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DISSERTATION

Titel der Dissertation

Molecular and chemical characterisation of microcystin producing cyanobacteria in Ugandan freshwater habitats

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To all in the team who made my dream a reality

Abstract (German)

Cyanobakterien (Blaualgen) kommen in Binnengewässern vor und dominieren häufig das Phytoplankton. Neben verschiedenen ökophysiologischen Anpassungen wie der Fähigkeit zum Schweben oder die effiziente Aufnahme von Bikarbonaten bei hohem pH-Wert, ist es vor allem die Bildung verschiedener Toxine und bioaktiver Substanzen, von der man annimmt, dass diese zum ökologischen Vorteil beiträgt. Bei den Toxinen handelt es sich einerseits um zyklische Peptide, wie z.B. den hepatotoxisch wirksamen Microcystinen und andererseits um Alkaloide, wie z.B. dem hepatotoxisch wirksamen Cylindrospermopsin oder den neurotoxisch wirksamen Saxitoxinen, Anatoxin-a oder Anotoxin-a (S).

Während bekannt ist, dass verschiedene Gattungen von Cyanobakterien in den Seen Ostafrikas eine wichtige Rolle spielen, ist über ihre Fähigkeit zur Produktion verschiedener Toxine praktisch nichts bekannt. Diese Wissenslücke ist in mehrerlei Hinsicht problematisch: Erstens verwendet ein beträchtlicher Anteil der Bevölkerung täglich Wasser aus den Seen als Trinkwasser, zweitens nützt ein ebenso beträchtlicher Anteil der Bevölkerung verschiedene phytoplanktivore Fische als Nahrungsquelle. Eine Abschätzung des Gesundheitsrisikos für die Bevölkerung erfordert jedoch nicht nur die Erfassung der Algenbiomasse, sondern auch das Verständnis, ob und wie es zur Toxinproduktion kommt und welche Faktoren diese begünstigen.

Es war daher das Ziel dieser Arbeit, in mehreren großen Süßwasserseen Ugandas saisonal die Produktion eines weit verbreiteten Toxins, dem Microcystin zu erfassen, sowie die verantwortlichen Umweltfaktoren bzw. die einzelnen Microcystinproduzenten zu identifizieren. Dafür wurden im Laufe eines Jahres fünf Seen in Uganda (der Victoriasee: Murchison Bay, Napoleon Gulf; drei seichte Seen im Einzugsgebiet des Ruwenzori Massivs: Lake George, Lake Edward, Lake Mburo; ein Kratersee: Lake Saka) in monatlichen Abständen beprobt und hinsichtlich ihrer physikalisch-chemischen Parameter analysiert. Die Phytoplanktonzusammensetzung wurde qualitativ und quantitativ im Umkehrmikroskop bestimmt und die Microcystinproduktion wurde mittels Hochleistungs-Durchflusschromatographie (HPLC-DAD) analysiert. Zusätzlich zur Freilandbeprobung wurden zahlreiche Stämme der Gattung *Microcystis* isoliert und hinsichtlich ihrer Fähigkeit zur Microcystinproduktion genetisch und chemisch-analytisch charakterisiert. Weiter wurde der Nachweis bestimmter artspezifischer Genregionen durch PCR sowohl für einzelne Isolate als auch für Wasserproben verwendet, um herauszufinden, welche der verschiedenen Gattungen der Cyanobakterien tatsächlich Microcystine produzieren.

In der ersten Publikation (Kapitel 2, Okello et al. 2009, Environmental Toxicology, 16 Juli 2009) konnte gezeigt werden, dass die Gattung *Microcystis* den alleinigen Produzenten von Microcystin in den untersuchten Gewässern darstellt. Sämtliche auf PCR basierenden Nachweismethoden in Wasserproben führten zur Identifizierung der für die Microcystinsynthese verantwortlichen Gene bei *Microcystis*. Weiter konnte gezeigt werden, dass Eutrophierung von Gewässern immer zur Dominanz von *Microcystis* und damit zwangsläufig zur Microcystinproduktion führt. Zusätzlich wurden zwei strukturelle Varianten des Microcystinmoleküls bestimmt, die in diesen Gewässern häufig vorkommen und zuvor nicht bekannt waren.

In der zweiten Publikation (Kapitel 3, Okello und Kurmayer, Water Research, zur Veröffentlichung eingereicht) wurde die Microcystinproduktion saisonal in den fünf genannten Seen über ein Jahr lang (April 2007-April 2008) erfasst. In praktisch allen Proben wurden Microcystine detektiert. In Übereinstimmung mit Publikation Nr. 1 konnte gezeigt werden, dass die im Mikroskop ermittelte Zellzahl von *Microcystis* hoch signifikant mit der gemessenen Konzentration an Microcystin korreliert. Andere potentiell Microcystin produzierende Gattungen wie *Anabaena* oder *Planktothrix* zeigten durchwegs Korrelationen mit geringerem Erklärungswert und müssen vor allem durch den fehlenden PCR Nachweis (gemäss Publikation Nr. 1) als nicht toxisch bezeichnet werden.

Unerwarteter Weise zeigten die einzelnen Seen saisonal relativ geringe Schwankungen in der Microcystinproduktion, unterschieden sich aber signifikant pro Liter durchschnittlich bis zum 28-fachen. Der Maximalwert wurde mit 10 µg/L Microcystin im Kraterseee L. Saka gemessen, während die Minimalwerte mit 0.02 µg/L durchwegs aus dem Lake George stammten. Diese für einzelne Seen spezifische Divergenz war jedoch nur zum Teil durch die Unterschiede in der durchschnittlichen Zellzahl von *Microcystis* begründet. Beispielsweise zeigte Lake George übers Jahr gemittelt die deutlich höchste Zellzahl an *Microcystis* (1.2 ± 0.3 (1 Standardfehler) Mio Zellen/ml), während die Microcystinkonzentrationen jedoch durchwegs sehr gering (0.2 ± 0.1 µg/L Microcystin) waren. Im Gegensatz dazu waren im morphometrisch und physikalisch sehr ähnlichem L. Mburo die Zellzahlen von *Microcystis* niedriger (0.17 ± 0.02 Mio Zellen/ml), die mittlere Microcystinkonzentration aber deutlich höher (1.6 ± 0.2 µg/L Microcystin). Eine quantitative Abschätzung der Abundanz der für die Microcystinsynthese verantwortlichen (*mcy*) Genotypen zeigte, dass die durchschnittliche Anzahl der *mcy* Genotypen im L. George (1.14±0.13 %) 17-fach niedriger als im L. Mburo (19.6±4.8%) war. Daraus folgt, dass die Populationen von *Microcystis* in den einzelnen Seen

sich in ihrem Anteil an Microcystin-Genotypen unterscheiden, wodurch die Unterschiede in der Microcystinproduktion pro *Microcystis*-Zelle erklärt werden können.

In der dritten Publikation (Kapitel 4, Okello & Kurmayer, in Vorbereitung zur Einreichung) wurden zunächst die morphologischen Charakteristika der Gattung *Microcystis* für einzelne Kolonien unter Freilandbedingungen mit den morphologischen Charakteristika der einzelnen Isolate unter Kulturbedingungen verglichen. Dabei zeigte sich, dass während in den Freilandproben die Morphotypen *M. aeruginosa*, *M. flos-aquae* und *M. wesenbergii* gefunden wurden, zwar unter Kulturbedingungen lediglich *M. aeruginosa* und *M. flos-aquae* identifiziert wurden, insgesamt jedoch die morphologischen Unterscheidungskriterien verlässlich sind. Nur 13 der insgesamt 96 *Microcystis* Isolate (14%) enthielten die Gene zur Synthese von Microcystin. Weiter wurden die Isolate hinsichtlich ihrer Phycocyanin-Genregion (600 bp) genetisch charakterisiert und in die bestehende Klassifikation für die Gattung *Microcystis* phylogenetisch eingeordnet. Dafür wurde aus insgesamt 128 Sequenz Einträgen in der Genbank-Database ein Kladogramm erstellt und die phylogenetische Zuordnung der insgesamt zwölf in dieser Arbeit isolierten Genotypen in den einzelnen Verwandschaftszweigen analysiert. Die phylogenetische Einordung der Isolate zeigte, dass diese in bestimmten Verwandschaftslinien überrepräsentiert waren (Cluster A, D) während sie in anderen sehr viele Stämme umfassenden Clustern (Cluster F) praktisch fehlten. Aus dieser Arbeit folgt, dass die Identifizierung von *Microcystis* direkt in den Freilandproben anhand verschiedener morphologischer Kriterien ausreichend ist, und selbst im Beisein ähnlicher Taxa wie *Gomphospaeria*, *Synechocystis*, *Chroococcus* eine verlässliche Zellzahlbestimmung durch mikroskopische Auszählung möglich ist. Aus den Publikationen Nr. 1-3 folgt, dass die Auszählung von *Microcystis* im Mikroskop eine sinnvolle Methode darstellt, um relativ schnell anhand der in dieser Studie erhobenen zellulären Microcystin Gehalte die Microcystinkonzentrationen in verschiedenen Gewässern Ugandas abschätzen zu können.

Abstract (English)

Cyanobacteria (blue-green algae) occur in freshwater all over the world and frequently dominate the phytoplankton community. Beside ecophysiological adaptations such as buoyancy due to the synthesis of gas vesicles or the efficient uptake of bicarbonate ions at high pH it is generally believed, that the production of toxins and other bioactive substances contributes to their ecological success. The toxins produced by cyanobacteria include cyclic peptides, most prominently the hepatotoxic microcystins and alkaloids, such as the hepatotoxic cylindrospermopsin or the neurotoxic saxitoxins, anatoxin-a or anatoxin-a(S).

While it is known that cyanobacteria in general play an important role in the saline and freshwater lakes in East Africa, the knowledge about their ability to produce toxins in freshwater in this region is very scarce. This lack of knowledge is of particular relevance, as a significant part of the population in Uganda daily uses the water as drinking water and consumes phytoplanktivorous fish harvested from the freshwater lakes. In order to estimate a potential health risk due to the toxin production by cyanobacteria in those countries, not only a sound estimate of the phytoplankton biovolume in the lakes is needed, but also the understanding, when toxins are produced and which are the factors that favour toxin production in those systems.

It was the aim of the study, to estimate the production of the most widely distributed toxin microcystin seasonally in several large freshwater lakes in Uganda as well as to identify not only the producers of microcystin but also the environmental factors favouring microcystin production. During one year five lakes in Uganda were sampled monthly (Lake Victoria: Murchison Bay, Napoleon Gulf; three shallow lakes within the catchment area of the Ruwenzori: Lake George, Lake Edward, Lake Mburo; one Crater lake: Lake Saka) and limnologically characterized using physical and chemical parameters. In parallel the composition of phytoplankton was determined qualitatively and quantitatively by counting from sedimentation chambers using the inverted microscope technique. The concentration of microcystin was analysed by means of high-performance liquid chromatography (HPLC-DAD). In addition clonal strains of the genus *Microcystis* were isolated from field samples and characterized both genetically and morphologically according to their ability to produce microcystin. PCR based diagnostic approaches were used to identify microcystin-producing genotypes both in field samples and in the laboratory.

In the first publication (chapter 2, Okello et al. 2009, Environmental Toxicology, 16 July 2009) it could be shown, that *Microcystis* is the only genus producing microcystin in Ugandan freshwaters. All PCR based tests revealed gene regions that only could be assigned to *Microcystis* and never to co-occurring other taxa such as *Anabaena* or *Planktolyngbia*. Further it could be shown that eutrophication of freshwater water bodies -either natural or human-induced - always result in dominance of *Microcystis* and therefore ultimately in the production of microcystin. Two structural microcystin variants have been determined that seem to be unique and dominant and have not been described previously.

In the second publication (chapter 3, Okello und Kurmayer, Water Research, submitted) the production of microcystin was analysed in the five freshwater lakes in Uganda during one year (April 2007-April 2008). In all samples microcystins were detected. Corresponding to publication No1 it could be shown that the cell number of *Microcystis* as determined in the microscope correlated significantly with the concentration of microcystin. Other potential microcystin-producing genera such as *Anabaena* or *Planktothrix* also showed partly significant correlations with the microcystin concentration in water. However since no evidence for the presence of microcystin genes that could be assigned to either *Anabaena* or *Planktothrix* was obtained during publication No1 and the correlation coefficients were always much lower when compared with those observed for *Microcystis* it is concluded that the significant correlations observed for *Anabaena* or *Planktothrix* are due to their co-occurrence with *Microcystis*.

Surprisingly the lakes showed a relatively narrow range in microcystin concentrations during the season, while differed significantly (on average up to 28-fold) in microcystin production. Samples from Lake Saka had the maximum MC concentration (10 $\mu g\ L^{-1}$) in July 2007. The minimum concentration (0.02 $\mu g\ L^{-1}$) was recorded in Lake George (Kahendero) in the months of May 07, June 07, January 08 and April 08. The difference in microcystin concentrations between lakes only partly could be explained by the variation in *Microcystis* cell numbers. For example, while on average the maximum *Microcystis* cell concentration was observed in L. George (1.2 ± 0.3 (1 standard error) Mio cells ml^{-1}), on average the microcystin concentrations were at the minimum (0.2 ± 0.1 $\mu g\ L^{-1}$). In contrast, in L. Mburo on average a rather low cell concentration was recorded (0.17 ± 0.02 Mio cells ml^{-1}) while the microcystin concentration on average was significantly higher (1.6 ± 0.2 $\mu g\ L^{-1}$). In order to test the hypothesis that populations of *Microcystis* differ in the proportion of genotypes containing the genes involved in microcystin synthesis (*mcy*) resulting in the significant variation in MC cellular contents between the study lakes, the absolute and relative

abundance of *mcy* genotypes was determined. The average proportion of *mcy* genotypes in L. George (1.14±0.13 (SE)% was 17-fold lower when compared with the average *mcy* genotype proportion in L. Mburo (19.6±4.8%). It is concluded that the variation in average MC content of *Microcystis* that is observed between lakes could be explained by the divergence in *mcy* genotype proportion between populations.

In the third publication (chapter 4, Okello and Kurmayer, in preparation) the morphological characteristics of the genus *Microcystis* in field samples were compared with those observed under culture conditions in the laboratory. While in field samples, the morphotypes *M. aeruginosa*, *M. flos-aquae* and *M. wesenbergii* occurred most frequently, only *M. aeruginosa*, *M. flos-aquae* could be found under culture conditions. However, altogether the morphological characters that were observed under field conditions were found reproducible. In total only 13 of 96 strains (14%) contained the *mcy*E/B gene indicative of microcystin production. In addition twelve isolates were sequenced for the intergenic spacer region of the phycocyanin region (PC-IGS) and assigned to the existing classification system of *Microcystis* by phylogenetic methods. To construct a phylogenetic tree 128 additional entries of *Microcystis* from the Genbank database (602 bp) were compared with the sequences obtained during this study. In general *Microcystis* strains showed a non-random distribution among the phylogenetic lineages, for example while the strains of this study occurred in two clades (cluster A, D) only but were absent from another clade (cluster F) containing the largest number of strains. It is concluded that the identification of *Microcystis* according to morphological criteria is reliable, and even in the presence of morphologically similar genera such as *Gomphospaeria*, *Synechocystis*, *Chroococcus* can be used to estimate *Microcystis* cell numbers under the microscope. Altogether from publications No1-3 it is concluded, that microscopical counting of *Microcystis* is a reliable tool, in order to provide a relatively quick and simple estimate of microcystin concentrations by multiplying the average cell numbers by the microcystin cell quotas as determined in this study.

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Dr. Gerhard Frena, my Austrian general medical practitioner routinely carried out check up that boosted my mental fitness to see my through sometimes harsh climate. At one incidence, during springtime not in the reality of cold winter, my *Eosinophil* counts went far above the normal range and Doctor suspected cases of either parasitic infection or allergy. After screening, he told me that "You are too healthy for me to prescribe any medication; you are just allergic to probably some pollen since many plants were blooming". That was the treatment I took.

To all my friends, I am grateful for the emotional support you gave to me. Being a social mammal as I am, when I can create some time, I like being with friends. You are that many but count yourself in there. Those jokes we cracked and funs we made to enlighten our positive thinking, must continue more often after this work in whichever part of the world we

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Lastly, I thank our family for the inspiration, sense of responsibility and encouragement they accorded to me while far away from home. Much as we miss the presence of all our parents now, we thank God who enabled them to rekindle the brighter candles that are burning in our souls. We should continue to take out the candles and go light up the world. Moreover, in the world, there are yet too many chances behind the mountains, with many treasures waiting for the young ones to discover as long as your feet are able to cross many rivers.

Table of Contents

List of Acronyms and Abbreviations

Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
Chl *a*	chlorophyll *a*
CID	collision-induced dissociation
DHB	2,5-dihydroxy-benzoic acid matrix solution
DNA	deoxyribonucleic acid
fg $cell^{-1}$	femto gram per cell
GF/C	glass fibres filter type C
HPLC-DAD	higher performance liquid chromatography with diode array detection
ITS	internal transcribed spacer
LC-MS	liquid chromatography mass spectrometry
LVEMP	Lake Victoria Environmental Management Project
MALDI-TOF-MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MC	microcystin
MC-LR	leucine and arginine in the positions of X and Z of microcystin
MC-RR	arginine and arginine in the positions of X and Z of microcystin
MC-YR	tyrosine and arginine the positions of X and Z of microcystin
mcy	microcystin synthetase gene
M+H	microcystin molecular weight
$mm^3 L^{-1}$	millimetre cube per litre
NH_4-N	ammonia-nitrogen
NO_3-N	nitrate-nitrogen
PC	phycocyanin
PC-IGS	phycocyanin - intergenic spacer
PCR	polymerase chain reaction
PO_4-P	phosphate-phosphorus
PSD	post source decay fragment analysis
RFLP	restriction fragment length polymorphism

SRP	soluble reactive phosphorus
SRSi	soluble reactive silica
UNESCO	United Nations Educational, Scientific and Cultural Organization
v/v	volume to volume
WHO	World Health Organization
µg	microgram
µg L^{-1}	microgram per litre
µg MC-LR eq.	microgram MC-LR equivalent
µL	micro litre
µm	micrometer

Chapter 1

Introduction

Far and away the best prize that life offer is the chance to work hard at work worth doing

(Theodore Roosevelt 1858-1919)

Introduction

The thesis

This thesis outlines the results of a doctoral study performed on freshwater habitats in Uganda. The research aims at generating information that will advance the knowledge on toxic cyanobacteria in Ugandan freshwater habitats. It begins with an introduction (this chapter) presenting the background information of freshwater in Uganda, cyanobacteria, microcystin production and introduces the objectives of and motivation for the study. Chapters 2 - 4 describe in detail the research carried out and its original outcomes. Chapter 5 presents a summary of the results from the previous chapters (2 - 4) and their discussions.

The Chapters 2 - 4 in which detailed finding are reported are written in form of manuscripts either accepted, submitted or in preparation for publication in international referred journals.

General background on Ugandan freshwater bodies, cyanobacteria and toxin production

In the 1960s, research on cyanotoxins started in small institutions in the temperate climatic region (USA, Europe, Japan) and Australia. With increasing eutrophication and occurrence of cyanobacteria, it extended and became a research field on its own (Chorus, 2005). To date, many of the lakes within the temperate regions have been studied and the levels of microcystin variations and variants have been documented (Rinehart et al., 1994; Hotto et al., 2005). In the tropical countries however, where lake productivity is highest with occasional cyanobacterial dominance and increasing eutrophication, not much is on record including those from Uganda.

Uganda, situated in mid-eastern Africa, astride the equator between latitude 1°30'S and 4°N and longitude 29°30'E and 35°E has rivers, lakes and wetlands that cover about 18% of its 241,000 km^2 total surface area. Uganda freshwater resources are considered a key strategic resource, which is vital for sustaining life, promoting development and maintaining the environment.

The country heavily depends on its water resources for most of its socio-economic development activities. These include among others: Domestic Water Supply, Livestock Water Supply, Industrial Water Supply, Hydropower generation, Agriculture, Marine transport, Fisheries, Waste discharge, Tourism and Environmental Conservation. In order to satisfy all the above water demands in a sustainable manner, the government has adopted a holistic approach to water resources management based on internationally recognised Integrated Water Resources Management principles (World Water Assessment Programme, UNESCO, 2006).

Approximately 16% of Uganda total surface area is covered by open freshwater bodies comprising of five major lakes and, numerous small and satellite lakes. Prominent on the list is Lake Victoria, the second world largest lake which stretches through Victoria Nile into Lake Kyoga. Lake Kyoga then drains into L. Albert through the Kyoga Nile. From Lake Albert, River Semiliki joins the Nile and drains Lake George and Lake Edward found in the rift valley and strong rainfalls area of the Ruwenzori mountains. Lake George and Lake Edward are connected through Kazinga Channel (World Water Assessment Programme, UNESCO, 2006).

Despite Uganda's being well-endowed with freshwater resources, some of these water bodies are either naturally eutrophic e.g. Lake George (Viner and Smith, 1973), or are becoming increasingly eutrophic due to human activities such as Lake Victoria (Mugidde, 1993, 2001; Kling et al., 2001;Verschuren et al., 2001; Oguttu et al., 2008). All these authors documented eutrophication processes particularly in Lake Victoria leading to increased biomass of phytoplankton dominated by Cyanobacteria.

Cyanobacteria are members of the Procaryotae kingdom in the division of Gracilicutes (bacteria with a gram-negative cell wall) assigned to Photobacteria class and the subclass Oxyphotobacteriae, which embraces them as Cyanobacteriales (Rippka, 1998). Cyanobacteria are known for the important insights they have provided into the origin of life and into the origin of organelles in the cells of higher organisms. According to Carmichael (1994), fossil records showed the existence of cyanobacteria 3.3 to 3.5 billion years ago and cyanobacteria in particular played a major role in oxygenation of the air, which then made other aerobic organisms to start evolving. In addition, they provide an extra ordinarily wide range of contribution to human affairs in everyday life that are both useful and harmful. They are primary producers with high nutritive value. Some people in Chad and Mexico are known to have consumed *Spirulina* for centuries (Carmichael, 1994). The nitrogen-fixing species contribute globally to soil and water fertility (Chorus & Bartram, 1999).

On the other hand, abundant growth of cyanobacteria in water reservoirs creates water quality problems with adverse implications for water suppliers, biodiversity and aquatic productivity. The development of strains producing toxins is a common experience in eutrophic inland water all over the world.

Cyanobacteria show an impressive diversity of genotypes which produce different bioactive oligopeptides (homo). For example most prominently the microcystins (Fig. 1.1), cylindrospermopsin and other toxins such as anatoxin-a (S) and saxitoxins. The microcystin and the saxitoxin can be further subdivided into isoforms, which share a common backbone but differ at variable positions.

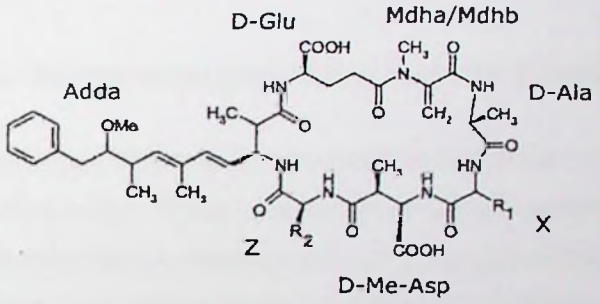

Fig. 1.1. The molecular structure of microcystins the variable L-amino acid residues at positions R_1 and R_2 are indicated by X and Z, respectively.

It is tempting to speculate the ecological success of cyanobacteria to be observed worldwide is at least partially because of producing bioactive compounds. A few examples on the biological role of the production of bioactive compounds have been described including mainly chemical defence against potential antagonists (Skulberg 2000, Burja et al. 2001).

For humans and vertebrates toxicity of microcystins is mediated through the active transport of MC into hepatocytes by the bile acid organic anion transport. This is followed by inhibiting eukaryotic serine/threonine protein phosphatases 1 and 2A (Kuiper-Goodman et al., 1999). After ingestion of MC-containing cyanobacteria, acute poisoning leading to death from massive hepatic hemorrhage has been reported in animals (Mez et al., 1997). A few incidents of human illness have been charged to MCs in drinking water or at recreational sites (Kuiper-Goodman et al., 1999). Uptake of sublethal doses has been further epidemiologically linked to primary liver cancer and colorectal cancer in humans (Zhou et al., 2002). Thus, for microcystins (and a few other potent toxins) the WHO has set up

guideline values to guarantee the safe use of drinking water (Codd et al., 2005). Similar to MC, cylindrospermopsin is a hepatotoxin causing severe hepatic haemorrhage in mammals. Other toxins such as (homo), anatoxin-a, anatoxin-a (S) and saxitoxin have been shown to inhibit the propagation of neuronal impulses. Saxitoxin blocks the transport of sodium ions (Na^{2+}) into the neuronal cell. Anatoxin-a inhibits the acetylcholine esterase thereby degrading acetylcholine while anatoxin-a (S) mimics acetylcholine. While saxitoxin result in the interruption in pulse propagation between neuronal cells, both anatoxin-a and anatoxin-a (S) tend to overstimulation and finally exhaustion. The symptoms in mammals include respiratory arrest (Carmichael, 1994).

Molecular characterisation of microcystin-producing cyanobacteria

The genotypes differing in cyanopeptide composition cannot be distinguished visually, that is by microscopy. Water samples often contain individual cells or colonies that show the same morphological characteristics, but contain different cyanotoxins. However, new molecular tools and refined chemical methods now distinguish these genotypes producing different toxins to enable studies of their biodiversity. There are notable scientific progresses made to explain the synthesis of hepatotoxic heptapeptides, the so-called microcystins.

The total gene cluster involved in the MC synthesis has been sequenced from *Microcystis* (Tillett et al., 2000), *Planktothrix* (Christiansen et al., 2003) and *Anabaena* (Rouhianinen et al., 2004). Thus, genotypes can now be discriminated and quantified on the molecular basis of toxin production in the laboratory and in the field (Kurmayer et al., 2002).

As Börner and Dittmann (2005) explained, the *mcy* gene cluster of *M. aeruginosa* spans about 55 kb of the chromosomal DNA and comprises 10 genes embedded in two bidirectionally transcribed operons (Tillett et al., 2000; Kaebernick et al., 2002). Downstream of the promoter region, *mcy*A-C encode three NRPS comprising 5 modules, whereas upstream *mcy*D-J encode polyketide synthases (*mcy*D), hybrid enzymes (*mcy*E, G) and additional tailoring enzymes (*mcy*F, I, J) as well as a component of a putative ABC transporter (*mcy*H) (Fig. 1.2). Taking into account that the multi-enzyme components of the *mcy* complex comprise the minimal set of domains needed for the assembly of seven amino acids and four acetate unit plus additional integrated domains with epimerase, methyltransferase and aminotransferase activities, the enzymes encoded by the *mcy* gene

cluster are thought to catalyse 45 out of 48 sequential steps of MC biosynthesis (Tillett et al., 2000).

The Adda-D-Glu precursor is synthesised by the activities of at least four enzymes, *Mcy*G, J, D and E. *Mcy*G is a hybrid enzyme that combines an unusual NRPS adenylation domain with similarity to Acyl-CoA ligases with a typical PKS elongation module. *Mcy*G presumably starts MC synthesis by activation of phenylacetate followed by transfer to the 4-phosphopantetheine of the first carrier domain. Subsequently, Adda is formed by the four PKS modules of *Mcy*G, D and E. *Mcy*J is needed for an O-methylation step. *Mcy*E is a hybrid protein composed of PKS and NRPS modules. An integrated domain with similarity to glutamate semialdehyde aminotransferases most likely provides the β-amino group of the Adda moiety. Finally, the first condensation domain of *Mcy*E condenses Adda with the activated glutamate thereby linking the PKS with the NRPS part of microcystin biosynthesis. The NRPS modules of *Mcy*A, B and C activate the remaining five amino acids and incorporate them into the growing peptide structure. It has been proposed that the TE domain of *Mcy*C (most probably together with a separate enzyme; Christiansen et al., 2003) is responsible for cyclisation and release of microcystin from the synthetase complex. *Mcy*F is a racemase though to provide D-aspartate and most likely also D-methyl-aspartate (Sielaff et al., 2003). Recently *Mcy*I has been shown to be involved in the methylation of the aspartate residue (Pearson et al., 2007). Since *Mcy*H shows significant similarity to exporters of the ABC transporter family it might play a role in MC export (Pearson et al., 2004).

