PCR BASED MARKERS FOR DETECTION AND IDENTIFICATION OF TOXIC CYANOBACTERIA

Report to the
Water Research Commission

by

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CD with Appendices is attached.

WRC Report No. 1502/1/08
ISBN 978-1-77005-702-9

March 2008

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EXCECUTIVE SUMMARY

INTRODUCTION

Toxic cyanobacteria found in eutrophic, municipal and residential water supplies are an increasing environmental hazard in South Africa. Cyanobacteria produce lethal toxins, and domestic and wild animal deaths are caused by drinking water contaminated by these toxins. Among the species causing death of livestock, blooms of *Microcystis aeruginosa* are the most common in South Africa. More than 65 microcystins have been isolated to date and they are the most abundant cyanobacterial toxins. Hazards to human health may result from chronic exposure via contaminated water supplies. Microcystins are powerful tumour promoters and inhibitors of protein phosphatase 1 and 2A and they are suspected to be involved in the promotion of primary liver cancer in humans.

Monitoring the quality of water destined to public supply includes identification of potentially toxic cyanobacteria and their population density. Identification of such microorganisms based on morphological features only, though widespread, has proven problematic, mainly for the genus *Microcystis*, due to its extensive phenotypic plasticity. Moreover, identification of a cyanobacterial genus by microscopic morphology or molecular analysis does not indicate the potential for toxin production. Different strains of one species can be morphologically identical but differ in genetics and toxigenicity as clearly demonstrated in the present study. It has been reported that *Microcystis aeruginosa* for example has both toxic and nontoxic strains.

Existing diagnostic technology does not provide for ease of analysis, since it is either specific but laborious and need specialized expensive equipment (i.e. mouse bioassays, HPLC, MALDI-TOFF) or non-specific and reasonably priced (i.e. ELISA and PPI2A assays). There have been numerous attempts to refine the identification of strains by using specific gene analysis. Examples include the use of PCR-based methods for amplification of the phycocyanin intergenic spacer (PC-IGS) between the α- and β- subunits of the phycocyanin operon in environmental samples, the 16S-23S rRNA internally transcribed spacer region, and the DNA-dependent RNA polymerase (*rpoC*I) gene. Although these molecular techniques have improved the accuracy of strain identification, they have not been able to distinguish toxigenic from nontoxigenic strains of the same species. Since the biosynthetic pathway for production of microcystin has now been elucidated, it has enabled the development of specific oligonucleotide primers for the gene clusters putatively involved in the production of microcystins. To better detect microcystin-producing cyanobacterial strains, several authors have developed genetic probes directed, respectively, to the *mcyB* gene and to adenylation domains within the microcystin synthetase gene cluster. The *mcy* gene cluster assembly has been reported to consist of 10 bidirectionally-arranged genes that reside in two operons (*mcyA-C* and *mcyD-J*) of *Microcystis aeruginosa*. The activities of these chromosomal gene products are primarily peptide synthetases (*mcyA-C*, *E, G*), polyketide synthases (*mcyD*, parts of *E* and *G*), and methylation (*mcyJ*), epimerization (*mcyF*), dehydration (*mcyI*), and localization (*mcyH*), resulting in nonribosomal toxin synthesis. It has been reported that the disruption of some of these genes (*mcyA, B, D*, or *E*) resulted in no detectable toxin production, and an *N*-metyltransferase (NMT) domain is usually associated with the *mcyA* and apparently with toxicity in strains. It was found that NMT-positive strains contained an open reading frame (OFR) of unknown function (*uma1*) at a conserved distance from *mcyC*. The results further suggested consistent linkage between *mcyC* and *uma1* in toxic and non-toxin strains. However, it was also found that *uma1* was not co-transcribed with the *mcyABC* cluster in non-toxic strains, suggesting that *mcyC* was also not transferred in non-toxic strains.

AIMS

The objectives of the study were to

- determine the genetic diversity and population structure in selected South African water reservoirs,
- determine the potential of using the *mcyB* gene sequence as a diagnostic tool in raw water to detect the presence of toxin-producing cyanobacterial spp. in South African water reservoirs.

MATERIAL AND METHODS

To elucidate the genetic diversity of geographically unrelated *M. aeruginosa* strains, we applied amplified fragment length polymorphisms as fingerprinting tool, as it has previously been shown efficient to discern between organisms on intra- and interspecies level. AFLP fragments have been used to unravel cryptic genetic variation for a wide range of taxa, which have previously been impossible to resolve with morphological characters. A combination of primers with two and three selective nucleotides on 23 *Microcystis aeruginosa* and outgroup axenic strains was used for this purpose (Figure E1).

Figure E1 Outline of research strategy.

The second objective of the study was to determine the potential of using the *mcyB* gene sequence as a diagnostic tool in raw water to detect the presence of toxin-producing cyanobacterial spp. in South African water reservoirs. To achieve the objective, two main strategies were followed. Firstly, the use of insertions and deletions (indels) present in the *mcyB* gene sequence as a fingerprint was tested, i.e. diagnostic tool to recognize the presence of "known" toxic strains in water reservoirs. Secondly, the use of nucleotide polymorphisms present in the *mcyB* gene sequence, with putative function in toxin production for association genetics and as a diagnostic tool was investigated (see Figure E1 for outline of strategy).

RESULTS

Molecular marker analysis is becoming increasingly capable of identifying informative genetic variation. Amplified fragment length polymorphism markers (AFLPs) are among the recent innovations in genetic marker technologies, and provide a greater capacity for genome coverage and more reproducible results than previous technologies. The usefulness of AFLP was investigated, that is based on the selective amplification of genomic restriction fragments by PCR, to differentiate between geographically unrelated *Microcystis* strains. In total 23 strains were subjected to the AFLP fingerprinting. After analysis of the data on the basis of the average linkage method, known as the Unweighted Pair Group Method using Arithmetic averages (UPGMA), a dendrogram with four clusters was obtained. Cluster 1 consisted mainly of the NIES strains that originated from Japan, while in cluster 2 the European strains grouped together. The South African strains that originated from the northern part of the country group together in cluster 3, while the strains collected from the central and southern regions group together with the US strains in cluster 4. The study provided evidence for the applicability of AFLP in cyanobacterial taxonomy, and furthermore clearly demonstrates the superior discriminative power of AFLP towards the differentiation of geographical unrelated *Microcystis aeruginosa* strains that belong to the same species.

Toxic freshwater cyanobacterial blooms are potential health hazards in water supply reservoirs and therefore predicting bloom events is an important goal of monitoring fresh water programmes. The recent identification of the *mcy* genes in the production of microcystin synthetase for the first time provides an avenue to study microcystin production at a genetic level. This study used PCR based technologies (i.e. PCR and quantitative real-time PCR) ELISA and PP2A methods for detection of strains present and determination of their toxigenicity. The presence of the toxic cyanobacterial strains was confirmed through the use of molecular markers that detect the presence of some of the *mcy* genes in the *mcy* gene cluster that is able to synthesize microcystin toxins in *Microcystis* spp.

CONCLUSIONS

In the present study it was shown that the population structure of *M. aeruginosa* strains from South Africa proved to be very diverse, and different from strains from other geographic regions (i.e. North America, Europe and Asia). It has also been found that polymerase chain reaction (PCR)-restriction fragment length polymorphisms (RFLPs) may be a cheap alternative for species and/or strain identification, provided that an array of enzymes are used to ensure proper identification. However, the combination of PCR and quantitative real time (qRT)-PCR proved superior in terms of accuracy, time and costs. In the study, it was shown that using the *mcyB* gene in PCR assays, applied directly to environmental samples provide a useful indicator of putative toxicity, since the genetic potential of a strain to produce microcystin is measured. Although high pressure liquid chromatography (HPLC) provides a direct measure of toxins present, it does require a large capital investment, considerable sample preparation and a level of expertise. The PCR-based assays detect toxigenic cells rather than toxins and require little sample preparation

and modest capital costs. Detection of toxic *Microcystis aeruginosa* strains through molecular markers for microcystin may have great end-use potential in routine analysis of aquatic ecosystems. Thus, it may make water monitoring more feasible and allow the early application of corrective action before cyanobacterial blooms start to decompose or disintegrate. The PCR-based assay is effective at a level of 10 cells.ml^{-1} and can indicate a possible toxic bloom well before the cell count reaches the action alert at a cell density of 2 000 cells.ml^{-1}, as recommended by the Australian Drinking Water Guideline (National Health and Medical Research Council/Agriculture and Resource Management Council of Australia and New Zealand, 1996) and a high alert level of 20 000 cells.ml^{-1}, where blooms may contain sufficient toxin to be of concern for human health.

RECOMMENDATIONS

- Future work should include wider collection of environmental samples to assess the application of the primers in management strategies.
- Development of more primers will contribute towards improved monitoring of toxigenic algae, since *Microcystis aeruginosa* is not the only toxin producing species in water reservoirs.
- Development of a short training module or workshop for training and transfer the technology to interested parties.

All the primary objectives set for the project were achieved. The population structure of *M. aeruginosa* strains from different geographical regions in South Africa and other countries was determined. In addition to the *mcyB* gene that resides within the one (*mcyA-C*) operon of *Microcystis aeruginosa*, other genes within the two operons (i.e. *mcyA*, *mcyC* and *mcyE*) were also investigated. Primers were designed, tested and shown to be reliable as diagnostic tools (i.e. used during PCR and qRT-PCR analysis), when applied to raw water during bloom management as indicators of the presence of toxigenic strains.

CAPACITY BUILDING

Ph.D. degree:

OBERHOLSTER PJ. 2006. Monitoring toxicity in raw water of Cache la Poudre River and Sheldon Lake, Colorado, USA using biomarkers and molecular marker technology. Ph.D. Pretoria University, pp. 218

PUBLICATIONS

OBERHOLSTER PJ, BOTHA A-M, & CLOETE TE (2005) An overview of toxic freshwater cyanobacteria in South Africa with special reference to risk, impact and detection by molecular marker tools. *Biokemistri* 17: 57-71.

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OBERHOLSTER PJ, BOTHA A-M, CLOETE TE (2006) Use of molecular makers as indicators for wintergrazing on toxic benthic cyanobacteria colonies by zooplankton in an urban Colorado lake. *Harmful Algae* 235: 1-12.

OBERHOLSTER PJ, BOTHA A-M, CLOETE TE (2006) Toxic cyanobacterial blooms in a shallow artificially mixed urban lake Colorado. *Lakes & Reservoirs: Research and Management* 11: 111-123.

OBERHOLSTER PJ, BOTHA A-M (2007) Use of PCR based technologies for risk assessment of a winter cyanobacterial bloom in Lake Midmar, South Africa. *African Journal of Biotechnology* 6: xxx-xxx (in press).

OBERHOLSTER PJ, BOTHA A-M, CLOETE TE (200X) Biological and chemical evaluation of sewage water pollution in the Rietvlei nature reserve wetland area, South Africa. Submitted to *Environmental Pollution*.

POSTER PRESENTATIONS

OBERHOLSTER PJ, BOTHA A-M & CLOETE TE (2006) Toxic Cyanobacterial blooms in a shallow artificially mixed urban lake in Colorado, USA. Society of Microbiology Meeting, CSIR Convention Centre, Pretoria, 9-11 April 2006

OBERHOLSTER PJ, BOTHA A-M, & CLOETE TE (2006) Using molecular makers as indicators for wintergrazing on toxic benthic cyanobacteria colonies by zooplankton in an urban Colorado lake. 54th North American Benthological Society Meeting, Anchorage Alaska, June 4-8, 2006.

OBERHOLSTER PJ, BOTHA A-M**. (**2007) Use of PCR based technologies for risk assessment of a cyanobacterial bloom in Lake Midmar, South Africa. Society of European Freshwater 5th Meeting, Palermo, Italy, July 8-13, 2007.

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The authors would like to express their sincere gratitude to Carin van Ginkel (Department of Water Affairs and Forestry) and Leanne Coetzee (Tshwane Metro Council) for the collection of some of the strains. Also, we want to thank the National Research Foundation and the Water Research Commission, South Africa, for provision of funding. We also would like to express our gratitude to the University of Pretoria for the provision of infrastructure. We especially want to thank Dr. Jiří Komárek for his assistance with the identification of some of the strains.

A special word of thanks to Mrs APM Moolman from the WRC who acted as chairperson for this project.

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Figure A1.3 Construction of genetic maps based on available restriction sites present in *mcyB* sequences obtained from UP37, PCC7813 and UV027 after analysis using the software programme Webcutter 2.0 (http://www.firstmarket/com/cgi-bin/cutter).

Figure A1.4 Sequences derived after cloning of fragments specific to *mcyA-C* and *mcyD* genes from USUP2.

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LIST OF ABBREVIATIONS

AFLP	amplified fragment length polymorphism
BLAST	Basic Local Alignment Search Tool
BLASTN	Basic Local Alignment Search Tool (nucleotide search)
BLASTX	Basic Local Alignment Search Tool (amino acid translation search)
bp	base pair
BSA	bovine serum albumin
C	constant current
CCAP	Institute of Freshwater Ecology, UK
Da	Dalton
DNA	Dioxyribose nucleic acid
dNTP	nucleotide triphosphate mix
EDTA	ethylenediamine tetra-acetic acid, disodium magnesium
ELISA	enzyme-linked immunosorbent assay
HPLC	high performance liquid chromatography
LD_{50}	lethal dose 50
LOD	limit of detection
M	molar
mA	miliampere
mcy	microcystin producing gene
MI	marker index
Min	minutes
ml	mililitre
mM	milimolar
NCBI	National Centre of Bioinformatics Institute
NIES	National Institute for Environmental Studies, Japan
ng	nanogram
OD	optical density
p	pico
PCC	Pasteur Culture Collection, France
PCR	polymerase chain reaction
PIC	polymorphic information content
PVP	Polyvinylpyrrolidone
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	Ribose nucleic acid
RFLP	restriction fragment length polymorphism
RP-HPLC	rapid iode array high performance liquid chromatography
rRNA	ribosomal RNA
s	seconds
SAG	Pflanzen Physiologisches Institut, Universität Gottingen
SDS	sodium dodecyl sulphate
TOX	Toxicity
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
TYG	Triptone
UP	University of Pretoria
UPGMA	Unweighted Pair Group Method Using Arithmetic Means algorithm
UV	University of the Free State
v/v	volume per volume
w/v	weight per volume
W	Watt
°C	Degrees Centigrade
µ	micro
µl	microlitre
$\%B_o$	(OD of sample or calibrator/OD of negative control) x 100.

1. LITERATURE REVIEW

The first fossilized evidence of living cells is found in rocks from Australia and South Africa which date back 3.5 billion years. These rocks reveal the presence of prokaryotic cells that are not noticeably different in appearance from prokaryotes living today. One such fossil consists of chains of cells that resembles modern photosynthetic cyanobacteria (Knoll & Golubic, 1990). Cyanobacteria are generally considered to be the oldest oxygenic organisms. Their early photoautotrophic efforts created the appropriate atmosphere for oxygen-dependent life. The diversity of Cyanobacteria is expressed by their morphological, biochemical, and physiological properties, which enable them to settle and persist in a wide range of habitats (Schopf & Walterand, 1983).

1.1 Morphology

Cyanobacterial cells are rather large and range in size from 1-50 μm in width. They can be unicellular, colonial or filamentous. Cyanobacteria lack any visible means of locomotion. The cell wall is multilayered composed of peptidoglycan and lipopolysaccharide lacking celluloses. The wall may be anywhere from 10 to 20 nanometers thick and sometimes is coated with a relatively thick, jellylike capsule or slime of proteinaceous material (Skulberg et al., 1993). Inside the wall, and tightly pressed against it, is the outer membrane of the prokaryotic cell, the plasmamembrane or plasmalemma, which may be smooth or have infoldings that extend into the cell, forming structures called mesosomes. Besides controlling what enters and leaves the cells, the membranes have other important functions. Many enzymatic reactions including photosynthesis and respiration take place on the proteins contained in membranes.

The nucleuplasmic region consists of DNA fibrils which are organized in a complex helical and folded configuration and are distributed uniformly throughout the centroplasm. The size of the genome varies widely in cyanobacteria, having a molecular weight between 1.6×10^9 and 8.6×10^9 daltons. Ribosomes in cyanobacteria are seen diffusely distributed throughout the cytoplasm and particularly concentrated in the nucleoplasmic region (Fray, 1983).

The strongly refractive appearance of the cells of cyanobacteria under the light microscope is due to the presence of gas-filled cylindrical vesicles, called gasvacuoles. The vesicles are formed by an extremely thin and rigid proteinaceous membrane enclosing a gas-filled space (Fray, 1983). Gasvesicles are the buoyancy-regulating organelles and responsible for the vertical migration of certain cyanobacteria (Walsby, 1969). The light reaction of photosynthesis occurs on a series of parallel membranes within the cytoplasm that are separate from the plasmallema. These membranes contain chlorophyll*a* (chl*a*) and several accessory pigments like phycoeryhrin, phycocyanin and allophycocyanin. The accessory pigments are grouped together as phycobilisomes that are attached to the outside of the membranes. These pigments harvest light of wavelengths 550 to 650 nm, and pass the energy on the chl*a* (Glazer, 1989).

1.2 Environmental factors and cyanobacteria

Like other organisms, the success of cyanobacteria species in a given habitat depends on a multitude of environmental factors. These factors also referred to as resources, comprise of physical and chemical characteristics of an aquatic environment. It has been often observed that the supply of one or more factor is just above the critical minimum required by a particular species. Increase in the level of this factor would lead to increased population of the species, until another factor becomes limiting (Darley, 1982).

1.2.1 Light

Being photoautotrophs, cyanobacteria are greatly influenced by changes in intensity and spectral quality of light. Attenuation of light takes place as it traverses down the water column and light intensity is greater at the surface of waters than the deeper layers. Concomitantly, changes in spectral quality also take place as most of the longer wavelengths of solar radiation are absorbed by the surface layers of water, and the shorter wavelengths like bluegreen light penetrate furthest in the photic zone (Falkowski, 1981).

Impairment of photosynthesis and photooxidative damage is common features of microalgae exposed to high light levels. However, in their study of the Neuse River (North Carolina, USA), Paerl et al. (1983) did not observe any impairment of photosynthesis in a *Microcystis aeruginosa* summer surface bloom. This contrasted with the inhibition of photosynthesis observed at the surface and at 0.25 m in the diatom-dominated spring phytoplankton community. In *Microcystis aeruginosa* surface blooms, photosynthetic efficiencies increased with the carotenoid:chl*a* ratio. The carotenoid pigments isolated from *Microcystis aeruginosa* showed an absorption peak in the near-UV region of the spectrum, suggesting that carotenoids played a photoprotective role in this surface-dwelling *Microcystis aeruginosa* population.

In a later study, Paerl et al. (1985) confirmed that photooxidation was not a common feature of natural populations of *Microcystis aeruginosa*. At the high surface irradiance levels encountered, these cyanobacterial populations were capable of more efficient photosynthesis than cultured strains or natural non-cyanobacterial populations. High light intensities in combination with the low CO_2 concentrations and supersaturated O_2 levels prevailing in surface waters during cyanobacterial bloom forming, could potentially lead to photooxidative damage of cyanobacterial cells. Carbon dioxide uptake or O_2 evolution is inhibited by high intensity irradiance (Harris, 1978). CO_2 presence appears to ameliorate the effects of super-saturating light intensities on the photosynthetic apparatus. The relative concentration of O_2 and CO_2 in the surrounding medium appeared to determine the extent of the damage caused by light energy in excess of that needed to saturate photosynthesis. In the absence of CO_2, the Calvin cycle is not coupled to photosynthetic electron transport. This allowed O_2 to function as the terminal electron acceptor and resulted in the formation of superoxide (O^{2-}), which could potentially lead to oxidative damage of the surrounding tissue (Abelivich & Shilo, 1972).

A variety of optimal light intensities for toxin production by *Microcystis aeruginosa* have been described by numerous authors (Rapala et al., 1997; Sivonen, 1990; Van der Westhuizen & Eloff, 1985; Watanabe & Oishi, 1985). These discrepancies are mainly due to differing measuring techniques employed in various studies. In a recent publication, it was found that light quality i.e. 16 qmol photons $m^{-2}s^{-1}$ in the red light spectrum, increased toxin production as well as gene transcription in *Microcystis aeruginosa* strains (Kaebernick et al., 2000). Increasing microcystin content was also observed when the light intensity was raised from approximately 2 to 40 qmol of photons $m^{-2}s^{-1}$. This led to the conclusion that light intensities influencing the toxicity of *M. aeruginosa* are less than about 40 qmol of photons $m^{-2}s^{-1}$. Such light intensity is found at a depth of about 1 m during bloom conditions, with intensities of 400 qmol of photons $m^{-2}s^{-1}$ measured at the surface (Walsh et al., 1997). This correlates to the highest toxicity measured in the surface waters of a *Microcystis* bloom during periods of calm weather (Annadotter et al., 1991). Possession of accessory pigments, such as phycoerythrin, allows several species of cyanobacteria to carry out photosynthesis at depths that receive only green light and where, in addition, nutrients are more abundant than on the surface. Cyanobacterial pigments as well as mycosporin-like amino acids are also involved in their great capacity to resist ultraviolet radiation in surface waters,

and this could give them another advantage over some phytoplankton (Castenholz & Garcia-Pichel, 2000).

1.2.2 Temperature

Temperature affects cyanobacteria directly by altering the rates of metabolic processes, and indirectly by changing the physico-chemical characteristics of the environment. During summer, high temperature causes stratification of temperate lakes into epilimnion, the upper, thoroughly mixed layer, and hypolimnion, the lower layer, separated by a zone called the thermocline. With the onset of winter, the surface water of hypolimnion cools down, becomes more dense, sinks to the bottom and the thermocline is disrupted. In general, cyanobacteria prefer warm conditions, and low temperatures are one of the major factors that end cyanobacterial blooms (Wetzel, 1983). Gorham (1964) found that toxin concentration of *Microcystis aeruginosa* NRC-1 increased approximately 10-fold when culture strains were incubated at 25°C instead of 32.5°C. *Microcystis* isolates were 4 times more toxic when grown at 18°C than at 29°C (Runnegar et al., 1983). Van der Westhuizen and Eloff (1985) in contrast, found less pronounced changes in the toxicity in the range 16-32°C. The above studies indicate that maximum toxicity of *Microcystis aeruginosa* culture strains is achieved at a temperature between 18 and 25°C. In Hartbeespoort Dam, South Africa, the greatest toxin concentration in *Microcystis aeruginosa* scum was found during the summer with surface water temperature up to 27°C, but negligible toxin concentrations were always found during the winter months with a minimum water temperature of 13°C (Van der Westhuizen & Eloff, 1985).

Krüger and Eloff (1978) found a correlation between the water temperature and the development of *Microcystis* blooms in small eutrophic reservoirs in the Vaal River, South Africa. They reported that *Microcystis* blooms started to develop in open lake water once temperatures reach 16-17°C. Their results show the effect of temperature on specific growth rate occurs after the upper temperature limit is surpassed. The mean value of the optimal temperature range was used to estimate the optimal temperature value. Strain UV 001 exhibited the lowest lower temperature limit of 10.5°C, while strain UV 006 from Hartbeespoort Dam, South Africa had the highest upper temperature limit of 40°C (Krüger & Eloff, 1978).

