Molecular assessment of the occurrence of toxic cyanobacteria and cyanotoxins in South African impoundments

by

Karin Ronel Conradie

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In the department of Botany
School of Environmental Sciences and Development
Faculty of Natural Sciences
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Supervisor: Dr. Sandra du Plessis
Co-supervisor: Prof. Kaarina Sivonen

The Roodeplaat Dam (www.earth.google.com)

The Hartbeespoort Dam (www.earth.google.com)

PREFACE

I hereby declare that this study presented for the degree Philosophiae Doctor at the North-West University, Potchefstroom campus, consists exclusively of my own original research and has not previously been presented for a degree at any other university.

Karin Ronel Conradie

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ABSTRACT

MOLECULAR ASSESSMENT OF THE OCCURRENCE OF TOXIC CYANOBACTERIA AND CYANOTOXINS IN SOUTH AFRICAN IMPOUNDMENTS

The theme of this thesis is the ecophysiological basis for the development of mass occurrences of *Microcystis* species in South African impoundments. Research concerning the reasons for harmful algal bloom (HAB) formation has been intensifying over the past few decades and the need for molecular investigations on natural occurring HAB species has been emphasised in recent literature.

The main objectives of this investigation were to:

- Verify the identity of specific bloom forming species by 16S rDNA analyses and to investigate the eco-physiological rationale for the mechanisms influencing growth and toxin production of the cyanobacteria.

In order to realize this objective we had to achieve the following aims:

- To verify the identity of the reference cultures in the culture collection of the North-West University by means of 16S rDNA sequencing and analysis.
- To measure the DNA copy number of the *Microcystis* specific 16S rDNA and microcystin producing genes, *mcyB* as well as *mcyE* in order to shed more light on toxin production in the sampled environmental water, as well as the occurrence of toxigenic strains.
- To investigate the *in vivo* expression of the *ntcA* and the *rbcL* genes in order to study the physiological rationale for the sudden increase in biovolume of a specific species during a bloom.

A polyphasic approach was used for the taxonomic identification of some of the bloom forming species grown in the culture collection of the North West University to be used as reference cultures during the study.

It was demonstrated that the isolate formerly known as "*Oscillatoria simplicissima*" should be reassigned to *Planktothrix pseudagardhii* under the order Oscillatoriales, family Phormidiaceae and subfamily Phormidioideae. Other strains in the culture collection were also not correctly identified, e.g. *Spirulina sp.* was in fact *Arthruspira sp.*

To obtain a reliable quantification method each real-time polymerase chain reaction (PCR) method had to be optimised. A reliable protocol for DNA isolation that does not discriminate between the different cyanobacterial species and cell types, and so influence results, was also developed.

The regulation of the gene expression of key metabolic enzymes was investigated in the Hartbeespoort Dam and the Roodeplaat Dam during the bloom season of 2004 to 2005. The *in vivo* expression of the *ntcA* and the *rbcL* genes were examined. The expression of these genes, *rbcL* (encoding the large subunit of Rubisco) and *ntcA* (encoding a nitrogen assimilation regulatory protein), reflect in part the photosynthetic and nitrogen metabolism activity of the cyanobacteria present in the sample. Together with this, DNA copy number of the *Microcystis sp.* specific 16S rDNA and toxin genes, *mcyE* as well as *mcyB* was also measured with real-time PCR. The trends of the ecological and molecular data were analysed using multivariate statistical analysis.

Although the Roodeplaat and the Hartbeespoort Dams are closely situated in the densely populated area of the Gauteng province in South Africa, and both are eutrophic water impoundments, they differ remarkably in their responses to environmental influences, most probably due to the difference in nutrient loading and water surface temperatures.

A very important variable in both dams is the inflow of the water into the system, representing nutrient loading. This nutrient loading is the main reason for the bloom occurrence to take place. The inflow of nutrients constitutes mainly of phosphorus and nitrogen. Clear relationships emerged between the total nitrogen in the water and the *Microcystis sp.* biomass, indicating that the decrease in nitrogen concentrations was caused by the increase in *Microcystis sp.* biomass.

The Hartbeespoort Dam's biomass consists of different *Microcystis sp.* strains, some of which are toxic and some that are non toxin producers. The biomass of the Roodeplaat Dam however, consists mainly of toxic *Microcystis* species, but other photosynthetic species are also present in the Roodeplaat Dam. Microcystin production is associated with higher temperatures, and the release of microcystin is most probably caused by cells that die off due to natural cycles or temperatures. An association was also observed between *ntcA* gene expression and microcystin synthesis. It is argued that the *ntcA* gene increased as a result of high microcystin concentrations in the water, and thus probably inhibited the synthesis of microcystin.

The Water Research Commission (WRC) has recently launched a program for the modulation of the production of harmful algal blooms. The data from this study is the first step to identify specific strains to be used for modulation and with this study, the first year's data is already submitted.

The information can then be applied by the industry to predict when toxic *Microcystis sp.* is going to form a bloom under certain conditions. Therefore water purification plants can prepare in advance for a blooming event lowering the risk of distributing water of low quality to the public.

OPSOMMING

MOLEKULÊRE ONDERSOEK VAN DIE VOORKOMS VAN TOKSIESE SIANOBAKTERIEË EN SIANOTOKSIENE IN SUID-AFRIKAANSE WATERBRONNE

Die tema van die proefskrif is die ekofisiologiese basis vir die ontwikkeling van opbloeie van *Microcystis* spesies in Suid-Afrikaanse damme. Navorsing oor die moontlike redes vir skadelike alg-opbloeivorming (HAB) het die laaste paar dekades sterk toegeneem en die noodsaaklikheid vir molekulêre navorsing oor skadelike alg-opbloeie in die natuur is in onlangse literatuur beklemtoon.

Die hoof doelwitte van die navorsing was om:

- Die identiteit van die spesifieke spesies wat die opbloeie veroorsaak te verifieer met behulp van 16S rDNA-analises en om die ekofisiologiese rasionaal vir die meganisme wat groei- en toksienproduksie van die sianobakterieë beïnvloed, na te vors.

Om hierdie doelwitte te bereik moet die volgende uitkomste bereik word:

- Om die identiteit van die verwysingskulture in die kultuurversameling van die Noordwes-Universiteit te verifieer met behulp van 16S rDNA-basispaar-volgordebepaling en analise.
- Om die DNA-kopiegetal van die *Microcystis sp.* spesifieke 16S rDNA en mikrosistien produserende gene, *mcyE* en *mcyB* te meet, om lig te werp op die toksienproduksie in die monsters wat versamel is, asook die voorkoms van toksigeniese lyne aan te dui.
- Om die *in vivo*-uiting van die *ntcA*- en die *rbcL*-gene na te vors om die fisiologiese rasionaal vir die skielike toename in biovolume van sekere spesies gedurende 'n opbloei te ondersoek.

'n Polifasiese benadering is dus gebruik vir die taksonomiese identifikasie van sekere van die opbloeivormende spesies wat in die kultuurversameling van die Noordwes-Universiteit gegroei word en wat as verwysingskulture gebruik sou kon word gedurende die studie.

Daar is bevind dat die isolaat voorheen bekend as "*Oscillatoria simplicissima*" herbenoem moet word na *Planktothrix pseudagardhii* binne die orde Oscillatoriales, familie Phormidiaceae en subfamilie Phormidioideae. Verder is daar gevind dat ander lyne in die kultuurversameling ook nie korrek geïdentifiseer is nie, soos bv. *Spirulina sp.* wat eintlik *Arthruspira sp.* is.

Vir die ontwikkeling van 'n betroubare kwantifiserende metode, moet elke intydse polimerase kettingreaksie (PCR) geoptimiseer word. 'n Betroubare protokol vir DNA-isolasie wat nie diskrimineer tussen verskillende sianobakteriese spesies en seltipes nie en dus ook nie die resultate sal beïnvloed nie, is ook ontwikkel.

Die regulering van die geenuiting van sleutel metaboliese ensieme is nagevors in die Hartbeespoortdam en die Roodeplaatdam gedurende die opbloeiseisoen van 2004 tot 2005. Die *in vivo*-uiting van die *ntcA*- en die *rbcL*-gene is ondersoek. Die uitdrukking van die gene, *rbcL* (wat vir die groot subeenheid van Rubisco kodeer) en *ntcA* (wat vir die stikstof assimilerende regulatoriese proteïen kodeer), reflekteer gedeeltelik die fotosintetiese- en stikstof metaboliese-aktiwiteit van die sianobakterieë teenwoordig in die monsters. Tesame hiermee, is die DNA-kopiegetal van die *Microcystis sp.* spesifieke 16S rDNA- en toksien-gene, *mcyE* en *mcyB* ook gemeet met intydse PCR. Die neigings van die ekologiese en molekulêre data was geanaliseer met meerveranderlike statistiese analise.

Alhoewel die Roodeplaatdam en die Hartbeespoortdam digby mekaar in die digbevolkte Gauteng-provinsie geleë is, en beide eutrofiese waterbronne is, verskil dit baie in die reaksie tot omgewingsfaktore, moontlik as gevolg van die verskille in voedingstofbeladings en wateroppervlakte temperatuur.

'n Baie belangrike veranderlike in beide damme is die invloei van water in die sisteem, wat voedingstofbelading verteenwoordig. Hierdie voedingstoftoevoegings is waarskynlik die hoofrede vir die opbloeie wat plaasvind. Die invloei van voedingstowwe bestaan hoofsaaklik uit fosfor en stikstof. Duidelike verbande kom na vore tussen die totale stikstof in die water en die *Microcystis*-biomassa, wat daarop dui dat die afname in die stikstofkonsentrasies veroorsaak word deur die toename in *Microcystis*-biomassa.

Die Hartbeespoortdam bevat verskillende *Microcystis*-lyne, waarvan sommiges toksies is en ander weer nie-toksienproduserend is nie. Die Roodeplaatdam daarenteen bevat hoofsaaklik toksiese *Microcystis* spesies, maar daar is ook ander fotosintetiese spesies teenwoordig. Mikrosistienproduksie word klaarlyklik gestimuleer deur hoër temperature, en die vrystelling van die mikrosistiene vind plaas deur selle wat afsterf as gevolg van natuurlike prosesse of deur hoër temperature. 'n Moontlike verband was ook waargeneem tussen die *ntcA*-geenuitdrukking en mikrosistiensintese. Die *ntcA*-geen vermeerder as gevolg van hoë mikrosistienkonsentrasies in die water en inhibeer moontlik die sintese van mikrosistiene.

Die Waternavorsingskommissie het onlangs 'n program geloods vir die modulering van die produksie van skadelike algopbloeie. Die data van hierdie studie dien as die eerste stap om die spesifieke lyne wat gebruik kan word vir modulering te identifiseer en die eerste jaar se data is reeds gepubliseer vir die studie.

Hierdie inligting kan dan deur die industrie gebruik word om te voorspel wanneer toksiese *Microcystis*-spesies 'n opbloei gaan vorm en onder watter omgewingstoestande dit kan gebeur. Dus kan watersuiweringsaanlegte hulleself voorberei vir 'n opbloei, wat die risiko vir die verspreiding van water van 'n lae kwaliteit aan die publiek kan verminder.

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LIST OF ABBREVIATIONS AND SYMBOLS

aa	Amino acid
AMT	aminotransferase
ANOVA	Analysis of variance
AP	Alkaline phosphatase
A_{260}/A_{280}	ratio of absorbency measured at 260 nm and 280 nm
bp	base pair
BPB	bromophenol blue: $C_{14}H_{10}Br_4O_5S$
BSA	bovine serum albumin
CCAP	Culture Collection of Algae and Protozoa, UK
Chl *a*	Chlorophyll *a*
CTAB	N-cetyl-N-N-N-trimethyl ammonium bromide
cDNA	complementary DNA
C_t	Threshold cycle
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
ddATP	2',3'-dideoxyadenosine-5'-triphosphate
ddCTP	2',3'-dideoxycytidine-5'-triphosphate
ddGTP	2',3'-dideoxyguanosine-5'-triphosphate
ddH_2O	double distilled water
ddNTP	2',3'-dideoxynucleotide-triphosphate
ddTTP	2',3'-dideoxythymidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
DIN	dissolved inorganic nitrogen
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide triphosphate
dsDNA	double stranded DNA
dTTP	2'-deoxythymidine-5'-triphosphate
DWAF	Department of Water Affairs and Forestry
EC	Electrical conductivity
EDTA	Ethylenediamine tetra-acetic acid, disodium magnesium: $C_{10}H_{16}N_2O_8$
ELISA	Enzyme-linked immunosorbent assay
E. coli	*Escherichia coli*

Eco R1	restriction endonyclease isolated from an *E. coli* strain that carries the cloned *Eco* R1 gene from *E. coli* RY 13, with recognition site 5'-G↓AATTC-3'
EtBr	ethidium bromide: $C_{21}H_{20}BrN_3$
EtOH	ethanol: CH_3CH_2OH
GLP	good laboratory practice
HAB	Harmful algal bloom
HPLC	High performance liquid chromatography
IAA	isoamyl alcohol
ICSP	International Committee on Systematics of Prokaryotes
IDT	Integrated DNA technologies
ITS	Internal transcribed spacer
kb	kilo base pair
KN	Kjeldahl nitrogen
LD_{50}	Lethal dose
MC	microcystin
MD	*Microcystis* dominance
mRNA	messenger RNA
MW	molecular weight
n	nano: 10^{-9}
N	nucleotide
NEMP	National Eutrophication Monitoring Program
NH_2	amino group, indicating the N-terminal of a protein molecule
NIVA	Norwegian Culture Collection of Algae
nm	nanometer: 10^{-9} meter
nM	nanomolar
N:P	Nitrate to phosphorus ratio
NR	Nitrate reductase
NRF	National Research Foundation
NRPS	Nonribosomal peptide synthetase
OD	optical density
ORF	Open reading frame
PBS	phosphate buffered saline
PCA	Principal component analysis
PCC	Pasteur Culture Collection

PCR	polymerase chain reaction
pH	indicates acidity: numerically equal to the negative logarithm of H^+ concentration expressed in molarity
P_i	inorganic phosphate
PKS	polyketide synthetases
pmol	pico mole
prot K	proteinase K: endopeptidase
Py	pyrimidine
qPCR	quantitative real-time polymerase chain reaction
qRT-PCR	quantitative real-time reverse transcription polymerase chain reaction
RBS	ribosome binding sites
RDA	redundancy analysis
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
RQS	Resource Quality Services
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP	Ribulose-1,5-bisphosphate
SDS	sodium dodecyl sulphate: $C_{12}H_{25}NaSO_4$
s	seconds
ssDNA	single stranded DNA
SSU	Small subunit of Rubisco
T_a	annealing temperature
T_m	melting temperature
Taq Polymerase	DNA deoxynucleotidyltransferase from *Thermus aquaticus*.
TBR	Tree bisection-reconnection
temp	temperature
TN	Total nitrogen
TP	Total phosphorus
Tris	Tris®: tris(hydroxymethyl)aminomethan: 2-Amino-2-(hydroxymethyl)-1,3-propanediol:$C_4H_{11}NO_3$
Tris-HCL	2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride: $C_4H_{11}NO_3.H_2O$

Triton X-100	Triton X-100®: octylphenolpoly(ethylene-glycolether)$_n$: $C_{34}H_{62}O_{11}$
tRNA	transfer ribonucleic acid
UCT	University of Cape Town
UV	ultraviolet
WCW	Water Care Works
WHO	World Health Organization
WRC	Water Research Commission

LIST OF UNITS

LD_{50}	Dose of toxin that kills 50% of the animals tested.
LC_{50}	Lethal concentration of toxin that kills 50% of the tested organisms.
LT_{50}	Lethal time that toxin take to kill 50% of the tested organisms.

Restriction Enzyme: One unit is the enzyme activity that completely cleaves 1 μg λDNA in 1 h at enzyme specific temperature in a total volume of 25 μl.

Taq DNA Polymerase: One unit is the quantity of enzyme required to catalyse the incorporation of 10 nmol of dNTP's into acid insoluble material in 30 minutes at 74°C.

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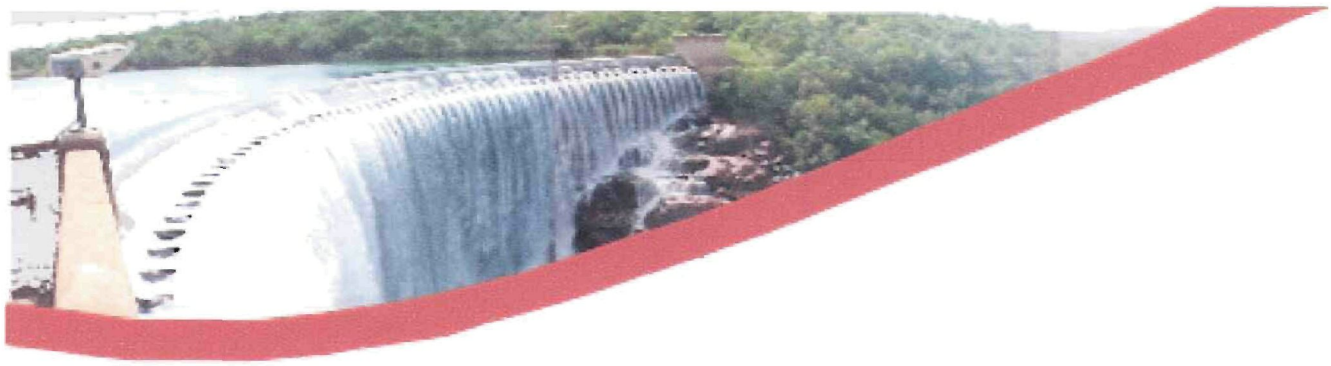


Chapter 1

Introduction

The severity, frequency, distribution and impact of harmful algal blooms (HAB) have all increased in the recent decades, but the underlying causes of such blooms are not entirely understood (Gobler *et al.,* 2007).

Specific detrimental effects of cyanobacterial blooms on the quality of drinking water include the production of taste- and odour-causing compounds and several toxic molecules (Neilan, 1995). As a result, blooms create major threats to animal and human health, tourism, recreation and aquaculture. Several species found in South Africa also produce cyanotoxins, eg. *Microcystis aeruginosa* is a common form of cyanobacteria in South Africa (van Ginkel *et al.,* 2006) and is capable of forming toxic heptapeptides (microcystins) that can cause illness or death (Nonneman and Zimba, 2002).

South Africa is an arid country with identified and serious future limitations for water quality and quantity (Harding and Paxton, 2001). In the absence of deliberate eutrophication management, the reality is that increasing numbers of the population will be exposed to waters containing cyanobacterial metabolites that pose acute and chronic implications for their health (Harding and Paxton, 2001). The development of an understanding of cyanobacterial growth and metabolism under different environmental conditions thus constitutes a crucial need in South Africa (Harding and Paxton, 2001).

Factors that may influence the growth and development of one cyanobacteria over another do not act singly, but rather in concert with a constantly changing suite of parameters – the combination of which determines the outcome of a successional phase of algal growth (Harding and Paxton, 2001). To gain insights into the internal dynamics of the freshwater cyanobacteria, this study aimed to trace ecophysiologically distinct, strains in their natural environment. Physiological knowledge of the bloom forming species is the key to our understanding of the bloom forming phenomena. Only with data obtained from fundamental investigations of the ecophysiological potential of problem species can we attempt to develop an early warning system of harmful algal blooms. While microscopic identification and toxin analysis have traditionally been employed for monitoring purposes, molecular biological methods may provide rapid and sensitive diagnoses for the presence of toxic and toxigenic cyanobacteria and are useful for general ecological studies (Ouellette and Wilhelm, 2003).

Furthermore, due to the complexity of an *in situ* community the physiological changes occurring in problem taxa can only be investigated with the use of molecular techniques. These techniques

were used during this study in order to ensure that the physiological characteristics of the problem species observed *in vitro* can also be investigated *in situ.*

In autotrophic organisms such as cyanobacteria the main metabolic processes of photosynthesis, respiration and nitrogen metabolism are tightly regulated and also in close interaction with each other. In order to investigate the bloom forming capabilities of these species it is important to look into the ecophysiological dynamics of each process. Several *in vitro* studies have been done on the subject but it still does not provide clear answers as to what is happening *in situ.* By identifying key metabolic enzymes and investigating their regulation of gene expression in field situations we might broaden our understanding of the regulation of these processes *in situ* and therefore growth potential as well.

In order to investigate the physiological rationale for the sudden increase in biovolume of a specific species during blooms the intent is to investigate the *in vivo* expression of the *ntcA* and the *rbcL* genes. The expression of these genes, *rbcL* (encoding the large subunit of Rubisco) and *ntcA* (encoding a nitrogen assimilation regulatory protein), reflect in part the photosynthetic and nitrogen metabolism activity of the cyanobacteria present in the sample. The existence of possible trends in the expression of the *ntcA* and the *rbcL* genes in specifically *Microcystis aeruginosa* (bloom former) in the Hartbeespoort Dam and Roodeplaat Dam was measured with reverse transcriptase real time PCR. Together with this, DNA copy number of the *Microcystis* specific 16S rDNA and toxin genes, *mcyE* as well as *mcyB,* were also measured with real time PCR.

Studies found that cyanobacteria form blooms in regulation to specific environmental conditions, eg. changes in temperature, phosphate concentrations or nitrate concentrations. Thus, through a combination of ecological and molecular research, it is hoped that experimental data may ultimately be extrapolated to the environment not only to understand the timing of toxin production but also the purpose of it (Kaebernick and Neilan, 2001).

During this study, the concentration of microcystins present as well as ecological data such as water temperature, pH, nutrient loads and chlorophyll *a* concentrations were recorded and obtained from the Department of Water Affairs and Forestry. The trends of the ecological and molecular data were analysed using multivariate statistical analysis (CONOCO for Windows, version 4). This produced insight into the mechanisms influencing growth of the cyanobacteria.

This study will only focus on the toxin gene cluster of *Microcystis sp.* as it was by far the most dominant species present in the environmental samples during the study.

To identify possible bloom formers with molecular techniques, before the bloom occur, can improve the current harmful algae management by South African water boards and prevent serious health risks. This information can then be applied by industry to predict which algae are going to form a bloom under certain conditions. Therefore water purification plants can prepare in advance for a blooming event lowering the risk of distributing water of low quality to the public. The experience gained is valuable for a risk assessment of microcystin in the environment and for future water management and dam-restoration strategies.

Chapter 2

Literature review

2.1 Introduction

Cyanobacterial mass occurrences have become an increasing worldwide problem in aquatic habitats such as lakes, rivers, estuaries and oceans as well as in man-made water storage systems (Sotero-Santos *et al.,* 2006). These oxygenic phototrophs rapidly form blooms as a result of various ecological stimuli, including eutrophication of their habitat and cause extensive physical and chemical damage to the aquatic environments affected (Reynolds and Walsby, 1975). Cyanobacteria are common in all kinds of habitats, including antarctic lakes, thermal springs, arid deserts and tropical acidic soils, but are mostly present in marine and freshwater environments (Kaebernick and Neilan, 2001).

In arid regions such as Southern Africa, cyanobacterial problems pose a considerable and significant threat to the sustainable use and management of fresh water resources (Harding and Paxton, 2001). As a result of increased nutrient loading worldwide in water systems, the frequency and severity of bloom events continue to rise (Harding and Paxton, 2001). Cyanobacterial blooms are usually caused by especially strains of the distantly related cyanobacterial genera *Microcystis*, *Anabaena*, *Planktothrix* and more rarely *Anabaenopsis, Haplosiphon* and *Nostoc* (Sivonen and Jones, 1999).

Cyanobacteria's ecological success is most probably due to their global distribution (Ouellette and Wilhelm, 2003). Their ability to exist in such diverse habitats is a reflection of their adaptability as a group to fix nitrogen, adapt their light harvesting pigments, regulate buoyancy and exhibit cellular differentiation for the purpose of reproduction or dormancy and giving them an advantage over many competitors (Kaebernick and Neilan, 2001). When water sources are subject to algal blooms, nontoxic taste and odour compounds that may be released by the cyanobacteria can compromise quality (Ouellette and Wilhelm, 2003). When the cyanobacteria produces a toxin, the threat can be considerable (Ouellette and Wilhelm, 2003). Water blooms formed by the genus *Microcystis*, have a relatively high frequency of toxicity (between 25% and 70%) and constitute a potential health hazard for livestock and humans worldwide (Rudi *et al.,* 1998b). For this reason bloom toxicity thus needs to be determined early in the bloom development in a drinking water reservoir (Baker *et al.,* 2002). Although the health effects of exposure to low levels of cyanotoxins remain unknown, chronic exposure to toxic cyanobacteria in water sources over a long period of time could be harmful (Carmichael, 2001).

Cyanobacterial blooms are also problematic in South African waters. The effects of these blooms can be seen in eutrophic water impoundments such as the Hartbeespoort Dam and the Roodeplaat Dam and constitute a health hazard for all South Africans.

2.2 South Africa

Large areas of South Africa rely on informal water sources such as dams, windmills and rivers for water as a result of no formal water supplies (Gehringer *et al.,* 2003). These water bodies may also be used at the same time for ablution purposes, leading to increased eutrophication and ultimately the formation of potentially toxic blooms (Falconer, 2001).

In November 2003 the total microcystin concentrations in the drinking water of two small towns in South Africa exceeded the World Health Organisation's total allowable daily intake (1 $\mu g.\ell^{-1}$) in the drinking water distribution system (Van Ginkel, 2004). Currently *Microcystis sp.* comprised more than 40% of the phytoplankton population in five different impoundments in South Africa, including the Hartbeespoort Dam and the Roodeplaat Dam for longer than 50% of the time and the maximum total microcystin concentration exceeded 100 $\mu g.\ell^{-1}$ more than 50% of the time (Van Ginkel *et al.,* 2006).

2.2.1 Hartbeespoort Dam

The Hartbeespoort Dam is a hypertrophic, warm, monomictic lake with high nitrogen and phosphorous loading from the surrounding suburbs. Even as early as 1987, Robarts and Zohary found that nitrogen and phosphorus availability were never found to be limiting in the Hartbeespoort Dam and were always in excess of requirements. Jarvis (1986) indicated that in hypertrophic conditions like in the Hartbeespoort Dam it is unlikely that large filter-feeders such as *Daphnia* are able to retard or limit the development of cyanobacterial blooms by high grazing pressure.

Since 1985, Zohary found that *M. aeruginosa* dominated (>80% by volume) the phytoplankton population up to 10 months of each year in the Hartbeespoort Dam because it maintained itself within shallow diurnal mixed layers and was thus ensured access to light, the major limiting resource. The post-maximum summer populations persisted throughout autumn and winter, despite suboptimal temperatures, by sustaining low losses (Zohary, 1985). Increased sedimentation losses caused a sharp decline of the population at the end of winter each year and

a short (2–3 months) successional episode followed, but by late spring *M. aeruginosa* was again dominant.

2.2.2 Roodeplaat Dam

The Roodeplaat Dam has shown increased incidences of toxic cyanobacterial blooms and these algal blooms are experienced for more than 20% of the time. This is as expected, since the nutrient concentrations are very high and the Roodeplaat Dam is a clear system with low light limitation to algal growth. Therefore, the Roodeplaat Dam is subject to potential toxic algal blooms (Van Ginkel, 2000a).

The cyanobacteria (especially *Microcystis* and *Anabaena* genera) formed the dominant algal group for most of the study period from 1989 to 2000. Chl *a* concentrations are constantly higher than 20 μg/L, indicating that the system does not recuperate at any time during the year. The cyanobacteria form more than 30% of the phytoplankton population annually. This is an indication that the Roodeplaat Dam is in a serious state of eutrophication and management measures should be taken to improve the situation (Van Ginkel, 2000a).

The eutrophication might be due to the fact that the Zeekoegat Water Care Works (WCW) discharges into the impoundment and the Baviaanspoort water care works discharges into the Pienaars River upstream of the impoundment. Informal settlements and livestock agricultural practices may also contribute to microbiological pollution during periods of higher rainfall when the surface wash-off could be a major contributor to microbiological and nutrient loads into the impoundment (Van Ginkel, 2000a).

2.3 Ecological roles for cyanotoxins

Does the production of cyanotoxins give cyanobacteria an ecological advantage? The biosynthesis of cyanotoxins is an energetically demanding process and the function is unclear (Dittmann *et al.*, 2001; Ouellette and Wilhelm, 2003). According to Ouellette and Wilhelm (2003), the toxic properties of cyanotoxins may have no connection to their functions.

There are thus far no conclusive studies about the purpose of microcystin synthesis but many studies (Carmichael, 1992; Chorus and Bartram, 1999; Dittmann *et al.*, 2001) suggest putative roles for microcystin as a chemical defence against zooplankton, or a suppressor of the growth of competing species. Others suggest that microcystin may act as an iron scavenging molecule

(Utkilen and Gjølme, 1995), or regulates endogenous protein posphatases or that it may be used as nitrogen reserve (De Figueiredo *et al.*, 2004), have some specific cell regulatory function (Shi *et al.*, 1995) or might function in light sensing with the expression of the light regulated *mrpA* and *mrpB* genes (Dittmann *et al.*, 2001). Downing (2007) suggests that microcystins may be involved in the enhancement of photosynthetic activity. To determine the function of cyanobacterial toxins it is necessary to understand the regulation of the production of these toxins.

2.4 Cyanobacterial toxins

Cyanobacterial toxins are classified by how they affect the human body or animals (Kaebernick and Neilan, 2001; De Figueiredo *et al.*, 2004). Most cyanotoxins are classified as either hepatotoxins or neurotoxins but there are also several dermatotoxins produced primarily by benthic marine cyanobacteria (Dittmann and Börner, 2005).

Hepatotoxic mass occurrences are more common than neurotoxic ones (Sivonen and Jones, 1999). Hepatotoxins (microcystins, nodularin and cylindrospermopsin) which affect the liver (Kaebernick and Neilan, 2001) are produced by some strains of the cyanobacteria *Microcystis*, *Anabaena*, *Planktothrix, Oscillatoria*, *Nodularia, Nostoc, Cylindrospermopsis* and *Umezakia* (figure 2.1) (Sivonen and Jones, 1999; Kaebernick and Neilan, 2001). Neurotoxins (anatoxin-a, homoanatoxin-a, anatoxin-a(s) and saxitoxins) which affect the nervous system are produced by some strains of *Aphanizomenon* and *Oscillatoria* (figure 2.1) (Sivonen and Jones, 1999; Kaebernick and Neilan, 2001). Currently the hepatotoxic *Oscillatoria* strains are called *Planktothrix agardhii* or *Planktothrix rubescence* (Anagnostidis and Komarek, 1988; Fujii *et al.*, 2000). Cyanobacteria from the species *Cylindrospermopsis raciborskii* may also produce toxic alkaloids, causing gastrointestinal symptoms or kidney disease in humans (Sivonen and Jones, 1999; Valério *et al.*, 2005). Cytotoxins (cell damaging) and toxins responsible for allergic reactions, dermatotoxins (lipopolysaccharides, lyngbyatoxin-a and aplysiatoxins) have all been isolated from cyanobacteria (Wilson *et al.*, 2000). Microcystins, cyclic heptapeptide hepatotoxins, are by far the most prevalent of the cyanobacterial toxins (Sivonen and Jones, 1999; Rantala *et al.*, 2004). Of the known toxins produced by cyanobacteria, microcystins and nodularins are the most significant threat to human and animal health (Dittmann and Börner, 2005).

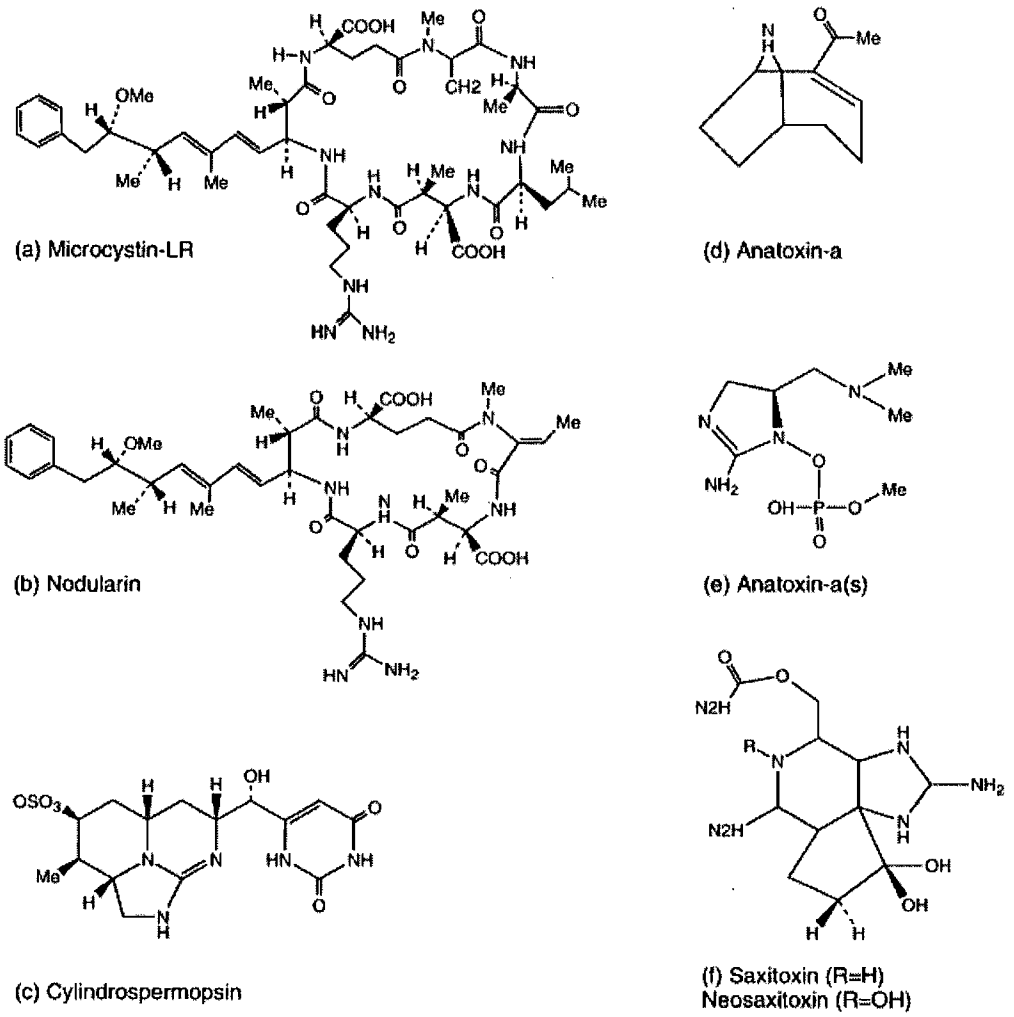


Figure 2.1: Chemical structures of the most common cyanobacterial hepatotoxins (a,b,c) and neurotoxins (d,e,f). Taken from Kaerbernick and Neilan (2001).

By inhibiting certain protein phosphatases these cyclic peptides exert their toxic effects (Kaebernick and Neilan, 2001). Their acute toxicity (given intraperitoneally to mice) varies between 50 and 800 μg/kg of body weight (Rinehart *et al.*, 1994).

2.5 Incidents of toxicity

One of the earliest reports of the toxic effects of cyanobacteria was 1000 years ago in China (Chorus and Bartram, 1999). Although relatively sound descriptions of cyanobacterial "blooms" exist from as far back as the 12th century, the first conclusive demonstration of and links to toxicity in this group of organisms were during the late 1800's in Australia (Harding and Paxton, 2001).

Incidents of toxicosis remain largely confined to the poisoning of domestic animals, livestock and occasionally, wild animals but there are no reliable figures for the number of people affected

worldwide (De Figueiredo *et al.,* 2004). According to Dittmann and Börner, (2005) only a few reported cases of human illnesses and proven effects of microcystin has been assigned to cyanobacterial toxins and this is most likely due to the lack of monitoring, especially in developing countries where drinking water treatment is ineffective (Dittmann and Börner, 2005).

In the last century various hepatotoxic blooms have been documented (De Figueiredo *et al.,* 2004). Illness in various regions of the world (North and South America, Africa, Australia, Europe, Scandinavia and China) have been linked to cyanobacteria (De Figueiredo *et al.,* 2004). However, an incident in 1996 involving 2 000 cases of gastroenteritis and the associated 60 deaths of dialysis patients in Brazil is the only verifiable and documented fatal case of cyanobacterially-linked human poisonings (Harding and Paxton, 2001).

In South Africa the first animal deaths suspected to be due to algal toxin poisoning were reported in 1927 from the Amersfoort district and another case was reported in Wakkerstroom in 1942 (Harding and Paxton, 2001). After the construction of the Vaal Dam in 1938, flooding of fertile farmland resulted in eutrophic conditions with the formation of a bloom covering up to 98% of the dams' surface (Harding and Paxton, 2001). As a result, numerous stock deaths were reported on farms adjacent to the dam during the summers of 1942 and 1943 (Harding and Paxton, 2001).

There has been a reported contamination of drinking water by *M. aeruginosa* (with confirmed MC-LR synthesis) in 1996, when an entire dairy herd were poisoned in Tsitsikamma-Kareedouw district (Harding and Paxton, 2001).

While there are, undoubtedly, many cases of sustained algal growths that result in persistent and often worrying problems, these remain largely hidden in the records of water quality managers and potable supply utilities (Harding and Paxton, 2001). It is also probable that numerous cases from the agricultural and rural sectors of many countries have gone unnoticed and the noxious effects undiagnosed (Harding and Paxton, 2001).

2.6 Exposure

Cyanotoxins may accumulate in the trophic web and produce diverse intoxication symptoms and chronic effects that are difficult to diagnose and prevent (Falconer, 2001; Bittencourt-Oliveira, 2003). Specifically, the neurotoxins and hepatotoxins associated with cyanobacterial blooms are

responsible for deaths in wild and domesticated animal populations and have various acute and chronic pathogenic effects on humans (Carmichael, 1992).

Human exposure to microcystins may occur through a direct route such as drinking water, recreational water, hemodialysis or through an indirect route such as food (De Figueiredo *et al.*, 2004 and references therein).

The most frequent and serious health effects are caused by drinking water containing the toxins (cyanobacteria), or by ingestion during recreational water contact (De Figueiredo *et al.*, 2004). Acute intoxication by microcystins frequently coincides with the lysis of the bloom forming cells by natural senescence or water treatment processes and hence the liberation of toxins into the water (De Figueiredo *et al.*, 2004). The inhalation of dry cyanobacteria cells or contaminated water is more dangerous than oral ingestion of contaminated water indicating the hazardous potential of practicing aquatic sports in recreational waters that suffer from a microcystin producing bloom (World Health Organisation, 2003). The low risk range for recreational exposure is 1-10 μg/L (Chorus and Bartram, 1999).

Disease due to cyanobacterial toxins varies according to the type of toxin and the type of water or water-related exposure (drinking, skin contact, etc.) (De Figueiredo *et al.*, 2004). Some of the symptoms characteristic to microcystin poisoning are skin irritation, weakness, anorexia, fever, sore throat, headache, muscle and joint pain, plaster of the mouth, pallor, apathy, respiratory problems, gastroenteritis, vomiting and diarrhoea (Codd, 2000; Dow and Swoboda, 2000) with necrosis of the liver that may lead to death by hemorrhagic shock or liver failure after some hours or days, depending on the species (Gorham and Carmichael, 1988). Animals, birds and fish can also be poisoned by high levels of toxin-producing cyanobacteria (De Figueiredo *et al.*, 2004).

Due to the specific binding of the organic anion transport system in hepatocyst cell membranes, these toxins target the liver (Dittmann and Börner, 2005). In the hepatocysts, they form adducts with Protein phosphatase-1 and Protein phosphatase-2A (PP1 and PP2A) from cytoplasm and nuclei, inhibiting them and leading to disruption of liver cell structures, intrahepatic haemorrhage and death if a high dose is administered (Fitzgerald, 2001). Microcystins seem not to be hydrolysed by stomach peptidases and microcystin-LR appears to be absorbed by the intestine (Dow and Swoboda, 2000).

2.6.1 Effect of toxins on humans

Children, old people and hepatitis-ß patients are groups that are more sensitive to microcystin poisoning and require special attention (Fitzgerald, 2001). Epidemiological studies have already related the presence of microcystins in drinking water to an increase in the incidence of colorectal cancer (Ueno *et al.,* 1996). Microcystins have been shown to be tumor promoters (Nishiwaki-Matsushima *et al.,* 1992; Falconer and Humpage, 1996) and pose a serious risk to populations exposed to chronic low-level doses (Baker *et al.,* 2002).

Due to the rapid, irreversible and severe damage that microcystins cause in the liver, therapy is difficult and prophylaxis is complicated (De Figueiredo *et al.,* 2004). A study by Gehringer *et al.* (2003) showed that the membrane-active antioxidant vitamin E, taken as a dietary supplement, may protect against toxicity of exposure (De Figueiredo *et al.,* 2004).

2.6.2 Effects of toxins on other organisms

Microcystins are known to affect many other organisms, from microalgae to mammals (De Figueiredo *et al.,* 2004).

Microcystins are poisonous to all *Daphnia* clones and the toxic effect could be directly correlated to the ingestion rate of *Microcystis* (De Figueiredo *et al.,* 2004). Mussels, crayfish and fish used for human consumption may also accumulate microcystins and cause health hazards to human consumers in such a way that microcystins should always be monitored during and after the occurrence of estuarine cyanobacterial blooms (De Figueiredo *et al.,* 2004). Some fish experience reproductive effects with substantial ecological consequences such as reducing population growth and changing species composition of the water body (De Figueiredo *et al.,* 2004 and references therein).

Crop plants for human consumption that are irrigated with microcystin-contaminated water may suffer growth and development effects – in addition to accumulating the toxins and therefore posing the potential risk of toxin transference to humans through the food chain (De Figueiredo *et al.,* 2004).

In order to prevent harmful cyanobacterial blooms and the concurrent toxic effects it can have on humans and the environment, knowledge of the structure and synthesis of microcystins are crucial.

2.6.3 Action

Microcystins and nodularins are potent inhibitors of eukaryotic protein phosphatases type 1 and 2A (MacKintosh *et al.,* 1990), with inhibition being dependent on particular structural variations (An and Carmichael, 1994) including the substitution of two variable L-amino acids and the methylation of aspartate (ß-iso-Asp) and dehydroalanine (Neilan *et al.,* 1999). The modified ß-amino acid, which is also found in the hepatotoxic pentapeptide nodularin, is conserved in all known toxic microcystins (Neilan *et al.,* 1999). Microcystins and related cyclic peptides are carried into hepatocytes via the bile acid transport system, where hyperphosphorylation of microfilaments, including cytokeratins, is the primary toxic effect (Neilan *et al.,* 1999 and references therein). Microcystins may also activate phospholipase A_2 and cyclo oxygenase in hepatocytes, while in macrophages they induce tumor necrosis factor alpha and interleukin 1 (Neilan *et al.,* 1999). Together with hyperphosphorylation of DNA, these functions have implicated microcystins as agents promoting hepatocellular carcinoma and tumor liver growth (Fujiki, 1992).

2.6.4 Microcystin structure and synthesis

Microcystins are synthesised nonribosomally through a mixed polyketide synthase/nonribosomal peptide synthetase system called microcystin synthetase (Nishizawa *et al.,* 1999; Kaebernick and Neilan, 2001; Dittmann and Börner, 2005) and their synthesis is an energy (ATP) dependent process (Bickel and Lyck, 2001). One strain may produce different microcystins and also other peptides simultaneously (Fastner *et al.,* 2001).

Gene clusters for microcystin biosynthesis have been identified and sequenced in the distantly related cyanobacterial genera, the unicellular *Microcystis aeruginosa* (Nishizawa *et al.,* 2000; Tillett *et al.,* 2000), the filamentous *Planktothrix agardhii* (Christiansen *et al.*, 2003) and filamentous *Anabaena* strain 90 (Rouhiainen *et al.*, 2004). Homologous genes have been detected in a nodularin producing *Nodularia* strain (in this case designated *nda* genes, M.C. Moffitt and B.A. Neilan, accession number AY210783).

The insertional inactivation of a peptide synthetase gene from the hepatotoxic strain *M. aeruginosa* PCC7806 resulted in transformation to the nontoxic state and a loss of microcystins, demonstrating that the gene, called *mcyB*, encodes a microcystin synthethase (Dittmann *et al.,* 1997). Christiansen *et al.* (2003) and references therein also demonstrated that mutations in *mcyA*, *-B, -D, -E* and *–F* of *Microcystis sp.* resulted in a complete loss of microcystin

biosynthesis by the cells and deletion of two key components (*mcyA* and *–B* of the *mcy* complex) but not of the tailoring enzyme (*mcyJ)*, which leads to the absence of the complete enzyme complex.

In *Microcystis*, *Anabaena* and *Nodularia* genera these genes are transcribed from a central bidirectional promoter region, whereas in *Planktothrix* all *mcy* genes except *mcyT* seem to be transcribed unidirectionally from a promoter located upstream of gene *mcyD* (Dittmann and Börner, 2005). Although the multi-enzyme components encoded by the different genera are highly similar, the arrangement of their genes clearly differs between *Anabaena* and *Nodularia* on one side and *Microcystis* and *Planktothrix* on the other side (Dittmann and Börner, 2005). The biosynthesis of microcystin has been elucidated for two strains of *M. aeruginosa* (Nishizawa *et al.,* 1999; Nishizawa *et al.,* 2000; Tillet *et al.,* 2000), but no definitive mechanism for the regulation of microcystin production by *M. aeruginosa* is known.

Transcriptional analysis of the *mcy* gene cluster not only should increase our understanding of microcystin synthetase regulation and toxin biosynthesis but may also provide useful insights into other nonribosomal systems, some of which are involved in antibiotic production (Kaebernick *et al.,* 2002).

2.6.5 Microcystin gene cluster of M. aeruginosa PCC 7806

The microcystin biosynthetic gene cluster from *M. aeruginosa* PCC 7806 is a cluster spanning 55 kb, consisting of six open reading frames (ORFs) with a mixed nonribosomal peptide synthetase/polyketide synthase nature (*mcyA* to *mcyE* and *mcyG*) and four smaller ORFs with putative precursor and tailoring functions (*mcyF* and *mcyH* to *mcyJ*) (figure 2.2) (Kaebernick *et al.,* 2002) and has been correlated with microcystin formation by gene disruption and mutant analysis (Tillet *et al.,* 2000; Dittmann *et al.,* 2001).

Genes encoding peptide synthetases are clustered in large operons with repetitive domains in which highly conserved core sequences have been identified (Dittmann *et al.,* 1996; Nishizawa *et al.,* 1999, 2000; Tillet *et al.,* 2000). Of the 48 sequential reactions involved in microcystin biosynthesis, 45 can be assigned to catalytic domains within six large multi-enzyme synthases/synthethases (*mcyA-E, G*). Nishizawa *et al.* (1999) identified the genes responsible for incorporation and activation of the five amino acid constituents of microcystin (*mcyABD, mcyD-G*).

The 10 ORFs are bidirectionally transcribed from a central 737 bp locus between *mcyA* and *mcyD* and are arranged in, what until now has been classified as two putative operons, *mcyABC* and *mcyDEFGHIJ* (Kaebernick *et al.,* 2002) (figure 2.2). Both polycistronic transcripts have alternate transcription start sites which appear to be light dependent (Kaebernick *et al.,* 2002). Catalytic domains in *mcyA* to *mcyE* and *mcyG* are responsible for incorporation of the precursors phenylacetate, malonyl coenzyme A, S-adenosyl-L-methionine, glutamate, serine, alanine, leucine, D-methyl-isoaspartate and arginine (Tillet *et al.,* 2000; Kaebernick *et al.,* 2002). The *mcyE* and *G* genes are hybrid enzymes, both including peptide synthetase and polyketide synthase modules (Dittmann and Börner, 2005). Polyketide synthases assemble acetate or propionate units into polyketide structures (Dittmann and Börner, 2005). The polyketide synthesis domains in *mcyD*, *mcyE* and *mcyG* gene products are responsible for the fatty acid side chain of Adda (Kaebernick and Neilan, 2001; Dittmann and Börner, 2005). The smaller ORF's encode monofunctional proteins which are putatively involved in O-methylation (*mcyJ*), epimerzation (*mcyF*), dehydration (*mcyI*) and cellular localisation (*mcyH*) (Oh *et al.,* 2000; Tillet *et al.,* 2000; Kaebernick *et al.,* 2002). Putative tailoring functions have been assigned to *mcyE* and *mcyJ* (Kaebernick and Neilan, 2001). The unusual polyketide amino acid Adda is formed by transamination of a polyketide precursor as enzyme-bound intermediate and not released during the process (Tillet *et al.,* 2000). According to Bertasi *et al.* (2004) the *mcyD* region of the microcystin cluster is responsible for the toxic region transcription. *mcyH* shows high identity to ABC transporter genes (Pearson *et al.,* 2004) but no functional activity has been described. The peptide synthetase employs the thio-template mechanism whereby individual sites of the multi-enzyme catalyse amino/hydroxyl acid activation and thioester formation in the order in which residues are added to the peptide chain; chain elongation is catalysed by the enzyme bound cofactor 4'-phosphopentetheine (Kaebernick and Neilan, 2001).

The ATG start codon and putative ribosome binding sites (RBS) of the second open reading frame, *mcyE*, is located 167 bp downstream of the TAA stop codon of *mcyD*. This large open reading frame encodes a 392703 Da polypeptide product of mixed polyketide synthetases (PKS) and nonribosomal peptide synthetase (NRPS) function. The amino-terminal region of *mcyE* contains a polyketide synthetase module and a putative aminotransferase (AMT) domain. This latter domain of about 430 amino acids, shows approximately 30% identity to a large group on nonintegrated amino transferases acting on glutamate semi-aldehyde or N-acetyl-ornithine. The most logical role of this module in microcystin biosynthesis would be to supply the amino group to Adda (Tillet *et al.,* 2000).

The ATG start codon and putative ribosome binding sites of the second ORF, *mcyB*, is located 15 base pairs (bp) downstream of the TAA stop codon of *mcyA* (Tillet *et al.*, 2000). This 6318 bp open reading frame encodes a peptide synthetase of 242334 Da containing two modules, each possessing adenylation, thiolation and condensation domains (Tillet *et al.*, 2000). The amino-terminal domain has been functionally identified by sequence alignment with known condensation domains as catalysing peptide bond formation between L-and D-aminoacyl residues (Tillet *et al.*, 2000).

More than 70 structural isoforms of microcystins with various toxicities have been identified having the common structure cyclo (Adda-D-Glu-Mdha-D-Ala-L-X-D-MeAsp-L-Z), where X and Z are variable L amino acids, Adda is 3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyl-4,6-decadienoic acid, D-MeAsp is 3-methylaspartic acid and Mdha is N-methyldehydroalanine (Sivonen and Jones, 1999; Christiansen *et al.*, 2003). Their acute toxicity (given intraperitoneally to mice) varies between 50 and 800 μg/kg of body weight (Rapala *et al.*, 1997 and references therein).

With the use of amino acid single letter code classification, each microcystin is designated a name based on the variable amino acids which complete their structure. For instance, the most frequent and studied variant is microcystin-LR with the variable amino acids leucine (L) and arginine (R) in these variable positions and is known to be produced by species belonging to the genera *Anabaena, Microcystis, Nostoc and Anabaenopsis* (Dow and Swoboda, 2000; WHO, 2003) and MC-YR is produced by *M. aeruginosa*, *M. viridis* and *Hapalosiphon* spp. (Dow and Swoboda, 2000; WHO, 2003). MC-RR has been isolated from *Oscillatoria agardhii, M. aeruginosa* and *M. viridis* and MC-LA from *M. aeruginosa* (Dow and Swoboda, 2000).

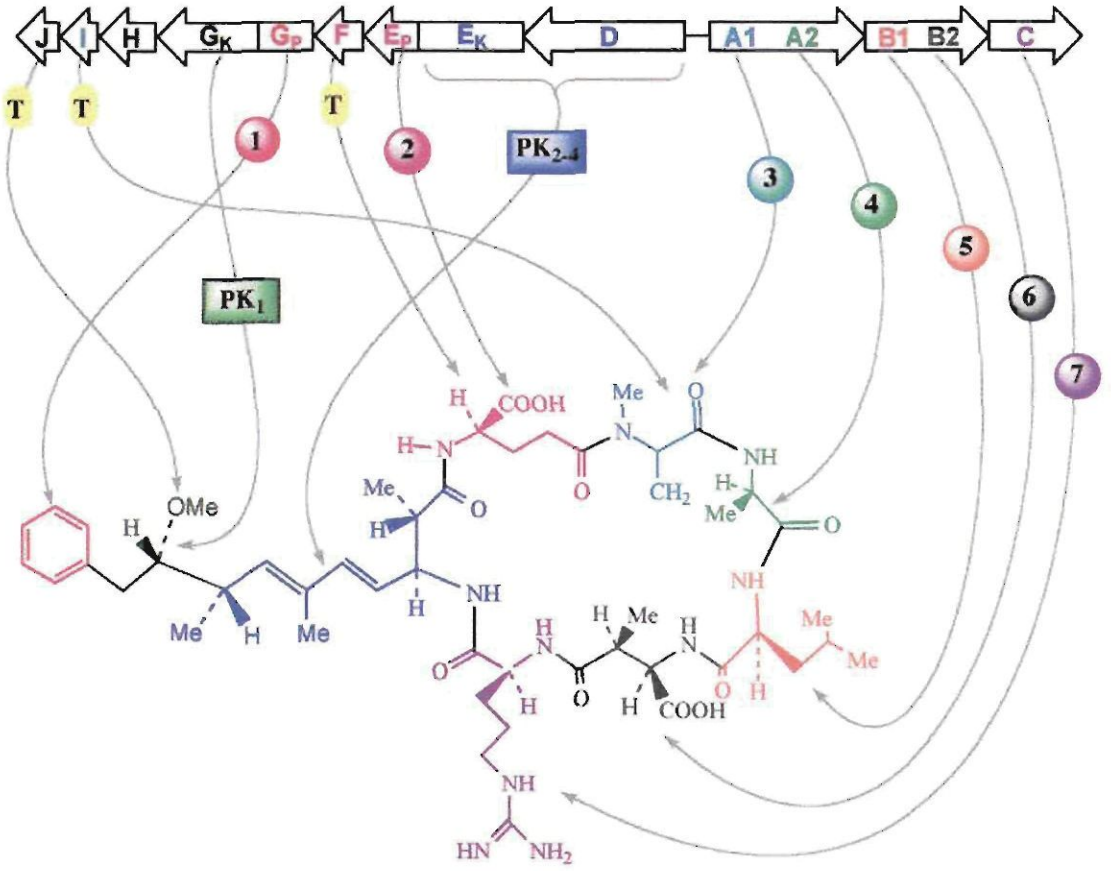


Figure 2.2: Proposed biosynthetic model for microcystin-LR, showing the organisation of the gene cluster *mcyA-J* and the toxin, microcystin. Biosynthesis is via a multienzyme complex consisting of both peptide synthetase and polyketide synthase modules (Tillet *et al.*, 2000). Numbered circles indicate the order of amino acids incorporated into the growing peptide chain by NRP genes (mcyA,B,C, Ep, Gp). mcyA and mcyB contain two modules, A1/A2 and B1/B2, respectively. A1 also encodes an N-methyltransferase domain. Numbered rectangles show the order of polyketide synthesis in the formation of Adda (mcyGK,EK,D). Additional open reading frames of putative microcystin tailoring function are indicated by 'T". mcyH shows high identity to ABC transporter genes (Tillet *et al.*, 2000). The relative sizes of the *mcy* ORFs have been approximated, with the entire gene cluster comprising some 55 kb. Taken from Kaebernick and Neilan (2001).

It is now thought that the difference between microcystin-producing (toxic) and non-producing (nontoxic) strains of cyanobacteria lies primarily in the presence or absence of the microcystin synthetase gene cluster (Neilan *et al.*, 1999; Nishizawa *et al.*, 2000; Tillet *et al.*, 2001; Kurmayer *et al.*, 2002).

In addition to these peptide toxins, cyanobacteria have been found to produce a wide variety of linear and cyclic peptides, which may not be acutely toxic but have other bioactivities such as serine protease inhibition (Golakoti *et al.*, 2000).

It is not clear how distantly related genera of cyanobacteria gained the ability to produce microcystins (Rantala *et al.,* 2004). Recently lateral gene transfer or a series of gene losses have both been proposed to explain the sporadic distribution of toxic strains of *Microcystis* (Neilan *et al.,* 1999; Tillet *et al.,* 2001). Results generated by Rantala *et al.* (2004) do not corroborate this horizontal transfer of genes for microcystin biosynthesis between the genera, as phylogenetic analyses by them indicate a coevolution of housekeeping genes and microcystin synthetase genes for the entire evolutionary history of the toxin (Rantala *et al.,* 2004). According to Christiansen *et al.* (2003), their data also do not support this idea of horizontal gene transfer of complete *mcy* gene clusters between the genera. Rantala *et al.* (2004) suggests that the sporadic distribution of microcystin synthetase genes in modern cyanobacteria suggests that the ability to produce the toxin has been lost repeatedly.

However, the data strongly suggest that the genes encoding nodularin synthetase are recently derived from those encoding microcystin synthetase (Rantala *et al.,* 2004). Similarities in the chemical structures and biological action of microcystins and nodularin indicate that these compounds are closely related (Sivonen and Jones, 1999).

Despite this evidence, they do not rule out the possibility that parts of the microcystin synthetase gene cluster are of more recent origin and might be laterally transferred between strains within a genus (Rantala *et al.,* 2004). In contrast, Christiansen *et al.* (2003) suggested that microcystin synthetase genes are derived from nodularin synthetase genes.

2.6.6 Microcystin synthetase gene cluster in Planktothrix

There are several interesting differences between the content of the *mcy* gene clusters of *Microcystis* and *Planktothrix*, including the general arrangement and transcriptional orientation of the *mcy* genes (Christiansen *et al.,* 2003) (figure 2.3).