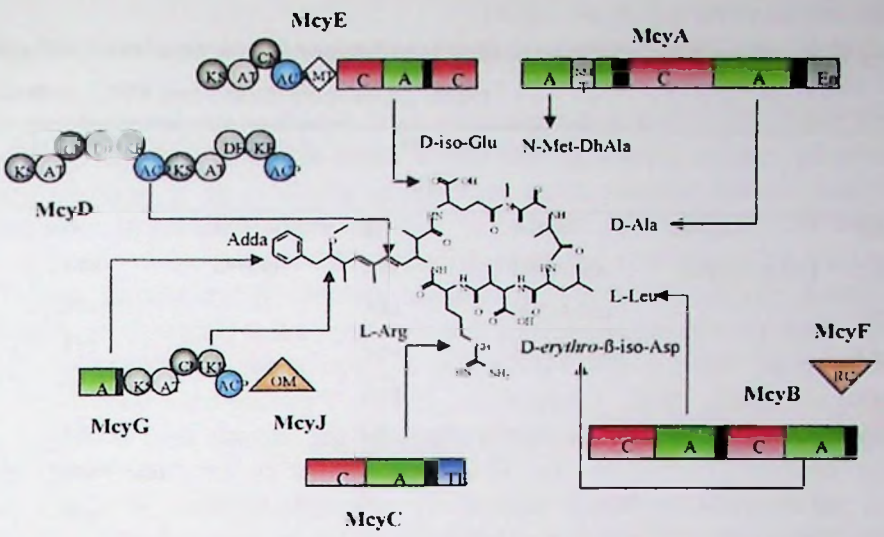

Fig. 1.2. The role of components of the microcystin synthetase (*mcy* proteins) extracted from Börner & Dittmann, (2005). Polyketide synthetases (PKS) domains: AT: acyltransferase; ACP: acyl carrier protein; KS: ß-ketoacyl synthase, KR: ketoacyl reductase; DH: dehydratase; CM: C-methyltransferase; AMT: aminotransferase; Non-ribosomal peptide synthetases (NRPS): A: aminoacyl adenylation; C: condensation; NMT: N-methyltransferase; Ep: epimerase, TE: thioesterase; *Mcy*F: racemase, OM (*Mcy*J): O-methyltransferase. Black bars represent the thiolation motif of NRPS modules. Arrows indicate the assignment of individual proteins to steps of microcystin biosynthesis.

Chemical characterisation of microcystin producing cyanobacteria

More than 65 structural variants of MC have been described. According to Sivonen and Jones (1999), usually individual strains produce more than one microcystin variant. Most of this variation is due to differences in individual amino acid positions 2 and 4 of the MC molecule normally represented in structure as X and Z, whereas the polyketide side chain of the Adda moiety is mostly conserved (Table 1.1). For example, MC-LR refers to leucine and arginine at position 2 and 4, respectively.

Table 1.1. Microcystin structural variability of individual amino acids produced by cyanobacteria (Rinehart et al., 1994 and Christiansen et al., 2003)

Structure Cyclo-						
(D-Ala[1]-	X[2]	D-MeAsp[3]-	Z[4]	Adda[5]	D-Glu[6]-	Mdha[7])
D-Ser	Leu	D-Asp	Ala	DMAdda	D-Glu(OCH_3)	Dha
D-Leu	Ala		Aba	(6Z)Adda		Ser
	Tyr		Leu	ADMAdda		Dhb
	Glu		Arg			MeSer
	Val		Glu			MeLan
	Glu(OMe)		Glu(OMe)			
	Arg					
	Phe					
	Met(O)					
	HPhe					
	HTyr					
	Trp					
	HArg					
	Hil					
	H_4Y					

Abbreviations: Aba: Amino-isobutyric acid; ADMAdda: O-acetyl-O-demethylAdda; Dha: Dehydroalanine; Dhb: Dehydrobutyric acid; DMAdda: O-DemethylAdda; E(Ome): Glutamatmethylester; HArg: Homoarginine; Hil: Homoisoleucine; HPhe: Homophenylalanine; HTyr: Homotyrosine; $(H_4)Y$: 1,2,3,4-tetrahydro-tyrosine; MLan: N-Methyllanthionine; MeSer: N-Methylserine; M(O): Methionin-S-oxide; (6Z)-Adda: Stereoisomer of Adda at the Δ^6 double bond.

Among chemical methods sensitive matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) and LC-MS methods have contributed significantly to the differentiation of cyanobacterial genotypes based on the toxins they produce (Erhard et al., 1997; Harada et al., 2004). With MALDI, it is possible to analyze single colonies or filaments of cyanobacteria isolated from water samples (Fastner et al., 2001; Kurmayer et al., 2004) and to identify toxins based on molecular weight and post source decay (PSD) fragmentation patterns. The high sensitivity coupled with the easy-to-use sample preparation makes MALDI a useful tool for field application. With LC-MS, it is possible to identify and quantify toxins according to their retention time and protonated mass. HPLC-DAD methods rely on retention time and the information obtained from the

UV spectrum. The microcystins have an extraordinary maximal absorption at 240 nm (due to the Adda side chain) which makes them unambiguous even when analysed in extracts from field samples containing plenty of unknown compounds.

The present study

Problem statement

During the Master of Science studies (Okello, 2004), it was found that many of the Ugandan water bodies had a high abundance of potentially MC producing cyanobacteria, mostly *Microcystis* and *Anabaena* (Lake George-Kahendero = 75 mm^3L^{-1}, Lake Mburo = 80 mm^3L^{-1}, Lake Edward-Katwe = 60 mm^3L^{-1} and Lake Victoria-Murchison Bay = 75 mm^3L^{-1}). Interestingly, the microcystin concentrations measured by HPLC and LC-MS were low, that is most field samples had no MCs and in only 5 out of 45 samples MCs were detected, i.e. 80-410 $\mu g\ mg^{-1}$ of dry weight (Okello, 2004). Ballot et al. (2005) made similar findings in two saline lakes in Kenya. In contrast, the median MC content for cyanobacterial samples dominated by *Microcystis* in Germany was 770 $\mu g\ g^{-1}$ DW (min 21- max 2736) and 98% of all samples contained MCs (Fastner et al., 1999). On the other hand most samples were found to be toxic, i.e. from the 45 extracts got from field samples, 19 were toxic as revealed by a sensitive bioassay using *Thamnocephalus platyurus* (Okello, 2004). The toxins present in these samples have not been identified. These results show that:

(i) The abundance of microcystin-producing genotypes and/or the regulation of microcystin production in Uganda may differ from the situation in Europe and

(ii) Other toxic peptides may be more abundant and contribute to the noted toxicity.

Biogeography of toxin production has recently been suggested for another toxin producing cyanobacterium *Cylindrospermopsis raciborskii*. For example, the toxic alkaloid cylindrospermopsin has been only reported from Australian and S. American *Cylindrospermopsis* strains but not from strains isolated from Germany (Neilan et al., 2003). So far, possible biogeographic patterns in toxin production in different climatic regions of the world have not been considered. This issue is relevant for the application of guideline values and risk assessment strategies to freshwater resources in developing countries.

The Lake Victoria region and its catchments in East Africa have undergone eutrophication over the last three decades (Verschuren et al., 2001). Based on microscopical counting of phytoplankton, increased cyanobacterial abundances were reported (Ochumba

& Kibaara, 1989; Hecky, 1993; Lungaiya et al., 2000; Kling et al., 2001 and Mugidde et al., 2003). In 1984, massive fish kills were observed in the Nyanza Gulf of Lake Victoria, Kenya, which coincided with the occurrence of cyanobacteria (Ochumba, 1990). By then, there was no identification of the MCs made. Reports in the Uganda national media reported the repetitive occurrence of cyanobacterial blooms throughout many parts of the northern Lake Victoria (Alweny, 2007). These blooms are a cause of concern to local water consumers, policy makers, as well as National Water and Sewerage Cooperation (NW&SC) that supplies treated water nationally.

Objectives

The aim of the study is to characterize potentially microcystin-producing cyanobacteria in Ugandan freshwater from a genetic and chemical perspective. The combination of both approaches will give a first comprehensive invaluable insight into toxin production in Ugandan freshwater.

1. *To identify and quantify toxic peptide diversity in relation to environmental factors (eutrophication, temperature and seasons).*

During the Master of Science thesis (Okello, 2004), various water bodies ranging from oligotrophic to hypertrophic conditions were found to inhabit cyanobacteria in samples taken between May and June 2004. Eutrophication has been shown to favour cyanobacteria in general; however, it is unknown whether MC producers are favoured with eutrophication or inactive MC genotypes increases in relative numbers. Data on the seasonal abundance of cyanobacteria in these water bodies as well as on microcystin production are not available. However, seasonality has been reported from the Lake Victoria (Talling, 1966; Akiyama et al., 1977) region and it is hypothesized that:

(i) Seasonality in phytoplankton and microcystin production can be observed in the tropical freshwater habitats explored in this study and

(ii) The number of genotypes producing microcystin depends on environmental conditions.

To quantify the abundance of microcystin producers and distinguish between active and inactive microcystin genotypes

As discussed above the MC concentration in Ugandan field samples dominated by *Microcystis* and/or *Anabaena* has been found to be low. It is unknown whether those low concentrations are due to the low abundance of MC-producing genotypes or a high abundance of inactive MC genotypes that is genotypes with MC genes but without detectable MC (i.e. Kurmayer et al., 2004). The genetic ability to produce MC is considered to be an old feature, i.e. all MC producing genotypes had a common MC ancestor about 2 billion years ago (Rantala et al., 2004). According to this hypothesis, genotypes without MC genes may have lost the corresponding genes and the process of MC gene loss may be accelerated at warmer temperatures with faster growth rates in the tropical region when compared to the temperate climatic zone. Consequently, it is hypothesized that the proportion of genotypes lacking the MC genes is significantly lower in the tropical region when compared with the temperate climatic region.

3. *Geographical differences between the temperate and the tropical climatic region in producing toxins*

Biogeographical patterns in producing bioactive peptides have been recognized only recently. A few studies have shown that MC produced in Finland differs structurally from the MCs produced more in the southern countries (Via-Ordorika et al., 2004). In Austria, populations of cyanobacteria occurring in individual lakes have been found that are characterized by MC variants that seem unique and dominant (Kurmayer et al., 2004). Those results show that populations of cyanobacteria may diverge in toxic genotype composition, for example because of geographical isolation (Papke and Ward, 2004). It is hypothesized that cyanobacteria in the tropical Africa not only differ from the temperate Europe with regard to the phytoplankton species producing MCs but also with regard to the MC structural variants.

Table 1.2. An outline of the research

Chapter	Specific objectives	Results and achievements
Chapter 2 Occurrence of microcystin-producing cyanobacteria in Ugandan freshwater habitats	1. To determine the environmental parameters that defines the trophic state of a given freshwater habitat. Estimate the phytoplankton species composition and abundance. 2. Assessing the microcystin concentrations on each site Performing PCR analyses using specific primers to identify all the potential microcystin-producing cyanobacteria.	1. Trophic status played a crucial role in the occurrence of microcystin-producing cyanobacteria. 2. Where there are blooms of *Microcystis*, there will be a potential for microcystin production. 3. Identification of *Microcystis* as the sole microcystin-producing organism.
Chapter 3 Seasonal development of cyanobacteria and microcystin production in five Ugandan freshwater lakes	1. To test the hypothesis that seasonality in the tropical freshwater habitats influences the chemical, physical and biological factors which in turns affects the developments of microcystin-producing cyanobacteria. 2. To improve the understanding of seasonal variability in microcystin concentration in Ugandan freshwater bodies and to indicate the dominant microcystin variant.	1. Eutrophication has been shown to favour cyanobacteria in general. 2. Data on the yearly seasonal abundance of cyanobacteria in Ugandan freshwater bodies as well as on microcystin production have been generated. 3. Significant linear correlations between *Microcystis* cell numbers and microcystin concentrations. 4. Between lakes the populations of *Microcystis* differ qualitatively and quantitatively in microcystin production due to genetic divergence.
Chapter 4 Morphological and genotypic variation of microcystin-	1. To characterise *Microcystis* morphologically both in the field	1. Four *Microcystis* sp. (*M. aeruginosa*, *M. botrys*, *M. flos-*

oducing cyanobacterium *licrocystis* in Uganda	and under culture conditions. 2. To characterise *Microcystis* genetically within a taxononomic marker gene and within two genes of the *mcy* gene clusters of *Microcystis* 3. To determine MC production from the *mcy* gene containing strains.	*aquae* and *M. wesenbergii*) identified from field samples. Only *M. aeruginosa* and *M. flos-aquae* were characterised from strains. 2. Ten strains out of 96 contained the *mcy* genes and were MC-producers. The MC variants fell into three groups and the newly discovered MC-RY dominated among the strains similar to field samples.

Originality

This thesis provides an insight into microcystin production and the ecology of harmful algal blooms in Uganda by:

a) Investigating microcystin production in a climatic region that has been so far unexplored (Codd et al., 2005).
b) Identification of MC-producing genotypes by state of the art technology in molecular, biological and chemical analyses.
c) Discovering two novel and abundant microcystin structural variants thus adding new perspectives to the global diversity of microcystin.
d) Performing a year round field study resulting in the compilation of a database characterising the limnology of the studied lakes.
e) Characterising the morphological variability of *Microcystis* in the studied lakes.

Applied aspects

This research is imperative in that all the sites chosen acts as drinking water supply to the surrounding local communities as well as the fish production system for Uganda. Improved knowledge on toxin production and the distribution of toxin producing cyanobacteria in these regions could therefore help to analyze the human health aspects

associated with the general increase of cyanobacteria because of the anthropogenic induced environmental changes. A lot remains unknown to the local and scientific communities as has been expressed in many national media. Two examples are provided here:

Kampala's green water may be toxic

1. Daily Monitor publication (Uganda national newspaper): August 9th , 2007

"Following the press reports about the quality of water and the public outcry, I would like to throw more light particularly on the green discolouration of water as reported and illustrated in Daily Monitor, August 2nd , 2007."

"The green effect is due to the presence of blue-green algae, which are bacteria and not fungi.... Characteristically they cause water discolouration ranging from yellowish-brown to blue-green, commonly termed as surface scum or a surface water bloom. There is no doubt a number of these blue-green algae are harmless to man or his animals. However, a subgroup known as *Microcystis* produce toxins and frequently release the poison into the water.

The poisons cause three side effects on human beings: skin allergy manisfested in skin irritation, a poisonous effect to the liver (hepatotoxicity) especially in children and poisoning of the nervous system.

The presence of *Microcystis* in our waters may be special public health concern because these blue-green algae are known to have tumour-promoting activity in the liver. More than 40 variants have been reported with varying toxicities.

Although little, (to my knowledge) none has been investigated in Uganda waters. Studies from elsewhere have shown a very close correlation between the incidence of primary liver cancer and microcystin content in drinking water.

This is of concern to our population when the variants of blue-green algae in our drinking water are unknown. National Water and Sewage Cooperation needs to clarify the following:

- Among the recommendations of drinking water is the maximum acceptable concentration of microcystins of 0.0015 mg/L in other countries. What is the acceptable limit in the drinking water in Uganda?

- What is the concentration of microcystins in the green water we are drinking today? Who knows, it may be that some of the incidents of liver cancers we are getting today are partly due to blue-green algae poisoning.

Otherwise the green water should be considered a human health hazard and unfit for consumption under the WHO recommendations."

Cancer risk from L. Victoria

2. Daily Monitor publication (Uganda national newspaper): September 27th , 2007

"LAKE Victoria is infested with bacteria that can cause liver cancer and diarrhea, environment experts have revealed. But one is only susceptible to the irritant diseases if they drink untreated lake waters. The special group of bacteria, the experts discovered, is scientifically called *Microcystis*....

...We have a higher amount of *Microcystis* but a few of them are producing toxins," said Mr. William Okello, a limnologist at the National Fisheries Resources Research Institute (NaFIRRI). The discovery, Daily Monitor has learnt, is a result of months of investigations by NaFIRRI. A report of the findings is yet to be published...

...This is not yet a conclusive thing because we are still carrying out the study. We will characterise them to find out which ones are active and which are inactive. The experts from NaFIRRI are investigating all classes of algae, which include cyanobacteria and the subgroups, present in water bodies in Uganda, as part of their mandate in understanding the productivity of algae in the water systems. Only recently, the algae turned piped water from Lake Victoria green, triggering panic."

The outcome of this study will therefore be used in the promotion of awareness to the local communities, scientist and policy makers. It could also be applied in guidance for designing water safety plans for Uganda to achieve the UN millennium development goals. The database generated will be made available to various institutions that include: Makerere University; Ministry of Water and Environment; Directorate of Water Development; National Environment Management Authority; National Water and Sewage Cooperation; Lake Victoria Basin Commission; Lake Victoria Management Authority; Uganda National Council for Science and Technology and Uganda Wildlife Authority.

Study area

In the first studies that form Chapter 2, twelve different sites where chosen as indicated in the map. However, after identifying the microcystin producers and most probable sites that might contain them all year round, other sites were dropped and only five remained. A new crater lake (Saka) was added after interview with the local community and a literature search and it was found to offer comparable ecological conditions suitable for

cyanobacterial proliferation (Chapter 2). Chapter 2 also contains a separate map showing the site locations where samples were repetitively taken monthly for one year.

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Chapter 2

Occurrence of microcystin-producing cyanobacteria in Ugandan freshwater habitats

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Occurrence of microcystin-producing cyanobacteria in Ugandan freshwater habitats

Abstract

Microcystins (MCs) are cyclic heptapeptides, which are the most abundant toxins produced by cyanobacteria in freshwater. The phytoplankton of many freshwater lakes in Eastern Africa is dominated by cyanobacteria. Less is known, however, on the occurrence of MC producers and the production of MCs. Twelve Ugandan freshwater habitats ranging from mesotrophic to hypertrophic conditions were sampled in May and June of 2004 and April of 2008 and were analyzed for their physicochemical parameters, phytoplankton composition, and MC concentrations. Among the group of the potential MC-producing cyanobacteria, *Anabaena* (0-10^7 cells ml^{-1}) and *Microcystis* (10^3-10^7 cells ml^{-1}) occurred most frequently and dominated in eutrophic systems. A significant linear relationship ($n = 31$, $r^2 = 0.38$, $P < 0.001$) between the *Microcystis* cell numbers and MC concentration (1.3-93 fg of MC $cell^{-1}$) was observed. Besides [$MeAsp^3$, $Mdha^7$]-MC-RR, two new MCs, [Asp^3]-MC-RY and [$MeAsp^3$]-MC-RY, were isolated and their constitution was assigned by LC-MS^2. To identify the MC-producing organism in the water samples, (i) the conserved aminotransferase domain part of the *mcy*E gene that is indicative of MC production was amplified by general primers and cloned and sequenced, and (ii) genus-specific primers were used to amplify the *mcy*E gene of the genera *Microcystis*, *Anabaena*, and *Planktothrix*. Only *mcy*E genotypes that are indicative of *Microcystis* sp. were obtained via the environmental cloning approach (337 bp, 96.1-96.7% similarity to the *Microcystis aeruginosa* strain PCC7806). Accordingly, only the *mcy*E primers, which are specific for *Microcystis*, revealed PCR products. We concluded that *Microcystis* is the major MC-producer in Ugandan freshwater.

Keywords: eutrophication; *Anabaena*; *Microcystis*; toxicity; genetic diversity; *mcy*E; *mcy*B gene; microcystin-RY

Introduction

Uganda, with a total area of 241 000 km^2 astride the equator between latitude 1°30′S and 4°N and longitude 29°30′E and 35°E, has a significant amount of freshwater resources covering ~16% of its surface area (World Water Assessment Programme, UNESCO, 2006). Some of these freshwater bodies are either naturally eutrophic, e.g. L. George (Viner and Smith, 1973), or are becoming increasingly eutrophic due to human activities, e.g. L. Victoria (Verschuren et al., 2001). In general, cyanobacteria have often been found to increase under eutrophic conditions.

Blooms formed by cyanobacteria have recently been reported throughout many parts of L. Victoria (Alweny, 2007). These blooms are a cause of concern to local water consumers, policy makers, as well as the National Water and Sewerage Cooperation (NW&SC) that supplies treated water nationally. In their statement to the Ugandan parliament, NW&SC indicated that 11 billion Ugandan shillings (7 million US dollars) would be required in 2008 to treat green water. The fishermen reported that areas covered by cyanobacteria accumulating on the surface could not be used for fishing anymore. Small dead fish were also seen floating close to the shoreline, while the larger fish caught within the area appeared weak and stressed. In East Africa, massive fish kills were observed in the Nyanza Gulf of Lake Victoria (Kenya) in 1984, coinciding with the occurrence of cyanobacterial blooms (Ochumba, 1990). The toxins produced by cyanobacteria include neurotoxins, hepatotoxins, and irritant-dermal toxins. All of these cause acute harm to humans, animals, and wildlife after exposure (Chorus et al., 2000; Carmichael, 2001). The most widely distributed hepatotoxins in freshwater are the microcystins (MCs). They are produced by the planktonic genera *Anabaena*, *Anabaenopsis*, *Microcystis*, *Nostoc*, and *Planktothrix* (Sivonen and Jones, 1999). MCs are cyclic heptapeptides and 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-erythroß-iso-methyl-aspartic acid, Adda is (2S, 3S, 8S, 9S)-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 at positions 2, 4, and 7 of the molecule resulting in over 60 structural variants that are characterized from field samples or isolated strains (Diehnelt et al., 2006). The elucidation and characterization of the gene cluster that is involved in MC synthesis significantly increased our understanding of the regulation of toxin synthesis in cyanobacteria (Dittmann et al., 1997; Tillett et al., 2000). It was only then that the investigation of the genetic

basis of MC production both in the laboratory and in the environment became possible. Considering the repetitive occurrence of algal blooms reported in Ugandan lakes, understanding the distribution of toxin-producing cyanobacteria in Ugandan freshwaters is essential. This will form the basis of estimating the health risks associated with cyanobacterial occurrence. A previous study reported the isolation of four MC-producing *Microcystis* sp. strains from Kenya and Uganda (Haande et al., 2007). Since it is generally known that the bias introduced by strain isolation may be substantial (Wilson et al., 2005), we believe that field studies are required to provide the information that is necessary to estimate a potential health risk. This article reports the first results on the occurrence of MC-producing cyanobacteria in relation to the environmental conditions and the production of MC in twelve Ugandan freshwaters ranging from mesotrophic to hypertrophic conditions.

Materials and methods

Sampling and Nutrient Analyses

Depth integrated water samples were taken in May and June 2004 from twelve freshwater bodies (site nos. 1-12, Table 2.1, Fig. 2.1) using a 2-L horizontal van Dorn sampler by sampling the water column every meter. To confirm the results obtained during this period, the site nos. 4, 5, 7, 10, and 11 were resampled in April 2008. In parallel, algae were concentrated by taking vertical hauls with a phytoplankton net (30 µm mesh size). Samples were filtered using Whatman GF/C filters. For chlorophyll a analysis, filters were stored frozen (-20°C). For MC and DNA analysis, filters were dried overnight (50°C) and stored frozen (-20°C). The filtrates were subsequently filtered through membrane filters (0.45 µm) for the analyses of dissolved nutrients. Soluble reactive phosphorus (SRP), nitrate (NO3-N), and ammonia (NH4-N) were determined by using the ammonium molybdate method (Wetzel and Likens, 2000), sodium-salycilate method (Müller and Wiedemann, 1955), and indophenol blue method (Krom, 1982), respectively. Soluble reactive silica was determined as yellow molybdate-silicic acid (Wetzel and Likens, 2000). The total phosphorus (TP) was determined by persulphate digestion from the aliquots of the samples and analyzed as SRP. The study (EC650) was approved by the Uganda National Council for Science and Technology in conjunction with the Uganda Wildlife Authority.

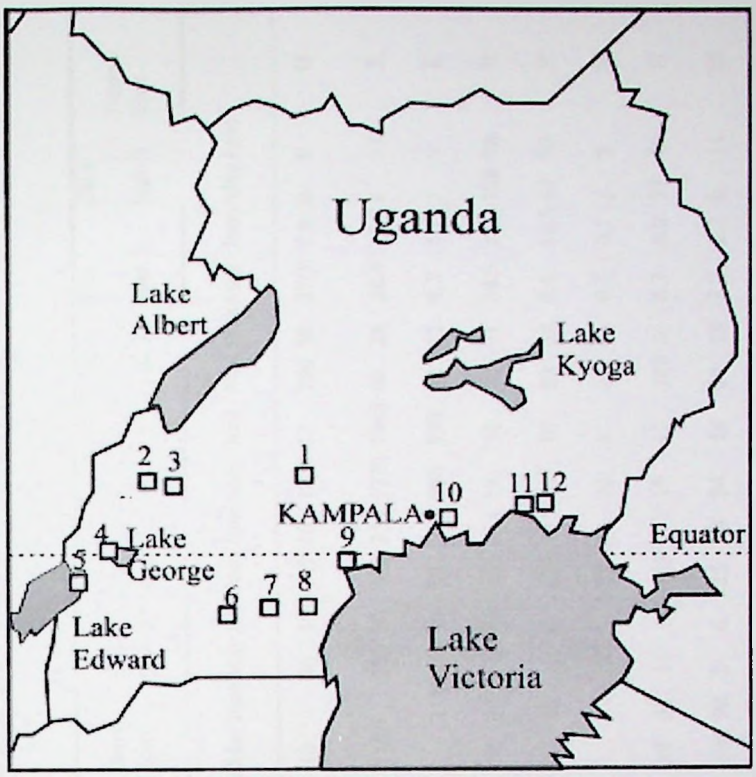

Fig. 2.1. Location of the study area and sampling sites in Uganda. Numbering of the sampling sites as in Table 2.1.

Lake Trophic States Definition

The trophic state was assigned according to Vollenweider and Kerekes (1982): Mesotrophic: TP (10-35 μg L^{-1}), chlorophyll a (3-8 μg L^{-1}), secchi disc depth (3-1.5 m); eutrophic: TP (35-100 μg L^{-1}), chlorophyll a (8-25 μg L^{-1}), secchi disc depth (1.5-0.7 m); hypertrophic: TP (≥100 μg L-1), chlorophyll a (≥25 μg L^{-1}), secchi disc depth (≤0.7 m).

Table 2.1. Environmental parameters in Ugandan freshwaters sampled during May and June 2008. Site, [illegible] silica, NH_4-N, ammonia nitrogen, NO_3-N, and nitrate nitrogen.