1.2.3 Buoyancy regulation

Klemer and Konopka (1989) proposed a conceptual model of buoyancy regulation based on a large body of evidence from laboratory investigations. Under conditions of balanced growth, cells were considered to be neutrally buoyant. However, if the rate of carbon (C) fixation, determined by the availability of light and C, does not meet the requirements for growth, the cyanobacteria would become positively buoyant. As the organisms float towards the surface water, the rate of C fixation would increase in response to the increase light availability. The organisms would remain buoyant and grow faster if nutrients such as N and P were sufficient, and the photosynthate could be used for the biosynthesis of proteins and nucleic acids. Other mechanisms that play an important role in the regulation of buoyancy of cyanobacterial cells are the form of stored carbohydrates and turgor pressure regulation. Compositional changes in the diel protein:carbohydrate ratios during buoyancy reversals suggest a complex relationship between light and nutrients (N:P) (Walsby et al., 2001). It however, seems that the regulation of gasvacuole synthesis is the most important factor. *Microcystis aeruginosa* could ascend, though buoyancy regulation, at rates of the order of 50 to 250 $m.d^{-1}$, respectively (Humphries & Lyne, 1988). The ability to negate sedimentation losses through buoyancy control should therefore be an important factor contributing to the development and maintenance of large epilimnetic populations of bloom-forming cyanophytes. Positively buoyant cyanobacteria should be favoured under conditions of increasing thermal stability. In addition to prolonging residence time in the upper layers of the water column, buoyancy regulation allowed cyanobacteria to position themselves

at depths offering optimal combinations of light and limiting nutrient concentrations (Paerl, 1988).

1.2.4 Optimum Nutrient Ratios

The differences in optimum ratios between different species of phytoplankton could provide a basis for their competitive exclusion or coexistence (Rhee, 1982). In chemostat competition experiments, it was shown repeatedly that species composition depended on the ratio of limiting nutrients (Sommer, 1989). Results from whole-lake and limnocorral nutrient loading manipulations (Flett et al., 1980) and from correlative studies (Smith, 1983) suggested that low N:P ratios (13:1 by atoms) in the nutrient supply were an important chemical factor favoring cyanobacteria to gain the selective advantage. Further studies indicated that even when the TN:TP ratio is 1:3 (by atoms), the relative biomass of cyanobacteria ranged from nearly 0 to 100 (by volume) (Smith, 1983). From this data, it is becoming apparent that factors other than N:P supply ratios might be important in determining the response of cyanobacteria to nutrient loading ratios. Light and temperature may modify the influence of N:P ratios on the dominance of cyanophytes in a waterbody. In Lake St. George, USA the proportion of cyanobacteria in the plankton was negatively correlated with the ratio NO_3-N:TP and positively correlated with temperature. However, when the water temperature was below 21°C and the ratio NO_3-N:TP exceeded 5:1, cyanophyte blooms never occurred (McQueen & Lean, 1987).

1.2.5 Effect of heavy metals

Aquatic pollutants affect phytoplanton at two levels of organization, namely inhibition of various physiological and biochemical processes of individual organisms, and alteration of structure and function of cyanobacteria communities. Lethal concentrations of heavy metals vary enormously between species and within ecotypes (Reed & Gadd, 1990). The toxicity of a metal is often determined as the concentration that lowers survival of test algae to 50 (LC_{50}), but this may be not a good measure of the sensitivities of other cellular activities. Certain metal ions such as Zn_2 and Fe_2 significantly influence toxin yield in *M. aeruginosa*. Zn_2 is involved in the hydrolysis of phosphate esters, the replication and transcription of nucleic acids, and the hydration and dehydration of CO_2 (Sunda, 1991). All cyanobacteria require Fe_2 for important physiological functions such as photosynthesis, nitrogen assimilation, respiration and chlorophyll synthesis (Boyer et al., 1997). It is not clear how Fe_2 deficiency modulates microcystin production, but it has been noted that as cyanobacteria experience iron stress, they appear to compensate for some of the effects of iron loss by synthesizing new polypeptides (Lukač & Aegerter, 1993).

1.3 Measures to control the growth of cyanobacteria

1.3.1 Chemical control

Chemical control is widely used to prevent the growth of nuisance cyanobacteria, and the commonest treatment is the application of copper sulphate at a concentration of 0.5-1.0 mg/L. Although such low concentrations are not toxic to aquatic animals, repeated administration may increase the copper in the waterbody to a toxic level. Cyanobacteria appear to be more sensitive to copper ions than diatoms or green algae, and hence lower concentrations of the salt are often adequate to suppress their growth. A number of other algicides are used effectively in water supplies, like phenolic compounds, amide derivatives, quaternary ammonium compounds and quinone derivatives (Fray, 1983). The hazards with administration of chemicals to waterbodies are the rapid lysing of dense cyanobacterial cells that may lead to a very high concentration of dissolved microcystins. Jones and Orr (1994) reported up to 1.8 mg/L from a lake which was treated with an organic copper algicide.

1.3.2 Biological control

Along with most autotrophs, cyanobacteria have evolved strategies to avoid being eaten by facultative heterotrophs. These include indigestibility or unmanageability as food (Richman and Dodson. 1983) or the production of herbivore deterrents (Ostrofsky et al., 1983). The lethality of toxic cyanobacteria to zooplankton has been demonstrated in laboratory experiments (Fulton and Paerl, 1987). It has been suggested that the production of toxins by cyanobacteria to reduce grazing pressure by zooplankton may be the main ecological role of these compounds (Lampert 1981; Carmichael, 1992). Not all zooplankton avoid cyanobacteria however, a number of flagellates, amoebae and ciliates can act as predators on them (Dryden and Wright, 1987) and the water flea *Daphnia pulex*, has been shown to feed on *Aphanizomenon flos-aquae* (Holm et al., 1983). Studies of Lake Kasumigaura in Japan have shown that *Microcystis* blooms can not only be degraded by the rotifer *Philodina* and the oligochaete *Aelosoma* (Inamori et al., 1987, 1988), but that these organisms can actually dominate the blooms.

Micro-organisms for example fungi, bacteria and viruses appear to play an important part in regulating the growth of cyanobacteria populations in freshwater bodies. In the English Lake District chytrids and fungal pathogens have been shown to attack planktonic cyanobacteria: certain chytrids specifically infest akinetes, and other heterocysts. Bacterial pathogens of cyanobacteria seem all to belong to the group of Myxobacteriales. They can affect rapid lysis of a wide range of unicellular and filamentous cyanobacteria. The bacteria attack always by means of end-on contact with the host cell: in this way they progress from cell to cell along a trichome (Canter, 1950, 1951, 1954).

Various viral pathogens that infect cyanobacteria cells have been isolated. All belong to the group of complex viruses or phages, and were named cyanophages. They exhibit some degree of host specificity. The virus LPP-1, for example, is effective only against strains of *Plectonema*, *Lyngbya* and *Phormidium*. Phage AR-1 attacks *Anabaenosis*, phage C-1 lyses only *Cylindrospermum*, whereas phages SM-1 and AS-1 are effective against the unicellular forms, *Microcystis* and *Synechococcus*. Viral numbers have been found to increase in lakes in response to the seasonal development of cyanobacteria populations, and may have an important role in controlling them in freshwater bodies (Hoffman & Stander, 1976; Lemke, 1976).

1.4 Cyanobacteria toxicity

Traditionally the cyanobacteria are classified into five orders namely Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales and Stigonematales. *Microcystis* spp. are classified as members of the *Microcystis* cluster of the genus *Synechocystis*, order Chroococcales (Skulberg et al., 1993).

Cyanobacteria commonly occur in fresh and brackish waters and may produce massive annual growths as a consequence of nutrient enrichment from natural waters, agricultural fertilizer run-off, or from domestic and industrial effluents. The cyanobacterial species which dominate these growths typically belong to the genera which produce toxins (Codd & Poon, 1988). A combination of biological and physical conditions usually led to cyanobacterial blooms and potentially animal poisoning. Death and illness occurs following oral ingestion of the cells or the released toxins, when toxin-forming cyanobacteria are suspended throughout the water column. Dilution of cells and toxins may occur so that an acute oral dose is not received during drinking. However, the planktonic cyanobacterial genera that include toxin-forming species can produce gas vesicles. These buoyancy devices often result in the surface accumulation of the cells to form dense blooms during calm weather (Reynolds & Walsby, 1975). This may result in concentrations of toxic cyanobacteria so that an acute oral dose can be provided in considerably less than the daily water requirement of animals (Richard et

al., 1983). Deaths of terrestrial animal have usually occurred in those circumstances where access to clearer water has not been possible and they have been obliged to drink in the vicinity of toxic blooms. Animal deaths may also occur following the bio-accumulation of cyanobacterial toxins via food chains (Richard et al., 1983).

1.4.1 Types of cyanobacterial toxins

Of the more than 50 genera of cyanobacteria, at least 19 of them, comprising 41 species, have been shown, or implied, to have toxic properties (Scott, 1991). Cyanobacteria produce a wide range of toxic compounds, all of which are secondary metabolities that are not used by the organism for its primary metabolism, but are produced for some ancillary purpose (Herbert, 1989). Toxins of cyanobacteria are grouped in two main categories by Carmichael (1994) namely, biotoxins and cytotoxins based on the types of bioassays used to screen for their activity. Cytotoxins are detected by mammalian cell lines and biotoxins are assayed with small animals, e.g. mice or aquatic invertebrates. Biotoxins of cyanobacteria are water-soluble and heat stable and they are released upon aging or lysis of the cells. The primary types of cyanobacterial biotoxins include hepatotoxin (microcystins, nodularins, cylindrospermopsins), neurotoxin (anatoxins, saxitoxins) and dermatotoxins (lyngbyatoxin A, aplysiatoxins, lipopolysaccharides) (Figure 1.1).

1.4.1.1 Hepatotoxins

Hepatotoxins are low molecular weight cyclic peptide toxins that affect the liver and have been the predominant toxins involved in the case of freshwater algae toxicosis. The toxic peptides of *Microcystis aeruginosa* have been the most intensively studied cyanobacterial toxins so far. Signs of poisoning in animals after drinking water containing toxic *Microcystis aeruginosa* or in mice after intraperitoneal bio-assay usually appear after 30 min to 24 hours, depending on dose. Acute dosage of mice causes death between about 30 min and 2-3 hours. Symptoms include weakness, vomiting, pilorection, diarrhoea, cold extremities, pallor, and heavy breathing. The most obvious internal sign of microcystin poisoning is a dark mottled liver, swollen with blood to about twice its normal weight (Carmichael, 1981). The most dramatic intoxication event documented to date occurred in 1992 along a 1000 km stretch of the Darling river, Australia where 10 000 livestock died after a massive bloom of *Anabaena circinalis* (Falconer, 1998).

An intensive study of the clinical and pathological consequences of microcystin poisoning in animals has been made in Australia. Deaths of 27 kg sheep occurred 18-48 hours after intraruminal inoculation with a *Microcystis aeruginosa* bloom, and were preceded by a sequence of depression, a transient rise in body temperature, increased heart rate, fluctuating respiration rate, cessation of ruminal sounds and cudding, persistent sternal then lateral recumbency, noisy laboured rapid breathing, muscle twitching, leg paddling, gasping respiration with periodic Cheyne-Stoke breathing cycles, mild nystagmus with eyelids widely opened, and finally loss of the eye preservation reflex (Jackson et al., 1988). The carcasses were jaundiced with yellow fluid in the body cavities and numerous haemorrhages. Lungs were slightly oedematous and the livers were swollen, with a blotchy pattern of congestion and petechial haemorrhages. Other organs appeared normal. Marked increased in serum activities of aspartate aminotransferase, lactate dehydrogenase, glutamate dehydrogenase, and alkaline phosphatase, an increased amount of bilirubin, and a decrease in serum glucose were observed (Jackson et al., 1988).

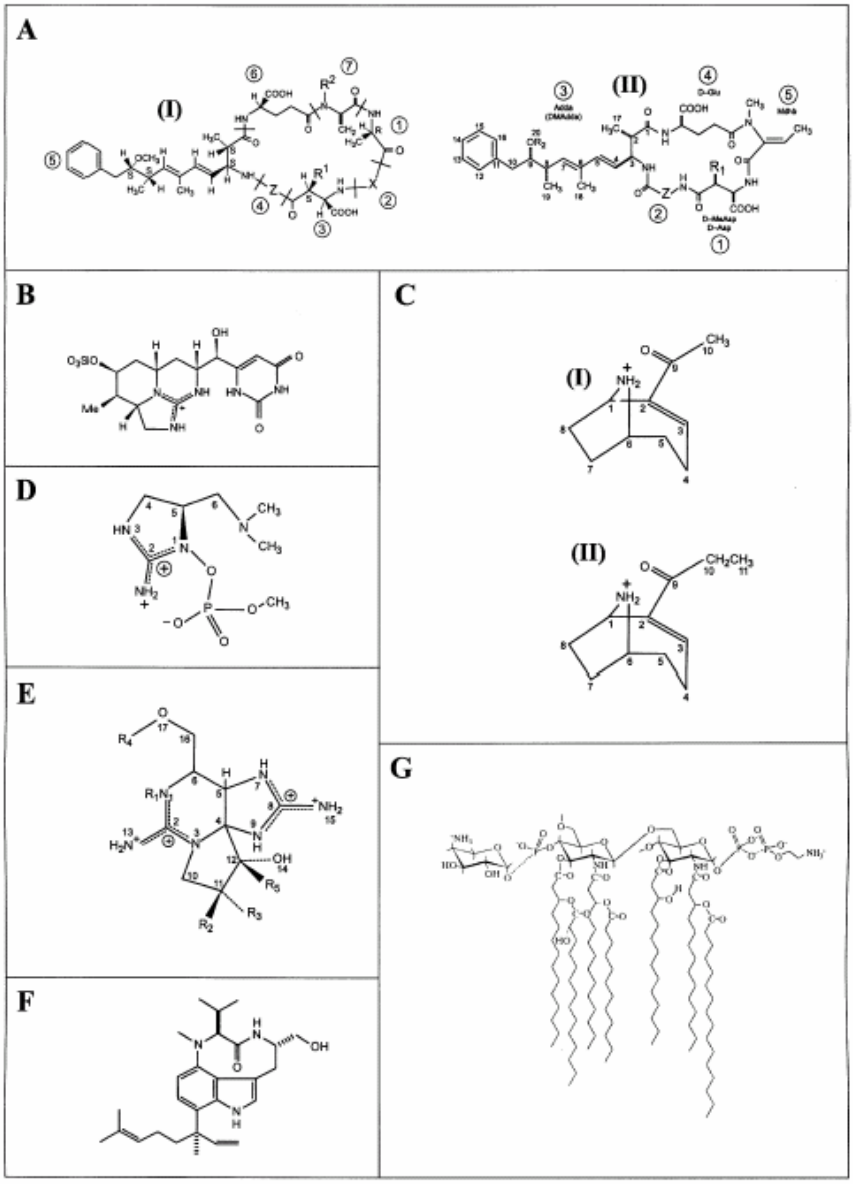


Figure 1.1 Chemical structures of **A**: microcystins (I) and nodularins (II) (X and Z are variable amino acids, R = H or CH3), **B**: cylindrospermopsin, **C**: anatoxin-a (I) and homoanatoxin-a (II), **D**: anatoxin-a(s), **E**: PSPs, **F**: lyngbyatoxin A, **G**: lipopolysaccharides (LPS) (from Zegura et al., 2002).

Massive hepatocyte necrosis was found in the livers of acutely poisoned sheep. The necrotic cells had eosinophilic, disintegrating cytoplasm and their nuclei showed pyknosis and karyorrhexis. The hepatocyte endoplasmic reticulum was aggregated and vacuolation was found in more severely affected cells. These observations are consistent with the *Microcystis* peptide toxins acting primarily on the liver (Jackson et al., 1988). Determination of the distribution of radioisotope-labelled microcystins between the major organs of rodents have been performed which have confirmed that the liver is the

main target organ for accumulation and excretion of the toxins. Thirty minutes after intraperitoneal administration of ^{125}I-labelled toxin to rats, 21.7% of the radioactivity was in the liver and 9.4% in the small intestine. The kidneys and urine contained only 5.6 and 2.9% respectively, of the ^{125}I administered (Falconer et al., 1986). Brooks and Codd (1987) have prepared ^{14}C-labelled microcystin and between 73 and 88% of that, administered to mice, was recovered from the liver between 1 and 180 min after dosage. Levels of ^{14}C in the lungs, kidneys, and all other organs range from 1 to 10% throughout. In addition to the microcystin-dependent changes in serum enzyme levels, which are indicative of liver damage (Jackson et al., 1988), the toxins cause major changes in hepatic microsomal cytochrome levels. Intraperitoneal administration of subacute doses to mice resulted in 80% decreases in cyt b_5 and P-450 and a fivefold increase in P-420 (Brooks &Codd, 1987).

In subacutely-affected animals, moderate to severe fatty change of hepatocytes with necrosis of individual or small foci of hepatocytes were observed. Hepatocytes often form intracytoplasmic eosinophilic globules, and accumulation of lipofuscinous pigment in Kupffer cells, especially in centrilobular areas were seen. The more chronic cases showed mild centrilobular fibrosis as well as mild bile ductular proliferation and portal fibrosis (Jubb et al., 1991; Kellerman et al., 1988).

1.4.1.1.1 Microcystins and nodularins

Microcystin are a cyclic heptapeptides with about 65 different isoforms identified, with diverse levels of toxicity (Sivonen & Jones, 1999). Nodularins are pentapeptides with only four forms been described. The hepatospecificity of these toxins is due to the requirement for uptake by a bileacid transporter. Microcystin and nodularins have been shown to be inhibitors of serine/threonine protein phosphatase 1 and 2A. This inhibition leads to hyperphosphorylation of proteins associated with the cytoskeleton in hepatocytes (Tiovola et al., 1997).

1.4.1.1.2 Cylindrospermopsins

Cylindrospermopsins is an alkaloid containing a tricyclic guanidine combined with hydroxymethyl uracyl and is stable to boiling. Studies on the mechanism of action of cylindrospermopsin have shown that in mouse hepatocytes *in vivo* the toxin disrupts protein synthesis (Tereo et al., 1994). The main target of this toxin is the liver, but unlike the microcystins, it can affect other organs such as the lungs, kidneys, adrenals and intestine (Falconer, 1998). Genotoxic activity is caused by the ability of cylindrospermopsins to induce strand breaks at the DNA level and loss of whole chromosomes (Humpage et al., 2000).

1.4.1.2 Neurotoxins

It is known that chemically-defined neurotoxins are produced by species and strains of *Anabaena*, *Aphanizomenon*, *Oscillatoria* and *Trichodesmium* (Carmichael, 1994). The neurotoxins are known to be produced by freshwater cyanobacteria strains include anatoxin-a, anatoxin-a(s) and saxitoxins. Neurotoxins producing death by paralysis of peripheral skeletal muscles, then respiratory muscles leading to respiratory arrest in a few minutes to a few hours following exposure.

1.4.1.2.1 Anatoxins

Anatoxin-a, produced by *Anabaena* and *Oscillatoria* is a secondary amine, 2-acetyl-9-azabicyclo(4.2.1)non-2-ene. This alkaloid is a potent post-synaptic cholinergic nicotinic agonist, which cause a depolarising neuromuscular blockade because it cannot be broken down by acetylcholinesterase. It, therefore, causes overstimulation of muscle followed by fatigue and paralysis. There is no known antidote and death is usually the result of respiratory arrest. The LD_{50} of purified toxin when given intraperitoneally (i.p.)

to mice is about 200 μg/kg body weight while the oral dose of dry weight cell material is much higher (hundred of mg/kg) (Carmichael, 1992).

1.4.1.2.2 Saxitoxins

Saxitoxin and neosaxitoxin are produced by species and strains of *Anabaena* and *Aphanizomenon* but are better known as the products of dinoflagellates, the marine algae responsible for red-tide paralytic shellfish poisoning. These alkaloids inhibit nerve conduction by blocking sodium channels in axons, thereby preventing the release of acetylcholine at neuromuscular junctions with resultant muscle paralysis. The LD_{50} i.p. in mice equals about 10 μg/kg body weight (Carmichael, 1992).

1.4.1.3 Dermatotoxins

Dermatotoxins, lyngbyatoxin A and aplysiatoxin are produced by the cyanobacterium *Lyngbya majuscula*, a marine benthic cyanobacterium with different metabolite constituents in deep and shallow water varieties. The deep water varieties is known to produce inflammatory substances and tumor promoters, while the shallow water forms produce lipophilic substances, malyngamides A, B and C. Known clinical signs include skin, eye and respiratory irritation (Osborne et al., 2001).

1.5 Toxicology of microcystins

Microcystins are monocyclic heptapeptides, characterised by some invariant amino acids, including two of unusual structure, which is essential for expression of toxicity. The two unusual amino acids are N-methyl-dehydroalanine (Mdha) and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) (Nishiwaki-Matsushima et al., 1992). Bishop and co-workers isolated the most common microcystin, microcystin-LR, in 1959 from a Canadian strain of *Microcystis aeruginosa* (Bishop et al., 1959). The total structure of microcystin-LR was established as cyclo-D-alanine-L-leucine-erythro-β-methyl-D-isoaspartic acid-L-arginine-Adda-D-isoglutamic acid-N-methyl-dehydroalanine (Mdha) (Rinehart et al., 1988). Structure variations of the microcystins were first observed in amino acids 2 and 4, L-leucine and L-arginine. Other microcystins are characterised largely by variations in the degree of methylation. The amino acid 3 has been found to be D-aspartic acid, replacing β-methylaspartic acid, and amino acid 7 to be dehydroalanine, replacing N-methyldehydroalanine (Rinehart et al., 1988).

Adda has been shown to be the key structural component for biological activity. Ozonalysis of the double bonds on Adda yields free Adda plus the corresponding cyclic peptide minus Adda. Mouse bioassay of these two structures shows that neither has toxicity (Dahlem, 1989). During purification of microcystins by HPLC, a small peak is often observed eluting close to the main toxin peak. When analyzed, this small peak was found to be a geometrical isomer of the parent toxin. The isomerization was located at the C-8 position of Adda. The toxins associated with these minor peaks were microcystin-LR and-RR. This isomer was found to be non-toxic up to 1 mg/kg i.p. in mouse bioassays (Harada et al., 1990a, b).

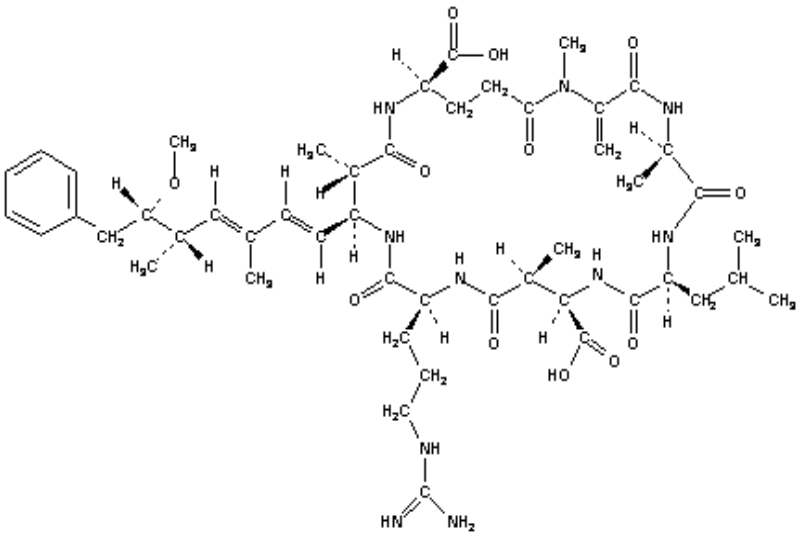


Figure 1.2 Chemical structure of microcystin-LR (from An and Carmichael, 1994).