Microcystin production in *Planktothrix* differs from that in *Microcystis* in the assortment of microcystin isoforms produced and in the cellular production rates of microcystin, which have been found to be higher in the filamentous strains in field studies (Christiansen *et al.,* 2003). At the 5'- end of the gene cluster and transcribed in the opposite direction, an additional open reading frame (*mcyT*), absent from the *mcy* gene cluster of *Microcystis*, was found showing homology to genes and gene domains, respectively, encoding thioesterases (Christiansen *et al.,* 2003). Two ORFs present in *Microcystis* are missing: the racemase gene *mcyF* and *mcyI*, an

ORF whose predicted product is similar to D-3-phosphoglycerate dehydrogenases genes (Christiansen *et al.*, 2003).

The arrangement of the *mcy* genes differs from that in *Microcystis* because the *mcy* genes of *Planktothrix* (except *mcyT*) are all on the same strand and may be transcribed as a single operon (Christiansen *et al.*, 2003).

2.6.7 Microcystin sythetase gene cluster in Anabaena

Rouhiainen *et al.* (2004) sequenced and characterised the cluster of microcystin synthetase genes from *Anabaena* strain 90 and propose a model for microcystin biosynthesis in *Anabaena* strain 90. According to Rouhiainen *et al.* (2004), the order of the microcystin synthetase genes in *Anabaena* strain 90 differs from the arrangement found in two other cyanobacterial species, *M. aeruginosa* and *Planktothrix agardhii* (figure 2.3).

In *Anabaena* strain 90, *M. aeruginosa* and *P. agardhii* the NRPS genes *mcyA*, *mcyB* and *mcyC* have the same order, but the organization of the other genes is different (Rouhiainen *et al.*, 2004). The *mcy* genes in *Anabaena* strain 90 and *M. aeruginosa* are in two clusters, which are transcribed in opposite directions (Rouhiainen *et al.*, 2004). In *Anabaena* strain 90 the arrangement of the genes from *mcyG* to *mcyI* and then from *mcyA* to *mcyC* is colinear with the structure of microcystin and Rouhiainen *et al.* (2004) speculated that the order of the microcystin synthetase genes in *Anabaena* is more original than the one in *Microcystis* and *Planktothrix*, in which reorganizations of these genes have taken place.

The average sequence match between the microcystin synthetase genes of *Anabaena* strain 90 and the corresponding genes of the other species is 74%.

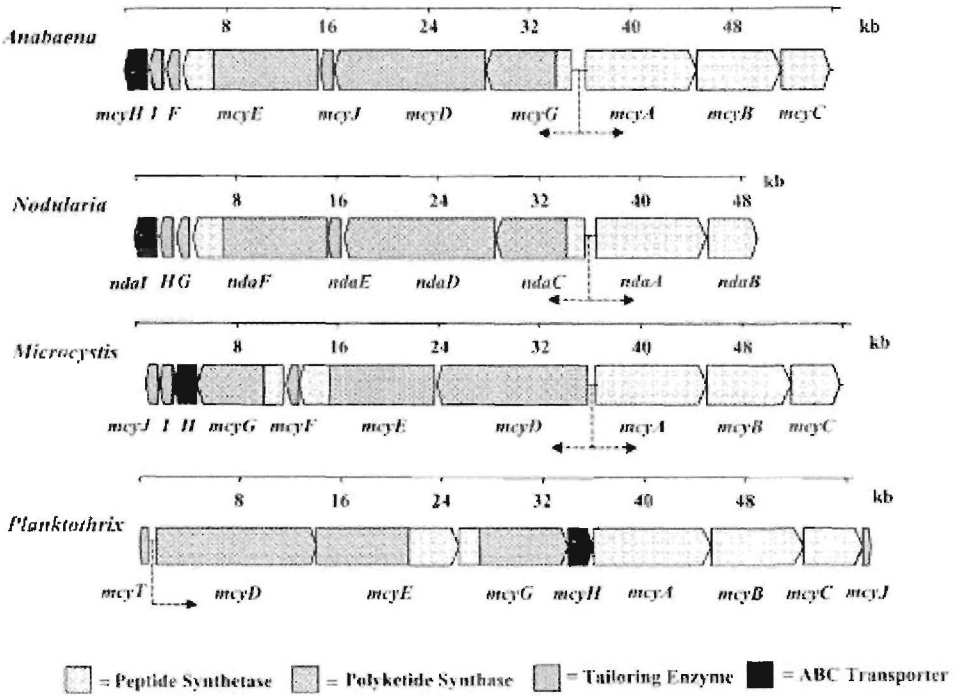


Figure 2.3: Gene clusters coding for the biosynthesis of microcystin in *Anabaena* (Rouhiainen *et al.*, 2004), *Microcystis* (Tillett *et al.*, 2000), *Planktothrix* (Christiansen *et al.*, 2003) and of nodularin in *Nodularia* (M.C. Moffitt and B.A. Neilan; accession no. AY210783). Arrows indicate the transcriptional start sites from the putative promoter regions. Taken from Dittmann and Börner (2005).

Because microcystins are regulated very similarly in different species, microcystins can be detected and compared in water samples by various methods but it is difficult to determine the strain causing the specific type and amount of toxin, especially in natural samples where different toxin producing species occur.

2.7 Detection of microcystins

Due to the results of toxicological studies, which have shown the adverse effects of microcystins to some mammals, many countries have started to monitor cyanobacterial cell densities and microcystin concentrations in raw water sources and recreational waters (Vaitomaa *et al.*, 2003).

The morphological discrimination between toxic and nontoxic cyanobacteria can be difficult, as some genera contain both toxic and nontoxic members (Neilan *et al.*, 1997; Tillet *et al.*, 2001; Baker *et al.*, 2002; Gugger *et al.*, 2002; Ouellette and Wilhelm, 2003; Vaitomaa *et al.*, 2003; Mbedi *et al.*, 2005). Some cyanobacteria are known to be toxic, some may be genetically capable of producing toxins (toxigenic) but do not under all conditions and some do not produce

toxins at all (Ouellette and Wilhelm, 2003). For example, *M. aeruginosa* has both toxic and non-toxic strains (Meissner *et al.,* 1996).

Several detection methods for toxins are currently in use: these methods include high performance liquid chromatography (HPLC) (Lawton *et al.,* 1994), small-animal bioassays (Campbell *et al.,* 1994) and enzyme inhibition assays (An and Carmichael, 1994; Ward *et al.,* 1997). However, such analysis does not indicate which cyanobacteria produce the toxins, since several genera of cyanobacteria may produce similar microcystin variants (Sivonen and Jones, 1999). There are also many plants that have been shown to be sensitive to microcystins and that may be used to assess toxicity of these toxins (De Figueiredo *et al.,* 2004).

Methods based on polymerase chain reaction are recent approaches to the detection of pathogenic micro-organisms in natural environments and they are being proposed as means both to rapidly determine whether a cyanobacterial bloom or a determined species is potentially toxic and to quantify toxic cyanobacteria by designing primers based on *mcy* genes (Rudi *et al.,* 1998b; Tillet *et al.,* 2001).

2.7.1 Molecular detection of toxic cyanobacteria

Now that the biosynthetic pathway for microcystin production has been elucidated (Tillet *et al.,* 2000) it has enabled the development of specific oligonucleotide primers for genes common to production of all microcystins (Tillet *et al.,* 2001; Ouellette and Wilhelm, 2003). While some species are not known to produce toxins, all strains containing the microcystin genes should be viewed as potential microcystin producers (i.e. toxigenic) (Ouellette and Wilhelm, 2003). The relatively quick PCR approach provides the required resolving power by targeting the toxin genes directly (Ouellette and Wilhelm, 2003). This approach is appealing as an early warning diagnostic and is very sensitive because of the amplification achieved by PCR (Ouellette and Wilhelm, 2003).

DNA based detection methods have become popular because of their potential specificity targeting genes involved in toxin biosynthesis, sensitivity and speed (Ouellette and Wilhelm, 2003). However, due to large variations in the sources and quality of environmental samples, problems with the preparation of cells and nucleic acids can be encountered (Steffan and Atlas, 1991). For these reasons, complete and reproducible assays – from water sampling to

quantification of target organisms - are required for routine environmental monitoring (Rudi *et al.,* 1998b).

PCR based methods are promising tools to obtain further insight into the distribution of microcystin-producers in cyanobacteria populations, but it could still have false positive and false negative detection (Mbedi *et al.,* 2005). This false detection can be because of DNA polymorphism in the *mcy* genes or the presence of *mcy* genes in non-microcystin producers (Mbedi *et al.,* 2005).

2.8 What causes microcystin synthesis?

No conclusive results regarding factors which influence toxin production, have been gained from any studies (Kaebernick and Neilan, 2001) and the stimuli for toxin production in species is still unknown (Dittmann and Börner, 2005). It is poorly understood what causes a water bloom to be toxic, or how the toxin content of a single strain varies (Rapala *et al.,* 1997). Surveys of water blooms have shown that 25 to 95% of them are toxic (Sivonen *et al.,* 1990). A large obstacle in understanding the regulation of toxicity in species has been that the toxicity varies substantially both in nature and in culture and seems to be expressed only under certain environmental conditions (Johansson and Granéli, 1999; Vaitomaa *et al.,* 2003).

Despite many contradictory studies, there are some factors that have been shown to influence microcystin synthesis (Ouellette and Wilhelm, 2003). These factors that have been investigated include varying nutrients (eg. phosphorus, nitrogen and iron), light and temperature (Ouellette and Wilhelm, 2003). The literature contains an abundance of such studies, which at times conflict (Ouellette and Wilhelm, 2003 and references therein).

Toxin data have been correlated to a variety of parameters, including dry weight, total protein and cell numbers, which led to widely varied results (Kaebernick and Neilan, 2001). Individual studies are not readily comparable due to the different bacterial strains, culturing methods and toxin analyses employed by the various laboratories (Kaebernick and Neilan, 2001).

Therefore, cellular responses unrelated to toxin production need to be considered when comparing results between studies (Kaebernick and Neilan, 2001). Molecular methods for studying microbial diversity and activity have gained widespread popularity and have revolutionized microbiology, in a large part because they can produce data without the need to

cultivate the organisms in question (Ouellette and Wilhelm, 2003). Many of the methods involve the amplification of genes, using the polymerase chain reaction technique (Saiki *et al.,* 1988).

2.8.1 Growth rate

According to Orr and Jones (1998) as well as Long *et al.* (2001), microcystin behaves as a primary biochemical compound and thus parallels cellular growth. This is also supported by other authors (Sivonen and Jones, 1999; Oh *et al.,* 2001), confirming that microcystin concentration in a body of water seems to be mostly dependent on the density of the hepatotoxic cells. Some studies (Hesse and Kohl, 2001) concluded that the differences found between toxicity of blooms of one species are mainly due to the growth rates and toxic characteristics of different strains (De Figueiredo *et al.,* 2004). Downing (2007) concluded that microcystin production rate is directly proportional to cell division rate where cell division rate is a function of nitrogen uptake. Aguiar and Azevedo (1998) reported that an increase in nutrient concentrations in aquatic environments would not be directly related to hepatotoxin production by *M. aeruginosa*, but the number of toxic cells can be increased under eutrophic conditions by higher cellular growth.

Gobler *et al.* (2007) suggest that the dominance of *Microcystis sp.* blooms during the summer is linked to nutrient loading, which can stimulate growth and cellular toxin synthesis and in turn can suppress grazing by mesozooplankton, allowing for the further accumulation of cells.

On the other hand, several earlier studies (Van der Westhuizen and Eloff, 1985; Johansson and Granéli, 1999) have demonstrated that toxin production in cyanobacteria is not a fixed component of growth. Instead, both the extent to which algae accumulate toxins and the number and quantity of individual toxins of algae are strongly influenced by environmental growth conditions (Plumley, 1997). Other studies (Kurmayer *et al.,* 2002; Ross *et al.,* 2006) also found that microcystin content cannot be correlated to the biomass of the toxin-producing cyanobacterial genera.

Dittmann *et al.* (2001) conclude that with the inability to compare results from various studies due to different culturing and analysis methods used, no conclusions can be made about the effect of growth rate or environmental factors regulating toxin production.

Nutrients may be one of the biggest contributing factors to the production of harmful cyanobacterial blooms (see section 5.2.1.1.1). Especially the nitrogen in the water has been shown to influence microcystin production. To gain insight into the assimilation of these nutrients, it is desirable to study the regulation of the genes that are linked to the assimilation of these nutrients.

2.8.2 Energy

Bickel and Lyck (2001) suggested that if microcystin synthesis requires energy (as ATP), the variation of toxin production should be mostly explained by the energetic state of the cyanobacterial cells, with nutrient limitation (P, N and Fe) and light variation having only an indirect influence, since cell energetic state changes under stress conditions. Under circumstances of low levels of energetic charge, viable energy in cells is primarily applied in essential protein synthesis and not in microcystin (secondary metabolite) synthesis (Bickel and Lyck, 2001).

2.8.3 Stress

A species' ability to dominate in a specific natural environment depends on its ability to compete successfully for the growth-limiting resource (Johansson and Granéli, 1999). Thus, from an ecological point of view, an increased toxin production when nutrients are limited would be a great advantage, giving the algae an opportunity to proliferate where they would otherwise be incapable of competing with competitively superior species of algae (Johansson and Granéli, 1999).

Johansson and Granéli (1999) suggest that the toxicity of *Prymnesium parvum* (harmful golden algae) is related to cellular physiological stress, due to nutrient limitation rather than to the direct involvement of either N or P in toxin synthesis. Ross *et al.* (2006) indicated that physical stressors induce oxidative stress in *M. aeruginosa*, which results in programmed cell death and a concomitant release of toxin into the surrounding media. They found that upon exposure to selected stressors, cell bound toxins were capable of being released into the immediate vicinity at levels over 90% above what was normally secreted by dense assemblages of *M. aeruginosa*.

Kaebernick *et al.* (2000) reported that transcript levels of *mcyB* and *mcyD* were reduced under certain artificial stress conditions. However, Sivonen (1990) found that toxin production by *Oscillatoria agardhii* strains is probably not a response to environmental stress conditions.

Sivonen (1990) found that hepatotoxins are largely kept within the cells during growth, although leakage of the toxins increased towards the end of the 3 to 4 week growth period. Active release of microcystins from the cell has not been shown (Kaebernick *et al.*, 2000). However, the recent identification of a putative ABC transporter gene (*mcyH*) located upstream of *mcyE* (Tillet *et al.*, 2000) may suggest the existence of a cytoplasmic or transmembrane microcystin transport system. This is further supported by Dittmann and Börner (2005) that suggested that this extracellular microcystin, though never exceeding 10% of the total microcystin content, is most likely the result of an active transport of microcystin rather than passive release due to cell lysis.

Dittmann *et al.* (1997) imply that microcystin plays some role in regulating its own biosynthesis and, taken with the putative effects of cell density, may also have a more general role as a signalling or quorum-sensing molecule. Sivonen (1990) found that co-existing bacteria do not seem to induce hepatotoxin production since with two or three strains the toxin production was higher in axenic cultures.

2.8.4 Environmental factors

Sivonen (1990) found that changes in environmental parameters did not induce toxin formation in the nontoxic strain of *O. agardhii*, nor did the toxin-producing strain lose their ability to produce toxin under different environmental conditions. Several environmental factors have, however, been described to influence the biosynthesis of cyanotoxins for several isolates (Kaebernick *et al.*, 2000). According to Dittmann and Börner (2005) the variations of the microcystin concentrations detected in the lakes might also be a result of population dynamics altering the proportion of toxic genotypes within the population of cyanobacteria which is important to keep in mind as is observed in this study.

Several authors found that conflicting results may be due to different behaviour of strains and species (Sivonen, 1990; Kaebernick *et al.*, 2000; Kurmayer *et al.*, 2002).

In the literature, ecological factors that have been investigated include varying nutrients (e.g. phosphorus, nitrogen and iron), light and temperature (Ouellette and Wilhelm, 2003).

2.8.5 Nutrients

As both the availability and composition of nutrients have a significant impact on the algal community structure and biomass and also on phytoplankton biochemistry, changes in nutrient

supply may ultimately affect the production of toxins (Johansson and Granéli, 1999). In view of this, Johansson and Granéli (1999) suggested a possible connection between nutrient limitation and high toxicity. According to Oh *et al.* (2001), microcystin production by cyanobacteria results from cyanobacterial blooms caused by an abundance of nutrients and favourable conditions for cyanobacterial growth.

There are many contradicting results concerning the effects of nitrogen and phosphorus concentrations on microcystin content of cyanobacteria but microcystin production in *Microcystis* strains seems to be influenced by a variation in nitrogen and phosphorus concentrations with different responses depending on the considered strain (De Figueiredo *et al.*, 2004 and references therein).

2.8.6 Phosphorus (P)

Rapala *et al.* (1997) suggested that microcystins in different cyanobacterial genera respond in a similar way to the extracellular phosphorus concentration: not only cyanobacterial growth but also the amount of intracellular hepatotoxins increase with phosphorus.

Because many cyanobacteria have the ability to fix nitrogen, it is assumed that P-loading promotes toxic cyanobacterial blooms (Paerl, 1988) and can often become a limiting nutrient controlling cyanobacterial growth (Watkinson *et al.*, 2005). Studies indicated that the growth of *Microcystis* spp. is enhanced by increasing phosphorus concentrations (Utkilen and Gjølme, 1995; Rapala and Sivonen, 1998; Kotak *et al.*, 2000; Oh *et al.*, 2000). Growth-limiting phosphorus concentrations have also been shown to decrease the content of microcystins in filamentous non-nitrogen-fixing *Oscillatoria agardhii* strains (Fitzgerald, 1969; Sivonen, 1990) as well as in the non-nitrogen fixing *M. aeruginosa* as measured by mouse bioassay (Watanabe and Oishi, 1985). This was confirmed by a positive correlation between total phosphorus with microcystin-LR in *M. aeruginosa* (Jacoby *et al.*, 2000; Kotak *et al.*, 2000). However, Codd and Poon (1988) reported that the removal of phosphorus from the growth medium did not influence the toxicity of unicellular non-nitrogen fixing *M. aeruginosa* as measured by mouse bioassay and Oh *et al.* (2000) documented higher values of microcystin content in *M. aeruginosa* under more P-limited conditions.

According to Downing (2007), cellular phosphorus plays an important role in photosynthetic carbon fixation, which in turn affects cellular C:N ratios and nitrogen assimilation.

Thus it would seem that higher phosphorus levels can yield higher, lower or unchanged toxin levels in cyanobacteria (Sivonen, 1990; Oh *et al.,* 2000). Thus, the various types of plankton from each local environment must be considered individually and no total environmental predictions made unless all situations have been evaluated (Fitzgerald, 1969).

2.8.7 Nitrogen/Nitrates/nitrites

Downing (2007) hypothesizes that cellular nitrogen status may be the main determinant of microcystin production state. Downing (2007) found that batch culture under identical conditions yielded increased microcystin when nitrogen uptake rate was relatively higher than growth rate, confirming the importance of nitrogen uptake in the modulation of microcystin content or a specific growth rate.

Graham *et al.* (2004) detected a non-traditional linear model between microcystin concentrations and nitrogen concentrations. According to these authors, maximal microcystin values (>2000 ng/L) occurred between 1500 and 4000 µg/L total nitrogen, but above 8000 µg/L nitrogen, the microcystin values were <150 ng/L.

Gobler *et al.* (2007) found that nitrogen loading is important in the occurrence of toxic cyanobacteria blooms in non-nitrogen fixing cyanobacteria such as *Microcystis*. Many laboratory and field studies indicate that increasing nitrogen loads will increase the growth and toxicity of non-nitrogen fixing cyanobacteria such as *Microcystis sp.* and *Oscillatoria agardhii* (Watanabe and Oishi, 1985; Codd and Poon, 1988; Sivonen, 1990; Lahti *et al.,* 1997) but may also be inversely correlated with nitrate concentrations (Kotak *et al.,* 1995). Codd and Poon (1988) found 10 times less toxin than in reference cells when they omitted the nitrogen source for *M. aeruginosa.* Downing (2007) showed that microcystin content was reduced under nitrogen limitation and increased under carbon or light limitation.

One should however keep in mind when comparing results of various authors how the concentration of microcystins is measured and expressed. This follows after studies reported that fast cell growth of *M. aeruginosa* under nitrogen-limited conditions is associated with smaller cells and consequently by higher intracellular microcystin production (Long *et al.,* 2001).

2.8.8 N:P ratio

The issue of N:P ratio as a controlling factor in cyanobacterial development only comes into play when one or both elements are present in concentrations that are limiting for further algal development (Harding and Paxton, 2001).

Investigation of initial medium N:P ratios and growth stage in batch culture by Lee *et al.* (2000) showed that N:P ratios of 16:1 and 50:1 yield maximum microcystin. They also found that more than 4.46 μmol phosphorus and 32.5 μmol nitrogen are required for the maximum growth of *M. aeruginosa*. It would seem that *M. aeruginosa* has an N and P uptake at a ratio of 11:21 plus a high variation in the cellular P content relative to the P concentration in the medium. Lee *et al.* (2000) found that the N content of *M. aeruginosa* is highly correlated with the total N content and in contrast, the microcystin content of *M. aeruginosa* which was slightly negatively correlated with the P content under N fixed conditions.

In the non-nitrogen fixing cyanobacterium, *M. aeruginosa*, microcystin content increases at higher N:P ratios (Utkilen and Gjølme, 1995; Lee *et al.*, 2000). Gobler *et al.* (2007) found a significant interaction between N and P and the nitrogen with the phosphorus treatment, yielded the highest growth rates and microcystin levels, but they also found that during autumn (October) nutrients did not significantly alter growth rates of any cyanobacterial population or microcystin levels. There was a clear relationship between the total P and total N content of the cells (Lee *et al.*, 2000) and it would seem that the cellular N and cellular P content are directly affected by their concentrations in the medium which then gradually decrease due to cell growth (Gobler *et al*,. 2007).

2.8.9 Micronutrients

Certain metal ions such as Zn^{2+} and Fe^{2+} significantly influence toxin yield (Oberholster *et al.*, 2004). All cyanobacteria require Fe^{2+} for important physiological functions such as photosynthesis, nitrogen assimilation, respiration and chlorophyll synthesis (Boyer *et al.*, 1987). Micronutrients, such as iron (Paerl *et al.*, 1994) and molybdenum (Paerl and Bowles, 1987), can also be potential limiting factors for N_2 fixation and consequently, cyanobacterial growth (Watkinson *et al.*, 2005). Iron has been identified as an important limiting nutrient in cyanobacterial productivity (Paerl *et al.*, 1994). Results by Watkinson *et al.* (2005) suggest that blooms of *Lyngbya majuscula*, may have been stimulated by bio-available iron, perhaps complexed by dissolved organic carbon and may have been sustained by additional inputs of

nutrients (N and P) and iron through sediment influx. Cyanobacterial toxins have been suggested to act like metal-complexing siderophores (Lehtimäki *et al.,* 1997 and references therein).

Toxin production of *M. aeruginosa* has been shown to be influenced by iron and the toxin producing strain of *M. aeruginosa* had a more efficient iron uptake system than the strain that did not produce toxin (Utkilen and Gjølme, 1992). Lukac and Aegerter (1993) found that low iron (Fe) concentrations decreased growth but increased toxin synthesis. Utkilen and Gjølme (1995) obtained contradictory results (probably due to the use of a different strain) in which a decrease in the iron concentration decreased the microcystin content and microcystin synthetase should be actively controlled by the amount of available free Fe^{2+}.

Lukac and Aegerter (1993) found that zinc (Zn) enhanced growth and microcystin production 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).

2.8.10 Light

Studies on light intensity and toxin production are highly variable, partly due to the different intensities and strains tested (Kaebernick and Neilan, 2001). Unfortunately, direct comparison of light intensities described by various studies is not possible due to different measuring techniques (Kaebernick *et al.,* 2000). In general, microcystin synthesis increases with light intensity or photosynthetically active radiation (Rapala *et al.,* 1997; Rapala and Sivonen, 1998; Nishizawa *et al.,* 1999; Kaebernick *et al.,* 2000; Kaebernick and Neilan, 2001) but light quality is also a determining factor (red light favours toxin production while blue light does not) (Kaebernick *et al.,* 2000). Wiedner *et al.* (2003) suggested that there are also maximum irradiance values above which microcystin production is inhibited. For example, Van der Westhuizen and co-workers (1986) found lower toxin production at very low and very high light intensities with *M. aeruginosa.* Graham *et al.* (2004) observed that microcystin–secchi maxima were characterised by exponential decline, with maximal microcystin values occurring at secchi depths < 2.5 m. This is also supported by Sivonen (1990), who found that high light intensities and high temperature had adverse effects on growth and promoted toxin leakage from the cells.

Kaebernick *et al.* (2000) found that the light-induced increase in *mcy* transcripts was not accompanied by a significantly higher level of microcystin in the cells. The authors propose that

the microcystin synthetase gene cluster is regulated by light quality, either directly of via another regulatory factor and that transcription requires different thresholds of light intensity for initiation and upregulation.

Yet, some studies (Böttcher *et al.,* 2001) concluded that light intensity variation in natural environments has little or no significant effect on microcystin cellular content and the differences found between toxicity of blooms of the same species are mainly due to the growth rates and toxic characteristics of different strains. This was however contradicted by studies from Kaebernick *et al.* (2000), who found that short term exposure to high light intensities revealed transcript increases within 2 hours of the move from low to high light and decreases after the move from light into the dark. Cell division rates are also likely to increase and decrease respectively under these conditions, and could thus be coupled to transcription (Kaebernick *et al.,* 2000). However, cells of *M. aeruginosa* divide less than once a day, which suggests that the changes in transcript amount seen after just 2 hours are due to a direct response to light and not entirely to cell division (Kaebernick *et al.,* 2000).

Light affect numerous cellular processes, but one cannot conclude that light is the single factor leading to the above mentioned thresholds (Kaebernick *et al.,* 2000). For instance cell division that is influenced by light may have a pronounced effect on transcription and/or toxin production. Hesse and Kohl (2001) speculated that microcystins could play a role in light adaptation processes.

Also important is the relationship between iron uptake and light intensity (Kaebernick and Neilan, 2001). High light intensities increase cellular iron uptake which may ultimately be responsible for higher toxin production (Utkilen and Gjølme, 1995).

2.8.11 Temperature

As water temperature increase, algal successional patterns typically follow a progression from diatoms through chlorophytes to cyanobacteria (Robarts and Zohary, 1987).

Sivonen (1990) found that toxin production at different temperatures seems to be strain dependent. Temperature has been shown to influence the type of toxin produced with high temperatures (>25°C) enhancing microcystin-RR production and lower temperatures favouring microcystin-LR synthesis in *Anabaena spp.* (Rapala *et al.,* 1997; Rapala and Sivonen, 1998).

According to Rapala *et al.* (1997) the overall toxin pool of nitrogen fixing *Anabaena* strains was highest at 25°C where lower and higher temperatures decreased their amounts. Sivonen and Jones (1999) found the same results, where microcystin and nodularin concentrations are highest between 18 and 25°C, as studied in *Anabaena*, *Microcystis* and *Nodularia,* with lower levels experienced at either higher or lower temperatures tested. The highest toxin production by *M. aeruginosa* occurred at 18-20°C rather than at higher temperatures (Van der Westhuizen and Eloff, 1985; Van der Westhuizen *et al.,* 1986).

Rapala *et al.* (1997) found that high temperatures (25 to 30°C) together with the highest levels of light (1-100 $\mu mol.m^{-2}.s^{-1}$) decrease the amount of toxin. Lee *et al.* (2000) reported that the condition most favourable to toxin production seems to be at a temperature slightly below the optimal growth conditions.

In South Africa, prevailing water temperatures are generally suitable for cyanobacterial growth during the greater portion of the year (Harding and Paxton, 2001).

2.9 Microcystin removal and elimination processes

2.9.1 Chemical control

The removal of cyanobacterial cells by flocculation or filtration methods has proven to be an effective method to reduce toxin levels in water but only if there is no cell lysis and liberation of microcystins to the water (De Figueiredo *et al.,* 2004). If toxins are released, other methods such as activated carbon absorption and ozonation are required to effectively eliminate dissolved microcystins from drinking water (De Figueiredo *et al.,* 2004). Hence, methods that lead to cell lysis are not advisable and should be avoided in drinking water treatment plants (De Figueiredo *et al.,* 2004).

In natural environments, photodegradation of microcystins occur indirectly via pigments or humic substances that absorb the sunlight (Welker *et al.,* 2001). Microcystins may also be photodetoxificated by UV irradiation (Kaya and Sano, 1998) and rapid photocatalytic degradation can be achieved through a reactor with immobilized titanium dioxide catalyst (Shephard *et al.,* 2002). Intracellular microcystin degradation mechanisms have not been identified, but both sunlight irradiation and photolysis with UV light have been implicated in microcystin decomposition and isomerisation to nontoxic forms (De Figueiredo *et al.,* 2004).

Presently, most drinking water treatment plants have methods such as ozonation, activated carbon filtration and chlorination that allow the removal of the majority of microcystins (but not all) in superficial waters (Tsuji *et al.*, 1997; De Figueiredo *et al.*, 2004).

Chlorination using adequate sodium hypochlorite doses after cell removal seems to be very effective for the elimination of microcystin-LR in raw water, with no formation of noxious product from the process (Tsuji *et al.*, 1997; De Figueiredo *et al.*, 2004). Microcystins may also be efficiently decomposed and removed from waters with high total organic carbon by ferrate oxidation-coagulation (Yuan *et al.*, 2002). Fenton oxidation of MC-LR have also been shown to be a promising method for rapid degradation of this kind of hepatotoxins (De Figueiredo *et al.*, 2004 and references therein).

The utilization of algicides, physical removal of surface scum using oil spill equipment and increasing the salinity of water reservoirs are all currently used as bloom remediation strategies that are capable of resulting in cyanobacterial stress and subsequently the release of toxin into the surrounding water column (Ross *et al.*, 2006). The key management plan for minimizing cyanobacterial blooms is to intervene at the source of the problem (Ross *et al.*, 2006).

2.9.2 Biological control

Microcystins can by biodegraded by complex natural populations of micro-organisms from diverse ecosystems (Oberholster *et al.*, 2004). Jones (1990) demonstrated that microcystins extracted from *M. aeruginosa* blooms are biodegraded in natural water bodies within 2-3 weeks and can be reduced to a few days if the water body has been previously exposed to microcystins.

Scott and Chutter (1981) suggest that viruses may be an important factor in controlling cyanobacteria but also suggest that myxobacteria are more important biological agents than viruses, since they are less host specific.

According to Harding and Paxton (2001), restructuring of the "top-down" control pathways through the deliberate management of the fish population can have profound and beneficial consequences.

2.10 Related metabolic processes

Because nitrogen is regarded as one of the biggest contributors to cyanobacterial growth, the expression of the *ntcA* gene is a good indicator of the regulation of ammonium and nitrogen assimilation by cyanobacteria.

2.10.1 ntcA gene

The *ntcA* gene encodes a positive transcriptional regulator of genes that are subjected to repression by ammonium and is widespread among cyanobacteria (Frías *et al.,* 1993) and seems to be highly conserved in all nitrogen fixing or non-fixing, unicellular or filamentous strains (Vega-Palas *et al.,* 1990; Frías *et al.,* 1993; Herrero *et al.,* 2001). It positively regulates the expression of genes related to nitrogen assimilation such as *glnA* which encodes glutamine synthetase (Muro-Pastor and Florencio, 2003). The *ntcA* gene has a complex expression pattern and may be regulated by its own gene product (Ramasubramanian *et al.,* 1996; Tanigawa *et al.,* 2002).

Nitrogen control consists of repression of the pathways for assimilation of some forms of nitrogen when more easily assimilated forms of nitrogen become available to the cell (Herrero *et al.,* 2001; Lindell and Post, 2001). In general, ammonium is the preferred source of inorganic nitrogen for cyanobacteria (Su *et al.,* 2005). It may be obtained from the environment by either passive diffusion or active uptake and is assimilated into organic matter via the activities of glutamine synthetase (GS) and glutamate synthase (GOGAT) (Flores and Herrero, 1994; Herrero *et al.,* 2001). Thus, in an ammonium limited environment, genes involved in the uptake and metabolism of alternative sources of nitrogen will be induced through the binding of *ntcA* to the *cis*-regulatory elements in the form of GTAN8TAC in the promoter regions of the genes (Su *et al.,* 2005). The assimilation of nitrate by cyanobacteria takes place in three successive steps: i) nitrate transport, ii) nitrate reduction to ammonium catalysed by nitrate reductase and nitrite reductase and iii) ammonium incorporation into carbon skeletons, which takes place mainly through the glutamine synthetase glutamate synthase cycle (Flores and Herero, 1994).

Luque *et al.* (2004) suggest that it is not ammonium itself that constitute the regulatory signal, but a metabolite resulting from ammonium incorporation into the carbon skeletons. Luque *et al.* (2004) observed that *ntcA* appears to respond, directly or indirectly, to a signal indicative of the C to N balance of the cell rather than simply to ammonium. Tanigawa *et al.* (2002) proposed that 2-oxoglutarate functions as a signalling molecule that transmits information on cellular

nitrogen status to *ntcA* and thereby regulates the transcription of genes related to nitrogen assimilation in cyanobacteria. The intracellular concentration of 2-oxoglutarate is thus inversely related to nitrogen availability (Tanigawa *et al.*, 2002). The GS-GOGAT pathway represents the connecting step between C and N metabolism and is tightly regulated in many organisms including cyanobacteria (Muro-Pastor and Florencia, 2003). Since cyanobacteria do not encode a 2-oxoglutarate dehydrogenase, the only fate of 2-oxoglutarate is to be converted into glutamate/glutamine. Hence, 2-oxoglutarate is widely believed to be an indicator of C/N balance in a cyanobacterial cell (Herrero *et al.*, 2001).

Ammonium entry into the cell takes place in two ways: diffusion of its gaseous form ammonia (NH_3), or operation of specific permeases for its charged form, ammonium (NH_4^+) (Muro-Pastor and Florencio, 2003).

During the adaptation of the cell to nitrogen stress, growth may continue transiently as many physiological changes take place, including the specific degradation of phycobiliproteins, which results in chlorosis (Görl *et al.*, 1998). This process would allow re-use of the nitrogen for the synthesis of proteins required for survival under conditions of nitrogen deprivation (Lindell and Post, 2001 and references therein). Growth is halted once both external and internal nitrogen supplies have been exhausted (Lindell and Post, 2001).

The nitrogen assimilation process is linked to light and photosynthesis (Alfonso *et al.*, 2001). Reduced ferrodoxin acts as an electron donor to the nitrate and nitrite reductases and reducing power is necessary for the action of glutamine synthetase and NADH glutamate synthase (Alfonso *et al.*, 2001). This strong coordination also occurs at the molecular level (Alfonso *et al.*, 2001).

The activities of *ntcA* are also subjected to modulations by the signal transducer protein PII which is in turn also controlled by ATP in the cell (Vazquez-Bermudez *et al.*, 2002; Vazquez-Bermudez *et al.*, 2003). Alfonso *et al.* (2001) as well as Jiang *et al.* (1997) suggest that the cellular redox status plays a central role in the autoregulatory mechanism of the *ntcA* protein. Vázquez-Bermúdez *et al.* (2002) reported that *ntcA* may respond to both nitrogen and carbon availability. This is supported by Su *et al.* (2006) that found that nitrogen assimilation affects the expression of many genes involved in photosynthesis, suggesting a tight coordination between nitrogen assimilation and photosynthetic processes. According to Su *et al.* (2006), *rbcL*

that encodes the large subunit of Ribulose bisphosphate carboxylase/oxygenase (Rubisco) is repressed by *ntcA*. Mori *et al.* (2002) also observed that at peak expression level of *ntcA* gene, the *rbcL* gene expression was minimal, providing support to its role as the negative regulator of the *rbcL* gene.

The *ntcA* gene is also required for the initiation of heterocyst differentiation in nitrogen fixing species (Frías *et al.*, 1994).

Equally important to nitrogen concentration and assimilation in the water by *Microcystis* species, is the phosphate concentrations in the water. The activation of cyanobacterial Rubisco is facilitated by inorganic phosphate. Therefore, to enhance our understanding of cyanobacterial carbon metabolism and bio-energetic status, that is directly influenced by phosphates, the gene expression of Rubisco in a natural environment need to be investigated.

2.10.2 Rubisco

In photosynthetic organisms, Rubisco enables the fixation of inorganic atmospheric carbon dioxide into organic matter for use as a source of energy after incorporation into cellular components (Carré-Mlouka *et al.*, 2006). Ribulose- 1,5-bisphosphate carboxylase oygenase catalyzes the first rate limiting step in the Calvin Cycle, the primary pathway for photosynthetic carbon reduction in the oceans (Pichard *et al.*, 1997).

Rubisco catalyzes two competing reactions: the reduction of CO_2 and the oxygenolysis of ribulose-1,5-bisphosphate (Selesi *et al.*, 2005). Despite the structural similarity between the higher plant and cyanobacterial Rubiscos, they differ in various aspects (Marcus and Gurevitz, 2000). Whereas the plant enzyme is soluble, the cyanobacterial enzyme is aggregated within subcellular complexes termed carboxysomes, which enables the elevation of CO_2 concentration and reduction of O_2 concentration at the carboxylation site (Marcus and Gurevitz, 2000 and references therein).

Rubisco exists in two natural forms which differ in their structures and primary sequences, their ability to fix carbon under varying oxygen tensions and their evolution (Watson and Tabita, 1996). The form I enzyme typically encountered in plants, eukaryotic algae and cyanobacteria (photo- and chemoautotrophic organisms (Selesi *et al.*, 2005)) is made up of eight large catalytic subunits and eight small subunits (L_8S_8) (Pichard *et al.*, 1997), with the *rbcL* gene encoding the

large subunit and the *RbcS* gene encoding the small subunit (Giri *et al.,* 2004). The form II enzyme consists only of a large subunit, usually a dimer (L_2) with 25-30% amino acid sequence identity to form I and is found in photo- and chemoautotrophs (Pichard *et al.,* 1997; Giri *et al.,* 2004).

Rubisco accumulates to very high levels in cyanobacteria and account for over 5 times more of the extractable cellular protein under culture conditions (MacDonald *et al.,* 2003). Rubisco is located in the carboxysomes, which are subcellular structures involved in efficient CO_2 utilization (Onizuka *et al.,* 2003). Although it is not a pigment-binding protein, it is a suspected target for UVB inhibition of cyanobacterial photosynthesis, particularly under long-term or repetitive exposure (MacDonald *et al.,* 2003). Wyman (1999) and Yang *et al.* (2002) found that the expression of the small subunit of the Rubisco gene (*rbcS*) was transcriptionally regulated by light so that synthesis of the enzyme decreased about two-fold in darkness. Besides transcriptional regulation, the activity of Rubisco is also subjected to post-translational regulation (Yang *et al.,* 2002). Mori *et al.* (2002) determined that the *rbcL* gene were repressed by cold (20°C) and osmotic (sucrose and salt) stress.

Carré-Mlouka *et al.* (2006) observed that in *M. aeruginosa* PCC 7806 the expression of both $rbcL_{\mathrm{I}}$ and $rbcL_{\mathrm{IV}}$ is sulphur-dependent and activation of cyanobacterial Rubisco is facilitated by inorganic phosphate (Marcus and Gurevitz, 2000).

There is convincing preliminary evidence that expression of the *rbcL* gene may be under circadian control (Wyman, 1999). Wyman (1999) found that mRNA levels of Rubisco increased in abundance during the night-time period and reach a maximum by midmorning. Transcript levels declined steadily thereafter before increasing once more from late afternoon to dusk (Wyman, 1999). The peak in mRNA abundance was observed about 4 hours post dawn. Diel rhythms in *rbcL* gene expression show a high degree of correlation with levels of carbon fixation (Pichard *et al.,* 1997). Onizuka *et al.* (2003) and references therein also found that the cellular concentration of Rubisco increases at low CO_2 levels.

2.11 Concluding remarks

Considerable ecological insights may be gained by the extrapolation to natural conditions of the results of physiological experiments carried out on cultures (Garcia-Pichel *et al.*, 1996).

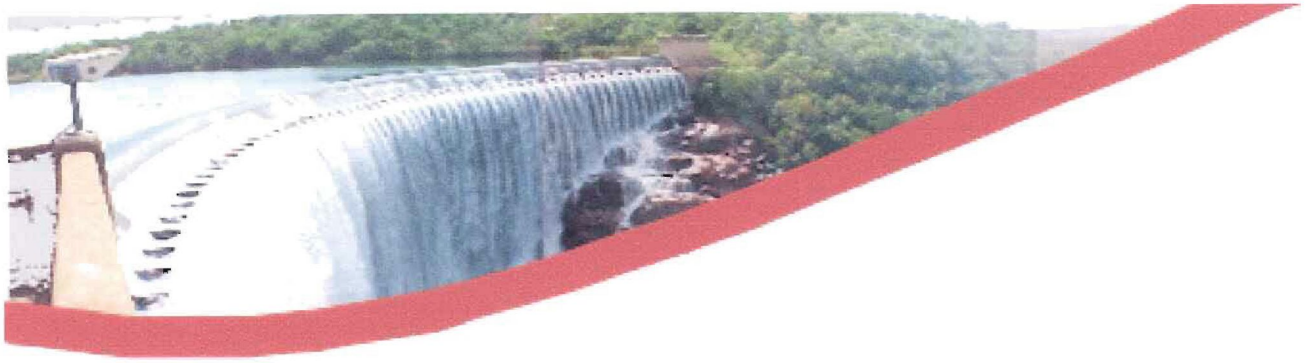
Microcystins are known to affect microalgae, zooplankton, aquatic and terrestrial insects, birds and mammals, among other organisms. Moreover, microcystin bioaccumulation have been reported for several organisms including crop plants irrigated with contaminated water and mussels, crayfish and fish used for human consumption. This poses a great concern for public health safety due to the cancer promotion potential of microcystins (MC-LR in particular) by the chronic ingestion of trace amounts of this toxin indirectly through food or directly through drinking water (De Figueiredo *et al.*, 2004).

Figueiredo *et al.* (2004) concluded that there is no global pattern in the blooms occurrence and the toxicity of a certain species. Rather than being due to the environmental parameter variation, the explanation for the differences found between the toxicity of blooms of species relies on the genotypic diversity of different strains and on the various growth and toxic characteristics of each one of those strains. However, despite all the controversial results, microcystin variants are secondary metabolites whose synthesis is known to be somehow regulated by factors such as light intensity and quality, temperature, nutrients and trace metal concentrations. Thus, the diversity of ecological features among toxic cyanobacteria makes the implementation of general cyanobacterial bloom control strategies difficult (De Figueiredo *et al.*, 2004).

Investigation should become more and more interdisciplinary in its approach with regard to the toxic cyanobacterial blooms' occurrence and toxicity, in order to achieve better results in the mitigation of the microcystin poisoning episodes. It is very important that the characterisation of the ecotoxicological features of a certain bloom is done in relation to the specific strain found as dominant. Only then may one establish specific patterns for blooms' occurrence of that strain worldwide and define specific strategies for its growth control, toxin elimination and lessening of toxic effects on organisms (De Figueiredo *et al.*, 2004).

Detection of toxic cyanobacteria through molecular markers for microcystin may have great potential in routine analysis of aquatic ecosystems. Thus, it may make water monitoring more feasible and allow the early application of corrective actions (Bittencourt-Oliveira, 2003).

With this study we aim to identify the samples in the culture collection of the North-West University by means of 16S rDNA sequencing and analysis, measure the DNA copy number of the *Microcystis* specific 16S rDNA and toxin genes (*mcyE* as well as *mcyB*) in order to shed more light on toxin production in the sampled environmental water and investigate the *in vivo* expression of the *ntcA* and the *rbcL* genes in order to investigate the physiological rationale for the sudden increase in biovolume of a specific species during a bloom.



Chapter 3

Optimisation of DNA extraction, PCR amplification and real-time PCR amplification

3.1 Introduction

Microcystis aeruginosa is the major toxin producing species in the Hartbeespoort Dam and the Roodeplaat Dam in South Africa (Van Ginkel *et al.*, 2006). These species are well known for the cyanotoxins, namely microcystin that they produce (Ouelette and Wilhelm, 2003). However, not all strains of *Microcystis aeruginosa* are toxin producers (Nakasugi and Neilan, 2005) and not all the toxic strains produce toxin all the time (Ouelette and Wilhelm, 2003). To distinguish between a toxin producing species and a non-toxin producing species is not possible using microscopy (Neilan *et al.*, 1997; Tillet *et al.*, 2001; Baker *et al.*, 2002; Gugger *et al.*, 2002; Ouellette and Wilhelm, 2003; Vaitomaa *et al.*, 2003; Mbedi *et al.*, 2005). This presents a problem to the monitoring programme of the water treatment works, as they need to identify the potential problem of toxins early in order to incorporate expensive treatment process steps to remove toxins from drinking water. The identification of potential toxin producing strains and the environmental conditions that can be associated with toxin production are therefore of the utmost importance to water management in South Africa. The *mcy* gene cluster in *M. aeruginosa* and other species have been identified as the site where toxin production is coded (Dittmann *et al.*, 1997). With the molecular analysis of the *mcy* gene cluster it is thus possible to determine if the species present in the sample are potential toxin producers or not.

During this thesis environmental samples collected from the Hartbeespoort Dam as well as the Roodeplaat Dam as part of the National Eutrophication Monitoring Program, would ultimately be used for the isolation of DNA and RNA, in order to quantify the presence and also expression of specific cyanobacterial genes including toxin producing genes with the use of quantitative real-time PCR analysis (qPCR). These environmental samples from both dams consists of different strains of cyanobacteria, but also of other organisms, such as bacteria, other picoplankton, green algae and zooplankton. DNA extracted from these environmental samples will vary significantly in quantity and quality with serious affects on the outcome of qPCR results.

qPCR has been shown to be an extremely powerful and sensitive method for quantitative detection of organisms. Several studies have used this method with success for the quantitative analysis of micro-organisms including harmful algal bloom species (Vaitomaa *et al.*, 2003; Gobler *et al.*, 2007; Werbrouck *et al.*, 2007). Although one can presume that the real-time quantitative reverse transcriptase-PCR (qRT-PCR) method is the result of an established and standardised technology (Werbrouck *et al.*, 2007), no standard operating protocol exists (Bustin, 2002). As pointed out by other authors (Bustin, 2002; Werbrouck *et al.*, 2007), to obtain a

reliable quantification method, each qRT-PCR method has to be optimized (Werbrouck *et al.*, 2007), with respect to DNA and mRNA isolation, cDNA synthesis, primer matching and of course the qRT-PCR itself.

The efficiency of DNA isolation determines the overall yield of DNA and thus the sensitivity of the procedure (Werbrouck *et al.*, 2007). The design and use of specific primers for this study necessitate the optimization of each PCR reaction and primer pair combination. Finally in order to be able to accurately quantify gene expression and the presence of certain genes, the qRT-PCR has to be optimized to ensure that each reaction is run at similar efficiency (Werbrouck *et al.*, 2007). While the methodology is straightforward, the application of qRT-PCR to environmental studies furthermore can produce variable results. The main reasons are:

1. Amplification efficiencies of laboratory cultures used for generation of standard curves may not accurately represent amplification efficiencies of DNA extracted from environmental samples.
2. qRT-PCR methods often fail to incorporate controls to assess accuracy of results.

The aim of this study was therefore to optimize each of the key steps in this multistep procedure in order to ensure accurate data that can be compared between samples as well as between sampling sites and to verify the use of laboratory cultures and controls for generating meaningful results using environmental samples.

3.2 Materials and methods

3.2.1 Strains used and DNA purification

The material that was used to test the isolation protocol had to include strains of both single celled as well as filamentous strains of cyanobacteria. Therefore, uni-algal strains (available in the NWU culture collection) of *Microcystis aeruginosa* UV027 (single cell cyanobacteria), *Oscillatoria simplicissima*, *Spirulina sp.*, *Arthruspira sp.* and *Planktothrix sp.* (filamentous cyanobacteria) were cultivated under controlled conditions (see Chapter 4) and used to evaluate the isolation procedure.

For this evaluation, the purity of the DNA was firstly assessed by A_{260}/A_{280} ratio measurement using a spectrophotometer (Spectronic, Genesys 2, Milton Roy) and the DNA concentration was determined using the following equation:

Equation 1: DNA concentration = $A_{(260)}$ x dilution x 50 µg/ml

where $A_{(260)}$ is the absorbance measured at 260 nm

(Sambrook *et al.*, 1989).

Secondly, the purified DNA was also evaluated through a conventional PCR analysis, since this procedure would ultimately be used to generate the results. PCR amplification of a part of the cyanobacterial 16S rRNA gene was done with primers designed by Nübel *et al.* (1997) (table 3.1).

The PCR products were run on a 1.5-2.0% agarose gel to visualize the amplified fragments to ensure that the fragments are of similar length, that only one fragment is obtained and that clear bands are visible. This is necessary for real-time applications to ensure high quality DNA for best amplification efficiency.

The protocols that were tested for the isolation of the DNA from different species of cyanobacteria were as follows:

3.2.1.1 Protocol 1

The first protocol used, was a modified method by Dellaporta *et al.* (1983) for the isolation and purification of DNA from plant material. This method was chosen as it is widely used as a standard procedure for the isolation of DNA from various sources of plant material.

Cyanobacterial cultures (15 ml) were filtered through glass fibre filters (47 mm-diameter GF 52 glass fibre paper filter) with a pore size of 1.2 µm (Schleicher & Schnell) at negative vacuum pressures. These filters were chosen because the pore size allows bacteria to be washed through the filters, but most cyanobacteria will remain on the filter (Kruskopf, 2002).

Filters with the cyanobacterial cells were homogenized (Heidolph DIAX 900) in an extraction buffer (50 mM tris(hydroxymethyl)aminomethan: 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris) (pH 8.0), 5 mM Ethylenediamine tetra-acetic acid, disodium magnesium (EDTA), 50 mM NaCl) to lyse the cells. After homogenisation, a final concentration of 1 mg/ml lisozyme was added which cause the degradation of the peptidoglycan walls and the mixture was incubated at 55°C for 30 minutes. Proteinase K with a final concentration of 50 µg/ml was subsequently added to the mixture and it was incubated for a further 10 minutes at 55°C. A final concentration

of 0.5% (w/v) SDS solution (sodium dodecyl sulphate) was added after the incubation period and the mixture was kept on ice for 10 minutes. After the 10 minute incubation period on ice, the suspension was centrifuged for 20 minutes at 12 000 x g to remove the precipitated proteins from the supernatant and the supernatant was transferred to a sterile micro centrifuge tube.

A phenol:chloroform:isoamyl (25:24:1) (v/v) extraction was done by adding an equal volume of the phenol:chloroform:isoamyl mixture to the aqueous DNA sample, forming a biphasic mixture and mixed by gentle inversion (30 times) until an emulsion formed.

After centrifugation (12 000 x g for 5 minutes at 25°C), the supernatant was transferred to a sterile microcentrifuge tube. This extraction was repeated twice, followed by an extraction with an equal volume of chloroform. DNA was precipitated with two volumes of 4 M NaCl and absolute (v/v) ethanol at -20°C overnight (Fiore *et al.*, 2000). The suspension was then centrifuged for 20 minutes at 12 000 x g and the supernatant removed, leaving a pellet of the crude DNA.

The precipitated pellet was then successively washed with 70% (v/v) and absolute (v/v) ethanol. The pellet with the DNA was air dried. One hundred micro litre TE (10 mM Tris [pH 8.0]; 1 mM EDTA) was added to the dried pellet and the dissolved DNA was stored at -20°C.

3.2.1.2 Protocol 2 (Modified method by Fiore *et al.* (2000))

Flasks containing 100 ml culture of *Microcystis sp.* and *Planktothrix sp.* respectively were subjected to sonic shock in an ultrasonic bath for 10 minutes to allow cell separation and filament breakage and centrifuged (12 000 x g for 5 minutes at 25°C) in a sterile 20 ml microcentrifuge tube. Pelleted cells were washed in 1.0 ml wash solution containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA and 50 mM NaCl to reduce extracellular impurities. Cell pellets were resuspended in a 200 µl solution of 50 mM Tris-HCl, (pH 8.0) and 50 mM EDTA. After resuspension, the cells were treated with proteinase K at a final concentration of 100 µg/ml at 55°C for 10 minutes before addition of the extraction buffer.

Subsequently, 600 µl of pre-warmed (55°C) extraction buffer (3% (w/v) cetyltrimethyl ammonium bromide (CTAB), 1% (w/v) sarkosyl, 20 mM EDTA, 1.4 M NaCl, 0.1 M Tris-HCl, pH 8.0, 1% (v/v) 2-mercaptoethanol, freshly prepared) were added and incubated at 55°C in a water bath for a further 30 minutes with gentle inversion every 5-10 minutes. The mixture was allowed to cool for 30 s before adding equal volumes chloroform (v/v) and mixed by gentle

inversion (30 times) until an emulsion formed. After centrifugation (12 000 x g for 5 minutes at 25°C), the supernatant was transferred to a sterile micro-centrifuge tube, 2 volumes of 4 M NaCl were added and the solution mixed by inversion (Fiore *et al.*, 2000).

After this step, 200 µl of silica suspension (100 mg/ml) was added, mixed by gentle inversion and incubated at 55°C in a water bath for 10 minutes.

The silica suspension was prepared with modification to that which was described in Boyle and Lew (1995), by adding 1 g silica to 50 ml phosphate buffered saline (PBS) (10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4). After adding the silica suspension the sample was mixed vigorously by vortexing and the silica was then allowed to settle for 1.5 hours. The supernatant was removed and 50 ml PBS was again added, repeating the procedure. After centrifugation (2000 x g for 2 minutes) the silica pellet was resuspended in 10 ml 4 M NaCl. The silica was recovered by centrifigation (12 000 x g for 30 s), the pellet washed twice with 1000 µl sterile wash solution (50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2.5 mM EDTA and 50% (v/v) ethanol) and dried by vacuum.

DNA was released from the silica in 100 µl ultrapure sterile water by incubation at 45°C for 10 minutes. After centrifugation (12 000 x g for 1 minute) the supernatant containing the DNA, was transferred to a sterile 0.5 ml micro-centrifuge tube.

This method was finally modified by first washing the algal cells three times with the wash solution, before homogenization to try to eliminate the slyme residues before the DNA was be released from the broken cells. This may prevent the impurities from forming a pellet around the DNA.

3.2.1.3 Protocol 3

The cell material was harvested as described above using filtration. The filters with the concentrated cyanobacterial cells were firstly washed three times with 10 ml wash solution (50 mM Tris-HCl, pH 8.0, 5 mM EDTA and 50 mM NaCl) to reduce extracellular impurities (Fiore *et al.*, 2000).

Filters with the concentrated cyanobacterial cells were incubated in a sterile buffer containing 50 mM Tris-HCl (pH 8.0) and 50 mM EDTA. The filters were then homogenized with a Heidolph DIAX 900 homogenizer for 1 minute. Proteinase K (100 µg/ml, final concentration) was added

to the mixture and the mixture was incubated at 55°C for 10 minutes. Subsequently, prewarmed (55°C) extraction buffer was added (3% (w/v) CTAB, 1% (w/v) Sarkosyl, 20 mM EDTA, 1.4 M NaCl, 0.1 M Tris-HCl, pH 8.0, 1% (v/v) 2-mercaptoethanol, freshly prepared) and incubated at 55°C in a water bath for 30 minutes with mixing by gentle inversion every 5 minutes (Fiore *et al.*, 2000). The mixture was allowed to cool for 30 s before centrifuging for 20 minutes at 12 000 x g. After centrifugation, an equal volume chloroform was added to the supernatant. The mixture was mixed by gentle inversion (30 times) until an emulsion formed. After centrifugation (12 000 x g for 5 minutes at 25°C), the supernatant was transferred to a sterile microcentrifuge tube and the chloroform extraction was repeated. After the second chloroform extraction, the supernatant was transferred to a sterile microcentrifuge tube, 2 volumes of 4 M NaCl were added and the solution mixed by gentle inversion (Fiore *et al.*, 2000).

An equal volume of isopropanol was then added to the mixture and incubated for 1 hour at room temperature to precipitate the DNA. The mixture was centrifuged for 20 minutes at 12 000 x g and the supernatant discarded. One millilitre 70% (v/v) ethanol was added and the DNA was centrifuged for 10 minutes at 12 000 x g. The supernatant was removed and the ethanol wash step was repeated with absolute (v/v) ethanol. The pellet was then air-dried in a desicator with silica gel crystals. The DNA-pellet was resuspended in 500 µl of TE buffer (10 mM Tris-HCL, pH 8.0, 1 mM EDTA).

Lastly, a final concentration of 750 mM ammonium acetate was added to the DNA containing solution followed by a chloroform extraction using equal volumes of chloroform. After centrifugation (5 minutes at 12 000 x g) the DNA in the supernatant was precipitated overnight with 1 ml of absolute ethanol and then washed twice with 70% (v/v) ethanol. The DNA was subsequently dried and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

3.2.2 Conventional PCR analysis

Conventional PCR analysis was done in order to verify the quality of the isolated DNA of different cyanobacterial species and also served as a quick verification of the potential for microcystin production of environmental samples and reference strains. Primer optimisation is, however, very important for accurate quantification and sensitivity of the PCR analysis. The primers were synthesized commercially by Integrated DNA Technologies, Inc. (IDT).

3.2.2.1 16S rDNA primer sequences for evaluation of DNA protocols

For optimising the reaction with the different primers, DNA isolated from cultured strains was used. In order to test which combination of the different 16S rDNA primers by Nübel *et al.* (1997) (table 3.1) will yield the best amplification product, a conventional PCR was done to amplify products for each combination. The best set of primers was chosen for each strain. These primer sets were chosen if only one fragment was obtained and the fragment was the correct size and a clear band was visible on the agarose gel.

