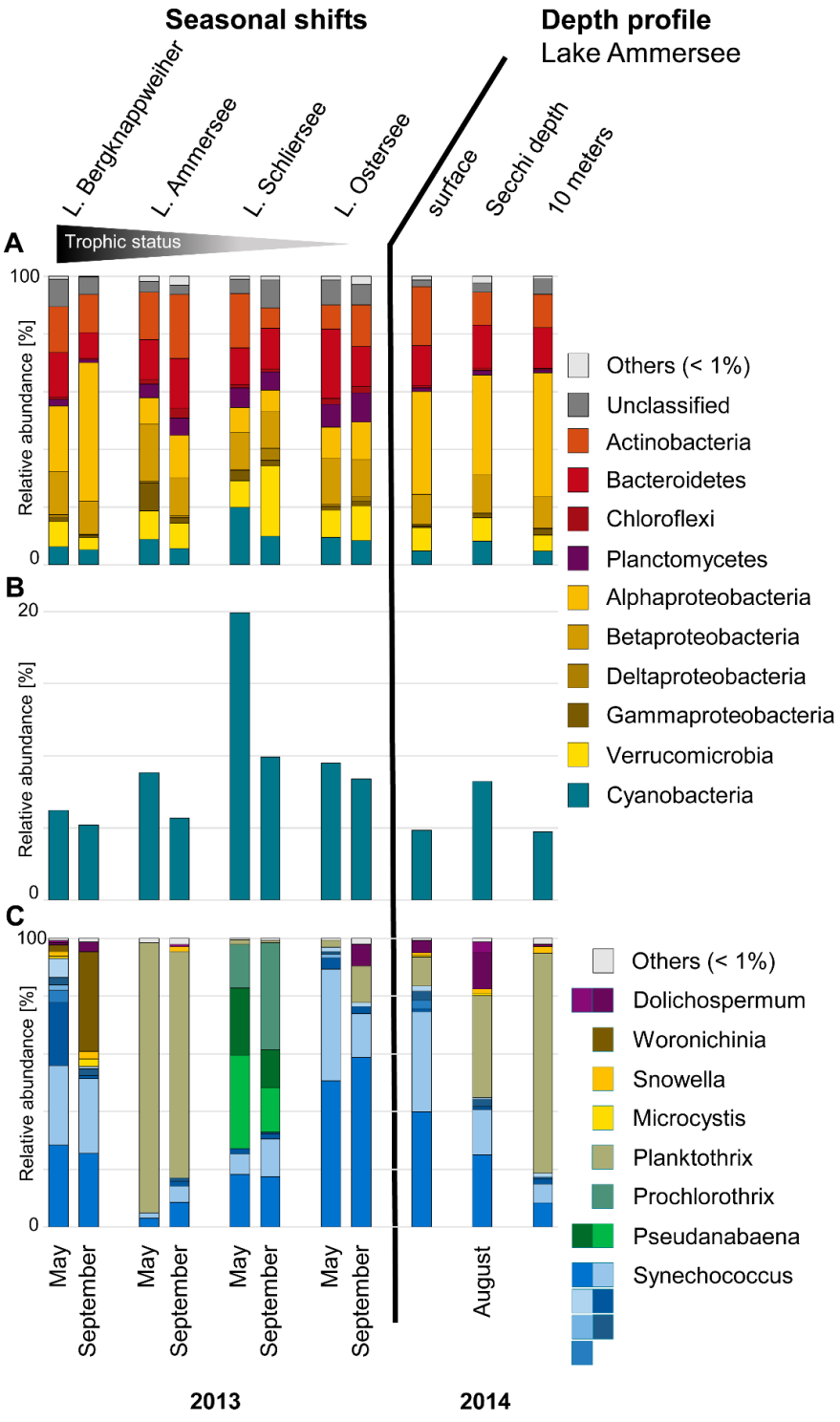

Figure 3.2 Microbial diversity across all lakes in May and September 2013 (left) and in the three depth layers of Lake Ammersee in August 2014 (right). **A** Relative abundance of major bacterial phyla. **B** Relative abundance of cyanobacteria. **C** Cyanobacterial diversity at OTU level (97%). The different shades of colour indicate different OTUs within a genus.

The dominant cyanobacterial genera differed in the four lakes investigated and each lake was dominated by one unique genus of cyanobacteria that contributed more than 50% of the total

cyanobacterial population at both sampling dates (except for Lake Schliersee in September). In Lake Ammersee, *Planktothrix* accounted for 94% (May 2013) and 78% (September 2013) of all cyanobacterial OTUs. In Lake Bergknappweiher, 94% of all the cyanobacterial OTUs in spring and 57% in late summer were classified as *Synechococcus*. A similar pattern was observed in Lake Ostersee, where 98% and 80% of cyanobacteria OTUs were *Synechococcus* in spring and late summer, respectively. In Lake Schliersee, *Pseudanabaena* was the dominant cyanobacterial genus present, accounting for 56% in spring and 29% in late summer, whereas *Prochlorothrix* was the dominant genus in September (37%). The differences among cyanobacterial communities were confirmed by SIMPER analysis (Bray Curtis). More than 90% of the overall average dissimilarity between the lake samples (= 77.9%) was explained by these six main OTUs: *Planktothrix* 33%, *Synechococcus* 33%, *Pseudanabaena* 17%, *Prochlorothrix* 11%, *Woronichinia* 2% and *Dolichospermum* 2%.

3.4.3 Distribution patterns of individual cyanobacterial OTUs

Synechococcus was present in all four lakes both in spring and in late summer 2013 (Fig. 3.2). *Planktothrix* was dominant in Lake Ammersee but was also found in lower abundance in Lake Ostersee and in Lake Schliersee in both spring and late summer. *Woronichinia* was unique to Lake Bergknappweiher. Similarly, OTUs of the genera *Pseudanabaena* and *Prochlorothrix* occurred only in Lake Schliersee.

Not all individual genera were clustered into discrete OTUs (presented as squares in Fig. 3.2). Sequences from *Woronichinia*, *Microcystis*, *Snowella*, *Planktothrix*, and *Prochlorothrix* clustered into single OTUs, whereas *Dolichospermum* and *Pseudanabaena* formed two OTUs and *Synechococcus* occurred as seven different OTUs. Four out of seven *Synechococcus* OTUs were found in all four lakes (Fig. 3.2), while only one *Synechococcus* OTU is restricted to Lake Bergknappweiher and Lake Ammersee.

The dominant cyanobacteria of Lake Ammersee were *Planktothrix* and *Synechococcus*. The relative abundance of *Synechococcus* decreased with depth and comprised 84%, 46% and 21% of all cyanobacteria at the surface, Secchi depth and 10 m, respectively. *Planktothrix* had an inverse relationship, increasing in abundance down the water column (10%, 35% and 76% of all cyanobacteria).

Beta diversity across the four lakes was explored with non-metric multidimensional scaling (NMDS) based on a Bray-Curtis distance matrix.

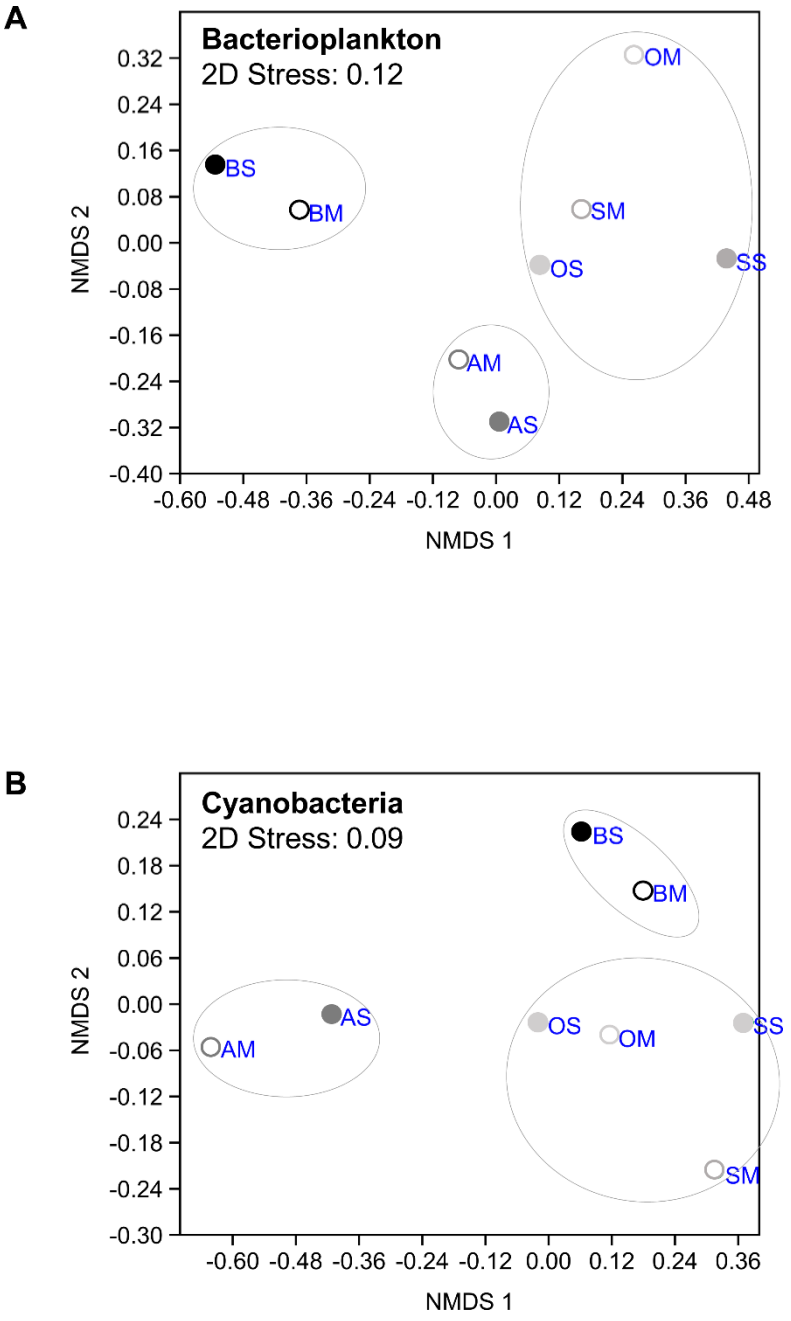

Figure 3.3 NMDS plots based on Bray-Curtis distance. **A** NMDS based on all bacterioplankton sequences (Stress: 0.1194, NMDS1: 0.7083, NMDS 2: 0.0531). **B** NMDS based on all cyanobacteria sequences (Stress: 0.09109, NMDS 1: 0.7382, NMDS 2: 0.00567). M, May; S, September; A, Lake Ammersee; B, Lake Bergknappweiher; O, Lake Ostersee; S, Lake Schliersee. Circles comprising lakes of the same trophic state have been drawn to the NMDS plots to clarify results.

Eutrophic Lake Bergknappweiher is the only lake well clustered at bacterioplankton level (Fig. 3.3 A), while the meso- (Lake Ammersee) and oligo-mesotrophic (Lakes Ostersee and Schliersee) are distributed along a gradient. On the level of cyanobacteria (Fig. 3.3 B), only Lake Ammersee is clearly separated from the other lakes, while oligo-mesotrophic and eutrophic lakes are mixed together. Differences between the samples of spring and late summer were

evident. Also, within the cyanobacteria, the NMDS indicated differences between the communities of spring and late summer (Fig. 3.3B). The highest seasonal variations on cyanobacteria level occurred in Lake Ammersee. The cyanobacterial communities of Lake Ostersee and Lake Bergknappweiher were similar and showed similar trends between spring and late summer. In general, differences between the lakes were more pronounced than the differences within one lake at different time points.

3.5 Discussion

In contrast to our expectation (i), the highest relative abundance of cyanobacteria occurred in one of the lakes with lowest nutrient levels (Lake Schliersee), whereas the eutrophic lake (Lake Bergknappweiher) showed the lowest relative abundances of cyanobacteria. In line with our hypothesis (ii), we found characteristic cyanobacterial communities in the different lakes. In general, all studied lakes (except Lake Schliersee in autumn) were dominated by a unique genus, accounting for more than 50% of the cyanobacterial sequence numbers from the respective sample. Furthermore (iii), the depth profile studied in Lake Ammersee revealed distinct variations in depth-distribution patterns.

In this study a typical distribution of bacterioplankton commonly found in freshwater was revealed (Zwart et al., 2002). No obvious difference at the level of bacterioplankton phyla was evident between the lakes. The cyanobacterial group was analysed at a higher resolution as they were the target of this study. In general, eutrophic lakes are expected to have a higher representation of cyanobacteria in contrast to more oligotrophic lakes (Van der Gucht et al., 2005). In our study, the nutrient-rich lake contained the lowest relative abundances of cyanobacteria compared to total bacterioplankton.

It has been described previously (Van der Gucht et al., 2005) that lakes have distinct bacterioplankton communities. We could demonstrate that these differences are also present at the cyanobacteria level in the studied lakes. Each of the studied lakes had a distinct community and a predominant cyanobacterial genus. Oligo-mesotrophic Lake Ostersee and eutrophic Lake Bergknappweiher were both dominated by the same genus, *Synechococcus*, underlying their close grouping in the NMDS (cyanobacteria). Freshwater *Synechococcus* are known to thrive in oligotrophic conditions (Sherr, Sherr & Wheeler, 2005) and this genus is widespread in the Osterseen Lake District (Zwirglmaier et al., 2015). In the marine environment, there are *Synechococcus* strains, which are predominantly found in meso-eutrophic waters (Zwirglmaier et al., 2007; Zwirglmaier et al., 2008). Generally, unicellular cyanobacteria such as *Synechococcus* are known to be associated with lower trophic status

(Mateo et al., 2015). Our results indicate a potential high-nutrient-adapted *Synechococcus* clade in freshwaters, as one *Synechococcus* OTU was only found in the eutrophic Lake Bergknappweiher and in the mesotrophic Lake Ammersee (A-SC, 2014), but not in the oligo-mesotrophic lakes Ostersee and Schliersee. Based on our data, *Synechococcus* as a genus is unsuitable as an indicator for trophic status, but the sub-genus level seems to show adaptations to different nutrient conditions. This assumption is supported by the already known use of *Synechococcus* as a sub-genus trophic marker within the marine environment (Scanlan & West, 2002). Some genera might therefore have greater potential as trophic indicators than others.

The predominant cyanobacterial genus in Lake Schliersee was identified as *Pseudanabaena* with our analysis pipeline and based on 400 nt Illumina sequence reads. These reads could actually belong to the genus *Limnothrix*, since these two genera are very closely related (Acinas et al., 2008) and Lake Schliersee is generally known for the occurrence of *Limnothrix redekei*, based on microscopic identification (Schaumburg et al., 2005). It has been shown, that *Limnothrix redekei* favours low light supplies, low soluble reactive phosphorus and low water temperature (Kangro & Nõges, 2003). Furthermore, *Limnothrix redekei* occurs in stratified lakes (Rücker, Wiedner & Zippel, 1997), conditions typical for Lake Schliersee. Additionally, *Limnothrix* sp. is known as eutraphent indicator species (Schaumburg et al., 2005). This categorization requires further investigation, as Lake Schliersee was determined as oligo-mesotrophic in our study based on TP. These results illustrate possible limits of phylogenetic identification based on NGS sequence reads of a few hundred bases, and potential discrepancies between the traditional, microscopy-based morphological identification and modern sequence-based approaches, which has resulted in the reclassification of a number of species in recent years (Wacklin, Hoffmann & Komárek, 2009; Komárek, 2010a; Komárek, 2010b; Komárek & Mareš, 2012).

The dominant cyanobacterial genus in mesotrophic Lake Ammersee was *Planktothrix* sp. (formerly part of *Oscillatoria*), which is a known member of the cyanobacterial plankton in this lake. The first quantitative record of *Planktothrix* in this lake has been reported in 1999 (Ernst, Hitzfeld & Dietrich, 2001). In our study, it was not possible to classify *Planktothrix* down to species level. Since we found only one OTU classified as *Planktothrix* species and Lake Ammersee is known for the occurrence of *P. rubescens* (Teubner et al., 2004; Ernst et al., 2006; Ernst et al., 2009) it is highly likely that this OTU represents *P. rubescens*. Over the last decades, *Planktothrix* sp. has increased in Lake Ammersee due to a gradual decrease of the very high nutrient status towards the current mesotrophic status. In 2013, high relative abundances of *Planktothrix* sp. were detectable. *Planktothrix* sp. are filamentous cyanobacteria able to regulate their buoyancy in response to light (Walsby, 2005). A future increase in abundance of

Planktothrix rubescens is therefore expected due to climate change influence and longer stratified periods. Another factor potentially promoting the high abundances of *Planktothrix rubescens* in the future might be increasing turbidity due to heavy rainfall events and brownification (Rasconi et al., 2015). A reduced light availability is considered as one of the main factors causing the increase of *Oscillatoriales*, such as *Planktothrix rubescens* (Reynolds, 1998).

The depth profile revealed a competition between *Planktothrix* and *Synechococcus* in Lake Ammersee. Previous studies on *Planktothrix rubescens* in a temperate deep prealpine lake (Lake Zurich, Switzerland) showed similar results, with coccoid cyanobacteria (presumably *Synechococcus spp*.) dominating in the epilimnion and *P. rubescens* dominating in the metalimnion during summer (Van den Wyngaert et al., 2011). An increase of *P. rubescens* during autumnal mixing in the upper water layers, which was accompanied by a decrease of coccoid cyanobacteria, was reported in the same study. This was not observed in the current study, possibly since the autumn samples were taken when the lake was still stratified. However, *P. rubescens* and *Synechococcus* sp. become competitors within the epilimnion, while *P. rubescens* can successfully occupy deeper water layers in stratified lakes because of its adaptation to low light conditions (Oberhaus et al., 2007). During stratification, the two genera manage to avoid competition, but during mixing they become competitors, since *Planktothrix* is carried towards the lake surface. The decrease of *Synechococcus* with increasing *Planktothrix* during mixing could also be explained by the potential ability of *Planktothrix* producing microcystins and their proven negative effect on *Synechococcus* growth (Hu, Liu & Li, 2004). These results suggest a focus on depth profiles of cyanobacterial communities in lakes as a further avenue of research to gain a better understanding of their ecology and ecological niches.

A general finding from our study is that *Synechococcus* is a widespread genus in the studied lakes, particularly in the upper layers of the lakes. Since we followed the standard protocol for sampling phytoplankton used by the Bavarian State Water Authority, which is based on a mixed water sample from surface to 20 m, this likely results in a dilution of *Synechococcus* counts from deeper lakes where they can still be the dominant genus in parts of the epilimnion.

The dominance of certain cyanobacteria species can be caused by several interacting factors, which are often difficult to separate (Reynolds, 1998; Dokulil & Teubner, 2000; Carey et al., 2012; Kosten et al., 2012; Marmen et al., 2016). The four ecotypes of cyanobacteria (Mur, Schreurs & Visser, 1993) are differently influenced and promoted: colony forming species, well-mixed species, stratifying species and nitrogen-fixing species. Thus, the behaviour in the

water column (buoyancy) might also be a key factor influencing the "signature species" (*Synechococcus* sp., *Planktothrix* sp. and *Limnothrix* sp.) that we found in the studied lakes.

We found unique genera only in Lakes Schliersee and Bergknappweiher, supporting previous studies that cyanobacterial indicator genera may exist only for lakes of higher nutrient status: meso-eutraphent: *Planktothrix rubescens*, eutraphent: *Limnothrix redekei*, *Lyngbya limnetica* and eu-polytraphent: *Aphanizomenon flos-aquae* (Schaumburg et al., 2005). However, it is interesting to note that *Limnothrix* was detected in oligo-mesotrophic Lake Schliersee and the unicellular picocyanobacterium *Synechocccus* was found in high abundance not only in oligo-mesotrophic Lake Ostersee, but also in eutrophic Lake Bergknappweiher.

Considering the differences observed within and between lakes in terms of cyanobacterial communities and temporal composition, these findings confirm that representative samplings must comprise several time points, particularly if community structures of the non-dominant species are to be evaluated. Trends related to the trophic status at the level of the cyanobacterial communities of the lakes were not noticeable, which may be due to too an undersized dataset and the dominance of one genus in each lake, stable during the two sampling campaigns (except for Lake Schliersee). Our study represents only a snapshot into the bacterioplankton and cyanobacteria community structures of four different lakes at two time points in the year. The communities remained relatively stable within each lake, consistent with findings of recent studies on the cyanobacterial communities in Lake Ostersee, which reported stable communities over several years (Zwirglmaier et al., 2015; Ruber et al., 2016). This stability between spring and autumn may be the result of stratification, still observed in autumn. These findings seem to indicate, that cyanobacteria as a group are not very effective for lake trophic classification. Nevertheless, the occurrence of specific cyanobacterial species, and their monitoring with modern NGS technologies, may have the potential to be used as an indicator for trophic or other environmental parameters influencing a lake. Further research, comparing a larger number of lakes and their cyanobacterial communities over space and time is needed to establish a reliable indicator system, which can be used for ecosystem monitoring and lake management.

3.6 Conclusions

The results of this study provide insights into the cyanobacteria communities of four lakes with different trophic status. We found evidence that the four studied lakes of different trophic status host characteristic cyanobacteria and cyanobacterial composition was depth-dependent.

We could identify highly abundant cyanobacterial genera characteristic for each lake, which should be verified in future studies including a larger number of lakes. The occurrence of specific genera clearly does not only depend on the trophic status, but also on other parameters such as stratification behaviour, light supplies and mean euphotic depth ratio or mean temperature. The decreasing cost of molecular genetic analyses will further increase potential applications of the NGS approach used in this study. These methods have the potential to identify the heretofore unknown and neglected world of cyanobacterial diversity. An approach based on NGS might reveal additional cyanobacterial species, which could be used not only for lake trophic classification but also for a deeper understanding of biodiversity and ecological function of cyanobacteria. A polyphasic approach, combining traditional methods and genomic molecular techniques might be a good option for future diversity studies on cyanobacteria.

4. Seasonal monitoring of potentially toxic cyanobacteria

A similar version of this chapter is in preparation: Bauer, F. R., U. Raeder, J. Geist & K. Zwirglmaier. Seasonal monitoring of potentially toxic cyanobacteria.

4.1 Abstract

Several European directives legally require the monitoring of eukaryotic phytoplankton and cyanobacteria to evaluate water quality and to protect swimmers from potential exposure to cyanotoxins and related injuries. The identification of organisms of interest is still often done by microscopy using morphological characteristics. In this study, we used *16S rRNA* gene-based methods to identify organisms. We studied the occurrence of two important cyanobacterial genera -*Limnothrix* sp. and *Planktothrix* sp.- during the vegetation period in 2014 in different depth layers of Lakes Ammersee and Schliersee. The data was analysed using quantitative real-time PCR. Furthermore, we revealed the cyanobacterial succession during a visible surface bloom by Illumina MiSeq sequencing in eutrophic Lake Bergkappweiher. We investigated the vertical distribution of *Limnothrix* sp. in Lake Schliersee in study year 2014, as it is known for *Planktothrix rubescens* in deep stratified lakes. Additionally, we could reveal, that a succession of diazotrophic and non-nitrogen-fixing genera occurred during the bloom in Lake Bergknappweiher. Knowing the actual community structure of cyanobacteria is a helpful tool to predict future changes in the specific lake ecosystem.

4.2 Introduction

Cyanobacteria are important representatives of phytoplankton in aquatic ecosystems. The presence of cyanobacteria can have positive and negative effects. On the one hand, cyanobacteria as ubiquitous phototrophic organisms are responsible for a large proportion of the worldwide CO_2 fixation. On the other hand, many cyanobacteria, especially when occurring in blooms, can produce toxic metabolites (cyanotoxins) and can therefore be a danger in bathing lakes, in fisheries and for drinking water supplies (Chorus et al., 2001; Paerl et al., 2001; Zurawell et al., 2005). Cyanobacteria in lakes follow the natural phytoplankton succession depending on nutrient supply and light intensity (Reynolds, 1984). In the temperate regions, especially during stratified periods, the risk for potential toxic cyanobacterial blooms is high (Paerl & Huisman, 2008). Several European directives legally require the monitoring of eukaryotic phytoplankton and cyanobacteria. A regular monitoring based on visual valuation is done in bathing waters, to protect swimmers from exposure to cyanotoxins and related

health problems (European Bathing Water Directive). The European Water Framework Directive requires the regular monitoring to evaluate general water quality. Currently, the identification based on morphological characteristics is still the common method to identify cyanobacteria.

Here, we use a house-in developed quantitative real-time PCR (qPCR) assay as an absolute quantification method for the analysis of monthly taken lake water samples (chapter 2). We were interested in the vertical distribution of *Planktothrix* sp. in Lake Ammersee and *Limnothrix* sp. in Lake Schliersee in three different depths, as these are the dominant cyanobacterial genera in the studied lakes (chapter 3). For both lakes, we studied surface and Secchi depth samples. In addition, we sampled 10 m depth in Lake Ammersee and the depth of the oxygen maximum (about 6 m) in Lake Schliersee. Additionally, we analysed the cyanobacterial composition during a visible surface bloom in eutrophic Lake Bergknappweiher by Illumina MiSeq sequencing to reveal the natural succession during the different stages of a cyanobacterial bloom.

We hypothesized that (1) we can reveal a vertical distribution of *Limnothrix* sp. in Lake Schliersee, as it is already known for *Planktothrix rubescens* in Lake Ammersee and (2) that in particular, *Limnothrix* sp. maxima will be found in the layers of oxygen maxima. As Lake Bergknappweiher is characterized by high P:N ratios, we hypothesized (3) that a succession of diazotrophic and non-nitrogen-fixing genera will occur.

4.3 Material and Methods

4.3.1 Study sites

The study was conducted at three pre-alpine lakes in Bavaria, Germany. Lakes Schliersee and Ammersee are both known for high abundances of potentially toxic cyanobacterial genera: *Limnothrix redekei* in Lake Schliersee and *Planktothrix rubescens* in Lake Ammersee (Schaumburg et al., 2005; Ernst et al., 2009). Lake Bergknappweiher is a small, shallow turbid lake, known for recurrent algal blooms during the summer months (Morscheid et al., 2006). The composition of the bacterial and cyanobacterial communities of these lakes has already been analysed (chapter 3). These results form the basis for the analyses in the current study. Furthermore, more information on the studied lakes has also been described in chapter 3.