1.6 Synthesis of microcystin

1.6.1 Chloroplast DNA

Shi et al. (1995) used a polyclonal antibody against microcystins in conjunction with immuno-gold labelling to localise microcystins in a toxin producing strain (PCC 7820) and non-toxin producing strain (UTEX 2063) of *Microcystis aeruginosa*. No specific labelling was found in the non-toxin producing strain, while most of the immuno-gold labelling in strain PCC 7820 occurred in the nucleoid and thylakoid region. The sheath area and the cell wall composed of peptidoglycan and lipopolysaccharide layers also displayed specific labelling, but to a lesser extent. No microcystins were found in cellular inclusions with storage products.

1.6.2 Plasmids

Vakeria et al. (1985) applied plasmid-curing agents to toxin-producing strains of *Microcystis aeruginosa* and did not find any significant decrease in toxicity. Also, to support this argument, Schwabe and co-wokers (1988) examined a number of toxic and non-toxic strains of *Microcystis aeruginosa*. Of the five toxic strains only one had no plasmids, while in the non-toxic strains no, or at most one plasmid of unstable occurrence, could be detected. Furthermore, they demonstrated that at least two of the plasmids they examined exhibited sequence homology with the cyanobacterial chromosomal DNA, indicating plasmids may be integrated into the host DNA. Results such as these, led Codd and Poon (1988) to conclude that plasmid DNA, which is not integrated into the cyanobacterial genome is likely to be involved in toxin production. This however, does not exclude the possibility that integrated plasmid DNA may contain the gene or genes coding for the peptide toxins (Codd & Poon, 1988). In contrast, evidence has been presented of a South African culture strain (WR70) that exhibited decreased toxicity after treatment with plasmid-curing agents (Hauman, 1982).

1.6.3 Thiotemplate mechanism

The thiotemplate mechanism, first characterized in *Bacillus brevis*, utilizes large, multifunctional enzymes to activate, modify, and condense amino acids into peptides. This system has been reviewed thoroughly in the literature (Kleinkauf & Gevers 1969; Laland & Zimmer 1973; Kleinkauf & von Döhren 1990; Nakano & Zuber 1990; Zuber

1991; Borchert et al., 1992; Marahiel et al., 1992). The activation step for the substrate amino acids is carried out through the formation of amino-acyl inter mediates that bind to the enzymes' surface. The hydrolysis of ATP to AMP and inorganic pyrophosphate provides the necessary energy. The standard method of elongation for the growing peptide chain is a 4-phosphopantetheine arm, for the transport of the activated amino acids to the condensation site. The condensation site catalyses a condensation reaction to form peptide bonds between amino acid adenylates (Stachelhaus et al., 1998).

The elongation of the peptide chain is not a repeated cycle of reactions as in polypeptide formation, rather it is a single cycle of sequential and similar reactions. The intermediates remain in an active state as thioesters and transfer of the growing peptide chain is mediated by the successive transthiolation of the cofactor 4-phosphopantetheine (Gilhuus-Moe et al., 1970; Kleinkauf et al., 1971). Peptide synthetases can employ one, two or three separate enzymes in the elongation and modification of the final product peptide. Arment and Carmichael (1995) were the first to speculate on the possibility of the thiotemplate mechanism being involved in the synthesis of microcystins. Towards the end of 1997 a peptide synthetase, termed *mcyB* was positively identified as a role-player in microcystin-LR production in culture strain PCC 7806 (Dittmann et al., 1997).

1.7 Mechanism of action by microcystin

1.7.1 Uptake

Microcystin is not cell permeant, and uptake occurs by carrier-mediated transport (Eriksson et al., 1990; Runnegar et al., 1991, 1995a, b). The properties of its transport, however, indicate that it is unlikely to be *via* one of the already well characterised bile salt organic anion transport proteins (Runnegar et al., 1995a, b). The elimination half-life of microcystin-LR in mouse plasma is about half an hour (Robinson et al., 1989).

1.7.2 Phosphatase inhibition

Microcystin-LR as an inhibitor of protein phosphatase type 1 and 2A was discovered at the National Cancer Center Research Institute, Tokyo (Yoshizawa et al., 1990; Matsushima et al., 1990). The toxin-phosphatase interactions are extremely strong, and binding is essentially stoichiometric (Suganuma et al., 1992). Recently the nature of the interaction of microcystins with protein phosphatase has been elucidated. Inhibition of the enzyme activity results from an initial non-covalent interaction, which is mediated by microcystins hydrophobic Adda side-chain and the glutamyl carboxyl (MacKintosh et al., 1995; Runnegar et al., 1995b). Inhibition of protein phosphatases leads to phosphorylation of cytoskeletal proteins and cytoskeletal-associated proteins and consequent redistribution of these proteins. Ghosh et al., (1995) showed that the collapse of cytoskeletal actin microfilament occurs in rat hepatocytes prior to the dislocation of the associated protein x-actinin and talin, rather than being caused by their dislocation. Macias-Silva and Garcia-Sainz (1994) proposed that the cytoskeletal disruption, which results from protein phosphatase inhibition, might cause alteration of the coupling between G-protein and phospholipase C.

1.7.3 Other biochemical effects

Microcystins are not only an inhibitor of protein phosphatases type 1 and 2A but also activate phosphorylase a (Runnegar et al., 1987). This may be the cause of the marked depletion of glycogen in the liver observed after administration of microcystins (Meriluoto et al., 1989; Miura et al., 1991). Depleted glutathione pools, and increased levels of serum sorbitol dehydrogenase and serum glucose have also been observed by Miura et al., (1991). Serum lactate dehydrogenase is another enzyme that increases in response to microcystin-LR following release of this enzyme from hepatocytes (Thompson & Pace, 1992). Increased levels of cytoplasmic calcium have also been observed in hepatocytes treated with microcystin (Runnegar et al., 1991). Time-

dependent decreases in hepatic microsomal membrane fluidity were observed when microcystin-LR was administered to mice (Hermansky & Stohs, 1991). These changes involve an indirect and secondary effect of the toxin, as no changes in membrane fluidity were observed when microcystin was incubated with control microsomes *in vitro*. LeClaire et al. (1995) suggested a potential cardiogenic component in the pathogenesis of shock, in addition to the effects on the liver. They observed a sustained, rapid decline in cardiac output and stroke volume in rats intoxicated with microcystin-LR. The acute hypotension was responsive to volume expansion with whole blood, and the acute drop in heart rate responded to both dopamine and isoproterenol. In response to hypotension a peripheral vasoconstriction appeared to occur.

1.8 Effects of cyanobacterial toxins on human activities and health

Much of the evidence implicating cyanobacterial toxins in humans health problems is circumstantial, although sufficient cases of human illness exist in which cyanobacterial poisoning is suspected. The involvement of cyanobacterial toxins in Haff disease remains speculative. Over a 1000 cases occurred in the 1920s and 1930s along the Baltic coast around Kaliningrad, USSR, among people eating fish, mainly burbot, from lagoons containing cyanobacterial blooms. Similar cases occurred along the Swedish coast (Berlin 1948). After eating fish, particularly the livers, from cyanobacterial bloom containing locations, symptoms of severe muscular pain, respiratory distress, vomiting, and brownish-black urine persisted for several days, though, of the large numbers of people affected, few fatalities were reported. However, it is known that microcystin peptide toxin principally causes liver damage when administered intraperitoneally to fish (Phillips et al., 1985). Only one incident involving parental exposure of humans to cyanobacteria is well documented. This involved a group of renal dialysis patients in a haemodialysis centre in Caruaru, Brazil, who, in early 1996, were inadvertently provided with dialysate improperly prepared from water taken from a reservoir which was contaminated with toxic cyanobacteria (Pouria et al., 1998: Jochimsen et al., 1998). The reservoir has been found to contain *Microcystis*, *Anabaena*, *Anabenopsis*, *Aphanizomenon* and *Oscillatoria* species (Pouria et al., 1998), each of which produced microcystins. All 126 patients became ill to various degrees and 60 of them eventually died, principally of liver failure, 6 had died within 2 weeks after exposure, 30 by 6 weeks, 44 by 10 weeks, and 55 by 27 weeks (Pouria et al., 1998). It was evident that these patients had suffered serious neurological and enterohepatic trauma, with the hepatotoxicity displaying many similarities to experimental animals injected with microcystin-LR (Ressom et al., 1994). The detection of microcystin-LR, microcystin-AR and microcystin-YR in liver tissue and serum samples at concentrations, which in experimental animals cause liver injury further implicated microcystins in the hepatotoxicosis (Pouria et al., 1998; Jochimsen et al., 1998).

Many cases of contact irritation have occurred after bathing in freshwaters containing toxic cyanobacteria. Symptoms of burning or itching of the skin, erythematous wheals, redness of the eyes and lips, often accompanied by sore throat, ear ache, and dizziness, have occurred among large numbers of bathers around the coasts of Hawaii, the Marshall Islands, Florida, and Okinawa (Hashimoto et al., 1996). Cyanotoxins such as microcystins, neurotoxins or cylindrospermopsin contained in the cells may reach target organs in the human body if the cells are accidentally ingested or aspired. This is relevant particularly for sports with intensive water contact such as sailboarding, water-skiing and competitive sailing in stormy weather. Swimmers can scarcely prevent nasal and oral uptake of water if they immerse their heads, and exposure estimates in recreational water toxicology are often based on the assumption of an average uptake of 100 ml per swimmer. Children are particularly at risk of ingesting major amounts of cyanobacterial cells when playing in the shallow zone along the shores where scum accumulates (Kay et al., 1994).

Epidemiological investigations of a population at Armidale, New South Wales, Australia, taking drinking water from a reservoir containing hepatotoxic blooms of

Microcystis aeruginosa, have indicated significant links between toxic cyanobacteria and human liver illness (Falconer et al., 1983). In this study, a pattern of liver damage, evidenced by increased levels of hepatic γ-glutamyltranspeptidase and alanine aminotransferase in the plasma, was found by examination of the results of routine assays of patients admitted to a local hospital. A seasonal increase in toxic liver injury coincided with the peaks of the toxic *Microcystis aeruginosa* bloom and this was only detected in patients who had taken water from the affected reservoir (Falconer et al., 1983). More importantly, microcystins are potent tumour promoters (Nishiwaki-Matsushima et al., 1992) and there is an indication that they may also act as tumour initiators (Ito et al., 1997). Epidemiological studies have suggested that microcystins are one of the risk factors for the high incidence of primary liver cancer in certain areas of China, where people have consumed pond-ditch water contaminated with cyanobacteria (Yu, 1995; Ueno et al., 1996).

Information regarding the genotoxicity of microcystins is relatively scarce. Repavich et al., (1990) reported that microcystins induced chromosomal aberrations in human lymphocytes *in vitro*. More recently it was found that they induce DNA damage in mouse livers *in vivo* (Rao & Bhattacharaya, 1996), and *in vitro* in cultured rat hepatocytes (Ding et al., 1999), baby hamster kidney cells and mouse embryo primary fibroblasts (Rao & Bhattacharaya, 1996). Moreover, it was found that *Microcystis* induced base substitution mutations at *K-ras* kodon 12 in human Rsa cells (Suzuki et al., 1998). More recently, it was demonstrated that microcystin-LR at doses that were assumed to be not cytotoxic (0.01-1 μg/ml), induced dose and time dependent DNA strand breaks in human hepatoma cell line HepG2. These DNA strand breaks were transient, reaching a maximum level after 4 h of exposure and declining with further exposure (Zegura et al., 2002).

1.9 Toxin persistence and degradation

In natural waters, it has been observed that dilution decrease the concentration of microcystins (Jones and Orr, 1994). Thus, a decrease in concentration at one site does not automatically allow the conclusion that a breakdown of the toxins has occurred. Cyclic peptide hepatotoxins are extremely stable due to their chemical structure. Complete chemical hydrolysis is achieved only by harsh treatments, for example with 6N hydrochloric acid or with trifluoroactetic acid under reflux (Harada, 1996). The Adda side-chain of microcystin, in particular its conjugated diene, is essential for toxic activity, but is also reasonably susceptible to the action of strong oxidants such as ozone or chlorine (Hrudey et al., 1999). The degration of microcystins can also be achieved by semiconductor photocatalysis under UV irradiation in the presence of titanium dioxide (Lawton et al., 1999: Feitz et al., 1999). The absorption of microcystins to particle surfaces has mostly been investigated under aspects of water treatment such as removal by activated carbon (Lambert et al., 1996). Some microcystin removal has been observed though adsorption onto suspended solids in water, but this seems to be only a low-affinity pathway for the removal of microcystin. Only about 10% of the initial 5μg/L microcystins were found adsorbed on natural seston particles after three days of contact (Rivasseau et al., 1998).

Stevens and Krieger (1991) showed that microcystin persisted for two to three months in cultures of *Microcystis* grown in water from the Vantaan River, Finland, while Jones and Orr (1994) were able to detect microcystin up to 21 days following algicide treatment of a bloom of *Microcystis*. Work on the environmental degradation of microcystin by Jones and Orr (1994) has revealed that bacteria are involved in the process. They found a Gram-negative rod bacterium that degraded microcystin at 1 mg/ml in water. Degradation began after a lag phase of 2-3 days, although the lag phase was absent upon subsequent re-addition of microcystin to water. The degradative activity was detected in the cell-free culture supernatant indicating that extracellular hydrolytic enzymes may be involved.

1.10 Detection and assessment of microsytin

1.10.1 Mouse bioassay

The mouse bioassay is world-wide the recognized standard in terms of establishing the LD_{50}, symptoms and effects of cyanotoxins, and thus by mouse bioassay the toxicity of cyanobacterial may be assessed (Chaivimol et al., 1994; Swoboda et al., 1994). Routinely, adult female and male mice are injected intraperitoneally with the study samples to be assayed. The mice are observed every 15 min for toxic symptoms and break points of toxicity are normally scored within the first 24 h. Toxic symptoms vary depending upon material and concentration, but are in general distinct for a category of toxins. The symptoms of death of the animal allow a clear distinction to be made as to whether the toxicity is due to neuro or hepatotoxin. Neurotoxicosis is rapid compared to hepatotoxicosis and does not cause liver damage to animals (Falconer & Yeung, 1992). A major disadvantage of the mouse bioassay is that it cannot detect low amounts of toxins and can therefore only be used for concentrated cell samples or concentrated toxin. In addition, it cannot distinguish between different types of neuro- or hepatotoxins, particularly when several are present in the same sample (Carmichael, 1992).

1.10.2 Immunoassays

The cyclic peptide toxins provide potentially antigenic molecules, which can be used to raise antibodies in test animals. Early studies in laboratories used microcystin covalently linked to human serum albumin by 1-ethyl 3-(3-dimethyl aminopropyl) carbodiimide as the antigen. This was emulsified with Freund's complete adjuvant and injected intradermally in young male rabbits. Second and third injections were given at 6 and 11 weeks. Antibodies were present in sera collected from 9 weeks after initial injection, until 32 weeks after injection. Microcystin-YM was the major antigen present, but failed to detect toxin present in cyanobacteria blooms rich in microcystin-RR and-LR. However Kfir et al., (1986) raised monoclonal antibodies against microcystin-LA that showed considerable cross-reactivity with other microcystins and offer a better approach to a general immunoassay for these cyanobacteria toxins.

An enzyme-linked immunosorbent assay (ELISA) technique has been developed by Chu et al., (1989), to the point where ng/ml of microcystin can be quantitated in supplies of domestic water. This immunoassay is based on polyclonal antisera raised in rabbits against bovine serum albumin conjugated to microcystin-LR (Chu et al., 1989). The antisera showed good cross-activity with microcystin-RR, -YR, -LR and nodularin, but less with -LA and -LY. For detection of binding to the antisera the enzyme horseradish peroxidase was conjugated to microcystin-LR. The sensitivity of the immunoassay showed approximately 50% binding of the enzyme at a toxin concentration of 1 ng/ml which is ideal for normal water quality testing.

1.10.3 Alternative Bioassays

Under cyanobacterial bloom conditions the amount of zooplanton is known to decrease (Lightner, 1978), consequently, bioassays have been devised using *Daphnia* sp. and *Artemia salina* (Kiviranta et al., 1991). Major difficulties in the use of alternative bioassays such as those based on *Artemia salina* are highlighted by Kiviranta et al. (1991). High to moderate concentrations of neuro-and hepatotoxins can be detected in bloom samples using this organism, but mouse bioassay is more reliable. In contrast, a laboratory-grown strain of *Oscillatora agardhii* was toxic to larvae of mosquito *Aedes aegyptii* (Kiviranta & Abdelhameed, 1994), *Artemia salina* and *Daphnia pulex*, but non-toxic to mice (Reinikainen et al., 1995). Also, toxicity differs for the larval and adult stages of the shrimp (*Artemia salina*), particularly for neurotoxins. It is possible that some compounds may enable the toxin to enter the larvae by affecting the biochemistry

of the crustacean or by hydrogen bonding to the chelates, which are more easily absorbed. Therefore the use of alternative bioassays must be approached with caution (Kiviranta & Abdelhameed, 1994).

1.10.4 Chromatographic Analysis

Initially to purify the hepatotoxins thin-layer chromatography was used, but lacked sensitivity and specificity. This was superceded by high performance liquid chromatography (HPLC) with which it has been possible to isolate hepatotoxins from several cyanobacterial species and environmental biomass with high sensitivity (Harada et al., 1996). Resolution of the toxic fraction from *Microcystis aeruginosa* PCC7806 into a closely related peptide has been achieved by reversed-phase (RP) HPLC (Swoboda et al., 1994). Integration of diode array spectroscopy with RP-HPLC has provided a rapid and sensitive method of assessing the nature and concentration of toxic peptides from cyanobacterial blooms. All these procedures involve solvent extraction of the cyanobacteria and clean-up prior to analysis, though reversed-phase fast protein liquid chromatography has been used to distinct toxic peptides in a single preparative scale run (Cremer & Henning, 1991).

1.10.4 Protein Phosphatase Inhibition Assay

The discovery that the hepatotoxic cyanobacterial toxins produce their toxic effects through the inhibition of protein phosphatases 1 and 2A has laid the foundation for toxin assays based on the inhibition of these enzymes (Sim & Mudge, 1994). This is an effective assay and reliable means of detecting all hepatotoxic cyanobacterial toxins and has been enhanced by the development of a colorimetric procedure (Ward et al., 1997).

1.10.5 Tissue Culture Cytotoxicity

It is well known that hepatotoxins cause deformation and ultrastructural changes of the cytoskeleton of mouse liver hepatocytes primarily due to the inhibition of phosphatase activity (Eriksson et al., 1990). Aune and Berg (1986) proposed the use of freshly prepared rat hepatocytes to study the toxicity of cyanobacterial blooms. They have shown that the *in vitro* toxicity to cells can be correlated with control animal experiments when crude cyanobacterial biomass is used. Similar effects have been observed with permanent cell lines and erythrocytes (Grabow et al., 1982). However, despite the remarkable toxic potential of hepatotoxins *in vivo*, no cell lysis, liberation of lactate dehydrogenase or haemolysis has been observed after application of pure hepatotoxin to primary or permanent cell lines (Eriksson et al., 1987).

1.10.6 Mass Spectrometry

The characteristics of the structure of hepatotoxins have been ascertained primarily by fast atom bombardment mass spectrometry and proton nuclear magnetic resonance (Kusumi, 1996). More recently, matrix assisted laser desorption ionisation mass spectrometry has permitted procedurally easier analysis of cyanobacterial biomass. This presents the possibility of rapid, sensitive analysis of environmental biomass for the presence of cyanotoxins (Chaivimol et al., 1994).

1.10.7 Molecular tools

Cyanobacteria display a great diversity of forms, which differ in their morphology, structure and function, and in their mode of response to environmental stimuli. Their characteristics are expressions of their genotype which itself is the result of many million years of evolutionary history. Biological macromolecules, primarily proteins and nucleic acids, are known to exhibit common group characteristics as well as high degree of specificity. Genetic information for the entire complement of phenotypically-expressed characteristics is incorporated in the nucleotide sequence of DNA, and thus

the relatedness between DNAs of different organisms should reflect their genealogical relationships (Fray, 1983).

There are several ways of using DNA for the assessment of genetic relatedness of cyanobacteria. One is the determination of genome size. It has been shown that genome size varies considerably among the cyanobacteria strains tested. The finding that most unicellular strains possess relatively small genomes, and that the pleurocapsalean and filamentous forms have larger genomes, seems to support the conclusion drawn from palaeontological evidence that the unicellular forms were first to evolve and that they gave rise in Precambrian times to other forms with more complex morphology (Fray, 1983).

Some of the molecular methods used for taxonomic studies of bacteria have also been applied to cyanobacteria (Wilmotte, 1994), sometimes with modifications applicable to phototrophs. Recent approaches include DNA-DNA hybridization (Wilmotte et al., 1997), fingerprinting based upon polymerase based reaction (PCR) with primers from short and long tandemly repeated elements (Rasmussen et al., 1998), PCR gene probe analysis (Neilan et al., 1999), classification of clone cultures based upon 16S rRNA sequences from the variable regions V6, V7 and V8 (Rudi et al., 1997); amplified ribosomal DNA restriction analysis of the Phycocyanin locus (Neilan et al., 1995) and the internally transcribed spacer (Scheldeman et al., 1999); amplified fragment length polymorphisms (AFLPs) (Oberholster, 2003); restriction fragment length polymorphism(RFLP)-PCR based analysis (Oberholster, 2003); and PCR-based genetic methods for screening environmental samples (Baker et al., 2001; Bittencourt-Oliveira, 2003; Mikalsen et al., 2003; Oberholster et al., 2005a, 2006a, b).