PCR amplification was performed as described by Nübel *et al.* (1997) in a 50 µl (total volume) reaction mixture containing 15 ng DNA, 1 x Super Taq Plus PCR buffer (Southern Cross biotechnology), 1 U of SuperTaq DNA polymerase (Southern Cross biotechnology), each deoxynucleotide triphosphate (dNTP) at a concentration of 0.2 mM, 0.5 µM primer and 1 µg bovine serum albumin (BSA).

Amplification was carried out with a PCR Thermal cycler (PCR Express, Hybaid) as described by Nübel *et al.* (1997). After the initial denaturation step (5 minutes at 94°C), ten cycles of 45 s at 94°C, 45 s at 57°C and 2 minutes at 68°C followed; and then 25 cycles of 45 s at 92°C, 45 s at 54°C and 2 minutes at 68°C followed by a final elongation step of 7 minutes at 68°C.

Table 3.1: PCR primers for the 16S rRNA gene by Nübel *et al.* (1997).

Primer	**Sequence (5'-3')**	**Position**
CYA106F	CGG ACG GGT GAG TAA CGC GTG A	106-127
CYA359F	GGG GAA TYT TCC GCA ATG GG	359-378
CYA781R(a)	GAC TAC TGG GGT ATC TAA TCC CAT T	781-805
CYA781R(b)	GAC TAC AGG GGT ATC TAA TCC CTT T	781-805

*Y is the symbol for either thymine or cytosine

The fragments generated were sent to the University of Cape Town to be sequenced. This was done to quickly verify the culture collection representatives.

The amplification of the 16S rRNA gene fragment concomitantly with microcystin genes during this phase of the research will act as an internal control to verify that the reaction was successful. This is necessary since not all strains present in the environmental samples contain microcystin genes.

3.2.2.2 mcyB and mcyE primers using conventional PCR

Table 3.2: *mcyB* and *mcyE* primers that were used to test for potential microcystin producers in a sample.

Primer	Sequence (5'-3')	Reference	Product size	Annealing temperatrue
mcyB FAA	CTATGTTATTTATACATCAGG	Neilan *et al.* 1999		50°C
mcyB RAA	CTCAGCTTAACTTGATTATC	Neilan *et al.* 1999	780 bp	
mcyE-F2	GAAATTTGTGTAGAAGGTGC	Vaitomaa *et al.* 2003		58°C
mcyE-micr-R8	CAATGGGAGCATAACGAG	Vaitomaa *et al.* 2003	247 bp	
mcyE-Osc R3	CTC AAT CTG AGG ATA ACG AT	Dr. Anne Rantala (personal communication)		
mcyE.fw *(Planktothrix* sp.*)*	TTACCTAATTATCCCTTTCAAAG	Mbedi *et al.* (2005)		50°C
mcyE.rev	CAATGGGTAAGGTTTGCTT	Mbedi *et al.* (2005)	589 bp	

The primers described in table 3.2 were tested for their specificity. To test these primers, strains of both toxic and non-toxic *Microcystis sp.* and *Planktothrix sp.* were used. These strains were obtained from different culture collections (see chapter 4).

The toxic strain for *Microcystis sp.* used was *M. aeruginosa* PCC 7806 and the non-toxic strain was *M. aeruginosa* CCAP 1450/1 (Nakasugi and Neilan, 2005; Pasteur Culture Collection (PCC), 2008). For *Planktothrix sp.* the toxic strain used was *P. agardhii* 126/8 and the non-toxic strain was *P. pseudagardhii* NIVA CYA 153/1.

PCR amplification was performed in a 25 µl (total volume) reaction mixture containing 15 ng DNA, 1 x Super Taq Plus PCR buffer (Southern Cross biotechnology), 1 U of SuperTaq DNA polymerase (Southern Cross biotechnology), each deoxynucleotide triphosphate at a concentration of 0.2 mM and 0.5 µM of each primer.

Amplification was carried out with a PCR Thermal cycler (PCR Express, Hybaid) and the following cycling parameters were used: After the initial denaturation step (10 minutes at 94°C), 35 cycles of 30 s at 94°C, 30 s at the specified annealing temperature (table 3.2) and 1 minute at 68°C, followed by a final elongation step of 7 minutes at 72°C.

3.2.3 Real-time PCR analysis

Signal intensities representing the expression levels of the experimental gene were compared to a standard curve generated by samples containing a concentration titration of an external standard. *M. aeruginosa* PCC 7806 was chosen as the external standard from which the standard curve would be generated. For qPCR analysis, the amplification efficiency of the reaction should be between 90-105% (DNAbiotec, 2006). It is necessary to maintain the same efficiency throughout, to be able to make more reliable comparisons of the data.

Initially the qPCR analysis was done on the Rotorgene (Celtic) (version 6) with 16S rDNA, *mcyE* and *mcyB* primers to determine the number of copies of the genes in all the environmental samples. However, due to the fact that the machine was not available for the duration of the study all the analyses completed were repeated and all subsequent analyses were done using the BioRAD iCycler courtesy of DNAbiotec in Pretoria.

qPCR amplification of cyanobacterial DNA was performed with the Bio-Rad iQ5 PCR thermal cycler, in a 25 µl (total volume) reaction mixture containing 12.5 µl iQ™ SYBR® Green Supermix Bio-Rad (100 mM KCl, 40 mM Tris-HCI, pH 8.4, 0.4 mM of each dNTP, 1 U iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, (20 nM fluorescent and stabilizers), 60 ng DNA, 0.5 µM primer F and 0.5 µM primer R (IDT).

qPCR amplification was carried out with the Bio-Rad iQ5 PCR Thermal cycler with the reaction mixture after the initial denaturation step (3 minutes at 95°C). The initial denaturation step was followed by 30 cycles of PCR, with each cycle consisting of 30 s at 95°C, 30 s at 60° (table 3.3) and 30 s at 72°C, followed by a final elongation step of 7 minutes at 72°C. Synthesis of the products was monitored after each extension step at 72°C, by measuring the fluorescence of double-stranded DNA binding SYBR green dye using IQ5 iCycler (Bio-Rad).

All samples were amplified in duplicate. The fluorescence threshold was set by the analytical software for the iCycler (Bio-Rad, version 2). Copy numbers of *mcyE* and *mcyB* gene of the dam water samples were determined by converting the obtained C_t values into the *mcyE* and *mcyB* copy numbers according to the regression equations of the external standards according to Vaitomaa *et al.* (2003).

Table 3.3: 16S rDNA, *mcyB* and *mcyE Microcystis sp.* specific primers that were used to test for potential microcystin producers in a sample using qPCR methods.

Primer	Sequence (5'-3')	Product size	Annealing temperature
16S_Micr_F	AGCCAAGTCTGCCGTCAAATCA		
16S_Micr_R	ACCGCTACACTGGGAATTCCTG	93 bp	60°C
mcyB-F	GCTGCGGTTTGGGAGTTATG		
mcyB-R	GCTGACGGTGATTTCTTGGG	126 bp	60°C
mcyE-F	AGTCATTTCGGGTTGGTTATG		
mcyE-R	CAGAAACTTGTGTGGGAGTATC	203 bp	60°C

3.2.3.1 16S rDNA primer

The 16S rDNA primers of Rudi *et al.* (1997) (table 3.3) were used for the amplification of the 16S rRNA gene. According to Rudi *et al.* (1997), the primers are *Microcystis sp.* specific and do not amplify with other strains of cyanobacteria and they generate a fragment of 93 bp which is desirable for qPCR analysis.

3.2.3.2 mcyB primers

The *mcyB* primer of Neilan *et al.* (1999) was aligned with *mcyB* sequences at NCBI using the BLAST network service (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and it was found that there was one base in the reverse primer that did not match the sequence of *M. aeruginosa* PCC 7806.

mcyB-R (5' CTCAGCTTAACTTGATTATC 3') (In the reference sequence of *M. aeruginosa* PCC 7806 the T in bold is substituted with a C).

As a result of this mismatch and since the amplified product for the *mcyB* primer of Neilan *et al.* (1999) is more than 200 bp in length, it was decided to design new primers with a shorter amplification product, specific for *Microcystis sp.* for the *mcyB* amplicon.

Microcystis sp. specific *mcyB* primers (table 3.3) were designed from the *mcyB* gene sequence of *M. aeruginosa* PCC 7806 (AAF00961) with Beacon designer software (version 3.0). The primers were tested for their specificity by performing a conventional PCR (see section 3.2.2) with these primers on different strains of cyanobacteria as well as non-toxic and toxic strains of *M. aeruginosa.*

3.2.3.3 mcyE primers

The *mcyE* primers were also tested with sequences of the *mcyE* genes available on BLAST. The existing primers were rated according to the Beacon designer software (version 3.0) and were only given a rating of 46.7%. The software calculates a percentage value for each primer and each primer combination to rate these primers according to its design (% GC content, melting temperature, length, formation of self-dimers or cross dimers and formations of hairpin structures). The criteria are as follows: poor = 0-50%; good = 50-75%; best 75-100%.

It was therefore decided to design new primers for the amplification of the *mcyE* gene in *Microcystis sp.* Primers were designed from the *mcyE* gene sequence of *M. aeruginosa* PCC 7806 (AF183408) using BLAST (http:/www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) and Beacon designer software (version 3.0). A rating of 72.8% was obtained for the new primer set. The primers amplify a 203 bp sequence of the *mcyE* gene and were specific for *Microcystis sp.*

3.2.3.4 Planktothrix mcyE primer

Two sets of primers were tested for its specificity, namely the *Planktothrix sp.* specific *mcyE* primer of Vaitomaa *et al.* (2003) and that of Mbedi *et al.* (2005).

The amplification product for the Vaitomaa *et al.* (2003) *mcyE* primer yielded only primer dimers. Therefore, the *Planktothrix* specific *mcyE* primers of Mbedi *et al.* (2005) were subsequently used for the amplification of toxic strains of *Planktothrix* in the environmental samples:

mcyE.fw (5' TTACCTAATTATCCCTTTCAAAG 3')
mcyE.rev (5' CAATGGGTAAGGTTTGCTT 3')
length of amplified fragment 589 bp

3.2.4 Reverse transcriptase real-time PCR analysis

The gene expression of the *ntcA* and *rbcL* genes were measured by qRT-PCR analysis.

For one-step qRT-PCR, the analysis was performed using the one step QuantiTect SYBR Green RT-PCR of Qiagen. The cycle conditions were 50°C for 30 minutes for the reverse transcriptase reaction to occur, 95°C for 15 minutes to activate the hot start taq, followed by 30 cycles with the following steps: 94°C for 15 s, 60°C for 30 s and 72°C for 30 s, with the addition of a melt curve protocol at the end of the program. In all cases, melt curves were used to confirm single

amplification products for the different reactions and in select cases amplification was also confirmed with agarose gel electrophoresis.

QRT-PCR amplification of *Microcystis sp.* RNA were performed in a 25 µl (total volume) reaction mixture containing 12.5 µl of 2x QuantiTect SYBR Green RT-PCR Master mix (HotStarTaq DNA polymerase, QuantiTect SYBR Green RT-PCR buffer (Contains Tris-HCl, KCL, $(NH_4)_2SO_4$, 5 mM $MgCl_2$, pH 8.7), dNTP mix, Fluorescent dyes), 0.25 µl QuantiTect RT mix (Omniscript and Sensiscript Reverse Transcriptases), 80 ng RNA, 0.5 µM primer F and 0.5 µM primer R. The QuantiTect SYBR Green RT-PCR master Mix together with the QuantiTect RT Mix allows both reverse transcription and PCR to take place in a single tube.

According to available literature (Ambion, 2007) the 16S rRNA gene is constant in its expression and is not influenced by environmental factors and is therefore a good internal reference for gene expression studies. Therefore, the 16S rRNA gene were amplified with qRT PCR as an internal control.

3.2.4.1 ntcA primer

For the determination of the expression of the gene that positively regulates the expression of genes related to nitrogen assimilation, the *ntcA* gene was amplified with qRT-PCR.

Very few *ntcA* primers could be located in literature and the only published primers were those for the amplification of the *ntcA* gene of *Synechocystis sp.* Primers of Alfonso *et al.* (2001) were used for the amplification of the *ntcA* gene with DNA of *M. aeruginosa.* Despite different temperatures and different $MgCl_2$ concentrations used, no amplification was observed (annealing temperatures from 48°C to 58°C were used with $MgCl_2$ concentrations from 1.5-3.0 mM) for *M. aeruginosa.*

*ntcA*1 : (5' ATA CTC GAG ATG GAT CAG TCC CTA ACC '3);
*ntcA*2: (5' TCA CTC GAG GGC ACT GGT CAT AGA GG 3 ').

Subsequently, degenerate primers were designed on an area which is the most conserved for all the available sequences:

ntcA-F: (5' GAT CCG GCN GAA MRG GTB TA '3);
ntcA-R1: (5' C YTC SGC RAT BGC YTG RTG '3);
ntcA-R2: (5' GTK GAD CCR ATS GCY TCS GC '3);

These primers were then used to amplify the DNA of *M. aeruginosa* via a touchdown PCR from 60°C to 48°C and the product was visible on an agarose gel.

The PCR product was send to the University of Cape Town (UCT) to be sequenced and from the sequenced data, primers, specific for *M. aeruginosa* were designed on IDT's website with the Primer Quest tool (http://www.idtdna.com) and analysed with the Oligo Analyser tool. The primers designed for the amplification of a part of the *ntcA* gene of *M. aeruginosa* were:

*ntcA*__micr-F (5' AGGAAATTACCGTGGCTCTACTGC 3');
*ntcA*__micr-R (5' CCAGTCAGTAAGGATAGCACACCA 3')
Size of amplification product: 63 bp.

3.2.4.2 rbcL primer

In order to determine the potential photosynthetic activity of the cyanobacteria, primers to amplify the *rbcL* gene of *M. aeruginosa* were needed. For the amplification of the *rbcL* gene, the primers designed by Macdonald *et al.* (2003) were used. However, these primers are not specific for *Microcystis sp.* and were designed to amplify a 576 bp part of the coding sequence of the *rbcL* gene of *Synecococcus sp.* PCC 7942. Despite the low specificity, the primers were used at very low annealing temperatures (54°C) (protocol described in section 3.2.2) with DNA of *M. aeruginosa* PCC 7806 to generate an amplification product of approximately 576 bp.
Primers by Macdonald *et al.* (2003):

*rbcL*_F: (5'GGY GGT YTK GAC TTC ACC AA 3');
*rbcL*_R: (5'GCC ANA CGT GGA TAC CRC CGG AAG C 3')

From these sequences, species specific primers were then designed. The amplification product was sent to UCT for sequencing. From these sequences, *Microcystis* specific primers were designed on IDT's website with the Primer Quest tool (http://www.idtdna.com) and analysed with the Oligo Analyser tool. These primers amplified a shorter region of 180 bp. This shorter sequence makes it more suitable for qPCR analyses.

*rbcL*_PCC_F (5' AATTCTGCCGCGACAAAGGGTTAC 3');
*rbcL*_PCC_R (5' TGATTCCGCGTTCACCTTCGAGTT 3')

These primers were subsequently tested for their specificity to *Microcystis sp.* and other cyanobacteria.

3.2.4.3 Statistical analysis

The efficiency for each primer set was evaluated and recorded during assay development by the Bio-Rad iCycler.

PCR efficiency (E) was determined using the following equation:

Equation 1: Calculation of reaction efficiency (E): $E = 10^{(-1/slope)}$

(Dyhrman *et al.*, 2006)

Equation 2 shows a mathematical model of relative expression ratio in qPCR under constant reference gene expression.

3.2.4.4 Quantification

The relative quantification normalised against unit mass (ΔC_t method) was used for the calculation of the ratio of the expression of the *ntcA* and the *rbcL* genes against the 16S rRNA gene expression.

Equation 3: The ΔC_t method for the calculation of the ratio of expression:

$$Ratio_{(test/control)} = Efficiency^{(Ct\ (test) - Ct\ (control))}$$

(Pfaffl, 2001)

Where control = ng value of 16S rRNA products gene expression
Where test = ng value of *ntcA* or *rbcL* products gene expression

3.2.5 Optimisation of qRT-PCR amplification

Total RNA was extracted with the Qiagen RNAeasy mini kit according to manufacturer's instructions. Glass fibre filters containing the cyanobacterial cells were first homogenised in the buffer to break the cell walls. The RNase free DNase set (150 Kunitz Units) were used to eliminate DNA contamination.

All the samples were then again treated with Ambion Turbo DNA free according to the manufacturer's instructions, in order to remove the DNA contamination that was still prevalent. The selected RNA samples were tested by doing a PCR and by running the products on a 2% agarose gel. No bands were observed, indicating that there were no DNA contamination.

All the environmental samples of the Hartbeespoort and the Roodeplaat Dams were also treated with Ambion Turbo DNA free and the RNA was measured spectrophotometrically to determine the concentration of the DNA. The measured RNA was diluted to a concentration of 8.42 ng/µl in order to have a final amount of 80 ng in the final reaction volume of 25 µl for the qPCR reaction.

A 3 fold dilution series was used. The efficiency of the reaction was stable with the 3 fold dilutions and amplification efficiencies ranged from 88% to 100%. The dilutions that were used were: 312.5 ng, 62.5 ng, 12.5 ng, 2.5 ng, 0.5 ng, 0.1 ng, 0.02 ng in a reaction of 25 µl.

3.2.6 Sequencing

3.2.6.1 Fragments generated for sequencing

Primers by Taton *et al.* (2003) as well as Wilmotte *et al.* (1993) (table 3.3) were used for amplifying nearly complete sequence of the 16S rRNA gene.

A temperature gradient between 46°C to 58°C was done with the different 16S rDNA primers (table 3.3) to determine which of these primer combinations were the best to use and at which annealing temperature did they yield the best results.

Sequencing was done automatically on a DNA sequencer, using cyclic sequencing by UCT. The sequencer is only able to sequence approximately 800-1000 bp at a time, thus, primers to amplify shorter fragments had to be used (table 3.4).

Table 3.4: Primers used for 16S rRNA gene sequencing

Primer	Sequence 5'-3'	position	Reference
16S27F	AGA GTT TGA TCC TGG CTC AG	7–27	Wilmotte *et al.* (1993)
16S781R (a/b)	(a) GAC TAC TGG GGT ATC TAA TCC CAT T (b) GAC TAC AGG GGT ATC TAA TCC CTT T	781-805	Nübel *et al.* (1997)
16S727F	RGG ATT AGA TAC CCC	7–27	Wilmotte *et al.* (1993)
CYA1514R	GTA CGG CTA CCT TGT TAC GAC	1494-1514	Taton *et al.* (2003); Wilmotte et al. (1993)

Figure 3.1: Map of primer sites in the 16S rDNA operon that were used in this study. Forward primers CYA106F, CYA359F, with reverse primer CYA781R(a) and (b) were used in the first PCR reaction and that product was sequenced using the same primers to obtain a sequence of approximately 450 bp. Primers 16S27F and 23S30R were used to generate a longer PCR product of the 16SrRNA plus ITS region and that product was sequenced using primers 16S27F, CYA 781Ra/b (green script), 16S727F and 16S1494R (red script) to obtain the sequence of whole genomic 16S rDNA gene (table 3.3).

3.2.6.2 Sequencing data processing

At first it was decided to use ARB (latin, "arbor" = tree) (http://www.arb-home) to manage the data generated. ARB is a free sequence database application for Unix. It includes a sequence editor, several sequence aligners, phylogeny reconstruction tools, probe/primers search. This was in part done to generate data compatible with other research groups in the field. The study was done in close collaboration with Prof. K. Sivonen (Viike Biocenter, University of Helsinki, Helsinki, Finland).

3.3 Results

3.3.1 DNA purification

Due to the cost of this study and the amount of samples that had to be processed, it would not have been financially viable to make use of commercially available kits. The amount of the product yielded with the use of a kit proved to be insufficient for the various cell types and the work intended and kits from different companies such as Peqlab and Bioline were tested. Hence several different manual protocols were tested to isolate high quality and quantity DNA from various cell types of cyanobacteria. According to table 3.5 protocol 3 yielded the best results for both single celled and filamentous cyanobacteria.

Table 3.5: Comparison of the results obtained after using different protocols for isolation of genomic DNA from *Planktotrix sp.*, a filamentous cyanobacterial species and *Microcystis sp.*, a single celled cyanobacterial species. Each method was repeated a minimum of 5 times.

Protocol	**$A_{260/280}$ ratio *Microcystis sp.***	**Yield (µg/µl) *Microcystis sp.***	**$A_{260/280}$ ratio *Planktothrix sp.***	**Yield (µg/µl) *Planktothrix sp.***	**PCR with *Microcystis sp.***	***PCR with Planktothrix sp.***
Protocol 1	1.875	1.2	1.2	0.09	Successful	Unsuccessful
Protocol 1 (with CTAB)	2.02	0.45	1.4	0.5	Successful	Unsuccessful
Protocol 2	0.8	0.08	1.0	0.018	Unsuccessful	Unsuccessful
Protocol 2 (without sonic shock)	2.0	0.45	1.5	0.03	Successful	Unsuccessful
Protocol 3	2.2	0.1	1.9	0.465	Successful	Successful

To remove any possible impurities from the extracted DNA, an extra cleanup procedure with ammonium acetate was followed after the extraction described (section 3.2.1.3). Then, as an additional step to evaluate the DNA quality obtained from each purification protocol, part of the 16S rRNA gene sequence were generated with PCR amplification.

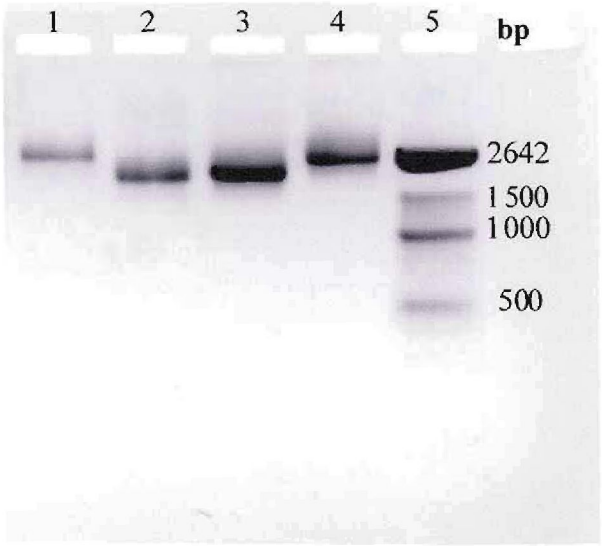


Figure 3.2: Agarose gel (1.5%) of different strains of cyanobacteria extracted with Protocol 3 were generated via conventional PCR using the 16S27F primer from Wilmotte *et al.* (1993) and the 23S30R primer from Taton *et al.* (2003) and references therein. Lane 1: PCR product with DNA from *M. aeruginosa*; Lane 2: PCR product of DNA from *Planktothrix sp.*; Lane 3: PCR product with DNA of *Spirulina sp.*; Lane 4: PCR product of DNA from *Synechococcus sp.* Lane 5: molecular weight marker XIV (Roche diagnostics).

From figure 3.2 it was clear that strains from different cyanobacterial species amplified successfully with the use of extraction protocol 3 (described in section 3.2.1.3) combined with the ammonium acetate clean-up. This sample preparation did not discriminate between the different types of fresh material from the different species tested and was used for all further DNA isolations.

3.3.2 Conventional PCR analysis

3.3.2.1 Evaluation of 16S rDNA primer sequences for DNA protocols

To evaluate the DNA purification protocol, a fragment of ±470 bp of the cyanobacterial 16S rRNA gene was amplified with primers designed by Nübel *et al.* (1997) (table 3.1). These amplification products were used to evaluate the quality of the DNA during the optimisation of the DNA extraction procedure.

Figure 3.3: Agarose gel (1.5%) of the 16S rDNA PCR product amplified at 55°C with different primers from Nübel *et al.* (1997) and DNA from different cyanobacteria species (see table 3.1); Lane 1: molecular weight marker XIV (Roche diagnostics); Lane 2: PCR with Primer CYA 359F and CYA 781R(a+b) with DNA from *M. aeruginosa*. Lane 3: PCR with Primer CYA 359F and CYA 781R(a+b) with DNA from *Oscillatoria sp.*; Lane 4: PCR with primer CYA 359F and CYA 781R(a+b) and DNA from *Spirulina sp.* (Gert); Lane 5: PCR with Primer CYA 359F and CYA 781R(a+b) with DNA from *Spirulina sp.* (BFN); Lane 6: PCR with Primer CYA 359F and CYA 781R(a+b) with DNA from *Arthruspira sp.* Lane 7: PCR with primer CYA 106F and CYA 781R(b) with DNA from *Microcystis sp.*; Lane 8: PCR with primer CYA 106F and CYA 781R(b) with DNA from *Oscillatoria sp.*

Figure 3.3 represented an amplification product in lane 2 of approximately 470 bp with DNA extracted with protocol 1 from a culture of *M. aeruginosa* using primers CYA359F and CYA781R (a+b) of Nübel *et al.* (1997). An amplification product is also seen in lane 7 and 8 with DNA from *M. aeruginosa* and *Oscillatoria sp.* using primers CYA106F and CYA 781R(b). From this figure it was clear that the DNA was best amplified with *Microcystis sp.* whereas the filamentous species did not amplify or amplified poorly.

3.3.2.2 mcyB primers (Neilan *et al.*, 1999) *and mcyE primers* (Vaitomaa *et al.*, 2003) *using conventional PCR*

Isolates from the local culture collection were firstly screened for potential microcystin producers to be able to use them as positive controls for subsequent analyses. In figure 3.6 DNA

from *Microcystis sp.* (Loch Vaal) and *Microcystis sp.* (UV027) were tested for potential microcystin production.

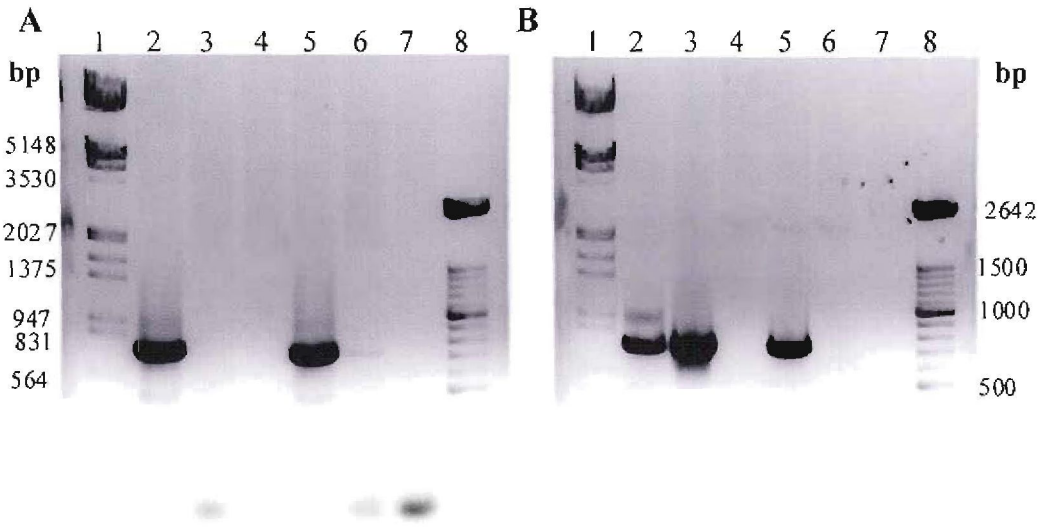


Figure 3.4: Agarose gel (1.5%) of the PCR products after amplification with the 16S rDNA, *mcyB* and *mcyE*-micr primers on *Microcystis sp.* DNA at 58°C. Lane 1: Molecular weight marker III (Roche diagnostics); Lane 8: Molecular weight marker XIV (Roche diagnostics). **A)** Lane 2: PCR product of the 16S primer and DNA of *Microcystis sp.* (Loch Vaal); Lane 3: PCR product of *mcyB* primer and *Microcystis sp.* (Loch Vaal); Lane 4: PCR product for *mcyE* primer pair of Vaitomaa *et al.* (2003) and *Microcystis sp.* (Loch Vaal); Lane 5: PCR product of *Microcystis sp.* (UV027) with the 16S primer; Lane 6: PCR product of *mcyB* primer and *Microcystis sp.* (UV027); Lane 7: PCR product for *mcyE* primer of Vaitomaa *et al.* (2003) and *Microcystis sp.* (UV027); **B)** Lane 2: PCR product of the 16S primers with *M. aeruginosa* PCC 7806. Lane 3: PCR product with *mcyB* primer and *M. aeruginosa* PCC 7806; Lane 4: PCR product of *mcyE* primer of Vaitomaa *et al.* (2003) with *M. aeruginosa* PCC 7806; Lane 5: PCR product of 16S with *M. aeruginosa* CCAP 1450/1 (non toxic). Lane 6: PCR product of *mcyB* and *M. aeruginosa* CCAP 1450/1; Lane 7: PCR product of *mcyE* primer of Vaitomaa *et al.* (2003) and *M. aeruginosa* CCAP 1450/1;

From figure 3.4A it was clear that although the 16S rDNA primer (positive control), amplified fragments for both *Microcystis sp.* (Loch Vaal) and *Microcystis sp.* (UV027), both species are potentially non-microcystin producers as indicated by the fact that no PCR product was observed for the *mcyB* and *mcyE*-micr primers. Thus it can be deduced that these strains in the local culture collection are not potential toxin producers. Therefore, there had to be resorted to the use of known toxin producers from international culture collections.

From figure 3.4B it can be observed that there was no amplification for the nontoxic strain, *M. aeruginosa* CCAP 1450/1 with both the *mcyE*-micr and *mcyB* primers (lane 6 and 7), indicating

that this species is not toxic. However, an amplification product was observed in lane 3 for the *mcyB* primer for the toxic *M. aeruginosa* PCC 7806 but only a very faint band was visible in lane 4 for the *mcyE*-micr primer. As it is known that *M. aeruginosa* PCC 7806 is a toxic strain, both primers were suppose to amplify a PCR fragment. With re-adjustment of the annealing temperature to 50°C both primers generated a single fragment (figure 3.5).

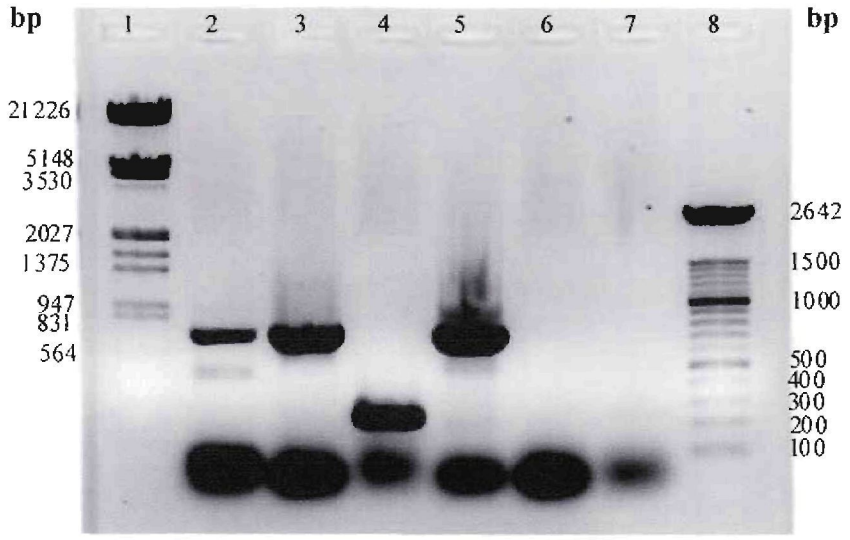


Figure 3.5. Agarose gel (1.5%) of the PCR product after amplification with primers 16S, *McyB* and *McyE* primer pairs and DNA from different cyanobacteria species at 50°C. Lane 1: Molecular weight marker III (Roche diagnostics); Lane 2: PCR product of 16S primer with DNA from *M. aeruginosa* PCC 7806 (toxic strain); Lane 3: PCR product of *McyB* primers with *M. aeruginosa* PCC 7806; Lane 4: PCR product of *Microcystis sp.* specific *McyE* primers, with DNA of *M. aeruginosa* PCC 7806. Lane 5: PCR product of 16S primer of *M. aeruginosa* (vd Bijl) Lane 6: PCR product for *McyB* primer and DNA of *M. aeruginosa* (vd Bijl); Lane 7: PCR product for *McyE*-micr primer and *M. aeruginosa* (vd Bijl); Lane 8: Molecular weigh marker XIV (Roche diagnostics).

Figure 3.5 clearly indicates that the *Microcystis* PCC 7806 is a toxic strain, and amplification for both the *McyB* and *McyE* amplicons occurred. In lanes 5-7 the DNA of *M. aeruginosa* isolated from Van der Bijl showed no amplification for both the *McyE* and *McyB* primers, but only for the 16S rDNA primers, indicating that this strain of *Microcystis sp.* (van der Bijl) are not toxic.

To confirm the potential microcystin production of the strains, the strains of all the *Microcystis sp.* from the local and international culture collections were tested by the Department of Water Affairs and Forestry by ELISA as indicated in table 3.6.

Table 3.6: Potential microcystin production of *M. aeruginosa* strains in culture.

Strain	Result (ppb)
Microcystis sp. Loch vaal	< 0.18
Microcystis sp. UV027	<0.18
M. aeruginosa PCC 7806	>2.5
M. aeruginosa CCAP 1450/1	<0.18
Microcystis sp. Vd Bijl	<0.18

<0.18 – not detected

All the environmental samples of the Hartbeespoort Dam as well as the Roodeplaat Dam were tested with the 16S rDNA primer (positive control), the *mcyB* primer and *mcyE*-micr primer to verify if they were potentially toxic or not and how the different fragments associated with each other.

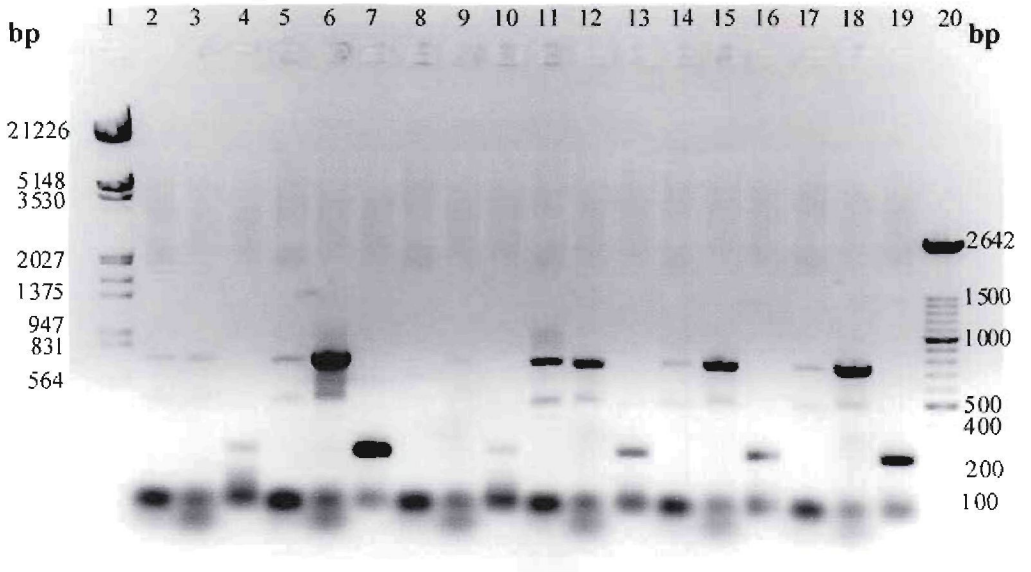


Figure 3.6: Agarose gel (1.5%) indicating the presence of toxic genes in the environmental samples. Lane 1: molecular weight marker III (Roche diagnostics). Lane 2,3 and 4 are respectively the 16S rDNA PCR product, the *mcyB* PCR product and the *mcyE* PCR product for one environmental sample. Lanes 5,6 and 7 are the same for the next environmental sample, and this was repeated for all the environmental samples. lane 20: Molecular weight marker XIV (Roche diagnostics).

The gel indicated in figure 3.6 was a typical example of one of the gels that was run for the PCR products of all the environmental samples.

It can be observed that the amplification of the *mcy* amplicons are not always consistent and substantial amounts of primer dimers were observed with most of the samples. This may be due to the primer design or enzymes that did not function properly.

3.3.3 Real-time PCR analysis

Primers that are badly designed will influence the data as well as the efficiency of the reaction. The efficiency of the reaction need to be between 90-105% (DNAbiotec, 2006) as this will ensure that false quantification caused by PCR inhibitors, badly designed primers or by competitive conditions in the PCR will not occur. Therefore proper design and testing of primers are crucial for any qPCR analysis (Mbedi *et al.*, 2005). Primer design and evaluation were initially evaluated using unialgal cultures from the North West culture collection.

3.3.3.1 16S rDNA primer

For optimal qPCR analysis, the amplified product should not exceed 250 bp in length as it may affect the efficiency of the reaction. The initial amplification products for the 16S rDNA, *mcyB* and *mcyE* amplicons were longer than 200 bp. This might explain why the efficiencies were not satisfactory enough.

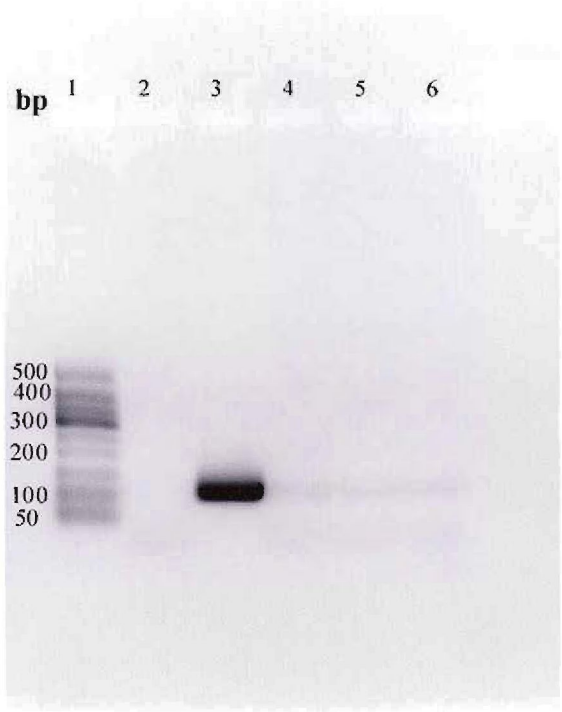


Figure 3.7: Agarose gel (1.5%) of the PCR amplification of the 16S primer at 60°C. Lane 1: 50 bp molecular weight marker (Qiagen); Lane 2: no template control; Lane 3: PCR amplification product for *M. aeruginosa* PCC 7806; Lane 4: PCR amplification product for *P. pseudagardhii*; Lane 5: PCR amplification product for *Planktothrix agardhii*; Lane 6: PCR amplification product of *Anabaena sp.*

From figure 3.8 it can be observed that the 16S primers by Rudi *et al.* (1997) are *Microcystis sp.* specific and a very clear, dark band could be observed at 93 bp. Very light bands are visible for the other species tested, but these bands were so faint that it was not detected by the iCycler software (figure 3.12).

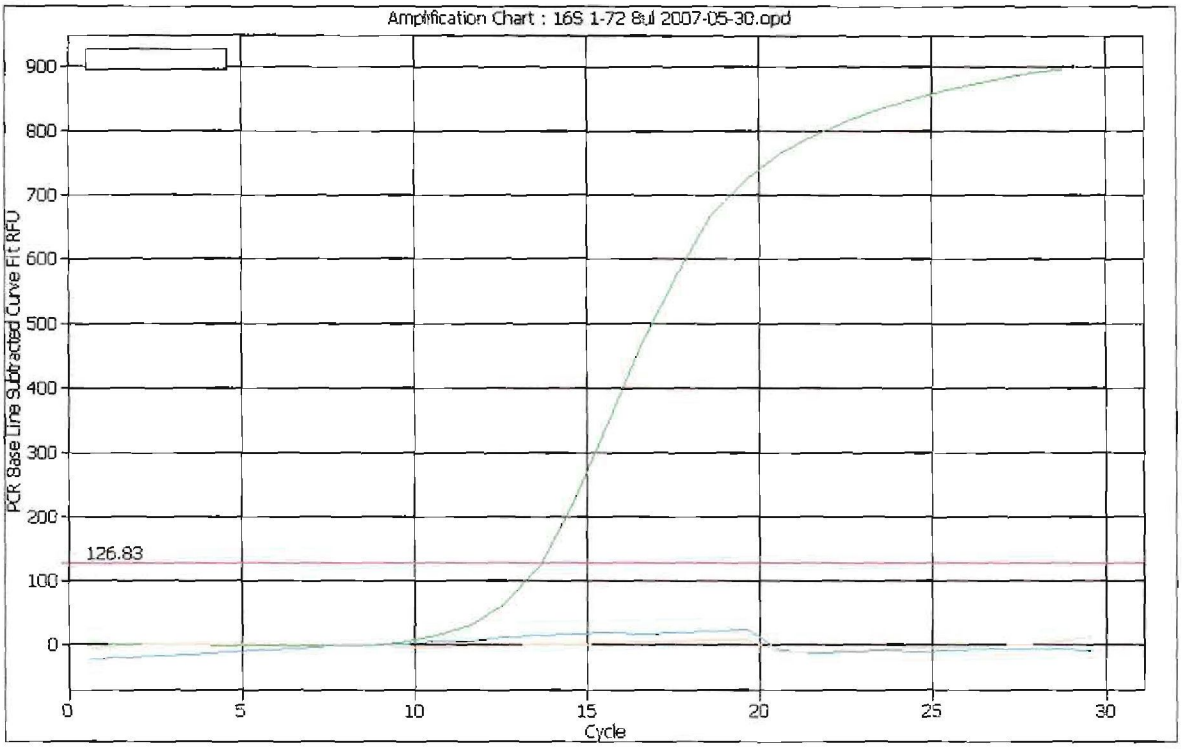


Figure 3.8: Amplification chart of a qPCR reaction with the iCycler with the 16S primers at 60°C. Green line: amplification of DNA from *M. aeruginosa* PCC 7806. Blue line: No template control containing the same reaction mixture but with no DNA. Grey line: Negative control containing DNA from *P. pseudagardhii* NIVA CYA 126/8.

From the amplification chart it is clear that the iCycler does not detect any product from the qPCR reaction with the 16S rDNA primers and DNA from *P. pseudagardhii.* The DNA from *M. aeruginosa* however amplified with the same reaction. Therefore it was safe to use the primers as species specific primers as there would be even less of these species present in the environmental samples than in the cultured samples. The 16S *micr* primers were used for all future amplifications of the 16S rRNA gene at a temperature of 60°C.

3.3.3.2 mcyB primers

The iCycler provides the option for optimisation using a temperature gradient. Therefore, a conventional PCR with primers (IDT) of *mcyB* and *mcyE* was done to determine the optimum reaction setup with a gradient on the Bio-Rad iCycler. Results not shown.

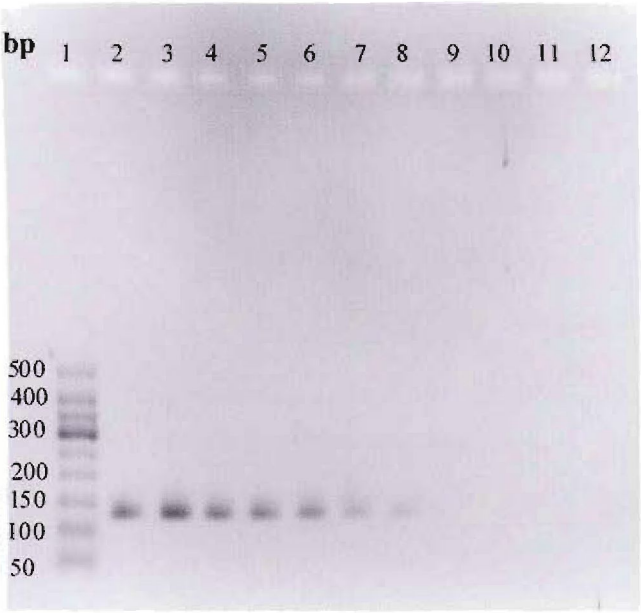


Figure 3.9: Agarose gel (1.5%) of the amplification products of a conventional PCR reaction with newly designed *mcyB* primers. Lane 1: 50 bp Molecular weight marker (Qiagen); Lanes 2-8: concentration gradient with *mcyB* primers and *M. aeruginosa* PCC 7806; Lane 9: Negative control, *M. aeruginosa* CCAP 1450/1; Lane 10: *P. agardhii*; Lane 11: *P. pseudagardhii*; Lane 12: No template control.

From figure 3.9 it was clear that the newly designed *mcyB* primer worked very well with the conventional PCR and the negative controls showed no amplification. These primers were then used for the determination of the copy number of the *mcyB* amplicon in *Microcystis sp.* The amplification efficiencies ranged between 90% and 100%.

3.3.3.3 mcyE primers

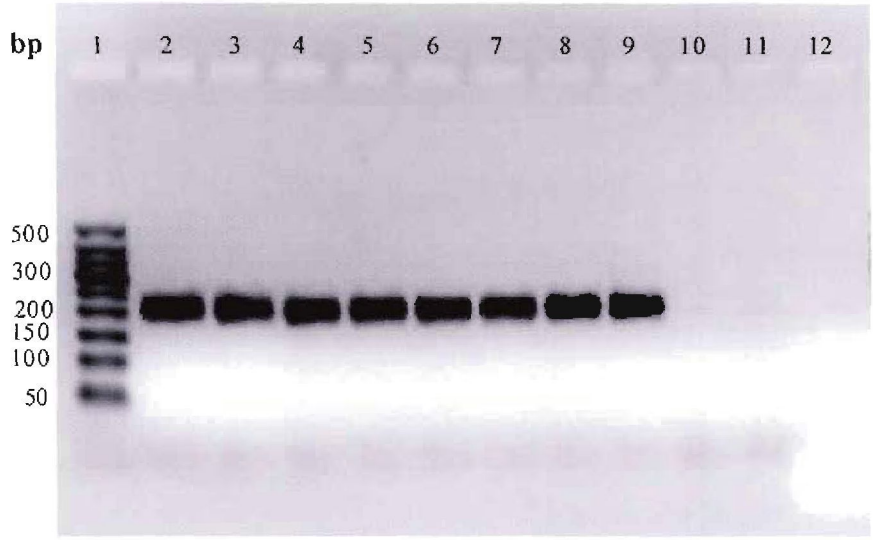


Figure 3.10: Agarose gel (1.5%) of the PCR product of newly designed *mcyE* primers. Lane 1: 50 bp molecular weight marker (Qiagen); Lanes 2-9: different concentrations of DNA used in the PCR reaction with *M. aeruginosa* PCC 7806; Lane 10: *M. aeruginosa* CCAP 1450/1; Lane 11: *P. pseudagardhii*; Lane 12: No template control.

From the results (figure 3.10) it was clear that for the conventional PCR the *mcyE* primers worked for toxic species of *M. aeruginosa* PCC 7806 (lanes 2-9) and did not amplify with other species or non-microcystin producing species (lane 10-12).

These primers were then used for the determination of the copy number of the *mcyE* amplicon in *M. aeruginosa*. The amplification efficiencies were between 90% and 100%.

3.3.4 Reverse transcriptase real-time PCR analysis (qRT-PCR)

It was decided to use the 16S rRNA gene as an internal standard. The ribosomal RNAs may be used as internal controls and are less likely to fluctuate under conditions that affect the expression of other mRNAs (Ambion, 2007). rRNA constitutes 80% of a total RNA sample, such that when the concentration of a total RNA sample is determined from spectrophotometric readings, the sample is essentially already being normalised to the amount of rRNA it contains (Ambion, 2007)

Only one 16S rDNA copy exists for each cell and can therefore be used as reference gene to calculate the ratio of expression of the other genes that are to be expressed (Ambion, 2007).

3.3.4.1 NtcA primer

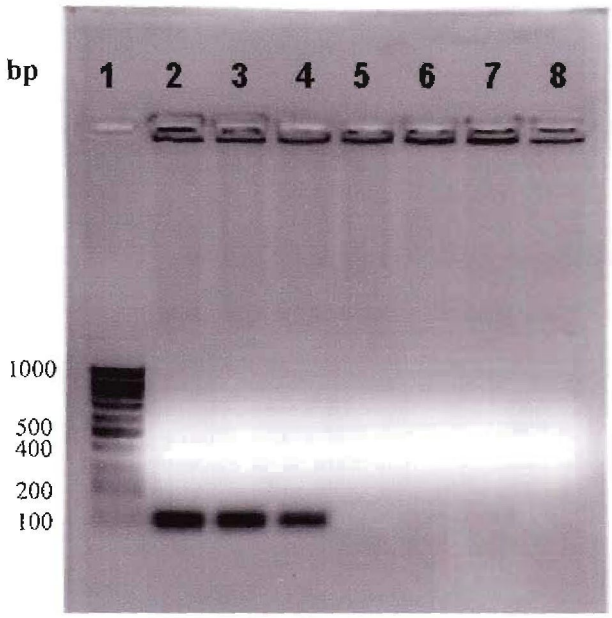


Figure 3.11: Agarose gel (1.5%) with PCR products of the *ntcA* primers with different DNA. First lane: 100 bp Molecular weight marker (Fermentas); Lane 2: PCR product after amplification of DNA from *M. aeruginosa* PCC 7806; Lane 3: PCR product after amplification of DNA from *M. aeruginosa* CCAP 1450/1; Lane 4: PCR product after amplification of DNA from environmental sample containing *Microcystis sp.*; Lane 5: PCR product after amplification of DNA from *P. agardhii* NIVA-CYA 126; Lane 6: PCR product after amplification of DNA from *P. pseudagardhii* NIVA-CYA 153/1; Lane 7: PCR product after amplification of DNA from *Synechococcus sp.*; Lane 8: No template control.

These primers were tested with *M. aeruginosa* PCC 7806 and with DNA from other species. Amplification was observed for *Microcystis sp.*, but no amplification was observed for any of the other species (figure 3.11). From figure 3.11 it can be seen that the *ntcA* primers are specific for *Microcystis* species.

The partial sequence for the *ntcA* gene for nitrogen regulatory protein of *Microcystis aeruginosa* PCC CCAP 1450/1 are deposited on the NCBI website (http://www.ncbi.nlm.nih.gov/entrez/viewer) under the accession number: AM411991.

3.3.4.2 RbcL primers

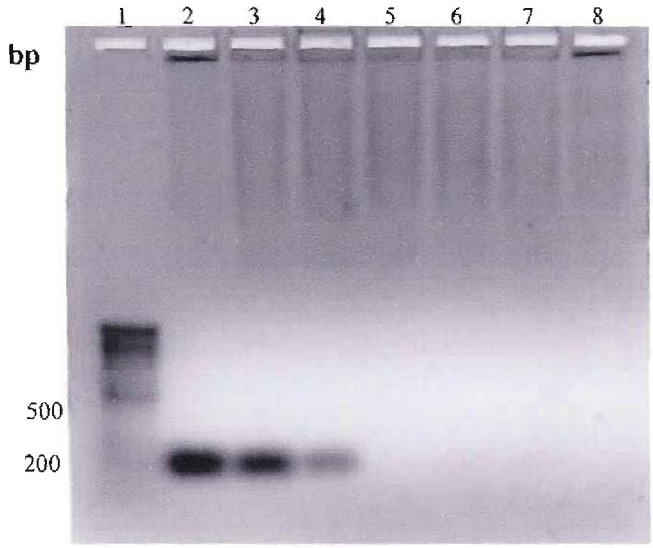


Figure 3.12: Agarose gel (1.5%) of the PCR amplification of part of the *rbcL* gene. Lane 1: 100 bp Molecular weight marker (Fermentas); Lane 2: PCR amplification product of the *rbcL* gene of RNA from *M. aeruginosa* PCC 7806; Lane 3: Amplification product of the *rbcL* gene of RNA from *M. aeruginosa* CCAP 1450/1. Lane 4: Amplification product of the *rbcL* gene of RNA of an environmental sample with *Microcystis* present. Lane 5: Amplification product of the *rbcL* gene of RNA from *Planktothrix agardhii* 126/8 (toxic species). Lane 6: Amplification product of the *rbcL* gene of RNA from *P. pseudagardhii* NIVA CYA 153/1 (non-toxic species). Lane 7: Amplification product of the *rbcL* gene of RNA from *Synechococcus sp.* Lane 8: No template control.

It was found that the *rbcL* primers amplified only with DNA from *Microcystis sp.* and no amplification was observed when DNA from other cyanobacteria and algae was used (figure 3.12). Thus it is clear that these primers are *Microcystis sp.* specific.

The partial *rbcL* gene sequence for *M. aeruginosa* PCC 7806 *rbcL* gene shared 100% homology in sequence with the *M. aeruginosa* PCC 7806 sequence (AM157793.1) deposited by T.N. de Marsac, on 13 Nov 2005 on Genbank and was therefore not submitted (see appendix).

The standard error for the amplification efficiencies for the RNA samples were: ±0.85 and the standard deviation ±3.5

Table 3.7: qRT-PCR efficiencies calculated by the iCycler software for each reaction using the newly designed primers for *ntcA* and *rbcL* and the 16S rDNA primers of Nübel *et al.* (1997).

16S	**Efficiency**	***ntcA***	**Efficiency**	***rbcL***	**Efficiency**
16S 11-59	90.1	*ntcA* 11-59	93	*rbcL* 11-58	96
16S 60-104	89.1	*ntcA* 57-80	94	*rbcL* 59-104	97.7
16S 105-150	91.8	*ntcA* 81-150	90	*rbcL* 105-150	95
16S 11-59	98.5	*ntcA* 11-58	96	*rbcL* 11-58	97.8
16S 59-104	100.8	*ntcA* 59-104	98.5	*rbcL* 59-104	91.2
16S 105-150	95.9	*ntcA* 105-150	98.9	*rbcL* 105-147	94.2
Average	94.37		95.07		95.32

As depicted in table 3.7 it can be observed that the reaction efficiencies for all the reactions were nearly the same and very good with a standard error of only 0.85.

3.3.5 Sequencing

In order to study the gene expression of specific genes of cyanobacteria, it is necessary to firstly identify the species that will be used as reference strains. Cyanobacterial species are often wrongly identified by microscopic examination and some of these species can also change certain morphological characteristics under different environmental circumstances or in culture conditions. Therefore it is necessary to identify the species by molecular and phylogenetic study (see chapter 4). For classification of species, it is recommended to amplify the whole 16S rDNA sequence. Therefore primers to amplify longer sequences were used for the sequencing. These primers were tested in order to find the best amplification product for sequencing.

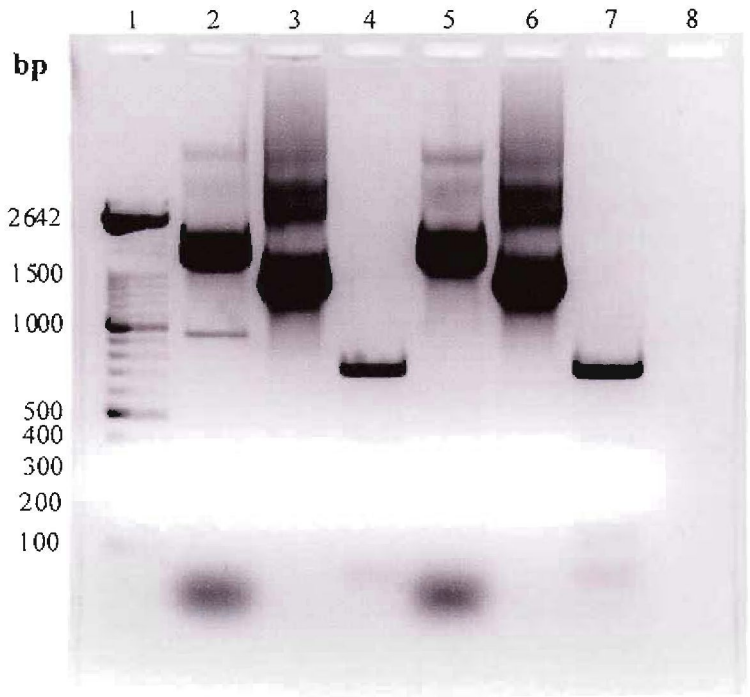


Figure 3.13: Agarose gel (1.5%) of the amplification product of the different 16S rDNA primers used with DNA from *Planktothrix pseudagardhii* (lanes 2-4) and *P. pseudagardhii* NIVA CYA 153/1 (lanes 5-7). Lane 1: Molecular weight marker XIV (Roche diagnostics); Lanes 2 and 5: PCR product of primer 16S27F and 23S30R; Lanes 3 and 6: PCR product of primer 16S27F and primer 1494R; Lanes 4 and 7: PCR product of primer 16S727F and primer 1494R.

Although all the primers have amplification products in figure 3.13 it was clear that primer 16S727F and primer 1494R (lane 4 and 7) have only one clear band with very little other products and these primers were chosen for the conventional PCR analyses of the 16S rDNA primers.

Table 3.8: List of all the primers used during this study.

Strain	Primer set	Sequence 5'-3'	Product Length	Reference	Tm (°C)
Microcystis sp. specific 16S rDNA primers	Micr 16S F	AGCCAAGTCTGCCGTCAAATCA	118bp	Rudi *et al.* 1997.	60°C
	Micr 16S R	ACCGCTACACTGGGAATTCCTG			
Microcystis sp. specific *mcyB* primers	*mcyB*-F	GCTGCGGTTTGGGAGTTATG	126 bp	Conradie, KR	55°C
	mcyB R	GCTGACGGTGATTTCTTGGG			
Microcystis sp. specific *mcyE* primers	*mcyE*-F	AGTCATTTCGGGTTGGTTATG	200 bp	Conradie, K.R.	58°C
	mcyE-R	CAGAAACTTGTGTGGGAGTATC			
Microcystis sp. specific *ntcA* primers	*ntcA*_CCAP_F	AGGAAATTACCGTGGCTCTACTGC	63 bp	Conradie, KR	60°C
	*ntcA*_CCAP_R	CCAGTCAGTAAGGATAGCACACCA			
Microcystis specific *rbcL* primer	*rbcL*_PCC_F	AATTCTGCCGCGACAAAGGGTTAC	180 bp	Conradie, K.R.	60°C
	*rbcL*_PCC_R	TGATTCCGCGTTCACCTTCGAGTT			
Planktothrix specific *mcyB* primer	*mcyB*.fw	ATTACAGCAGAGAAAATCCAAGCA	93 bp	Mbedi *et al.* 2005	50°C
	mcyB.rw	TCGCAATAGCGGGATCA			

3.4 Discussion

Optimisation of DNA extraction procedures, PCR amplification and PCR conditions can be very time consuming and costly, but it is absolutely essential for proper data sets and good scientific practice. Therefore, much time and attention have been given during this study to optimise each step in this multistep process.