4.3.2 Physical and chemical measurements

Depth profiles of temperature, oxygen content, oxygen saturation, pH and conductivity were measured on each sampling day using a multi-parameter probe (MPP 930 ITS, WTW, Germany). Physical parameters were measured every metre from the surface to 10 m depth and every two metres from 10 m to 20 m depth in the deep lakes. In the shallow Lake Bergknappweiher, physical parameters of the whole water column comprising about 2.0 to 2.5 m were determined in 0.5 m steps. For the analysis of chemical parameters, a surface water sample was taken on each sampling day. Sampled water was filtered through a 0.2 µm pore size membrane filter (Whatman/GE Healthcare, UK) prior to measurement of nitrate-nitrogen (NO_3^--N) and ammonium-nitrogen (NH_4^+-N) concentrations. Total phosphorus (TP) concentration was calculated from unfiltered water samples. TP values were determined following established methods by the German chemists' association GdCH (2013), NO_3^--N values were determined using the method described by Navone (1964). Ammonia-nitrogen was determined using ion chromatography.

4.3.3 Field sampling for molecular analyses

Samples for the monitoring of cyanobacteria were taken monthly between February and December 2014 in Lake Schliersee and between March and December 2014 in Lake Ammersee. For molecular analyses, one litre from the surface and the Secchi depth was sampled, respectively. As Lake Ammersee is known for the mass occurrence of the potentially toxic *Planktothrix rubescens* in deeper water layers (metalimnion) during stratified stages (Ernst et al., 2009), additional samples from 10 m depth were taken from Lake Ammersee during the monitoring. In Lake Schliersee an additional oxygen maximum apart from the surface was detected in most of the samples, indicating photosynthesis. Therefore, in these lakes, water from the depths of the oxygen maxima was sampled, additionally.

In Lake Bergknappweiher, a visible surface bloom (VSB) from the beginning of July until the beginning of October 2014 occurred. To get an overview of the natural cyanobacterial succession in the lake, water samples were taken before the VSB, during the VSB and after the VSB. For molecular analyses, one litre of surface water was sampled and cooled until further processing. Within two hours after sampling, the water was filtered through 47 mm diameter cellulose nitrate membrane filters with a pore size of 0.2 µm (Whatman, UK). Filters were stored at -80 °C until DNA was extracted.

4.3.4 DNA extraction

DNA was extracted using a phenol-chloroform-based protocol as described previously (Zwirglmaier et al., 2015).

4.3.5 Quantitative real-time PCR

The occurrence of genera, which had been classified to be important in the studied lakes (chapter 3), was quantified using qPCR. The abundances of *Planktothrix* in Lake Ammersee and *Limnothrix* in Lake Schliersee were quantified by qPCR targeting the *16S rRNA* gene using primer pairs PlanC_f (5' CTGAGAGGATGATCAGCC 3') and PlanC_r (5' CTGAGAAAAGGGGTTGAC 3') for targeting *Planktothrix* and Limn667_f (5' GGATGCAAGCGTTATCCG 3') and Limn667_r (5' CACCTGGAATTCCTCCTG 3') for targeting *Limnothrix.* Primer pairs have been developed earlier in this thesis (chapter 2). Cycling conditions for these reactions were as follows: 3 min, 95 °C; 39 cycles of 15 s, 95 °C and 30 s 64.5 °C (for *Planktothrix*) and 65.0 °C (for *Limnothrix*). The qPCR amplifications were performed with a Biorad CFX96 cycler using the SSO Advanced Universal SYBR Green Super Mix (Biorad, Hercules, CA, United States) using 10-µl reactions with 0.2 µM primers. Melting curves and gel electrophoresis were carried out to verify the specific amplification of the target. Cell numbers were calculated in consideration of the detection limits determined earlier (chapter 2). The detection limit for the primer specific for *Planktothrix* was 10^6 (SQ value), the detection limit for the primer specific for *Limnothrix* was 10^4 (SQ value). The SQ value is the absolute gene copy number in the reaction.

4.3.6 Sequencing

In Lake Bergknappweiher, a visible surface bloom was present throughout most of sampling period in 2014. To identify the composition of these blooms, the *16S rRNA* gene of the samples was sequenced bidirectionally using Illumina MiSeq v3 2x300 paired end sequencing. A detailed description is given in chapter 3.

4.3.7 Data analysis

The qPCR data was analysed using the Bio-Rad CFX Manager software. Sequencing data was analysed with USEARCH v 7.0 and associated python scripts (Edgar, 2010). Quality control and classification was done as described previously (chapter 3). PAST software (Hammer, Harper & Ryan, 2001) was used for statistical calculations. Shannon indices were determined as a measure of cyanobacterial and total microbial diversity during the visible surface bloom. Non-

metric multidimensional scaling analyses were carried out to represent similarities between the Lake Bergknappweiher samples during the visible surface bloom on phyla and cyanobacteria level.

4.4 Results

4.4.1 Physical and chemical measurements

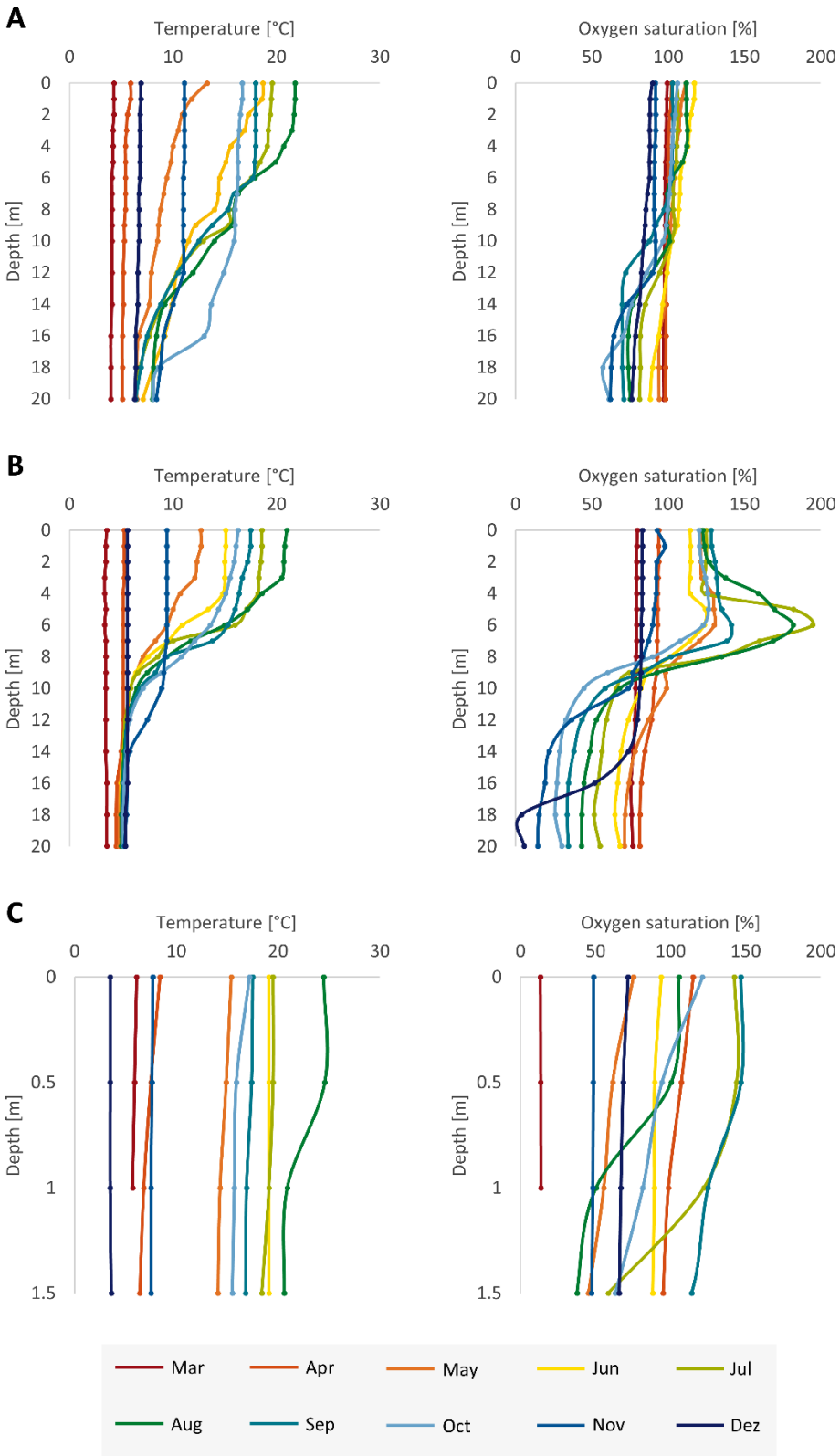

Figure 4.1 Depth profiles of temperature and oxygen saturation in the monitored lakes determined in the study year 2014. **A** Lake Ammersee. **B** Lake Schliersee. **C** Lake Bergknappweiher.

Lake Ammersee was stratified from June to October 2014 (Fig. 4.1). The metalimnion extended between six and 16 metres depth. The highest oxygen saturations were detected at the surface on each sampling date. The maximum oxygen saturation of 117% was measured in June. Oxygen saturation never dropped below 57% in 20 metres depth.

The highest surface temperature in Lake Ammersee was measured in August (21.8 °C).

Lake Schliersee was also stratified from June to October. The metalimnion during the stratification period was between four and nine metres depth. During the summer months, the highest oxygen saturations were between five and six metres depth. The maximum of 195% oxygen saturation was reached in July. In Lake Schliersee, oxygen saturation decreased towards 20 metres depth and the minimum of 4% was measured in December in 18 metres depth.

The highest surface temperature in Lake Schliersee was measured in August (21.0 °C).

Lake Bergknappweiher had no stable stratification throughout the study period. The oxygen maximum of 146.9% was detected in August at the surface. The highest water temperature of 23.0 °C was also measured in August at the surface.

Table 4.1 Mean hydrochemical parameters measured at the surface of the studied lakes in the study year 2014.

	Lake Ammersee	Lake Schliersee	Lake Bergknappweiher
Secchi depth [m]	3.9	2.3	0.4
Total phosphorus [µg/l]	14	11	209
Soluble reactive phosphorus [µg/l], detection limit < 5 µg/l	Below detection limit	Below detection limit	14
Nitrate-nitrogen [mg/l]	1.0	0.1	0.5
Ammonium nitrogen [mg/l], detection limit < 0.1 mg/l	Below detection limit	Below detection limit	Below detection limit

Chemical parameters are presented in Table 4.1. Highest mean total phosphorus contents were measured in Lake Bergknappweiher (209 µg/l). Mean soluble reactive phosphorus content in Lake Bergknappweiher was about 14 µg/l. Lake Schliersee and Lake Ammersee were lower in total (14 µg/l and 11 µg/l) and soluble reactive phosphorus contents (below detection limit in both lakes). Highest concentrations of nitrate-nitrogen were measured in Lake Ammersee (1.0 mg/l). Lake Bergknappweiher and Lake Schliersee had mean nitrate-nitrogen values of 0.5 mg/l and 0.1 mg/l. Ammonium-nitrogen was below the detection limit in all analysed lake surface samples. Mean Secchi depths were calculated from monthly-determined Secchi depths during the experimental period. Mean Secchi depths were 3.9 m in Lake Ammersee, 2.3 m in Lake Schliersee and 0.4 m in Lake Bergknappweiher.

4.4.2 Vertical distribution of the main cyanobacterial genera

The abundance of *Planktothrix* in Lake Ammersee (Fig. 4.2) ranged from values below the detection limit to 3.57 x 10^9 cells/ml. The highest cell counts of *Planktothrix* were detected in August at a depth of 10 m. During the stratified months June to October, the cell numbers were below 4 x 10^8 cells/ml (except for August, 10 metres depth). In the spring months March to May, the cell numbers varied between 4 x 10^8 and 2 x 10^9 per millilitre. Distribution of *Planktothrix* sp. was similar in September and October. In the winter months, November and December, the cell numbers ranged between 2 x 10^8 and 5 x 10^8 per millilitre. An increase of cell number with depth was observed in five of ten studied months. In June, the number of

Planktothrix cells was below the detection limit in all three depth layers. At the surface, the abundance of *Planktothrix* in the summer months was comparably low, with 3.57 x 10^9 cells/ml except for August.

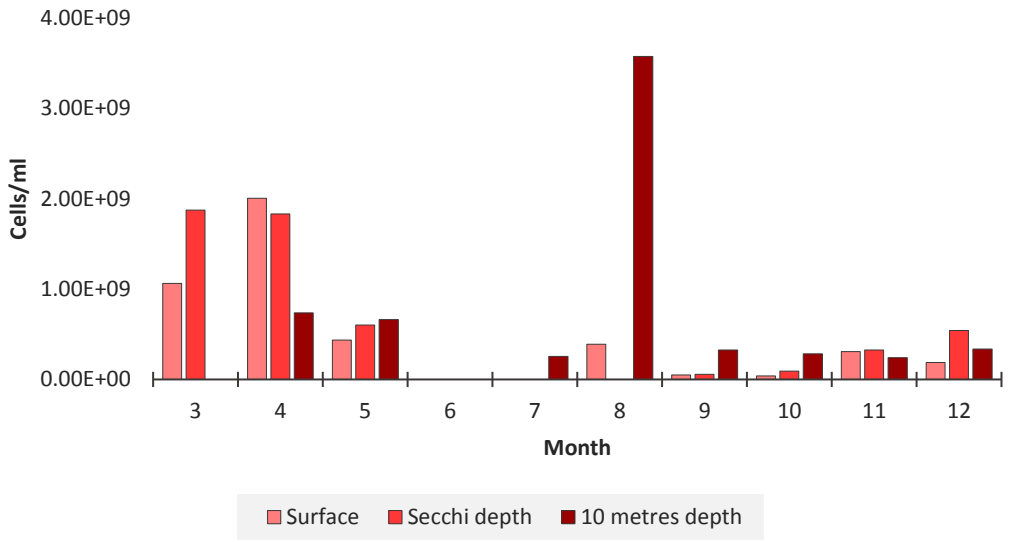

Figure 4.2 *Planktothrix* sp. cell counts per ml determined by qPCR in the different depths of Lake Ammersee between March ("3") and December ("12") 2014. Quantification limit: SQ value ≤ 10^6.

The abundance of *Limnothrix* in Lake Schliersee (Fig. 4.3) ranged from values below the detection limit to 1.91 x 10^8 cells/ml. The highest cell counts of *Limnothrix* were detected in February at the Secchi depth. During the stratified months June to October, the cell numbers were below 9 x 10^8 cells/ml. An increase of cells in the oxygen rich layer could be observed in May, August, September and October. The lowest numbers of *Limothrix* were detected in July in all three studied depth layers. The abundance of *Limnothrix* during the summer months is comparably low at the surface, ranging from values below the detection limit to 1.5 x 10^8 cells/ml.

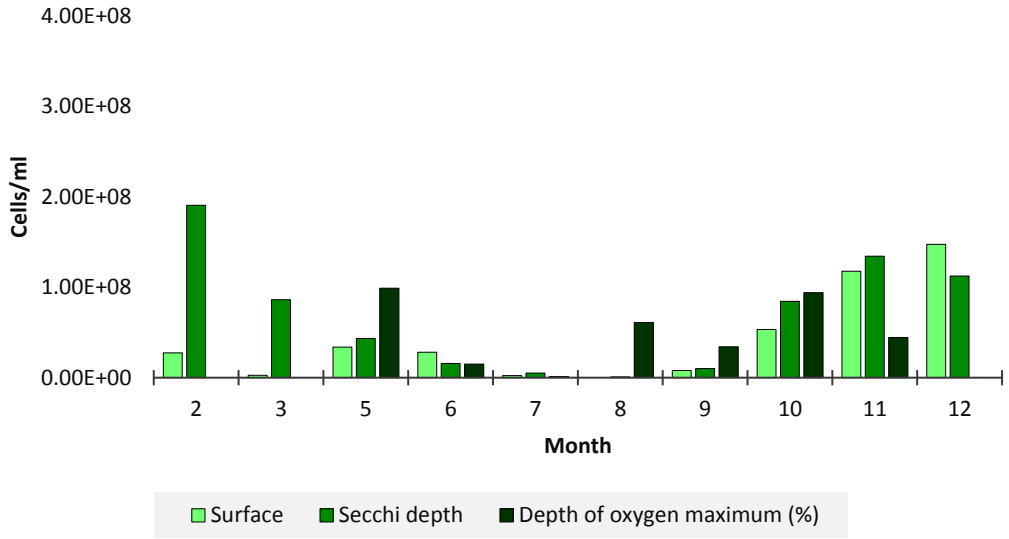

Figure 4.3 *Limnothrix* sp. cell counts per ml determined by qPCR in the different depths of Lake Schliersee between February („2“) and December („12“) 2014. April data is missing. Quantification limit: SQ value ≤ 10^4.

4.4.3 Cyanobacterial succession during a natural visible surface bloom

From May to December 2014, the relative abundance of cyanobacteria in the field samples of Lake Bergknappweiher varied between 0.5% in spring and 66% at the climax of the visible surface algal bloom (Fig. 4.4 A).

The relative abundance of cyanobacteria decreased from the beginning of the bloom in early July until the stage after the algal bloom in November. The only exception was the climax of the visible surface bloom, when cyanobacteria comprised 66% of the bacterial community. The visible surface bloom was dominated by cyanobacteria. Chloroplast sequences decreased during the bloom, indicating a low abundance of eukaryotic phytoplankton (Fig. 4.5 A). At the level of cyanobacteria, the algal bloom was divided in two stages. The first stage was dominated by *Dolichospermum* species and the second stage was dominated by *Microcystis* species. During the peak of the visible algal bloom, the cyanobacterial community comprised exclusively *Dolichospermum* sp. and *Microcystis* sp. (Fig. 4.4 B). *Microcystis* was represented during the dominated stages by only one OTU, *Dolichospermum* was represented by two OTUs, one was the dominating OTU during the first stages of the bloom, and the other one was dominating during the peak of the VSB and the later stages of the bloom.

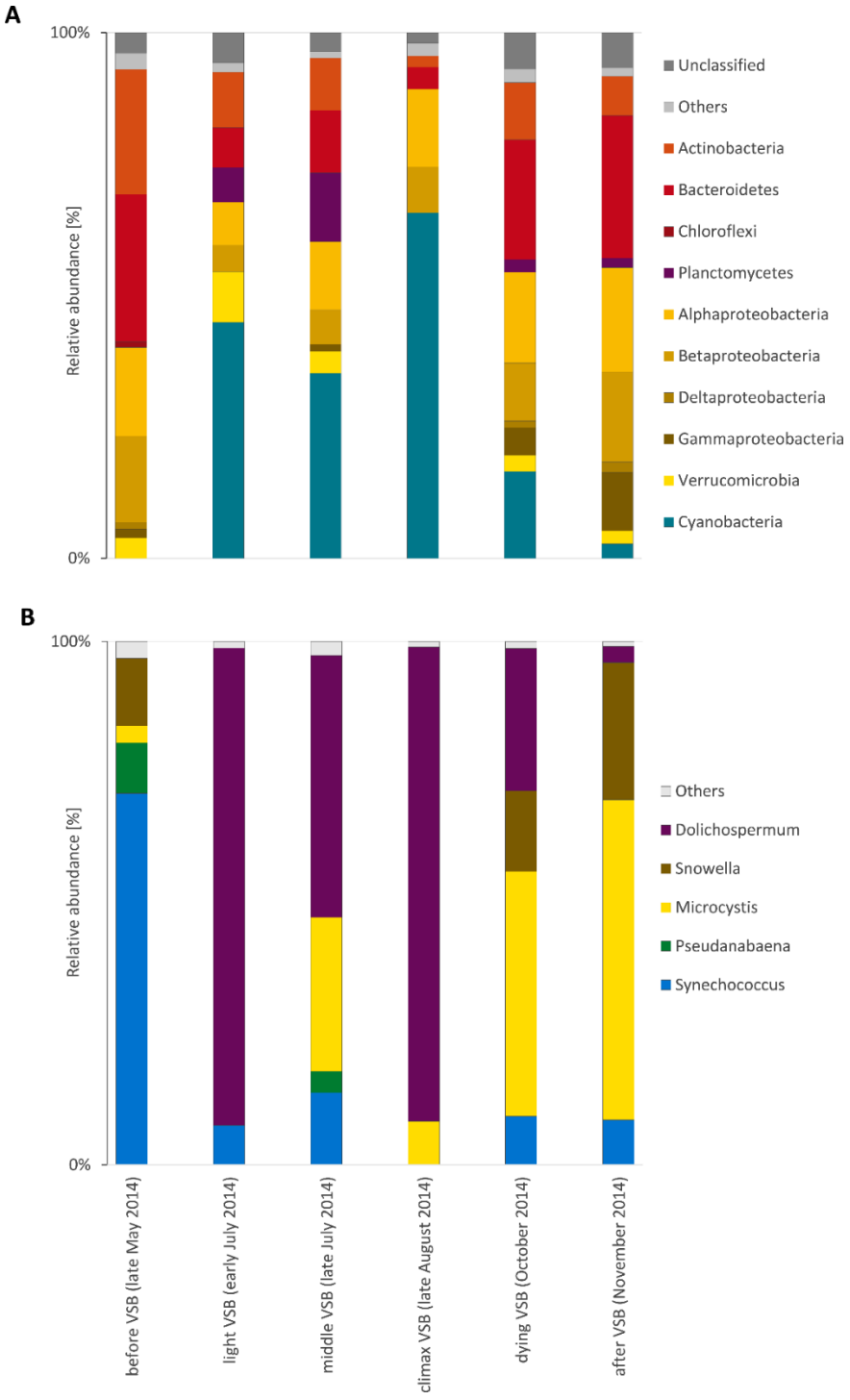

Figure 4.4 Visible surface bloom in Lake Bergknappweiher in 2014. **A** Bacterioplankton composition. **B** Relative proportion of Cyanobacteria.

Shannon indices of the cyanobacterial diversity in the sampling season 2014 are shown in Fig. 4.5 B and varied between 2.0 (before VSB) and 0.5 (climax of the VSB). The cyanobacterial diversity indices of the different sampling days (different stages of the bloom) were inverse

unimodally related to the stage of the bloom (beginning algal bloom, middle algal bloom, climax of algal bloom, collapsing algal bloom, after visible algal bloom). The diversity indices of the microbial communities before, during and after the algal bloom followed the same trend as the cyanobacterial communities. The values ranged from 2.1 (bloom climax) to 4.8 (dying bloom). The lowest diversity was determined in the bacterial as well as in the cyanobacterial communities during the peak of the VSB.

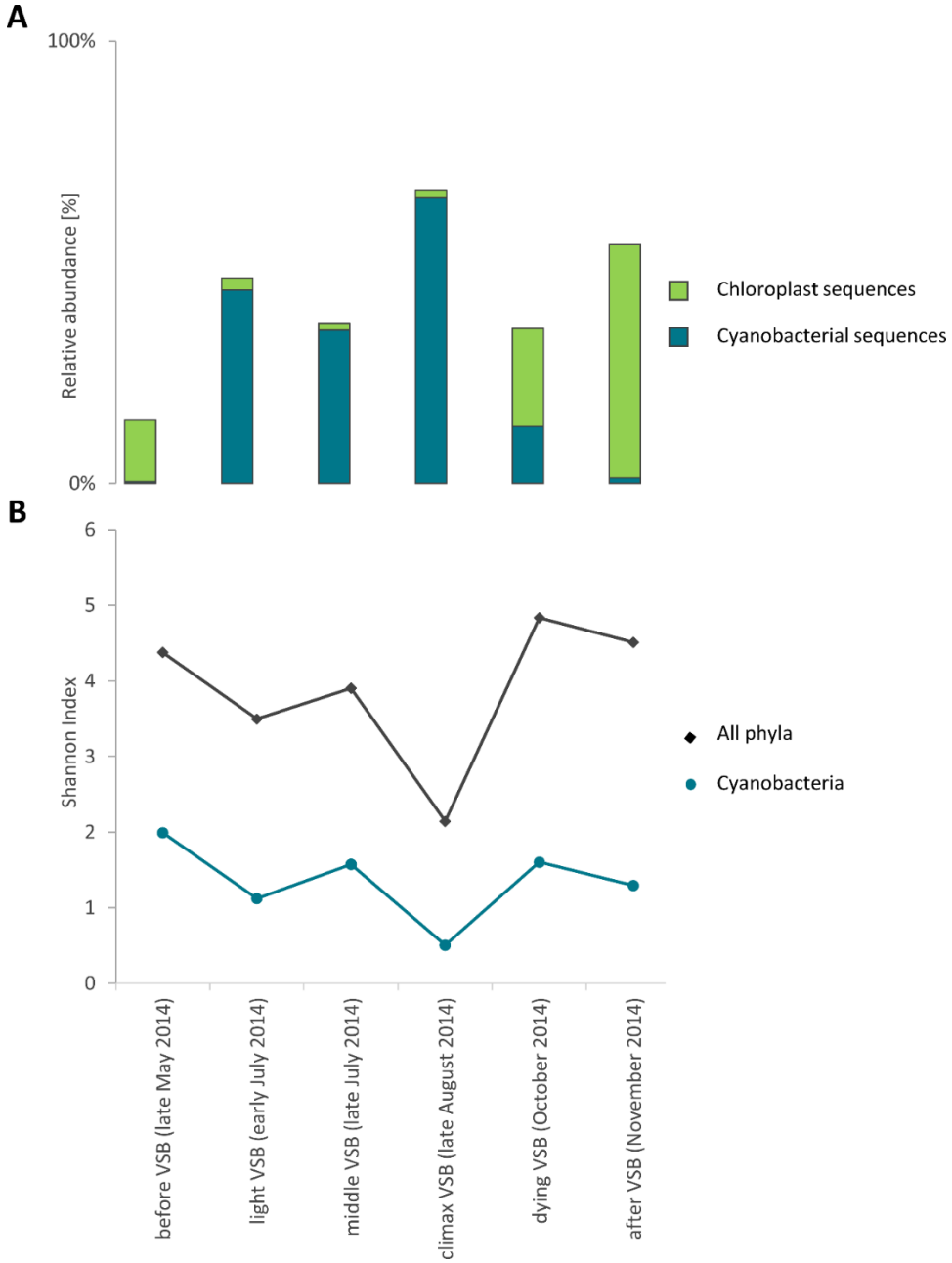

Figure 4.5 Visible surface bloom (VSB) in Lake Bergknappweiher in 2014. **A** Relative abundance of cyanobacterial vs. chloroplast sequences (% of total sequences). **B** Shannon's diversity indices (H_S) of all bacterial phyla (black) and cyanobacteria only (blue) during the bloom stages.