1.11 *Mcy* genes

Over 70 structural variations of microcystins have been isolated from cyanobacteria. Peptides containing non-protein amino acids are synthesized non-ribosomally by a large multifunctional enzyme complex, utilizing a thio-template mechanism, called non-ribosomal peptide synthetase and polyketide synthase modules. Individual peptide synthetase modules catalyze amino/hydroxy acid activation and thioester formation reactions in the same order in which their residues are incorporated into the growing heptapeptide chain. The polyketide synthase is proposed to be involved in the production of the fatty acid side chain of the unique amino acid Adda, essential for toxicity of the microcystin peptide. Genes for the enzyme complex form a large gene cluster containing at least two operons, *mcyABC* (peptide synthetase) and *mcyDE* (hybrid polyetide-peptide synthetase) (Tillett et al., 2000). The messages are transcribed in opposite directions by a central sequence of approximately 900 bp containing the promotor and putative regulator *cis* elements (Kaebernick et al., 2000). The *mcyA,B* and *C* genes encoding five modules that activate the five amino acid constituents, and a putative open reading frame 743 bp upstream of *mcyA*, which was in the opposite orientation to *mcyABC*. Analysis of the cloned nucleotide sequence revealed three huge open reading frames and three small open reading frames with the same direction of transcription. The first open reading frame, *mcyD*, is 11,718 bp in length, encoding a polypeptide of 3,906 amino acids with a predicted molecular mass of 435,915 Da. The putative Shine-Dalgarno sequence (AAG—GA) was found 9 nucleotides upstream of the start codon. The second open reading frame, *mcyE*, is located 167 bp downstream of the TAA stop codon of *mcyD*. A possible Shine-Dalgarno sequence (AGAGAA) is located 6 bp upstream of the ATG codon. This open reading frame (10,461 bp) encodes a putative protein of 3,487 aa with a predicted molecular mass of 392,319 Da. The third open reading frame, *mcyF*, is located 35 bp downstream of *mcyE* and is 753 bp in length, encoding a 251 residue polypeptide with a predicted molecular mass of 27,990 Da. The putative Shine-Dalgarno sequence (AGGAGA)) was found 4 nucleotides

before the putative initiation codon. The fourth open reading frame, *mcyG*, is located 74 bp downstream of *mcyF* and is 7,896 bp in length, encoding a 2,632 residue polypeptide with a predicted molecular mass of 294,299 Da. The putative Shine-Dalgarno sequence (AAGAGG) was found 10 nucleotides before the putative initiation codon (Nishizawa et al., 2000). The insertion inactivation of microcystin peptide synthetase gene *mcyB* of *Microcystis aeruginosa* hepatotoxic strain (PCC7806) resulted in loss of microcystin production, showing their involvement in microcystin synthesis (Dittmann et al., 1997). It was also observed in this study that all isoforms of this cyclic heptapeptide were disrupted by inactivation of the microcystin synthetase gene sequence *mcyB* (Nishizawa et al., 2000).

With the identification of the *mcy* genes in the production of microcystin synthetase for the first time provide an avenue to study the regulation of microcystin production at a genetic level (Figure 1.3). Kaebernick et al. (2000) used *M. aeruginosa* strain PCC7806 grown, either under continuous light of various intensities or under low light with subsequent short-term exposure to different light intensities and qualities, as well as various stress factors, to observe the different levels of *mcyB* and *mcyD* transcription under each condition. RNase protection assays were employed. Both *mcyB* and *mcyD* transcript levels were increased under red light and high light intensities, while artificial stress factors like NaCl and methylviologen, as well as blue light led to reduced transcript amounts.

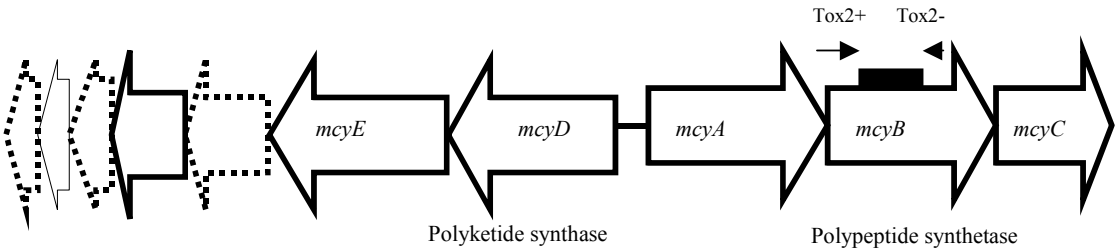


Figure 1.3 Microcystin synthetase gene clusters *mcyABC* and *mcyED* (Kaebernick et al., 2000; Christiansen et al., 2003; Mikalsen et al., 2003; Hisbergues et al., 2003).

1.12 Objectives

The study aimed to:

- determine the genetic diversity and population structure in selected South African water reservoirs (Chapter 2);
- determine the potential of using the *mcyB* gene sequence as a diagnostic tool in raw water to detect the presence of toxin-producing cyanobacterial spp. in South African water reservoirs (Chapter 3).

2. GENETIC DIVERSITY OF GEOGRAPHICALLY UNRELATED *MICROCYSTIS AERUGINOSA* STRAINS USING AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS (AFLPs)

2.1 INTRODUCTION

The phylum Cyanobacteria is large and diverse, containing over 1000 species of oxyphototrophs, and its members are classified by using both botanical and bacteriological taxonomic codes (Castenholz et al., 1989; Komárek, 1991a, b; Komárek & Anagnostidis, 1986, 1989; Rippka, 1988; Rippka et al., 1979). The taxonomy and classification of cyanobacteria has been under investigation since about the middle of the 19th century using morphological and cellular criteria, similar to other microalgae (Kützing, 1849; Nägeli, 1849 as cited by Komárek, 2003). Because of morphological simplicity of most prokaryotes, their classification was previously based largely on physiological properties, as expressed in pure laboratory cultures (Doers & Parker, 1988). While field studies relied mostly on morphological analyses of natural populations, laboratory studies concentrated on culture characterisations. The principle of morphological studies includes the use of characteristics observable and measurable under a light microscope, such as shape of colony, presence of sheaths and envelopes, colour of colonies, differentiation and cell content. Based on these criteria, the traditional taxonomic classification systems of cyanophytes placed a high value on cell division patterns, colony formation and relationship to extracellular envelopes and sheaths. Cell shapes and dimensional differences were used largely to distinguish between species within each genus (Doers & Parker, 1988). This method caused difficulties in their classification by introducing organisms with different cell organizations but similar cell arrangements to the same generic identity. Furthermore, it required considerable expertise to identify species since both morphological and developmental characteristics can vary with the growth conditions (Evans et al., 1976). The main problems met in applying morphological criteria in cyanophyte classification arise from the considerable variability in morphological features with indifferent environmental conditions (Komárek, 1991a, b).

Diversification through ecological acclimation, adaptation and stabilization of diverse morpho- and ecotypes, as well as changes caused by mutation and possibly also genome transfers (Rudi et al., 1998) can give rise to new cyanobacterial types in different geographical locations. Nearly all populations of cyanobacteria from different geographical locations differ to some degree from each other, and these deviations may stabilize in long term cultures (Waterbury & Rippka 1989; Kato & Watanabe, 1993). This process indicates that new forms continually develop and are stabilized under new constant conditions. Diversification within the cyanobacteria is a continuing process in which new types develop from continually modified cyanobacterial genotypes under different environmental conditions at different geographical locations. Many investigations showed that blooms of *Microcystis* spp. can differ in important characteristics (Cronberg & Komárek, 1994). The cyanobacteria *Microcystis aeruginosa* is characterized by the existence of a wide variety of genotypes that differ in their content of secondary metabolites (Martin et al., 1993; Moore, 1996), plasmid content (Kohl et al., 1988; Schwabe et al., 1988) and interaction with zooplankton (Hanazato, 1996). Recent data also reveal variations within cyanobacterial genomes, such as changes in the toxicity of certain strains (Neilan et al., 1997) and possible interchanges of genetic material (Rudi et al., 1998). Other well-adapted phytoplankton forms, so called traditional species like *Aphanothece, Chroococcus* and *Hyella* appears to persist over the long-term and contain a wide spectrum of stable types for long periods (Komárek & Anagnostidis, 1998). Due to the development of recent molecular techniques new approaches have been introduced to the phylogeny and taxonomy of cyanobacteria. Several methods of molecular biology have to date been successfully applied in aid of cyanobacterial taxonomy: determinations of DNA base ratios (Herdman et al., 1979a, b), DNA-DNA hybridisations (Stam, 1980; Wilmotte & Stam,

1984; Stam et al., 1985) and gene sequencing (Masui et al., 1988; Nielan et al., 1997; Lyra et al., 2001; Gugger et al., 2002).

The use of DNA-based genetic markers (Bodstein et al., 1980) has forever changed the practice of genetics. Over the past 20 years since that discovery, many different types of DNA-based genetic markers have been used for the analysis of genetic diversity, as well as for applied diagnostic purposes (Powell et al., 1996). The use of modern molecular biological techniques to determine the degree of sequence conservation between bacterial genomes has led to the development of methods based solely on the detection of naturally occurring DNA polymorphisms. These polymorphisms are a result of point mutations or rearrangements (i.e. insertions and deletions) in the DNA and can be detected by scoring band presence versus absence in banding patterns that are generated by restriction enzyme digestion and DNA amplification procedures. The underlying idea is that variation in banding patterns are a direct reflection of the genetic relationship between the bacterial strains examined and therefore, that these banding patterns can be considered as genomic fingerprints allowing numerical analysis for characterization and identification. AFLP markers have been used to scan genome-wide variations of strains, or closely related species, that have been impossible to resolve with morphological features or other molecular systematic characters. Therefore, AFLP has broad taxonomic applicability and have been used effectively in a variety of taxa including bacteria (Huys et al., 1996).

AFLP analysis is based on selective amplification of DNA restriction fragments (Vos et al., 1995). It is technically similar to restriction fragment length polymorphism analysis, except that only a subset of the fragments are displayed and the number of fragments generated can be controlled by primer extensions. The advantage of AFLP over other techniques is that multiple bands are derived from all over the genome. This prevents over interpretation or misinterpretation due to point mutations or single-locus recombination, which may affect other genotypic characteristics. The main disadvantage of AFLP markers is that alleles are not easily recognized (Majer et al., 1998). PCR has proven to be successful in detecting genetic variation amongst plant-pathogenic fungi, as well as bacteria (Majer et al., 1996; Janssen et al., 1996). The utility, repeatability and efficiency of the AFLP technique are leading to broader application of this technique in the analysis of cyanobacteria populations (Janssen et al., 1996). The purpose of this study was to investigate the usefulness of AFLP, which is based on the selective amplification of genomic restriction fragments by PCR, to differentiate between geographical unrelated strains of *Microcystis* spp.

2.2 MATERIALS AND METHODS

2.2.1 *Chemicals, Strains and Culture Conditions*

Analytical reagent grade chemicals were purchased from various commercial sources and were used without further purification. *Microcystis aeruginosa* strains used in the study represented a wide variety of geographically unrelated strains (Table 2.1). Strains PCC7806 and PCC7813 were obtained from the Pasteur Institute Culture Collection, France; UV027 from the University of the Free State Culture Collection, South Africa; CCAP1450/1 was obtained from the Culture Collection of Algae and Protozoa, Institute of Freshwater Ecology, UK; NIES88, NIES89, NIES91, NIES99 and NIES299 from the National Institute for Environmental Studies, Japan; and SAG1 from the Pflanzen Physiologisches Institut, Universität Gottingen, Germany. All these strains were received as axenic, maintained as such and microscopically verified prior to further experiments.

Unicellular strains UP01, UP03, UP04, UP10, UP13, UP26, UP37 and UP38 were collected by the authors, representatives of the Water Research Commission and

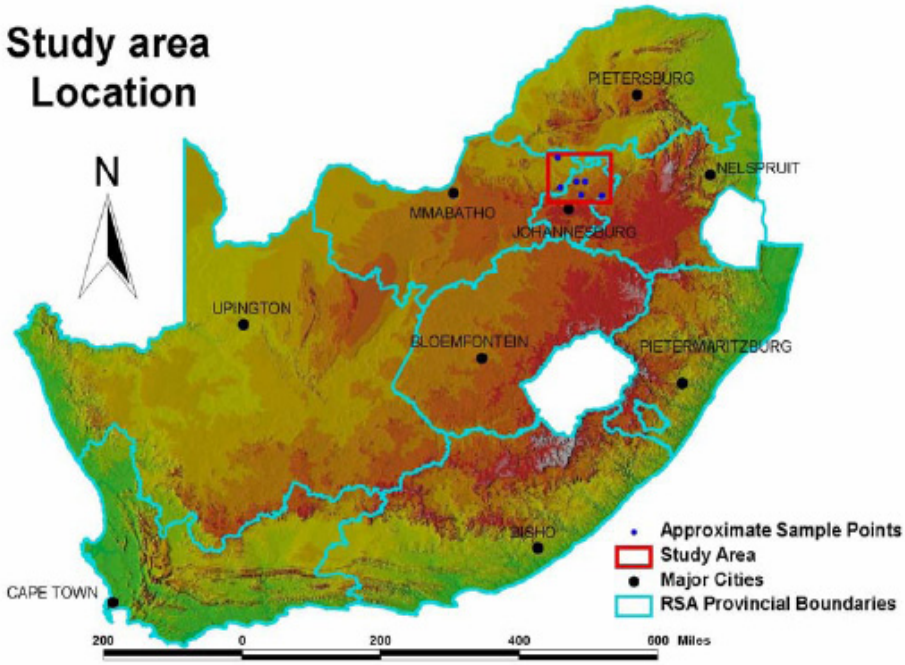
Tswhane Metro Council, respectively. The UPUS1 and UPUS2 were collected at Sheldon Lake, Colorado by the authors. Outgroups included in the study were the division Cyanophyta: UPUS2 *Woronichinia naegeliana* (Smith, 1950); UP03 *Microcystis wesenbergii* (Teiling, 1941; Wojciechowski, 1971); UP13 *Chroococcidiopsis cubana* (Komárek & Hindák, 1975) and the division Chlorophyta: UP26 *Scenedesmus acutus* (Ettl & Gärtner, 1995).

Water samples were collected from blooms of *Microcystis* spp. in seven eutrophic or hypertrophic artificial reservoirs in Gauteng and North-West provinces South Africa (Figure 2.1), used principally for public water supply and recreation, using a 25 µm plankton net or Van Dorn bottle. In some reservoirs collections were carried out at several points, both on the surface and down the water column, at different seasons of the year. It was attempted, as much as possible, to sample more than one population per locality.

Table 2.1 Different strains used in the study and their origin.

Strain	Source	Origin
Microcystis spp.		
M. aeruginosa strain PCC7806	Pasteur Culture Collection, France	The Netherlands
M. aeruginosa strain PCC7813	Pasteur Culture Collection, France	Scotland
M. aeruginosa strain UV027	University of the Free State Culture Collection	Israel
M. aeruginosa strain NIES88	National Institute for Environmental Studies	Japan
M. aeruginosa strain NIES89	National Institute for Environmental Studies	Japan
M. aeruginosa strain NIES91	National Institute for Environmental Studies	Japan
M. aeruginosa strain NIES99	National Institute for Environmental Studies	Japan
M. aeruginosa strain NIES299	National Institute for Environmental Studies	Japan
M. aeruginosa strain SAG1	Pflanzen Physiologisches Institut, Universität Gottingen	Germany
M. aeruginosa strain CCAP1450/1	Institute of Freshwater Ecology	UK
M. aeruginosa strain UP01	University of Pretoria Culture Collection	Rietvlei, ZA
M. aeruginosa strain UP04	University of Pretoria Culture Collection	Hartbeespoort, ZA
M. aeruginosa strain UP06	University of Pretoria Culture Collection	Paardekraal, ZA
M. aeruginosa strain UP09	University of Pretoria Culture Collection	Hartbeespoort, ZA
M. aeruginosa strain UP10	University of Pretoria Culture Collection	Roodeplaat, ZA
M. aeruginosa strain UP15	University of Pretoria Culture Collection	Bon Accord, ZA
M. aeruginosa strain UP37	University of Pretoria Culture Collection	Krugersdrift, ZA
M. aeruginosa strain UP38	University of Pretoria Culture Collection	Hartbeespoort, ZA
M. aeruginosa strain UPUS1	University of Pretoria Culture Collection	Ft Collins USA
M. wesenbergii strain UP02	University of Pretoria Culture Collection	Rietvlei, ZA
Other genera		
Chroococcidiopsis cubana strain UP13	University of Pretoria Culture Collection	Klipvoor, ZA
Woronichinia naegeliana strain UPUS2	University of Pretoria Culture Collection	Ft Collins USA
Scenedesmus acutus strain UP26	University of Pretoria Culture Collection	Klipvoor, ZA

A



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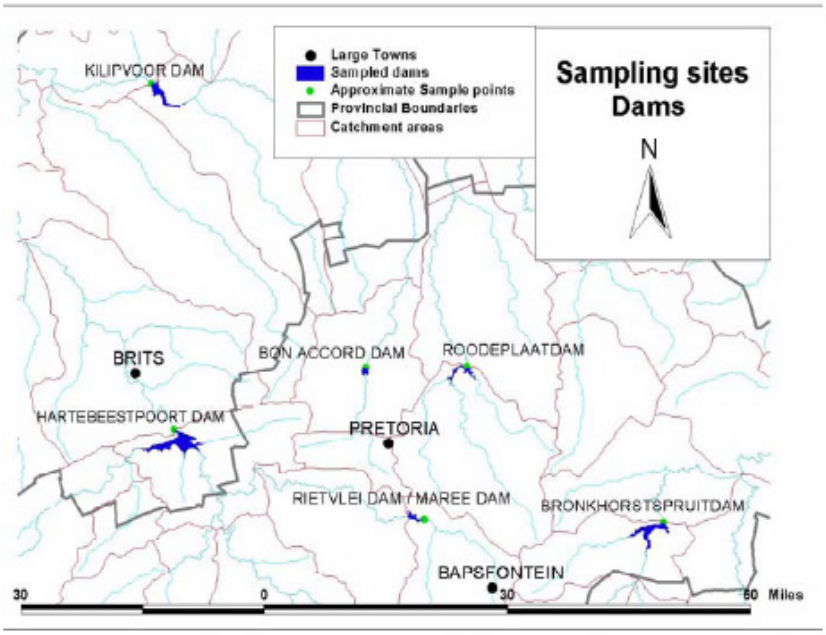


Figure 2.1. (a) Study area (in red) where the study is conducted in South Africa. (b) The approximate sampling points relative to the provincial boundaries, urban and catchment areas.

After collection, the water samples were placed on ice in a darkened cooler during transport to the laboratory. Holding time for samples was less than 48 h in all cases. An aliquot of the samples were then transferred into 100-mL vessels and treated with overpressure of 0.2 mPa for 2 min. In consequence, gas vesicles, and in some cases, cells of most cyanobacteria (e.g. *Aphanizomenon*) collapsed, whereas *Microcystis* cells were still intact and buoyant. After the pressure treatment, the suspension was centrifuged (3 000 g) to separate *Microcystis* colonies from the destroyed cells of other cyanobacterial spp.

The screw-cap tubes containing the collected samples of cyanobacteria were supplemented with water from the habitat to fill the sample tubes almost completely, this insure that the samples are well submerged by the aqueous phase typical of their natural environment, but also minimize the growth of aerobic bacteria during transport of the samples to the laboratory by limiting their supply of oxygen. This generally does not harm cyanobacteria, but increases considerably the changes of rapid purification. In addition, major changes of pH and nutrient composition resulting from other bacterial metabolic activities, potentially harmful for the cyanobacterial samples, is minimized.

2.2.1.1 Isolation and purification

Since *Microcystis* have a significant portion of their cell volume taken up by gas vesicles, positive buoyancy is a general feature of at least those species that are circulated within the epilimnia of lakes. Because of this property, contaminants may be separated from gas-vesiculate types by centrifugation. The procedure is well described by Walsby (1969). A general outline of the procedure as followed is given (Figure 2.2). Since very high centrifugation speeds results in gas vesicle collapse with applied pressures of about 1 bar or more, a mixed collection (e.g. $\pm 10^4$ cells ml^{-1}) were placed in tubes in a swing-out rotor and turned for the first 2 min at 100*g* or less, after which time the buoyant cells have risen part way in the tube. At this point, the applied pressure of 1 bar would not be exceeded at that depth until an acceleration of 200*g* is used. Therefore, the rotor can be increased 100*g* every 2 min.

After 10 min when all buoyant cells have reach the surface meniscus, the centrifuge is been increased to its maximum safe setting for another 5 min. After a complete stop and standing for a few minutes to allow gas vacuolated cells to float to the edge of the meniscus, the meniscus edge is touch with the top of a flat-ended syringe needle and a small amount of crude material is withdraw and transfer to a separator funnel with sterilized water. The lower aqueous layer was discarded. The obtained buoyant cells in the top layer were suspended in sterilized water. This procedure was repeated five times to wash the cells. Then, the washed cells were vigorously stirred with a vortex mixer for 1 to 2 min and serially diluted 10-fold in sterilized water to break the blooms into single cells and to separate the *Microcystis* cells from other cyanobacteria.

2.2.1.2 Preparation of solid media

Analytical reagent grade chemicals were purchased from various commercial sources and were used without further purification. Unless otherwise stated, standard methods described in Sambrook et al. (1989) were used. BG-11, designed by M.M Allen in 1968 was used as growth media (1989). BG-11 consists of very high nitrates concentrations and relatively low phosphate (molar ratio 99:1). The liquid BG-11 nutrient medium containing 17.65 mM $NaNO_3$, 0.18 mM $K_2HPO_4.3H_2O$, 0.30 mM $MgSO_4.7H_2O$, 0.25 mM $CaCl_2.2H_2O$, 0.03 mM citric acid, 0.03 mM ferric ammonium citrate, 0.003 mM EDTA (ethylenediamine tetra-acetic acid, disodium magnesium), 0.19 mM Na_2CO_3, 0.05 mM H_3BO_3, 9.15 mM $MnCl_2.4H_2O$, 0.77 mM $ZnSO_4.7H_2O$, 1.61 mM $Na_2MoO_4.2H_2O$, 0.37 mM $CuSO_4.5H_2O$ and 0.17 mM $Co(NO_3)_2.6H_2O$ dissolved in 1L distilled water. Freshly poured petri plates were left to cool, solidify, and dry in a laminar flow hood sterilized by UV irradiation. The warm plates were stacked on top of each other to prevent the formation of condensation below their covers. The medium was left to dry at 37 ºC for 24 hr.