3.4.1 DNA purification and cell type differences

It is imperative to find a protocol of DNA isolation that does not discriminate between the different cyanobacterial species. The isolation of all the different cyanobacterial species represented in the dams should ultimately result in the same amount of isolated DNA as well as the same amount and quality of PCR fragments amplified to ensure accurate results.

Cyanobacteria are gram-negative bacteria with a thick peptidoglucan layer that ensures resistance to routine cell disruption methods (Pan *et al.*, 2002) and like plant material, it is not easy to lyse the cells to release the cell content with the DNA.

From the results it was clear that the DNA of filamentous strains of cyanobacteria does not amplify as easily as the single celled strains. The filamentous strains of cyanobacteria usually have a thick slyme layer (glycocalyx) that surrounds the cell wall which consists of sugars and proteins (Stal, 2000). This slyme layer is visible under a light microscope and aid in their motility (gliding) (Stal, 2000; Conradie *et al.*, 2008). The polysaccharide content may thus be very high in the sample and DNA that is negatively charged might bind to positively charged polysaccharides and impurities and be trapped in the insoluble polysaccharide. These polysaccharides and other impurities that surround the cell are very difficult to eliminate with the DNA extraction protocols and tend to block the binding of primers and other enzymes to the DNA because they form a shield around the DNA. It is thus clear that the DNA extraction from cyanobacteria can be quite difficult and time consuming and special care must be taken to remove all the polysaccharides and proteins in the sample that might impede any enzyme reaction. Therefore, a method to eliminate the polysaccharides and impurities from the DNA needed to be found to perform efficient PCR reactions.

The use of ammonium acetate has proven to be a successful extra step in the purification of DNA from both single celled and filamentous strains of cyanobacteria.

3.4.2 Conventional PCR amplification

Baker *et al.* (2002) showed that bloom components can be identified and monitored for toxigenicity by PCR more effectively than by other methods such as microscopy and mouse bioassay. This technique also has the advantage of being specific for cyanobacteria in the presence of other organisms and can even be specific towards a specific species. By using primers for PCR amplification, even toxigenic strains in bloom samples can be distinguished (Tillett *et al.*, 2001).

Since DNA from other organisms were also present in the samples, the primers should also be specific for cyanobacteria and should not amplify DNA from other organisms that might be present in the samples. The amplification of the 16S rRNA gene fragment concomitantly with microcystin genes during this phase of the research will act as an internal control to verify that

the reaction was successful. This is necessary since not all strains present in the environmental samples contain microcystin genes.

3.4.2.1 mcyB and mcyE primers

The *mcyB* amplicon is well described in literature and generally used as an indicator of toxicity in cyanobacterial strains (Neilan *et al.,* 1999). Vaitomaa *et al.* (2003) reported that the *mcyE* gene can be used as a surrogate for microcystin-producing cyanobacteria. Mbedi *et al.* (2005) found that the *mcyE* amplicon is the most suitable for the detection of microcystin-producing *Planktothrix* strains and confirmed that *mcyE* is a very solid molecular marker for microcystin producing *Planktothrix sp.,* since it does not occur in non-microcystin-producing species and the amplicons are not variable. Mbedi *et al.* (2005) found that the *mcyB* and *mcyE* amplicons were conserved to a differing degree. There were 19 variable base pairs detected in the 555 bp *mcyB*-amplicon, while only one variable base pair was found in the 589 bp long *mcyE*-amplicon.

According to their data (Mbedi *et al.,* 2005), the *mcyT* and *mcyTD* amplicons are inadequate regions for the detection of microcystin-producing *Planktothrix sp.* since they also occur in non-producers. Furthermore Mbedi *et al.* (2005) also found that the *mcyA* gene fragment was not detected in nine of the twelve microcystin producing strains of *Planktotrix* and that the *mcyTD* and *mcyEG* amplicons are too variable for the identification of microcystin production. Therefore, the *mcyE* and the *mcyB* amplicons (primers used – table 3.2) were tested on environmental samples in the Hartbeespoort Dam and the Roodeplaat Dam.

3.4.3 Real-time reverse transcriptase PCR analysis (qRT-PCR)

3.4.3.1 16S rRNA gene expression

According to available literature (Ambion, 2007) the 16S rRNA gene is constant in its expression and is not influenced by environmental factors and is therefore a good internal reference for gene expression studies.

3.4.3.2 ntcA and rbcL gene expression

Studies have shown that nitrogen and carbon assimilation may be two of the main role players in toxin production (Rapala *et al.,* 1997; Johansson and Granéli, 1999; Su *et al.*, 2005; Gobler *et al.,* 2007). Therefore the gene expression of the *ntcA* gene (regulating nitrogen assimilation) and the *rbcL* gene (indicative of carbon assimilation) were measured to determine the *in situ* regulation of these genes in the environmental samples.

Most studies were done under cultured conditions and focused on *in vivo* measurements. This study might shed some light on the regulation of these genes *in situ* and the major mechanisms responsible for toxin production.

The newly designed primers (*ntcA* and *rbcL*) described in section 3.2.4 worked very well and were therefore used for the molecular ecology study (see chapter 5).

3.4.4 Primers deposited on NCBI

The primers that were designed for amplification of a part of *ntcA* gene were specific for *Microcystis sp.* and one clear band was obtained. Therefore the primers for *Microcystis aeruginosa* CCAP 1450/1 partial *ntcA* gene for nitrogen regulatory protein were deposited at http://www.ncbi.nlm.nih.gov/entrez/viewer under the accession number AM411911.

3.4.5 Sequencing

According to Wilmotte (1994), the extensive variations in the ITS sequence render the alignment of cyanobacterial 16S rRNA-23S rRNA spacer regions impossible. Iteman *et al.* (2000) have observed multiple ITS products in PCR amplifications of many cyanobacteria, in particular among the filamentous heterocystous strains. However, no comparative analyses are available for the ITS regions that have been sequenced so far and the differences between the spacer regions of different lengths within a single cyanobacterial genome have not been examined (Iteman *et al.*, 2000). Therefore, the ITS sequence was not used for the phylogenetic analysis and was therefore not sequenced with the 16S rRNA gene. Using the primers described in table 3.4, the almost complete 16S rRNA gene of the species described in section 3.2 were amplified and used in the taxonomic study as described in Chapter 4.

3.4.5.1 Data processing

As noted previously ARB was initially used as program for sequence data processing. ARB (latin, "arbor" = tree) (http://www.arb-home) is a UNIX-based, graphically interfaced program for handling large sequence databases (Ludwig *et al.*, 2004). It is a tree-based hierarchical system to keep sequences in order, also available online and made use of a Linux operating system (Ludwig *et al.*, 2004).

PhredPhrap was used to align and edit the sequences. The software was available online (http://www.phrap.org) and also made use of a Linux operating system.

These packages were used for alignment and editing of the sequences, as they are easy to use and are capable of handling many sequences. However, with the second set of sequences obtained from UCT, problems were encountered with the software and the new sequences could no longer be manipulated in PhredPhrap.

All the sequences were then exported to BioEdit v 7.0.9 (Biological sequence alignment editor for Windows) (Hall, 2007) and all further alignment and phylogenetic analysis were done with BioEdit. BioEdit is an easy-to-use sequence alignment editor and sequence analyser (Hall, 2007). BioEdit supply a single program that can handle most simple sequence alignment editing and manipulation functions, as well as a few basic sequence analyses (Hall, 2007) (see appendix for sequences).

3.5 Conclusion

As supported in literature (Werbrouck *et al.*, 2007) it is clear that every step of this multistep procedure can have a significant influence on the outcome of the data.

We have described modifications to the extraction procedure that improves the amount and quality of the DNA and also permits the extraction of DNA from environmental samples with different species present. qRT-PCR is a very sensitive technique and it is therefore absolutely crucial to standardize all the DNA or RNA samples to be compared.

The design and sensitivity of the primers play an important role in qRT-PCR analysis. New species-specific primers were designed for the qRT-PCR reactions to ensure better amplification and efficiency of the reaction.

We have found that the sensitivity of the methods described here permits the accurate quantification of the gene expression of cyanobacterial species in environmental samples. From the results environmental variables that influence gene expression and potential microcystin production can then be identified.

Chapter 4

Phylogenetic study

The contents of this chapter have been published in the South African Journal of Botany, 74(2008): 101-110 (accepted 7 September 2007).

4.1 Introduction

In order to study the gene expression of specific genes of cyanobacteria occurring naturally in the water systems of South Africa, it is necessary to firstly identify the species. Cyanobacterial species are often wrongly identified by microscopic examination, since cyanobacteria have only a few morphological characteristics and can easily be misidentified. Some of these species can also change certain morphological characteristics under different environmental circumstances or in culture conditions. To understand the ecophysiology of cyanobacteria, it is desirable to match isolated strains and their counterparts in nature (Nübel *et al.,* 1997). Only then can physiological data gained from culture studies be confidently extrapolated to natural conditions (Garcia-Pichel *et al.,* 1996).

A polyphasic approach was used for the taxonomic identification of some of the bloom forming species grown in the Culture collection of the North-West University to be used as reference cultures later during this study.

In South Africa there is a real need to identify specific bloom formers in order to develop an early warning system for bloom formation. Cyanobacteria represents only a small proportion of all algal groups present in the Vaal River, but they probably are one of the most problematic because of their toxin-producing, filter-clogging, scum-forming, unaesthetic properties (Venter *et al.,* 2003).

The taxonomy of the Cyanophyceae has often been revised since the last century as a consequence of their relative morphological simplicity and, thus, the restricted set of their characteristics usable for classification (Wilmotte *et al.,* 1992). Furthermore, the stability of phenotypic characters and their use in taxonomy have been questioned by a number of authors (reviewed by Anagnostidis and Komárek, 1988; Suda *et al.,* 2002).

Komárek and Anagnostidis (1989) concluded that the features of more than 50% of strains in collections do not correspond to the diagnoses of the taxa to which they are assigned (Rudi *et al.,* 1997). This is because the morphological and cytological characters of cyanobacteria in laboratory cultures can change with growth conditions and it is therefore often considerably different from the original morphology of the environmental isolates and do not necessarily

reflect evolutionary changes (Pearson and Kingsbury 1966; Rudi *et al.,* 1997). Thus, classification based on microscopic observations alone is not always evolutionary significant (Rudi *et al.,* 1997). Molecular structures and sequences are generally more revealing of evolutionary relationships than what classical phenotypes are (particularly so among micro-organisms) (Woese *et al.,* 1990). Consequently, the basis for the definition of taxa has progressively shifted from the organismal to the cellular to the molecular level.

The primary genetic analysis of these organisms in their natural habitats, has technically been challenging (Giovannoni *et al.,* 1990; Schmidt *et al.,* 1991; Rudi *et al.,* 1997). Therefore, a phylogenetic system based on the 16S rRNA gene sequence information retrieved from organisms in pure cultures has been developed to classify organisms (Giovannoni *et al.,* 1988). The small subunit rRNA (16S rRNA) represents the best-studied sequence (Giovannoni *et al.,* 1988; Schmidt *et al.,* 1991; Nelissen *et al.,* 1994, 1995, 1996). Sequences of 16S rRNA genes are independent from different growth conditions and can be retrieved by PCR from small amounts of DNA extracted from laboratory cultures or natural environments (Giovannoni *et al.,* 1990). The molecular differences found by sequencing 16S rRNA make it possible to accurately distinguish between for example *Microcystis* and *Synechococcus sp.* (Rudi *et al.,* 1997).

The amplification of a mixture of 16S rRNA genes using various "universal" primer sets which, for example, target appropriately conserved regions of the gene is now routine (Ouellette and Wilhelm, 2003). The limit of resolution of 16S rRNA sequence comparison is probably to the genus level (Stackebrandt and Goebel, 1994).

According to Komárek (2005), molecular (phylogenetic) data should be accepted as a basic criterion for classification. The correct classification is impossible without the careful combination of genetic data with morphological diversity and variation, ecological and ecophysiological characteristics, ultra structural studies and without the correct application of convenient formal prescriptions for designation of taxa and strains (Komárek, 2005). This combined methodology is described by Komárek (2005) as a polyphasic approach. Biochemical and molecular data are still too scarce to give a clue to the taxonomy of the entire family of the cyanophyceae. Thus, there is a real need for information concerning the genotypic relationships among cyanophyceae species and the correlation of these relationships with morphology and other characters (Wilmotte *et al.,* 1992).

An identification system based on sequence variation and group-specific PCR amplifications has several practical advantages (Rudi *et al.*, 1997):

(i) the analysis is simple to perform,

(ii) it reflects phylogeny and evolution and

(iii) the results are easy to interpret and allow for automation (that is, the results are represented by, e.g., presence or absence of PCR fragments and not complex morphological or cytological characters) (Rudi *et al.*, 1997).

Bacterial rRNA genes are commonly organised in an operon in the order 16S rRNA-23S rRNA-5S-rRNA, with each rRNA gene being separated by an internal transcribed (ITS) region (Iteman *et al.*, 2000). According to Boyer *et al.* (2001) some investigators have questioned whether sufficient variability exists in 16S RNA to allow discrimination among species of a genus or strains of a species. It has been suggested that the ITS region separating 16S and 23S sequences in the rRNA operon might be useful for these fine levels of discrimination (Boyer *et al.*, 2001). As a result, researchers have increasingly turned to the more variable 16S-23S ITS (Boyer *et al.*, 2001).

The use of the 16S-23S ITS region in studies of phylogeny, molecular evolution or population genetics is a potentially powerful tool (Boyer *et al.*, 2001). However, investigators need to be aware of potential problems imposed by the possibility of multiple non-identical rRNA operons (Boyer *et al.*, 2001).

Comparison of the cyanobacterial ITS sequences shows that they vary considerably in size (283-545 nucleotides). Consistent with other investigations (Neilan *et al.*, 1997), Iteman *et al.* (2000) have observed multiple ITS products in PCR amplifications of many cyanobacteria, in particular among the filamentous heterocystous strains. According to Wilmotte (1994), the extensive variations in sequence render the alignment of cyanobacterial 16S rRNA-23S rRNA spacer regions impossible, therefore the ITS sequence was not used in this study.

Most of this study concentrated on the re-identification of the organism formerly known as *Oscillatoria simplicissima* (Conradie *et al.*, 2008). Blooms of *O. simplicissima* result in the production of unpleasant odours and tastes in water and a general decline of water quality. Although this cyanobacteria is presumably non-toxic in small concentrations, the magnitude of its blooms cause many logistical problems in extracting and purifying water from the Vaal River,

leading to increases in purification costs and the loss of large volumes of water (Venter *et al.*, 2003).

4.2 Materials and Methods

4.2.1 Strain and strain cultivation

Five strains of cyanobacteria were used to compile a phylogeny based on near complete 16S rDNA sequences: the presumed *Oscillatoria simplicissima* (*"O. simplicissima"*) (figure 4.1), two different cultures of *Spirulina sp.* Turpin (1829) and Gomont (1892) (figure 4.2 and 4.3), *Arthrospira sp.* Stizenberger (1852) and Gomont (1892) (figure 4.4) and *Microcystis aeruginosa* UV 027 (figure 4.5a and 4.5b). These organisms were chosen because of their regional abundance in the Vaal River and their potential to produce toxins (Janse Van Vuuren and Pieterse, 2000; Janse van Vuuren, 2001). The two *Spirulina sp.* cultures were obtained from two different *Spirulina* "farms" where the strains are grown in order to produce the commercially available *"Spirulina"* tablets used as a health source.

In order to establish the phylogeny of the locally isolated strains, true representatives of the various genera were obtained from known culture collections, namely *Microcystis aeruginosa* PCC 7806 from the Pasteur Culture Collection (PCC), *Microcystis aeruginosa* CCAP 1450/1 from the Culture Collection of Algae and Protozoa (UK), *Arthruspira sp.* from Carolina™ (Science and Math. ER-15-1721, www.carolina.com), *Planktothrix pseudagardhii* NIVA CYA 153 from the Norwegian culture collection and the strain of *Planktothrix sp.* 126/8 was provided by K. Sivonen (University of Helsinki, Helsinki, Finland).

Figure 4.1: Light micrograph of "*Oscillatoria simplicissima*" in the culture collection of the NWU isolated from the Vaal River, South Africa.

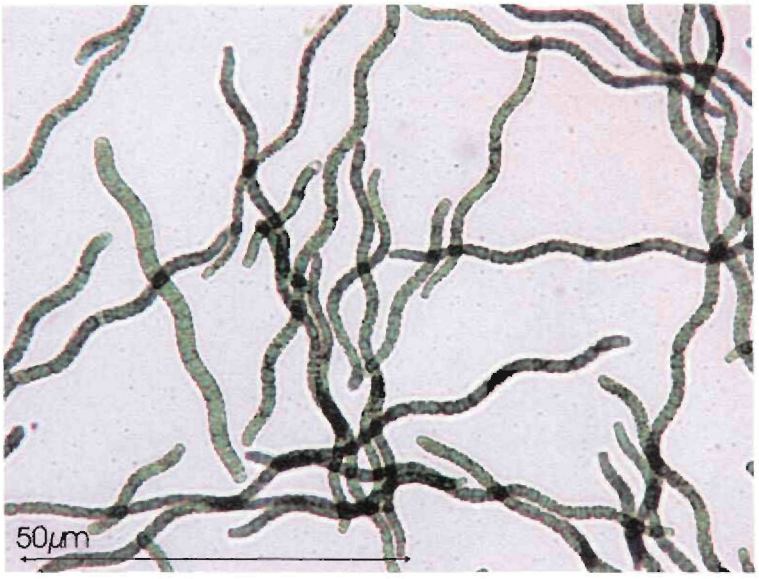


Figure 4.2: Light micrograph of *Spirulina sp.* obtained from the University of the Orange Free State Culture Collection.

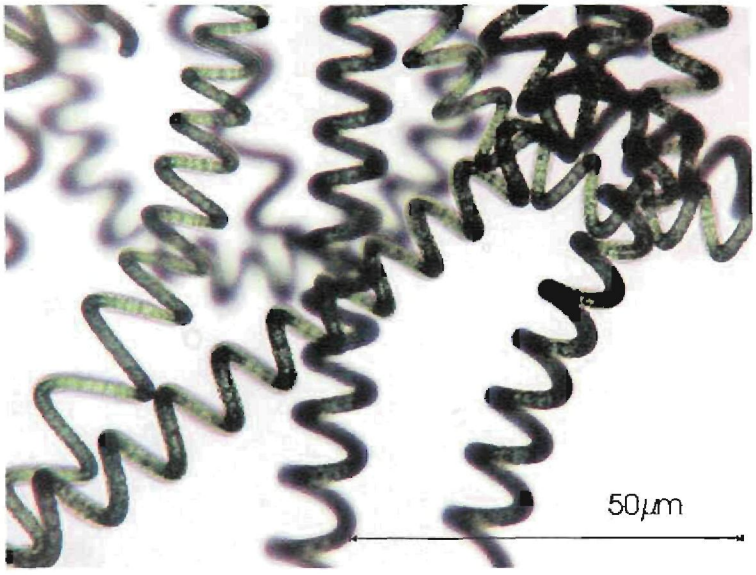


Figure 4.3: Light micrograph of *Spirulina sp.* obtained from a *Spirulina* farm.

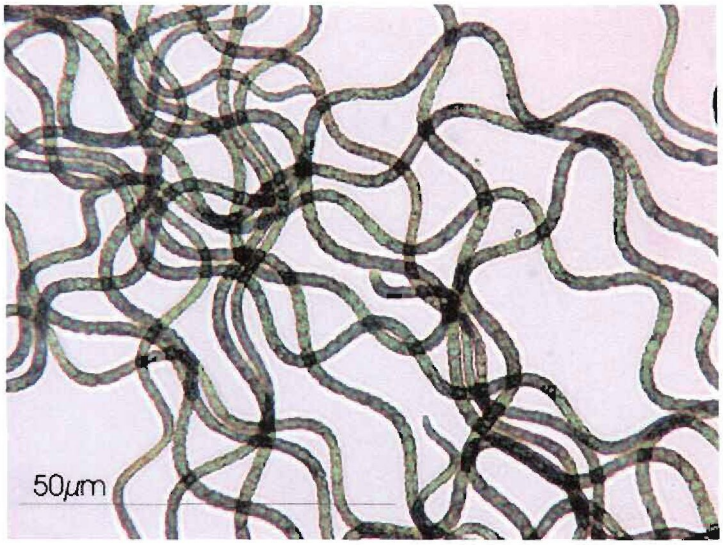


Figure 4.4: Light micrograph of *Arthrospira sp.* obtained from Carolina Culture Collection.

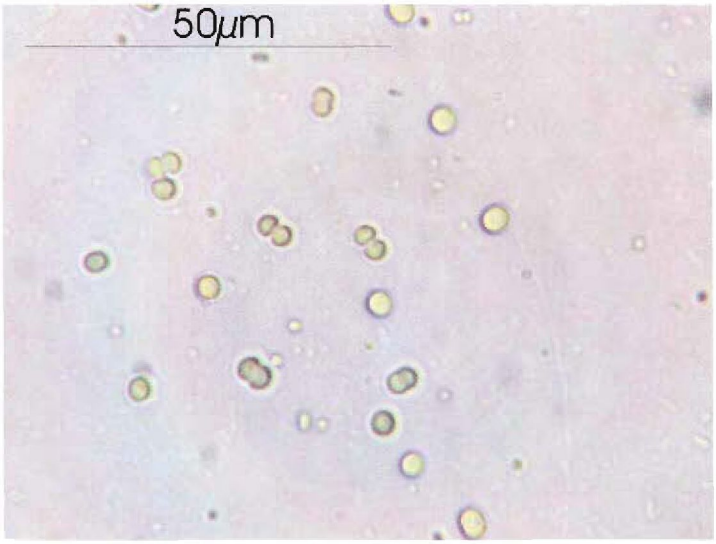


Figure 4.5a: Light micrograph of *Microcystis aeruginosa* UV 027 obtained from University of the Orange Free State Culture Collection.

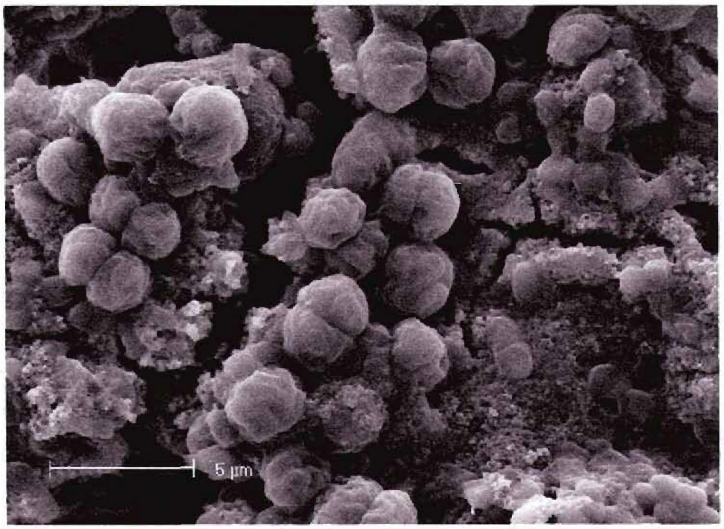


Figure 4.5b: Scanning electron micrograph of *Microcystis aeruginosa* UV 027 obtained from University of the Orange Free State Culture Collection.

Cultures of "*O. simplicissima*" and *P. pseudagardhii* were established in glass flasks containing 100 ml EM medium (table 4.1) (Venter, 2000) and strains of *Arthrospira sp.* and *Spirulina sp.* were cultivated in glass flasks containing 100 ml Zarrouk medium (table 4.2), (Fox, 1996) at constant temperature (24°C) and light (20 $\mu mol.m^{-2}.s^{-1}$). Cultures of *M. aeruginosa* were established in glass flasks containing 100 ml GBG11 medium (table 4.1), (Krüger, 1978) at constant temperature (18°C) and light (20 $\mu mol.m^{-2}.s^{-1}$) (figure 4.6). Cultures were routinely checked for purity by microscopic examination.

Table 4.1: Modified GBG11 medium and EM medium (Krüger, 1978; Venter, 2000).

Macro-nutrients	**Final concentration GBG-11 (pH 9.4)**	**Final concentration EM**
1. $NaNO_3$	150 500 µg l^{-1}	270 000 µg l^{-1}
2. $K_2HPO_4 \cdot 3\ H_2O$	69 300 µg l^{-1}	67 200 µg l^{-1}
3. $MgSO4 \cdot 7\ H_2O$	75 000 µg l^{-1}	77 000 µg l^{-1}
4. $CaCl_2 \cdot 2\ H_2O$	36 000 µg l^{-1}	36 000 µg l^{-1}
5. Na_2CO_3	20 000 µg l^{-1}	40 000 µg l^{-1}
6. EDTA	1 000 µg l^{-1}	0 µg l^{-1}
7. Citric acid	12 000 µg l^{-1}	12 000 µg l^{-1}
8. $FeSO_4 \cdot 7\ H_2O$	10 000 µg l^{-1}	10 000 µg l^{-1}
Micro-nutrients		
1. H_3BO_3	1.43 µg l^{-1}	1.43 µg l^{-1}
2. $MnCl_2 \cdot 4\ H_2O$	0.57 µg l^{-1}	0.57 µg l^{-1}
3. $ZnSO_4 \cdot 7\ H_2O$	0.11 µg l^{-1}	0.11 µg l^{-1}
4. $NaMoO_4 \cdot 5\ H_2O$	0.20 µg l^{-1}	0.20 µg l^{-1}
5. $Co(NO_3)_2 \cdot 6\ H_2O$	0.02 µg l^{-1}	0.02 µg l^{-1}
6. $CuSO_4 \cdot 5\ H_2O$	0.04 µg l^{-1}	0.04 µg l^{-1}
Vitamins		
1. Cyanocoblamin (B12)	0 µg l^{-1}	33.0 µg l^{-1}
2. Thiamine – HCL	0 µg l^{-1}	33.0 µg l^{-1}
3. Biotin	0 µg l^{-1}	330.0 µg l^{-1}

Table 4.2: Zarrouk medium (Fox, 1996).

Macro-nutrients	**Final concentration (g/ℓ)**
$NaNO_3$	2.5 $g.\ell^{-1}$
NaCl	1 $\mu g.\ell^{-1}$
$NaHCO_3$	16.8 $g.\ell^{-1}$
K_2SO_4	1 $g.\ell^{-1}$
K_2HPO_4	0.5 $g.\ell^{-1}$
$MgSO_4.7H_2O$	0.2 $g.\ell^{-1}$
$CaCl_2$	0.04 $g.\ell^{-1}$
$FeSO_4.7H_2O$	0.01 $g.\ell^{-1}$
$EDTA.Na_2$	0.08 $g.\ell^{-1}$
Micro-nutrients	**Final concentration (µg/ℓ)**
1. H_3BO_3	11 44 $\mu g.\ell^{-1}$
2. $MnCl_2 \cdot 4\ H_2O$	4 520 $\mu g.\ell^{-1}$
3. $ZnSO_4 \cdot 7\ H_2O$	880 $\mu g.\ell^{-1}$
4. $NaMoO_4 \cdot 5\ H_2O$	1 560 $\mu g.\ell^{-1}$
5. $Co(NO_3)_2 \cdot 6\ H_2O$	98 $\mu g.\ell^{-1}$
6. $CuSO_4 \cdot 5\ H_2O$	316 $\mu g.\ell^{-1}$

Figure 4.6: Cultures grown in 100 ml of culture medium in regulated chambers at a constant temperature (18°C) and light (20 $\mu mol.m^{-2}.s^{-1}$).

4.2.2 DNA purification

The sample preparation did not discriminate between the different types of fresh material from the different species tested and DNA from all different species was similarly extracted.

Two weeks after inoculation, cyanobacterial cells were concentrated by filtration of 30 ml culture onto glass fibre paper filters. A modified method of Fiore *et al.* (2000) was followed for the purification of DNA.

The filters with the concentrated cyanobacterial cells were firstly washed three times with 10 ml of a solution containing 50 mM Tris-HCl, pH 8.0, 5 mM EDTA and 50 mM NaCl to reduce extracellular impurities.

Filters with the concentrated cyanobacterial cells were incubated in a sterile buffer containing 50 mM Tris-HCl (pH 8.0) and 50 mM EDTA. The filters were then homogenized with a Heidolph DIAX 900 homogenizer for 1 minute. Proteinase K (100 µg/ml) was added to the mixture and the mixture was incubated at 55°C for 10 minutes. Subsequently, prewarmed (55°C) extraction buffer was added (3% (w/v) CTAB (Cetyltrimethyl ammonium bromide), 1% (w/v) Sarkosyl, 20 mM EDTA, 1.4 M NaCl, 0.1 M Tris-HCl, pH 8.0, 1% (v/v) 2-mercaptoethanol, freshly prepared) and incubated at 55°C in a water bath for 30 minutes with mixing by gentle inversion every 5 minutes. The mixture was allowed to cool for 30 s before centrifuging for 20 minutes at 12 000 x g. After centrifugation 1:1 (v/v) of chloroform was added to the supernatant. The mixture was mixed by gentle inversion (30 times) until an emulsion was formed. After centrifugation (12 000 x g for 5 minutes at 25°C), the supernatant was transferred to a sterile micro-centrifuge tube and the chloroform extraction was repeated. After the second chloroform extraction, the supernatant was transferred to a sterile micro-centrifuge tube, 2 volumes of 4 M NaCl were added and the solution mixed by gentle inversion.

Isopropanol 1:1 (v/v) was added to the mixture and incubated for 1 hour at room temperature to precipitate the DNA. The mixture was centrifuged for 20 minutes at 12 000 x g and the supernatant discarded. One millilitre of 70% ethanol was added and the DNA was centrifuged for 10 minutes at 12 000 x g. The supernatant was removed and the ethanol wash step was repeated with absolute ethanol. The pellet was then air-dried in a desicator with silica gel crystals. The DNA-pellet was resuspended in 500 µl of TE buffer (10 mM Tris-HCL, pH 8.0, 1 mM EDTA).

To remove excessive impurities from the DNA sample, a final concentration of 750 mM ammonium acetate was added to the DNA solution followed by a chloroform extraction (1:1) (v/v). After centrifugation the DNA in the supernatant was precipitated overnight and washed twice with ethanol. The DNA was dried and dissolved in TE buffer (10 mM Tris-HCL, pH 8.0, 1 mM EDTA).

4.2.3 PCR amplification

For classification of species, it is recommended to amplify the whole 16S rDNA sequence. Therefore, primers by Taton *et al.* (2003) as well as Wilmotte *et al.* (1993) (table 4.3) were used for amplifying the near complete sequence of the 16S rDNA gene.

Table 4.3: The primers used for sequencing the 16S rRNA genomic fragment

Primer	Sequence (5' to 3')	Target site	Reference
16S27F	AGA GTT TGA TCC TGG CTC AG	7-27	Wilmotte *et al.*,(1993)
CYA781R(a)	GAC TAC TGG GGT ATC TAA TCC CAT T	781-805	Nübel *et al.*,(1997)
CYA781R(b)	GAC TAC AGG GGT ATC TAA TCC CTT T	781-805	Nübel *et al.*,(1997)
16S727R	RGG ATT AGA TAC CCC	727-741	Wilmotte *et al.*,(1993)
CYA1514R	GTA CGG CTA CCT TGT TAC GAC	1494-1514	Taton *et al.*,(2003) and references therein

The primers were synthesized commercially (Roche) (table 4.3). PCR amplification of a cyanobacterial 16S rRNA gene was performed in a 50 µl (total volume) reaction mixture containing 15 ng DNA, 1 x Super Taq Plus PCR buffer (Southern Cross biotechnology), each deoxynucleotide triphosphate at a concentration of 0.2 mM, 0.5 µM primer 16S27F, 0.5 µM primer 23S30R and 1 mg of bovine serum albumin.

Amplification was carried out with a PCR Thermal cycler (PCR Express, Hybaid) as follows. To minimize non-specific annealing of the primers to non-target DNA, 1 U of SuperTaq DNA polymerase (Southern Cross biotechnology) was added to the reaction mixture after the initial denaturation step (5 minutes at 94°C), at 80°C. Ten cycles of 45 s at 94°C, 45 s at 57°C and 2 minutes at 68°C followed; and then 25 cycles of 45 s at 92°C, 45 s at 54°C and 2 minutes at 68°C followed by a final elongation step of 7 minutes at 68°C.

PCR products were run on a 1.5 % (w/v) agarose gel, the PCR fragments excised from the gel and purified with an agarose gel DNA extraction kit and subsequently used for sequencing.

4.2.4 Sequencing

Sequencing was done on an automated DNA sequencer, using cyclic sequencing by the University of Cape Town (UCT). The primers used for sequencing of the 16S rRNA gene are shown in table 4.4.

4.2.5 Alignment and phylogenetic analysis

Publically-available 16S rDNA sequences deposited on the NCBI database (Altschul *et al.*, 1997), with similarities greater than 96 % (closely related strains) to "*O. simplicissima*" were

aligned with the sequences of the species isolated from the Vaal River, giving a total of fifty strains. The strains used in the present study are listed in table 4.4.

Table 4.4: Strains used in the cladistic analysis and listed in the cladogram.

Taxon	Accession number	Location of isolation	Reference	Base pairs
Arthrospira	-	Carolina	Carolina™ Science and Math. ER-15-1721. www.carolina.com	1350
Arthrospira fusiformis	AF260510	taxon: 54297 Strain Ethi-B2	Li, R., Debella, H.J. and Carmichael, W.W. Taxonomic re-evaluation of species of *Arthrospira* (Cyanobacteria). Unpublished.	1294
Arthrospira maxima	AF260509	taxon: 129910 Strain Ethi-A2	Li, R., Debella, H.J. and Carmichael, W.W. Taxonomic re-evaluation of species of *Arthrospira* (Cyanobacteria). Unpublished.	1293
Arthrospira sp.	X70769	taxon: 35824 Strain PCC 8005	Nelissen *et al.*, (1994)	1959
Arthrospira sp.	AF329392	taxon: 153965 Strain FACHB438	Mao, Y., Yang, G., Zhang, B. and Zhang, X. Application of the sequences analysis of the 16S rRNA gene and ITS of 16S-23S rRNA to the systematic study of the genus *Arthrospira* and *Spirulina*. Unpublished.	1963
Gloeobacter violaceus	AF132790	Strain PCC 7421	Turner *et al.*, (1999)	1407
Leptolyngbya sp.	AB039012	Taxon: 118166 Strain: PCC 7104	Ishida *et al.*, (2001)	1440
Lyngbya aestuarii	AJ000714	taxon: 65095 Strain PCC 7419	Nübel *et al.*, (1997) Garcia-Pichel *et al.*, (1998)	1451
Lyngbya aesuarii	AB039013	taxon: 118322 Strain PCC 7419	Ishida *et al.*, (2001)	1432
Lyngbya hieronymusii	AB045906	China: Lake Dalai, Inner Mongolia Strain CN4-3	Suda *et al.*, (2002)	1368
Microcystis (Loch Vaal)	-	Loch Vaal: South Africa		1485
Microcystis sp (UV 027)	-	Fich Pond near Beit Shean, Israel		1290
Oscillatoria neglecta	AB003168	taxon: 71189 strain="M-82"	Ishida *et al.*, (1997)	1438
Oscillatoria princeps^{T}	AB045961	Thailand:Chao Phya, Bangkok Strain NIVA CYA 150	Suda *et al.*, (2002)	1367
Oscillatoria simplissisima	AM236076	Vaal River	Venter (2000)	1350
Oscillatoria sp.	AJ133106	Netherlands:Lake Loosdrecht	Zwart G., Van Agterveld M., Gons H., van der Werff I. and Hagen F. cyanobacterial diversity in a shallow eutrophic lake. Unpublished	1410
Planktothrix sp.	AB045914	Finland:Lake Langsjon, Aland *NIVA CYA 126*	Suda *et al.*, (2002)	1494
Planktothrix agardhii	X84811	taxon:1160 Strain CYA 18	Nelissen *et al.*, (1996)	1463
Planktothrix agardhii	AB045958	Netherlands: Veluwemeer Strain NIES 596	Suda *et al.*, (2002)	1373
Planktothrix agardhii	AB045915	Finland: Lake Vesijari, Lahti Strain NIVA CYA 127	Suda *et al.*, (2002)	1371
Planktothrix agardhii	AB045918	Norway: Lake Ogderen, Akershus Strain NIVA CYA 133	Suda *et al.*, (2002)	1369
Planktothrix agardhii	AB045938	Norway: Lake Steinsfjorden, Buskerud Strain NIVA CYA 56/3	Suda *et al.*, (2002)	1369
Planktothrix agardhii	AB045935	Norway: Lake Kolbotnvatnet, Akershus Strain NIVA CYA 34	Suda *et al.*, (2002)	1369
Planktothrix agardhii	AB045947	Sweden: Lake Oren, Atvidanberg, Ostergotland Strain NIVA CYA 88/3	Suda *et al.*, (2002)	1371
Planktothrix agardhii	AB045924	United Kingdom: Windermere, Cumbria Strain NIVA CYA 168	Suda *et al.*, (2002)	1370
Planktothrix agardhii^{T*}	AB045954	Japan:Lake Kasumigaura, Ibaraki Strain NIES 204	Suda *et al.*, (2002)	
Planktothrix mougeotii	AB045971	Thailand: Nakhon Pathon Strain TR1-5	Suda *et al.*, (2002)	1360
Planktothrix mougeotii	AB045969	Thailand: Bangkok Strain TK4-5	Suda *et al.*, (2002)	1370
Planktothrix pseudagardhii	ABO45922	Chao Phya, Bangkok, Thailand by R. *StrainNIVA CYA 153*	Suda *et al.*, (2002)	1544

Planktothrix pseudagardhii (NIVA 153)	ABO45922	Thiland: Chao Phya, Bangkok Strain NIVA CYA 153	Suda *et al.*, (2002)	1352
Planktothrix pseudagardhii	AB045907	China: Lake Dalai, Inner Mongolia Strain CW4-5	Suda *et al.*, (2002)	1355
Planktothrix pseudagardhii	AB045966	Thailand: King Palace, Bangkok Strain T19-6'-8	Suda *et al.*, (2002)	1355
Planktothrix pseudagardhii^T	AB045968	Thailand:Bangkok Strain "T1-8-4"	Suda *et al.*, (2002)	1355
Planktothrix rubescens	AB045921	Denmark: Lake Almind so Strain NIVA CYA 151	Suda *et al.*, (2002)	1370
Planktothrix rubescens	AB045959	Norway: Lake Gjersjoen Strain NIES 610	Suda *et al.*, (2002)	1369
Planktothrix rubescens	AB045946	Sweden: Lake Levrasjon, Skane Strain NIVA CYA 87	Suda *et al.*, (2002)	1369
Planktothrix rubescens	AB045950	Norway: Lake Steinsfjorden, Buskerud Strain NIVA CYA 97/5	Suda *et al.*, (2002)	1370
Planktothrix rubescens	AB045937	Norway: Lake Steubsfjorden, Buskerud Strain NIVA CYA 55	Suda *et al.*, (2002)	1369
Planktothrix rubescens	AB045934	Norway: Lake Kolbotnvatnet, Akershus Strain NIVA CYA 320	Suda *et al.*, (2002)	1370
Planktothrix rubescens	AJ132250	Switzerland Strain BC-Pla 9401	Beard *et al.*, (1999)	1443
Planktothrix sp	AJ133168	taxon: 213625 Strain NIVA-CYA 127	Lyra *et al.*, (2001)	1446
Planktothrix sp.	AJ133166	taxon 213624 Strain NIVA CYA 126	Lyra *et al.*, (2001)	1448
Planktothrix sp.	AJ133169	taxon: 213626 Strain NIVA CYA 128/R	Lyra *et al.*, (2001)	1446
Spirulina platensis	AB074508	taxon: 1156 Strain IAM M-135	Seo, P. and Yokota, A. The phylogenetic relationships of cyanobacteria inferred from gyrB sequences. Unpublished.	1441
Spirulina sp. (BI·N)	-	Kamferpan, Kimberley	Roos, J. (Unpublished)	1393
Spirulina sp. (G)	-	Kamferpan, Kimberley	Jordaan, G. (Unpublished)	1356
Synechococcus sp.	AF330251	Germany: Lake Constance Strain BO0014	Ernst *et al.* (2003)	2309
Synechococcus sp.	AY172832	taxon: 69042 Strain WH5701	Fuller *et al.* (2003)	1440
Synechococcus sp.	AY151249	taxon: 210768 Isolate MW101C3	Crosbie *et al.* (2003)	1552
Synechococcus sp.	AY151251	taxon: 210717 Isolate MW97C4	Crosbie *et al.* (2003)	1554

*T: type species

Alignment and phylogenetic analysis were done with BioEdit (Windows) (http://www.mbio.ncsu.edu) (see appendix for sequences). The computer-generated alignments were refined manually and gaps removed as suggested by Suda *et al.* (2002) to obtain a set containing 1269 positions that was used for phylogenetic analysis of the 50 cyanobacterial strains.

The cyanobacterium *Gloeobacter violaceus* (PCC 7421) (AF132790) was used as outgroup to root the tree. *Synechococcus* (single cell) and *Lyngbya* (filamentous) were used to test the monophyly of the ingroup and to give better resolution between the groups in the tree.

All phylogenetic analysis was done using NONA via WINCLADA version 1.00.08 (Nixon, 2002). Cladograms were generated with equally weighted heuristic searches using tree bisection-reconnection (TBR) branch-swapping algorithm and a random addition sequence with

a 1000 replicates (Steepest descent option in effect). Uninformative characters were deactivated and the consensus was strict. Reliability of internal nodes of trees was ascertained by 1000 bootstrap replications.

Phylogenetic reconstruction is highly sensitive to different alignment options (e.g., gap-to-substitution cost or alignment algorithms), which can lead to very different phylogenetic hypotheses (Giribet and Wheeler, 1999; Sanchis *et al.,* 2001).

According to Kawakita *et al.* (2003), inclusion of gap characters consistently improved support for nodes recovered by substitutions and inclusion of ambiguously aligned regions. They conclude that gaps are an exceptionally reliable source of phylogenetic information that can be used to corroborate and refine phylogenies hypothesized by base substitutions, at least at lower taxonomic levels. Despite recent progress in analysing gap characters, they have not been widely accepted as phylogenetic markers due, in part, to insufficient empirical study of the quality of gaps as characters (Kawakita *et al.*, 2003). Some authors assume that gaps are less homoplastic and therefore more phylogenetically reliable than base substitutions, since gaps generally occur less frequently, but other authors emphasize the potential for gaps to be misleading (Kawakita *et al.,* 2003 and references therein).

The influence of gap characters on phylogenetic inference was investigated by conducting an additional parsimony analysis with gap information included as coded characters (http://www.home.duq.edu/~youngnd/GapCoder). The bootstrap values for this tree was lower and the data matrix was maintained without gaps.

4.2.6 Microscopic study

A comparative morphological study was also done between *"Oscillatoria simplicissima" and Planktothrix pseudagardhii NIVA CYA 153* under different environmental conditions. Strains of both *"Oscillatoria simplicissima"* and *Planktothrix pseudagardhii*, NIVA CYA 153 cultures were grown at three different temperatures (18°C, 25°C and 30°C) each at three different light intensities (5, 10 and 20 $\mu mol.m^{-2}.s^{-1}$). Six replicates of each treatment were grown for each strain.

For light microscopic studies, eight-day old living cultures of *"O. simplicissima"* and *P. pseudagardhii* were filtered to increase cell density and studied under the light microscope.

For the confocal microscopic studies, samples of eight-day old cultures were fixed in 2% (v/v) formaldehyde for 24 hours. The samples were stained with Nile Red (for phospholipids) and studied and digital images captured using a PCM 2000 confocal microscope connected to a Nikon (TE300) microscope, with a 60x/1.40 Apo Planar oil objective. The system used the helium-neon ion laser to excite the Nile Red colorant at 505 nm. Emission waves were gained at 565 nm.

A ten percent neutral density filter was used to distinguish between the plasma membrane potential and the mitochondria membrane potential. Only a small opening was used to let the light through from the confocal microscope to the light source to prevent photo-discolouring. The intensity of the fluorescence was always less than 250 intensity units which enables photometric measurement. A scanning tempo of one frame was selected and the average of eight frames was used to give the final image.

4.3 Results and Discussion

4.3.1 Sequencing of Microcystis sp.

Different strains of presumed *Microcystis sp.* from the North-West culture collection were sequenced to confirm their phylogeny. However, it was found that they were not *Microcystis aeruginosa* strains (results not shown). Therefore, true representatives were obtained from known culture collections. *Microcystis aeruginosa* PCC 7806 from the Pasteur Culture Collection (PCC) as well as *Microcystis aeruginosa* CCAP 1450/1 from the Culture Collection of Algae and Protozoa were obtained as true representatives for *Microcystis sp.* for subsequent studies.

For the identification of *Oscillatoria simplicissima*, two additional strains were used as references, one of *Planktothrix pseudagardhii* (Suda *et al.*, 2002), NIVA CYA 153 from the Norwegian culture collection and the other of *Planktothrix sp.* 126/8, provided by K. Sivonen (University of Helsinki, Helsinki, Finland) .

The filamentous cyanobacteria that first appeared in the Vaal River during the early nineties was originally identified by Pieterse and Steynberg (1993) and Venter *et al.* (2003) as *O. simplicissima* under the order Nostocales, family *Oscillatoria*ceae, according to the criteria of Desikachary (1959).

A cladogram (figure 4.8) based on the 16S rDNA sequences revealed two distinct groups, the filamentous and the single celled cyanobacteria. The filamentous group is divided into four distinct clades, namely, a cluster of the family Pseudanabaenaceae, a cluster with *Oscillatoria princeps*, a cluster with *Arthrospira/Lyngbya* and a cluster with *Planktothrix* species. The last cluster is clearly divided into two subclusters, *P. pseudagardhii* and *P. agardhii/rubescens*.

In the first cluster, the strains labelled as *Oscillatoria* (AJ133106) and *O. neglecta* (AB003168) according to the BLAST database do no longer belong to the genus "*Oscillatoria*". According to the modern system, *O. neglecta* is classified to the genus *Jaaginema*, which is close to *Leptolyngbya* (Anagnostidis and Komárek, 1988). All strains from this cluster, thus *Spirulina* (Gert) as well, belong to the family Pseudanabaenaceae, not *Oscillatoria*ceae.

"*Spirulina platensis*" belongs to the genus *Arthrospira*, not *Spirulina* (Ballot *et al.*, 2002). The strain called "*Spirulina* BFN" (figure 4.2), which is included in the cluster together with *Arthrospira*, is clearly *Arthrospira*. Both of these genera are quite different according to molecular and cytological criteria (Nelissen *et al.*, 1994).

As the results indicated, other researchers (Mark Graham - personal communication) also found that the *Spirulina* "tablets" which are being marketed as *Spirulina* (with all of its supposed associated health benefits) is actually *Arthrospira sp.*

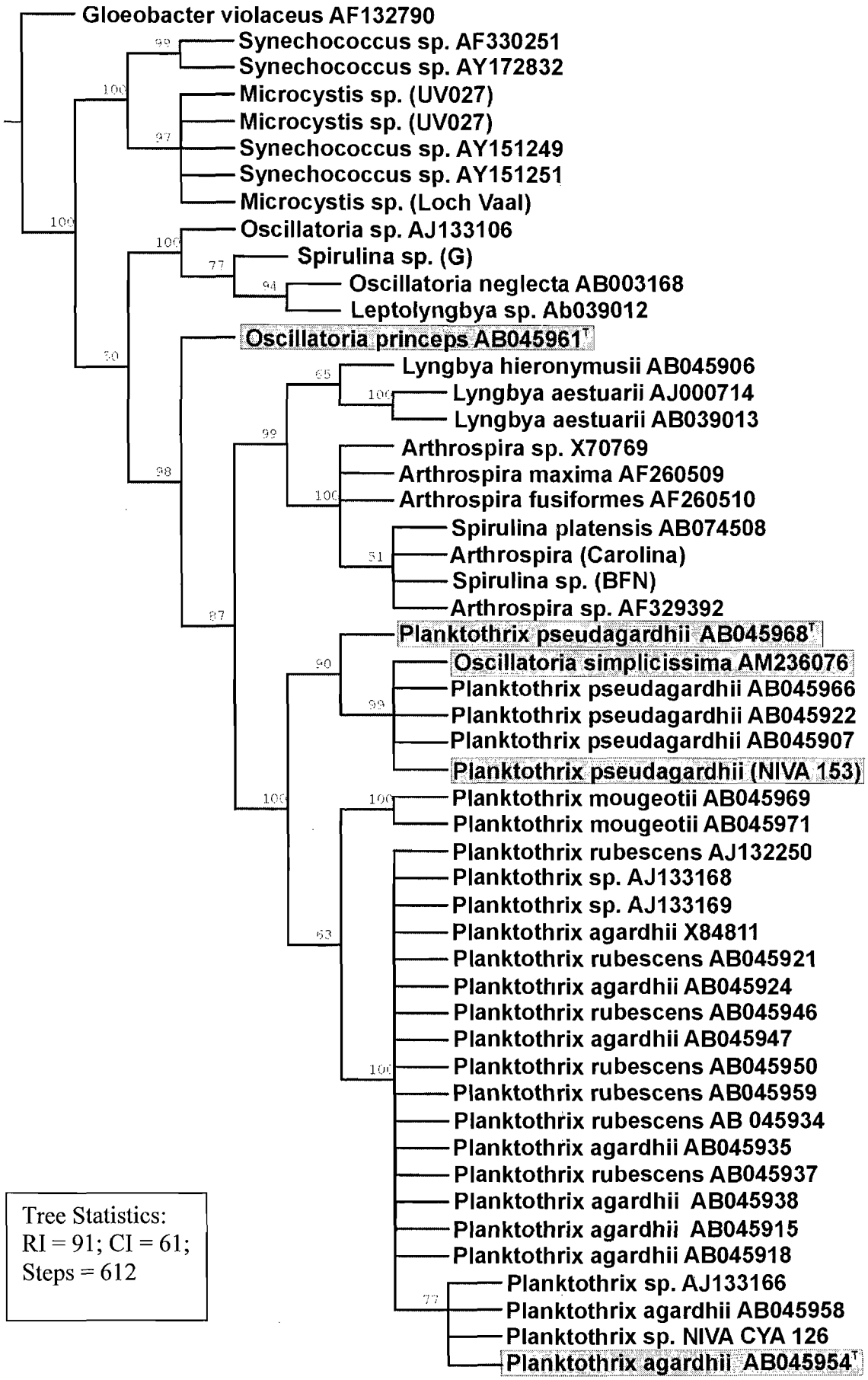


Figure 4.7: Cladogram based on partial 16S rRNA gene sequences. *Gloeobacter violaceus* (PCC 7421) sequence was used as a outgroup. Bootstrap values are indicated at the nodes. Abbreviations: table 4.5.

The organism used as health food is *Arthrospira sp*, but has always been marketed as "*Spirulina*" because of popularity of the commercial name. *Spirulina* is in fact a totally different genus (confirmed by biochemical and molecular studies), but with superficial similarity to *Arthrospira*.

Rantala *et al.* (2004) suggests that the ability for cyanobacteria to produce toxin has been lost repeatedly in the more derived lineages of cyanobacteria and strains of cyanobacteria not previously suspected of producing microcystins may retain the genes necessary for their synthesis. Because microcystins and nodularins are highly toxic to humans and pose a serious health risk to water users, more taxa should be carefully checked for the presence of these toxins (Rantala *et al.,* 2004). This would be particularly important for strains of genera like *Spirulina* and *Arthrospira* which are commonly used in health food supplements (Gilroy *et al.,* 2000).

Arthrospira has not been taxonomically amalgamated with *Spirulina*. These two genera are morphologically and also genetically totally different. The taxa are closely related, but distinct. Although the genera were described originally as different, Geitler (1925a) merged them and some subsequent workers followed him. Since *Spirulina* was the earlier genus, Geitler (1925b) changed also Gomont's (1892) *Arthrospira platensis* to *Spirulina platensis* (Gom.) Geitl. (Komárek and Lund, 1990). However, modern studies have led to a better understanding of both cell and trichome structure and found that differences exist in structure of the cell walls, also in position of thylakoids, type of fragmentation of trichomes, motility, spirality etc. and Komárek and Lund (1990), therefore followed the separation of both genera.

There has been considerable back and forth movement of taxa between the two genera over the years, many of which was previously known as *Spirulina* have been transferred to *Arthrospira*. But essentially, the cell walls are very indistinct in *Spirulina*, which is generally highly motile and more benthic, while *Arthrospira* has very distinct crosswalls and is largely non-motile, occurring in both planktonic and benthic habitats.

From figure 4.2 it is clear that *Sprirulina* BFN has distinct crosswalls and this support the molecular study that *Spirulina* BFN should be classified as *Arthrospira*. It is thus clear that *Spirulina* BFN is incorrectly marketed as *Spirulina* for its commercial name and should be named *Arthrospira*.

The strain of *Spirulina* (Gert) however is clearly not *Arthropira* or *Spirulina*. *Spirulina* (Gert) is in a cluster (see figure 4.8) that belongs to the order *Oscillatoria*les family Pseudanabaenaceae

(Anagnostidis and Komárek, 1988), not the family *Oscillatoria*ceae. These strains are quite different from *Spirulina* or *Arthrospira* and can not be used as a food supplement.

In the last cluster, molecular sequencing indicates that all the strains belong to the genus *Planktothrix*, of which the species are only slightly distinguishable according to phenotype but are clearly divided into two clusters namely the *pseudagardhii* and the *agardhii/rubescens* cluster. Similar results were found by Humbert and Le Berre (2001). These authors found no polymorphism between *P. agardhii/rubescens* using partial 16S rDNA sequences. Rudi *et al.* (1998a) suggested that sequence homogeneity can be due to frequent transfers of genetic material between strains. Humbert and Le Berre (2001) subsequently also suggested that recombination occurred between these species (*P. agardhii/rubescens*) and that the existence of recombinations between the strains suggest that they are conspecific (Humbert and Le Berre, 2001).

Oscillatoria princeps (AB045961^T) and *Planktothrix agardhii* (AB045954^T) NIES 204 (Anagnostidis and Komárek, 1988) were selected as reference strains of the genera *Oscillatoria* and *Planktothrix* respectively and *Planktothrix pseudagardhii* (AB045968^T) (Suda, personal communication) was selected as reference strain of the species *Planktothrix pseudagardhii*. Our findings corroborate those of Suda *et al.* (2002) that species of *Oscillatoria* are polyphyletic and none are related to the reference strains. The bacteriological requested limit of similarity below 95% is clearly expressed between these entities. Suda *et al.* (2002) suggested that *O. princeps* should not be listed under the genus name *Oscillatoria* and that its generic name should be changed, as proposed previously by Anagnostidis and Komárek (1988) on the basis of purely phenotypic characteristics. This is also clearly supported by this study's findings (figure 4.8).

The *"O. simplicissima"* isolate from the Vaal River, had a nearly identical sequence of 16S rDNA with that of the reference strain *Planktothrix pseudagardhii* and forms part of a distinct *Planktothrix pseudagardhii* clade (figure 4.8). Computation was performed at NCBI using the BLAST network service (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and the sequences of the 16S rDNA of all representatives within this clade show a 99% similarity. The high bootstrap values for that clade strongly support the re-identification of the organism found in the Vaal River as *Planktothrix pseudagardhii.*

Use of 16S rDNA sequence analyses is a powerful tool for taxonomy; however, characterization based on the sequence alone is not enough for the classification of species of some organisms, especially those that are closely related species (Suda *et al.,* 2002). For example, *Planktothrix agardhii* and *Planktothrix rubescens* could not be separated by 16S rDNA sequence analysis (Humbert and Le Berre, 2001; Suda *et al.,* 2002), morphology, fatty acid composition, or G+ C content, but were distinguishable by phycobilin pigment composition, growth at different temperatures and salinities and DNA-DNA hybridization (Suda *et al.,* 2002). The resolution power of DNA hybridization is significantly higher than that of sequence analysis and DNA hybridization remains the optimal method for measuring the degree of relatedness between highly related organisms. However, it is interesting that at sequence homology values below about 97.5%, it is unlikely that two organisms have more than 60-70% DNA similarity and hence that they are related at the species level (Stackebrand and Goebel, 1994). On the other hand, Suda *et al.* (2002) reported that the closely-related species *P. agardhii* and *P. pseudagardhii* were well defined by 16S rDNA sequence analysis, although they shared the same morphology as well as fatty acid composition, phycobilin pigment and G+C content. The present results (figure 4.8) confirm this as *P. agardhii* clearly clustered separately from the group *P. pseudagardhii.* These results therefore, once again emphasize the use of a polyphasic approach in identifying cyanobacterial strains.