Site	Site No	Coordinates		Area (km^2)	depth (m)	Temp (°C)		Conductivity ($\mu S\ cm^{-1}$)		pH		Secchi (cm)		SRP (μgL^{-1})		TP (μgL^{-1})		NH_4-N (μgL^{-1})		NO_3-N (μgL^{-1})		SRSi (mgL^{-1})		Chl a (μgL^{-1})		Trophic State
		Latitude	Longitude			May	June	May	June	May	June	May	June	May	June	May	June	May	June	May	June	May	June	May	June	
Swamp (between Kiranzi and Nabingora)	1	00°29.533'N	031°09.800'E	0.001	0.5	28	20	66	93	7		10		26	15	167	109	18	17	290	59	27.3	7.8	0	0	H
Nyabikere Crater Lake	2	00°29.990'N	032°19.729'E	0.04	20	25	24	287	288	7		110		119	70	425	335	1729	1983	40	24	28.4	17.7	8	13	E
Nkuruba Crater Lake	3	00°31.020'N	030°18.178'E	0.03	16	24	24	387	373	7	8		314	41	34	82	71	506	850	20	11	4.3	5.7	7	9	E
Lake George (Kahendero)	4	00°03.026'N	030°03.274'E	270	2.4	28	25	343	345	10	10	10	20	14	41	138	191	18	52	205	71	34.9	27.2	128	78	H
Lake Edward (Katwe)	5	00°09.096'S	029°53.073'E	11.5	4	28	26	590	592	10	9	30	40	12	8	132	115	26	10	80	64	8.6	10.5	44	23	H
Nkugute Crater Lake	6	00°33.086'S	030°10.362'E	0.025	20	25	25	103	120	9	8			16	7	57	23	24	8	20	11	0.7	0.1	5	5	M
Lake Mburo	7	00°39.106'S	030°56.537'E	158	4	26	24	122	136	10	8	30	30	14	12	125	161	19	13	140	46	8.3	8.0	37	40	H
Lake Nabugabo	8	00°21.283'S	031°52.789'E	214	4.5	25	25	20	20	8	8	100	90	2	8	25	29	94	18	80	19	3.2	2.2	8	11	M
Lake Victoria (Bunjako)	9	00°04.340'S	032°07.520'E	60.1	5	23	25	82	108	7	9	120	120	3	5	57	51	11	8	25	16	0.6	0.1	10	8	E
Lake Victoria (Murchison)	10	00°16.911'N	032°38.398'E	18	4	26	26	100	100	6		80	80	5	8	122	105	28	25	150	199	1.0	0.4	13	23	E
Lake Victoria (Napoleon)	11	00°40.281'N	033°29.806'E	66.1	18	27	26	94	98		9	100	110	3	7	70	75	19	5	45	14	0.3	0.1	11	10	E
Pond (Jinja)	12	00°43.248'N	033°23.289'E	0.001	1	31	26	1134	1095		9	11	20	25	12	222	213	24	18	60	46	18.2	21.8	33	22	H

M = mesotrophic; E = eutrophic; H = hypertrophic;

Phytoplankton Composition and Abundance

Chlorophyll a analysis was based on hot ethanol extraction (International Organisation for Standardisation, 1992).Algae were counted from Lugol fixed samples by using the inverted microscope, as described (Wetzel and Likens, 2000). Cyanobacteria were identified according to the morphological keys published by Talling (1987), Komarek and Kling (1991), and Komarek and Anagnostidis (1999). At least 400 specimens of the dominant phytoplankton genera were counted at 400× magnification. Most of the genera were counted as single cells [*Anabaena*, *Chroococcus*, *Merismopedia*, *Microcystis*; the pennate diatom *Nitzschia* and unidentified centric diatoms, green algae (including desmids) and cryptomonads]. Filamentous cyanobacteria were counted as filaments (*Planktolyngbya*, *Pseudanabaena*). *Aphanocapsa* was counted as colonies: Cells from 20 of these colonies were counted to determine the average number of cells per colony. *Microcystis* was counted as single cells and could be discriminated from other cyanobacteria by morphological characters, such as cell size [2.5-3.6±0.2-4.9] and the formation of small colonies. To compare the reproducibility of the *Microcystis* counting method between two different labs (Kampala vs. Mondsee), a number of depth-integrated and plankton net samples (nos. 4, 5, 7, and 11) were repeatedly counted in September 2004 (Kampala) and in November 2006 (Mondsee). On average, the *Microcystis* cell numbers as determined in November 2006 accounted for 76±40% (1 SD, n = 13) of the cell numbers as determined in September 2004. This implies that *Microcystis* cells could be reliably identified among other phytoplankton under an inverted microscope. The biovolume was calculated based on the measured dimensions of cells/filaments (Wetzel and Likens, 2000).

Microcystin Analysis

The extraction of MC from filters was performed as described (Kurmayer et al., 2003). One hundred microliters of the extracts were injected into high performance liquid chromatography coupled to diode array detection (HPLCDAD), and MCs were identified by their retention time and characteristic absorption spectra according to Lawton et al. (1994) and Fastner et al. 1999). MCs were quantified at 240 nm, and the concentrations of all the MC variants were determined as concentration equivalents of [D-MeAsp, D-Mdha]-MC-LR (Cyanobiotech GmbH, Berlin, Germany). HPLC peaks identified as MC were collected manually and were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as described by Kurmayer et al. (2004) and identified by the postsource decay (PSD) fragment structure analysis as described by Erhard et al. (1997) and

stner et al. (1999). The constitution of the new MC variants was assigned by ESI-MS and I-MS^2 experiments that were performed on a Q-TOF Ultima mass spectrometer (Waters, ilford, MA) equipped with a nanospray source and operated in the positive ionization mode der the control of MassLynx 4.1. The sample was loaded into a PicoTip Emitter (New bjective, Woburn, MA). Single MS measurements were followed by MS^2 experiments on the lected precursor ions. Fragmentation was operated by manually increasing the collision ergy (4-60) to provide optimum fragment ion coverage over the mass range. External ilibration was performed with glu-fibinopeptide-B (standard error 50 ppm), and an additional ostcalibration was applied when needed. Assignment of fragments by collision-induced ssociation (CID) enhanced PSD was carried out following a published procedure (Diehnelt et ., 2006).

enetic Analysis of the Field Samples

For DNA extraction, 25-200 mL of the depth integrated samples and 25-60 mL of the lankton net samples were filtered using Whatman GF/C filters. The filters were dried in the ven at 50°C overnight. DNA was extracted by the phenol-chloroform method as described Kurmayer et al., 2003). The DNA was diluted to 2-20 ng μL^{-1} for PCR analyses.

A part of the mcyE gene region was amplified by PCR using the HEPF/HEPR primers Jungblut and Neilan, 2006). These primers bind to the conserved regions of the aminotransferase domain and, therefore, amplify the *mcy*E genes of all the MC-producing cyanobacteria (472 bp). Subsequently, the genus-specific primers *mcy*E-F2/F8 (specific for *Microcystis*; 370 bp), *mcy*E-F2/12R (specific for *Anabaena*; 370 bp), and *mcy*E-F2/plaR3 (specific for *Planktothrix*; 370 bp) were used to detect the respective MC-producing species (Rantala et al., 2006). These primers bind to the adenylation domain of the *mcy*E gene, which is responsible for the activation of the strictly conserved glutamic acid residue during MC synthesis (Tillett et al., 2000).

For three sites (nos. 4, 7, and 11) sampled in 2008, the PCR products that were obtained by using the HEPF/HEPR primer pair were cloned using the pDrive Cloning Vector system (Qiagen, VWR, Austria) according to the manufacturer's instructions. For the construction of a clone library, clones were picked for each of the samples and analyzed by using restriction fragment length polymorphism (RFLP) using two restriction enzymes (*BsuR*I and *Tru1*I) following standard protocols. Using the NEBcutter program (Vincze et al., 2003), the restriction of *mcy*E of the genus *Microcystis* (472 bp, PCC7806, AF183408) resulted in three

agments (restriction type A: 176/160, 92, 18/16/8 bp) that were visualized using ethidium-
omide staining and electrophoresis in 2.0% agarose in 0.53 TBE-buffer. In contrast, the same
striction analysis for *mcy*E (472 bp) of the genus *Anabaena* (strain *Anabaena* 90, AY212249)
sulted in two fragments only [352, 110, 8 bp (invisible)], denoted as the restriction type B.
/hile the *mcy*E gene is generally more conserved and useful to detect all MC-producing
yanobacteria, this gene region cannot reveal the genetic diversity within a certain genus on a
ubpopulation level (Rantala et al., 2006; Jungblut and Neilan, 2006). Consequently, the
ox4F/tox4R primers (Kurmayer et al., 2002) amplifying the first adenylation domain of the
*mcy*B gene in *Microcystis* (*mcy*BA1, 1313 bp) were used to estimate the genetic diversity
vithin the MC-producing Microcystis subpopulation. The tox4F/tox4R PCR products were
loned and the clones were screened by restriction using *BsuR*I. Following the NEBcutter
rogram, the *BsuR*I digestion of *mcy*BA1 (1313 bp) of the *Microcystis* strain PCC7806
AF183408, representing *mcy*B1(B), Mikalsen et al., 2003] resulted in two fragments
restriction type I: 973, 340 bp). In contrast, the recombination type *mcy*B1(C) (e.g., strain
HUB524, Z28338) revealed one much longer and one much shorter fragment (restriction type
II: 1236, 77 bp). From the same sites, twelve *Microcystis* strains were isolated using standard
plating procedures as has been described (Okello, 2004). Six strains were found to contain
*mcy*B (strains nos. 1B5, 2D6, 18A8, 20A2, 6C5, and 20A5; access nos. EU014158-
EU014163), and this *mcy*B genotype was not digested by *BsuR*I (restriction type III). Both
*mcy*E and *mcy*B environmental sequences that were obtained from various environmental
clones identified by RFLP were submitted to the DDBJ/EMBL/GenBank database (access nos.
FJ429838-FJ429844).

To analyze all the field samples for the occurrence of potentially MC-producing *Microcystis*, a PCR test that allows for the detection of lowest *Microcystis* cell densities was required. To check for the quality of the extracted DNA and the presence of potential PCR inhibitors, a gene region part of a housekeeping gene was amplified in parallel to the PCR amplifying the *mcy*B gene. Primers specified to amplify the intergenic spacer region of the phycocyanin operon (PC-IGS) of *Microcystis* sp. were designed by aligning the sequences of PC-IGS (576 bp) from the twelve freshly isolated *Microcystis* strains (strains nos. R14, 1B5, 2D6, 18A8, R50, 2A3, 20A2, 21A1, 17B7, 6C5, 19G6, and 20A5; access nos. EU014146-EU014157). In addition, 128 *Microcystis* sequences of PC-IGS from the DDBJ/EMBL/GenBank (June 10, 2007) were included in a multiple sequence alignment (ClustalW2) to design the primer pair: MaPCnewfwd, 5'-GGAGCTTCCGTAGCTGC-3'

(57.6°C), and MaPCnewrev, 5′-TGCAATAAGTTTCCTACGGT-3′ (53.5°C), yielding a total amplification fragment of 192 bp. Pilot experiments showed that these primers were specific to PC-IGS of *Microcystis*. The six freshly isolated *Microcystis* strains that were found to contain *mcy*B (1312 bp) were aligned with eleven sequences from entries in the DDBJ/EMBL/GenBank (June 10, 2007) representing the two recombination types that were reported within *mcy*B of *Microcystis* sp. [*mcy*B1 (B), *mcy*B1(C), Mikalsen et al., 2003]. The following primers were designed: Ma*mcy*Bnewfwd, 5′-GGATATCCTCTCAGATTCGG-3′ (57.3°C), and Ma*mcy*Bnewrev, 5′-CTGATGTATAAATAACATAGGCTAAA-3′ (55.3°C), yielding a total amplification fragment of 188 bp for both recombination types (Mikalsen et al., 2003). For both PC-IGS and the *mcy*B primers, the lower limit of detection was equivalent to two cells of *Microcystis* strain 18A8.

In general, PCR amplifications from field samples were carried out in 20 µL volumes containing 2 µL PCR buffer (Qiagen, Austria), 1.2 µL $MgCl^2$ (25 mM), 0.6 µL deoxynucleotide triphosphates (10 µM each, MBI Fermentas, Germany), 1 µL of each primer (10 pmol), 0.1 µL Taq DNA polymerase (Qiagen), 13.6 µL sterile Millipore water, and 0.5 µL of 2–20 ng μL^{-1} DNA. The PCR cycling conditions for the two separate regions of the *mcy*E gene were performed as described (Jungblut and Neilan, 2006; Rantala et al., 2006). For the PCR amplifying *Microcystis*-specific PC-IGS and mcyB, the thermal cycling protocol included an initial denaturation step at 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. PCR products (4 µL of the reaction mix) were visualized by ethidium–bromide staining and electrophoresis in 2.0% agarose in 0.5× TBE-buffer.

Results

Trophic Characterization of the Sampling Sites

In the swamp sample (no. 1), the turbidity was high (Secchi depth < 10 cm; Table 2.1) and no chlorophyll a was detected. The three Crater lakes (nos. 2, 3, and 6) and L. Nabugabo (no. 8) showed low concentrations of chlorophyll a (5-13 $\mu g\ L^{-1}$) resulting in high transparency (>1 m) suggesting a mesotrophic status. In two of the Crater Lakes (nos. 2 and 3), however, high TP concentrations were recorded, co-occurring with high SRP and NH_4-N concentrations. Therefore, they were classified as eutrophic lakes. All three sites (nos. 9-11) from L. Victoria were classified as eutrophic. The highest chlorophyll a values were recorded in L. George (no. 4), L. Edward (no. 5), and L. Mburo (no. 7), and in Jinja pond (no. 12), which indicates

hypertrophic conditions. Correspondingly, the secchi depths were the lowest (<0.5 m) and the pH was the highest under hypertrophic conditions.

Phytoplankton Species Composition and Abundance

The total biovolume estimated via the microscopical counting correlated significantly with the chlorophyll a concentration (R^2 = 0.76, n = 37). Five phytoplankton classes were observed: Cyanobacteria, green algae, desmids, diatoms, and cryptomonads [Fig. 2.2(A)]. Cyanobacteria occurred in all the water bodies. The other phytoplankton classes were generally of minor importance. The Crater Lakes (nos. 2 and 3) and mesotrophic L. Nabugabo (no. 8) had the lowest biovolume of cyanobacteria and mostly contained diatoms, i.e., centric diatoms and Nitzschia (nos. 2 and 3) or green algae/desmids, i.e., Chlorella, Cosmarium, and Staurastrum (no. 8). Cyanobacterial dominance was only observed under eutrophic and hypertrophic conditions (nos. 4, 5, 7, and 9-12), including the genera *Anabaena*, *Aphanocapsa*, *Chroococcus*, *Merismopedia*, *Microcystis*, *Planktolyngbya*, and *Pseudanabaena* [Fig. 2.2(B)]. *Anabaena* was present in the Crater lakes (nos. 2 and 3) and in the deep eutrophic L. Victoria (nos. 9-11), while *Aphanocapsa* occurred in both the hypertrophic shallow lakes (nos. 4, 5, and 7) and the deep eutrophic L. Victoria (nos. 9-11). *Microcystis* was most abundant in the hypertrophic shallow lakes (nos. 4, 5, and 7). In summary, the dominance of cyanobacteria was positively related with the trophic situation.

Microcystin Analyses of the Field Samples

MCs were detected in plankton net samples only, i.e., in five plankton net samples in 2004 (no. 4, May; no. 5, May and June; no. 7, May and June) and in all five plankton net samples in 2008. MC 1 [molar weight 1038 ($M+H^+$)] occurred in all samples containing MC and eluted at 15.6 min and was identified by PSD analysis of the fractionated peak as [D-MeAsp^3, Mdha^7]-MC-RR. In addition, the MCs extracted from the plankton net samples (L. Edward, L. George, L. Mburo, June 2004, 2008) eluted at 21.7 min [MC 2, molar weight 1031 ($M+H^+$)] and 23.2 min [MC 3, molar weight 1045 ($M+H^+$)]. MCs 2 and 3 were also identified in aqueous methanolic extracts of the *Microcystis* strains 2D6, 6C5, and 18A8 isolated from L. Edward and L. Mburo. The original UV spectrum as well as the first-order derivative of the peak apex showed a close match with [MeAsp^3, Mdha^7]-MC-YR, which is available from the spectrum library. However, MC 2 and MC 3 differed in the retention time from [MeAsp^3, Mdha^7]-MC-YR. The sequence and constitution of MC 2 and MC 3 were investigated by LC-MS^2. CID-

nhanced PSD produced 34 and 33 fragments for MC 2 and MC 3, respectively, detected with mass accuracy of <50 ppm (Tables 2.2 and 2.3).

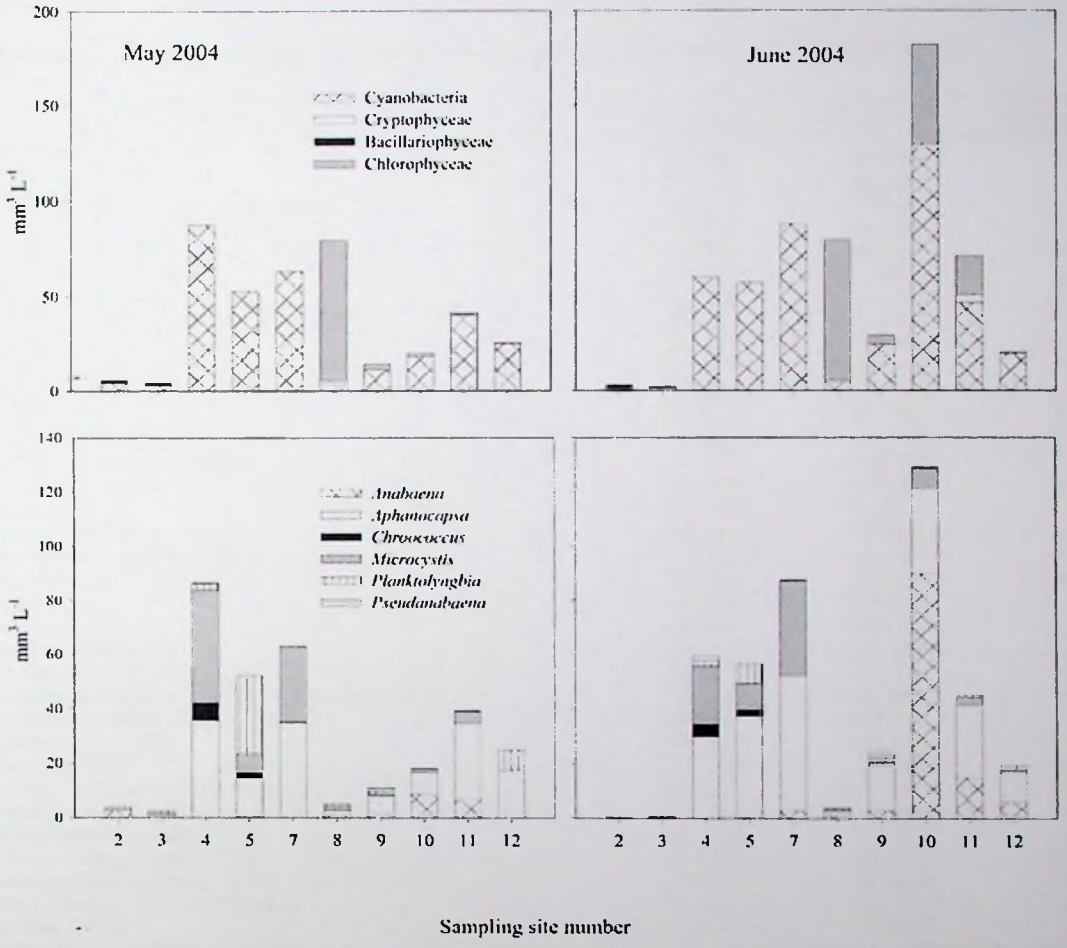

Fig. 2.2. (A) Phytoplankton composition and (B) cyanobacterial composition in various Ugandan freshwater habitats during May and June 2004. Numbering of the sampling sites as in Table 2.1. No phytoplankton was detected at sites (1) and (6). Only taxa contributing >5% of the total phytoplankton biovolume (upper panel) or cyanobacterial biovolume (lower panel) are shown.

able 2.2. Composition of ions in the MS^2 spectrum of the $[M+H]^+$ ion of $[Asp^3]$-MC-RY

Composition and Sequence	Calculated *m/z*	Measured *m/z*	Error (ppm)
M (+ H^+)	1031.5197	1031.5197	0.0
M (− CO) (+ H^+)	1003.5248	1003.5244	−0.4
Glu-(Mdha or Dhb)-Ala-Arg-Asp (+ H^+)	555.2522	555.2643	21.8
Glu-(Mdha or Dhb)-Ala-Arg (+ H^+)	440.2252	440.2386	30.4
Glu-(Mdha or Dhb)-Ala-Arg (− COOH) (+ H^+)	395.2276	395.2124	−38.5
Glu-(Mdha or Dhb) (+ H^+)	213.0870	213.0879	4.2
(Mdha or Dhb)-Ala-Arg-Asp (+ H^+)	426.2096	426.2177	19.0
(Mdha or Dhb)-Ala-Arg-Asp (− NH_3) (+ H^+)	409.1831	409.1936	25.7
(Mdha or Dhb)-Ala-Arg (+ H^+)	311.1826	311.1879	17.0
(Mdha or Dhb)-Ala-Arg (− CO) (+ H^+)	283.1877	283.1771	−37.4
(Mdha or Dhb)-Ala-Arg (− NH_3) (+ H^+)	294.1561	294.1605	15.0
(Mdha or Dhb)-Ala (+ H^+)	155.0815	155.0824	5.8
(Mdha or Dhb)-Ala (− CO) (+ H^+)	127.0866	127.0899	26.0
Ala-Arg-Asp-Tyr-Adda (+ H^+)	819.4400	819.4226	−21.2
Ala-Arg-Asp (+ H^+)	343.1724	343.1826	29.7
Ala-Arg (+ H^+)	228.1455	228.1474	8.3
Arg-Asp-Tyr (+ H^+)	435.1987	435.2142	35.6
Arg-Asp-Tyr (− COOH) (+ H^+)	390.2010	390.1989	−5.4
Arg-Asp (+ H^+)	272.1353	272.1411	21.3
Arg (+ H^+)	157.1084	157.1098	8.9
Asp-Tyr (+ H^+)	279.0975	279.1034	21.1
Asp-Tyr (− CO) (+ H^+)	251.1026	251.1096	27.9
Tyr-Adda-Glu-(Mdha or Dhb)-Ala-Arg (+ H^+)	916.4927	916.4935	0.9
Tyr-Adda-Glu-(Mdha or Dhb)-Ala-Arg (− CO) (+ H^+)	888.4978	888.5056	8.8
Tyr-Adda-Glu-(Mdha or Dhb) (+ H^+)	689.3545	689.3567	3.2
Tyr-Adda-Glu (+ H^+)	606.3174	606.3105	−11.4
Tyr-Adda (+ H^+)	477.2748	477.2565	−38.3
Adda-Glu-(Mdha or Dhb)-Ala-Arg-Asp (− NH_3) (+ H^+)	851.4298	851.4353	6.5
Adda-Glu-(Mdha or Dhb)-Ala-Arg-Asp (− NH_3) (− 134 Adda) (+ H^+)	717.3565	717.3604	5.4
Adda-Glu-(Mdha or Dhb) (− NH_3) (+ H^+)	509.2647	509.2621	−5.1
Adda-Glu-(Mdha or Dhb) (− NH_3) (− 134 Adda) (+ H^+)	375.1915	375.1996	21.6
Adda-Glu-(Mdha or Dhb) (− CO) (− NH_3) (− 134 Adda) (+ H^+)	347.1966	347.2035	19.9
Adda (− NH_3) (− 134 Adda) (+ H^+)	163.1118	163.1127	5.5
134Adda (+ H^+)	135.0804	135.0824	14.8

The amino acid at position 7 was assigned as either Mdha or Dhb. The distinction between these isomers is difficult by MS, as D labelling experiments on MC 2 and MC 3 were inconclusive. The assignment of MC 2 as $[Asp^3]$-MC-RY was based on fragments such as $[Ala-Arg-Asp+H]^+$ (343.1826), $[Ala-Arg+H]^+$ (228.1474), $[Mdha\ or\ Dhb-Ala-Arg+H]^+$ (311.1879), $[Tyr-Adda-Glu+H]^+$ (606.3105), $[Tyr-Adda+H]^+$ (477.2565), and many others (Table 2.2). The assignment of MC 3 as $[MeAsp^3]$-MC-RY was based on fragments such as $[Ala-Arg-MeAsp1H]^+$ (357.2023), $[Ala-Arg-NH_3+H]^+$ (211.1247), $[Mdha\ or\ Dhb-Ala-Arg+H]^+$ (311.1938), $[MeAsp-Tyr+H]^+$ (293.1255), $[Tyr-Adda+H]^+$ (477.2628), [Arg-MeAsp-

'yr+H]$^+$ (449.2364), and many others (Table 2.3). These two MC variants have not yet been escribed in the literature, to the best of our knowledge, and thus constitute new MCs.

'able 2.3. Composition of ions in the MS^2 spectrum of the $[M+H]^+$ ion of [$MeAsp^3$]-MC-RY

Composition and Sequence	Calculated *m/z*	Measured *m/z*	Error (ppm)
M (+ H^+)	1045.5353	1045.5353	0.0
M (− CO) (+ H^+)	1017.5404	1017.5602	19.5
Glu-(Mdha or Dhb)-Ala-Arg-MeAsp (+ H^+)	569.2678	569.2905	39.9
Glu-(Mdha or Dhb)-Ala-Arg (− NH_3) (+ H^+) or (Mdha or Dhb)-Ala-Arg-MeAsp (− NH_3) (+ H^+)	423.1987	423.2173	44.0
Glu-(Mdha or Dhb)-Ala-Arg (− COOH) (+ H^+)	395.2276	395.2204	−18.2
Glu-Mdha + H	213.0870	213.0916	21.6
(Mdha or Dhb)-Ala-Arg-MeAsp-Tyr-Adda (+ H^+) or Tyr-Adda-Glu-(Mdha or Dhb)-Ala-Arg (+ H^+)	916.4927	916.5134	22.6
(Mdha or Dhb)-Ala-Arg-MeAsp-Tyr (− NH_3) (+ H^+)	586.2620	586.2884	45.0
(Mdha or Dhb)-Ala-Arg-MeAsp (+ H^+) or Glu-(Mdha or Dhb)-Ala-Arg (+ H^+)	440.2252	440.2413	36.6
(Mdha or Dhb)-Ala-Arg-MeAsp (− CO) (+ H^+) or Glu-(Mdha or Dhb)-Ala-Arg (− CO) (+ H^+)	412.2303	412.2466	39.5
(Mdha or Dhb)-Ala-Arg (+ H^+)	311.1826	311.1938	36.0
(Mdha or Dhb)-Ala-Arg (− NH_3) (+ H^+)	294.1561	294.1656	32.3
(Mdha or Dhb)-Ala-Arg (− CO) (+ H^+)	283.1877	283.1843	−12.0
(Mdha or Dhb)-Ala (+ H^+)	155.0815	155.0850	22.6
(Mdha or Dhb)-Ala (− CO) (+ H^+)	127.0866	127.0922	44.1
Ala-Arg-MeAsp-Tyr-Adda (+ H^+)	833.4556	833.4562	0.7
Ala-Arg-MeAsp (+ H^+)	357.1880	357.2023	40.0
Ala-Arg (− NH_3) (+ H^+)	211.1190	211.1247	27.0
Arg-MeAsp-Tyr (+ H^+)	449.2143	449.2364	49.2
Arg-MeAsp-Tyr (− COOH) (+ H^+)	404.2167	404.2209	10.4
Arg-MeAsp (+ H^+)	286.1509	286.1632	43.0
Arg (+ H^+)	157.1084	157.1132	30.6
MeAsp-Tyr (+ H^+)	293.1131	293.1255	42.3
Tyr-Adda-Glu-(Mdha or Dhb)-Ala-Arg (+ H^+)	916.4927	916.5134	22.6
Tyr-Adda-Glu-(Mdha or Dhb)-Ala-Arg (− CO) (+ H^+)	888.4978	888.5134	17.6
Tyr-Adda (+ H^+)	477.2748	477.2628	−25.1
Adda-Glu-(Mdha or Dhb)-Ala-Arg-MeAsp (− NH_3) (+ H^+)	865.4454	865.4628	20.1
Adda-Glu-(Mdha or Dhb)-Ala-Arg-MeAsp (− NH_3) (− 134 Adda) (+ H^+)	731.3722	731.3859	18.7
Adda-Glu-(Mdha or Dhb) (− NH_3) (+ H^+)	509.2647	509.2813	32.6
Adda-Glu-(Mdha or Dhb) (− NH_3) (− 134 Adda) (+ H^+)	375.1915	375.2071	41.6
Adda-Glu-(Mdha or Dhb) (− CO) (− NH_3) (− 134 Adda) (+ H^+)	347.1966	347.2095	37.2
Adda (− NH_3) (− 134 Adda) (+ H^+)	163.1118	163.1154	22.1
134Adda (+ H^+)	135.0804	135.0852	35.5

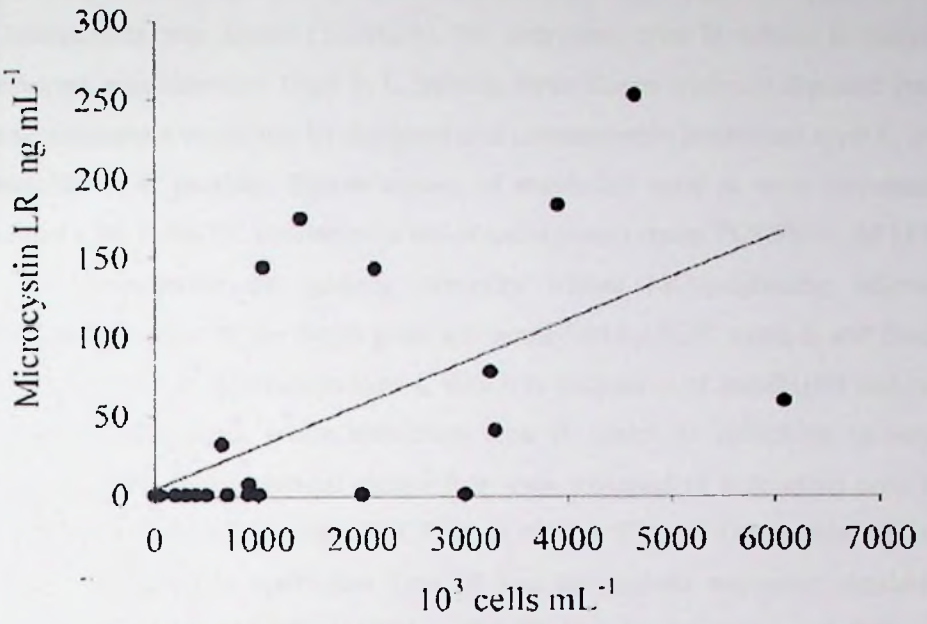

Fig. 2.3. Relationship between the MC concentrations (in ng mL^{-1} of MC-LR equivalents) and the concentration of cells mL^{-1} of *Microcystis* during May and June 2004 and April 2008. For details on the regression curve, see the text.

