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DISSERTATION

Titel der Dissertation

„Environmental regulation of microcystin genotype abundance and the resulting net production of the toxic heptapeptide microcystin among bloom-forming cyanobacteria“

verfasst von

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The mind is not a vessel to be filled, but a fire to be kindled.

Plutarch

Abstract

The toxic heptapeptide microcystin (MC) produced by cyanobacteria has gained much attention, as high amounts of this toxin occur within cyanobacterial blooms, which constitutes a considerable health risk to humans. Within a population, commonly toxin-producing and nontoxic genotypes co-occur due to the loss or the inactivation of the MC synthetase (*mcy*) gene cluster. Little is known, however, about the environmental factors regulating the toxigenic genotype composition in a habitat. Understanding the controls is one of the most challenging questions with regard to the regulation of toxin production in aquatic systems. The aim of this thesis is the elucidation of environmental factors that influence the distribution and abundance of toxic and nontoxic genotypes among planktonic cyanobacteria over different spatial and temporal scales. As a method of choice quantitative real-time PCR (qPCR) is not only highly sensitive but also enables the quantification of specific toxic and nontoxic genotypes directly in the field. It allows drawing conclusions on the role of environmental factors regulating MC genotype abundance and the resulting MC production in habitats.

In the first chapter, the abundance and the proportion of the MC-producing genotype was investigated in populations of the cyanobacterium *Microcystis* occurring in five lakes in Uganda (East Africa) in dependence on environmental factors during one year. The results showed that the MC genotype proportion differed consistently and independently of the season between lakes, suggesting that local factors differing spatially between lakes might determine the MC genotype abundance. In addition, the average MC content per cell was correlated linearly to the proportion of the MC-producing genotype in the different lakes.

In the second chapter, the abundance and proportion of three *mcy* genotypes encoding the synthesis of different MC structural variants were investigated in populations of the cyanobacterium *Planktothrix* occurring in twelve deep stratified lakes of the Alps (Austria, Germany, Switzerland) during two years. The concentration and proportion of the abundant MC structural variants [Asp, Dhb]-MC-RR, [Asp, Mdha]-MC-RR and [Asp, Dhb]-MC-HtyR were significantly explained by the respective MC genotype abundance and proportion. In general, the genotype encoding the synthesis

of [Asp, Dhb]-MC-RR showed the highest proportion irrespective of the trophic state and over a wide range in population density. The results of chapters 1 and 2 show that MC net production could be quantitatively and qualitatively explained by the abundance of specific *mcy* genotypes, suggesting that the (eco-)physiological influence on MC production through transcriptional regulation was of minor importance.

In the third chapter, the average abundance of the same genotypes encoding the synthesis of [Asp, Dhb]-MC-RR, [Asp, Mdha]-MC-RR and [Asp, Dhb]-MC-HtyR were analyzed in deep-stratified Lake Zürich (Switzerland) during its re-oligotrophication period of ca. thirty years (1977-2008). This long-term observational approach was possible by extracting the DNA from heat-desiccated phytoplankton and the quantification of the *mcy* genotype abundance and proportion by means of qPCR. The historical DNA showed the occurrence of heat-induced substitutions that could be repaired by enzyme treatment. However, the major DNA destruction resulted from fragmentation. Both fragmentation and the occurrence of heat-induced substitutions were found evenly distributed among the various gene loci used for qPCR. Thus, it was concluded that the estimates of genotype proportions as determined by qPCR were relatively robust against heat-desiccation induced damage of the DNA. Similar to the study described in chapter 2, the proportion of the genotype encoding the synthesis of [Asp, Dhb]-MC-RR was higher when compared with the [Asp, Mdha]-MC-RR genotype during the whole observation period, although it decreased in proportion. This decline could be related to the minor increase in proportion of an inactive MC genotype carrying a deletion within this gene region, but presumably did not affect the overall toxicity of the population.

In the fourth chapter, using the same DNA samples as in chapter 3, the proportion of the toxigenic MC genotypes was compared to the proportion of the nontoxic genotypes. Despite major changes in trophy and the *Planktothrix* population density in deep-stratified Lake Zürich (Switzerland) the proportion of the toxigenic MC genotypes was close to hundred percent. Nontoxic genotypes inactive due to insertions or a deletion within the *mcy* gene cluster occurred in low proportions only. A genotype that is known from shallow polymictic lakes, and which lost ninety percent of the *mcy* gene cluster and differs in pigmentation co-occurred, but was never

abundant. This remarkable stability of MC genotype dominance could be explained by its adaptation to deep-mixing events, for example through the gene expression of highly resistant gas vesicles.

Zusammenfassung

Das von Blaualgen (Cyanobakterien) produzierte toxische Heptapeptid Microcystin (MC) rückte zunehmend in den Fokus öffentlichen Interesses, da die in Binnengewässern im Zuge einer Bildung von Algenblüten auftretenden hohen Konzentrationen dieses Toxins ein Gesundheitsrisiko für den Menschen darstellen können. Innerhalb einer Population kommen für gewöhnlich toxische und nicht-toxische Genotypen gemeinsam vor, wobei letztere durch den Verlust oder die Inaktivierung des MC Synthetase (*mcy*) Genclusters entstanden sind. Die Umweltfaktoren, welche die Genotypenzusammensetzung innerhalb eines Habitats regulieren, sind allerdings unbekannt. Die Entschlüsselung dieser Regulationsmechanismen ist in Bezug auf das Verständnis der Toxinproduktion in aquatischen Systemen eine große Herausforderung. Das Ziel dieser Arbeit war es, die Umweltfaktoren, die die Verbreitung und die Abundanz von toxischen und nicht-toxischen Genotypen innerhalb planktischer Cyanobakterien beeinflussen, unter Berücksichtigung verschiedener räumlicher und zeitlicher Skalen zu identifizieren. Die quantitative real-time PCR (qPCR) ist als Methode der Wahl nicht nur höchst sensitiv, sondern ermöglicht auch die direkte Quantifizierung von toxischen und nicht-toxischen Genotypen aus Freilandproben. Die qPCR-Methode erlaubt es, Schlussfolgerungen über die Rolle der Umweltfaktoren zu ziehen, die die MC Genotypenzusammensetzung und die daraus resultierende MC Produktion regulieren.

Im ersten Kapitel wurden die Abundanz und der Anteil des MC produzierenden Genotyps in Populationen des Cyanobakteriums *Microcystis* in fünf verschiedenen Binnengewässern in Uganda (Ostafrika) in Abhängigkeit von Umweltfaktoren über den Verlauf eines Jahres untersucht. Die Ergebnisse zeigten, dass der Anteil des MC Genotyps durchgehend und unabhängig von den Jahreszeiten zwischen den Seen variierte, was auf lokale Einflussfaktoren schließen lässt, die für die jeweiligen Seen unterschiedlich sind und somit die MC Genotypenhäufigkeit beeinflussen.

Im zweiten Kapitel wurden die Abundanz und der Anteil von drei verschiedenen *mcy* Genotypen bestimmt, welche für die Synthese von unterschiedlichen MC Strukturvarianten ([Asp, Dhb]-MC-RR, [Asp, Mdha]-MC-RR und [Asp, Dhb]-MC-HtyR) bei dem Cyanobakterium *Planktothrix* kodieren. Dazu wurden zwölf

Populationen des Cyanobakteriums *Planktothrix*, welches in tiefen, geschichteten Seen der Alpen (Österreich, Deutschland, Schweiz) vorkommt, über einen Zeitraum von zwei Jahren untersucht. Die Konzentration und der Anteil der drei häufigen MC Strukturvarianten konnte durch die Häufigkeit und den Anteil der entsprechenden Genotypen hoch signifikant erklärt werden. Generell hatte der Genotyp, der die Synthese von [Asp, Dhb]-MC-RR kodiert den höchsten Anteil, der von der Trophiestufe eines Gewässers und der Populationsdichte nicht beeinflusst wurde. Die Ergebnisse von Kapitel eins und zwei zeigen, dass die MC Netto-Produktion vor allem quantitativ und qualitativ durch die Abundanz der entsprechenden MC Genotypen erklärt werden konnte. Daraus folgt weiter, dass der (öko-)physiologische Einfluss von Umweltfaktoren, z.B. von Licht auf die Transkription der jeweiligen Gene von nur geringer Bedeutung war.

Im dritten Kapitel wurde die durchschnittliche Abundanz derselben Genotypen, die die Synthese von [Asp, Dhb]-MC-RR, [Asp, Mdha]-MC-RR und [Asp, Dhb]-MC-HtyR kodieren, im tiefen und geschichteten Zürichsee (Schweiz) über den Zeitraum der Re-oligotrophierungsphase von ca. 30 Jahren (1977-2008) untersucht. Diese einzigartige Langzeituntersuchung wurde durch Extraktion der DNA aus Phytoplanktonproben, die durch Hitzebehandlung haltbar gemacht und seither aufbewahrt wurden, und dem Einsatz der bereits etablierten qPCR Methodik ermöglicht. In der durch Hitzebehandlung konservierten DNA wurden einzelne DNA Substitutionen gefunden, die durch enzymatische Behandlung repariert werden konnten. Die Hauptschädigung des genetischen Materials bestand jedoch wahrscheinlich aus Doppelstrangbrüchen, die die DNA Menge insgesamt sehr deutlich reduzierten. Nachdem jedoch die Schädigung der DNA über alle der für qPCR verwendeten Loci gleichmäßig verteilt war, wurde davon ausgegangen, dass die relative Abschätzung der Anteile einzelner Genotypen von der Art und Weise der Konservierung der DNA Proben unabhängig sein sollte. Ähnlich der in Kapitel zwei beschriebenen Studie, war der Anteil des Genotyps, der für die Synthese von [Asp, Dhb]-MC-RR kodiert, verglichen mit dem Anteil des [Asp, Mdha]-MC-RR Genotyps über den gesamten Untersuchungszeitraum stabil am höchsten, obwohl eine prozentuale Abnahme detektiert wurde. Diese Abnahme konnte auf eine leichte Zunahme eines inaktiven MC Genotyps zurückgeführt werden, der eine Deletion im untersuchten Genbereich aufweist, jedoch wahrscheinlich die Toxizität der Gesamtpopulation nicht beeinflußte.

Im vierten Kapitel wurde unter Verwendung derselben DNA aus Kapitel drei der Anteil des toxischen Genotyps mit jenem der nicht-toxischen Genotypen über den Zeitraum der Re-oligotrophierungsphase von ca. 30 Jahren (1977-2008) im tiefen, thermisch geschichteten Zürichsee (Schweiz) verglichen. Trotz großer Schwankungen in der Trophiestufe und in der Populationsdichte von *Planktothrix* war der Anteil des toxischen MC Genotyps fast einhundert Prozent. MC Genotypen, die durch Insertionen oder eine Deletion in der MC Synthese inaktiviert wurden, hatten nur geringen Anteil an der Gesamtpopulation. Ein aus seichten und polymiktischen Gewässern bekannter nicht-toxischer Genotyp, der neunzig Prozent des *mcy* Genclusters verloren hat und eine andere Pigmentierung aufweist, konnte über den gesamten Beobachtungszeitraum detektiert werden. Allerdings war dessen Häufigkeit erstaunlich gering. Diese bemerkenswerte Stabilität in der Dominanz des MC Genotyps konnte durch dessen Adaption an regelmäßig stattfindende Durchmischungsereignisse erklärt werden, die aufgrund des hohen Wasserdrucks die Ausbildung von maximal druckresistenten Gasvesikeln erfordern.

List of Abbreviations

A domain	amino acid activation domain
ANOVA	analysis of variance
Arg	arginine
Dha	dehydroalanine
Dhb	dehydrobutyrine
C_t value	number of cycles at the threshold fluorescence
Fur	ferric uptake regulator
HPLC	high-performance liquid chromatography
Hty	homotyrosine
i.p.	intraperitoneal
LD_{50}	median lethal dose; dose required to kill half the members of a tested population after a specified duration
Leu	leucine
LPS	lipopolysaccharides
MC	microcystin
mcy	microcystin synthetase genes
*mcy*AAd1, *mcy*BAd1	first adenylation domain of *mcy*A or *mcy*B
Mdha	N-methyldehydroalanine
nda	nodularin synthetase genes
NFQ	non-fluorescent quencher
NRPS	non-ribosomal peptide synthetase
NtcA	global nitrogen uptake regulator
OATP	organic anion transporting polypeptide
PC	phycocyanin
PC-IGS	phycocyanin intergenic spacer
PKS	polyketide synthetase
PSA	P700 apoprotein
qPCR	quantitative real-time polymerase chain reaction
Tyr	tyrosine
SNP	single nucleotide polymorphism
16S rDNA	ribosomal RNA of the small subunit of prokaryotic ribosomes
16S-ITS	16S – 23S ribosomal DNA internal transcribed spacer

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PART II

PART I

1. Ecology of Cyanobacteria

The name cyanobacteria is ascribed to the frequently observed bluish or greenish color in surface blooms (Greek: kyanós = blue) and cyanobacteria are therefore also named blue-green algae. The characteristic color is due to the presence of phycobiliproteins, a complex formed by phycobilins, which function as chromophores, and associated proteins. Phycobiliproteins are located in the phycobilisomes on the thylakoid membrane, acting as light collecting antennae that allow for the use of a broad range of the light spectrum.

The cells can contain phycocyanin, absorbing orange and red light and allophycocyanin, absorbing red light, making the cells look blue-green. The facultative phycoerythrin, absorbing blue-green and yellow light, gives the cells a red or brownish colour. The proportion of the pigments, including phycoerythrin, can be variable, depending on the available light spectrum. The presence of these pigments that are accessory to chlorophyll makes cyanobacteria very efficient light harvesters. Other accessory pigments for example include the carotenoids zeaxanthin and oscillaxanthin that also absorb light energy for photosynthesis and protect the photosystems from photooxidation.

Cyanobacteria form a monophyletic group within the domain of *Eubacteria* and are among the oldest organisms on earth. As the only bacteria that possess photosystem I and II they are capable of producing oxygen via photosynthesis using H_2O as hydrogen donor like higher plants. Under anaerobic conditions also other electron donors like H_2S or $S_2O_3^{2-}$ can be used for photosynthesis using photosystem I only, like it is found among purple bacteria.

It is widely accepted that early cyanobacteria changed the reducing atmosphere by the release of oxygen derived from oxygenic photosynthesis, yet there is controversy about when oxygen began to accumulate. According to the general hypothesis, the accumulation of oxygen started about 2.2 to 2.3 billion years ago (Canfield *et al* 2000).

Schopf (1993) reported the detection of cyanobacteria-like filaments in chert of the Apex Basalt from Western Australia that was dated to be 3.5 billion years old, which suggests that oxygen-producing photoautotrophy might have evolved at that time. Fossil records of cyanobacteria from stromatolites, which are lithified biosediments that arise from the binding and cementation of sediment particles by biofilms of

microoganisms, in particular of cyanobacteria, were however reported to be 2.6 billion years old (Kazmierczak and Altermann 2002).
The general success of cyanobacteria that are found in numerous of different habitats, is most likely related to their long evolutionary history and their ability to adapt to a wide range of habitats. They are found in the desert and the Polar Regions, in marine and freshwater systems and can also colonize most extreme habitats. They are among the most successful organisms in highly saline environments and show a high tolerance to desiccation; e.g. for herbarium specimens of *Nostoc commune* it was shown that vegetative cells remain viable after several decades of storage (Wynn-Williams 2000). Among others, for example *Nostoc* can tolerate several stresses at the same time, e.g. desiccation, oxidation and UV irradiation, resembling conditions that are present on rocks. Within geothermal habitats like hot springs cyanobacteria can tolerate temperatures up to 73°C (Castenholz 1996) and they are often the most important primary producers in microbial mats in coastal lagoons of changing salinity and temperature (Stal *et al* 1996).

Generally, mass occurrences of cyanobacteria in aquatic ecosystems have been associated with eutrophication and increased water temperatures. Phosphorus is thought to be the main nutrient controlling growth, as many cyanobacteria can avoid nitrogen limitation by fixing atmospheric nitrogen in specialized cells, the so-called heterocysts. In marine habitats cyanobacteria play an important role in carbon and nitrogen cycling because of their contribution to primary production by CO_2-fixation and by the reduction of N_2 to NH_3, which can be used as a nitrogen source by other organisms. Yet, compared to eukaryotic algae, various cyanobacteria have been shown to have a high phosphate uptake affinity and high phosphate storage capacity by the formation of polyphosphate granules (Isvánovics *et al* 2000) enabling further cell divisions (Carey *et al* 2012). It has been shown, that cyanobacteria can store phosphorus sufficient for two to four further cell divisions, which is equivalent to a 4 – 32 fold increase in biomass (Mur *et al* 1999).

Besides their high degree of adaptation to different habitats, cyanobacteria often do not suffer from zooplankton grazing. The aggregation of cells into colonies in association with excretion of exopolysaccharides is common and can impede grazing losses (Jarvis *et al* 1987). Also a large filament size can pose a mechanical barrier

against grazing. Generally the food quality of cyanobacteria is considered to be low, e.g. for some species the absence of sterols (Basen *et al* 2012) was shown. Further, a variety of strains of different cyanobacteria were shown to be toxic to zooplankton by inhibiting eukaryotic protein phosphatases, e.g. due to the presence of MCs (Honkanen *et al* 1995). It was calculated that more than 74% of cyanobacteria blooms from European countries were either toxic in mouse bioassays or contained cyanotoxins, implying a high probability that mass occurrences of cyanobacteria are toxic (Quesada *et al* 2004).

There is great morphological diversity within the phylum of cyanobacteria, ranging from unicellular growth to multicellular filaments; cells can appear singly or grouped together in colonies. The colonial structure is often maintained by exopolysaccharides like mucilage or alternatively a firm sheath. The taxonomic classification has been revised several times (e.g. Turner 1997). In the common classification system, the phylum is divided into 5 orders that are referred to as subsections, based on traditional morphological and molecular analysis of axenic clonal cultures (e.g. Rippka *et al* 1979, Rippka 1988, Castenholz and Waterbury 1989, Castenholz 2001). The system distinguishes the non-filamentous sections Chroococcales and Pleurocapsales from the filamentous sections Oscillatoriales, Nostocales and Stigonematales (Figure 1).
The section Chroococcales is distinguished from the section Pleurocapsales by the mode of cell division: Chroococcales divide in one to three planes, symmetric or asymmetric or by budding, while Pleurocapsales reproduce by multiple fissions resulting in daughter cells that are smaller than the parent cells.
Oscillatoriales and Nostocales feature binary cell division in one plane and may form false branches; however Nostocales may also form heterocysts for nitrogen fixation and some of them also form akinetes, resting cells formed under unfavorable environmental conditions. Heterocysts and akinetes do not occur within the order Oscillatoriales.
The common division in more than one plane, which gives rise to multiseriate trichomes, trichomes with true branches or both, marks the order Stigonematales. They may form akinetes, but always posses the ability to form heterocysts.

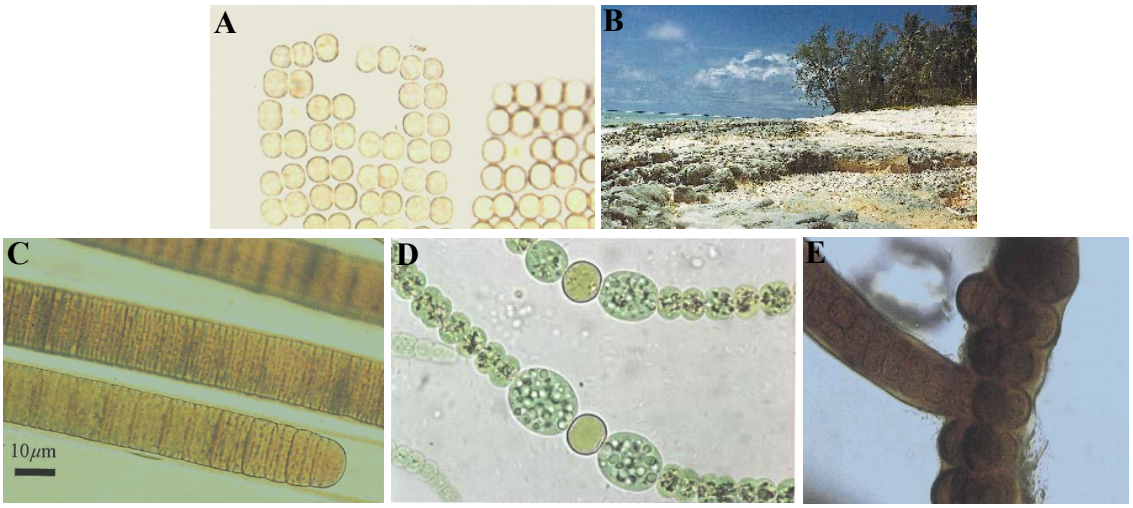

Figure 1

Cyanobacteria representative of the different orders: **A**, *Merismopedia* (Chroococcales); **B**, *Hyella* (Pleurocapsales); **C**, *Lyngbya* (Oscillatoriales); **D**, *Anabaena* (Nostocales); **E**, *Stigonema* (Stigonematales), reprinted from Whitton BA and Potts M (2000), with kind permission from Springer Science+Business Media B.V.

1.1 Cyanobacterial Bloom Formation

In 1878 George Francis published the first scientific report on the toxicity of a surface bloom formed by cyanobacteria. He had observed the rapid death of sheep, horses, dogs and pigs within 1 to 24 hours after the consumption of surface water from Lake Alexandria, South Australia, which was covered by a thick scum of *Nodularia spumigena*. In the journal "Nature" he stated: "Thus floating, it is wafted to the lee shores, and forming a thick scum like green oil paint, some two to six inches thick, and as thick and pasty as porridge, it is swallowed by cattle when drinking, especially such as suck their drink at the surface like horses. This acts poisonously, and rapidly causes death; symptoms – stupor and unconsciousness, falling and remaining quiet, as if asleep, unless touched, when convulsions come on, with head and neck drawn back by rigid spasm, which subsides before death." (Francis 1878).

In general, the term bloom defines a phytoplankton biomass, which is significantly higher than the average of a lake and usually comprises only one or two species. Surface blooms are however only formed by species that are buoyant or motile and accumulate at the water surface under appropriate environmental conditions forming a scum. There is a number of gas-vacuolate species of cyanobacteria among different genera that vary in size and shape from filaments to spherical colonies. Gas vacuoles

are comprised of cylindrical air-filled structures, the gas vesicles. They provide buoyancy and allow for vertical movement in the water column. Buoyancy by increased synthesis of gas vesicles is counteracted by the accumulation of carbohydrate reserves by photosynthesis (Reynolds *et al* 1987). Loss of gas vesicles or a decrease in production can also contribute to buoyancy loss in some species (Oliver and Walsby 1984). Additionally, by actively regulating buoyancy cyanobacteria can benefit from the higher irradiance in the upper water layer and on the other hand from increased nutrient availability at greater depths.

Surface blooms of cyanobacteria are common in freshwater, but they are also observed in tropical marine systems and in brackish waters like the Baltic Sea, where extensive surface blooms have already been described in the 19th century. Evidence is given for the existence of cyanobacteria in the Baltic Sea about 8000 before present from investigations of sediments (Bianchi *et al* 2000).
In nutrient poor marine environments only few taxa including *Trichodesmium*, *Nodularia*, *Aphanizomenon* and endosymbiont taxa such as *Richelia* are observed to form blooms. These taxa usually posses the ability to fix N_2 in heterocysts, but *Trichodesmium* is capable of fixing N_2 in the light without heterocysts. *Trichodesmium*, a cyanobacterium that is distributed throughout the oligotrophic tropical and subtropical oceans, is known for extensive blooms, which can be easily observed on satellite images (Subramaniam and Carpenter 1994). As an N_2-fixing species, it is thought to be an important contributor to the global nitrogen cycle (Capone *et al* 1997). During Captain James Cook's voyage of discovery along the Eastern coast of Australia (1770) the sailors first called the phenomenon "sea-sawdust" due to the yellowish thick appearance at the water surface. A key feature for the occurrence of *Trichodesmium* blooms is the presence of gas vesicles that provide buoyancy and help to maintain the population in the high-irradiated upper water column. The formation of aggregates provides highest buoyancy, these so-called "puffs" are commonly found in surface blooms. Besides the capability to fix N_2 and the presence of gas vesicles, other factors involved in the success are the highly efficient photosynthetic apparatus and negligible losses by grazing. At periods of low wind, however, the buoyancy can induce the accumulation of an initially dispersed population at the surface, leading to massive blooms, the "red tides". These blooms

were shown to be toxic to zooplankton (Guo and Tester 1994, Hawser *et al* 1992) and can affect phytoplankton succession.

Species typically found in freshwater blooms are *Planktothrix* (Figure 2A), *Anabaena*, *Aphanizomenon* and *Microcystis* (Figure 2B). Like in the marine environment, surface blooms can appear within hours as a result of the upward migration of a pre-existing population that was initially dispersed in the water column. They are not the result of a sudden increase of the total population as assumed earlier (Reynolds 1971). When the cells are entrained in the water column by wind mixing, especially after a period of deep mixing, they become buoyant as a response to the lower irradiance. Under calm weather conditions however, when then the turbulence is reduced, the cells will appear at the water surface and accumulate there. Aggregated cells or filaments can form macroscopic colonies of red or green colour.

The thickness of a scum depends not only on the abundance of the population but also on the size of the cells and colonies. Following Stokes' law, those particles with greatest surface to volume ratios show the slowest floating and sinking velocities. Colony size is a key factor, as the floating and sinking velocity increases with the square of the radius. In fact, the combination of the production of gas vesicles and a big particle size, like for example in mucilage-bound colony forming species will increase the floating velocity. In contrast, single filaments of *Planktothrix* show only slow floating velocities. While *Microcystis* can have floating rates up to 250 m d^{-1} (Oliver and Ganf 2000), the maximum floating rate of *Planktothrix* is about 0.6 m d^{-1} (Walsby 2005).

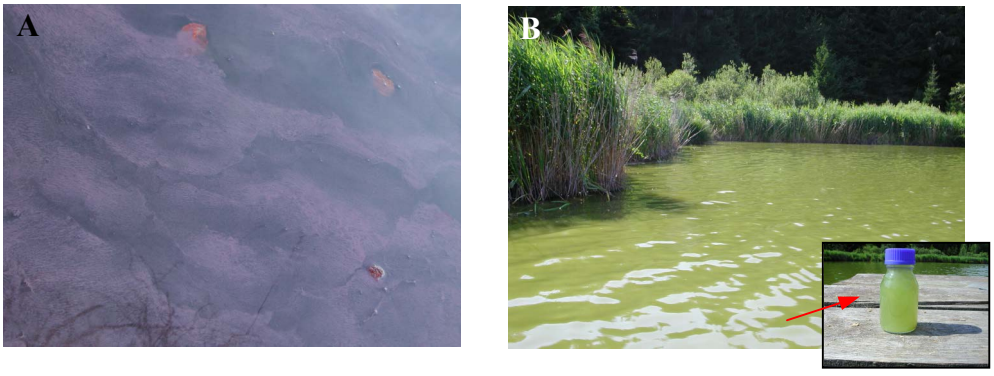

Figure 2

Surface bloom of *Planktothrix rubescens* in Mindelsee, Germany (**A**) and *Microcystis aeruginosa* in Deixelfurther Weiher, Germany (**B**).

1.2 Ecology of *Microcystis* spp.

The most commonly reported species among toxigenic cyanobacteria in lakes and reservoirs worldwide are of the genus *Microcystis* (Carmichael 1996). *Microcystis aeruginosa* is a very successful species distributed in both temperate and tropical regions and has been shown to develop dense blooms in eutrophic freshwaters worldwide. Due to the colony size, sinking and floating velocities are enhanced and dense surface accumulations of *Microcystis* can appear rapidly under calm weather conditions. In temperate regions *Microcystis* blooms usually occur in summer, in tropical and subtropical regions, however, they can persist throughout the year. According to Komárek and Anagnostodis (1998) the genus is characterized by great morphological variability with spherical to oval free-floating cells differing in size from 1 to 8.5 µm in diameter for different species. Cells can be sparsely or densely arranged in mucilage bound colonies. These colonies with irregularly agglomerated cells can be micro- or macroscopic in size and vary in morphology during the vegetation cycle. The vegetative stages of the cells always contain gas vesicles. *Microcystis* species are often classified by morphology-based taxonomy including characters like the shape of the colonies, mucilage structure, cell size and content of pigments. Due to the morphological variability populations often show an overlap in limiting criteria, so the taxonomic classification is difficult (Komárek and Komárková 2002).

Investigations of *Microcystis* populations by molecular approaches are independent of morphological characteristics and these methods are applied increasingly to detect the genetic variability within and between species. Haande *et al* (2007) showed that 24 *Microcystis aeruginosa* strains isolated from Uganda and Kenya varied in morphologic and genetic characteristics. While only four morphotypes were identified by the morphological approach, ten genotypes could be detected by investigation of the PC-IGS and the 16S-ITS regions. Van Gremberghe *et al* (2011) investigated the 16S-ITS regions of *Microcystis* populations in man-made reservoirs in Ethiopia and found low ITS diversity in general, however the local ITS diversity was lower than the regional diversity. A limited influence of the local environmental conditions on the genetic population structure was found, and in addition colonization dynamics with regard to priority effects were supposed to influence the *Microcystis* population structure.

Wilson *et al* (2006) reported physiological differences of *Microcystis aeruginosa* strains isolated from 12 lakes in Michigan, USA. The authors found the maximum population growth rate of strains to differ significantly among genotypes but not among lakes, furthermore variation in morphological characteristics of the strains was found. The surface areas of the colonies differed significantly among the lakes and the strains, while cell diameters differed among the strains only. Similar to van Gremberghe *et al* (2011) it was supposed that different strains are influenced differentially by local environmental conditions.

1.3 Ecology and Ecotypes of *Planktothrix* spp.

The filamentous cyanobacteria of the genus *Planktothrix* have been described to regularly form blooms in Europe and other parts of the world (Komárek and Anagnostidis 2005). *Planktothrix* filaments are always straight, and can be comprised of less than ten to hundreds of cells that may be millimeters long (Hayes *et al* 2002) and contain gas vacuoles for buoyancy regulation. Filaments are motile with positive phototaxis enabled by the secretion of mucilage and gliding via surface undulation of the cells (Häder 1987).

Two main species of *Planktothrix* occur in temperate climatic regions, which can be considered as different ecotypes (Mur *et al* 1993). The phycoerythrin-rich (high ratio of phycoerythrin to phycocyanin) and therefore reddish appearing ecotype *P. rubescens* (Figure 3A) has been described to occur mainly in Europe, but is also known from North America (Komárek 2003) and Canada (Nürnberg and LaZerte 2003). It commonly inhabits deep, physically stratified oligo- to mesotrophic lakes. The green-pigmented, phycocyanin-rich ecotype *P. agardhii* (Figure 3B) is known from temperate and tropical zones (Komárek 2003) and inhabits more shallow, eutrophic water bodies that are polymictic. According to Komárek (2003) trichomes of *P. rubescens* are usually between 6 – 8.2 µm wide, while filaments of *P. agardhii* have average trichome widths of 4 – 6 µm.

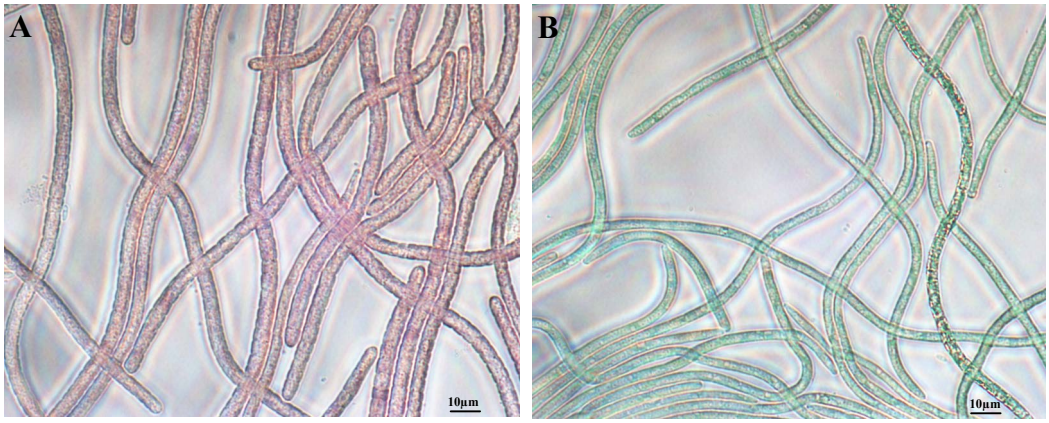

Figure 3

Filaments of *Planktothrix rubescens* (**A**) and *P. agardhii* (**B**) at magnification 400x.

Both ecotypes are known to grow under lowest light conditions, but *P. rubescens* was shown to grow at greater depths as its compensation light intensity for growth is lower compared to that of *P. agardhii* (Davis *et al* 2003). In addition, the green-pigmented ecotype was shown to be less sensitive to high irradiance when compared to the red-pigmented ecotype. From experimental data of differently pigmented strains, a combined effect of temperature and light quality was suggested to influence growth. While at lower temperatures (15°C) and green light the red-pigmented strain was more competitive, the green-pigmented strain was shown to have higher growth rates at higher temperatures (25°C) and was less specialized to light quality (Oberhaus *et al* 2007). The possible preference of green light and lower temperatures may explain the frequently observed dense layers of *P. rubescens* in the metalimnion (at 10-12m depth) during the thermal stratification period of lakes. When a lake is wind-mixed in autumn, filaments will be entrained in the hypolimnion and establish higher buoyancy that can lead to the upward flotation and surface accumulation during calm days (Walsby *et al* 2006).

It has been shown, that red- and green-pigmented *Planktothrix* ecotypes produce gas vesicles that differ in strength (Beard *et al* 2000). Gas vesicle strength is particularly important in deep lakes, where the high hydrostatic pressure during lake mixing may collapse gas vesicles and prevent filaments from rising to the euphotic zone after deep-mixing. The *gvpC* gene determines the strength of the gas vesicle as it stabilizes the outer surface of the gas vesicle (Hayes *et al* 1992). Genotypes synthesizing the longer variant $gvpC^{28}$, possessed gas vesicles that collapsed at a relatively low hydrostatic pressure, while genotypes containing also the shorter $gvpC^{20}$ variant

produced stronger gas vesicles. Among red-pigmented *Planktothrix* strains the strong gas vesicle type was found to be dominant, while among green-pigmented strains the weaker gas vesicles were more common. It was suggested that strains containing the stronger gas vesicle form were favored in deep lakes because of the higher resistance against hydrostatic pressure (Beard *et al* 2000, D'Alelio *et al* 2011).

Planktothrix blooms commonly consist of either the red- or the green-pigmented ecotype, however also mixed pigmented populations have been described e.g. from Norway (Rohrlack *et al* 2008) or the English Lake District (Davis *et al* 2003). Blooms can appear sporadically or persist all year round; even in winter dense accumulations of *Planktothrix* have been observed under the ice-cover (Skulberg 1964). Dense *Planktothrix* populations can dominate the phytoplankton community of a lake over decades, like it was shown for *P. rubescens* in Lake Zürich (Switzerland).
Even when the input of phosphorus, which was thought to be one of the most important nutrients controlling the proliferation of cyanobacteria was reduced, *P. rubescens* has been shown to persist or even increase in abundance for years, counteracting lake restoration efforts (Jacquet *et al* 2005, Posch *et al* 2012). The authors provided several explanations for the observed phenomenon, most importantly i) the reduction of available phosphorus leads to the decrease of other phytoplankton species, i.e. green algae and thus improves the light regime in the water column, while *P. rubescens* is less nutrient limited during stratification and can take advantage of the improved light quality and ii) elevated temperatures enhance stratification and elongate the stratification period of lakes and furthermore reduce holomixis, which leads to optimum growth conditions for *Planktothrix rubescens*.

Surface accumulations of cyanobacteria like *Planktothrix* deteriorate the recreational and economic value of lakes by causing taste and odor problems but more importantly, due to the high concentration of toxins that are usually found in those dense blooms (Fastner *et al* 1999). When these lakes or reservoirs are used as a drinking water supply, recurrent cyanobacteria blooms can pose a serious health risk to the population.

2. Cyanotoxins

Cyanobacteria are known to produce a wide range of bioactive compounds that have in part been correlated with animal and human poisonings or have potential for pharmaceutical use. Some of the compounds also act on viruses or other bacteria. High concentrations of toxins are commonly found among bloom-forming species of cyanobacteria, i.e. in blooms and scums of planktonic species or mats and biofilms of benthic and littoral species (Codd *et al* 2005). The term toxic is rather context specific and may refer to the effects on human and livestock. Commonly the toxicity (µg kg^{-1} body weight) is defined by mammalian or vertebrate test systems. Other compounds synthesized by cyanobacteria are harmless to mammals but have toxic effects e.g. on invertebrates. Oscillapeptin J for example, which is produced by *Planktothrix rubescens*, showed toxicity to crustaceans, but no effect on mice was observed (Blom *et al* 2006).

More than 600 peptides or compounds with peptidic substructure have been described from various taxa (Welker and von Döhren 2006). Toxins can be grouped according to the symptoms and organs, tissues or cells that are primarily affected: neurotoxins, hepatotoxins, cytotoxins and irritants including gastrointestinal toxins (Codd *et al* 2005).

2.1 Neurotoxins: Anatoxin-a, Homoanatoxin-a, Anatoxin-a(S) and Saxitoxins

These neurotoxins are alkaloids, a group of heterocyclic compounds containing ring structures with at least one carbon-nitrogen bond. They are diverse in their chemical structure and show differential toxicities in mammals.

Anatoxin-a (Figure 4A), a secondary amine, (2-acetyl-9-azabicyclo(4-2-1)non-2-ene) has been isolated from planktonic species of *Anabaena*, *Aphanizomenon*, *Planktothrix* and *Raphidiopsis* but also from mat-forming *Oscillatoria* and *Phormidium*. Homoanatoxin-a is the methylated variant of anatoxin-a and has a similar toxicity. The binding of anatoxin-a to neuronal nicotinic acetylcholine receptors leads to continued propagation of neural pulses by the neurons causing nerve depolarisation, as the toxin cannot be removed by the acetylcholine esterase. It can quickly lead to paralysis and death by respiratory arrest (Carmichael 1994, 1997).

Anatoxin-a(S) (Figure 4B) inhibits the enzyme acetylcholine esterase from releasing acetylcholine from neurons and therefore leads to symptoms similar to intoxication by anatoxin-a. So far, it has only been described from strains of *Anabaena flos-aquae*

and *A. lemmermannii* (Matsunga *et al* 1989). The suffix "S" was added because of the observed hypersalivation and lachrymation in mammals.

Saxitoxins are a group of >30 carbamate alkaloid neurotoxins that can either be singly or doubly sulphated or non-sulphated (Figure 4C). These highly potent toxins (0.5-1 mg is the lethal dose for an adult human) were originally described as the cause of paralytic shellfish poisoning. The consumption of shellfish that was contaminated, as it filter-feeds on eukaryotic algae (dinoflagellates) producing the toxin, lead to human illness and deaths (Kao 1993). Saxitoxin structural variants have been isolated from *Anabaena*, *Aphanizomenon*, *Planktothrix*, *Cylindrospermopsis* and *Lyngbya* genera. Saxitoxins block voltage-gated sodium channels of the excitable membranes of nerves and inhibit impulse generation in nerves and muscle fibres. This results in paralysis and death by respiratory arrest within minutes. Saxitoxins are classified as chemical weapons (Metcalf and Codd 2009).

Hepatotoxins: Microcystins and Nodularins

The hepatotoxic MCs are the most frequently found toxins in cyanobacterial freshwater blooms. Globally, the most important producers are the planktonic genera *Microcystis*, *Planktothrix* and *Anabaena*, however also *Nostoc* and *Anabaenopsis* (Sivonen and Jones 1999) as well as the mat-forming species *Oscillatoria limnosa* and *Phormidium konstantinosum,* marine *Synechococcus* (Carmichael and Li 2006) and the terrestrial *Hapalosiphon* (Prinsep *et al* 1992) were shown to produce MCs. Microcystins share the common structure cyclo(-D-alanine^{1}-X^{2}-D-MeAsp^{3}-Y^{4}-Adda^{5}-D-glutamate^{6}-Mdha^{7}), where X and Y are variable L-amino acids, D-MeAsp^{3} is D-erythro-ß-methyl aspartic acid, Adda is a novel ß-amino acid (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4) and Mdha is N-methyldehydroalanine (Figure 4D). The positions 2 and 4 (X and Y) of the molecule show high variability compared to the other positions, which is also the basis for the nomenclature (Carmichael *et al* 1988); e.g., MC-LR refers to L-leucine and L-arginine in the variable amino acid positions 2 and 4 of the peptide. The positions 3 and/or 7 can be subjected to demethylation. In most MC variants, a N-methylated variant of dehydroalanine (Dha), N-methyldehydroalanine (Mdha), is found in position 7, or alternatively the unmethylated Dha. Both variants originate from dehydration of serine. Analogously a threonine derivative, 2-amino-2-butenoic acid (Dhb), is commonly found in this position. While N-methylated Dha^{7} MC variants (Mdha^{7}) are well known, N-

methylated Dhb[7] MCs have only been described from *Nodularia*. Almost 90 structural variants of these cyclic heptapeptides have already been described from different genera (Welker and van Döhren 2006).

The cyclic pentapeptides nodularins share the structure cyclo-(D-MeAsp[1]-Z[2]-Adda[3]-D-glutamate[4]-Mdhb[5]), where Mdhb is a methylated Dhb moiety, 2-(methylamino)-2-dehydrobutyric acid (Figure 4E). These peptides show in general less structural variability than MCs and have only been characterized from *Nodularia* (Sivonen and Jones 1999).

Most of the MC and nodularin structural variants are highly toxic to mammals, however a differential toxicity of MC structural variants was shown (Feurstein *et al* 2009). Both MC and nodularin uptake occurs via an organic anion transporting polypeptide (OATP) system into liver cells (Runnegar *et al* 1991). In case of an acute intoxication, the macrostructure of the liver is destroyed and the majority of the blood is retained in the liver, so the supply necessary for other organs to remain viable is insufficient (haemorrhage in the liver). Furthermore both peptides are potent liver and colon cancer tumour promoters and inhibit the serine and threonine specific eukaryotic protein phosphatases PP1 and PP2A participating in the control of a wide range of cellular processes (MacKintosh *et al* 1990). The LD_{50} (intraperitoneal in mice) of MC-LR varies from 25-150 µg kg^{-1} body weight (Kuiper-Goodman *et al* 1999).

From countries all over the world reports of human and animal poisonings after exposure to MCs lead to public awareness repeatedly. Some severe incidents with different exposure routes include e.g. a gastro-enteritis epidemic after the flooding of Itaparica Dam Reservoir (Brazil) in 1988 that served as a drinking water reservoir, leaving 88 of 2000 affected people dead within 42 days. The cause was probably an extremely high concentration of *Microcystis* and *Anabaena*, which was detected in the water. In the UK, in 1989, ten out of 20 army recruits became ill (diarrhoea, vomiting, blistering of the lips) after swimming in water with a bloom of *Microcystis* sp.; two developed severe pneumonia due to aspiration of the toxin (Turner *et al* 1990). After haemodialysis treatment in a hospital in Brazil, 117 patients developed severe signs of intoxication and 50 of the 136 patients treated by dialysis died. The examination of the fatal incident revealed MCs in the water used for dialysis (Jochimsen *et al* 1998).

In 1997 the WHO published a guideline value of a maximum concentration of 1 µg l^{-1} for MC-LR in drinking water (WHO 1997).

Cytotoxins: Cylindrospermopsin

Cylindrospermopsin (Figure 4F) is a cyclic guanidine alkaloid of which three structural variants have been identified: cylindrospermopsin and 7-epicylindrospermopsin, that have similar toxicities, and deoxycylindrospermopsin, which is considered nontoxic (Norris *et al* 1999). Originally identified from *Cylindrospermopsis raciborskii* of tropical and subtropical climatic regions, it was also detected in *Raphidiopsis curvata* and *Umezakia natans*, but it is also produced by *Anabaena* species and *Aphanizomenon ovalisporum* from temperate climatic regions. Cylindrospermopsin causes multiple organ damage; it mainly affects the liver but also the kidneys, spleen, thymus and heart (Falconer and Humpage 2005). It inhibits protein synthesis and may also be carcinogen (Humpage 2008). The LD_{50} of this toxin is 200-2100 μg kg^{-1} body weight (i.p. mouse).

Irritants and Gastrointestinal Toxins: Aplysiatoxins, Lyngbyatoxins and Lipopolysaccharides (LPS)

Aplysiatoxin (Figure 4G) and debromaplysiatoxin are phenolic bislactones produced by *Lyngbya majuscula, Schizothrix calcicola* and *Oscillatoria nigroviridis*, respectively. They cause severe dermatitis with blistering, erythema and desquamation after skin contact e.g. by swimming. The symptoms are also known as "Swimmers' Itch". Lyngbytoxins (Figure 4H) are indole alkaloid compounds produced by *Lyngbya majuscula* and are similar in toxicity to aplysiatoxins. These three cyanobacterial toxins are tumor promoting (Smith *et al* 2008).

Lipopolysaccharides consist of a core oligosaccharide, an outermost O-polysaccharide component and an innermost lipid A region. The fatty acid lipid A component is primarily the cause of allergenic responses like hypertension, inflammatory response, vomiting and diarrhoea in humans, as the cyanobacterial cell wall, like the cell wall of all gram negative bacteria, contains lipopolysaccharides as an integral component. Compared to LPS of other bacteria, e.g. *Salmonella*, cyanobacterial LPS are considered to be less toxic (Martin *et al* 1989).

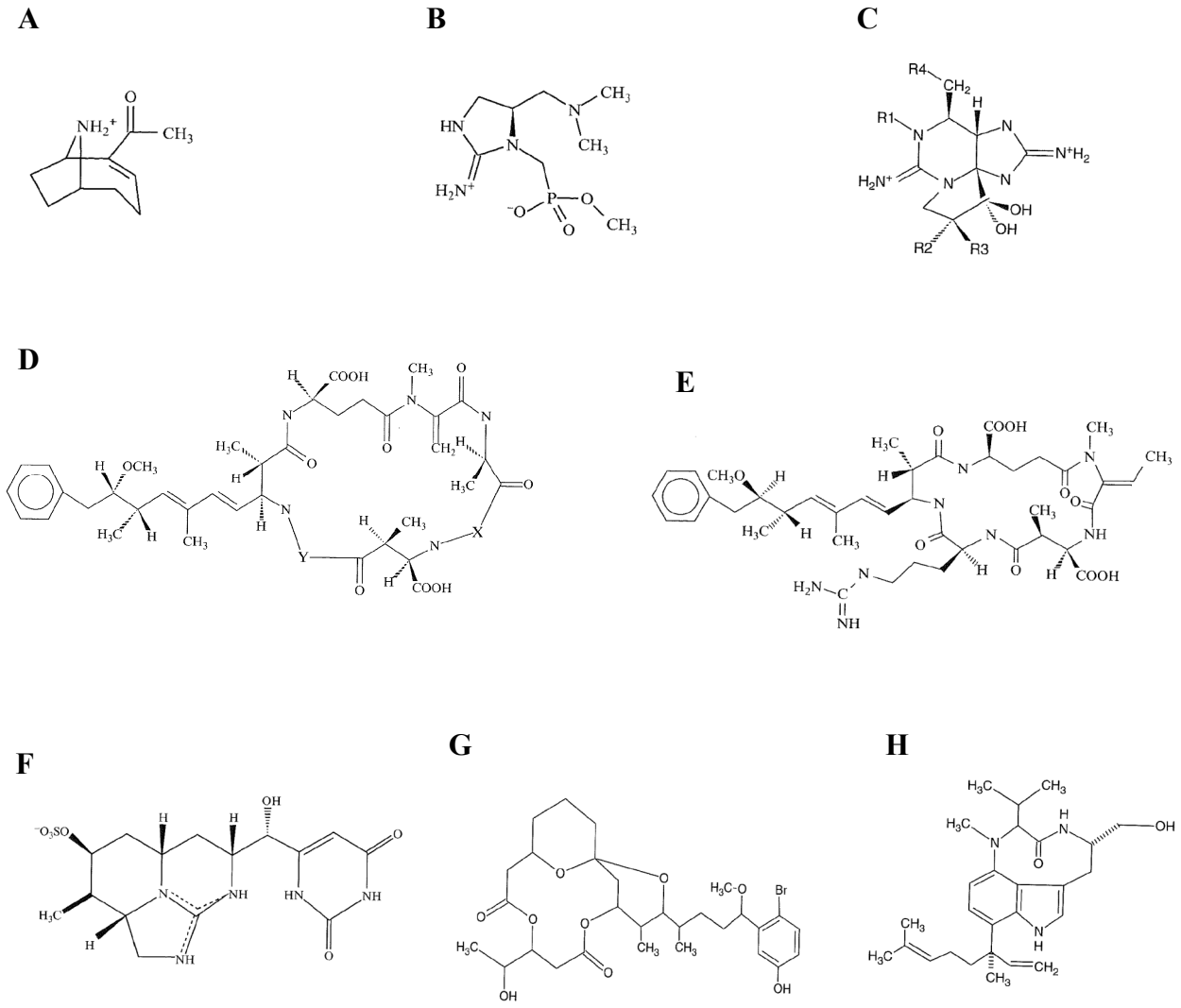

Figure 4

General structure of representatives of major cyanotoxins: **A**, anatoxin-a, **B**; anatoxin-a(S); **C**, saxitoxin; **D**, microcystin; **E**, nodularin; **F**, cylindrospermopsin; **G**, aplysiatoxin; **H**, lyngbyatoxin A, reprinted from Metcalf and Codd (2012), with kind permission from Springer Science+Business Media B.V.

2.2 Toxin Synthesis

The majority of cyanobacterial secondary metabolites are synthesized by giant multi-domain enzyme complexes, the nonribosomal peptide synthetases (NRPS), which can be mixed with polyketide synthase (PKS) pathways. In contrast to the ribosomal peptide synthesis, NRPS operates autonomous (Finking and Marahiel 2004). NRPS are built up modular, with each module being responsible for the incorporation of a single amino acid.

Generally, the order of the modules corresponds to the order of amino acids in the final product (colinearity rule). A module consists of catalytic domains with a minimal module consisting of an amino acid activation domain (A domain), a peptidyl carrier domain (PCP domain, also called T domain) that carries the phosphopantetheine cofactor and a condensation domain (C domain). The A domain is the main factor for specificity by substrate recognition and catalyzation of the activation of specific amino acids because the geometric shape of the binding pocket in the enzyme only allows a certain amino acid to enter the catalytic site. The PCP domain is the 4'-phosphopantethein cofactor-binding site; all substrates of the growing peptide are covalently bound there. The C domain catalyzes the formation of a peptide bond between two intermediates that are PCP bound. Furthermore NRPS modules can contain additional domains for substrate modifications, e.g. epimerization, N-methylation and heterocyclization domains (Marahiel and Essen 2009).

The PKS systems are also built up modular but in contrast to NRPS, small carboxylic acids are activated and assembled.

2.3 The MC Synthetase Gene Cluster

The MC synthetase gene cluster has been sequenced from three different genera of cyanobacteria: *Microcystis* (Tillett *et al* 2000), *Planktothrix* (Christiansen *et al* 2003) and *Anabaena* (Rouhianinen *et al* 2004), and additionally the closely related nodularin synthetase gene cluster from *Nodularia* has been elucidated (Moffit and Neilan 2004). Inactivation of the *mcy* gene cluster by insertional mutagenesis revealed its involvement in MC synthesis and additionally, the production of several structural variants by the same *mcy* gene cluster was demonstrated by this approach (Dittmann *et al* 1997).

The individual *mcy* gene clusters have been found similar, yet the organization of the clusters and the arrangement of genes were found to differ. In *Microcystis* and *Planktothrix* the gene cluster spans 55kb, and is comprised of 10 and 9 genes, respectively. The modules *mcy*A, B and C encode five NRPS modules, *mcy*D encodes two PKS modules and *mcy*E and *mcy*G encode hybrid NRPS/PKS modules. The genes *mcy*F, *mcy*H, *mcy*I and *mcy*J encode a racemase, an ABC transporter, a dehydrogenase and an O-methyltransferase, respectively. The *mcy* gene cluster in *Planktothrix* does not contain the genes *mcy*F and *mcy*I but it encodes an additional

thioesterase, *mcy*T. The *nda* gene cluster encodes 9 genes and spans 48kb. Two of the NRPS modules are deleted and the remaining have been fused to a bimodular NRPS instead of the *mcy*A and *mcy*B genes. Additionally, a change in the substrate binding pockets was observed. The deletion event and the change in substrate specificity point towards the evolution of the *nda* gene cluster from the *mcy* gene cluster (Moffit and Neilan 2004).
Except for *mcy*T, all *mcy* genes in *Planktothrix* are transcribed unidirectionally from a promotor located upstream of *mcy*D. In contrast, the gene clusters in the three other genera are transcribed from a central bi-directional promotor region. Only the genes of the *nda* gene cluster and the *mcy* gene cluster of *Anabaena* follow the common colinearity rule of NRPS gene clusters, that is, the order of modules are in agreement with the amino acid sequence in the final peptide.

The sequencing of the *mcy* and *nda* gene clusters and the elucidation of the correlation between different genotypes and the synthesis of specific MC structural variants enabled the detection and quantification of potential toxin producers in dependence on environmental factors in the field using molecular techniques.

2.4 Function of MC

In general, MCs are considered to be secondary metabolites, i.e. compounds that are not used for the primary metabolism (e.g. cell division or metabolism) of an organism (Carmichael 1992). The impact of environmental conditions on toxin production revealed that MC production by a single strain is rather consistent with a variation around a factor of four, while more pronounced differences between strains were observed (Sivonen and Jones 1999). Orr and Jones (1998) supposed that there is a linear correlation between cell division and toxin production, and according to their theory environmental conditions influence toxin production mostly indirectly by the effects on the cell division rate. They concluded that MC is not a secondary metabolite according to the established criteria but rather displays the attributes of an essential intracellular compound. This conclusion is however at odds with the observation of strains lacking MCs.