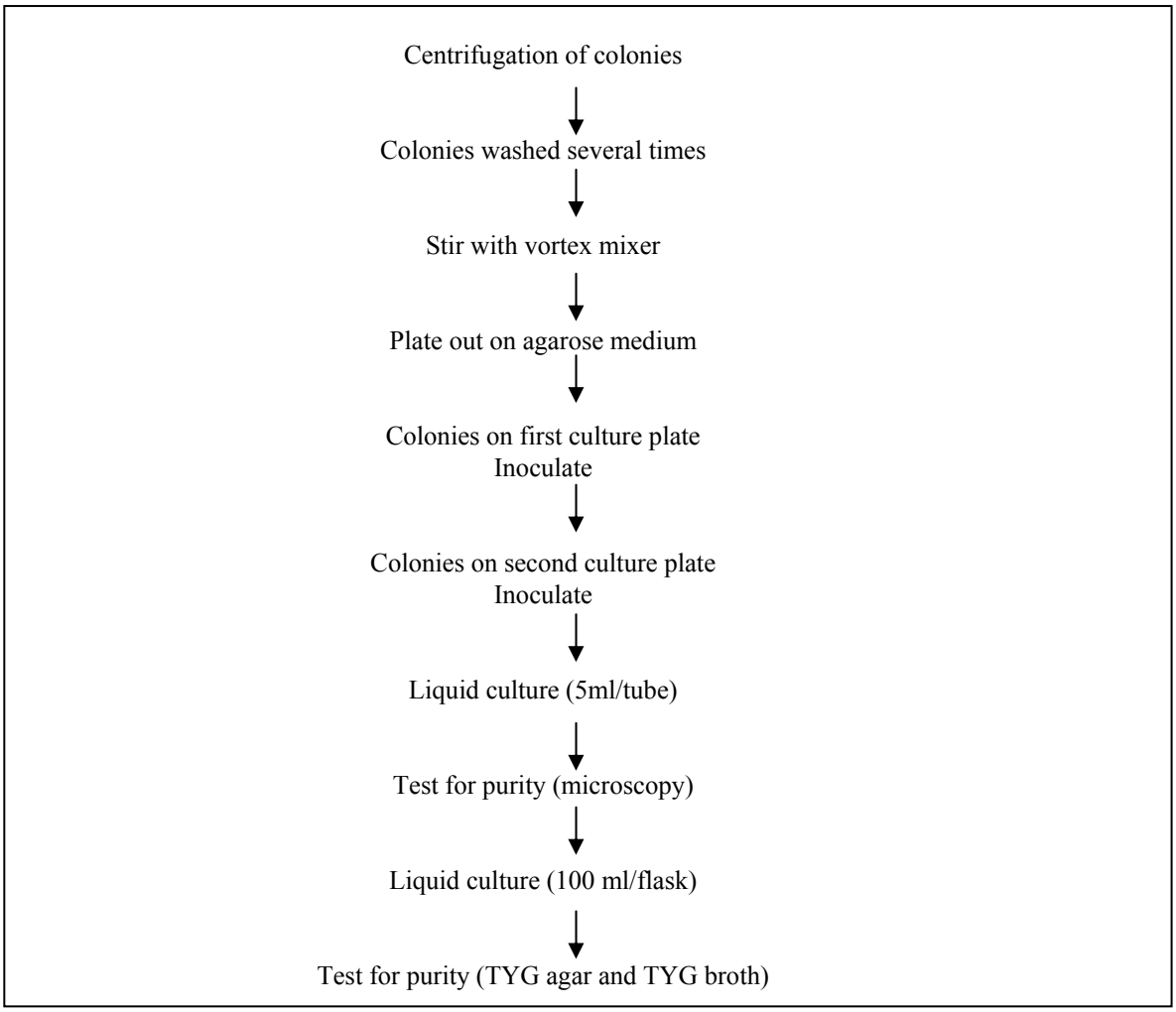


Figure 2.2 Isolation procedure for axenic *Microcystis* strains.

When the agar medium was solidified in Petri dishes a bacterial loop was mainly employed in the initial stages of isolation. With the loop, heated to redness in the flame of a Bunsen burner and cooled at one edge of the agar plate, the crude material deposited is densely spread over the first quarter of homogenize aggregates of colonies. The loop is then flamed again, cooled and used to perform several parallel streaks traversing at an angle of 90° to the previously spread material. The incubation was done at 25°C under continuous illumination of approximately 60 µmol photons.m^{-2}.s^{-1} from cool white fluorescent tube for 7 to 10 days (first culture). When the colony formation was observed, the *Microcystis aeruginosa* cells in the colonies were identified using Komárek's (1958) criteria for colony morphology and cell diameter (Table 2.2). These microcystis cells were then transferred by a sterilized toothpick to fresh agarose medium and incubated again (second culture). Long-term first culture has been avoided because of the possibility for contamination by bacteria and fungi increases. The cells in fully propagated colonies in the second culture were inoculated into BG-11 medium (5 ml per tube) and cultured for 1 to 2 weeks. A phase-contrast microscope was then used to determine whether any contamination had occurred during culturing. If contamination was observed, the culture was returned to the first step of the isolation procedure. The cells from the cultures that were confirmed to be contamination free by microscopic examination were encircle by an autoclaved toothpick and an incision through the agar was made. The tiny piece of agar bearing the micro colony of *M. aeruginosa* was inoculated into 100 ml of BG-11 medium in a 250-ml Erlenmeyer flask and then cultured. The purity of the resulting cultures was verified by the absence of bacterial growth on TYG agar and TYG broth (tryptone (Difco), 5.0 g; yeast extract

(Difco), 2.5g; glucose, 1.0 g per litre) after incubation of two weeks at 26°C. The final proof of purity was verified by microscopic examination.

2.2.1.3 Morphological identification

Cultures of *Microcystis aeruginosa* were harvested at the end of exponential growth phase (± three weeks) by centrifugation at 6 000 *g* for 10 min at room temperature. The cultures were then freeze-dried and stored at –20 °C. The strains isolated from the blooms were identified following the procedure as described by Komárek, 1991b (Table 2.2).

Table 2.2 Diacritical features (morphological) of three species (Komárek, 1991b).

Cell size (µm)	Mucilage	Form of colony	Holes	Names
(2.5) 3.5-4.8 (5.6)	very narrow diffluent, ± 1µm overlapping groups of cells	irregularly spherical with very densely and regularly distributed cells	-	*M. flos-aquae*
3.5-6.5	diffluent; (2)-5-(10) µm wide	irregular, with homogeneously distributed cells elongated, lobate	+	*M. aeruginosa*
4 –7	limited, distinctly refractive, smooth 3-6 µm wide	spherical up to elongated with scarcely up to densely distributed cells, intensely lobate	+	*M. wesenbergii*

Cultures of *Microcystis aeruginosa* were harvested at the end of exponential growth phase (~three weeks) by centrifugation at 6 000 g for 10 min at room temperature. The cultures were then freeze-dried and stored at –20 °C.

2.2.2 DNA Extraction

Genomic DNA was isolated from a freeze-dried culture according to a modified method of Raeder and Broda (1985). One volume extraction buffer (200 mM Tris-HCl (pH 8.00), 150 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 1% (w/v) Polyvinylpyrrolidone, PVP) was added to each gram of freeze-dried culture, and homogenized in the presence of washed sand, thereafter the homogenate was placed at 60 °C for 10 min. The homogenate was then centrifuged at 6 000 g for 15 min. After removal of the supernatant, the extraction was further purified by the addition of equal volumes of chloroform:phenol (1:1). The resulting mixture was vortexed and centrifuged again at 6 000 g for 15 min, where after the upper layer was carefully removed. This was followed by a DNA precipitation step using two volumes of ice-cold absolute ethanol and stored at –20 °C for at least 1 h. The DNA mixture was centrifuge at 6 000 g for 15 min, and the resulting pellet was washed with 70% ethanol. The ethanol wash-step was repeated three times, and dried after removal of the liquid. The resulting DNA was resuspended in distilled water and stored at –80 °C until further use (Oberholster, 2003).

Quantification of the DNA concentrations was done through spectrophotometric measurements at absorbances of 260 and 280 nm, using a Beckman DU650 Spectrophotometer. The DNA quality was also assessed and the concentration determined by visualisation under UV light, on 1% TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) agarose gels containing ethidium bromide (Sambrook et al., 1989).

2.2.3 AFLP analysis

To determine the usefulness of AFLP analysis to discern between the individual strains, as well as their geographical origin, AFLP analysis was conducted using the IRDye™ Fluorescent AFLP® Kit (LI-COR Biosciences, Lincoln, USA) following the

manufacturer's instructions. The principle of the AFLP procedure is presented in Figure 2.3.

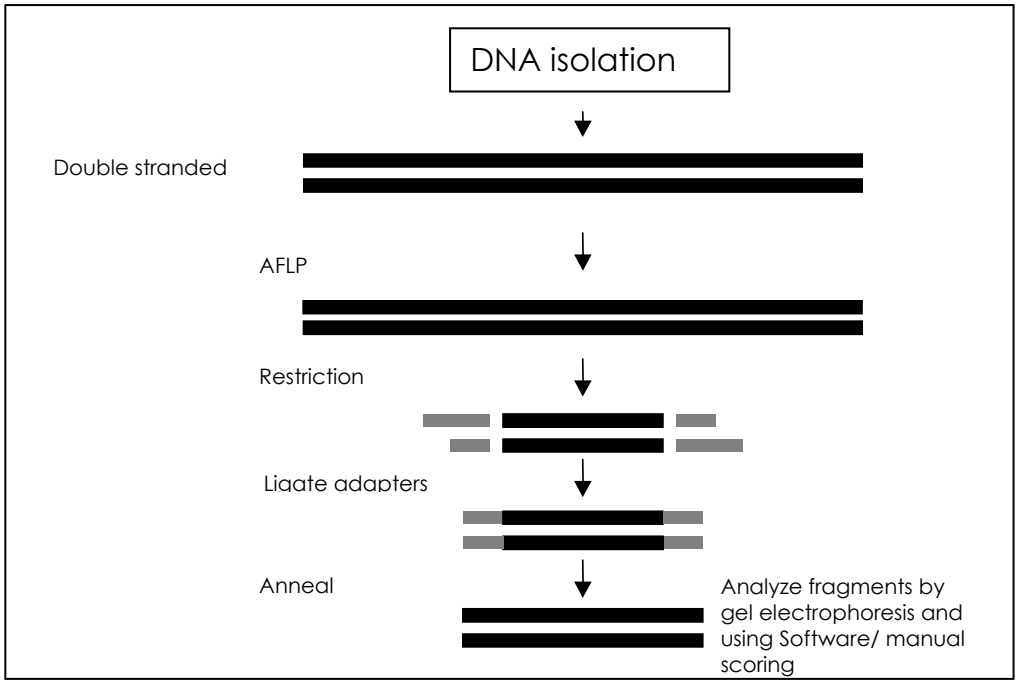


Figure 2.3 A representation explaining the principle of the AFLP procedure.

DNA is subjected to restriction digestion to enable for genome wide coverage, and for the purpose of this study restriction digestions were done with primer combinations *Eco*R1/*Mse*1 (Table 2.3). The genomic DNA (75 ng) was incubated for 2 h at 37 °C with 1.25 U of *Mse*1, 1.25 U of *Eco*R1, 1 U of T4 DNA ligase, 40 pmol of *Mse*1 adapters and 10 pmol *Eco*R1 adapters. This reaction was done in a volume of 50 µL restriction–ligase buffer containing 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM dithriothreitol (DTT), 0.1 mM EDTA, 50% (v/v) glycerol, 0.15% (v/v) Triton X-100 and 200 ng/µL BSA. Termination of the reaction was done by heating at 70˚C for 15 min, where after it was placed on ice. For adaptor ligation, 25 µL of the Adapter mix (containing *Mse*1 adaptors and *Eco*R1 adaptors, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, and 50 mM K-acetate, and 1 U of T4 DNA ligase) was added followed by incubation at 37 °C for 3 h. An aliquot (10-µL) of the adapter-ligated DNA was diluted (1:10) with TE buffer (10 mM Tris-HCl pH 8.0, 1.0 mM EDTA) to serve as template in the preselective amplification PCR. The remaining portion was used to verify whether the digestion was complete (Oberholster, 2003).

The preselective amplification reaction contained 2.5 µL of adapter-ligated DNA (diluted 1:10), 2.5 U of Taq DNA polymerase (Roche Molecular Biochemicals), 2.5 µL of 10 x PCR reaction buffer (Roche Molecular Biochemicals), 15 mM $MgCl_2$, 500 mM KCl and 100 µM of RDye700^{TM}-labeled *Eco*R1 or IRDye800^{TM}-labeled *Eco*R1 and *Mse*1 primers (containing dNTPs) with every selective nucleotide, in a total volume of 25.5 µL. The amplification procedure consisted of twenty cycles of 30s at 94°C, 1 min at 56°C, 1 min at 72 °C, and soaked at 4 °C. The selective PCR contained 2 µL of the diluted (1:10) product of the preselective PCR, 0.5 U of Taq DNA polymerase (Roche Molecular Biochemicals), 2 µL of 10 x Taq DNA polymerase buffer (Roche Molecular Biochemicals), KCl and $MgCl_2$ as mentioned above, and 100 µM of IRDye700^{TM}-labeled *Eco*R1 or IRDye800^{TM}-labeled *Eco*R1 and *Mse*1 primers (containing dNTPs) with every selective nucleotide, in a total volume of 11 µL. Eight *Eco*R1-*Mse*1 primer pairs (LI-COR Biosciences, Lincoln, USA) were used for selective amplification (Table 2.3). The first amplification cycle was carried out for 30s at 94 °C, 30s at 65 °C and 1 min at 72 °C. At each of the following 12 cycles, the annealing temperature was

reduced by 0.7 °C per cycle. The last 23 cycles of annealing were carried out at 72 °C for 1 min; and then soaked at 4 °C (Oberholster, 2003).

2.2.4 Gel electrophoresis and scoring

An equal volume of loading solution (LI-COR Biosciences, Lincoln, USA) was added to each selective amplification reaction. Samples were denatured at 95 ºC for 3 min and placed on ice for 10 min before loading. A volume of 0.8 µL was loaded with an 8-channel syringe (Hamilton, Reno, Nevada) onto 25-cm 8% Long Ranger gels (BMA, Rockland, ME, USA). Electrophoresis and detection of AFLP fragments were performed on LI-COR IR2 (model 4200S) automated DNA analysers. The electrophoresis parameters were set to 1 500V, 40mA, 40W, 50 ºC, and a scan speed of 3. The run-time was set to 4 h and gel images were saved as TIF files for further analysis.

The gel images were scored using a binary scoring system that recorded the presence and absence of bands as 1 and 0, respectively. Semi-automated scoring was performed with AFLP Qauntar Pro (Version 1.0, LI-COR) and followed by manual editing to make adjustments to the automated score where necessary. A locus was scored as polymorphic when the frequency of the most common allele (band present or absent) was less than 0.97 (absent or present in at least two individuals). Bands with the same mobility were considered as identical products (Waugh et al., 1997), receiving equal values regardless of their fluorescence intensity.

2.2.5 Data analysis

Polymorphic bands scored as plus (+) and minus (-) and converted to 1 and 0 were compiled in a data matrix. The data matrix was used to perform cluster analysis on the basis of the average linkage method, known as the Unweighted Pair Group Method using Arithmetic averages (UPGMA) and PAUP 4.0 software (Hintze, 1998). The average polymorphic information content (PIC) was calculated across each primer combination according to Riek et al. (2001) as follows:

$$PIC = 1-[f^2+(1- f)^2]$$

Where f is the frequency of the marker bands in the data set. Marker index (MI) was determined by multiplying PIC values with percentage polymorphism for each primer combination (Lübberstedt et al., 2000). The raw data matrix was used to estimate genetic similarities among different geographical cyanobacterial strains. Estimates of genetic similarity between all pairs of strains were calculated in the form of dissimilarity and expressed as Euclidean genetic distance (Jacoby et al., 2003). The 'goodness of fit' of the clustering to the data matrix was determined by calculating the cophenetic correlation coefficient between the dissimilarity matrix and the cophenetic matrix derived from the dendrogram (Sneath & Sokal, 1973; Sneath, 1989).

2.3 RESULTS

2.3.1 Fast screening of AFLP primer combinations

After screening 20 primer combinations on a subset of strains using either IRDye700™-labeled *Eco*R1 or IRDye800™-labeled *Eco*R1 primers, eight IRDye700™-labeled *Eco*R1 primer pairs were selected for analysis (Table 2.3).

Primer pair *Eco*R1-ATC/*Mse*1-CCA amplified the largest number of bands (53), but the lowest PIC (0.287) and a MI of 28.156. Primer pair *Eco*R1-AAA / *Mse*1-CCC amplified the lowest number of bands (18) and had the lowest MI (27.671). While

primer pair *Eco*R1-CC / *Mse*1-CT had the highest PIC (0.397) and MI (39.700). The generated fingerprints were evaluated for repeatability and overall clearness of the banding pattern. The number of informative fragments was also taken into account. We found no significant difference in MI through the inclusion of primer pairs with two rather than three selective nucleotides, since the mean MI of the primer pairs with three nucleotides was 30.881 ± 3.506 and the MI of primers with two selectives was 34.655 ± 5.044. A total of 276 bands were amplified from the eight primer combinations, of which 266 were informative, 10 non-informative, with an average of 95.87 polymorphic bands per primer combination.

Table 2.3 Degree of polymorphism and average polymorphism information content (PIC) and marker index (MI) for the eight AFLP primer combinations used to analyse the 23 strains (Oberholster et al., 2005b).

No	Primer combination	Total number of bands	Number of polymorphic bands	% of polymorphic bands	PIC	MI
1	*Eco*R1-ATC / *Mse*1-CCA	53	52	98.11	0.287	28.156
2	*Eco*R1-AAA / *Mse*1-CCC	18	17	94.44	0.293	27.671
3	*Eco*R1-ACG / *Mse*1-CCC	34	33	97.05	0.324	31.444
4	*Eco*R1-ACG / *Mse*1-CAC	19	18	94.73	0.363	34.387
5	*Eco*R1-ACT / *Mse*1-CAC	47	45	95.74	0.342	32.743
6	*Eco*R1-AT / *Mse*I-CG	28	25	89.28	0.326	29.105
7	*Eco*R1-AT / *Mse*1-CT	43	42	97.67	0.360	35.161
8	*Eco*R1-CC / *Mse*1-CT	34	34	100.00	0.397	39.700
	Total	276	266	NA	NA	
	Mean	34.5	33.25	95.87	0.337	32.308

2.3.2 Genetic diversity as defined by AFLP fingerprinting

The genetic relationship among all the *Microcystis aeruginosa* strains based on the combination of data obtained with the eight primer combinations is represented in the dendrogram (Figure. 2.4). The dendrogram consists of four clusters. Cluster one contains NIES88, NIES299 and NIES90 basal to this group. The mean character difference between the NIES88 and NIES299 strains were 0.163, while they differ in pairwise distance from NIES90 with a mean of 0.243. The mean character difference of 0.163 is also the lowest obtained mean value in the data set. The largest mean character differences of 0.558 were obtained between the strains *Scenedesmus acutis* (UP26) and *M. aeruginosa* (NIES88), and *M. wesenbergii* (UP02) and *M. aeruginosa* (NIES90), respectively.

In cluster 2 there are two groupings. In cluster 2A, the NIES strains 89 and 99 group together with a mean character difference of 0.243, while in 2B strains PCC7806 and CCAP1450/1 fall into a group with a mean character difference of 0.207, and UV27 group with PCC7813 (mean character difference of 0.192), with SAG1 basal to this group.

Cluster 3 consists exclusively of *Microcystis aeruginosa* strains from the geographical area of Gauteng and North-West provinces in South Africa, and includes strains UP04, UP09, UP38, UP09 and UP15, with UP01 basal to this group. The strains from Hartbeespoort reservoir (UP04, UP09 and UP38) also group together, while UP10 (Roodeplaat) and UP15 (Bon Accord) forms a subgroup. The geographical distances between the Hartbeespoort reservoir and Bon Accord and Roodeplaat reservoirs are 36.6 km and 53.8 km, respectively (Table 2.4). UP01 was collected from the Rietvlei reservoir that is located in a south-eastern direction to all the above reservoirs. The mean character difference between UP01 and the Hartbeespoort strains was 0.361.

Table 2.4 Geographical distance in kilometers between the reservoirs in Gauteng and North-West Provinces, South Africa (Oberholster et al., 2005b).

Reservoir	Klipvoor	Hartbeespoort	Bon Accord	Rietvlei	Roodeplaat
Klipvoor	0.0	65.9	66.6	96.5	78.4
Hartbeespoort	65.9	0.0	36.3	48.1	53.8
Bon Accord	66.6	36.3	0.0	30.7	18.1
Rietvlei	96.5	48.1	30.7	0.0	30.0
Roodeplaat	78.4	53.8	18.1	30.0	0.0

In cluster 4, UP37 and UP06 of the geographical area of the Free State and Western Cape provinces of South Africa group together with a mean character difference of 0.275. The strains UPUS1 and UPUS2 of Colorado U.S. fall in a separate group, while strain UP13 is basal to cluster 4.

2.4 DISCUSSION

AFLP fragments have been used to unravel cryptic genetic variation for a wide range of taxa, including plants (Mackill et al., 1996), fungi (Majer et al., 1996, 1998) and bacteria (Huys et al., 1996), which have previously been impossible to resolve with morphological characters. In the present study, complex AFLP banding patterns were obtained. Janssen et al. (1996) have showed that the choice of the restriction enzymes, and the length and composition of the selective nucleotide will determine the complexity of the final AFLP fingerprint. Primer selectivity is good for primers with one or two selective nucleotides in simple genomes such as fungi, bacteria and some plants and still acceptable with primers having three selective nucleotides, but is lost with the addition of a fourth nucleotide (Vos et al., 1995). We used a combination of primers with two and three selective nucleotides on 23 *Microcystis aeruginosa* and outgroup strains, and a total of 276 bands were amplified, constituting 95.87% informative bands and 4.13% monomorphic bands (Table 2.3). However, we did not gain advantage in number of amplified loci when using two rather than three selective primers sets.

In the dendrogram, the Japanese strains (NIES88 NIES90, NIES299) group separate from the other NIES strains (cluster 1), with NIES88 and NIES299, genetically closest to each other, while NIES strains 89 and 99 group together with the European strains (cluster 2). In cluster 3, all the *Microcystis aeruginosa* strains of Gauteng and North-West province of South Africa group together, except for the outgroup strain *Chroococcidiopsis cubana* from Klipvoor dam. This phenomenon may be due to the fact that geographical distance between the *Microcystis* strain locations in cluster 3 is close and are located in the northern geographical region of South Africa (Table 2.4; Figure 2.1).

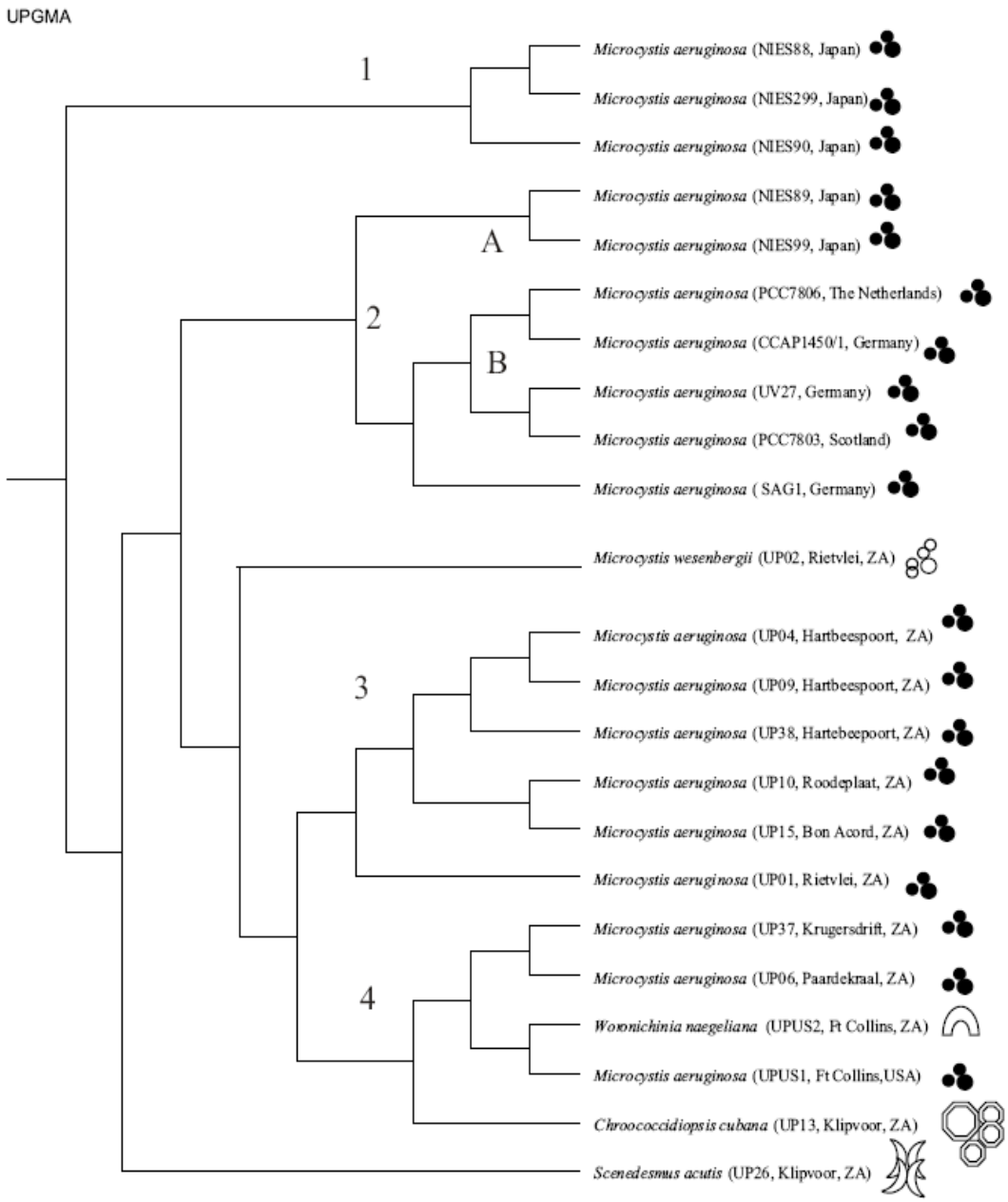


Figure 2.4 Combined cluster analysis derived from AFLP analysis of 23 *Microcystis aeruginosa* and outgroup strains using eight AFLP primer combinations (Oberholster et al., 2005b).