There was a linear relationship between the *Microcystis* cell concentrations and the total MC concentrations: $y = 0.03x + 3.54$ ($R^2 = 0.38$, $n = 31$, $P < 0.001$, Fig. 2.3), where y is the MC concentration in ng mL^{-1} and x is the cell concentration mL^{-1}. In contrast, the MC concentrations were found to vary independently from the *Anabaena* cell concentrations.

Identification of microcystin producers and genetic diversity

With the exception of a plankton net sample from L. Mburo (June 2004), all the samples that contained detectable MCs revealed PCR products for *mcy*E by using HEPF/HEPR primers. To identify the MC-producing genus, further PCR analyses were performed using the *Microcystis* (*mcy*E-F2/F8)-, *Anabaena* (*mcy*E-F2/12R)-, and *Planktothrix* (*mcy*EF2/plaR3)-specific primers. In all of the cases, the *mcy*E products that are indicative of *Microcystis* were obtained from the plankton net samples (Table 2.4), while no PCR products that are indicative of *mcy*E of *Anabaena* were obtained.

Three samples (lakes George, Mburo, and Victoria-Napoleon, nos. 4, 7, and 11) revealed abundant *Microcystis* but also showed a frequent occurrence of *Anabaena* [Fig. 2.2(B)]. Using the HEPF/HEPR primers, the *mcy*E gene was amplified and the clones were analyzed by

RFLP. In the vast majority of the clones (n = 165), only restriction type A, which is indicative of *Microcystis*, was found (Table2.5). No restriction type B, which is indicative of *mcy*E of *Anabaena*, was obtained. Only in L. Mburo, three clones were not digested (restriction type C). Those sequences could not be assigned and consequently restriction type C was considered an unspecific PCR product. Eleven clones of restriction type A were sequenced (337 bp) and revealed a 96.1–96.7% similarity to the *M. aeruginosa* strain PCC7806, AF183408.

To characterize the genetic diversity within MC-producing *Microcystis*, the first adenylation domain of the *mcy*B gene was amplified by PCR, cloned, and then the clones were screened by RFLP. Restriction type I, which is indicative of *mcy*B1(B) and restriction type III (undigested), occurred, while restriction type II, which is indicative of *mcy*B1(C), was not detected. Three environmental clones that were assigned to restriction type I had the highest sequence similarity to the strain PCC7806 (1190 bp, 97.2-98.1% similarity). In contrast, clones that were assigned to restriction type III had the highest sequence similarity to the strains 18A8, 20A2, 20A5, and 2D6 (1190 bp, 97.4-98.4%). Restriction type I differed by 24.7-25.7% of the nucleotides (1190 bp) from restriction type III.

Distribution of Microcystin-Producing Microcystis

Using the PC-IGS primers that were designed in the present study, the distribution of *Microcystis* was studied in all the field samples that have been analyzed for phytoplankton composition under the microscope (Table 2.4). PCR products were obtained from all the samples containing *Microcystis* as inferred from the microscope. *Vice versa*, in the swamp (no. 1) and in Nyabikere Crater Lake (no. 2), no PC-IGS of *Microcystis* was detected, which was in accordance with the microscopical analysis. Except for the depth integrated sample from the Nkuruba Crater Lake, all the samples containing PC-IGS, indicative of *Microcystis*, were found to contain *mcy*B.

(+) or absence (-) of microcystin (MC) producers (HEP), *mcy*B of *Microcystis* and for the MCs by HPLC

Sample ID	Site No.	Filtered Volume (mL)			Cyanobacterial Biovolume ($mm^3 L^{-1}$)			*Microcystis* (cells mL^{-1})			HEP-PCR Product			*Microcystis* *mcy*E-F2/F8[a] PCR Product			PC-IGS PCR Product			*mcy*B PCR Product			fg MC-LR Equivalent $Cell^{-1}$		
		M4	J4	A8	M4	J4	A8	M4	J4	A8	M4	J4	A8	M4	J4	A8	M4	J4	A8	M4	J4	A8	M4	J4	A8
Depth integrated samples																									
Kiranzi swamp	1	250	250					–	–		–	–		–	–		–	–		–	–		–	–	
Nyabikere Crater Lake	2	400	700		4	0.4		–	–		–	–		–	–		–	–		–	–		–	–	
Nkuruba Crater Lake	3	500	500		3	1		5×10^3	5×10^3		–	–		–	–		+	+		–	–		–	–	
Lake George	4	25	50		88	60		4×10^5	7×10^5		+	+		+	+		+	+		+	+		–	–	
Lake Edward (Katwe)	5	150	150		53	57		2×10^5	2×10^5		+	+		+	+		+	+		+	+		–	–	
Nkugute Crater Lake	6	700	500																						
Lake Mburo	7	100	200		63	88		9×10^5	9×10^5		+	+		+	+		+	+		+	+		–	–	
Lake Nabugabo	8	250	250		5	5		5×10^4	2×10^4		–	–		–	–		+	+		+	+		–	–	
Lake Victoria (Bunjako Bay)	9	800	500		11	24		2×10^4	4×10^4		–	–		–	–		+	+		+	+		–	–	
Lake Victoria (Murchison Bay)	10	500	500		18	129		4×10^4	2×10^5		+	+		+	+		+	+		+	+		–	–	
Lake Victoria (Napoleon Gulf)	11	500	500		40	46		3×10^4	3×10^4		+	+		+	+		+	+		+	+		–	–	
Jinja Pond	12		200			19			3×10^4			–			–			–			–			–	
Plankton net samples																									
Kiranzi swamp	1	40	30		Not applicable			–	–		–	–		–	–		–	–		–	–		–	–	
Nyabikere Crater Lake	2	50	50		Not applicable			–	–		–	–		–	–		–	–		–	–		–	–	
Nkuruba Crater Lake	3	50	50		Not applicable			–	–		–	–		–	–		–	–		–	–		–	–	
Lake George	4	25	50	60	Not applicable			3×10^6	3×10^6	6×10^6	+	–	+	+	–	+	+	+	+	+	+	+	8.1	–	9.6
Lake Edward (Katwe)	5	35	40	60	Not applicable			1×10^6	4×10^6	2×10^6	+	+	+	+	+	+	+	+	+	+	+	+	92.9	31.2	67.1
Lake Mburo	7	35	50	60	Not applicable			3×10^6	3×10^6	4×10^6	+	–	+	+	–	+	+	+	+	+	+	+	5.2	15.8	52.4
Lake Nabugabo	8	40	40		Not applicable			3×10^5	5×10^5		–	–		–	–		+	+		+	+		–	–	
Lake Victoria (Bunjako Bay)	9	50	50		Not applicable			2×10^5	2×10^5		–	–		–	–		+	+		+	+		–	–	
Lake Victoria (Murchison Bay)	10	50	25	60	Not applicable			4×10^5	4×10^6	1×10^7	+	+	+	+	+	+	+	+	+	+	+	+	–	–	1.3
Lake Victoria (Napoleon Gulf)	11	50	50	60	Not applicable			1×10^6	7×10^5	6×10^5	+	+	+	+	+	+	+	+	+	+	+	+	–	–	49.1
Jinja Pond	12		50		Not applicable																				

M4, May 2004; J4, June 2004; A8, April 2008.

Only the plankton net samples from the site nos. 4, 5, 7, 10, and 11 were analyzed in April 2008.

[a]Using *mcy*E-F2/12R for *Anabaena* and plaR3 for *Planktothrix*, no PCR products were obtained.

Table 2.5. Number of restriction types as observed in clone libraries obtained from HEP-PCR products (*mcy*E, restriction, restriction types A-C) and tox4F/4R PCR products (*mcy*B, restriction types I-III)

			HEPF/HEPR-PCR (*mcy*E, 470 bp)					Tox4f/4r-PCR (*mcy*B, 1330 bp)				
Sites/Restriction Type	Site No.	Date	No of Clones	A (*Microcystis*)	B (*Anabaena*)	C Undigested	Not Assigned	No. of Clones	I (PCC7806)	II (HUB524)	III (Strain 18A8)[a]	Not Assigned
L. George	4	26 April 2008	20	20	0	0	0	20	0	0	20	0
L. Mburo	7	27 April 2008	75	71	0	3	1	24	8	0	15	1
L. Victoria (Napoleon)	11	26 April 2008	70	70	0	0	0	35	11	0	20	4

For each restriction type, the respective genus (A, B) or *Microcystis* strain (I-III) is indicated in parentheses.

[a] In addition, twelve *Microcystis* strains were isolated from the same sites (Okello, 2004). Six strains were found to contain *mcy*B (strain nos.: 1B5, 2D6, 18A8, 20A2, 6C5, 20A5; access nos. EU014158–EU014163).

[b] Sequencing revealed a 382 bp (FJ429839) with a 96% similarity to *Microcystis* strain PCC7806.

Discussion

Environmental Parameters and Phytoplankton Composition

We recorded high concentrations of nutrients, but low concentrations of chlorophyll a and phytoplankton in the two Crater lakes (nos. 2 and 3). Correspondingly, Kizito et al. (1993) reported low chlorophyll a, but high nutrient concentrations for Lake Nkuruba (no. 3). The highest NH_4-N concentration that was observed in the Crater lakes may indicate significant vertical stratification, since the nitrification of ammonia to nitrate requires oxygen. During a 2-year study, Chapman et al. (1998) reported that the average anoxia (0 mg L^{-1} O_2) in Lake Nkuruba (no. 3) was down to 9 m. In this study, it is possible that a part of the anoxygenic water column with the highest ammonium concentrations was sampled resulting in a higher average ammonium concentration due to the vertical integration down to 15 m.

Despite its shallowness, L. Nabugabo (no. 8) also had the lowest phytoplankton density, which was dominated by green algae and desmids. The soils of the catchment area of Lake Nabugabo have a very low salt content, possibly resulting in low calcium carbonate levels and the lowest conductivity (Beadle, 1981). A belt of mosses (*Sphagnum, Miscanthidium violaceum*) that is surrounding the lake has been suggested to indicate more acidic conditions (Kateyo, 2006). The general low ionic content in combination with a low nutrient concentration might be responsible for the dominance of green algae and desmids.

The cyanobacterial dominance in L. Victoria (nos. 9-11) as well as in Jinja Pond was linked to a high concentration of nutrients. Kling et al. (2001) reported that the eutrophic condition in L. Victoria supported a high algal biomass that has risen by a factor of 4–5 since the 1960s. Hecky (1993), Lipiatou et al. (1996), and Verschuren et al. (2001) reported a shift in dominance from diatoms and green algae to cyanobacteria. Mugidde et al. (2003) suggested that because of their ability to fix nitrogen cyanobacteria may particularly increase in L. Victoria in response to phosphorus loading and increasing nitrogen limitation.

In all the hypertrophic shallow lakes (nos. 4, 5, and 7), *Microcystis* was abundant. These three lakes situated within the national park had a high pH ranging from 8 to 10. Cyanobacteria are better competitors due to their efficient carbon dioxide-concentrating mechanism at a higher pH (Shapiro, 1984). In addition, these shallow waters are polymictic, while at calm conditions a high insolation will penetrate the whole water column. In general, these physical conditions favor the genus *Microcystis* sp. at the expense of other genera, and very often *Microcystis* sp. is able to dominate phytoplankton for long times (Reynolds et al., 2002). The

dominance of *Microcystis* sp. probably remained unaltered in Lake George for decades (Ganf, 1974).

Microcystin Analyses

In the present study, MCs were found in plankton net samples that were obtained from five sites (nos. 4, 5, 7, 10, and 11). In contrast, no MCs were found in aqueous methanolic extracts obtained from depth-integrated samples. In general, the high silt content that was clogging the filters during the filtration process resulted in low filtration volumes (50-200 mL), increasing the lower limit of detection for MC by HPLC significantly above 1 $\mu g\ L^{-1}$. It is expected that MC concentrations would have been in the measurable range, if more phytoplankton biomass would have been extracted. Indeed in the phytoplankton net samples with higher *Microcystis* cell numbers ranging from 2×10^5 to 1×10^7 cells mL^{-1}, MCs were recorded more frequently. Nevertheless, in plankton net samples, the cellular MC content was found to be within the range that was also observed in other field studies previously (1.3-93 fg MCLR equivalent $cell^{-1}$; Kurmayer et al., 2003). It is, therefore, concluded that the net samples were useful for analyzing the mean cellular MC content. These results are in agreement with other studies that document, by means of plankton net samples, that whole water conditions can be adequately represented (Rogalus and Watzin, 2008).

Genetic diversity and microcystin net production

In most of the samples, the genera *Anabaena*, *Aphanocapsa*, *Chroococcus*, *Merismopedia*, *Microcystis*, *Planktolyngbya*, and *Pseudanabaena* were identified under the microscope. Among those, *Anabaena*, *Chroococcus*, *Microcystis*, and *Pseudanabaena* have been reported to produce MCs (Jungblut and Neilan, 2006; Sivonen and Börner, 2008). We used the conserved HEPF/HEPR primer pair to amplify all the potential MC-producing cyanobacteria. Surprisingly, only PCR products that are indicative of *mcy*E of *Microcystis* were obtained. Correspondingly, the independent application of the genus-specific *mcy*E primers revealed the occurrence of MC-producing *Microcystis* only. Consequently, *Anabaena* and other taxa, although rather abundant, did not have the potential to produce MC. In other studies, the populations of *Microcystis* from all five continents typically have been found to contain *mcy* genotypes as well as MCs (Sivonen and Börner, 2008). In contrast, other taxa show a more irregular pattern of MC production, for example, MC-producing *Anabaena* have been reported from Europe, Canada, and North Africa, but not from Australia (Sivonen and Jones, 1999; Sivonen and Börner, 2008).

Using the more sensitive primers, all the *Microcystis* populations were found to be able to produce MCs. This result is in agreement with other studies showing that *Microcystis* populations always contain the *mcy* genotype. For example, in a European survey including nine water bodies in seven countries, *mcy*A and *mcy*B genes as a part of the *Microcystis* population were always detected (Via-Ordorika et al., 2004). Within *mcy*BA1, the highest genetic diversity was found (24.7-25.7%), which has also been reported for populations sampled in Europe, e.g., Lake Wannsee (Kurmayer et al., 2002), and in Scandinavia (Mikalsen et al., 2003). It is generally accepted that this highest genetic diversity resulted from frequent recombination processes involving the exchange of shorter fragments (<1000 bp) of DNA. Besides for the *mcy*BA1 restriction type I showing the highest similarity to the *Microcystis* strain PCC7806, a new *mcy*BA1 restriction type III that is indicative of a *mcy*BA1 genotype, which was so far unknown from the strains of the northern temperate hemisphere (Mikalsen et al., 2003), was found. In contrast, restriction II *mcy*B (C) was not detected. Notably, from the same *mcy*BA1 restriction type, two new MCs, [Asp^3]-MC-RY and [$MeAsp^3$]-MC-RY, were isolated. The genetic variation that is causing the biosynthesis of MC-RY remains to be elucidated.

Although the linear relationship between the *Microcystis* cell numbers and the total MC concentration was highly significant, as much as 62% of the variation remained unexplained (Fig. 2.3). Other studies documenting a significant linear relationship between the *Microcystis* cell concentration and the total MC concentration also report a relatively high proportion of unexplained variation (Kotak et al., 2000; Ozawa et al., 2005; Hotto et al., 2008). It is emphasized that so far no effort has been made to identify the contribution of systematic errors in cell counting and/or analysis of MC concentrations to the unexplained part of variation that is observed. However, in a consecutive field study sampling the same water bodies for 1 year, consistent lakespecific differences in MC cell quotas were observed, e.g., MC cell quotas obtained from Lake George were on average twenty-fold lower when compared with MC cell quotas obtained from Lake Mburo. We are currently investigating this phenomenon; however, lake-specific differences in MC cell quotas could explain the relatively large part of unexplained variation observed.

Conclusion

The trophic conditions and water depth were of profound importance for the occurrence of MC-producing cyanobacteria. Seven of the sampling sites were nutrient rich. However, only

e shallow sites had favorable environmental conditions that enabled MC-producing yanobacteria to flourish. From the extensive genetic analysis, it is concluded that *Microcystis*). constitute the major MC-producing taxon in Ugandan freshwaters. Besides MC-RR, the ew MC-RY variants constitute abundant structural variants in the phytoplankton dominated y *Microcystis* sp. in Uganda. However, the annual MC production rate in tropical freshwater ystems will exceed that in comparable systems in the temperate region due to the generally igher primary production rate.

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Chapter 3

Seasonal development of cyanobacteria and microcystin-production in Ugandan freshwater lakes

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Seasonal development of cyanobacteria and microcystin-production in Ugandan freshwater lakes

Abstract

This study is the first that investigates the seasonal development of phytoplankton and potential microcystin (MC)-producing cyanobacteria and MC concentrations in freshwater lakes in Uganda. During one year (May 2007-April 2008) chemical and physical characteristics, the phytoplankton composition and the MC concentrations were recorded monthly in a hypertrophic crater lake (Lake Saka), in shallow eutrophic lakes (Lakes Mburo, George, Edward), and in Lake Victoria (Murchison Bay, Napoleon Gulf). During the whole study period cyanobacteria (composed of the genera *Anabaena*, *Aphanocapsa*, *Chroococcus*, *Cylindrospermopsis*, *Microcystis*, *Planktolyngbya*, *Planktothrix*) dominated and always contributed >50% to total phytoplankton biovolume. With the exception of few sampling dates in Lake George, Lake Victoria (Murchison and Napoleon), all the other samples from all sampling sites were found to contain MC. Samples from Lake Saka had the maximum MC concentration (10 µg L^{-1}) in July 2007. The minimum concentration (0.0 µg L^{-1}) was recorded in Lake George (Kahendero) in the months of 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 µg MC-LR eq. L^{-1}) were recorded. For all sampling sites highly significant positive linear relationships between the total MC concentration and *Microcystis* cell numbers were obtained. Relating the total MC concentrations to *Microcystis* cells revealed a >100-fold variation in the average MC contents per cell between lakes. While *Microcystis* from Lake George showed the lowest MC cell quotas (0.03-1.24 fg $cell^{-1}$) *Microcystis* from Lake Saka consistently showed maximum MC cell contents (14-144 fg $cell^{-1}$). It is concluded that at all sites MC production is due to the occurrence of *Microcystis*, however between sites the populations differ consistently and independently of the season in their average MC content per cell.

Keywords: Blue-green algae, Cyanotoxins, Phytoplankton, *Microcystis*, Water monitoring

Introduction

During the last decades cyanobacteria in freshwater have been of general awareness due to their ability to produce various hepatotoxic and neurotoxic substances. It is generally agreed that the hepatotoxic microcystin (MCs) are probably the most abundant toxins produced by cyanobacteria in freshwater (Hudnell, 2008; Chorus and Bartram, 1999). While the number of taxa that has been found to produce MCs is constantly increasing, the MC-producing genera that are of major importance in phytoplankton have been already identified during the nineties: *Anabaena*, *Microcystis*, and *Planktothrix* (Sivonen and Jones, 1999; Sivonen and Börner, 2008).

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 favoured under more shallow, eutrophic conditions which is in correspondence to the general theory on how abiotic and biotic factors govern phytoplankton associations that have been defined since 1984 (Reynolds *et al.*, 2002). In contrast to *Microcystis* other genera known to produce MCs were found in lower abundance, e.g. *Anabaena* was abundant in Lake Victoria and one of the Crater Lakes while *Planktothrix* was not observed.

However, in this earlier study we were unable to monitor the phytoplankton community during different seasons. This issue is of relevance as usually dry seasons with precipitation minima and wet seasons with maximum precipitation have been correlated with changes in phytoplankton composition. Usually precipitation varies annually from 30 mm to 132 mm in Kasese, western Uganda and 60 mm to 184 mm in Kampala, the L. Victoria basin (Meteorological Department, Entebbe). This also results in annual changes of water temperature by 5°C in Kasese and 3°C in Kampala. The highest water temperatures are usually recorded during the dry season in February (33°C in Kasese and 29°C in Kampala). During the rainy season (from March to May and August to 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. In deeper lakes such as Lake Victoria, the mixing regime will change, as a higher stability of the water column has been described during the dry season June to July (Hecky, 1993). These physical changes have a significant effect on phytoplankton community composition. For example, Akiyama et al. (1977) reported highest cell densities

of *Anabaena* in Lake Victoria (Mwanza Gulf) in the dry period from November - January when the water was calm and *Anabaena* accumulated on the surface. In Lake Victoria, typically after the first algal maximum that occurred between July and August and that is composed of diatoms, the second and broader algal maximum occurs between November and January and it is chiefly composed of cyanobacteria (Talling, 1986). In contrast, in shallow lakes much less seasonality in phytoplankton composition is observed.

This study investigated the seasonal development of phytoplankton and potential MC-producing cyanobacteria and MC concentration in six lakes in Uganda. During one year (May 2007-April 2008), chemical and physical characteristics, the phytoplankton composition and the MC concentrations were recorded. The data on phytoplankton and MC concentrations are of importance towards creating awareness and guidance for designing water safety plans for Uganda to achieve the UN millennium development goals.

Materials and methods

Description of the study sites

Six sites were selected from five freshwater lakes of varying limnological characteristics (Fig. 3.1): (1) Station in the middle of L. Saka (N0°41.670', E30°14.667'), mean depth of 3.6 m. L. Saka is a small crater lake (1.4 km^2) at an altitude of 1520 m. (2) Lake George (228.5 km^2) part of the western rift valley at an altitude of 914 m, Kahendero (N0°03.004', E30°03.439'), mean depth of 1.8 m. This site was hydrologically separated from the main basin of the lake due to the formation of three large islands. (3) Lake Edward, Katwe (0°09.198'S, 29°53.056'E) which is a fish landing site (mean depth of 3.2 m). Originally Katwe was a salt mining area. It is located in a bay at the northeastern shoreline where the Kazinga channel and the river Nyamugasani are inflowing (close to Mweya Lodge in Queen Elisabeth National Park). (4) Lake Mburo, in the centre of the lake (S0°38.513', E30°56.869'), mean depth of 2.8 m. L. Mburo inhabits hippopotami and other wildlife. The site was selected in order to keep the influence from the animals and the inflowing river Rinzi to a minimum

Two sites were selected from the northern part of L. Victoria: (5) Murchison Bay (N0°15.461', E32°38.481'), Kampala, mean depth of 5.5 m. (6) Napoleon Gulf (N0°24.177', E33°14.756'), Jinja, mean depth of 17.8 m, it is also close to the Source of River Nile. Both sites were located inshore and considered pilot zones for the Lake Victoria Environmental Management Project (LVEMP).

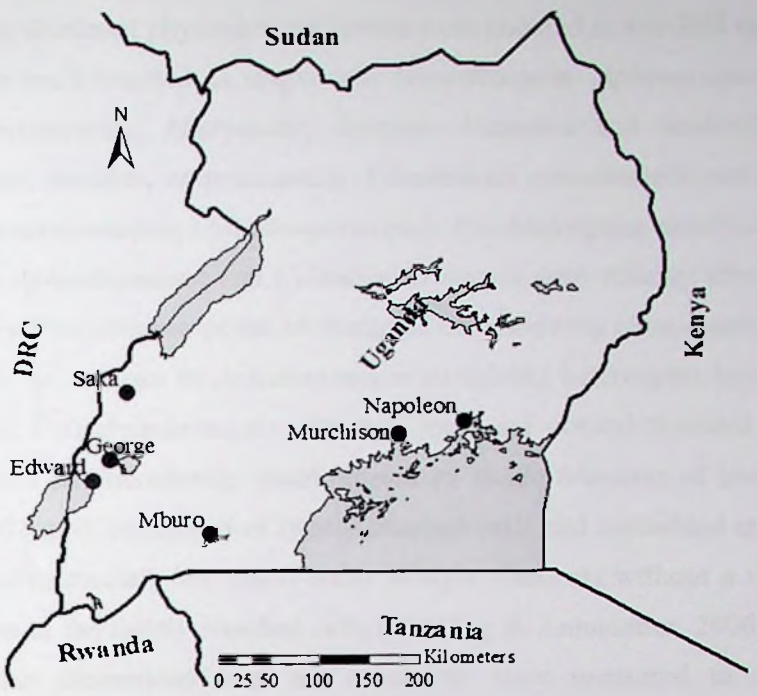

Fig. 3.1. Map of Uganda showing the six sampling sites (black circles).