As MCs are very potent toxins, their function as feeding deterrents against herbivorous zooplankton has been investigated. Many studies have confirmed the

detrimental effects of MC on zooplankton (e.g. Rohrlack *et al* 1999, Blom *et al* 2001), however the primary function of MC as a defense against eukaryotic grazers remains questionable. Rantala *et al* (2004) showed that the *mcy* genes are ancient and were already present in the last common ancestor of a large number of cyanobacteria (see below). The frequently used argument that MC-producing cyanobacteria evolved before their potential predators is still a controversial issue and the early role of MC as a feeding deterrent remained unclear.

Allelopathic effects of MCs e.g. concerning the reduction of photosynthetic activity or the growth rate of phototrophic organisms have been discussed, but a general ability of MCs to act as allelopathic compounds is considered to be unlikely (Babica *et al* 2006). The authors argued that only a limited number of studies showed detrimental effects of MCs in concentrations that are typically found in the environment. Schatz *et al* (2007) proposed that MCs function as intraspecific info-chemicals and found that the release of MCs by lysed cells lead to enhanced toxin production by the remaining cells possibly to increase their fitness.

Further the role of MC as a metal chelator has been discussed and MCs were shown to have the ability to bind copper and zinc (Humble *et al* 1997). Additionally it was suggested that MCs chelate intracellular Fe, thus providing a protective function or serve for Fe storage (Utkilen and Gjølme 1992).

Recent studies also indicate an intracellular function of MC, e.g Zilliges *et al* (2011) showed the covalent binding of MC to proteins involved in redox and carbon metabolism. The binding was found to be enhanced under high-light and oxidative stress conditions implying an effect on the stability and activity of proteins. Similarly Alexova *et al* (2011) showed the differential expression of proteins associated with carbon-nitrogen metabolism and redox maintenance in toxic and nontoxic strains of *Microcystis*.

2.5 Regulation of MC Production by Environmental Factors

The variation in the toxicity of a cyanobacterial bloom can depend on the composition and proliferation of MC-producing and nontoxic genotypes, on variations in genotypes encoding the synthesis of different MC structural variants or the physiological state of the cells. Numerous studies have investigated the influence of environmental parameters on MC production of cyanobacterial strains. High MC production has been correlated with low nitrogen concentrations for *Microcystis*

(Long *et al* 2001), and intermediate temperature (25°C) and low light intensity (Sivonen 1990) for *Planktothrix*. The total MC production rate in a *P. agardhii* strain was found to increase with light intensity and simultaneously a change in production of MC structural variants was observed (Tonk *et al* 2005).

In P-limited cultures of *Microcystis* an increased MC content per dry weight was found (Oh *et al* 2000) and higher MC production was observed under the deficiency of inorganic carbon (Jähnichen *et al* 2007).

As it was proposed earlier, the MC production rate seems to be positively correlated with cell growth, which is in turn influenced by environmental parameters. Very little is known however about the parameters that differentially influence the growth of toxic and nontoxic strains. Under high nutrient levels, toxic strains of *Microcystis* grew faster than nontoxic strains (Vézie *et al* 2002) and nontoxic strains were shown to outgrow toxic strains in light-limited chemostats (Kaardinal *et al* 2007a). In competition experiments using *Planktothrix* strains, toxic strains were at an advantage over nontoxic strains at growth-limiting conditions, i.e. due to low light intensity, low temperature and N-deficiency (Briand *et al* 2008a).

The investigation of strains in the laboratory may be biased due to physiological differences between strains. A few studies have investigated the competitiveness of a wild type strain and its mutant impaired in toxin production, e.g. Van de Waal *et al* (2011) reported a selective advantage of the wild type at low levels of CO_2 whereas the nontoxic strain dominated under light-limited and high CO_2 conditions.

Yet, with the limited number of available strains for this approach it is difficult to draw conclusions regarding the diverse composition of genotypes in the field.

3. Evolution of Genes Encoding MC Synthesis

Investigations on the distribution of MC synthesis genes showed that not all genera of cyanobacteria can produce MCs and that within one species MC-producing strains often co-exist with strains that do not have the ability to produce MC. It was suggested that horizontal gene transfer was involved in the distribution of the *mcy* genes. For example, a functional type VI pilus system was detected in *Microcystis*, which is known to be involved in DNA uptake via lateral gene transfer in many bacteria and its possible role in the uptake of the *mcy* gene cluster has been discussed (Nakasugi *et al* 2007).

The sequencing and phylogenetic analysis of biosynthetic genclusters encoding toxin synthesis from different genera of cyanobacteria allowed to draw conclusions about the evolutionary history as well as about ongoing diversification processes.

The phylogenetic comparison of data sets comprised of genes involved in primary metabolism and the genes directly involved in MC and nodularin synthesis showed congruency, which points to a phylogenetic history revealed by housekeeping and *mcy* genes (Rantala *et al* 2004). The authors suggested the existence of a common ancestor of the cyanobacteria containing the *mcy* gene cluster and that the absence of the gene cluster among different cyanobacterial genera was due to repeated loss events, while other genera retained the *mcy* gene cluster. This is also reflected in the patchy distribution of the *mcy* gene cluster among different genera. Furthermore, the patchy distribution among single strains of a cyanobacterial species also reflects those loss processes, however, on a much shorter evolutionary time scale (Christiansen *et al* 2008a). These results are in contrast with the earlier hypothesis that plasmid-mediated horizontal gene transfer was involved in the distribution of the *mcy* gene cluster.

3.1 Structural Changes of the *mcy* Genes in *Planktothrix*

In *Planktothrix*, the *mcy* gene cluster is comprised of nine genes arranged in a 55kb gene cluster encoding peptide synthetases, polyketide synthases and modifying enzymes (Figure 5A, Christiansen *et al* 2003). The adenylation domains of *mcy*A, B and C are responsible for the activation of amino acids that will be incorporated in the MC molecule in position 7 and 1, 2 and 3, and 4, respectively (Figure 5B). In position 7 of the MC molecule three different amino acid residues activated by the first adenylation domain of *mcy*A (*mcy*AAd1) have been described: dehydroalanine, dehydrobutyric acid and serine (Luukkainen *et al* 1993, Sano and Kaya 1995).

Two distinctive *mcy*AAd1 genotypes were reported from *Planktothrix*, one with the insertion of an N-methyltransferase domain that could be correlated with the synthesis of N-methyldehydroalanine in position 7 of the molecule ($Mdha^7$), while the other genotype was correlated with the synthesis of dehydrobutyric acid (Dhb^7) at the same position (Kurmayer *et al* 2005). $Mdha^7$ is derived from activation and dehydration of L-serine, while Dhb^7 derives from activation of L-threonine, which is then dehydrated.

In position 2 of the MC molecule three different amino acids activated by the first adenylation domain of *mcy*B (*mcy*BAd1) have been described: leucine (Leu), arginine

(Arg) and homotyrosine (Hty), (Luukkainen *et al* 1993, Henriksen and Moestrup 1997, Kurmayer *et al* 2005). It was observed that the same *Planktothrix* isolate can produce two different MC structural variants (with either Leu or Hty in position 2) and it was proposed that the *mcy*BAd1 domain can activate two chemically different amino acids, probably due to a relaxed substrate specificity (Christiansen *et al* 2008b). Genotypes that produce MC variants with either Leu or Arg in position 2 or either Hty or Leu could be identified by analysis of the *mcy*BAd1 domain (Kurmayer and Gumpenberger 2006).

Within the A-domain of the *mcy*C gene, five nucleotide substitutions were shown to correlate with a replacement of Arg in position 4 of the MC molecule by Hty or tyrosine (Tyr) in the same position. Two of these substitutions were located within *mcy*CA resulting in the change of one amino acid only (Christiansen *et al* 2008b). The *m*cyC gene generally shows very little sequence variation but high diversity to *mcy*B, possibly preventing homologous recombination (Tooming-Klunderud *et al* 2008).

The modular structure of NRPS is thought to promote intragenic and intergenic recombination enabling the evolution of new genetic variants and peptides or peptide variants.

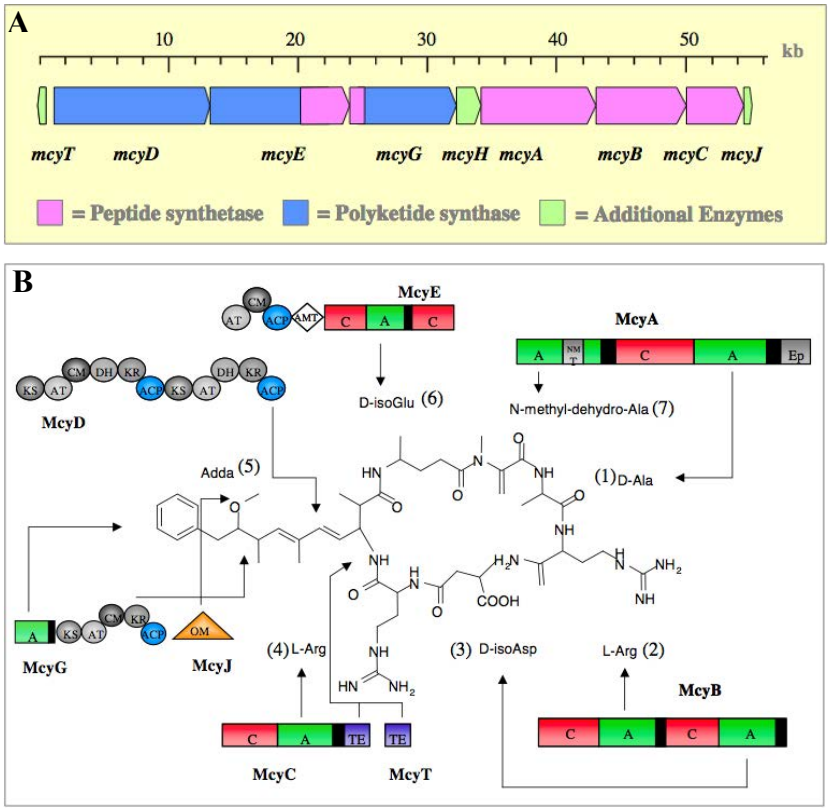

Figure 5

A, Organization of the *mcy* gene cluster in *Planktothrix agardhii*. Nonribosomal peptide synthases in pink, polyketide synthases in blue and additional enzymes in green (from Christiansen 2002).

B, Role of *mcy* proteins: PKS domains: AT, acteyltransferase; ACP acyl carrier protein; KS β-ketoacyl synthase; KR ketoacyl reductase; DH dehydratase; CM C-methyltransferase; AMT aminotransferase; NRPS domains: A aminoacyl adenylation; C condensation; NMT N-methyltransferase; Ep epimerase; Te thioesterase; *Mcy*F racemase; *Mcy*J (OM) O-methyltransferase. Black bars represent thiolation motif of NRPS modules; arrows indicate the assignment of individual proteins to steps of MC biosynthesis, reprinted from Börner and Dittmann (2005), with kind permission from Springer Science+Business Media B.V.

3.2 Inactive MC Genotypes

Several studies on strains of *Microcystis* showed the presence of *mcy* genes but a lack of detectable MCs (Nishizawa *et al* 1999, Tillett *et al* 2001). The reason for the lack of MCs was unclear and it was proposed that mutations that possibly occurred during cultivation inactivated the *mcy* gene cluster (Kaebernick *et al* 2001). Following these observations, also cyanobacterial field populations were investigated and it could be shown that populations of a cyanobacterial species are commonly comprised of genotypes that are MC producers and genotypes that lost the ability to produce MC due to insertions or deletions within the *mcy* gene cluster.

Christiansen *et al* (2008a) reported the lack of *mcy* genes among strains of *Planktothrix*. The authors showed that the flanking regions of the gene clusters from different strains occurred with remnants of the *mcy* genes that were flanked by copies of the same transposable element. They anticipated that site-specific recombination between the two copies of the mobile element at both ends of the *mcy* gene cluster lead to the gene loss in a 2-step process. In a first step the *mcy* gene cluster would be inactivated and lost by site-specific recombination in a second step.

Similarly (Fewer *et al* 2011) reported the inactivation of the *mcy* genes from *Anabaena* by the insertion of a transposable element. Christiansen *et al* (2006) identified several deletions and insertions in strains of *Planktothrix rubescens* as a cause for the inactivation of MC biosynthesis. Specifically, deletions in *mcy*B (400 bp) and within *mcy*HA (1,869 bp) and three insertions (1,429 bp in *mcy*D, 1,433 bp within *mcy*EG and 1,433 bp in *mcy*A) of identical transposable elements in different genes and transcribed in opposite directions were identified in different strains (Figure 6). The authors considered the inactivation of the *mcy* gene cluster as an intermediate step in the rearrangement of genes, resulting in novel structural variants, as discussed above.

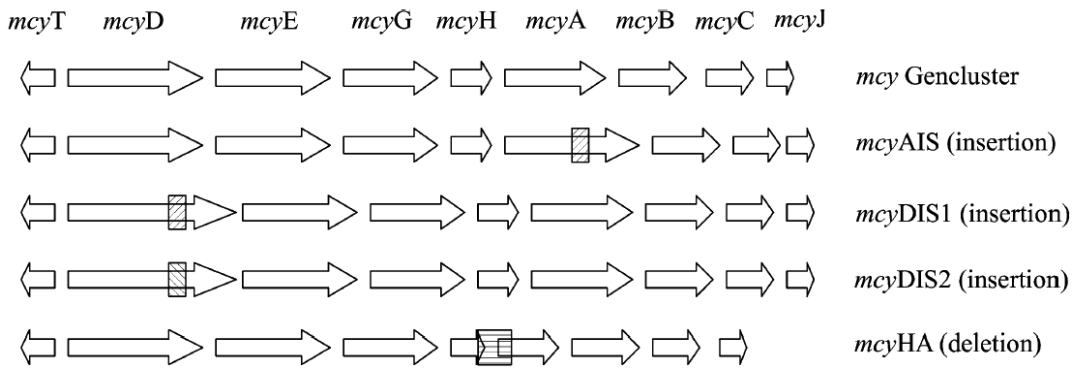

Figure 6

The *mcy* gene cluster of *Planktothrix* and locations of mutations resulting in the inactivation of MC biosynthesis. The insertions *mcy*AIS, *mcy*DIS1, *mcy*DIS2 are caused by a transposable (IS) element into *mcy*A and *mcy*D of the *mcy* gene cluster and are transcribed in both directions. The deletion *mcy*HA affects parts of the *mcy*H and *mcy*A genes, reprinted from Ostermaier and Kurmayer (2009), with kind permission from Springer Science+Business Media B.V.

4. Detection of *mcy* Genotypes by Quantitative Real-Time PCR

For a long time cyanobacterial assemblages and blooms were quantified by traditional light microscopy with counting of cells to calculate the biovolume present in a water sample. However, MC-producing genotypes typically cannot be distinguished from nontoxic genotypes by microscopy, and likewise genotypes producing different structural variants of toxins can also not be differentiated by this approach. It was observed that many lakes show large seasonal variation in MC content (Sivonen and Jones 1999) and it was unclear whether environmental factors lead to altered MC production rates or whether this was due to changing proportions of MC-producing and nontoxic genotypes within a population. PCR based methods with primers targeted to the *mcy* genes of *Microcystis* or *Planktothrix* were developed to differentiate MC-producing from nontoxic strains both in the laboratory and in the field (Kurmayer *et al* 2002, 2004, Pan *et al* 2002). These methods were suitable for qualitative or semiquantitative results, for example to be obtained from individual colonies or filaments.

The introduction of quantitative real-time PCR (qPCR) to quantify toxin-producing cyanobacteria in environmental water samples opened up a new possibility for rapid and sensitive monitoring of cyanobacteria (Foulds *et al* 2002). Primers and probes for the relative and absolute quantification of toxin-producing genotypes were developed to monitor the succession of MC-producing genera or the wax and wane of a genotype in relation to the total population (Vaitomaa *et al* 2003, Kurmayer and Kutzenberger 2003). Commonly conserved domains of the *mcy* gene cluster are targeted and related to the total population that is quantified via the 16S rDNA locus.

A possible limitation of this approach might be the overestimation of potential toxicity in populations that contain genotypes with insertions or deletions within *mcy* genes that hinder toxin syntheses but are not targeted by qPCR. Yet, the discovery of specific *mcy* mutants and the elucidation of the affected loci enabled the direct targeting of inactive *mcy* genotypes in the field by PCR (e.g. Christiansen *et al* 2006) and thus the opportunity to observe their growth also quantitatively by qPCR. qPCR offers a highly sensitive tool to monitor the dynamics of cyanobacterial (sub-)populations in the field that can be related to environmental parameters and it provides the possibility of early warning of toxic bloom formation.

4.1 Variability of *mcy* Genotypes in the Field

The knowledge about the occurrence of different *mcy* genotypes in the same population lead to several studies investigating the composition and changes in abundance of MC-producing and nontoxic genotypes to gain knowledge about the environmental factors influencing these dynamics. Briand *et al* (2008b) followed the dynamics of a potentially MC-producing genotype in a *P. agardhii* bloom over the course of two years by qPCR. The authors found the percentage of the MC-producing genotype to be high and variable (from 31 to 83%) and reported a negative correlation between this genotype and the *P. agardhii* cell density, implying a competitive advantage for inactive genotypes under favorable growth conditions. Kurmayer *et al* (2011) investigated the *Planktothrix* populations in 23 European lakes and found the proportion of the MC genotype to differ spatially between populations but independently from population density. Furthermore this study showed a higher proportion of nontoxic genotypes among green-pigmented populations occurring in shallow lakes when compared to red-pigmented populations from deep, stratified lakes.

Despite the fact that higher MC contents per µg dry weight are commonly found among *Planktothrix* blooms when compared to blooms of *Microcystis* (Fastner *et al* 1999) and that *Planktothrix* has been shown to dominate phytoplankton communities for years or decades (Jacquet *et al* 2005, Posch *et al* 2012) only few studies investigated the dynamics of genotypes.

A positive correlation between the relative abundance of MC-producing genotypes and higher nitrate concentrations were reported for *Microcystis* (Yoshida *et al* 2007, Ha *et al* 2009). Yeh *et al* (2009) observed the highest abundance of the MC-producing genotype during bloom development while Briand *et al* (2009) found the MC producing genotype to be dominant before and after a bloom, but under environmental conditions favorable for *Microcystis* growth the nontoxic genotype was favored.

Also differences in the succession of MC-producing and nontoxic genotypes were observed between a deep, stratified lake and mixed lakes: while in the stratified lake a genotype shift from toxic at the beginning of a bloom to nontoxic at the end of the bloom was observed, toxic genotypes occurring in the unstratified lake dominated throughout the bloom (Kaardinal *et al* 2007b). In contrast Kurmayer and

Kutzenberger (2003) found the proportion of the MC-producing genotype to be rather low and stable in a shallow lake from winter to summer. Sabart *et al* (2010) investigated the proportion of the potentially MC-producing genotype in *Microcystis* populations in close geographic proximity along the River Loire. In general, only little variation was observed, with the MC genotype predominating in all populations except for one, which was always dominated by nontoxic genotypes. These differences in toxin genotype abundance in more or less connected habitats point to the importance of local environmental parameters not directly linked to MC production that regulate genotype composition.

These contrasting results point out the need for further investigations on the effect of environmental factors on subpopulation dynamics. It has been hypothesized that climate warming could in general lead to the dominance of toxic genotypes in cyanobacterial blooms, e.g. by increasing growth rates of toxic strains or higher transcription rates (Davis *et al* 2009).

Studies investigating the occurrence and distribution of genotypes in the field often cover a limited period only, and little is known about the long-term development in genotype composition, especially with regard to the competitive ability of nontoxic genotypes.

4.2 Genetic Regulation of MC Production

It was supposed earlier, that MC production could be regulated on the cellular level, i.e. that environmental factors promote the enhanced transcription of *mcy* genes and therefore lead to increased MC production and concentrations.

Wood and colleagues (2011) showed significant changes in up- and downregulation of the *mcy*E gene transcript that were correlated to changes in total intracellular MC per cell. A 462-fold variation of *mcy*E gene expression was observed within 12 hours in Lake Rotorua (New Zealand) coinciding with a 30-fold increase in *Microcystis* cell concentration. The authors suggested that the MC production was influenced by changes in the *Microcystis* cell concentrations. Kaebernick *et al* (2000) observed increased transcript levels of both *mcy*B and *mcy*D genes in *Microcystis* under high light intensities. Based on these findings, Kaebernick *et al* (2002) reported the presence of two alternative transcriptional start sites in *Microcystis* for the operons *mcy*A and *mcy*D, and *mcy*D-J under high light and low light intensities, respectively. The promotor region between *mcy*A and *mcy*D contains motifs for transcription factor

binding sites, i.e. the global nitrogen regulator (NtcA) and ferric uptake regulator (Fur) implying a role of nitrogen and iron in the regulation of MC synthesis. While increased transcription of *mcy*D was correlated to an increase in MC levels due to iron starvation (Sevilla *et al* 2008), the effect of nitrogen on MC levels is unclear. Sevilla *et al* (2010) found that excess nitrogen promotes cell growth, however no direct influence on *mcy*D gene expression and MC production was observed.
As it was shown that NtcA expression is influenced by the cellular redox state (Alfonso *et al* 2001), it was proposed that binding of NtcA to the *mcy* promotor may regulate *mcy* transcription as a response to the redox status rather than to nitrogen availability (Kaplan *et al* 2012).

These results only partly support the conclusion of Orr and Jones (1998) that environmental parameters influence the net production of MC indirectly via the growth rate of the cells. MC concentrations are found to vary considerably in a habitat and the prediction of the MC production of all genotypes within a cyanobacterial bloom is difficult. A differential influence of environmental parameters on subpopulation dynamics cannot be excluded, yet several studies showed the correlation between toxic genotype abundance and the corresponding toxin concentrations in water, implying a rather stable synthesis that is related to the growth rate. For *Microcystis* in Lake Wannsee (Germany), Kurmayer *et al* (2003) reported that smaller cell sizes of colonies contained the lowest proportion of the MC-producing genotype and lowest MC cell quotas, whereas larger size classes of colonies showed highest proportions of the MC-producing genotype and highest MC cell quotas. Almost a 1:1 relationship between the increase and decrease in MC concentrations and the growth rate of the cells of larger colony size classes was found, implying that the MC production mainly resulted from the progression of the cells from larger colonies. Koskenniemi *et al* (2007) investigated the potentially nodularin-producing genotype of the *Nodularia* population in the Baltic Sea in July 2004 by estimating the *nda*F gene copy numbers. Although the copy numbers varied 1,500 fold, the authors found a significant correlation with the nodularin concentrations in the water and concluded that toxin production by *Nodularia* was relatively constant. Moreover, a positive correlation between saxitoxin gene copy numbers and saxitoxin concentrations in a bloom of toxic *Anabaena* from Australia was reported (Al-Tebrineh *et al* 2010).

5. Aims of the Thesis

Populations of cyanobacteria are commonly comprised of MC-producing and nontoxic genotypes, the knowledge about the factors regulating their abundance is however rare. Molecular methods like qPCR offer a sensitive and reliable tool to detect potential MC-producing genotypes in the environment and enable the correlation of their proportion with abiotic and biotic factors. The primary aim of this thesis was the elucidation of environmental factors that influence the distribution and abundance of toxic and nontoxic genotypes among populations of planktonic cyanobacteria over different spatial and temporal scales.

The specific aims of this study were:

- To investigate the abundance and proportion of the potentially MC-producing genotype in populations of *Microcystis* in Ugandan lakes with regard to the seasonal development of the populations
- To explore the correlation between the proportion of the potentially MC-producing genotype and the average intracellular MC content of *Microcystis* found in these lakes
- To investigate the abundance and proportion of three individual *mcy* genotypes, each encoding the synthesis of a specific MC structural variant in populations of the cyanobacterium *Planktothrix* in dependence of the population density
- To determine the relationship between the abundance of those *mcy* genotypes and the concentrations of the dominant MC variants detected in the lakes
- To extract DNA from historical heat-desiccated phytoplankton samples from Lake Zürich, taken between 1977 and 2008, in order to extend the observation period
- To investigate the reliability of genotype estimations by qPCR from DNA of heat-desiccated phytoplankton samples
- To investigate the stability of the same *mcy* genotypes encoding the synthesis of structural variants during a long-term observation period of thirty years

- To develop a molecular approach for the differentiation of *Planktothrix* genotypes that lost or that still contain the *mcy* gene cluster.
- To investigate the abundance of potentially MC-producing genotypes and genotypes that are either inactive due to insertions or a deletion within the *mcy* gene cluster or that lost the *mcy* gene cluster over the same long-term observation period
- To explain the abundance of the potentially MC-producing genotypes and the nontoxic genotypes by ecological adaptations that are related or unrelated to MC production

5.1 List of Publications

The thesis is based on the following articles:

Chapter 1

Okello W, Ostermaier V, Portmann C, Gademann K, Kurmayer R (2010): Spatial isolation favours the divergence in microcystin net production by *Microcystis* in Ugandan freshwater lakes. Water Research 44:2803-2014.
This manuscript was partly published in the PhD thesis of William Okello (2009)

Chapter 2

Ostermaier V and Kurmayer R (2010): Application of real-time PCR to estimate toxin production by the cyanobacterium *Planktothrix* sp. Applied and Environmental Microbiology 76:3495-3502.

Chapter 3

Ostermaier V, Christiansen G, Schanz F, Kurmayer R (2013): Genetic variability of microcystin biosynthesis genes in *Planktothrix* as elucidated from samples preserved by heat desiccation during three decades. Submitted to PLoS ONE (August 07, 2013).

Chapter 4

Ostermaier V, Schanz F, Köster O, Kurmayer R (2012): Stability of toxin gene proportion in cyanobacteria during 29 years of re-oligotrophication in Lake Zürich. BMC Biology 10:100.

The Author's Contribution

Chapter 1

The author performed all pPCR measurements, analyzed the data, prepared figures 2, 3 and 5 and contributed to the manuscript.

Chapter 2

The author contributed to the design of the primers and probes, performed the field sampling, the DNA and microcystin extractions, the qPCR, HPLC and ELISA analyses, the phytoplankton counting, data analysis and statistics, prepared the figures and wrote the manuscript under the supervision of Rainer Kurmayer.

Chapter 3

The author performed the experiments using the PreCR Repair Mix, did the DNA extractions, all qPCR measurements and PCRs, the cloning and the sample preparation for sequencing, analyzed the data, prepared the figures and wrote the manuscript under the supervision of Rainer Kurmayer.

Chapter 4

The author performed the DNA extractions, qPCR and Custom TaqMan genotype assay measurements, the data analysis and statistics, prepared the figures and wrote the manuscript under the supervision of Rainer Kurmayer.

5.2 Results

In Chapter 1, the *Microcystis* populations of five lakes (six sampling sites in total) in Uganda were investigated by qPCR with regard to the abundance and proportion of the potentially MC-producing genotype to determine the seasonal and spatial influence on genotype proportion. Additionally, the respective MC concentrations were determined by HPLC-DAD and correlated with the proportion of the potentially MC-producing genotype.

Between the lakes, a 50-fold difference in the average *Microcystis* biovolume (min 1.6 ± 0.3 mm^3 L^{-1} and max 81 ± 21 mm^3 L^{-1}) was found. In all samples, *Microcystis* and potentially MC-producing *Microcystis* cells were detected by qPCR via the phycocyanin locus (PC-IGS) and the *mcy*B gene, respectively. In general, a good correlation was found between cell numbers estimated by microscopic counting and the PC-IGS genotype quantified by qPCR (R^2 = 0.62). In contrast, no relationship between cell numbers estimated by microscopic counting and the cell numbers of the *mcy*B genotype was observed (R^2 = 0.13). In all lakes, potentially MC-producing genotypes were detected, and on average comprised 19.9 ± 1.8 % of the total population (min = 0.5, max = 66.6%). There were significant differences in the proportion of the *mcy*B genotype between lakes. Consequently, the lack of correlation between microscopically determined cell numbers and the *mcy*B genotype cell numbers determined by qPCR was probably caused by spatial differences in *mcy*B genotype proportion. The highest *mcy*B proportion was e.g. detected in Lake Saka (min – mean ± SE – max, 24.4 – 37.0 ± 3.3 – 66.6%) and the lowest proportion in Lake George (0.49 – 1.4 ± 0.2 – 3.6%).

HPLC analyses revealed the presence of MCs in most of the samples, with significant differences in the relative abundance of MC structural variants between the lakes. In general, MC-RY was most abundant, followed by MC-RR and MC-YR. On average, a 28-fold higher concentration of MCs was detected in Lake Saka (4.7 ± 0.9 µg L^{-1}) when compared to Lake George (0.2 ± 0.1 µg L^{-1}). Within the lakes, MC concentrations varied seasonally between 12- and 30-fold. A significant relationship between the MC concentrations and the *mcy*B genotype cell numbers was found (R^2 = 0.62) and between the proportion of the *mcy*B genotype and the average cellular MC content per cell (R^2 = 0.58). The average *Microcystis* MC quotas differed significantly between populations, and in accordance with *mcy*B genotype proportions, consistently lowest MC cell quotas (0.03 – 1.24 fg $cell^{-1}$) were detected for *Microcystis* from Lake

George and highest MC cell quotas (14 – 144 fg cell^{-1}) were found for Lake Saka. The multiple regression analysis using the forward selection of explanatory variables revealed, that the *mcy*B genotype abundance was the most significant predictor variable to explain MC concentrations (R^2 = 0.73).

In Chapter 2, the abundances and proportions of three *Planktothrix* genotypes encoding the synthesis of different MC structural variants (denoted the Dhb, Mdha and Hty genotype) were investigated by qPCR in 12 lakes of the Alps that differed in trophic state and in *Planktothrix* population densities over a period of two years. The concentrations and proportions of the corresponding MC structural variants, i.e. the [Asp, Dhb]-MC-RR, [Asp, Mdha]-MC-RR and [Asp, Dhb]-MC-HtyR variants, were determined from aliquots of the water samples by means of HPLC.

The lakes differed in trophic state from oligotrophic to mesoeutrophic, and microscopic counting revealed average proportions of *Planktothrix* in total phytoplankton ranging from a minimum of 0.2% to a maximum of 95.6% between the lakes. There was a linear correlation between the total *Planktothrix* biovolume estimated by qPCR (16S rDNA) and the biovolume determined in the microscope (R^2 = 0.82). On average, the genotype encoding the synthesis of MC with Dhb in position 7 showed the highest proportion (28.7 ± 2%) in the populations, while the proportion of the genotype encoding the synthesis of Mdha in the same position was significantly lower (13.7 ± 1%). The genotype encoding Hty in position 2 of the MC molecule showed the lowest proportion in the populations (10.6 ± 2%). On a logarithmic scale, the abundance of each of the MC genotypes was significantly positively related to the total population determined by 16S rDNA (R^2 = 0.92, 0.93 and 0.88 for the Mdha, Dhb and Hty genotype, respectively). In general, highest proportions of [Asp, Dhb]-MC-RR (44.2 ± 3%) were detected by HPLC when compared to [Asp, Mdha]-MC-RR (32.6 ± 2%), [Asp]-MC–HtyR (7 ± 1%) and [Asp]-MC-LR (14.9 ± 2%). Correspondingly, on a logarithmic scale, there was a linear correlation between the abundance of each of the three genotypes and the concentrations of the respective MC structural variant (R^2 = 0.72, 0.8 and 0.84 for the Mdha, Dhb and Hty genotype, respectively) and between the average proportion of MC genotypes and the concentrations of the corresponding MC structural variant (R^2 = 0.54). Four of the investigated lakes (oligotrophic and oligomesotrophic lakes, e.g. Ammersee in Germany) showed higher average proportions of the Mdha genotype when compared

to the Dhb genotype, and correspondingly, higher average proportions of [Asp, Mdha]-MC-RR were recorded when compared to [Asp, Dhb]-MC-RR.

In Chapter 3, a long-term monitoring approach enabled by the analysis of DNA extracted from heat-desiccated phytoplankton samples was performed. In a first step, the quality and usefulness of the preserved DNA for qPCR was investigated. The effect of heat-desiccation on the qPCR estimate of the Dhb and Mdha genotype abundance was evaluated by drying aliquots of strains of these genotypes at different temperatures. The DNA of the heat-desiccated biomass of strains was compared to DNA extracted from aliquots of these strains stored frozen. In addition, an enzyme mix (PreCR Repair Mix) designed to improve the DNA quality in damaged templates was used, in order to find out which damages occurred and whether they could be repaired. The gene loci analyzed by qPCR were sequenced to identify DNA substitutions introduced by the heat-desiccation treatment or storage time and to investigate their distribution. Subsequently, the abundance and proportion of the same three *mcy* genotypes encoding the synthesis of different MC variants (see above) was monitored in deep, stratified Lake Zürich (Switzerland) during the re-oligotrophication period for almost 30 years (1980 – 2008). Despite the overall reduction in nutrient introduction (total phosphorus), the *Planktothrix* population increased significantly during this period. This approach offered the possibility to monitor the abundance of the dominant MC-producing genotypes over a period of time that exceeds conventional observation periods considerably, and during which major changes in the population size of *Planktothrix* occurred.

The DNA extracted from heat-desiccated lake samples was highly fragmented, with the biggest share of fragments within the size range of 50 – 300bp. The heat-desiccation of biomass from strains showed significant DNA loss when compared to aliquots stored frozen. For both the Dhb and Mdha genotype, increased proportions as estimated by qPCR were detected among heat-desiccated DNA samples when compared to estimates of genotype proportions obtained from DNA samples stored frozen, however, this effect was not sigificant.

The treatment of the heat-desiccated DNA with the PreCR Repair Mix prior to the sequencing of the loci used for the detection of *mcy* genotypes and the 16S rDNA locus revealed the presence of substitutions that accounted for a rate of 1.4×10^{-3} per nucleotide. The sequencing of non-repaired heat-desiccated DNA revealed a rate of

substitutions that accounted for 2.5×10^{-3} substitutions per nucleotide. As a control, the number of substitutions per nucleotide from DNA stored frozen was determined, which showed the lowest rate of substitutions (1.04×10^{-3}). The highest number of substitutions detected were transitions (87% of the substitutions). However, nucleotide substitutions were found to be evenly distributed among the investigated loci. No significant improvement in the DNA yield by qPCR through the application of the PreCR Repair Mix was observed and it was concluded that the major destruction of DNA by heat-desiccation occurred quantitatively, while the qualitative changes were of minor importance.

On a logarithmic scale, a linear correlation was found between each of the *mcy* genotypes and the total *Planktothrix* population as revealed by qPCR (R^2 = 0.8, 0.6 and 0.6 for the Dhb, Mdha and Hty genotype, respectively). On average, the abundance of the Dhb genotype was always higher than the abundance of the Mdha or the Hty genotype. Except for three years, the abundance of the Mdha genotype was higher than the abundance of the Hty genotype. In general, also the proportion of the Dhb genotype was highest, however a decrease in proportion was observed from the first decade (1977-1989, mean 76 ± 11%) to the two subsequent decades (1990 – 1999, mean 57% ± 5% and 2000 – 2008, mean 32 ± 4%) and no corresponding increase of the Mdha genotype was observed. The decrease in proportion of the Dhb genotype could partly be explained by the presence and slight increase of an inactive *mcy* genotype carrying a deletion within the Dhb gene (see Chapter 4) that would prevent its amplification in qPCR. The Dhb genotype proportion was significantly negatively related to the proportion and abundance of this inactive genotype and there was no correlation between the Mdha and Hty genotypes and this inactive genotype. The overall relation between the three *mcy* genotypes was however stable and the changes were considered to be minor compared with the 5,000-fold increase in total population density.

In Chapter 4, the same DNA as analyzed in Chapter 3 was used to quantify the abundance and proportion of the *mcy*-containing genotype (via the presence of the *mcy*B gene), of a red- or green-pigmented toxic lineage and a green-pigmented nontoxic lineage of *Planktothrix* (Christiansen *et al* 2008). Additionally, the proportion of nontoxic genotypes inactive due to insertions or a deletion within the *mcy* gene cluster was determined (Ostermaier and Kurmayer 2009). Furthermore, a

TaqMan Genotyping Assay using a single nucleotide polymorphism (SNP) that constitutes a remnant of the *mcy* gene cluster in nontoxic strains, was applied to samples of Lake Zürich and other European lakes with red- or green-pigmented *Planktothrix* populations, to estimate the proportions of *Planktothrix* genotypes that lost or still contain the *mcy* genes. This long-term investigation was performed to reveal whether nontoxic genotypes, which are distributed all over Europe were able to immigrate and increase in abundance, as environmental conditions changed dramatically during the study period.

The total population changed from a minimum population density with almost complete disappearance of *Planktothrix* to its stable dominance of the phytoplankton community. For example the green-pigmented nontoxic lineage 1 (sensu Christiansen *et al* 2008) that is known from shallow polymictic lakes and which lost the *mcy* gene cluster, was expected to occur under more eutrophic conditions like observed in the early eighties.

On a logarithmic scale, a highly significant positive relationship was found between the total *Planktothrix* biovolume detected by qPCR and by microscopic counting (R^2 = 0.9) and between the abundance of the *mcy*-containing genotype and the total population (R^2 = 0.94). The mean proportion of the *mcy*-containing genotype was 106 ± 8% of the total population, indicating that the population was constantly dominated by the *mcy*-containing genotype. The majority of the samples contained inactive *mcy* genotypes in minimum concentrations that were below the limit of quantification but could be detected in undiluted DNA extracts; only the genotype carrying the deletion occurred in higher proportions. From 1987 to 2008 it occurred with an annual average proportion of 3.3 ± 0.4% of the total population and showed no significant increase or decrease in abundance. It was concluded that inactive *mcy* genotypes occurred consistently but never constituted a significant part of the total population.

The average proportion of the nontoxic green-pigmented lineage 1 of *Planktothrix* that lost the *mcy* gene cluster was very low (0.9 ± 0.2%) while the red-and green-pigmented lineage 2 that retained the *mcy* gene cluster showed much higher proportions (7.3 ± 1%). The green-pigmented lineage 1 showed no significant change in proportion over the investigated period, in contrast the red- and green-pigmented lineage 2 increased significantly in proportion. The comparison of the intercept and slope of the regression curves obtained between the abundance of the green-pigmented lineage and the total population, and the regression curve of the *mcy*-

containing genotype and the total population revealed a significant difference in intercept and slope. The same regression curve obtained from the red-pigmented lineage differed in intercept only. This implies that the green-pigmented lineage showed a tendency to decrease in abundance, while the red-pigmented lineage showed a parallel increase with the abundance of the total population. It was therefore concluded that the lineage of the red- or green-pigmented toxic ecotype of *Planktothrix* consistently outgrew the green-pigmented nontoxic lineage.

The TaqMan Genotyping Assay showed a clear separation of the genotypes containing the *mcy* gene cluster and genotypes that lost these genes visualized by an allelic discrimination plot. Distinct clusters were separated, indicating populations only comprised of the toxic genotype (samples dominated by the red-pigmented ecotype and a mixed-pigmented population), populations of both the toxic and nontoxic genotype (green-pigmented and mixed-pigmented populations), and populations composed entirely of the nontoxic genotype (green-pigmented populations). It was shown that the nontoxic genotype never became abundant in Lake Zürich, and that this genotype typically shows a higher abundance in populations of green-pigmented or mixed-pigmented populations.

5.3 Discussion

In Chapter 1, the variation in the average MC content per *Microcystis* cell between the sites (16- to 150-fold in integrated samples) was substantially higher than the variation observed from single strains in the laboratory under variable environmental conditions. Sivonen and Jones (1999) reported up to 5-fold variation in MC production as a response to environmental factors, a higher variation (up to 30-fold) was observed in extreme cases only. This implies that the observed variation in MC content may be due to differential proportions of the *mcy*B genotype within populations. In particular, the significant relationship between the average MC content per cell and the proportion of the *mcy*B genotype for the individual populations supports this hypothesis. It cannot be excluded that environmental factors like lower light intensity in dense populations had an impact on the transcriptional rate of the *mcy* gene cluster, yet our data suggests a major influence of the proportion of potentially MC-producing genotypes on MC production, as the genetic differences in *mcy* genotype proportion could explain the variation in the average MC content per cell among the lakes.

This study revealed that in populations of *Microcystis* the MC net production differed quantitatively and qualitatively. Furthermore, the seasonal variation in the average proportion of the MC-producing genotype within each of the investigated sites did not outweigh its variation between the spatially isolated sites, leading to a rather stable differentiation in the genetic population structure of *Microcystis*. It was concluded that the genotype composition was regulated by local abiotic or biotic factors, which differed between the lakes.

In Chapter 2, the concentrations of total MCs and each of the three dominant structural variants could be explained from the corresponding *mcy* genotype concentrations in populations of *Planktothrix*. In a previous study (Ostermaier and Kurmayer 2009) we could show that the abundance of inactive genotypes within the same populations was low (on average 6.5 ± 1.1%) and it was suggested that the populations are dominated by active MC-producing genotypes. The dominance of these MC-producing genotypes probably contributed to the highly significant correlations between *mcy* genotype abundance and MC concentration. Since *Planktothrix* was the main MC producer in these lakes, no other MC-producing cyanobacteria had to be considered. If other MC-producing taxa would have co-

occurred in the lakes of this study, more general primers and probes, e.g. targeted to the *mcy*E gene (Rantala *et al* 2004), which showed little genetic variability among genera, would be required.

Overall, the Dhb genotype and the corresponding [Asp, Dhb]-MC-RR showed the highest proportion, and only in four lakes higher proportions of the Mdha genotype and the corresponding [Asp, Mdha]-MC-RR were detected. Earlier, Ernst *et al* (2009) reported [Asp, Mdha]-MC-RR to be the main MC structural variant produced by *P. rubescens* in Ammersee during 1998 – 2004. The authors found far lower concentrations of [Asp, Dhb]-MC-RR and MC-LR, which is in accordance to our measurements obtained between 2005 and 2007. This implies that the composition of Mdha and Dhb MC-producing genotypes was rather stable between 1998 and 2007.

The good correlations between the total *Planktothrix* population and the abundance and proportion of these *mcy* genotypes imply that the *mcy* genotype composition is rather stable and that each of the *mcy* genotypes grows rather constantly with the total population.

Additionally, we showed that the application of qPCR for water quality monitoring can be used in order to predict MC concentrations in water. It might be useful to differentiate genotypes encoding the synthesis of different structural MC variants that exhibit differential toxicities because the current risk assessment schemes based on MC-LR may be insufficient (Dietrich and Hoeger 2005). For example, it was shown that the toxicity of [Asp, Dhb]-MC-RR was among the highest compared to MCs lacking Dhb in position 7, while the inhibition of the protein phosphatase by the same variant was lowest (Blom and Jüttner 2005).

In Chapter 3, the improvement of heat-desiccated DNA templates could be shown by the application of PreCR Repair Mix by a reduction of nucleotide substitutions. Yet, no significant improvement in terms of DNA yield was observed when comparing heat-desiccated repaired and non-repaired aliquots by qPCR. The presence of DNA polymorphisms and a high degree of fragmentation in heat-desiccated or ancient samples is common (e.g. Bruskov *et al* 2002, Eigner *et al* 1961) and it was concluded that it was rather the heat-induced major fragmentation of the DNA than heat-induced substitutions that primarily decreased DNA quality. Furthermore it was concluded that estimations of genotype proportions by qPCR were relatively robust against the heat-induced DNA damage.

Despite major changes in *Planktothrix* population density and the trophic state of the lake, the overall MC genotype composition was rather stable, which implies a stable *mcy* genotype subpopulation composition over the study period. In Lake Zürich, it is therefore unlikely that one of the three dominant *mcy* genotypes will vanish or reappear within shorter periods of time. Nevertheless, a decrease in proportion of the Dhb genotype was detected. This decrease was in part explained by the parallel increase of an inactive *mcy* genotype that carries a deletion within the Dhb primer binding region and therefore was not detected by qPCR. As the proportion of this inactive genotype was rather low, the presence of so far undetected inactive genotypes carrying a deletion within *mcy*A could however not be excluded.
Yet, compared with the population increase by three orders of magnitude, the variation in *mcy* genotype composition appeared to be relatively minor over the 30 years observation period. It was further concluded that the increase of the inactive *mcy* genotype had a negligible effect on the overall toxicity of the population only (see Chapter 4).

In Chapter 4, the increase in the total *Planktothrix* population density during the re-oligotrophication period was explained by the improved light regime in the water column due to the disappearance of eukaryotic algae blooms, like it has already been documented for other lakes (Jacquet *et al* 2005). Unexpectedly, the genetic population structure was rather stable over a period of almost 30 years. Nontoxic genotypes occurred consistently in low proportions. It was concluded that these genotypes grow rather slowly and thus sudden changes in abundance within short periods of time are rather unlikely to occur in Lake Zürich.
The observed stability in the dominance of the *mcy*-containing genotype points to a role of abiotic and biotic parameters of the habitat, e.g. lake morphometry, light regime, grazers and viruses that regulate genotype composition. As Lake Zürich is deep, gas vesicle strength is of particular importance. It was shown that the resistance of gas vesicles to hydrostatic pressure is of major importance during lake mixing because the surviving filaments will form the basis for the population in the following spring (Walsby *et al* 1998). Furthermore it was reported that gas vesicles resisting strong hydrostatic pressures are more common among red-pigmented populations of *Planktothrix* when compared to green-pigmented populations that contained a weaker gas vesicle type (Beard *et al* 2000). Our own preliminary results showed that strains

of the green-pigmented nontoxic lineage 1 always contained the weaker type of gas vesicles, while strains of the red-pigmented lineage 2 additionally contained a stronger gas vesicle type. It was concluded that the stability in toxic genotype proportion could be explained by the adaptation to deep-mixing of a genotype that retained the *mcy* gene cluster during evolution, and that regular deep-mixing events selectively reduced the abundance of the nontoxic genotype in Lake Zürich.

5.4 Conclusions

From the present study the following conclusions could be drawn:

- *Microcystis* populations in Uganda differed genetically in the proportion of the MC-producing genotype between the lakes, independently of the season, implying that local abiotic or biotic factors that differ between the lakes regulate genotype composition.
- The observed between site variation in the average proportion of the MC-producing genotype was higher when compared to the variation within sites, leading to a divergence in genotype composition of spatially isolated populations.
- The proportion of the MC-producing genotype explained the observed variation in the average MC content per *Microcystis* cell among the lakes.
- The production of different MC structural variants in *Planktothrix* populations can be explained quantitatively and qualitatively by the abundance and proportion of the respective *mcy* genotypes encoding the synthesis of the individual MC structural variants.
- The abundance and proportion of the specific *mcy* genotypes explained the concentrations of the respective MC structural variants over a wide range of population densities.
- The use of qPCR as a monitoring tool is suitable for the prediction of the concentrations of specific MC structural variants in water, which are known to show varying toxicities.
- The highly significant correlations between the proportion of the MC-producing genotype(s) and the MC net production in *Microcystis* and *Planktothrix* populations under natural conditions imply that for MC net production the abundance of the *mcy* genotype is of major influence, while the influence of the transcriptional rate of the *mcy* gene cluster is of minor importance.
- Preserved phytoplankton biomass can be utilized to analyze the long-term development of toxigenic cyanobacteria by qPCR; estimates of genotype

proportions were rather robust against the DNA damage induced by heat-desiccation and storage, and DNA fragmentation caused the major damage resulting in quantitative DNA destruction.

- The overall relationship between the proportions of *mcy* genotypes encoding the synthesis of different MC structural variants in the *Planktothrix* population of Lake Zürich was relatively stable over a re-oligotrophication period of almost 30 years and inactive genotypes occurred in lowest proportions only.
- The decrease in proportion of the Dhb genotype was related to the increase in proportion of an inactive *mcy* genotype carrying a deletion within the primer binding region of the Dhb genotype.
- The MC-producing genotype was always dominant in Lake Zürich, implying that despite major changes in population density, the environmental factors did not change to an extend that would favor nontoxic genotypes.
- The stability in abundance of the MC-producing genotype was explained by the adaptation (i.e. by the synthesis of gas vesicles that are more resistant to hydrostatic pressure) to deep-mixing of a genotype that retained the MC synthetase gene cluster during evolution, while the green-pigmented lineage of *Planktothrix* that lost the *mcy* genes was consistently outgrown.
- The TaqMan Genotyping Assay based on a single nucleotide polymorphism is a highly sensitive tool for the discrimination of *Planktothrix* genotypes that lost or still contain the *mcy* gene cluster.

6. References

Alexova R, Haynes PA, Ferrari BC, Neilan BA (2011): Comparative protein expression in different strains of the bloom-forming cyanobacterium *Microcystis aeruginosa*. Molecular and Cellular Proteomics 10:M110.003749.

Alfonso M, Perewoska I, Kirilovsky D (2001): Redox control of *ntc*A gene expression in *Synechocystis* sp. PCC 6803. Nitrogen availability and electron transport regulate the levels of the NtcA protein. Plant Physiology 125:969-981.

Al-Tebrineh J, Mihali TK, Pomati F, Neilan BA (2010): Detection of saxitoxin-producing cyanobacteria and *Anabaena circinalis* in environmental water blooms by quantitative PCR. Applied and Environmental Microbiology 76:7836-7842.

Babica P, Bláha L, Maršálek B (2006): Exploring the natural role of microcystins - a review of effects on photoautotrophic organisms. Journal of Phycology 42:9-20.

Basen T, Rothhaupt KO, Martin-Creuzburg D (2012): Absence of sterols constrains food quality of cyanobacteria for an invasive freshwater bivalve. Oecologia 170:57-64.

Beard SJ, Davis PA, Iglesias-Rodriguez D, Skulberg OM, Walsby AE (2000): Gas vesicle genes in *Planktothrix* spp. from Nordic lakes: strains with weak gas vesicles possess a longer variant of *gvp*C. Microbiology 146:2009-2018.

Bianchi TS, Engelhaupt E, Westman P, Andren T, Rolff C, Elmgren R (2000): Cyanobacterial blooms in the Baltic Sea: Natural or human-induced. Limnology and Oceanography 45:716-726.

Blom JF, Baumann HI, Codd GA, Jüttner F (2006): Sensitivity and adaptation of aquatic organisms to oscillapeptin J and [D-Asp^3,(*E*)-Dhb^7]microcystin-RR. Archive für Hydrobiologie 167:547-559.

Blom J and Jüttner F (2005): High crustacean toxicity of microcystin congeners does not correlate with high protein phosphatase inhibitory activity. Toxicon 46:465-470.

Blom JF, Robinson JA, Jüttner F (2001): High grazer toxicity of [D-Asp^3,(*E*)-Dhb^7] microcystin-RR of *Planktothrix rubescens* as compared to different microcystins. Toxicon 39:1923-1932.

Börner T and Dittmann E (2005): Molecular biology of cyanobacterial toxins: Genetic basis of microcystin production. In Harmful cyanobacteria. Mathhijs HCP and Visser PM (eds), Springer, The Netherlands, pp.25- 40.

Briand E, Escoffier N, Straub C, Sabart M, Quiblier C, Humbert JF (2009): Spatiotemporal changes in the genetic diversity of a bloom-forming *Microcystis aeruginosa* (cyanobacteria) population. ISME Journal 3:419-429.

Briand E, Yéprémian C, Quiblier C (2008a): Competition between microcystin- and non-microcystin-producing *Planktothrix agardhii* (cyanobacteria) strains under different environmental conditions. Environmental Microbiology 10:3337-3348.

Briand E, Gugger M, François JC, Bernard C, Humbert JF, Quiblier C (2008b): Temporal variations in the dynamics of potentially microcystin-producing strains in a bloom-forming *Planktothrix agardhii* (Cyanobacterium) population. Applied and Environmental Microbiology 74:3839-3848.

Bruskov VI, Malakhova LV, Masalimov ZK, Chernikov AV (2002): Heat-induced formation of reactive oxygen species and 8-oxoguanine, a biomarker of damage to DNA. Nucleic Acids Research 30:1354-63.

Canfield DE, Habicht KS, Thamdrup B (2000): The Archean sulfur cycle and the early history of atmospheric oxygen. Science 288:658-661.

Capone DG, Zehr JP, Paerl HW, Bergman B, Carpenter EJ (1997): *Trichodesmium*, a globally significant marine cyanobacterium. Science 276:1221-1229.

Carey CC, Ibelings BW, Hoffmann EP, Hamilton DP, Brookes JD (2012): Ecophysiological adaptations that favour freshwater cyanobacteria in a changing climate. Water Research 46:1394-1407.

Carmichael W and Li R (2006): Cyanobacteria toxins in the Salton Sea. Saline Systems 2:5.

Carmichael WW (1997): The cyanotoxins. Advances in Botanical Research 27:211-256.

Carmichael WW (1996): Toxic *Microcystis* and the environment. In Toxic *Microcystis*. Watanabe MF, Harada KI, Carmichael WW, Fujiki H (eds), CRC Press, Boca Raton, FL, pp.1-11.

Carmichael WW (1994): The toxins of cyanobacteria. Scientific American 270:78-86.

Carmichael WW (1992): Cyanobacteria secondary metabolites - the cyanotoxins. Journal of Applied Bacteriology 72:445-459.

Carmichael WW, Beasly V, Bunner DL, Eloff JN, Falconer I, Gorham P, Harada KI, Krishnamurty T, Min-Juan Y, Moore RE, Rinehart K, Runnegar M, Skulberg OM, Watanabe M (1988): Naming cyclic heptapeptide toxins of cyanobacteria (blue-green algae). Toxicon 26:971-973.

Castenholz RW (2001): Phylum BX. Cyanobacteria. In Bergey's manual of systematic bacteriology. 2nd Edition, vol. 1, Boone DR and Castenholz RW (eds), Springer, New York, pp.473-487.

Castenholz RW (1996): Endemism and biodiversity of thermophilic cyanobacteria. Nova Hedwigia Beiheft 112:33-47.

Castenholz RW, Waterbury JB (1989): Group I. Cyanobacteria. In Bergey's manual of systematic bacteriology. vol. 3, Krieg NR and Holt JG (eds), Williams & Wilkins, Baltimore, pp.1710-1728.

Christiansen G, Molitor C, Philmus B, Kurmayer R (2008a): Nontoxic strains of cyanobacteria are the result of major gene deletion events induced by a transposable element. Molecular Biology and Evolution 25:1695-1704.

Christiansen G, Yoshida WY, Blom JF, Portmann C, Gademann K, Hemscheidt T, Kurmayer R (2008b): Isolation and structure determination of two microcystins and sequence comparison of the *mcy*ABC adenylation domains in *Planktothrix* species. Journal of Natural Products 71:1881-1886.