The measured total phosphorus content in Lake Bergknappweiher ranged between 175 µg/l and 394 µg/l (Fig. 4.6). The TP increased during the VSB and reached the maximum of 394 µg/l at the peak of the bloom. The nitrate-nitrogen content accounted for 0.4 mg/l before the VSB and increased after the first observation of the bloom. The maximum of 2.1 mg/l nitrate-nitrogen was reached during the bloom climax. When the bloom was collapsing, the nitrate-nitrogen content decreased again. However, it increased again to 1.8 mg/l until the sampling in early winter after the VSB. The Secchi depth varied between 0.3 and 0.5 metres during the sampling period.

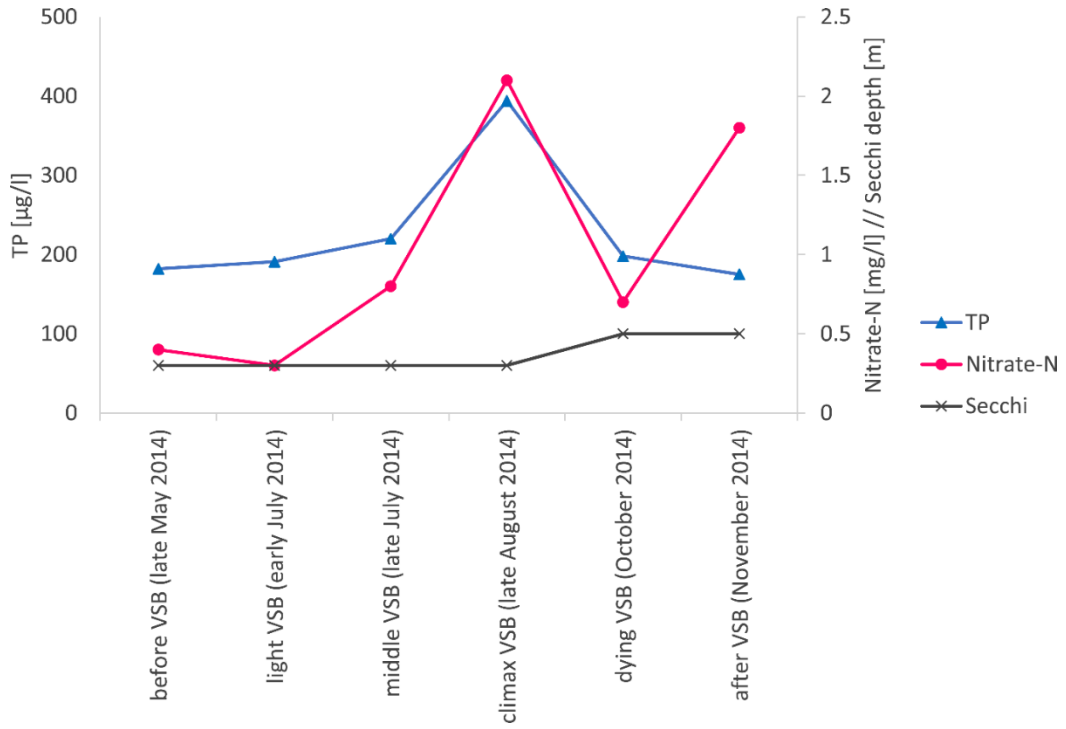

Figure 4.6 Secchi depth, total phosphorus (TP) and nitrate-nitrogen concentrations before, during and after the visible surface bloom (VSB) in Lake Bergknappweiher in 2014.

The NMDS of the bacterioplankton sequences (Fig. 4.7 A) shows a high similarity between the bacterioplankton communities before and after the VSB. Furthermore, the community of the stadium of the middle VSB was similar to these communities. The communities of bloom climax and dying VSB were also similar to each other but varied greatly from all the other communities. The community of the light VSB differed from all the other communities. Considering only the cyanobacterial sequences in the NMDS (Fig. 4.7 B), a high dissimilarity between the community before the VSB and all the other communities studied is evident. The

cyanobacterial communities of the middle VSB and after the bloom form are very similar and thus, the cyanobacterial communities of the stages in between form a loop.

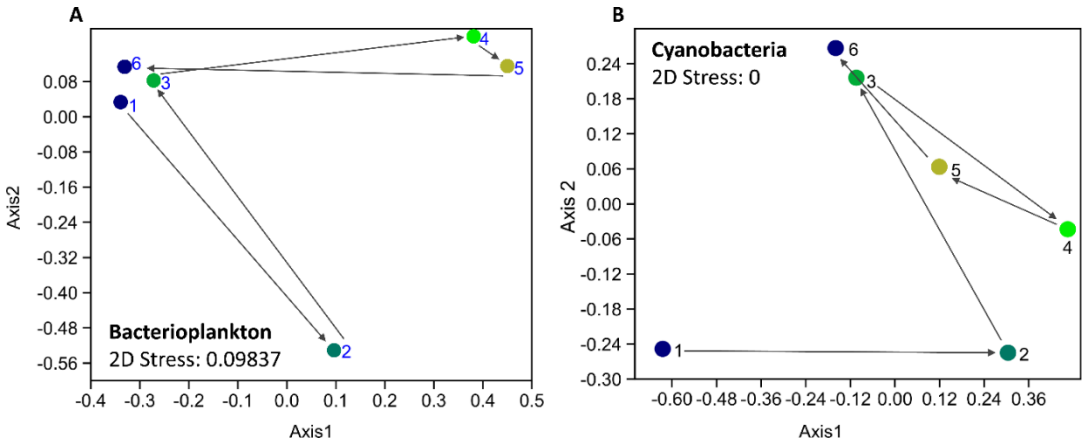

Figure 4.7 Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis distance. A Sequence-based bacterioplankton community similarities in the samples before, during and after the VSB. Axis 1: 0.5414, Axis 2: 0.1284, Stress: 0.09837. B Cyanobacteria community similarities in the samples before, during and after the VSB. Axis 1: 0.7685, Axis 2: 0, Stress: 0. Data points are interconnected by auxiliary lines to clarify temporal connections. 1: before VSB, 2: light VSB, 3: middle VSB, 4: climax VSB, 5: dying VSB, 6: after VSB.

4.5 Discussion

4.5.1 Vertical distribution

Vertical differences in abundances of *Planktothrix* and *Limnothrix* could be revealed in both lakes. In general, the cell numbers exceeded maximum values reported in previous studies. Ernst et al. (2009) reported a maximum of 8 x 10^4 cells/ml in the years 1999 to 2004. In our study, most of the samples exceeded this value by four orders of magnitude. The cell densities in the study of Ernst et al. (2009) were calculated via fluorescence image analyses. Our results indicate a general increase in the abundance of *Planktothrix* sp. during the last years. The differences in the results might also be caused by the different applied methods for the determination of cell counts. During stratification, the maximum of *Planktothrix* abundance is expected in deeper layers of the water column (Walsby et al., 2004; Ernst et al., 2009). We found the maximum abundance of *Planktothrix* sp. cells in August at a depth of ten metres, indicating that this sample was really taken from the layer of *Planktothrix* mass occurrence. It has been shown, that internal waves have a major impact on the distribution of *Planktothrix rubescens* in deep stratified lakes (Cuypers et al., 2011), which might be a reason for the lower cell numbers in ten metres in the other months of stratification. An increase of *Planktothrix*

rubescens has been reported for many other lakes, due to decreasing phosphor concentrations in consequence of lake restoration (Ernst, Hitzfeld & Dietrich, 2001; Jacquet et al., 2005; Legnani et al., 2005). Other studies have shown a minor impact of nutrient load, but a higher impact of light supplies (Walsby & Schanz, 2002). The depth of mass occurrences of *P. rubescens* is explained by buoyancy regulation in relation to the irradiance (Walsby et al., 2004).

Limnothrix was found in abundances of about 1 x 10^8 cells/ml in Lake Schliersee. It is known, that the occurrence of *Limnothrix redekei* is favoured by low soluable reactive phosphorus contents, low light supply and low water temperature (Rojo & Cobelas, 1994; Kangro & Nõges, 2003). Furthermore, stratification events positively influence the abundance of *Limnothrix redekei* (Rückner et al., 1997). We found a high abundance of *Limnothrix* at the Secchi depth in spring. During the stratified period of Lake Schliersee, the abundance of *Limnothrix* decreased in all analysed depth layers. In contradiction to hypothesis (2), this result indicates that *Limnothrix* is not responsible for the oxygen production in the layer of the oxygen maximum. The high oxygen values might be caused by high abundances of diatoms or other phototrophic phytoplankton. Future research on *Limnothrix* sp. in Lake Schliersee requires the analysis of samples from a more detailed depth profile. Furthermore, it might be helpful to use a fluorometric probe to detect cyanobacteria maxima for future studies on buoyancy-regulated cyanobacteria. For the analysis of future data, it should be noted that Lake Schliersee is equipped with an air ventilation system at the bottom of the lake.

4.5.2 Visible surface bloom

During the peak of the bloom, cyanobacteria dominated the total bacterial community. When including the chloroplast sequences in the analysis, results confirmed that the visible surface algal bloom in Lake Bergknappweiher consisted mainly of cyanobacteria and that eukaryotic phytoplankton played only a minor role. Although numerous phyla of freshwater phytoplankton can form blooms, cyanobacteria are the most notorious bloom formers (Paerl et al., 2001). The reasons for this phenomenon are that some cyanobacteria have higher growth rates, and some are resistant to grazing due to their morphological properties and toxin production (Van Donk & Hessen, 1993).

During the main phases of the natural algal bloom, *Dolichospermum* sp. was the dominant cyanobacterial genus. The genus *Dolichospermum* sp. comprises all planktonic morphospecies of the former genus *Anabaena* (Wacklin, Hoffmann & Komárek, 2009). It is one of the ubiquitous bloom-forming nitrogen fixating cyanobacterial genera and some of the species can

produce toxic metabolites, such as cyanotoxins (Li, Dreher & Li, 2016). During the later stages of the bloom, *Microcystis* sp. dominated the cyanobacterial community, accompanied by minor proportions of *Dolichospermum* species. Previous studies have shown that *Microcystis* species can alternate dominance with *Aphanizomenon*, a genus with the ability to fix nitrogen (Beversdorf, Miller & McMahon, 2013) and estimated N inputs from nitrogen-fixation were large enough to supplement or even support the *Microcystis* blooms. We conclude that during the bloom in Lake Bergknappweiher in 2014, *Microcystis* species benefited from the co-occurrence of nitrogen-fixing *Dolichospermum* species. The predominance of *Dolichospermum* sp. and *Microcystis* sp. is reflected in the decreasing diversity indices during the bloom. The picocyanobacterium *Synechococcus* sp. plays a minor role during the bloom but is still co-occurring with the potential toxic bloom-forming cyanobacteria at all sampling days, except the bloom-maximum. The measured TP values represent the different stages of the VSB with the highest TP concentrations at the climax of the VSB. The measured nitrate-nitrogen contents substantiate that diazotrophic *Dolichospermum* species fix nitrogen, as an increase of nitrate-nitrogen is measurable after the beginning of the first stage of the bloom, which is dominated by *Dolichospermum*. After the beginning of the second stage dominated by *Microcystis*, the nitrate-nitrogen contents decrease, indicating a consumption of available nitrogen by *Microcystis* species.

The NMDS analyses show, that the VSB influenced the whole bacterioplankton community on phyla level. The entire community structure changed during the VSB. After the bloom, the community was again very similar to the community before the VSB. A different result has been revealed on the level of cyanobacteria. The community changed during the bloom, which is also evident in the NMDS. The loop in between the middle bloom (dominated by *Dolichospermum*) and the stage after the bloom indicates that the bloom would have changed back to a *Dolichospermum* dominated stage after the stage, dominated by *Microcystis*. Due to the beginning of winter and the accompanying decrease in cyanobacterial cells, a further blooming was prevented. Nevertheless, the few cyanobacteria left represent a bloom-forming community composition.

4.6 Conclusion

The community composition of cyanobacteria in lakes is a crucial factor determining the state of a lake and its future potential for alteration due to climate change or other processes of change in the lake. To predict future changes, comprehensive knowledge on the actual community structures of cyanobacteria is essential. Thus, it is very important to further extend the current knowledge on this group of organisms related to the respective lake. This study proves that molecular biology, e. g. *16S rRNA* gene-based methods, are a helpful tool to gain this task. Most of the questions are fully addressable by using the qPCR assays applied here, others by the application of comprehensive high throughput sequencing. Long-term monitoring is important, as it furthermore provides the evaluation of the efficiency of environmental protection measures.

5. Impact of global warming on freshwater cyanobacteria in Bavarian lakes

A similar version of this chapter is submitted: Bauer, F. R., A. D. Millard, N. Gross, U. Raeder, J. Geist & K. Zwirglmaier, 2018. Impact of climate change on freshwater cyanobacteria in Bavarian lakes.

5.1 Abstract

Global warming may have ecological consequences for undesired blooms of cyanobacteria. We investigated the impact of temperature on the prevalent cyanobacterial communities in selected Bavarian lakes, testing the hypotheses that 1) bloom-forming cyanobacteria will occur more frequently and at higher abundance at warmer temperatures, 2) effects will be more pronounced in nutrient-rich waters and 3) effects are dependent on the season when temperature increase occurs. We conducted aquarium experiments with water from an oligo-mesotrophic and a eutrophic lake. Water was incubated in climate chambers with increased and control temperatures at three time points in the growing period. The changes in community structures were analysed by Illumina MiSeq sequencing of the partial *16S rRNA* gene. Increased temperature favoured cyanobacterial communities, and bloom-forming cyanobacteria in water from both lakes, although the effects were less pronounced under nutrient-poor conditions. Cyanobacterial blooms are expected to occur earlier in the year in nutrient-rich lakes. These findings suggest that problems due to harmful cyanobacterial blooms become more likely with climate change in the study region.

5.2 Introduction

Predicted climate change and global warming are expected to have substantial impact on aquatic ecosystems (Parmesan, 2006; Daufresne, Lengfellner & Sommer, 2009; Hewitson et al., 2014; Rasconi et al., 2015). For instance, warming of the upper ocean layers and changes in salinity and hydrologic changes result in changes in water circulation as well as in carbon and oxygen contents (Rhein et al., 2013). Climate change will not only affect oceans but also freshwater ecosystems, like rivers, reservoirs and lakes (Paerl & Paul, 2012). Analogously to the oceans, climate change scenarios predict rising surface temperatures of lakes and more intense and longer periods of vertical stratification and hydrologic changes. Those effects are accompanied by altered nutrient loading, more intense watercolour (due to increased dissolved organic carbon and humic acids) and increasing reactive oxygen stress (Monteith et

al., 2007; Paerl & Huisman, 2009; Carey et al., 2012; Paerl & Paul, 2012; Lürling et al., 2013; Paerl & Otten, 2013), although a great regional variation is expected (Hamilton & Keim, 2009; Hewitson et al., 2014). The changes will have an impact on aquatic communities, including cyanobacteria. As the earth's oldest oxygen-producing photosynthetic microorganisms, cyanobacteria have had a major impact on shaping earth's atmosphere and biosphere (Raven, 1995; Field et al., 1998; Paerl & Huisman, 2009). It is expected, that the extremely diverse group of cyanobacteria will respond in different ways to climate change. Due to various eco-physiological traits, evolved over their long evolutionary history, cyanobacteria can grow at warmer temperatures and some are able to regulate their buoyancy (Paerl & Huisman, 2009; Carey et al., 2012). Over the last decade, many studies focussed on the influence of climate change on cyanobacteria and research has confirmed that climate change is likely to favour cyanobacterial growth and cyanobacterial dominance in a wide range of aquatic ecosystems (Paerl & Huisman, 2009; Carey et al., 2012; Flombaum et al., 2013). Consequently, cyanobacterial blooms are predicted to occur more frequently in the future (Ekvall et al., 2013). Especially surface blooms often result in the release of toxins (Paerl & Otten, 2013), cause oxygen depletion at night, and can potentially pose a risk to ecosystem services such as drinking water production, fishing and recreational use of lakes. However, the expected impacts of climate change will vary on global, regional and local scales (Heino & Mykrä, 2008), as regional and anthropogenically-driven changes are expected (Paerl & Paul, 2012). Furthermore, cyanobacterial community composition varies due to the adaptation to local conditions, impact of the surroundings and local climate of the respective lake. This makes it challenging to generalise recent findings on the future development of cyanobacteria. The species-specific responses are critical to understand ecosystem changes and to predict potential adverse effects (Ekvall et al., 2013).

The Bavarian alpine upland is an important recreational area and the use of its various lakes is essential for tourism. In future, the recreational use of many lakes might be threatened due to changes in the cyanobacterial community and to the potential increase in toxic blooms. In general, there are two major different types of recreational waters in Bavaria: There are nutrient poor, mostly larger and deeper lakes with clear water, and smaller shallow lakes, which are mostly nutrient rich and more turbid.

The aim of this study was to investigate the cyanobacterial communities of these two different categories of lakes in Bavaria and to identify potential shifts in their composition in the future due to climate change. Therefore, we artificially increased the water temperature to simulate the possible effects of global warming in aquarium experiments with lake water sampled at three different time points in the growing period, i. e. when growth of macrophytes and

phytoplankton can be expected. Sampling in May, July and October, we wanted to capture the communities in spring (lower water temperature, unstratified water column, low abundance of cyanobacteria cells expected), in summer (warmer water temperatures, stratified water column, higher density of cyanobacteria cells expected) and in autumn (decreasing water temperature, end of stratified period, maxima of cyanobacteria density expected, usually season of blooms). The water for the experiments derived from two different lakes in Upper Bavaria: Lake Bergknappweiher, a small, eutrophic and turbid shallow lake, and Lake Ostersee, a larger lake, which is oligo-mesotrophic and poor in humic acids. In our study, we focussed on increased temperature under the aspect of different starting conditions regarding nutrient content of the studied lakes. It has been shown that temperature and nutrient content are responsible for most bacterioplankton community changes (Lindström, 2000; Suikkanen et al., 2013). Specifically, we hypothesized that 1) bloom-forming cyanobacteria will have an advantage at increased water temperatures, and 2) that temperature effects will vary depending on the season. Furthermore, we expected that these effects are more pronounced under nutrient-rich conditions. We evaluated the adequacy of aquarium experiments for the study of climate change effects, as mesocosms have been shown to be suitable when it comes to the study of algal responses to nutrient enrichment (Spivak, Vanni & Mette, 2011; Ekvall et al., 2013). Using *16S rRNA* gene sequencing to quantify changes in bacterial taxa, we identified changes in bacterial phyla, with a focus on the phylum Cyanobacteria.

5.3 Material and Methods

5.3.1 Study design

At three time points in the 2014 sampling period, water samples were taken from two water bodies differing in their nutrient content and natural turbidity. Aquaria experiments were conducted to analyse potential differences in the composition and relative abundance of cyanobacteria based on *16S rRNA* gene sequence analyses. Samples were taken on day 0 (natural lake water, starting point of each experiment), day 27 (after four weeks in the aquarium at a specific temperature) and day 55 (after eight weeks in the aquarium at a specific temperature).

5.3.2 Experimental setup

Twelve aquaria (50 cm length x 30 cm width x 30 cm height) were placed in temperature-controlled climate chambers at the Limnological Research Station in Iffeldorf (LSI), Germany. Six of the aquaria were filled with 40 L of surface water that originated from Lake

Bergknappweiher, the other six aquaria were filled with 40 L of surface water sampled from Lake Großer Ostersee. The water was not prefiltered, as we wanted to mimic natural starting conditions and did not want to create an artificial system by removing grazers or filamentous (cyano)bacteria. Lakes Bergknappweiher and Ostersee are situated in Bavaria (Germany) and differ in their trophic state: Lake Bergknappweiher has previously been classified as eutrophic (Teubner et al., 2004) and Lake Ostersee has been classified as oligo-mesotrophic (Zwirglmaier et al., 2015). We could confirm this classification by measurements of several hydrochemical parameters. The total phosphorus content in Lake Bergknappweiher was 190 µg/l, nitrate nitrogen 0.5 mg/l and ammonium nitrogen 40 µg/l in the field samples (measured at the surface). Total phosphorus content in Lake Ostersee was 15 µg/l, nitrate nitrogen 1.2 mg/l and ammonium nitrogen 30 µg/l. Furthermore, the two lakes vary in their watercolour. Watercolour was measured spectrophotometrically (420 nm wavelength) according to Rasconi et al. (2015). Lake Bergknappweiher has a distinct brownish watercolour (absorption 0.053), indicating humic acids, whereas the transparency of Lake Ostersee is clearly higher (absorption 0.004). Lake Bergknappweiher is a small pond with 5 ha surface area and a maximum depth of 2.5 m. Lake Ostersee has a surface area of ~118 ha and a maximum depth of 29.5 m. Both lakes are surrounded by forests and farmland, Lake Ostersee is situated in a nature conservation area. Both, Lake Bergknappweiher and Lake Ostersee, are used as bathing lakes.

After collecting water samples in plastic barrels, the water was equally distributed into each of the aquaria. The temperature treatments of the water in the aquaria (two replicates each) consisted of the surface temperature of the lake at the sampling date (T_C), as control, slightly elevated temperature ($T_5 = T_C + 5$ °C), and strongly elevated temperature ($T_{10} = T_C + 10$ °C). To simulate the effects of increased temperature at different times during the growth period, the experiments were conducted in spring (May), in summer (July) and in autumn (October) 2014. Each of the individual experiments ran over eight weeks. At the end of the experiments the total phosphorus contents of the aquaria had decreased compared to the field samples (Lake Ostersee below the detection limit of 5 µg/l; Lake Bergknappweiher about 50 µg/l). Nitrate nitrogen and ammonium nitrogen values were similar to values measured in the field. Sterile air diffusers permanently and equally aerated each of the aquaria and a cling film at the top protected each of the aquaria from external input of particles and evaporation. The temperature in the aquaria was controlled by the adjustable temperature in the climate chamber and by using aquarium heater elements (Eheim, Germany) for the higher temperatures in T_5 and T_{10} aquaria. All aquaria received the same light conditions (illumination from above, same distance of lightbulbs from all aquaria).

Physical parameters, i.e. temperature, dissolved oxygen, oxygen saturation, pH and conductivity (temperature corrected) were concurrently measured in the field at the surface at the beginning of each experiment and in situ in the aquaria once a week with a multi-parameter probe (MPP 930 IDS, WTW, Germany). Additionally, water temperature was monitored throughout the whole experiment by using HOBO data loggers (HOBO Pendant® Temperature/Light Data Logger, ONSET, USA), in each aquarium. The aquaria were illuminated by 20-30 µEinstein m^{-2} s^{-1} simulating long-day conditions with 16 hours light and 8 hours dark (MASTER TL5 HO 39W/865 1SL, Philips, Netherlands). The replicates were similar concerning their hydrophysical properties (temperature monitoring data measured with HOBO data loggers is shown in Supplementary Figs. 11.8 – 11.10), microbial communities from one replicate of each treatment was sequenced.

Samples for molecular analyses were taken at the start of the experiment on day 0, day 27 (four weeks) and on day 55 (eight weeks) of each experiment. These intervals were chosen to get an overview over the whole experimental period, i.e. initial community composition, halfway through the experiment, where we expected effects from the temperature treatment would be obvious, and at the end of the experiment.

For day 0 natural surface water was sampled (field sample), for days 27 and 55 a sample was taken from each aquarium treatment. Before sampling, the aquarium was stirred, and then 0.5 L were removed. Immediately after sampling, 0.25 L of water was filtered through a 0.2 µm pore size (47 mm diameter) cellulose nitrate membrane filter (Whatman/GE Healthcare, UK). Filters were stored at -80 °C until DNA was extracted, the remaining filters were stored as backups.

5.3.3 Molecular analyses

DNA from the filters was extracted using a phenol-chloroform-based protocol as described previously (Zwirglmaier et al., 2015). Samples were sequenced bidirectionally using Illumina MiSeq v3 2 x 300 paired end sequencing. Samples were prepared according to the manufacturer's recommendations ("Illumina 16S Metagenomic Sequencing Library Preparation", Illumina 2013). A detailed description on sample preparation, sequencing, and quality control is given in chapter 3, with the exception, that the sequences were not normalized in this study. After quality control a total of 804,570 sequences were analysed. Sequences per sample (Supplementary Table 11.1) and rarefaction curves (Supplementary Fig. 11.7) are in the supplementary to data. Afterwards, operational taxonomic units (OTUs) were clustered at 97% sequence identity and subsequently classified with SINA online. For

classification, greengenes (DeSantis et al., 2006) taxonomy was used, which is implemented in SINA online. Unless indicated otherwise, chloroplast sequences (as identified by greengenes classification) were excluded for all analyses. The sequences, which could not be classified using greengenes, were inserted in a phylogenetic tree based on the LTP tree (tree_LTPs106.SSU, August 2011) using ARB (Ludwig et al., 2004). For the deeper classification of cyanobacteria, we used an in-house cyanobacteria-based tree containing 30,000 cyanobacterial sequences. As more than 1000 OTUs were unclassified, we selected the four most abundant OTUs of each sample. These 103 OTUs explain a major part of the unclassified sequences. For further information, we have added graphs showing the development of the four most important OTUs of each sample during each experiment including a table with the classification of these OTUs in the supplementary data (Supplementary Fig. 11.11).

Samples of day 0, day 27 and day 55 were sequenced and the data analysed. In parallel, the development of a natural algal bloom occurring in the eutrophic lake was analysed by sequence analysis of samples taken at three time points. The data were combined with the field samples of the aquarium experiments. By combining the data, it was possible to compare the results of the aquarium experiments with the development of a natural algal bloom. To avoid loss of data, the samples have not been normalized (McMurdie & Holmes, 2014). Instead, lower sequence numbers were taken into account during the interpretation of the data.

Sequences have been submitted to GenBank (accession PRJNA361041).

5.3.4 Statistical analysis

Shannon indices were calculated as a measure of cyanobacterial and total microbial diversity during the aquarium experiments. Non-metric, multidimensional scaling (NMDS) analyses based on Bray Curtis distance of abundance data was computed for the samples of the aquaria experiments, to visualize potential differences between the control and the treatments over time and to separate temperature effects from aquarium effects. The samples of Lake Bergknappweiher and Lake Ostersee were separated for these NMDS analyses but all samples within one lake were analysed together (however, data points of NMDS are presented separately per each of the three experiments to avoid confusion). All statistical analyses to determine the alpha and beta diversity were carried out using the PAST software (Hammer, Harper & Ryan, 2011).

5.4 Results

5.4.1 Hydrophysical measurements

Surface water temperature at the beginning of each experiment varied by about 0.5 °C between Lake Bergknappweiher and Lake Ostersee. The starting experimental temperatures (T_C) recorded were 13 °C in the spring, 22 °C in the summer and 16 °C in the autumn. In general, the conductivity of Lake Ostersee (mean 438 µS/cm) exceeded the conductivity of Lake Bergknappweiher (mean 278 µS/cm). Mean pH value was 8.2 in Lake Ostersee and 8.5 in Lake Bergknappweiher. In Lake Ostersee, oxygen saturation varied between 101.2% and 108.0%, whereas oxygen saturation in Lake Bergknappweiher varied between 103.9% and 121.3% in the field. The mean Secchi depth of Lake Ostersee (3.4 m) exceeded the Secchi depth of Lake Bergknappweiher (0.4 m).