Sampling and limnological characteristics

Depth integrated water samples were taken monthly from May 2007 until April 2008 (Fig. 3.1). A two-litre horizontal van Dorn sampler was used for sampling the water column at a one-meter interval. Samples were filtered using 47mm diameter GF/C filters (Whatman, Kent, Great Britain). The filters were stored frozen for the analysis of chlorophyll a. Filtrates were again filtered through 47mm diameter membrane filters (0.45 µm, Millipore Corporation, Bellerica, United States) for the analyses of dissolved nutrients: soluble reactive phosphorus (SRP), nitrate (NO_3-N) and ammonia (NH_4-N). These were determined using the ammonium molybdate method (Wetzel and Likens, 2000), the sodium-salycilate method (Müller and Wiedemann, 1955) and the indophenol blue method (Krom, 1982), respectively. Total phosphorus (TP) was determined as SRP subsequent to persulphate digestion from aliquots of the samples.

Phytoplankton community and abundance

Chlorophyll a analysis was based on hot ethanol extraction (ISO, 1992). Algae were counted by the inverted microscope technique from Lugol fixed samples as described in Utermöhl (1958). Cyanobacteria were identified according to the descriptions by Talling

(1987), Komarek and Kling (1991) and Komarek and Anagnostidis (1999). For each sample 400 specimen of the dominant phytoplankton genera were counted at 400-fold magnification. Most of the genera were counted as single cells (cyanobacteria: *Aphanocapsa*, *Anabaena, Chroococcus, Merismopedia, Microcystis*; diatoms: *Nitzschia* and unidentified centric diatoms, green algae, desmids, cryptomonads). Filamentous cyanobacteria were counted as single filaments (*Aphanizomenon*, *Cylindrospermopsis*, *Planktolyngbya,* and *Planktothrix*). In general the genera *Aphanizomenon* and *Cylindrospermopsis* were reliably identified by the formation of solitary filaments composed of elongated cells showing clear constrictions at the attaching cell walls. In contrast to *Aphanizomenon* containing heterocytes located between the cells the genus *Cylindrospermopsis* only had long and conical terminal heterocytes. *Planktolyngbya* could be undoubtedly discriminated by single filaments of lowest diameter (mean±1SE 2.4±0.03 µm), composed of tightly attached cells and embedded in a colourless sheath. In contrast *Planktothrix* had much wider straight filaments without a visible sheath and no constrictions at the tightly attached cells (Cronbeg & Annadotter, 2006). From each genus cells/filaments dimensions from ten specimens were measured to calculate the biovolume according to standard geometric shapes (Hillenbrand et al., 1999; Wetzel & Likens, 2000).

Microcystin analysis

A volume of 250 to 2400 mL of integrated water samples depending on the algal concentration were filtered using 47mm GF/C filters and the filters were dried at 50°C. Phytoplankton collected on filter was extracted in 1.5 mL of 50% methanol (v/v) and MCs were extracted following Fastner et al. (1998). The mixture was agitated for 60 minutes at 250rpm and centrifuged at 15000 × *g* for another 10 minutes. Clear supernatants were pipetted into new reaction tubes and the extraction procedure was repeated twice. Extracts were concentrated to dryness at 30°C using a vacuum centrifuge (Eppendorf AG, Hamburg, Germany). The residues were resuspended in 350 µL of 50% methanol mixed, centrifuged at 15000 × *g* for 10 minutes and the clear supernatants were analysed by high performance liquid chromatography-diode array detection (HPLC-DAD) on a LiChrosper 100, octydecyl silane, 5-µm LiChroCART 250-4 cartridge column (Merck, Darmstadt, Germany). A linear gradient of aqueous acetonitrile containing 0.05% trifluoroacetic acid (TFA) was used at a flow rate of 1 mL^{-1} increasing from 30% acetonitrile to 70% acetonitrile in 42 minutes (Lawton et al. 1994). MCs were quantified at 240 nm and the concentration of all MC variants was determined as concentration equivalents of [D-MeAsp, D-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 ng of MC-LR and x was mAU recorded at 240 nm.

HPLC fractions identified as MC were collected manually and concentrated to dryness. The collected fractions were re-dissolved in 10 µL of 100% methanol. 1 µL of each sample was then loaded onto a steel template (CellPath, Newtown Powys, Great Britain) and 1 µL of matrix solution (2,5-dihydroxy-benzoic acid (DHB) solublized in 50% acetonitrile, 0.03 % TFA) was loaded immediately and the mixture left to dry. Samples were then analysed by matrix-assisted laser desorption/ionisation voyager elite time-of-flight mass spectrometry (MALDI-TOF), (PerSeptive BioSystems, Framingham MS, USA) as described in Erhard *et al.* (1999) and Welker *et al.* (2002). Identification of particular MCs was by the post source decay (PSD) fragment analysis as described in Erhard *et al.* (1997) and Fastner *et al.* (1999).

Results

Limnological characteristics

The limnological parameters characterising either eutrophic or hypertrophic state varied significantly among the five sampling sites (Table 3.1). Averaged over the study period TP concentrations varied four fold and chlorophyll a concentrations varied ten fold between the lakes ($p<0.0001$, Kruskal Wallis One Way ANOVA on Ranks). Lake Saka, the only Crater Lake sampled in this study had the highest TP (193 ± 15.3 (mean ± 1SE) and chlorophyll a concentration (192 ± 17.6) resulting in low transparency (0.3 ± 0). Surprisingly the relatively low pH 7.9 was not in accordance with the highest primary production. Lakes George (Kahendero), Edward (Katwe) and Mburo also showed high TP concentrations, 117.3 ± 15.2, 110.9 ± 19 and 101.7± 13.3, respectively that accordingly resulted in high chlorophyll a concentrations (70.3 - 94.9 µg L^{-1}) characteristic of a hypertrophic state with pH 8.5 - 10.4. The two sampling sites in Lake Victoria (Murchison Bay and Napoleon Gulf) showed the lowest TP concentrations resulting in relatively low chlorophyll a concentrations, highest transparency and intermediate pH 7.8 - 9.9.

Table 3.1. Limnological characteristics (min-mean±SE-max) during the sampling period, May 2007 to April 2008. For all characteristics, sample size = 12

	Secchi depth (m)	Chlorophyll-a (μgL^{-1})	pH	Temperature (°C)	TP (μgL^{-1})	SRP (μgL^{-1})	Nitrate (μgL^{-1})	Ammonia (μgL^{-1})
	min-mean±SE-max	min-mean±SE-max	min-mean±SE-max	min-mean±SE-max	min-mean±SE-max	min-mean±SE-max	min-mean±SE-max	min-mean±SE-max
L. Saka	0.2 - 0.3±0 - 0.5	86.6 - 192±17.6 - 273	7.6 - 7.9±0.1- 8.7	20.7 - 22.4±0.4 - 24.4	99 - 193.0±15.3 - 301	2.0 - 10.0±2.8 - 33.0	11.1 - 65.4±11.3 - 113	0.0 - 17.1±4.9 - 51.0
L. George	0.2 - 0.2±0 - 0.4	30.0 - 94.9±1.8 - 177	9.8 - 10.4±0.1 - 10.9	24.3-26.8±0.3-2933	52 - 117.3±15.2 - 243	2.0 - 6.8±1.3 - 16.0	11.1 - 86.4±12.0 - 167	3.0 - 11.3±1.6 - 19.0
L. Edward	0.3 - 0.4±0 - 0.6	36.5 - 70.3±7.1 - 122	9.7 - 9.9±0 - 10.2	25.4 - 27.2±1.2 - 29.0	60 - 110.9±19.0 - 293	2.0 - 8.3±3.3 - 41.0	11.1 - 65.5±9.3 - 122	0.0 - 8.6±2.1 - 26.0
L. Mburo	0.3 - 0.4±0 - 0.5	31.3 - 77.9±8.3 - 129	7.5 - 8.5±0.2 - 9.5	24.2 - 23.8±1.2 - 26.2	36 - 101.7±13.3 - 206	1.0 - 7.1±1.4 - 16.0	44.4 - 94.7±9.8 - 160	1.0 - 15.5±5.2 - 69.0
Murch†	0.5 - 0.6±0 - 0.7	30.0 - 64.5±5.3 - 98.3	7.8 - 8.7±0.2 - 9.6	24.3 -25.8±1.0 - 27.4	35 - 87.2±11.8 - 172	0.0 - 5.6±0.0 - 14.0	0.0 - 78.6±24.4 - 325	0.0 - 21.8±7.0 - 79.0
Napo†	1.2 - 1.4±0 - 1.6	7.8 - 19.4±3.4 - 47.0	8.8 - 9.5±0.1 - 9.9	25.5 -26.7±0.7 - 28.0	30 - 49.3±5.5 - 90	2.0 - 7.7±1.7 - 21.0	0.0 - 33.7±15.7 - 175	2.0 - 12.3±2.0 - 24.0

Min = Minimum; Max = Maximum; SRP = Soluble reactive phosphorus; TP = Total Phosphorus; SE = Standard error; Murch† = Murchison Bay, L. Victoria; Napo† = Napoleon Gulf, L. Victoria.

Cyanobacterial composition

In total 36 genera of phytoplankton were identified that belonged to six classes (cryptomonads, cyanobacteria, diatoms, dinoflagellates, euglenoids and green algae). The total phytoplankton community composition will be described in detail elsewhere. Only four classes (cryptomonads, cyanobacteria, diatoms and green algae) contributed to ≥ 5% of total biovolume (Fig. 3.2). The total biovolume ranged from 83 $mm^3 L^{-1}$ in Lake Saka, 433 $mm^3 L^{-1}$ in Lake George (Kahendero) to 47 $mm^3 L^{-1}$ in Lake Victoria (Murchison Bay). The lowest biovolume would have been recorded in Lake Victoria (Napoleon Gulf) at an average of 5 $mm^3 L^{-1}$ in July 2007. However in June 2007 extremely high cell numbers of *Anabaena* were recorded. In summary, cyanobacteria dominated during the whole study period and always contributed >50% to phytoplankton biovolume.

Cyanobacterial biovolume ranged from 427 $mm^3 L^{-1}$ (maximum) in Lake George (Kahendero) to 3.2 $mm^3 L^{-1}$ (minimum) in Lake Victoria (Napoleon Gulf). Seven cyanobacterial genera were identified each contributing ≥ to 5% of total cyanobacterial biovolume (Fig. 3.3). However, not all the genera were present in all the samples. In samples from lakes George (Kahendero), Edward (Katwe) and Mburo, filamentous cyanobacteria (*Planktolyngbya*) dominated and co-occurred with *Anabaena*, *Aphanocapsa*, *Chroococcus* and *Microcystis*. The genus *Cylindrospermopsis* was abundant and contributed to the total biovolume of cyanobacteria in Lake Mburo. In contrast to lakes George (Kahendero), Edward (Katwe) and Mburo, *Planktothrix* contributed 24 - 92% to the cyanobacterial biovolume in Lake Saka. In contrast to *Cylindrospermopsis* and *Planktothrix*, *Microcystis* appeared throughout the sampling period in all the lakes. *Anabaena* occurred consistently in Lake Victoria (Murchison and Napoleon) ranging in biovolume from 1.0 $mm^3 L^{-1}$ to 50 $mm^3 L^{-1}$. We recorded the highest *Microcystis* biovolume in samples from Lake George (Kahendero) (273 $mm^3 L^{-1}$) and the lowest (0.3 $mm^3 L^{-1}$) in samples from Lake Victoria (Napoleon).

In summary, three genera of potentially microcystin-producing cyanobacteria (*Anabaena*, *Microcystis* and *Planktothrix*) were identified. This indicated that the phytoplankton in all the five lakes had the potential for microcystin production (Table 3.2).

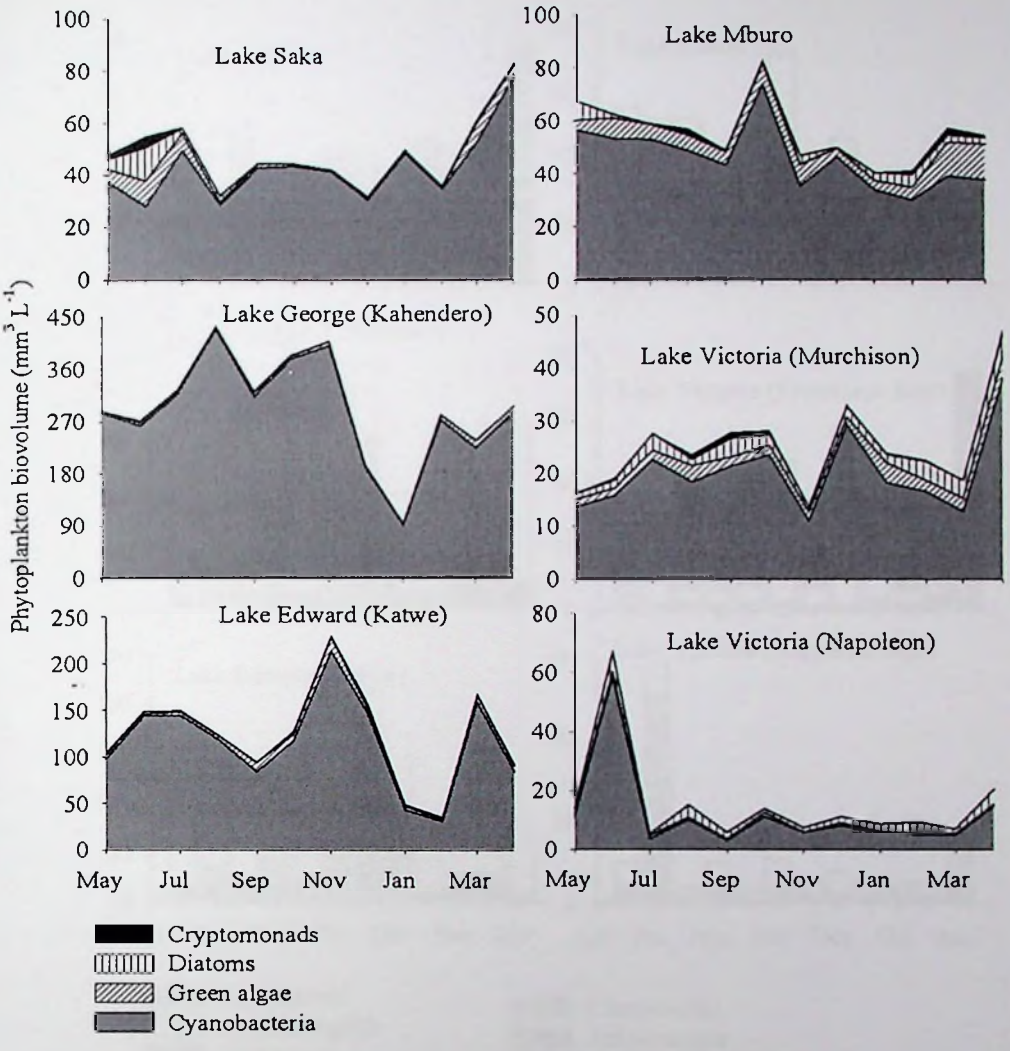

Fig. 3.2. Phytoplankton composition at the six sampling sites from May 2007 to April 2008. Note that the scales on the y-axis differ between sites.

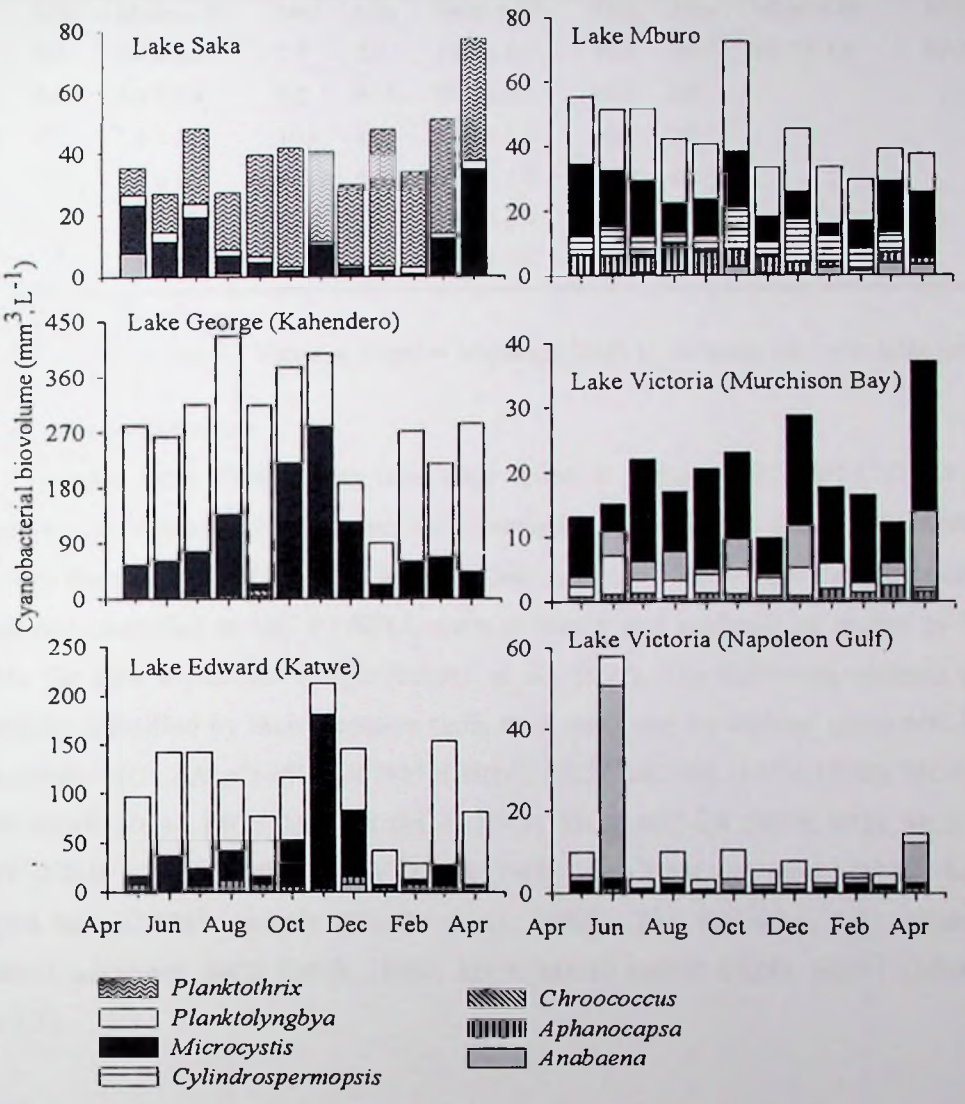

Fig. 3.3. Cyanobacteria composition at the six sampling sites from May 2007 to April 2008. Note that the scales on the y-axis differ between sites.

Table 3.2. Proportion (percentage) of the possible microcystin producing cyanobacteria in the five lakes from May 2007 to April 2008. Sample size = 12.

	Anabaena			*Microcystis*			*Planktothrix*		
	Min	Mean ± SE	Max	Min	Mean ± SE	Max	Min	Mean ± SE	Max
Saka	0.0	0.8 ± 0.6	7.7	2.3	18.3 ± 4.0	39.5	60.5	81.7 ± 4.0	97.7
George	0.0	0.8 ± 0.4	5.2	94.8	99.2 ± 0.4	100	nd		
Edward	0.0	2.6 ± 1.1	10.4	89.6	97.4 ± 1.1	100	nd		
Mburo	0.0	3.8 ± 0.9	9.3	52.9	64.3 ± 1.8	74.9	nd		
Murch†	15.0	27.1 ± 2.0	42.0	58.0	72.9 ± 2.0	85.0	nd		
Napo†	53.4	66.5 ± 3.6	93.2	6.8	33.5 ± 3.6	46.6	nd		

Murch† = Murchison Bay, L. Victoria; Napo† = Napoleon Gulf, L. Victoria, nd = not detected.

Microcystin net production

All samples from all sampling sites 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 MC-RR, MC-YR, MC-LR as available from the spectrum library. All fractions identified as MC by HPLC were collected and analysed by means of MALDI-TOF MS for their molecular weight (Erhard et al., 1997). 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 by $LC\text{-}MS^2$ recently (Okello et al., 2009). The following MC variants were considered unknown: MC3 (M+H 1063), MC7, MC10 (M+H 1024), MC11 (M+H 1031), (Table 3.3).

Table 3.3. Microcystin variants retention time, their percentage composition (in parentheses) and frequency of occurrence underneath for each site from May 2007 to April 2008. Number of samples per site = 24.

	MC1	MC2	MC3	MC4	MC5	MC6	MC7	MC8	MC9	MC10	MC11
M+H	1024	1038	1063	1031	1045	995	nd	1031	1045	1024	1031
	[Asp^3]MC-RR	MC-RR	Unknown	[Asp^3]MC-YR	MC-YR	MC-LR		[Asp^3]MC-RY	MC-RY	Unknown	Unknown
Saka	14.0-14.5	15.2-15.5	16.0	17.7	18.0-18.9	19.0-19.9		21.0-21.7	23.1-23.9	24.7	
	(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)	
	3.7	22.4	2.8	4.7	15.0	14.0		15-0	18.7	3.7	
George		15.4						21.7-21.8	23.1-23.4		
		(1.6±1.6)						(12.0±4.4)	(78.0±6.6)		
		3.2						25.8	71.0		
Edward	14.1-14.3	15.1-15.4		17.0	18.6	19.9		21.6-21.8	23.0-23.2	24.0-25.0	27.8-27.9
	(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)
	7.2	19.3		10.8	13.3	1.2		3.6	27.7	7.2	9.6
Mburo	14.0-14.4	15.2-15.5		17.3-17.5	18.3-18.7	19.0-19.9	20.9	21.6-21.7	23.1-23.2	24.0-24.2	27.9
	(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)
	7.9	12.9		7.3	12.4	7.9	9.0	10.7	13.5	11.2	7.3
Murch*		15.1-15.4	16.3-16.4		18.0	19.3-19.9			23.0-23.5	24.3-24.4	
		(3.9±1.9)	(7.8±3.0)		(2.9±1.4)	(12.4±2)			(48.8±6.3)	(6.7±1.7)	
		18.1	8.4		7.2	22.9			25.3	18.1	
Napo*		15.3-15.7	16.3-16.4		18.0-18.8	19.2-19.8		21.1	23.3	24.6	
		(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)	
		11.8	61.8		11.8	5.9		2.9	2.9	2.9	
RT	14.0-14.5	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	23.0-23.6	24.0-24.9	27.8-27.9
	(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)
	4.7	16.1	6.0	5.2	11.4	9.7	3.3	9.1	21.5	8.9	4.1

M+H = molecular weight; nd = molecular weight not determined; MC = microcystin variant; RT = retention time in minutes; Murch* = Murchison; Napo* = Napoleon.

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 L. Saka, L. Mburo and L. Edward and in Murchison Bay. In contrast MC-RY and [Asp^3]-RY were dominant at all sites except Napoleon Gulf. (Table 3.4). Surprisingly in the samples from Napoleon Gulf an unknown MC3 (M+H 1063) variant occurred most frequently. Taking all sampling sites together MC-RY was most frequently occurring, followed by MC-RR and MC-YR. Notable MC-LR only occurred in 11% of all the samples.

Table 3.4. Relative frequency of occurrence of each of the MC variants (Table 3.3) in the 24 samples obtained from each lake (n=24). For each variant the hypothesis of random distribution of the MC variants was rejected based on the Chi-square statistic (P).

MC No	MC Name	Saka	George	Edward	Mburo	Murch†	Napo†	In Total	P
MC1	Asp-MC-RR	17	0	25	58	0	0	17	<0.01
MC2	MC-RR	100	4	67	96	63	17	58	<0.01
MC3	unknown	13	0	0	0	29	88	22	<0.01
MC4	Asp-MC-YR	21	0	38	54	0	0	19	<0.01
MC5	MC-YR	67	0	46	92	25	17	41	<0.01
MC6	MC-LR	63	0	4	58	79	8	35	<0.01
MC7	unknown	0	0	0	67	0	0	11	<0.01
MC8	Asp-MC-RY	67	33	13	79	0	4	33	<0.01
MC9	MC-RY	83	92	96	100	88	4	77	<0.01
MC10	unknown	17	0	50	17	33	4	20	<0.01
MC11	unknown	0	0	33	54	0	0	15	<0.01

Murch† = Murchison Bay, L. Victoria; Napo† = Napoleon Gulf, L. Victoria,

In general, the contribution of each MC variant to the total MC concentration was closely related to 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. The MC observed in Napoelon was dominated by the unknown MC3 variant. The phytoplankton further differed significantly in the production of MC in total (calculated as MC-LR equivalents) ($p<0.0001$, Kruskal Wallis One Way ANOVA on Ranks). On average, the MC concentrations were 28-fold higher in Lake Saka (4.7±0.9 μg L^{-1}) when compared with the mean (0.2±0.1 μg L^{-1}) MC concentration measured in L. George (Kahendero) (Fig. 4.4). Samples from Lake Saka had the maximum MC concentration (10 μg L^{-1}) in July 2007. The minimum concentration

(0.02 μg L^{-1}) was recorded in Lake George (Kahendero) in the months of 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 μg MC-LR eq. L^{-1}) were recorded. Within the lakes, the total MC concentration varied seasonally from 12 fold to 30 fold.

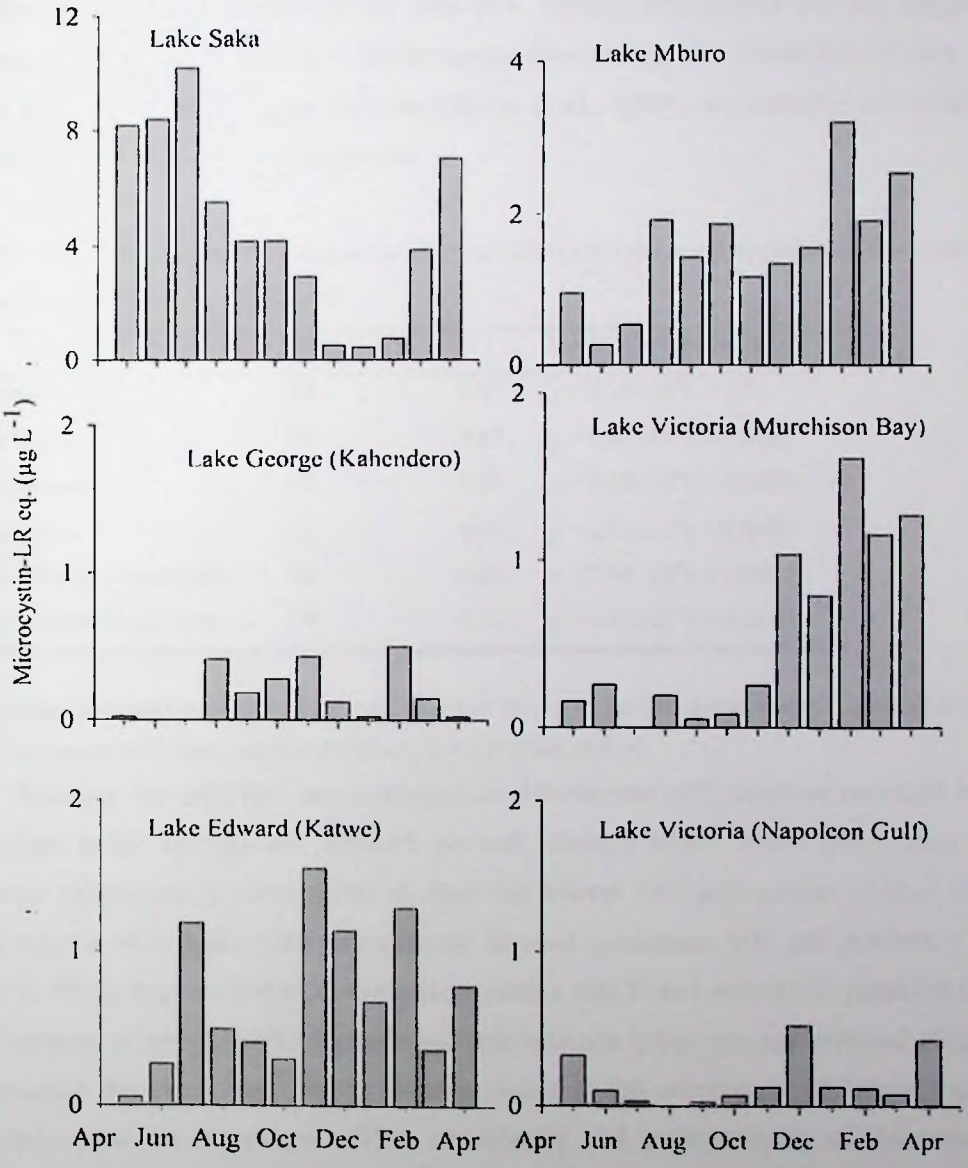

Fig. 3.4. Microcystin concentrations (μg MC-LR eq.) at the six sampling sites from May 2007 to April 2008. Note that the scales on the y-axis differ between sites.