Christiansen G, Kurmayer R, Liu Q, Börner T (2006): Transposons inactivate biosynthesis of the nonribosomal peptide microcystin in naturally occurring *Planktothrix* spp. Applied and Environmental Microbiology 72:117-123.

Christiansen G, Fastner J, Erhard M, Börner T, Dittmann E (2003): Microcystin biosynthesis in *Planktothrix*: genes, evolution, and manipulation. Journal of Bacteriology 185:564-572.

Christiansen G (2002): Untersuchungen zum Microcystinsynthetase-Gencluster aus dem Cyanobakterium *Planktothrix agardhii*. Dissertation, Humboldt-Universität zu Berlin, Germany, 154pp.

Codd GA, Morrison LF, Metcalf JS (2005): Cyanobacterial toxins: risk management for health protection. Toxicology and Applied Pharmacology 203:264-272.

D'Alelio D, Gandolfi A, Boscaini A, Flaim G, Tolotti M, Salmaso N (2011): *Planktothrix* populations in subalpine lakes: selection for strains with strong gas vesicles as a function of lake depth, morphometry and circulation. Freshwater Biology 56:1481-1493.

Davis TW, Berry DL, Boyer GL, Gobler CJ (2009): The effects of temperature and nutrients on the growth and dynamics of toxic and non-toxic strains of *Microcystis* during cyanobacteria blooms. Harmful Algae 8:715-725.

Davis PA, Dent M, Parker J, Reynolds CS, Walsby AE (2003) The annual cycle of growth rate and biomass change in *Planktothrix* spp. in Blelham Tarn, English Lake District. Freshwater Biology 48:852-867.

Dietrich D and Hoeger S (2005): Guidance values for microcystins in water and cyanobacterial supplement products (blue-green algal supplements): a reasonable or misguided approach? Toxicology and Applied Pharmacology 203:273-289.

Dittmann E, Neilan BA, Erhard M, von Döhren H, Börner T (1997): Insertional mutagenesis of a peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. Molecular Microbiology 26:779-787.

Eigner J, Boedtker H, Michaels G (1961): The thermal degradation of nucleic acids. Biochimica et Biophysica Acta 51:165-8.

Ernst B, Hoeger SJ, O'Brien E, Dietrich DR (2009): Abundance and toxicity of *Planktothrix rubescens* in the pre-alpine Lake Ammersee, Germany. Harmful Algae 8:329-342.

Falconer IR and Humpage AR (2005): Health risk assessment of cyanobacterial (blue-green algal) toxins in drinking water. International Journal of Environmental Research and Public Health 2:43-50.

Fastner J, Neumann U, Wirsing B, Weckesser J, Wiedner C, Nixdorf B, Chorus I (1999): Microcystins (hepatotoxic heptapeptides) in German fresh water bodies. Environmental Toxicology 14:13-22.

Feurstein D, Kleinteich J, Heussner AH, Stemmer K, Dietrich DR (2009): Investigation of microcystin congener-dependent uptake into primary murine neurons. Environmental Health Perspectives 118:1370-1375.

Fewer DP, Halinen K, Sipari H, Bernardová K, Mänttäri M, Eronen E, Sivonen K (2011): Non-autonomous transposable elements associated with inactivation of microcystin gene clusters in strains of the genus *Anabaena* isolated from the Baltic Sea. Environmental Microbiology Reports 3:189-194.

Finking R and Marahiel MA (2004): Biosynthesis of nonribosomal peptides 1. Annual Review of Microbiology 58:453-488.

Foulds IV, Granacki A, Xiao C, Krull UJ, Castle A, Horgen PA (2002): Quantification of microcystin-producing cyanobacteria and *E. coli* in water by 5'-nuclease PCR. Journal of Applied Microbiology 93:825-834.

Francis G (1878): Poisonous Australian lake. Nature 18:11-12.

Guo C and Tester PA (1994): Toxic effect of the bloom-forming *Trichodesmium* sp. (Cyanophyta) to the copepod *Acartia tonsa*. Natural Toxins 2:222-227.

Ha JH, Hidaka T, Tsuno H (2009): Quantification of toxic *Microcystis* and evaluation of its dominance ratio in blooms using real-time PCR. Environmental Science and Technology 43:812-818.

Häder DP (1987): Photomovement. In The Cyanobacteria. Fay P and Van Baalen C (eds), Elsevier, Amsterdam, pp.325-345.

Hawser SP, O'Neil JM, Roman MR, Codd GA (1992): Toxicity of blooms of the cyanobacterium *Trichodesmium* to zooplankton. Journal of Applied Phycology 4:79-86.

Haande S, Ballott A, Rohrlack T, Fastner J, Wiedner C, Edvardsen B (2007): Diversity of *Microcystis aeruginosa* isolates (Chroococcales, Cyanobacteria) from East-African water bodies. Archives of Microbiology 188:15-25.

Hayes PK, Barker GLA, Batley J, Beard SJ, Handley BA, Vacharapiyasophon P, Walsby AE (2002): Genetic diversity within populations of cyanobacteria assessed by analysis of single filaments. Antonie Van Leeuwenhoek 81:197-202.

Hayes PK, Buchholz B, Walsby AE (1992): Gas vesicles are strengthened by the outer-surface protein, GvpC. Archives of Microbiology 157:229-234.

Henriksen P and Moestrup O (1997): Seasonal variations in microcystin contents of Danish cyanobacteria. Natural Toxins 5:99-106.

Honkanen RE, Caplan FR, Baker KK, Baldwin CL, Bobzin SC, Bolis CM, Cabrera GM, Johnson LA, Jung JH, Larsen LK, Levine IA, Moore RE, Nelson CS, Patterson GML, Tschappat KD, Tuang GD, Boynton AL, Arment AR, An J, Carmichael WW, Rodland KD, Magun BE, Lewin RA (1995): Protein phosphatase inhibitory activity in extracts of cultured blue-green algae (Cyanophyta). Journal of Phycology 31:478-486.

Humble AV, Gadd GM, Codd GA (1997): Binding of copper and zinc to three cyanobacterial microcystins quantified by differential pulse polarography. Water Research 31:1679-1686.

Humpage AR (2008): Toxin types, toxicokinetics and toxicodynamics. Advances in Experimental Medicine and Biology 619:383-415.

Isvánovics V, Shafik HM, Présing M, Juhos S (2000): Growth and phosphate uptake kinetics of the cyanobacterium *Cylindrospermopsis raciborskii* (Cyanophyceae) in throughflow cultures. Freshwater Biology 43:257-275.

Jacquet S, Briand JF, Leboulanger C, Avois-Jacquet C, Oberhaus L, Tassin B, Vincon-Leite B, Paolini G, Druart JC, Anneville O, Humbert JF (2005): The proliferation of the toxic cyanobacterium *Planktothrix rubescens* following restoration of the largest natural French lake (Lac du Bourget). Harmful Algae 4:651-672.

Jähnichen S, Ihle T, Petzoldt T, Benndorf J (2007): Impact of inorganic carbon availability on microcystin production by *Microcystis aeruginosa* PCC 7806. Applied and Environmental Microbiology 73:6994-7002.

Jarvis A C, Hart RC, Combrink S (1987): Zooplankton feeding on size fractionated *Microcystis* colonies and *Chlorella* in a hypertrophic lake (Hartbeesport Dam, South Africa): implications to resource utilization and zooplankton succession. Journal of Plankton Research 9:1231-1249.

Jochimsen EM, Carmichael WW, An J, Cardo DM, Cookson ST, Holmes CEM, Antunes, MB de C, Filho DA de M, Lyra TM, Barreto VST, Azevedo SMFO, Jarvis WR (1998): Liver failure and death after exposure to microcystins at a haemodialysis center in Brazil. New England Journal of Medicine 338:873-878.

Kaardinal WEA, Tonk L, Janse I, Hol S, Slot P, Huisman J, Visser PM (2007a): Competition for light between toxic and nontoxic strains of the harmful cyanobacterium *Microcystis*. Applied and Environmental Microbiology 73:2939-2946.

Kaardinal WEA, Janse I, Kamst-van Agterfeld M, Meima M, Snoek J, Mur LR, Huisman J, Zwart G, Visser P (2007b): *Microcystis* genotype succession in relation to microcystin concentrations in freshwater lakes. Aquatic Microbial Ecology 48:1-12.

Kaebernick M, Dittmann E, Börner T, Neilan BA (2002): Multiple alternate transcripts direct the biosynthesis of microcystin, a cyanobacterial nonribosomal peptide. Applied and Environmental Microbiology 68: 449-455.

Kaebernick D, Rohrlack T, Christoffersen K, Neilan BA (2001): A spontaneous mutant of microcystin biosynthesis: genetic characterization and effect on *Daphnia*. Environmental Microbiology 3:669-679.

Kaebernick M, Neilan BA, Börner T, Dittmann E (2000): Light and the transcriptional response of the microcystin biosynthesis gene cluster. Applied and Environmental Microbiology 66:3387-3392.

Kao CY (1993): Paralytic shellfish poisoning. In Algal Toxins in Seafood and Drinking Water. Falconer I (ed), Academic Press, London, pp.75-86.

Kaplan A, Harel M, Kaplan-Levy RN, Hadas O, Sukenik A, Dittmann E (2012): The languages spoken in the water body (or the biological role of cyanobacterial toxins). Frontiers in Microbiology 3:138.

Kazmierczak J and Altermann W (2002): Neoarchean biomineralisation by benthic cyanobacteria. Science 298:2351.

Komárek J and Anagnostidis, K (2005): Cyanoprokaryota. Part 2: Oscillatoriales. In Süsswasserflora von Mitteleuropa 19/2. Büdel B, Krienitz L, Gärtner G, Schagerl M (eds), Spektrum Akademischer Verlag, Heidelberg, 759pp.

Komárek and Anagnostidis (1998): Cyanoprokaryota. Part 1: Chroococcales. In Süsswasserflora von Mitteleuropa 19/1. Ettl H, Gärtner G, Heynig H, Mollenhauer D (eds), Gustav Fischer, Jena-Stuttgart-Lübeck-Ulm, 548pp.

Komárek J and Komárková J (2002): Review of the European *Microcystis*-morphospecies (Cyanoprokaryotes) from nature. Czech Phycology 2:1-24.

Komárek J (2003): Planktic oscillatorialean cyanoprokaryotes (short review according to combined phenotype and molecular aspects). Hydrobiologia 502:367-382.

Koskenniemi K, Lyra C, Rajaniemi-Wacklin P, Jokela J, Sivonen K (2007): Quantitative real-time PCR detection of toxic *Nodularia* cyanobacteria in the Baltic Sea. Applied and Environmental Microbiology 73:2173-2179.

Kuiper-Goodman T, Falconer I, Fitzgerald J (1999): Human health aspects. In Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management. Chorus I, Bartram J (eds), E & FN Spon, London and New York, Chapter 4.

Kurmayer R, Schober E, Tonk L, Visser PM, Christiansen G (2011): Spatial divergence in the proportions of genes encoding toxic peptide synthesis among populations of the cyanobacterium *Planktothrix* in European lakes. FEMS Microbiology Letters 317:127-137.

Kurmayer and Gumpenberger (2006): Diversity of microcystin genotypes among populations of the filamentous cyanobacteria *Planktothrix rubescens* and *Planktothrix agardhii*. Molecular Ecology 15:3849-3861.

Kurmayer R, Christiansen G, Gumpenberger M, Fastner J (2005): Genetic identification of microcystin ecotypes in toxic cyanobacteria of the genus *Planktothrix*. Microbiology 151:1525-1533.

Kurmayer R, Christiansen G. Fastner J, Börner T (2004): Abundance of active and inactive microcystin genotypes in populations of the toxic cyanobacterium *Planktothrix* spp. Environmental Microbiology 6:831-841.

Kurmayer R, Christiansen G, Chorus I (2003): The abundance of microcystin-producing genotypes correlates positively with colony size in *Microcystis* sp. and determines its microcystin net production in Lake Wannsee. Applied and Environmental Microbiology 69:787-795.

Kurmayer R and Kutzenberger T (2003): Application of real-time PCR for quantification of microcystin genotypes in a population of the toxic cyanobacterium *Microcystis* sp. Applied and Environmental Microbiology 69:6723–6730.

Kurmayer R, Dittmann E, Fastner J, Chorus I (2002): Diversity of microcystin genes within a population of the toxic cyanobacterium *Microcystis* spp. in Lake Wannsee (Berlin, Germany). Microbial Ecology 43:107-118.

Long BM, Jones GJ, Orr PT (2001): Cellular microcystin content in N-limited *Microcystis aeruginosa* can be predicted from growth rate. Applied and Environmental Microbiology 67:278-283.

Luukkainen R, Sivonen K, Namikoshi M, Färdig M, Rinehart KL, Niemelä SI (1993): Isolation and identification of eight microcystins from thirteen *Oscillatoria agardhii* strains and structure of a new microcystin. Applied and Environmental Microbiology 59:2204-2209.

MacKintosh C, Beattie KA, Klumpp S, Cohen P, Codd GA (1990): Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. FEBS Letters 264:187-192.

Marahiel MA and Essen LO (2009): Nonribosomal peptide synthetases: Mechanistic and structural aspects of essential domains. Methods in Enzymology 458:337-351.

Martin C, Codd GA, Siegelman HW, Weckesser J (1989): Lipopolysaccharides and polysaccharides of the cell envelope of toxic *Microcystis aeruginosa* strains. Archives of Microbiology 152:90-94.

Matsunga S, Moore RE, Niemczura WP, Carmichael WW (1989): Anatoxin-a(S), a potent anticholinesterase from *Anabaena flos-aquae*. Journal of the American Chemical Society 111:8021-8023.

Metcalf JS and Codd GA (2012): Cyanotoxins. In The ecology of cyanobacteria II: Their diversity in time and space. Whitton BA (ed), Springer, Dordrecht, pp.651-676.

Metcalf JS and Codd GA (2009): The status and potential of cyanobacteria and their toxins as agents of bioterrorism. In Handbook on cyanobacteria: biochemistry, biotechnology and applications. Gault PM, Marler HJ (eds), Nova Publishers, New York.

Moffitt MC and Neilan BA (2004): Characterization of the nodularin synthetase gene cluster and proposed theory of the evolution of cyanobacterial hepatotoxins. Applied and Environmental Microbiology 70:6353-6362.

Mur LR, Skulberg OM, Utkilen H (1999): Cyanobacteria in the environment. In Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management. Chorus I and Bartram J (eds), E & FN Spon, London and New York, Chapter 2.

Mur LR, Schreurs H, Visser P (1993): How to control undesirable cyanobacterial dominance. In Strategies for lake ecosystems beyond 2000. Proceedings of the 5th International Conference on the Conservation and Management of Lakes. Giussani G and Callieri C (eds), Stresa, Italy, pp.565-569.

Nakasugi K, Alexova R, Svenson CJ, Neilan BA (2007): Functional analysis of PilT from the toxic cyanobacterium *Microcystis aeruginosa* PCC 7806. Journal of Bacteriology 189:1689-1697.

Nishizawa T, Asayama M, Fujii K, Harada K, Shirai M (1999): Genetic analysis of the peptide synthetase genes for a cyclic heptapeptide microcystin in *Microcystis* spp. The Journal of Biochemistry 126:520-529.

Norris RL, Eaglesham GK, Pierens G, Shaw GR, Smith MJ, Chiswell RK, Seawright AA, Moore MR (1999): Deoxycylindrospermopsin, as analog of cylindrospermopsin from *Cylindrospermopsis raciborskii*. Environmental Toxicology 14:163-165.

Nürnberg GK and LaZerte BD (2003): An artificially induced *Planktothrix rubescens* surface bloom in a small kettle lake in Southern Ontario compared to blooms world-wide. Lake and Reservoir Management 19:307-322.

Oberhaus L, Briand JF, Leboulanger C, Jaquet S, Humbert JF (2007): Comparative effects of the quality and quantity of light and temperature on the growth of *Planktothrix agardhii* and *P. rubescens*. Journal of Phycology 43:1191-1199.

Oh HM, Lee SJ, Jang MH, Yoon BD (2000): Microcystin production by *Microcystis aeruginosa* in a phosphorus-limited chemostat. Applied and Environmental Microbiology 66:176-179.

Okello W (2009): Molecular and chemical characterisation of microcystin producing cyanobacteria in Ugandan freshwater habitats. Dissertation, University of Vienna, Austria, 109pp.

Oliver RL and Ganf GG (2000): Freshwater blooms. In The ecology of cyanobacteria: their diversity in time and space. Whitton BA and Potts M (eds), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp.150-178.

Oliver RL and Walsby AE (1984): Direct evidence for the role of light-mediated gas vesicle collapse in the buoyancy regulation of *Anabaena flos-aquae* (Cyanobacteria). Limnology and Oceanography 29:879-886.

Orr PT and Jones GJ (1998): Relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures. Limnology and Oceanography 43:1604-1614.

Ostermaier V and Kurmayer R (2009): Distribution and abundance of nontoxic mutants of cyanobacteria in lakes of the Alps. Microbial Ecology 58:323-333.

Pan H, Song L, Liu Y, Börner T (2002): Detection of hepatotoxic *Microcystis* strains by PCR with intact cells from both culture and environmental samples. Archives of Microbiology 178:421-427.

Posch T, Köster O, Salcher MM, Pernthaler J (2012): Harmful filamentous cyanobacteria favoured by reduced water turnover with lake warming. Nature Climate Change 2:809-813.

Prinsep M, Caplan F, Moore R, Patterson G, Honkanen R, Boynton A (1992): Microcystin-LA from a blue-green alga belonging to the Stigonematales. Phytochemistry 31:1247-1248.

Quesada A, Sanchis D, Carrasco D (2004): Cyanobacteria in Spanish reservoirs. How frequently are they toxic? Limnetica 23:109-118.

Rantala A, Fewer DP, Hisbergues M, Rouhiainen L, Vaitomaa J, Börner T, Sivonen K (2004): Phylogenetic evidence for the early evolution of microcystin synthesis. Proceedings of the National Academy of Sciences of the United States of America 101:568-573.

Reynolds CS, Oliver RL, Walsby AE (1987): Cyanobacterial dominance: the role of buoyancy regulation in dynamic lake environments. New Zealand Journal of Marine and Freshwater Research 21:379-390.

Reynolds CS (1971): The ecology of the planktonic blue-green algae in the North Shropshire Meres, England. Field Studies 3:409-432.

Rippka R (1988): Recognition and identification of cyanobacteria. Methods in Enzymology 167:28-67.

Rippka R, DeReuelles J, Waterbury JB, Herdman M, Stanier RY (1979): Generic assignments, strain histories and properties of pure cultures of cyanobacteria. Journal of General Microbiology 111:1-61.

Rohrlack T, Edvardsen B, Skulberg R, Halstvedt CB, Utkilen HC, Ptacnik R, Skulberg, OM (2008): Oligopeptide chemotypes of the toxic freshwater cyanobacterium *Planktothrix* can form subpopulations with dissimilar ecological traits. Limnology and Oceanography 53:1279-1293.

Rohrlack T, Dittmann E, Henning M, Börner T, Kohl JG (1999): Role of microcystins in poisoning and food ingestion inhibition of *Daphnia galeata* caused by the cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 65:737-739.

Rouhiainen L, Vakkilainen T, Siemer BL, Buikema W, Haselkorn R, Sivonen K (2004): Genes coding for hepatotoxic heptapeptides (microcystins) in the cyanobacterium *Anabaena* strain 90. Applied and Environmental Microbiology 70:686-692.

Runnegar MTC, Gerdes RG, Falconer IR (1991): The uptake of the cyanobacterial hepatotoxin microcystin by isolated rat hepatocytes. Toxicon 29:43-51.

Sabart M, Pobel D, Briand E, Combourieu B, Salençon MJ, Humbert JF, Latour D (2010): Spatiotemporal variations in microcystin concentrations and in the proportions of microcystin-producing cells in several *Microcystis aeruginosa* populations. Applied and Environmental Microbiology 76:4750-4759.

Sano T and Kaya K (1995): A 2-amino-2-butenoic acid (Dhb)-containing microcystin isolated from *Oscillatoria agardhii*. Tetrahedron Letters 36:8603–8606.

Schatz D, Keren Y, Vardi A, Sukenik A, Carmeli S, Börner T, Dittmann E, Kaplan A (2007): Towards clarification of the biological role of microcystins, a family of cyanobacterial toxins. Environmental Microbiology 9:965-970.

Schopf JW (1993): Microfossils of the early Archean apex chert: New evidence of the antiquity of life. Science 260: 640-646.

Sevilla E, Martin-Luna B, Vela L, Bes MT, Peleato ML, Fillat MF (2010): Microcystin-LR synthesis as response to nitrogen: transcriptional analysis of the *mcy*D gene in *Microcystis aeruginosa* PCC7806. Ecotoxicology 19:1167-1173.

Sevilla E, Martin-Luna B, Vela L, Bes MT, Fillat MF, Peleato ML (2008): Iron availability affects *mcy*D expression and microcystin-LR synthesis in *Microcystis aeruginosa* PCC7806. Environmental Microbiology 10:2476-2483.

Sivonen K and Jones G (1999): Cyanobacterial toxins. In Toxic cyanobacteria in water. A guide to their public health consequences, monitoring and management. Chorus I and Bartram J (eds), E & FN Spon, London, pp.41-112.

Sivonen K (1990): Effects of light, temperature, nitrate, orthophosphate, and bacteria on growth of and hepatotoxin production by *Oscillatoria agardhii* strains. Applied and Environmental Microbiology 56:2658-2666.

Skulberg OM (1964): Algal problems related to the eutrophication of European water supplies, and a bio-assay method to assess fertilizing influences of pollution on inland waters. In Algae and Man. Jackson DF (ed), Plenum Press, New York, pp.262-299.

Smith JL, Boyer GL, Zimba PV (2008): A review of cyanobacterial odorous and bioactive metabolites: impacts and management alternatives in aquaculture. Aquaculture 280:5-20.

Stal LJ, Behrens SB, Villbrandt M, Van Bergeijk S, Kruyning F (1996): The biogeochemistry of two eutrophic marine lagoons and its effect on microphytobenthic communities. Hydrobiologia 329:185-198.

Subramaniam A and Carpenter EJ (1994): An empirically derived protocol for the detection of blooms of the marine cyanobacterium *Trichodesmium* using CZCS imagery. International Journal of Remote Sensing 15:1559-1569.

Tillett D, Parker DL, Neilan BA (2001): Detection of toxigenicity by a probe for the microcystin synthetase A gene (*mcy*A) of the cyanobacterial genus *Microcystis*: comparison of toxicities with 16S rRNA and phycocyanin operon (phycocyanin intergenic spacer) phylogenies. Applied and Environmental Microbiology 67:2810-2818.

Tillett D, Dittmann E, Erhard M, von Dohren H, Börner T, Neilan BA (2000): Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system. Chemistry & Biology 7:753-764.

Tonk L, Visser PM, Christiansen G, Dittmann E, Snelder EOFM, Wiedner C, Mur LR, Huisman J (2005): The microcystin composition of the cyanobacterium *Planktothrix agardhii* changes toward a more toxic variant with increasing light intensity. Applied and Environmental Microbiology 71:5177-5181.

Tooming-Klunderud AT, Fewer DP, Rohrlack T, Jokela J, Rouhiainen L, Sivonen K, Kristensen T, Jakobsen KS (2008): Evidence for positive selection acting on microcystin synthetase adenylation domains in three cyanobacterial genera. BMC Evolutionary Biology 8:256.

Turner PC, Gammie AJ, Hollinrake K, Codd GA (1990): Pneumonia associated with cyanobacteria. British Medical Journal 300:1440-1441.

Turner S (1997): Molecular systematics of oxygenic photosynthetic bacteria. Plant Systematics and Evolution Supplement 11:13-52.

Utkilen H and Gjølme N (1992): Toxin production by *Microcystis aeruginosa* as a function of light in continuous cultures and its ecological significance. Applied and Environmental Microbiology 58:1321-1325.

Vaitomaa J, Rantala A, Halinen K, Rouhiainen L, Tallberg P, Mokelke L, Sivonen K (2003): Quantitative real-time PCR for determination of microcystin synthetase E copy numbers for *Microcystis* and *Anabaena* in lakes. Applied and Environmental Microbiology 69:7289-7297.

Van de Waal DB, Verspagen JMH, Finke JF, Vournazou V, Immers AK, Kardinaal WEA, Tonk L, Becker S, Van Donk E, Visser PM, Huisman J (2011): Reversal in

competitive dominance of a toxic versus non-toxic cyanobacterium in response to rising CO_2. ISME Journal 5:1438-1450.

Van Gremberghe I, Van der Gucht K, Vanormelingen P, Asmelash T, Dejenie T, D'hondt S, Declerck S, De Meester L, Vyverman W (2011): Genetic diversity of *Microcystis* blooms (Cyanobacteria) in recently constructed reservoirs in Tigray (Northern Ethiopia) assessed by rDNA ITS. Aquatic Ecology 45:289-306.

Vézie C, Rapala J, Vaitomaa J, Seitsonen J, Sivonen K (2002): Effect of nitrogen and phosphorus on growth of toxic and nontoxic *Microcystis* strains and on intracellular microcystin concentrations. Microbial Ecology 43:443-454.

Walsby AE, Schanz F, Schmid M (2006): The Burgundy-blood phenomenon: a model of buoyancy change explains autumnal waterblooms by *Planktothrix rubescens* in Lake Zürich. New Phytologist 169:109-122.

Walsby AE (2005): Stratification by cyanobacteria in lakes: a dynamic buoyancy model indicates size limitations met by *Planktothrix rubescens* filaments. New Phytologist 168:365-376.

Walsby AE, Avery A, Schanz F (1998): The critical pressures of gas vesicles in *Planktothrix rubescens* in relation to the depth of winter mixing in Lake Zürich, Switzerland. Journal of Plankton Research 20:1357-1375.

Welker M and von Döhren H (2006): Cyanobacterial peptides – Nature's own combinatorial biosynthesis. FEMS Microbiology Reviews 30:530-563.

Whitton BA and Potts M (eds) (2000): The ecology of cyanobacteria: their diversity in time and space. Kluwer Academic Publishers, Dordrecht, The Netherlands, Color Plates 1b and 16b (Potts M), 1f, 1i (Boulton PNG and Whitton BA) and 3a (Schopf JW).

WHO (1997): Report of the Working Group on chemical substances in drinking water, Geneva, 22-26 April 1997, Section 5.2, microcystin-LR. World Health Organisation, Geneva.

Wilson AE, Wilson WA, Hay ME (2006): Intraspecific variation in growth and morphology of the bloom-forming cyanobacterium *Microcystis aeruginosa*. Applied and Environmental Microbiology 72:7386-7389.

Wood SA, Rueckert A, Hamilton DP, Craig SC, Dietrich DR (2011): Switching toxin production on and off: intermittent microcystin synthesis in a *Microcystis* bloom. Environmental Microbiology Reports 3:118-124.

Wynn-Williams DD (2000): Cyanobacteria in Deserts – Life at the Limit? In The ecology of cyanobacteria: their diversity in time and space. Whitton BA and Potts M (eds), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp.341-366.

Ye W, Liu X, Tan J, Li D, Yang H (2009): Diversity and dynamics of microcystin-producing cyanobacteria in China's third largest lake, Lake Taihu. Harmful Algae 8:637-644.

Yoshida M, Yoshida T, Takashima Y, Hosoda N, Hiroishi S (2007): Dynamics of microcystin-producing and non-microcystin producing *Microcystis* populations is correlated with nitrate concentration in a Japanese lake. FEMS Microbiology Letters 266:49-53.

Zilliges Y, Kehr JC, Meissner S, Ishida K, Mikkat S, Hagemann M, Kaplan A, Börner T, Dittmann E (2011): The cyanobacterial hepatotoxin microcystin binds to proteins and increases the fitness of *Microcystis* under oxidative stress conditions. Plos ONE 6, e17615.

PART II

Chapter 1

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Spatial isolation favours the divergence in microcystin net production by *Microcystis* in Ugandan freshwater lakes

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ABSTRACT

It is generally agreed that the hepatotoxic microcystins (MCs) are the most abundant toxins produced by cyanobacteria in freshwater. In various freshwater lakes in East Africa MC-producing *Microcystis* has been reported to dominate the phytoplankton, however the regulation of MC production is poorly understood. From May 2007 to April 2008 the *Microcystis* abundance, the absolute and relative abundance of the *mcy*B genotype indicative of MC production and the MC concentrations were recorded monthly in five freshwater lakes in Uganda: (1) in a crater lake (Lake Saka), (2) in three shallow lakes (Lake Mburo, George, Edward), (3) in Lake Victoria (Murchison Bay, Napoleon Gulf). During the whole study period *Microcystis* was abundant or dominated the phytoplankton. In all samples mcyB-containing cells of *Microcystis* were found and on average comprised 20 ± 2% (SE) of the total population. The proportion of the mcyB genotype differed significantly between the sampling sites, and while the highest mcyB proportions were recorded in Lake Saka (37 ± 3%), the lowest proportion was recorded in Lake George (1.4 ± 0.2%). Consequently *Microcystis* from Lake George had the lowest MC cell quotas (0.03–1.24 fg MC cell^{-1}) and resulted in the lowest MC concentrations (0–0.5 μg L^{-1}) while *Microcystis* from Lake Saka consistently showed maximum MC cell quotas (14–144 fg cell^{-1}) and the highest MC concentrations (0.5–10.2 μg L^{-1}). Over the whole study period the average MC content per *Microcystis* cell depended linearly on the proportion of the mcyB genotype of *Microcystis*. It is concluded that *Microcystis* populations differ consistently and independently of the season in mcyB genotype proportion between lakes resulting in population-specific differences in the average MC content per cell.

1. Introduction

During the last decades cyanobacteria in freshwater have been of interest due to their ability to produce various hepatotoxic and neurotoxic substances. It is generally agreed that the hepatotoxic microcystins (MCs) are the most abundant toxins produced by cyanobacteria in freshwater (WHO, 2006; Erdner et al., 2008; Hudnell, 2008). MCs are cyclic

Abbreviations: MC, microcystin; *mcy*, gene encoding the MC synthetase; HPLC, high performance liquid chromatography; DAD, diode array detection; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PC, the phycocyanin gene.

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heptapeptides that share the common structure cyclo (-D-Ala(1)–X(2)–D-MeAsp(3)–Z(4)–Adda(5)–D-Glu(6)–Mdha(7)), where X and Z are variable L-amino acids (e.g. MC-LR refers to leucine and arginine in the variable positions of this peptide), D-MeAsp is D-erythro-β-iso-methyl-aspartic acid, Adda is (2*S*, 3*S*, 8*S*, 9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, and Mdha is *N*-methyl-dehydroalanine. Structural variation has been reported most frequently in positions 2, 4, and 7 of the MC molecule resulting in over 80 structural variants that have been characterized from field samples or isolated strains (Krüger et al., 2009). In a recent paper, we could show that cyanobacteria contribute significantly to the phytoplankton of freshwater lakes in Uganda while other algal groups like diatoms, green algae, and cryptomonads are of a relatively minor importance (Okello et al., 2009). We further concluded that in Uganda the genus *Microcystis* is the only MC-producing genus which is favoured under more shallow, eutrophic conditions which is in correspondence to the general theory on how physical factors govern phytoplankton associations (Reynolds et al., 2002). In this earlier study we did not monitor *Microcystis* populations as well as the MC net production of the phytoplankton community during different seasons. This is of relevance as it is known that beside the absolute population abundance it is the proportion of MC-producing genotypes (those containing the *mcy* gene cluster encoding MC synthesis) vs. non-MC-producing genotypes (those lacking the *mcy* gene cluster) that has a decisive influence on MC net production (Sivonen and Jones, 1999; Kurmayer and Christiansen, 2009). So far research was unable to document an adaptive value of MC production, thus making it difficult to identify proximate factors triggering MC synthesis (Kaebernick and Neilan, 2001; Schatz et al., 2007). Although the transcription rate of the *mcy* gene cluster is increased from low to high irradiance conditions it is generally agreed that MC synthesis is constitutive (Kaebernick and Neilan, 2001). In the course of seasonal studies several researchers reported the occurrence of seasonal shifts in the proportion of MC-producing vs. non-MC-producing genotypes in dependence on various abiotic or biotic environmental factors (Briand et al., 2008; Hotto et al., 2008). In East Africa usually dry seasons with precipitation minima and wet seasons with maxima of precipitation have been correlated with changes in phytoplankton composition (Talling, 1986). During the rainy season (from March to May and August–November), the phytoplankton in shallow lakes will be affected directly by a reduced water temperature (2.5 °C in Kasese and 3 °C in Kampala), reduced light availability in the water column as well as increased terrestrial run-off. Only in deep lakes such as the main basin of Lake Victoria, the mixing regime will change, as a higher stability of the water column has been described during the dry season (Talling, 1986). These physical changes in the water column have a significant effect on phytoplankton community composition (Talling, 1987). Generally in shallow lakes less seasonality in phytoplankton composition is observed when compared with the main basin of Lake Victoria.

In contrast to the seasonal influence we recently emphasized that it is rather the spatial isolation of populations than the seasonal influence of biotic or abiotic factors that leads to differences in the population structure of MC-producing genotypes (Kurmayer and Gumpenberger, 2006). We further concluded that the structural variation within the position 2 of the MC molecule is selectively neutral (Kurmayer and Gumpenberger, 2006) implying that genetic drift resulting from geographic isolation has the potential to lead to new MC variants that appear unique and dominant in particular waterbodies, i.e. [Asp^3, Dhb^7]-MC-HtyY and [Asp^3, Dhb^7]-MC-HtyHty (Christiansen et al., 2008a). For lakes located in the Alps of Austria, Germany and Switzerland we hypothesized that populations diverge in their *mcy* genotype composition at least during consecutive years (Ostermaier and Kurmayer, 2009). However, we also observed that the genetic population structure changed between years, for example due to the extinction of the population during winter and a subsequent re-immigration of new genotypes (Kurmayer and Gumpenberger, 2006).

In this study we aimed to investigate the seasonal development of *Microcystis* and potential MC-producing genotypes and the resulting MC concentrations in five freshwater lakes in Uganda. *Microcystis* has been reported to dominate in shallow eutrophic lakes such as Lake George at least for decades (Ganf, 1974). While the shallow lakes close to the Ruwenzori mountain (L. George, L. Edward, L. Mburo) are naturally eutrophic, the genus *Microcystis* also has become abundant in bays of Lake Victoria as a result of human induced eutrophication (Hecky, 1993; Mugidde, 1993; Verschuren et al., 2002). It is hypothesized that if spatial isolation leads to genetic divergence in MC production then the variation in *mcy* genotype proportion between sites should significantly exceed the variation in *mcy* genotype proportion that is observed within sites during the season. *Vice versa* if spatial isolation is of minor importance, the seasonal variation in *mcy* genotype proportion as caused by unknown biotic and abiotic factors that is observed within sites should significantly exceed the between site variation.

2. Materials and methods

2.1. *Description of the study sites*

From five freshwater lakes in Uganda six sampling sites were chosen (Fig. 1): (1) A site in the center of Lake Saka (N0°41.670′, E30°14.667′), mean depth of 3.6 m. Lake Saka is a small crater lake (1.4 km^2) located at an altitude of 1520 m.a.s.l.. The other sampling sites included the shallow eutrophic lakes (2) Lake George, (3) Lake Edward, (4) Lake Mburo as well as Lake Victoria, (5) Murchison Bay near Kampala, and (6) Napoleon Gulf near Jinja, which were described previously (Okello et al., 2009).

2.2. *Field sampling and analysis of* Microcystis *abundance*

Depth-integrated water samples and plankton net samples (30 μm mesh size) were taken monthly from May 2007 until April 2008 as described (Okello et al., 2009). For DNA and MC analysis aliquots (250–2400 ml) were filtered onto GF/C filters (∅ 47 mm, Whatman, Kent, Great Britain) and the filters were dried at 50 °C. *Microcystis* were counted by the inverted

Fig. 1 – Map of Uganda showing the six sampling sites (black circles).

microscope technique from Lugol fixed samples following standard techniques (Wetzel and Likens, 2000). The genus *Microcystis* was discriminated from other cyanobacteria according to Komárek and Anagnostidis (1999). Following the taxonomic revision for the genus *Microcystis* Kützing ex Lemmerman 1907 (Otsuka et al., 2001) all morphospecies were considered morphological varieties of individuals of *Microcystis aeruginosa* comb. nov. Kützing 1833. For each sample 400 specimens of *Microcystis* and other dominant phytoplankton genera were counted at 400-fold magnification. Only the data on *Microcystis* cell numbers and *Microcystis* biovolume are reported here while the phytoplankton community composition will be described in another publication. The average biovolume of a *Microcystis* cell was $76 \pm 12\ \mu m^3$ (1SE).

2.3. Quantification of the microcystin genotype

To estimate the abundance and the proportion of the MC-producing genotype, DNA was extracted from aliquots of the samples analyzed for *Microcystis* cell numbers and for MC as described previously (Kurmayer et al., 2003). The absolute and relative cell numbers of *Microcystis* and the *Microcystis* genotype containing the mcyB gene that is indicative of MC production were determined by means of quantitative real-time PCR as described (Kurmayer and Kutzenberger, 2003) and the same primers and probes have been used. To quantify the total population of *Microcystis* the linear regression was $y = -3.4552x + 36.229$ ($n = 6$, $R^2 = 0.998$), where y was the cycle of threshold (C_t value) at the set fluorescence threshold level obtained for the intergenic spacer region of the phycocyanin gene (PC) and x was the amount of starting DNA (given as $\log_{10}$ cell number equivalents of *Microcystis* strain HUB524). To quantify the *Microcystis* cells containing the mcyB gene only the linear regression curve was $y = -3.9759x + 40.712$ ($n = 6$, $R^2 = 0.994$), where y was the C_t value at the set fluorescence threshold level obtained for the mcyB gene and x was the amount of starting DNA aliquots used for PC (given as $\log_{10}$ cell number equivalents of *Microcystis* strain HUB524). The relative abundance of the mcyB genotype of *Microcystis* was determined by dividing the cell numbers of the mcyB genotype through the cell numbers of the total population (as estimated from the PC genotype). All measurements were done in triplicate using an Eppendorf mastercycler ep realplex system (Eppendorf, Vienna). Both gene regions that were amplified by PCR have been shown to be specific for *Microcystis* in the presence of other MC-producing cyanobacteria such as *Anabaena* or *Planktothrix* (Kurmayer and Kutzenberger, 2003). In order to include the whole study period all depth-integrated samples from all six sampling sites ($n = 72$) as well as plankton net samples from Lake George ($n = 4$) and Lake Mburo ($n = 4$) were analyzed.

2.4. Microcystin analysis

Phytoplankton collected on filter was extracted in aqueous methanol as described (Okello et al., 2009). The clear supernatants of extracts were analyzed by high performance liquid chromatography-diode array detection (HPLC-DAD) as described using a HP1100 Chemstation (Lawton et al., 1994; Kurmayer et al., 2003). MCs were quantified at 240 nm and the concentration of all MC variants was determined as concentration equivalents of [MeAsp, Mdha]-MC-LR (Cyanobiotech GmbH, Berlin, Germany). The concentration of MC-LR was calculated from the regression curve $y = 1885.3x - 6.8775$, ($R^2 = 0.99$), where y was the absorption (mAU) recorded at 240 nm and x was the μg of MC-LR injected.

HPLC fractions identified as MC were collected manually and analyzed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS), (PerSeptive BioSystems, Framingham MS, USA) as described (Erhard et al., 1997). The constitution of the new MC variant [$NMeSer^7$]-MC-YR was assigned by ESI-MS and $ESI\text{-}MS^2$ experiments that were performed on a Q-TOF Ultima mass spectrometer (Waters, Milford, MA.) equipped with a nanospray source and operated in the positive ionization mode under the control of MassLynx 4.1.

2.5. Statistical analysis

The linear regression curves were fitted using the least square approximation and the associated statistical tests of Sigma Plot 2000 (V 6.10). The data were $\log_{10}$ transformed in order to achieve normal distribution and constant variances. The linear regressions between the total *Microcystis* cell number (as estimated from the microscope) and the abundance of the PC genotype and the mcyB genotype were compared in slope and intercept using a general factorial model of analysis of variance (ANOVA). The data were modeled as $y = \mu + \beta x + \varepsilon$, where y is the measured abundance of the PC genotype or the mcyB genotype, μ is the overall mean level, β is the effect of the PC or the mcyB genotype, x is the effect of the cell number as determined from the microscope as a covariate, and ε is the

random deviation. To compare the mcyB proportions between sampling sites one-way ANOVA was used.

Multiple linear regression analysis was used to test the relationship between the MC concentration (in $\mu g\,MC\,ml^{-1}$) as dependent variable and the influence of *Microcystis* cell numbers as determined by the microscope or real-time PCR via PC and mcyB as independent variables. A forward stepwise analysis was employed selecting for the independent variable for inclusion that makes the most significant unique contribution to the prediction of the data. Calculations were performed using SPSS 15.0 for Windows and the F value to enter the respective model was set default ($p < 0.05$).

3. Results

3.1. Microcystis *abundance*

Microcystis appeared throughout the sampling period in all the lakes. Only on 30 September 2007 *Microcystis* abundance was below the detection limit in the depth-integrated sample obtained from Lake Victoria (Napoleon Gulf). During the study period *Microcystis* cell numbers varied between 10^4 –10^6 (Lake Saka), 10^5 –10^6 (L. George), 10^5 –10^6 (L. Edward), 10^4 –10^5 (L. Mburo), 10^4–10^5 (L. Victoria, Murchison bay), and 10^3–10^4 cells ml^{-1} (L. Victoria, Napoleon Gulf). We recorded the highest *Microcystis* biovolume in samples from Lake George (273 $mm^3\,l^{-1}$) and the lowest (0.3 $mm^3\,l^{-1}$) in samples from Lake Victoria (Napoleon Gulf). On average *Microcystis* constituted between 18 and 46% of the total phytoplankton biovolume (Table 1). This implied that the phytoplankton at all the sampling sites had the potential for MC production.

3.2. *Quantification of the microcystin genotype*

In order to test the hypothesis that populations of *Microcystis* differ in the proportion of the *mcy* genotype, the absolute abundance of both the total population as well as the subpopulation containing the mcyB gene were determined by means of real-time PCR. Overall, both the microscope as well as real-time PCR showed congruent results in estimating the total *Microcystis* population number that on average differed by one order of magnitude in lakes George and Edward, and less than an order of magnitude in the other lakes (Fig. 2). Consequently over the study period a linear relationship between cell numbers estimated via the microscope and real-time PCR of the PC genotype was found (Fig. 3A): The regression curve was $y = 0.595x + 1.661$ ($n = 80$, $R^2 = 0.62$), where y is the $\log_{10}$ cell number as determined by the real-time PCR (PC) and x is the $\log_{10}$ cell number as counted in the microscope.

In all samples mcyB-containing cells of *Microcystis* were found and on average comprised 19.9 ± 1.8 (SE) % of the total population (min = 0.5, max = 66.6%). In contrast to PC a relatively weak relationship between cell numbers as counted in the microscope and cell numbers of the mcyB genotype was found (Fig. 3B): $y = 0.3222x + 2.1935$ ($n = 80$, $R^2 = 0.13$) and the two regression curves were not parallel (ANOVA, $p < 0.001$). Particularly the measurements obtained from Lake Saka and Lake George showed a much wider scatter when compared with the measurements on the PC genotype. The proportion of the mcyB genotype differed significantly between the sampling sites (Fig. 3C): While the highest mcyB proportions were recorded in Lake Saka (min – mean ± SE – max, 24.4 – 37.0 ± 3.3 – 66.6%) and in Napoleon Gulf (12.9 – 31.6 ± 3.8 – 59.3%), the by far lowest proportion was recorded in Lake George (0.49 – 1.4 ± 0.2 – 3.6%). At the other three sites, Lake Edward (7.5 – 14.9 ± 2.8 – 36.7%), Lake Mburo (6.1 – 14.0 ± 2.2 – 32.3%), and Murchison Bay (3.3 – 16.4 ± 3.1 – 39.2%) an intermediate proportion was recorded. It is concluded that the significant differences in the proportion of the mcyB genotype between the lakes resulted in a poor correlation between microscopically determined cell numbers and the cell numbers of the mcyB genotype.

3.3. *Microcystin net production*

3.3.1. *Composition of microcystins*

The vast majority of the samples were found to contain MC. HPLC-DAD analyses documented the occurrence of eleven MC structural variants that showed an unequivocal match with the spectrum of either MC-RR, or MC-YR, or MC-LR. All fractions identified as MC by HPLC were collected and analyzed by means of MALDI-TOF for their molecular weight. The following variants could be undoubtedly identified by their retention time, their mass and by spiking using MC-RR, YR, LR standards: MC1, [Asp^3]-MC-RR ($M + H^+$ 1024), MC2, MC-RR ($M + H^+$ 1038), MC4, [Asp^3]-MC-YR ($M + H^+$ 1031), MC5, MC-YR ($M + H^+$ 1045), MC6, MC-LR ($M + H^+$ 995), MC8, [Asp^3]-MC-RY ($M + H^+$ 1031), MC9, MC-RY ($M + H^+$ 1045). [Asp^3]-MC-RY and MC-RY have been identified recently (Okello et al., 2009). The MC3 variant was determined as [$NMeSer^7$]-MC-YR by LC-MS^2 (Suppl. Table 1). The others were considered unknown: MC7 ($M + H^+$ 1013), MC10 ($M + H^+$ 1024), MC11 ($M + H^+$ 1031).

Table 1 – Cell numbers (min – mean ± SE – max, cells ml^{-1}), biovolume ($mm^3\,l^{-1}$) and proportion (%) of *Microcystis* (in total phytoplankton) in depth-integrated samples from the six sampling sites from May 2007 to April 2008 (n = 12).

	Cell numbers	Biovolume	Proportion
Lake Saka	$8 \times 10^3 - 1.4 \times 10^5 \pm 3.3 \times 10^4 - 4.5 \times 10^5$	0.6 – 10.5 ± 2.6 – 34.6	1.7 – 18.3 ± 3.8 – 41.7
Lake George	$1.5 \times 10^5 - 1.0 \times 10^6 \pm 2.7 \times 10^5 - 3.6 \times 10^6$	11.6 – 81 ± 21 – 273	3.6 – 27.5 ± 5.2 – 67
Lake Edward	$4.5 \times 10^4 - 4.1 \times 10^5 \pm 1.6 \times 10^5 - 2.3 \times 10^6$	3.5 – 31 ± 12 – 177	3.8 – 20 ± 5.4 – 77
Lake Mburo	$5.5 \times 10^4 - 1.7 \times 10^5 \pm 2 \times 10^4 - 2.9 \times 10^5$	4.2 – 12.6 ± 1.5 – 22	10.5 – 21.5 ± 2.1 – 37.2
Murchison Bay	$4 \times 10^4 - 1.5 \times 10^5 \pm 2 \times 10^4 - 3 \times 10^5$	3.1 – 11.4 ± 1.5 – 23.2	21.6 – 45.6 ± 3.3 – – 59.9
Napoleon Gulf	$4.4 \times 10^3 - 2 \times 10^4 \pm 4 \times 10^3 - 4.5 \times 10^4$	0.3 – 1.6 ± 0.3 – 3.5	0 – 21.5 ± 3.7 – 44.1

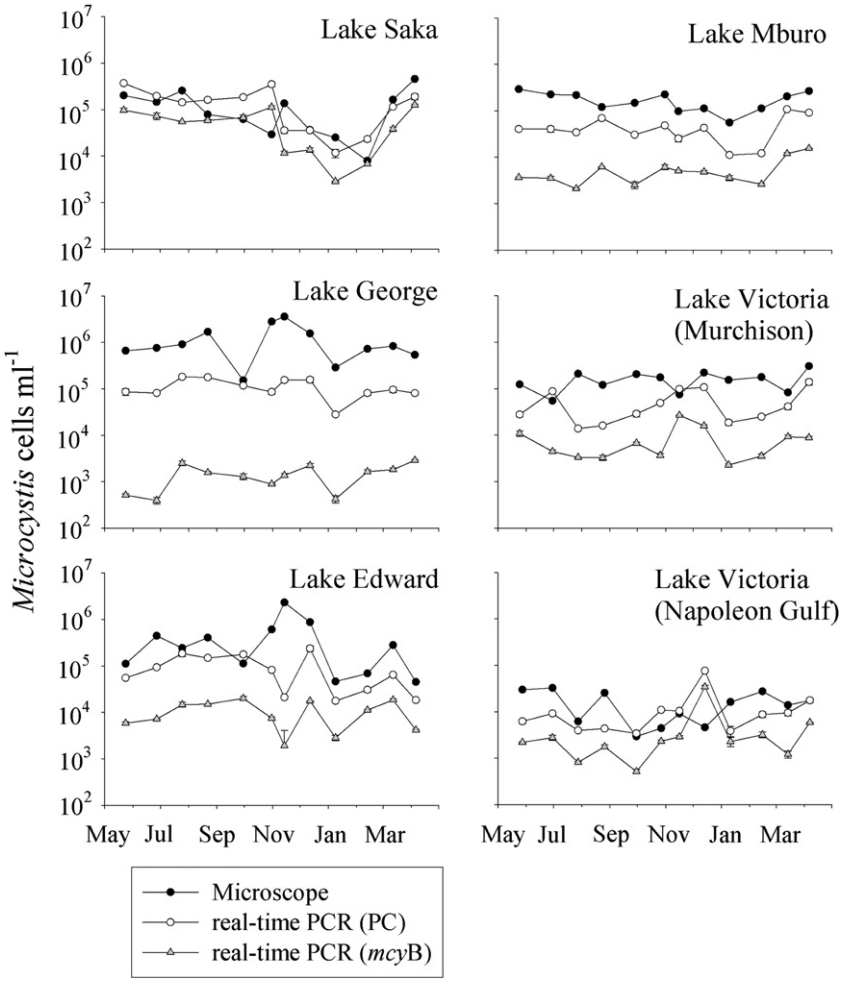

Fig. 2 – *Microcystis* cells (ml^{-1}) as estimated by the microscope (black circles), and by real-time PCR via the phycocyanin (PC) gene (white circles) and the mcyB gene (grey triangles) at the six sampling sites from May 2007 to April 2008. For the PCR estimates the mean ± SE is shown.

The sampling sites differed significantly in the relative abundance of all the MC variants (Chi square test, $p < 0.01$). For example MC-RR and [Asp^3]-MC-RR were most frequent in lakes Saka, Mburo and Edward and in Murchison Bay. In contrast MC-RY and [Asp^3]-RY were dominant at all sites except in Napoleon Gulf (Table 2). Surprisingly in the samples from Napoleon Gulf the new [$NMeSer^7$]-MC-YR variant occurred most frequently. Taking all sampling sites together MC-RY was most abundant, followed by MC-RR and MC-YR. In contrast MC-LR only occurred in 11% of all the samples.

3.3.2. *Concentration of microcystins*

The contribution of each MC variant to the total MC concentration (calculated as MC-LR equivalents) closely matched the frequency of occurrence. For example, MC-RR contributed on average ≥50% to the total MC in lakes Saka and Mburo. MC-RY contributed >50% to the total MC in lakes George and Edward and in Murchison Bay. All MC detected in Napoleon Gulf was dominated by the [$NMeSer^7$]-MC-YR variant (Table 2). The phytoplankton further differed significantly in the concentration of MC in total ($p < 0.0001$, ANOVA). On average, the MC concentrations were 28-fold higher in Lake Saka ($4.7 \pm 0.9\ \mu g\ l^{-1}$) when compared with the average ($0.2 \pm 0.1\ \mu g\ l^{-1}$) MC concentration measured in Lake George (Fig. 4). Samples from Lake Saka had the maximum MC concentration ($10\ \mu g\ l^{-1}$) in July 2007. The minimum concentrations ($0.02\ \mu g\ l^{-1}$) were recorded in Lake George in May 07, June 07, January 08 and April 08. At the sampling sites in the other three lakes intermediate MC concentrations (0.1–$2.5\ \mu g\ l^{-1}$) were recorded. Within the lakes, the total MC concentration varied seasonally from 12-fold to 30-fold.

3.4. *Genotype determined microcystin production*

3.4.1. *Relation of the PC and mcyB genotypes to microcystin concentrations*

Both PC and mcyB genotype cell numbers were highly significantly related to MC concentrations: $y = 1.33 \times 10^{-8}x + 0.000195$ ($R^2 = 0.28$) for the PC genotype and $y = 6.16 \times 10^{-8}x + 0.00038+$ ($R^2 = 0.62$) for the mcyB genotype. With the exception of Lake George the cells of the mcyB genotype showed an increase of the

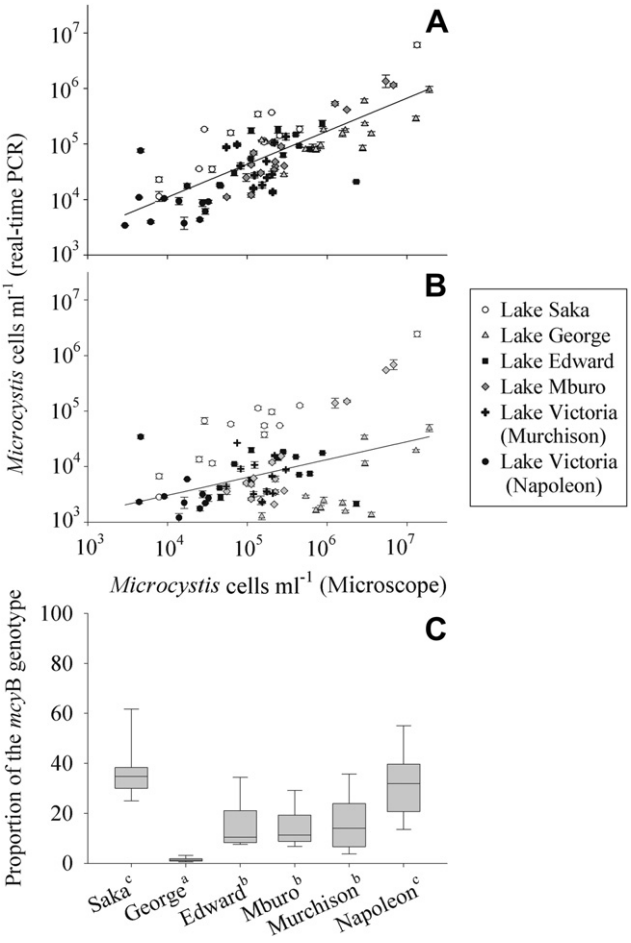

Fig. 3 – Relationship between the *Microcystis* cell numbers as estimated in the microscope and the cell numbers as estimated via real-time PCR (mean ± SE) for the (A) phycocyanin gene (indicative of the total *Microcystis* population), (B) the mcyB gene (indicative of MC production) at the six sampling sites from May 2007 to April 2008. The details on the regression curves are given in the text. (C) Proportion of the mcyB genotype at the six sampling sites for the same data set. The whiskers of each box indicate the 10th and 90th percentiles (n = 12). The differences were tested by one-way ANOVA followed by the Tukey post-hoc comparison procedure ($p < 0.001$). Superscripts indicate homogeneous subsets ($p > 0.05$).

average MC content by a factor of 2.9–7.8 when compared with the MC content of the PC genotype (Fig. 5A). Corresponding to the lowest proportion of the mcyB genotype in Lake George (Fig. 3C) the MC content of the mcyB genotype from Lake George showed a 69-fold increase when compared with the MC content of the PC genotype. Consequently the mcyB genotype occurring at the six sampling sites rather differed in absolute numbers than in the *in situ* activity or in the regulation of MC net production.