The position of the UPUS1 and 2 strains in their respective cluster is surprising, since these strains originated from Colorado, U.S. However, they group together with the unrelated strains from the central and southern geographical areas of South Africa (i.e. the Western Cape and the Free State provinces), but not with the *Microcystis* strains from the Gauteng and North-West provinces or the European strains. There are two plausible explanations for this observation. Firstly, this occurrence is most likely the result of a reflection of ease of dispersal; yet for the majority of species there is little information on their mechanism of transport (Round, 1981). Among the factors shaping the geographical distribution of cyanobacterial species, temperature is usually considered to be of prime importance. Other factors that also play an important role are migratory barriers for example oceans and mountain ranges, as well as passive dispersal

agents involving water, air and different animals. The probability of successful dispersal to other geographical areas depends strongly on the effectiveness of the carrier, and the ability of cyanobacteria to tolerate the transport conditions (Kristiansen, 1996a, b). Water beetles and aquatic mammals may be effective over a distance of a few kilometres, whereas dragonflies have been shown to transport viable algal material almost 1 000 km. Atkinson's (1980) reported in his experiments that viable remains of *Asterionella* was found in the faeces of waterfowl after a maximum of 20 hours, perhaps corresponding to a flight of 220 km. Atkinson (1980) has also shown that transport by the digestive tract of birds may be more efficient because desiccation is ruled out. Specialised life cycle forms for example spores, cysts and akinetes, as well as thick, resistant cell walls like in the case of *Microcystis* spp. will better survive longer transport, either externally or internally. Many ducks migrate up to 4 800 km, but with many stops. Non-stop flight distances of the order of 3 200 km in 48 hours have been noted for the white-fronted goose (*Anser albifrons*) (Owen & Black, 1990). For airborne transport, it is very difficult to give maximal distances. Since viable *Melosira* were found at 3 000 m altitude, this dust containing diatom frustules can certainly be blown across the Atlantic Ocean (Kristiansen, 1996b) and with it, possible some species of cyanobacteria.

A second explanation for the grouping of UPUS1 and 2 together with the South African strains, rather than with the European and NIES strains, may be due to the fact that the so called traditional cultured strains PCC, NIES, CCAP1450/1, UV and SAG1 are decades in prolonged subcultures. In contrast, the UP and UPUS are much shorter in subculture and thus 'wild-type' strains. This phenomenon is most likely due to the fact that although nearly all populations of cyanobacteria from different locations differ to some degree from each other, they may stabilize in long-term cultures (Waterbury & Rippka, 1989; Kato & Watanabe, 1993; Van der Westhuizen & Eloff, 1982). Carr (1999) has argued convincingly about the bias introduced to research by use of strains selected for their ability to grow exponentially. There exists abundant evidence that morphological changes take place during prolonged subculture, with almost as much evidence for physiological changes (Doers & Parker, 1988). Fray (1983) stated that records of mutations (i.e. permanent changes of the genome) are well substantiated in laboratory cultures. These include long-term subculture strains that fail to differentiate heterocysts and to synthesize nitrogenase (*Anabaena variabilis*); which lack the ability to produce akinetes (*Anabaena cylindrical*); which are incapable of forming gas vesicles (*Anabaena flos-aquae*); synthesizing toxin (*Microcystis aeruginosa*) or depositing sheath material. Such mutants generally outgrow the 'wild type' strains because energy previously invested in the formation of structures, which confer no advantage in particular conditions, can be diverted to cell growth.

We have also noted two outgroups in cluster 4, namely *Chroococcidiopsis cubana* (UP13) belonging to the division Cyanophyta, which cells are solitary or aggregated in irregular groups, enveloped by thin, firm, colourless sheaths. This species is non-heterocystous but able to fix N_2. The cells are spherical, oval to irregular rounded of varying sizes between 1.5-20 µm in diameter and are usually attach to stony substrata in the aquatic habitat. The other outgroup in this cluster is *Woronichinia naegeliana* which comprises of spherical or irregular, free-living colonies surrounded by a fine, colourless mucilage. Cells are at or within the ends of mucilaginous stalks and radially arranged with dimensions of (1)2.5-7 x 1-4(5) µm. Stalks are densely packed, causing radial lamellation, but sometimes are diffuse near the center. Of the 15 described species, 3 contain gas vesicles of which *Woronichina naegeliana* is one, and may form cyanobacterial surface blooms in eutrophic North American waters (Komárek & Anagnostidis, 1998). The other outgroups that don't fall in any cluster is (UP3) *Microcystis wesenbergii* and the green algae (UP26) *Scenedesmus acutis* which belong to the division Chlorophyta. Colonies of *Scenedesmus* are 2-4-8-16-32 celled, flattened, with long axes of cells parallel, laterally adjoined and arranged in single linear or alternating series. Cells are ellipsoidal or tapering toward each end, while the cell wall is smooth and spines are absent (Shubert, 2003).

Nielan et al. (1995) assessed the usefulness of AFLPs and restriction fragment length polymorphism (RFLP) analyses on members of the genera *Microcystis*, *Anabaena*, *Aphanizomenon*, *Cylindrospemopsis*, *Nodularia*, *Nostoc* and *Planktothrix* with regard to cyanobacterial systematics. In their study, they were able to discern the individual strains when using AFLP analysis, but not always when using RFLPs. They observed delineation of the *Microcystis* strains with regard to geographical origin when using both AFLP and RFLP analyses, but the technologies were not able to discriminate between strains on the basis of their levels of toxicity. But more importantly, they found no relationship between the PCR amplicons and restriction fragment profiles obtained with the respective genera and the taxonomic placement of the respective genera in cyanobacterial systematics.

In view of the present study, AFLP analysis is useful for the identification of genetic diversity of geographical unrelated strains and analysis of *Microcystis aeruginosa* strains. AFLPs seem to overcome the major pitfalls present in other PCR based methods, e.g. DAF or RAPD analysis, and appear to be as reproducible, heritable and intraspecific as RFLPs (Law et al., 1998). The use of the AFLP fingerprinting method resulted in a high degree of discrimination and identification of geographically unrelated *Microcystis aeruginosa* strains, and was found useful and practical. The results from this study is concurrent with the results by Nielan et al. (1995) that AFLP analysis is not suitable if evolutionary phylogeny is the objective, since the data did not support the taxonomic placing of the genera, but rather the geographical origin.

3. PCR BASED MARKERS FOR DETECTION OF TOXIC CYANOBACTERIA

3.1 INTRODUCTION

The recent discovery of the *mcy* genes coding for subunits of the microcystin synthetase in *Microcystis* (Dittmann et al., 1997; Nishizawa et al., 1999, 2000; Tillett et al., 2000) made it possible for the design and construction of primer sets, which can then been used to identify environmental strains bearing *mcy* genes (Baker et al., 2001; Bittencourt-Oliveira, 2003; Mikalsen et al., 2003; Oberholster et al., 2005, 2006a, b). This 55-kb gene cluster consists of six open reading frames (ORFs) with a mixed nonribosomal peptide synthetase/polyketide synthase nature (*mcyA* to *mcy E* and *mcyG*) and four smaller ORFs with putative precursor and tailoring functions (*mcyF* and *mcyH* to *mcyJ*) (Tillett et al., 2000). It could be demonstrated that the occurrence of *mcy* genes in cells is correlated with their ability to synthesize microcystin and, *vice versa*, that microcystin-free cells usually do not contain *mcy* genes (Kurmayer et al., 2002). This approach is appealing as an early warning diagnostic and is very sensitive due to the amplification achieved by PCR. The objective of this chapter was to determine the potential of using the *mcyB* gene sequence as a diagnostic tool in raw water to detect the presence of toxin-producing cyanobacterial spp. in South African reservoirs. To achieve the objective, two main strategies were followed. Firstly, we studied the use of insertions and deletions (indels) present in the *mcyB* gene sequence as a fingerprint, i.e. diagnostic tool to recognize the presence of "known" toxic strains in water reservoirs; and secondly, the use of PCR-based technologies for detecting toxic cyanobacteria and the expression of the *mcy* genes as representatives of the microcystin peptide synthetase and polyketide synthase genes, respectively.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals, Strains and Culture Conditions

Analytical reagent grade chemicals were purchased from various commercial sources and were used without further purification. Unless otherwise stated, standard methods described in Sambrook et al. (1989) were used. *Microcystis aeruginosa* strains used in the study represented a wide variety of geographically unrelated strains (Table 3.1). Strains PCC7806 and PCC7813 were obtained from the Pasteur Institute Culture Collection, France; UV027 from the University of the Free State Culture Collection, South Africa; CCAP1450/1 was obtained from the Culture Collection of Algae and Protozoa, Institute of Freshwater Ecology, UK; NIES88, NIES89, NIES91, NIES99 from the National Institute for Environmental Studies, Japan; and SAG1 from the Pflanzen Physiologisches Institut, Universität Gottingen, Germany. All these strains were received as axenic, maintained as such and microscopically verified prior to further experiments. Unicellar stains were collected by the authors, representatives of the Water Research Commission and Tswhane Metro Council, respectively. Water samples were placed on ice in a darkened cooler during transport to the laboratory. Holding time for samples was less than 48 h in all cases. After the samples were vigorously spun with a vortex mixer to break the blooms, the samples were diluted in sterilized, deionized and distilled water and placed in 100 ml of liquid BG-11 medium in 200-ml flasks.

Table 3.1 Different strains used in the study and their origin.

Strain	Source	Origin
M. aeruginosa		
PCC7806	Pasteur Culture Collection, France	The Netherlands
PCC7813	Pasteur Culture Collection, France	Scotland
UV027	University of the Free State Culture Collection	Israel
NIES88	National Institute for Environmental Studies	Japan
NIES89	National Institute for Environmental Studies	Japan
NIES91	National Institute for Environmental Studies	Japan
NIES99	National Institute for Environmental Studies	Japan
NIES299	National Institute for Environmental Studies	Japan
SAG1	Pflanzen Physiologisches Institut, Universität Gottingen	Germany
CCAP1450/1	Institute of Freshwater Ecology	UK
UP01	University of Pretoria Culture Collection	Rietvlei Dam, ZA
UP03	University of Pretoria Culture Collection	Rietvlei Dam, ZA
UP04	University of Pretoria Culture Collection	Hartbeespoort Dam, ZA
UP05	University of Pretoria Culture Collection	Hartbeespoort Dam, ZA
UP06	University of Pretoria Culture Collection	Paardekraal Dam, ZA
UP07	University of Pretoria Culture Collection	Hartbeespoort Dam, ZA
UP08	University of Pretoria Culture Collection	Hartbeespoort Dam, ZA
UP09	University of Pretoria Culture Collection	Hartbeespoort Dam, ZA
UP10	University of Pretoria Culture Collection	Roodeplaat Dam, ZA
UP11	University of Pretoria Culture Collection	Roodeplaat Dam, ZA
UP12	University of Pretoria Culture Collection	Roodeplaat Dam, ZA
UP13	University of Pretoria Culture Collection	Roodeplaat Dam, ZA
UP14	University of Pretoria Culture Collection	Klipvoor Dam, ZA
UP15	University of Pretoria Culture Collection	Bon Accord Dam, ZA
UP16	University of Pretoria Culture Collection	Bon Accord Dam, ZA
UP17	University of Pretoria Culture Collection	Bon Accord Dam, ZA
UP18	University of Pretoria Culture Collection	Bronkhorstspruit Dam, ZA
UP19	University of Pretoria Culture Collection	Bronkhorstspruit Dam, ZA
UP20	University of Pretoria Culture Collection	Bronkhorstspruit Dam, ZA
UP23	University of Pretoria Culture Collection	Bronkhorstspruit Dam, ZA
UP24	University of Pretoria Culture Collection	Bronkhorstspruit Dam, ZA
UP27	University of Pretoria Culture Collection	Klipvoor Dam, ZA
UP28	University of Pretoria Culture Collection	Klipvoor Dam, ZA
UP29	University of Pretoria Culture Collection	Hartbeespoort Dam, ZA
UP30	University of Pretoria Culture Collection	Hartbeespoort Dam, ZA
UP31	University of Pretoria Culture Collection	Hartbeespoort Dam, ZA
UP32	University of Pretoria Culture Collection	Hartbeespoort Dam, ZA
UP33	University of Pretoria Culture Collection	Hartbeespoort Dam, ZA
UP34	University of Pretoria Culture Collection	Hartbeespoort Dam, ZA
UP35	University of Pretoria Culture Collection	Krugersdrift Dam, ZA
UP36	University of Pretoria Culture Collection	Krugersdrift Dam, ZA
UP37	University of Pretoria Culture Collection	Krugersdrift Dam, ZA
UP38	University of Pretoria Culture Collection	Hartbeespoort Dam, ZA
UP39	University of Pretoria Culture Collection	Midmar Dam ZA
UP40	University of Pretoria Culture Collection	Midmar Dam ZA
UP41	University of Pretoria Culture Collection	Midmar Dam ZA
UP42	University of Pretoria Culture Collection	Midmar Dam ZA
UP43	University of Pretoria Culture Collection	Roodeplaat, ZA
UP44	University of Pretoria Culture Collection	Krugersdrift Dam, ZA
UPUS1	University of Pretoria Culture Collection	Ft Collins USA
M. wesenbergii		
UP02	University of Pretoria Culture Collection	Rietvlei Dam, ZA
C. cubana		
UP13	University of Pretoria Culture Collection	Klipvoor Dam, ZA
S. acutis		
UP26	University of Pretoria Culture Collection	Klipvoor Dam, ZA
W. naegaliana		
UPUS2	University of Pretoria Culture Collection	Ft Collins USA

Unicellular and axenic strains were maintained at a temperature of approximately 24°C in liquid BG-11 nutrient medium containing 17.65 mM $NaNO_3$, 0.18 mM $K_2HPO_4.3H_2O$, 0.30 mM $MgSO_4.7H_2O$, 0.25 mM $CaCl_2.2H_2O$, 0.03 mM citric acid, 0.03 mM ferric ammonium citrate, 0.003 mM EDTA (ethylenediamine tetra-acetic

acid, disodium magnesium), 0.19 mM Na_2CO_3, 0.05 mM H_3BO_3, 9.15 mM $MnCl_2.4H_2O$, 0.77 mM $ZnSO_4.7H_2O$, 1.61 mM $Na_2MoO_4.2H_2O$, 0.37 mM $CuSO_4.5H_2O$ and 0.17 mM $Co(NO_3)_2.6H_2O$ dissolved in 1L distilled water. Cultures were grown under constant light of approximately 60 µmol photons.m^{-2}.s^{-1} (PAR) at pH 8.0. The purity of cyanobacterial cultures was verified weekly by the absence of bacterial growth on TYG agar and in TYG broth (5.0 g tryptone (Difco); 2.5 g yeast extract (Difco); glucose, 1.0 g per litre) after incubation of two weeks at 26°C.

The final proof of purity was verified by microscopic examination. Cultures of *Microcystis aeruginosa* were harvested at the end of exponential growth phase (three weeks) by centrifugation at 6 000 g for 10 min at room temperature. The cultures were then freeze-dried and stored at –20°C. The strains isolated from the blooms were identified following the procedure as described by Komárek (1958).

3.2.2 Toxicity as measured using Protein Phosphatase Inhibition and ELISA Assays

Toxicity was determined by using the same methods as described in Boyer et al. (2004). Briefly, 5-liter samples were collected where the cyanobacterial bloom occurred. The water was poured gently through a 934 AH glass fibre filters in the field, frozen on dry ice, and returned to the laboratory in a cool box for toxin analysis. Filters for toxin analysis were extracted by grinding with 10 ml of 50% methanol containing 1% acetic acid and clarified by centrifugation. This extract was used for analysis of microcystins using the protein phosphatase inhibition assay (PPIA) as described in Carmichael and An (1999).

ELISA assay was conducted with a Quanti™ Kit for microcystins (EnviroLogix, USA). The limit of detection (LOD) of the kit is 0.147 ppb. The LOD was determined by interpolation at 81.3% Bo from a standard curve. 81.3% Bo was determined to be 3 standard deviations from the mean of a population of negative water samples. 100% Bo equals the maximum amount of microcystin-enzyme conjugate that is bound by the antibody in the absence of any microcystin in the sample. %Bo = (OD of sample or calibrator/OD of negative control) x 100. The results were obtained by reading the plate on a multiskan ascent (Thermo Labsystems, USA).

3.2.3 Genome analysis

3.2.3.1 DNA Extraction

Genomic DNA was extracted according to a modified method of Raeder and Broda (1985). The extraction buffer consisted of 200 mM Tris-HCl (pH 8.00), 150 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 1% (w/v) Polyvinylpyrrolidone (PVP). A volume of extraction buffer were added to each 1 gram of freeze-dried culture, and homogenized in the presence of washed sand. The homogenate was placed at 60°C for 10 min. The homogenate was then centrifuged at 12 000 rpm for 15 min. The supernatant was removed and equal volumes of chloroform:phenol (1:1) was added, vortexed and centrifuged again at 12 000 rpm for 15 min. The upper layer was carefully removed. The DNA in the aqueous layer was precipitated with two volumes of ice-cold absolute ethanol and stored at –20°C for at least 1 h. Following a centrifugation step (12 000 rpm, 15 min), the resulting pellet was washed with 70% ethanol (this step was repeated three times), and dried after removal of the liquid. The DNA was resuspended in distilled water and stored at –80°C.

DNA concentrations were determined by visualisation under UV light, on 1% TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) agarose gels containing ethidium bromide (Sambrook et al., 1989), as well as through spectrophotometric measurements at absorbances of 260 and 280 nm, using a Beckman DU650 Spectrophotometer.

3.2.3.2 Polymerase chain reaction (PCR)

3.2.3.2.1 Pretreatments of environment samples for whole-cell PCR

For whole-cell PCR, cyanobacterial colonies were collected from the environmental samples, by placing the samples mixed with distilled water in a glass cylinder under fluorescent light. Under these conditions, the cyanobacterial cells floated to the surface and the lower water layers were siphoned off. Before resuspension in distilled water to define volume, the cells were washed three times with distilled water and subjected to a freeze-thaw treatment for PCR template preparation (Baker et al., 2001). DNA was extracted from the environment samples as well as from the reference culture strain PCC 7806 using DNAzol®-Genomic DNA Isolation reagent following the manufacturer's procedures (Molecular Research Center, Inc., USA). Extracted DNAs were purified once (culture strain) or twice (environmental strains) with a Prep-A-Gene DNA Purification Kit (Bio-Rad) according to the manufacturer's instructions and eluted in 60 µl.

3.2.3.2.2 PCR amplification

PCR was performed in a GeneAmp2400 thermocycler (Perkin-Elmer Cetus, Emeryville, Calif., USA). The thermal cycling protocol included an initial denaturation at 94°C for 2 min, followed by 35 cycles. Each cycle began with 10 s at 93°C followed by 20 s at the annealing temperature at T_m°C for the specific primer pairs (Table 3.2), and ended with 1 min at 72°C. When extracted DNA was used, the amplification reactions contained a 10x amplification buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmol of each primer and 1 U Taq DNA polymerase, and 3-5 ng purified DNA in a final volume of 50 µl (Dittmann et al., 1999). The PCR amplification with whole cells started with 6 µl of crude sample, pretreated subsample with an approximate cell density of $8x10^6$ cells/ml, or 0.1µg lyophilized cyanobacterial cells. The sample was added directly to a 20 µl-reaction solution containing bovine serum albumin (0.1 mg/ml) or skim milk (0.1-100 mg/ml, w/v), and a 10x amplification buffer that contained 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmol of each primer, and 0.5 U Taq DNA polymerase (Howitt, 1996). The PCR amplifications conditions were identical to those for the samples described above. An extra ramp rate of 3 s/°C between the denaturing and annealing steps was set when a GeneAmp 9600 cycler instead of GeneAmp 2400 was used for PCR amplification. The dosage for the skim milk ranging from 1 to 100 mg/ml was determined to be appropriate based on the results of PCR.

3.2.3.2.3 Sequencing

PCR fragments generated with primer pairs Tox 3P/2M, Tox 1P/1M, Tox 7P/3M and Tox 10P/4M from UP37, PCC7813 and UV027 were subsequently cloned into the pGem®T-Easy vector (Promega), transformed into *E. coli* cells (JM 109; > 108 cfu/µl) and blue/white screening was carried out in order to determine transformation efficiency. The cells were resuspended in 100 µl LB-media (Luria Bertrani; 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L Difco agar, pH 7.0, Sambrook et al., 1989), plated out on LB/IPTG (0.5 mM isopropylthio-β-D-galactoside)/X-gal (80 µg/µl 5-bromo-4-chloro-3-indolyl-β-D-galactoside) plates and incubated overnight at 37°C.

Table 3.2 Oligonucleotides used for qRT-PCR and PCR analysis.