For all sampling sites highly significant positive linear relationships between the total MC concentration and *Microcystis* cell numbers were obtained (Table 3.5). For other potentially MC-producing taxa (*Anabaena* and *Planktothrix*) occurring in Lakes Saka, George, Edward only marginal significant relationships (with a low explained variation) between cell numbers and MC concentrations were found. In contrast significant relationships were observed for the sites in L. Mburo, Murchison bay and Napoelon Gulf. 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.

Table 3.5. Linear regression output of *Microcystis* cells versus microcystin concentration for each lake from May 2007 to April 2008

	Sample size	r^2	Equation of the slope
Lake Saka	24	0.97	$y = 3.18\times10^{-8}x + 0.01$
Lake George	24	0.87	$y = 1.12\times10^{-9}x - 0.0009$
Lake Edward	24	0.66	$y = 2.38\times10^{-8}x + 0.0005$
Lake Mburo	24	0.94	$y = 2.11\times10^{-8}x - 0.0009$
Lake Victoria (Murchison)	24	0.86	$y = 2.64\times10^{-9}x + 0.0009$
Lake Victoria (Napoleon)	24	0.87	$y = 6.33\times10^{-9}x + 0.0001$

Both integrated and net samples were combined (n = 24). y is the MC concentration (µg MC-LR eq. mL^{-1}) and x is *Microcystis* cell concentration (Cells mL^{-1}). $p < 0.001$ in all sites.

Relating the total MC concentrations to *Microcystis* cell numbers revealed a >100-fold variation in the average MC contents per cell between lakes. While *Microcystis* from Lake George (Kahendero) consistently showed the lowest MC cell quotas (0.03-1.24 fg $cell^{-1}$) *Microcystis* from Lake Saka consistently showed maximum MC cell contents (14 -144 fg $cell^{-1}$). While in general the between site variation was found reduced in plankton net samples the ranking of average MC contents per cell between lakes was not affected (Fig. 3.5). It is concluded that at all sites MC production is due to the occurrence of *Microcystis*, however between sites the populations differ consistently and independently of the season in their average MC content per cell.

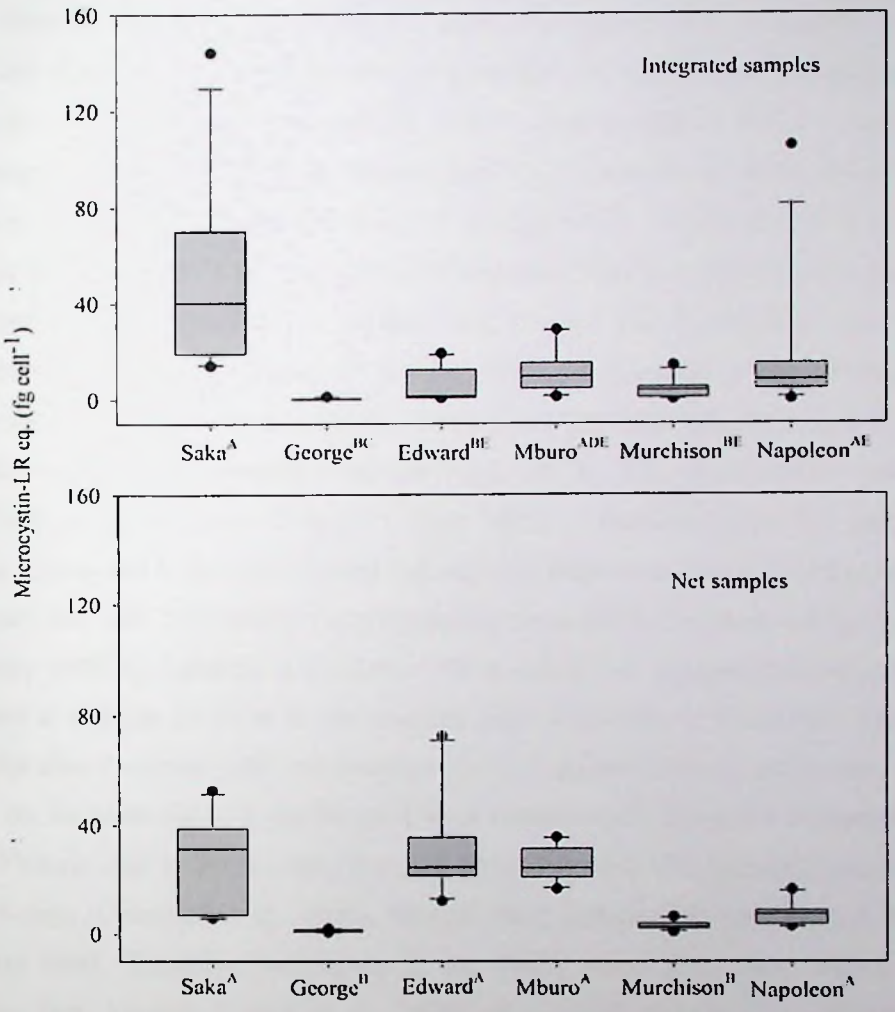

Fig. 3.5. MC cell quotas of *Microcystis* sp. at the six sites from May 2007 to April 2008. The lower and upper borders of each box indicate the 25th and 75th percentiles; the line inside the box represents the median; the whiskers indicate the 10th and 90th percentiles and the black dots are the outliers. N = 12 per sampling site for both net and integrated samples. Superscripts indicate homogeneous subsets ($p < 0.05$) after testing the overall significance differences (Kruskal Wallis, One Way ANOVA).

Discusion

Natural and human induced eutrophication

The shallow lakes George, Edward, Mburo are all of a hypertrophic state with TP and chl a values > 100 µg L^{-1} and 70µg L^{-1}, each. The high trophy is probably natural as those lakes are shallow, located closely to the Ruwenzori Mountain where massive run offs and nutrients are introduced via several rivers (Nyamwamba, Rukoli, Mubuku, Ruimi, Nyamugasani, Ishasha, Rutshuru, Rwindi and Rinzi) particularly during the rainy season. In addition abundant wildlife contributes to eutrophication. Schönborn (2003) reported that hippopotami introduce large amounts of excrements that cause considerable nutrient loading. Hippopotami also occur in L. Edward and George and particularly the bulls use the excrements as territorial marks. In contrast Lake Saka located at the Northern end of the Crater lakes does not contain wildlife, however has been eutrophied by agricultural activities and fish stocking for decades (Crisman et al. 2001). The same authors reported highest dissolved oxygen maxima 15 mg L^{-1} (max 180% saturation) during the period of the dry season. Compared with Lake George, Edward and Mburo the pH in L. Saka was found to be relatively low (pH 7.9) which is corresponding to the pH 7.7 as observed from January 1995 - January 1998 by Crisman et al. (2001). Since rather low oxygen concentrations have been reported at a depth of 10 m in the southern part of the lake it is possible that a low redox potential also counteracts the pH increase due to a highest primary production in the surface layer. As for Lake Saka, a significant human induced eutrophication comparable to bays of Lake Victoria due to human activities has been observed. For example, studies on Winam Gulf, Kenya (Calamari et al., 2005), Nyanza Gulf, Kenya (Gikuma-Njura & Hecky, 2005), Mwanza Gulf, Tanzania (Sekadende et al., 2005), Murchison Bay, Napoleon Gulf and Fielding Bay, Uganda (Silsbe et al., 2006) all reported the trend in nutrient enrichment. However, either natural or human-induced eutrophication both finally resulted in a dominance of cyanobacteria.

Seasonal and spatial variation in phytoplankton composition

Over the whole study period, total phytoplankton biovolume was found to be relatively stable, and cyanobacteria never contributed less than 53 % to the phytoplankton in total. Only at the station in Lake Victoria, Napoleon Gulf a distinct maximum of *Anabaena* in June was recorded. In this study Napoleon Gulf was the deepest sampling site (18 m) and the dry

weather conditions might have favoured the growth of *Anabaena* at the expense of the diatoms. Buoyant cyanobacteria, like *Anabaena* and *Microcystis* are found in higher cell numbers during the dry season when compared with the wet season (Talling 1986). In general, in Kampala there are seasons of minimum rainfall from January-February and June-July (Meteorological Department, Entebbe) contributing to a higher degree of stratification in the water column. Buoyant cyanobacteria are expected to have a selective advantage compared with non-motile algae under stratifying conditions (Talling, 1986).

In contrast to the seasonal phytoplankton biovolume the composition of cyanobacteria was more variable, for example phytoplankton of Lake Saka was dominated by *Planktothrix* spp. during December – February, while *Microcystis* was more abundant during the rest of the year. For unknown reasons *Planktothrix* was recorded in Lake Saka only. There have been generally rather few records of the occurrence of *Planktothrix* from the tropical climatic zone. Up to date *Planktothrix* has been reported to occur in another tropical lake in Cameroon (Kemka et al., 2003). The genus *Planktothrix* is more frequently and dominates phytoplankton composition in deep and shallow lakes within the northern temperate climatic zone (VanLiere et al., 1979; Oliver & Ganf, 2000). Interestingly *Planktothrix* occurring in this area is morphologically indistinguishable from *P. agardhii* occurring in Europe and Asia (Suda et al., 2002). It is likely that *Planktothrix* in L. Saka is *P. pseudagardhii* that is known to grow under higher temperature regimes when compared with *P. agardhii* (Suda et al., 2002; Conradie et al., 2008). Consequently, while in most freshwater lakes in Uganda *Planktothrix* seems to be out competed by other genera (*Planktolyngbya*, *Microcystis* and *Anabaena*), it is still able to dominate in habitats of a more specific physical-chemical characteristic. Both habitats, Lake Saka and Yaounde Municipal Lake in Cameroon are relatively small lakes, shallow and anoxic in the lower layers (Crisman et al., 2001; Kemka et al., 2003). The observation that *Planktothrix cf. ornata* living at the chemocline of a sulphate-rich karstic lake in Spain can sustain high sulphide concentrations found around the chemocline in the lake (Camacho et al., 2000) may provide a hint on specific ecological adaptations of this species in tropical Africa.

When compared with the phytoplankton in Lake Saka the genus *Planktolyngbya* replaced *Planktothrix* in the shallow hypertrophic lakes George, Edward and Mburo. In contrast to *Planktothrix*, *Microcystis* and *Anabaena* the genus *Planktolyngbya* belongs to the group of the Pseudanabaenaceae and never forms gas vesicles (Komarek, 2003). Since the

genus *Planktolyngbya* grows in rather thin filaments, probably no compensation of sinking losses is required. *Planktolyngbya* is a typical member of the group of shade-adapted species (Reynolds et al., 2002) resulting in the so-called third stable state to which shallow lakes in Europe may develop (Scheffer et al., 1997). It is known that together with *Planktothrix agardhii*, *Limnothrix redekei*, *Pseudanabaena limnetica*, *Planktolyngbya contorta* can form the so-called association "S" that is characterised by highly light deficient conditions. In Lakes George, Edward and Mburo co-occurring *Microcystis* sp. can escape these light-limiting conditions by vertical migration to the surface.

In contrast to the shallow lakes Mburo, George and Edward the genus *Anabaena* is much more abundant at stations of Lake Victoria. While *Anabaena* flos-aquae are also capable of vertical migration the higher proportion of *Anabaena* might be due to the prevalence of N-limiting conditions.

Effects of phytoplankton composition on microcystin production

For all lakes the abundance of *Microcystis* was significantly 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. We reported previously that in the same lakes we were unable to amplify genotypes of the *mcy*E gene encoding microcystin synthesis that are indicative of the genera *Anabaena* or *Planktothrix* (Okello et al., 2009). In contrast in the same study the occurrence of *mcy*E and *mcy*B genotypes assigned to the genus *Microcystis* showed a perfect correspondence with MC production. In addition nine strains of Planktothrix sp. were isolated from Lake Saka in April 2008 and analysed for MC production as described (Kurmayer et al., 2004). None of the strains were found to contain MCs and/or the *mcy*E/*mcy*B gene part of the *mcy* gene cluster (R. K., unpublished results).

Surprisingly, the average MC *Microcystis* cell quotas differed significantly between populations (Fig. 3.5). In general 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 40–80 fg cell^{-1} under light conditions from 10-100 µmol m^{-2} s^{-1}. Long et al. (2001) observed a variation in MC content per cell of Microcystis strain MASH 01-A19 from 0.052-0.116 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 (Orr & Jones, 1998), while larger variation (up to 30-fold) at 30°C vs 12.5°C has been reported in exceptional cases only (Rapala et al., 1997). In this study the average MC contents differed between populations by 17-175 folds in integrated samples and 2.5-23 folds in plankton net samples. The range of variation substantially increases the variation observed for single strains under variable conditions in the laboratory. Consequently it is possible that genetic differences between populations such as the variable proportion of *mcy* genotypes contributes to the between site variation that is observed. In order to test this hypothesis phytoplankton from Lakes George and Mburo was extracted for DNA at four sampling dates and analysed for the proportion of mcy genotypes following exactly the quantitative real-time PCR (Taq nuclease assay, TNA) protocol that has been described (Kurmayer & Kutzenberger, 2003). In general the Microcystis cells that were counted in the microscope correlated significantly with the Microcystis cell number as estimated by the TNA (n=16, R^2=0.70). The average proportion of *mcy* genotypes in Lake George (1.14±0.13 (SE)% and 5.62±0.38% in integrated and net samples, each) was 17-fold lower in integrated samples and 7-fold lower in net samples when compared with the average *mcy* proportion in Lake Mburo (19.6±4.8% and 40.5±7.4 % in integrated and net samples, each). This difference in *mcy* genotype proportion compares with the average difference in cellular MC content both in integrated samples (0.3±0.1 vs 11.7±2.6 fg $cell^{-1}$ of Microcystis in Lake George and Lake Mburo, each) and in net samples (1.2±0.1 vs 24.4±1.9 fg $cell^{-1}$ in Lake George and Mburo, each). It is concluded that the variation in average MC content of *Microcystis* that is observed between lakes could be explained by the between site differences in *mcy* genotype proportion. Notably the variation in average MC content that was observed within sites could not outweigh this between site variations in MC content.

Conclusion

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 a relatively minor variation 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. This approach is considered mostly reliable as the

microscopical enumeration technique is well established and the maintenance of technically sophisticated equipment is avoided.

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Chapter 4

Morphological and genotypic variation of the microcystin-producing cyanobacterium *Microcystis* in Uganda

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Morphological and genotypic variation of the microcystin-producing cyanobacterium *Microcystis* in Uganda

Abstract

Microcystis has been shown to be involved in the formation of toxic algal blooms due to its production of microcystins (MCs). Environmental programmes frequently use the microscopical examination of water samples as an early warning tool in order to predict the occurrence of hazardous cell concentrations. The reliable identification of *Microcystis* sp. under the microscope is particularly challenging if the co-occurrence of other morphologically similar taxa cannot be excluded. In this study, the morphological variability of *Microcystis* was analysed (i) directly in field water samples by picking individual colonies and (ii) in the laboratory by isolating cultures via streaking on agar. In total, 895 colonies were picked from field samples from Lakes Saka; George; Edward; Mburo; Victoria-Murchison Bay and Victoria-Napoleon Gulf field samples in Uganda. Four morphospecies were identified from the field samples, these includes: *M. aeruginosa*, *M. botrys*, *M. flos-aquae* and *M. wesenbergii*. During the analysis of 96 laboratory strains from the same lakes, *M. aeruginosa*, and *M. flos-aquae* were isolated while *M. wesenbergii* was not successfully cultured. The strains could be undoubtedly assigned to *Microcystis* based on morphological and genetic characters (PC-IGS). Only 13.5% of the strains were found to contain the *mcy*B/E genes indicative of microcystin production. More than 60% of the strains producing microcystins either contained MC-RY or MC-RR as the major microcystin structural variant but MC-LR only in minor proportion (0.4 - 2.3%). It is concluded that *Microcystis* can be reliably identified under the microscope. The occurrence of *mcy* genotypes producing MC-RY/MC-RR only can explain the scarcity of MC-LR in Uganda. This finding is relevant as MC-LR is known to show highest toxicity to livestock and humans in general.

Keywords: genotypes, MC-LR, mcy genes, morphospecies

Introduction

The colony forming cyanobacterium *Microcystis* has frequently been shown to form toxic algal blooms in freshwater. Among the toxins, cyclic heptapeptides microcystins (MCs) have been shown to be the most frequent and several planktonic and benthic genera could be shown to produce MCs (Sivonen and Jones, 1999). Most prominently *Microcystis*, *Anabaena*, *Planktothrix* have been shown to contain the *mcy* gene cluster that is responsible for the MC production (Tillett et al., 2000; Christiansen et al., 2003; Rouhianinen et al., 2004).

The genus *Microcystis* has a cosmopolitan distribution, although particular species have been found or shown to occur in specific climatic regions only (Komarek and Anagnostidis, 1999). For example *M. panniformis* has been described to occur in tropical countries only. As early as 1979, Reynolds et al. followed the seasonal variation in morphology in consequence to growth conditions. Typically, colonies form compact aggregates of cells during growth, while they tend to disintegrate when growth conditions deteriorate (Reynolds et al., 1979). The concept of morphologically based classification in Microcystis has been challenged by the observation that various morphological characters such as the formation of mucilage is unstable under suitable culture conditions. In particular, morphotypes assigned to *M. wesenbergii* have been observed to transform into *M. aeruginosa* under culture condition in the course of weeks to months (Otsuka et al., 2001).

In general, *Microcystis* morphology has been investigated intensively in the temperate climatic regions, however much is yet to be known from tropical countries. Haande et al., 2007 reported the isolation of several morphotypes from Uganda (Murchison Bay, Lake Victoria) and Kenya. The morphotypes mainly differ in cell size and the density of cells per colony. However, as these morphotypes were described under culture conditions, only limited conclusions could be obtained on the morphological variation that is observed in the field. The reliable microscopical identification of *Microcystis* sp. is important both for ecological studies and for applied aspects such as water quality monitoring. For example several alert level frame works are based on the number of countable cells per millilitre of water volume, i.e. the most severe alert level set by WHO is at >100,000 cells mL^{-1} (Chorus and Bartram, 1999). This issue is particularly relevant when *Microcystis* co-occurs with other morphologically similar genera such as *Gomphosphaeria*, *Synechocystis*, *Chroococcus*.

It was the aim of this study to compare the morphological variation of *Microcystis* in the field with the morphological characteristics as observed under culture conditions. This

was achieved by analysing the morphological variability (i) directly from colonies from field samples and (ii) from laboratory isolated strains. According to the most recent taxonomic revision of the genus *Microcystis* (Otsuka et al., 2001) all morphospecies are considered morphotypes of a single species *M. aeruginosa* KÜTZING and LEMMERMANN 1907. To strengthen taxonomic assignments, the strains were analysed genetically for the intergenic spacer region of the phycocyanin gene (PC-IGS) and the *mcy*B/E gene part of the *mcy* gene cluster of *Microcystis* (Tillett et al., 2000). The strains containing the *mcy* genes were further characterised for MC production.

Materials and methods

Isolation, purification and growth conditions of Microcystis

Integrated and net samples were first obtained from the 12 sites sampled in May and June 2004 (Kirinzi swamp, Nyabikere Crater Lake, Nkuruba Crater Lake, Lake George, Lake Edward, Nkugute Crater Lake; Lake Mburo; Lake Nabugabo; Lake Vicroria-Bunjako Bay; Lake Victoria-Murchison Bay; Lake Victoria-Napoleon Gulf and Jinja Pond) in Okello et al. (2009). During the one year study period, (May 2007 to April 2008), chapter 2; net samples were again taken in April 2008 from Saka Crater Lake, Lake George-Kahendero, Lake Edward-Katwe, Lake Mburo, Lake Victoria-Murchison Bay and Lake Victoria-Napoleon Gulf.

Microcystis sp. were isolated using a modification of BG_{11} medium ($2N_{10}$) medium containing 0.6% agarose (Bacto™ Agar, Becton, Dickinson and Company, Sparks, USA) by streaking and dilution (Rippka, 1988). Cultures were purified from epibiontic algae (*Planktolyngbya*, *Planktothrix* and *Pseudanabaena*) by repeating the streaking procedure. After each streaking step, the pure colonies were transferred into wells of tissue culture test plates 24 (Nunclon, Switzerland) filled with a mixture of $2N_{10}$ (2.5 µg mL^{-1} of cycloheximide). Incubation was at 22°C under a 12:12 h light/dark cycle with a light intensity of approximately 40 µmol m^{-2} s^{-1} provided by daylight fluorescent lamps. After three weeks the cultures were transferred to tissue culture flasks (25 cm^3 Nunclon, Switzerland) filled with a mixture of 25 mL of $2N_{10}$ (2.5 µg mL^{-1} of cycloheximide). The cultures were grown for one month and then harvested for morphological, molecular and microcystin analyses.

For genetical analysis, 1.5 mL of pure culture was sampled into a 2 mL reaction tube, centrifuged at 15000 × *g* for 10 minutes. The pellet was then used for DNA extraction (see

below). Positively screened *Microcystis* cultures for *mcy*E and *mcy*B genes were further cultured in 250 mL of $2N_{10}$ (2.5 μg mL^{-1} cycloheximide) medium in a 500 mL flat bottom conical flask and then harvested for HPLC analysis.

To avoid cultivation bias due to the isolation procedure, single colonies of *Microcystis* were isolated directly from field samples. Serial dilution was made on the field samples using BG11 medium until the *Microcystis* colonies were approximately 5 colonies per drop of medium onto the microscope glass slide. All the sub sampled colonies per drop selected were picked up by forceps under the dissecting microscope and described morphologically according to their cell size, colony diameter and type of colony formation.

Genetic analysis of strains

Extraction of DNA from *Microcystis* strains was performed as follows: 1.5 mL of culture was centrifuged at 15000 × *g* for 10 minutes and the supernatant decanted. The pellets were then suspended in 0.5 mL of TES buffer (25% w/v Saccharose; 50 mM Tris-HCl and 100 mM EDTA with pH of 8.0) and incubated on ice for 2 hours. 50μL of lysozyme (~ 5 mg mL^{-1}) was added (37°C, 1 hour) followed by 55 μL of 20% SDS and 2 μL of Proteinase K (~ 50 mg mL^{-1}). The tube was incubated for an hour at 60°C. Then 1 mL of Roti Phenol-Chloroform-Isoamyl alcohol was added, mixed and centrifuged at 15000 × *g* for ten minutes. The water phase with DNA was pipetted into a new 2 mL tube and another 1 mL of Roti Phenol-Chloroform-Isoamyl alcohol was added. The mixture was centrifuged at 15000 × *g* for ten minutes and the water phase containing the DNA was again transferred to a new tube. Finally, 1 mL of Chloroform Isoamyl alcohol was added to the transferred water phase, vortexed then centrifuged at 15000 × *g* for 10 minutes. The water phase was taken off into a new tube and 1 mL of 90% ethanol (-20°C) was added and the tube incubated on ice in the fridge for one hour to precipitate the DNA. Precipitated DNA was centrifuged at 15000 × *g* for 20 minutes and the supernatant discarded. The remaining pellet was washed with 0.5 mL of 70% ethanol (-20°C), centrifuged at 15000 × *g* for 20 minutes (4°C) and the supernatant was discarded. The pellet was air dried and resuspended in 50 μL Millipore water and the final double stranded DNA (dsDNA) concentration was determined at a wavelength of 260nm (1.O. D_{260nm} = 1 μg DNA) using a Nanodrop Spectrophotometer (ND 1000 Spectrophotometer, PEQLAB Biotechnologie GmbH, Germany).

Microcystis sp. strains were analyzed for (i) the intergenic spacer region (IGS) within the phycocyanin (PC) operon and (ii) the *mcy*B and the *mcy*E regions part of the MC biosynthesis gene cluster (Tillett et al., 2000). Primers to amplify PC-IGS (685 bp, Neilan et

al., 1995) and the first adenylation domain of *mcy*B (1,312 bp, Kurmayer et al., 2002) and the aminotransferase located in *mcy*E (472 bp, Jungblut and Neilan, 2006) have been used. The PCR thermal cycling protocol included an initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, at an annealing temperature of 50°C for 30 s, and at 72°C for 30 s was used. 50 µL of PCR products were loaded in 1% agarose gel and separated by electrophoresis during 45 minutes at 120 mV to clone the PCR product. Single PCR bands were cut out using a long wave UV lamp and then purified according to the manufacturer's protocol.

1 µL of purified DNA was added to 1.5 µL of Ligase solution and incubated overnight in a refrigerator. The following day, the mixture of ligation solution was added to thawed chemically competent *E. coli* cells, mixed briefly and put back on ice for 20 minutes. The cells were then heat shocked by incubating the tube for 60 seconds at 42°C and placed back on ice for another 60 seconds.

500 µL of SOC-media was later added to the reaction tube and the tube incubated at 37°C for 45 minutes in an orbital shaker. The transformed *E. coli* cells were spanned for 15 seconds and all the pelleted cells were plated onto LB-Agar (containing 40 µL X-gal and 40 µL IPTG). The plate was incubated overnight at 37°C and the following morning, four white colonies were isolated, each dissolved in 20 µL Millipore water and mixed several times. To the 20 µL of the suspended isolates, 3 mL of LB-media (100 g mL^{-1}) ampicilin was added and the mixture incubated overnight in an orbital shaker (250 rpm) at 37°C. Purification of the high-copy plasmids was then performed by alkaline lyses according to the QIAGEN PCR Cloning Handbook 2001 and the DNA was sequenced (accession numbers: **EU014146-EU014163**, Table 4.4). Out of the 96 strains, only 12 strains could be sequenced from the PCR products.

Phylogenetic analysis

PC-IGS Sequences (576 bp) were aligned together with all available sequences from the DDBJ/EMBL/GenBank database (128 entries, 10 June 2007) using multiple sequence alignment (Clustal W 1.8). The following entries were used: **AF195158-AF195179** (Tillett et al., 2001), **AF385368-AF385391** (Bittencourt et al., 2001), **AJ003167-AJ003183** (unpublished), **AM048614-AM048621** (Haande et al., 2007), **AY117040-AY117047** (Baker et al., 2002), **AY271724-AY271741** (Sanchis et al., 2005), **AY524848-AY524851** (Schatz et al., 2005), **AY568036** (unpublished), **AY568681-568710** (Wu et al., 2007), **AY672735-AY672742**, **AY999930- AY999932** (unpublished). The origin of isolation was inferred from

the literature: Europe (38 strains), Asia (29), Sub-saharan Africa (24), Australia (10), North America (21) and South America (16). Two entries were of unknown origin. In addition 22 *mcy*B sequences (645 bp) were obtained from the database: **AB019578** (Nishizawa et al., 1999), **AB092806- AB092807** (Yoshida et al., 2003), **AF183408** (Tillett et al., 2000), **AJ492552-AJ492560** (Mikalsen et al., 2003), **AY034601-AY034602**, **AY147795**, **AY147796** (Bittencourt, 2003), **AY568035**, **DQ184674** (Boaru et al., 2006), **DQ218313** (Ross et al., 2006).