3.4.2. *Relation of* Microcystis *cells to microcystin concentrations*

For all sampling sites highly significant positive linear relationships between the total MC concentration and *Microcystis* cell numbers were obtained (Table 3). However, relating the total MC concentrations to *Microcystis* cell numbers revealed a >100-fold variation in the average MC contents per cell between lakes (Fig. 5B). Corresponding to its lowest mcyB genotype proportion *Microcystis* from Lake George consistently showed the lowest MC cell quotas (0.03–1.24 fg cell^{-1}) while *Microcystis* from Lake Saka showed maximum MC cell contents (14–144 fg cell^{-1}). While the between site variation was found reduced in plankton net samples the ranking of sampling sites by their average MC contents per *Microcystis* cell was not affected (data not shown). It is concluded that at all sites MC production was related to the occurrence of *Microcystis* as enumerated in the microscope while between sites the populations differ consistently and independently of the season in their average MC content per cell.

3.4.3. *Relation of the mcyB genotype proportion to the microcystin content*

Over the study period the proportion of the mcyB genotype was linearly related to the average cellular MC content per cell: $y = 1.2884x - 0.7835$ ($n = 77$, $R^2 = 0.58$), where x is the $\log_{10}$ proportion of the mcyB genotype and y is the $\log_{10}$ MC content in fg MC cell^{-1} (Fig. 5C). In order to explain MC concentrations the forward multiple regression analysis revealed a significant inclusion of the mcyB genotype abundance as the first and most significant predictor variable ($R^2 = 0.68$) and subsequently the microscopically determined *Microcystis* cell numbers as the second predictor variable ($R^2 = 0.73$): $y = 0.862x + 0.256z - 7.805$ ($R^2 = 0.73$, $n = 77$), where x is the $\log_{10}$ abundance of the mcyB genotype (cells ml^{-1}), z is the $\log_{10}$ cell number (ml^{-1}) determined in the microscope and y is the $\log_{10}$ MC concentration (μg ml^{-1}). It is concluded that the *Microcystis* populations differ genetically in the mcyB proportion which can indeed explain the variation in the average MC content of *Microcystis* cells observed among the lakes during the study period.

4. Discussion

4.1. *Correlation of* Microcystis *cell numbers with microcystin net production*

For all lakes the abundance of *Microcystis* cells was significantly positively related to MC production. In contrast MC production was negatively related to the abundance of *Planktothrix* in Lake Saka and to the abundance of *Anabaena* in Lake Victoria in Napoleon Gulf and Murchison Bay. Significant relationships between the total MC concentration and *Anabaena* cell numbers were observed for the sites in lakes Mburo, Murchison Bay and Napoleon Gulf (data not shown). However, as we were unable to detect genes involved in MC production of any other taxa than *Microcystis* in the same habitats (Okello et al., 2009), we consider this relationship as due to the co-occurrence of these taxa and *Microcystis* (Okello et al., 2009). In addition nine strains of *Planktothrix* sp. were isolated from Lake Saka in April 2008 and analyzed for MC production. None of the strains were found to contain MCs and/or the mcyE/mcyB gene part of the *mcy* gene cluster (Rainer Kurmayer, unpublished results).

Table 2 – Relative frequency of occurrence (%) and proportion (mean ± SE) in HPLC chromatograms of each microcystin variant in the depth-integrated and the plankton net samples at the six sampling sites from May 2007 to April 2008 (n = 24). For each site the most abundant MC variant is marked in Bold.

	MC1	MC2	MC3	MC4	MC5	MC6	MC7	MC8	MC9	MC10	MC11
$M+H^+$	1024	1038	1063	1031	1045	995	1013	1031	1045	1024	1031
Structural variant	[Asp^3]MC-RR	MC-RR	[$NMeSer^7$]-MC-YR	[Asp^3]MC-YR	MC-YR	MC-LR	Unknown	[Asp^3]MC-RY	MC-RY	Unknown	Unknown
Retention time (min)	14–14.5	15.1–15.7	16.0–16.4	17.0–17.7	18.0–18.9	19.0–19.9	20.9	21.0–21.8	23.0–23.9	24.0–25.0	27.8–27.9
Lake Saka											
Frequency	17	**100**	13	21	67	63	0	67	83	17	0
Proportion	0.8 ± 0.6	**58.7 ± 4.1**	3.2 ± 2	0.4 ± 0.2	17.3 ± 3.5	4.4 ± 1.3		8.0 ± 2.8	7.0 ± 1.3	0.1 ± 0.07	
Lake George											
Frequency	0	4	0	0	0	0	0	33	**92**	0	0
Proportion		1.6 ± 1.6						12.0 ± 4.4	**78.0 ± 6.6**		
Lake Edward											
Frequency	25	67	0	38	46	4	0	13	**96**	50	33
Proportion	0.6 ± 0.2	8.3 ± 1.7		0.3 ± 0.1	1.8 ± 0.5	0.03 ± 0		5.9 ± 4.3	**81.1 ± 4.5**	1.0 ± 0.2	0.3 ± 0.1
Lake Mburo											
Frequency	58	96	0	54	92	58	67	79	**100**	17	54
Proportion	2.6 ± 0.9	**46.9 ± 2.8**		0.8 ± 0.2	12.9 ± 1.5	1.6 ± 0.4	1.8 ± 0.4	2.2 ± 0.5	**27.7 ± 3.5**	2.6 ± 0.4	0.4 ± 0.1
Murchison Bay											
Frequency	0	63	29	0	25	79	0	0	88	33	0
Proportion		3.9 ± 1.9	7.8 ± 3.0		2.9 ± 1.4	12.4 ± 2			**48.8 ± 6.3**	6.7 ± 1.7	
Napoleon Gulf											
Frequency	0	17	**88**	0	17	8	0	4	4	4	0
Proportion		3.9 ± 1.9	**82.1 ± 6.9**		4.7 ± 2.4	0.5 ± 0.3		0.5 ± 0.5	2.1 ± 2.1	2 ± 2	
Total											
Frequency	17	58	22	19	41	35	11	33	77	20	15
Proportion	0.7 ± 0.2	23.0 ± 2.1	15.5 ± 2.8	0.3 ± 0.1	6.6 ± 0.9	3.3 ± 0.6	0.3 ± 0.09	4.8 ± 1.2	40.5 ± 3.2	2 ± 0.5	0.1 ± 0.02

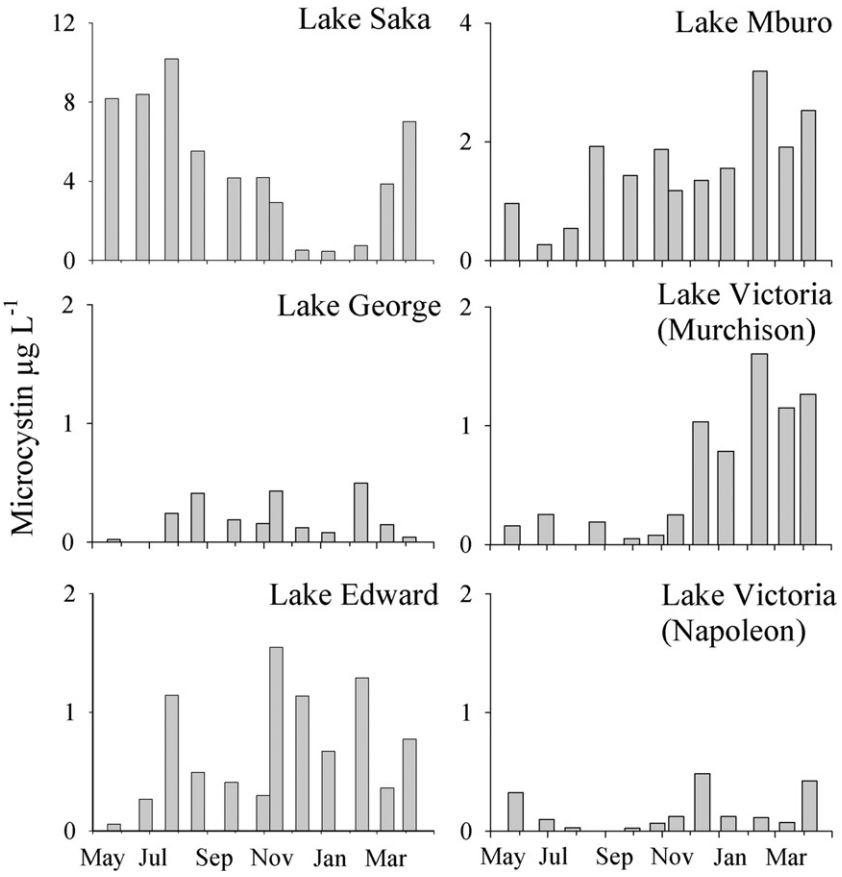

Fig. 4 – Microcystin concentrations (µg MC l^{-1}) at the six sampling sites from May 2007 to April 2008. Note that the scales on the y-axis differ.

Microcystis probably constitutes the most widely distributed MC-producing organism on earth. MC-producing strains have been isolated from all continents (Sivonen and Börner, 2008). A phylogenetic analysis of 164 *Microcystis* strains revealed the occurrence of two phylogenetic clades only that contained the *mcy* gene cluster, while the other four clades did not (Tanabe et al., 2007). The same authors concluded that those phylogenetic clades either containing or lacking the *mcy* gene cluster constitute cryptic ecotypes that are adapted to various environmental conditions. Notably these MC-producing ecotypes seem to have a wide geographic distribution, as typically MC-producing genotypes occur in waters inhabiting *Microcystis* (Kurmayer and Christiansen, 2009). In contrast *Anabaena* – although occurring frequently worldwide – shows a more restricted pattern of MC production as so far only MC-producing strains from Europe, North America and North Africa have been reported (Sivonen and Jones, 1999). When compared with *Microcystis* the genus *Anabaena* shows a much wider genetic variation, for example the taxonomic discrimination of the two morphologically distinct genera *Anabaena* and *Aphanizomenon* cannot be confirmed by 16S rDNA sequence analysis (Gugger et al., 2002). Recently, MC-producing *Anabaena* occurring even in brackish water in the Gulf of Finland in the Baltic Sea has been reported (Halinen et al., 2007) implying that the genus *Anabaena* is composed of MC-producing ecotypes showing resistance to increased salinity (5.03–6.67 practical salinity units). Consequently, although *Microcystis* cell numbers as determined in the microscope typically correlate with MC production, *Anabaena* cells cannot be used to infer MC concentrations in water.

4.2. *Differences in microcystin net production between sites*

The average MC cell quotas of *Microcystis* differed significantly between populations (Fig. 5A, B). Environmental conditions such as light availability and nitrogen availability have been shown to increase MC production in *Microcystis*. For example Wiedner et al. (2003) reported a linear increase in MC content per cell of *Microcystis* strain PCC7806 from 40 to 80 fg $cell^{-1}$ under light conditions from 10 to 100 µmol m^{-2} s^{-1}. Long et al. (2001) observed a variation in MC content per cell of *Microcystis* strain MASH 01-A19 from 0.052 to 0.116 fmol $cell^{-1}$ under nitrogen limiting and nitrogen-replete conditions. Typically, environmental factors have been shown to modulate MC production per cell up to 5-fold, while larger variation (up to 30-fold) at 30 °C vs. 12.5 °C has been reported in exceptional cases only (Sivonen and Jones, 1999). In this study the average MC contents differed between *Microcystis* populations by 16–150-fold in integrated samples and 2.5–23-fold in plankton net samples. This range of variation substantially exceeds the variation observed for single strains under variable environmental conditions in the laboratory. Consequently it is more likely that genetic differences between populations such as

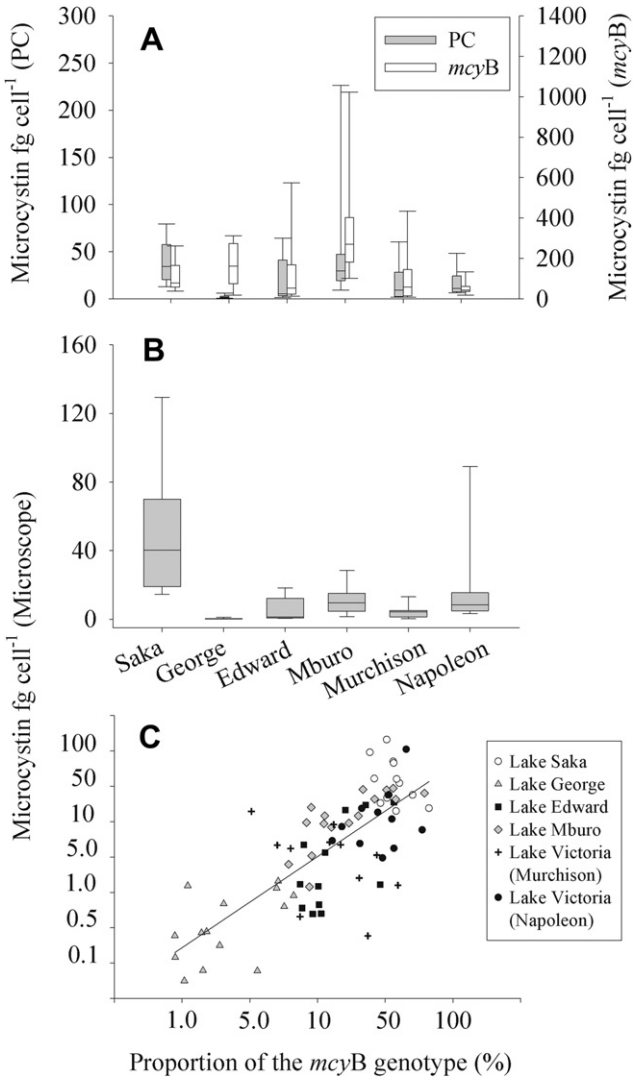

Fig. 5 – Microcystin cell quotas (fg MC cell^{-1}) of (A) the PC and the mcyB genotype of *Microcystis* and (B) of *Microcystis* cells as determined in the microscope at the six sampling sites from May 2007 to April 2008 (n = 12). (C) Dependence of the microcystin content (fg MC cell^{-1}) on the proportion of the mcyB genotype of *Microcystis* for the same data set.

the variable proportion of the *mcy* genotype contributed to the variation in MC content that is observed. Indeed by applying real-time PCR to estimate the proportion of the *mcy* genotype in the individual *Microcystis* populations it could be shown that the average proportion of the *mcy* genotype was significantly related to the average MC content per cell (Fig. 5C). As suggested by one reviewer it might be that the inclusion of an estimate of the transcriptional rate of the mcyB genotype leads to an even higher correlation coefficient as observed in this study ($R^2 > 0.58$). According to the results observed in this study, however it is unlikely that the recording of the transcriptional rate of the mcyB genotype only is able to explain the variability in the average MC content between sites. It is concluded that the differences in the *mcy* genotype proportion between sites have a major impact on MC production while possible environmental influences (such as a higher irradiance in a less densely populated water column) cannot be excluded, but are of minor importance.

Table 3 – Linear regression curves on the dependence of microcystin concentrations on *Microcystis* cell numbers as determined in the microscope in the depth-integrated and the plankton net samples at the six sampling sites from May 2007 to April 2008.

	Sample size	R^2	Linear regression curve[a]
Lake Saka	24	0.97	$y = 3.18 \times 10^{-8}x + 0.00654$
Lake George	24	0.81	$y = 1.3 \times 10^{-9}x - 0.000787$
Lake Edward	24	0.66	$y = 2.38 \times 10^{-8}x + 0.000475$
Lake Mburo	24	0.94	$y = 2.44 \times 10^{-8}x - 0.000919$
Lake Victoria (Murchison Bay)	24	0.87	$y = 2.74 \times 10^{-9}x + 0.000599$
Lake Victoria (Napoleon Gulf)	24	0.87	$y = 6.33 \times 10^{-9}x + 0.000128$
Total	144	0.40	$y = 1.41 \times 10^{-8}x - 0.00245$

a y is the microcystin concentration (μg MC ml^{-1}) and x is the *Microcystis* cell concentration (cells ml^{-1}).

4.3. *Differences in microcystin genotype proportion between sites*

We have shown previously that populations of cyanobacteria in lakes may diverge in *mcy* genotype composition even if they are located only a few kilometres apart due to spatial isolation (Kurmayer and Gumpenberger, 2006). While this geographical isolation may result in the evolution of MC structural variants that appear to be unique and dominant (Christiansen et al., 2008a), this study is the first that demonstrates, that in consequence MC net production may differ quantitatively between sites as well. The structural analysis of protein phosphatase 1 – MC complexes did not provide evidence that the most variable amino acid residues at positions 2 and 4 of the MC molecule are of functional consequence (Bagu et al., 1997; Maynes et al., 2005). In contrast a quantitative change in MC production might be of a selective consequence. For example it has been shown that dissolved MC affects the growth of several submersed and emersed macrophytes negatively (Wiegand and Pflugmacher, 2005) and allelopathic effects on other phytoplankton and zooplankton species have been repeatedly suggested (Gross, 2003; Leflaive and Ten-Hage, 2007; Martins and Vasconcelos, 2009). However, it has also been shown that herbivorous organisms may develop behavioural or physiological resistance to MC production (Kurmayer and Jüttner, 1999). Following the concept of co-evolutionary interactions between herbivores and plants producing defensive compounds (Futuyama, 1983; Jongsma and Bolter, 1997) one might speculate that particularly in those *Microcystis* populations showing lowest mcyB proportion the allelopathic role of MC is increasingly replaced by bioactive compounds other than MCs (Welker and von Döhren, 2006). If this conclusion is true then one might expect that MC production is becoming selectively neutral to individual *Microcystis* colonies. While it is likely that only strong selective pressure led to the evolution of the *mcy* gene cluster in cyanobacteria it is known that the *mcy* gene cluster

probably evolved about two billion years ago (Rantala et al., 2004). According to this hypothesis the majority of the modern cyanobacterial lineages had lost the *mcy* gene cluster during their evolution. Unexpectedly within species such as *Planktothrix* the loss of the *mcy* gene cluster in strains happened on a much shorter time scale in evolution, yet has been found to be a rather rare event that happened a few million of years ago (Christiansen et al., 2008b). It was further concluded that in the meantime both the genotype retaining the *mcy* gene cluster and the genotype that lost the *mcy* gene cluster diverged and adapted to various other environmental conditions. Consequently it is impossible to compare costs and benefits of MC production between strains unless these strains have been genetically characterized in total (by comparative genome analysis) in order to elucidate potential hidden ecophysiological differences. It is likely that the *Microcystis* populations investigated in this study also diverged in other phenotypic characters not directly linked to MC production.

The results are of relevance with regard to the question of whether biogeography can influence toxin production in cyanobacteria. For example, in this study MC-LR that is most frequently found in populations of *Microcystis* in Europe (Via-Ordorika et al., 2004) could only be rarely detected in Ugandan freshwater lakes. MC-LR is known to have a ten-fold higher toxicity to vertebrates when compared with MC-RR and therefore those Ugandan water samples also should be less toxic to livestock and humans when compared with European habitats. In summary, the seasonal variation in average *mcy* proportion within each of the sites could not outweigh the between site variation in *mcy* genotype proportion, thus leading to a rather stable divergence in MC production of *Microcystis* between the spatially isolated populations. This lake-specific divergence might lead to a divergence in MC production on a wider geographic scale affecting MC production both qualitatively and quantitatively resulting in a so far unrecognised bio geographic pattern.

5. Conclusions

The finding that *Microcystis* is a consistent MC producer has important implications for water monitoring. By counting *Microcystis* cells under the microscope, *Microcystis* cell numbers can be used as a proxy to predict MC concentrations in surface water. Since for a specific sampling site a relatively minor variation in the average MC content both during dry and rainy seasons has been found, worst case MC concentrations could be calculated from cell numbers using the maxima of cellular MC quotas as reported for each sampling site. The microscopical approach is considered feasible as the microscopical enumeration technique is well established and the maintenance of technically sophisticated equipment is avoided. However, quantifying the mcyB genotype directly could make more accurate predictions of MC concentrations. In contrast the influence of the transcriptional rate of the mcyB gene on the observed variation in MC net production between sites is considered of minor importance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.watres.2010.02.018.

REFERENCES

Bagu, J.R., Sykes, B.D., Craig, M.M., Holmes, C.F.B., 1997. A molecular basis for different interactions of marine toxins with protein phosphatase-1-molecular models for bound motuporin, microcystins, okadaic acid, and calyculin A. Journal of Biological Chemistry 272 (12), 5087–5097.

Briand, E., Gugger, M., Francois, J.C., Bernard, C., Humbert, J.F., Quiblier, C., 2008. Temporal variations in the dynamics of potentially microcystin-producing strains in a bloom-forming *Planktothrix agardhii* (Cyanobacterium) population. Applied and Environmental Microbiology 74 (8), 3839–3848.

Christiansen, G., Yoshida, W.Y., Blom, J., Portmann, C., Gademann, K.G., Hemscheidt, T., Kurmayer, R., 2008a. Isolation and structure determination of two microcystins and sequence comparisons of *Mcy*ABC adenylation domains in *Planktothrix* species. Journal of Natural Products 71 (11), 1881–1886.

Christiansen, G., Molitor, C., Philmus, B., Kurmayer, R., 2008b. Nontoxic strains of cyanobacteria are the result of major gene deletion events induced by a transposable element. Molecular Biology and Evolution 25 (8), 1695–1704.

Erdner, D.L., Dyble, J., Parsons, M.L., Stevens, R.C., Hubbard, K.A., Wrabel, M.L., Moore, S.K., Lefebvre, K.A., Anderson, D.M., Bienfang, P., Bidigare, R.R., Parker, M.S., Moeller, P., Brand, L.E., Trainer, V.L., 2008. Centers for oceans and human health: a unified approach to the challenge of harmful algal blooms. Environmental Health 7 (Suppl. 2), S2.

Erhard, M., von Döhren, H., Jungblut, P., 1997. Rapid typing and elucidation of new secondary metabolites of intact cyanobacteria using MALDI-TOF mass spectrometry. Nature Biotechnology 15 (9), 906–909.

Futuyama, D.J., 1983. Evolutionary interactions among herbivorous insects and plants. In: Futuyama, D.J., Slatkin, M. (Eds.), Coevolution. Sinauer Associates Inc., pp. 207–231.

Ganf, G.G., 1974. Phytoplankton biomass and distribution in a shallow eutrophic lake (Lake George, Uganda). Oecologia 16 (1), 9–29.

Gross, E.M., 2003. Allelopathy of aquatic autotrophs. Critical Reviews in Plant Sciences 22 (3–4), 313–339.

Gugger, M., Lyra, C., Henriksen, P., Couté, A., Humbert, J.-F., Sivonen, K., 2002. Phylogenetic comparison of the

cyanobacterial genera *Anabaena* and *Aphanizomenon*. International Journal of Systematic and Evolutionary Microbiology 52 (5), 1867–1880.
Halinen, K., Jokela, J., Fewer, D.P., Wahsten, M., Sivonen, K., 2007. Direct evidence for production of microcystins by *Anabaena* strains from the Baltic Sea. Applied and Environmental Microbiology 73 (20), 6543–6550.
Hecky, R.E., 1993. The eutrophication of Lake Victoria. Verhandlungen der Internationalen Vereinigung für Theoretische und Angewandte Limnologie 25 (1), 39–48.
Hotto, A.M., Satchwell, M.F., Berry, D.L., Gobler, C.J., Boyer, G.L., 2008. Spatial and temporal diversity of microcystins and microcystin-producing genotypes in Oneida Lake, NY. Harmful Algae 7 (5), 671–681.
Hudnell, H.K. (Ed.), 2008. Proceedings of the Interagency, International Symposium on Cyanobacterial Harmful Algal Blooms (ISOC-HAB): State of the Science and Research Needs. Advances in Experimental Medicine and Biology 924pp.
Jongsma, M.A., Bolter, C., 1997. The adaptation of insects to plant protease inhibitors. Journal of Insect Physiology 43 (10), 885–895.
Kaebernick, M., Neilan, B.A., 2001. Ecological and molecular investigations of cyanotoxin production. FEMS Microbiology Ecology 35 (1), 1–9.
Komárek, J., Anagnostidis, K., 1999. Cyanoprokaryota, 1. Teil Chroococcales. Gustav Fischer Verlag, Jena, pp. 225–236.
Krüger, T., Christian, B., Luckas, B., 2009. Development of an analytical method for the unambiguous structure elucidation of cyclic peptides with special appliance for hepatotoxic desmethylated microcystins. Toxicon 54 (3), 302–312.
Kurmayer, R., Jüttner, F., 1999. Strategies for the co-existence of zooplankton with the toxic cyanobacterium *Planktothrix rubescens* in Lake Zürich. Journal of Plankton Research 21 (4), 659–683.
Kurmayer, R., Christiansen, G., Chorus, I., 2003. The abundance of microcystin-producing genotypes correlates positively with colony size in *Microcystis* and determines its microcystin net production in Lake Wannsee. Applied and Environmental Microbiology 69 (2), 787–795.
Kurmayer, R., Kutzenberger, T., 2003. Application of real-time PCR for quantification of microcystin genotypes in a population of the toxic cyanobacterium *Microcystis* sp. Applied and Environmental Microbiology 69 (11), 6723–6730.
Kurmayer, R., Gumpenberger, M., 2006. Diversity of microcystin genotypes among populations of the filamentous cyanobacteria *Planktothrix rubescens* and *Planktothrix agardhii*. Molecular Ecology 15 (12), 3849–3861.
Kurmayer, R., Christiansen, G., 2009. The genetic basis of toxin production in cyanobacteria. Freshwater Reviews 2 (1), 31–50.
Lawton, L.A., Edwards, C., Codd, G.A., 1994. Extraction and high-performance liquid cromatographic method for the determination of microcystins in raw and treated waters. Analyst 119 (7), 1525–1530.
Leflaive, J., Ten-Hage, L., 2007. Algal and cyanobacterial secondary metabolites in freshwaters: a comparison of allelopathic compounds and toxins. Freshwater Biology 52 (2), 199–214.
Long, B.M., Jones, G.J., Orr, P.T., 2001. Cellular microcystin content in N-limited *Microcystis aeruginosa* can be predicted from growth rate. Applied and Environmental Microbiology 67 (1), 278–283.
Martins, J.C., Vasconcelos, V.M., 2009. Microcystin dynamics in aquatic organisms. Journal of Toxicology and Environmental Health-Part B-Critical Reviews 12 (1), 65–82.
Maynes, J., Luu, H., Cherney, M., Andersen, R., Williams, D., Holmes, C., James, M., 2005. Crystal structures of protein phosphatase-1 bound to motuporin and dihydromicrocystin-LA: elucidation of the mechanism of enzyme inhibition by cyanobacterial toxins. Journal of Molecular Biology 356 (1), 111–120.
Mugidde, R., 1993. The increase in phytoplankton primary productivity and biomass in Lake Victoria (Uganda). Verhandlungen der Internationalen Vereinigung für Theoretische und Angewandte Limnologie 25 (2), 846–849.
Okello, W., Portmann, C., Erhard, M., Gademann, K., Kurmayer, R., 2009. Occurrence of microcystin-producing cyanobacteria in Ugandan freshwater habitats. Environmental Toxicology. doi: 10.1002/tox.20522.
Ostermaier, V., Kurmayer, R., 2009. Distribution and abundance of nontoxic mutants of cyanobacteria in lakes of the Alps. Microbial Ecology 58 (2), 323–333.
Otsuka, S., Suda, S., Shibata, S., Oyaizu, H., Matsumoto, S., Watanabe, M.M., 2001. A proposal for the unification of five species of the cyanobacterial genus *Microcystis* Kützing ex Lemmermann 1907 under the rules of the bacteriological code. International Journal of Systematic and Evolutionary Microbiology 51, 873–879.
Rantala, A., Fewer, D.P., Hisbergues, M., Rouhiainen, L., Vaitomaa, J., Börner, T., Sivonen, K., 2004. Phylogenetic evidence for the early evolution of microcystin synthesis. Proceedings of the National Academy of Sciences USA 101 (2), 568–573.
Reynolds, C.S., Huszar, V., Kruk, C., Naselli-Flores, L., Melo, S., 2002. Towards a functional classification of the freshwater phytoplankton. Journal of Plankton Research 24 (5), 417–428.
Schatz, D., Keren, Y., Vardi, A., Sukenik, A., Carmeli, S., Börner, T., Dittmann, E., Kaplan, A., 2007. Towards clarification of the biological role of microcystins, a family of cyanobacterial toxins. Environmental Microbiology 9 (4), 965–970.
Sivonen, K., Jones, G., 1999. Cyanobacterial toxins. In: Chorus, I., Bartram, J. (Eds.), Toxic Cyanobacteria in Water. A Guide to their Public Health Consequences, Monitoring and Management. WHO, E & FN Spon, London, pp. 41–112.
Sivonen, K., Börner, T., 2008. Bioactive compounds produced by cyanobacteria. In: Herrero, A., Flores, E. (Eds.), The Cyanobacteria: Molecular Biology, Genomics and Evolution. Caister Academic Press, UK, Norfolk, pp. 159–197.
Talling, J.F., 1986. The seasonality of phytoplankton in African lakes. Hydrobiologia 138 (1), 139–160.
Talling, J.F., 1987. The phytoplankton of Lake Victoria (East Africa). Archiv für Hydrobiologie Beiheft Ergebnisse Limnologie 25, 229–256.
Tanabe, Y., Kasai, F., Watanabe, M., 2007. Multilocus sequence typing (MLST) reveals high genetic diversity and clonal population structure of the toxic cyanobacterium *Microcystis aeruginosa*. Microbiology 153, 3695–3703.
Verschuren, D., Johnson, T.C., Kling, H.J., Edgington, D.N., Leavitt, P.R., Brown, E.T., Talbot, M.R., Hecky, R.E., 2002. History and timing of human impact on Lake Victoria, East Africa. Proceedings of the Royal Society B: Biological Sciences 269, 289–294.
Via-Ordorika, L., Fastner, J., Kurmayer, R., Hisbergues, M., Dittmann, E., Komárek, J., Erhard, M., Chorus, I., 2004. Distribution of microcystin-producing and non-microcystin-producing *Microcystis* sp. in European freshwater bodies: detection of microcystins and microcystin genes in individual colonies. Systematic and Applied Microbiology 27 (5), 592–603.
Welker, M., von Döhren, H., 2006. Cyanobacterial peptides – Nature's own combinatorial biosynthesis. FEMS Microbiology Reviews 30 (4), 530–563.
Wetzel, R.G., Likens, G.E., 2000. Limnological Analyses, third ed. Springer-Verlag, New York. 429pp.

Wiedner, C., Visser, P.M., Fastner, J., Metcalf, J.S., Codd, G.A., Mur, L.R., 2003. Effects of light on the microcystin content of *Microcystis* strain PCC 7806. Applied and Environmental Microbiology 69 (3), 1475–1481.

Wiegand, C., Pflugmacher, S., 2005. Ecotoxicological effects of selected cyanobacterial secondary metabolites: a short review. Toxicology and Applied Pharmacology 203, 201–218.

World Health Organization, 2006. Guidelines for Drinking-water Quality. First Addendum to Third Edition. In: Recommendations, third ed., vol. 1. World Health Organization, Geneva. 515pp.

Supplemental Material Chapter 1

Suppl. Table 1. Composition of ions in the MS^2 spectrum of the $[M+H]^+$ ion of [$NMeSer^7$]-MC-YR[a]

Composition and Sequence	Calculated m/z	Measured m/z	Error [ppm]
M (+H)	1063.5459	1063.5437	-2.1
M (-H2O) (+H)	1045.5353	1045.5543	18.2
M (-CO) (+H)	1035.551	1035.548	-2.9
Arg-Adda-Glu-NMeSer-Ala-Tyr (+H) or M (-Glu) (+H)	934.5033	934.4994	-4.2
MeAsp-Arg-Adda-Glu (+H)	728.3978	728.4411	59.4
NMeSer-Ala-Tyr-MeAsp-Arg (+H)	621.2991	621.3149	25.4
NMeSer-Ala-Tyr-MeAsp-Arg (-H2O) (+H)	603.2885	603.3096	35.0
Arg-Adda-Glu (+H) or MeAsp-Arg-Adda (+H)	599.3552	599.3609	9.5
Ala-Tyr-MeAsp-Arg (+H)	520.2514	520.2705	36.7
Glu-NMeSer-Ala-Tyr (+H) or Thr-Ala-Tyr-MeAsp (+H)	465.198	465.2186	44.3
Tyr-MeAsp-Arg (+H)	449.2143	449.2236	20.7
NMeSer-Ala-Tyr (+H)	336.1554	336.1542	-3.6
Tyr-MeAsp (+H)	293.1132	293.1317	63.1
MeAsp-Arg (+H)	286.151	286.1591	28.3
Glu-NMeSer (+H)	231.0975	231.1012	16.0
Glu-NMeSer (-H2O) (+H)	213.087	213.0916	21.6
NMeSer-Ala (+H)	173.0921	173.0909	-6.9
Adda (-NH3) (-134 Adda) (+H)	163.1118	163.1133	9.2
Arg (+H)	157.1084	157.1095	7.0
NMeSer-Ala (-H2O) (+H)	155.0815	155.0824	5.8
134Adda (+H)	135.0804	135.0811	5.2

[a] The amino acid at position 7 can be either NMeSer or Thr, it was not possible to differentiate these two amino acids by MS^2.

Chapter 2

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Application of Real-Time PCR To Estimate Toxin Production by the Cyanobacterium *Planktothrix* sp.▽†

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Quantitative real-time PCR methods are increasingly being applied for the enumeration of toxic cyanobacteria in the environment. However, to justify the use of real-time PCR quantification as a monitoring tool, significant correlations between genotype abundance and actual toxin concentrations are required. In the present study, we aimed to explain the concentrations of three structural variants of the hepatotoxin microcystin (MC) produced by the filamentous cyanobacterium *Planktothrix* sp., [Asp, butyric acid (Dhb)]-microcystin-RR (where RR means two arginines), [Asp, methyl-dehydro-alanine (Mdha)]-microcystin-RR, and [Asp, Dhb]-microcystin–homotyrosine-arginine (HtyR), by the abundance of the microcystin genotypes encoding their synthesis. Three genotypes of microcystin-producing cyanobacteria (denoted the Dhb, Mdha, and Hty genotypes) in 12 lakes of the Alps in Austria, Germany, and Switzerland from 2005 to 2007 were quantified by means of real-time PCR. Their absolute and relative abundances were related to the concentration of the microcystin structural variants in aliquots determined by high-performance liquid chromatography (HPLC). The total microcystin concentrations varied from 0 to 6.2 μg liter^{-1} (mean ± standard error [SE] of 0.6 ± 0.1 μg liter^{-1}) among the samples, in turn resulting in an average microcystin content in *Planktothrix* of 3.1 ± 0.7 μg mm^{-3} biovolume. Over a wide range of the population density (0.001 to 3.6 mm^3 liter^{-1} *Planktothrix* biovolume), the Dhb genotype and [Asp, Dhb]-MC-RR were most abundant, while the Hty genotype and MC-HtyR were found to be in the lowest proportion only. In general, there was a significant linear relationship between the abundance/proportion of specific microcystin genotypes and the concentration/proportion of the respective microcystin structural variants on a logarithmic scale. We conclude that estimating the abundance of specific microcystin genotypes by quantitative real-time PCR is useful for predicting the concentration of microcystin variants in water.

During the last decade, genetic methods have significantly increased our understanding of the distribution of genes that are involved in the production of toxins within cyanobacteria that occur in fresh and brackish water (45). Although genetic methods can indicate only the potential risk of toxin synthesis and do not provide information about the actual toxin concentrations, quantitative real-time PCR has been increasingly applied for monitoring the toxin-producing genotypes of cyanobacteria in water (26, 33, 44). The development of real-time PCR methods was driven primarily by its potential (i) as an early-warning tool as well as to monitor toxin-producing cyanobacteria and (ii) to identify those factors that lead to a dominance/repression of toxin-producing genotypes versus nontoxic genotypes. For the first aim, it is essential that the abundance of toxin-producing cyanobacteria can be related to the concentration of the respective toxic substance in water. A few studies showed that the concentration of certain toxic genotypes was linearly related to the respective toxin concentrations, e.g., for the most common group of hepatotoxins, the microcystins (MCs) (7, 12, 14), and for the related nodularin (19). Both microcystins and nodularins are known to be potent inhibitors of eukaryotic protein phosphatases 1 and 2A, resulting in a health hazard to humans and the environment (9). In contrast, no correlation was found (37, 50), or even the opposite was reported, by other studies, i.e., that the measurement of microcystin-producing genotypes is not a satisfactory method for use in monitoring programs in order to predict the toxic risk associated with cyanobacterial proliferation (3). For microcystins, these contrasting results may be due to several reasons: (i) several genera producing microcystins frequently coexist in water bodies, and therefore, not all microcystin producers may have been identified; (ii) the semilogarithmic calibration curves limit the accuracy in estimations of genotype numbers and proportions (for example, the only laboratory comparison carried out so far revealed that among the three laboratories tested, the proportions of toxic genotypes were overestimated or underestimated by 0 to 72% and 0 to 50%, respectively [42]); and (iii) inactive mutants that contain the respective genes, however, which have been inactivated in toxin production through the insertion of transposable elements, may co-occur and decrease toxin production in a given population (6). Nevertheless, the real-time PCR technique is the only quantitative technique available for estimating the proportion of potential toxin-producing genotypes in water. The development of automated and field-applicable real-time PCR methods (e.g., see reference 35), in particular, may contribute to a more widespread integration of real-time PCR into routine monitoring programs in the future.

In the present study, we attempted to quantify microcystin-producing genotypes in total as well as quantify the specific

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genotypes that were shown to encode different microcystin structural variants characterized for strains isolated from lakes in the Alps (23): (i) the methyl-dehydro-alanine residue (Mdha) genotype, which was found to synthesize structural variants containing only Mdha in position 7; (ii) the butyric acid (Dhb) genotype, which was found to contain Dhb instead of Mdha in the same position; and (iii) the homotyrosine (Hty) genotype, which was found to contain Hty and Leu in position 2 but never Arg. The Hty variant has always been found to co-occur with Dhb in position 7 of the molecule (24). Consequently, the Hty genotype forms a subgroup of the microcystin-producing population composed of the Mdha and Dhb genotypes. The following hypotheses were tested: (i) as only one microcystin-producing organism (*Planktothrix* sp.) is of quantitative importance in those lakes (32), the total microcystin concentration should be predictable from the sum of Mdha and Dhb genotypes; (ii) given that all *Planktothrix* genotypes are amenable to cultivation, all the structural microcystin variants found in the field samples should have been described for the strains isolated previously (23); and (iii) as, on average, the proportion of the inactive microcystin genotypes was found to be low and rather stable (<6.5% [32]), their occurrence should not reduce the ability to predict microcystin concentrations from genotype abundance. For this purpose, the phytoplankton in 12 lakes of the Alps in Austria, Germany, and Switzerland was monitored both with an inverted microscope as well as by means of real-time PCR over the course of 2 years (2005 to 2007). In parallel, microcystin concentrations in aliquots were determined by means of high-performance liquid chromatography (HPLC). We show that the abundance of specific microcystin genotypes can be related to the corresponding microcystin concentrations in water on a logarithmic scale over a range of trophic conditions. The proportion of certain genotypes encoding the synthesis of a specific microcystin variant significantly correlates with the concentration of the respective microcystin variant. We argue that these genotype-toxin concentration relationships are of great importance for the justification of real-time PCR use in monitoring programs.

MATERIALS AND METHODS

Sampling procedure. Phytoplankton sampling was performed in 12 lakes of the Alps (Afritzersee, Attersee, Fuschlsee, Irrsee, Mondsee, Offensee, Schwarzensee, Wolfgangsee, and Wörthersee in Austria; Ammersee in Germany; and Hallwilersee and Zürichsee in Switzerland) differing in trophic states from oligotrophic to mesoeutrophic (for a detailed morphometric and trophic description of the lakes, see Table 1 in reference 32). Water samples were taken between spring 2005 and autumn 2007 by pooling 1 liter of water every 2 m from the surface to a depth of 20 m (depth-integrated samples). Since some lakes had a rather low population density (0 to 0.0026, 0 to 0.0058, and 0.0011 to 0.0136 mm^3 $liter^{-1}$ [minimum to maximum {min-max}] for Attersee, Wolfgangsee, and Schwarzensee, respectively), three vertical plankton net hauls (30-μm mesh size) from a depth of 20 m to the surface were taken in parallel (net samples). As estimated from the diameter (0.5 m) and length (1.5 m) of the conical net, taking one net haul from a depth of 20 m up to the surface results in the filtering of 1,300 liters. In total, 80 depth-integrated and 79 net samples were analyzed. Aliquots of each sample (2 to 4 liters of integrated samples and 20 to 100 ml of net samples) were filtered onto glass fiber filters (BMC; Ederol, Vienna, Austria) under vacuum pressure and stored frozen (−20°C) for subsequent DNA and microcystin analyses. For microscopic counting, aliquots (100 ml) of the samples were preserved with Lugol solution and formaldehyde (2% final concentration). To characterize the trophic state, the total phosphorus concentration and the chlorophyll *a* concentration were determined according to standard methods (48).

Culturing of strains. *Planktothrix rubescens* strains PCC 7821 (a microcystin-producing genotype with dehydrobutyrine in position 7 from Lake Gjersjøen, Norway), number 21/1 (a microcystin-producing genotype synthesizing Hty/Leu in position 2, from Lake Figur, Austria), and No. 40 (a microcystin-producing genotype synthesizing Mdha in position 7, from Lake Mondsee, Austria) were all grown under continuous light (5 to 15 μmol m^{-2} s^{-1}) (Osram type L30W/77; Fluora) at 20°C in BG11 medium (38). Cells from cultures in the exponential growth phase were harvested as described above. Aliquots of cultures (100 ml) were preserved by the addition of formaldehyde, and the cells were enumerated by use of epifluorescence microscopy. Cell numbers (converted to biovolume equivalents) were related to the threshold cycle (C_T) value measured by real-time PCR from a series of DNA dilutions of the respective strains.

Microscopic counting. Lugol-fixed, integrated samples were counted in sedimentation chambers by use of an inverted microscope. At least three transects per chamber were screened to enumerate *Planktothrix* at a ×100 magnification and the other phytoplankton at a ×400 magnification. Filaments were assigned to the genus *Planktothrix* according to morphological criteria (18). *Planktothrix* filaments in the net samples were enumerated by use of epifluorescence microscopy (Zeiss Axioscop 40). The biovolume was then calculated by approximating the geometrical shape (48). Other phytoplankton species were enumerated from integrated samples only. Only phytoplankton taxa that were visible at a 400-fold magnification were considered, while small cyanobacteria such as *Synechococcus* sp. could not be detected quantitatively.

Genetic analysis. The DNA that was collected on filters was isolated quantitatively by use of a standard chloroform-phenol protocol described previously (21). In order to quantify the three genotypes differing in microcystin syntheses as well as the total population of *Planktothrix*, a TaqMan assay was used as described previously (32). The TaqMan assay for the detection of each microcystin genotype was designed for genotypes that were characterized for their microcystin production (23): (i) the Mdha genotype (long *mcyAA1* variant [GenBank accession no. AJ749248.1 to AJ749259.1 and AJ441056.1]), (ii) the Dhb genotype (short *mcyAA1* variant [accession no. AJ749260.1 to AJ749266.1 and AJ749254.1 to AJ749256.1]), and (iii) the Hty genotype (accession no. AJ890275.1 to AJ890279.1, AJ749273.1, AJ749275.1, AJ749278.1, and AJ863131.1 to AJ863134.1). Those DNA sequences were aligned (ClustalW 1.8), and the target regions with complete identity within a genotype and the maximum difference from the other genotypes were identified. For each of these gene regions, primers and TaqMan probes were designed by use of Primer Express software (version 2.0; ABI). Prior to synthesis, all oligonucleotides were tested for stem-loop formation, the formation of primer dimers, and heat stability and were modified, if necessary, by TIB Molbiol (Berlin, Germany). This resulted in the design of the following forward primers, TaqMan probes, and reverse primers, respectively: (i) mdha+ (5′-AAGTCAA TCTCTTACATCCTTGT-3′), Mdha (5′-ACCGGCTAATCTAGCTAAAATT ATCTGC-3′), and mdha− (5′-CAATGAGATCCCAATCACTAT-3′) (amplicon length of 77 bp) for Mdha; (ii) dhb+ (5′-CCTCAACATCAAGCGAGTA TTAT-3′), Dhb (5′-TACAGAATGGGAAAAAATTACTCAAGAGAA-3′), and dhb− (5′-CCACTTTCCGGGTTTG-3′) (83 bp) for Dhb; and (iii) HtyS (5′-AAACCGATGATCCCGTCATTTCT-3′), Hty (5′-CCCATTGCCAATAA CCAAATATACATTCTTGATCC-3′), and HtyA (5′-CACAATGCCGACAGG AACG-3′) (98 bp) for Hty. Concentrations of the forward primer, reverse primer, and TaqMan probe were optimized according to the manufacturer's instructions (ABI TaqMan universal PCR master mix) and adjusted to 200, 200, and 100 fmol μl^{-1} for the Mdha and Hty genotypes and 200, 200, and 250 fmol μl^{-1} for the Dhb genotype, respectively. All samples were measured in triplicate with an Eppendorf Master Cycler Ep Realplex system. The reaction mixtures were 25 μl in total volume, consisting of 12.5 μl master mix (TaqMan Universal PCR master mix; ABI, Austria), 5 μl of template DNA, and the optimized concentrations of the primers and probe. Following the initial denaturation step of 10 min at 95°C, 50 cycles of a two-step PCR were run, with alternating steps of 15 s of denaturation at 95°C, and a 1-min annealing and elongation step at 60°C for Hty and at 55°C for Dhb and Mdha. The calibration curves were established as described previously (32) and are listed in Table S1 in the supplemental material. The specificity of the TaqMan assays was tested by the addition of DNAs of *Microcystis* strains HUB53 and HUB524, which were added at the same concentration as the target DNA (equivalent to 460 cells per template) and also at a 100-fold-lower concentration (32). In no case did the C_T values of the calibration curve show a significant deviation in the presence of the DNA background (see Table S2 in the supplemental material). In addition, the specificity of the primer sets was tested by endpoint PCR by using the DNA of 18 *Planktothrix* strains differing in the production of specific microcystin variants and the DNA of different species of cyanobacteria (*Aphanizomenon gracile*, *Aphanizomenon flexuosum*, *Microcystis aeruginosa* HUB524, *Microcystis flos-aquae*, *Nostoc* sp. strain PCC 7120, and *Synechococcus* sp. strain MW-10). All

primer pairs showed the specific PCR products only. For each TaqMan assay, the lower limit of detection, corresponding to 1 copy template^{-1}, was determined by measurements of a dilution series of purified PCR products from the strains, as described previously (32).

Microcystin analysis. Microcystins were extracted from filters by using 75% (wt/vol) aqueous methanol (10). Extracts were analyzed by HPLC with diode array detection (DAD) for their microcystin compositions by using a linear gradient of acetonitrile (0.05% trifluoroacetic acid) against water (27). Microcystin variants were identified by their respective retention times and molecular masses as described previously (22, 23). Microcystin variants were quantified as equivalents of [D-Asp, Mdha]-microcystin-LR (Cyanobiotech GmbH, Berlin, Germany). All aqueous methanolic extracts that were found to be negative for microcystin, as revealed by HPLC, were subsequently tested by an indirect competitive enzyme-linked immunosorbent assay (ELISA; Abraxis, United Kingdom) that was targeted at the ADDA group of the molecule (11) detecting microcystin variants independent of structural variation. The ELISA has good cross-reactivity with a range of microcystin structural variants, such as microcystin-LR, -RR, -YR, -LW, -LF, D-Asp-LR, D-Asp-RR, and even nodularin. The sensitivity of the assay ranged from 0.15 to 5 ng ml^{-1}. To prevent the inhibition of the assay by methanol (30), the extracts were dried and resuspended in Millipore water. The ELISA was performed in triplicate for each sample according to the manufacturer's instructions.

RESULTS

Phytoplankton composition. According to the total phosphorus and chlorophyll *a* concentrations, the 12 lakes were classified as oligotrophic, oligomesotrophic, mesotrophic, and mesoeutrophic (see Table 1 in reference 32). In the vast majority of the samples, *Planktothrix* filaments were observed. There was no *Planktothrix* detected in a sedimentation volume of 25 ml for only 10 integrated samples, which were obtained from the lakes showing the lowest population densities (Attersee [four samples], Irrsee [three samples], Offensee [one sample], and Wolfgangsee [two samples]). During the study period, the 12 lakes varied in phytoplankton compositions and in the proportions of *Planktothrix* biovolume, ranging from 0.2% (Attersee) to 95.6% (Hallwilersee) (Fig. 1). Other cyanobacteria (*Anabaena*, *Lyngbya*, and *Aphanizomenon* spp.) made up no more than 3.6% of the total amount of phytoplankton. The second most common phytoplankton group was diatoms (*Aulacoseira*, *Fragillaria*, *Tabellaria*, and *Stephanodiscus* spp.), ranging from 1% (Hallwilersee) to 71.9% in Wolfgangsee. Cryptomonads (*Cryptomonas* sp.) and dinoflagellates (*Ceratium* sp.) contributed 0.8% (Hallwilersee) to 22.5% (Schwarzensee) and 1.3% (Afritzersee) to 11.8% (Zürichsee) to the total phytoplankton counts, respectively. On average, chrysomonads (*Dinobryon* sp.) and green algae (*Ankistrodesmus* and *Elakatothrix* spp.) never constituted more than 10% of the total phytoplankton community.

Abundance of microcystin-producing genotypes. For a total of 159 samples, a linear correlation between the total *Planktothrix* biovolume estimated by a 16S rRNA gene assay and the biovolume calculated from counting with a microscope was found: $y = -0.47 + 0.93x$ ($R^2 = 0.82$; $n = 145$; $P < 0.001$) (where x is the $\log_{10}$ biovolume [mm^3 liter^{-1}] calculated by counting by microscopy and y is the $\log_{10}$ biovolume estimated by 16S rRNA gene assay) (see Fig. S1 in the supplemental material). Compared to microscopy, real-time PCR showed a higher sensitivity: no *Planktothrix* could be detected for only three integrated samples (Attersee, Fuschlsee, and Wolfgangsee) and three net samples (one sample from Wolfgangsee and two from Attersee).

In order to find out whether the microcystin-producing genotypes of *Planktothrix* sp. contributed equally in all populations, the proportions of the sum of the Mdha genotype and the Dhb genotype were compared between lakes. On average, the Mdha and Dhb genotypes contributed 42.4% ± 3% (min-max, 16.3 to 63.2%) to the total population. In general, the proportion of the Dhb genotype was the highest (mean, 28.7% ± 2%; min-max, 6.1 to 49.8%), and on average, the populations showed a significantly lower proportion of the Mdha genotype ($P < 0.001$ by Kruskal Wallis one-way analysis of variance [ANOVA]; mean, 13.7% ± 1%; min-max, 0.3 to

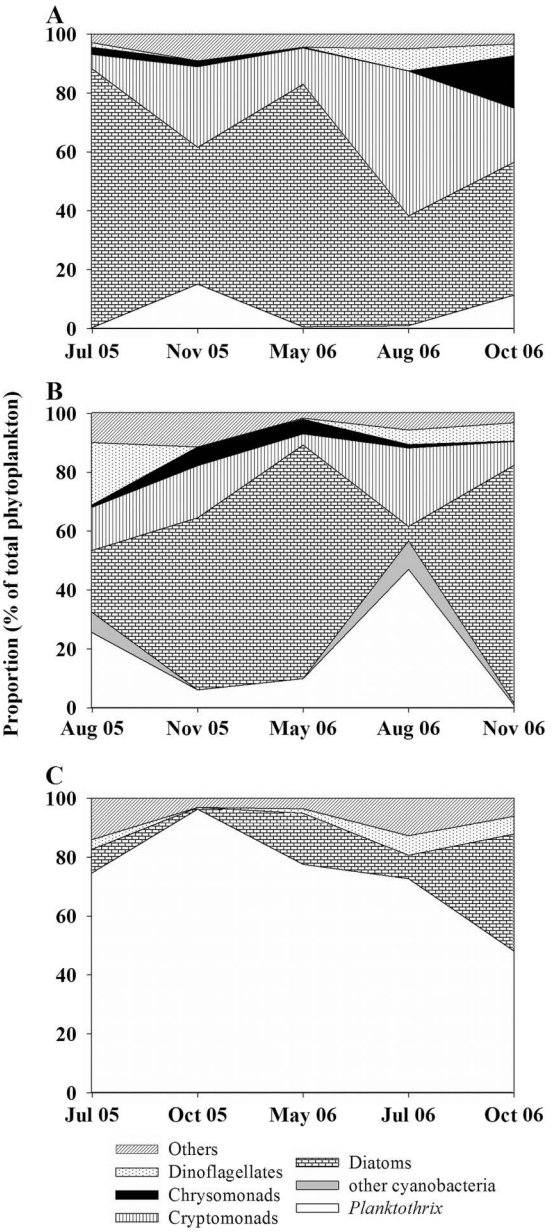

FIG. 1. Phytoplankton compositions in the study lakes classified into three groups depending on the average proportions of *Planktothrix* in the total phytoplankton biovolume ($n = 5$): <10% of the average proportion of *Planktothrix* in total phytoplankton, e.g., Schwarzensee (A); 10 to 50%, e.g., Mondsee (B), and 50 to 90%, e.g., Wörthersee (C). Phytoplankton groups that contributed ≤5% to the total biovolume are shown as "others."