Gene region and primer	Sequence	T_m (°C)	Authors
McyB			
Tox 1P	5'-CGATTGTTACTGATACTCGCC-3'	57.9	Oberholster, 2004
Tox 1M	5'-TAAGCGGGCAGTTCCTGC-3'	58.2	Grobbelaar et al., 2004 Grobbelaar, 2005
Tox 3P	5'-GGAGAATCTTTCATGGCAGAC-3'	62.4	
Tox 2M	5'-CCAATCCCTATCTAACACAGTACCTCGG-3'	65.1	
Tox 7P	5'-CCTCAGACAATCAACGGTTAG-3'	53.7	
Tox 3M	5'-CGTGGATAATAGTACGGGTTTC-3'	58.4	
Tox 10Pf	5'-GCCTAATATAGAGCCATTGCC-3'	59.8	
Tox 4Mr	5'-CCAGTGGGTTAATTGAGTCAG-3'	57.9	
McyB1-L	5'-AGGCAAGCAGAAATTCAGGA-3'	55.9	Oberholster et al., 2006
McyB1-R	5'-ATAGCAACCACCGTCAAAGG-3'		
McyB2-L	5'-CTCTAGTACAGGCCGCTTGG-3'	54.9	
McyB2-R	5'-GAATTTCTGCTTGCCTTTGC-3'		
McyB3-L	5'-TCATCCCAACGTTGAACAAA-3'	55.2	
McyB3-R	5'-CACCATATAAGCGGGCAGTT-3'		
McyB4-L	5'-GCGTCAACAAGGGTTTGAAT-3'	53.8	
McyB4-R	5'- GGTTTTGCTCCTGTAACCA-3'		
McyB5-L	5'-CCTCGGTAATGCGTCTGTTT-3'	53.3	
McyB5-R	5'-GGAACCCGTTCATAGGGAAT-3		
McyB6-L	5'-GATTTTTAGGGCGCATTGA-3'	54.8	
McyB6-R	5'-ACCATATAAGCGGGACGTTG-3'		
McyB7-L	5'-GAATTTTTAGGGCGCATTGA-3'	52.9	
McyB7-R	5'-ACCATATAAGCGGGCAGTTG-3'		
McyB8-L	5'-GGCGCATTGATAATCAGGTT-3'	53.4	
McyB8-R	5'-CACCATATAAGCGGGCAGTT-3'		
McyB9-L	5'-AGCTGAGGGGATTACGGATT-3'	54.3	
McyB9-R	5'-CACCATATAAGCGGGCAGTT-3'		
McyB10-L	5'-GCGTTATCCGGAATTATTGG-3'	55.5	
McyB10-R	5'-CCACTAAGAACGAGGGTTGC-3'		
Tox2+	5'-AGGAACAAGTTGCACAGAATCCGCA-3'	50	Kaebernick et al., 2000.
Tox2-	5'-ACTAATCCCTATCTAAACACAGTAACTCA-3'	50	
FAA	5'-CTATGTTATTTATACATCAGG-3'	40	Neilan et al., 1999
RAA	5'-CTCAGCTTAACTTGATTATC-3'		
McyD			
McyDF2	5'-GGTTCGCCTGGTCAAAGTAA-3'	50	Kaebernick et al., 2000.
McyDR2	5'-CCTCGCTAAAGAAGGGTTGA-3'	50	
***McyA* NMT**			
MSF	5'-ATCCAGCAGTTGAGCAAGC-3'	59	Tillett et al., 2001
MSR	5'-TGCAGATAACTCCGCAGTTG-3'	60	
MSI	5'-GAGAATTAGGGACACCTAT-3'	48	
uma1			
UMF	5'-CCTATCGTCGTATTTGGAGT-3'	54	Tillett et al., 2001
UMR	5'-AAGGAATGGACACGATAGGC-3'	59	

Single white colonies were used to inoculate 5 ml LB-media containing 2.5 mg ampicillin and incubated at 37°C overnight with shaking. The cells were centrifuged at 10 000 rpm for 2 minutes and the supernatant discarded. The pellet was resuspended in 300 µl STET buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 5% (v/v) Triton X-100). Lysozyme (0.15 mg) was added and the cells incubated at room temperature for 5

minutes. To facilitate lyses the cells were then incubated at 95°C for 1 minute and centrifuged at 14 000 rpm for 15 minutes at 4°C. The pellet was removed, 5% (w/v) CTAB (N-cetyl-N-N-N-trimethylammonium bromide) was added to the supernatant and centrifuged at 14 000 rpm for 5 minutes. The supernatant was discarded, the pellet resuspended in 300 µl 1.2 M NaCl and 750 µl cold absolute ethanol added. This mixture was then centrifuged at 14 000 rpm for 10 minutes at 4°C. The supernatant was discarded, 1 ml cold 70% ethanol added and centrifuged at 14 000 rpm for 2 minutes at 4°C. The supernatant was removed, the pellet vacuum-dried in a SpeedVac Concentrator SVC 100H (Savant) and resuspended in 30 – 50 µl ddH_2O. The inserts were verified by restriction analysis with approximately 1 µg plasmid DNA, 5 U *Eco*RI, 50 mM Tris-HCl, 10 mM $MgAc_2$, 10 mM $MgCl_2$, 66 mM KAc, 100 mM NaCl and 0.5 mM DDT at pH 7.5 all from Roche. The entire reaction was loaded onto a 1% TAE agarose gel (Techcomp Ltd.) containing 0.15 mg ethidium bromide (Sigma), separated at 85 mV and visualized under UV-light.

Sequencing of the fragments was performed using the ABI Prism® Big Dye® Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). Sequencing reactions were performed according to the manufacturers' instructions and contained 200–500 ng plasmid template and 3.2–5 pmol of the appropriate primer. Reactions were cycled on a GeneAmp PCR System 2400 (PE Biosystems) thermal cycler and the products precipitated with NaOAc and EtOH according to the manufacturers' instructions. Samples were dried in a SpeedVac Concentrator SVC 100H (Savant) and resuspended in formamide and 25 mM EDTA buffer.

Approximately 30–50% of each reaction was loaded onto a 4% acrylamide gel, separated at 1.6 kV for 7 h at 51°C and data collected on an ABI Prism 377 DNA Sequencer (PE Biosystems). The data were analysed using Sequencing Analysis V 3.3. Sequences were reverse-complemented and compared by using Sequence Navigator V 1.0.1 and assembled using AutoAssembler V 1.4.0 and DNAssist V 1.02. Analyzed sequences were used to search the Genbank Database (http://www.ncbi.nlm.nih.gov/).

3.2.3.2.4 Composition of the genetic map

The obtained sequences were utilized and imported into the Webcutter 2.0 software programme (http://www.firstmarket/com/cgi-bin/cutter) to obtained potential nucleotide restriction sites that can be used to differentiate between the strains.

3.2.3.2.5 RNA extraction and quantitative real-time (qRT)-PCR

Cells were homogenized using liquid nitrogen and RNA extracted using the Qiagen RNAeasy kit (Qiagen Inc., USA) according to the manufacturers' instructions, and using DEPC-treated equipment and solutions.

Quantitative PCR was performed using 5 ng of total RNA per reaction and with 10 µM of each primer (Table 3.2). Quantitative real-time PCR was performed using the iScript One-Step RT-PCR Kit (Bio-Rad, USA) and analysed using the iCycler iQ Real-Time PCR Detection Instrument (Bio-Rad). The cycling parameters consisted of 1 cycle at 95°C for 10 min; 40 cycles starting with 1 cycle at 95°C for 10 s, primer specific annealing T°C for 5 s, 72°C for 10 s; followed by the melting curve analysis (95°C for 0 s, 65°C for 15 s, 95°C for 0 s), and cooling (40°C for 30s). A minimum of 7 reactions was done for each fragment analyzed, standard curves were generated using dilution series (1:1, 1:10, 1:100, 1:1000) and repeated. After primer design from TDF sequence information using Primer Designer 5 (ver. 5.03, Scientific and Educational Software) purified salt-free primers were synthesized (IDT). In order to calculate relative expression ratios for target genes, these were normalised with the expression of the unregulated 16S chloroplast rRNA transcript (Pfaffl, 2001).

3.3 RESULTS

3.3.1 Measurement of Toxicity

Medium to high levels of toxicity were measured during a survey of reservoirs in South Africa (Figure 3.1). Toxicity was measured by ELISA and using the PPIA assay. The *Microcystis aeruginosa* strain UP37 with the highest measured toxicity was collected from Krugersdrift Dam during December 2004. Other strains with high levels of toxicity were collected from Hartbeespoort Dam (UP09), Roodeplaat Dam (UP13), Bon Accord Dam (UP15) (all collected during 2000-2001); Hartbeespoort Dam (UP33) and Krugersdrift Dam (UP36) and a UPUSA1 strain from Fort Collins (USA)(collected during 2004-2005). Different strains collected from the same reservoir gave varying toxicity (compare toxicities of strains UP04-UP05 and UP07-UP09). No toxicity was measured in UP26. This was expected since strain UP26 is an axenic strain of *Scenedesmus acutis* which belong to the division Chlorophyta. (Shubert, 2003).

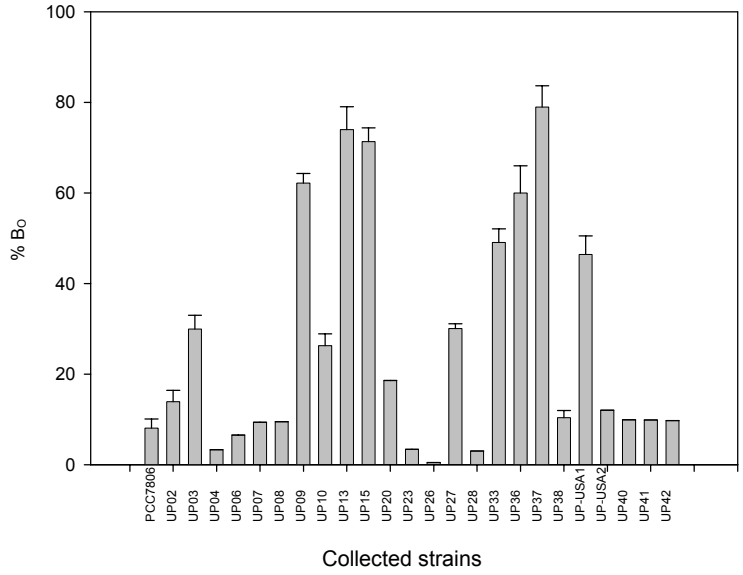


Figure 3.1 Toxicity as measured in microcystin (ppb). ELISA assay was conducted with a Quanti™ Kit for microcystins (EnviroLogix, USA). The limit of detection (LOD) of the kit is 0.147 ppb. The LOD was determined by interpolation at 81.3% B_o from a standard curve ($r^2 = 0.9998$). 81.3% B_o was determined to be 3 standard deviations from the mean of a population of negative water samples. 100% B_o equals the maximum amount of microcystin-enzyme conjugate that is bound by the antibody in the absence of any microcystin in the sample. $\%B_o$ = (OD of sample or calibrator/OD of negative control) x 100.

3.3.2 PCR Markers as tools for Risk Management

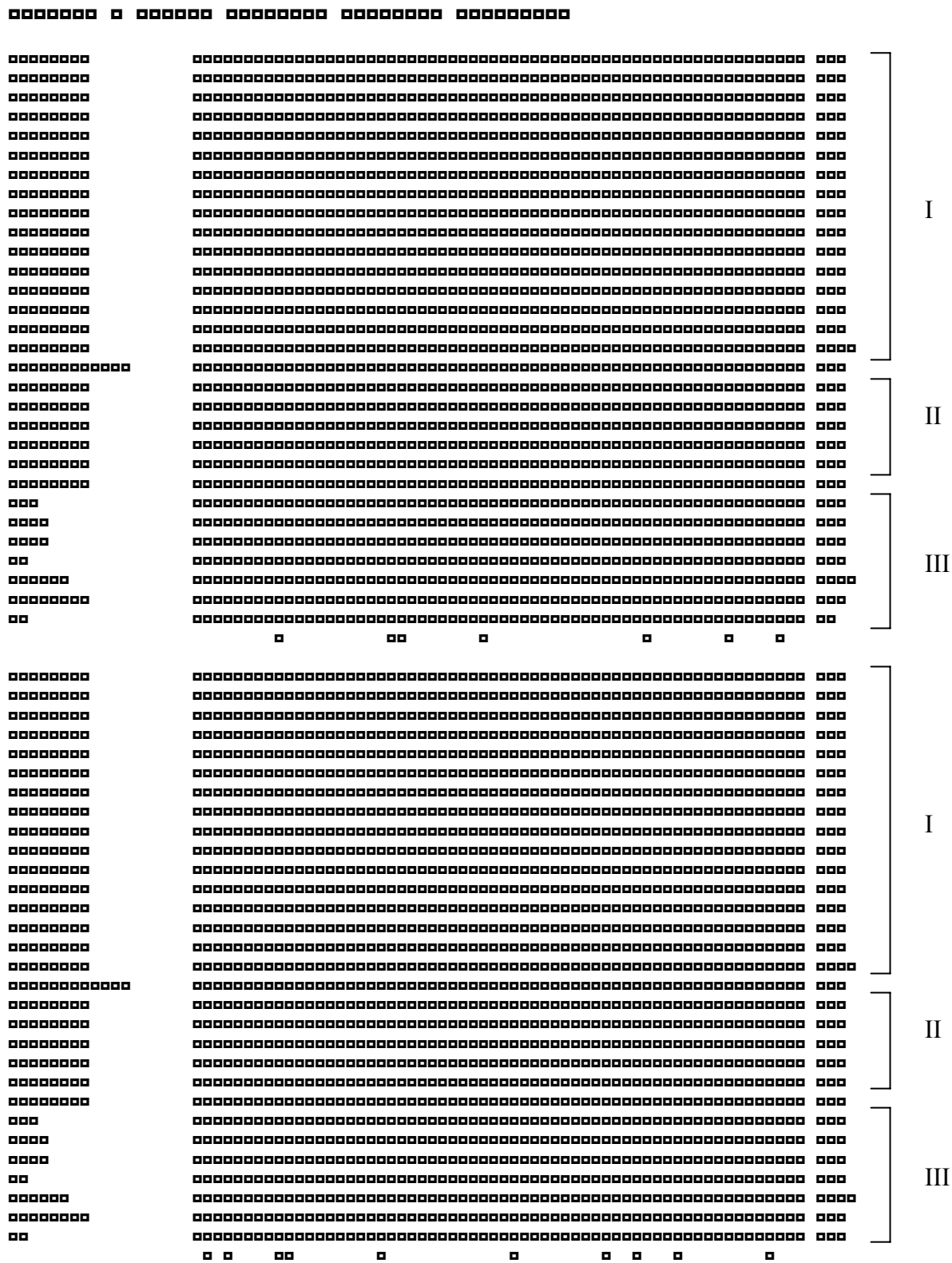
3.3.2.1 Sequencing and primer design

Using previously designed primer pairs Tox 3P/2M, Tox 1P/1M, Tox 7P/3M and Tox 10P/4M, the *mcyB* gene from UP37, PCC7813 and UV027 were sequenced (Grobbelaar et al., 2004), resulting in fragments of 2126, 2174 and 2170 base pairs in size, respectively. The obtained sequences were analysed using nucleotide BLASTN and BLASTX annotation of the Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990) at http://www.ncbi.nlm.nih.gov/BLAST. When aligning the obtained sequences from UP37, PCC7813 and UV27 with other published *mcyB* sequences from

GenBank, including sequences from *M. aeruginosa, M. viridis* (AJ492557, AB092807), *M. botrys* (AJ492559, AJ492556) and *Planktothrix rubescens* (AJ863134), high homologies were obtained indicating that the obtained sequences indeed represent that of *mcyB* [E-values from 0.0 (representing a perfect match) to $5e^{-14}$ (representing a significant match)](Figure 3.2).

Annotation of sequences obtained from strain USUP2 with the designed primer set to amplify the *mcyA-C* gene cluster (Appendices, Figure A1.4: sequence AS5-M13F), resulted in E-values of 0.0 (98% homology, 856/868) with the *mcyA-C* gene cluster, while with primer set designed for *mcyB* (Appendices, Figure A1.4: sequences AS10-M13F), resulted in E values of 0.0 (98% homology, 346/347) with *mcyB*.

However, the primer set design to amplify *McyD* (Appendices, Figure A1.4: AS8-M13F) from USUP2 (*W. naegaliana*) had no significant homology to the known *mcyD* sequence from cyanobacteria on GenBank. These differences led us to investigate the potential of using differences in sequence with regard to (1) nucleotide polymorphisms when using PCR and qRT-PCR technology, and (2) restriction sites, and thus insertions/deletions (indels) in nucleotide sequence to discriminate between the strains.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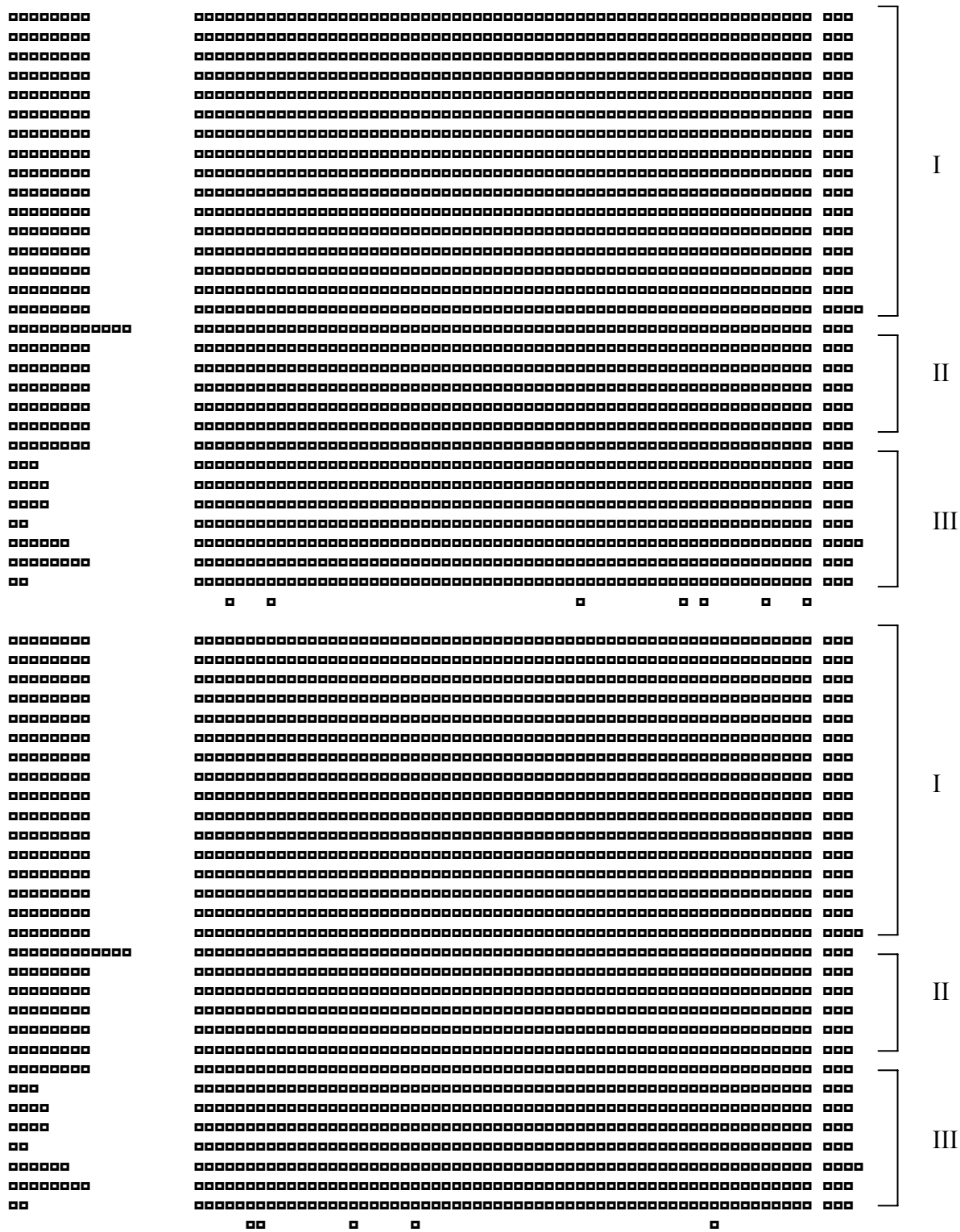
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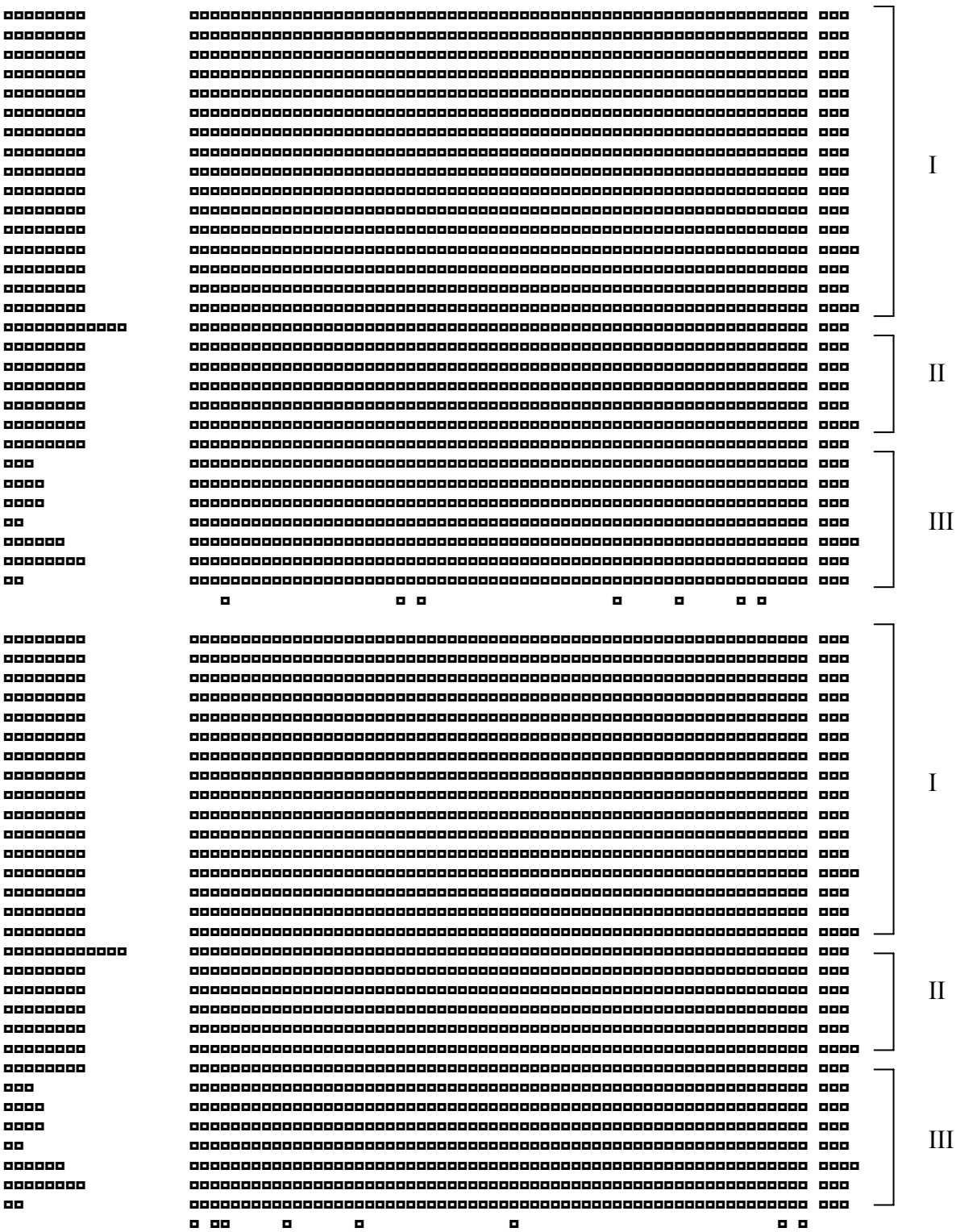


Figure 3.2 Alignment of obtained *mcyB* sequences to all publicly available sequences (http://www.NCBI.nlm.nih.gov, October 2005).