5.4.2 Microbial Diversity

5.4.2.1 Trends in major bacterial phyla

The most abundant bacterial phyla across the field and aquaria samples were Actinobacteria, Bacteroidetes, Planctomycetes, Proteobacteria (Alpha- and Betaproteobacteria), Verrucomicrobia and Cyanobacteria (Fig. 5.1). The relative abundance of cyanobacteria varied between 0.5% (spring) and 35% (autumn) in the field samples of Lake Bergknappweiher and between 4% (summer) and 19% (spring) in the field samples of Lake Ostersee. During the aquaria experiments the relative abundance of cyanobacteria varied between 2% (spring, day 27, T_C) and 48% (summer, day 27, T_C) in Lake Bergknappweiher and between 0.1% (summer, day 27, T_5) and 17% (spring, day 55, T_C) in Lake Ostersee. In Lake Bergknappweiher in spring, cyanobacterial sequences increased slightly with increasing temperature, whereas in summer the inverse trend was detected. In autumn, the proportions of cyanobacteria remained constant, but on day 55 an increase in T_{10} was observed. Converserly to Lake Bergknappweiher, in Lake Ostersee, the cyanobacterial sequences decreased in spring with increasing temperature.

Figure 5.1 Microbial diversity across all aquarium samples in Lake Bergknappweiher and Lake Ostersee in spring, summer and autumn. Presented are the results of the analyses on day 0 (left), after 27 days (centre) and after 55 days of experiment (right).

In the field samples, the percentages of unclassified bacterial phyla in Lake Bergknappweiher varied between 4% (spring, summer) and 7% (autumn) and in Lake Ostersee between 1% (spring) and 4% (summer, autumn). In the experiment with water of Lake Bergknappweiher, the maximum percentage of unclassified sequences was 15%, and in the Lake Ostersee experiments the maximum was 55%, both values were reached in summer (day 55, T_C), respectively. In the autumn experiments of Lake Ostersee, the proportions of unclassified sequences increased with time and increasing temperature. The unclassified sequences could be further classified mainly as Proteobacteria, Bacteroidetes and Cyanobacteria using ARB software (data in the supplementary, Supplementary Fig. 11.11).

5.4.2.2 Trends in cyanobacterial composition

The dominant cyanobacterial phyla in the field samples of Lake Bergknappweiher varied at the three starting points of the aquaria experiments (Fig. 5.2). In spring the dominant genus was *Synechococcus* (71% of cyanobacterial OTUs), in summer *Dolichospermum* (50%) and in

autumn *Microcystis* (47%). In Lake Ostersee in the field samples, the dominant genus was *Synechococcus* in spring (100%), summer (98%) and autumn (70%). Other genera found in field samples of Lake Bergknappweiher, which occurred in smaller quantities, were *Snowella* (up to 15%) and *Pseudanabaena* (up to 10%). In Lake Ostersee in the field sample of autumn, *Synechococcus* was accompanied by *Dolichospermum* (16%) and *Snowella* (14%).

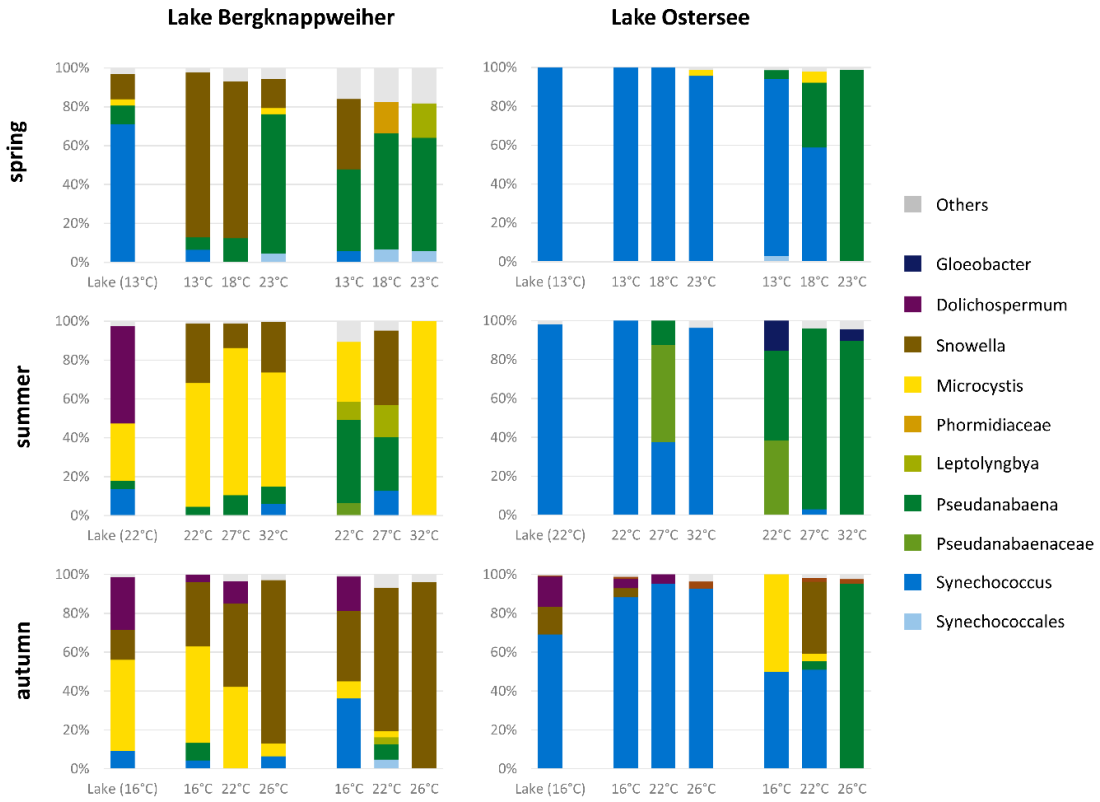

Figure 5.2 Cyanobacterial composition across all aquarium samples in Lake Bergknappweiher and Lake Ostersee in spring, summer and autumn. Presented are the results of the analyses on day 0 (left), after 27 days (centre) and after 55 days of experiment (right).

In Lake Bergknappweiher, in the experiments conducted in spring, during the first four weeks an increase of *Snowella* was evident in T_C and T_5, and in T_{10} *Pseudanabaena* was dominant. After eight weeks *Pseudanabaena* was the dominant cyanobacterial genus in all aquaria (spring 42%, 60% summer, 58% autumn). Furthermore, in T_C an increase in *Snowella*, in T_5 an increase in Phormidiacaeae and in T_{10} an increase in *Leptolyngbya* genera was detected. In the experiments conducted in summer, after four weeks the dominant cyanobacterial genus in the control and the two temperature treatments was *Microcystis* (64%, T_C; 76%, T_5; 59%, T_{10}). Other abundant genera were *Snowella* (up to 31%), *Pseudanabaena* (up to 10%) and *Synechococcus* (up to 6%). By week eight there was a shift towards *Pseudanabaena* and

Leptolyngbya in T_C and T_5, whereas in T_C *Microcystis* and in T_5 *Snowella* were also highly abundant. In T_{10} only *Microcystis* was detected (but this result is only supported by a small amount of data in this sample, Supplementary Table 11.1). In the experiments conducted in autumn, after four weeks a decrease of *Microcystis* could be detected with increasing temperature (50%, T_C; 42%, T_5; 7% T_{10}). Conversely, an increase in *Snowella* could be detected with increasing temperature (33%, T_C; 43%, T_5; 84% T_{10}). This trend continued until day 55: T_{10} contained 96% *Snowella*, T_5 74% and T_{10} 36%.

In Lake Ostersee, in the experiments conducted in spring after four weeks, *Synechococcus* was still the dominant taxa (more than 96%) in each of the samples. After eight weeks, an increase of *Pseudanabaena* correlated with increasing temperature (5%, T_C; 33%, T_5; 98%, T_{10}). In T_5 in addition to *Pseudanabaena* and *Synechococcus*, 6% of *Microcystis* could be detected. In the experiments conducted in summer, a drecrease of *Synechococcus* and an increase of *Pseudanabaena* (13%) and Pseudanabaenaceae (50%) could be detected in T_5 after four weeks. In T_C and T_{10} *Synechococcus* was still highly abundant (> 96%). After eight weeks, the community shifted towards *Pseudanabaena* and Pseudnanabaenaceae in the control (T_C, 46% and 38%). In T_5 and T_{10} the communities shifted towards *Pseudanabaena* (93% and 90%). In the experiments conducted in autumn, after four weeks the community of the control was similar to the starting community, even though *Synechococcus* had increased (89%). In T_5 *Synechococcus* increased (95%) accompanied by *Dolichospermum* (5%), a very similar result was detected in T_{10} (96% and 4%). After eight weeks, in the control *Synechococcus* and *Microcystis* were detected (50%, respectively). The cyanobacterial community of T_{10} comprised 98% Pseudanabaenaceae.

The most abundant genera in Lake Bergknappweiher were *Synechococcus*, *Snowella*, *Microcystis*, *Dolichospermum* und *Pseudanabaena*. The most important genera overall the Lake Ostersee samples were *Synechococcus* and *Pseudanabaena*, as well as *Dolichospermum*, *Microcystis*, *Snowella* and *Gloeobacter* to a lesser extent. *Lyngbya* could only be detected Lake Bergknappweiher, whereas *Gloeobacter* could only be detected in Lake Ostersee.

The maximum relative abundance of cyanobacteria in the Lake Bergknappweiher samples was found in summer (day 27, T_C) and comprised mainly *Microcystis* and *Snowella*. In the aquaria with elevated temperature, the highest relative abundance of cyanobacteria could be detected in autumn (day 55, T_{10}) and comprised mainly *Snowella*. For Lake Ostersee the maximum relative abundance of cyanobacteria was detected in spring (day 0, field sample) and comprised only *Synechococcus*. In the aquaria with elevated temperature, the highest relative

abundance of cyanobacteria could be detected in spring (day 55, T_C) and comprised mainly *Synechococcus*.

5.4.2.3 Alpha diversity of cyanobacteria

Shannon indices for cyanobacterial diversity ranged from 0.25 (autumn, day 55, T_{10}) to 2.22 (spring, day 55, T_5) in Lake Bergknappweiher and from 0 (summer, day 27, T_C) to 1.52 (summer, day 55, T_C) in Lake Ostersee (Fig. 5.3).

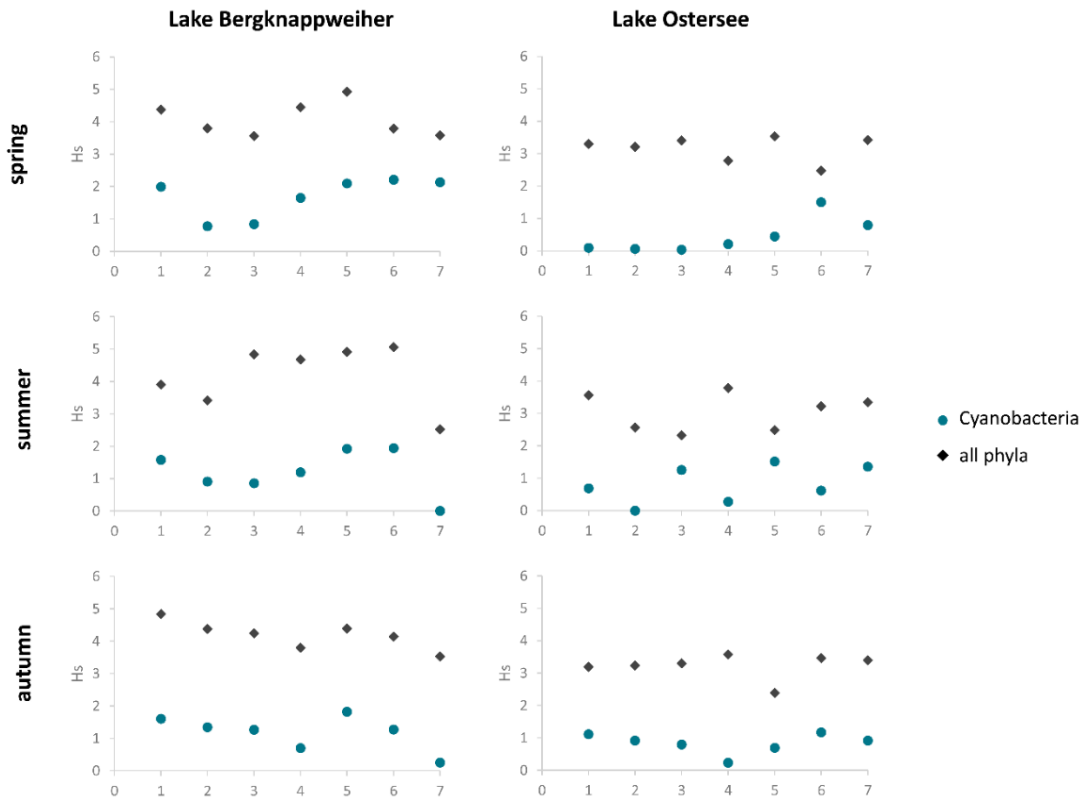

Figure 5.3 Shannon's Diversity Indices of all phyla and cyanobacteria during the aquarium experiments. 1: control/Lake, 2: T_C (day 27), 3: T_5 (day 27), 4: T_{10} (day 27), 5: T_C (day 55), 6: T_5 (day 55), 7: T_{10} (day 55).

The cyanobacterial diversity decreased in all experiments in the first four weeks compared to day 0 (lake sample) and increased in most experiments between day 27 and day 55. The only exception was the autumn experiment of Lake Ostersee, where the cyanobacterial diversity in the control decreased again between day 27 and day 55. In Lake Bergknappweiher in autumn the cyanobacterial diversity decreased with increasing temperature. In Lake Ostersee in spring the highest diversity was found on day 55 in T_5 (27 °C). The total microbial diversity ranged between 3.4 and 5.1 in the samples of Lake Bergknappweiher. In these samples, the same

tendencies as for the cyanobacterial diversity could be observed for the microbial diversity in all three experiments. The Shannon indices for the total microbial diversity in Lake Ostersee ranged between 2.3 and 3.8. The diversity indices of the microbial communities generally showed an opposite trend than the diversity indices of the cyanobacterial communities.

5.4.2.4 Beta diversity of cyanobacteria

As evident from the NMDS (Fig. 5.4), the field samples of Lake Bergknappweiher at the three starting times of the aquaria experiments differed in their cyanobacterial composition, however, the field sample from summer was similar to the field sample of autumn.

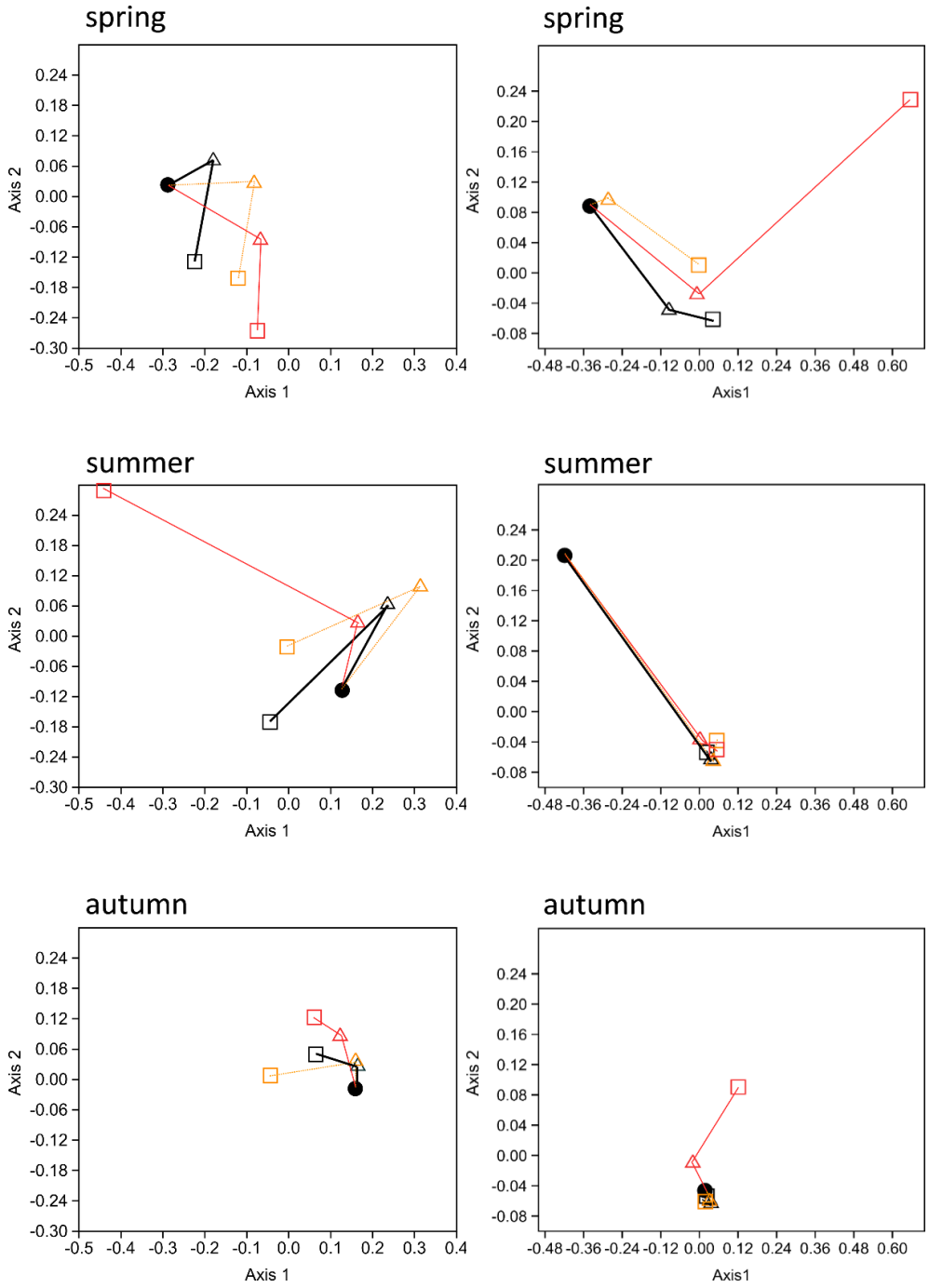

Figure 5.4 NMDS plots based on Bray-Curtis distance. Sequence-based cyanobacterial community similarities in the samples of the spring, summer and autumn aquarium experiments of Lake Bergknappweiher (left) and Lake Ostersee (right). Dot: lake/day 0, triangle: day 27, box: day 55; black: T_C, orange: T_5, red: T_{10}. Left: Axis 1 *0.4498*, Axis 2 *0.2257*, Stress *0.1156*, right: Axis 1 *0.3504*, Axis 2 *0.2865*, Stress *0.1283*. To clarify temporal connections, data points of the same treatment are interconnected by auxiliary lines.

In general, the cyanobacterial communities of the Lake Bergknappweiher aquaria developed similarly and the cyanobacteria composition in the control and in the treatments showed similar trends. However, it has to be stated that the trends varied within the three different time points when the aquaria experiments were conducted. In the spring experiment, the

control samples in week four were most similar to the field sample. The strongest differences compared to the field sample and the control occurred in T_{10}. The result of summer must not be overinterpreted due to the low number of sequences in one of the samples (day 55, T_{10}). In autumn, the cyanobacterial community of T_5 (day 27) was very similar to that from the control. Whereas, after eight weeks, T_5 differed. The cyanobacterial communities of T_{10} (spring, day 55), T_C (summer, day 55) and T_5 (autumn, day 55) were very similar (Axis 1) and represented communities of water samples incubated at matching temperature values (23 °C, 22 °C and 21 °C).

The field samples of Lake Ostersee were similar in spring and in summer. In autumn, the cyanobacterial community differed. In spring, the community changed most rapidly in the T_{10}. The communities of T_C (day 27 and day 55), as well as the communities of T_5 (day 55) and T_{10} (day 27) were similar. In contrast, T_{10} on day 55 comprises a changed cyanobacterial community. In autumn, T_C and T_5 comprised communities similar to the starting community of the lake, both after four and after eight weeks after the beginning of the experiments. The communities of spring (exception T_{10}, day 55) and especially the cyanobacterial communities of summer in week four and week eight were very similar to the field sample of autumn.

5.5 Discussion

The aim of the study was to analyse the potential influence of global warming as a part of climate change on two different lakes situated in Bavaria (Southern Germany), one shallow eutrophic and one deeper oligo-mesotrophic lake. As hypothesized, the aquarium experiments conducted at different seasons demonstrated, 1) that bloom-forming cyanobacteria will have an advantage in the course of the predicted temperature increase (climate change) in the studied lakes and 2) that temperature effects vary depending on the season. Furthermore, it could be shown 3) that these effects are more pronounced and severe under nutrient-rich conditions.

5.5.1 Aquarium experiments

As a core finding of this study, it could be demonstrated, that increased temperature has a strong influence on the development of the cyanobacterial communities causing shift towards bloom-forming cyanobacteria in the studied lakes. This finding is in line with the general assumption that cyanobacterial blooms are likely to occur more frequently in the future (Paerl & Huisman, 2009; Elliott, 2012; Ekvall et al., 2013). Additionally, we found that the timing of temperature increase influences these effects: increased temperature seems to be a

strong risk for bloom-formation in spring but does not necessarily increase bloom-formation in summer in nutrient rich lakes. The incubation at 27 °C and especially 32 °C seemed to be at the upper limit for the growth of cyanobacteria, although in literature the mean optimum growth temperatures for cyanobacteria are reported to range between 27.2 °C and 29.2 °C (Lürling et al., 2013). Thus, the finding of an enhanced growth of cyanobacteria with rising temperature only seems to hold true for colder waterbodies that are below such a temperature limit.

Due to incorporation of the control samples in the NMDS, temperature effects (caused by increased temperature) could be distinguished from aquarium effects (caused by aquarium conditions). If the treated samples (T_5 and T_{10}) react in a similar manner as the control (T_C), changes in the communities are likely to be caused by aquarium effects. If T_5 and T_{10} react in a dissimilar manner as T_C, this suggests community shifts due to temperature effects. Any dissimilarities between the starting sample (Lake/Day 0) and T_C indicate effects caused by aquarium conditions.

In autumn, a temperature dependent shift (according to the NMDS analyses) towards *Snowella* sp. occurred. In the aquarium experiments of the oligo-mesotrophic humic acid poor Lake Ostersee, temperature effects were less pronounced than in eutrophic Lake Bergknappweiher. However, in Lake Ostersee the aquarium effects on the cyanobacterial communities were more pronounced than effects caused by temperature treatments. Slight effects caused by increased temperatures could be observed in the experiments conducted in spring and in summer. A cyanobacterial succession depending on temperature was evident in the spring experiment. In summer, mainly effects caused by aquarium conditions were observable, with little additional influence of temperature effects. In the autumn experiment, slight effects in cyanobacterial community caused by increased temperature appeared. Interestingly, in Lake Ostersee the percentages of unclassified sequences increased with temperature. These unclassified sequences were further analysed using ARB (Ludwig et al., 2004) and reported in the supplementary (Supplementary Fig. 11.11). In the spring experiment, these increasing sequences were mainly classified as Proteobacteria, which might be an indicator for the beginning of the dying phase of primary producers at the end of the experiment. In the summer experiment, a shift towards *Leptolyngbya* sp. could be detected with time, but especially in the control, not representing a temperature effect. As also observed in the spring experiment, Proteobacteria and Bacteroidetes increased. During the autumn experiment, besides a shift towards Proteobacteria and Bacteroidetes, an increase in *Synechococcus* sequences could be detected in the two temperature treatments after eight weeks of the experiment. These results might indicate a shift from naturally occurring

Synechococcus towards a higher temperature adapted *Synechococcus*. In addition to the other results, these findings suggest that oligo-mesotrophic Lake Ostersee is less sensitive towards bloom-formation than eutrophic Lake Bergknappweiher, also during global warming.

5.5.2 Bloom-forming cyanobacteria

In the field samples of both the eutrophic Lake Bergknappweiher and oligo-mesotrophic Lake Ostersee, bloom-forming genera were detected to a varying extent and dependent on the sampling date. *Microcystis* is a globally abundant toxic cyanobacteria genus (Marmen et al., 2016). The optimal light-intensity of *Microcystis* species varies between 7.5 and 22 µE $^{m-2\ s-1}$ (Krüger & Eloff, 1977) and *Microcystis* species are tolerant to high insolation (Reynolds et al., 2002). The optimal habitat for *Microcystis* species are mixed layers of small eutrophic lakes (Reynolds et al., 2002). The growth rate of *Microcystis* cells increases with higher temperatures up to 30 °C (Chu et al., 2007). Furthermore, it has been shown, that temperature is a significant factor influencing the competition of *Microcystis* and other genera, such as *Planktothrix* (Chu et al., 2007). Therefore, it is already well-known, that *Microcystis* species will have an advantage when water temperature increases above 20 °C, due to their physiology (Carey et al., 2012). This could also be confirmed by the results of this study and indicates that an increase of toxin-producing cyanobacteria appears likely. *Snowella* is a genus of the family of Coelosphaeriaceae but the whole family is currently being revised due to phylogenetic relations (Komárek, 2016). Recently, it has been reported that this genus is not known to produce any toxins (Gallina, Anneville & Beniston, 2011) but other studies identified *Snowella* as potentially microcystin producing cyanobacterium (Kouzminov, Ruck & Wood, 2007). In the UK *Snowella* is even accounted as one the most common cyanobacterial genera causing toxin problems in freshwater (Carvalho et al., 2011). It is one of the most common colonial picocyanobacteria in freshwaters and does not form blooms (Callieri & Stockner, 2002; Jungblut, Lovejoy & Vincent, 2010). *Snowella* is a sister group of the closely related *Woronichinia naegeliana* (Rajaniemi-Wacklin et al., 2006) and it is most commonly detected in oligo-mesotrophic lakes. Conversely, our results show that *Snowella* also occurs in eutrophic freshwaters, suggesting that this genus has a greater plasticity than currently assumed. The development of *Pseudanabaena* was promoted during the experiments. Less is known on the toxicity of the *Pseudanabaena* genus so far, but some studies on the toxicity of *Pseudanabaena* have been published (Oufdou et al., 2000; Oudra et al., 2001; Oudra et al., 2002; Kouzminov, Ruck & Wood, 2007) suggesting that some strains are able to produce toxins. The preferred habitat of *Pseudanabaena* are turbid, mixed layers, and *Pseudanabaena* is tolerant to highly deficient light conditions (Reynolds et al., 2002). The cosmopolitan genus of *Pseudanabaena* might have the potential to fix nitrogen

(Acinas et al, 2009; Stal, 2009). In addition to *Pseudanabaena*, the abundance of *Leptolyngbya* and Phormidiacaea also increased in the aquaria with the eutrophic lake water. *Leptolyngbya* is also known as potential nitrogen fixing genus (Mohamed et al., 2008). Some strains of Phormidium are able to produce bioactive compounds, cyanotoxins, as anatoxin and saxitoxin (Gugger et al., 2005; Teneva et al., 2005). *Dolichospermum*, which was highly abundant in some of the field samples, was not promoted by the increased temperature during the aquarium experiments.