TABLE 1. Average proportions of microcystin genotypes synthesizing different structural variants determined by real-time PCR and the corresponding microcystin variants detected by HPLC analysis over the study period

Lake	Trophy[a]	No. of samples used for real-time PCR[b]	Mean % biovolume of genotype ± SE			No. of samples used for HPLC[b]	Mean % proportion of microcystin variant ± SE					Mean total MC (μg liter^{-1}) ± SE[c]
			Mdha	Dhb	Hty		[Asp, Mdha]-microcystin-RR	[Asp, Dhb]-microcystin-RR	[Asp]-microcystin-HtyR	[Asp]-microcystin-LR	Unknown MCs	
Attersee	O	6	32.3 ± 10	25.2 ± 11.4	12.3 ± 11.9	1	0	0	0	100	0	0[d]
Wolfgangsee	O	9	6.0 ± 2.9	22.7 ± 1.4	7.8 ± 3.2	0	0	0	0	0	0	0[d]
Schwarzensee	O	13	0.3 ± 0.2	28.1 ± 4.6	4.8 ± 2.0	7	0	100	0	0	0	0[d]
Offensee	O	13	2 ± 1.8	44.4 ± 11.7	35.3 ± 4.9	8	0	0	42.2 ± 2.2	57.8 ± 2.2	0	0.05 ± 0.02
Ammersee	O-M	12	29.3 ± 6.8	17.7 ± 3.5	3 ± 0.4	12	67.3 ± 5.4	25.3 ± 5.3	0.8 ± 0.5	6.6 ± 2.9	0	0.11 ± 0.04
Fuschlsee	O-M	12	8.5 ± 1.8	23.1 ± 11.5	16.7 ± 13.6	12	41.0 ± 1.9	43.8 ± 3.4	1.2 ± 0.6	10.8 ± 3.1	3.3 ± 2.2	0.11 ± 0.04
Irrsee	O-M	13	22.2 ± 6.4	19.7 ± 5.2	7.6 ± 6.0	6	15.2 ± 15.2	48.6 ± 21.8	10.2 ± 10.2	26.0 ± 15.9	0	0.04 ± 0.03
Mondsee	O-M	12	10.2 ± 2.6	6.1 ± 1.9	2.1 ± 0.9	8	56.2 ± 2.1	38.8 ± 2.8	0.7 ± 0.7	4.3 ± 2.2	0	0.05 ± 0.02
Wörthersee	M	12	15.1 ± 3.1	32 ± 8.9	5.4 ± 1.3	12	27.9 ± 1.7	48.9 ± 1.9	6.4 ± 0.6	14.1 ± 1	2.7 ± 1.4	1.09 ± 0.29
Afritzersee	M	12	17.9 ± 4.5	34.4 ± 7.6	6.3 ± 0.9	12	39.9 ± 2.7	38.1 ± 2.5	7.3 ± 1.4	12.6 ± 0.7	2 ± 1	0.85 ± 0.24
Zürichsee	M	15	15.5 ± 1.1	36.7 ± 3.9	9.1 ± 1.1	15	33.7 ± 2.1	52.8 ± 2.3	3.3 ± 0.8	7.3 ± 1.7	2.9 ± 1.3	2.08 ± 0.73
Hallwilersee	M-E	11	13.4 ± 4.7	49.8 ± 8.3	16 ± 2.6	11	20.6 ± 5.8	57 ± 5.8	7.9 ± 1.0	14.4 ± 1.2	0.2 ± 0.1	3.82 ± 0.80
Total		140	13.7 ± 1.4	28.7 ± 2.3	10.6 ± 1.6	104	32.6 ± 2.3	44.2 ± 2.6	7.0 ± 1.2	14.9 ± 1.9	1.4 ± 0.4	0.60 ± 0.14

[a] O, oligotrophic; O-M, oligomesotrophic; M, mesotrophic; M-E, mesoeutrophic.
[b] Depth-integrated and plankton net samples were combined.
[c] Total microcystin concentrations (μg liter^{-1}) from integrated samples.
[d] Microcystin positive as revealed by ELISA.

32.3%). Extremely low proportions of the Mdha genotype occurred in oligotrophic lakes, i.e., Offensee, Wolfgangsee, and Schwarzensee (Table 1). Consequently, the proportions of the Mdha genotype were significantly positively related with either total phosphorus concentrations or chlorophyll *a* concentrations ($P < 0.05$). However, the correlation coefficients were always low ($R^2 \leq 0.20$). The Hty genotype generally constituted the lowest proportion in all the populations (10.6% ± 2%). Only for Offensee, Fuschlsee, and Hallwilersee was a higher proportion of the Hty genotype recorded. The proportion of the Hty genotype did not depend on the total phosphorus or chlorophyll *a* concentrations.

Altogether, the abundance of the three microcystin genotypes was significantly positively related to the total *Planktothrix* biovolume revealed by 16S rRNA gene analysis. For the sum of the Mdha and Dhb genotypes, the linear relationship was $y = -0.52 + 0.99x$ ($R^2 = 0.94$; $n = 138$; $P < 0.001$), where x is the $\log_{10}$ biovolume estimated by 16S rRNA gene assay and y is the sum of the $\log_{10}$ biovolume of the Mdha and the Dhb genotypes (Fig. 2). The regression curves for each of the three genotypes considered separately were $y = -0.97 + 0.94x$ ($R^2 = 0.92$; $n = 111$; $P < 0.001$) for Mdha, $y = -0.67 + 0.97x$ ($R^2 = 0.93$; $n = 126$; $P < 0.001$) for Dhb, and $y = -1.22 + 0.94x$ ($R^2 = 0.88$; $n = 113$; $P < 0.001$) for Hty, where x is the $\log_{10}$ biovolume estimated by 16S rRNA gene assay and y is the $\log_{10}$ biovolume of the respective microcystin genotype (see Fig. S2 in the supplemental material). Consequently, the population density was a significant factor related to the abundance of all microcystin genotypes.

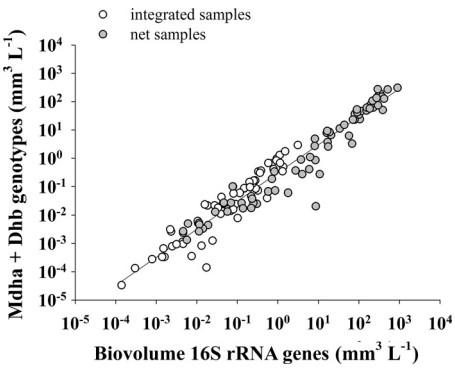

FIG. 2. Relationship between the total *Planktothrix* biovolume (mm^3 liter^{-1}) determined by real-time PCR via 16S rRNA genes and the microcystin genotypes consisting of the Mdha and Dhb genotypes (synthesizing either Mdha or Dhb in position 7 of the microcystin molecule). For details on the regression curves, see the text.

Microcystin concentrations. Microcystin concentrations varied from 0 to 6.2 μg liter^{-1} (mean, 0.6 ± 0.1 μg liter^{-1}; $n = 79$) among the integrated samples and from 0 to 4,994 μg liter^{-1} (mean, 420 ± 90 μg liter^{-1}; $n = 80$) among the net samples. No microcystins were detected for 34% of all the integrated samples and 35% of all the net samples by means of HPLC (see Table S2 in the supplemental material). Subsequent ELISAs revealed the presence of microcystins in all of those samples. The microcystin concentrations determined by ELISA varied from 0.001 to 10.5 μg liter^{-1} (mean, 0.412 ± 0.4 μg liter^{-1}; $n = 27$) for the integrated samples and from 0.084 to 13.3 μg liter^{-1} (mean, 2.1 ± 0.6 μg liter^{-1}; $n = 28$) for the net samples. The average microcystin content in *Planktothrix* was 3.1 ± 0.7 μg mm^{-3} biovolume (0.2% ± 0.05% of the cellular biovolume). There was no statistically significant difference in the average microcystin contents between integrated and net samples ($P = 0.96$ by t test).

Microcystins consisted of [Asp, Dhb]-microcystin-RR (44.2% ± 3%), [Asp, Mdha]-microcystin-RR (32.6% ± 2%), [Asp]-microcystin–homotyrosine-arginine (HtyR) (7% ± 1%), and [Asp]-microcystin-LR (14.9% ± 2%). Unknown microcystins generally contributed less than 1.4%. Corresponding to the low proportion of the Mdha genotype in oligotrophic lakes, the proportion of [Asp, Mdha]-microcystin-RR was significantly posi-

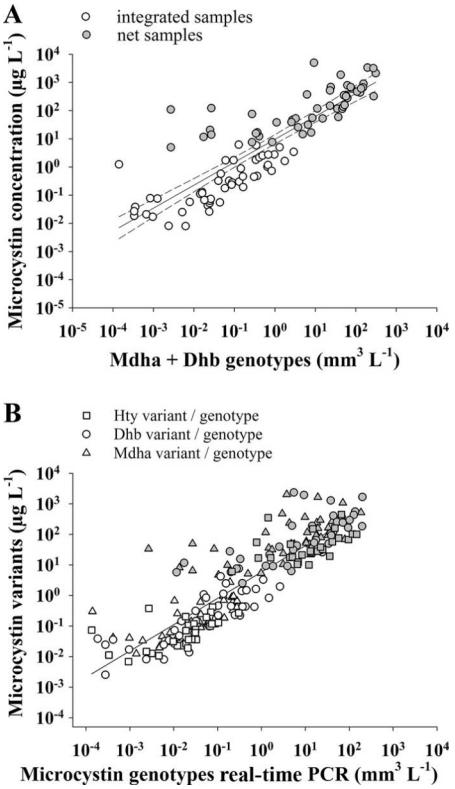

FIG. 3. (A) Relationship between the total of the microcystin genotypes (Mdha and Dhb) determined by real-time PCR (biovolume in mm^3 $liter^{-1}$) and the total microcystin concentration estimated by HPLC (μg $liter^{-1}$) from integrated (white symbols) and net (gray symbols) samples. Broken lines indicate 95% confidence limits. (B) Relationship between each of the microcystin genotypes and the corresponding microcystin variants for the same data set. For details on the regression curves, see the text.

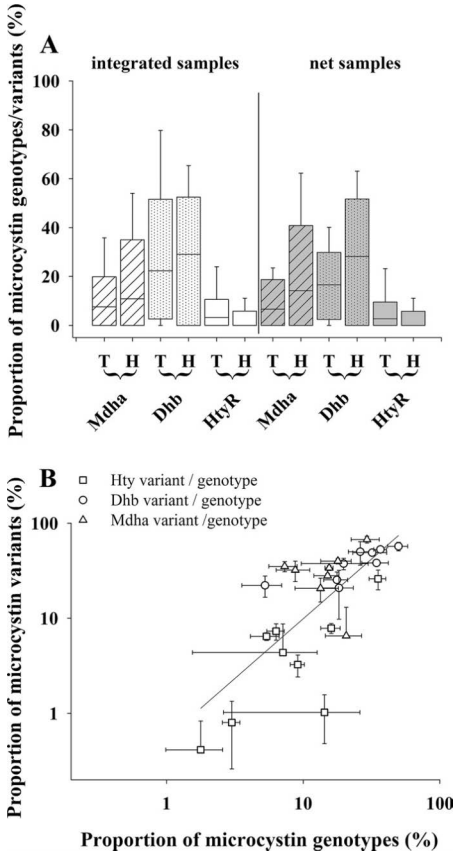

FIG. 4. (A) Proportions of genotypes encoding the synthesis of a specific microcystin variant (Mdha, Dhb, and Hty) revealed by real-time PCR (T) and of the respective microcystin structural variants revealed by HPLC (H). Box plots show the median and 25% to 75% percentiles. (B) Relationship between the average proportion (±SE) of microcystin variants determined by HPLC and the average proportion (±SE) of microcystin genotypes estimated by real-time PCR for each lake. For details on the regression curve, see the text.

tively related to the trophic state, as indicated by the total phosphorus or chlorophyll *a* concentrations ($P < 0.05$; $R^2 \leq 0.27$). The proportions of the other MC variants did not show any relation to the trophic state.

Relationship between microcystin genotype abundance and microcystin concentration. There was a linear correlation between the abundance of each of the three genotypes and the concentrations of the respective microcystin variants ($y = 0.97 + 0.85x$ [$R^2 = 0.72$; $n = 79$; $P < 0.001$] for Mdha, $y = 0.75 + 0.89x$ [$R^2 = 0.8$; $n = 80$; $P < 0.001$] for Dhb, and $y = 0.5 + 0.85x$ [$R^2 = 0.84$; $n = 54$; $P < 0.001$] for HtyR, where x is the $\log_{10}$ of the respective microcystin genotype measured by real-time PCR [mm^3 $liter^{-1}$] and y is the $\log_{10}$ of the corresponding microcystin variant measured by HPLC [μg $liter^{-1}$]). The sum of Mdha and Dhb genotypes was significantly related to the total concentration of microcystin following the regression $y = 0.98 + 0.81x$ ($R^2 = 0.73$; $n = 100$; $P < 0.001$) (Fig. 3A). Furthermore, the abundance of each of the microcystin genotypes was significantly related to the microcystin concentration assigned to the corresponding microcystin variant, $y = 0.77 + 0.86x$ ($R^2 = 0.77$; $n = 213$; $P < 0.001$), where x is the $\log_{10}$ biovolume of a specific genotype and y is the $\log_{10}$ of the concentration of the corresponding microcystin variant (μg $liter^{-1}$) (Fig. 3B). Corresponding to the highest Dhb genotype proportion (36.8% ± 4% among the integrated samples and 20.9% ± 2% among the net samples), the Dhb microcystin constituted 46.2% ± 4% of the integrated samples and 43.9% ± 4% of the net samples (Fig. 4A). Correspondingly, the lower Mdha genotype proportion (14.2% ± 2% of the integrated samples and 13.2% ± 2% of the net samples) resulted in a lower proportion of the Mdha microcystin (30.8% ± 3% of the integrated samples and 35.5% ± 4% of the net samples). In contrast, the lowest Hty genotype proportion (10.4% ± 2% of the integrated samples and 10.7% ± 3% of the net samples) resulted in a low proportion of the Hty microcystin both among the integrated samples (8% ± 2%) and among the net samples (6.3% ± 2%). On average, the proportion of microcystin genotypes in the lakes was related to the concentrations of the corresponding microcystin variant following the regression $y = -0.26 + 1.26x$ ($R^2 = 0.54$; $n = 26$), where x is the $\log_{10}$ of the average proportion of the genotypes and y is the $\log_{10}$ of the average proportion of the corresponding microcystin variants of the lakes (Fig. 4B).

DISCUSSION

Correlation between microcystin concentrations and the abundance of microcystin genotypes. In the present study,

the concentrations of microcystins in total as well as each of the three structural variants could be predicted from the corresponding genotype concentrations. We did not find that a specific genotype concentration was significantly better at predicting the corresponding microcystin concentration than the total population, as determined via 16S rRNA gene assay or via microscopic counting. For all predicting variables, the slope of the regression curve was close to 1, and the coefficient of determination was close to 0.9. This is probably because the populations that were investigated were absolutely dominated by the microcystin-producing genotype (22). Accordingly, Briand et al. (4) previously reported a highly significant correlation between the individual growth rate and the microcystin production rate of *Planktothrix* in Lac du Bourget, France. In that study, the high correlation coefficients observed were favored by the occurrence of only one microcystin-producing organism. Since the real-time PCR assays that were applied were *Planktothrix* specific, the presence of other microcystin-producing taxa would have deteriorated the quality of the regression curves. If other taxa containing the *mcy* gene cluster would be found to be co-occurring, more general probes and primers, for example, targeted at the *mcyE* gene (16, 34), would need to be applied. In general, the polyketide part of the *mcy* synthetase gene cluster shows less genetic variation between genera and is most suitable for the design of primers binding to the *mcyE* gene of all taxa. The *mcyE* abundance would then need to be referred to by applying another real-time PCR assay targeted at cyanobacterial 16S rRNA genes (36). The sequencing of the *mcyE* products would then lead to the identification of the corresponding microcystin-producing organisms.

In addition, from the results of the present study, it must be concluded that all microcystin variants produced by *Planktothrix* spp. that were observed under field conditions were also observed in culture, and the proportion of the unexplained microcystin variants occurring under natural conditions was low ($\leq$5.1%) (see Table S1 in the supplemental material). Recently, by comparing peptide compositions between the single isolates and field samples, the isolation of all major chemotypes occurring in Lake Steinsfjorden in Norway was reported (39). This high correlation in peptide occurrence between the laboratory and field conditions is remarkable, as the cultivation of bacteria in general is considered biased, i.e., favoring those species and/or genotypes that are able to cope with the high concentrations of nutrients that are provided (1). It was speculated that the positive phototaxis of *Planktothrix* filaments on agar, which allows the cutting out of single filaments by means of a microspade (38), leads to a much higher rate of success in the isolation of individual strains compared to immotile cyanobacteria. It is speculated that due to its accessibility to cultivation, the genus *Planktothrix* is highly suitable for the analysis of the genetic regulation of toxin production both in the laboratory and in the field.

Low proportion of inactive microcystin genotypes. In a previous study, we reported the proportion of inactive microcystin genotypes carrying either deletions or insertions within the *mcy* gene cluster in samples from the same lakes (32). On average, a rather low proportion of the mutant genotypes carrying either the deletion (3.7%) or several insertions (2.8%) was found. In this study, the high correlation coefficient between the individual microcystin genotypes and the concentration of the corresponding microcystins (R^2 = 0.72 to 0.84) implies that the proportion of inactive microcystin genotypes did not vary significantly and that the occurrence of selective sweeps leading to a dominance of inactive genotypes did not occur. Recently, Manganelli et al. (28) suggested an increase in *Planktothrix* genotypes lacking the *mcy* gene cluster under conditions that favor blooms in an Italian lake. The highest densities recorded were 44.4×10^6 cells liter^{-1}, corresponding to 1.47 mm^3 liter^{-1} (assuming a cellular *Planktothrix* biovolume of 33.4 μm^3). In this study, these higher *Planktothrix* cell densities were observed frequently in depth-integrated samples of Lake Wörthersee, Lake Zürichsee, and Lake Hallwilersee. For a much more densely growing green-pigmented *Planktothrix* population flourishing in Lake Base Nautique de Viry (Paris, France), an increase in the non-*mcy*-containing genotype from a blooming period (50 mm^3 liter^{-1}; 36 to 60% of the *mcyA* genotype) to a period with the lowest cell numbers (0.7 to 8 mm^3 liter^{-1}; 56 to 73%) was reported (3). From this data set, the change in the proportion of the *mcy*-containing genotype seems rather minor compared with the large variation in population cell density.

At present, it is difficult to explain which factors keep the proportion of the inactive microcystin genotypes low. It is frequently argued that the production of secondary metabolites, including enzymes and toxins, can be considered a common good that is beneficial to the whole population of a particular prokaryotic organism, e.g., *Myxococcus xanthus* or *Bacillus subtilis* (the multicellular organism hypothesis [43]). In these populations, the occurrence of mutants that are deficient in secondary metabolite production but that show faster growth rates that lead to an ultimate outgrowth of the wild type has been frequently reported (47). Recently, it was shown that so-called nontoxic cheaters occurring in populations of *Pseudomonas fluorescens* show a negative frequency-dependent fitness, implying that the nontoxic mutants indeed rely on the exotoxins produced by wild-type bacteria (15). For *Planktothrix*, it could be argued that predators such as aquatic crustaceans keep the proportion of inactive microcystin genotypes low. Unfortunately for this particular hypothesis, the vast majority of *Planktothrix* filaments exceed the ingestible size range for pelagic herbivorous crustaceans, and the ingestion rates that have been determined for *Planktothrix* filaments are close to zero compared with those of other food items (25). Another selective factor promoting microcystin production in *Planktothrix* sp. might be chronic infection by parasites, such as by the chytrid fungus *Rhizophidium megarrhizum* Sparrow (5). According to that study, infected filaments can be identified by one or several zoospores settling at the apex of the filament. In our study, a low proportion of infected filaments was observed (only two filaments were found to carry zoospores during the enumeration of *Planktothrix* in the 159 samples). It remains to be seen whether it is indeed the inactive microcystin genotype that was infected by the parasite. In the future, single filaments should be isolated and probed for the presence of *mcy* gene cluster mutations and the co-occurrence of potential parasites by means of single-filament PCR (22).

Water quality monitoring. The results of the present study are of relevance to justify the application of toxic genotype monitoring by means of real-time PCR in order to predict

microcystin concentrations in water. Following the overall regression curve (depicted in Fig. 3A), a biovolume of 10 mm^3 $liter^{-1}$ of microcystin genotypes corresponds to a microcystin concentration of 62.3 ± 1 μg (confidence interval, 45.8 to 84.8 μg) microcystin-LR equivalents. Since microcystin-producing strains of *Microcystis* and *Planktothrix* do not differ principally in their microcystin contents (20), such a regression curve may also be applicable to water samples that are dominated by *Microcystis*. While for both taxa, comparable ranges of cellular microcystin contents have been reported (e.g., see data in reference 49 versus data in reference 46), we hereby emphasize, however, that the applicability of the regression curve (Fig. 3A) to freshwater that is dominated by *Microcystis* still needs to be confirmed.

Currently, drinking water/recreational water guideline values are calculated based on microcystin-LR equivalents. It has been repeatedly shown that microcystin structural variants can differ profoundly in their toxicodynamic and toxicokinetic properties (2, 13), and therefore, it was suggested that risk assessment schemes that are based solely on microcystin-LR are insufficient (8). According to a report by Blom et al. (2), the inhibition of protein phosphatases 1 and 2A was much weaker for [Asp, Dhb]-microcystin-RR than for the other structural variants, while the toxicity was one of the highest. Consequently, it might be useful to differentiate the genotypes encoding those microcystin variants, such as [Asp, Dhb]-microcystin-RR, which differ in toxicity compared with microcystin-LR.

Frequently, the length of the shorelines that need to be observed constitutes a major factor that increases the monitoring costs and response time due to sample transportation (45). Since portable real-time PCR instruments have been shown to be field applicable (35), it is conceivable that during future monitoring, those measurements will be confirmed directly on site subsequent to the filtration of water samples and the rapid extraction of the DNA from filters according to previously established protocols (41). The results would then be directly forwarded to the respective authorities and distributed to the public via websites, newspapers, and radio (9). This approach would reduce not only the effort of sample transportation but also the sample analysis time until an early warning is possible. Various real-time PCR assays quantifying the microcystin-producing genotypes of the other microcystin-producing genera *Microcystis* and *Anabaena* and the nodularin-producing genotypes of the genus *Nodularia* have been developed (see reference 33 for a review). Analogously, in parallel to the description of the gene cluster involved in cylindrospermopsin synthesis (31, 40), real-time PCR assays for the identification of the cylindrospermopsin-producing genera *Cylindrospermopsis* and *Aphanizomenon* have been applied (35). The gene clusters encoding the synthesis of other toxic metabolites have been elucidated more recently, i.e., saxitoxin (17) and anatoxin-a (29). This sequence information forms the basis for the establishment of a real-time-based monitoring approach that targets all the toxic genotypes of cyanobacteria in surface water.

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REFERENCES

1. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Mol. Biol. Rev. **59:**143–169.
2. **Blom, J. F., J. A. Robinson, and F. Jüttner.** 2001. High grazer toxicity of [D-Asp^3,(E)-Dhb^7]microcystin-RR of *Planktothrix rubescens* as compared to different microcystins. Toxicon **39:**1923–1932.
3. **Briand, E., M. Gugger, J. C. Francois, C. Bernard, J. F. Humbert, and C. Quiblier.** 2008. Temporal variations in the dynamics of potentially microcystin-producing strains in a bloom-forming *Planktothrix agardhii* (cyanobacterium) population. Appl. Environ. Microbiol. **74:**3839–3848.
4. **Briand, J.-F., S. Jacquet, C. Flinois, C. Avois-Jacquet, C. Maisonnette, B. Leberre, and J. F. Humbert.** 2005. Variations in the microcystin production of *Planktothrix rubescens* (cyanobacteria) assessed from a four-year survey of Lac du Bourget (France) and from laboratory experiments. Microb. Ecol. **50:**418–428.
5. **Canter, H. M., and J. W. G. Lund.** 1951. Studies on plankton parasites. III. Examples of the interaction between parasitism and other factors determining the growth of diatoms. Ann. Bot. **15:**359–371.
6. **Christiansen, G., R. Kurmayer, Q. Liu, and T. Börner.** 2006. Transposons inactivate biosynthesis of the nonribosomal peptide microcystin in naturally occurring *Planktothrix* spp. Appl. Environ. Microbiol. **72:**117–123.
7. **Davis, T. W., D. L. Berry, G. L. Boyer, and C. J. Gobler.** 2009. The effects of temperature and nutrients on the growth and dynamics of toxic and non-toxic strains of *Microcystis* during cyanobacteria blooms. Harmful Algae **8:**715–725.
8. **Dietrich, D., and S. Hoeger.** 2005. Guidance values for microcystins in water and cyanobacterial supplement products (blue-green algal supplements): a reasonable or misguided approach? Toxicol. Appl. Pharmacol. **203:**273–289.
9. **Erdner, D. L., J. Dyble, M. L. Parsons, R. C. Stevens, K. A. Hubbard, M. L. Wrabel, S. K. Moore, K. A. Lefebvre, D. M. Anderson, P. Bienfang, R. R. Bidigare, M. S. Parker, P. Moeller, L. E. Brand, and V. L. Trainer.** 2008. Centers for Oceans and Human Health: a unified approach to the challenge of harmful algal blooms. Environ. Health **7**(Suppl. 2)**:**S2.
10. **Fastner, J., I. Flieger, and U. Neumann.** 1998. Optimised extraction of microcystins from field samples—a comparison of different solvents and procedures. Water Res. **32:**3177–3181.
11. **Fischer, W. J., I. Garthwaite, C. O. Miles, K. M. Ross, J. B. Aggen, A. R. Chamberlin, N. A. Towers, and D. R. Dietrich.** 2001. Congener-independent immunoassay for microcystins and nodularins. Environ. Sci. Technol. **35:** 4849–4858.
12. **Ha, J. H., T. Hidaka, and H. Tsuno.** 2009. Quantification of toxic *Microcystis* and evaluation of its dominance ratio in blooms using real-time PCR. Environ. Sci. Technol. **43:**812–818.
13. **Hoeger, S. J., D. Schmid, J. F. Blom, B. Ernst, and D. R. Dietrich.** 2007. Analytical and functional characterization of microcystins [Asp(3)]MC-RR and [Asp(3),Dhb(7)]MC-RR: consequences for risk assessment? Environ. Sci. Technol. **41:**2609–2616.
14. **Hotto, A. M., M. F. Satchwell, D. L. Berry, C. J. Gobler, and G. L. Boyer.** 2008. Spatial and temporal diversity of microcystins and microcystin-producing genotypes in Oneida Lake, NY. Harmful Algae **7:**671–681.
15. **Jousset, A., L. Rochat, M. Pechy-Tarr, C. Keel, S. Scheu, and M. Bonkowski.** 2009. Predators promote defence of rhizosphere bacterial populations by selective feeding on non-toxic cheaters. ISME J. **3:**666–674.
16. **Jungblut, A. D., and B. A. Neilan.** 2006. Molecular identification and evolution of the cyclic peptide hepatotoxins, microcystin and nodularin, synthetase genes in three orders of cyanobacteria. Arch. Microbiol. **185:**107–114.
17. **Kellmann, R., T. K. Michali, Y. J. Jeon, R. Pickford, F. Pomati, and B. A. Neilan.** 2008. Biosynthetic intermediate analysis and functional homology reveal a saxitoxin gene cluster in cyanobacteria. Appl. Environ. Microbiol. **74:**4044–4053.
18. **Komarek, J.** 2003. Planktic oscillatorialean cyanoprokaryotes (short review according to combined phenotype and molecular aspects). Hydrobiologia **502:**367–382.
19. **Koskenniemi, K., C. Lyra, P. Rajaniemi-Wacklin, J. Jokela, and K. Sivonen.** 2007. Quantitative real-time PCR detection of toxic *Nodularia* cyanobacteria in the Baltic Sea. Appl. Environ. Microbiol. **73:**2173–2179.
20. **Kosol, S., J. Schmidt, and R. Kurmayer.** 2009. Variation in peptide net production and growth among strains of the toxic cyanobacterium *Planktothrix* spp. Eur. J. Phycol. **44:**49–62.
21. **Kurmayer, R., G. Christiansen, and I. Chorus.** 2003. The abundance of microcystin-producing genotypes correlates positively with colony size in *Microcystis* and determines its microcystin net production in Lake Wannsee. Appl. Environ. Microbiol. **69:**787–795.

22. **Kurmayer, R., G. Christiansen, J. Fastner, and T. Börner.** 2004. Abundance of active and inactive microcystin genotypes in populations of the toxic cyanobacterium *Planktothrix* spp. Environ. Microbiol. **6:**831–841.
23. **Kurmayer, R., G. Christiansen, M. Gumpenberger, and J. Fastner.** 2005. Genetic identification of microcystin ecotypes in toxic cyanobacteria of the genus *Planktothrix*. Microbiology **151:**1525–1533.
24. **Kurmayer, R., and M. Gumpenberger.** 2006. Diversity of microcystin genotypes among populations of the filamentous cyanobacteria *Planktothrix rubescens* and *Planktothrix agardhii*. Mol. Ecol. **15:**3849–3861.
25. **Kurmayer, R., and F. Jüttner.** 1999. Strategies for the co-existence of zooplankton with the toxic cyanobacterium *Planktothrix rubescens* in Lake Zürich. J. Plankton Res. **21:**659–683.
26. **Kurmayer, R., and G. Christiansen.** 2009. The genetic basis of toxin production in cyanobacteria. Freshw. Rev. **2:**31–50.
27. **Lawton, L. A., C. Edwards, and G. A. Codd.** 1994. Extraction and high-performance liquid chromatographic method for the determination of microcystins in raw and treated waters. Analyst **119:**1525–1530.
28. **Manganelli, M., S. Scardala, M. Stefanelli, S. Vichi, D. Mattei, S. Bogialli, P. Ceccarelli, E. Corradetti, I. Petrucci, S. Gemma, E. Testai, and E. Funari.** 2010. Health risk evaluation associated to *Planktothrix rubescens*: an integrated approach to design tailored monitoring programs for human exposure to cyanotoxins. Water Res. **44:**1297–1306.
29. **Méjean, A., S. Mann, T. Maldiney, G. Vassiliadis, O. Lequin, and O. Ploux.** 2009. Evidence that biosynthesis of the neurotoxic alkaloids anatoxin-a and homoanatoxin-a in the cyanobacterium *Oscillatoria* PCC 6506 occurs on a modular polyketide synthase initiated by L-proline. J. Am. Chem. Soc. **131:**7512–7513.
30. **Metcalf, J. S., P. Hyenstrand, K. A. Beattie, and G. A. Codd.** 2000. Effects of physicochemical variables and cyanobacterial extracts on the immunoassay of microcystin-LR by two ELISA kits. J. Appl. Microbiol. **89:**532–538.
31. **Mihali, T. K., R. Kellmann, J. Muenchhoff, K. D. Barrow, and B. A. Neilan.** 2007. Characterization of the gene cluster responsible for cylindrospermopsin biosynthesis. Appl. Environ. Microbiol. **74:**716–722.
32. **Ostermaier, V., and R. Kurmayer.** 2009. Distribution and abundance of nontoxic mutants of cyanobacteria in lakes of the Alps. Microb. Ecol. **58:**323–333.
33. **Pearson, L. A., and B. A. Neilan.** 2008. The molecular genetics of cyanobacterial toxicity as a basis for monitoring water quality and public health risk. Curr. Opin. Biotechnol. **19:**281–288.
34. **Rantala, A., D. P. Fewer, M. Hisbergues, L. Rouhiainen, J. Vaitomaa, T. Börner, and K. Sivonen.** 2004. Phylogenetic evidence for the early evolution of microcystin synthesis. Proc. Natl. Acad. Sci. U. S. A. **101:**568–573.
35. **Rasmussen, J. P., S. Giglio, P. T. Monis, R. J. Campbell, and C. P. Saint.** 2008. Development and field testing of a real-time PCR assay for cylindrospermopsin-producing cyanobacteria. J. Appl. Microbiol. **104:**1503–1515.
36. **Rinta-Kanto, J., A. Ouelette, G. Boyer, M. Twiss, T. Bridgeman, and S. Wilhelm.** 2005. Quantification of toxic *Microcystis* spp. during the 2003 and 2004 blooms in Western Lake Erie using quantitative real-time PCR. Environ. Sci. Technol. **39:**4198–4205.
37. **Rinta-Kanto, J. M., E. A. Konopko, J. M. Debruyn, R. A. Bourbonniere, G. L. Boyer, and S. W. Wilhelm.** 2009. Lake Erie *Microcystis*: relationship between microcystin production, dynamics of genotypes and environmental parameters in a large lake. Harmful Algae **8:**665–673.
38. **Rippka, R.** 1988. Isolation and purification of cyanobacteria. Methods Enzymol. **167:**3–27.
39. **Rohrlack, T., B. Edvardsen, R. Skulberg, C. B. Halstvedt, H. C. Utkilen, R. Ptacnik, and O. M. Skulberg.** 2008. Oligopeptide chemotypes of the toxic freshwater cyanobacterium *Planktothrix* can form subpopulations with dissimilar ecological traits. Limnol. Oceanogr. **53:**1279–1293.
40. **Schembri, M. A., B. A. Neilan, and C. P. Saint.** 2001. Identification of genes implicated in toxin production in the cyanobacterium *Cylindrospermopsis raciborskii*. Environ. Toxicol. **16:**413–421.
41. **Schober, E., and R. Kurmayer.** 2006. Evaluation of different DNA sampling techniques for the application of the real-time PCR method for the quantification of cyanobacteria in water. Lett. Appl. Microbiol. **42:**412–417.
42. **Schober, E., M. Werndl, K. Laakso, I. Korschinek, K. Sivonen, and R. Kurmayer.** 2007. Interlaboratory comparison of Taq nuclease assays for the quantification of the toxic cyanobacteria *Microcystis* sp. J. Microbiol. Methods **69:**122–128.
43. **Shapiro, J. A.** 1998. Thinking about bacterial populations as multicellular organisms. Annu. Rev. Microbiol. **52:**81–104.
44. **Sivonen, K.** 2008. Emerging high throughput analyses of cyanobacterial toxins and toxic cyanobacteria, p. 539–557. *In* H. K. Hudnell (ed.), Cyanobacterial harmful algal blooms: state of the science and research needs. Springer, New York, NY.
45. **Sivonen, K., and T. Börner.** 2008. Bioactive compounds produced by cyanobacteria, p. 159–197. *In* A. Herrero and E. Flores (ed.), The cyanobacteria: molecular biology, genomics and evolution. Caister Academic Press, Norfolk, United Kingdom.
46. **Tonk, L., P. Visser, G. Christiansen, E. Dittmann, E. Sneldfer, C. Wiedner, L. Mur, and J. Huisman.** 2005. The microcystin composition of the cyanobacterium *Planktothrix agardhii* changes toward a more toxic variant with increasing light intensity. Appl. Environ. Microbiol. **71:**5177–5181.
47. **Travisano, M., and G. J. Velicer.** 2004. Strategies of microbial cheater control. Trends Microbiol. **12:**72–78.
48. **Wetzel, R. G., and G. E. Likens.** 2000. Limnological analyses, 3rd ed., p. 147–174. Springer-Verlag, New York, NY.
49. **Wiedner, C., P. Visser, J. Fastner, J. S. Metcalf, G. A. Codd, and L. R. Mur.** 2003. Effects of light on the microcystin content of *Microcystis* strain PCC 7806. Appl. Environ. Microbiol. **69:**1475–1481.
50. **Ye, W. J., X. L. Liu, J. Tan, D. T. Li, and H. Yang.** 2009. Diversity and dynamics of microcystin-producing cyanobacteria in China's third largest lake, Lake Taihu. Harmful Algae **8:**637–644.

Supplemental Material Chapter 2

Suppl. Table 1: Linear calibration curves to estimate cell numbers of the total *Planktothrix* population and of three genotypes differing in the amino acid residue in pos. 7 (Mdha vs. Dhb) or pos. 2 (Hty) of the microcystin molecule by real-time PCR (TNA).

TNA	Gene locus	Strain	Calibration curve[a]	*E* (%)[b]	R^2	Sample size
16S	16S rDNA	PCC7821	$y = 36.537 - 3.5123x$	92.6	0.997	12
McyH	*mcy*A, Dhb Pos.7	PCC7821	$y = 39.319 - 3.8369x$	82.2	0.987	11
Mdha	*mcy*A, Mdha Pos.7	No.40	$y = 38.435 - 3.3371x$	99.4	0.996	10
Hty	*mcy*B, Hty Pos.2	No.21/1	$y = 37.206 - 3.4666x$	94.3	0.996	11

[a] $y = C_t$ value (threshold cycle of PCR at fluorescence 100), x = amount of template DNA (expressed as $\log_{10}$ of cell number equivalents)

[b] Amplification efficiencies (E) were calculated from $E = (10^{-1/\text{slope}} - 1) \times 100$

Suppl. Table 2: Mean ± SE C_t values as determined by real-time PCR from DNA extracts of *Planktothrix* strain PCC7821 both in the absence and in the presence of background DNA. Background DNA was added in the ratio 1:100 and 1:1 equivalent to the DNA of *Planktothrix*. For the preparation of background DNA see Ostermaier & Kurmayer (2009).

	Dhb	Mdha	Hty
Planktothrix DNA	30.48 ± 0.32	31.76 ± 0.2	28.2 ± 0.1
Planktothrix DNA / background DNA ratio 1:100	30.61 ± 0.1	31.62 ± 0.27	28.24 ± 0.25
Planktothrix DNA / background DNA ratio 1:1	30.39 ± 0.03	31.94 ± 0.13	27.6 ± 0.01

Reference

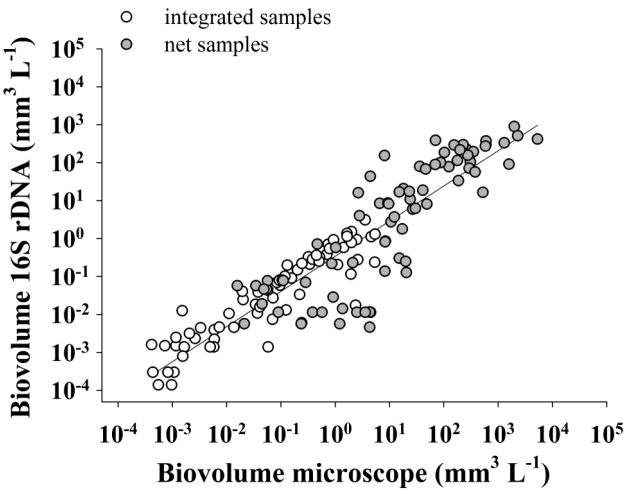
Ostermaier, V. and R. Kurmayer. 2009. Distribution and abundance of nontoxic mutants of cyanobacteria in lakes of the Alps. Microb. Ecol. **58**:323-333.

Suppl. Table 3: Average (± SE) proportions (in %) and retention times (in min) of microcystin variants as measured by HPLC from integrated and net samples

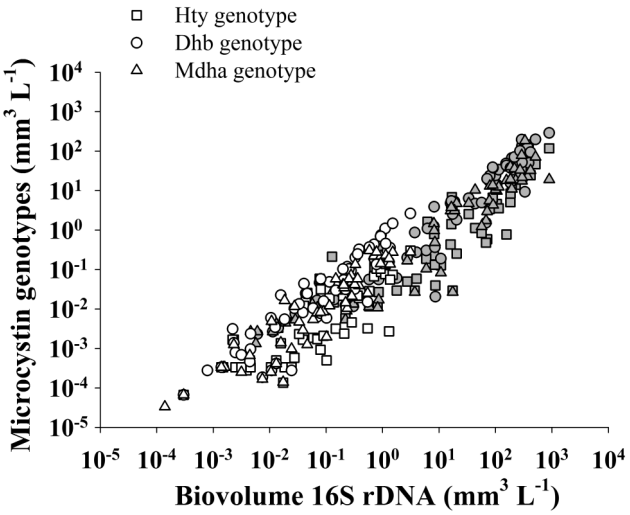
Lake	Country	Type	N[a]	D-Asp-Mdha-RR	Retention time	D-Asp-Dhb-RR	Retention time	D-Asp-HtyR	Retention time	D-Asp-LR	Retention time	unknown microcystins	Retention time
Afritzersee	AT	int	6	38 ± 4.2	13.9-14.4	40 ± 3.3	14.7-15.2	8.6 ± 2.1	18.7-18.9	12.6 ± 1.2	19.6-20.0	0.9 ± 0.9	>20
Afritzersee	AT	net	6	41.8 ± 3.5	12.9-14.5	36.2 ± 3.8	14.0-15.0	6 ± 2	18.3-18.8	12.7 ± 0.9	19.3-19.5	3.2 ± 1.8	>20
Ammersee	D	int	6	64.2 ± 7.2	13.9-14.5	25 ± 8.2	14.7-15.2	0.8 ± 0.8	18.6-18.6	10.1 ± 5.5	19.6-19.8	0	-
Ammersee	D	net	6	70.4 ± 8.4	13.1-13.9	25.6 ± 7.5	13.9-14.6	0.8 ± 0.8	18.4-18.4	3.2 ± 1.7	19.2-19.6	0	-
Attersee	D	int	1	0	-	0	-	0	-	100	19.7-19.7	0	-
Attersee*	D	net	0	0	-	0	-	0	-	0	-	0	-
Fuschlsee	AT	int	6	41.6 ± 3.4	13.9-14.5	45.5 ± 4.7	14.7-15.2	1.6 ± 1.0	18.8-18.9	8.5 ± 3.9	19.8-19.9	2.9 ± 2.9	>20
Fuschlsee	AT	net	6	40.3 ± 1.8	13.3-14.0	42.1 ± 5.2	14.1-14.7	0.8 ± 0.8	18.5-18.5	13.1 ± 4.9	19.3-19.6	3.7 ± 3.7	>20
Hallwilersee	CH	int	5	16 ± 1.9	13.9-14.5	62.4 ± 2	14.6-15.6	7.3 ± 1.4	18.6-19.0	14.2 ± 2	19.6-20.1	0	-
Hallwilersee	CH	net	6	24.5 ± 10.7	12.6-14.2	52.5 ± 10.6	13.4-14.8	8.3 ± 1.4	18.1-19.0	14.5 ± 1.7	18.9-20.1	0.3 ± 0.2	>20
Irrsee	AT	int	4	0	-	47.9 ± 27.7	14.9-15.0	15.3 ± 15.3	18.7-18.7	36.8 ± 22.7	19.8-19.8	0	-
Irrsee	AT	net	2	45.7 ± 45.7	13.8-13.8	50 ± 50	14.6-14.6	0	-	4.3 ± 4.3	19.5-19.5	0	-
Mondsee	AT	int	4	54.3 ± 1.2	14.2-14.5	37.5 ± 4	15.0-15.2	1.4 ± 1.4	18.7-18.7	6.8 ± 3.9	19.7-19.8	0	-
Mondsee	AT	net	4	58.1 ± 4	13.8-13.9	40.1 ± 4.4	14.5-14.6	0	-	1.8 ± 1.8	19.5-19.5	0	-
Offensee	AT	int	4	0	-	0.0	-	25.1 ± 8.9	18.7-18.9	32.1 ± 11.4	19.7-20.0	0	-
Offensee	AT	net	4	0	-	0.0	-	40.6 ± 4.2	18.2-18.5	59.4 ± 4.2	19.4-19.6	0	-
Schwarzensee	AT	int	3	0	-	100	14.7-15.2	0	-	0	-	0	-
Schwarzensee	AT	net	4	0	-	100	14.1-14.8	0	-	0	-	0	-
Wolfgangsee[b]	AT	int	0	0	-	0	-	0	-	0	-	0	-
Wolfgangsee[b]	AT	net	0	0	-	0	-	0	-	0	-	0	-
Wörthersee	AT	int	6	26.3 ± 0.9	13.9-14.4	51.8 ± 0.8	14.6-15.2	6.1 ± 0.6	18.6-18.9	14.4 ± 1	19.6-20.0	1.4 ± 1.4	>20
Wörthersee	AT	net	6	29.6 ± 3.2	12.9-14.0	46 ± 3.4	13.6-14.3	6.8 ± 1.1	18.1-18.7	13.8 ± 1.8	19.2-19.5	3.9 ± 2.4	>20
Zürichsee	CH	int	7	32.1 ± 2.8	13.5-15.1	54.5 ± 3.4	14.3-15.8	2.5 ± 1.3	18.3-18.9	5.9 ± 1.9	19.3-20.0	5.1 ± 2.6	>20
Zürichsee	CH	net	8	35.2 ± 3.1	11.3-14.6	51.3 ± 3.3	12.2-15.3	3.9 ± 1.2	18.1-19.0	8.6 ± 2.8	19.1-19.6	1 ± 0.8	>20

[a] Number of samples used to calculate the proportions

[b] MCs detected only by ELISA in all samples



Suppl. Fig. S1: Relationship between the *Planktothrix* biovolume (mm^3 L^{-1}) as estimated from microscopical counting and as estimated by real-time PCR via the 16S rDNA. For details on the regression curve, see text. Ten samples that were negative in the microscope were excluded in the regression curve.



Suppl. Fig. S2: Relationship between the total *Planktothrix* biovolume ($mm^3 L^{-1}$) as estimated by real time PCR via 16S rDNA and the biovolume ($mm^3 L^{-1}$) of each of the three microcystin genotypes for integrated (white symbols) and net samples (grey symbols). For each of the microcystin genotypes the details on the linear regression curve are reported in the text.

Chapter 3

Genetic variability of microcystin biosynthesis genes in *Planktothrix* as elucidated from samples preserved by heat desiccation during three decades

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Keywords:

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Abstract

Historic samples of phytoplankton can provide information on the abundance of the toxigenic genotypes of cyanobacteria in dependence on increased or decreased eutrophication. The analysis of a time-series from preserved phytoplankton samples by quantitative PCR (qPCR) extends observation periods considerably. The analysis of DNA from heat-desiccated samples by qPCR can be aggravated by point substitutions or the fragmentation of DNA introduced by the high temperature. In this study, we analyzed whether the heat desiccation of the cellular material of the cyanobacterium *Planktothrix* sp. introduced potential errors to the template DNA that is used for qPCR within (i) 16S rDNA and phycocyanin genes and (ii) *mcy*A genes indicative of the incorporation of either dehydrobutyrine (Dhb) or N-methyl-dehydroalanine (Mdha) in position 7 or the *mcy*B gene, which is indicative of homotyrosine (Hty) in position 2 of the microcystin (MC) molecular structure. Due to high temperature desiccation, the major deterioration of the DNA template quality was due to fragmentation. In contrast, the nucleotide substitutions reduced the template DNA quality only to a minor extent. By using the heat-desiccated samples of Lake Zürich (Switzerland) the abundance of the Dhb, Mdha and Hty genotypes was determined during three decades (1977-2008). Despite major changes in the trophic state of the lake resulting in a major increase of the total *Planktothrix* population density, the proportion of these genotypes resulting in different MC congeners showed high stability. The decline of most of the abundant genotype that is responsible for the synthesis of Dhb in position 7 of the MC molecule, however, could be related to the gradual incline in the proportion of a mutant carrying a 1.8kbp deletion of this gene region. The increase of this *mcy* (Dhb) gene deletion mutant has been minor so far, however, and likely did not affect the overall toxicity of the population.

Introduction

The microcystin (MC) synthetase (*mcy*) gene cluster encodes a mixed nonribosomal peptide synthetase (NRPS)/polyketide synthase (PKS) enzyme complex that produces the heptapeptide MC, which is the most frequent toxin occurring in blooms formed by cyanobacteria in freshwater. The *mcy* gene cluster has been sequenced from different cyanobacterial genera, e.g. *Microcystis*, *Planktothrix* and *Anabaena* [1-3]. NRPS are composed of modules consisting of adenylation, thiolation, and condensation domains, which are responsible for the activation and condensation of amino acid substrates to the growing peptide chain. Additional enzymes regularly found in NRPS catalyze modification such as N-methylation (N-methylation domain, NMT), O-methylation, and the racemization of precursor amino acid substrates. The modular structure of NRPS has been linked to the high rate of evolution as gene collectives, as single genes or domains of different NRPS gene clusters can be horizontally exchanged and functionally interact to give rise to novel peptides (e.g. [4]). For *Microcystis*, the occurrence of different MC structural isoforms could be assigned to major genetic differences within the first adenylation (A1) -domain of *mcy*B [5], which were related to a recombination event between A-domains of *mcy*B and *mcy*C [6]. In *Planktothrix*, two distinct *mcy*A-A1-domain genotypes of the *mcy*A gene are known that differ in sequence and in the presence of a gene region encoding an N-methyltransferase domain [7]. It was concluded earlier that genetic recombination led to a complete replacement of a gene region coding for the first A-domain of *Mcy*A activating serine, which is later dehydrated to dehydroalanine (Dha). An integrated N-methyltransferase domain catalyzes the transfer of a methyl group to Dha resulting in N-methyl-dehydroalanine (Mdha) in position 7 of the MC molecule. Through recombination, a second genotype of the *mcy*A-A1-domain activates threonine, which is later dehydrated to dehydrobutyrine (Dhb) at the same position of the MC molecule [7]. Further evidence for sequence variability and recombination events between the

A-domains of *mcy*A, *mcy*B, and *mcy*C was given by Fewer et al. [8] and Tooming-Klunderud et al. [9].

The introduction of structural variability can have functional consequences, e.g. altered enzyme inhibition activity [10-13]. As evaluated by the *Thamnocephalus platyurus* toxicity assay, the toxicity of the MC variant with Dhb in position 7 ([D-Asp^3, (*E*)-Dhb^7]-MC-RR) was shown to be significantly higher compared to other MCs with Mdha in position 7. In contrast, the inhibition of the protein phosphatase 1 and 2A by the same variant was the lowest when compared with structural variants with Mdha in position 7 [11]. In mouse bioassays, the LD_{50} values of the MC-RR variants with Mdha in position 7 ([Asp^3]-MC-RR) or Dhb in the same position ([Asp^3, Dhb^7]-MC-RR) were 500-800 and 250 µg kg^{-1} body weight, while the MC variant with homotyrosine in position 2 ([D-Asp^3,(*E*)Dhb^7]-MC-HtyR) showed a lower LD_{50} value of 70 µg kg^{-1} body weight [14-15].

While toxigenic cyanobacteria are routinely monitored by means of qPCR, e.g. [16], the quantification of individual genotypes encoding specific MC structural variants has received less attention. In a previous study, the proportion of genotypes indicative of Dhb or Mdha in position 7 or of homotyrosine (Hty) in position 2 of the MC molecule were compared among the populations of the cyanobacterium *Planktothrix* in lakes of the Alps over a period of two years (2005-2007). On average, the Dhb genotype was the most abundant and its abundance correlated with the higher concentration of the [Asp, Dhb]-MC-RR variant of the same samples [17]. Like other lakes in the Alps, Lake Zürich (Switzerland) underwent major changes in its trophic state during the last 30 years and the population of red-pigmented *Planktothrix rubescens* increased from rare to a stable dominance of the plankton community [18-19]. During this re-oligotrophication, phase phytoplankton samples have been preserved by high temperature desiccation for the purpose of documentation. By utilizing these samples we showed that the proportion of toxigenic genotypes in total remained one hundred percent and genotypes that lost the *mcy* gene cluster [20] never became abundant [19]. However, in

the previous study, the results on *mcy* genotype composition as well as the applicability of qPCR to preserved DNA samples have not been reported. As the samples from the last thirty years were preserved at 110°C for 2 h and stored at room temperature in the dark for decades, we were interested in the suitability of the DNA isolated from dried biomass for qPCR. Thus, the effect of high temperature desiccation on the applicability of the DNA for qPCR was evaluated experimentally. In order to examine the DNA damage from heat-desiccated samples, an enzyme cocktail formulated to reverse specific DNA base modifications (New England Biolabs, Germany) was applied. Finally, we aimed to correlate the *mcy* genotype abundance to the general increase in *Planktothrix* total population density, thus enabling conclusions on the success of certain *mcy*A or *mcy*B genotypes during 1977 to 2008. We analyzed the proportion of three different *mcy* genotypes that are indicative of Dhb or Mdha in position 7 or of Hty in position 2 of the MC molecule. Previously, [21] a natural deletion mutant *mcy*HA (Dhb) carrying a 1.8 kbp deletion of the Dhb gene was described and this *mcy*HA mutant genotype would reduce the detectability of the Dhb genotype. In order to analyze the gradual decline of the proportion of the most abundant Dhb genotype, we used the data on the abundance of this *mcy*HA deletion genotype [19].

Methods

DNA isolation from heat-desiccated samples

As described previously, the DNA extracted from *Planktothrix* cells (and other phytoplankton) collected on filters from Lake Zürich (n = 111) during 1977-2008 has been used [19]. The phytoplankton biomass was heat-desiccated (110°C, 2h) and stored at room temperature in the dark. For the high-temperature experiments, all DNA extractions were performed using a standard chloroform-phenol method [22].

Experimental evaluation of high temperature-induced effects on DNA quality

Strains PCC7821 (Dhb genotype) and No. 40 (Mdha genotype) were grown in BG11 medium [23] at 20°C under continuous light (5 to 15 µmol photons m^{-2} s^{-1}). The cells were harvested on glass-fiber filters (BMC, Ederol, Vienna, Austria) during the exponential growth phase. The effect of heat desiccation on the DNA quality of the harvested cells was evaluated by means of qPCR using the 16S rDNA, Dhb, and Mdha gene loci (see below). Aliquots from cell cultures (PCC7821: 107 ± 21 (SE) mm^3 L^{-1}, No. 40: 98 ± 26 mm^3 L^{-1}) were filtered and dried at three different temperatures (80°C, 110°C, and 150°C) for either 2 h or 12 h, each. As a control, one aliquot was frozen directly (-20°C). The experiment was repeated twice.