3.3.2.2 Restriction digestion of fragments derived from *McyB*.

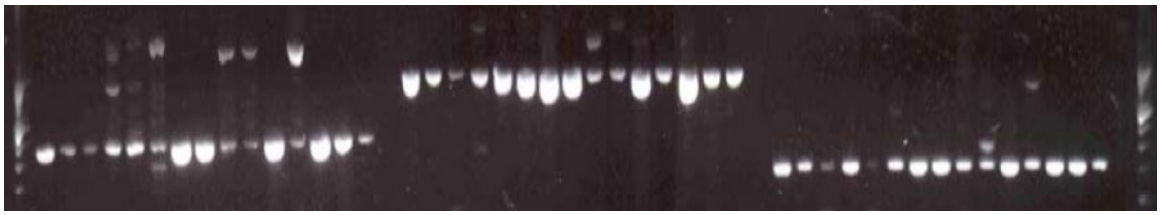
The sequences were also submitted to the Webcutter 2.0 software programme (http://www.firstmarket/com/cgi-bin/cutter, October 2005) to obtain all the available

restriction sites present in the strains (Appendices, Table A1). A vast number of restriction sites were identified with differences, for example compare the restriction sites of *Aci*I (10; 186, 314, 845, 857, 1397, 1793, 1846, 2184, 2743, 3148) in UP37 with that in PCC7813 (6; 26, 550, 800, 928, 1459, 1947 bp) and in UV027 (5; 26, 799, 927, 1458 bp). Unique restriction sites have also been identified, e.g. *Acc*B1I (2; 1149 and 2191 bp), *Acc*II (3; 505,1701, 1843 bp) and *Acc*III (1; 1045 bp) in UP37, and then *Mva*I (1 fragment, 276 bp) and *Eco*130I (1, 1101 bp) in UV027; and *Aoc*I (1, 652 bp), *Bse*21I (1, 652 bp) and *Bsp*HI (1, 873 bp) in PCC7813, to name a few (Appendices Table A1). Selected strains were subjected to restriction digestion with *Rsa*1, *EcoR*I, *Sau*3AI and *Alu*I and amplicons analyzed (Table 3.3). Restriction enzymes *Rsa*I, *Eco*RI and *Alu*I were unable to produces any polymorphic fragments, while *Sau*3AI discerned some.

Table 3.3 Number of indels observed after restriction of the *mcyB* gene with selected enzymes of *Microcystis aeruginosa* strains.

***Microcystis* strains**	***Rsa*I**	***EcoR*I**	***Sau*3AI**	***Alu*I**
PCC7813	2	1	4	1
PCC7806	2	1	4	1
SAG1	2	1	5	1
CCAP1450/1	2	1	5	1
UV027	2	1	5	1
UP01	2	1	5	1
UP03	2	1	5	1
UP04	2	1	5	1
UP37	2	1	4	1

Other strains were selected for PCR amplification of other designed primer sets, where after the obtained amplicons were subjected to restriction digestion using the *Pst*I, *Eco*RI and *Hind*III enzymes, respectively (Figure 3.3). Cost efficiency was taken in consideration when these enzymes were selected for testing as diagnostic tools. The selected restriction enzymes were however unable to discern between the different strains on geographic region.



M *Pst*I *Pst*I *Eco*RI

M

Figure 3.3 Amplicons obtained after amplification with primer sets Uma-1 (*Pst*I, *Eco*RI) and McyB-1 (PR22) (*Pst*I). Sample order: UP03, UP04, UP09, UP10, UP13, UP14, UP17, UP18, UP20, UP23, UP24, UP27, UP28, UP29, UP31.

3.3.2.3 PCR and qRT-PCR as diagnostic tools

Since toxicity is not only confounded to the *mcyB* gene (Nishizawa et al., 2000), other genes within the *mcy* gene clusters were also investigated. After cloning and sequencing of fragments that were derived from amplicons obtained *via* PCR amplification of the *mcy* gene clusters (i.e. *mcyA-C*, *mcyD-E*, *mcyG-I*) primers were designed. The

feasibility of these primers to be used as diagnostic tools were tested though amplification using a subset of samples, i.e. toxic strains and non-toxic strains. Only primers that were discerning between non-toxic and toxic strains (i.e. amplifying the *mcy* fragment in a known toxigenic strain) were used for the PCR and qRT-PCR analysis (Table 3.2).

After screening the different primer sets, it became evident the primer sets differed in their robustness with regard to indication of toxicity. Developed primers McyB1-10 were able to discern toxic from non-toxigenic strains in all cases tested (not shown), however, of the existing primer sets, only the primer set designed to amplify the *McyD* gene gave consistently positive results (Table 3.4).

3.3.2.4 Case studies

The developed primer pairs were tested in several case studies, i.e. Midmar Dam, Krugersdrift Dam, Sheldon Lake (USA), and under varying environmental conditions. Amplification products obtained from the cyanobacteria spp. sampled in Lake Midmar provided for supporting evidence that the environment strains *Microcystis aeruginosa*, contained representative genes within the *mcy* gene cluster present in the *Microcystis aeruginosa* culture strain PCC7806, and that is normally associated with toxin production (Figure 3.4, Table 3.4). Low expression levels of the *mcyA-D* genes at sampling sites 1 and 2 were observed after analysis of RNA from the strains isolated from the environmental samples using quantitative real-time PCR (Figure 3.5). The toxicity of the environmental strains was also determined using ELISA and inhibition of PP2A assays and the toxicity levels were compared to the toxin levels present in the cultured PCC7806 strain. Although we found variations in the toxigenic levels of the cyanobacterial samples, microcystin-LR was detectable in all samples of sites 1 and 2, varying from 0.09 to 0.17 $\mu g\ L^{-1}$. The levels of microcystin-LR in Lake Midmar never exceeded the World Health Organization (WHO) drinking water threshold fixed at $1 \mu g.L^{-1}$ during the study period.

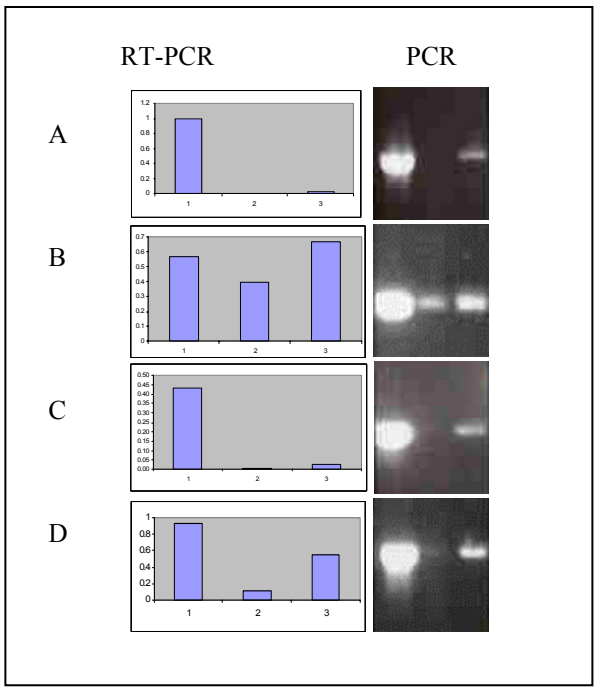


Figure 3.4 qRT-PCR and PCR from *Microcystis aeruginosa* strains. UPUS1 (1), UP40 (2) and UP10 (3). (A) *McyB* primer set Tox3P/2M; (B) *McyB* primer set McyB2-FAA/FBB; (C) *McyD* primer set McyD-R2/F2; and (D) *Uma1* with primer set Uma1-UMR. 16S RNA was included as standard. PCC7806 (1), UP40 (2) and UP10 (3). Separation of PCR amplicons obtained after PCR of *Microcystis aeruginosa* strains on a 2% agarose gel. M = Hyperladder^{TM} IV, Bioline, USA.

Table 3.4 Comparison of PCR with different primers, quantitative PCR, ELISA and Protein Phosphatase inhibition (PP2A) assay as determinants of toxicity in strains from different geographical regions. (+ = positive/product; - = negative/no product; / = not assayed, ? = result unclear).

Strain	Geographic origin	PCR										RT-PCR[a]	PP2A	ELISA
M. aeruginosa		*McyB*-Tox3P/2M	*McyB*-Tox1P/1M	*McyB*-Tox7P/3M	*McyB*-Tox10P/4M	*McyB*-Tox2+/-	*McyB2*-FAA/RAA	*McyA*-MSR/MSF	*McyD*-F2/R2	*McyA*-MSI	*Uma1*-UMF/R			
PCC7806	The Netherlands	+	+	+	+	+	+	+	+	+	+	/	+	+
UP10	Roodeplaat ZA	+	-	-	-	-	+	-	+	-	+	+	+	+
UP40	Midmar, ZA	-	-	-	-	+	-	+	+	-	+	+	+	+
UPUS1	Colorado, USA	-	+	-	-	-	-	+	+	-	+	+	+	+
UP03	Rietvlei, ZA	+	+	+	+	+	+	+	+	-	+	/	+	+
UP04	Hartbeespoort, ZA	+	-	+	+	+	+	+	+	+	+	/	+	+
UP09	Hartbeespoort, ZA	-	+	-	-	+	+	+	?	-	+	/	+	+
UP10	Roodeplaat, ZA	-	-	+	+	+	+	+	+	-	+	/	+	+
UP13	Roodeplaat, ZA	-	-	+	+	+	+	+	+	-	+	/	+	+
UP14	Klipvoor, ZA	+	-	+	+	+	+	-	+	-	+	/	+	+
UP17	Bon Accord, ZA	-	+	-	+	+	+	+	+	-	+	/	+	+
UP18	Bronkhorstspruit, ZA	-	-	+	+	+	+	+	+	-	+	/	+	+
UP20	Bronkhorstspruit, ZA	+	+	-	+	+	-	+	+	+	+	/	+	+
UP23	Bronkhorstspruit, ZA	-	-	+	-	-	-	+	+	-	+	/	+	+
UP24	Bronkhorstspruit, ZA	+	+	-	+	+	+	+	+	-	+	/	+	+
UP27	Klipvoor, ZA	-	-	+	+	+	-	+	+	-	+	/	+	+
UP28	Klipvoor, ZA	+	+	+	+	+	+	+	?	+	+	/	+	+
UP29	Hartbeespoort, ZA	-	+	-	-	+	-	+	+	-	+	/	+	+
UP31	Hartbeespoort, ZA	-	+	-	-	-	-	-	+	-	+	/	+	+
UP37	Krugersdrift, ZA	-	+	+	-	-	-	-	+	-	+	+	+	+
UP45	Krugersdrift, ZA	+	-	-	+	-	+	-	+	-	-	/	+	+
W. naegaliana														
UPUS2	Colorado,USA	-	+	-	-	-	-	+	+	-	+	+	+	+

[a] Results obtained with primers used for RT-PCR correspond in all experiments to that obtained with PCR, results were comparable with regard to presence of amplicon

3.2.2.5 Correlation of PCR and qRT-PCR results and Toxicity as measured by ELISA and PPIA

The toxicity of the strain was also confirmed with ELISA. In order to correlate toxicity with RT-PCR quantitation different samples with known toxicities were subjected to qRT-PCR analysis. Direct correlations were found between the level of expression as quantified by qRT-PCR, the presence of the PCR amplicons derived from selected genes within the *mcy* gene cluster (Figure 3.4), and level of measured toxicity as quantified using ELISA assays (Figure 3.1) (also see Oberholster et al., 2006a, 2006b).

3.4 DISCUSSION

3.4.1 Measured Toxicity

Surveys of different cyanobacterial blooms for given geographical areas have shown that the frequencies of toxic cyanobacterial blooms in fresh water ranged from 22 to 95% (Sivonen & Jones, 1999). For example, the frequency is an average of 74% for some Mediterranean countries including Portugal, France (Brittany) and Greece (Lanaras et al. 1989; Vasconcelos 1994; Vezie et al., 1997). The screening of cyanobacterium strains isolated from rice fields, irrigation and drainage water canals in the Nile Delta in Egypt showed that 23% of these isolates were found as active producers of microcystins with an amount of more than 500 $ng.L^{-1}$ (Yanni & Carmichael, 1998). A survey of cyanobacterial water blooms carried out from 2000 to 2004 in South Africa reservoirs by Oberholster and co-workers confirmed an average frequency of 95% toxicity tested by the ELISA method.

The severe and exceptional high toxicity of strain UP 37 (Krugersdrift dam, Figure 3.1) that was sampled in December 2004 might be explained by the particular climatic conditions (global drought period in the watershed, thermal and hydrologic stability, high radiation, minimal water flow) that characterized this sampling period as some of these conditions are known to affect cell toxicity and toxin production (Watanabe, 1996).

3.4.2 PCR-based markers

An interesting observation that derived from the sequence data is that fact that the sequences were three distinct "groupings", with the sequences deposited by Mikalsen et al. (2003) generated in a study on the natural variation within the *mcyABC* gene cluster from *M. aeruginosa* that fall within their own "group", the group of sequences from other species that group together, and then the sequences from this study and other studies (Grobbelaar et al., 2004; Jacobs et al., 1997) proofed more similar. The latter "group" was then aligned separately and several base pair differences were observed between the strains on nucleotide (Appendices, Figure A1.1) and amino acid level (Appendices, Figure A1.2). This may imply some evolutionary divergence for the Southern African strains.

Previous data (Oberholster, 2003) indicated that selective use of restriction sites can be use for strain identification. Since sequencing is expensive, laborious and time consuming when comparing to PCR-RFLPs, it is not well suited for high throughput application. However, even though PCR-RFLPs can be applied for this purpose not all restriction enzymes is useful for this purpose (e.g. indels obtained from UP13 i.e. *Cococales* gave similar results than *M. aeruginosa.* when restricted with *Pst*I, but *M. wesenbergii* was dissimilar to *M. aeruginosa* after digestion with this enzyme), and an accurate, well tested identification key is therefore imperative during application to prevent misclassifications. However, the use of an array of enzymes will be needed to ensure proper identification.

In our study, we found the PCR-based methods highly suitable as a method for early detection of putative toxigenic strains, even at very low cell counts. In all our studies we confirmed the presence of the PCR products and correlate the presence of PCR and/or qRT-PCR products with levels of toxicity. Toxicity was measured using ELISA and PPIA, and data was then correlated to data obtained using PCR and qRT-PCR analysis. During the study, PPIA occasionally gave "false" positives, indicating the measurement /sensitivity for other toxic compounds besides microcystins. This was never observed with our suite of PCR primers (i.e. primers derived from *mcyB* and *mcyD*). Hawkins et al. (2005) recently ranked methods for assessing the public health risk from microcystin in the aquatic environment, using the *mcyA* component of the microcystin synthetase gene, ELISA, PPi and HPLC during a survey of aquatic systems in temperate Australia and tropical Thailand. They concluded that off all methods ranked, ELISA ranked highest because of its superior specificity for microcystin. They found PCR highly sensitive, but found three results with false negatives when using only *mcyA* specific primers, whereas PPi was less specific than ELISA, although very cheap. Hawkins et al. (2005) stated that although HPLC is the standard protocol of the Queensland Health Services (QLD Health Method # 15605), is it extremely costly in comparison to other testing methods, timely and laborious. HPLC was also unable to prove or disprove the presence of the *mcyA* gene.

4. RECOMMENDATIONS

4.1 Use of PCR and qRT-PCR as diagnostic tools during Risk Assessment and Management strategies

The need for a reliable framework of risk assessment and management of contamination of drinking water by cyanobacteria toxin in South Africa exist. The application of the Du Preez and Van Baalen model (2006) is limited to Rand Water, due to shortage in skills, infrastructure and expertise at most water treatment plants. The major disadvantage of the Du Preez and Van Baalen Cyanobacterial Incident Management Framework (Du Preez & Van Baalen, 2006) is the use of the Mouse Test as indicator of toxicity on both ALERT LEVELS 1 and 2 during drinking water management. The use of mouse bioassays are restricted by law to specified, accredited facilities, and presently in South Africa, mouse bioassays are only conducted at the Onderstepoort Veterinary Institute in Pretoria (Du Preez & Van Baalen, 2006); and thus results are not readily available for rapid assessment by most water boards and purification facilities, especially those in rural areas. Moreover, there are moral concerns with the use of mammals as toxigenic indicators. The intraperitoneal mouse bioassay, which has been the most extensively used biotest to determine the toxicity of cyanobacterial blooms, is useful as an initial screening test, but requires expensive husbandry, is strictly regulated in some countries, and is opposed on moral grounds (Bell & Codd, 1996). However, such analysis does not indicate which cyanobacterial strains produce the toxins. Microcystin concentration in a body of water seems to be mostly dependent on the density of the hepatotoxic cells (Sivonen & Jones, 1999). The mouse bioassay is thus a generic test for toxicity and not specific for microtoxins present in cyanobaterial cells. Thus, rendering the Du Preez and Van Baalen Cyanobacterial Incident Management Framework (Du Preez & Van Baalen, 2006) rather ineffective and impractical. An alternative to this model is proposed using a combination of PCR-based technology (ALERT LEVEL 1) and ELISA and /or HPLC (ALERT LEVEL 2) as a toxic hazard indicator (Figure 4.1).

The proposed risk assessment model consists of a Pro-active Cyanobacterial Risk Assessment Monitoring Framework (Figure 4.1). The proposed strategy entails the monitoring of cell-bound cyanotoxins, total cyanotoxin concentrations and the presence of potential toxin producing cells. The strategy enables for early detection (i.e. good quality DNA can be extracted from as little as 10 cells.ml^{-1}) and pro-active / preventative measurements, since cyanotoxins are water-soluble, costly and difficult to remove during water purification processes. Since PCR-based technology is reasonably priced (~R 5-00/reaction) and allows for high-throughput, the presence of nuisance causing, harmful cells can be detected early. ALERT LEVEL 1 is reached with the confirmation of the potential hazard through PCR and/or qRT-PCR, coinciding with the exponential increase in cell numbers (i.e. increase in cell counts from 2 000 to 20 000 cells.ml^{-1}), normally associated with the occurrence of a bloom. This action is followed-up by total toxin concentration measurements (i.e. application of method of choice and according to budget capabilities, e.g. ELISA and / or HLPC). If total toxin concentration levels are $\geq$ 0.8 µg.L^{-1} then ALERT LEVEL 2 is reached. If the duration of the bloom is more than 8 consecutive days, then operational response measures must be applied. During this period, the water purification must take steps to reduce the amount of cyanotoxins according to guideline value (<5 µg.L^{-1} for 8 days and $\geq$ 5 µg.L^{-1} for 2 days). If the cyanotoxin concentration exceeds the guideline limits then the Health Authorities and general public must be notified and alternative drinking water supply be provided.

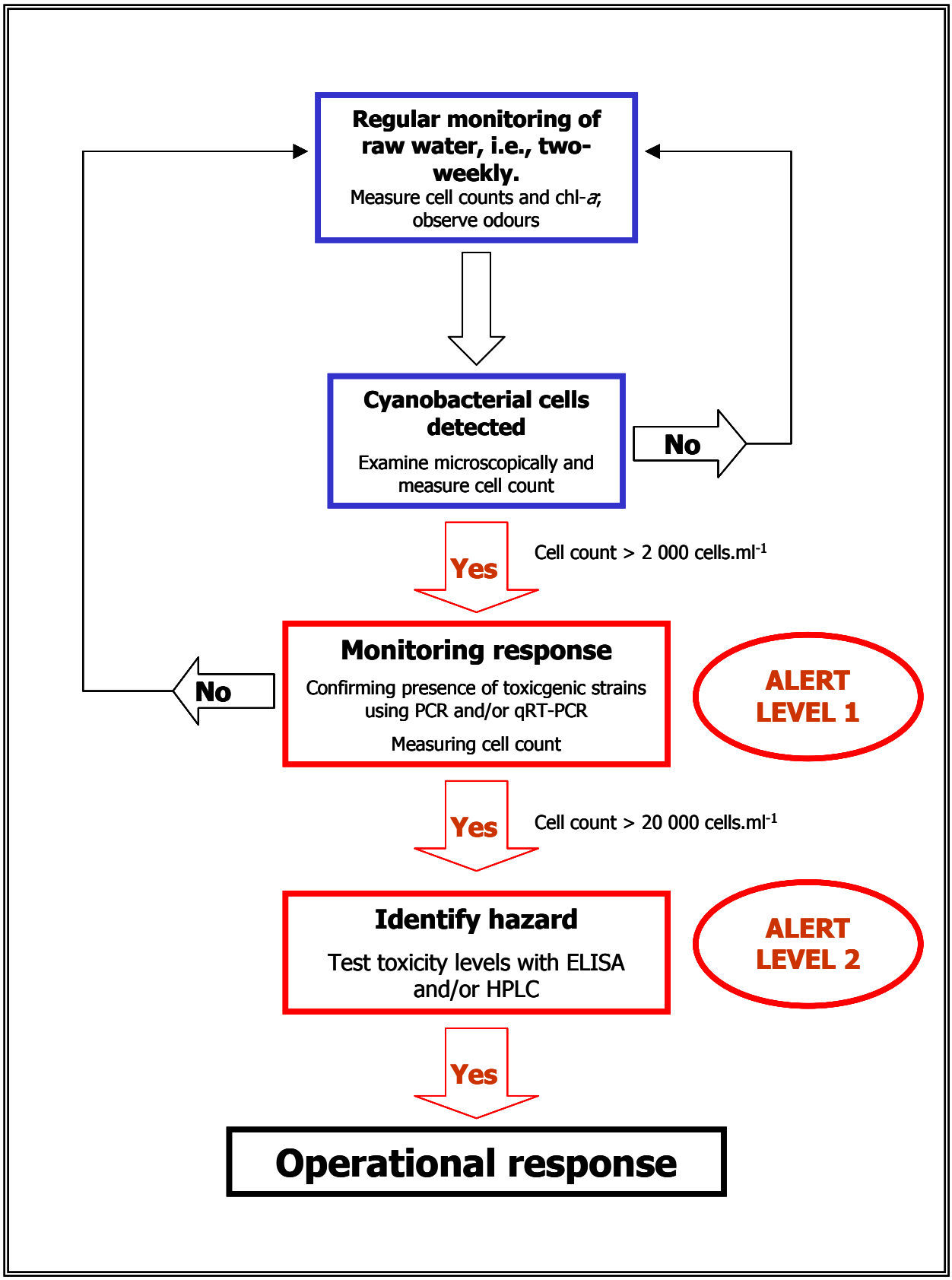


Figure 4.1 Cyanobacterial Pro-active Risk Assessment Monitoring Framework using the presence of cyanobacterial cells and PCR amplicons as primary indicators of water quality.

5. **REFFERENCES**

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