5.5.3 The predicted potential of occurrence of blooms

During no-bloom stages, in both lakes, the cyanobacterial community was generally dominated by *Synechococcus* species. The results suggest that the future potential of the mass occurrence of bloom-forming species due to global warming is considerably higher in the eutrophic lake, which is already known for recurrent algal blooms (Teubner et al., 2004). In addition, increased temperature led to a shift in cyanobacterial community in the oligo-mesotrophic lake, even though the community did not shift towards a dominance of bloom-forming species. Nevertheless, our results show, that potentially toxic cyanobacteria such as *Microcystis* will also increase in the oligo-mesotrophic lake, especially if nutrient levels further increase. In addition to the visible surface blooms, which are expected in future, in Lake Ostersee the potential of mass occurrences of other cyanobacterial genera might increase. Summer stratified Lake Ostersee might in future experience longer periods of stratification. A strong relationship between stratification, nutrient availability and cyanobacterial abundance has been reported (Wagner & Adrian, 2009; Elliott, 2012). Cyanobacteria depending on stratified water bodies, such as *Planktothrix rubescens* might increase in Lake Ostersee, especially as a mass occurrence of *Planktothrix rubescens* (former *Oscillatoria*) has already been reported in this lake in the past (Raeder, 1990).

5.5.4 Aquarium experiment vs. culture experiment

A core question in predicting effects of climate change on cyanobacteria is if aquarium experiments are suitable and efficient to appropriately analyse their responses. Naturally, the experimental set up is not able to perfectly imitate the real environmental conditions and it is always necessary to be aware of the limitations of extrapolating findings from experimental studies to natural waters (Carpenter, 1996; Cáceres & Schwalbach, 2001). However, the results clearly show a development of the cyanobacterial communities of the two lakes. Furthermore, the bloom forming genera occurring in the aquaria (Lake Bergknappweiher) are similar to

those increasing under natural conditions. By means of the NMDS analyses, it was possible to detect aquarium effects and to separate these effects from temperature effects. Besides, the revealed temperature effects are different for the two studied lakes. In comparison, culture experiments are able to simulate how the behaviour of a single homogeneous group of cyanobacteria reacts to changing conditions. In addition, it has been shown, that interactions between algae are stronger and more complex in lake simulator systems than in flask systems (Chu et al., 2007). Culture experiments in the past focused on the temperature- and light-dependent growth of invasive and native cyanobacterial species (Mehnert et al., 2010), or on the quality and quantity of light and temperature on the growth of *Planktothrix agardhii* and *Planktothrix rubescens* (Oberhaus et al., 2007). Other studies concentrated on the growth rates of native and invasive cyanobacteria species under various light and temperature conditions (Wiedner et al., 2003) or investigated the potential of algal development growing on dissolved nitrogen compounds (Berman & Charva, 1999).

Furthermore, the effects of temperature as well as nutrients like nitrogen and phosphorus were studied (Vezie et al., 2002; Davis et al., 2009; Davis et al., 2010). Apart from growth behaviour of cyanobacteria cells, the gene expression of microcystin synthesis genes in response to selected environmental factors associated with global warming were investigated (Kim et al., 2005; Scherer et al., 2017a). Depending on the aim of the study, culture experiments might often be the method of choice. However, in these kinds of standardized experiments it is not possible to study the interactions between naturally occurring organisms of the whole aquatic ecosystem. Other studies conducting mesocosm experiments have shown, that it is important to understand effects on both biodiversity and ecosystem functioning (Ekvall et al., 2013; Rasconi et al., 2015). A combination of the results of both kinds of experiments might be expedient. In addition to the improvement of experimental set ups, it would also be necessary to expand the molecular data set of cyanobacteria and to develop methods for an easier and more efficient identification to allow a more detailed monitoring and analysis of interrelationships between organisms.

5.6 Conclusion

Based on the findings of this study, increasing temperatures and higher nutrient status can both synergistically favour potentially harmful cyanobacteria in the studied lakes. Thus, climate change effects in the study region are likely to result in increasing problems with cyanobacterial blooms. At the current stage, it is difficult to predict how other changes in the community structure will interact or even counterbalance these effects. While temperature can hardly be manipulated in larger lakes, the concentrations of nutrients are easier to be addressed as evident from the re-oligotrophication of several (sub)alpine lakes (Schindler, 2006; Geist, 2014; Geist, 2015). Thus, the current policy of reducing nutrient loading is a useful mitigation measure.

6. General discussion

The studies presented in this thesis provide novel information on the diversity of cyanobacteria in Bavarian lakes, their seasonal variations during vegetation periods and blooms, as well as information on future perspectives on the potential impacts of global warming on cyanobacteria. The application of molecular biology-based methods, like next-generation sequencing (NGS), PCR and qPCR, which were developed and compared during this study, allowed answering the questions mentioned above. The results provide the basis for further analyses of cyanobacterial communities on local and regional scales, by the implementation of the established methods and results.

This study has shown that cyanobacterial communities vary between lakes, whereas community structure is not only dependent on nutrient content and trophic status but also on lake structure and surroundings. Each of the studied lakes hosted a unique cyanobacterial community, characterized by a predominant main genus. Besides, it has been shown, that the impact of climate change will be varying on a local level and that the communities of nutrient-poor lakes predominated by picocyanobacteria such as *Synechococcus* are more stable against temperature stress.

6.1 Methods for the detection of cyanobacteria

For some time, leading specialists in the field of cyanobacteria research voiced the idea of a polyphasic approach, combining the study of phenotypic and molecular criteria (Komárek, 2006). Up to now, the detection of cyanobacteria is often still done with traditional (i.e. microscopically) methods. This approach does not seem sufficient anymore, as the supplementation with molecular biology-based methods is nowadays feasible. The results of this study extend the molecular data basis and methodologies needed for the realisation of polyphasic approaches in the future. Different methods for the DNA-based identification of cyanobacteria have been established, which were used in parallel to analyse cyanobacterial communities from Bavarian lakes. Comprehensive analyses have shown that lakes vary greatly on the level of cyanobacteria community structures, even when similarity on the phylum level is high (chapter 3). Recent studies revealed that molecular genetic identification of cyanobacteria offers a great potential for research and monitoring purposes in the future (Salmaso et al., 2016).

The choice of the taxonomic identification method depends on the required resolution and accuracy of the analysis. A differentiation between the analysis of cyanobacteria as aggregates in blooms and the determination of cyanobacteria on genus, species or strain level is often

necessary, i.e. when it comes to identifying taxa of toxicological relevance. Chapter 2 in this study provides a detailed description of the characteristics of different methods. This chapter also includes a discussion of the applicability of different methods based on the analysis of the *16S rRNA* gene depending on the target organism and the aim of the study.

Based on the analyses and results of this thesis, some specific recommendations for future detection and identification of cyanobacteria are summarized here: For a first overview over the cyanobacterial community composition, microscopy should be the preferred strategy. This approach allows a first overview assessment and classification of the cyanobacterial genera present, even for non-specialists in this field. Sometimes, it is even possible to determine the main genus of a bloom by eye directly at the sampling site, as there are genera which form macroscopically visible characteristic trichomes, e. g. some species of the *Aphanizomenon* genus. However, many genera and species of cyanobacteria are hard to identify based on their morphological characteristics alone, especially small coccoid (e. g. *Synechococcus* sp.) but also filamentous types (e.g. *Tychonema* sp.) which play an important role in aquatic ecosystems (Flombaum et al., 2013; Shams et al., 2015). Especially *Synechococcus* species are highly abundant in Bavarian lakes and were found to be the dominant genus in 50% of the studied lakes (chapter 3). In addition to this study, other studies confirmed the importance of *Synechococcus* in Bavarian lakes (Zwirglmaier et al., 2015; Ruber et al., 2016). The use of molecular methods (chapter 3) was essential for the detection and further break down of this genus. The results of this thesis build a data basis for future detection of *Synechococcus* with *16S rRNA*-based methods.

After a first insight into cyanobacterial composition based on morphological characterization, it is recommended to proceed with subsequent molecular analyses, to confirm and refine the first results in a second step. In this thesis, different assays were established for the detection of several cyanobacteria genera and the respective methods compared (chapter 2). Based on the preceding morphological characterization, it is easier to decide which molecular analyses are most appropriate in answering the research question. Next-generation sequencing is always the best method to get comprehensive information over the whole cyanobacterial community structure, as it has been shown in chapter 3. The disadvantage is that NGS is still a comparably expensive and time-consuming method. Nevertheless, NGS should be considered whenever the total bacterial community is of interest. NGS has been used in this study to analyse the whole bacterial and cyanobacterial community and diversity in field samples (chapter 3) and to investigate changes in bacterial and cyanobacterial community structure

caused by simulation of global warming in aquarium experiments (chapter 5). Precise instructions for the evaluation of the NGS data are also presented in chapters 3 and 5.

Besides NGS, also other molecular methods, which are easier to apply, should be used in future research assays on cyanobacteria. Therefore, several methods based on the analysis of the *16S rRNA* gene have been established and optimized in this study (chapter 2). Those methods allow the detection and quantification of different specific cyanobacteria genera to a varying extent: In some cases, it may be sufficient to just look at presence-absence data. Then, the preferred approach would be a simple polymerase chain reaction (PCR) assay without further quantification. If quantification is necessary, the favoured method would be qPCR. For semi-quantitative detection or without access to a real-time PCR thermocycler, the developed PCR-ELISA (PCR-based enzyme-linked-immuno-sorbent assay) would be the adequate choice. PCR-ELISA is a simple procedure, which enables the analysis of data on a semi-quantitative level based on the usage of comparable simple laboratory instruments. The method delivers semi-quantitative results and approximately relative abundances, so that the analysis and comparison of differences among samples are possible (e. g. time series). In comparison, qPCR allows for quantification and determination of concrete cell numbers. The performance of this method is very fast but there is the need of cost intensive reagents and a specific thermocycler, making this method more expensive. All three methodologies have been applied and optimized in this study (chapter 2) and can be used with high reliability for future studies on cyanobacteria.

Molecular identification of cyanobacteria has been used in recent studies to address various research questions. The first PCR primers for the detection of cyanobacteria based on the *16S rRNA* genes were published in the late 1990s (Nübel, Garcia-Pichel & Muyzer, 1997). Since then, the number of cyanobacterial sequences in public sequence databases like EMBL or GenBank has increased dramatically. In recent years, it has been shown that sequence-based diversity is much greater than diversity based on traditional morphologic analysis (Taton et al., 2003). Cyanobacteria can also be detected and classified using other genes than the *16S rRNA* gene, i.e. the phycocyanin operon (Neilan, Jacobs & Goodman, 1995), but there is far less sequence data available for these genes. Up to date PCR primers for the detection of specific cyanobacteria genera are published in this study (chapter 2).

Two aspects, which have not been taken into consideration in the present study, are the application of molecular methods for the detection of cyanotoxin genes and remote sensing for the detection of cyanobacteria blooms. The characterization of cyanobacterial communities as discussed in this thesis also provides a possibility of linking high-resolution information on

spatio-temporal dynamics of cyanobacteria with early detection tools of toxin gene transcription and expression, as well with remote sensing technology. Molecular analysis should in the future also be reconsidered for the detection of cyanotoxins and their genes. Cyanotoxins are directly measured using ELISA assays or liquid chromatography so far. These measurements are often imprecise. Furthermore, the direct detection of the toxins does not provide further information on the producing taxa. In contrast to that, identifying the cyanotoxin genes present in the water makes an identification of the toxin-producing genus possible and allows the detection of the main toxin producer (Scherer et al., 2017b). Thus, molecular analyses of cyanotoxin genes can provide more target-oriented analyses and monitoring programs. For differentiating between the presence of cyanotoxins and the potential for cyanotoxin production, a combination of methods is recommended. In addition to DNA analyses of cyanotoxin genes, the analysis of messenger RNA in combination with direct detection of toxins makes sense, as the amount of produced and released toxins depends on the particular cells.

Remote sensing is a method, which can be considered for a future detection of cyanobacterial blooms, as cyanobacteria can be detected as a whole group without any further identification. Recent studies have shown that cyanobacterial mapping based on spectroscopic characteristics (remote sensing) is still difficult in limnic systems. A high potential for future monitoring is expected, after an adjustment of algorithms for local characteristics (Matthews et al., 2010). Mapping of potentially toxic cyanobacterial blooms in marine systems is possible, but the biomass needed for a detection is often higher than the number of cells already considered as a bloom, as it has been shown for the Baltic Sea (Kutser et al., 2006).

Figure 6.1 summarizes the future perspectives in methods for the detection of cyanobacteria. It clearly shows that a combination of different methods will become more and more important and unavoidable for future studies on cyanobacteria.

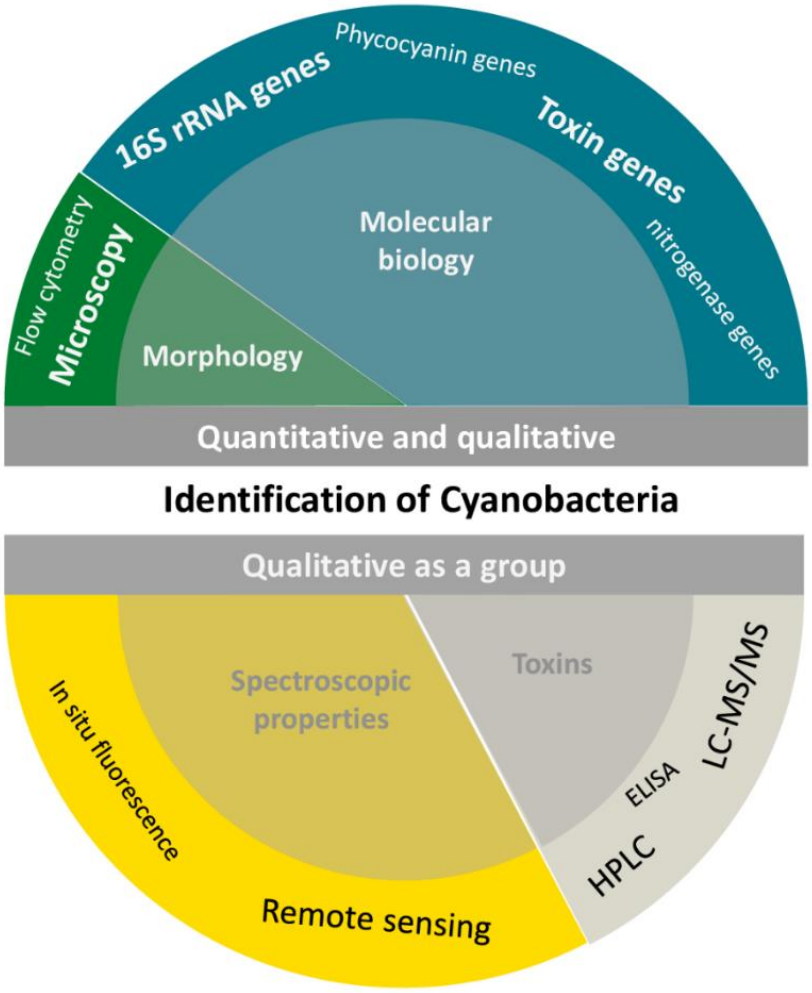

Figure 6.1 Perspectives in methods for the future detection of cyanobacteria. A polyphasic approach consisting of molecular biology and morphological characteristics seems advisable for the identification and quantification of cyanobacteria. Analyses based on spectroscopic properties as well as toxin analyses are methodological approaches, which allow the characterization of cyanobacteria as a group.

6.2 Monitoring of cyanobacteria in their specific habitats

Depending on the research question, it is important to define monitoring strategies, as different cyanobacterial genera or species occupy different ecological niches due to their various physiological adaptations and characteristics (chapter 3). The monitoring strategy depends on the aim of the study. Thus, it has to be determined if the study fulfils water state authority purposes, i. e. control and monitoring of potentially harmful cyanobacteria blooms, or more scientific questions, i. e. the study of the ecology of a certain genus or impact of stress factors on the community structures in the lake. The results of this study provide data, which enable to optimize future strategies for the monitoring of cyanobacteria, especially in temperate regions of Europe (chapter 4).

In general, a number of individual taxa are of interest due to certain characteristics: bloom-formation or mass occurrences, potential toxicity, nitrogen fixation, invasion or even harmless qualities, association to a stable state of the lake (chapter 3). Depending on their habitat preferences, cyanobacteria occur as benthic or pelagic organisms, a further factor which influences monitoring strategies.

The genera, which have turned out to be the most important due to their high abundance in the presented study area were *Limnothrix*, *Planktothrix* and *Synechococcus* (chapter 3). Both, *Limnothrix* and *Planktothrix* are potential cyanotoxin producers (Christiansen et al., 2003; Bernard et al., 2009), whereas *Synechococcus* is a harmless but widely distributed picocyanobacterial genus (Zwirglmaier et al., 2008; Flombaum et al., 2013). Important companion genera of the main genera in the lakes were *Microcystis*, *Woronichinia* and *Dolichospermum*, which are also known as potential cyanotoxin producers (Sivonen et al., 1990; Li, Dreher & Li, 2016).

Synechococcus is a picocyanobacterium, which occurs in the pelagic zone of freshwater lakes. This has been shown in this study (chapter 3) but also in other studies (Postius & Ernst, 1999; Ruber et al., 2016). *Synechococcus* is very stress resistant (chapter 5) and its genetically diverse strains can cope with various stress situations resulting in a physiologically highly diversified population, as has been shown in Lake Constance (Postius & Ernst, 1999). There are some genotypes, which have maxima in deep lake layers (Becker, Richl & Ernst, 2007). In the study presented here, the coexistence of *Synechococcus* and *Planktothrix* could be revealed, which coexisted in different depth layers of mesotrophic Lake Ammersee (chapter 3). *Microcystis* species generally prefer mixed layers of small eutrophic lakes and have high light requirements (Reynolds et al., 2002). These habitat requirements were confirmed by the results of this study, as *Microcystis* was detected in the upper layers of the mesotrophic lake and the mixed layer of

the eutrophic lake by NGS (chapter 3). *Woronichina* species are known to be adapted to summer epilimnia of mesotrophic lakes (Reynolds et al., 2002). In this study, *Woronichina* species were also detected in the mixed layers of the eutrophic lake (chapter 3). This result indicates that this location is also an important habitat for this genus. *Dolichospermum* species, with the ability to fix elementary nitrogen, are dependent on sufficient light and their occurrence is promoted when nitrogen contents are low (De Tezanos Pinto & Litchman, 2010; Mantzouki et al., 2016). This is confirmed by the results of this study, as *Dolichopermum* could be detected in the upper layers of the mesotrophic lake and in nitrogen limited Lake Bergknappweiher (chapter 3). *Limnothrix* and *Planktothrix* are filamentous cyanobacteria genera of the order Oscillatoriales. *Limnothrix redekei* and *Planktothrix agardhii* both are known to occur in turbid mixed shallow lakes (Rücker, Wiedner & Zippel, 1997; Reynolds et al., 2002). In contrast, *Planktothrix rubescens* is dependent on stable epilimnia and can regulate buoyancy (Reynolds et al., 2002; Walsby, Schanz & Schmid, 2006). *Planktothrix rubescens* occurs mainly in the metalimnion of stratified lakes. Our study has shown that *Planktothrix* sp. and *Synechococcus* sp. co-exist as main genera in lakes. Nevertheless, these two genera avoid each other, as they are adapted to different depth layers (chapter 3).

In this study (chapter 3), every lake was characterized by a distinct cyanobacterial community, and therefore, monitoring strategies must not only be determined dependent on the research question, they also have to include the high local variability of cyanobacteria.

Depending on the lake type (small, shallow and nutrient-rich versus large and nutrient-poor), two different sampling strategies for representative characterization of cyanobacterial communities can be distinguished. Type 1 is typical for bloom-forming nuisance cyanobacteria like *Microcystis*, *Dolichospermum* or *Woronichina*, whereas lake type 2 can be characterized by buoyancy-regulating cyanobacteria such as *Planktothrix rubescens*. In lakes of type 1, surface blooms are expected, thus, sampling for monitoring purposes should take place at the lake surface. For lakes of type 2, an additional sampling of the metalimnion is recommended, as buoyancy-regulating cyanobacteria, like *Planktothrix rubsecens,* accumulate in this thermal layer during stratification.

In addition to the consideration of the lake type, a further essential factor is the choice of the optimal time and time span of investigation. Seasonal changes in the lake characteristics like mixing and thermal stratification lead to a different supply of resources (nutrient, light) and cause the natural phytoplankton succession. Cyanobacteria succession in the studied lakes has been shown in chapter 4. Cyanobacterial surface blooms can be expected during the warmer months in the year, as cyanobacteria have a competitive advantage at higher temperatures

compared to other phytoplankton species, like diatoms or green algae (Elliott, Jones & Thackeray, 2006; Reynolds, 2006; Jöhnk et al., 2008; Paerl & Huisman, 2008). Surface blooms also occurred in the study area, while the sampling strategy was unable to detect mass occurrences in deeper depth layers (chapter 4). Mass occurrences of cyanobacteria in deeper layers of the lakes can only be expected during the stages of stratification and are therefore dependent on the lake stratification behaviour. In this period, the rhythm of monitoring is often restricted to monthly or twice-monthly water sampling (Pobel, Robin & Humbert, 2011). Nevertheless, the presented and other previous studies have shown, that a high sampling frequency should be preferred (chapter 4). Besides, some species like *Aphanizomenon flos-aquae* have growth maxima, which can only be detected by more frequent sampling strategies (Pobel, Robin & Humbert, 2011). As has been shown in chapter 3, the spatial distribution of the sampling points within a lake is also highly important. The considerations stated here, should serve as a good start for finding the optimal strategy for future monitoring.

6.3 Environmental factors influencing cyanobacteria

In this study, different limnic systems have been studied, which varied in their size, surroundings, and nutrient supplies (trophic status). It has been shown, that each lake had its own cyanobacterial community represented by a main genus, which contributed with more than 50% to the cyanobacterial community and remained stable during the study period (chapter 3).

The composition of cyanobacterial communities underlies a variety of environmental factors that will most likely undergo changes because of climate change. The environmental factors that have major impact on cyanobacteria taxa composition are trophic conditions, lake temperature, light climate (impacted by watercolour), size and volume of the lake, stratification behaviour as well as anthropogenic impact and management of the lake. The key factors causing cyanobacterial dominance are the extent of summer mixing, phosphate concentrations and light climate (Rücker, Wiedner & Zippel, 1997; Mischke, 2003). The interaction of environmental factors influences cyanobacteria in a number of ways and individual cyanobacteria species will therefore be affected differently by future changes. Simulations indicate that cyanophytes are likely to be favoured by future climate (Trolle et al., 2011). Climate change scenarios predict a future eutrophication of lakes, especially during summer months, in lakes of currently different trophic status (Trolle et al., 2011). Increase in phosphorus will be caused by more intensive run-off due to higher rainfall and temperature-mediated higher P release from lake sediments (Jeppesen et al., 2009). Especially shallow lakes

are sensitive to wash out and sediment resuspension. Furthermore, climate change will affect water temperature and stratification behaviour as surface and epilimnic water temperature are correlated with regional-scale air temperatures (Adrian et al., 2009). These changes will affect intensity and duration of stratification, favouring buoyancy-regulating cyanobacteria such as the potentially toxic *Planktothrix rubescens*. In addition, the third key factor responsible for cyanobacteria dominance, the light climate, will change in the course of climate change. Changes in water turbidity will be caused by increasing run-off due to heavy rain events, but also by changes in phytoplankton growth. In comparison to other phytoplankton, cyanobacteria as a group are superior competitors under low light conditions and can even promote these conditions by self-shading (Scheffer et al., 1997). It has been shown earlier, that reduced light availability caused by self-shading, watercolour or increased mixing, affects cyanobacterial community resulting in a possible subsequent replacement of Nostocales by Oscillatoriales (Mischke, 2003).

As climate change effects will vary on regional and local scales (Heino & Mykrä, 2008), aquaria experiments were conducted as a part of this study (chapter 5). These experiments were carried out to directly simulate predicted climate change effects on the present cyanobacterial communities in the studied lakes. The lakes whose water was used for the aquarium experiments, were selected due to their differences in trophic status and turbidity. Furthermore, the selected lakes are known to harbour different cyanobacterial communities and one of the lakes is prone to summer blooms (chapter 5). The major result of the aquarium experiments was that lakes dominated by picocyanobacteria, like *Synechococcus*, have more stable communities and might be less prone to changes driven by climate change (chapter 5). Furthermore, it could be shown for the first time that the season when temperature increase occurs has major impact on the changes in cyanobacterial community composition (chapter 5).

Climate change will influence various environmental factors and therefore will have major impact on the composition and distribution of cyanobacteria. In addition to the natural environmental factors, which influence the occurrence and distribution of cyanobacteria, anthropogenic impact and management play an important role and need to be considered.

6.4 Outlook

The methods developed in this study can be applied to future studies on the ecology of cyanobacteria and their monitoring. For instance, the developed detection range can now be easily expanded for the identification of further cyanobacterial genera or species based on the methodologies and procedures published here. In the course of climate change, greater focus should be laid on the establishment of methods for the molecular detection of *Cylindrospermopsis raciborskii*, an invasive, thermophilic and potentially toxic species originating in tropic regions, which has already been detected in Northern Germany in recent years (Fastner et al., 2003; Stüken et al., 2006). Furthermore, assays could be established for the detection of widespread genera like *Synechococcus* or *Woronichinia*. The established set-up of aquarium experiments could be further applied for the study of climate change effects on cyanobacterial communities originating from other lakes.