DNA treatment to reverse DNA base modifications

Since heat-induced base modifications resulting from the heat desiccation of DNA could not be excluded, the effect of an enzyme cocktail formulated to reverse DNA modifications (PreCR Repair Mix, New England Biolabs, Germany) was evaluated. The PreCR Repair Mix contains different enzymes for the repair of the most common lesions in DNA (abasic sites, nicks, thymidine dimers, blocked 3' ends, oxidized guanine, oxidized pyrimidines, deaminated cytosine). In a first step, the efficiency of DNA treatment was evaluated by the application of the PreCR Repair Mix to aliquots of the DNA of a heat-preserved sample from 1980 under various incubations (37°C for 20 min, 4°C for 20 min, and 4°C overnight, respectively). A total volume of 25 µl included 1× ThermoPol buffer, dNTPs (100 µM final concentration), 1× NAD^+, 0.5 µl of the Repair Mix and a final DNA amount of 125 ng. The treated DNA and aliquots of the untreated DNA were compared by qPCR using the 16S rDNA, Dhb, Mdha, and Hty gene loci (see below). No significant difference was found in the C_t values obtained by qPCR under the various incubation conditions (data not shown). Subsequently, all samples were incubated at 37°C (20 min). Aliquots of DNA isolated from four heat-desiccated samples from 1980, 1982, 1986, and 1988 were treated with the PreCR

Repair Mix (37°C, 20 min). The efficiency of the PreCR Repair Mix was again tested by means of qPCR (see below).

Nucleotide variation among high temperature-desiccated samples

In order to find out whether high-temperature caused DNA modifications within the gene loci to be analyzed by qPCR, PCR products of a heat-desiccated sample (from 1980) were sequenced before and after PreCR Mix treatment and then compared to sequences from PCR products obtained from a DNA sample stored frozen (from 2007). For comparing the variability within the 16S rDNA, 16S ITS rDNA, Dhb, Mdha, and Hty genotype loci, the reference sequences of *Planktothrix* strains CCAP1459/30 and No. 3 (16S: GQ994995.1, Dhb: AJ749260.1, Mdha: AJ749248.1, Hty: AJ749276.1, 16S ITS rDNA: EU266211.1) were used.

For sequencing, the following gene regions were amplified using primers flanking the regions to be amplified by qPCR: 16S rDNA and 16S ITS rDNA, the Dhb, Mdha, and the *mcy*B gene fragments. The PCR reactions were performed using Dream Taq polymerase (Thermo, St. Leon-Rot, Germany) according to the protocol of the manufacturer. For primer sequences, annealing temperatures, and the length of the PCR product, see Suppl. Table 1. PCR products were cloned and sequenced using vector primers according to standard procedures.

Quantification of toxigenic genotypes by qPCR

The Dhb and Mdha genotype, indicating the production of either Dhb or Mdha at position 7 and a genotype indicating Hty at position 2 of the MC molecule, were quantified by qPCR using the TaqMan assay. Primers and TaqMan probes were described previously [17, 24]. For the quantification of genotypes, calibration curves were established by relating the dilution series of predetermined DNA concentrations (expressed in equivalents of cellular biovolume) from *Planktothrix* strains PCC7821 (Dhb genotype) and strains No. 40 (Mdha genotype) and

No. 21/1 (Hty genotype) to the measured C_t values as described [17, 24]. The lowest dilution of the calibration series was defined as the limit of quantification, which corresponded to a four cells template^{-1}. All of the samples were measured in triplicate on an Eppendorf Master Cycler Ep Realplex System (Eppendorf, Vienna, Austria) in a total volume of 25µl.

Results

Size range of DNA isolated from high temperature-desiccated samples

In order to estimate the degree of DNA fragmentation introduced by heat desiccation, the DNA was visualized by ethidium bromide staining using agarose gel electrophoresis. In general, the DNA extracted from the heat-desiccated samples of the years 1980, 1985, 1990, and 1995 was fragmented. Nevertheless, larger DNA fragments were visible (> 300 bp). Within the heat-desiccated samples, no correlation between the age of the isolated DNA and the grade of fragmentation was found (Suppl. Fig. 1).

Experimental evaluation of the high temperature-induced effects on the qPCR results

Since heat-induced DNA modifications, such as nucleotide substitutions, could introduce a bias to the quantification of genotypes by means of qPCR, the DNA extracted from heat-desiccated samples was compared quantitatively to the DNA that was extracted from samples stored frozen. In general, through high temperature, a significant reduction in DNA template concentration (expressed in equivalents of cellular biovolume) occurred for all gene loci (One Way ANOVA, $p < 0.01$). For the 16S rDNA locus, the lowest DNA concentration was measured when the filters were dried at 150°C (12 h): 0.1-15% of the biovolume of the control (Fig. 1). The duration of high temperature incubation significantly reduced the DNA template concentration further (pairwise *post-hoc* test, $p < 0.05$). Overall, the DNA template concentration of heat-desiccated filters of the strains PCC7821 and No. 40 accounted for 18 ± 7% (min 0.4, max 120%) and 7 ± 2% (min 0.1, max 22%), respectively.

For both the Dhb and Mdha genotype, the high temperature incubation led to increased proportions, but this preservation effect was not statistically significant (One Way ANOVA, Dhb: $p = 0.12$, Mdha: $p = 0.4$, Suppl. Table 2). In contrast to the heat-induced decrease in DNA template concentration, there was no significant difference between incubation times (2 h vs 12 h, Dhb: $p = 0.33$, Mdha: $p = 0.84$). Altogether, these results indicate that the average proportion of both the Dhb and Mdha genotype seemed to be relatively unbiased through high-temperature desiccation.

Evaluation of DNA treatment to reverse DNA modifications

Four randomly selected DNA samples were treated with the PreCR Repair Mix and the DNA template concentration as estimated by qPCR was compared quantitatively to untreated aliquots for four different loci (16Sr DNA, Dhb, Mdha, and Hty). In the majority of samples the difference in C_t values between treated DNA and untreated DNA was small (≤ 0.5 C_t-value). No significant difference between the two data sets was found (One Way ANOVA, $p = 0.872$, $n = 16$) and no consistent improvement in DNA template concentration was observed. A linear correlation with a slope close to one was found between the C_t values of the DNA treated with the Repair Mix and the C_t values of the untreated DNA aliquots: $y = -0.875 + 1.037x$, $R^2 = 0.96$, $n = 16$, $p < 0.001$, where x is the C_t value of the untreated DNA and y is the C_t value of the treated DNA as measured by qPCR (Suppl. Fig. 2). Thus, the application of the DNA repair Mix did not lead to increased genotype abundance from the heat-desiccated samples.

Polymorphisms occurring in treated and untreated heat-desiccated DNA

In order to find out whether the PreCR Repair Mix had a qualitative effect reducing the number of nucleotide substitutions, gene fragments targeted by qPCR (16S rDNA, 16S ITS rDNA, Dhb, Mdha, and Hty) were amplified, cloned, sequenced, and compared with the

sequences obtained from untreated DNA aliquots. From the sample from 1980 without treatment, 21% of the sequences (n = 81) showed nucleotide substitutions and in one case a nucleotide deletion. In contrast, from the treated DNA aliquot, only 12% (n = 57) contained nucleotide substitutions. From the frozen DNA sample, a similar proportion of polymorphisms (9%) were detected (n = 79), (Fig. 2A). This resulted in an error rate (number of substitutions per nucleotide) of 2.5×10^{-3}, 1.4×10^{-3}, and 1.04×10^{-3} for the untreated (heat-desiccated) DNA, treated (heat-desiccated) DNA and frozen DNA, respectively. The same trend was observed when comparing only the binding region of the primers and the TaqMan probe: For each of the gene fragments, an increased number of substitutions were found in the heat-desiccated DNA sample (19% of the sequences, n = 81) when compared with the treated aliquot (9%, n = 57). The smallest proportion was observed from the DNA sample stored frozen (8%, n = 79), Fig. 2B.

Overall, the highest numbers of DNA modifications were composed of transitions (87%), of which 55% were A → G and T → C changes, and 32% were C → T and G → A changes. From the untreated and the treated DNA aliquot, 82% (n = 17) and 86% (n = 7) of the substitutions were transitions, while within sequences from the DNA stored frozen only transitions were found (n = 7), (Table 1). The rates of total transitional substitutions per nucleotide were 2.05×10^{-3}, 1.2×10^{-3}, and 1.04×10^{-3} for the sequences obtained from the heat-desiccated DNA, treated heat-desiccated DNA, and frozen DNA, respectively. No transversion occurred in the sequences obtained from the frozen sample but 2% (n = 57) and 1% (n = 81) of sequences obtained from the treated and untreated DNA aliquot, respectively were affected by transversions.

Between different loci of the heat-desiccated DNA the rate of substitutions was similar: 23% of the sequences of 16S rDNA (n = 13), 28% of Dhb (n = 18), 23% of Mdha (n = 22), and 31% of Hty (n = 13). It is concluded that as a result of heat desiccation qualitative changes in DNA nucleotide sequences occurred that could be partly reverted by the PreCR Repair Mix.

However, since nucleotide substitutions were evenly distributed between gene loci, the chance that DNA nucleotide sequence substitutions influence the quantification of genotype proportions by qPCR was considered negligible.

Quantification of microcystin genotype composition in Lake Zürich

Within the period 1977–2008 the abundance of three different *Planktothrix* genotypes indicative of Dhb or Mdha in position 7 or of Hty in position 2 of the MC molecule was determined and compared with the changes in the total *Planktothrix* population density. Except for 1984, the year with the lowest *Planktothrix* biovolume, all three genotypes were detected in all the years (Fig. 3A). Taking all of the samples together, 88% were positive for the Dhb, Mdha, and Hty genotype. On average, the abundance of the Dhb genotype was the highest (min 0.013, mean ± SE, 0.9 ± 0.14, max 2.6 mm^3 biovolume L^{-1}, $n = 27$), while the Mdha genotype occurred in lower concentrations only (0.001, 0.4 ± 0.3, 1.4 mm^3 L^{-1}, $n = 27$). Except for the years 1985-1987 the Hty genotype (0.0003, 0.1 ± 0.01, 0.49 mm^3 L^{-1}, $n = 27$) occurred with the lowest abundance (Suppl. Table 3).

During the study period the annual average abundance of the three different genotypes varied three (Mdha genotype), two (Dhb genotype), and three (Hty genotype) orders of magnitude and there was a highly significant linear relation of each genotype abundance with the total population density as revealed by 16S rDNA (Fig. 3B, Table 2). In particular, the abundance of Dhb and Mdha genotypes were related to the total population density following the linear regression: $y = - 0.3282 + 0.725x$, ($n = 27$, $R^2 = 0.83$) and $y = - 0.85+ 0.888x$, ($n = 27$, $R^2 = 0.59$), where x is the annual average $\log_{10}$ biovolume determined by 16S rDNA (mm^3 L^{-1}) and y is the annual average $\log_{10}$ biovolume of the Dhb or Mdha genotype, respectively. Similarly, the relationship between the Hty genotype and the total population followed the linear regression: $y = - 1.402 + 0.745x$, ($n = 27$, $R^2 = 0.62$), where x is the annual average $\log_{10}$ biovolume determined by 16S rDNA (mm^3 L^{-1}) and y is the annual average $\log_{10}$ biovolume

of the Hty genotype. The linear regressions differed in intercept but not in slope (One Way ANOVA, group effect: $p < 0.001$, interaction group × covariate: $p > 0.3$).

Consequently, there was a relatively high stability in overall *mcy* genotype composition over the whole study period. With the exception of year 2007, the Dhb genotype occurred in the highest proportion (min, mean ± SE, max, 16%, 55 ± 5%, 141% of the total population, $n = 27$). On average, the Mdha genotype occurred in lower proportion (0.1%, 20 ± 2%, 57.3%). The Hty genotype occurred in the lowest proportion (0.1%, 19.3 ± 0.9%, 6.6%), Fig. 4A. Nevertheless, over the study period, the Dhb genotype decreased in its proportion from the first decade (1977-1989: mean ± SE, 76 ± 11%, $n = 9$) to the two subsequent decades (1990–1999: 57 ± 5%, $n = 9$; 2000–2008: 32 ± 4%, $n = 9$), $p < 0.01$ (Kruskal-Wallis One Way ANOVA). Unexpectedly, for the Mdha genotype proportion no corresponding increase from the first decade (1977-1989: 17 ± 4%, $n = 9$) to the two subsequent decades was found (1990-1999: 22 ± 5%, $n = 9$, 2000-2008: mean 22 ± 2%, $n = 9$, $p = 0.49$). The lowest Hty genotype proportion was found to be stable between the decades (1977-1989: mean 7 ± 2%, $n = 9$, 1990-1999: mean 7 ± 1%, $n = 9$, 2000-2008: mean 6 ± 0.6%, $n = 9$, $p = 0.47$), Suppl. Table 3. In order to explain the decline of the Dhb genotype, each of the *mcy*A/B genotypes was compared in abundance to the only genotype carrying a 1.8 kbp deletion of the Dhb gene [19]. Notably, this 1.8 kbp deletion ranged from part of *mcy*H to *mcy*A and would prevent the amplification of the Dhb genotype by qPCR [21, 24]. From the year 1987 onwards the *Mcy*HA (Dhb) deletion genotype occurred consistently and showed a significant relation in its abundance with the total population density (Table 2, Fig. 4B). From 1987 onwards the proportion of the *mcy*HA deletion mutant varied from 0.3–7.2% (mean ± SE, 3.3% ± 0.4). Only the proportion of the Dhb genotype was significantly negatively related with the *mcy*HA deletion genotype biovolume as well as its proportion (Table 2). In contrast, the proportions of the Mdha and Hty genotypes were not related to the biovolume of the *mcy*HA deletion genotype. Consequently, it is concluded that part of the decline of the Dhb genotype

proportion that occurred since 1987 could be related to its *mcy*HA deletion mutant. The overall relationship between the three different *mcy* genotypes, however, was stable and showed a minor change only when compared with the more than 5,000–fold increase (min 0.001, max 5.3 mm^3 L^{-1}) of the total population.

Discussion

DNA quality isolated from heat-desiccated samples

In this study, the most common substitutions were transitions, i.e. the highest number of these was A → G and T → C or C → T and G → A conversions. The rate of these substitutions was higher compared to the DNA isolated from frozen samples (Fig. 2). In studies investigating ancient DNA, nucleotide substitutions are commonly observed, specifically transitions A → G or T → C and C → T and G → A [25]. It has been shown that elevated temperatures promote the hydrolytic deamination of adenine to hypoxanthine [26] and the conversion of cytosine to uracil in DNA [27]. Lindahl and Nyberg [27] estimated that in DNA incubated at 100°C (10 min), one uracil residue per 5,000 cytosine residues will be generated. In addition, Bruskov et al. [28] showed that the heat-mediated oxidation of guanine to 8-oxoguanine occurs, which can pair with adenine. Karran and Lindahl [26] showed that the rate of deamination of adenine is a rather slow process compared to the deamination of cytosine, which occurs at a 40-fold faster rate in DNA heated to 110°C. Since in this study the phytoplankton biomass used for DNA isolation was dried at 110°C (2h) it was reasonable to expect that the PreCR Repair Mix used to reverse the high-temperature induced substitutions resulted in a significant increase in genotype abundance as estimated by qPCR. However, this was not the case (Suppl. Fig. 2). Furthermore, no improvement in the detectability of rare inactive *Planktothrix* genotypes such as the *mcy*HA deletion mutant [24] could be achieved after the treatment of the heat-desiccated DNA with the PreCR Repair Mix compared to untreated DNA (data not shown). Nevertheless, the activity of the PreCR Repair enzyme mix

was demonstrated when comparing the number of nucleotide substitutions between the heat-desiccated DNA and the treated aliquot. The 6.6-fold reduced occurrence of A → G nucleotide substitutions and 1.7-fold reduced occurrence of all the transitions found in the treated DNA when compared with the untreated DNA complies with the enzymatic reversion of e.g. uracil to cytosine or the repair of abasic sites. In total, the observed nucleotide substitutions occurred with a frequency far too low ($< 10^{-3}$) implying that those errors could not account for the observed reduction in DNA template concentration. Instead we found the heat-desiccated DNA to be highly fragmented, which is in accordance to earlier studies [29]. It is concluded that through the high temperature, rather the fragmentation of DNA into small fragments, than the heat-induced substitutions was the reason for decreased DNA quality.

Abundance of individual microcystin genotypes

Over a study period of thirty years, a highly significant positive correlation was found between the abundance of two *mcy*A and one *mcy*B genotypes and the total *Planktothrix* population density. While such a relationship has been found when comparing *mcy* genotype composition between lakes [17], this study shows that even during a thirty-year period of re-oligotrophication and a population increase by three orders of magnitude the *mcy* genotype composition was stable. This implies that the individual *mcy* genotype subpopulations constitute a stable part of the total population and are not likely to disappear or reappear within short time periods.

Nevertheless, a decrease in the proportion of the Dhb genotype was observed between the first and the following two decades (Fig. 4A, B). In contrast, the Mdha genotype did not show a corresponding increase in abundance, suggesting that a significant part of the *Planktothrix* population remained undetected by means of qPCR. A natural factor that may explain a possible decrease in the proportion of the Dhb genotype over time would be the parallel increase of an unknown genotype affected by mutation within the gene region encoding for

the first A-domain of the *mcy*A gene. One such genotype that was described earlier showed a 1.8 kbp deletion within *mcy*HA affecting the Dhb primer binding region and thus prevented detection by qPCR [24]. Overall, the proportion of this genotype was rather low (Fig. 4) and as such cannot explain the decrease of the Dhb genotype during the last decade completely. So far, we cannot exclude additional (unknown) *mcy*A deletion genotypes that occurred in Lake Zürich. For example, due to the accuracy of the TaqMan Assay, even *mcy*HA deletion mutants carrying single substitutions would have led to a reduced detectability.

However, when compared with the decline of the Dhb genotype proportion over time, the proportion of its *mcy*HA deletion mutant clearly showed the opposite trend (Fig. 4). Furthermore, the abundance of the *mcy*HA deletion mutant was positively related with the total *Planktothrix* population density. Among twelve lakes in the Alps (Austria, Germany, Switzerland) that were sampled during 2005-2007 [24], an overall increase of the abundance of mutants carrying either the *mcy*HA deletion or insertion of transposable elements with the total *Planktothrix* population density has been observed. Consequently, it is reasonable to conclude that the abundance of mutations in the *mcy* gene cluster and in particular the abundance of the *mcy*HA genotype is generally favored by the total population density.

On the other hand, the evidence that also the proportion of the *mcy*HA deletion mutant is favored by an increase in total population density is scarce. In the same study [24], when comparing sparse and dense *Planktothrix* populations among twelve lakes of the Alps it could be shown that the same mutations that were absent from depth-integrated water samples could be found when using enriched plankton net samples (Fig. 3 in [24]). Through the observation period of this study, the decrease of the Dhb proportion from the 1980s to the 1990s indirectly could also show a proportional increase of the *mcy*HA deletion. While *Microcystis* strains, which contained deletions of several *mcy* genes, have been observed to outgrow the original strain under culture conditions [30, 31], those observations so far could not be made under field conditions. No further increase of the *mcy*HA mutant since its first detection in 1987

until 2008 was observed. Consequently, it is likely that the proportional increase of the *mcy*HA deletion genotype in lakes of the Alps is rather slow, requiring even longer observation periods than applied in this study. The reasons for this overall slow increase are possibly selective pressure preventing a further increase, for example through grazing or parasitism [19]. Another possible explanation is that through the deletion of part of the *mcy*HA genes the advantage in growth rate resulting from the release from the energetic costs involved in MC biosynthesis is too small to become relevant.

Potential microcystin biosynthesis

In this study, the average abundance of the Dhb genotype responsible for the incorporation of Dhb in position 7 of the MC molecule was significantly higher when compared with the Mdha genotype responsible for the incorporation of Mdha in position 7 of the MC molecule. Recently, we could show that the abundance and proportion of the Dhb or Mdha genotypes was significantly related to the concentration of the respective MC structural variants [17]. Frequently, the MCs isolated from samples of red-pigmented *P. rubescens* were dominated by MCs carrying Dhb in position7 ([D-Asp^3, Dhb^7]-MC-RR: in Lake Steinsfjorden (Norway) [32], in Hallwiler See (Switzerland) [33], in Lac du Bourget (France) [34], in Talsperre Weida, Talsperre Pöhl, Arendsee (Germany) [35]. On the other hand, Ernst et al. [36] reported higher amounts of [D-Asp^3]-MC-RR compared with [D-Asp^3, Dhb^7]-MC-RR from *P. rubescens* in Lake Ammersee (Germany) during 1999–2004. Correspondingly, a survey of lakes of the Alps from 2005 until 2007 [17] showed that the average proportion of the Mdha genotype exceeded the Dhb genotype proportion in Lake Ammersee resulting in the dominance of [D-Asp^3, Mdha^7]-MC-RR and a minor proportion of [D-Asp^3, Dhb^7]-MC-RR [17]. It is not known as to whether this spatial variation in Dhb vs Mdha in position 7 of the MC molecule among spatially isolated *P. rubescens* populations is of adaptive significance. With regard to the much more pronounced structural variation of variable amino acids in

position 2 of the MC molecule a similar distinct spatial variation has been reported [37]. Earlier, from comparing the genetic information from the A-domains of isolated *Planktothrix* strains, it was concluded that the genetic variation could imply either the relaxation of selective constrains or adaptive significance [37]. However, from the population genetic structure as recorded directly from field samples it could be shown that populations in different habitats were sufficiently isolated to allow a random genetic drift to occur. As observed during this study, the results on the rather stable genetic population structure over three decades support this hypothesis.

The increase in the frequency of the *mcy*A (Dhb) deletion mutant during the 30 year observation period is of significance with regard to the overall MC net production of the population in Lake Zürich. During the earlier survey on *mcy* mutant genotype abundance [24], a total of 103-114 individual filaments of *Planktothrix* were isolated from a depth-integrated sample from each of the twelve lakes and analyzed for MC production by means of matrix assisted laser desorption ionization-time of flight mass spectrometry [38]. As a result from a total of 108 filaments isolated from Lake Zürich (20 January 2006) only six filaments did not show MC. Thus, the proportion of inactive *mcy* genotypes (5.6%) compares with the range in the proportion of the *mcy*HA deletion mutant (Fig. 4B, 0.3-7.2%). Consequently, so far the increase of the *mcy*HA gene deletion mutant has a minor effect on the overall toxicity of the *Planktothrix* population in L. Zürich.

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References

1. Tillett D, Dittmann E, Erhard M, vonDöhren H, Börner T, et al. (2000) Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an

integrated peptide-polyketide synthetase system. Chem Biol 7: 753–764.

2. Christiansen G, Fastner J, Erhard M, Börner T, Dittmann E (2003) Microcystin biosynthesis in *Planktothrix*: genes, evolution, and manipulation. J Bacteriol 185: 564–572.

3. Rouhiainen L, Vakkilainen T, Siemer BL, Buikema W, Haselkorn R, et al. (2004) Genes coding for hepatotoxic heptapeptides (microcystins) in the cyanobacterium *Anabaena* strain 90. Appl Environ Microbiol 70: 686–692.

4. Fischbach MA, Walsh CT, Clardy J (2008) The evolution of gene collectives: How natural selection drives chemical innovation. Proc Natl Acad Sci U S A 105: 4601–4608.

5. Kurmayer R, Dittmann E, Fastner J, Chorus I (2002) Diversity of microcystin genes within a population of the toxic cyanobacterium *Microcystis* spp. in Lake Wannsee (Berlin, Germany). Microb Ecol 43: 107–118.

6. Mikalsen B, Boison G, Skulberg OM, Fastner J, Davies W, et al. (2003) Natural variation in the microcystin synthetase operon *mcy*ABC and impact on MC production in *Microcystis* strains. J Bacteriol 185: 2774–2785.

7. Kurmayer R, Christiansen G, Gumpenberger M, Fastner J (2005) Genetic identification of microcystin ecotypes in toxic cyanobacteria of the genus *Planktothrix*. Microbiology 151: 1525–1533.

8. Fewer DP, Rouhiainen L, Jokela J, Wahlsten M, Laakso K, et al. (2007) Recurrent adenylation domain replacement in the microcystin synthetase gene cluster. BMC Evol Biol 7, 183.

9. Tooming-Klunderud A, Mikalsen B, Kristensen T, Jakobsen KS (2008) The mosaic structure of the *mcy*ABC operon in *Microcystis*. Microbiology 154: 1886–1899.

10. Blom JF, Robinson JA, Jüttner F (2001) High grazer toxicity of [D-Asp^3,(E)-Dhb^7]microcystin-RR of *Planktothrix rubescens* as compared to different microcystins. Toxicon 39: 1923–1932.

11. Blom J, Jüttner F (2005) High crustacean toxicity of microcystin congeners does not correlate with high protein phosphatase inhibitory activity. Toxicon 46: 465–470.

12. Hoeger SJ, Schmid D, Blom JF, Ernst B, Dietrich DR (2007) Analytical and functional characterization of microcystins [Asp(3)]MC-RR and [Asp(3),Dhb(7)]MC-RR: Consequences for risk assessment? Environ Sci Technol 41: 2609–2616.

13. Ufelmann H, Kruger T, Luckas B, Schrenk D (2012) Human and rat hepatocyte toxicity and protein phosphatase 1 and 2A inhibitory activity of naturally occurring desmethyl-MCs and nodularins. Toxicology 293: 59–67.

14. Sano T, Kaya K (1998) Two new (E)-2-butenoic acid (Dhb)-containing microcystins isolated from *Oscillatoria agardhii*. Tetrahedon 54: 463–470.

15. Namikoshi M, Rinehart KL, Sakai R, Stotts RR, Dahlem AM, et al. (1992) Identification of 12 hepatotoxins from a Homer Lake bloom of the cyanobacteria *Microcystis aeruginosa*, *Microcystis viridis*, and *Microcystis wesenbergii*: nine new microcystins. J Org Chem 57: 866–872.

16. Humbert JF, Quiblier C, Gugger M (2010) Molecular approaches for monitoring potentially toxic marine and freshwater phytoplankton species. Anal Bioanal Chem 397: 1723–32.

17. Ostermaier V, Kurmayer R (2010) Application of real-time PCR to estimate toxin production by the cyanobacterium *Planktothrix* sp. Appl Environ Microbiol 76: 3495–502.

18. Posch T, Köster O, Salcher MM, Pernthaler J (2012) Harmful filamentous cyanobacteria favoured by reduced water turnover with lake warming. Nat Clim Chang 2: 809–813.

19. Ostermaier V, Schanz F, Köster O, Kurmayer R (2012) Stability of toxin gene proportion in red-pigmented populations of the cyanobacterium *Planktothrix* during 29 years of re-oligotrophication of Lake Zurich. BMC Biol 10, 100.

20. Christiansen G, Molitor C, Philmus B, Kurmayer R (2008) Nontoxic strains of cyanobacteria are the result of major gene deletion events induced by a transposable

element. Mol Biol Evol 25: 1695–1704.

21. Christiansen G, Kurmayer R, Liu Q, Börner T (2006) Transposons inactivate biosynthesis of the nonribosomal peptide microcystin in naturally occurring *Planktothrix* spp. Appl Environ Microbiol 72: 117–123.

22. Kurmayer R, Christiansen G, Chorus I (2003) The abundance of microcystin-producing genotypes correlates positively with colony size in *Microcystis* and determines its microcystin net production in Lake Wannsee. Appl Environ Microbiol 69: 787–795.

23. Rippka R (1988) Isolation and purification of cyanobacteria. Meth Enzymol 167: 3–27.

24. Ostermaier V, Kurmayer R (2009) Distribution and abundance of nontoxic mutants of cyanobacteria in lakes of the Alps. Microb Ecol 58: 323–33.25. Hansen A, Willerslev E, Wiuf C, Mourier T, Arctander P (2001) Statistical evidence for miscoding lesions in ancient DNA templates. Mol Biol Evol 18: 262–265.

26. Karran P, Lindahl T (1980) Hypoxanthine in deoxyribonucleic acid: generation by heat-induced hydrolysis of adenine residues and release in free form by a deoxyribonucleic acid glycosylase from calf thymus. Biochemistry 19: 6005–11.

27. Lindahl T, Nyberg B (1974) Heat-induced deamination of cytosine residues in deoxyribonucleic acid. Biochemistry 13: 3405–10.

28. Bruskov VI, Malakhova LV, Masalimov ZK, Chernikov AV (2002) Heat-induced formation of reactive oxygen species and 8-oxoguanine, a biomarker of damage to DNA. Nucleic Acids Res 30: 1354–1363.

29. Eigner J, Boedtker H, Michaels G (1961) The thermal degradation of nucleic acids. Biochim Biophys Acta 51: 165–168.

30. Kaebernick M, Rohrlack T, Christoffersen K, Neilan BA (2001) A spontaneous mutant of microcystin biosynthesis: genetic characterization and effect on *Daphnia*. Environ Microbiol 3: 669–679.

31. Schatz D, Keren Y, Hadas O, Carmeli S, Sukenik A, et al. (2005) Ecological implications

of the emergence of non-toxic subcultures from toxic *Microcystis* strains. Environ Microbiol 7: 798–805.

32. Miles CO, Sandvik M, Haande S, Nonga H, Ballot A (2013) LC-MS analysis with thiol derivatization to differentiate [Dhb7]- from [Mdha7]-microcystins: analysis of cyanobacterial blooms, *Planktothrix* cultures and European crayfish from Lake Steinsfjorden, Norway. Environ Sci Technol 47: 4080–4087.
33. Baumann H, Jüttner F (2008) Inter-annual stability of oligopeptide patterns of *Planktothrix rubescens* blooms and mass mortality of *Daphnia* in Lake Hallwilersee. Limnologica 38: 350–359.
34. Briand JF, Jacquet S, Flinois C, Avois-Jacquet C, Maisonette C, et al. (2005) Variations in the microcystin production of *Planktothrix rubescens* (Cyanobacteria) assessed from a four-year survey of Lac du Bourget (France) and from laboratory experiments. Microb Ecol 50: 418–428.
35. Fastner J, Erhard M, Carmichael WW, Sun F, Rinehart KL, et al. (1999) Characterization and diversity of microcystins in natural blooms and strains of the genera *Microcystis* and *Planktothrix* from German freshwaters. Arch Hydrobiol 145: 147–163.
36. Ernst B, Hoeger SJ, O'Brien E, Dietrich DR (2009) Abundance and toxicity of *Planktothrix rubescens* in the pre-alpine Lake Ammersee, Germany. Harmful Algae 8: 329–342.
37. Kurmayer R, Gumpenberger M (2006) Diversity of microcystin genotypes among populations of the filamentous cyanobacteria *Planktothrix rubescens* and *Planktothrix agardhii*. Mol Ecol 15: 3849–3861.
38. Kurmayer R, Christiansen G, Fastner J, Börner T (2004) Abundance of active and inactive microcystin genotypes in populations of the toxic cyanobacterium *Planktothrix* spp. Environ Microbiol 6: 831–841.

Table 1: Rate of nucleotide variability (per 100 bp) detected in DNA sequences obtained from heat-desiccated DNA (from 1980), an aliquot treated with the PreCR Mix, and DNA from frozen samples (from 2007).

Sample type and analyzed locus	Number of analyzed sequences	Variability in primer and probe binding sites	Variability within the total gene fragment	A→G	G→A	T→C	C→T	C→A	T→G	Gap
16S rDNA (81 bp, 48.8% GC) [a]										
Heat-desiccated	13	0.28	0.28	0.09	0	0	0.19	0	0	0
Heat-desiccated^{+} [b]	12	0.1	0.21	0	0.1	0.1	0	0	0	0
Frozen	17	0	0	0	0	0	0	0	0	0
Dhb (*mcy*AA1, 82 bp, 37.3% GC)										
Heat-desiccated	18	0.34	0.34	0.14	0.07	0	0	0.07	0.07	0
Heat-desiccated^{+}	23	0.05	0.11	0.05	0	0	0	0.05	0	0
Frozen	18	0.14	0.2	0.07	0	0.07	0.07	0	0	0
Mdha (*mcy*AA1, 77 bp, 37.7% GC)										
Heat-desiccated	22	0.25	0.31	0.25	0	0	0	0	0	0.06
Frozen	15	0.09	0.09	0	0	0.09	0	0	0	0
Hty (*mcy*BA1, 98 bp, 42.9% GC)										
Heat-desiccated	13	0.31	0.31	0.16	0.08	0	0.08	0	0	0
Heat-desiccated^{+}	22	0.14	0.14	0	0.09	0	0.05	0	0	0
Frozen	11	0.19	0.19	0.09	0	0.09	0	0	0	0
16S ITS rDNA (88 bp, 38.6% GC)										
Heat-desiccated	15	0	0	0	0	0	0	0	0	0
Frozen	18	0.06	0.06	0.06	0	0	0	0	0	0

[a] analyzed gene locus, and length of the analyzed region (bp) and guanine – cytosine content (%) given in brackets
[b] $^{+}$ indicates the treatment of the DNA sample with PreCR Repair Mix

Table 2: Correlation coefficients between biovolume ($mm^3\ L^{-1}$) and the proportion of the Dhb, Mdha, and Hty genotypes and the genotype carrying the *mcy*HA deletion (data from [19]). Significant values are marked in bold $p < 0.05$, * $p < 0.01$, ** $p < 0.001$.

	Total population ($mm^3\ L^{-1}$)	Dhb ($mm^3\ L^{-1}$)	Dhb (%)	Mdha ($mm^3\ L^{-1}$)	Mdha (%)	Hty ($mm^3\ L^{-1}$)	Hty (%)	Del ($mm^3\ L^{-1}$)	Del%
Total population ($mm^3\ L^{-1}$)	-								
Dhb ($mm^3\ L^{-1}$)	**0.61****	-							
Dhb (%)	-0.31	-0.08	-						
Mdha ($mm^3\ L^{-1}$)	**0.58****	**0.7****	**-0.48***	-					
Mdha (%)	0.12	-0.14	-0.21	0.27	-				
Hty ($mm^3\ L^{-1}$)	0.27	**0.5****	-0.37	**0.48**	0.04	-			
Hty (%)	-0.23	-0.15	0.1	-0.17	0.17	**0.44**	-		
*mcy*HA-Del ($mm^3\ L^{-1}$)	0.35	**0.4**	**-0.66****	**0.71****	0.19	**0.42**	-0.2	-	
*mcy*HA-Del (%)	0.26	0.23	**-0.6****	**0.48**	**0.41**	0.39	-0.14	**0.79****	-

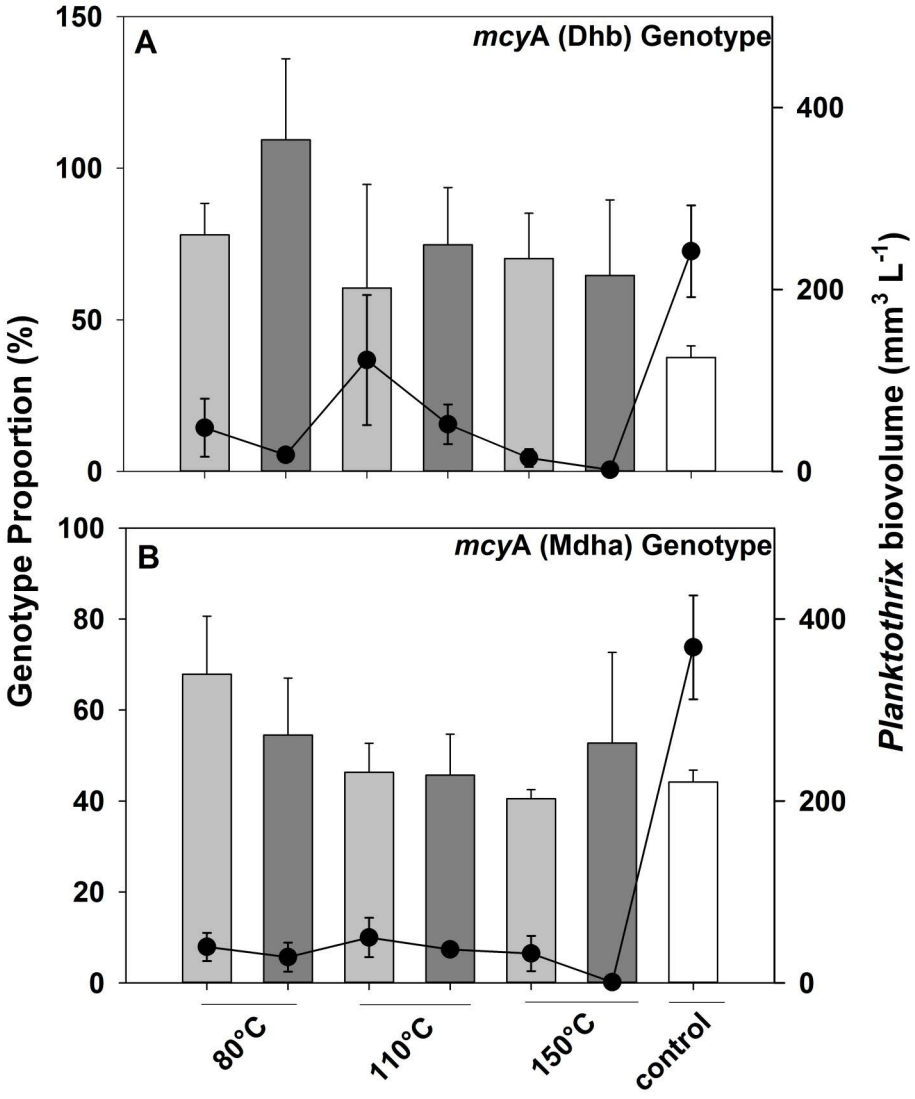


Figure 1. Influence of different desiccation temperatures on mean ± SE biovolume (black line) and mean ± SE proportion of the (A) Dhb and (B) Mdha genotype (bars). The experiment was repeated twice.

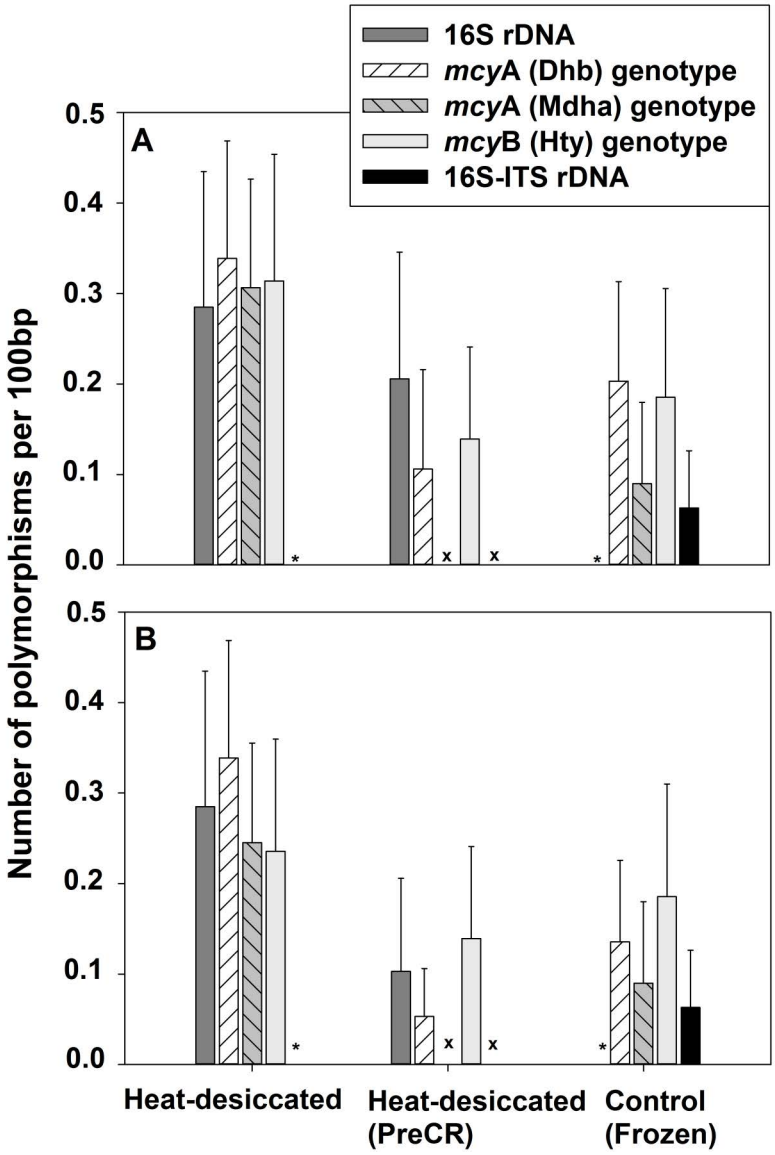


Figure 2. A) Number of substitutions (per 100bp) and B) number of substitutions in the primer sites used for qPCR and detected by sequencing different gene loci from heat-desiccated DNA, an aliquot treated with the PreCR Mix, and DNA from a frozen sample. *, no substitutions detected; x, no data.

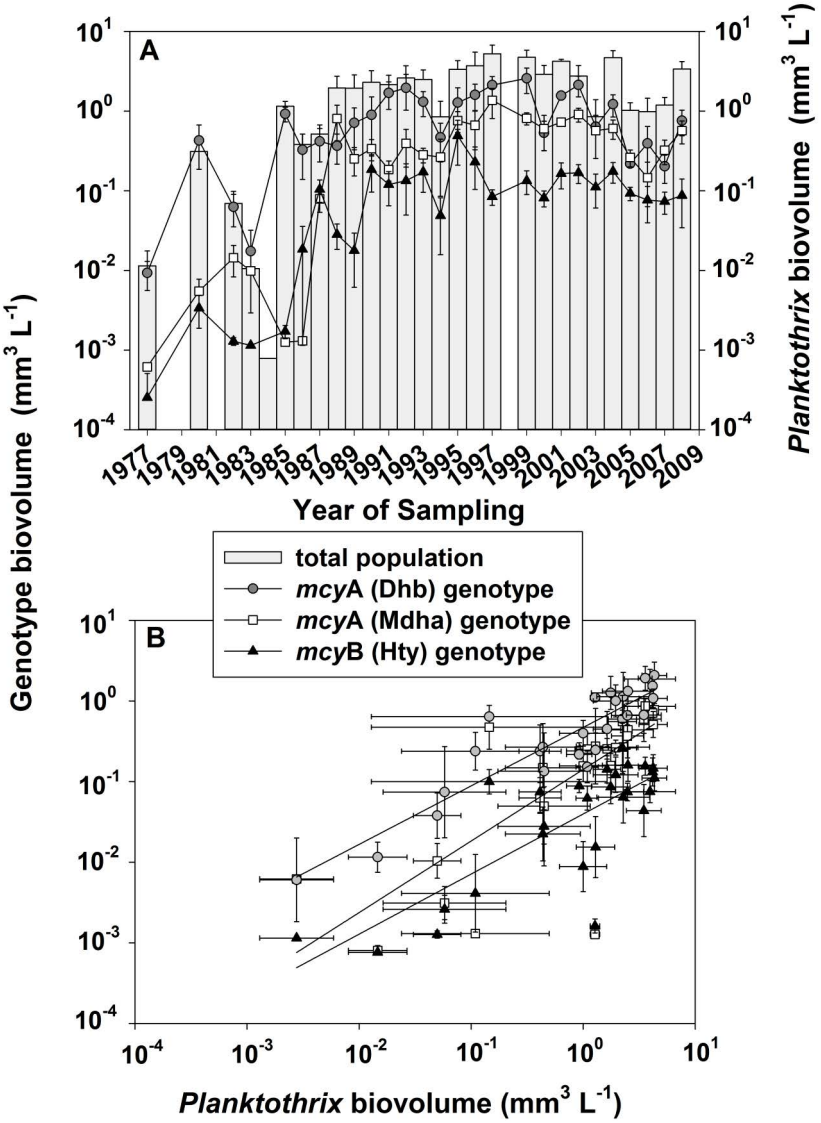


Figure 3. Time course of annual (A) mean ± SE abundance of the Dhb, Mdha, and Hty genotype and the total population biovolume (grey bars) of *Planktothrix* (mm^3 L^{-1}) as determined by qPCR in Lake Zürich. (B) Regression curves between Dhb, Mdha, and Hty genotype and the total population. For the regression curve details, see the text.

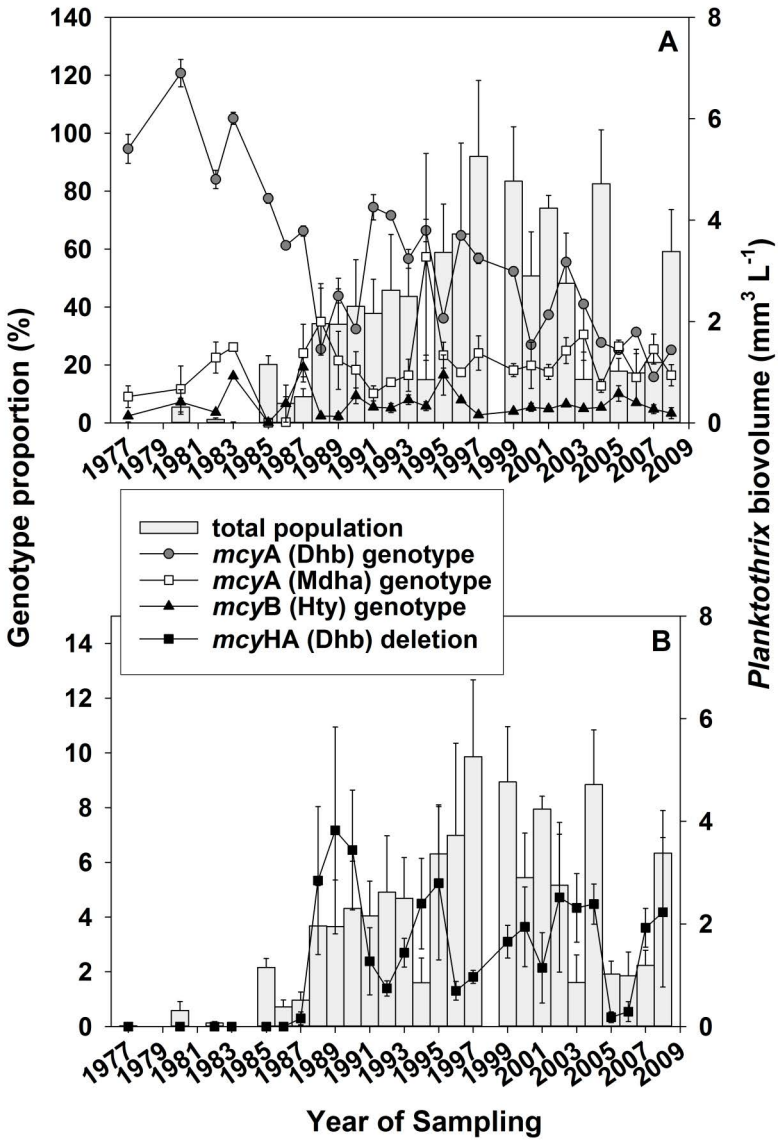


Figure 4. Time course of (A) mean ± SE proportion of the Dhb, Mdha, and Hty genotype and (B) mean ± SE proportion of the *mcy*HA deletion genotype and the total population biovolume (gray bars) of *Planktothrix* (mm^3 L^{-1}) as determined by qPCR in Lake Zürich.

Supplemental Material Chapter 3

Suppl. Table 1. Oligonucleotide primers used to amplify the target regions of the DNA fragments analyzed by qPCR from heat-desiccated DNA samples. The primer pairs were designed to amplify the whole qPCR targeted gene fragment and subsequently the PCR products were cloned and sequenced to detect heat-induced DNA damages.

Application	Target region	Forward primer / reverse primer (5'-3')	Annealing T (°C)	Elongation time (s)	Amplicon length (bp)
Amplification of gene fragments targeted by qPCR					
	16S rDNA	ggcgtaaagagtccgtaggtagt / ttcaccgctacaccaggaat	60	12	129
	Dhb	ttaatcttaactcaagaaaaattagtcgagt / gataatatgttaaattatcgggtttagcc	60	12	143
	Mdha	gcagatacccaagtcaaaatattactca / caaaattgtctggattttcctttg	61	12	134
	Hty	acctgctggcaatgtccac / ccgccaatatgtaattcgcc	62	12	139
	16S ITS rDNA	gagtccgcttacctccac / gaacttttccagcatttactct	55	12	89

Suppl. Table 2: Influence of heat-desiccation of *Planktothrix* cells on qPCR quantification. Cell aliquots of strains PCC7821 and No.40 were desiccated at different temperatures (80, 110 and 150°C) for two and twelve hours, respectively. As a control aliquots of the strains were stored frozen (-20°C). DNA was quantified by qPCR with regard to mean ± SE biovolume (16S rDNA) and the influence on mean ± SE proportions of Dhb and Mdha genotypes. The experiment was repeated twice. P-values for testing treatment effects (factor °C, factor h) were estimated by ANOVA ($n = 21$).

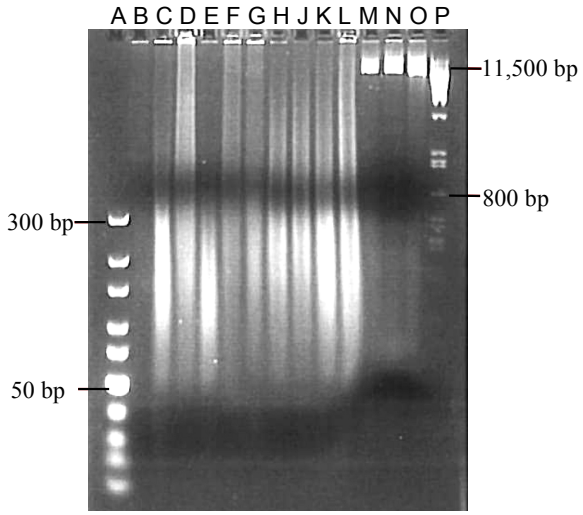
Strain	Treatment	16S rDNA (mm^3L^{-1})	Dhb or Mdha genotype (%)
Dhb genotype (PCC7821)			
	80°C (2h)	48 ± 32^{ab}	78 ± 10
	110°C (2h)	123 ± 72^{ab}	60 ± 34
	150°C (2h)	15 ± 10^{ab}	70 ± 15
	80°C (12h)	18 ± 7^{ab}	109 ± 27
	110°C (12h)	52 ± 22^{ab}	61 ± 34
	150°C (12h)	2 ± 0.5^{b}	65 ± 25
	Control	242 ± 50^{a}	38 ± 4
	Factor °C	$P < 0.001$	$P = 0.12$
	Factor h	$P < 0.001$	$P = 0.33$
	°C × h	$P = 0.72$	$P = 0.29$
Mdha genotype (No.40)			
	80°C (2h)	40 ± 15^{ab}	68 ± 13
	110°C (2h)	50 ± 22^{ab}	46 ± 6
	150°C (2h)	32 ± 19^{ab}	41 ± 2
	80°C (12h)	28 ± 16^{ab}	55 ± 12
	110°C (12h)	37 ± 6^{ab}	46 ± 9
	150°C (12h)	0.8 ± 0.2^{b}	53 ± 20
	Control	369 ± 57^{a}	44 ± 3
	Factor °C	$P < 0.001$	$P = 0.4$
	Factor h	$P < 0.001$	$P = 0.84$
	°C × h	$P = 0.91$	$P = 0.53$

If ANOVA found the overall effect signifiant, pairwise post-hoc comparison (Tukeys test) was used. Estimates sharing identical superscripts did not differ at $p > 0.05$.

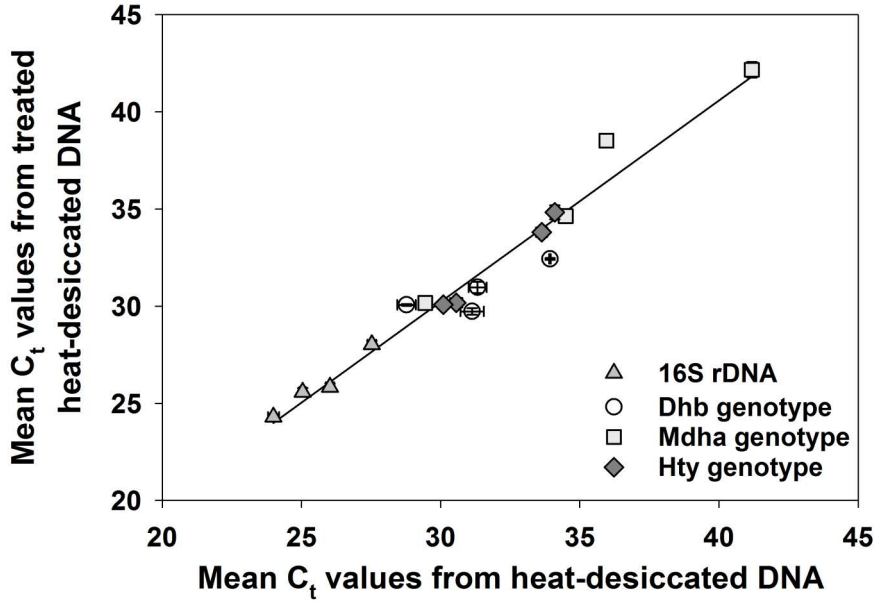
Suppl. Table 3: Average ± SE biovolume per year of the total *Planktothrix* population and biovolume and proportions of *mcy* genotypes in Lake Zürich between 1977 and 2008 as estimated by qPCR. Biovolume in $mm^3 L^{-1}$, genotype proportions in % of the total population as estimated by 16S rDNA.