To verify the molecular results, a comparative morphological study was also done between "*O. simplicissima"* and *P. pseudagardhii* NIVA CYA 153 under different environmental conditions. Venter *et al.* (2003) found that "*O. simplicissima"* has typical characteristics of the *Oscillatoria*ceae as described by Geitler (1932) and Desikachary (1959). This purely clarifies that *"O. simplicissima"* is a filamentous bacterium with uniseriate cells which are not constricted at the cross walls. Terminal cells are hemispherical with a slightly-thickened membrane on the outer cell envelope. The trichomes are not attenuated or capitated at the apices. Venter *et al.* (2003) found gas vesicles (aerotopes) present in the trichome and found that the straight, unbranched trichome is covered with a thin hyaline sheath, which is in contrast with the description by Anagnostidis and Komárek (1988) of the genus *Oscillatoria.*

The family *Oscillatoria*ceae, as reviewed by Anagnostidis and Komárek (1988), is characterised by straight or slightly waved trichomes. One or more trichomes can be present in a sheath. The trichomes have a thicker outer cell wall sometimes with a calyptra and the trichomes are rarely solitary and mainly compact. In the genus *Oscillatoria,* the trichomes are usually without sheaths, but a sheath can occur in suboptimal conditions and no gas vesicles are present.

Trichomes of the current material, similarly to what was found by Venter *et al.* (2003) were always straight, with only one trichome in each sheath and the trichomes were solitary. Gas vesicles were present. Thus this species cannot be classified as a member of the genus *Oscillatoria* under the family *Oscillatoria*ceae according to the criteria of Anagnostidis and Komárek (1988).

Anagnostidis and Komárek (1988) however reassigned *O. simplicissima* to *Phormidium simplicissimum,* in the family Phormidiaceae, which is characterized by slightly- or intensely-waved or coiled trichomes that form mats. No gas vesicles (aerotopes) are present in the trichomes of this family and the sheath, the production of which is dependent on environmental conditions, remains open at the ends and contains one or more trichomes (Anagnostidis and Komárek, 1988). In the genus *Phormidium*, the trichomes are slightly- to intensely waved or coiled (Anagnostidis and Komárek, 1988). These phenotypical characteristics furthermore clearly exclude the current material of *"O. simplicissima"* from the family Phormidiaceae and the genus *Phormidium.*

Anagnostidis and Komárek (1988) established the new genus *Planktothrix* for gas-vacuolated *Oscillatoria* species. According to Suda *et al.* (2002), gas vesicles in *P. pseudagardhii* are relatively large and scattered at the periphery of the cells. Venter *et al.* (2003) found that gas vesicles in *"O. simplicissima"* formed conspicuous beehive-like structures in the older cells of filaments but that filaments grown in culture for a long time, lose these gas vesicles. The finding of this study showed that gas vesicles are present in both "*Oscillatoria simplicissima"* (figure 4.9a) as well as *Planktothrix pseudagardhii* even under different light and temperature conditions (figure 4.9b).

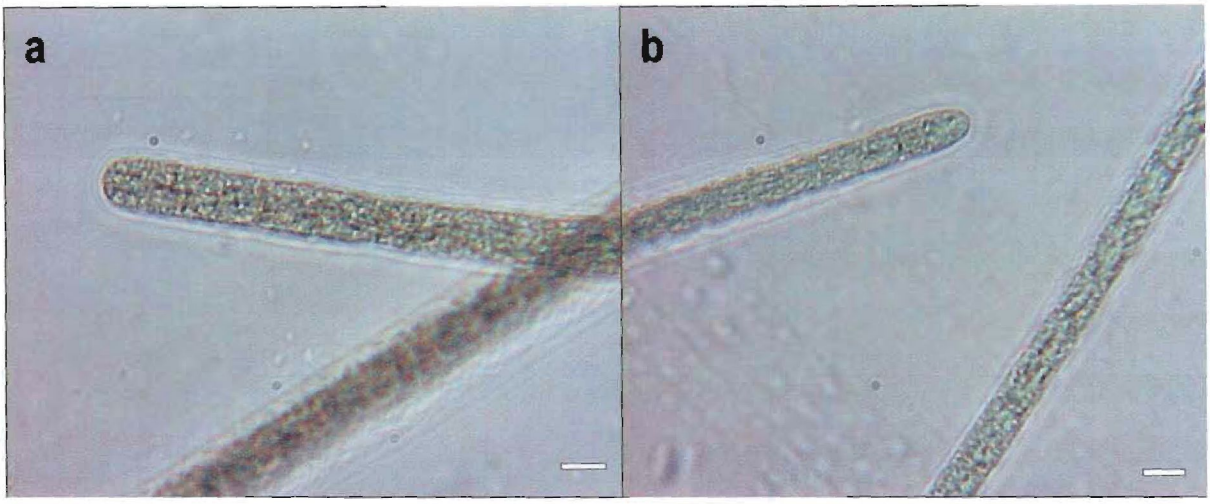


Figure 4.8: Light micrograph of a) *O. simplicissima* and b) *P. pseudagardhii*, grown at 25°C with a light intensity of 20 μmol.m^{-2}s^{-1}. Scalebar = 10 μm.

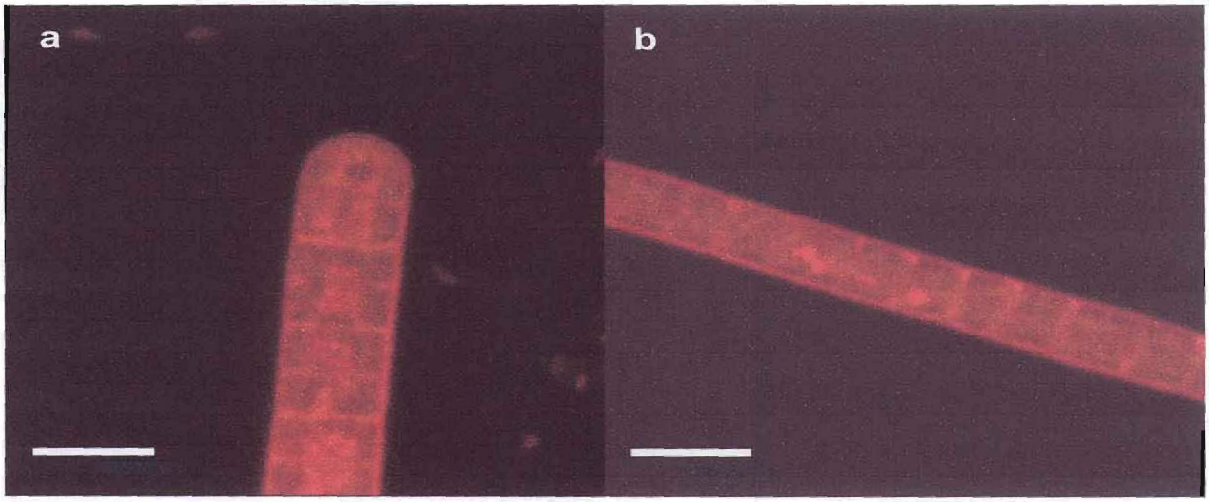


Figure 4.9: Confocal micrograph of a) *O. simplicissima* and b) *P. pseudagardhii* grown at 25°C with a light intensity of 20 μmol.m^{-2}s^{-1}. Scalebar = 5 μm.

The average width of the filament of *P. pseudagardhii* (3.05 μm) was found to be narrower than that of "*O. simplicissima*" (3.65 μm) (figures 4.8 and 4.9). Cells of *Planktothrix pseudagardhii* are 3.0-6.4 μm wide and 1.2-4.2 μm long (Suda *et al.*, 2002), but Desikachary (1959), Drouet (1968) and Anagnostidis and Komárek (1988) respectively reported widths of 8-9 μm, 1.5-8 μm and 1-12 μm in this species. *Planktothrix pseudagardhii* NIVA 153 has a width of 2–4 μm. Venter *et al.* (2003) found that the width of *"O. simplicissima"* cells varies between 8.0-12.0 μm, while current observations range from 3-5 μm. It is thus clear that width does not characterise this species.

The following findings furthermore illustrate why morphology alone cannot be used to distinguish between closely related cyanobacterial species. The trichomes of *Planktothrix* are sometimes attenuated towards the end and apical cells show variable shapes, such as rounded,

tapered, bluntly conical and occasionally capitate, with and without calyptra (Suda *et al.*, 2002). Trichomes of *Planktothrix* are slightly tapering or not tapering to the end and the end cells (when fully developed) with a calyptra or with thickened outer cells (Anagnostidis and Komárek, 1988). According to Venter *et al.* (2003) the trichome of *"O. simplicissima"* is not attenuated or capitated at the apex and the terminal cells are hemispherical with a slightly thickened outer cell wall on the outer cell envelope. Our results indicate that the trichomes are hemispherical to the end without a calyptra (figure 4.10). No transient forms have been observed.

The cross walls of *P. agardhii* may be slightly constricted or not constricted at the end (Anagnostidis and Komárek, 1988; Suda *et al.*, 2002). Venter *et al.* (2003) found that the cells of *O. simplicissima* are not constricted at the cross walls. Our results indicate that filaments may be constricted or not constricted under different environmental conditions.

No clear differences between *"O. simplicissima"* and *P. pseudagardhii* NIVA CYA 153 were found using light and confocal microscopy, which further supports results obtained from the 16S rRNA study. According to Komárek (2005) the molecular (phylogenetic) data should be accepted as a basic criterion for taxonomical studies.

In conclusion, the presented morphological results such as the presence of gas vesicles and especially the molecular results which indicate that the isolate formerly known as *"O. simplicissima"* grouped with the genus *Planktothrix* and more precisely the species of *Planktothrix pseudagardhii,* suggested that *"Oscillatoria simplicissima"* (sensu Pieterse and Steynberg, 1993; Venter *et al.*, 2003) should be reassigned to *Planktothrix pseudagardhii* under the order *Oscillatoriales*, family Phormidiaceae and subfamily Phormidioideae (Suda – personal communication). *Planktothrix pseudagardhii* (Suda *et al.*, 2002) is however illegitimate because the genus name *Planktothrix* has never been validly published under the Rules of the *Bacteriological Code* (1990 Revision) and the species was therefore not validly published according to Rule 16, Note 2 (De Vos and Trüper, 2000). *Planktothrix agardhii* (Gomont) (Anagnostidis and Komárek, 1988) is the only *Planktothrix* validly published under the International Code of Botanical Nomenclature.

In spite of this, it is suggested that *"O. simplicissima"* must then be reassigned to the name of *Planktothrix pseudagardhii* according to the results of the 16S rDNA sequencing. This follows after the still ongoing dispute that exists in the international community on whether the cyanobacteria should be described and published as blue-green algae under the International

Code of Botanical Nomenclature or be recognized as having been published under the *Bacteriological Code.*

In June 1985 the Subcommittee on the taxonomy of Phototrophic Bacteria proposed that names of cyanobacteria described and validly published as blue-green algae under the International Code of Botanical Nomenclature are recognized as having been published under the *Bacteriological Code* (1990 Revision) (Trüper, 1986).

In September 1986, the Judicial Commission agreed unanimously to recommend the following to the International Committee on Systematic (ICSB), now International Committee on Systematics of Prokaryotes (ICSP): those names of taxa of Cyanobacteria and Cyanophyta that are valid under the Botanical Code be considered valid under the *Bacteriological Code* for the purpose of preparing an acceptance list comparable to the *Approved Lists of Bacterial Names* (Jones, 1987). However, "Declarations of intent" by the Judicial Commission were not taken up by the ICSB (now ICSP). In 2003, the "Subcommittee on the taxonomy of phototrophic bacteria" (Imhoff and Madigan, 2004) proposed the preparation of a list of approved names of cyanobacteria under the *Bacteriological Code* and a small committee was appointed to prepare such a list. However, such a list is not yet available.

Consequently, names of cyanobacteria described and validly published as blue-green algae under the International Code of Botanical Nomenclature have no standing in bacterial nomenclature, unless they are again described under the *Rules of the Bacteriological Code* (1990 revision).

Chapter 5

Molecular ecophysiology of toxic *Microcystis* species

5.1 Introduction

Predicting bloom events is an important goal of water monitoring programs and is of fundamental interest to those examining the ecology of aquatic ecosystems (Ouellette and Wilhelm, 2003). While microscopic identification and toxin analyses have traditionally been employed for monitoring purposes, molecular biological methods may provide rapid and sensitive diagnoses for the presence of toxic and toxigenic cyanobacteria that are useful for general ecological studies (Ouellette and Wilhelm, 2003). Knowledge of the occurrence and dynamics of microcystin producers and non-producers is essential to explain the variability of microcystin concentrations in natural waters, as well as to explore the function of microcystin, that is still not known (Mbedi *et al.*, 2005).

More precise investigations of potential regulatory mechanisms of cyanotoxin biosynthesis require knowledge of the genes and enzymes involved (Kaebernick *et al.*, 2000). Such studies are now possible with the discovery of the genes and biosynthetic pathway required for the production of microcystins in *M. aeruginosa* (Dittmann *et al.*, 1997).

However, it is desirable to match isolated strains and their counterparts in nature to understand the ecophysiology of cyanobacteria (Nübel *et al.*, 1997). To date, laboratory-based ecological investigations have focused on the isolation of individual environmental effectors, which is a crucial step in the identification of toxin regulation mechanisms (Kaebernick and Neilan, 2001). Yet, such results can not be extrapolated to the natural environment, where a greater array of factors exist (Kaebernick and Neilan, 2001).

Molecular studies have the potential to identify the individual environmental factors which affect gene transcription and expression directly, opposed to those factors that may affect other cellular processes which lead to changes in toxin production (Kaebernick and Neilan, 2001). Thus, through a combination of ecological and molecular research, it is hoped that experimental data may ultimately be extrapolated to the environment not only to understand the timing of toxin production but also the purpose of it (Kaebernick and Neilan, 2001). Understanding conditions that regulate cyanotoxin production may shed light on potential functions (Ouellette and Wilhelm, 2003).

Despite many contradictory studies, there are some factors that have been shown to influence microcystin synthesis. The literature contains an abundance of such studies, which at times conflict (Ouellette and Wilhelm, 2003).

Factors that may influence the growth and development of one cyanobacteria over another do not act singly, but rather in concert with a constantly changing suite of parameters – the combination of which determines the outcome of a successional phase of algal growth (Harding and Paxton, 2001). To gain insights into the internal dynamics of the freshwater cyanobacteria, this study aimed to trace ecophysiologically distinct strains in their natural environment.

Due to the complexity of an *in situ* community, the physiological changes occurring in problem taxa *in situ* can only be investigated with the use of molecular techniques. In autotrophic organisms such as cyanobacteria the main metabolic processes of photosynthesis, respiration and nitrogen metabolism are tightly regulated and are also in close interaction with each other. In order to investigate the bloom forming capabilities of these species it is important to look into the ecophysiological dynamics of each process. By identifying key metabolic enzymes and investigating their regulation of gene expression in field situations we might broaden our understanding of the regulation of these processes *in situ* and therefore growth potential as well. Studies found that cyanobacteria form blooms in relation to specific environmental conditions, eg. changes in temperature, phosphate concentrations or nitrate concentrations (Reynolds and Walsby, 1975).

The aim of this study was thus, through the use of a combination of ecological and molecular research methods and the use of environmental samples, to provide insight into both the timing of bloom forming and toxin production. The expression of the genes, *rbcL* (encoding the large subunit of Rubisco) and *ntcA* (encoding a nitrogen assimilation regulatory protein), will hopefully in part reflect the photosynthetic and nitrogen metabolism activity of the cyanobacteria present in the sample. The existence of possible trends in the expression of the *ntcA* and the *rbcL* genes, specifically in *Microcystis aeruginosa* (bloom former) in the Hartbeespoort Dam and Roodeplaat Dam, was measured with qRT PCR. Together with this, DNA copy number of the *Microcystis* specific 16S rRNA and toxin genes, *mcyE* as well as *mcyB,* were also measured with qPCR. During this study, the concentration of microcystins present as well as ecological data such as water temperature, pH, nutrient loads and chl *a* concentrations were also recorded and obtained from the Department of Water Affairs and Forestry.

5.2 Materials and Methods

5.2.1 Strains and strain cultivation

5.2.1.1 Cultures

Microcystis aeruginosa PCC 7806 obtained from the Pasteur Culture collection, as well as *Microcystis aeruginosa* CCAP 1450/1 obtained from the Culture Collection of Algae and Protozoa were used respectively as toxic and non-toxic reference strains for *Microcystis sp.* in the PCR reactions. Together with this, a strain of *Planktothrix pseudagardhii* NIVA-CYA 153/1 (Norweigan culture collection) (Suda *et al.*, 2002), as well as *P. agardhii* NIVA-CYA 126 provided by Prof. Kaarina Sivonen (University of Helsinki, Helsinki, Finland) were used respectively as toxic and non-toxic reference strains for *Planktothrix sp.* in the PCR reactions for this study.

Cultures of *M. aeruginosa* were established in glass flasks containing 100 ml of GBG11 medium (Krüger, 1978) at a constant temperature (18°C) and light intensity (20 $\mu mol.m^{-2}.s^{-1}$) and strains of *Planktothrix sp.* were cultivated in glass flasks containing 100 ml of EM medium (Venter, 2000) at a constant temperature (24°C) and light intensity (20 $\mu mol.m^{-2}.s^{-1}$). Cultures were routinely verified for purity by microscopic examination.

5.2.2 *Study area*

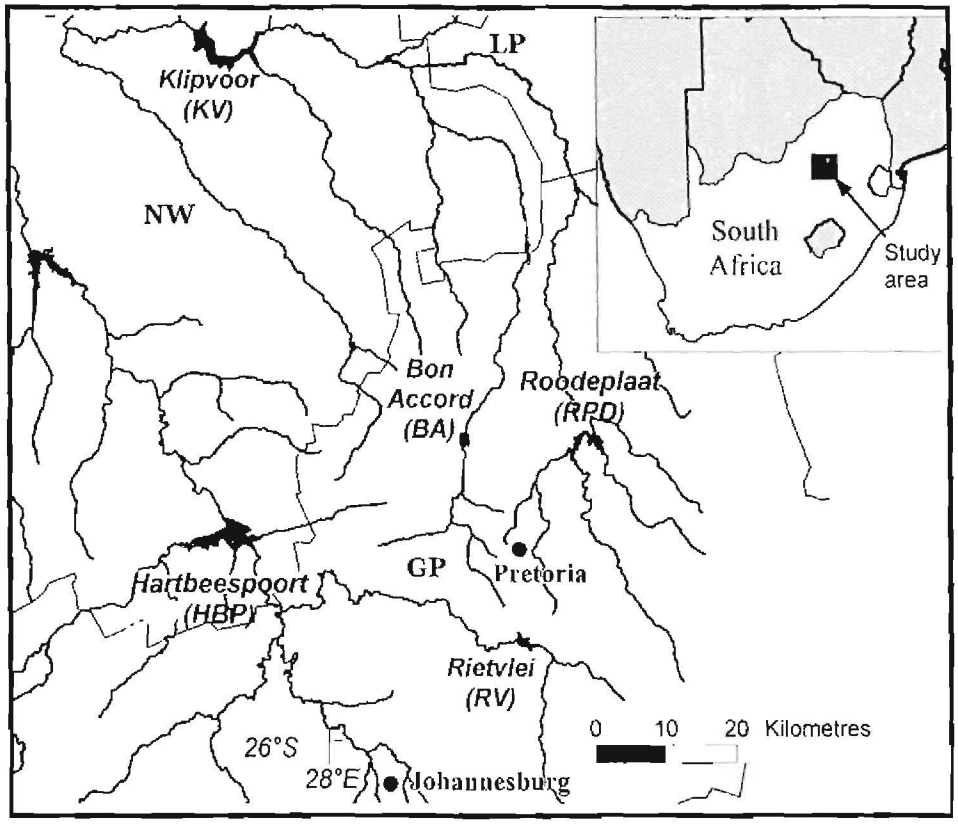


Figure 5.1: Map displaying the study area (Van Ginkel *et al.*, 2006). The Hartbeespoort Dam (HBP) is located in the North-West Province, while the Roodeplaat Dam (RPD) is located in Gauteng Province, South Africa; Pretoria is the major city closest to both dams and both of the reservoirs are used for drinking water abstraction, and are used extensively for recreation.

5.2.2.1 The Roodeplaat Dam

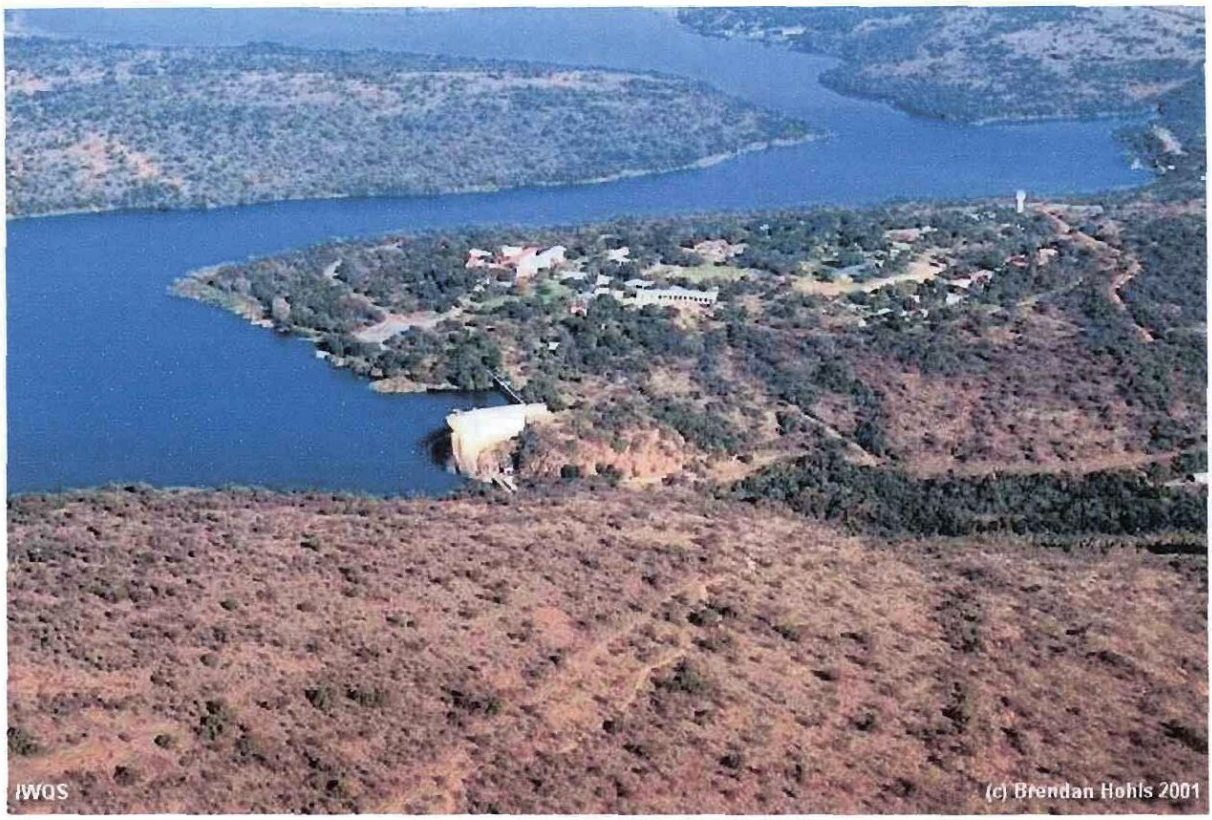


Figure 5.2: The Roodeplaat Dam with the dam wall visible in the front of the picture (Hohls, 2001). Roodeplaat Dam is situated north-east of Pretoria in the Gauteng Province.

The Roodeplaat Dam is situated 20 km north-east of Pretoria, at the confluence of the Pienaars River, the Moreleta/Hartbees Spruit and the Edendale Spruit. These rivers drain the highly populated areas of northern and eastern Pretoria. The catchment includes two point sources of nutrient loading, namely a) the Baviaanspoort Water Care Works (WCW) situated on the Pienaars River and b) the Zeekoegat WCW that discharges directly into the Roodeplaat Dam (Van Ginkel, 2000b). Diffuse sources of pollution include formal and informal settlements, as well as agricultural activities along the banks of the tributaries (Van Ginkel, 2000a). Walmsley *et al.* (1978) stated that the Roodeplaat Dam is highly eutrophic.

The impoundment is an important recreational resource (fishing, canoeing and other water related uses) for the greater Gauteng area. The water is, therefore, used for direct contact and semi-contact recreation (Van Ginkel, 2000b).

5.2.2.2 The Hartbeespoort Dam

Figure 5.3: Picture of the dam wall of the Hartbeespoort Dam, with cyanobacterial scums clearly visible (Venter, 2005). The Hartbeespoort Dam is situated to the west of Pretoria in the North West Province of South Africa.

The Hartbeespoort Dam is situated in the North West Province of South Africa and lies in a valley to the south of the Magaliesberg mountain range and north of the Witwatersberg mountain range, about 35 kilometres west of Pretoria. The dam was originally designed for irrigation which is currently its primary use.

The dam was completed in 1923 and soon became a water source for primary consumption and also an attractive recreational destination for many water sports enthusiasts, anglers and local and international tourists (Department of Water affairs and Forestry, 2007).

Due to rapid urban development and industrial growth in the Hartbeespoort Dam catchment area, the volumes of water and the loads of plant nutrients (phosphates and nitrogen) reaching the dam have increased. This trend may continue in the future as the urban areas in the catchment are expected to grow steadily (Department of Water affairs and Forestry, 2007).

5.2.3 Sample collection

5.2.3.1 Environmental samples

One of the sampling sites for the two Reservoirs in this study, Hartbeespoort and Roodeplaat Dams, was at a site close to the dam wall of each dam, which is also the sampling site for the National Eutrophication Monitoring Program (NEMP) of South Africa (DWAF, 2002). One litre of subsurface grab samples were taken from each of 5 different sites by the Department of Water Affairs and Forestry (DWAF) from the Hartbeespoort Dam as well as the Roodeplaat Dam, every second week during the bloom season of October 2004 to May 2005, at different locations in each dam. Wind action and currents cause cyanobacteria to accumulate in patches, therefore, during each sampling event (once every two weeks) five different sites where cyanobacteria accumulated around the edges of a reservoir were found and sampled. This means that the sites for the toxins were not necessarily the same sites during the study period. A total of 150 samples were taken over the sampling period. The average values of the parameters measured were determined and used as average values in subsequent figures.

5.2.3.2 Limnological sampling

The samples at each reservoir were subdivided into a macro inorganic sample, an algal identification sample and a Chl *a* sample. The macro inorganic samples (major inorganic chemicals) were analyzed on automatic analyzers at Resource Quality Services of the Department of Water Affairs and Forestry.

The macro chemical variables measured by the Resource Quality Services of the Department of Water Affairs and Forestry were: calcium, chloride, dissolved major salts (DMS), electrical conductivity (EC), fluoride, potassium, magnesium, ammonium, nitrate and nitrite, sodium, ortho-phosphorus, sulphate, silica, total alkalinity and pH (see table 5.1). Additional to the normal macro chemical variables, the Kjeldahl nitrogen (KN) and total phosphorus (TP) were also measured using digestion methods (DWAF, 2006a and 2006b). To determine the dissolved inorganic nitrogen (DIN) concentrations, the sum of the ammonium, nitrate and nitrite was calculated. To determine the total nitrogen (TN) concentrations the sum of the Kjeldahl nitrogen, nitrate and nitrite concentrations was calculated by the Department of water affairs and Forestry.

Algal identification samples, done by the Resource Quality Services of the Department of Water Affairs and Forestry, were preserved with Lugol's solution. For identification, 10 ml samples

were poured into a counting chamber and left to settle overnight or for a minimum of 6 hours. Algal identification was done with an inverted microscope and results were expressed as a percentage of the total algal population. The method is described in detail in the methods manual of the RQS (Department of Water Affairs and Forestry, 1997).

For Chl *a* determination, done by the Resource Quality Services of the Department of Water Affairs and Forestry, at least 500 ml of sample water were filtered through a 45 µm Whatman filter paper. The Chl *a*, was then extracted from the filter paper with 10 ml ethanol. The Chl *a* content was measured using a spectrophotometer (Department of Water Affairs and Forestry, 2003a).

On each sampling occasion done by the Resource Quality Services of the Department of Water Affairs and Forestry, *in situ* temperature and oxygen measurements were taken at 1 m depth intervals with an YSI meter. Secchi disc readings were taken regularly at the sampling site in each reservoir by lowering the 20 cm black and white disc into the water and measuring the depth at which the disc is not visible anymore. The Secchi disc reading was used to determine the extinction coefficient as follows:

Extinction coefficient – 1.7/SD

Lawrence *et al.* (2000)

The rationale when sampling for total microcystin was that a whole Reservoir was considered one site. This method of sampling was decided on, as a regular sampling site does not always portray toxicity of cyanobacteria because of the phenomenon of shifting masses of cyanobacteria. One litre, subsurface grab samples (Gerber *et al.*, 2004) were taken to the laboratory of the RQS at Roodeplaat for analysis. The samples were then frozen and unfrozen three times to release the intra-cellular toxins. Total microcystin concentration was determined for the shifting five sites with the ELISA method (Department of Water Affairs and Forestry, 2003b) to determine if toxins are present and to determine maximum, minimum and mean concentrations in a Reservoir. This was done to determine correlations between the regular sampling results and the presence of cyanobacterial toxins. This method was also followed for other studies such as Van Ginkel (2008).

5.2.3.3 Sampling for molecular analyses

Water samples (500 ml) for molecular analysis from Hartbeespoort Dam and Roodeplaat Dam were filtered through a 47 mm-diameter GF 52 glass fibre paper filter with a pore size of 1.2 µm (Schleicher & Schnell) at negative vacuum pressure to concentrate the cells and as previously noted (section 3.2.1) to remove bacteria. According to Wyman (1999), this fractionation procedure results in the retention of greater than 90% of the original biomass. These filters were then immediately frozen in liquid nitrogen and sent on ice to the North-West University, Potchefstroom campus, where it was kept frozen at -80°C until use.

5.2.3.4 Cells in Culture (control samples)

Two weeks after inoculation, cyanobacterial cells of the different control species were concentrated by filtration of 30 ml culture onto glass fibre paper filters and the DNA extracted.

5.2.4 DNA extraction

The filters with the concentrated cyanobacterial cells were firstly washed three times with 10 ml of a solution containing 50 mM Tris-HCl, pH 8.0, 5 mM EDTA and 50 mM NaCl to reduce extracellular impurities.

Filters with the concentrated cyanobacterial cells were incubated in a sterile buffer containing 50 mM Tris-HCl (pH 8.0) and 50 mM EDTA. The filters were then homogenised with a Heidolph DIAX 900 homogeniser for 1 minute. Proteinase K (100 µg/ml) was added to the mixture and the mixture was incubated at 55°C for 10 minutes. Subsequently, prewarmed (55°C) extraction buffer was added (3% (w/v) CTAB, 1% (w/v) Sarkosyl, 20 mM EDTA, 1.4 M NaCl, 0.1 M Tris-HCl, pH 8.0, 1% (v/v) 2-mercaptoethanol, freshly prepared) and incubated at 55°C in a water bath for 30 minutes with mixing by gentle inversion every 5 minutes. The mixture was allowed to cool for 30 s before centrifuging for 20 minutes at 12 000 x g. After centrifugation an equal volume of chloroform was added to the supernatant. The mixture was mixed by gentle inversion (30 times) until an emulsion was formed. After centrifugation (12 000 x g for 5 minutes at 25°C), the supernatant was transferred to a sterile micro-centrifuge tube and the chloroform extraction was repeated. After the second chloroform extraction, the supernatant was transferred to a sterile micro-centrifuge tube, 2 volumes of 4 M NaCl were added and the solution mixed by gentle inversion.

An equal volume of isopropanol was added to the mixture and incubated for 1 hour at room temperature to precipitate the DNA. The mixture was centrifuged for 20 minutes at 12 000 x g and the supernatant discarded. One millilitre of 70% (v/v) ethanol was added and the DNA was centrifuged for 10 minutes at 12 000 x g. The supernatant was removed and the ethanol wash step was repeated with absolute ethanol. The pellet was then air-dried in a desiccator with silica gel crystals. The DNA-pellet was resuspended in 500 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

To remove excessive impurities from the DNA sample, a final concentration of 750 mM ammonium acetate was added to the DNA solution, followed by an extraction with an equal volume of chloroform. After centrifugation the DNA in the supernatant was precipitated overnight with absolute ethanol and washed twice with 70% (v/v) ethanol. The DNA was dried and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

5.2.5 RNA extraction

All plastic and glassware were treated with 0.1% (v/v) DEPC, incubated overnight at 37°C and then autoclaved twice to inactivate the DEPC. Non-autoclavable plastic ware was treated with a solution containing 0.1 M NaOH and 1 mM EDTA and rinsed in sterile water. Special care was taken to remove all possible contamination by cleaning all working surfaces and pipettes with 70% (v/v) ethanol and Ambion's RNase ZAP.

The Qiagen RNeasy mini kit was used for the extraction of total RNA from cyanobacterial cells according to the manufacturers' instructions. Glass fibre filters were first homogenised in the RLT buffer in order to break the cell walls and the mixture of homogenized glass fibre filter, cyanobacterial cells and buffer were centrifuged to remove the excess material. The supernatant was further used according to the manufacturers' instructions. RNA was treated with Qiagen's RNase free DNase set according to the manufacturers' instructions to remove DNA contamination from the RNA samples.

The total RNA concentration was measured with a spectrophotometer set at 260 nm (Sambrook *et al.*, 1989). Purity was determined by calculating the ratio of the absorbance measured at 260 nm (A_{260}) to the absorbance measured at 280 nm (A_{280}). The concentration of the RNA was determined with the following equation:

RNA concentration = A_{260} x dilution x 40 $\mu g.ml^{-1}$

(Sambrook *et al.*, 1989)

5.2.6 Conventional PCR amplification

PCR amplification of a cyanobacterial DNA was performed in a 25 µl (total volume) reaction mixture containing 1x Buffer (Southern Cross), 1.5 mM $MgCl_2$, 250 µM of all four deoxynucleoside triphosphates, 0.5 U of Hot start *Taq* DNA polymerase (Southern Cross), 60 ng DNA, 0.5 µM primer F and 0.5 µM primer R.

Amplification was carried out with the PCR Thermal cycler (Hybaid PCR Express) on the reaction mixture after the initial denaturation step (15 minutes at 95°C according to the manufacturers, Southern Cross). The initial denaturation step was followed by 30 cycles of PCR, with each cycle consisting of the first step of 30 s at 95°C, the second step of 30 s at the specified temperature (chapter 3, table 3.7) and the third step of 30 s at 72°C, followed by a final elongation step of 10 minutes at 72°C.

PCR products were run (Bio-Rad Power Pac 1000) on a 1.5% (w/v) TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) agarose gel (10 x 15 cm) at 85 V for 1 hour, stained with 2 µg/µl ethidium bromide and visualised and photographed under UV illumination with a gel documentation system i.e. GeneSnap from Syngene v6.08.04 (Synoptics Ltd. Ingenius Bio Imaging, Cambridge, England).

5.2.7 Real-time PCR amplification

5.2.7.1 Real-time PCR amplification to determine copy numbers

Genomic DNA of *M. aeruginosa* PCC 7806 (toxic) was used as an external standard to determine *mcyB* and *mcyE* copy numbers. A spectrophotometer was used to measure the concentration of the DNA at 260 nm. The purity of the DNA was determined by calculating the ratio of the absorbance measured at 260 nm (A_{260}) to the absorbance measured at 280 nm (A_{280}). For *mcyB* and *mcyE* copy number calculation, an approximate genome size of 4.7 Mb for *Microcystis* was used (Vaitomaa *et al.*, 2003). These genome sizes were estimated on the basis of the genome size of *Microcystis sp.* strain PCC 7941 (Vaitomaa *et al.*, 2003). By using the following equation, the *mcyB* and *mcyE* copy numbers of the DNAs of the standard strains were calculated assuming that each genome had only one *mcyB* or one *mcyE* gene and that the molecular weight of 1 bp was 660 $g.mol^{-1}$ (Vaitomaa *et al.*, 2003).

Equation 1: calculation of copy number (Vaitomaa *et al.*, 2003):

$$\text{number of copies per microlitre} = \frac{(6 \times 10^{23})(\text{DNA concentration})}{\text{molecular weight of one genome}}$$

- Where 6×10^{23} is the number of copies per mole,
- DNA concentration is given in grams per microlitre,
- The molecular weight of one genome is given in grams per mole.

Series of 3 fold dilutions of genomic DNA of the standard strain *M. aeruginosa* PCC 7806 were prepared and these dilutions were amplified with *Microcystis* specific *mcyE* and *mcyB* primers (see appendix for partial sequences of the *mcyE* and *mcyB* genes).

Linear regression equations for the cycle threshold values obtained (C_t values, ie., the first turning points of the fluorescence curves as a function of cycle numbers) were calculated as a function of known *mcyE* and *mcyB* copy numbers according to Vaitomaa *et al.* (2003).

qPCR amplification of a cyanobacterial DNA was performed with the Bio-Rad iQ5 PCR thermal cycler, in a 25 µl (total volume) reaction mixture containing 12.5 µl iQ™ SYBR® Green Supermix (Bio-Rad) (100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 50 units/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, (20 nM fluoresien and stabilizers)), 60 ng DNA, 0.5 µM primer F and 0.5 µM primer R (IDT).

Amplification was carried out with the Bio-Rad iQ5 PCR Thermal cycler. The initial denaturation step (3 minutes at 95°C) was followed by 30 cycles of PCR, with each cycle consisting of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C, followed by a final elongation step of 7 minutes at 72°C. Synthesis of the products was monitored after each extension step at 72°C, by measuring the fluorescence of double-stranded DNA binding SYBR green dye using the IQ5 iCycler (Bio-Rad). All samples were amplified in duplicate. The fluorescence threshold was set by the analytical software for the iCycler (Bio-Rad, version2). Copy numbers of *mcyE* and *mcyB* gene of the dam water samples were determined by converting the obtained C_t values into the *mcyE* and *mcyB* copy numbers according to the regression equations of the external standards as outlined by Vaitomaa *et al.* (2003).

Amplification efficiencies, e ($e = 10^{-1/s} - 1$, where S is the slope of the linear regression) of the qPCR with standard strains (*M. aeruginosa* PCC 7806) were calculated as a function of known *mcyE* and *mcyB* copy numbers. A slope of -3.32 indicates the PCR reaction is 100% efficient (Ginzinger, 2002).

In order to determine the melting temperatures for the amplification products of the standard strains and of the environmental samples, the temperature was raised from 45°C to 95°C and fluorescence was detected after each step. The characteristic melting temperatures of the *mcyE* and *mcyB* qRT-PCR were determined with iCycler software (version 2). In all cases, melt curves were used to confirm single amplification products for the different reactions and in selected cases, amplification was also confirmed with agarose gel electrophoresis.

The standard curve was linear ($r^2 = 0.998$) over two orders of magnitude and exhibited good efficiency (E = 95-99% which is in the recommended range (Pfaffl, 2001)) and replication between duplicate amplifications of each standard. The standard error between the efficiencies of different runs was ±0.27.

5.2.7.2 Reverse transcriptase real-time PCR to determine level of gene expression

For quantitative, real-time, one-step Reverse Transcriptase PCR, the analysis was performed using the one step QuantiTect SYBR Green RT-PCR of Qiagen. The cycle conditions were 50°C for 30 minutes for the Reverse transcriptase reaction to occur, 95°C for 15 minutes to activate the hot start *Taq*, followed by 30 cycles with the following steps: 94°C for 15 s (denaturing of strands), 60°C for 30 s for annealing and 72°C for 30 s for extension, with the addition of a melt curve protocol at the end of the program. In all cases, melt curves were used to confirm single amplification products for the different reactions and in selected cases amplification was also confirmed with agarose gel electrophoresis.

qRT-PCR amplification of *Microcystis sp.* RNA was performed in a 25 µl (total volume) reaction mixture containing 12.5 µl of 2x QuantiTect SYBR Green RT-PCR Master mix (HotStarTaq DNA polymerase, QuantiTect SYBR Green RT-PCR buffer (contains Tris-HCl, KCL, $(NH_4)_2SO_4$, 5 mM $MgCl_2$, pH 8.7), dNTP mix, Fluorescent dyes),, dNTP mix, Fluorescent dyes), 0.25 µl QuantiTect RT mix (Omniscript and Sensiscript Reverse Transcriptases), 80 ng RNA, 0.5 µM primer F and 0.5 µM primer R. The QuantiTect SYBR Green RT-PCR master

Mix together with the QuantiTect RT Mix allows both reverse transcription and PCR to take place in a single tube.

The fluorescence threshold was set by the analytical software for the iCycler (Bio-Rad). The PCR cycle during which this threshold was crossed for each sample was designated the C_t. The reported C_t is the average of triplicate field samples, standards or internal controls. Sample C_t can be compared to the C_t of standards with a known cell count to specify the number of cells present in the sample. To assess the potential for PCR inhibition by components of the field sample, a dilution series of DNA from cultured *M. aeruginosa* cells were made. A three fold dilution series with the following DNA concentrations was used: 312.5 ng, 62.5 ng, 12.5 ng, 2.5 ng, 0.5 ng, 0.1 ng, 0.02 ng. No significant PCR inhibition was detected. The efficiency for each primer set was evaluated and recorded during assay development by the Bio-Rad iCycler.

PCR efficiency (E) was determined using the following equation:

Equation 2: Calculation of reaction efficiency (E): $E = 10^{(-1/slope)}$ (Dyhrman *et al.*, 2006)

Equation 2 shows a mathematical model of relative expression ratio in qPCR under constant reference gene expression. C_t values in the sample and control are equal and represent ideal housekeeping conditions. Efficiency describes how much of the sequence of interest was being produced with each cycle. A hundred percent efficiency means that the sequence of interest was doubled with each cycle (Dyhrman *et al.*, 2006).

Using qPCR the number of cells in a field sample were determined through comparison to a standard curve. Standards of 3x dilutions from *Microcystis aeruginosa* PCC 7806 were prepared via dilution of a concentrated cell stock. A three fold dilution series with the following DNA concentration was used: 312.5 ng, 62.5 ng, 12.5 ng, 2.5 ng, 0.5 ng, 0.1 ng, 0.02 ng.

The standard curve was linear (r^2 = 0.99) over two orders of magnitude and exhibited good efficiency (E= 90-99%), (±SE 0.8) and replication between duplicate amplifications of each standard. The relative quantification normalised against unit mass (ΔC_t method) was used for the calculation of the ratio of the expression of the *ntcA* and the *rbcL* genes against the 16S rRNA gene expression.

Equation 3: The ΔC_t method for the calculation of the ratio of expression:

$Ratio_{(test/control)} = Efficiency^{(Ct\ (test) - Ct\ (control))}$ (Pfaffl, 2001)

Where control = ng value of 16S rRNA products gene expression

Where test = ng value of *ntcA* or *rbcL* products gene expression

5.2.8 Statistical Analysis

Canoco for Windows, v4.5 was used for the statistical analysis of the environmental data sets.

Principal Component Analysis (PCA's) was performed on the molecular data and the environmental data to detect possible relationships between environmental variables and the presence of expression of different genes tested with the molecular analysis.

PCA is a technique based on a secondary matrix – a correlation matrix (Walker, 1998a). PCA is based on the assumption that there are linear correlations among the data being reduced, i.e., that the species or samples are linearly correlated (Walker, 1998b). The eigenvalues are correlation coefficients and therefore can be used directly to measure the variance explained by the ordination (Walker, 1998b). Higher order axes are uncorrelated with the first axis, except that in an oblique solution this is not enforced (Walker, 1998b).

Only environmental variables that were measured at all localities were included in the analysis. The PCA ordination plot was used to eliminate variables that are not prominent to simplify the graph.

Redundancy Analysis (RDA's) were chosen as the multivariate analysis tool to use to perform the ordinations on environmental and species abundance data. Molecular data was treated as species data and analysed in combination with the chemical data which was treated as environmental data. The graphs were then created to find possible interaction between the molecular data and the environmental data.

Statistica version 7.0 was used to determine correlations between the different data sets. The Kolmogorof-Smirnov and Lilliefors test for normality was used, as well as the Shapiro-Wilk's W to determine if the datasets were distributed parametrically. Most of the data was parametrically

distributed and correlations were determined between these datasets. For non-parametric data, correlations were done with the use of the Spearman Rank R test.

Excel (Office 2007) and SigmaPlot (version 6) were used to create histograms and flowcharts.

5.3 Results

Changes in environmental and molecular data were investigated for a period of 8 months, from October 2004 to May 2005 during the bloom season of cyanobacteria, in particular *Microcystis aeruginosa,* in the Hartbeespoort Dam as well as the Roodeplaat Dam. Certain physico-chemical and biological indicators of water quality that appeared to be related to the microcystin concentration and cyanobacterial growth were analysed for their statistical relationships.

When compared with other phytoplankton groups present in the dams, it can be seen that cyanobacteria dominate during the summer (figure 5.4). In the Hartbeespoort Dam the dominance is mostly later in the season, while chlorophyceae are also present during spring, with chrysophyceae becoming dominant in autumn. It is interesting to notice that the sharp decline in the cyanobacterial biomass in the middle of the bloom season in the Roodeplaat Dam coincide with a sharp incline in the presence of chrysophyceae species.

The number of cyanobacteria increased dramatically in February 2005, with *Microcystis* reaching 100% dominance in both dams during February 2005 (figure 5.5). In the Roodeplaat Dam the *Microcystis* dominance occurred at the beginning of the season (October 2004) and is followed by a slight increase in *Anabaena sp* and *Oscillatoria sp* dominance from February to April 2005 (figure 5.5). This is in accordance with data from the summer of 2003 and 2004 where *Microcystis sp.* comprised more than 40% of the phytoplankton population in both dams for longer than 50% of the time (Van Ginkel *et al.,* 2006).

Although *Planktothrix sp.* was dominant in both dams during autumn (April 2005), as the species counts indicated (figure 5.5), and also confirmed by qRT-PCR of the 16S rRNA cyanobacterial specific gene, it can be assumed that there was no toxin producing species of *Planktothrix sp.* present in the two dams because there was no amplification with the qRT-PCR for the *mcyB* primers specific for *Planktothrix sp.* throughout the bloom season. The specificity of these primers was confirmed previously with the reference strains in chapter 3. For the rest of

this chapter, there would only be focused on the molecular data of *Microcystis sp.* due to the presence of the toxin genes and overall species dominance of *Microcystis sp.*

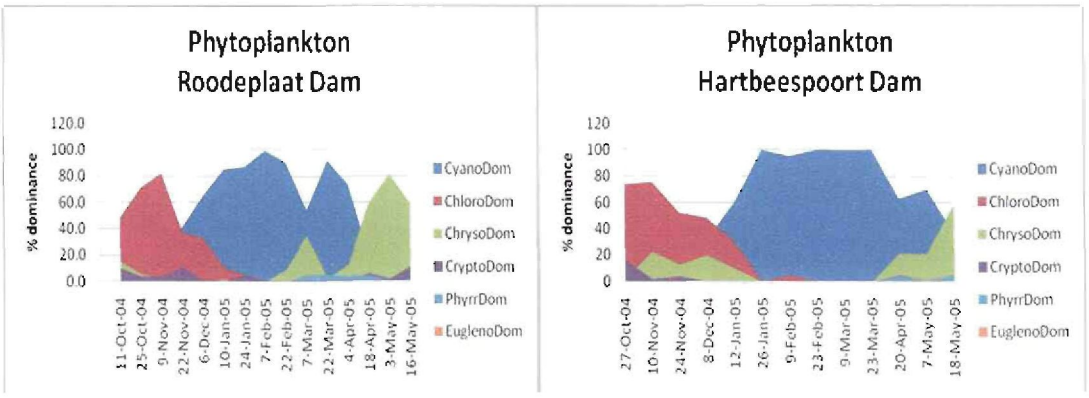


Figure 5.4: Percentile variation of the phytoplankton composition by major taxonomic groups as determined during the bloom season from October 2004 to May 2005 in Roodeplaat and Hartbeespoort Dams respectively. (CyanoDom = Cyanophyceae, ChloroDom = Chlorophyceae, ChrysoDom = Chrysophyceae, CryptoDom = Cryptophyceae, PhyrrDom = Phyrrhophyta, EugleneDom = Euglenophyceae.

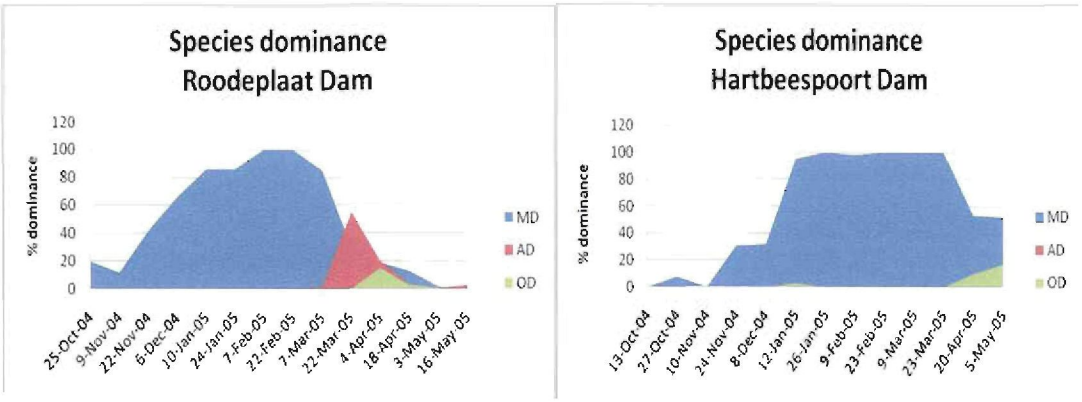


Figure 5.5: Percentile variation observed in the different cyanobacterial species composition that was present during the bloom season from October 2004 to May 2005 in the Roodeplaat and Hartbeespoort Dams respectively. (MD = dominance of *Microcystis*, AD = dominance of *Anabaena*, OD = dominance of *Oscillatoria*).

Table 5.1 is a list of all the environmental and molecular data measured during the time of sampling (See appendix for environmental and molecular data).

The chl *a* concentration varied within a range of 17 to 113 µg/L in the Roodeplaat Dam and from 20 to 173 µg/L in the Hartbeespoort Dam. The maximum chl *a* concentration was recorded in February and March 2005 in the Hartbeespoort Dam and the Roodeplaat Dam respectively. From these values it was clear that both dams are in a highly eutrophic state. The water temperature, which is an important factor in supporting algal growth, varied from 19.44°C to

25.7°C in the Roodeplaat Dam and was a little higher in the Hartbeespoort Dam where it varied from 18.63 to 28.3°C. Microcystin concentrations increased in spring in the Roodeplaat Dam and reached a maximum of 217 µg/L in November 2004. In the Hartbeespoort Dam microcystin concentrations were much higher and reached a maximum of 3200 µg/L during autumn 2005.

In the Roodeplaat Dam the total phosphorus (TP) values ranged from 67-202 µg/L and the total nitrogen (TN) values ranged from 665-2915 µg/L. In the Hartbeespoort Dam, the TP values ranged from 38 to 181 µg/L and the TN values ranged from 1568 to 2902 µg/L. The maximum ammonium concentration reached was 0.136 mg/L and the average over the period was 0.0487 mg/L. This is only half of the ammonium present in the Roodeplaat Dam and it is speculated that the large amount of *Microcystis sp.* cells in the water use all the NH_4.

The TN:TP ratio in the Roodeplaat Dam was higher than 16:1 from October to December 2004, but the ratio decreased to below 16:1 later in the season. In the Hartbeespoort Dam however, the TN:TP ratio was almost always higher than 16:1 and reached its peak also at the beginning of the season (October to December 2004) with values as high as 49.9:1 in December 2004, whereafter it gradually declined with its lowest ratio at the end of March 2005 (11:1).

Abbreviations for environmental, species and chemical data are given in Table 5.1.

Table 5.1: List of abbreviations and units of measurement used for environmental variables and species and chemical data in ordination diagrams (see appendix for raw data).

Description	Unit	Abbreviation
Molecular data		
16S rRNA minimum copy number based on DNA	Copy number	16S min
16S rRNA average copy number based on DNA	Copy number	16S avg
16S rRNA maximum copy number based on DNA	Copy number	16S max
mcyB minimum copy number based on DNA	Copy number	*mcyB* min
mcyB average copy number based on DNA	Copy number	*mcyB* avg
mcyB maximum copy number based on DNA	Copy number	*mcyB* max
mcyE minimum copy number based on DNA	Copy number	*mcyE* min
mcyE average copy number based on DNA	Copy number	*mcyE* avg
mcyE maximum copy number based on DNA	Copy number	*mcE* max
ntcA minimum ratio (RNA expression)	Ratio	*ntcA* min
ntcA average (RNA expression)	Ratio	*ntcA* avg
ntcA maximum ratio (RNA expression)	Ratio	*ntcA* max
rbcL minimum ratio (RNA expression)	Ratio	*rbcL* min
rbcL average (RNA expression)	Ratio	*rbcL* avg

rbcL maximum ratio (RNA expression)	Ratio	*rbcL* max
Biological data		
average microcystin concentration	µg/L	MCN avg
minimum microcystin concentration	µg/L	MCN min
maximum microcystin concentration	µg/L	MCN max
Chl *a* concentration	µg/L	Chl *a*
Species data		
Micocystis dominance	%	MD
Anabaena dominance	%	AD
Oscillatoria dominance	%	OD
Cylindrospermopsis dominance	%	CD
Environmental data		
Nutrient associated parameters		
Total nitrogen	mg/L	TN
KJEL-N	mg/L	KJEL-N
Ammonium NH_4-N	mg/L	NH4-N
Total phosphorus	mg/L	TP
Total Nitrogen:Total Phosphorus ratio	ratio	TN:TP
Phosphate:Ammonium ratio	ratio	PO_4:NH_4
Ortho-phosphate PO_4-P	mg/L	PO_4-P
Dissoloved inorganic nitrogen	mg/L	DIN
Calcium	mg/L	Ca
Chlorine	mg/L	Cl
Fluorine	mg/L	F
Potassium	mg/L	K
Magnesium	mg/L	Mg
NO_3+NO_2	mg/L	NO_3+NO_2
Sodium	mg/L	Na
Sulphate	mg/L	SO_4
Silicon	mg/L	Si
Dissolved molecular solvents	mg/L	DMS
Total alkalinity	mg/L	TAL
Physical parameters		
Flow	Megalitre per day	Flow
Watersurface temperature	°C	WST
pH	mS/m	pH
Electrical conductivity	ratio	EC
Dissolved inorganic nitrogen:Dissolved inorganic phosphorus	ratio	DIN:DIP
Nitrogen: phosphorus	mgJ.m^2	N:P
Radiation on the day of sampling	m	Radiation
Secchi depth reading		Secchi

5.3.1 PCA site plot

The statistical models used on the multivariate data set from the Hartbeespoort Dam as well as the Roodeplaat Dam included an indirect gradient analysis using the most prominent or the prominent component analysis (PCA) and the direct gradient analysis using the redundancy analysis (RDA).

The first step in the multivariate analysis was to perform a PCA in order to detect relationships and potential co-linearity.

The results of the PCA site plot (table 5.2) indicated that the first axis explains 25% of the variance in the data and the second axis, 22% of the variance in the data. From the PCA site plot (figure 5.6) it can be seen that the dams clearly differ in their association and response to the environmental factors and molecular variables. This is indicated by the distance between the symbols which approximate the dissimilarity in the environmental composition. Water surface temperature correlates very strongly with microcystin concentration, *mcyE* and *mcyB* and flow, indicating the importance of the water temperature and presence of nutrients in toxin production. These factors also closely associate with *Microcystis sp* specific 16S rRNA gene copy numbers. The correlations determined indicated that samples obtained from the Hartbeespoort Dam associate more strongly with the different variables when compared to that of the Roodeplaat Dam.

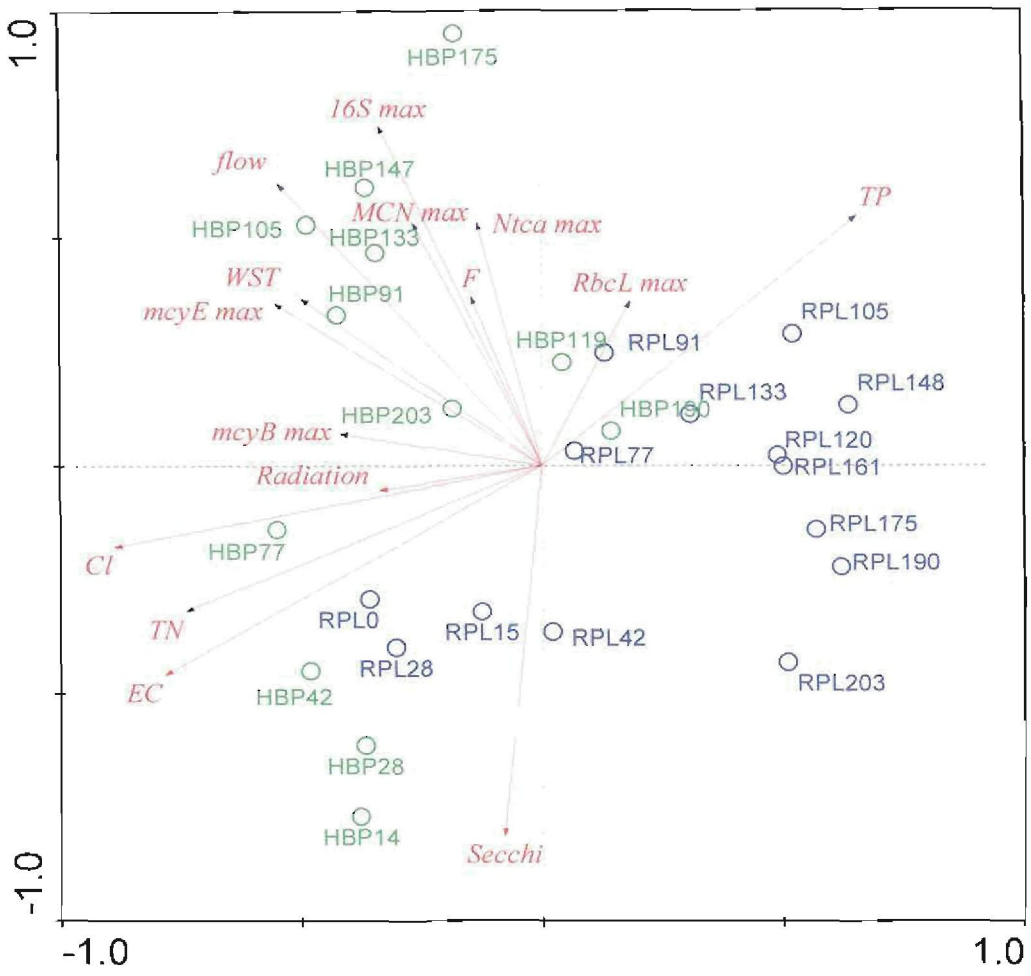


Figure 5.6: PCA site plot displaying the correlation between gene expression and selected environmental factors in both Hartbeespoort and Roodeplaat Dams. Green: represents different samples and sampling dates from the Hartbeespoort Dam. Blue: represents the different samples and sampling dates from the Roodeplaat Dam. The red vectors indicate the correlation of the different environmental and molecular variables measured in relation to each other as well as the sample site and time.