Similarities between nucleotide sequences were calculated using the PHYLIP package (Version 3.6 alpha; Felsenstein, 1989). Maximum likelihood analysis was used to estimate nucleotide substitution parameters under a general time-reversible nucleotide substitution model by estimating the gamma distribution for variable rates among sites. Ambiguous sites were removed and the discrete gamma algorithm was used to approximate the continuous gamma distribution using five categories of rates (ncatG = 5) in the program BASEML in the PAML package (Version 3.14, Yang, 1997). Phylogenetic trees were also constructed using (i) neighbour-joining from the nucleotide sequences distance matrix (calculated using Kimura's substitution model) and (ii) maximum parsimony from nucleotide sequences using the PHYLIP package.

The best fit of the phylogenetic tree (Fig. 4.2) showed (log-) likelihood lnL = -1837 with estimates for the transition/transversion ratio κ = 2.3588 and the shape parameter α = 0.13857. Numbers at nodes indicate the percent bootstrap frequency (100 replicates) obtained from maximum likelihood/neighbor-joining/maximum parsimony calculated using the PHYLIP package.

The best fit of the maximum likelihood model (Fig. 4.3) showed (log-) likelihood lnL = -3310 with estimates for the transition/transversion ratio κ = 1.5473 and the shape parameter α = 0.6048. Numbers at nodes indicate the percent bootstrap frequency (100 replicates) obtained from maximum likelihood/neighbor-joining/maximum parsimony calculated using the PHYLIP package.

The frequency of the occurrence of PC-IGS genotypes among clusters A-F (*sensu* Sanchis et al., 2005) of the phylogeny (Fig. 4.2) was statistically compared using chi-square statistics for 6×2 contingency tables. Proportions with an expected frequency <1 were not tested (Jongman et al., 1995).

Microcystin analysis

The pure cultures were filtered onto a pre-weighed GF/C filter dried at 50°C overnight for the determination of the dry weight (>10 mg). Cells collected on filter were extracted in 1.5 mL of 50% methanol (v/v). The mixture was agitated for 60 minutes at 250rpm and centrifuged at 15000 × *g* for another 10 minutes. The clear supernatant was pipetted into a new eppendorf tube and the extraction procedure was repeated twice. The extract was concentrated to dryness at 30°C using a vacuum centrifuge (Eppendorf AG, Hamburg, Germany). The residues were resuspended in 600 µL of 50% methanol, centrifuged at 15000 × *g* for 10 minutes and the clear supernatant was injected into HPLC-DAD on a LiChrosper 100, octydecyl silane, 5-µm LiChroCART 250-4 cartridge column (Merck, Darmstadt, Germany). A linear gradient of aqueous acetonitrile (Carl Roth GmbH, Kalrsruhe, Germany) mixed with 0.05% trifluoroacetic acid (TFA) was used at a flow rate of 1 mL min^{-1} increasing from 30% acetonitrile to 70% during 42 minutes (Lawton et al., 1994). MCs were quantified at 240 nm and the concentration of all MC variants was determined as concentration equivalents of [D-MeAsp, D-Mdha]-MC-LR (Cyanobiotech GmbH, Berlin, Germany). The concentration of MC-LR was calculated from the regression curve y=1885.3x-6.8775, where y is µg of MC-LR eq. and x is mAU recorded at 240 nm.

HPLC peaks identified as MCs were collected manually and concentrated to dryness. The collected fractions were re-dissolved in 10 µL of 100% methanol. 1 µL of each sample was then loaded onto a steel template (CellPath, Newtown Powys, Great Britain) and 1 µL of matrix solution (2,5-dihydroxy-benzoic acid (DHB) solubilised in 50% acetonitrile, 0.03 % trifluoroacetic) was loaded immediately. Samples were then analysed by matrix-assisted laser desorption/ionisation voyager elite time-of-flight mass spectrometry (PerSeptive BioSystems, Framingham MS, USA) as described in Erhard et al. (1999) and Welker et al. (2002). Identification of particular MCs was done by post source decay (PSD) fragment analysis as described in Erhard et al. (1997) and Fastner et al. (1999).

Results

Morphological analysis

A total of 96 *Microcystis* strains were isolated and purified (Table 4.1). All strains were growing in microscopic to macroscopic colonies. Under the microscope, the cells were spherical and irregularly distributed in the colourless mucilage and contained gas vesicles.

Following the Rules of the Bacteriological Code, morphospecies were considered morphological variations of individuals of one species (Otsuka et al., 2001). The following morphospecies were distinguished: *Microcystis aeruginosa*, *M. botrys* and seven unidentified *Microcystis* strains(Table 4.1).

Table 4.1. Number of pure strains of *Microcystis* (morphospecies) isolated from six sites in Ugandan freshwaters (May and June 2004 and April 2008).

	Microcystis aeruginosa	*Microcystis flos-aquae*	*Unidentified*	Total	MC-producer
Lake Saka	15	11	1	27	0
Lake George (Kahendero)	10	5	0	15	2
Lake Edward (Katwe)	7	1	2	10	7
Lake Mburo	8	3	4	15	1
Lake Victoria (Murchison)	2	3	0	5	0
Lake Victoria (Napoleon)	15	9	0	24	0
Total	57	32	7	96	10

From the field, a total of 895 single colonies were isolated between March 2007 to April 2008 (Table 4.2) and included the following morphospecies: *M. aeruginosa, M. botrys, M. flos-aquae* and *M. wesenbergii*. Although *M. wesenbergii* (Fig. 4.1) was not found within cultures, its colonies of were isolated from field samples from L. Saka, L. George (Kahendero) and L. Edward (Katwe). In the majority of samples, *M. flos-aquae* occurred most frequently (>41%). In contrast in Lake Victoria (Napoleon Gulf), *M. botrys* was found to be more dominant (67.7%). Overall, *M. flos-aquae* dominated (41.9%), while *M. aeruginosa* (25.5%) and *M. wesenbergii* (8.0%) was less abundant.

Table 4.2. *Microcystis* (morphospecies) field isolates from six sites in Ugandan lakes obtained between March 2007 to February 2008. Percentage proportion in parenthesis. Mean cell size ± Standard Error.

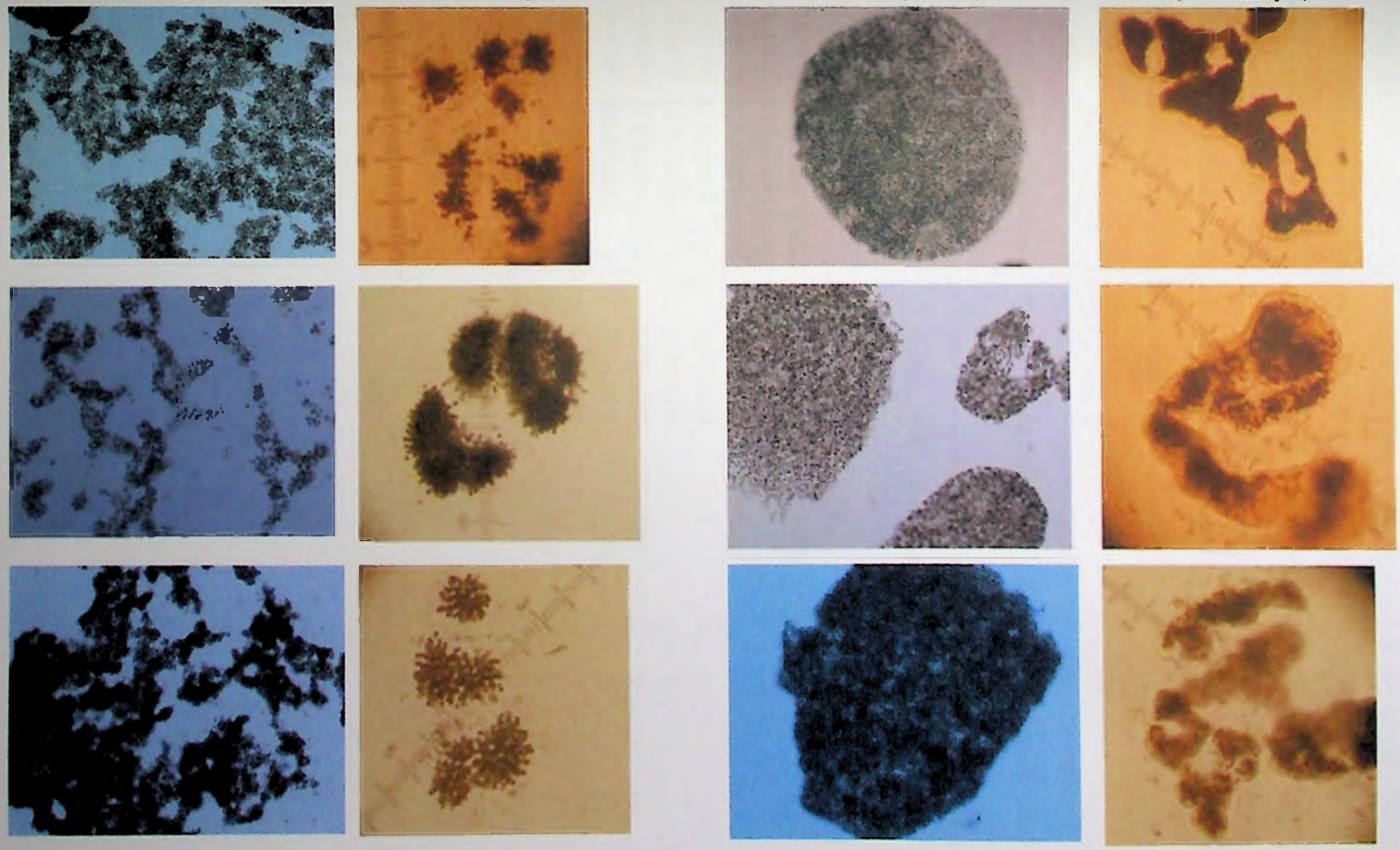
		Microcystis aeruginosa	*Microcystis botrys*	*Microcystis flos-aquae*	*Microcystis wesenbergii*	Total (n)
	Colony diameter	386±13.6	366±11.8	372±10.8	360±21.0	
	Cell size (µm)	5.4±0.1	6.1±0.1	5.0±0.1	5.3±0.1	
Lake Saka	Mar 07	11 (11.2)	0	47 (48.0)	40 (40.8)	98
Lake George (Kahendero)	Mar 07, Apr 07	58 (29.0)	43(21.5)	90 (45.0)	9 (4.5)	200
Lake Edward (Katwe)	Apr 07, Sept 07	52 (26.3)	35 (17.7)	88 (44.4)	23 (11.6)	198
Lake Mburo	Sept 07, Feb 08	74 (37.0)	44 (22.0)	82 (41.4)	0	200
Lake Victoria (Murchison)	Sept 07	26 (26.0)	31 (31.0)	43 (43.0)	0	100
Lake Victoria (Napoleon)	Sept 07	7 (7.1)	67 (67.7)	25 (25.3)	0	99
Total (n)		228 (25.5)	220 (24.6)	375 (41.9)	72 (8.0)	895

Table 1: Comparison of the field and culture traits of *Microcystis* (morphospecies) isolated from the Ugandan freshwaters

	Microcystis aeruginosa	*Microcystis botrys*	*Microcystis flos-aquae*	*Microcystis wesenbergii*
Figure	Fig. 1 A	Fig. 1 B	Fig. 1 C	Fig. 1 D
Colonies shape	net like, irregular outline with distinct holes	more or less spherical sub-colonies without holes	spherical to irregular without holes, no lobes	spherical to elongate
Cells	spherical, blue-green in colour with many aerotopes and sparsely to densely distributed	spherical with many aerotopes, arranged irregularly and densely aggregated in mucilaginous sub-colonies	spherical with aerotopes, most densely packed cells	spherical with many aerotopes, more or less evenly spread, sparsely distributed
Margin	colourless mucilage with distinct narrow margin around the cells	wide, colourless mucilage radiating from sub-colonies	smooth edge and indistinct thin mucilage	distinct, refractive mucilage edge

Microcystis wesenbergii.

A 200× (Laboratory strain) B 400× (Field sample) C 200× (Laboratory strain) D 400× (Field sample)



Genetic analysis of strains

All of the 96 *Microcystis* strains showed a PCR product of the PC-IGS region (~700 bp). Notably only 13 (13.5%) of the strains revealed a PCR product of the *mcy*B (1400 bp) or the *mcy*E (400 bp) gene that are indicative of *mcy* gene clusters (Table 4.4). The majority of the *mcy* containing strain originated from L. Edward (seven). Two *mcy* containing strains originated from L. George, while one *mcy* containing strain was isolated from L. Mburo and a pond in Jinja each. All of the strains isolated in 2004 were sequenced for PC-IGS (602 bp) and for *mcy*B gene (645 bp).

Table 4.4. *Microcystis* strains isolated from eight sites in Ugandan freshwaters, screened for the presence/absence of *mcy*B and *mcy*E indicative of potential MC production, and analyzed by HPLC-DAD for microcystin (MC) contents.

Strain ID	Sampling date	Origin	Accession No. PC-IGS	Accession No. *mcy*B	*mcy* B	*mcy* E	HPLC	DW (mg)	µg MC-LR eq. mg^{-1} DW
IR14	May 04	L. George (Kahendero)	EU014155		-	-	-		
MB5	May 04	L. Edward (Katwe)	EU014146	EU014163	+	+	-		
ED6	May 04	L. Edward (Katwe)	EU014147	EU014158	+	+	+	12.3	4.4
II7B7	June 04	Nkugute Crater Lake	EU014149		-	-	-		
II8A8	June 04	L. Edward (Katwe)	EU014150	EU014160	+	+	+	34.1	2.3
IR50	May 04	L. Edward (Katwe)	EU014156		-	-	-		
2A3	May 04	L. Edward (Katwe)	EU014148		-	-	-		
20A2	June 04	L. Edward (Katwe)	EU014152	EU014161	+	+	-		
20A5	June 04	Pond (Jinja)	EU014153	EU014162	+	+	-		
21A1	June 04	L. Edward (Katwe)	EU014154		-	-	-		
6C5	May 04	Lake Mburo	EU014157	EU014159	+	+	+	56.1	0.4
19G6	June 04	L. Victoria (Napoleon)	EU014151		-	-	-		
M02	April 08	L. Saka			-	-			
M03	April 08	L. Saka			-	-			
M04	April 08	L. Saka			-	-			
M05	April 08	L. Saka			-	-			
M06	April 08	L. Saka			-	-			
M07	April 08	L. Saka			-	-			
M09	April 08	L. Saka			-	-			
M12	April 08	L. Saka			-	-			
M13	April 08	L. Saka			-	-			
M14	April 08	L. Saka			-	-			
M15	April 08	L. Saka			-	-			
M17	April 08	L. Saka			-	-			
M19	April 08	L. Saka			-	-			
M20	April 08	L. Saka			-	-			
M21	April 08	L. Saka			-	-			
M22	April 08	L. Saka			-	-			
M25	April 08	L. Saka			-	-			

Strain ID	Sampling date	Origin	Accession No. PC-IGS	Accession No. *mcy*B	*mcy* B	*mcy* E	HPLC	DW (mg)	µg MC-LR eq. mg^{-1} DW
26	April 08	L. Saka			-	-			
27	April 08	L. Saka			-	-			
29	April 08	L. Saka			-	-			
30	April 08	L. Saka			-	-			
31	April 08	L. Saka			-	-			
32	April 08	L. Saka			-	-			
34	April 08	L. Saka			-	-			
40	April 08	L. Saka			-	-			
41	April 08	L. Saka			-	-			
43	April 08	L. Saka			-	-			
07	April 08	L. Saka			-	-			
10	April 08	L. Saka			-	-			
I 02	April 08	L. George (Kahendero)			-	-			
I 08	April 08	L. George (Kahendero)			+	+	+	47.4	0.2
I 19	April 08	L. George (Kahendero)			-	-			
I 22	April 08	L. George (Kahendero)			-	-			
I 33	April 08	L. George (Kahendero)			-	-			
I 37	April 08	L. George (Kahendero)			-	-			
I 38	April 08	L. George (Kahendero)			-	-			
I 40	April 08	L. George (Kahendero)			-	-			
I 42	April 08	L. George (Kahendero)			-	-			
I 46	April 08	L. George (Kahendero)			-	-			
II 10	April 08	L. George (Kahendero)			-	-			
II 14	April 08	L. George (Kahendero)			-	-			
II 18	April 08	L. George (Kahendero)			-	-			
II 21	April 08	L. George (Kahendero)			-	-			
II 46	April 08	L. George (Kahendero)			+	+	+	54.6	0.1
d 01	April 08	L. Edward (Katwe)			+	+	+	68.7	0.3
d 03	April 08	L. Edward (Katwe)			-	-			
d 06	April 08	L. Edward (Katwe)			-	-			
d 08	April 08	L. Edward (Katwe)			+	+	+	19.0	0.4
d 10	April 08	L. Edward (Katwe)			+	+	+	26.2	0.1
d 11	April 08	L. Edward (Katwe)			+	+	+	45.6	0.3
d 12	April 08	L. Edward (Katwe)			+	+	+	39.0	0.1
d 13	April 08	L. Edward (Katwe)			-	-			
b M01	April 08	L. Mburo			-	-			
b M02	April 08	L. Mburo			-	-			
b M05	April 08	L. Mburo			-	-			
b M06	April 08	L. Mburo			-	-			
b M07	April 08	L. Mburo			-	-			
b M09	April 08	L. Mburo			-	-			
b M11	April 08	L. Mburo			-	-			
b M13	April 08	L. Mburo			-	-			
b M14	April 08	L. Mburo			-	-			
b M19	April 08	L. Mburo			-	-			
b M20	April 08	L. Mburo			-	-			

Strain ID	Sampling date	Origin	Accession No. PC-IGS	Accession No. *mcy*B	*mcy* B	*mcy* E	HPLC	DW (mg)	µg MC-LR eq. mg^{-1} DW
Mb M21	April 08	L. Mburo			-	-			
Mu 07	April 08	L. Victoria (Murchison)			-	-			
Mu 09	April 08	L. Victoria (Murchison)			-	-			
Mu 11	April 08	L. Victoria (Murchison)			-	-			
Mu 17	April 08	L. Victoria (Murchison)			-	-			
Mu 18	April 08	L. Victoria (Murchison)			-	-			
Nap 02	April 08	L. Victoria (Napoleon)			-	-			
Nap 03	April 08	L. Victoria (Napoleon)			-	-			
Nap 05	April 08	L. Victoria (Napoleon)			-	-			
Nap 06	April 08	L. Victoria (Napoleon)			-	-			
Nap 10	April 08	L. Victoria (Napoleon)			-	-			
Nap 12	April 08	L. Victoria (Napoleon)			-	-			
Nap 13	April 08	L. Victoria (Napoleon)			-	-			
Nap 16	April 08	L. Victoria (Napoleon)			-	-			
Nap 17	April 08	L. Victoria (Napoleon)			-	-			
Nap 19	April 08	L. Victoria (Napoleon)			-	-			
Nap 23	April 08	L. Victoria (Napoleon)			-	-			
Nap 24	April 08	L. Victoria (Napoleon)			-	-			
Nap 25	April 08	L. Victoria (Napoleon)			-	-			
Nap 27	April 08	L. Victoria (Napoleon)			-	-			
Nap 28	April 08	L. Victoria (Napoleon)			-	-			
Nap 29	April 08	L. Victoria (Napoleon)			-	-			
Nap 31	April 08	L. Victoria (Napoleon)			-	-			
Nap 32	April 08	L. Victoria (Napoleon)			-	-			

Phylogenetic analysis

To assign the strains to the existing phylogenetic classification (Bittencourt et al., 2001; Sanchis et al., 2005; Haande et al., 2007) a phylogenetic tree was constructed using PC-IGS obtained from 12 strains (variability 6.8%, 576 bp) and all available PC-IGS sequences from the database (128 entries). The total variability was due to single nucleotide polymorphism (8.64%). The phylogenetic tree resulted in six main clusters A-F (Fig. 4.2), that have been identified previously (Sanchis et al., 2005). The strains isolated during this study were assigned to cluster A (variability 2.95%) and cluster D (variability 4.5%). Two strains remained unassigned (R14, 21A1). Taking all strains together, strains isolated from Sub-Saharan Africa showed a non-random distribution, i.e. the occurrence of strains of cluster D (n=43) significantly depended on the origin of isolation: 17 of 24 strains isolated from Sub-Saharan Africa occurred in cluster D (χ^2 (df=5), $p<0.01$). In contrast the occurrence of strains of cluster F (n=61) also depended on the origin of isolation (χ^2 (df=5), $p<0.01$), i.e. 39 of 67 strains isolated from Europe and Asia occurred in cluster F.

Among the strains isolated in 2004, six strains were found to contain *mcy*B and *mcy*E indicative of the *mcy* synthetase gene cluster (variabilility 5.9%, 1,312 bp). Using 22 additional entries from the database, a phylogenetic tree (645 bp) was constructed reflecting a total variability of 49% (Fig. 4.3). All the six strains isolated from Uganda formed one distinct cluster in the phylogenetic tree, that was clearly distinct from the *mcy*B1(B) cluster and the *mcy*B1(C) cluster identified by Mikalsen et al. (2003). Unique genotypes were also observed for the corresponding gene region of *mcy*C (variability 6.2%, 1,314 bp), however, only three sequence entries from Genbank were available for comparison (data not shown).

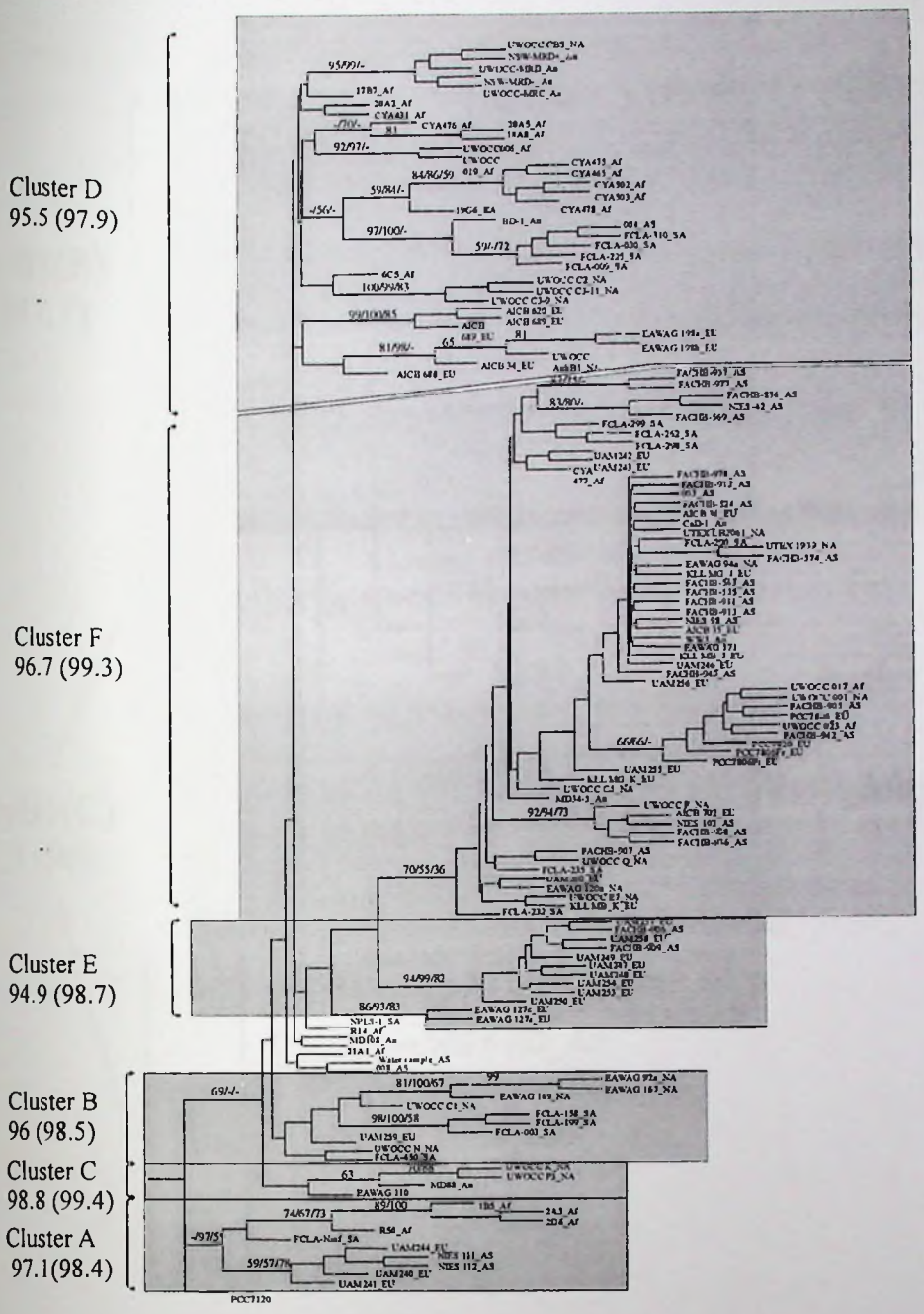


Fig. 4.2. Maximum-likelihood tree of *Microcystis* strains (140 sequences) inferred from *PC*-IGS gene sequences (602 bp). Only bootstrap values of >50% are shown. Minimum and mean (parentheses) pairwise percent similarities are shown for each cluster. *Nostoc* strain PCC7120 was used as an outgroup. For each strain the last two letters indicate the origin of isolation (EU, Europe, AS, Asia, Af, Sub-saharan Africa, Au, Australia, NA, North America, SA, South America). Strains in bold have been sequenced during this study.