Year	16S rDNA	Dhb genotype		Mdha genotype		Hty genotype		*mcy*HA deletion genotype	
	$mm^3 L^{-1}$	$mm^3 L^{-1}$	%	$mm^3 L^{-1}$	%	$mm^3 L^{-1}$	%	$mm^3 L^{-1}$	%
1977	0.02 ± 0.01	0.01 ± 0.01	80 ± 12	0.001 ± 0.0001	7 ±2	0.0003 ± 0.0003	2.4	nd	
1980	0.27 ± 0.16	0.37 ± 0.2	142 ± 33	0.004 ± 0.002	15 ± 11	0.003 ±0.001	7.2 ± 4.3	nd	
1982	0.07 ± 0.03	0.06 ± 0.03	84 ± 18	0.014 ± 0.01	23 ± 5	0.001 ± 0.0001	3.7 ± 0.8	nd	
1983	0.01 ± 0.01	0.01 ± 0.01	94 ± 15	0.007	24	0.001	16	nd	
1984	0.0008 ± 0.0002	nd		nd		nd		nd	
1985	1.31 ± 0.12	1.11 ± 0.07	86 ± 4	$0.001 \pm 3\times10^{-5}$	0.1 ± 0.01	0.002 ± 0.0003	0.1 ± 0.02	nd	
1986	0.38 ± 0.14	0.33 ± 0.19	61 ± 31	0.001 ± 0.0001	0.26 ± 0.03	0.02 ± 0.02	6.7 ± 6.4	nd	
1987	0.52 ± 0.16	0.42 ± 0.19	66 ± 18	0.08 ± 0.03	24 ± 10	0.1 ± 0.03	19.3 ± 3.3	0.0005 ±	0.3 ±0.2
1988	1.62 ± 0.54	0.35 ± 0.16	26 ± 9	0.65 ± 0.3	35 ± 12	0.03 ± 0.01	2.4 ± 0.6	0.08 ± 0.05	5.3 ± 2.7
1989	1.36 ± 053	0.52 ± 0.23	45 ± 13	0.22 ± 0.1	22 ± 10	0.02 ± 0.01	2.2 ± 1.2	0.06 ± 0.03	7.2 ± 3.8
1990	1.94 ± 0.57	0.74 ± 0.5	34 ± 12	0.29 ± 0.1	18 ± 6	0.19 ± 0.1	9.4 ± 2.7	0.12 ± 0.05	6.5 ± 2.2
1991	2.16 ± 0.7	1.69 ± 0.6	75 ± 12	0.19 ± 0.1	10 ± 3	0.12 ± 0.1	5.4 ± 0.9	0.03 ± 0.01	2.4 ± 1.3
1992	2.5 ± 1.1	1.96 ± 0.9	72 ± 19	0.4 ± 0.4	14 ± 1.3	0.13 ± 0.1	5.2 ± 1.6	0.03 ± 0.01	1.4 ± 0.3
1993	2.5 ± 0.8	1.31 ± 0.5	57 ± 12	0.28 ± 0.1	17 ± 6	0.17 ± 0.1	8.0 ± 1.7	0.05 ± 0.01	2.7 ± 0.5
1994	0.85 ± 0.5	0.5 ± 0.2	66 ± 14	0.27 ± 0.2	57 ± 36	0.05 ± 0.03	5.8 ± 1.5	0.04 ± 0.03	4.5 ± 1.7
1995	2.6 ± 0.7	1.1 ± 0.5	39 ± 16	0.65 ± 0.2	26 ± 3	0.49 ± 0.3	16.5 ± 7	0.08 ± 0.01	5.2 ± 2.8
1996	3.73 ± 1.8	1.6 ± 0.6	65 ± 20	0.67 ± 0.5	18 ± 0.5	0.23 ± 0.1	7.9 ± 0.8	0.04 ± 0.02	1.3 ± 0.3
1997	5.26 ± 1.5	2.13 ± 0.6	57 ± 20	1.37 ± 0.8	24 ± 6	0.08 ± 0.02	2.8 ± 0.9	0.08 ± 0.03	1.8 ± 0.2
1999	4.8 ±1.1	2.58 ± 0.9	52 ± 11	0.82 ± 0.1	18 ± 2	0.13 ± 0.04	4 ± 0.2	0.09 ± 0.02	3.1 ± 0.6
2000	2.9 ± 0.9	0.71 ± 0.2	27 ± 5	0.6 ± 0.4	20 ± 8	0.08 ± 0.02	5.4 ± 1.4	0.11 ± 0.07	3.6 ± 1.5
2001	4.24 ± 0.3	1.56 ± 0.2	37 ± 5	0.73 ± 0.1	18 ± 3	0.16 ± 0.1	4.9 ± 1	0.07 ± 0.05	2.1 ± 1.3
2002	3.67 ± 0.5	2.13 ± 0.6	56 ± 10	0.91 ± 0.3	25 ± 5	0.17 ± 0.04	6.5 ± 0.6	0.1 ± 0.08	4.7 ± 2.7
2003	1.14 ± 0.7	0.64 ± 0.02	41 ± 12	0.57 ± 0.3	31 ± 9	0.11 ± 0.1	4.9 ± 0.3	0.09 ± 0.02	4.3 ± 1.3
2004	4.72 ± 1.1	1.22 ± 0.4	28 ± 6	0.61 ± 0.2	13 ± 2	0.18 ± 0.1	5.4 ± 0.6	0.1 ± 0.04	4.5 ± 0.7
2005	1.02 ± 0.3	0.22 ± 0.01	25 ± 5	0.26 ± 0.1	27 ± 2	0.09 ± 0.02	10.2 ± 2.7	0.003 ± 0.002	0.34 ± 0.2
2006	0.99 ± 0.5	0.39 ± 0.3	31 ± 6	0.15 ± 0.1	16± 8	0.08 ± 0.04	7 ± 0.6	0.01 ± 0.01	0.5 ± 0.4
2007	1.19 ± 0.3	0.2 ± 0.1	16 ± 5	0.32 ± 0.2	26 ± 5	0.07 ± 0.02	4.7 ± 1.5	0.07 ± 0.04	3.6 ± 0.7
2008	4.26 ± 1.6	1.29 ± 0.5	28 ± 8	1.1 ± 0.9	20 ± 6	0.09 ± 0.1	3.4 ± 1.9	0.1 ± 0.08	4.2 ± 2.7

nd, not detected



Suppl. Figure 1. DNA isolated from heat-desiccated vs. frozen phytoplankton samples obtained from Lake Zürich. Samples from the years 1980, 1985, 1990, 1995 (heat-desiccated) and 2005, 2006 (frozen) are shown. Lane A: Gene Ruler DNA Ladder, Ultra Low Range (Fermentas), Lane B, C and E: DNA from 1980, lane F, G: DNA from 1985, lane D, K and L: 1990, lane H, J: 1995, lane M, N: 2005 and lane O: 2006, lane P: λ PstI DNA marker.



Suppl. Figure 2. Mean ± SE C_t values as determined by qPCR using four different gene loci of *Planktothrix* (16S rDNA, Dhb, Mdha and Hty genotype) from heat-desiccated DNA and treated heat-desiccated DNA. The aliquots of the heat-desiccated untreated DNA were measured twice to control for random errors and high reproducibility was found between repeated measurements (data not shown).

Chapter 4

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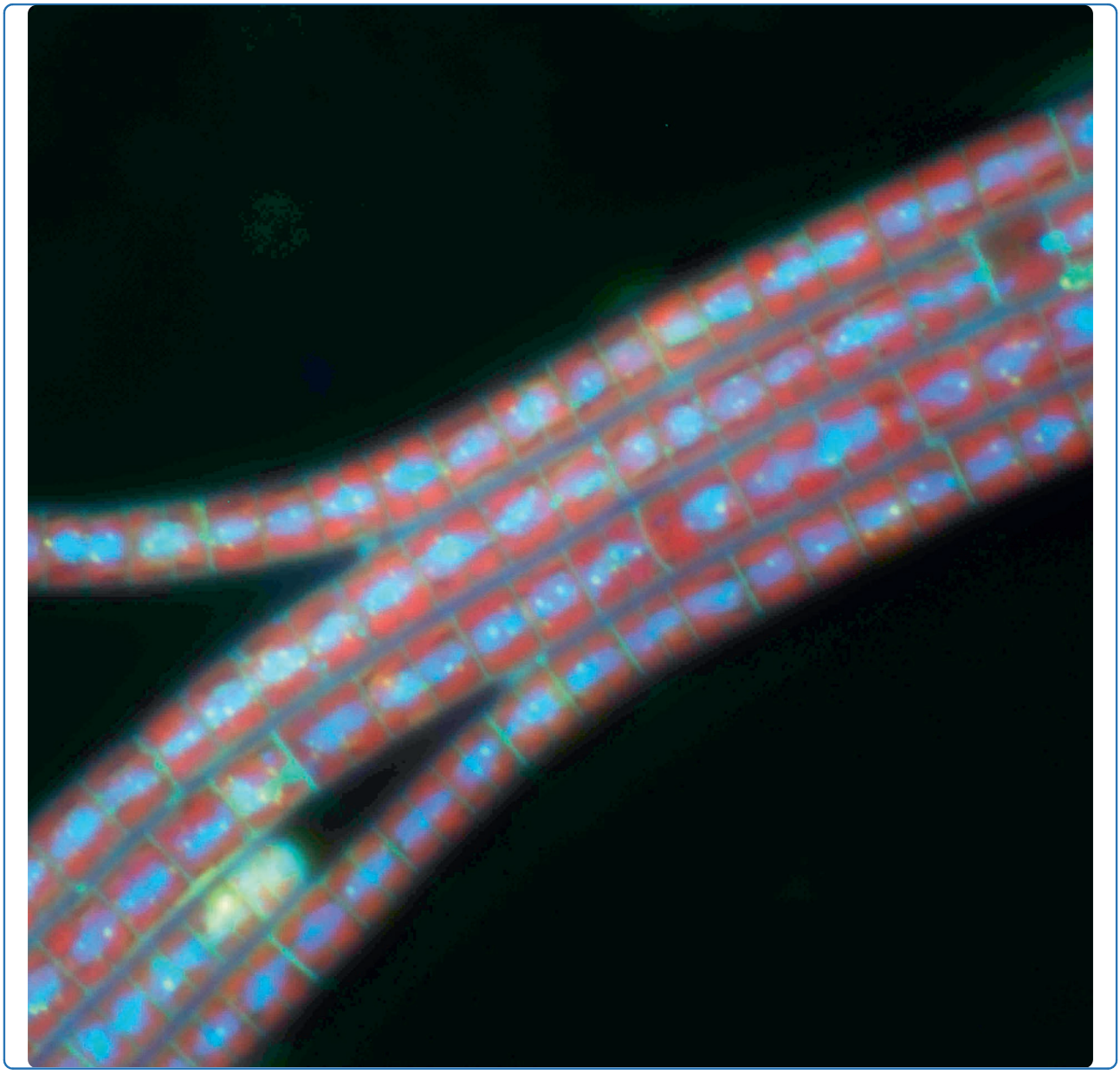

Stability of toxin gene proportion in red-pigmented populations of the cyanobacterium Planktothrix during 29 years of re-oligotrophication of Lake Zürich

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RESEARCH ARTICLE Open Access

Stability of toxin gene proportion in red-pigmented populations of the cyanobacterium *Planktothrix* during 29 years of re-oligotrophication of Lake Zürich

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Abstract

Background: Harmful algal blooms deteriorate the services of aquatic ecosystems. They are often formed by cyanobacteria composed of genotypes able to produce a certain toxin, for example, the hepatotoxin microcystin (MC), but also of nontoxic genotypes that either carry mutations in the genes encoding toxin synthesis or that lost those genes during evolution. In general, cyanobacterial blooms are favored by eutrophication. Very little is known about the stability of the toxic/nontoxic genotype composition during trophic change.

Results: Archived samples of preserved phytoplankton on filters from aquatic ecosystems that underwent changes in the trophic state provide a so far unrealized possibility to analyze the response of toxic/nontoxic genotype composition to the environment. During a period of 29 years of re-oligotrophication of the deep, physically stratified Lake Zürich (1980 to 2008), the population of the stratifying cyanobacterium *Planktothrix* was at a minimum during the most eutrophic years (1980 to 1984), but increased and dominated the phytoplankton during the past two decades. Quantitative polymerase chain reaction revealed that during the whole observation period the proportion of the toxic genotype was strikingly stable, that is, close to 100%. Inactive MC genotypes carrying mutations within the MC synthesis genes never became abundant. Unexpectedly, a nontoxic genotype, which lost its MC genes during evolution, and which could be shown to be dominant under eutrophic conditions in shallow polymictic lakes, also co-occurred in Lake Zürich but was never abundant. As it is most likely that this nontoxic genotype contains relatively weak gas vesicles unable to withstand the high water pressure in deep lakes, it is concluded that regular deep mixing selectively reduced its abundance through the destruction of gas vesicles.

Conclusions: The stability in toxic genotype dominance gives evidence for the adaptation to deep mixing of a genotype that retained the MC gene cluster during evolution. Such a long-term dominance of a toxic genotype draws attention to the need to integrate phylogenetics into ecological research as well as ecosystem management.

Keywords: allelic discrimination assay, eutrophication, genetic population structure, harmful algal blooms, historic samples, long-term monitoring, microcystin

Background

There is growing concern about observations of harmful algae blooms promoted by nutrient enrichment both in freshwater and estuarine systems. The dominant species frequently include bloom-forming cyanobacteria such as the genera *Anabaena*, *Microcystis* and *Planktothrix*, whose growth may also be favored by elevated temperature [1]. However, efforts of lake restoration, such as the reduction of nutrient input, can also lead to the proliferation of stratifying cyanobacteria such as *P. rubescens* because of an increased underwater light regime [2].

It is widely agreed that the production of microcystin (MC), which is the most abundant toxin in freshwater, is directly related to the cell division rate of a particular isolate

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grown under controlled laboratory conditions [3]. In nature, blooms of cyanobacteria are typically composed of toxic and nontoxic genotypes, the latter resulting from the loss [4] or the inactivation of the MC synthetase (*mcy*) gene cluster [5,6]. Some indices show that the production of MC has a selective advantage for the producers [7]. However, besides the fact that MC is a potent inhibitor of eukaryotic protein phosphatases 1 and 2A, the cellular function of MC is not known. To elucidate parameters that influence the competitive ability of toxic and nontoxic genotypes, several growth experiments with toxic and nontoxic strains of *Microcystis* or *Planktothrix* have been performed under controlled laboratory conditions. Vézie *et al.* [8] reported that under high nutrient levels (nitrogen, phosphorus) toxic strains of *Microcystis* grew faster than nontoxic strains, while Briand *et al.* [9] found an advantage of toxic over nontoxic *Planktothrix* strains when environmental conditions limited growth (through dim light, low temperature or nitrogen-limiting conditions). Furthermore, toxic *Microcystis* strains were shown to be the better competitor at high irradiances [10] when compared with nontoxic strains. The rather contrasting conclusions obtained from different toxic and nontoxic strains possibly result from physiological adaptations of the individual genotypes to specific environmental conditions, which are not related to MC production. Indeed, it has been shown that, within the genus *Planktothrix*, the origin of nontoxic strains is rather ancient, and that toxic and nontoxic strains evolved independently and differentiated physiologically in response to environmental factors not directly related to toxin production [4]. Only one monophyletic lineage of green-pigmented strains of the genus *Planktothrix* that lost the *mcy* gene cluster (henceforth referred to as lineage 1) could be found, which invaded shallow, polymictic lakes throughout Europe [4]. A second lineage containing both red- and green-pigmented strains (henceforth referred to as lineage 2) retained the *mcy* gene cluster. This phylogenetic evidence can explain why red-pigmented (phycoerythrin-rich) populations of *Planktothrix*, which typically occur in deep, stratified lakes and reservoirs, are commonly composed solely of the genotype containing the *mcy* gene cluster [5,11]. By contrast, green-pigmented populations that dominate in shallow, eutrophic and polymictic water bodies have a much higher proportion of the nontoxic genotype. A recent survey on toxic genotype abundance in European lakes revealed that red-pigmented populations of *Planktothrix* show a significantly higher proportion of the toxic genotype when compared with green-pigmented populations [12].

Only few studies investigated the selective advantage of red- versus green-pigmented *Planktothrix* ecotypes under field conditions. Davis *et al.* [13] investigated a mixed-pigmented *Planktothrix* population in Blelham Tarn, Lake District, England and analyzed the vertical distribution of the biomass of the two ecotypes. For both ecotypes, the biovolume was increasing under stratified conditions of the water column. However, the red-pigmented ecotype could be shown to grow at greater depths under stratifying and mixed conditions, as its compensation light intensity for growth was lower compared with the green-pigmented ecotype. It is known that *P. rubescens* is adapted to low light conditions whereas *P. agardhii* is more tolerant to high light intensities [14,15]. Oberhaus *et al.* [15] suggested further that the combined effects of temperature and light quality and quantity influence the proliferation of *P. rubescens* and *P. agardhii*. They found the red-pigmented strain to be more competitive at lower temperatures (15°C) and low intensities of green light, resembling the conditions present in the metalimnion, whereas the green-pigmented strain was more competitive at higher temperatures (25°C) and generally less specialized to light quality. Similarly, Stomp and colleagues [16] showed that the underwater light regime was an important factor for niche differentiation of red- and green-pigmented picocyanobacteria and reported their coexistence in waters of intermediate turbidity, whereas red-pigmented picocyanobacteria dominated in clear waters and green-pigmented picocyanobacteria were dominant in turbid waters. Additionally, Walsby and coworkers [17] suggested that the resistance of gas vesicles against hydrostatic pressure is of major importance during lake mixing, especially in deep lakes when filaments become entrained in the hypolimnion. A selective difference between red- and green-pigmented strains producing different types of gas vesicles has been suggested [18], which could further influence the dominance of the red-pigmented ecotype in deep habitats and the common abundance of the green-pigmented ecotype in more shallow water bodies. However, still not much is known about the temporal stability of those contrasting ecotype strategies in ecosystems subject to severe shifts in local environmental conditions.

Here, we report the detailed analysis of the genotypic population structure of *Planktothrix* spp. in Lake Zürich, Switzerland, covering a time span of almost 30 years, which was facilitated by the isolation of DNA from phytoplankton preserved on filters. Because Lake Zürich represents an important drinking water source for about 900,000 inhabitants, its planktonic phytoplankton composition has been monitored intensively. Lake Zürich underwent a well-documented history of eutrophication that reached its maximum around 1965. The re-oligotrophication process was initiated by reducing the input of phosphorus [19]. Except for the period of maximum eutrophy (1965 to 1975), *Planktothrix* occurred in Lake Zürich during the whole century. The first *Planktothrix*

bloom was recorded in 1897. During the eutrophic period, eukaryotic algae frequently formed surface blooms, which subsequently disappeared as a result of re-oligotrophication measures, while *Planktothrix* consistently increased [20].

The aim of the study was to find out whether (i) the abundance of the toxic *Planktothrix* genotype changed during the observation period, that is from the period with minimum population density and almost complete disappearance to a stable dominance of the phytoplankton community; (ii) nontoxic genotypes (including inactive mutants) increased in proportion during the observation period: following the hypothesis of Briand *et al.* [21] we would expect an increase of nontoxic genotypes parallel to the increase of the total population density; (iii) the green-pigmented ecotype was present and was of selective advantage under certain environmental conditions, for example at the beginning of the 1980s when the euphotic zone was rather shallow and the red-pigmented ecotype was disfavored because of the high absorption coefficient in the water column.

Results

Long-term changes in phytoplankton composition in Lake Zürich

Although the mean total phytoplankton biovolume per year showed little change between 1980 and 2008 (minimum 1.5 $mm^3\ L^{-1}$, maximum 3.0 $mm^3\ L^{-1}$, mean 2.3 ± 0.1 $mm^3\ L^{-1}$), the mean *Planktothrix* biovolume per year underwent pronounced fluctuation (Figure 1). During a population collapse in 1984, with a minimum of 0.001 $mm^3\ L^{-1}$, *Planktothrix* accounted for only 0.1% of the total phytoplankton and 6.0% of the total cyanobacterial biovolume. Subsequently, the population recovered and increased up to a maximum of 2.0 ± 0.2 $mm^3\ L^{-1}$ in 2001. Within the period between 1985 and 2008, *Planktothrix* contributed on average 39 ± 2.9% to the total phytoplankton (minimum 11.5%, maximum 68.4%) and 87 ± 2.8% to the total cyanobacterial biovolume (minimum 47.6%, maximum 98.2%). Excepting 2005, the share of *Planktothrix* of the total phytoplankton exceeded 70% in the period from August to March during the years 1995 to 2008 (n = 43).

Quantification of toxic *Planktothrix* in Lake Zürich

Planktothrix was detected by means of quantitative PCR (qPCR; using a Taq nuclease assay (TNA)) in all samples except for two (in the years 2002 and 2003). The mean *Planktothrix* biovolume per year was 1.6 ± 0.2 $mm^3\ L^{-1}$. The lowest annual *Planktothrix* biovolume was found in 1984 (0.001 $mm^3\ L^{-1}$) and the maximum annual biovolume was measured in 1997 (4.2 $mm^3\ L^{-1}$) (Figure 2). There was a highly significant positive and linear relationship between the *Planktothrix* biovolume quantified by 16S rDNA and the biovolume as determined by microscopic counting, $y = -0.289 + 0.737x$, $n = 27$, $R^2 = 0.9$, $P < 0.001$, where x is the average $\log_{10}$ biovolume ($mm^3\ L^{-1}$) per year as determined by 16S rDNA and y is the average $\log_{10}$ biovolume as determined by microscopic counting (Figure S1A in Additional file 1). The mean *Planktothrix* biovolume as calculated from microscopic counting was 0.71 ± 0.1 $mm^3\ L^{-1}$. The 16S rDNA abundance estimates were validated using a second TNA quantifying the phycocyanin intergenic spacer region (PC-IGS). Comparison of the biovolume as estimated by 16S rDNA and by the PC-IGS locus revealed a positive linear relationship following the equation $y = -0.225 + 0.980x$, $n = 28$, $R^2 = 0.96$, $P < 0.001$, where x is the average $\log_{10}$ biovolume ($mm^3\ L^{-1}$) per year determined by 16S rDNA and y is the respective $\log_{10}$ biovolume as determined via the PC-IGS (Figure S1B in Additional file 1). At four dates, the TNA for PC-IGS was negative, while the TNA for 16S rDNA was positive (three samples from 1984, and one sample from 1986). In all these cases the *Planktothrix* biovolume was very low (< 0.0006 $mm^3\ L^{-1}$), and the introduced variability was negligible. It is concluded that the TNA estimates on the *Planktothrix* abundance constitute a reliable estimate of the *Planktothrix* population density observed in the lake.

The TNA targeting the *mcy*B gene fragment indicative of the MC-producing genotype was found positive in 100 samples (87.8%). In 11 samples showing the lowest *Planktothrix* biovolume (< 0.0006 $mm^3\ L^{-1}$), no *mcy*B signal was recorded. A highly significant linear relationship was found between the abundance of the genotype carrying the *mcy*B gene fragment and the total population, $y = -0.0358 + 0.960x$, $n = 27$, $R^2 = 0.94$, $P < 0.001$ (Figure 3), where x is the average annual $\log_{10}$ biovolume ($mm^3\ L^{-1}$) as determined by 16S rDNA and y is the average annual $\log_{10}$ biovolume of the *mcy*B genotype. The mean proportion of the *mcy*B genotype was 106 ± 8% (minimum 38.9%, maximum 247.3%), indicating that the *Planktothrix* population was constantly dominated by the *mcy*B genotype (minimum 0.003 $mm^3\ L^{-1}$, maximum 4.8 $mm^3\ L^{-1}$, mean 1.6 ± 0.3 $mm^3\ L^{-1}$, Figure 2).

Quantification of nontoxic *Planktothrix* in Lake Zürich

To elucidate whether the changes in environmental conditions affecting the phytoplankton composition also influenced the genotypic structure of the population, two types of nontoxic genotypes were quantified. First, genotypes containing the *mcy* gene cluster but inactive in MC production due to insertions by mobile elements or partial deletions of the *mcy* gene cluster [22]; and second, genotypes that lack the *mcy* gene cluster due to evolutionary gene loss [4].

In general, all of the four genotypes containing either insertions or a deletion within the *mcy* gene cluster were detected frequently, but the majority in the lowest

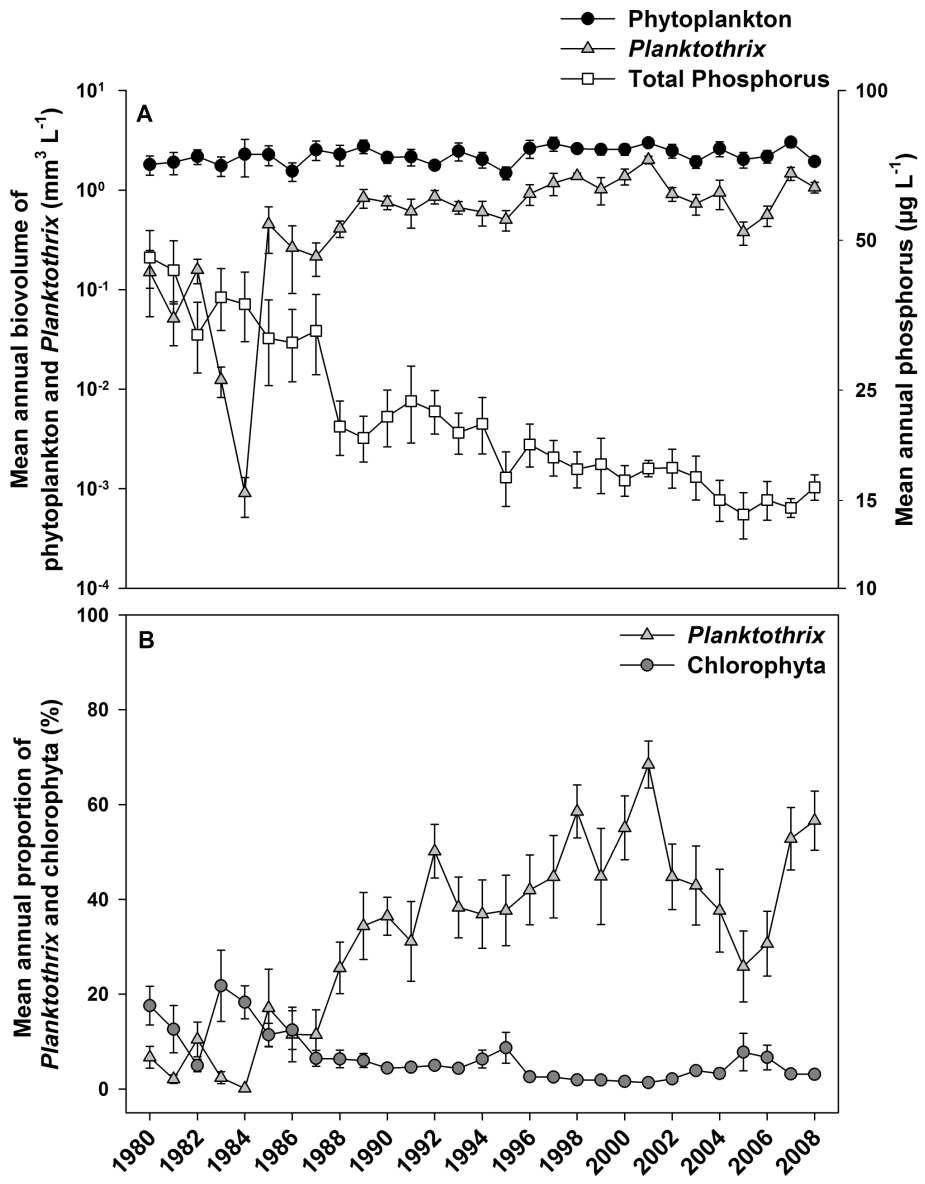

Figure 1 History of phytoplankton and total phosphorus concentrations between 1980 and 2008. (A) Annual mean ± SE biovolume of total phytoplankton, *Planktothrix* and concentration of total phosphorus in Lake Zürich from 1980 to 2008. Biovolume in mm^3 L^{-1}, determined by microscopic counting, total phosphorus in µg L^{-1}. (B) Annual mean ± SE percentage of *Planktothrix* and chlorophyta out of total phytoplankton.

abundance (Figure 4). Only the genotype carrying a deletion between *mcy*H and *mcy*A occurred in sufficient amounts that could be quantified by the respective TNA. The *mcy*HA deletion was detected in 1977, although its numbers were below the quantification limit. It was not detected between 1980 and 1986, but occurred consistently later on: from 1987 to 2008, the genotype carrying the *mcy*HA deletion had an annual average proportion of 3.3 ± 0.4% (minimum 0.3%, maximum 7.2%) of the total population density as determined by 16S rDNA. In total, it was detected in 74% of the samples. Comparing its proportion between three decades (1977 to 1989, 1990 to 1999, 2000 to 2008) revealed no significant increase of this genotype ($P = 0.16$, Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks). The other three inactive genotypes containing insertions were present in minimum concentrations only and could be detected in the undiluted DNA extract. The genotypes carrying insertions within *mcy*D, that is, *mcy*DIS1 and *mcy*DIS2, were detected in 87% and 88% of all the samples, respectively. These genotypes were detected throughout the investigated period except for the years 1983 and 1984, the years showing the lowest *Planktothrix* biovolume. By contrast, the *mcy*AIS genotype was detected as late as in

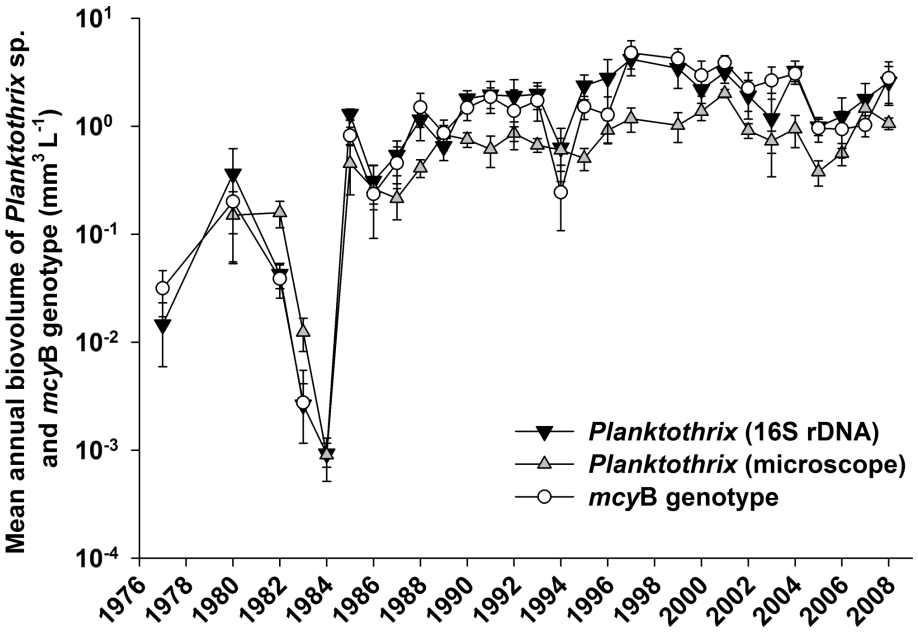

Figure 2 Abundance of the total *Planktothrix* population and the toxic genotype. Mean ± SE biovolume of *Planktothrix* determined in Lake Zürich from 1977 to 2008 using 16S rDNA and the *mcy*B gene by means of qPCR and microscopic counting. Biovolume in $mm^3\ L^{-1}$.

1996 and was then continuously present until 2008 (32% of the samples). It is concluded that inactive *mcy* genotypes occurred consistently, but never constituted a significant part of the total population.

Genotypes that lost the *mcy* gene cluster were quantified by amplifying a SNP in the *mcy*T gene, which constitutes a remnant of the *mcy* gene cluster in nontoxic strains [4]. In a first step, the sensitivity and efficiency of the TaqMan

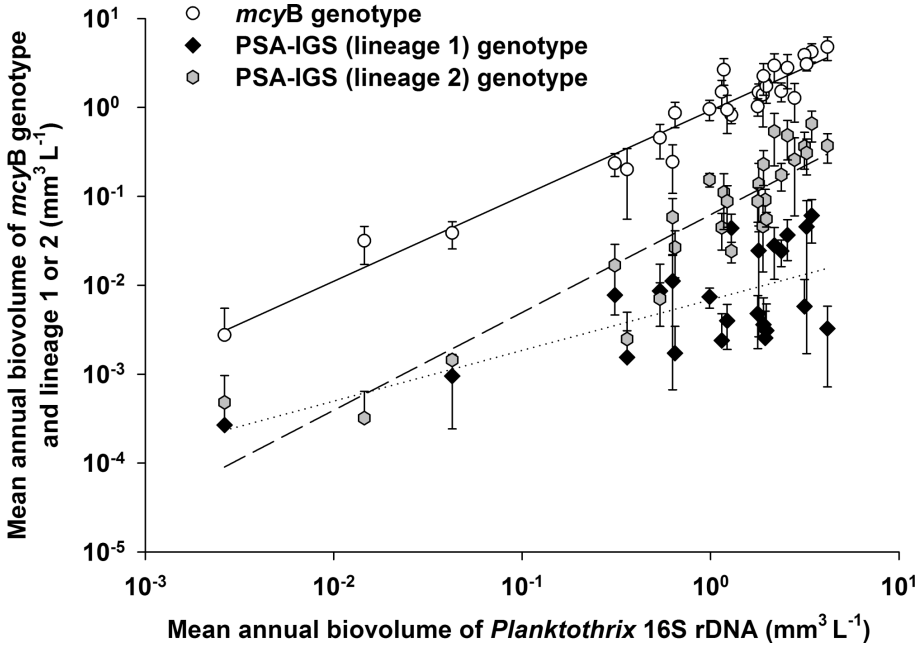

Figure 3 Correlations of the biovolumes of the toxic genotype and two lineages differing in pigmentation with the total biovolume of *Planktothrix*. Relationship between the mean ± SE biovolume of *Planktothrix* as determined by 16S rDNA, the *mcy*-containing genotype and two clonal complexes of *Planktothrix*: lineage 1 (green-pigmented strains that lost the *mcy* gene cluster) and lineage 2 (red- or green-pigmented strains that always contain the *mcy* gene cluster). Biovolume in $mm^3\ L^{-1}$. Details on the regression curves are given in the text. PSA-IGS: P700 apoprotein subunit Ia and Ib intergenic spacer.

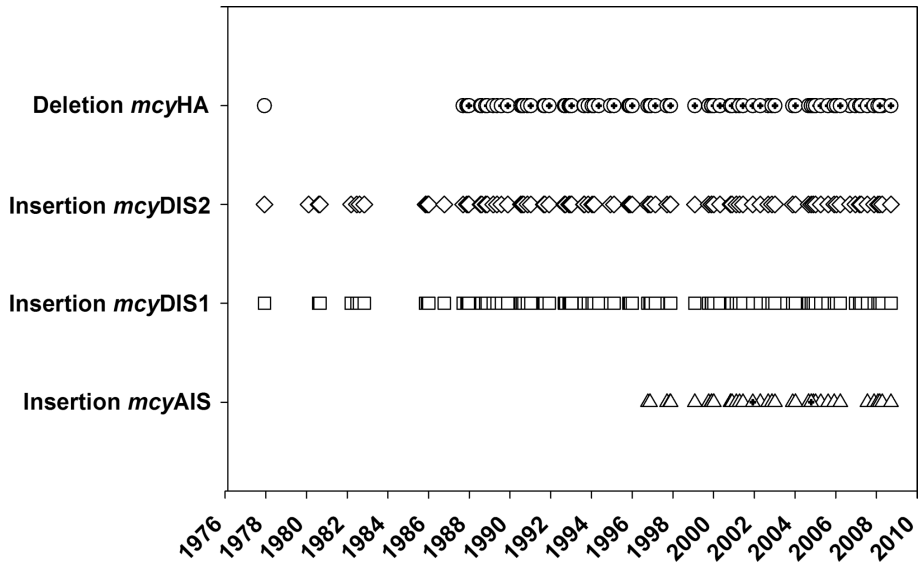

Figure 4 Occurrence of inactive *mcy* genotypes between 1977 and 2008. Occurrence of four different inactive *mcy* genotypes containing either insertions or a deletion within the *mcy* gene cluster. Genotypes were detected by means of qPCR. The cross in the symbols indicates numbers of the respective genotype that were above the quantification limit.

Genotyping Assay for *mcy*T was tested by the serial dilution of DNA isolated from axenic strains PCC7811 (allele 1, nontoxic genotype that lost the *mcy* gene cluster) and PCC7821 (allele 2, toxic genotype that still contains the *mcy* gene cluster) and analyzed using an allelic discrimination plot (Figure S2 in Additional file 1). The SNP assay showed a clear separation of the nontoxic versus toxic genotype and the two SNP probes were considered to be specific. The lowest DNA concentration that could be depicted was equivalent to two cells per template. Mixtures of the DNA (calculated from cell equivalents) from the two strains containing a 2% concentration of DNA from nontoxic strain PCC7811 showed a slight deviation of the delta Rn values compared with those of pure DNA from the toxic strain PCC7821. The delta Rn values of DNA mixtures with concentrations of 10% and 20% of nontoxic strain PCC7811 were clearly different from pure DNA of the toxic strain PCC7821, while the concentration of 50% of DNA of the nontoxic strain PCC7811 was displayed in the center of the allelic discrimination plot (Figure S2 in Additional file 1). It is concluded that the nontoxic genotype was detected as soon as its share exceeded 2% of the total population.

The analysis of field samples obtained from Lake Zürich and other shallow eutrophic lakes (Table 1) by an allelic discrimination plot revealed four distinct clusters (Figure 5). The first cluster comprised samples clustering along the *y*-axis (allele 2, toxic genotype), indicating populations only composed of the toxic genotype. This cluster included samples dominated by the red-pigmented ecotype: Lake Zürich, Irrsee and Mondsee and one lake with a population of the red- and the green-pigmented ecotype (Steinsfjorden). Samples of the green-pigmented populations (Wannsee, Slotermeer, Tjeukemeer and Havel) formed a second cluster in the center of the allelic discrimination plot, indicating populations comprising both the toxic and the nontoxic genotype. The *Planktothrix* population tested from Klinkenberger Plas comprising the red- and the green-pigmented ecotype was also found in this central cluster. By contrast, samples from shallow polymictic lakes (for example, Lake Frederiksborg Slotssø, Zeegerplas and the Albufera Lagoon) with a green-pigmented *Planktothrix* population formed a third cluster along the *x*-axis (allele 1, nontoxic genotype), indicating populations composed entirely of the nontoxic genotype. It is concluded that, in Lake Zürich, the nontoxic genotype carrying the *mcy*T gene as a remnant of the *mcy* gene cluster never became abundant during the entire observation period. By contrast, *Planktothrix* populations that were either only green-pigmented or both red- and green-pigmented typically showed a higher proportion of the nontoxic genotype.

Quantification of the pigmentation types of *Planktothrix* in Lake Zürich

To find out whether the abundance of the green-pigmented (nontoxic) ecotype was influenced by the trophic change during the study period, two phylogenetic lineages differing in pigmentation and the presence of the *mcy* gene cluster were quantified using the P700 apoprotein subunit Ia (*psa*A) and Ib (*psa*B) intergenic spacer region (PSA):

Table 1 Origin of field samples analyzed by the TaqMan Genotyping Assay (SNP) for allelic discrimination of the *mcy*T gene both as a remainder of the *mcy* gene cluster (nontoxic strains) and as part of the *mcy* gene cluster (toxic strains) [4,12].

Lake	Country	Number of samples	Lake area (km^2)	Z_{mean} (m)	Z_{max} (m)	Sampling period
Red-pigmented populations[a]						
Lake Zürich	CH	51	68	52	136	1977-2008
Irrsee	AT	4	3.5	15	32	Sep-Dec 2003
Mondsee	AT	22	16	36	68	Apr 2003-Dec 2004
Green-pigmented populations						
Albufera Lagoon	ES	1	21	1	3	Aug 2004
Frederiksborg Slotssø	DK	8	0.2	3	9	Jul-Oct 2003
Havel (Potsdam)	DE	1	-	3	4	Sep 2004
Slotermeer	NL	1	12.4	1.2	6	May 2004
Tjeukemeer	NL	1	20	2	5	Oct 2004
Zeegerplas	NL	3	0.7	18	34	Aug, Sep and Oct 2004
Wannsee	DE	6	2.7	6	9	Feb-Mar and May-Aug 2000
Mixed-pigmented populations						
Klinkenberger Plas	NL	3	0.3	-	30	May, Jul and Aug 2004
Steinsfjorden	NO	6	13.9	10	24	Jul-Sep 2003, Jul 2004

[a]Pigmentation type was determined by visual inspection in the microscope. AT: Austria; CH: Switzerland; DE: Germany; DK: Denmark; ES: Spain; NL: Netherlands; NO: Norway.

lineage 1, the green-pigmented, nontoxic genotype that lost the *mcy* gene cluster (PSA I), and lineage 2, both red- and green-pigmented toxic genotype that always carried the *mcy* gene cluster (PSA II) [4]. The green-pigmented lineage 1 was detected during the entire observation period although in a smaller number of samples (43%). By contrast, lineage 2 (red- and green-pigmented ecotypes containing the *mcy* genes) was detected in 83% of all the samples. A few samples with low *Planktothrix* biovolume (< 0.09 mm^3 L^{-1}) did not show a TNA signal indicative of lineage 2 (17% of the samples). The average proportion of the genotype of lineage 1 was generally low (minimum 0%, maximum 3.8%, mean 0.9 ± 0.2%) and no increase or decrease was observed when comparing the mean proportion of this genotype between the three decades: 1977 to 1989: 1.3 ± 0.5% (n = 9), 1990 to 1999: 0.6 ± 0.2% (n = 9) and 2000 to 2008: 0.7 ± 0.2% (n = 9); P = 0.26. Maximum abundances were measured in 1983 and 1985 (3.8% and 3.5% of the total population), coinciding with the time of the *Planktothrix* population breakdown and the following recovery. The mean proportion of the genotype of lineage 2 was 7.3 ± 1% of the total population (minimum 0.2%, maximum 18.5%). A significant increase of this genotype was detected when comparing the mean proportion between the three decades: 1977 to 1989: 2.3 ± 0.5% (n = 9), 1990 to 1999: 7.5 ± 1.5 (n = 9) and 2000 to 2008: 12.1 ± 1.2% (n = 9); $P < 0.001$.

The abundances of genotypes of lineage 1 and 2 were both linearly correlated to the total population as follows: lineage 1, $y = -2.165 + 0.57x$, $n = 23$, $R^2 = 0.45$, $P < 0.001$ (dotted line, Figure 3); lineage 2, $y = -1.207 + 1.1x$, $n = 27$, $R^2 = 0.83$, $P < 0.001$, (dashed line, Figure 3), where x is the $\log_{10}$ biovolume of the total population determined by 16S rDNA (mm^3 L^{-1}) and y is the $\log_{10}$ biovolume of *Planktothrix* lineage 1 or 2 (mm^3 L^{-1}), respectively. The regression curve of lineage 1 significantly differed in intercept and slope from the regression curve obtained between the *mcy*B genotype and the total population ($P < 0.001$, n = 27). By contrast, the regression curve of lineage 2 significantly differed in intercept but not in the slope (P = 0.179, n = 27) from the *mcy*B regression curve. Thus, where lineage 2 showed a parallel increase with the abundance of the total population, lineage 1 showed a tendency to decrease in proportion with a higher *Planktothrix* population density. The same results were obtained when the samples from the years with lowest *Planktothrix* density (1977 and 1982 to 1984) were excluded (data not shown). It is concluded that the genotype of lineage 2 consistently outgrew the genotype of lineage 1 under conditions of favorable growth for the *Planktothrix* population.

Discussion

Dominance of red-pigmented *Planktothrix* in Lake Zürich

The observed increase of the *Planktothrix* population in Lake Zürich over the last 30 years can be explained by the improved light regime in the water column that occurred following re-oligotrophication, through the disappearance of eukaryotic algae blooms. This phenomenon has already been documented for other lakes [2]. It is well known that *P. rubescens* stratifies in the metalimnion of thermally stratified lakes at approximately 12 m depth, and grows at the lowest irradiance [23]. Furthermore, *Planktothrix* can take

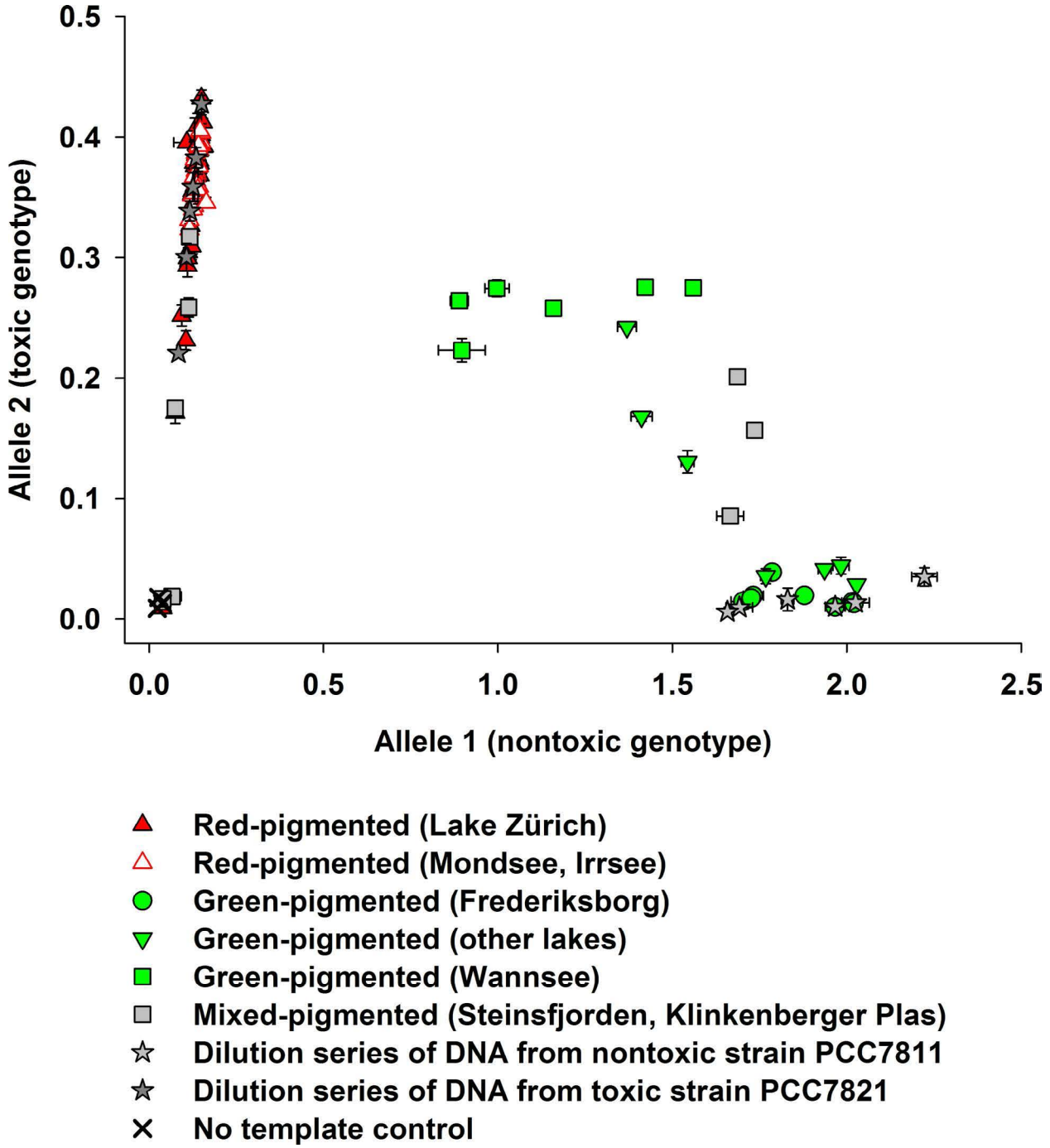

Figure 5 Composition of the toxic and nontoxic genotype in populations of *Planktothrix* across Europe. Allelic discrimination plot of toxic and nontoxic *Planktothrix* genotypes of populations differing in pigmentation as determined by a custom TaqMan SNP genotyping assay within *mcy*T, indicative of a genotype that lost (allele 1) or still contains (allele 2) the *mcy* gene cluster. Delta Rn values represent the difference in fluorescence from the post-PCR and pre-PCR reads of the normalized reporter (VIC, FAM). The symbols represent the mean ± SE of measurements in triplicate. When not visible, the error bars are hidden behind the symbol. Values located close to the no-template control failed to amplify.

advantage of higher concentrations of the limiting macronutrient (soluble reactive phosphorus) in the metalimnion as well as intracellularly stored phosphate for three or four further cell divisions [1,24]. The population breakdown in 1984 was probably the consequence of various factors that weakened the population. First, higher biomasses of eukaryotic algae occurred during the years 1983 and 1984 and might have reduced deep light penetration and forced

Planktothrix to enter the mixed epilimnion [25]. Mur [26] reported a higher growth rate of the green algae (*Scenedesmus*) at high light intensities whereas *Planktothrix* grew more efficiently at the lowest light intensities, thereby outcompeting *Scenedesmus*. Second, Posch *et al.* [27] reported that holomixis occurred in Lake Zürich from 1980 to 1987, probably leading to major losses in the *P. rubescens* population. Walsby and co-workers [17] reported a major decrease in population biomass in spring after a strong winter 1996 to 1997) when compared with population biomass in spring after a warmer winter (1994 to 1995). Posch *et al.* [27] further suggested that the increased epilimnetic water temperatures since 1988 led to a more stabilized stratification of the water column and allowed the survival of a bigger share of the population over winter due to reduced loss processes during lake mixing. However, it is unclear whether changes in environmental parameters (for example, depth of the euphotic zone or higher stability of the water column due to rising water temperatures) may not only have an impact on the growth of the total population but also on the subpopulation dynamics of specific ecotypes.

Factors indirectly causing the stability of genotype composition

Strikingly, the genotypic composition of *Planktothrix* was found to be rather stable over the last 30 years. The population was constantly dominated by the toxic *mcy*-containing genotype, while the share of the nontoxic (*mcy*-lacking) genotype was low. Sabart *et al.* [28] found the proportions of toxic and nontoxic genotypes in the populations of *Microcystis* along the catchment of River Loire to be generally stable over time but variable between sampling sites. Okello *et al.* [29] reported differing proportions of toxic and nontoxic genotypes from spatially isolated *Microcystis* populations in Uganda, and these differences in proportions were much more pronounced than the observed seasonal variation within lakes. These findings are in accordance with the present study and imply the role of both abiotic and biotic parameters of the habitat (for example, light regime, lake morphometry, zooplankton grazers, viruses) that regulate genotype composition [12]. It is important to note that these factors are not directly related to MC production and that effects of re-oligotrophication on the *Microcystis* genotype structure will differ considerably from those on the *Planktothrix* genotypic composition because of the overall difference between the ecological niches of the two taxa [14].

Probably the best example for an ecological trait not directly related to MC production is green versus red pigmentation. In Lake Zürich, the green-pigmented nontoxic *Planktothrix* lineage 1 was detected throughout the study period, but in very low proportions only. Interestingly, in the period before and after the population breakdown, a maximum proportion of the green-pigmented lineage 1 was observed. During this eutrophic period, blooms formed by non-stratifying phytoplankton still occurred (Figure 1). Under these conditions, the green-pigmented ecotype might have been at a selective advantage because of the variable irradiances, as it is found to be characteristic of shallow polymictic lakes [14]. By contrast, the red-pigmented ecotype was shown to be a better competitor for light only under mesotrophic and stratified conditions as its compensation depth for growth was lower compared with that of the green-pigmented ecotype [13]. Consequently, under eutrophic strong light shading conditions, the red-pigmented *Planktothrix* are seldom abundant [14,30].

Because Lake Zürich is a deep lake, the resistance of gas vesicles to hydrostatic pressure may be a critical factor for survival during conditions of lake mixing, as gas vesicles may collapse and filaments cannot return to the euphotic zone. It has previously been shown that the red- and green-pigmented ecotypes of *Planktothrix* vary in the presence of gas vesicle proteins, which could be attributed to lake depths [18]. The filaments remaining buoyant after mixing will, therefore, form the basis of the population for the next season [17]. Gas vesicles resisting strong hydrostatic pressures are more commonly found among the red-pigmented strains of *Planktothrix*, as in the population of Lake Zürich, whereas gas vesicles that collapse at a relatively low critical pressure are more common within green-pigmented populations [18]. Additionally, the genotype that produces gas vesicles that resist the highest hydrostatic pressures, which is the dominant genotype in Lake Zürich, was not detected in isolates obtained from 21 Norwegian lakes with a maximum depth of 67 m [18]. Accordingly, in red-pigmented populations, stronger gas vesicle types were found to be positively related to lake depth [31].

It is likely that the green-pigmented *Planktothrix* ecotype of Lake Zürich produces gas vesicles that do not resist the critical pressure during lake mixing and that only a small percentage of filaments survive entrainment in the hypolimnion. Therefore, in spring, the basis for the population development of the green-pigmented ecotype would be low when compared with the red-pigmented ecotype, of which a larger proportion can survive mixing during the winter (> 90% of the filaments [17]). Our own preliminary results showed that strains of nontoxic lineage 1 almost exclusively contained the weakest gas vesicle genotype ($gvpC^{28}$) while strains of toxic lineage 2 typically contained two gas vesicle genotypes ($gvpC^{28}$ and $gvpC^{20}$). Some of the strains of lineage 2 also showed the genotype encoding the smallest gas vesicle ($gvpC^{16}$), which is known to resist high hydrostatic pressure [18].

Potential factors directly favoring microcystin production

The inactive genotypes formed a stable but very small subpopulation throughout the study period. By contrast, the genotype carrying an insertion within *mcy*A (*mcy*AIS) was detected for the first time in 1996. It is conceivable that this genotype arrived in the lake at a later time than the other inactive genotypes, given that all of these four inactive genotypes occurred regularly in different populations in the Alps [22]. It is concluded that these inactive *mcy*-containing genotypes grew very slowly and still unknown factors keep their abundance low.

The role of MC as a feeding deterrent has been investigated and evidence has been gathered that MCs are indeed toxins resulting in a significantly reduced survival rate of herbivorous crustaceans [7,32,33]. The frequently cited hypothesis that MC-producing cyanobacteria evolved before their potential predators (the metazoans), which is based on phylogenetic analyses [34], ignores the fact that chemical defense still could be an additional (secondary) function simply because MCs are very effective toxins (for example, see [35]). In addition, the evolutionary age of eukaryotes has been a matter of debate, and the coexistence of eukaryotes with prokaryotes has been inferred from biomolecules in fossil oil droplets with an age of 2.4 billion years [36]. Thus, the possible co-occurrence of cyanobacteria and eukaryotes before snowball earth (2 billion years ago) is a matter of ongoing research. We suggest that, in nontoxic *P. agardhii*, other bioactive but nontoxic peptides functionally replace MC. For example, both lysogenic bacteria [37] and parasitic fungi have been described; for example, Sønstebø and Rohrlack [38] reported a relationship between chytridiomycete infectability of strains and the presence of certain peptides, such as cyanopeptolins and anabaenopeptins. The higher variability in toxic and bioactive peptide genotype proportion among nontoxic green-pigmented populations could be because of an ongoing co-evolutionary arms race between host and parasite [38].