Future monitoring should be lake-specific, in consideration of spatial and temporal distributions of cyanobacteria. As elaborated earlier in this study, the combination of traditional and molecular biology-based methods should be considered, and methods need to be further evolved. In addition to the methods developed in this study, modern instruments should be used, e. g. fluorometric probes or probes for the measurements of turbidity, which allow the detection of highly populated lake layers, reveal layers potentially inhabited by buoyancy-regulating cyanobacteria and support the design of the optimal monitoring strategy. The molecular analyses can then be applied for water samples taken in these depth layers. Even though microscopy is a very traditional method, its value should not be neglected as it could still serve as a strong and useful supplement to molecular analyses in future. Furthermore, the detection of cyanotoxins and their genes needs extension and improvement, as the abundance and distribution of cyanotoxin producing cyanobacteria is likely to increase with climate change (Paerl & Huisman, 2009). Due to future changes in community structures, the importance of the analysis of cyanotoxins other than microcystin, like cylidrospermospin and anatoxin-a will increase. Recently, radical changes in the cyanobacterial communities of the largest lakes south of the Alps have already been reported, with the spreading of *Tychonema bourellyi* in Lake Garda (Shams et al., 2015; Salmaso et al., 2016). This anatoxin-a producing cyanobacterial species has been identified with a polyphasic approach and it has been found that this highly diversified cyanobacterial species is dominant in lakes previously dominated by potentially microcystin-producing *Planktothrix* species (Pinto et al., 2017). Future changes might not only comprise new cyanotoxins but also the interaction between differing cyanotoxins and allelopathy (Leflaive & Ten-Hage, 2007). The results of this study will contribute to future cyanobacteria research, as they provide basic methodologies for the

detection of cyanobacteria on molecular level and advice for more concrete monitoring programmes.

In conclusion, in light of a worldwide changing environment, the study of cyanobacteria, i. e. their community compositions, physiological adaptions, as well as their potentially toxic metabolites is now more important than ever and demands high awareness.

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8. Publication list

The following papers were included in this thesis:

Bauer, F. R., A. D. Millard, U. Raeder, J. Geist & K. Zwirglmaier. Cyanobacterial diversity in four Bavarian lakes of different trophic status. (submitted)

Bauer, F. R., A. D. Millard, N. Fleischmann, U. Raeder, J. Geist & K. Zwirglmaier. Impact of global warming on freshwater cyanobacteria in Bavarian lakes. (submitted)

Bauer, F. R., U. Raeder, J. Geist, K. Zwirglmaier. Development and comparison of methods for the detection of cyanobacteria. (in preparation)

Bauer, F. R., U. Raeder, J. Geist & K. Zwirglmaier. Seasonal monitoring of potentially toxic cyanobacteria. (in preparation)

Co-authorships (not included in this thesis):

Ruber, J., F. R. Bauer, A. D. Millard, U. Raeder, J. Geist & K. Zwirglmaier, 2016. *Synechococcus* diversity along a trophic gradient in the Osterseen Lake District, Bavaria. Microbiology 162: 2053-2063.

Poster presentations related to the PhD thesis:

Bauer, F. R., M. Engel, J. Geist & K. Zwirglmaier, 2015. Phytoplankton diversity in four Bavarian lakes of different trophic level. 9th European workshop on the molecular biology of cyanobacteria. September 7-11 2014, Royal Netherlands Institute for Sea Research (NIOZ), Texel, Netherlands.

Bauer, F. R., J. Geist & K. Zwirglmaier, 2015. Phytoplankton diversity in Bavarian lakes: Impact of trophic level and seasonal shifts. Fresh blood for fresh water. April 15-17 2015, Research Department for Limnology, Mondsee, University of Innsbruck, Austria.

Oral contributions related to the PhD thesis:

Bauer, F., A. D. Millard, J. Geist & K. Zwirglmaier, 2015. Phytoplankton diversity in Bavarian lakes: Impact of trophic level and seasonal shifts. 15th International symposium on phototrophic prokaryotes, August 2-6 2015, University Tübingen, Tübingen, Germany.

Bauer, F. R., A. D. Millard, M. Engel, J. Geist & K. Zwirglmaier, 2015. Cyanobakterien in bayerischen Seen: Einfluss von Trophie und saisonalen Unterschieden auf die Zusammensetzung der mikrobiellen Gemeinschaft. Jahrestagung der Deutschen Gesellschaft für Limnologie und der deutschsprachigen Sektionen der SIL, September 21-25 2015, University Duisburg-Essen, Germany.

Oral contributions with a peer-reviewed abstract:

Bauer F. R., J. Geist & K. Zwirglmaier, 2016. Impact of global warming on freshwater cyanobacteria in Bavaria. 20th IAC Cyanophyte/Cyanobacteria research symposium. August 28 – September 2 2016, Institute of Botany, University of Innsbruck, Austria.

9. Author contribution statements

Franziska R. Bauer (**FB**), Prof. Dr. Jürgen Geist (**JG**), Nadja Fleischmann (**NF**), Dr. Andrew D. Millard (**AM**), Dr. Uta Raeder (**UR**), Dr. Katrin Zwirglmaier (**KZW**)

Chapter 2 – Development and comparison of methods for the detection of cyanobacteria

This study was primarily designed by FB and KZW, with critical revision by JG and UR. The development of methods was carried out by FB, with critical support and feedback by KZW, UR and JG. Analyses and interpretation of data were prepared by FB and discussed with KZW, JG and UR. The manuscript was drafted by FB with continuous input and revision by KZW, UR and JG.

Chapter 3 – Cyanobacterial diversity in four Bavarian lakes of different trophic status

The study was primarily designed by KZW and FB, with input of knowledge by UR and JG. Sampling, laboratory analyses and preparation of sequencing was done by FB. Sequencing was carried out at the University of Warwick by AM. Analysis of data was done by FB with the help of KZW and AM. Interpretation of data was prepared by FB and KZW. Results were critically discussed with UR and JG. The manuscript was drafted by FB, with continuous input and revision by KZW, AM, UR and JG.

Chapter 4 – Seasonal monitoring of potentially toxic cyanobacteria

Study conception and design was done by FB and KZW in consultation with UR and JG. Acquisition of data in terms of sampling and laboratory analyses was done by FB. Analyses and interpretation of data was done by FB, and critically and continuously discussed with KZW, UR and JG. The manuscript was drafted by FB, with critical revision of KZW, UR and JG.

Chapter 5 – Impact of global warming on freshwater cyanobacteria in Bavarian lakes

The study was primarily designed by FB and KZW. The experimental set up was designed by FB and continuously discussed with KZW. Experiments and laboratory analyses were primarily carried out by NF with critical feedback and revision by FB. Sequencing was done by AM. Data analyses and interpretation were primarily done by FB and NF and discussed with KZW. The manuscript was drafted by FB with continuous input and revision by KZW, AM, UR, NF and JG.

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11. Supplementary

11.1 Supplementary to chapter 3

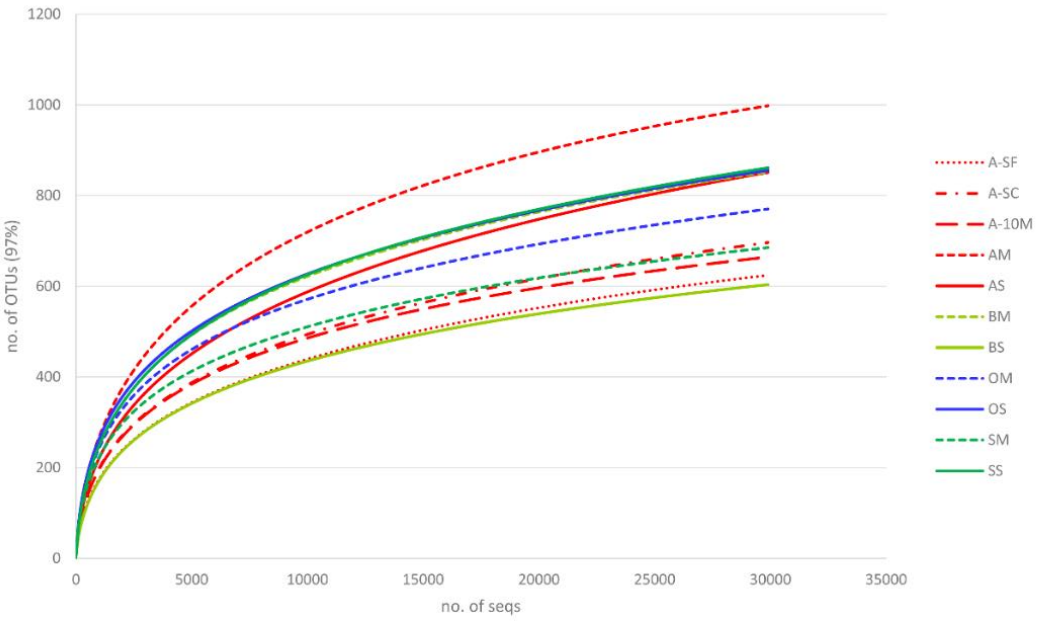


Figure 11.1 Rarefaction curves of all the sequences after normalization. Cutoff = 0.03. M, May; S, September; A, Lake Ammersee; B, Lake Bergknappweiher, O, Lake Ostersee; S, Lake Schliersee; SF, surface; SC, Secchi depth; 10M, 10 metres depth.

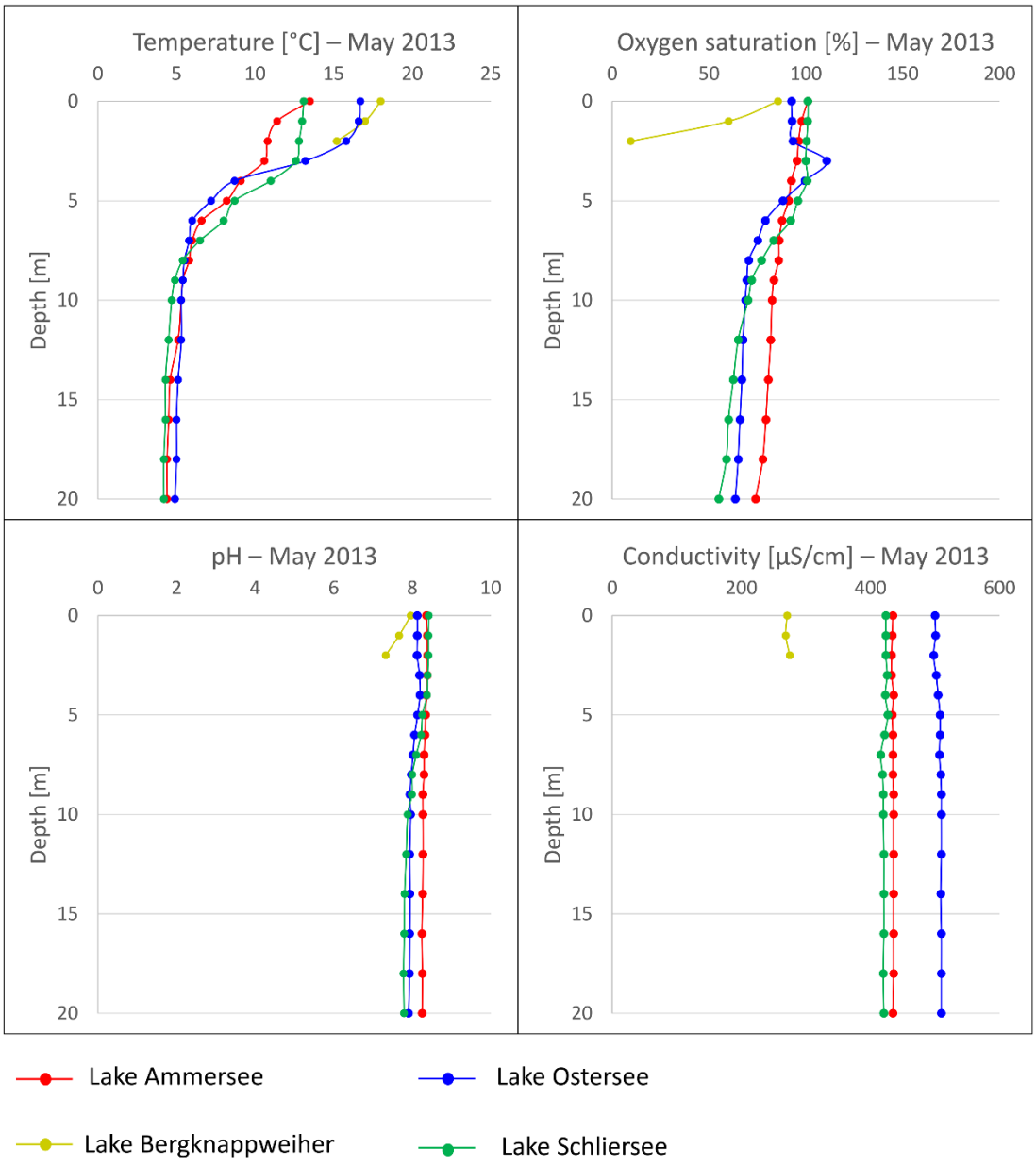


Figure 11.2 Depth profiles of hydrophysical parameters of the studied lakes determined in May 2013.

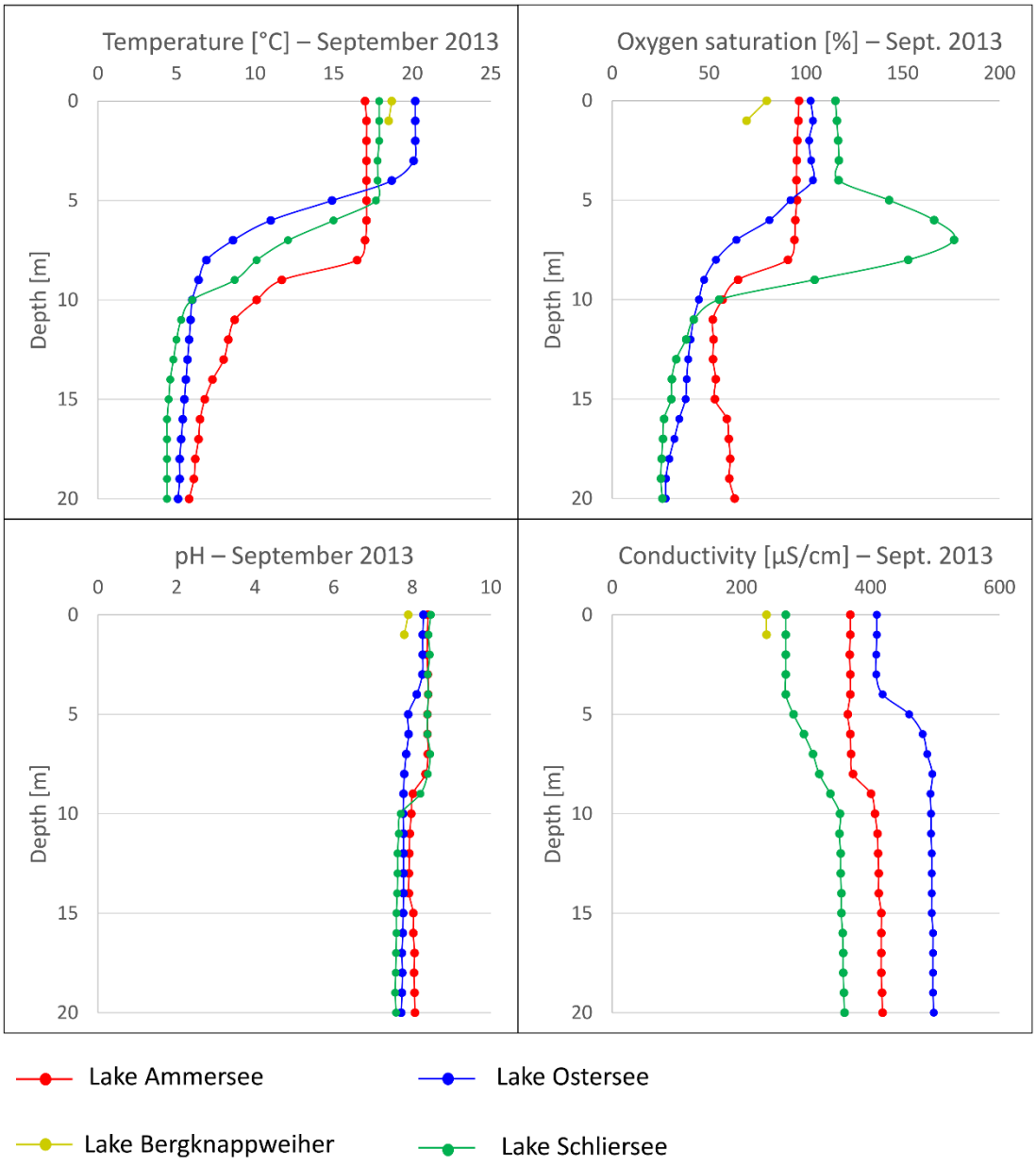


Figure 11.3 Depth profiles of hydrophysical parameters of the studied lakes determined in September 2013.

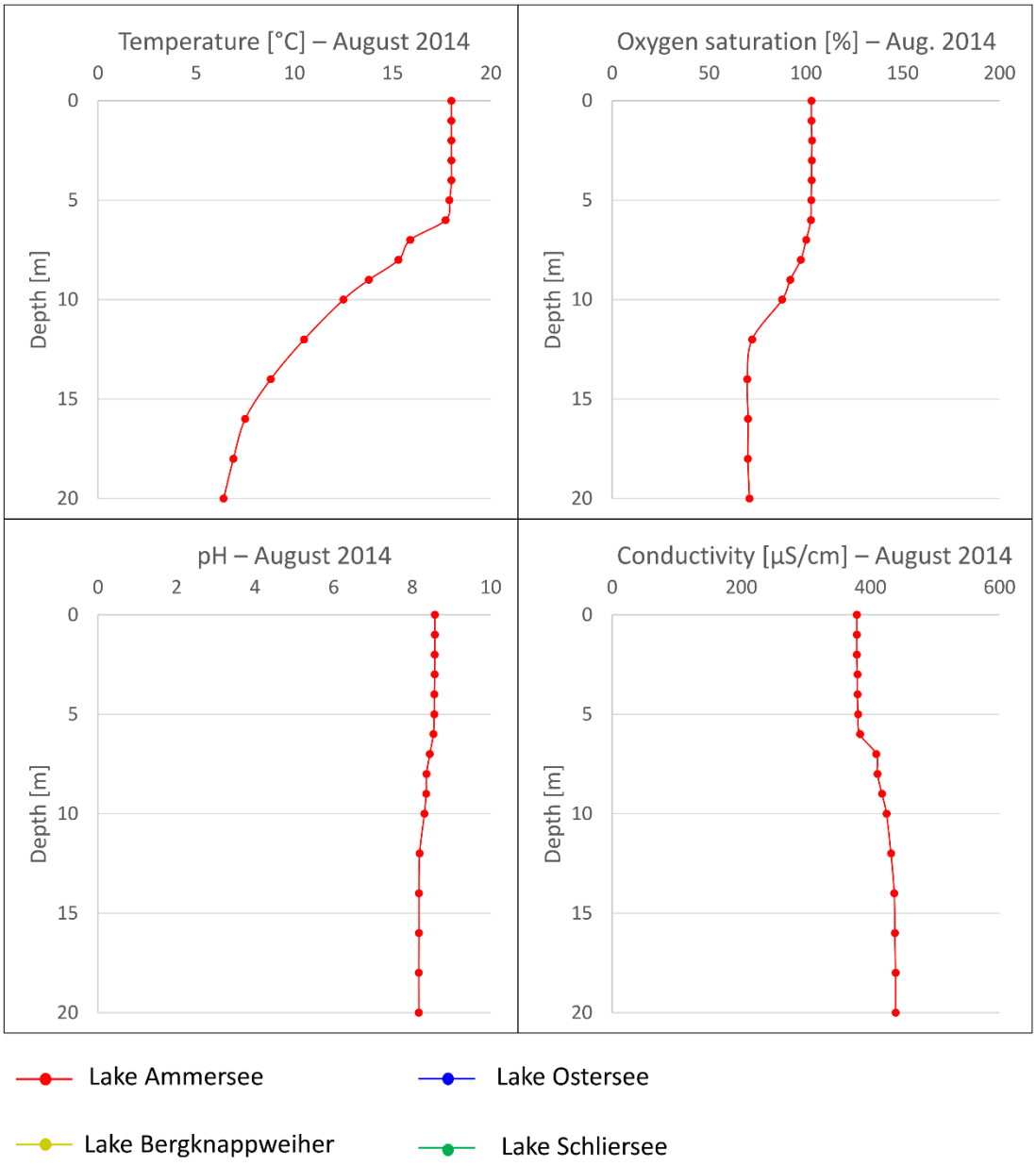


Figure 11.4 Depth profiles of hydrophysical parameters of Lake Ammersee determined in August 2014.

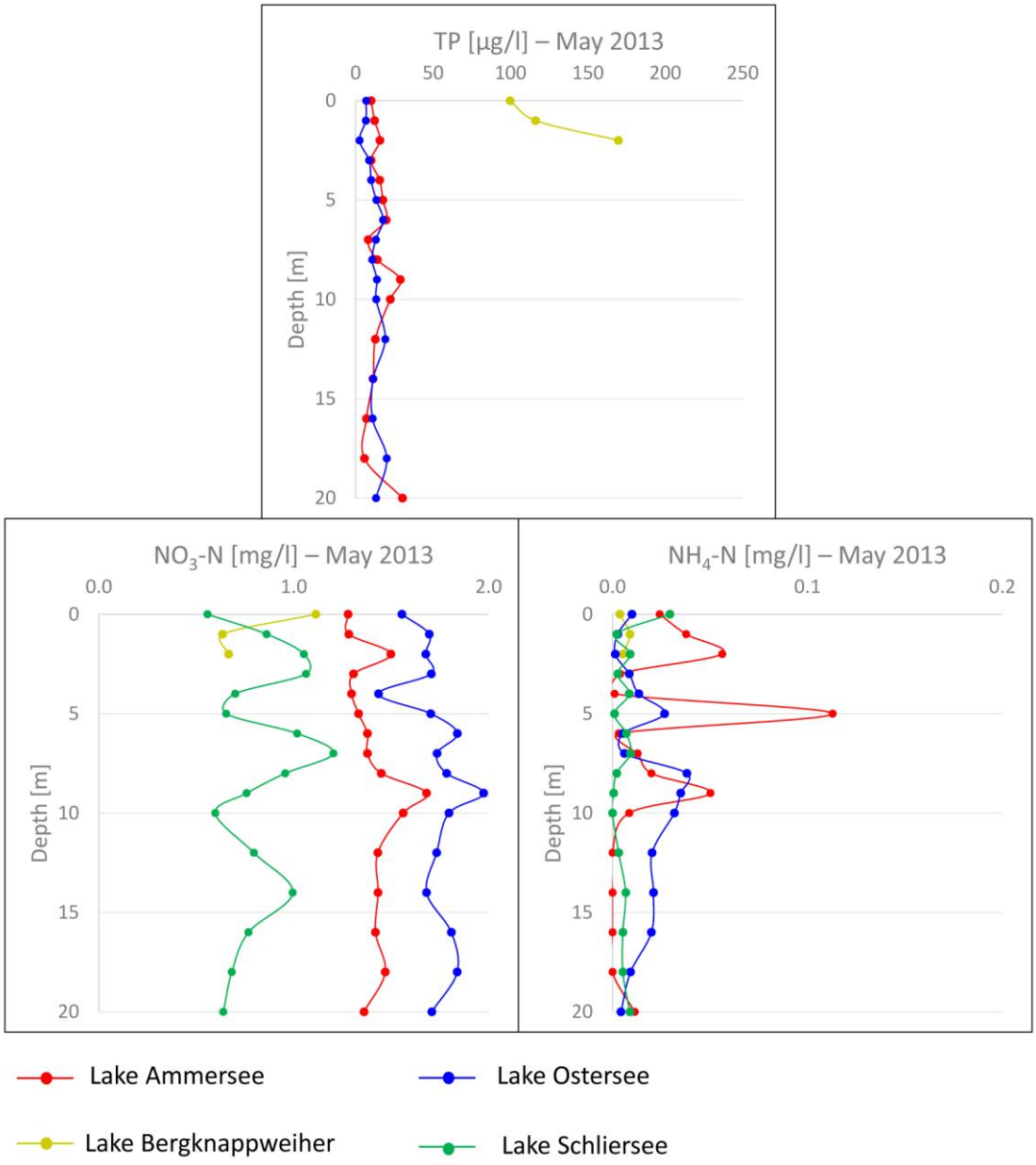


Figure 11.5 Depth profiles of hydrochemical parameters of the studied lakes determined in May 2013.

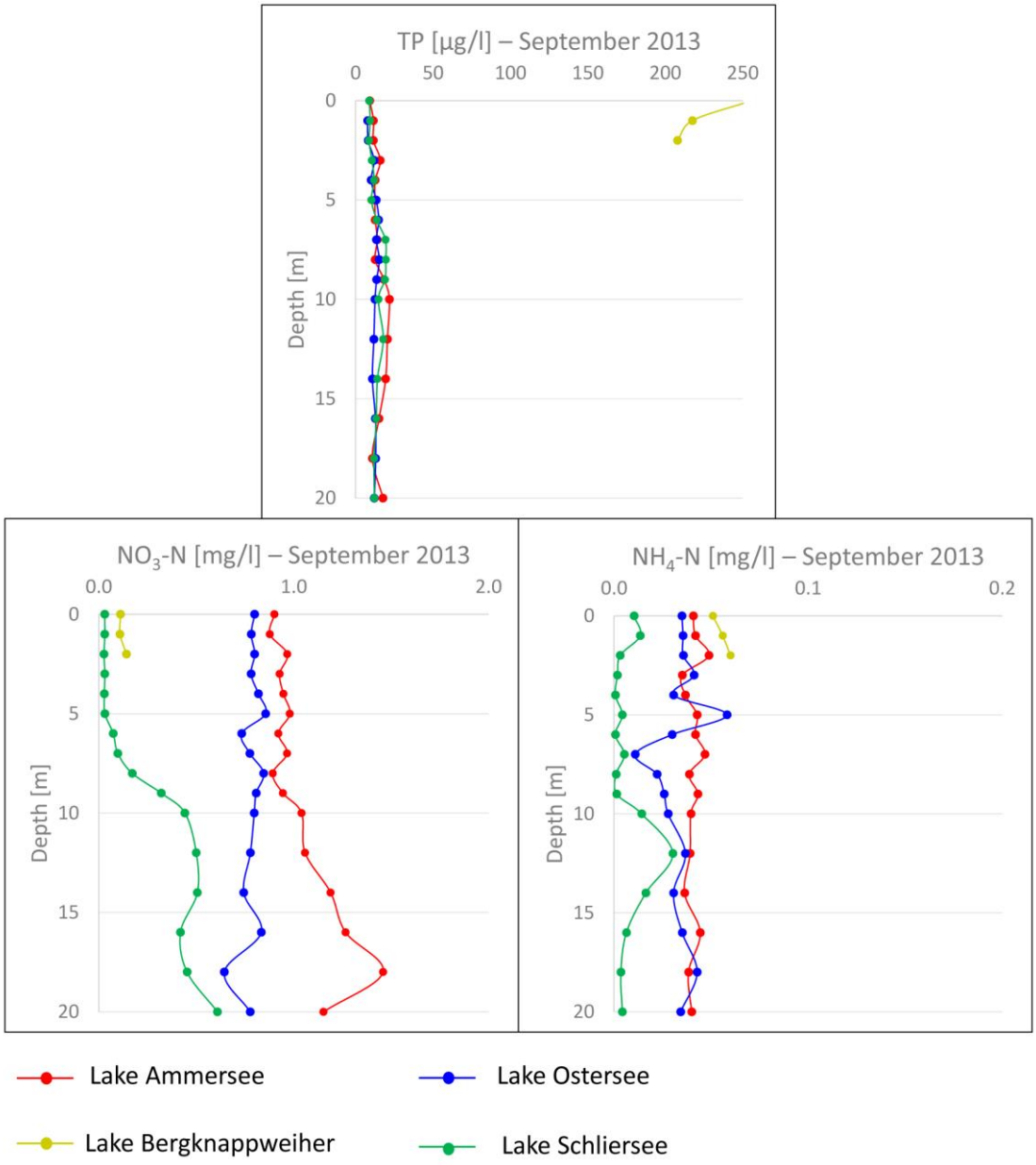


Figure 11.6 Depth profiles of hydrochemical parameters of the studied lakes determined in September 2013.