Table 5.2: Results from the PCA analysis depicted in figure 5.6 indicating the percentage of variance explained by the different axes.

Axes	**1**	**2**	**3**	**4**	**Total variance**
Eigenvalues	0.254	0.224	0.139	0.095	1.000
Cumulative percentage variance of species data	25.4	47.8	61.7	71.2	
Sum of all eigenvalues					1.000

5.3.2 RDA biplots of environmental data

According to the RDA biplot of the environmental data, the Hartbeespoort Dam and the Roodeplaat Dam (figure 5.7) could also be separated due to differences in the principal environmental components. This separation is not very distinct and most of the data overlap. However, because of slight differences in the data sets, the data was treated separately for further analyses.

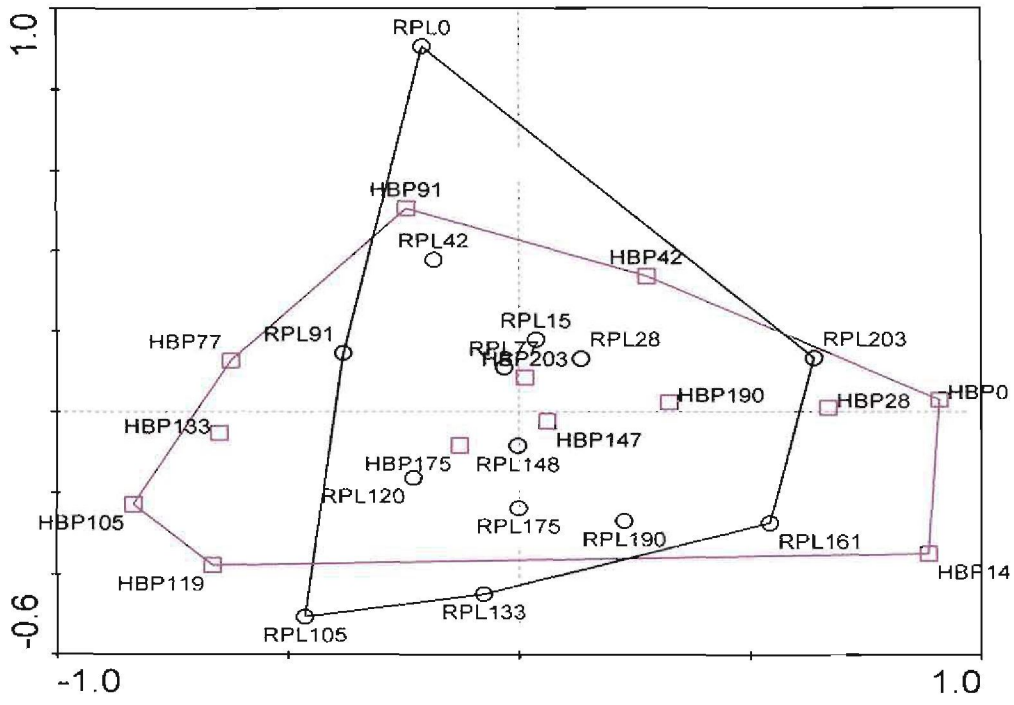


Figure 5.7: RDA environmental site plot showing sampling localities against environmental data in the two different dams, the Hartbeespoort Dam (HBP - open square) and the Roodeplaat Dam (RPL – open circle). Sample points: the distance between the symbols in the diagram approximates the dissimilarity of their environmental composition, measured by their euclidean distance.

5.3.2.1 RDA biplots of the Roodeplaat and the Hartbeespoort Dams

In figure 5.8 and figure 5.9, the RDA environmental biplots of the Roodeplaat Dam and the Hartbeespoort Dam are displayed separately as well as the species dominance information.

In the Roodeplaat Dam, the *Microcystis sp.* specific 16S rRNA gene copy number does not associate with chl *a* (figure 5.8). As can be seen in figure 5.9, in the Hartbeespoort Dam, the percentage of *Microcystis* dominance (MD) was closely associated with the chl *a* measured and the values correlated significantly ($p < 0.05$; $r = 0.67$; $n = 13$), indicating that *Microcystis sp.* was

propably the most abundant species in the Hartbeespoort Dam during the sampling period. A negative correlation was evident in the Hartbeespoort Dam between N:P and *Microcystis sp.* specific 16S rRNA gene copy number (figure 5.9).

The RDA biplots with the species dominance had very high inflation values as a result of the TN:TP and TN which inflated each other and therefore the species data as well as the TN:TP values were omitted in figure 5.10 and figure 5.11 to ensure a better statistical correlation resulting in lower inflation values.

5.3.3 Species dominance in the Roodeplaat Dam

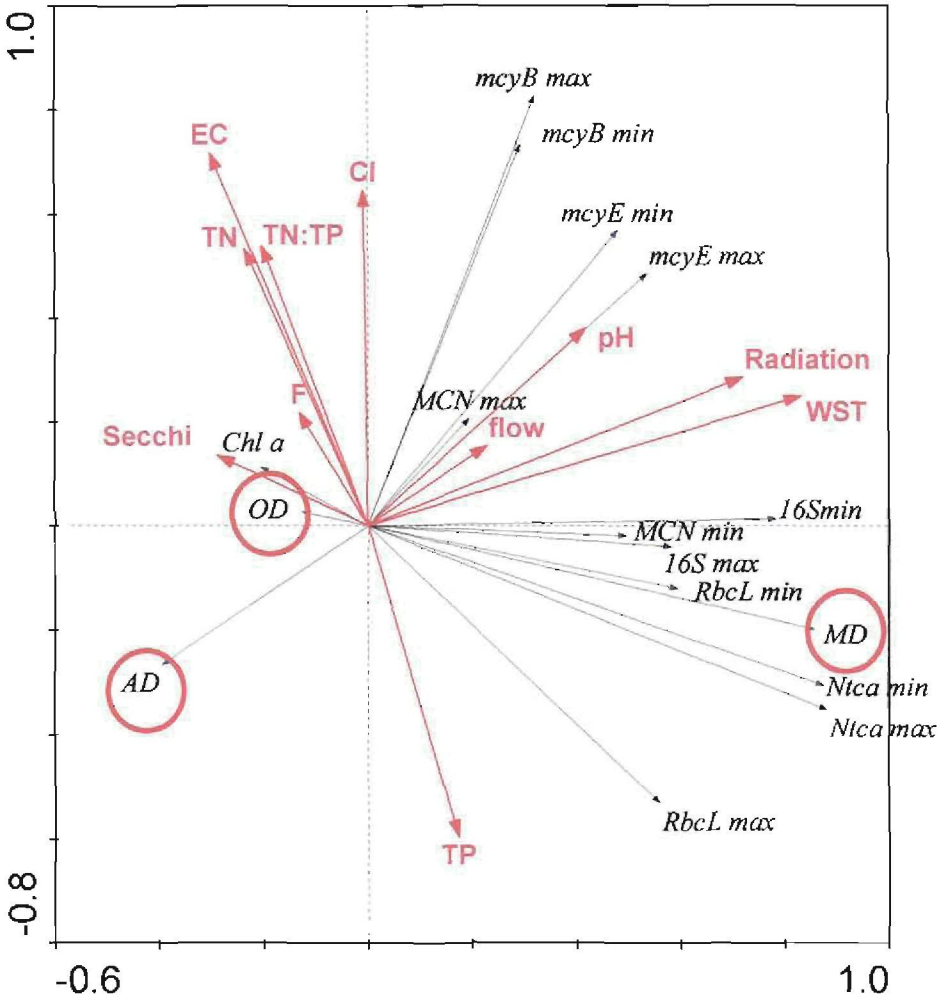


Figure 5.8: RDA environmental biplot of the Roodeplaat Dam together with molecular data of samples from October 2004 to May 2005 and including the species dominance data. Environmental data are indicated in red and molecular data are indicated in black (Encircled: OD=*Oscillatoria sp.* dominance; MD=*Microcystis sp.* dominance and AD=*Anabaena sp.* dominance).

Table 5.3: Results from the RDA analysis of the Roodeplaat Dam depicted in figure 5.8 indicating the percentage of variance explained by the different axes.

Axes		**1**	**2**	**3**	**4**	**Total variance**
Eigenvalues		0.319	0.154	0.122	0.098	1.000
Species-environmental correlations		0.940	0.928	0.992	0.963	
Cumulative percentage variance	**Of species data (molecular data)**	31.9	47.3	59.5	69.3	
	Of species-environmetal relation	37.1	54.9	69.1	80.5	
Sum of all eigenvalues						1.000
Sum of all canonical eigenvalues						0.861

Summary of Monte Carlo test

Test of significance of first canonical axis: eigenvalue = 0.319

F-ratio = 0.937

P-value = 0.6620

Test of significance of all canonical axes: Trace = 0.861

F-ratio = 1.127

P-value = 0.4240

5.3.4 *Species dominance in the Hartbeespoort Dam*

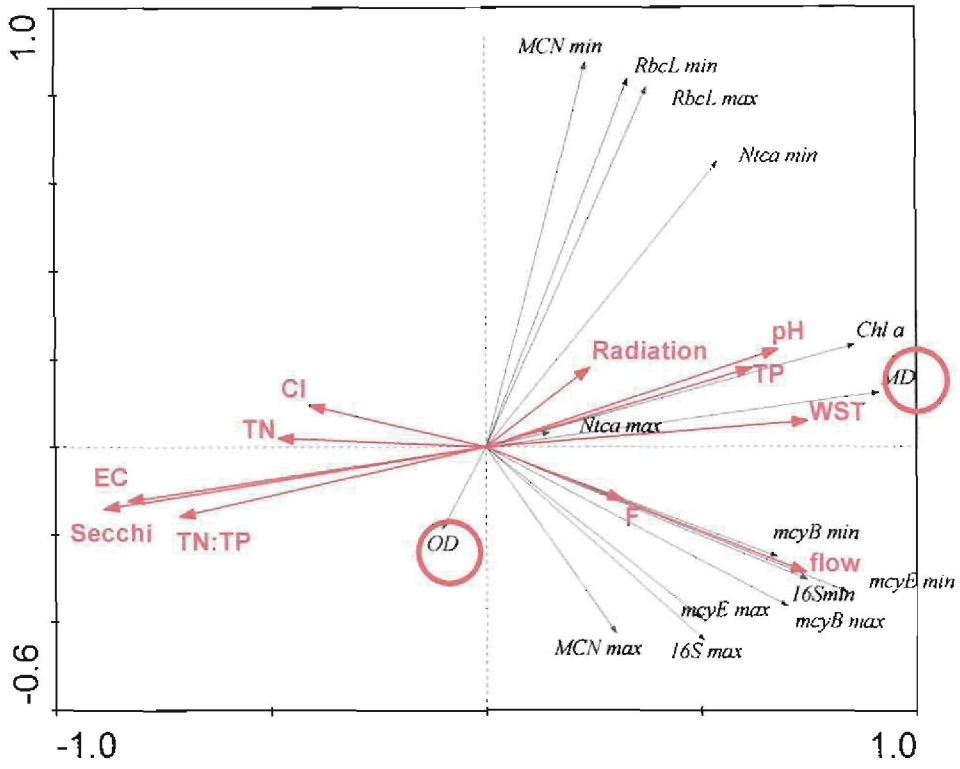


Figure 5.9: RDA biplot with species dominance of the Hartbeespoort Dam together with molecular data of samples from October 2004 to May 2005. Environmental data are indicated in red and molecular data are indicated in black. (Encircled: OD=*Oscillatoria sp.* dominance and MD=*Microcystis sp.* dominance).

Table 5.4: Results from the RDA analysis of the Hartbeespoort Dam depicted in figure 5.9 indicating the percentage of variance explained by the different axes.

Axes		**1**	**2**	**3**	**4**	**Total variance**
Eigenvalues		0.335	0.240	0.141	0.072	1.000
Species-environmental correlations		0.999	0.947	0.969	0.946	
Cumulative percentage variance	**Of species data (molecular data)**	33.5	57.5	71.5	78.8	
	Of species-environmetal relation	36.3	62.4	77.6	85.5	
Sum of all eigenvalues						1.000
Sum of all canonical eigenvalues						0.922

Summary of Monte Carlo test

Test of significance of first canonical axis: eigenvalue = 0.335

F-ratio = 0.503

P-value = 0.2220

Test of significance of all canonical axes : Trace = 0.922

F-ratio = 1.069

P-value = 0.5140

5.3.5 RDA biplot of the Roodeplaat Dam

In figure 5.10 and figure 5.11 the RDA environmental biplots of the Roodeplaat Dam and the Hartbeespoort Dam are displayed.

The most important environmental variables for both the dams (indicated by the length of the arrow) were water surface temperature, total nitrogen, total phosphorus, pH, electrical conductivity, chlorine and radiation.

The WST in both dams was closely associated with radiation as can be expected. In the Roodeplaat Dam (figure 5.10) water surface temperature and *mcyE* copy number were closely associated. Similarly a significant correlation between water surface temperature and *mcyE* max copy number exists in the Hartbeespoort Dam (figure 5.11).

The pH of the Roodeplaat Dam and the Hartbeespoort Dam was quite high, ranging from 8.0 to 9.75 in the Roodeplaat Dam and from 7.8 to 9.2 in the Hartbeespoort Dam. In the RDA biplots of both dams (figures 5.10 and 5.11), pH is highly significant as indicated by the length of the arrow. From the RDA biplot of the Roodeplaat Dam, an association between pH and *mcyE* copy number could be observed and a statistically significant ($p < 0.05$; $r = 0.54$; $n = 14$) correlation between pH and *mcyB* copy number, however this was not determined in the Hartbeespoort Dam.

From the RDA biplot of the Hartbeespoort Dam (figure 5.11) a close association between total phosphates, pH and chl *a* could be observed indicating the relationship between nutrients and biomass. In the Roodeplaat Dam (figure 5.10) total phosphates associated very closely with the maximum *rbcL* gene expression, indicating more specifically the association between nutrients and the onset of primary production that might lead to an increase in biomass.

The total nitrogen in the Roodeplaat Dam increased due to the inflow as shown in the RDA biplot of the Roodeplaat Dam (figure 5.10). This is also supported by the positive association of the flow with total phosphates as observed in the RDA biplot (figure 5.10).

From figure 5.10 and 5.11 it is also clear that although the max *mcyB* and max *mcyE* copy number are closely related in the RDA in both dams, according to the statistical analysis, they are not co-linear. According to figure 5.10 and figure 5.11, there was a closer association between the *mcyE* copy number and *Microcystis sp.* specific 16S rRNA gene copy number, than the *mcyB* copy number and the *Microcystis sp.* 16S rRNA gene copy number.

In both dams a positive association exists between the influx (flow) of nutrients and *mcyE/mcyB* copy numbers (figure 5.10 and 5.11). In the Roodeplaat Dam, the *mcyB* and *mcyE* copy numbers do not correlate with phosporus and nitrogen or phosphates and nitrate concentrations (figure 5.10) as was the case in the Hartbeespoort Dam (figure 5.11). In contrast to the Roodeplaat Dam, the *mcyE* and *mcyB* copy number in the Hartbeespoort Dam correlated negatively with the TN:TP ratio as well as dissolved inorganic nitrogen (DIN) (figure 5.9). However, a positive correlation between *mcyE* copy number and Kjel-N occurred in both dams. The results of the Hartbeespoort Dam (figure 5.11) indicate that *mcyE* max copy number clusters with the flowdata and KJEL-N (organic nitrogen).

This can also be seen in the positive correlation of *ntcA* gene expression and NH_4-N in the RDA biplot (figure 5.11). The RDA biplot of the Roodeplaat Dam indicates that the *ntcA* gene expression correlates negatively with NH_4 concentration.

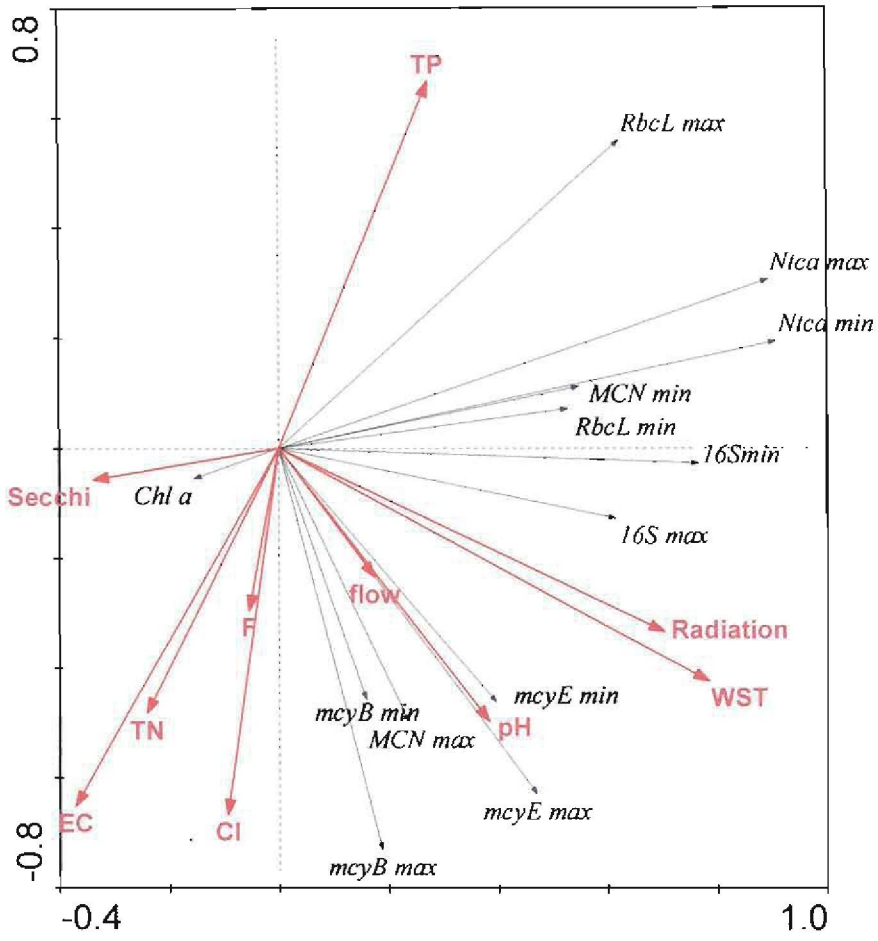


Figure 5.10: RDA environmental biplot of the Roodeplaat Dam including molecular data of the samples from October 2004 to May 2005, without the species dominance. Environmental data are indicated in red and molecular data are indicated in black.

Table 5.5. Results from the RDA analysis of the Roodeplaat Dam depicted in figure 5.10 indicating the percentage of variance explained by the different axes.

Axes		**1**	**2**	**3**	**4**	**Total variance**
Eigenvalues		0.314	0.161	0.124	0.095	1.000
Species-environmental correlations		0.924	0.840	0.945	0.978	
Cumulative percentage variance	**Of species data (molecular data)**	31.4	47.4	59.8	69.3	
	Of species-environmetal relation	39.7	60.1	75.8	87.8	
Sum of all eigenvalues						1.00
Sum of all canonical eigenvalues						0.789

Summary of Monte Carlo test

Test of significance of first canonical axis: eigenvalue = 0.314

F-ratio = 1.371

P-value = 0.9640

Test of significance of all canonical axes: Trace = 0.789

F-ratio = 1.125

P-value = 0.4780

5.3.6 RDA biplot of the Hartbeespoort Dam

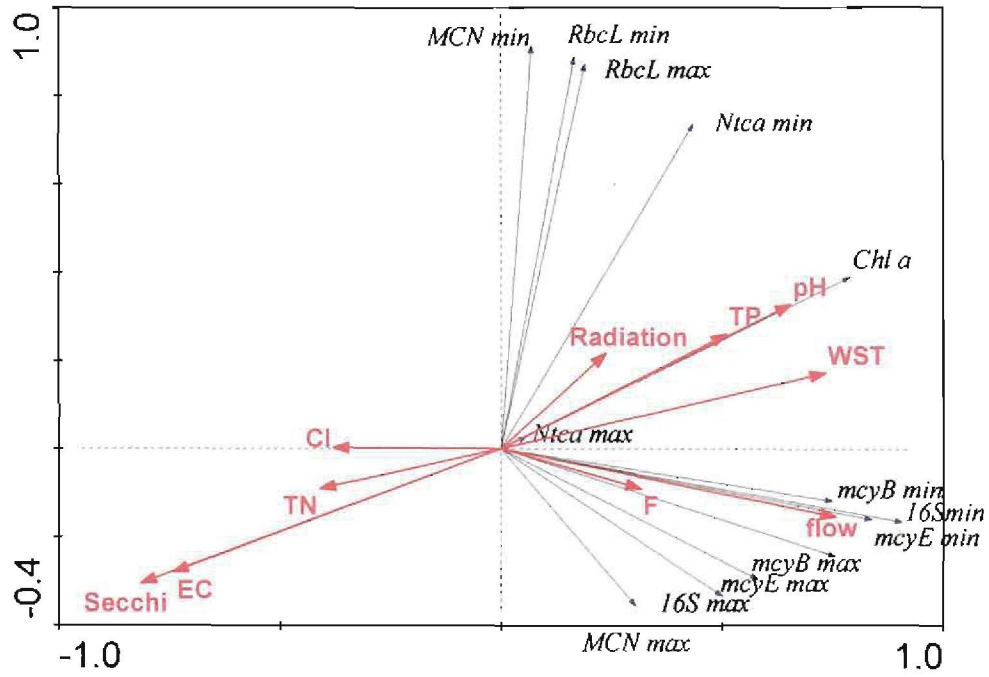


Figure 5.11: RDA environmental biplot of the Hartbeespoort Dam including molecular data of samples from October 2004 to May 2005 without the species dominance. Environmental data are indicated in red and molecular data are indicated in black.

Table 5.6. Results from the RDA analysis of the Hartbeespoort Dam depicted in figure 5.11 indicating the percentage of variance explained by the different axes.

Axes		1	2	3	4	Total variance
Eigenvalues		0.326	0.272	0.129	0.067	1.000
Species-environmental correlations		0.990	0.955	0.923	0.997	
Cumulative percentage variance	**Of species data (molecular data)**	32.6	59.7	72.7	79.3	
	Of species-environmetal relation	36.1	66.3	80.6	88.1	
Sum of all eigenvalues						1.000
Sum of all canonical eigenvalues						0.901

Summary of Monte Carlo test

Test of significance of first canonical axis: eigenvalue = 0.326

F-ratio = 0.966

P-value = 0.3020

Test of significance of all canonical axes : Trace = 0.901

F-ratio = 1.822

P-value = 0.0920

5.3.7 Seasonal variation

The bloom period was divided into three seasons, namely: spring (October 2004 to November 2004), summer (December 2004 to February 2005) and autumn (March 2005 to May 2005).

5.3.7.1 Seasonal variation in Microcystis sp. 16S rRNA gene copy number and nutrients

The *Microcystis sp.* 16S rRNA gene copy numbers in the Hartbeespoort Dam are very high, reaching a maximum of 1,669,500 copies and an average of 324,085 during the bloom season. This is more than twice the amount of copies present in the Roodeplaat Dam (figure 5.12).

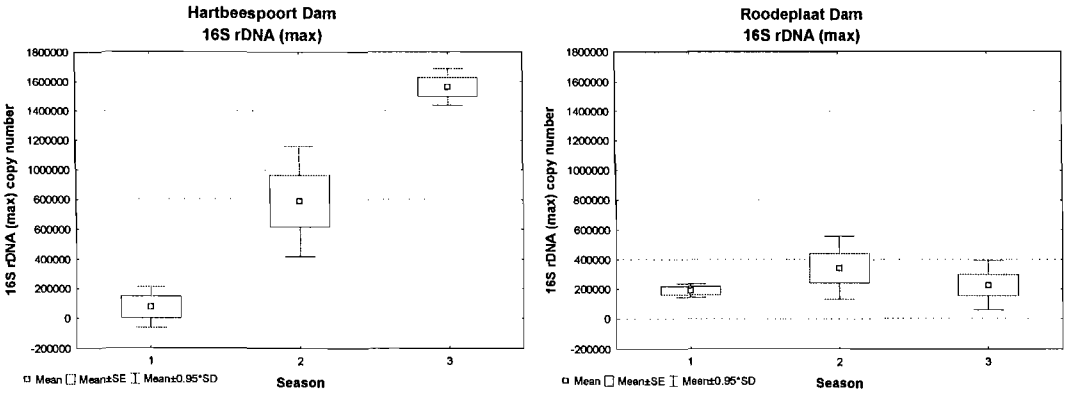


Figure 5.12: Boxplot representing the seasonal variation in the maximum *Microcystis* specific 16S rRNA gene copy number in the Hartbeespoort and Roodeplaat Dams between October 2004 and May 2005, representing spring (1), summer (2) and autumn (3).

A clear negative association could be seen in the RDA biplots of both dams (figure 5.10 and 5.11) between the total nitrogen and the amount of *Microcystis sp.* cells in the water as indicated by the *Microcystis sp.* specific 16S rRNA gene copy numbers. In the Hartbeespoort Dam, the total nitrogen steadily decreased during the sampling period while there was a steady increase in the *Microcystis sp.* specific 16S rRNA gene copy number throughout the sampling period (figure 5.13). In the Roodeplaat Dam, the *Microcystis sp.* specific 16S rRNA gene copy number increased from spring to summer and concomitantly the total nitrogen decreased from a maximum during spring to a minimum during summer, after which *Microcystis sp.* specific 16S rRNA gene copy numbers decreased and the total nitrogen levels increased. The maximum *Microcystis sp.* specific 16S rRNA gene copy number observed in the Roodeplaat Dam coincides with the minimum total nitrogen as was the case in the Hartbeespoort Dam (figure 5.13).

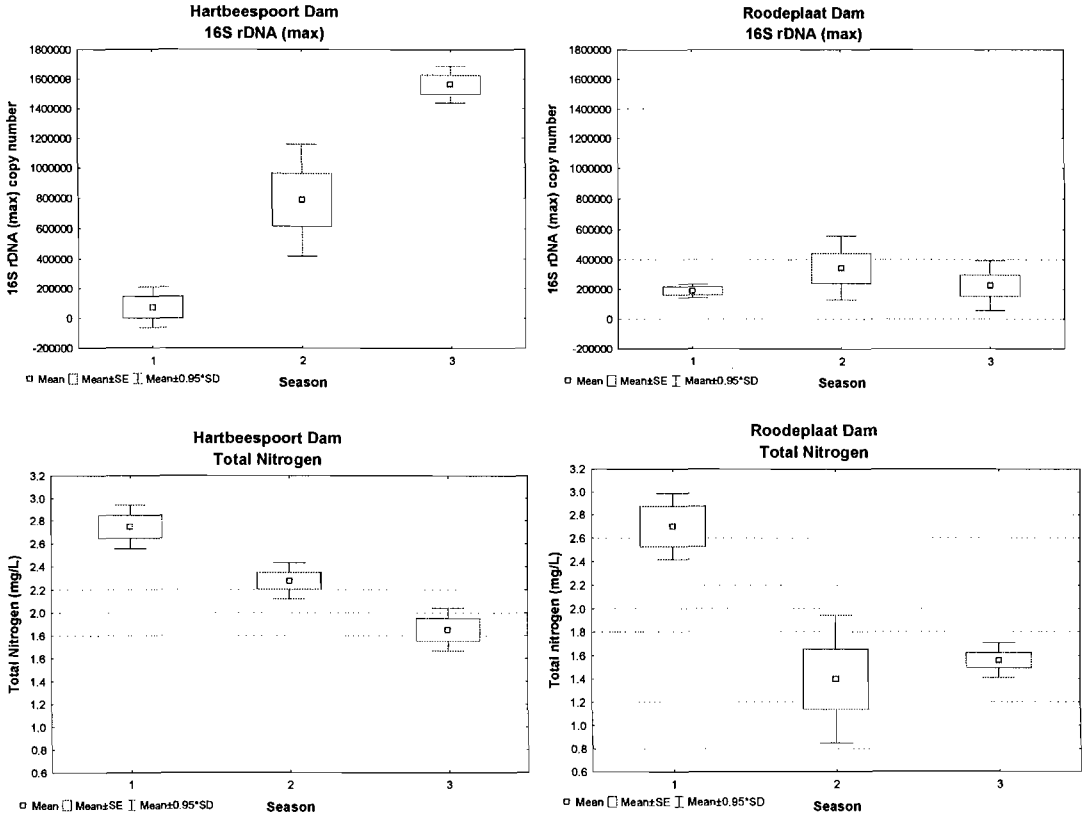


Figure 5.13: Boxplot representing the variation in the max 16S rRNA gene copy number and total nitrogen (TN) concentration measured in the Hartbeespoort and Roodeplaat Dams between October 2004 and May 2005, representing spring (1), summer (2) and autumn (3).

No such trends were observed between TN and chl *a* or *mcyB* and *mcyE* (figure 5.14) containing *Microcystis sp.* strains in either of the dams.

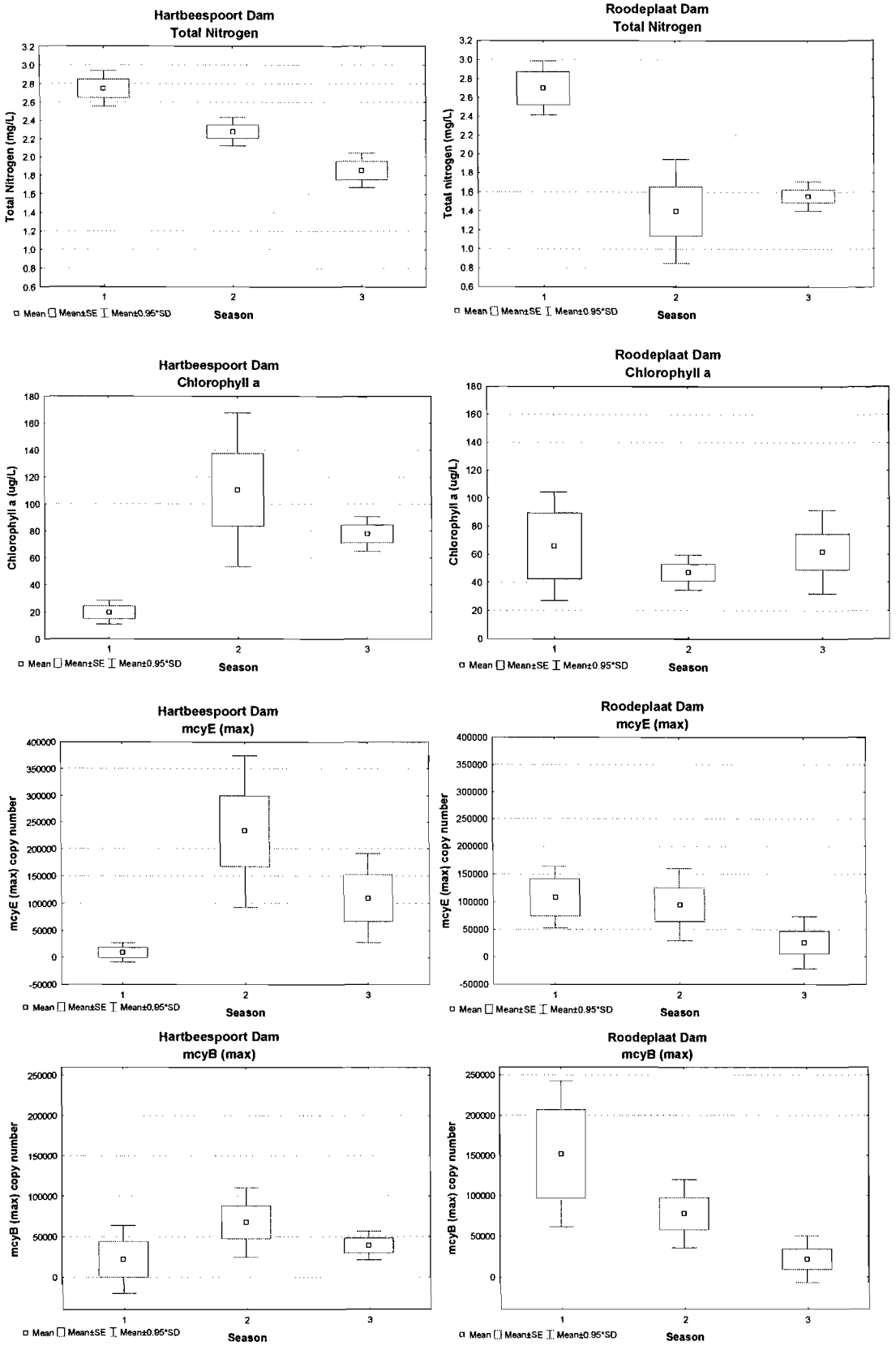


Figure 5.14: Boxplot representing the variation in the Total Nitrogen (TN), chl *a* concentration, *mcyE* max copy number and *mcyB* max copy number measured in the Hartbeespoort and Roodeplaat Dams between October 2004 and May 2005, representing spring (1), summer (2) and autumn (3).

A positive association between the *Microcystis sp.* specific 16S rRNA gene copy number and PO_4-P:NH_4-N ratio was observed in the Hartbeespoort Dam. No statistically significant correlations were however detected between total phosphorus or PO_4 levels and inflow into both dams. Maximum values for total phosphate and 16S rRNA gene copy number only coincide in the Hartbeespoort Dam, while maximum 16S rRNA gene copy number in the Roodeplaat Dam only coincides with maximum inflow, which can be linked to nutrients (figure 5.15).

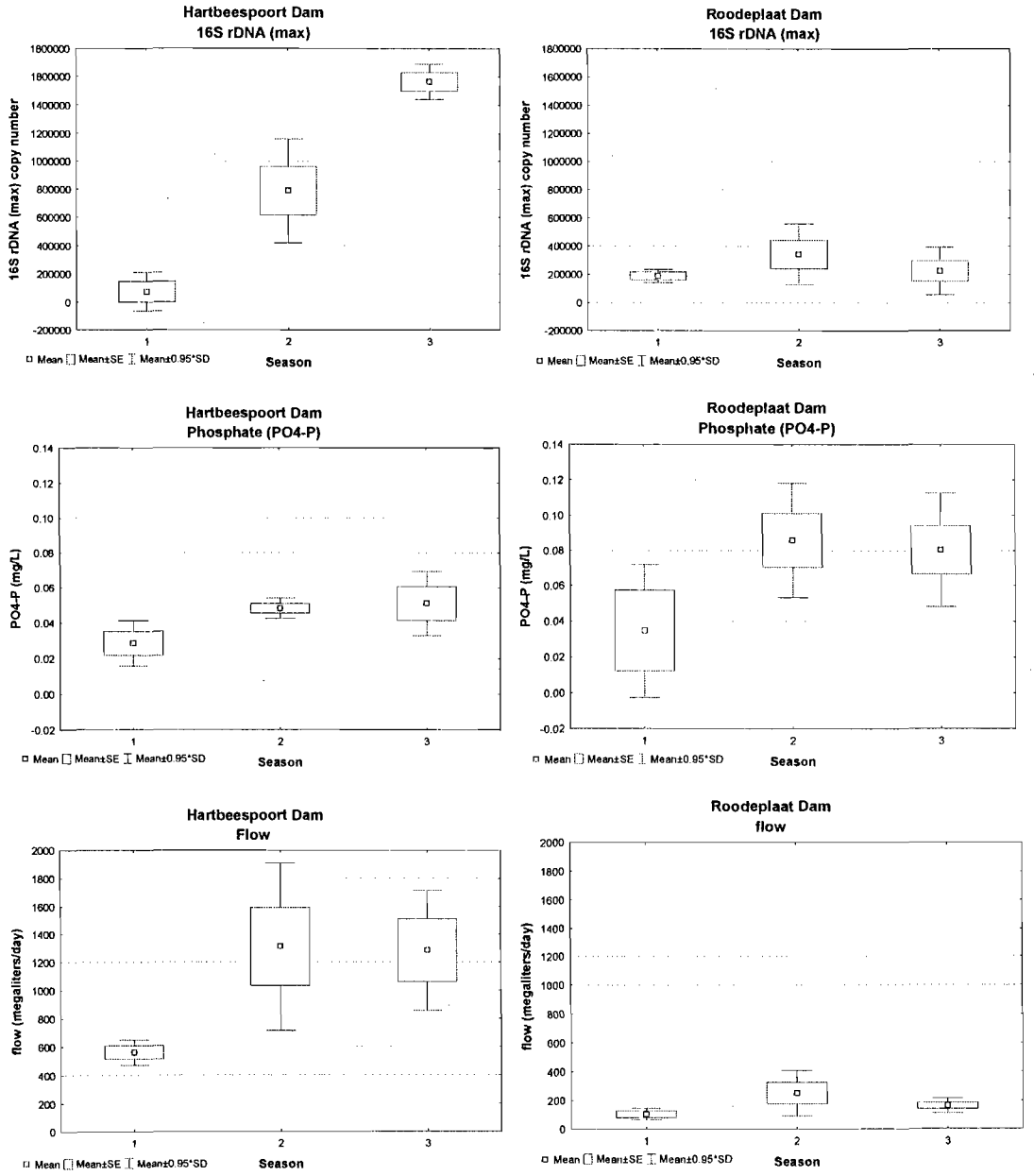


Figure 5.15: Boxplot representing the variation in the max 16S rRNA gene copy number, the phosphate (PO_4-P) concentration and the flow measured in the Hartbeespoort and Roodeplaat Dams between October 2004 and May 2005, representing spring (1), summer (2) and autumn (3).

16S rRNA gene copy number showed a similar trend to chl *a* concentration in the Hartbeespoort Dam, although, the *Microcystis* specific 16S rRNA gene copy number does not correlate significantly with the chl *a* concentration. This is also emphasised by the time series in figure 5.16, indicating that the percentage of *Microcystis sp.* dominance reached its maximum during the middle of the bloom season. No such trends were observed in Roodeplaat Dam (figure 5.17).

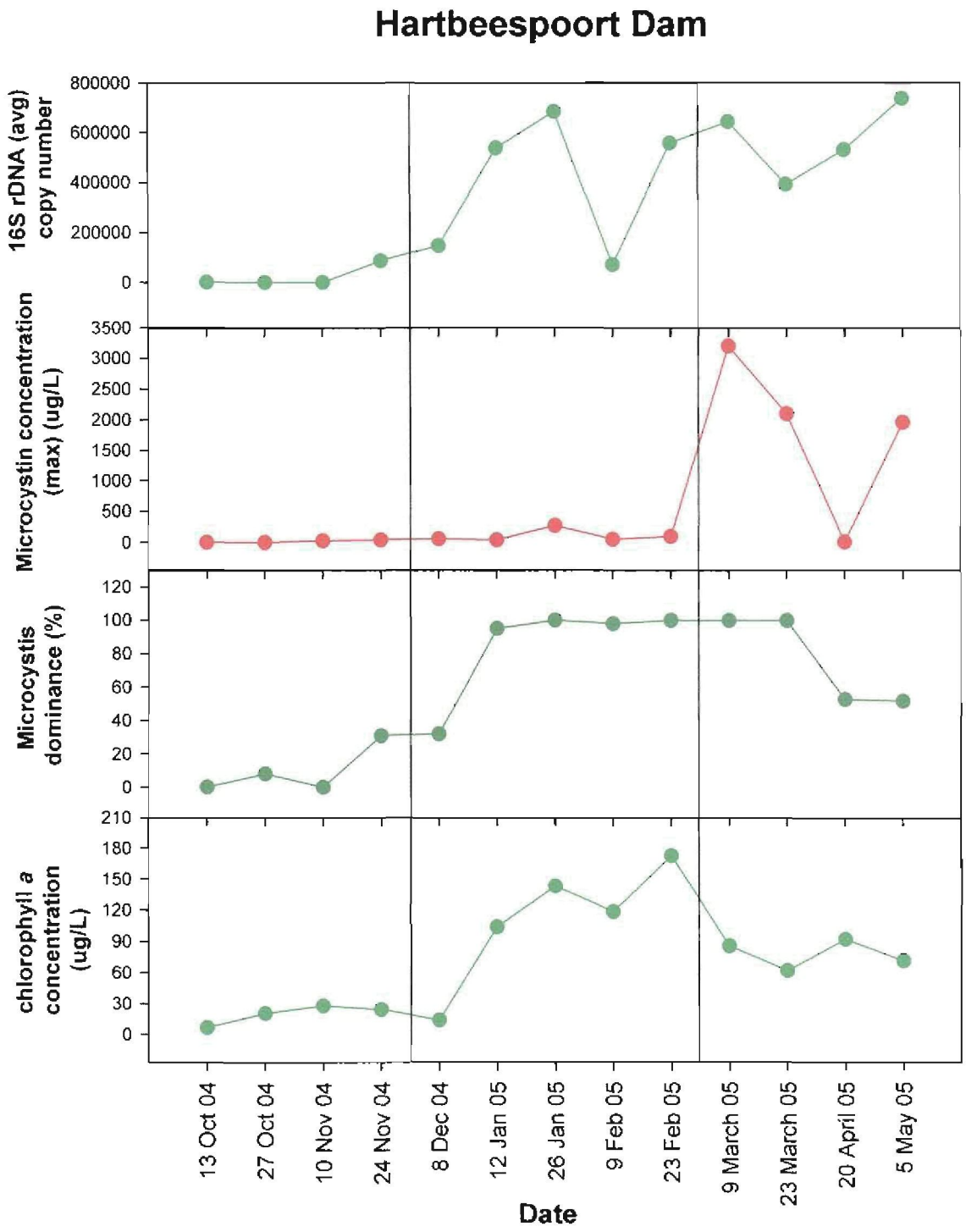


Figure 5.16: Time series representing 16S rDNA copy number, maximum microcystin concentration, chl *a* concentration and *Microcystis* dominance in the Hartbeespoort Dam from October 2004 to May 2005.

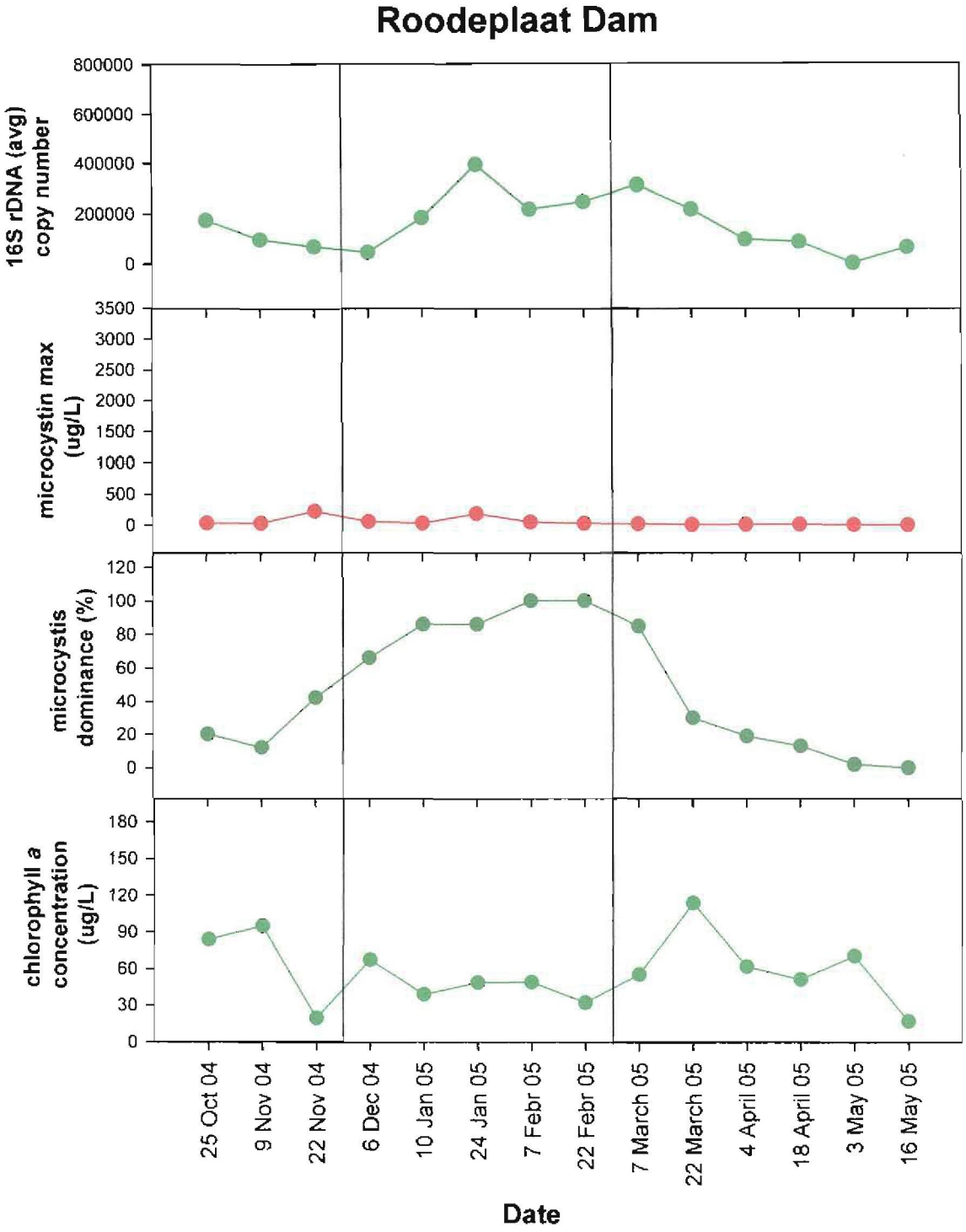


Figure 5.17: Time series representing 16S rDNA copy number, microcystin concentration, chl *a* concentration and *Microcystis* dominance in the Roodeplaat Dam from October 2004 to May 2005.

5.3.7.2 Seasonal variation in microcystin and toxigenic strains

The ratio of *mcyE* to *mcyB* during the periods of maximum presence of toxigenic strains was 1.5:1 in the Roodeplaat Dam, compared to 4:1 in the Hartbeespoort Dam, suggesting the presence of different strains causing toxicity in the dams. According to the statistical analysis of the data, a significant correlation was found between the *Microcystis sp.* 16S rRNA gene copy

number and *mcyB* copy number ($p < 0.05$; $r = 0.7$; $n = 13$), as well as the *mcyE* copy number in the Hartbeespoort Dam. Similarly there was a significant correlation between the *mcyE* and 16S rRNA gene copy number in the Roodeplaat Dam ($p < 0.05$; $r = 0.99$; $n = 13$). After maximum values were observed in the summer for these variables, both the copy number for *mcyB* and *mcyE* decreased during autumn at the end of the sample period. In contrast with this, the 16S rRNA gene copy numbers increased during the sample period to reach a maximum at the end of the sampling period during autumn. Copy numbers calculated for the *mcyB* and *mcyE* and 16S rRNA genes are considerably lower in the Roodeplaat Dam than in the Hartbeespoort Dam (figure 5.18, 5.19 and 5.20).

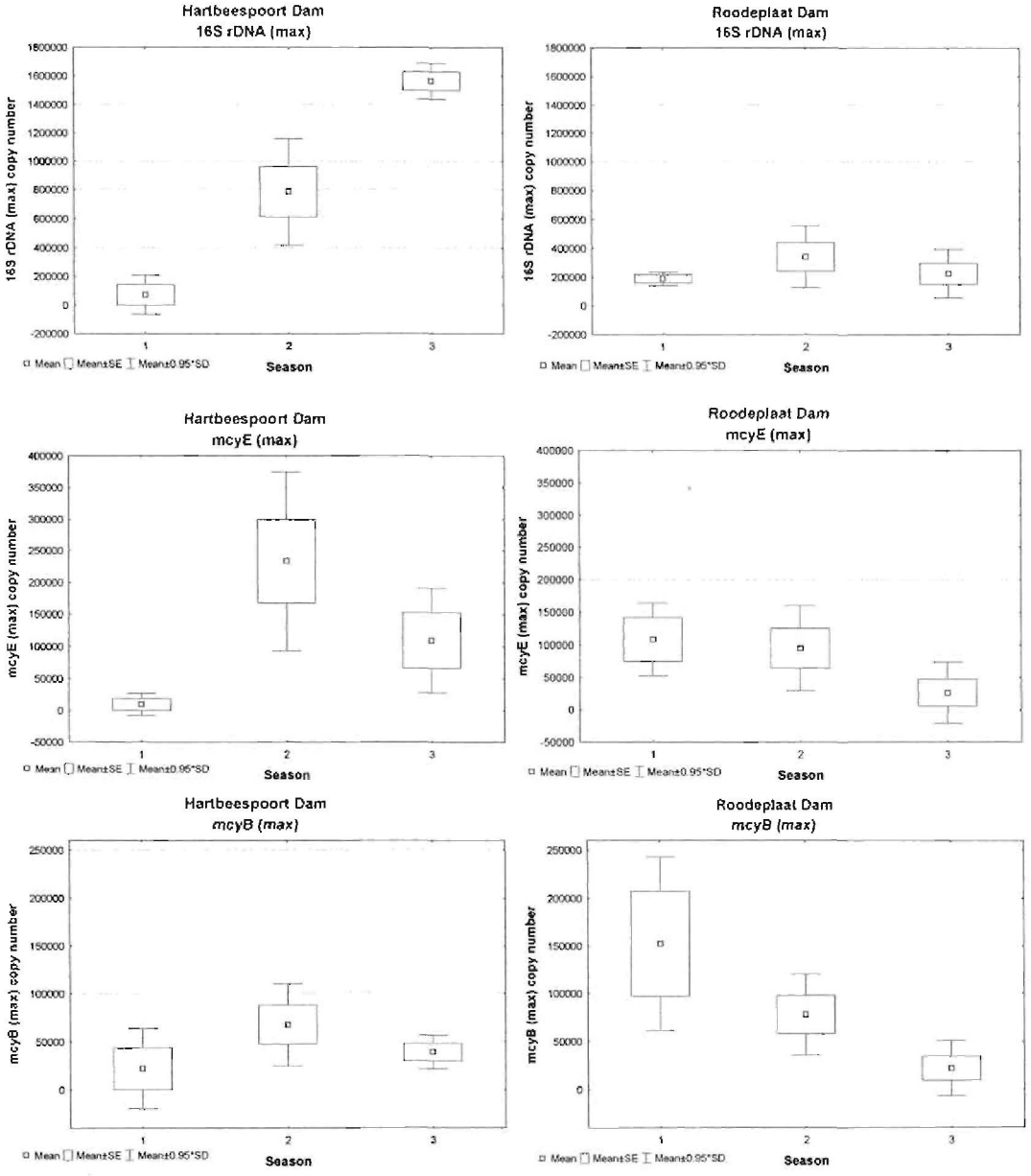


Figure 5.18: Boxplot representing the seasonal variation in the maximum *Microcystis* specific 16S rRNA gene copy number, *mcyE* and *mcyB* max copy number in the Hartbeespoort and Roodeplaat Dams between October 2004 and May 2005, representing spring (1), summer (2) and autumn (3).

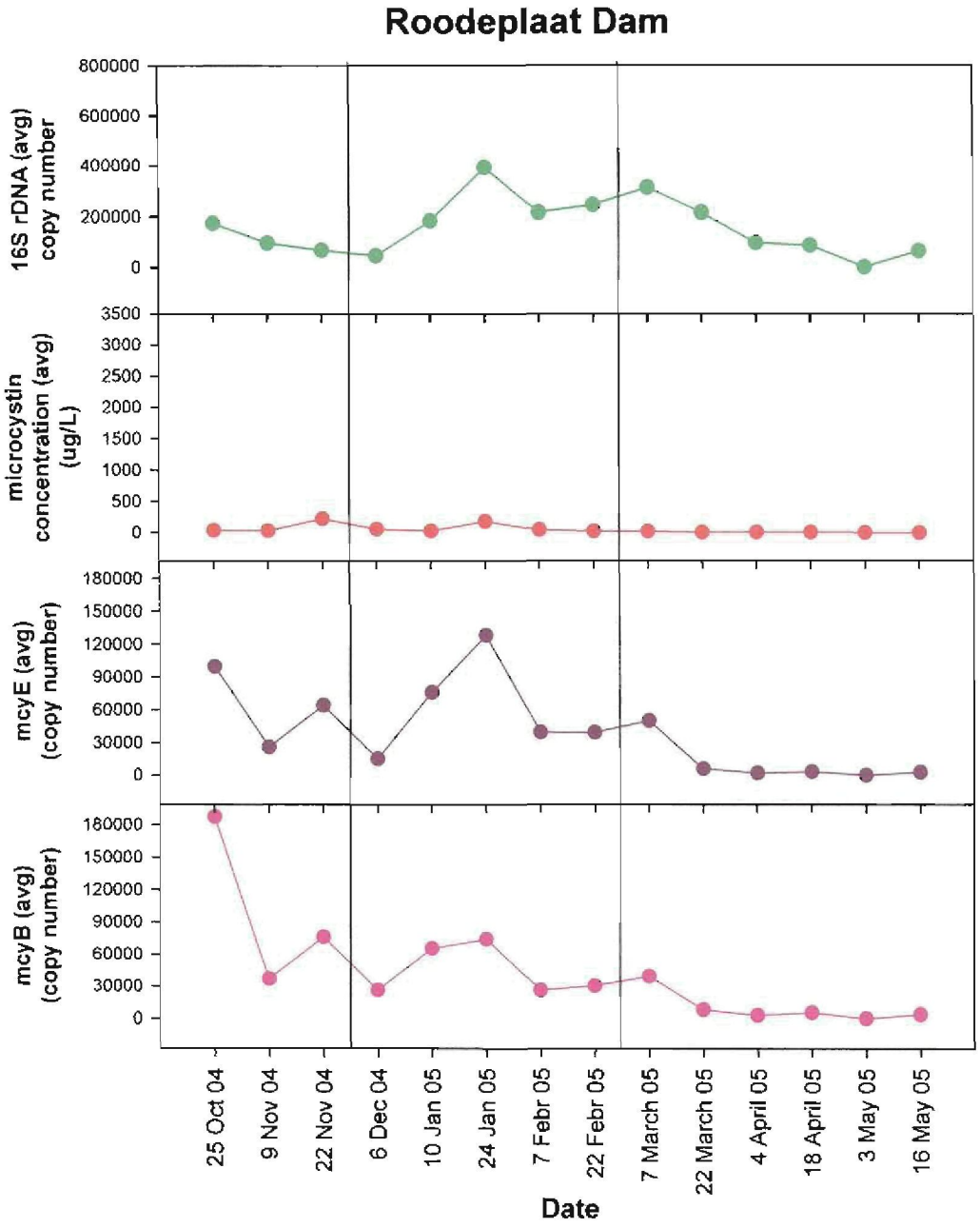


Figure 5.19: Time series representing 16S rRNA gene copy number, microcystin concentration, *mcyE* and *mcyB (avg)* copy number in the Roodeplaat Dam from October 2004 to May 2005.

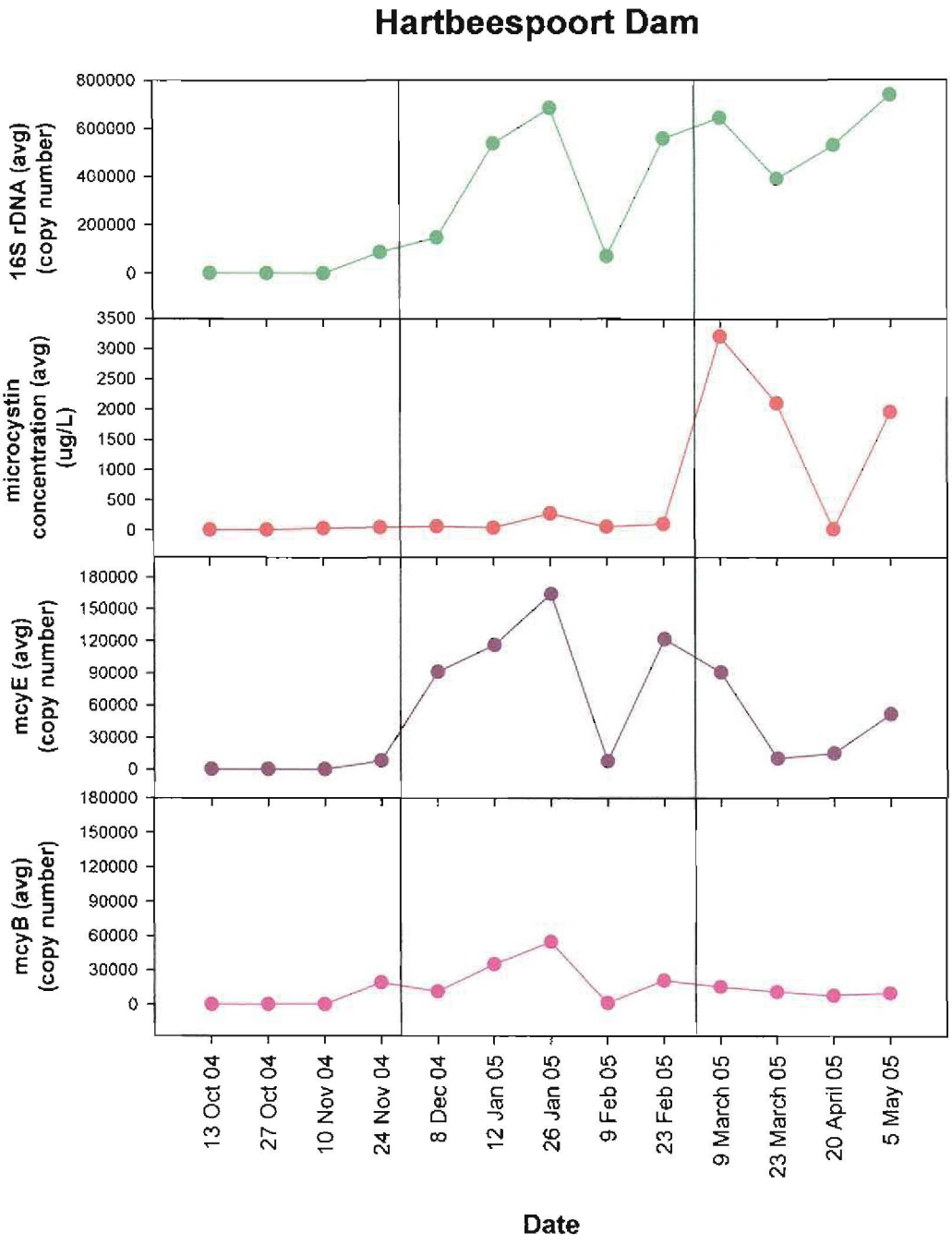


Figure 5.20: Time series representing 16S rRNA gene copy number, microcystin concentration, *mcyE* and *mcyB* (avg) copy number in the Hartbeespoort Dam from October 2004 to May 2005.