As suggested for *Microcystis* [39], MC is considered to have an intracellular impact on the stability and activity of proteins involved in carbon-nitrogen metabolism by interfering with their redox state control. The enhanced binding of MC to proteins is thought to be part of a general response to oxidative stress [39,40]. The binding of MC to cysteine residues of proteins might be stimulated by conformational changes, which delays the degradation of redox-sensitive proteins. Zilliges *et al.* [39] showed that a MC-deficient mutant of *Microcystis* was more susceptible to high irradiance compared with the MC-producing wild type. It is conceivable that, in particular for red-pigmented *Planktothrix*, conditions of enhanced oxidative stress occur, for example when the low-light-adapted buoyant *Planktothrix* population accumulates at the surface in the autumn [41]. It is known that, among all phytoplankton species, red-pigmented *Planktothrix* is most efficient in light harvesting but this capability increases the chance of light damage to the cells under high irradiance, such as during calm days at the water surface [41]. Green-pigmented *P. agardhii* has been found more resistant to high light intensities [15]. In light of these findings, it can be speculated that MC production would be of selective advantage in red-pigmented *Planktothrix* populations, whereas MC production might be of less selective value in green-pigmented populations due to the general lower sensitivity to high light damage. Further, *mcy* genotypes inactive in MC production in red-pigmented populations would be selectively reduced during surface blooms under high irradiance conditions. It is possible that *mcy* genotypes inactive in MC synthesis grow under physically stable stratified conditions of the water column, as losses due to accidental accumulation at the surface will be low.

Methodology

Field studies on toxic genotype composition within a population often cover one season only and rarely exceed a period of several years. Long-term studies on the genotypic composition of a population with regard to environmental parameters would, therefore, be of relevance to understand the influence of lake restoration measures on bloom toxicity. A longer study period can also aid in predicting the toxic genotype composition during future blooms with regard to changing environmental conditions. Historical analyses investigated the occurrence of cyanobacteria by extracting DNA or MC from dried biomass archived in herbaria or from water samples and sediment cores [42-44]. These samples offer a reservoir of precious information about preserved plankton organisms. A set of continuously taken and preserved samples spanning decades is, however, scarce.

It is known that analyzing DNA from ancient samples causes difficulties either because of DNA degradation or contamination by contemporary DNA. Ancient DNA is often highly fragmented (100 to 500 bp) and contains modifications that hinder the amplification by Taq polymerases. Rapid desiccation can, however, delay enzymatic or microbial degradation processes [45]. Schober and Kurmayer [46] investigated the influence of freeze-drying on the quantification of cells by qPCR. When compared with cell numbers estimated from aliquots stored at -20°C, no differences were detected between the two treatments. We chose qPCR for estimating the proportions of genotypes from preserved samples for a number of reasons: fragmentation of DNA is considered of minor importance as the size of the amplification products is generally small (< 100 bp); estimating proportions of individual genotypes (relative to the total population) should be robust against

the bias due to point mutations in primer-binding regions; and the absolute cell concentrations as determined by qPCR could be validated by the cell numbers as determined in the microscope. On an absolute scale, a rather high correlation was observed between the biovolume of the total population estimated by microscopic counting and as estimated by 16S rDNA or the PC-IGS (Figure S1 in Additional file 1). The qPCR approach to relate the subpopulation of the toxic genotype to the total population was developed a decade ago [47], and typically the subpopulation of the nontoxic genotype has been inferred only indirectly. In this study, the first attempt was made to quantify the subpopulation of the nontoxic genotype directly by using a SNP that is indicative of the nontoxic genotype that lost the *mcy* gene cluster (Table S1 in Additional file 1[4]). In fact, the lowest proportion of the nontoxic genotype in all the samples from Lake Zürich as revealed by the allelic discrimination plot confirms that the population was constantly dominated by the toxic genotype (Figures 2 and 5). In addition, the allelic discrimination plot results highly correlate with the proportions of the *mcy*B gene as estimated in other red- and green-pigmented populations [12].

For unknown reasons, the sum of the PSA I and PSA II genotypes did not make up 100% of the population, but on average constituted only 8.2 ± 1.7%. It is speculated that the relatively larger amplicon size of both the PSA I and PSA II TNAs (167 bp and 158 bp) could be responsible for the relative underestimation of those genotypes. The fact that isolated DNA from ancient tissues is generally fragmented has already been described [48,49]. In our study, the isolated DNA of samples from different years was inspected on an agarose gel and showed a high degree of fragmentation. We therefore conclude that the underestimation of both PSA-IGS genotypes can be attributed to the relatively long amplification size that, except for the *mcy*AIS TNA, exceeds the size of all other TNAs (Table 2). However, since TNAs for both PSA-IGS genotypes were designed from the same primer-binding region of the same locus and the calibration curves could not be discriminated in slope or in intercept, the proportions of both PSA-IGS genotypes are considered reliable.

Conclusions

The stability in genotype composition provides evidence for the competitive superiority of a phylogenetic lineage that was shown to retain the MC gene cluster during evolution [4]. From an evolutionary point of view, it is important to see that the evolution of toxin synthesis genes within this population of cyanobacteria is slow. The long-term stability of toxic genotype composition, however, is useful to forecast the toxicity of blooms formed by *Planktothrix* in deep mesotrophic water bodies. In future, it will be necessary to consider the evolutionary history of the toxic genotypes of cyanobacteria to unravel their selective adaptations to the various aquatic environments.

Methods

Study site

Lake Zürich in Switzerland (47°15'N, 8°38'E, 406 m above sea level) is a deep (mean depth 51 m, maximum depth 136 m), monomictic lake with a surface area of 68 km^2. The first signs of Lake Zürich eutrophication were observed before 1890 (problems with the clogging of drinking water filters due to high zooplankton biovolumes). At the peak of eutrophication in 1965, the total phosphorus concentration reached a maximum of 100 μg L^{-1} at spring overturn [50] and the *Planktothrix* abundance was close to zero from 1965 to 1975. Since 1975, the *Planktothrix* population has recovered and has an average share of 33% per year (minimum 0.1%, maximum 68%) of the total phytoplankton biovolume between 1980 and 2008. Today, Lake Zürich is classified as mesotrophic with average total phosphorus concentrations of 23 μg L^{-1} (minimum 3.9 μg L^{-1}, maximum 78.1 μg L^{-1}) between 1980 and 2008. For the analysis of both phytoplankton and total phosphorus concentrations (Figures 1 and 2 and Figure S1A in Additional file 1), the average of the countings between the surface and 20 m in depth (0, 1, 2.5, 5, 7.5, 10, 12.5, 15 and 20 m) of the monthly sampling data was used.

Extraction of DNA from archived phytoplankton samples

A total of 111 samples from 1977 to 2008 were analyzed (in 1981 and 1998, no sampling was performed). The samples originated from a biweekly monitoring program targeting the euphotic zone by separate filtration of two liters from depths of 0, 5, 10 and 20 m through glass fiber filters, which were subsequently dried at 110°C for 2 h and stored in a desiccator until the next day. The filters were glued on paper and stored at room temperature (Figure S3 in Additional file 1). Filters were inspected and four filters from each year that showed maximum biomass were used for DNA extraction. Thus, the sampling depth varied according to the occurrence of the maximum population density. From the majority of the samples, one half of the filter with the preserved phytoplankton was used for DNA extraction, from ten samples only one quarter of the filter was used, and from three samples (600 mL from different depths, 7 December 1977) the phytoplankton of the complete filter was used for DNA extraction.

DNA was extracted from filters using the chloroform-phenol method as previously described [51]. The extracted DNA was precipitated from the aqueous phase by adding half the sample volume of ammonium acetate (7.5 M), one tenth of the sample volume of magnesium chloride (0.1 M), 1 μL of glycogen (Fermentas, St. Leon-Rot, Germany)

Table 2 Calibration curves and strains used for the Taq nuclease assay and the TaqMan genotyping assay (SNP).

TNA	Gene locus	Strain	Calibration curve[a]	*E* (%)[b]	R^2 [c]	n	Quantification limit (cells per template)[d]	Amplicon length (bp)
16S	16S rDNA	PCC7821	$y = 11.021 - 3.5123x$	92.6	0.997	12	4	82
PC-IGS	Intergenic spacer of the phycocyanin operon	PCC7821	$y = 9.9401 - 3.5673x$	90.7	0.994	12	4	72
*mcy*B	First adenylation domain of *mcy*B	PCC7821	$y = 12.151 - 3.3745x$	97.9	0.993	11	4	76
*mcy*DIS1	3'-end of IS-element within *mcy*D	No.110	$y = 7.941 - 3.8823x$	81.0	0.994	12	4	93
*mcy*DIS2	3'-end of IS-element within *mcy*D	No.139	$y = 11.956 - 3.1314x$	108.6	0.980	11	18	110
*mcy*AIS	3'-end of IS-element within *mcy*A	No.40	$y = 11.126 - 3.6644x$	87.5	0.991	11	4	168
*mcy*HA	Deletion between *mcy*H and *mcy*A	No.62	$y = 14.648 - 3.751x$	84.8	0.996	12	4	71
PSA I (lineage 1)[e]	Intergenic spacer between *psa*A and *psa*B	PCC7811	$y = 14.144 - 3.4482x$	93.2	0.996	11	3	167
PSA II (lineage 2)[e]	Intergenic spacer between *psa*A and *psa*B	PCC7821	$y = 13.681 - 3.3161x$	100.2	0.978	9	4	158
SNP Assay allele 1	SNP within *mcy*T (genotypes that lost the *mcy* gene cluster)	PCC7811	$y = 10.449 - 3.6685x$	98.2	0.996	15	2	66
SNP Assay allele 2	SNP within *mcy*T (genotypes containing the *mcy* gene cluster)	PCC7821	$y = 15.875 - 3.3579x$	98.5	0.996	15	2	66

[a] Calibration curve = regression line; y = number of PCR cycles at the threshold fluorescent value (C_t), x = amount of template DNA (expressed as $\log_{10}$ of mm^3 biovolume per template). [b] Amplification efficiencies (E) were calculated from $E = (10^{-1/x} - 1) \times 100$ (%), x = slope of calibration curve. [c] R^2 = coefficient of determination of calibration curve. [d] Quantification limit given as cell number equivalents represents the lower end of the calibration curve. [e] According to [4].

and one sample volume of isopropanol before overnight incubation (-20°C). After centrifugation (16,000 g, 4°C, 1 h) the supernatant was discarded and samples were washed twice with 70% ethanol by centrifugation (20 min, 4°C). The DNA pellets were dried in a sterile hood and resuspended in 50 μL of sterile Millipore water.

Design of qPCR TaqMan assays (Taq nuclease assay)

Samples were analyzed by TNA for the total population of *Planktothrix* by the 16S rDNA locus [11] and the PC-IGS [46]; for the abundance of the *mcy*B genotype encoding MC synthesis [12]; for the abundance of various mutant *mcy* genotypes carrying either a deletion or insertion inactivating MC biosynthesis [22], such as *mcy*DIS1, *mcy*DIS2, *mcy*AIS (insertion of a transposable element into the *mcy* gene cluster) and *mcy*HA (deletion of 1.8 kbp between *mcy*H and *mcy*A); for the abundance of the nontoxic genotype that lost the *mcy* gene cluster [4]; and for the abundance of red-pigmented versus green-pigmented ecotypes via the IGS between *psa*A and *psa*B (PSA-IGS; see below). The nontoxic genotype that lost the *mcy* gene cluster, except for *mcy*T, was differentiated from the genotype still containing the *mcy* gene cluster using a SNP. The primers and TaqMan probes for the 16S, PC-IGS, the *mcy*B gene and the four inactive *mcy* genotypes have been published previously (Table 2). For the design of TNAs targeting the PSA-IGS locus, sequences from 62 *Planktothrix* strains (GenBank:EU258202 to EU258263[4]) were aligned together with 78 sequences of *Planktothrix* strains isolated from Europe, North America and East Africa (R. Kurmayer, unpublished) by means of Clustal W 2.0. A target region was identified that discriminates all the strains of lineage 1 (n = 56; containing strains that are green-pigmented and lost the *mcy* gene cluster) from strains of lineage 2 (n = 84; containing both red-pigmented and green-pigmented strains that always retained the full *mcy* gene cluster). The following forward primers, TaqMan probes and reverse primers were designed: lineage 1 (167 bp): forward primer PSA I + (5'-CCAGCAATTCAACCTCGC-3'), TaqMan probe PSA I (5'-TGGTGTAGCTCACTA CCTCTTAGGAGGCAT-3') and reverse primer PSA I- (5'-AAAGTAGATTAGATTTCCTCCCACCT-3'); lineage 2 (158 bp): forward primer PSA II + (5'-CCAGC AATTC AACCTCGC-3'), TaqMan probe PSA II (5'-CGT GC GGTTGGTGTAGCTCACTACC-3') and reverse primer PSA II- (5'-ATTCAGCCTTTCCCAGTCCC-3'). Both probes were labeled with 6-carboxyfluorescein (FAM) at the 5'-end and BlackBerry Non-Fluorescent Quencher (NFQ) at the 3'-end.

Detection of the nontoxic genotype by single nucleotide polymorphism (custom TaqMan genotype assay)

In total, 113 *mcy*T sequences (GenBank: EU266304 to EU266364, and RK, unpublished; Table S1 in Additional file 1) of both toxic and nontoxic strains were aligned. A SNP was found to discriminate all the strains that lost the *mcy* gene cluster from strains that still contain the full *mcy* gene cluster. A custom TaqMan SNP genotyping assay based on this SNP was created using the Custom TaqMan Assay Design Tool (Applied Biosystems, ABI, Vienna, Austria) to differentiate the two genotypes. The following forward and reverse primers and two TaqMan probes labeled with 4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein (VIC) or FAM at the 5'-end and a NFQ at the 3'-end were obtained: *mcy*T SNP + (5'-ACAGAGAAAGC CGAGTTGGTT-3'), *mcy*T SNP-(5'-AGATTTGAAACC-TAACGCCTTGGA-3'), TaqMan Probe allele 1 (VIC 5'-TGTTCCCACCAAGCTT-3' NFQ) and TaqMan Probe allele 2 (FAM 5'- CCCGCCAAGCTT-3' NFQ) (66 bp). The specificity and sensitivity of the assay were tested by measuring the mixtures of DNA from axenic strains PCC7811 (loss of the *mcy* gene cluster except the *mcy*T gene) and PCC7821 (containing the *mcy* gene cluster).

Setup of qPCR TaqMan assays

All the samples were measured in triplicate in a total volume of 25 μL including 5 μL of template DNA. The initial denaturation of 10 min at 95°C was followed by 50 cycles of a two-step PCR with an annealing and elongation temperature of 60°C or 55°C on an Eppendorf Master Cycler Ep Realplex system (Eppendorf, Vienna, Austria), as described [11]. To increase the DNA concentration in the template as much as possible, the inactive *mcy* genotypes were measured in a total volume of 12.5 μL including 1 μL of the pure DNA extract and 1 μL (50 μg μL^{-1}) of bovine serum albumin. Because these results were frequently below the limit of quantification, only a presence/absence analysis was performed.

TaqMan assays were tested for specificity and sensitivity using dilution series of DNA isolated from *Planktothrix* strains 110, 139, 40 and 62, as described (Table 2) [22]. Calibration curves were also prepared from a dilution series of DNA from strains PCC7811 and PCC7821 for the TNA PSA I and PSA II, representing lineage 1 or lineage 2 [4]. Strains PCC7811 and PCC7821 were grown at 20°C in a BG11 medium under continuous light (5 to 15 μmol photons m^{-2} s^{-1}) and the cells were harvested during logarithmic growth. For each TNA calibration curve, the predetermined DNA concentrations in the template (expressed as biovolume per template) were diluted and related to the measured C_t values [22]. The limit of quantification was defined as the lowest concentration of the calibration curve, which corresponded to 3 to 18 cells per template for the individual assays (Table 2). In the case that the C_t values of a particular gene locus were below the limit of quantification, only the results from the 16S rDNA locus were included in the analysis (n = 8).

TaqMan Genotype Assay (single nucleotide polymorphism)

All the samples were measured in triplicate on a ViiA7 Real-Time PCR System (ABI, Vienna, Austria), using the ViiA7 software (version 1.2.1) in a total volume of 12.5 μL including 2.5 μL of template DNA, ABI TaqMan Universal PCR Master Mix, the SNP Genotyping Assay Mix and sterile water according to the manufacturer's recommendations. A two-step PCR of 55 cycles was run with denaturation (92°C, 15 s) and annealing and extension (60°C, 1 min). The protocol included an initial denaturation step at 92°C for 10 min and a pre-read and post-read step at 60°C for 30 s, each. The relative threshold cycle was automatically set to 0.04 for all reads. The results were analyzed based on the delta Rn values (with Rn = fluorescence signal of reporter dye normalized to the fluorescence signal of the passive reference ROX) for allele 1 and allele 2, which represent the difference of the normalized fluorescence signal (VIC, FAM) between the pre-PCR and the post-PCR read.

To test the sensitivity and the specificity of the SNP assay, DNA of the axenic nontoxic strain PCC7811 was mixed down to 0.2, 0.5, 1, 2, 10, 20 and 50% of DNA from the toxic strain PCC7821 per template (calculated as cell equivalents). Dilution series from DNA of axenic strains PCC7811 and PCC7821 reaching from 2 to 180,000 and 2 to 230,000 cells per template, respectively, were established. At least one sample per year (1977 to 2008, $n = 35$), as well as depth integrated and net samples (0 to 20 m) of Lake Zürich from the years 2005, 2006 and 2007 ($n = 16$ [11]) were analyzed. In addition, samples of various European lakes composed of either green- or mixed-pigmented *Planktothrix* populations [12] were included (Table 1).

Statistical analyses

To calculate regression curves, the raw data were $\log_{10}$-transformed and tested for normal distribution (Shapiro-Wilks, $P > 0.05$) and for constant variance by computing Spearman's rank correlation between the absolute values of the residuals and the observed value of the dependent variable ($P > 0.05$). The residuals were tested for their independence from each other by the Durbin-Watson statistic.

The linear regressions between the total population density (as estimated from 16S rDNA) and the abundance of the *mcy*B gene and the two PSA-IGS genotypes (representing lineage 1 and lineage 2) were compared in terms of the slope and intercept using a general factorial model of ANOVA [52]. A SPSS statistical package (V 19.0 for Windows) was used. To compare the genotype proportions from samples between the three decades (1977 to 1989, 1990 to 1999, 2000 to 2008), the nonparametric Kruskal-Wallis one-way ANOVA on ranks was used.

Additional material

Additional file 1: Additional file 1, Table S1 and Figures S1 to S3. Supplementary file providing additional Table S1 and Figures S1 to S3 in one pdf file.

Abbreviations

MC: microcystin; *mcy*: microcystin synthetase genes; NFQ: non-fluorescent quencher; PC-IGS: phycocyanin intergenic spacer; PCR: polymerase chain reaction; PSA: P700 apoprotein; qPCR: quantitative real-time polymerase chain reaction; SNP: single nucleotide polymorphism; TNA: TaqNuclease assay.

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Authors' contributions

VO performed the DNA extractions and qPCR measurements, analyzed the data, and drafted the manuscript. FS collected and archived all the samples from Lake Zürich. OK provided the data on phytoplankton and nutrients. RK designed the experiments, analyzed the data, and drafted the manuscript. All of the authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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References

1. Carey CC, Ibelings BW, Hoffmann EP, Hamilton DP, Brookes JD: **Eco-physiological adaptations that favour freshwater cyanobacteria in a changing climate.** *Water Res* 2012, **46**:1394-1407.
2. Jacquet S, Briand JF, Leboulanger C, Avois-Jacquet C, Oberhaus L, Tassin B, Vincon-Leite B, Paolini G, Druart JC, Anneville O, Humbert JF: **The proliferation of the toxic cyanobacterium *Planktothrix rubescens* following restoration of the largest natural French lake (Lac du Bourget).** *Harmful Algae* 2005, **4**:651-672.
3. Orr PT, Jones GJ: **Relationship between microcystin production and cell division rates in nitrogen-limited *Microcystis aeruginosa* cultures.** *Limnol Oceanogr* 1998, **43**:1604-1614.
4. Christiansen G, Molitor C, Philmus B, Kurmayer R: **Nontoxic strains of cyanobacteria are the result of major gene deletion events induced by a transposable element.** *Mol Biol Evol* 2008, **25**:1695-1704.
5. Kurmayer R, Christiansen G, Fastner J, Börner T: **Abundance of active and inactive microcystin genotypes in populations of the toxic cyanobacterium *Planktothrix* spp.** *Environ Microbiol* 2004, **6**:831-841.
6. Fewer DP, Halinen K, Sipari H, Bernardová K, Mänttäri M, Eronen E, Sivonen K: **Non-autonomous transposable elements associated with inactivation of microcystin gene clusters in strains of the genus *Anabaena* isolated from the Baltic Sea.** *Environ Microbiol Rep* 2011, **3**:189-194.

7. Rohrlack T, Dittmann E, Henning M, Börner T, Kohl JG: **Role of microcystins in poisoning and food ingestion inhibition of *Daphnia galeata* caused by the cyanobacterium *Microcystis aeruginosa*.** *Appl Environ Microbiol* 1999, **65**:737-739.
8. Vézie C, Rapala J, Seitsonen J, Sivonen K: **Effect of nitrogen and phosphorus on growth of toxic and nontoxic *Microcystis* strains and on intracellular microcystin concentrations.** *Microb Ecol* 2002, **43**:443-454.
9. Briand E, Yéprémian C, Humbert JF, Quiblier C: **Competition between microcystin- and non-microcystin-producing *Planktothrix agardhii* (cyanobacteria) strains under different environmental conditions.** *Environ Microbiol* 2008, **10**:3337-3348.
10. Kardinaal W, Tonk L, Janse I, Hol S, Slot P, Huisman J, Visser P: **Competition for light between toxic and nontoxic strains of the harmful cyanobacterium *Microcystis*.** *Appl Environ Microbiol* 2007, **73**:2939-2946.
11. Ostermaier V, Kurmayer R: **Application of real-time PCR to estimate toxin production by the cyanobacterium *Planktothrix* sp.** *Appl Environ Microbiol* 2010, **76**:3495-3592.
12. Kurmayer R, Schober E, Tonk L, Visser PM, Christiansen G: **Spatial divergence in the proportions of genes encoding toxic peptide synthesis among populations of the cyanobacterium *Planktothrix* in European lakes.** *FEMS Microbiol Lett* 2011, **317**:127-137.
13. Davis PA, Dent M, Parker J, Reynolds CS, Walsby AE: **The annual cycle of growth rate and biomass change in *Planktothrix* spp. in Blelham Tarn, English Lake District.** *Freshw Biol* 2003, **48**:852-867.
14. Reynolds CS: *The Ecology of Freshwater Phytoplankton* Cambridge: Cambridge University Press; 1984, 281-310.
15. Oberhaus L, Briand JF, Leboulanger C, Jaquet S, Humbert JF: **Comparative effects of the quality and quantity of light and temperature on the growth of *Planktothrix agardhii* and *P. rubescens*.** *J Phycol* 2007, **43**:1191-1199.
16. Stomp M, Huisman J, Vörös L, Pick FR, Laamanen M, Haverkamp T, Stal LJ: **Colourful coexistence of red and green picocyanobacteria in lakes and seas.** *Ecol Lett* 2007, **10**:290-298.
17. Walsby AE, Avery A, Schanz F: **The critical pressures of gas vesicles in *Planktothrix rubescens* in relation to the depth of winter mixing in Lake Zürich, Switzerland.** *J Plankton Res* 1998, **20**:1357-1375.
18. Beard SJ, Davis PA, Iglesias-Rodriguez D, Skulberg OM, Walsby AE: **Gas vesicle genes in *Planktothrix* spp. from Nordic lakes: strains with weak gas vesicles possess a longer variant of gvpC.** *Microbiology* 2000, **146**:2009-2018.
19. Schanz F: **Oligotrophication of Lake Zürich as reflected in Secchi depth measurements.** *Ann Limnol* 1994, **30**:57-65.
20. Gammeter S, Zimmermann U: **Changes in phytoplankton productivity and composition during reoligotrophication in two Swiss lakes.** *Verh Internat Verein Limnol* 2000, **27**:2190-2193.
21. Briand E, Gugger M, Francois JC, Bernard C, Humbert JF, Quiblier C: **Temporal variations in the dynamics of potentially microcystin-producing strains in a bloom-forming *Planktothrix agardhii* (cyanobacterium) population.** *Appl Environ Microbiol* 2008, **74**:3839-3848.
22. Ostermaier V, Kurmayer R: **Distribution and abundance of nontoxic mutants of cyanobacteria in lakes of the Alps.** *Microb Ecol* 2009, **58**:323-333.
23. Walsby AE, Ng G, Dunn C, Davis PA: **Comparison of the depth where *Planktothrix rubescens* stratifies and the depth where the daily insolation supports its neutral buoyancy.** *New Phytol* 2004, **162**:133-145.
24. Healey FP: **Phosphate.** In *The Biology of Cyanobacteria.* Edited by: Whitton NG, Carr BA. Berkeley and Los Angeles: Blackwell Scientific Publications; 1982:105-124.
25. Walsby AE, Schanz F: **Light-dependent growth rate determines changes in the population of *Planktothrix rubescens* over the annual cycle in Lake Zürich, Switzerland.** *New Phytol* 2002, **154**:671-687.
26. Mur LR: **Cyanobacteria in the environment.** In *Toxic cyanobacteria in Water A Guide to Their Public Health Consequences, Monitoring and Management.* Edited by: Chorus I, Bartram J. London and New York: E 1999:15-40.
27. Posch T, Köster O, Salcher MM, Pernthaler J: **Harmful filamentous cyanobacteria favoured by reduced water turnover with lake warming.** *Nat Clim Chang* 2012, **2**:809-813.
28. Sabart M, Pobel D, Briand E, Combourieu B, Salencon MJ, Humbert JF, Latour D: **Spatiotemporal variations in microcystin concentrations and in the proportions of microcystin-producing cells in several *Microcystis aeruginosa* populations.** *Appl Environ Microbiol* 2010, **76**:4750-4759.
29. Okello W, Ostermaier V, Portmann C, Gademann K, Kurmayer R: **Spatial isolation favours the divergence in microcystin net production by *Microcystis* in Ugandan freshwater lakes.** *Water Res* 2010, **44**:2803-2814.
30. Scheffer M, Rinaldi S, Gragnani A, Mur L, van Nes EH: **On the dominance of filamentous cyanobacteria in shallow, turbid lakes.** *Ecology* 1997, **78**:272-282.
31. D'Alelio D, Gandolfi A, Boscaini A, Flaim G, Tolotti M, Salmaso N: ***Planktothrix* populations in subalpine lakes: selection for strains with strong gas vesicles as a function of lake depth, morphometry and circulation.** *Freshw Biol* 2011, **56**:1481-1493.
32. Kurmayer R, Jüttner F: **Strategies for the co-existence of zooplankton with the toxic cyanobacterium *Planktothrix rubescens* in Lake Zürich.** *J Plankton Res* 1999, **21**:659-683.
33. Rohrlack T, Christoffersen K, Dittmann E, Nogueira I, Vasconselos V, Börner T: **Ingestion of microcystins by *Daphnia*: intestinal uptake and toxic effects.** *Limnol Oceanogr* 2005, **50**:440-448.
34. Rantala A, Fewer DP, Hisbergues M, Rouhiainen L, Vaitomaa J, Börner T, Sivonen K: **Phylogenetic evidence for the early evolution of microcystin synthesis.** *Proc Natl Acad Sci USA* 2004, **101**:568-573.
35. Hessen DO, Anderson TR: **Excess carbon in aquatic organisms and ecosystems: physiological, ecological, and evolutionary implications.** *Limnol Oceanogr* 2008, **53**:1685-1696.
36. George SC, Volk H, Dutkiewicz A, Ridley J, Buick R: **Preservation of hydrocarbons and biomarkers in oil trapped inside fluid inclusions for > 2 billion years.** *Geochim Cosmochim Acta* 2008, **72**:844-870.
37. Rashidan KK, Bird DF: **Role of predatory bacteria in the termination of a cyanobacterial bloom.** *Microb Ecol* 2001, **41**:97-105.
38. Sønstebø H, Rohrlack T: **Possible implications of chytrid parasitism for population subdivision in freshwater cyanobacteria of the genus *Planktothrix*.** *Appl Environ Microbiol* 2011, **77**:1344-1351.
39. Zilliges Y, Kehr JC, Meissner S, Ishida K, Mikkat S, Hagemann M, Kaplan A, Börner T, Dittmann E: **The cyanobacterial hepatotoxin microcystin binds to proteins and increases the fitness of *Microcystis* under oxidative stress conditions.** *PLoS One* 2011, **6**:e17615.
40. Vardi A, Schatz D, Beeri K, Motro U, Sukenik A, Levine A, Kaplan A: **Dinoflagellate-cyanobacterium communication may determine the composition of phytoplankton assemblage in a mesotrophic lake.** *Curr Biol* 2002, **12**:1767-1772.
41. Walsby A, Schanz F, Schmid M: **The Burgundy-blood phenomenon: a model of buoyancy change explains autumnal water blooms by *Planktothrix rubescens* in Lake Zürich.** *New Phytol* 2005, **169**:109-122.
42. Metcalf JS, Beattie KA, Purdie EL, Bryant JA, Irvine LM, Codd GA: **Analysis of microcystins and microcystin genes in 60-170-year-old dried herbarium specimens of cyanobacteria.** *Harmful Algae* 2012, **15**:47-52.
43. Rinta-Kanto JM, Saxton MA, De Bruyn JM, Smith JL, Marvin CH, Krieger KA, Sayler GS, Boyer GL, Wilhelm SW: **The diversity and distribution of toxigenic *Microcystis* spp. in present day and archived pelagic and sediment samples from Lake Erie.** *Harmful Algae* 2009, **8**:385-394.
44. Savichtcheva O, Debroas D, Kurmayer R, Villar C, Jenny JP, Arnaud F, Perga ME, Domaizon I: **Quantitative PCR enumeration of total/toxic *Planktothrix rubescens* and total cyanobacteria in preserved DNA isolated from lake sediments.** *Appl Environ Microbiol* 2011, **77**:8744-8753.
45. Pääbo S, Poinar H, Serre D, Jaenicke-Despres V, Hebler J, Rohland N, Kuch M, Krause J, Vigilant L, Hofreiter M: **Genetic analyses from ancient DNA.** *Annu Rev Genet* 2004, **38**:645-679.
46. Schober E, Kurmayer R: **Evaluation of different DNA sampling techniques for the application of the real-time PCR method for the quantification of cyanobacteria in water.** *Lett Appl Microbiol* 2006, **42**:412-417.
47. Kurmayer R, Kutzenberger T: **Application of real-time PCR for quantification of microcystin genotypes in a population of the toxic cyanobacterium *Microcystis* sp.** *Appl Environ Microbiol* 2003, **69**:6723-6730.
48. Pääbo S: **Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification.** *Proc Natl Acad Sci USA* 1989, **86**:1939-1943.
49. Sawyer S, Krause J, Guschanski K, Savolainen V, Pääbo S: **Temporal patterns of nucleotide misincorporations and DNA fragmentation in ancient DNA.** *PLoS One* 2012, **7**:e34131.
50. Bossard P, Gammeter S, Lehmann C, Schanz F, Bachofen R, Bürgi HR, Steiner D, Zimmermann U: **Limnological description of the Lakes Zürich, Lucerne, and Cadagno.** *Aquat Sci* 2001, **63**:225-249.
51. Kurmayer R, Christiansen G, Chorus I: **The abundance of microcystin-producing genotypes correlates positively with colony size in *Microcystis***

and determines its microcystin net production in Lake Wannsee. *Appl Environ Microbiol* 2003, **69**:787-795.

52. Sokal R, Rohlf F: *Biometry The Principles and Practice of Statistics in Biological Research*. 3 edition. New York: W.H. Freeman and Company; 1995.

doi:10.1186/1741-7007-10-100
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Supplemental Material Chapter 4

Table S1. Alignment of *Planktothrix mcy*T sequences of nontoxic strains (loss of 90% of the *mcy* gene cluster) and toxic strains (containing the *mcy* gene cluster) used for designing a Custom TaqMan SNP Genotyping Assay

Strain	*mcy* gene cluster[a]	Pigmentation[b]	Lineage[c]	Origin[d]	Lake depth (m)[e]	Sequence[f]
Nontoxic strains						
No.2A	n	green	1	Markusbölefjärden (FI)	9	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**A**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.41	n	green	1	Jägerteich (AT)	2	ACAGAGAAAGCCGAGTTGGTTTGTT**T**CC**A**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.63, 66	n	green	1	Jägerteich (AT)	2	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**A**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.250, 251, 253, 254, 255, 256, 257	n	green	1	Albufera Lagoon (ES)	3	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**A**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.259, 263, 274, 281	n	green	1	Wannsee (DE)	9	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**A**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.299, 320	n	green	1	Klinkenberger Plas (NL)	30	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**A**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.307	n	green	1	Klinkenberger Plas (NL)	30	ACAGAGAAAGCCGAGTTGGTT**C**GTTCCC**A**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.760, 788, 790	n	green	1	St. Domingos (PT)	42.5	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**A**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.781, 828, 829, 836, 837	n	green	1	Nero (RU)	4.7	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**A**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
PCC7805	n	green	1	Veluwemeer (NL)	5	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**A**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
PCC7811	n	green	1	Paris, Vert le Petit (FR)	2	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**A**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
PH22	n	green	1	Bagsværd Sø (DK)	3	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**A**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
SAG5.81	n	green	1	Kiessee (DE)	2	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**A**CCAAGCTTCTA**C**CTCCAAGGCGTTAGGTTTCAAATCT
No.277	n	green	1	Wannsee (DE)	9	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**A**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.364	n	green	1	Moose Lake (CA)	19.8	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**A**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.371, 372, 396, 552, 553, 557	n	greenbrown	1	Moose Lake (CA)	19.8	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**A**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
CCAP1459/15	n	green	1	Lough Neagh (UK)	34	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**A**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
Toxic strains						
No.31/1, 32, 39	y	green	1	Wannsee (DE)	9	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.79	y	green	1	Arresø (DK)	40	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
SAG6.89	y	green	1	Plußsee (DE)	30	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
NIVA-CYA126/8	y	green	1	Langsjön (FI)	18	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
CCAP1459/11A	y	green	2	Windermere (UK)	64	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
CCAP1459/14	y	red	2	Loughrigg Tarn (UK)	10.3	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
CCAP1459/16, CCAP1459/17	y	green	2	Blelham Tarn (UK)	15	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
CCAP1459/21	y	green	2	Esthwaite Water (UK)	16	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
CCAP1459/30	y	red	2	Plöner See (DE)	60	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
CCAP1459/31	y	green	2	White Lough (UK)	10.7	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
CCAP1460/5	y	green	2	Kasumigaura (JP)	10	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.3, 97, 111	y	red	2	Mondsee (AT)	68	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.21-	y	red	2	Figur (AT)	12	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.40, 91/1, 110	y*	red	2	Mondsee (AT)	68	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.64	y	red	2	Wörthersee (AT)	86	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.80	y	red	2	Schwarzensee (AT)	54	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.108	y	red	2	Irrsee (AT)	32	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.139, 145, 161, 166, 169, 170, 178	y*	red	2	Grabensee (AT)	13	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.260	y	green	2	Wannsee (DE)	9	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.403, 405, 406, 496, 549, 550, 551	y*	red	2	Moore (Crane) Lake (CA)	26	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.761	y	red	2	Reservoir Garcia (IT)	43	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.775	y	red	2	Reservoir Nicolletti (IT)	36	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTT~ATCTCCAAGGCGTTAGGTTTCAAATCT
No.777, 778, 779	y	red	2	Reservoir Nicolletti (IT)	36	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.803, 808, 804, 805, 806, 807, 811	y	green	2	Winnecook Lake (US)	12.5	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.813, 814, 815	y	green	2	China Lake (US)	25.9	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.838, 840	y	red	2	Reservoir Garcia (IT)	43	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
PCC7821	y	red	2	Gjersjøen (NO)	64	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
CCAP1459/36	y*	green	2	Gjersjøen (NO)	64	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.758, 763, 764, 765, 766, 769, 770, 772	y	green	2	Hormanjärvi (FI)	21	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT
No.822, 863, 865, 872, 873	y	green	2	Pyhäjärvi (FI)	35	ACAGAGAAAGCCGAGTTGGTTTGTTCCC**G**CCAAGCTTCTATCTCCAAGGCGTTAGGTTTCAAATCT

[a] presence/absence of the *mcy* gene cluster, a star indicates the inactivation of the *mcy* gene cluster by insertion or deletion [1]

[b] as recorded under culture conditions (at 15°C and continuous light 5-10 µmol m^{-2} s^{-1}, Osram Type L30W/77 Fluora), [1]

[c] *Planktothrix* strains were assigned to two different lineages according to Christiansen et al [2]

[d] Countries: FI = Finland, DE = Germany, DK = Denmark, UK = United Kingdom, AT = Austria, ES = Spain, NL = Netherlands, PT = Portugal, RU = Russia, FR = France, CA = Canada, JP = Japan, IT = Italy, US = USA, NO = Norway

[e] maximum lake depth

[f] *P. agardhii* microcystin biosynthesis gene cluster (AJ441056.1, bp 552-617)

Figure S1. (A) Relationship between the total *Planktothrix* biovolume as determined by 16S rDNA, and the biovolume of the *Planktothrix* population determined by microscopical counting. (B) Relationship between the total *Planktothrix* biovolume determined by 16S rDNA and the biovolume of the same cells carrying the PC-IGS gene fragment. Biovolumes in $mm^3\ L^{-1}$, symbols represent the mean values of four measurements per year and standard errors. For details on the regression curve see text.

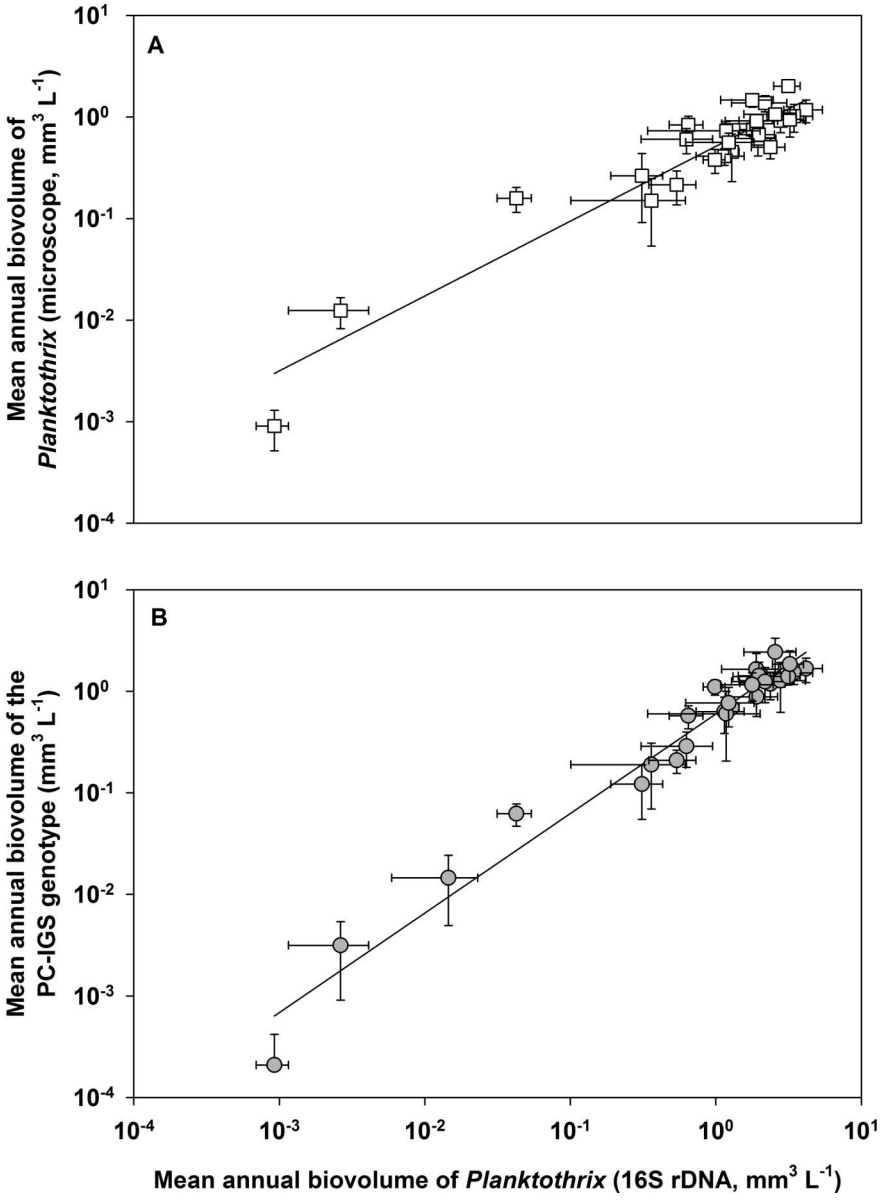


Figure S2. Allelic discrimination plot differentiating genotypes that lost (allele 1) or still contain (allele 2) the *mcy* gene cluster along the axis by a single nucleotide polymorphism within the *mcy*T gene. Serial dilutions of pure DNA of axenic strain PCC7811 (loss of the *mcy* gene cluster except of the *mcy*T gene), and PCC7821 (containing the *mcy* gene cluster) and mixtures of both strains containing 0.1, 0.2, 0.5, 1, 10, 20 and 50% of DNA of nontoxic strain PCC7811 (calculated as cell equivalents) are shown. When not visible, error bars are hidden behind the symbol.

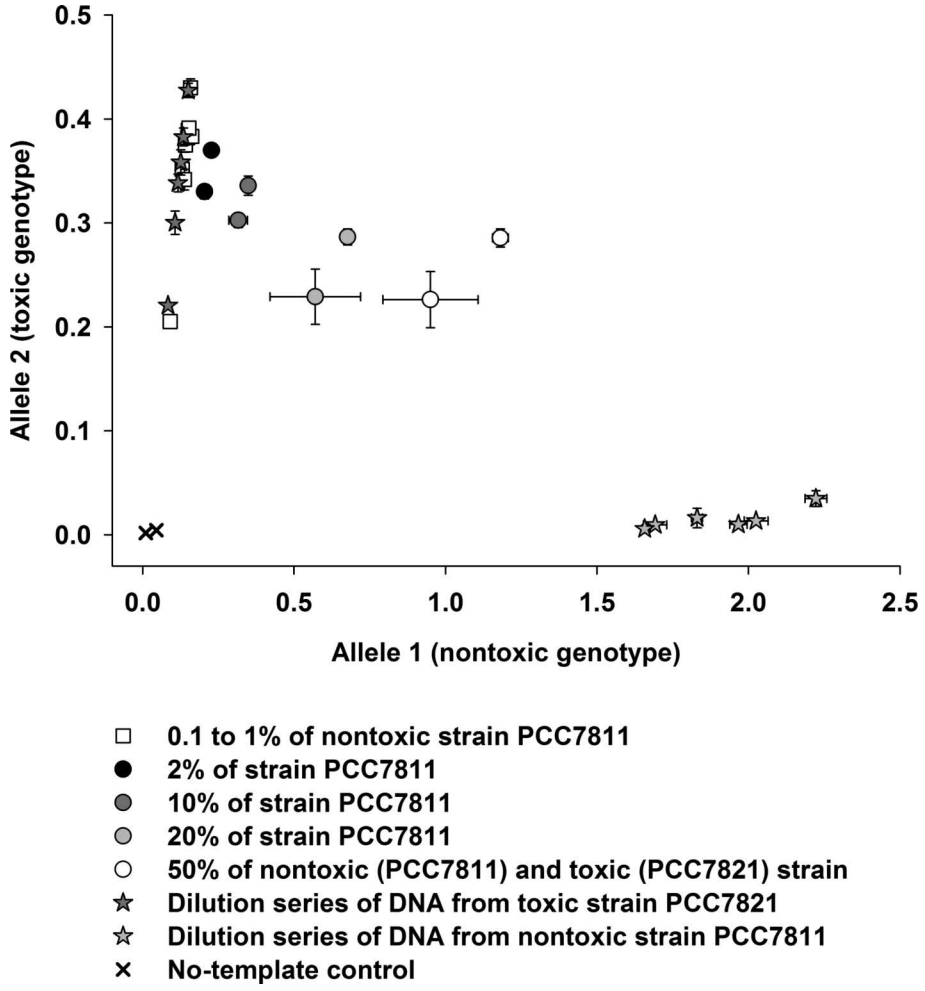
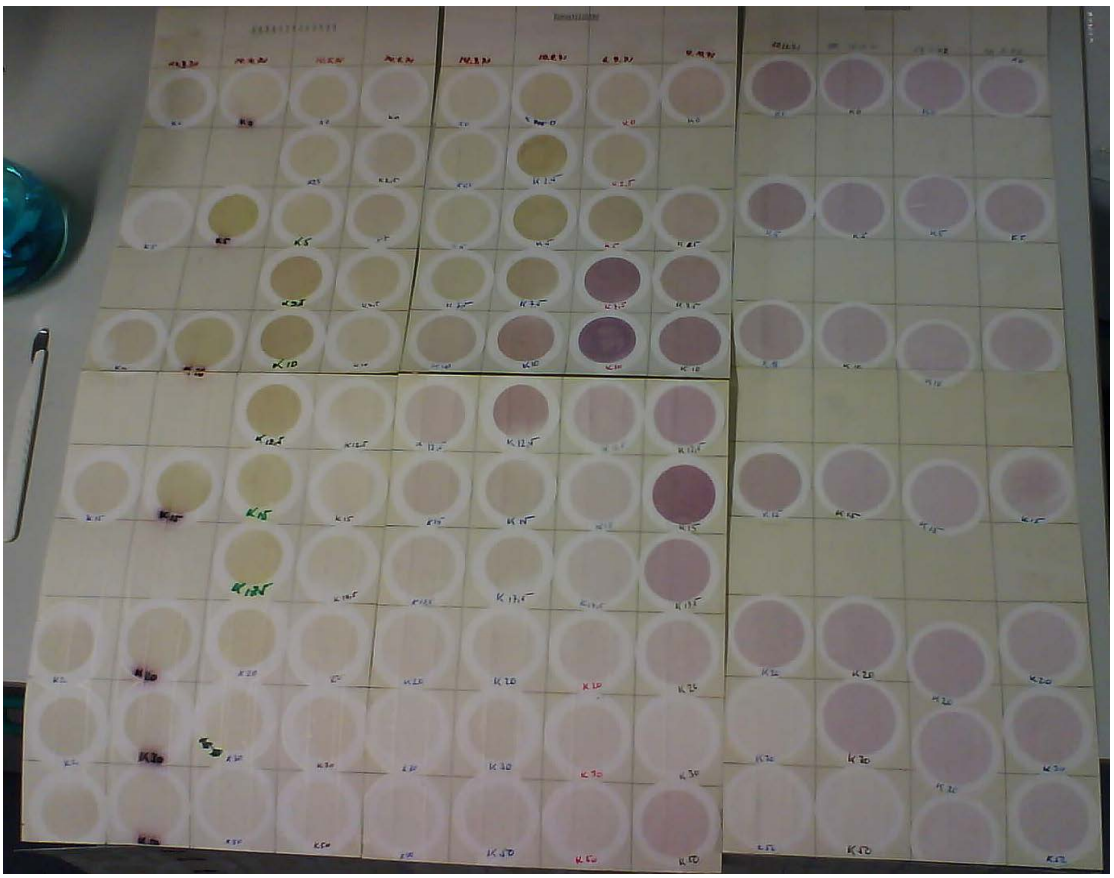


Figure S3. Photograph of filters with phytoplankton harvested from different depths and stored dry at room temperature. The darker reddish color is indicative of the stratification of *Planktothrix* at a depth of 12 – 18m.



References

1. Kurmayer R, Christiansen G, Fastner J, Börner T: **Abundance of active and inactive microcystin genotypes in populations of the toxic cyanobacterium *Planktothrix* spp.** *Environ Microbiol* 2004, **6:**831-841.

2. Christiansen G, Molitor C, Philmus B, Kurmayer R: **Nontoxic strains of cyanobacteria are the result of major gene deletion events induced by a transposable element.** *Mol Biol Evol* 2008, **25:**1695-1704.

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Curriculum Vitae

Name:	Veronika Ostermaier
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Education

Since April 2009	Doctoral Program in Ecology, University of Vienna, Austria
2007	Diploma in Biology Diploma Thesis: "Seasonal abundance patterns of toxic genotypes in populations of aquatic cyanobacteria"
2001 – 2007	Studies in Biology, Ludwig-Maximilians-University, Munich, main subject: ecology; minors: genetics, zoology, anthropology and human genetics
1991 – 2000	High-school, Munich

Working Experience

Since Sept. 2007	Scientific employee and since 2009 doctoral candidate, working group "Molecular ecology and physiology of cyanobacteria", Research Institute for Limnology, University of Innsbruck (formerly Austrian Academy of Sciences), Mondsee, Austria
Aug. 2006 – Aug. 2007	Degree candidate, Institute for Limnology, Austrian Academy of Sciences, Mondsee
Nov. 2003 – May 2004	Student employee, German Research Center for Environmental Health, Neuherberg, Germany
Dec. 2000 – May 2003	Part-time employee, Patent Attorneys Dr. Münich & Colleagues, Munich

Research Stays Abroad

Mai – Oct. 2013	Research stay at the Norwegian Institute for Water Research (NIVA), Oslo, Norway. Title of the project: "Tracing toxigenic cyanobacteria from preserved genetic material".

Aug. – Sept. 2004 Practical work, University of Palangka Raya and field work, Borneo, Indonesia. Title of the project: "Ecology of tropical rainforests; investigation of causes and prevention of forest fires".

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Awards

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2004 Fellowship of the program „Hochschule International" (University International) of the Ludwig-Maximilians-University, Munich, Germany, (partial financing of the stay in Indonesia), August 2004.

List of Publications

Ostermaier V, Christiansen G, Schanz F, Kurmayer R (2013): Genetic variability of microcystin biosynthesis genes in *Planktothrix* as elucidated from samples preserved by heat desiccation during three decades. Submitted to PLoS ONE (August 07, 2013).

Stoeck T, Breiner H-W, Filker S, Ostermaier V, Kammerlander B, Sonntag B (2013): A morpho-genetic survey on ciliate plankton from a mountain lake pinpoints the necessity of lineage-specific barcode markers in microbial ecology. Environmental Microbiology doi:10.1111/1462-2920.12194.

Ostermaier V, Schanz F, Köster O, Kurmayer R (2012): Stability of toxin gene proportion in red-pigmented populations of the cyanobacterium *Planktothrix* during 29 years of re- oligotrophication of Lake Zürich. BMC Biology 10:100.

Ostermaier V and Kurmayer R (2010): Application of real-time PCR to estimate toxin production by the cyanobacterium *Planktothrix*. Applied and Environmental Microbiology 76:3495-3502

Okello W, Ostermaier V, Portmann C, Gademann K, Kurmayer R (2010): Spatial isolation favours the divergence in microcystin net production by *Microcystis* in Ugandan freshwater lakes. Water Research 44:2803-2814.

Ostermaier V and Kurmayer R (2009): Distribution and abundance of non-toxic mutants of cyanobacteria in lakes of the Alps. Microbial Ecology 58:323-333.

Ostermaier V (2007): Saisonale Abundanzmuster von toxischen Genotypen in Populationen aquatischer Cyanobakterien. Ludwig-Maximilians-University, Munich, Germany, diploma thesis.

Contributions to Conferences

Ostermaier V, Schanz F, Köster O and Kurmayer R (2010): Historical analysis of toxic and nontoxic genotype composition in cyanobacteria. Poster presentation, International Evaluation of the Institute for Limnology, November 2010, Institute for Limnology, Mondsee, Austria.

Ostermaier V, Schanz F, Köster O, Kurmayer R (2010): Historical analysis of toxic and nontoxic genotype composition in cyanobacteria. Poster presentation, 3rd meeting of the Scientific Advisory Board of the Institute for Limnology of the Austrian Academy of Sciences, October 2010, Institute for Limnology, Mondsee, Austria.

Ostermaier V and Kurmayer R (2010): Time in microevolution of microcystin synthesis in *Planktothrix*. Oral presentation, International Conference on Toxic Cyanobacteria (ICTC 8), August 2010, Istanbul, Turkey.

Ostermaier V and Kurmayer R (2010): Zeitliche Aspekte der Microcystinsynthese. Oral presentation, Limnological Station of the University of Zürich, May 2010, Kilchberg, Switzerland.

Ostermaier V and Kurmayer R (2009): Transposon induced variation in toxin synthesis of cyanobacteria. Oral presentation, 2nd meeting of the Scientific Advisory Board of the Institute for Limnology of the Austrian Academy of Sciences, September 2009, Mondsee, Austria.

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Ostermaier V and Kurmayer R (2009): Genotype determined microcystin net production of *Planktothrix rubescens* in alpine lakes. Oral presentation, Symposium for European Freshwater Sciences, (SEFS 6), August 2009, Sinaia, Romania.

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Ostermaier V and Kurmayer R (2008): Genotype determined microcystin net production of *Planktothrix rubescens* in alpine lakes. Oral presentation, American Society of Limnology and Oceanography (ASLO summer meeting), June 2008, St John's, Canada.

Ostermaier V and Kurmayer R (2008): Distribution and abundance of nontoxic mutants in populations of cyanobacteria in the Alps. Poster presentation, mySIL meeting, May 2008, Lunz am See, Austria.

Ostermaier V and Kurmayer R (2007): Saisonale Abundanzmuster von toxischen Genotypen in Populationen aquatischer Cyanobakterien. Oral presentation, November 2007, Institute for Limnology, Mondsee, Austria.