11.2 Supplementary to chapter 5

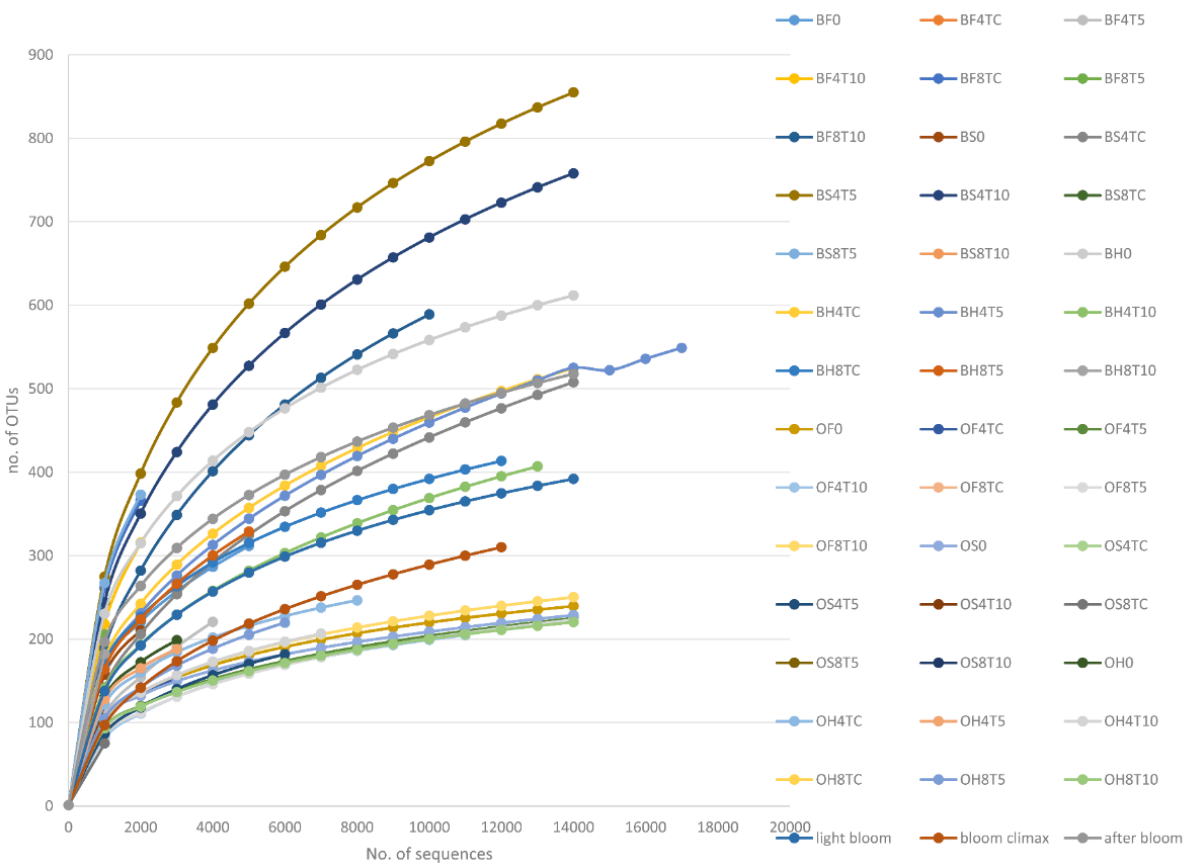


Figure 11.7 Rarefaction curves of all samples and sequences (excluding chloroplast sequences). Cutoff = 0.03.

Table 11.1 Number of sequence reads per sample after quality control.

Sample ID	Sample	total sequences (chloroplast excluded)
BKW_w0_F	Lake Bergknappweiher, spring, day 0	6023
BKW_w4_F_T1	Lake Bergknappweiher, spring, day 27, 13 °C	1981
BKW_w4_F_T2	Lake Bergknappweiher, spring, day 27, 18 °C	5350
BKW_w4_F_T3	Lake Bergknappweiher, spring, day 27, 23 °C	3456
BF_w8_T1	Lake Bergknappweiher, spring, day 55, 13 °C	3081
BF_w8_T2	Lake Bergknappweiher, spring, day 55, 18 °C	2290
BF_w8_T3	Lake Bergknappweiher, spring, day 55, 23 °C	11437

BKW_w0_S	Lake Bergknappweiher, summer, day 0	3213
BKW_w4_S_T1	Lake Bergknappweiher, summer, day 27, 22 °C	18522
BKW_w4_S_T2	Lake Bergknappweiher, summer, day 27, 27 °C	98041
BKW_w4_S_T3	Lake Bergknappweiher, summer, day 27, 32 °C	19255
BS_w8_T1	Lake Bergknappweiher, summer, day 55, 22 °C	874
BS_w8_T2	Lake Bergknappweiher, summer, day 55, 27 °C	3909
BS_w8_T3	Lake Bergknappweiher, summer, day 55, 32 °C	38
BKW_w0_H	Lake Bergknappweiher, autumn, day 0	15850
BKW_w4_H_T1	Lake Bergknappweiher, autumn, day 27, 16 °C	20279
BKW_w4_H_T2	Lake Bergknappweiher, autumn, day 27, 21 °C	19036
BKW_w4_H_T3	Lake Bergknappweiher, autumn, day 27, 26 °C	14472
BH_w8_T1	Lake Bergknappweiher, autumn, day 55, 16 °C	13096
BH_w8_T2	Lake Bergknappweiher, autumn, day 55, 21 °C	6375
BH_w8_T3	Lake Bergknappweiher, autumn, day 55, 26 °C	2164
OS_w0_F	Lake Ostersee, spring, day 0	15955
OS_w4_F_T1	Lake Ostersee, spring, day 27, 13 °C	4322
OS_w4_F_T2	Lake Ostersee, spring, day 27, 18 °C	29342
OS_w4_F_T3	Lake Ostersee, spring, day 27, 23 °C	12646
OF_w8_T1	Lake Ostersee, spring, day 55, 13 °C	394
OF_w8_T2	Lake Ostersee, spring, day 55, 18 °C	96538
OF_w8_T3	Lake Ostersee, spring, day 55, 23 °C	106392
OS_w0_S	Lake Ostersee, summer, day 0	83606
OS_w4_S_T1	Lake Ostersee, summer, day 27, 22 °C	164
OS_w4_S_T2	Lake Ostersee, summer, day 27, 27 °C	7897
OS_w4_S_T3	Lake Ostersee, summer, day 27, 32 °C	2329

OS_w8_T1	Lake Ostersee, summer, day 55, 22 °C	2170
OS_w8_T2	Lake Ostersee, summer, day 55, 27 °C	1070
OS_w8_T3	Lake Ostersee, summer, day 55, 32 °C	1322
OS_w0_H	Lake Ostersee, autumn, day 0	4666
OS_w4_H_T1	Lake Ostersee, autumn, day 27, 16 °C	9490
OS_w4_H_T2	Lake Ostersee, autumn, day 27, 21 °C	4723
OS_w4_H_T3	Lake Ostersee, autumn, day 27, 26 °C	8655
OH_w8_T1	Lake Ostersee, autumn, day 55, 16 °C	1678
OH_w8_T2	Lake Ostersee, autumn, day 55, 21 °C	7077
OH_w8_T3	Lake Ostersee, autumn, day 55, 26 °C	41030
startbloom	02/07/2014, lake sample	23800
middlebloom	26/08/2014, lake sample	13504
endbloom	10/11/2014, lake sample	15294

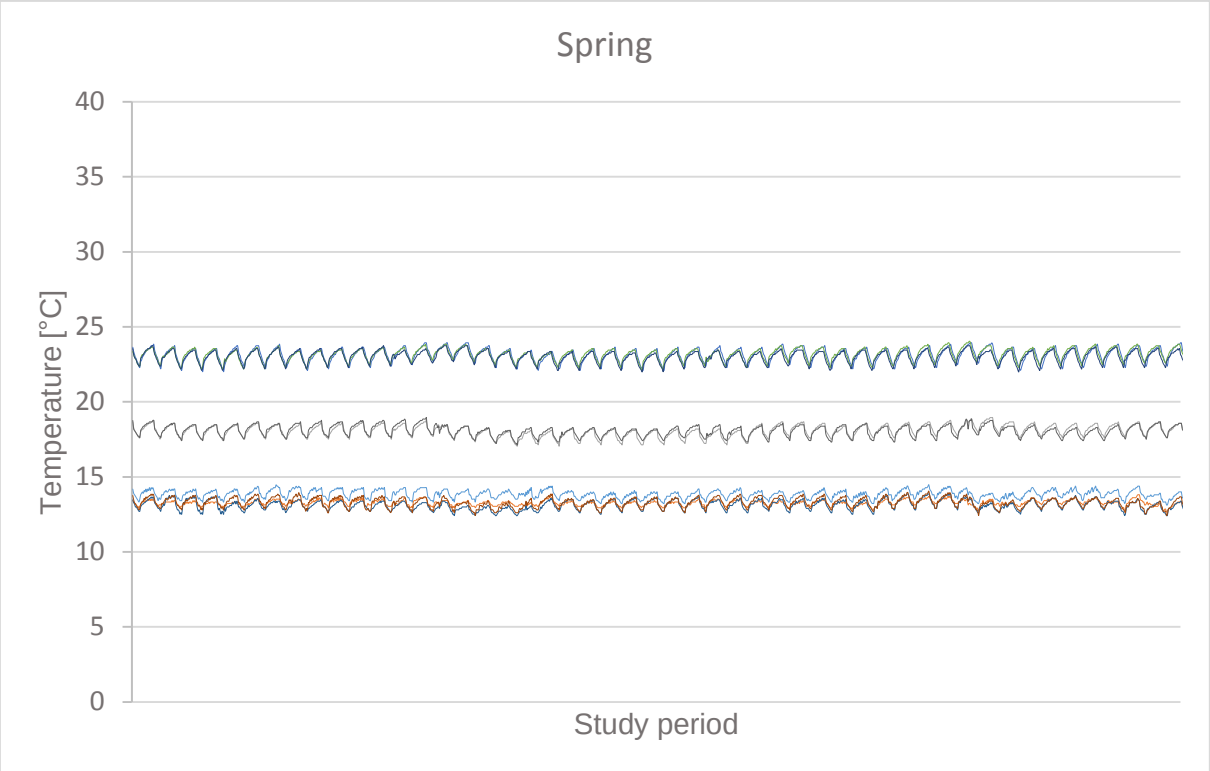


Figure 11.8 Temperature data measured with HOBO loggers during the spring experiments. Variation (within replicates) 0.4 °C, variation (during day) 0.7 °C. Adjusted temperatures: T_C = 13 °C, T_5 = 18 °C, T_{10} = 23 °C.

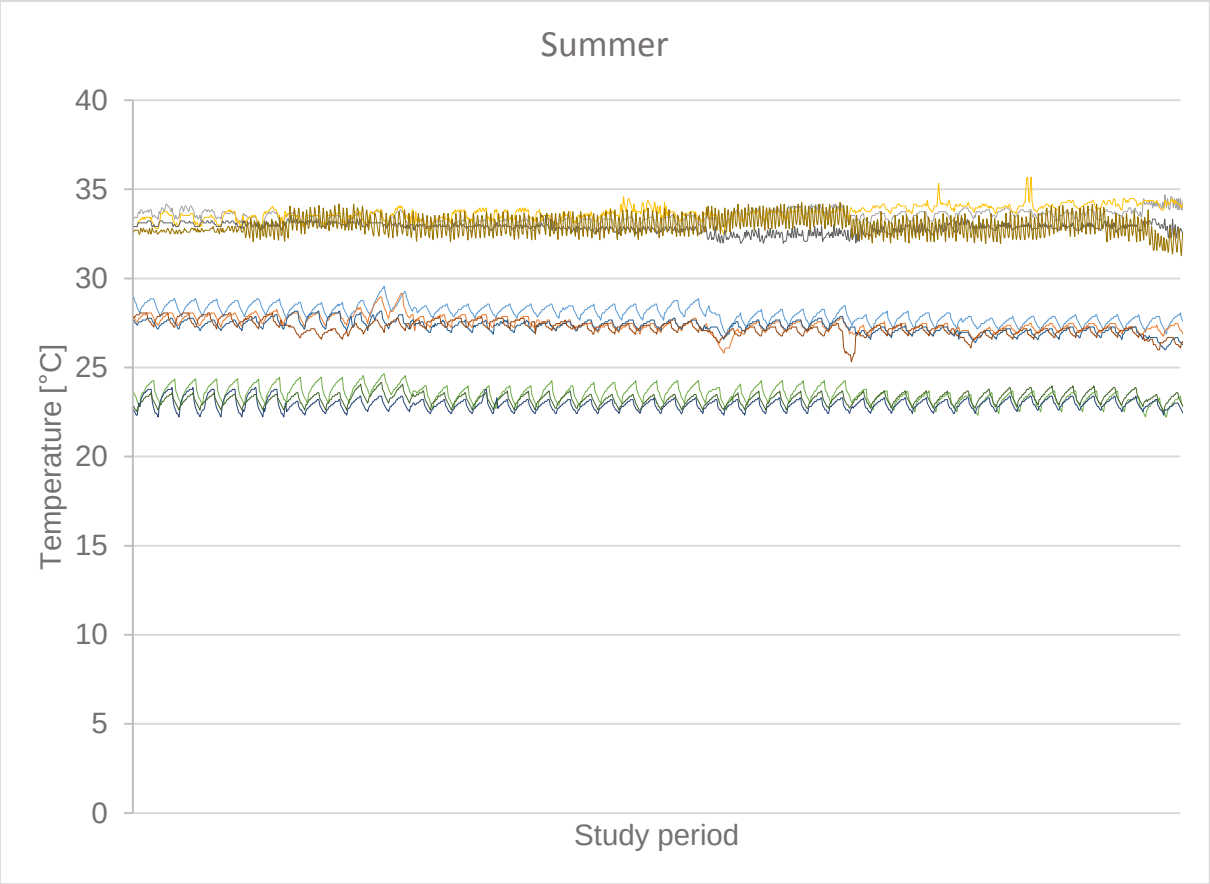


Figure 11.9 Temperature data measured with HOBO loggers during the spring experiments. Variation (within replicates) 0.3 °C, variation (during day) 0.9 °C. Adjusted temperatures: T_C = 22 °C, T_5 = 27 °C, T_{10} = 32 °C.

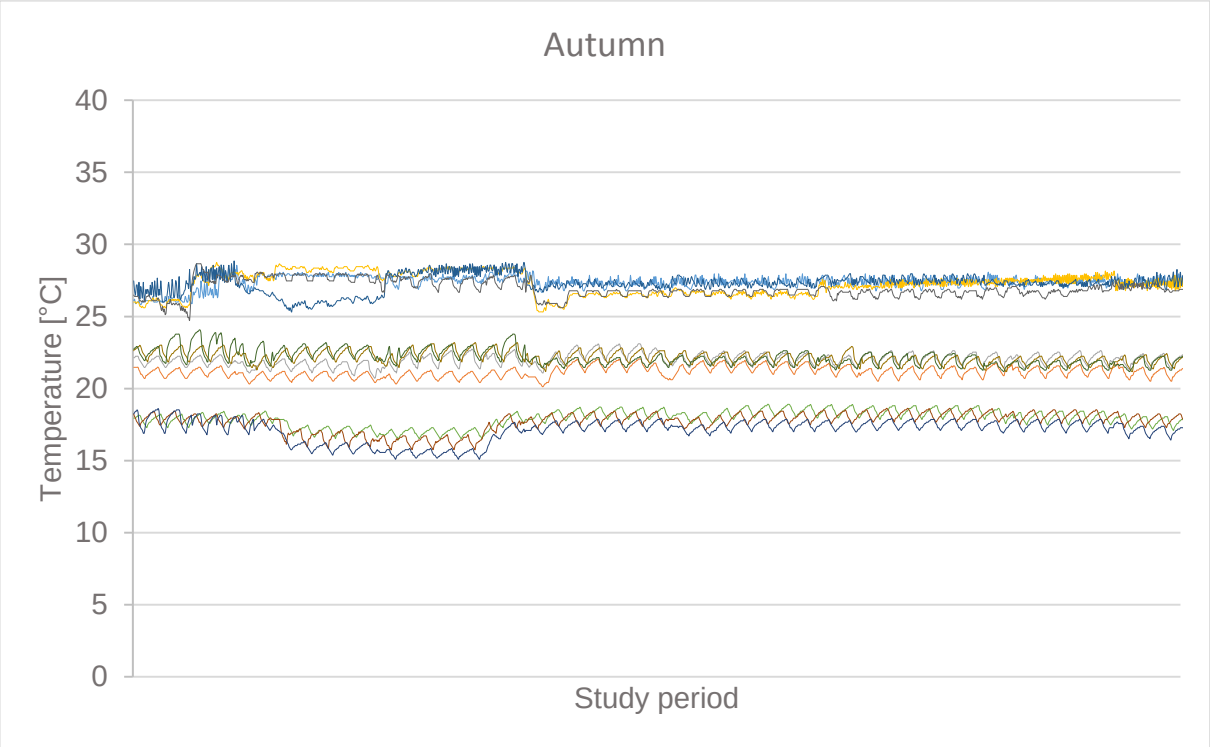


Figure 11.10 Temperature data measured with HOBO loggers during the spring experiments. Variation (within replicates) 0.3 °C, variation (during day) 0.8 °C. Adjusted temperatures: T_C = 16 °C, T_5 = 21 °C, T_{10} = 26 °C.

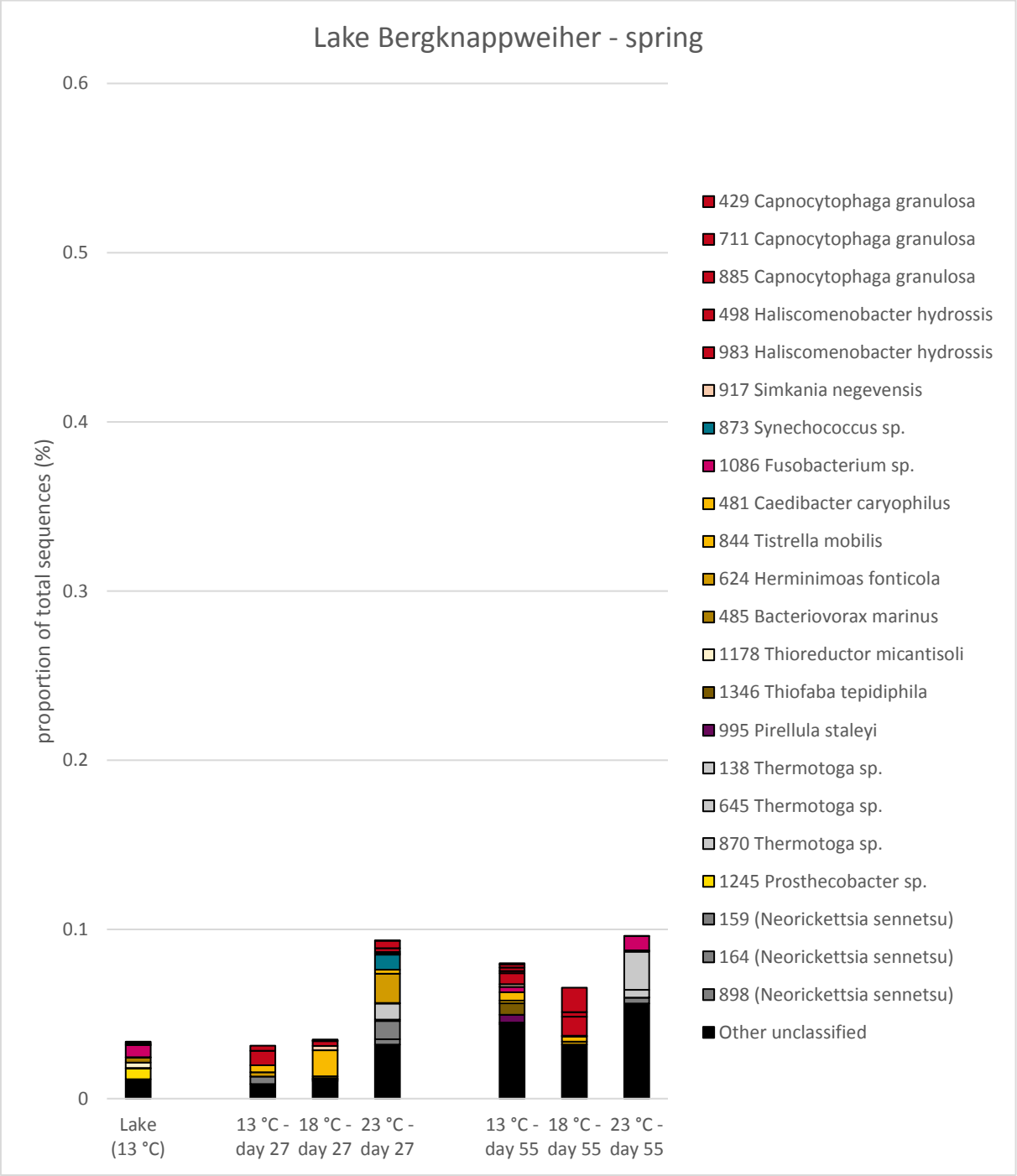
A. Classification

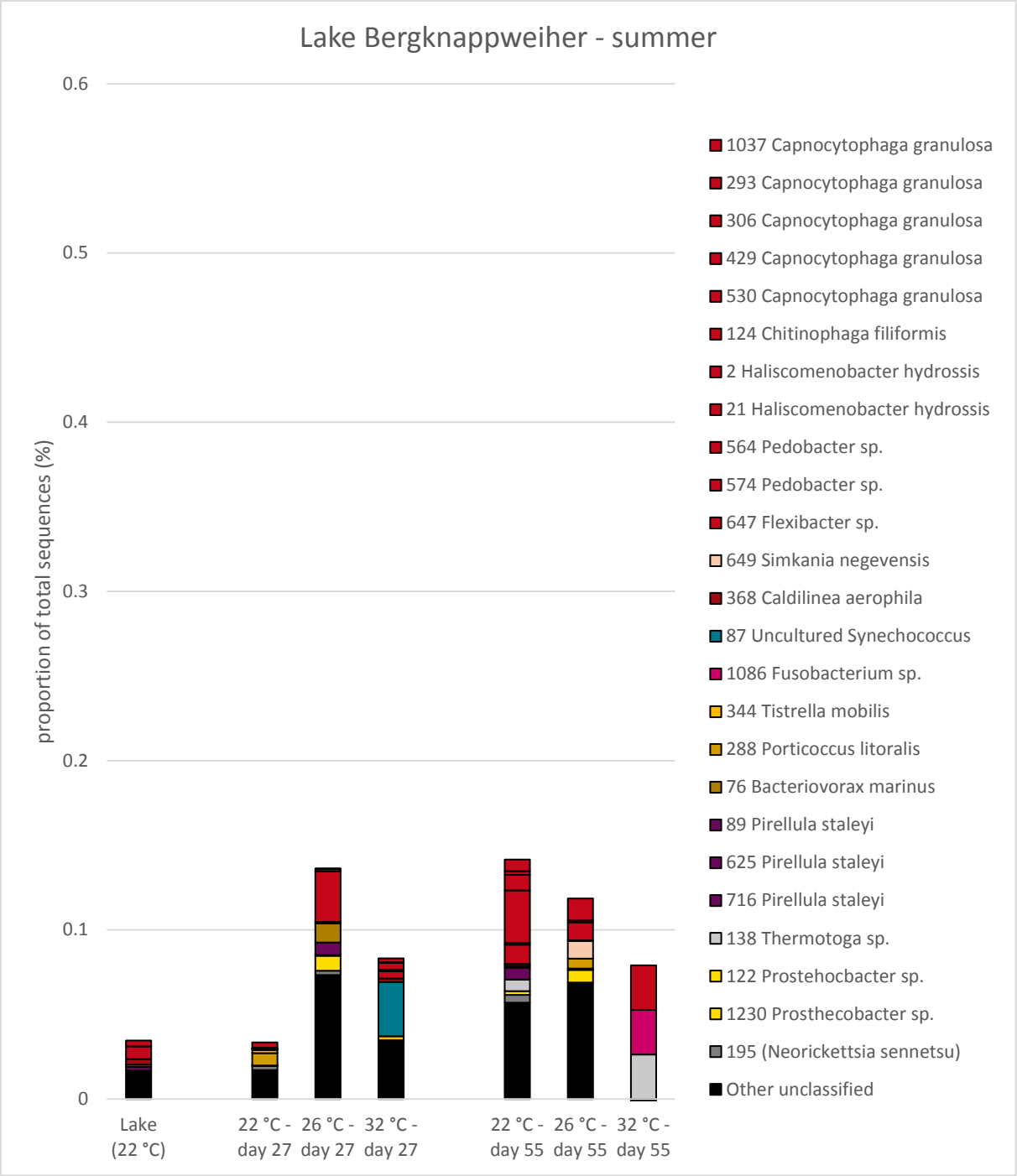
OTU no.	Identity	Genus/Species	Phylum
547	0.99	*Acidothermus cellulolyticus*	Actinobacteria
2	0.99	*Haliscomenobacter hydrossis*	Bacteroidetes
21	0.99	*Haliscomenobacter hydrossis*	Bacteroidetes
25	0.99	*Pedobacter sp.*	Bacteroidetes
80	0.99	*Flexibacter sp.*	Bacteroidetes
85	0.99	*Filimonas lacunae*	Bacteroidetes
88	0.99	*Flexibacter sp.*	Bacteroidetes
124	0.99	*Chitinophaga filiformis*	Bacteroidetes
167	0.99	*Pedobacter sp.*	Bacteroidetes
204	0.99	*Pedobacter sp.*	Bacteroidetes
206	0.99	*Pedobacter sp.*	Bacteroidetes
221	0.99	*Filimonas lacunae*	Bacteroidetes
293	0.99	*Capnocytophaga granulosa*	Bacteroidetes
306	0.99	*Capnocytophaga granulosa*	Bacteroidetes
426	0.99	*Capnocytophaga granulosa*	Bacteroidetes
498	0.99	*Haliscomenobacter hydrossis*	Bacteroidetes
530	0.99	*Capnocytophaga granulosa*	Bacteroidetes
564	0.99	*Pedobacter sp.*	Bacteroidetes
574	0.99	*Pedobacter sp.*	Bacteroidetes
647	0.99	*Flexibacter sp.*	Bacteroidetes
660	0.99	*Pedobacter sp.*	Bacteroidetes
711	0.99	*Capnocytophaga granulosa*	Bacteroidetes
764	0.99	*Flexibacter sp.*	Bacteroidetes
885	0.99	*Capnocytophaga granulosa*	Bacteroidetes
944	0.99	*Flexibacter sp.*	Bacteroidetes
983	0.99	*Haliscomenobacter hydrossis*	Bacteroidetes
1037	0.99	*Capnocytophaga granulosa*	Bacteroidetes
1128	0.99	*Filimonas lacunae*	Bacteroidetes
1214	0.99	*Flexibacter sp.*	Bacteroidetes
1589	0.99	*Haliscomenobacter hydrossis*	Bacteroidetes
156	0.99	*Simkania negevensis*	Chlamydiae
649	0.99	*Simkania negevensis*	Chlamydiae
917	0.99	*Simkania negevensis*	Chlamydiae
368	0.99	*Caldilinea aerophila*	Chloroflexi
44	0.99	*Synechococcus sp.*	Cyanobacteria
47	0.99	*Leptolyngbya sp.*	Cyanobacteria
87	0.99	*Uncultured Synechococcus*	Cyanobacteria
166	0.99	*Synechococcus sp.*	Cyanobacteria
303	0.99	*Synechococcus sp.*	Cyanobacteria
336	0.99	*Uncultured Synechococcus*	Cyanobacteria
482	0.99	*Synechococcus sp.*	Cyanobacteria