In the seasonal variation of the temperature depicted in figure 5.21, 5.22 and 5.23, it can be seen that the highest temperatures in both the Hartbeespoort Dam and the Roodeplaat Dam were experienced in the middle of the sampling period, namely during summer. In the Hartbeespoort Dam the average temperature during spring was much lower compared to the Roodeplaat Dam (22°C vs. 24°C). Then, when the temperature increased to 26.5°C during the summer in the Hartbeespoort Dam, the copy number of the strains containing *mcyB* and *mcyE* (toxic strains) also increased, together with the 16S rRNA gene copy number. At the end of the sampling

period (autumn), the temperature decreased again to 22°C in the Hartbeespoort Dam, and subsequently the *mcyE* and *mcyB* copy numbers also decreased. This decrease is not related to the copy number of all the *Microcystis sp.* in the dam, as the 16S rRNA gene copy number still increased at the end of the season. In the summer, the temperature in the Roodeplaat Dam did not increase very much and the *mcyE* and *mcyB* strains did not increase either. However, when the temperature decreased in autumn (21.5°C) the *mcyE* and *mcyB* copy numbers also decreased. This is related to the copy number of all the *Microcystis* species in the dam, as the 16S rRNA gene copy number also decreased at the end of the season.

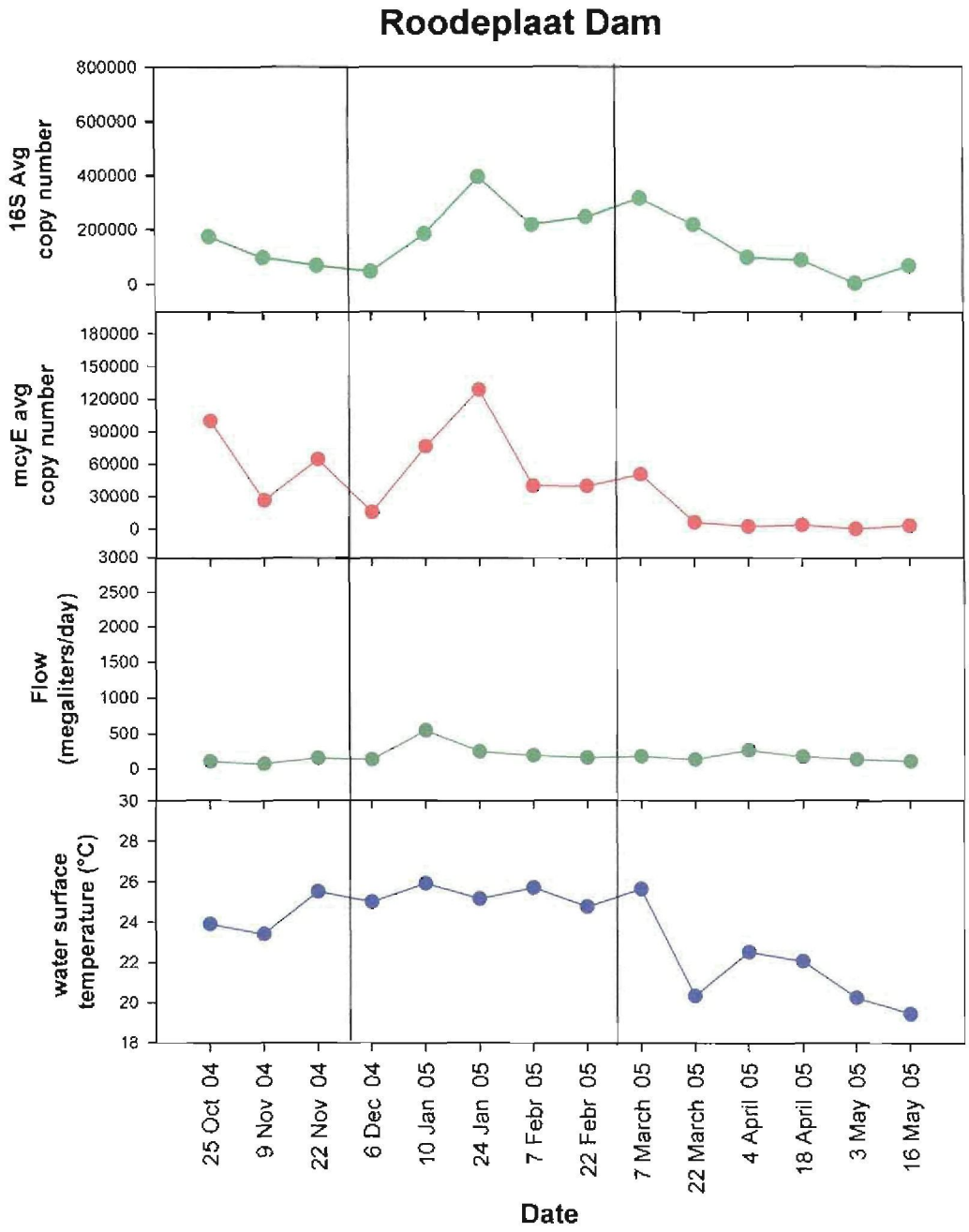


Figure 5.21: Time series representing the average 16S rRNA gene copy number, the average *mcyE* copy number, the flow and the water surface temperature in the Roodeplaat Dam from October 2004 to May 2005.

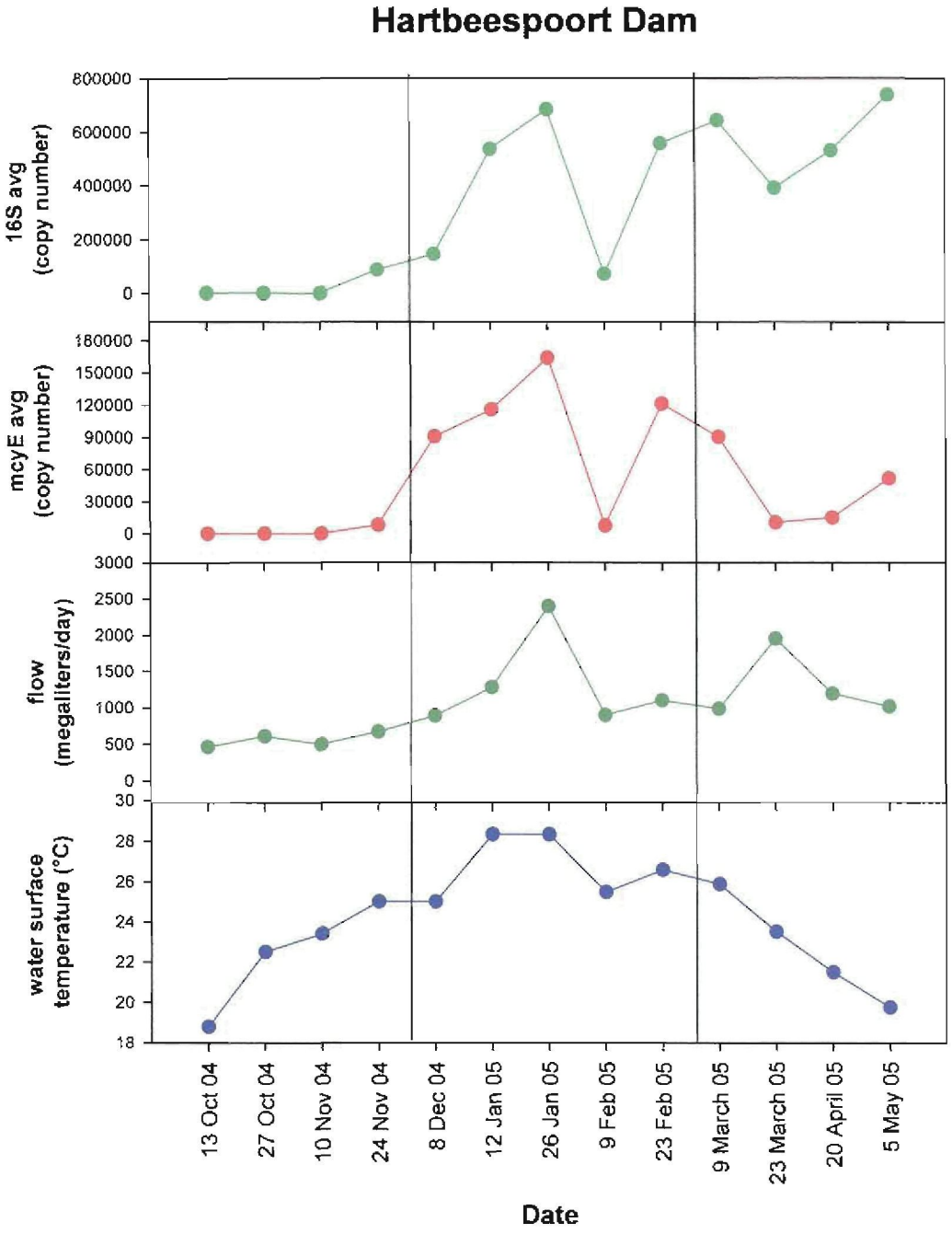


Figure 5.22: Time series representing the average 16S rRNA gene copy number, the average *mcyE* copy number, the flow and the water surface temperature in the Hartbeespoort Dam from October 2004 to May 2005.

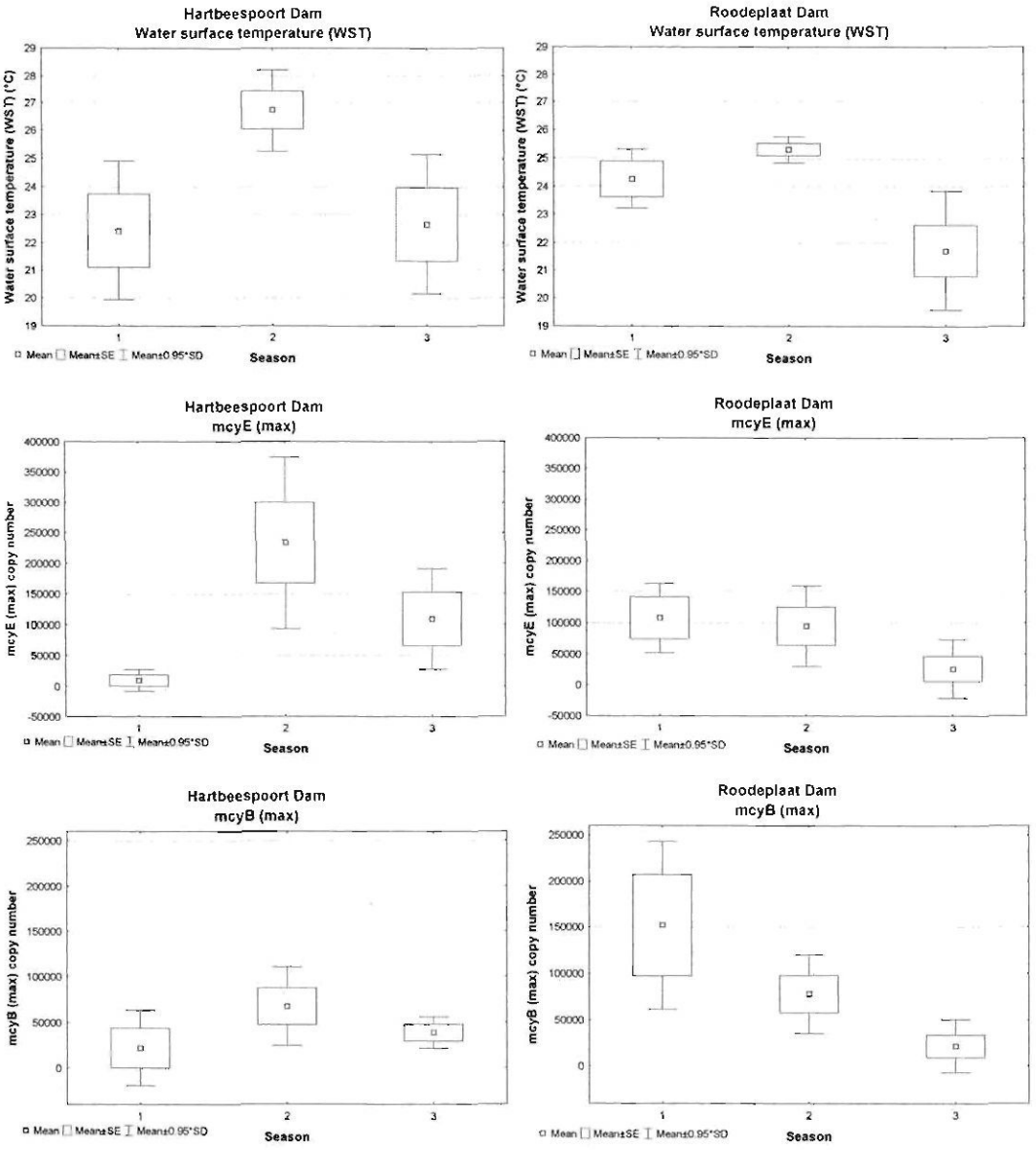


Figure 5.23: Boxplot representing the variation in the water surface temperature (WST), *mcyE* (max) copy number and *mcyB* (max) copy number measured in the Hartbeespoort and Roodeplaat Dams between October 2004 and May 2005, representing spring (1), summer (2) and autumn (3).

These results also have an affect on the microcystin levels (figure 5.24). The disappearance of cells containing *mcyE* and *mcyB* copy numbers together with the change in water surface temperature (figure 5.21 and 5.22) coincides with maximum microcystin concentrations (Roodeplaat Dam - 217.4 µg/L; Hartbeespoort Dam - 3 200 µg/L) found in the water (figure 5.24).

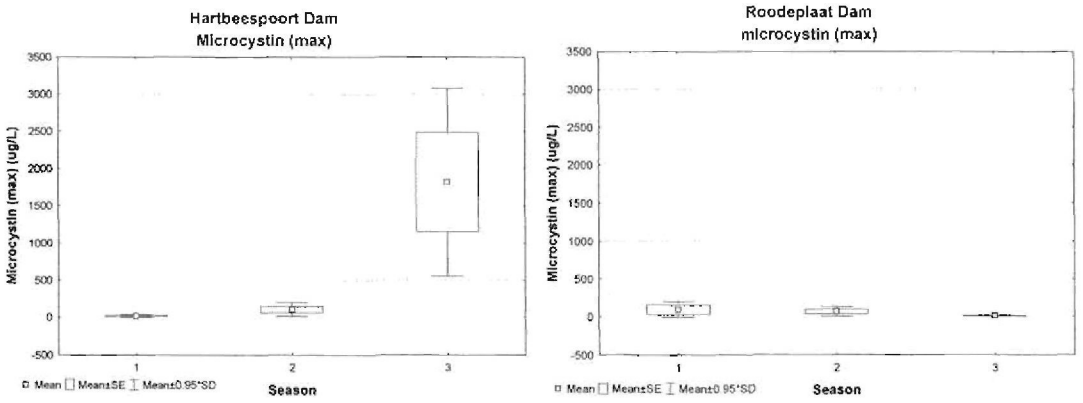


Figure 5.24: Boxplot representing the variation in the max microcystin (MCN) concentration measured in the Hartbeespoort and Roodeplaat Dams between October 2004 and May 2005, representing spring (1), summer (2) and autumn (3).

When comparing a time series of *mcyE* and *mcyB* copy numbers (figure 5.25 and 5.26) it was clear that toxigenic *M. aeruginosa* blooms occured in two almost separate events. In the Roodeplaat Dam the *mcyE* copy number and *mcyB* copy number occured in high numbers together with microcystin at the beginning of the sampling period. The second event is characterised by a high copy number of *mcyE* during the summer period. Similar trends can be observed in the 16S rRNA gene copy number.

In the Hartbeespoort Dam (figure 5.26) two separate events of toxigenic *Microcystis* can be observed as revealed by *mcyB* and *mcyE* copy numbers, with a similar trend for 16S rRNA gene copy number. Maximum microcystin concentration is however only detected after the peaks in *mcyB* and *mcyE* copy numbers started to decline.

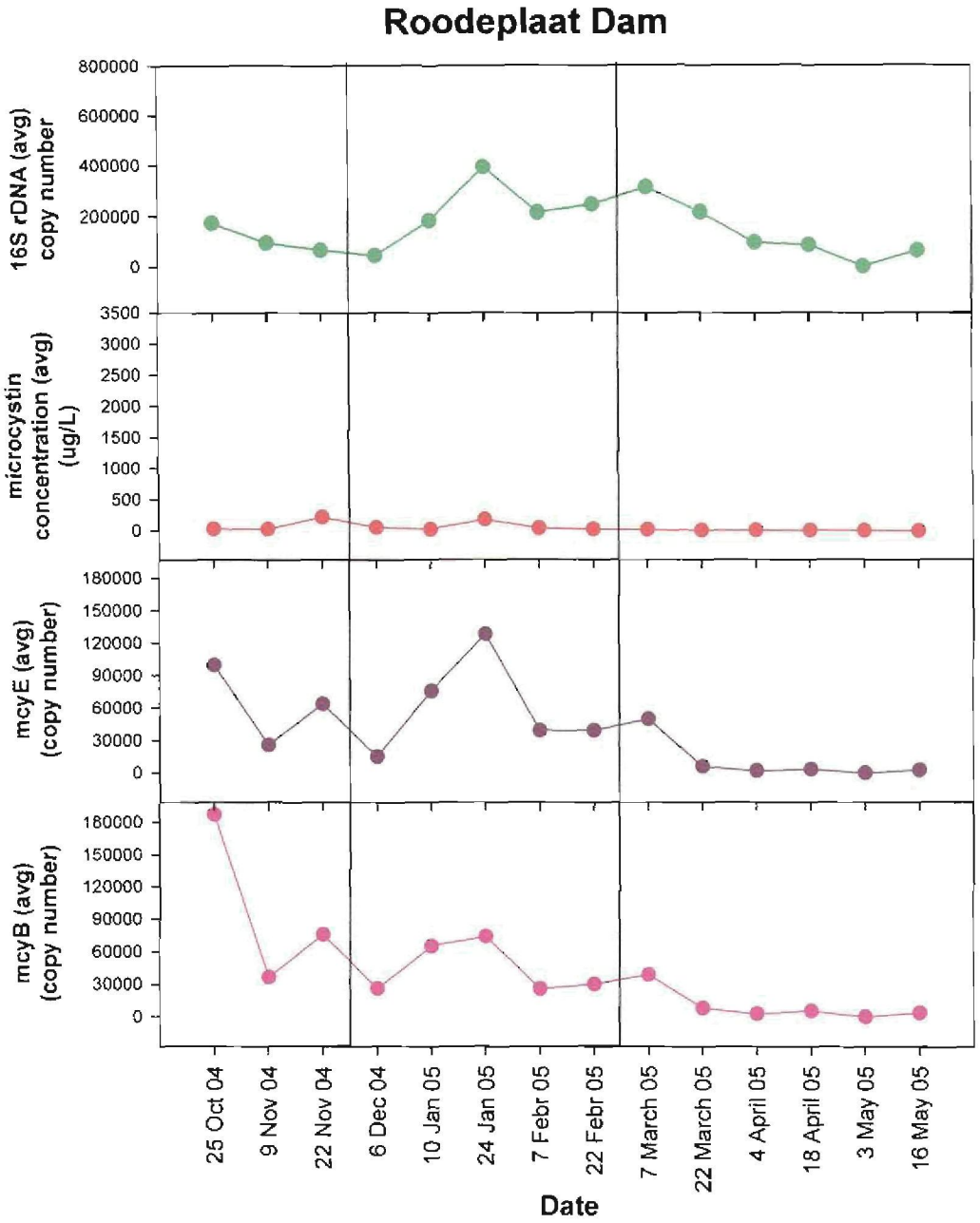


Figure 5.25: Time series representing 16S rRNA gene copy number, microcystin concentration, *mcyE* and *mcyB* copy number in the Roodeplaat Dam from October 2004 to May 2005.

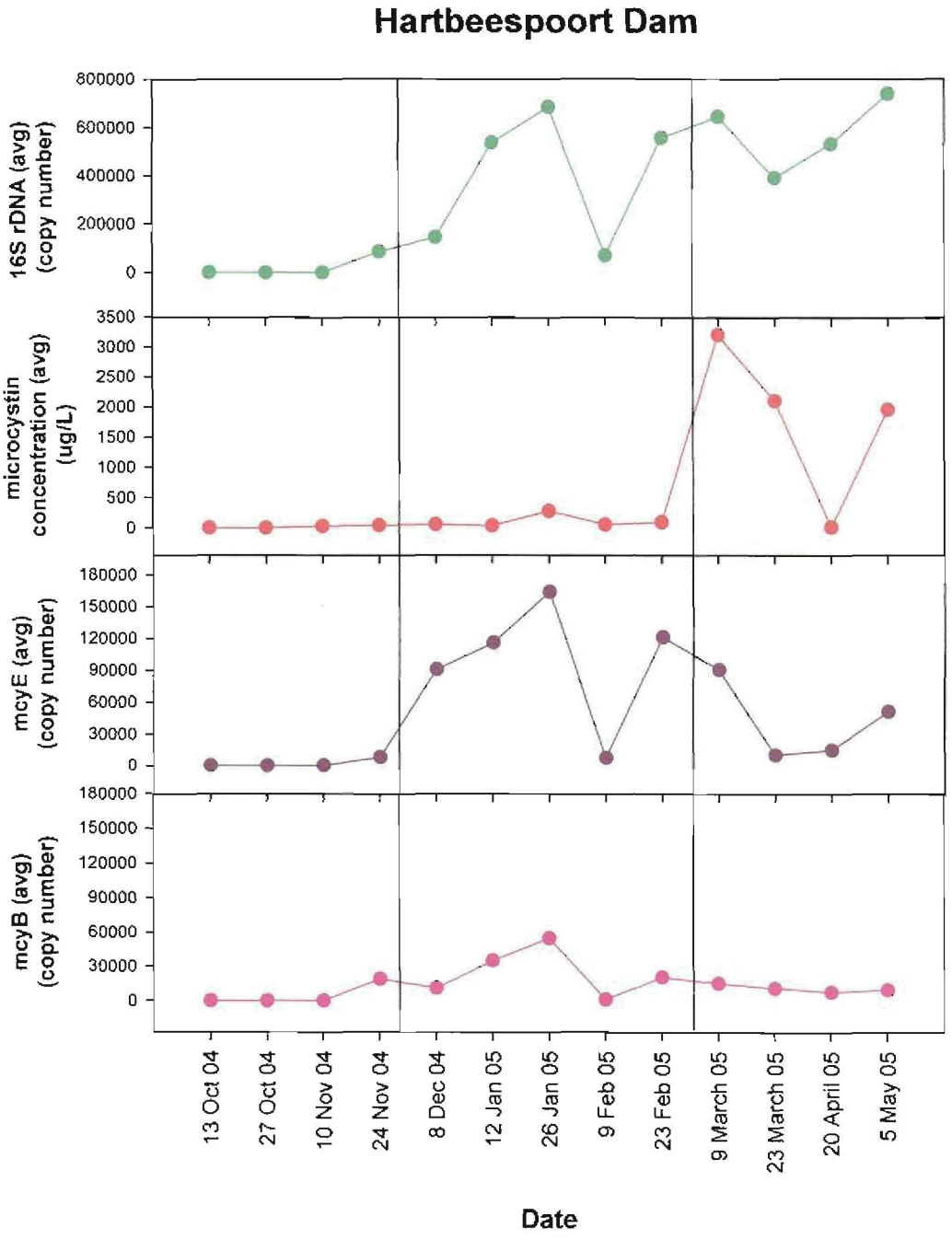


Figure 5.26: Time series representing 16S rRNA gene copy number, microcystin concentration, *mcyE* and *mcyB* copy number in the Hartbeespoort Dam from October 2004 to May 2005.

In the Roodeplaat Dam it can clearly be seen that as the NH_4 concentration decreases in the middle of the sampling period, the *ntcA* levels increased. However, when the NH_4 levels increased in April 2005 to a value of 0.25 mg/L, the *ntcA* gene expression decreased. In the Hartbeespoort Dam, the ammonium levels reached an average value of 0.08 mg/L and a maximum of 0.15 mg/L and one can speculate that during the bloom period (summer) most of the ammonium is used by the cyanobacteria (figure 5.27 and 5.28).

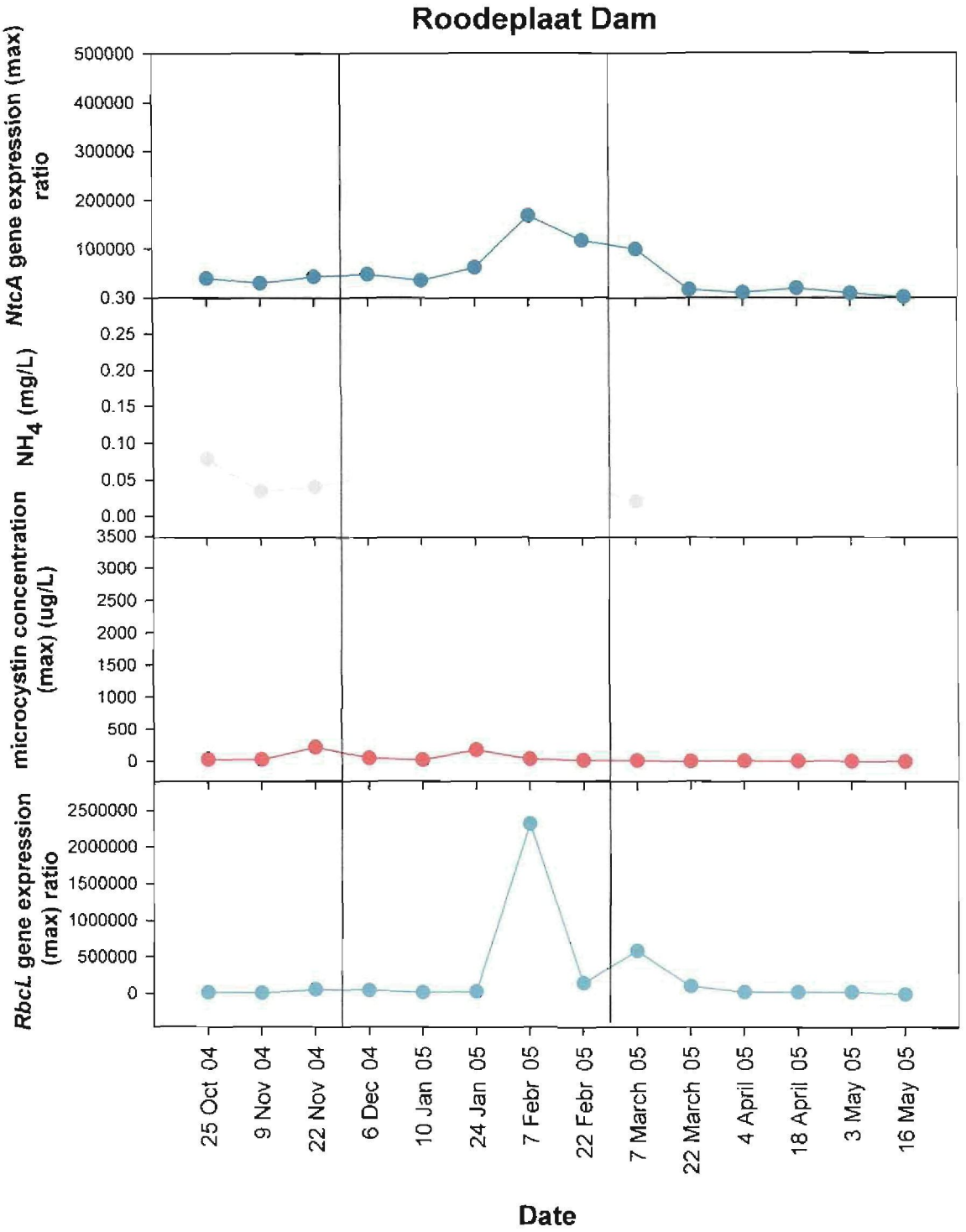


Figure 5.27: Time series representing the maximum *ntcA* ratio, NH_4 concentration, maximum microcystin concentration and maximum *rbcL* ratio in the Roodeplaat Dam from October 2004 to May 2005.

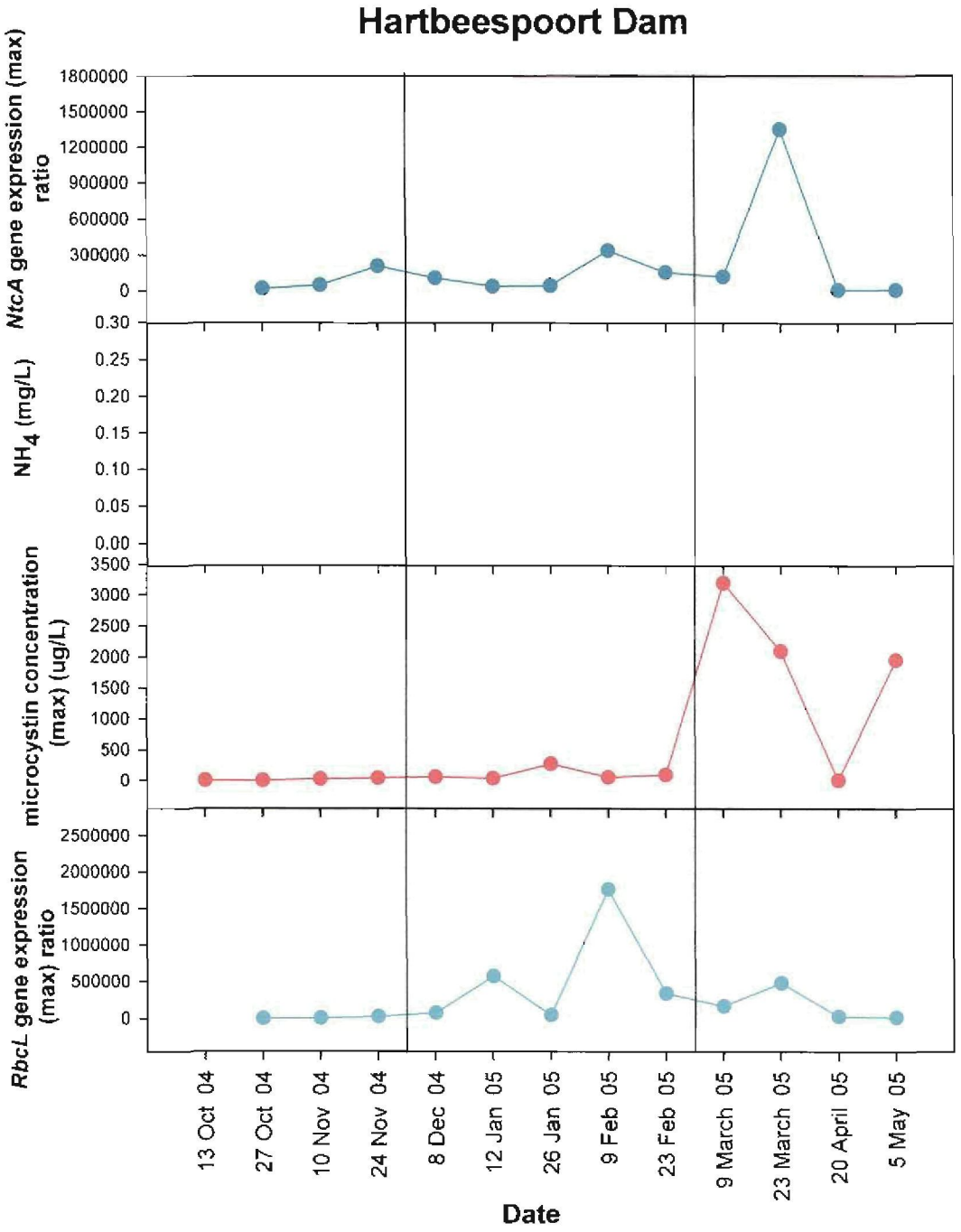


Figure 5.28: Time series representing the maximum *ntcA* ratio, NH_4 concentration, maximum microcystin concentration and maximum *rbcL* ratio in the Hartbeespoort Dam from October 2004 to May 2005.

No significant correlations could be observed between *rbcL* gene expression and the environmental data.

5.4 Discussion

From the data presented during this study it was clear that both the Hartbeespoort and Roodeplaat Dams are in a highly eutrophic state. Cyanobacteria, in particular *Microcystis sp.* dominated the phytoplankton community in both dams during the summer period. During

periods with lower temperatures such as spring and autumn other phytoplankton species belonging to the chlorophyceae and chrysophyceae can become dominant. Cyanobacterial blooms are well known for the production of cyanobacterial toxins such as microcystins and these toxins pose a serious health risk to both humans and animals.

The WHO guideline values for cyanotoxins which are of health significance in drinking water is 1 μg/L microcystin-LR and the values recorded in these two dams were much higher than this guideline value. The low risk range for recreational exposure to microcystin is at concentrations of 1-10 μg/L (Chorus and Bartram, 1999; Harada *et al.,* 1999). However, values in the Roodeplaat Dam as well as the Hartbeespoort Dam were almost always higher than that range and exceeded 10 μg/L from December 2004 to March 2005.

When comparing the seasonal variation in microcystin detected in the water, the Roodeplaat Dam and the Hartbeespoort Dam showed opposite trends. Microcystin reached maximum concentrations at the end of the sampling period in the Hartbeespoort Dam while maximum concentrations were measured at the beginning of the season in the Roodeplaat Dam. Figure 5.10 and 5.11 indicated that 16S rDNA gene copy number, although positively associated with *mcyE* and *mcyB* copy number is not on the same axis as the *mcy* genes in the multivariate analysis, which leads to the assumption that the toxins are released when the cells are dying. This is also very clearly depicted in the time series of the Hartbeespoort Dam (figure 5.20) where it was clear that microcystins are released after the *mcyE* and *mcyB* copy numbers reached a peak and not during the same time. This is also supported by Oh *et al.* (2001) and references therein, who suggested that the high percentage of extracellular microcystin in filtered lake water at the end of the bloom period would seem to suggest that the release of microcystins occurs during the senescence and decomposition periods of *Microcystis sp.* cells. According to Lahti *et al.* (1997) dissolved microcystins have seldom been detected in water during cyanobacterial blooms and if detected, the concentrations have been very low compared to the toxins in the particulate matter. Therefore, Oh *et al.* (2001) suggested that the removal of particles, including cyanobacterial cells, from water resources is recommended to effectively reduce the risk of microcystin in drinking water.

When one compares seasonal trends for both the *mcyB* and *mcyE* copy number and 16S rRNA gene copy numbers together with microcystin detected in the water (figure 5.18 and 5.24), it can be seen that in the Hartbeespoort Dam similar trends for *mcyB* and *mcyE* were observed during

the sample period. Sivonen (1990) found that hepatotoxins are largely kept within the cells during growth, although leakage of the toxins increased towards the end of the growth period. Therefore this suggests that the toxic strains of *Microcystis sp.* in the Hartbeespoort Dam have died and released the toxins into the surrounding water. However, according to the increase in 16S rRNA gene copy numbers, the possible nontoxic strains survived and grew further.

A similar observation can be made for the Roodeplaat Dam. Both the *mcyE* and *mcyB* copy number occur at a maximum during spring in the beginning of the sampling period and then decrease to a minimum in autumn at the end of the sampling period (figure 5.23). The 16S rRNA gene copy numbers increase to a maximum during summer and decrease during autumn. Harding and Paxton (2001) also observed that temperature has the most pronounced effect on toxicity. That seems to be true for this study and might therefore explain why the *mcyE* and *mcyB* copy numbers are very low in the Hartbeespoort Dam at the beginning of the sampling period during spring compared to those found in the Roodeplaat Dam where the copy numbers are much higher during spring, indicating that toxic strains die off while non-toxic strains carry on living, possibly due to the direct effect of lower water surface temperature.

Rapala and Sivonen (1998) observed that maximal production of microcystins correlated with maximal growth rates. Studies by Harding and Paxton (2001) and references therein suggested that the highest growth rate does not correlate with highest toxicity. The results of this study indicate that the growth rate according to 16S rRNA gene copy number does not correlate with *mcyE* and *mcyB* copy numbers or microcystin in the water in both dams and supports results by Harding and Paxton (2001) that growth rate and toxicity are not correlated.

The Hartbeespoort Dam and the Roodeplaat Dam are mostly similar in their responses but there are also a few differences in the responses to the environmental factors. In summary there is a strong positive association between chl *a*, TP and pH and a negative correlation with TN in Hartbeespoort Dam. In the Roodeplaat Dam there is a strong positive correlation between chl *a* and TN. In both dams 16S rRNA gene copy numbers associated very strongly with WST.

According to Oh *et al.* (2001), microcystin production by cyanobacteria is related to the abundance of nutrients and conditions favourable for cyanobacterial growth. However, both the algal species' composition and the cellular components are affected by changes in the environmental factors in a water system and changes in the water quality and nutrient

distribution will affect the microcystin concentration (Oh, *et al.,* 2001). According to Vaitomaa *et al.* (2003) lower nutrient concentrations could favour the growth of nontoxic *Microcystis* strains instead of toxic ones.

According to White (1989) a Chl *a*:TP ratio «1 indicates that algae are not P-limited, while ratios ≈ 1 are indicative of potential P-limitation. In this study, chl *a*:TP ratios in the Roodeplaat Dam were always « 1 indicating no P-limitation and chl *a*:TP ratios in the Hartbeespoort Dam were most of the time «1 with only occasionally higher values.

Rapala and Sivonen (1998) detected that hepatotoxic *Microcystis* blooms showed tendencies towards the highest concentrations of PO_4-phosphorus whereas *Anabaena* blooms with unknown neurotoxicity were associated with low PO_4-phosphorus and high NO_3-nitrogen concentrations. This was also evident in this study.

According to Gobler *et al.* (2007) no study has directly examined the impact of N and P loading on the growth and toxicity of wild cyanobacterial blooms. They (Gobler *et al.* 2007) have stated that although it has often been assumed that phosphorus-loading promotes toxic cyanobacteria blooms, the precise impact of phosphorus on the growth and toxicity of cyanobacteria is complex and varies among genera. Laboratory studies have found that higher phosphorus levels can yield higher, lower or unchanged toxin levels in cyanobacteria (Sivonen, 1990; Rapala *et al.,* 1997; Oh *et al.,* 2001; Gobler *et al.,* 2007). According to Gobler *et al.* (2007), nitrogen loading may be equally important in the occurrence of toxic cyanobacterial blooms. However, in the Roodeplaat Dam, mainly represented by toxic species, there is no statistical significant correlation between the influx of nutrients (flow) and the *mcyE* and *mcyB* copy numbers (figure 5.10), suggesting the influx of nutrients alone does not have a significant effect on the growth of toxic species. This is also supported by the data in the Hartbeespoort Dam where the *mcyE* and *mcyB* copy numbers decrease at the end of the season (figure 5.18) while there is still a high influx (flow) of nutrients (figure 5.15; 5.22; 5.26). Although this suggests that it may be the influx of nutrients that contribute to this toxin production and especially the KJEL-N concentrations, no statistically significant correlation could be observed between the phosphates, nitrates and *mcyE* and *mcyB* copy numbers.

Oh *et al.* (2001) observed that an increase in the nitrogen concentration in the cells exhibited a potential increase in the microcystin concentration in the cells as supported by Downing (2007).

They found that the microcystin concentration in the water varied with the particulate N:P ratio. At low N:P ratios (<8), the microcystin concentration was lower and at a high ratio (>8), the microcystin concentration was higher, but variable. However, these results may be due to an increase in the *Microcystis* cell numbers as a result of more nutrients available in the system, as described earlier.

Kotak *et al.* (1995) also found an inverse correlation with nitrate concentrations. As previously mentioned, a possible explanation might be that because of all the cyanobacteria present in the water, nitrogen is utilised by the cyanobacteria and this could explain the negative correlation between *mcyE* copy number and the N:P ratio. Downing (2007) found that microcystin content was increased where nitrogen uptake rate exceeded growth rate and suggested cellular nitrogen status or the regulation of nitrogen metabolism as the primary modulator of microcystin content.

Results by Gobler *et al.* (2007) indicated that the *Microcystis* population expressed the *mcyE* gene during summer months and that the gene expression declined to undetectable levels during autumn as *in situ* cell densities in the lake declined. They indicate that *Microcystis* and other cyanobacterial populations did not respond to nutrient amendments during early summer, but experienced significantly increased growth rates and toxin concentrations during late summer and early autumn in nitrogen enrichment experiments. Similar results were obtained during this study (figure 5.24 and 5.25). As such, Gobler *et al.* (2007) found that the dominance of *Microcystis sp.* blooms during the summer was linked to nutrient depleted conditions. The decline of the bloom was associated with N-limitation which reduced growth rates and toxin production by *Microcystis*.

Lee *et al.* (2000) reported that N values of 1000 µg/L and P values of 200 µg/L are normal values for eutrophic water and since the values are higher than this, the water of both dams could once again be considered as eutrophic due to the nutrients present. When nitrate is actively transported into the cyanobacterial cell, this intracellular nitrate is being photosynthetically reduced via nitrite to ammonium (the inorganic form of nitrogen that is incorporated into organic compounds) (Herrero and Flores, 1997). Ammonium is an effective inhibitor of nitrate assimilation in cyanobacteria and an amount of ammonium close to 90% of the nitrate or nitrite taken up, is found to be released by the cells (Herrero and Flores, 1997). If the cells continue to be exposed to ammonium, even when nitrate is simultaneously present, a long-term regulatory system operates which results in the repression of the proteins involved in nitrate assimilation

and this effect is reversible (Herrero and Flores, 1997). *ntcA* (ntc- translating to nitrogen control) controls the expression of these nitrate-assimilation proteins by interacting with the promoter region of the regulated genes in order to operate activation of their transcription (Herrero and Flores, 1997).

Lindell and Post (2001) observed that *ntcA* transcription was induced when ammonium levels dropped below 1 μM and reached maximal levels within 2 h. Furthermore, the addition of more than 1 μM ammonium led to a rapid decline in *ntcA* mRNA. This suggests that there are certain threshold values of NH_4 at which *ntcA* is activated or not. This could be seen in our data, showing that although there is NH_4 present in the water, the values are too low and the *ntcA* gene is still expressed at times (figure 5.27 and 5.28).

The *ntcA* gene expression in the Roodeplaat Dam and the Hartbeespoort Dam responded differently to the ammonium concentrations. This can be explained by the fact that *ntcA* binds, in the absence of ammonium, to the promoter regions of a suite of N-regulated genes (Wyman, 1999).

In the Hartbeespoort Dam, as a result of the large amount of the biovolume present, the NH_4 levels are too low for the cells to use the ammonium as their only nitrogen source, thus the *ntcA* gene is still expressed during this time (figure 5.28) in order for the cells to actively take up nitrates from the water.

In both the Roodeplaat and the Hartbeespoort Dams, *ntcA* gene expression peaks after the *mcyE* and microcystin concentration reached its maximum (figure 5.27 and 5.28). It has been suggested (Downing, 2007) that *ntcA* binds to the promoter region of *mcyA* and prevents microcystin production. It can thus be speculated that the *ntcA* is activated by microcystin in the water and by binding to the *mcyA* promoter, inactivates microcystin synthesis. One can speculate that *ntcA* might act in a similar fashion in this study and play a regulatory role in microcystin synthesis.

Rubisco catalyses the first, rate-limiting step in the Calvin cycle (light independent), which is the primary pathway for photosynthetic carbon reduction. According to Su *et al.* (2005), *ntcA* promoters are found for many genes involved in various stages of photosynthesis and photosynthesis is known to be tightly coordinated with nitrogen assimilation, although very little

is known about the underlying mechanism. According to Wyman (1999), the pathways of carbon and nitrogen assimilation are metabolically linked at the level of glutamate synthetase pathway in cyanobacteria as the enzyme is extensively regulated in response to a variety of environmental signals. Glutamate synthase is rapidly inactivated by ammonium ions in the model system *Synechocystis sp.* strain PCC6803 and several other cyanobacteria (Wyman, 1999). As with glutamate synthase, activation of PII is regulated by nitrogen availability and is dependent upon photosynthetic electron transport from the light cycle.

Diel rythms in *rbcL* regulation have been reported by Wyman (1999). According to the author, Rubisco mRNA showed a temporal variation and oscillated during the night to reach a maximum by midmorning (~08:00). In this study, sampling took place each week at the same time (~09:00) to ensure that *rbcL* levels were not variable as a result of the diel rythms in gene expression.

ntcA and *rbcL* gene expression correlates with PO_4:NH_4 ratio, confirming that the combination of PO_4:NH_4 is also necessary for photosynthetic CO_2 assimilation in the Calvin cycle to form ATP (Adenosine + 3(PO_4) = ATP).

The maximum expression of the *rbcL* gene correlates negatively with microcystin concentration in the water, indicating that growth and senescence are separated. This could be explained by the fact that these genes first need to be expressed in order to produce the proteins necessary for photosynthesis and N-metabolism so that growth can take place.

In the Roodeplaat Dam (figure 5.25), the increase in microcystin concentration on 22 November 2004 and then again on 24 January 2005 are a result of the increase in the *mcyE* and *mcyB* copy numbers. On this date the increase in *mcyB* and *mcyE* copy numbers are accompanied by a sudden decrease in chl *a* concentration, suggesting that the other species in the dam were affected by the microcystin in the water. This effect could also be seen in the Hartbeespoort Dam with the sudden increase in microcystin concentration in the water on the 9th of March and a decrease in the chl *a* concentration (figure 5.16). The 16S rRNA gene copy number decreased a little on the 23th of March, but increased till the end of autumn. Once again one should also keep in mind the effect of changing water surface temperature towards the end of the sampling period.

5.5 Conclusion

Although the Roodeplaat and the Hartbeespoort Dams are closely situated to each other in the highly populated area of the Gauteng province in South Africa and both are eutrophic water impoundments, they differ remarkably in their responses to environmental influences, most probably due to the difference in nutrient loading and water surface temperatures.

A very important variable in both dams is the inflow of the water into the system, representing nutrient loading. This nutrient loading is the main reason for the bloom occurrence taking place. The inflow of nutrients consists mainly of phosphorus and nitrogen. Clear relationships emerged between the total nitrogen in the water and the *Microcystis* biomass, indicating that the decrease in nitrogen concentrations is caused by the increase in *Microcystis* biomass.

The discovery of the genes and biosynthetic pathway required for the production of microcystins in *M. aeruginosa* has made it possible now to study the ecophysiology of cyanobacteria in natural populations. Knowledge of the occurrence and dynamics of microcystin producers and non-producers can explain the variability of microcystin concentrations in natural waters.

In both dams the 16S rRNA gene copy number differs from the *mcyE* and *mcyB* copy number. From the data it could be observed that the Hartbeespoort Dam contains different *Microcystis* strains, some which are toxic and some that are non toxin producers. The Roodeplaat Dam however, constitutes mainly toxic *Microcystis* species, but other photosynthetic species are also present.

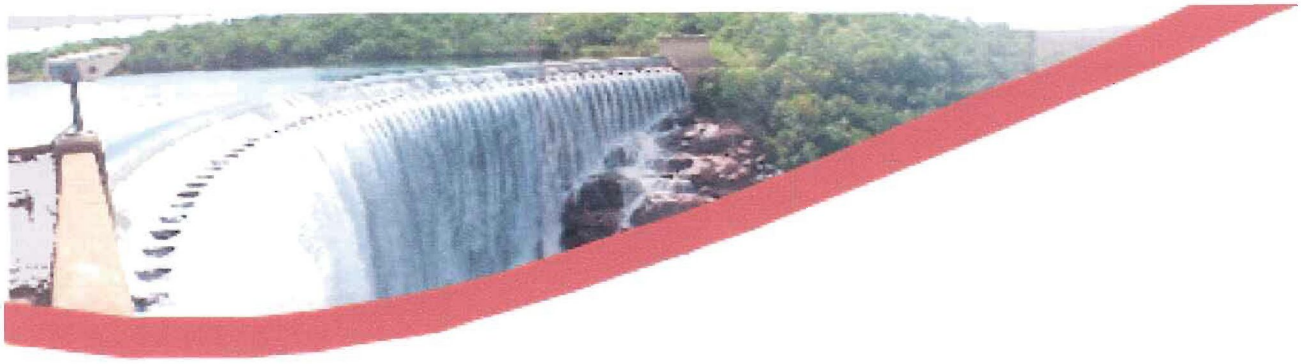
Microcystins are released mainly when the cells are dying. This is influenced by the water surface temperature. Microcystin production is stimulated by temperatures higher than 24°C and the release of microcystin is caused by cells that die off due to natural cycles or temperatures.

A connection was also observed between *ntcA* gene expression and microcystin synthesis in the Hartbeespoort Dam. The *ntcA* gene expression increased as a result of high microcystin concentrations in the water and thus probably inhibited the synthesis of microcystin.

It is thus clear that to prevent serious water problems in the dams, it would be crucial to limit the inflow of nutrients into the system to prevent toxic blooms from happening. The decreased inflow of nitrogen as well as phosphorus can result in less *Microcystis* blooms.

One can assume that the chl *a* was not only represented by *Microcystis* but that other photosynthetic species were also present in the water during the sampling time. The chl *a* concentration may thus not always be a good indicator for the biomass in the water. Scott *et al.* (1981) suggested that *M. aeruginosa* has a lower cellular chl *a* content when they adapt to high light intensity. It is thus better to determine the biomass or cell count of *Microcystis* species in the water or alternatively the *Microcystis* specific 16S rRNA gene copy number.

Most of the efforts to understand the regulation of toxin production have relied on laboratory experiments. However, toxicity varies substantially both in nature and in culture and seems to be expressed only under certain environmental conditions. Molecular methods can produce data without the need to cultivate the organisms in question. Investigating the production of toxins by molecular analysis and in field studies, can provide us with a better understanding of the interchangeable variables influencing toxin biosynthesis.



Chapter 6

Conclusion

In developing countries, such as South Africa, resistance to the provision of funding for cyanobacterial research is often based on the argument that there are far greater health problems and that funding needs to be directed to the alleviation of diseases such as HIV-AIDS and tuberculosis (Harding and Paxton, 2001). While this is inarguably legitimate, the threats posed by cyanobacteria are also very real (Harding and Paxton, 2001). In the absence of deliberate eutrophication management, the reality is that increasing numbers of the population will be exposed to waters containing cyanobacterial metabolites that pose acute and chronic implications to their health (Harding and Paxton, 2001). The development of an understanding of cyanobacterial growth and metabolism under different environmental conditions thus constitutes a crucial need in South Africa.

Through a combination of ecological and molecular research, it is hoped that experimental data may ultimately be extrapolated to the environment not only to understand the timing of toxin production but also the purpose of it (Kaebernick and Neilan, 2001). It is thus imperative to identify the species involved.

In order to study the gene expression of specific genes of cyanobacteria occurring naturally in the water systems of South Africa, it is necessary to firstly verify the species. A polyphasic approach was therefore used for the taxonomic identification of some of the bloom forming species grown in the culture collection of the North West University to be used as reference cultures later during this study.

The results indicated that the isolate formerly known as "*Oscillatoria simplicissima*" should be reassigned to *Planktothrix pseudagardhii* under the order Oscillatoriales, family Phormidiaceae and subfamily Phormidioideae. Other strains in the culture collection were also not correctly identified, eg. *Spirulina sp.* was in fact *Arthruspira sp.*

To obtain a reliable quantification method each real-time PCR method has to be optimised. The efficiency of DNA isolation determines the overall yield of DNA and thus the sensitivity of the procedure. Therefore, it is imperative to find a protocol of DNA isolation that does not discriminate between the different cyanobacterial species and cell types. Although not documented in all studies one should ensure that the efficiency in the real-time PCR is comparable between reactions in order to generate usable results.

In order to investigate the physiological rationale for the sudden increase in biovolume of a specific species during blooms it is important to look into the ecophysiological dynamics of each process. The regulation of the gene expression of key metabolic enzymes was investigated in the Hartbeespoort Dam and the Roodeplaat Dam during the bloom season of 2004 to 2005.

The *in vivo* expression of the *ntcA* and the *rbcL* genes were examined. The expression of these genes, *rbcL* (encoding the large subunit of Rubisco) and *ntcA* (encoding a nitrogen assimilation regulatory protein), reflect in part the photosynthetic and nitrogen metabolism activity of the cyanobacteria present in the sample.

Together with this, DNA copy number of the *Microcystis sp.* specific 16S rDNA and toxin genes, *mcyE* as well as *mcyB* was also measured with real-time PCR. The trends of the ecological and molecular data were analysed using multivariate statistical analysis.

Although the Roodeplaat and the Hartbeespoort Dams are closely situated in the highly populated area of the Gauteng Province in South Africa, and both are eutrophic water impoundments, they differ remarkably in their responses to environmental influences, most probably due to the difference in nutrient loading and water surface temperatures.

A very important variable in both dams is the inflow of the water into the system, representing nutrient loading. The inflow of nutrients constitutes mainly of phosphorus and nitrogen. Clear relationships emerged between the total nitrogen in the water and the *Microcystis sp.* biomass, indicating that the decrease in nitrogen concentrations was caused by the increase in *Microcystis sp.* biomass.

The Hartbeespoort Dam constitutes of different *Microcystis* strains, some which are toxic and some that are not toxin producers. The Roodeplaat Dam however, constitutes mainly of toxic *Microcystis* species, but other photosynthetic species are also present in the Roodeplaat Dam. Microcystin production is stimulated by higher temperatures, and the release of microcystin is caused by cells that die off due to natural cycles or temperatures.

A connection was also observed between *ntcA* gene expression and microcystin synthesis. The *ntcA* gene increased most probably as a result of high microcystin concentrations in the water, and thus probably inhibited the synthesis of more microcystin.

The water research commission has recently launched a program for the modulation of harmful algal blooms. The data from this study is the first step to identify specific strains to be used for modulation and with this study, the first year's data is already recorded.

The information can then be applied by the industry to predict when *Microcystis sp.* is going to form a bloom under certain conditions and whether it is toxic or not. Therefore water purification plants can prepare in advance for a blooming event lowering the risk of distributing water of low quality to the public.

Future research:

Ecophysiological studies concerning HAB species are still scarce, but are now more possible than ever with the continuous discovery of new methods in molecular research. It is now possible and of great importance to follow the environmental changes in the natural environment together with changes in species, strains and toxigenicity throughout the years so that all possible relationships can be measured.

The study conducted here can be improved with the use of reverse transcriptase real-time PCR analysis on the genes from the *mcy* gene cluster. Thereby, the expression of microcystin genes can be measured in natural occurring samples to determine the cause of microcystin production.