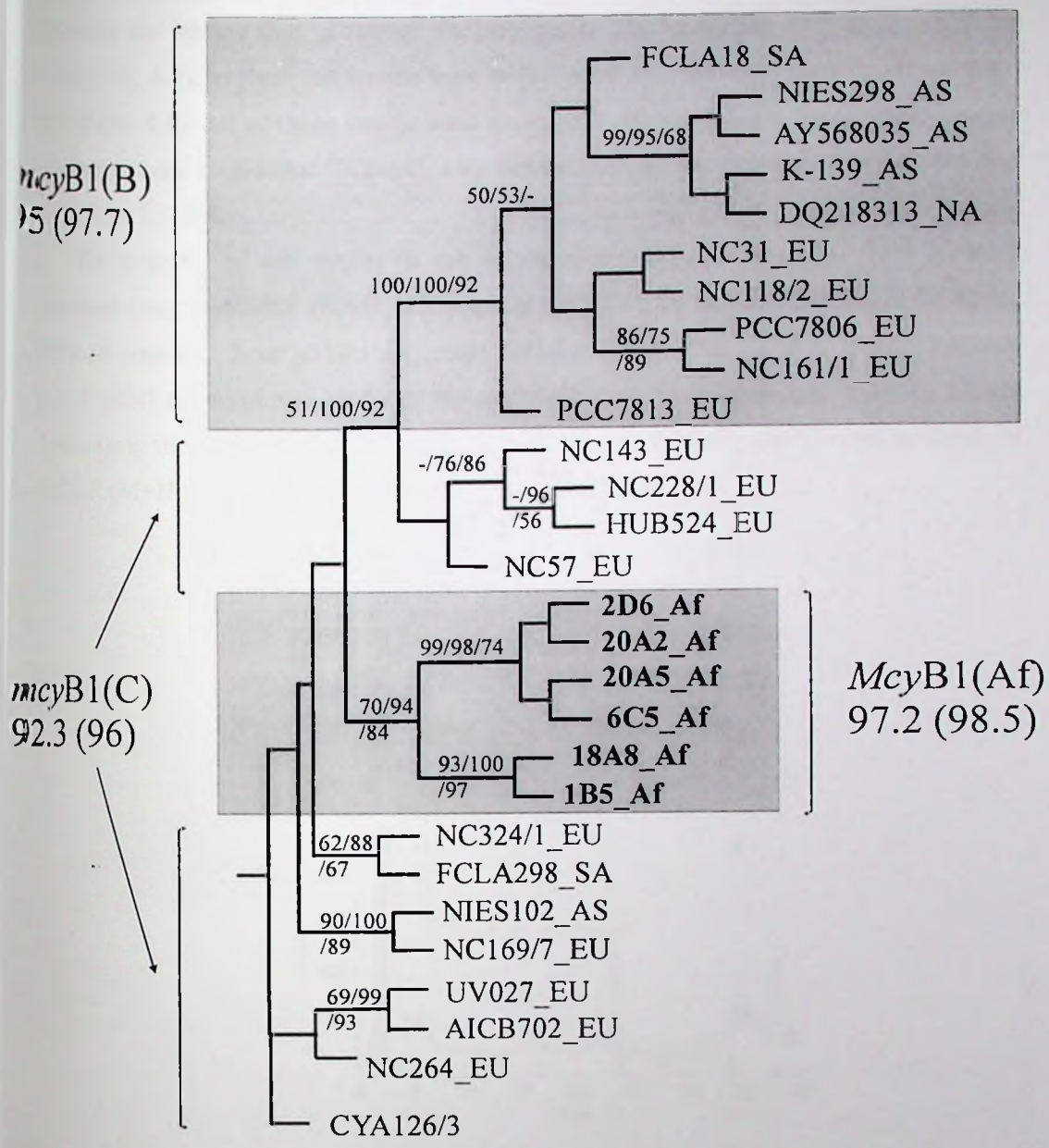


Fig. 4. 3. Maximum-likelihood tree of *Microcystis* strains (28 sequences) inferred from *mcy*B (645 bp) part of the MC synthetase gene cluster. Only bootstrap values of >50% are shown. Minimum and mean (parentheses) pairwise percent similarities are shown for each cluster. *Planktothrix* CYA126/8 was used as an outgroup. The origin of isolation is given for each strain as in Fig. 4.2. Strains in bold have been sequenced during this study.

Microcystin production in Microcystis strains

Typically the strains that contained the *mcy* genes also contained MC as revealed by HPLC (Fig. 4.4). In total, ten strains were found to produce 0.1 - 4.4 μg MC-LR eq. mg^{-1} DW (Table 4.4). All of those strains were assigned to *M. aeruginosa*. Seven of the strains originated from L. Edward (Katwe), two strains from L. George (Kahendero) and one strain from L. Mburo.

The majority of the strains (6 out of 10) contained high amount of the recently described [Asp^3]-MC-RY (M+H 1031) and/or MC-RY (M+H 1045) ≥80% of the total MC). In contrast, three strains contained MC-RR (M+H 1038) and/or [Asp^3]-MC-RR (M+H 1024) constituting ≥90% of the total MC. One strain contained MC-YR (M+H 1045). Only the strains that contained MC-RY were also found to contain low amounts of MC-LR (M+H 995) at ≤2.3% of the total MC (Table 4.5).

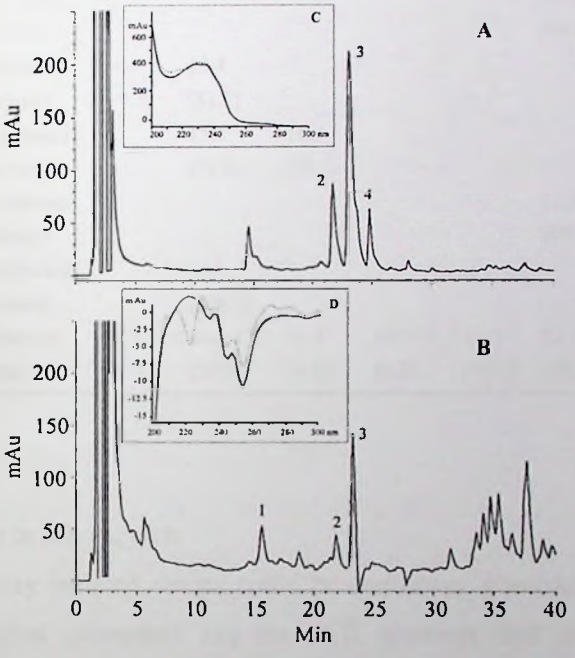


Fig 4.4. Chromatogram of an extract of (A) *Microcystis* sp. strain 18A8 and (B) a water sample from L. Edward (5 June 2004, net sample) analysed by HPLC-DAD for MCs. The signals were recorded at 240 nm using a linear gradient of aqueous acetonitrile (Lawton et al. 1994). The peaks at min 15.6 (1), 21.7 (2), 23.2 (3), 24.8 (4) constitute the following MCs variants: (1) MC-RR (M+H 1038); (2) [Asp^3]-MC-RY (M+H 1031); (3) MC-RY (M+H 1045) and (4) Unknown MC (M+H 1024). Inserts: (C) original spectrum of the peak at 23.3 min (dotted line) and spectrum of [$MeAsp^3$, $Mdha^7$]-MC-YR (solid line). (D) Comparison of the first order derivative of the peak at 23.3 min (dotted line) with the first order derivative of [$MeAsp^3$, $Mdha^7$]-MC-YR (solid line).

Table 4.5. Microcystin variants retention time and their proportion (in parentheses) in total MC content for all MC-producing strains of *Microcystis* sp

Group	Strain ID	Sampling date	Origin	MC1 1024 [Asp³] MC-RR	MC2 1038 MC-RR	MC3 1045 MC-YR	MC4 995 MC-LR	MC5 1031 [Asp³] MC-RY	MC6 1045 MC-RY	MC7 1024 NK	MC8 1031 NK
1	2D6	May 04	L. Edward (Katwe)	13.7 (0.6)	15.3 (0.7)			21.7 (41.1)	23.1 (49.6)	24.8 (6.8)	
1	18A8	June 04	L. Edward (Katwe)				19.7 (0.4)	21.7 (20.3)	23.1 (65.5)	24.8 (12.3)	28.0 (1.3)
1	6C5	May 04	Lake Mburo	13.7 (1.9)	15.3 (2.5)		19.8 (0.5)	21.7 (45.4)	23.2 (49.7)		
1	GI 08	April 08	L. George (Kahendero)				20.4 (2.3)		23.2 (97.7)		
2	GII 46	April 08	L. George (Kahendero)	14.4 (31.9)	15.4 (63.1)				23.2 (5.0)		
1	Ed 01	April 08	L. Edward (Katwe)						23.2 (94.8)		28.2 (2.5)
2	Ed 08	April 08	L. Edward (Katwe)	14.4 (18.3)	15.4 (81.7)						
3	Ed 10	April 08	L. Edward (Katwe)		15.5 (51.8)	18.5 (48.2)					
2	Ed 11	April 08	L. Edward (Katwe)						23.2 (99.3)		28.2 (0.7)
1	Ed 12	April 08	L. Edward (Katwe)		15.7 (100.0)						
			Retention Time	13.7-14.4 (5.3)	15.3-15.7 (30.0)	18.5 (4.8)	19.7-20.4 (0.3)	21.7 (10.7)	23.1-23.2 (46.2)	24.8 (1.9)	28.0-28.4 (0.5)

Discussion

Morphological variation in Microcystis

In this study, all laboratory isolated strains could be undoubtedly assigned to *Microcystis* due to both morphological characters and the PCR products that were obtained and sequenced. Since a total of 96 strains were isolated, it seems unlikely that a possibly co-occurring *Microcystis*-like cyanobacteria such as *Synechocystis* have been missed. *Synechocystis* PCC 6803 is known to grow well in BG_{11} medium and under the culture conditions applied (Rippka, 1988). Secondly, the overall accordance in morphospecies composition both in the field samples and in the culture collections implies that the identification of *Microcystis* under the microscope is reliable.

Consequently, microscopic counting of *Microcystis* in the field can provide a reasonable estimate of *Microcystis* cell numbers. This issue is of relevance as it was shown

hat *Microcystis* so far is the major MC producer in Ugandan freshwater habitats (Okello et al., 2009). *Microcystis* cell number therefore can be used to predict MC concentration in field samples. Okello et al. (2009) believes that a monitoring approach that is based on regular phytoplankton counting under the microscope combined with occasional determination of MC concentration (twice a year) will be most effective in those countries where more skilful technology for chemical analyses cannot be maintained. Since MC concentration can be reliably determined from dried samples, samples can be sent for analysis in reference laboratories on a routine basis.

Geographic variation in Microcystis

Two morphospecies *M. novaceckii* and *M. viridis*, both occurring in the temperate climate zone were not observed in this study. While *M. novaceckii* can only be discriminated from *M. botrys* by the absence of radial structure in the mucilage (Via-Odorika et al., 2004), *M. viridis* always grows in packet-like subcolonies that cannot be observed within other morphospecies (Komarek and Anagnostidis, 1999). Consequently it is unlikely that *M. viridis* was overlooked in the course of the colony description isolated from field samples under the microscope.

The PC-IGS genotype that has been sequenced showed a non random distribution among the phylogenetic clusters A-F that have been reported from other studies (Bittencourt et al., 2001; Sanchis et al., 2005; Haande et al., 2007). Particularly, cluster F containing the maximum number of *Microcystis* strains (n = 61) was found to be underrepresented and contained only one strain NIVA-CYA 477 isolated from Murchison Bay by Haande et al. (2007). Such a non random distribution could be explained by taking into account the clonal growth of populations and the resulting linkage disequilibrium in the genetic population structure (Maynard-Smith et al., 1993). For example, for the filamentous toxic cyanobacterium *Nodularia* occurring in the Baltic Sea, distinct geographic differences in allele frequency in populations separated over 800 km have been reported (Barker et al., 2000). The same authors concluded that genetic exchange occurs among geographically separated populations, but the frequency is not high enough for the loci to be in linkage equilibrium. Notably, high genetic exchange was reported for the benthic filamentous cyanobacterium *Microcoleus chthonoplastes* and no evidence for geographically caused differences in genetic population structure was found (Lodders et al., 2005). From the results of this study, it is concluded that specific alleles of PC-IGS in *Microcystis* showed a geographic dependence in their frequency of occurrence.

Variation of microcystin production

Only 13 strains (13.5%) of the 96 strains were found to contain the *mcy* genes. Similarly Haande et al. (2007) also reported a low percentage of MC-producing strains isolated from East Africa. Overall studies reporting the isolation of *Microcystis* from various geographic regions indicated a rather low proportion of MC-producing strains: L. Kasumigaura (n = 68, 39%, Shirai et al., 1991); L. Grand-Lieu (n = 98, 16%, Vezie et al., 1998); L. Wannsee (n = 22, 45%, Rohrlack et al., 2001); L. Mikata (n = 61, 20%, Yoshida et al., 2005).

Although the cultivation of strains *mcy* lead to a biased representation of the proportion of MC-producing strains (Wilson et al. 2005), the results in general imply that MC-production in water is due to the minor part of the *Microcystis* population.

Within the MC-producing strains, three groups of strains in the MC variants have been found: (1) strains containing MC-RY (M+H 1031, 1045), (2) strains containing MC-RR (M+H 1024, 1038) and (3) strains containing MC-RR (M+H 1038)/MC-YR (M+H 1045). In contrast, Kurmayer et al. (2002) and Mikalsen et al. (2003) reported the occurrence of either strains producing MC-LR (M+H 995)/MC-YR (M+H 1045) only (the *mcy*B genotype, strain PCC7806) or strains producing mainly MC-RR (M+H 1038), (the *mcy*B(C) genotype, strain HUB524). The identification of unique *mcy*B and *mcy*C genotype in strains producing MC-RY may be linked to this structural variation. It has been shown previously that specific genotypes of the *mcy*B gene, that is involved in the activation and condensation of the variable amino acid in the MC-XR position can indeed be linked to specific MC-variants (Mikalsen et al., 2004). It remains to be shown whether the discovery of new Ugandan *mcy*B genotypes can explain the minor production of MC-LR in the habitats studied. This issue is of relevance as MC-LR is known to show a 10-fold higher toxicity to humans and mammals when compared with MC-RR.

Conclusion

Four major *Microcystis* species (*M. aerugionsa*, *M. botrys*, *M. flos-aquae* and *M. wesenbergii*) were identified from the six freshwater sites sampled in this study. Counting *Microcystis* cell numbers via the inverted microscope provided reliable estimation that it is recommended for use as a monitoring tool in Uganda where the state of art technology is still lacking for the MC concentration determination.

The *Microcystis* strains from Ugandan freshwater habitats occupied cluster D on the phylogenetic tree that was dependent on the origin of isolation from the sub-saharan Africa. This geographical variation linked well with the newly discovered MC-RY which

ontinued to dominate in the strain samples. MC-LR the most important MC variant in the emperate region constituted the least proportion of the MC in Ugandan water samples.

Out of the 11 MC variants identified from field samples, nine of them occurred in the train samples. All the MC-producers were morphologically identified as *M. aeruginosa* confirming our earlier finding of *Microcystis* as the major MC-producer in Ugandan freshwater habitats. However, the MC-production in Ugandan water in general is due to the minor part of the *Microcystis* population despite their higher biovolume.

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Chapter 5

Cyanobacteria and microcystin production: Synthesis

"Where shall I begin, your majesty?" he asked.
"Begin at the beginning, "the King said gravely,
"and go on till you come to the end; then stop."

(*Lewis Carroll - Alice in the Wonderland*)

obacteria and microcystin production: Synthesis

previous chapters, the results of three studies were presented and discussed. In nthesis chapter, the key results that provide invaluable insights into MC tion in Ugandan freshwaters habitats are discussed and conclusions and mendations are presented.

rance of eutrophication to cyanobacterial dominance and microcystin ction

phication was recognised as a pollution problem in many western European and North can lakes and reservoirs in the middle of the twentieth century. Since then, it has me more widespread, on a global scale and has caused deterioration in the aquatic nment and serious problems for water use, particularly for drinking-water treatment rus and Bartram, 1999). Dokulil and Teubner (2000) reviewed some of the causes and equences of cyanobacterial dominance with several hypotheses noting that when lakes me eutrophic, frequently the diversity of the phytoplankton assemblage decreases ately leading to the dominance of cyanobacteria. They quoted the increased input of ents as the primary cause for cyanobacterial dominance. In this study, the long term inance by filamentous species (e.g. *Planktolyngbya*) is related to shallow lake depth, e colony forming species (e.g. *Anabaena* and *Microcystis*) are typically dominating in er lakes (Scheffer et al., 1997). This theory has also been defined by Reynolds et al. 2) as due to their buoyancy, large colonies are able to migrate vertically. Therefore they exploit the light in the water column more efficiently than single cells that are entrained e water column.

Cyanobacterial dominance causes negative effects such as reduced transparency, eased biodiversity, elevated primary production and oxygen depletion, which may result massive fish kills. Cyanobacteria further produce odour and taste compounds as well as ins (Chorus and Bartram, 1999; Reynolds, 1991). The most prominent MC producing obacteria genera are *Anabaena*, *Microcystis* and *Planktothrix* (Carmichael, 1992; Chorus Bartram, 1999). Of these genera, *Microcystis* globally has been demonstrated to be the

inant MC producer (Kaebernick et al., 2000; Lehman et al., 2005; Li et al., 2007; 2008; Xu et al., 2008).

results in Chapter 2, 3 and 4 of this thesis conform to general theory on terial dominance in lakes. Nutrient concentrations were high in most lakes including rally hypertrophic shallow lakes (e.g. Lake George) and the human-induced ed lakes (e.g. Lake Victoria and Saka Crater Lake). Only two out of the thirteen lakes were mesotrophic while the rest were either eutrophic or hypertrophic, showing cterial dominance (>50% of the phytoplankton in total). The more shallow phic lakes (George, Edward, Mburo and Saka) inhabited *Microcystis*. *Planktothrix* d in the shallow Saka Crater Lake only. In contrast, *Anabaena* occurred consistently Victoria (Murchison Bay and Napoleon Gulf). Since all lakes inhabited *Microcystis*, as therefore a clear potential for microcystin production within each of these habitats

With the exception of very few sampling dates in Lakes George, Lake Victoria-ison Bay and Lake Victoria-Napoleon Gulf, MC was detected in all samples all year There were only minor variations in MC concentrations seasonally when comparing ny and the dry season. There was however, a significant difference between sites both regards to MC production and the relative abundance of MC structural variants (Chi e test, $p<0.01$). For example, MC-RY and [Asp^3]-MC-RY the two newly identified MC nts from this study occurred most frequently, while MC-RR and MC-YR were less ent. MC-LR had the lowest frequency occurring only in 11% of all the samples during me year study period. One yet unknown MC variant ($M+H^+$ 1063) persistently occurred ake Victoria-Napoleon Gulf. The finding that MC-LR occurred less frequently in Uganda compared with the frequency of occurrence in the temperate climatic regions supports idea that geographic differences should be considered when interpreting consequences of n production on a global scale (Codd et al., 2005).

The MC concentrations from all the sampling sites showed highly significant positive ear relationships with the *Microcystis* cell numbers. On the other hand, the study revealed iations in the average MC contents per cell between the lakes (Saka, George, Edward, uro, Victoria-Murchison Bay and Victoria-Napoleon Gulf). Indeed from the genetical alyses that were performed, all indicated that the cyanobacterium *Microcystis* is the only C producer in these habitats. Two major lakes (i.e. L. Kyoga and L. Albert) have not been cluded in this database however, since both lakes are interconnected to Lake Victoria via e Nile, it is unlikely that the conclusions of chapters 2 and 3 will change.

ce the *Microcystis* cells counts were highest from samples from the shallow Lake one would have expected the maximum MC concentrations in Lake George. In *Microcystis* consistently showed the lowest MC cell quotas (0-1.24 fg cell^{-1}) while aka had the highest MC cell contents (14-144 fg cell^{-1}). On average, the other four re all intermediate. Notably Lake George is known to contain high amounts of *stis* at least since 1973 (Ganf, 1974). In contrast, Lake Saka has only been recently ed by human activities such as land clearing for agriculture and introduction of Nile risman et al., 2001). One could speculate that the number of *Microcystis* genotypes ng MC might be influenced by the relative age of the *Microcystis* population in the habitats.

and inactive microcystin genotypes in cyanobacterium Microcystis and ance of microcystin producers

icrocystis is a common planktonic freshwater cyanobacterium which frequently forms evelopments in surface waters used for recreation and as drinking water source (Via-ka, 2004). In their natural populations, they are often referred to as *Microcystis nosa* although taxonomists distinguish many more species separated by characters such size and shape of the cells and the colonies (Otsuku et al., 2001; Visser et al., 2005). forming *Microcystis* have frequently been characterised to produce MC which is of concern (Carmichael, 1992; Jochimsen et al., 1998; Kuiper-Goodman et al., 1999). onitoring and potentially predicting MC concentrations in water has therefore become portant task not only for public health, but also for limnologists (Via-Odorika, 2004). rticular interest is the question of regulation of MC concentrations in natural tions of MC-producing cyanobacteria such as *Microcystis*. It has been noted that the *ystis* populations not always contain actively producing MC genotypes but also *mcy* pes that stopped to produce MC (Via-Ordorika et al. 2004). Typically the inactivation C synthesis has been caused by mutations, such as deletions or insertions of parts of the involved in MC synthesis (Christiansen et al. 2006). In this study three strains ned the *mcy* genes, however did not produce MC (Chapter 5). Unfortunately the strains to be inactive in this study were over grown by green algae and lost. The occurrence of inactive *mcy* genotypes in the field, however, might interfere with the applicability of based detection of *mcy* genotypes in field samples and the potential aim to predict MC trations. However, similar to earlier reports on *Microcystis* sp. the proportion of these

mcy genotypes was low (references in Via-Ordorika et al. 2004). In chapters 2, 3 the *mcy*-type detection could be shown to be a reliable predictor of MC concentration in samples. In addition the quantification of the *mcy* genotypes showed a good linear correlation with the MC concentrations that have been observed. If a high number of inactive *mcy* genotypes would have occurred in field samples, then the variability in MC concentration that could not be explained by the *mcy* genotype proportion should have been substantial.

Only a low percentage (13.5%) out of the 96 cultivated strains were actively producing *mcy* genotypes. In conformity to this finding, Haande et al. (2007) did a similar study on *Microcystis* strains from East Africa and also reported a low percentage of MC-producing strains (<30%). Consequently as reported for the Northern temperate climatic region *mcy* genotypes of *Microcystis* typically constitute the minority of the *Microcystis* population (Kurmayer and Kutzenberger, 2009, Freshwater Reviews 2). Nevertheless, highest proportions of *mcy* genotypes and MC concentrations were found for the samples obtained from L. Saka and L. Funga. Fortunately, those lakes are not used for the preparation of drinking water: L. Saka belongs to a private missionary and this does not use it for domestic water supply. Lake Funga is inhabited by lots of wildlife, however so far no report concerning health conditions of animals around Lake Mburo National Park has been documented.

Biogeographical differences between the temperate and the tropical climatic region in toxin producing toxins

Because to their small size and the extreme abundance of microorganisms it is frequently assumed that microbial species are globally ubiquitous, but they are detected only where they encounter conditions suitable for population growth (the so-called Baas Becking hypothesis, Fenchel et al. 2002). As cyanobacteria are typically growing in cells of a few micrometer in size it is often argued that - in contrast to higher plants - geographic barriers do not restrict the global occurrence of cyanobacteria. Indeed cyanobacteria are in general cosmopolitan and not restricted to geographic regions (Chorus and Bartram 1999). The findings in this study, however, suggest that the size of organisms in general cannot be the only factor explaining biogeographic patterns and spatial variation also occur among such small sized organisms as cyanobacteria. This argument is relevant with regard to the diversity in toxin production in general, for example toxic compounds produced by *Cylindrospermopsis* differ among different parts in the world (Neilan et al., 2003). Although *Cylindrospermopsis* is found in

and in Europe it has not been found to produce another hepatotoxin, the so-called ermopsin (Haande et al. 2008).

97 the WHO established a provisional guideline on allowable concentrations of the MC variant MC-LR in drinking water (Chorus Bartram, 1999). While this guideline only threshold that has been published for organic contaminants in drinking water s are inorganic), it basically reflects the state of knowledge during the mid-nineties t century. While MC-LR certainly is one of the more abundant MCs it seems that it e most abundant toxin in all parts of the world and instead is replaced by other l variants such as MC-RR. MC-RR is known to be substantially less toxic to humans elbrates but nothing so far is known about the toxicity of the most abundant MC-RY Definitely MC-RY would need to be purified in several milligram amounts and ed with MC-LR and MC-RR experimentally both with regard to the inhibition of the phosphatase 1 and 2A (the target enzymes) as well as in situ using appropriate test ms. The results of this study emphasize the conclusion of the CYANONET initiative, was attempting a first global estimate of toxin production in cyanobacteria that arly the tropical and subtropical regions of the world remain severely underexplored et al. 2005) and consequently such global estimates are highly speculative. The only ility to fill this gap of knowledge is to set up monitoring programs that have been ly in use in Europe, North-America and Australia for years and in principle could be rred to regions like in East Africa by means of follow-up programs.

llusions

is research has shown that the chemical characteristics of each individual freshwater dy in Uganda were different ranging from mesotrophic to hypertrophic. Those water dies that were either eutrophic or hypertrophic harboured abundant MC-producers *crocystis*).

sing the molecular techniques, *Microcystis* was identified as the major MC-producer in ganda. This was further confirmed by finding highly significant positive linear lationships between the *Microcystis* cell numbers and the MC concentration.

espite the abundance of the potential MC-producers, partly forming surface blooms, the MC concentrations were found to be relatively low. Only in L. Saka and L. Mburo the MC concentrations were found above the threshold of 1 µg L^{-1} recommended for drinking ater by the WHO.

4. Even though the monthly MC concentrations were highest in L. Saka and L. Mburo, they were composed of the MC structural variant with the lowest toxicity (MC-RR). In contrast the 10-fold more toxic MC-LR variant occurred with low frequency only (< 10% of all the samples in L. Saka and L. Mburo). Since both lakes are not used for drinking purposes, the potential health risk is considered not as high as for drinking water.
5. There were two new structural variants (MC-RY and [Asp^3]-MC-RY) described from this study which differed from those described earlier in the temperate regions. The persistence of those two variants in Africa but not in European habitats might be explained by geographic isolation between the climatic zones.

Recommendations

1. The nutrient loading from the catchment areas into the lakes is probably the most important factor in stimulating algal blooms in the lakes. The appropriate authorities concerned with environmental integrity should start taking action in averting nutrient loading from various sources into the lakes, such as Murchison Bay or Lake Saka.
2. The database reporting the phytoplankton density and its composition and MC concentrations is considered unique and should be used by authorities and even expanded over the next years. The database may assist the transfer of the application of regulations from other countries in order to achieve a more safe water use. By recording the variation in phytoplankton composition and resulting MC production over years a monitoring programme might be established that would be essential to interpret the changes in water quality that are observed on a short term.
3. Where access to more sophisticated toxin detection technology does not exist, *Microcystis* cell counts under the microscope could be used for the estimation of MC concentration as a monitoring tool. The MC determination via standardized HPLC-DAD analyses would then be done twice a year by sending filters to reference laboratories. In that way, there will be no need to purchase the expensive instrumentation that in addition requires regular maintenance and services not easily available in developing countries.
4. As *Microcystis* has been found in all water bodies (at least in trace amounts) it is essential to monitor other water bodies that are known to be used for the preparation of drinking water. Particularly, in the case of drinking water use the awareness of the population would need to be raised and in the case of high abundance of *Microcystis* alternative water bodies should urgently be recommended.

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Curriculum vitae

William Okello was born on the 18th October 1969 in Dokolo district, Northern Uganda. He went through his studies in primary and secondary school then first obtained a diploma in Science and Technology bias Chemistry and Biochemistry in 1994 before joining Makerere University in 1999 for a 3-year Bachelor of Biomedical Laboratory Technology.

While still a student on training, William worked on contract as a Laboratory Technician with Fisheries Research Institute (FIRI), Jinja later on as Research Assistant with the Department of Zoology, Makerere University. In 2002, he was invited to lecture on a part-time basis at the Department of Zoology, Makerere University.

Between 2003 to October 2004, William secured a fellowship for a Master of Sciences in Environmental Science and Technology (Limnology and Wetland Ecosystem Management) a taught programme between UNESCO-IHE, The Netherlands and International Post-graduate Programme in Limnology (IPGL), The Austrian Academy of Sciences, Austria. Thereafter William returned to Uganda and National Agricultural Research Organisation under the Ministry of Agriculture, Animal Industry and Fisheries appointed him as a Research Officer (Limnologists) to work with National Fisheries Resources Research Institute (NaFIRRI), Jinja-Uganda. He also continued to lecture on a part time basis at the Department of Zoology, Makerere University, Kampala. In June 2006, William got an award for a 3-year PhD scholarship from the Austrian Agency for International Cooperation in Education and Research (OeAD GmbH) tenable at the Institute of Ecology and Conservation Biology, University of Vienna, Austria.

William's 11 years experience in water quality research showed him rising upto his current position in the Ugandan Ministry of Agriculture. His major duty involved: planning and implementing priority research on physico-chemical factors and algal dynamics affecting fish stocks, water quality and the environment for each fishery area; analysing and synthesising information on physico-chemical characteristics of the aquatic environment/fish

production systems and their influence on fish production, distribution and abundance; harmonising work programmes and research results with regional working groups (Uganda, Kenya and Tanzania) on shared water bodies and reviewing and making requisition for laboratory supplies.

International conferences

2009. Attended the Fifth World Water Forum, held from 16 - 22 March 2009 in Istanbul, Turkey.

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