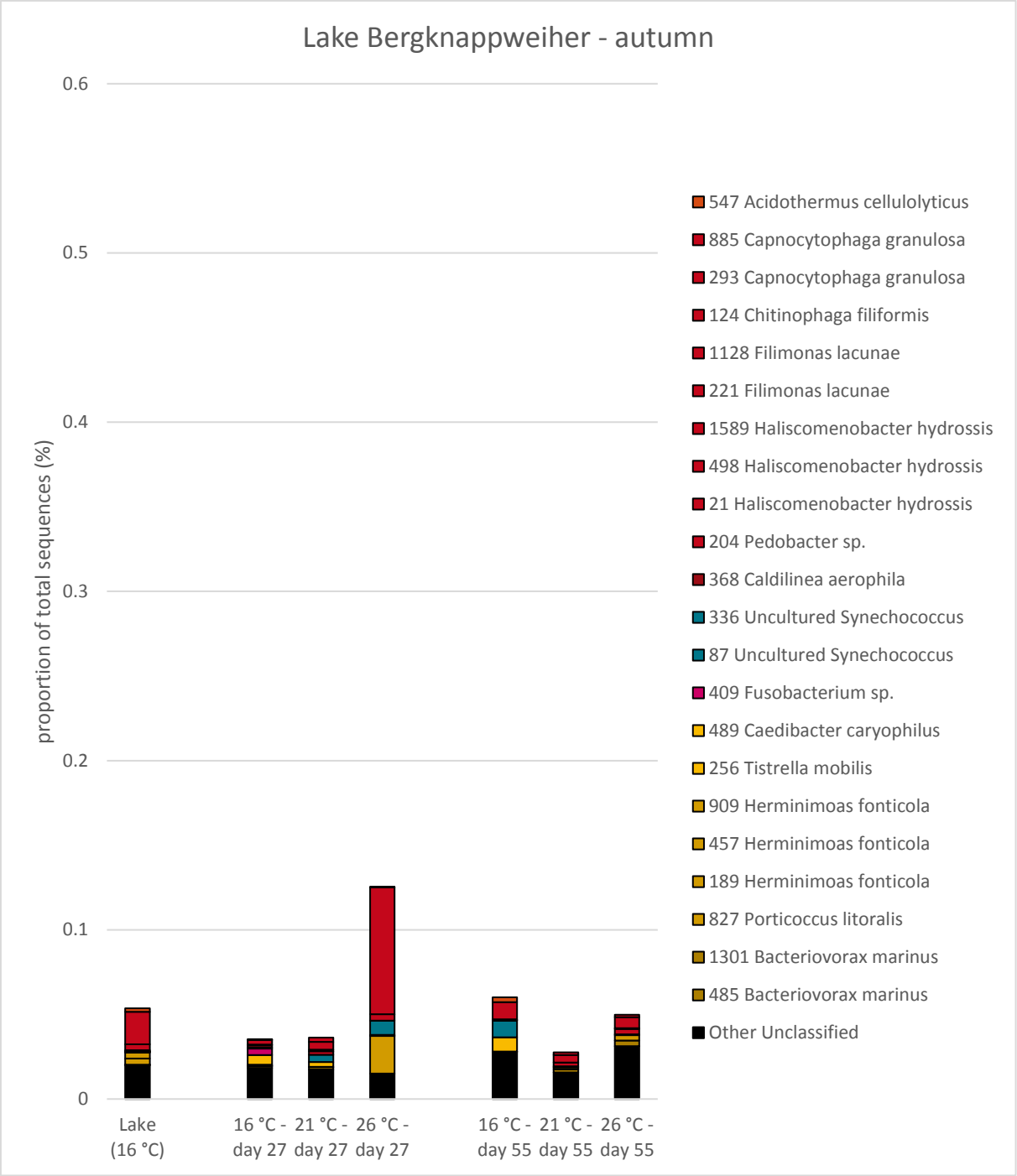
571	0.99	*Synechococcus sp.*	Cyanobacteria
770	0.99	*Synechococcus sp.*	Cyanobacteria
873	0.99	*Synechococcus sp.*	Cyanobacteria
148	0.99	*Asteroleplasma anaerobium*	Firmicutes
409	0.99	*Fusobacterium sp.*	Firmicutes
550	0.99	*Fusobacterium sp.*	Firmicutes
1086	0.98	*Fusobacterium sp.*	Firmicutes
89	0.99	*Pirellula staleyi*	Planktomycetes
180	0.99	*Pirellula staleyi*	Planktomycetes
467	0.99	*Pirellula staleyi*	Planktomycetes
625	0.99	*Pirellula staleyi*	Planktomycetes
716	0.99	*Pirellula staleyi*	Planktomycetes
995	0.99	*Pirellula staleyi*	Planktomycetes
20	0.99	*Kiloniella laminariae*	Alphaproteobacteria
59	0.99	*Meganema perideroedes*	Alphaproteobacteria
159	0.99	*Neorickettsia sennetsu*	Alphaproteobacteria
195	0.99	*Neorickettsia sennetsu*	Alphaproteobacteria
214	0.99	*Pleomorphomonas koreensis*	Alphaproteobacteria
253	0.99	*Caedibacter caryophilus*	Alphaproteobacteria
256	0.99	*Tistrella mobilis*	Alphaproteobacteria
301	0.99	*Tistrella mobilis*	Alphaproteobacteria
344	0.99	*Tistrella mobilis*	Alphaproteobacteria
367	0.99	*Neorickettsia sennetsu*	Alphaproteobacteria
385	0.99	*Rickettsia bellii*	Alphaproteobacteria
397	0.99	*Neorickettsia sennetsu*	Alphaproteobacteria
447	0.99	*Rickettsia bellii*	Alphaproteobacteria
464	0.99	*Meganema perideroedes*	Alphaproteobacteria
468	0.99	*Neorickettsia sennetsu*	Alphaproteobacteria
481	0.99	*Caedibacter caryophilus*	Alphaproteobacteria
489	0.99	*Caedibacter caryophilus*	Alphaproteobacteria
844	0.99	*Tistrella mobilis*	Alphaproteobacteria
863	0.99	*Neorickettsia sennetsu*	Alphaproteobacteria
898	0.99	*Neorickettsia sennetsu*	Alphaproteobacteria
93	0.99	*Rhodocyclus tenuis*	Betaproteobacteria
189	0.99	*Herminimoas fonticola*	Betaproteobacteria
288	0.99	*Porticoccus litoralis*	Betaproteobacteria
457	0.99	*Herminimoas fonticola*	Betaproteobacteria
577	0.98	*Herminimoas fonticola*	Betaproteobacteria
624	0.98	*Herminimoas fonticola*	Betaproteobacteria
827	0.99	*Porticoccus litoralis*	Betaproteobacteria
909	0.99	*Herminimoas fonticola*	Betaproteobacteria
1284	0.98	*Herminimoas fonticola*	Betaproteobacteria
2723	0.99	*Herminimoas fonticola*	Betaproteobacteria
76	0.99	*Bacteriovorax marinus*	Deltaproteobacteria

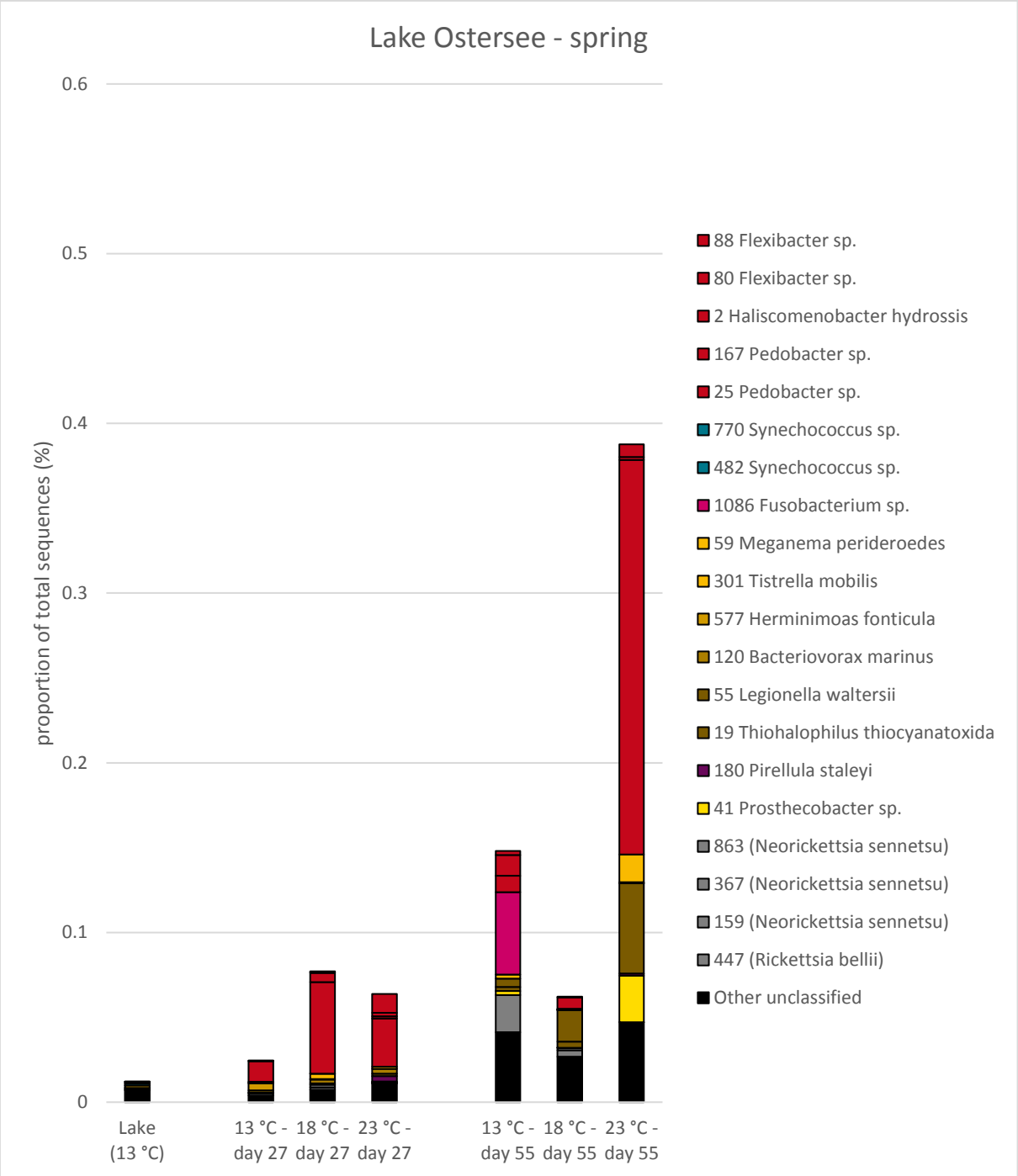
120	0.99	*Bacteriovorax marinus*	Deltaproteobacteria
485	0.99	*Bacteriovorax marinus*	Deltaproteobacteria
494	0.99	*Bdellovibrio bacteriovorus*	Deltaproteobacteria
1301	0.99	*Bacteriovorax marinus*	Deltaproteobacteria
1178	0.99	*Thioreductor micantisoli*	Epsilonproteobacteria
19	0.99	*Thiohalophilus thiocyanatoxyda*	Gammaproteobacteria
55	0.99	*Legionella waltersii*	Gammaproteobacteria
1346	0.99	*Thiofaba tepidiphyla*	Gammaproteobacteria
138	0.99	*Thermotoga sp.*	Thermotogae
645	0.99	*Thermotoga sp.*	Thermotogae
870	0.99	*Thermotoga sp.*	Thermotogae
41	0.99	*Prosthecobacter sp.*	Verrucomicrobia
122	0.99	*Prosthecobacter sp.*	Verrucomicrobia
1230	0.99	*Prosthecobacter sp.*	Verrucomicrobia
1245	0.99	*Prosthecobacter sp.*	Verrucomicrobia

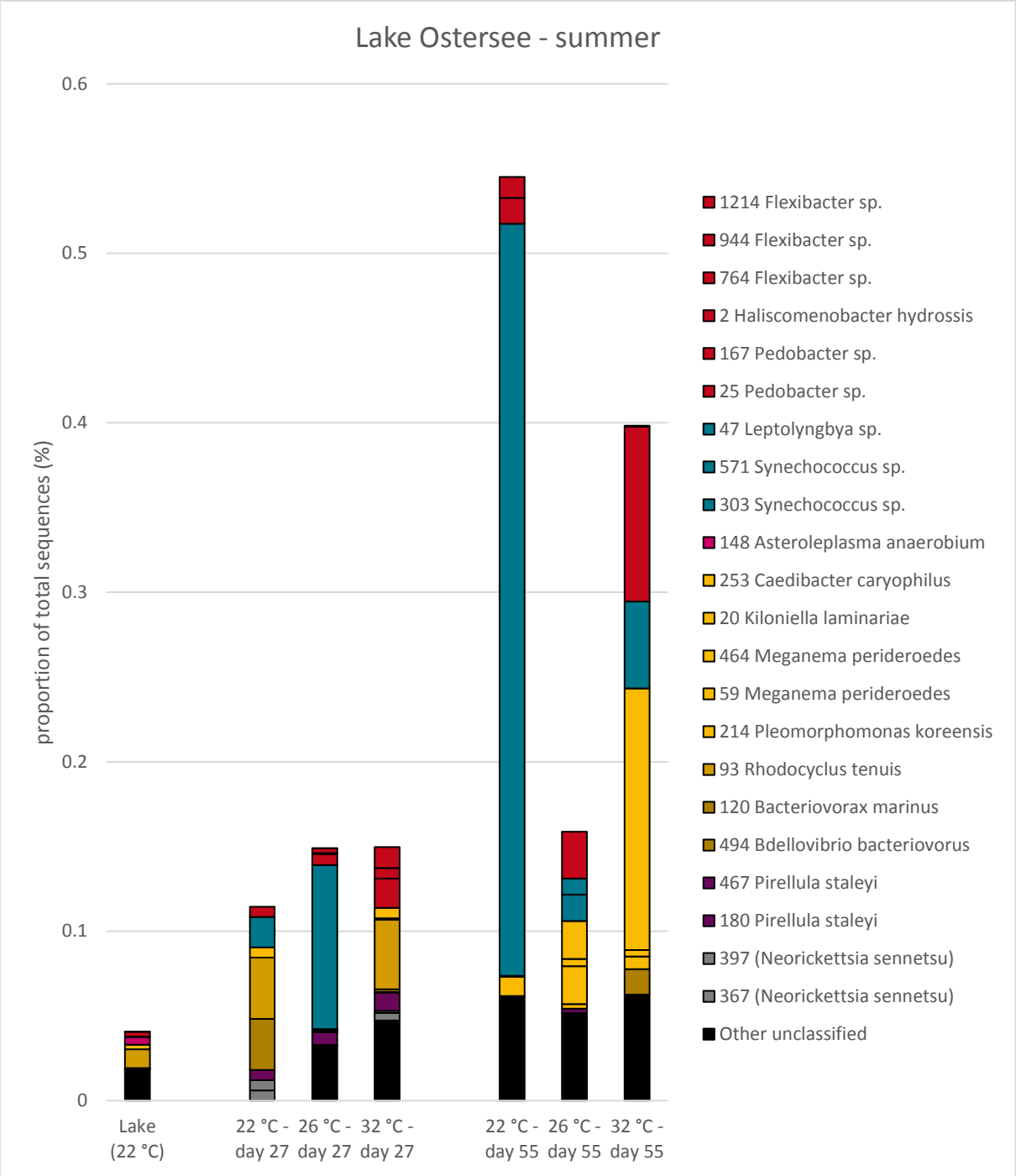
B. Graphical presentation




Lake Bergknappweiher - summer
proportion of total sequences (%)
0.6
0.5
0.4
0.3
0.2
0.1
0
Lake (22 °C)
22 °C - day 27
26 °C - day 27
32 °C - day 27
22 °C - day 55
26 °C - day 55
32 °C - day 55
1037 Capnocytophaga granulosa
293 Capnocytophaga granulosa
306 Capnocytophaga granulosa
429 Capnocytophaga granulosa
530 Capnocytophaga granulosa
124 Chitinophaga filiformis
2 Haliscomenobacter hydrossis
21 Haliscomenobacter hydrossis
564 Pedobacter sp.
574 Pedobacter sp.
647 Flexibacter sp.
649 Simkania negevensis
368 Caldilinea aerophila
87 Uncultured Synechococcus
1086 Fusobacterium sp.
344 Tistrella mobilis
288 Porticoccus litoralis
76 Bacteriovorax marinus
89 Pirellula staleyi
625 Pirellula staleyi
716 Pirellula staleyi
138 Thermotoga sp.
122 Prostehocbacter sp.
1230 Prosthecobacter sp.
195 (Neorickettsia sennetsu)
Other unclassified


Lake Bergknappweiher - autumn
proportion of total sequences (%)
0.6
0.5
0.4
0.3
0.2
0.1
0
Lake (16 °C)
16 °C - day 27
21 °C - day 27
26 °C - day 27
16 °C - day 55
21 °C - day 55
26 °C - day 55
547 Acidothermus cellulolyticus
885 Capnocytophaga granulosa
293 Capnocytophaga granulosa
124 Chitinophaga filiformis
1128 Filimonas lacunae
221 Filimonas lacunae
1589 Haliscomenobacter hydrossis
498 Haliscomenobacter hydrossis
21 Haliscomenobacter hydrossis
204 Pedobacter sp.
368 Caldilinea aerophila
336 Uncultured Synechococcus
87 Uncultured Synechococcus
409 Fusobacterium sp.
489 Caedibacter caryophilus
256 Tistrella mobilis
909 Herminimoas fonticola
457 Herminimoas fonticola
189 Herminimoas fonticola
827 Porticoccus litoralis
1301 Bacteriovorax marinus
485 Bacteriovorax marinus
Other Unclassified


Lake Ostersee - spring
proportion of total sequences (%)
0.6
0.5
0.4
0.3
0.2
0.1
0
Lake (13 °C)
13 °C - day 27
18 °C - day 27
23 °C - day 27
13 °C - day 55
18 °C - day 55
23 °C - day 55
88 Flexibacter sp.
80 Flexibacter sp.
2 Haliscomenobacter hydrossis
167 Pedobacter sp.
25 Pedobacter sp.
770 Synechococcus sp.
482 Synechococcus sp.
1086 Fusobacterium sp.
59 Meganema perideroedes
301 Tistrella mobilis
577 Herminimoas fonticula
120 Bacteriovorax marinus
55 Legionella waltersii
19 Thiohalophilus thiocyanatoxida
180 Pirellula staleyi
41 Prosthecobacter sp.
863 (Neorickettsia sennetsu)
367 (Neorickettsia sennetsu)
159 (Neorickettsia sennetsu)
447 (Rickettsia bellii)
Other unclassified


Lake Ostersee - summer
proportion of total sequences (%)
0.6
0.5
0.4
0.3
0.2
0.1
0
Lake (22 °C)
22 °C - day 27
26 °C - day 27
32 °C - day 27
22 °C - day 55
26 °C - day 55
32 °C - day 55
1214 Flexibacter sp.
944 Flexibacter sp.
764 Flexibacter sp.
2 Haliscomenobacter hydrossis
167 Pedobacter sp.
25 Pedobacter sp.
47 Leptolyngbya sp.
571 Synechococcus sp.
303 Synechococcus sp.
148 Asteroleplasma anaerobium
253 Caedibacter caryophilus
20 Kiloniella laminariae
464 Meganema perideroedes
59 Meganema perideroedes
214 Pleomorphomonas koreensis
93 Rhodocyclus tenuis
120 Bacteriovorax marinus
494 Bdellovibrio bacteriovorus
467 Pirellula staleyi
180 Pirellula staleyi
397 (Neorickettsia sennetsu)
367 (Neorickettsia sennetsu)
Other unclassified

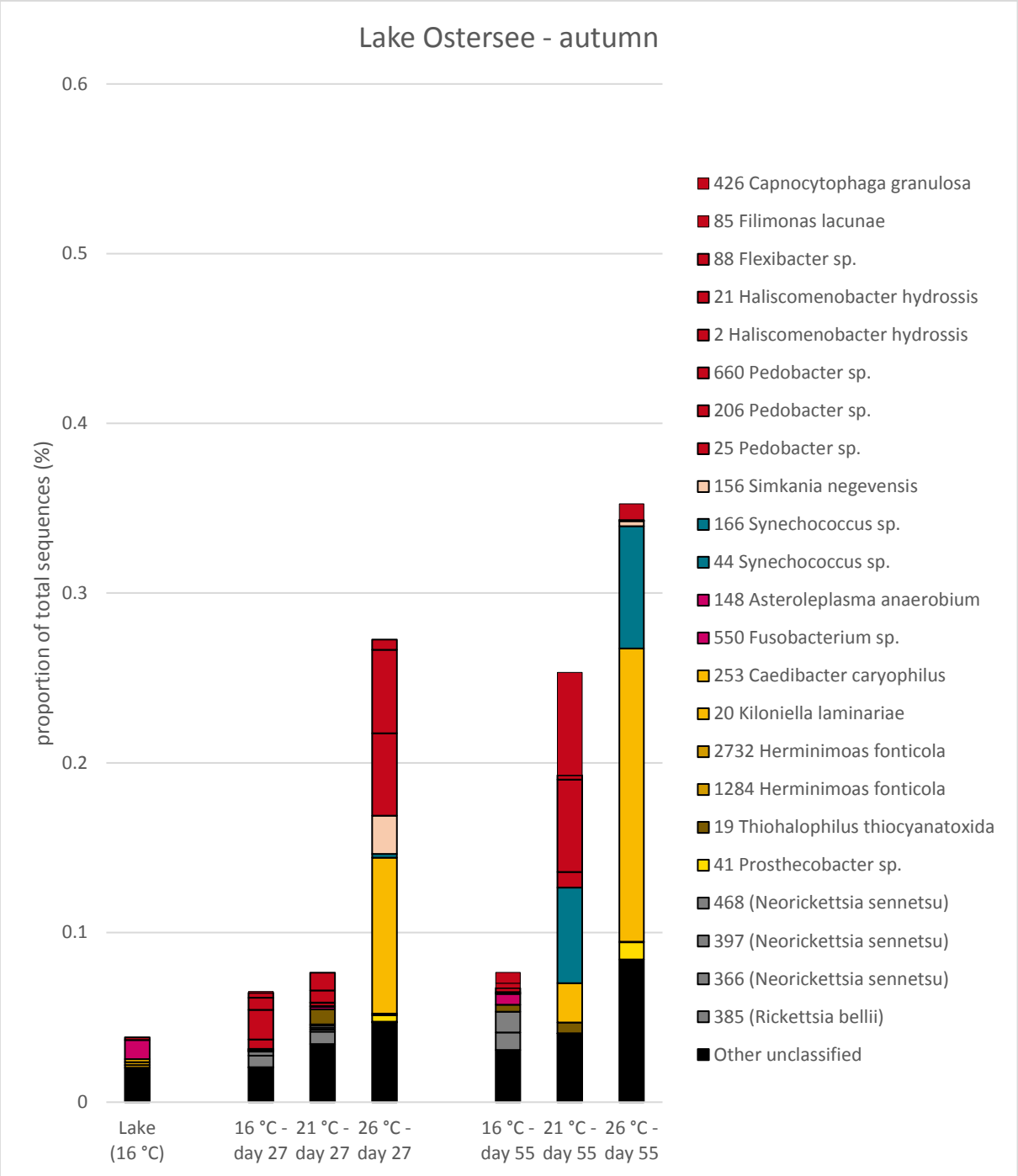


Figure 11.11 Classification was done by inserting the sequences in a phylogenetic tree based on the LTP tree (tree_LTPs106.SSU, August 2011) using ARB (Ludwig et al., 2004). For the deeper classification of cyanobacteria, we used an *in-house* cyanobacteria-based tree containing 30000 sequences. The sequences identified as *Rickettsia* sp. and *Neorickettsia* sp. are of eukaryotic origin (mitochondria) and can therefore be ignored.