Chapter 7

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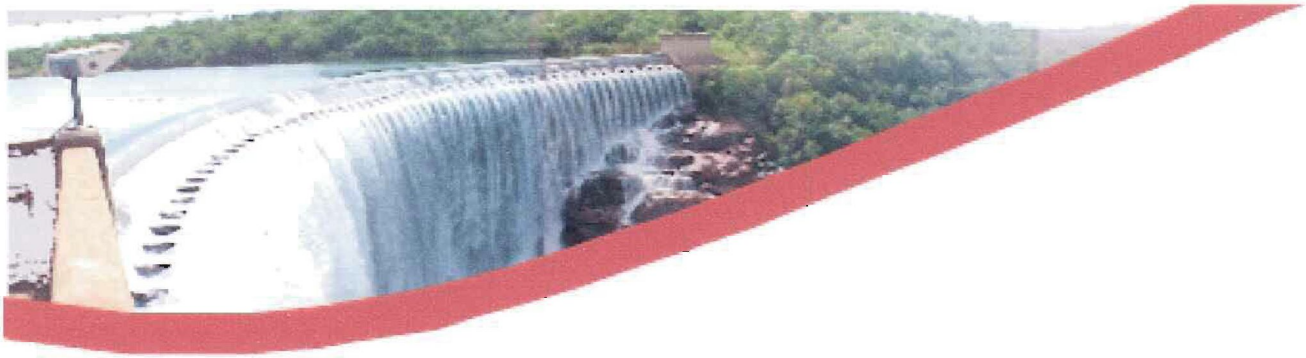

Appendix

Molecular data

date	RPL	16S min copy number	16S max copy number	16S avg copy number	mcyB min copy number	mcyB max copy number	mcyB avg copy number	mcyE min copy number	mcyE max copy number	mcyE avg copy number
25-Oct-04	RPL	134640.00	221150.00	173528.00	135800.00	250350.00	187070.00	73865.00	120450.00	99553
09-Nov-04	RPL	7942.50	217050.00	95564.50	602.60	59870.00	36587.52	100.65	44355.00	25827.13
22-Nov-04	RPL	9878.50	132270.00	67228.70	8325.50	146400.00	76183.10	2847.00	159250.00	63813.2
06-Dec-04	RPL	19632.00	111810.00	45774.40	11715.00	57040.00	26195.00	3509.50	35330.00	15080.5
10-Jan-05	RPL	126270.00	245500.00	183892.00	32585.00	130550.00	65324.00	38165.00	126200.00	75866
24-Jan-05	RPL	226050.00	713600.00	393890.00	34663.33	121266.67	74120.67	63845.00	200750.00	128283
07-Feb-05	RPL	180000.00	292550.00	217840.00	18315.00	38090.00	26057.00	20745.00	59225.00	39322
22-Feb-05	RPL	134850.00	355250.00	247090.00	19145.00	42520.00	30225.00	28630.00	53685.00	39143
07-Mar-05	RPL	169600.00	492950.00	315570.00	10296.50	82475.00	39215.30	4753.00	128300.00	49920.6
22-Mar-05	RPL	105430.00	384550.00	218426.00	3284.50	16350.00	8186.10	2115.50	11305.00	5934.8
04-Apr-05	RPL	49505.00	163600.00	97781.00	2290.00	4689.00	3149.10	858.65	3293.00	2012.23
18-Apr-05	RPL	44845.00	158800.00	88644.00	1194.35	12657.00	5781.17	644.30	7553.50	3447.96
03-May-05	RPL	1364.00	11023.50	4004.00	166.13	440.10	271.00	0.00	213.30	55.97
16-May-05	RPL	576.40	145900.00	67020.48	85.17	15402.90	4055.67	0.00	8546.67	2765.81333
13/10/2004	HBP	51.00	1217.55	361.62	0.00	86.97	27.49	0.00	0.00	0
27-Oct-04	HBP	7.52	1107.20	300.46	0.00	223.55	129.61	0.00	0.00	0
10-Nov-04	HBP	2.65	318.75	86.86	29.66	131.42	85.35	0.00	0.00	0
24-Nov-04	HBP	5541.00	289000.00	85972.20	1098.60	87705.00	19036.57	126.90	37350.00	7967.25
08-Dec-04	HBP	622.55	703200.00	146119.12	284.75	47980.00	11304.44	0.00	448950.00	91014.51
12-Jan-05	HBP	95490.00	1087000.00	536588.00	10695.00	91300.00	35150.00	8219.50	209200.00	116100.9
26-Jan-05	HBP	409950.00	925600.00	682800.00	23865.00	122100.00	54845.00	107430.00	230750.00	164006
09-Feb-05	HBP	24480.00	150090.00	70727.00	384.45	2778.50	1274.22	293.80	32450.00	7390.3
23-Feb-05	HBP	251450.00	1077500.00	556920.00	2630.00	72925.00	21137.80	27610.00	247450.00	121650
09-Mar-05	HBP	282350.00	1563500.00	642850.00	1778.50	63375.00	15619.40	29020.00	224050.00	90614
23-Mar-05	HBP	20975.00	1669500.00	392044.00	308.40	39850.00	11259.00	678.05	46305.00	10392.24
20-Apr-05	HBP	22540.00	1378500.00	531166.00	334.25	19500.00	8026.71	341.35	40525.00	15037.47
05-May-05	HBP	133750.00	1641000.00	737290.00	958.35	32985.00	10235.70	1779.50	126900.00	51786.9

Molecular data

date	RPL	Ntca min ratio	Ntca max ratio	Ntca avg ratio	RbcL min ratio	RbcL max ratio	RbcL avg ratio
25-Oct-04	RPL	13647.07088	40028.1445	24364.68708	1689.962972	15592.38816	5588.861558
09-Nov-04	RPL	8560.187573	30459.68578	17558.00712	795.9644365	10700.91403	4580.035401
22-Nov-04	RPL	10145.45663	43215.79865	22889.36848	2730.364787	58325.33226	19958.37126
06-Dec-04	RPL	18114.23412	47758.14349	33252.88785	7198.60152	47335.74573	21571.21977
10-Jan-05	RPL	450.2832328	35504.18825	12750.68048	1474.202588	20018.19287	9818.861936
24-Jan-05	RPL	47917.51261	61929.8684	54544.48064	12267.07145	30971.30946	20760.96149
07-Feb-05	RPL	56602.51829	168809.1232	112526.323	0.131049041	2323009.22	654376.8748
22-Feb-05	RPL	46969.21343	117406.3186	96365.604	37824.16532	150731.3894	90903.09673
07-Mar-05	RPL	36706.91492	99391.66885	72840.75641	43504.70247	592721.5709	263723.2797
22-Mar-05	RPL	2091.579101	17116.85102	8651.16738	937.1051446	107664.8394	28874.14315
04-Apr-05	RPL	2813.469051	10489.14035	5653.871619	1365.463466	26394.39667	8353.116619
18-Apr-05	RPL	4309.593932	20152.01737	10853.91528	10524.14265	29658.60915	17916.10403
03-May-05	RPL	2220.839787	9397.114409	4842.761748	2184.153116	28878.60049	14556.64645
16-May-05	RPL	918.5596067	1735.608735	1344.27959	273.1878069	1727.916377	742.3591405
13/10/2004	HBP						
27-Oct-04	HBP	0	17288.78048	3457.756096	0	2303.734131	1283.038169
10-Nov-04	HBP	19556.76698	47283.20937	27459.93913	1678.740352	8307.336707	3882.261329
24-Nov-04	HBP	17598.708	205474.9436	74504.74845	842.3445491	28782.55299	6914.515611
08-Dec-04	HBP	13876.2973	106003.9442	35908.09346	2832.277496	76903.14332	18985.39488
12-Jan-05	HBP	8560.187573	37698.36572	18211.16916	2895.885147	580991.4674	124669.294
26-Jan-05	HBP	21043.95264	43215.79865	30038.54959	5459.605516	49375.98059	18939.73577
09-Feb-05	HBP	68667.60174	338675.1513	185052.6	73643.64988	1767709.489	699282.0868
23-Feb-05	HBP	37950.38471	156357.54	89757.46586	10594.49806	347822.7397	114960.4315
09-Mar-05	HBP	35860.80852	120979.8212	71031.14368	6623.34519	175110.0377	44753.27835
23-Mar-05	HBP	3401.820847	1349639.197	396380.9328	14733.85975	488578.7869	219135.1482
20-Apr-05	HBP	1753.041981	9944.675587	4671.164837	5459.605516	32342.11034	15962.17072
05-May-05	HBP	793.32	11174.54	5500.817913	1219.236268	22270.16433	11947.26743

Environmental data

	RPL	Microcystin min	Microcystin max	Microcystin avg	Chlorophyll-a	Microcystis dominance (%)	Anabaena dominance (%)	Oscillatoria dominance (%)	Cylindrosp ermopsis dominance (%)	flow	Water Surface Temp	Total Nitrogen (TN) (mg/l)	KJEL N (mg/L)	NH4-N (mg/L)	TP (mg/L)	TAL (mg/L)	TN:TP ratio	PO4:NH4	PO4-P (mg/L)
25-Oct-04	RPL	4.82	28.77	11.59	83.73	20.00	0.00	0.00	0.00	100.65	23.90	2.92	1.36	0.08	0.11	107.51	27.24	1.03	0.08
09-Nov-04	RPL	2.29	27.49	12.02	94.96	12.00	0.00	0.00	0.00	63.43	23.40	2.83	1.42	0.03	0.10	97.03	29.81	0.35	0.01
22-Nov-04	RPL	1.79	217.40	53.18	19.46	42.00	0.00	0.00	0.00	147.46	25.50	2.36	1.17	0.04	0.07	96.06	35.19	0.30	0.01
06-Dec-04	RPL	1.99	52.20	36.00	67.25	66.00	0.00	0.00	0.00	126.27	25.00	2.23	1.43	0.05	0.08	94.52	27.47	0.81	0.04
10-Jan-05	RPL	2.28	27.00	9.35	38.88	86.00	0.00	0.00	1.00	537.60	25.90	1.10	1.04	0.05	0.12	89.98	9.38	1.20	0.06
24-Jan-05	RPL	1.80	179.60	70.09	48.70	86.00	1.00	0.00	0.00	241.35	25.16	1.52	1.46	0.09	0.14	90.75	10.82	1.00	0.09
07-Feb-05	RPL	7.00	42.00	26.90	49.06	100.00	0.00	0.00	0.10	186.04	25.70	1.48	1.42	0.04	0.20	97.79	7.32	3.56	0.13
22-Feb-05	RPL	4.30	21.40	9.28	32.21	100.00	0.10	0.00	0.00	155.66	24.77	0.67	0.58	0.04	0.16	96.17	4.29	2.50	0.11
07-Mar-05	RPL	0.90	19.50	11.22	55.53	85.00	2.00	0.00	0.00	171.51	25.64	1.59	1.55	0.02	0.14	95.09	11.12	3.00	0.06
22-Mar-05	RPL	1.58	6.15	3.65	113.88	30.00	55.00	0.00	0.10	131.74	20.34	1.77	1.73	0.06	0.18	111.11	9.83	2.38	0.14
04-Apr-05	RPL	4.04	7.61	5.20	62.02	19.00	20.00	15.00	3.00	259.35	22.52	1.29	1.25	0.23	0.15	103.72	8.72	0.29	0.07
18-Apr-05	RPL	5.40	9.60	7.73	51.53	13.00	3.00	3.00	0.00	169.83	22.08	1.64	1.24	0.27	0.19	99.97	8.58	0.34	0.09
03-May-05	RPL	0.23	3.50	1.98	70.92	2.00	1.00	1.00	1.00	133.01	20.26	1.56	0.85	0.05	0.17	105.01	9.42	1.63	0.08
16-May-05	RPL	0.28	2.20	0.87	17.41	0.00	3.00	0.00	2.00	103.65	19.44	1.51	0.92	0.18	0.11	109.36	13.36	0.27	0.05
13/10/2004	HBP	0.09	0.31	0.22	6.52	0.00	0.00	0.00	0.00	466.24	18.80	2.90	0.93	0.13	0.07	124.57	44.65	0.22	0.03
27-Oct-04	HBP	0.17	0.24	0.22	20.29	8.00	0.00	0.00	0.00	609.52	22.50	2.72	0.87	0.02	0.08	126.12	34.86	1.93	0.03
10-Nov-04	HBP	1.14	25.89	6.77	27.74	0.00	0.00	0.00	0.00	501.44	23.40	2.90	1.09	0.05	0.06	118.95	49.95	0.27	0.01
24-Nov-04	HBP	1.47	39.05	9.72	24.11	31.00	0.00	0.00	0.00	670.83	25.00	2.47	0.86	0.05	0.05	119.12	48.45	0.83	0.05
08-Dec-04	HBP	1.16	57.40	18.66	13.78	32.00	0.00	0.00	0.00	888.19	25.00	2.49	1.17	0.06	0.08	107.96	29.94	0.78	0.04
12-Jan-05	HBP	3.00	38.00	23.00	103.85	95.00	0.00	3.00	0.00	1279.97	28.33	2.19	1.88	0.06	0.13	80.88	17.34	0.93	0.05
26-Jan-05	HBP	0.59	273.00	93.29	143.43	100.00	0.00	0.00	0.00	2397.76	28.31	2.39	2.27	0.05	0.13	85.51	17.97	1.22	0.06
09-Feb-05	HBP	33.40	53.00	42.85	118.79	98.00	0.00	0.00	0.00	898.70	25.47	2.08	1.97	0.02	0.13	78.31	16.21	2.05	0.04
23-Feb-05	HBP	1.20	97.90	71.63	173.03	100.00	0.00	0.00	0.00	1099.86	26.57	2.25	2.21	0.02	0.15	82.72	15.01	2.55	0.05
09-Mar-05	HBP	2.00	3200.00	649.16	85.74	100.00	0.00	0.00	0.10	985.77	25.87	1.94	1.90	0.02	0.11	83.61	17.66	2.35	0.05
23-Mar-05	HBP	2.20	2100.00	422.56	62.11	100.00	0.00	0.10	0.10	1950.30	23.50	2.02	1.82	0.14	0.18	94.30	11.15	0.54	0.07
20-Apr-05	HBP	3.87	9.52	5.79	92.01	53.00	0.00	10.00	0.00	1191.78	21.50	1.89	1.52	0.02	0.14	97.80	13.88	2.75	0.06
05-May-05	HBP	1.43	1960.00	516.50	71.29	52.00	0.00	17.00	1.00	1015.65	19.76	1.57	0.82	0.02	0.04	104.61	41.26	1.40	0.03

Environmental data

	RPL	DIN (mg/L)	Ca (mg/L)	Cl (mg/L)	DMS (mg/L)	EC (mS/m)	F (mg/L)	K (mg/L)	Mg (g/L)	NO3+NO 2 (mg/L)	Na (mg/L)	SO4 (g/L)	Si (mg/L)	pH	DIN:DIP ratio	N:P
25-Oct-04	RPL	1.63	25.65	50.93	317.65	48.70	0.29	8.77	14.62	1.56	41.59	37.49	3.08	9.56	20.41	19.44
09-Nov-04	RPL	1.45	20.26	48.39	294.55	47.70	0.29	8.44	14.93	1.42	42.13	35.46	4.12	9.71	120.83	118.00
22-Nov-04	RPL	1.22	17.55	55.89	309.30	46.70	0.28	9.64	14.03	1.18	48.18	41.29	3.77	9.58	102.00	98.67
06-Dec-04	RPL	0.85	17.25	47.06	289.89	45.60	0.27	8.52	13.99	0.79	44.44	39.42	4.72	9.76	19.20	17.98
10-Jan-05	RPL	0.10	16.70	46.96	262.36	42.80	0.28	7.83	12.65	0.06	37.37	30.37	5.47	9.41	1.76	0.93
24-Jan-05	RPL	0.15	17.58	35.71	245.74	40.80	0.30	6.98	11.24	0.06	32.32	30.32	4.62	9.04	1.59	0.59
07-Feb-05	RPL	0.09	21.95	36.08	256.55	38.90	0.27	6.62	12.52	0.06	27.61	31.59	5.56	9.36	0.71	0.43
22-Feb-05	RPL	0.13	21.13	42.08	277.87	39.30	0.26	6.70	13.95	0.09	30.89	44.84	5.83	8.88	1.26	0.86
07-Mar-05	RPL	0.06	21.51	33.90	249.91	40.00	0.26	6.15	12.44	0.04	27.59	31.74	4.54	9.14	1.00	0.67
22-Mar-05	RPL	0.10	23.42	34.28	271.67	39.90	0.33	6.86	14.05	0.04	30.75	25.82	5.42	9.07	0.70	0.28
04-Apr-05	RPL	0.27	24.26	37.24	265.81	40.10	0.32	6.33	12.92	0.04	29.52	28.09	5.24	8.69	4.08	0.62
18-Apr-05	RPL	0.67	23.83	34.24	259.29	42.10	0.27	6.69	13.31	0.40	29.10	27.57	4.54	8.04	7.37	4.40
03-May-05	RPL	0.75	25.15	36.60	269.36	41.70	0.25	6.69	13.47	0.70	29.84	25.94	1.74	8.19	9.96	9.35
16-May-05	RPL	0.77	24.26	35.90	276.33	43.20	0.25	6.55	14.29	0.59	30.42	28.33	0.85	8.03	16.04	12.35
13/10/2004	HBP	2.10	36.44	60.16	380.91	58.10	0.30	8.97	16.06	1.97	47.49	50.64	1.39	8.13	75.04	70.46
27-Oct-04	HBP	1.87	34.52	59.47	384.51	57.50	0.27	9.70	15.31	1.85	47.56	55.62	1.73	8.53	64.31	63.79
10-Nov-04	HBP	1.86	31.87	53.73	361.32	56.50	0.29	9.31	16.01	1.81	48.02	48.96	2.33	8.76	154.58	150.83
24-Nov-04	HBP	1.66	28.86	56.93	368.69	54.80	0.27	9.73	14.84	1.61	50.31	55.17	1.55	8.46	36.93	35.73
08-Dec-04	HBP	1.37	25.62	58.22	348.81	52.80	0.27	9.52	15.20	1.32	50.04	52.28	0.75	8.99	31.93	30.65
12-Jan-05	HBP	0.36	16.22	54.59	284.10	48.20	0.28	9.20	13.73	0.31	45.31	44.58	1.54	9.22	7.10	6.02
26-Jan-05	HBP	0.17	17.97	46.37	275.60	46.70	0.30	8.72	12.34	0.12	40.54	44.35	2.11	9.28	3.04	2.22
09-Feb-05	HBP	0.13	17.78	51.84	274.09	44.90	0.27	9.03	13.44	0.11	37.99	47.65	2.74	9.14	3.10	2.61
23-Feb-05	HBP	0.06	17.99	58.24	290.27	44.50	0.32	8.68	12.79	0.04	43.52	47.52	2.96	9.20	1.18	0.78
09-Mar-05	HBP	0.06	20.92	49.88	281.60	44.70	0.28	8.81	13.20	0.04	40.26	45.97	3.72	9.06	1.28	0.85
23-Mar-05	HBP	0.33	23.26	46.46	295.09	46.50	0.31	8.19	13.40	0.20	40.74	46.48	3.93	8.32	4.47	2.64
20-Apr-05	HBP	0.39	24.34	41.64	296.42	46.90	0.26	8.20	13.61	0.37	40.53	46.80	3.88	8.58	7.04	6.67
05-May-05	HBP	0.77	26.11	50.13	317.94	47.10	0.27	7.99	13.21	0.75	39.95	49.28	3.68	7.93	27.61	26.89

Correlations (Roodeplaatdam ekstras normaalv.sta)
Marked correlations are significant at p < .05000
N=14 (Casewise deletion of missing data)

Variable	date	1/16s min N	1/16s max	1/16s avg	mcyB min N	1/mcyb max	mcyB avg N	mcyE min N	1/mcye max	1/mcyE avg	Ntca min N	Ntca max N	Ntca avg N	RbcL min N	RbcL max N	RbcL avg N	MCN min N	MCN max N	MCN avg N	1/Chl a	AD N	OD N	Mer_D N	fl
date	1.00	0.50	0.35	0.35	-0.54	0.41	-0.73	-0.45	0.40	0.38	-0.15	-0.19	-0.14	0.13	0.03	0.08	-0.17	-0.47	-0.47	0.15	0.31	0.32	0.51	
1/16s min N	0.50	1.00	0.35	0.35	-0.24	0.33	-0.31	-0.30	0.33	0.33	-0.34	-0.40	-0.37	-0.24	-0.17	-0.19	-0.49	-0.23	-0.31	0.59	-0.11	-0.11	0.48	
1/16s max	0.35	0.35	1.00	1.00	-0.18	0.99	-0.25	-0.24	1.00	1.00	-0.28	-0.29	-0.28	-0.18	-0.13	-0.14	-0.36	-0.18	-0.23	-0.14	-0.12	-0.00	0.18	
1/16s avg	0.35	0.35	1.00	1.00	-0.19	0.99	-0.28	-0.24	1.00	1.00	-0.26	-0.28	-0.27	-0.17	-0.13	-0.14	-0.38	-0.18	-0.22	-0.14	-0.11	-0.02	0.17	
mcyB min N	-0.54	-0.24	-0.18	-0.19	1.00	-0.19	0.93	0.85	-0.18	-0.17	0.13	0.10	0.08	-0.07	-0.04	-0.05	0.32	0.07	0.09	-0.20	-0.20	-0.18	-0.24	
1/mcyb max	0.41	0.33	0.99	0.99	-0.19	1.00	-0.28	-0.23	1.00	1.00	-0.26	-0.28	-0.27	-0.15	-0.11	-0.12	-0.34	-0.22	-0.26	-0.18	-0.08	0.06	0.22	
mcyB avg N	-0.73	-0.31	-0.25	-0.26	0.93	-0.28	1.00	0.81	-0.27	-0.26	0.13	0.10	0.07	-0.05	-0.10	-0.11	0.19	0.36	0.31	-0.06	-0.30	-0.28	-0.36	
mcyE min N	-0.45	-0.30	-0.24	-0.24	0.85	-0.23	0.81	1.00	-0.22	-0.21	0.39	0.25	0.27	0.04	0.00	-0.00	0.28	0.28	0.36	-0.14	-0.25	-0.24	-0.28	
1/mcye max	0.40	0.33	1.00	1.00	-0.18	1.00	-0.27	-0.22	1.00	1.00	-0.25	-0.28	-0.26	-0.15	-0.11	-0.13	-0.35	-0.21	-0.25	-0.17	-0.07	0.03	0.20	
1/mcyE avg	0.38	0.33	1.00	1.00	-0.17	1.00	-0.26	-0.21	1.00	1.00	-0.24	-0.26	-0.25	-0.14	-0.11	-0.12	-0.36	-0.20	-0.24	-0.17	-0.09	-0.00	0.18	
Ntca min N	-0.15	-0.34	-0.28	-0.26	0.13	-0.26	0.13	0.39	-0.25	-0.24	1.00	0.91	0.96	0.54	0.63	0.69	0.38	0.29	0.48	-0.05	-0.31	-0.27	-0.45	
Ntca max N	-0.19	-0.40	-0.29	-0.28	0.10	-0.28	0.10	0.25	-0.28	-0.26	0.91	1.00	0.98	0.48	0.80	0.85	0.51	0.13	0.27	-0.04	-0.30	-0.29	-0.46	
Ntca avg N	-0.14	-0.37	-0.28	-0.27	0.08	-0.27	0.07	0.27	-0.26	-0.25	0.96	0.98	1.00	0.58	0.72	0.78	0.46	0.14	0.30	-0.03	-0.29	-0.28	-0.45	
RbcL min N	0.13	-0.24	-0.18	-0.17	-0.07	-0.15	-0.05	0.04	-0.15	-0.14	0.54	0.48	0.58	1.00	0.02	0.17	-0.05	-0.05	-0.01	-0.00	-0.19	-0.15	-0.32	
RbcL max N	0.03	-0.17	-0.13	-0.13	-0.04	-0.11	-0.10	0.00	-0.11	-0.11	0.63	0.80	0.72	0.02	1.00	0.99	0.54	-0.04	0.09	-0.07	-0.11	-0.13	-0.19	
RbcL avg N	0.05	-0.19	-0.14	-0.14	-0.05	-0.12	-0.11	-0.00	-0.13	-0.12	0.69	0.85	0.78	0.17	0.99	1.00	0.50	-0.08	0.08	-0.07	-0.13	-0.15	-0.22	
MCN min N	-0.17	-0.49	-0.38	-0.38	0.32	-0.34	0.19	0.29	-0.35	-0.36	0.38	0.51	0.46	-0.05	0.54	0.50	1.00	-0.12	-0.02	-0.24	-0.12	0.23	-0.16	
MCN max N	-0.47	-0.23	-0.18	-0.18	0.07	-0.22	0.36	0.28	-0.21	-0.20	0.29	0.13	0.14	-0.05	-0.04	-0.06	-0.12	1.00	0.93	0.37	-0.24	-0.21	-0.33	
MCN avg N	-0.47	-0.31	-0.23	-0.22	0.09	-0.26	0.31	0.36	-0.25	-0.24	0.48	0.27	0.30	-0.01	0.09	0.08	-0.02	0.93	1.00	0.17	-0.28	-0.23	-0.40	
1/Chl a	0.15	0.59	-0.14	-0.14	-0.20	-0.18	-0.06	-0.14	-0.17	-0.17	-0.05	-0.04	-0.03	-0.00	-0.07	-0.07	-0.24	0.37	0.17	1.00	-0.29	-0.16	0.23	
AD N	0.31	-0.11	-0.12	-0.11	-0.20	-0.06	-0.30	-0.25	-0.07	-0.09	-0.31	-0.30	-0.29	-0.19	-0.11	-0.13	-0.12	-0.24	-0.28	-0.29	1.00	0.25	0.18	
OD N	0.32	-0.11	-0.00	-0.02	-0.18	0.08	-0.28	-0.24	0.03	-0.00	-0.27	-0.29	-0.28	-0.15	-0.13	-0.15	0.23	-0.21	-0.23	-0.16	0.25	1.00	0.74	
Mer_D N	0.51	0.48	0.18	0.17	-0.24	0.22	-0.36	-0.26	0.20	0.18	-0.45	-0.48	-0.45	-0.32	-0.19	-0.22	-0.16	-0.33	-0.40	0.23	0.16	0.74	1.00	
flow_N	0.03	-0.26	-0.14	-0.14	0.03	-0.11	0.04	0.28	-0.12	-0.12	-0.05	0.03	-0.03	-0.04	0.00	-0.00	0.06	0.04	0.04	0.02	-0.06	0.18	0.28	
1/TN	0.38	-0.04	-0.05	-0.05	-0.17	-0.00	-0.28	0.10	-0.01	-0.01	0.35	0.36	0.43	0.52	0.05	0.10	0.18	-0.21	-0.21	0.20	-0.05	0.11	0.17	
1/kjeln	0.28	0.30	0.29	0.28	-0.10	0.28	-0.17	0.01	0.29	0.29	0.10	0.11	0.18	0.35	-0.18	-0.15	-0.01	-0.18	-0.28	0.41	-0.29	-0.06	0.16	
NH4-N	0.51	0.24	-0.12	-0.13	-0.15	-0.08	-0.28	-0.18	-0.10	-0.12	-0.38	-0.47	-0.44	-0.21	-0.27	-0.30	0.24	-0.23	-0.23	0.13	0.12	0.63	0.53	
TN:TP N	-0.77	-0.05	-0.11	-0.12	0.26	-0.20	0.49	-0.01	-0.19	-0.18	-0.26	-0.23	-0.28	-0.31	-0.27	-0.30	-0.18	0.46	0.36	0.12	-0.24	-0.23	-0.29	
1/PO4:NH4	0.08	0.39	-0.15	-0.16	-0.29	-0.17	-0.18	-0.41	-0.18	-0.19	-0.53	-0.56	-0.57	-0.38	-0.38	-0.42	-0.08	0.14	-0.02	0.49	-0.09	0.45	0.48	
1/P04-P	-0.54	0.00	-0.08	-0.08	-0.20	-0.14	0.11	-0.30	-0.13	-0.12	-0.24	-0.18	-0.23	-0.21	-0.21	-0.22	-0.24	0.46	0.27	0.28	-0.23	-0.13	-0.18	
1/DIN	0.19	-0.34	-0.29	-0.28	-0.10	-0.23	-0.12	0.09	-0.23	-0.23	0.52	0.60	0.59	0.55	0.50	0.58	0.04	-0.12	-0.04	-0.16	0.23	-0.14	-0.17	
Cl N	-0.84	-0.21	-0.13	-0.14	0.40	-0.21	0.61	0.23	-0.20	-0.19	-0.19	-0.08	-0.14	-0.24	-0.26	-0.30	0.02	0.43	0.28	0.19	-0.38	-0.22	-0.24	
K N	-0.86	-0.21	-0.13	-0.13	0.38	-0.21	0.60	0.20	-0.20	-0.19	-0.24	-0.17	-0.23	-0.38	-0.27	-0.32	-0.04	0.52	0.41	0.12	-0.26	-0.31	-0.37	
NO3_NO2 N	-0.61	0.14	0.15	0.14	0.39	0.07	0.51	0.06	0.09	0.10	-0.35	-0.33	-0.36	-0.37	-0.31	-0.35	-0.08	0.18	0.06	0.05	-0.33	-0.24	-0.23	
Na N	-0.84	-0.18	-0.14	-0.14	0.30	-0.23	0.53	0.13	-0.22	-0.20	-0.26	-0.22	-0.27	-0.32	-0.36	-0.40	-0.14	0.50	0.41	0.14	-0.26	-0.26	-0.30	
1/Si	0.44	0.99	0.31	0.31	-0.11	0.29	-0.18	-0.21	0.29	0.30	-0.35	-0.41	-0.38	-0.24	-0.19	-0.21	-0.47	-0.20	-0.29	0.59	-0.14	-0.13	0.45	
DIN:DIP N	-0.56	-0.00	-0.06	-0.05	-0.11	-0.11	0.17	-0.25	-0.10	-0.10	-0.25	-0.20	-0.24	-0.27	-0.21	-0.24	-0.17	0.40	0.23	0.20	-0.22	-0.16	-0.22	
N:P N	-0.59	-0.01	-0.05	-0.05	-0.10	-0.11	0.18	-0.24	-0.10	-0.09	-0.23	-0.18	-0.22	-0.26	-0.20	-0.22	-0.17	0.41	0.23	0.18	-0.22	-0.18	-0.24	
10^(WST)	-0.21	-0.28	-0.22	-0.22	0.03	-0.23	0.15	0.18	-0.22	-0.21	0.26	0.47	0.36	0.11	0.45	0.47	0.08	0.20	0.20	0.15	-0.27	-0.25	-0.12	
2^(md)	0.04	-0.17	-0.14	-0.14	-0.01	-0.12	-0.12	0.13	-0.13	-0.12	0.71	0.83	0.83	0.31	0.68	0.69	0.60	-0.09	-0.01	0.08	-0.17	-0.14	-0.21	
2^(TAL)	0.27	0.15	-0.11	-0.10	-0.11	-0.08	-0.19	-0.18	-0.08	-0.08	-0.30	-0.29	-0.29	-0.22	-0.11	-0.14	-0.25	-0.24	-0.29	-0.10	0.90	-0.13	0.00	
2^(Ca)	0.14	0.30	0.46	0.45	0.56	0.47	0.35	0.24	0.47	0.46	-0.37	-0.39	-0.39	-0.30	-0.20	-0.23	0.07	-0.38	-0.44	-0.19	0.05	0.19	0.27	
2^(EC)	-0.71	-0.14	-0.12	-0.13	0.78	-0.16	0.81	0.46	-0.15	-0.14	-0.16	-0.14	-0.17	-0.25	-0.19	-0.22	0.19	0.06	-0.00	-0.19	-0.21	-0.17	-0.27	
10^(Mg)	-0.53	0.07	-0.13	-0.12	0.22	-0.15	0.30	0.02	-0.15	-0.14	-0.23	-0.21	-0.22	-0.25	-0.22	-0.25	-0.03	-0.11	-0.15	-0.18	-0.11	-0.20	-0.19	
2^(SO4)	0.02	-0.13	-0.11	-0.10	-0.01	-0.10	-0.05	0.12	-0.10	-0.09	0.41	0.40	0.49	0.58	-0.06	0.00	0.21	-0.04	-0.08	0.20	-0.13	-0.11	-0.18	
pH	-0.91	-0.59	-0.40	-0.40	0.38	-0.45	0.54	0.31	-0.44	-0.42	0.25	0.35	0.28	-0.05	0.17	0.18	0.12	0.38	0.43	-0.25	-0.10	-0.29	-0.48	
TP	0.65	-0.12	0.15	0.15	-0.20	0.23	-0.43	-0.07	0.22	0.21	0.28	0.31	0.32	0.14	0.49	0.49	0.43	-0.43	-0.33	-0.35	0.35	0.17	0.02	
DMS	-0.64	0.04	-0.02	-0.03	0.44	-0.09	0.53	0.08	-0.08	-0.08	-0.34	-0.25	-0.29	-0.31	-0.32	-0.38	0.00	0.17	0.01	0.16	-0.11	-0.17	-0.17	
F	-0.17	-0.41	-0.36	-0.36	0.15	-0.31	0.15	0.16	-0.32	-0.34	-0.19	-0.27	-0.28	-0.38	-0.15	-0.21	0.13	0.12	0.12	-0.39	0.70	0.47	0.20	

Variable	flow_N	1/TN	1/kjeln	NH4-N	TN:TP N	1/PO4:NH4	1/P04-P	1/DIN	Cl N	K N	NO3_NO2 N	Na N	1/Si	DIN:DIP N	N:P N	10^(WST)	2^(md)	2^(TAL)	2^(Ca)	2^(EC)	10^(Mg)	2^(SO4)	pH	TP	DMS	F
date	0.03	0.38	0.28	0.51	-0.77	0.08	-0.54	0.19	-0.84	-0.86	-0.61	-0.84	0.44	-0.58	-0.59	-0.21	0.04	0.27	0.14	-0.71	-0.53	0.02	-0.91	0.65	-0.64	-0.17
1/16s min N	-0.26	-0.04	0.30	0.24	-0.05	0.39	0.00	-0.34	-0.21	-0.21	0.14	-0.18	0.99	-0.00	-0.01	-0.28	-0.17	0.15	0.30	-0.14	0.07	-0.13	-0.59	-0.12	0.04	-0.41
1/16s max	-0.14	-0.05	0.29	-0.12	-0.11	-0.15	-0.08	-0.29	-0.13	-0.13	0.15	-0.14	0.31	-0.05	-0.05	-0.22	-0.14	-0.11	0.48	-0.12	-0.13	-0.11	-0.40	0.15	-0.02	-0.36
1/16s avg	-0.14	-0.05	0.28	-0.13	-0.12	-0.16	-0.08	-0.28	-0.14	-0.13	0.14	-0.14	0.31	-0.05	-0.05	-0.22	-0.14	-0.10	0.45	-0.13	-0.12	-0.10	-0.40	0.15	-0.03	-0.36
mcyB min N	0.03	-0.17	-0.10	-0.15	0.26	-0.29	-0.20	-0.10	0.40	0.38	0.39	0.30	-0.11	-0.11	-0.10	0.03	-0.01	-0.11	0.56	0.78	0.22	-0.01	0.36	-0.20	0.44	0.15
1/mcyb max	-0.11	-0.00	0.28	-0.08	-0.20	-0.17	-0.14	-0.23	-0.21	-0.21	0.07	-0.23	0.29	-0.11	-0.11	-0.23	-0.12	-0.08	0.47	-0.16	-0.15	-0.10	-0.45	0.23	-0.09	-0.31
mcyB avg N	0.04	-0.26	-0.17	-0.28	0.49	-0.18	0.11	-0.12	0.61	0.60	0.51	0.53	-0.18	0.17	0.18	0.15	-0.12	-0.19	0.35	0.81	0.30	-0.05	0.54	-0.43	0.53	0.15
mcyE min N	0.28	0.10	0.01	-0.18	-0.01	-0.41	-0.30	0.09	0.23	0.20	0.06	0.13	-0.21	-0.25	-0.24	0.18	0.13	-0.18	0.24	0.46	0.02	0.12	0.31	-0.07	0.08	0.18
1/mcye max	-0.12	-0.01	0.29	-0.10	-0.19	-0.18	-0.13	-0.23	-0.20	-0.20	0.09	-0.22	0.29	-0.10	-0.10	-0.22	-0.13	-0.08	0.47	-0.15	-0.15	-0.10	-0.44	0.22	-0.08	-0.32
1/mcyE avg	-0.12	-0.01	0.29	-0.12	-0.18	-0.19	-0.12	-0.23	-0.19	-0.19	0.10	-0.20	0.30	-0.10	-0.09	-0.21	-0.12	-0.08	0.48	-0.14	-0.14	-0.09	-0.42	0.21	-0.08	-0.34
Ntca min N	-0.05	0.35	0.10	-0.38	-0.26	-0.53	-0.24	0.52	-0.19	-0.24	-0.35	-0.26	-0.35	-0.25	-0.23	0.26	0.71	-0.30	-0.37	-0.16	-0.23	0.41	0.25	0.28	-0.34	-0.19
Ntca max N	0.03	0.36	0.11	-0.47	-0.23	-0.56	-0.18	0.60	-0.08	-0.17	-0.33	-0.22	-0.41	-0.20	-0.18	0.47	0.83	-0.29	-0.39	-0.14	-0.21	0.40	0.35	0.31	-0.25	-0.27
Ntca avg N	-0.03	0.43	0.18	-0.44	-0.28	-0.57	-0.23	0.59	-0.14	-0.23	-0.36	-0.27	-0.38	-0.24	-0.22	0.36	0.83	-0.29	-0.39	-0.17	-0.22	0.49	0.28	0.32	-0.29	-0.28
RbcL min N	-0.04	0.52	0.35	-0.21	-0.31	-0.38	-0.21	0.55	-0.24	-0.36	-0.37	-0.32	-0.24	-0.27	-0.26	0.11	0.31	-0.22	-0.30	-0.25	-0.25	0.58	-0.05	0.14	-0.31	-0.38
RbcL max N	0.00	0.05	-0.18	-0.27	-0.27	-0.38	-0.21	0.50	-0.26	-0.27	-0.31	-0.36	-0.19	-0.21	-0.20	0.45	0.68	-0.11	-0.20	-0.19	-0.22	-0.06	0.17	0.49	-0.32	-0.15
RbcL avg N	-0.00	0.10	-0.15	-0.30	-0.30	-0.42	-0.22	0.58	-0.30	-0.32	-0.35	-0.40	-0.21	-0.24	-0.22	0.47	0.69	-0.14	-0.23	-0.22	-0.25	0.00	0.18	0.49	-0.36	-0.21
MCN min N	0.06	0.18	-0.01	0.24	-0.18	-0.08	-0.24	0.04	0.02	-0.04	-0.08	-0.14	-0.47	-0.17	-0.17	0.08	0.60	-0.25	0.07	0.19	-0.03	0.21	0.12	0.43	0.00	0.13
MCN max N	0.04	-0.21	-0.18	-0.23	0.46	0.14	0.46	-0.12	0.43	0.52	0.18	0.50	-0.20	0.40	0.41	0.20	-0.09	-0.24	-0.38	0.06	-0.11	-0.04	0.38	-0.43	0.17	0.12
MCN avg N	0.04	-0.21	-0.28	-0.23	0.38	-0.02	0.27	-0.04	0.28	0.41	0.06	0.41	-0.29	0.23	0.23	0.20	-0.01	-0.29	-0.44	-0.00	-0.15	-0.08	0.43	-0.33	0.01	0.12
1/Chl a	0.02	0.20	0.41	0.13	0.12	0.49	0.28	-0.18	0.19	0.12	0.05	0.14	0.59	0.20	0.18	0.15	0.08	-0.10	-0.19	-0.19	-0.16	0.20	-0.25	-0.35	0.16	-0.39
AD N	-0.06	-0.05	-0.29	0.12	-0.24	-0.08	-0.23	0.23	-0.36	-0.26	-0.33	-0.26	-0.14	-0.22	-0.22	-0.27	-0.17	0.90	0.05	-0.21	-0.11	-0.13	-0.10	0.35	-0.11	0.70
OD N	0.18	0.11	-0.06	0.63	-0.23	0.45	-0.13	-0.14	-0.22	-0.31	-0.24	-0.28	-0.13	-0.16	-0.16	-0.25	-0.14	-0.13	0.19	-0.17	-0.20	-0.11	-0.29	0.17	-0.17	0.47
Mer_D N	0.28	0.17	0.16	0.53	-0.29	0.48	-0.16	-0.17	-0.24	-0.37	-0.23	-0.30	0.45	-0.22	-0.24	-0.12	-0.21	0.00	0.27	-0.27	-0.19	-0.18	-0.48	0.02	-0.17	0.20
flow_N	1.00	0.38	0.04	0.03	-0.39	-0.17	-0.26	0.39	0.00	-0.10	-0.52	-0.10	-0.29	-0.36	-0.36	0.73	-0.04	-0.19	-0.28	-0.35	-0.47	-0.07	0.07	0.07	-0.44	0.14
1/TN	0.38	1.00	0.80	0.00	-0.69	-0.28	-0.42	0.37	-0.25	-0.49	-0.62	-0.46	-0.10	-0.48	-0.47	0.16	0.63	-0.11	-0.25	-0.51	-0.42	0.82	-0.30	0.38	-0.38	-0.22
1/kjeln	0.04	0.80	1.00	-0.03	-0.33	-0.06	-0.13	-0.12	0.04	-0.19	-0.11	-0.16	0.26	-0.14	-0.14	-0.11	0.49	-0.22	0.05	-0.21	-0.13	0.83	-0.38	0.06	0.05	-0.51
NH4-N	0.03	0.00	-0.03	1.00	-0.27	0.64	-0.25	-0.37	-0.38	-0.35	-0.15	-0.33	0.25	-0.24	-0.27	-0.42	-0.26	-0.01	0.26	-0.17	-0.19	-0.19	-0.67	0.26	-0.20	0.18
TN:TP N	-0.39	-0.69	-0.33	-0.27	1.00	0.37	0.78	-0.56	0.82	0.91	0.87	0.92	0.00	0.81	0.81	-0.12	-0.40	-0.14	0.02	0.68	0.60	-0.25	0.61	-0.83	0.82	0.05
1/PO4:NH4	-0.17	-0.28	-0.06	0.64	0.37	1.00	0.53	-0.64	0.19	0.21	0.35	0.25	0.39	0.51	0.48	-0.34	-0.40	-0.13	0.02	0.08	0.27	-0.23	-0.27	-0.38	0.28	0.11
1/P04-P	-0.26	-0.42	-0.13	-0.25	0.78	0.53	1.00	-0.41	0.67	0.69	0.64	0.71	-0.01	0.98	0.98	-0.00	-0.25	-0.21	-0.30	0.35	0.61	-0.10	0.43	-0.67	0.56	-0.00
1/DIN	0.39	0.37	-0.12	-0.37	-0.56	-0.64	-0.41	1.00	-0.46	-0.51	-0.74	-0.53	-0.36	-0.49	-0.48	0.60	0.34	0.18	-0.41	-0.45	-0.44	0.11	0.13	0.45	-0.64	0.04
Cl N	0.00	-0.25	0.04	-0.38	0.82	0.19	0.67	-0.46	1.00	0.95	0.72	0.94	-0.17	0.68	0.66	0.15	-0.12	-0.29	-0.02	0.65	0.47	0.10	0.68	-0.81	0.83	-0.00
K N	-0.10	-0.49	-0.19	-0.35	0.91	0.21	0.69	-0.51	0.95	1.00	0.78	0.98	-0.16	0.71	0.72	0.08	-0.26	-0.16	-0.03	0.66	0.48	-0.11	0.69	-0.79	0.82	0.08
NO3_NO2 N	-0.52	-0.62	-0.11	-0.15	0.87	0.35	0.64	-0.74	0.72	0.78	1.00	0.75	0.21	0.74	0.74	-0.34	-0.33	-0.18	0.40	0.83	0.74	-0.18	0.33	-0.65	0.87	-0.11
Na N	-0.10	-0.48	-0.16	-0.33	0.92	0.25	0.71	-0.53	0.94	0.98	0.75	1.00	-0.14	0.72	0.72	0.04	-0.32	-0.18	-0.09	0.60	0.49	-0.09	0.69	-0.87	0.80	0.07
1/Si	-0.29	-0.10	0.26	0.25	0.00	0.39	-0.01	-0.36	-0.17	-0.16	0.21	-0.14	1.00	-0.00	-0.01	-0.29	-0.21	0.14	0.38	-0.03	0.10	-0.16	-0.56	-0.16	0.10	-0.40
DIN:DIP N	-0.36	-0.48	-0.14	-0.24	0.81	0.51	0.98	-0.49	0.68	0.71	0.74	0.72	-0.00	1.00	1.00	-0.12	-0.23	-0.17	-0.19	0.46	0.72	-0.10	0.43	-0.64	0.63	0.02
N:P N	-0.36	-0.47	-0.14	-0.27	0.81	0.48	0.98	-0.48	0.68	0.72	0.74	0.72	-0.01	1.00	1.00	-0.11	-0.22	-0.17	-0.19	0.47	0.73	-0.10	0.45	-0.64	0.63	0.02
10^(WST)	0.73	0.16	-0.11	-0.42	-0.12	-0.34	-0.00	0.60	0.15	0.08	-0.34	0.04	-0.29	-0.12	-0.11	1.00	0.19	-0.27	-0.48	-0.23	-0.35	-0.12	0.39	-0.04	-0.30	-0.18
2^(md)	-0.04	0.63	0.49	-0.26	-0.40	-0.40	-0.25	0.34	-0.12	-0.26	-0.33	-0.32	-0.21	-0.23	-0.22	0.19	1.00	-0.16	-0.23	-0.20	-0.17	0.67	0.06	0.44	-0.15	-0.27
2^(TAL)	-0.19	-0.11	-0.22	-0.01	-0.14	-0.13	-0.21	0.18	-0.29	-0.18	-0.18	-0.18	0.14	-0.17	-0.17	-0.27	-0.16	1.00	0.10	-0.10	0.01	-0.12	-0.11	0.24	0.01	0.45
2^(Ca)	-0.28	-0.25	0.05	0.26	0.02	0.02	-0.30	-0.41	-0.02	-0.03	0.40	-0.09	0.38	-0.19	-0.19	-0.48	-0.23	0.10	1.00	0.52	0.14	-0.18	-0.34	0.11	0.34	0.03
2^(EC)	-0.35	-0.51	-0.21	-0.17	0.68	0.08	0.35	-0.45	0.65	0.66	0.83	0.60	-0.03	0.46	0.47	-0.23	-0.20	-0.10	0.52	1.00	0.72	-0.12	0.48	-0.48	0.79	0.15
10^(Mg)	-0.47	-0.42	-0.13	-0.19	0.60	0.27	0.61	-0.44	0.47	0.48	0.74	0.49	0.10	0.72	0.73	-0.35	-0.17	0.01	0.14	0.72	1.00	-0.06	0.37	-0.44	0.61	0.11
2^(SO4)	-0.07	0.82	0.83	-0.19	-0.25	-0.23	-0.10	0.11	0.10	-0.11	-0.18	-0.09	-0.16	-0.10	-0.10	-0.12	0.67	-0.12	-0.19	-0.12	-0.06	1.00	-0.04	0.08	0.09	-0.27
pH	0.07	-0.30	-0.38	-0.67	0.61	-0.27	0.43	0.13	0.68	0.69	0.33	0.69	-0.56	0.43	0.45	0.39	0.06	-0.11	-0.34	0.48	0.37	-0.04	1.00	-0.52	0.44	0.28
TP	0.07	0.36	0.06	0.26	-0.83	-0.38	-0.67	0.45	-0.81	-0.79	-0.65	-0.87	-0.16	-0.64	-0.64	-0.04	0.44	0.24	0.11	-0.48	-0.44	0.08	-0.52	1.00	-0.68	0.05
DMS	-0.44	-0.38	0.05	-0.20	0.82	0.28	0.56	-0.64	0.83	0.82	0.87	0.80	0.10	0.63	0.63	-0.30	-0.15	0.01	0.34	0.79	0.61	0.09	0.44	-0.68	1.00	0.04
F	0.14	-0.22	-0.51	0.18	0.05	0.11	-0.00	0.04	-0.00	0.08	-0.11	0.07	-0.40	0.02	0.02	-0.18	-0.27	0.48	0.03	0.15	0.11	-0.27	0.26	0.05	0.04	1.00

Correlations (Hartbeespoortdam.sta)
Marked correlations are significant at p < .05000
N=13 (Casewise deletion of missing data)

Variable	date	16S min	1/16Smax	1/16S avg	mcyB min	1/mcyB max	1/mcyB avg	mcyE min	mcyE max	mcyE avg	1/NtcA min	Ntca max	Ntca avg	RbcL min	RbcL max	RbcL avg	MCN min	MCN max	MCN avg	1/Chl a	MD	OD	Mer_D	2^(W
date	1.00	0.38	-0.53	-0.52	0.06	-0.68	-0.61	0.13	0.16	0.25	0.63	0.16	0.15	0.12	0.06	0.09	0.23	0.46	0.56	-0.67	0.37	0.66	0.58	-
16S min	0.38	1.00	-0.32	-0.32	0.73	-0.38	-0.32	0.89	0.46	0.83	-0.16	0.55	0.67	0.79	0.73	0.78	0.71	0.43	0.41	-0.42	0.56	-0.22	0.06	-
1/16Smax	-0.53	-0.32	1.00	1.00	-0.22	0.71	0.47	-0.20	-0.42	-0.41	-0.22	-0.06	-0.07	-0.21	-0.24	-0.22	-0.19	-0.25	-0.30	0.42	-0.48	-0.25	-0.16	-
1/16S avg	-0.52	-0.32	1.00	1.00	-0.21	0.70	0.46	-0.20	-0.42	-0.41	-0.22	-0.05	-0.07	-0.21	-0.24	-0.22	-0.19	-0.25	-0.29	0.42	-0.46	-0.24	-0.16	-
mcyB min	0.06	0.73	-0.22	-0.21	1.00	-0.26	-0.22	0.90	0.34	0.75	-0.25	0.12	0.29	0.91	0.82	0.85	0.82	-0.10	-0.09	-0.30	0.44	-0.24	-0.14	-
1/mcyB max	-0.68	-0.38	0.71	0.70	-0.26	1.00	0.95	-0.24	-0.49	-0.48	-0.26	-0.15	-0.17	-0.25	-0.29	-0.27	-0.25	-0.30	-0.35	0.55	-0.63	-0.29	-0.19	-
1/mcyB avg	-0.61	-0.32	0.47	0.46	-0.22	0.95	1.00	-0.21	-0.42	-0.41	-0.22	-0.16	-0.18	-0.22	-0.25	-0.23	-0.22	-0.26	-0.30	0.50	-0.56	-0.25	-0.16	-
mcyE min	0.13	0.89	-0.20	-0.20	0.90	-0.24	-0.21	1.00	0.36	0.75	-0.21	0.35	0.53	0.97	0.91	0.95	0.87	0.13	0.11	-0.29	0.48	-0.25	-0.14	-
mcyE max	0.16	0.46	-0.42	-0.42	0.34	-0.49	-0.42	0.36	1.00	0.81	-0.19	0.23	0.26	0.29	0.49	0.40	0.26	0.25	0.23	-0.52	0.70	-0.14	-0.03	-
mcyE avg	0.25	0.83	-0.41	-0.41	0.75	-0.48	-0.41	0.75	0.81	1.00	-0.25	0.31	0.42	0.65	0.69	0.67	0.58	0.33	0.32	-0.55	0.74	-0.25	-0.04	-
1/NtcA min	0.63	-0.16	-0.22	-0.22	-0.25	-0.26	-0.22	-0.21	-0.19	-0.25	1.00	-0.06	-0.16	-0.20	-0.24	-0.21	-0.10	0.32	0.43	-0.23	-0.12	0.95	0.34	-
Ntca max	0.16	0.55	-0.06	-0.05	0.12	-0.15	-0.18	0.35	0.23	0.31	-0.06	1.00	0.98	0.28	0.32	0.39	0.08	0.39	0.33	-0.10	0.39	-0.28	-0.17	-
Ntca avg	0.15	0.67	-0.07	-0.07	0.29	-0.17	-0.18	0.53	0.26	0.42	-0.16	0.98	1.00	0.46	0.49	0.55	0.24	0.37	0.31	-0.16	0.47	-0.36	-0.22	-
RbcL min	0.12	0.79	-0.21	-0.21	0.91	-0.25	-0.22	0.97	0.29	0.65	-0.20	0.28	0.46	1.00	0.95	0.98	0.91	-0.07	-0.07	-0.30	0.42	-0.21	-0.13	-
RbcL max	0.06	0.73	-0.24	-0.24	0.82	-0.29	-0.25	0.91	0.49	0.69	-0.24	0.32	0.49	0.95	1.00	0.99	0.85	-0.07	-0.07	-0.34	0.53	-0.24	-0.18	-
RbcL avg	0.09	0.78	-0.22	-0.22	0.85	-0.27	-0.23	0.95	0.40	0.67	-0.21	0.39	0.55	0.98	0.99	1.00	0.87	-0.05	-0.05	-0.31	0.49	-0.24	-0.16	-
MCN min	0.23	0.71	-0.19	-0.19	0.82	-0.25	-0.22	0.87	0.26	0.58	-0.10	0.08	0.24	0.91	0.85	0.87	1.00	-0.18	-0.15	-0.30	0.21	-0.02	0.23	-
MCN max	0.46	0.43	-0.25	-0.25	-0.10	-0.30	-0.26	0.13	0.25	0.33	0.32	0.39	0.37	-0.07	-0.07	-0.05	-0.18	1.00	0.99	-0.27	0.39	0.17	0.00	-
MCN avg	0.56	0.41	-0.30	-0.29	-0.09	-0.35	-0.30	0.11	0.23	0.32	0.43	0.33	0.31	-0.07	-0.07	-0.05	-0.15	0.99	1.00	-0.33	0.38	0.29	0.11	-
1/Chl a	-0.67	-0.42	0.42	0.42	-0.30	0.55	0.50	-0.29	-0.52	-0.55	-0.23	-0.10	-0.16	-0.30	-0.34	-0.31	-0.30	-0.27	-0.33	1.00	-0.67	-0.30	-0.21	-
MD	0.37	0.56	-0.46	-0.46	0.44	-0.63	-0.56	0.46	0.70	0.74	-0.12	0.39	0.47	0.42	0.53	0.49	0.21	0.39	0.38	-0.67	1.00	-0.17	-0.28	-
OD	0.66	-0.22	-0.25	-0.24	-0.24	-0.29	-0.25	-0.25	-0.14	-0.25	0.95	-0.28	-0.36	-0.21	-0.24	-0.24	-0.02	0.17	0.29	-0.30	-0.17	1.00	0.47	-
Mer_D	0.58	0.06	-0.16	-0.16	-0.14	-0.18	-0.16	-0.14	-0.03	-0.04	0.34	-0.17	-0.22	-0.13	-0.18	-0.16	0.23	0.00	0.11	-0.21	-0.28	0.47	1.00	-
2^(WST)	-0.19	-0.11	-0.19	-0.19	0.19	-0.26	-0.24	-0.08	0.67	0.44	-0.30	-0.18	-0.16	-0.06	0.11	0.00	-0.13	-0.17	-0.17	-0.34	0.54	-0.23	-0.24	
1/Kjel-N	-0.05	-0.42	0.46	0.46	-0.42	0.53	0.47	-0.40	-0.59	-0.61	0.42	-0.31	-0.40	-0.39	-0.48	-0.44	-0.13	-0.13	-0.06	0.46	-0.84	0.44	0.53	-
NH4-N	-0.08	0.19	0.07	0.08	-0.13	-0.12	-0.18	-0.00	0.27	0.14	-0.11	0.85	0.75	-0.07	0.04	0.06	-0.23	0.22	0.15	0.12	0.22	-0.31	-0.21	
2^(TAL)	-0.45	-0.22	0.16	0.14	-0.15	0.79	0.94	-0.14	-0.28	-0.28	-0.15	-0.14	-0.16	-0.16	-0.17	-0.16	-0.16	-0.18	-0.21	0.39	-0.41	-0.17	-0.11	-
TN:TP	-0.51	-0.52	0.69	0.69	-0.32	0.63	0.46	-0.35	-0.61	-0.61	0.14	-0.33	-0.38	-0.36	-0.42	-0.39	-0.29	-0.17	-0.16	0.59	-0.76	0.09	-0.05	-
1/PO4:NH4	-0.37	-0.17	0.24	0.25	-0.23	0.16	0.01	-0.18	-0.16	-0.24	-0.14	0.20	0.13	-0.20	-0.19	-0.17	-0.20	-0.11	-0.15	0.31	-0.44	-0.22	-0.03	-
1/PO4-P	-0.17	-0.24	0.14	0.14	-0.19	0.27	0.21	-0.19	-0.38	-0.35	0.06	-0.32	-0.34	-0.18	-0.25	-0.23	0.01	-0.20	-0.17	0.20	-0.70	0.11	0.37	-
DIN	-0.65	-0.55	0.64	0.63	-0.41	0.78	0.69	-0.41	-0.63	-0.69	-0.07	-0.23	-0.31	-0.40	-0.46	-0.43	-0.30	-0.38	-0.38	0.82	-0.92	-0.10	-0.00	-
2^(Ca)	-0.51	-0.25	0.20	0.18	-0.17	0.82	0.96	-0.16	-0.33	-0.32	-0.17	-0.16	-0.18	-0.18	-0.20	-0.18	-0.18	-0.20	-0.24	0.43	-0.49	-0.19	-0.13	-
2^(Cl)	-0.54	-0.38	0.19	0.17	-0.25	0.69	0.81	-0.26	-0.45	-0.48	-0.29	-0.17	-0.17	-0.21	-0.21	-0.21	-0.26	-0.32	-0.35	0.59	-0.39	-0.31	-0.21	-
DMS	-0.64	-0.57	0.66	0.65	-0.47	0.83	0.75	-0.44	-0.70	-0.75	-0.06	-0.18	-0.28	-0.43	-0.46	-0.45	-0.38	-0.30	-0.34	0.84	-0.87	-0.12	-0.10	-
EC	-0.76	-0.56	0.61	0.62	-0.39	0.83	0.74	-0.42	-0.53	-0.62	-0.21	-0.23	-0.30	-0.41	-0.44	-0.42	-0.33	-0.43	-0.48	0.81	-0.86	-0.23	-0.10	-
F	0.10	0.16	-0.18	-0.17	-0.02	-0.21	-0.21	-0.02	0.08	0.12	-0.25	0.53	0.54	-0.00	0.04	0.06	-0.21	0.15	0.15	-0.32	0.45	-0.39	-0.10	
Mg	-0.72	-0.42	0.52	0.51	-0.33	0.67	0.58	-0.24	-0.48	-0.57	-0.24	-0.15	-0.20	-0.24	-0.25	-0.24	-0.18	-0.36	-0.43	0.83	-0.82	-0.25	-0.15	-
1/NO3_NO2	0.24	0.37	-0.30	-0.30	0.18	-0.34	-0.29	0.25	0.23	0.37	-0.31	0.05	0.18	0.21	0.22	0.20	0.03	0.42	0.41	-0.47	0.67	-0.31	-0.20	
Na	-0.62	-0.67	0.64	0.63	-0.49	0.63	0.50	-0.51	-0.41	-0.64	-0.38	-0.19	-0.26	-0.47	-0.41	-0.44	-0.48	-0.43	-0.50	0.83	-0.60	-0.40	-0.31	-
2^(SO4)	-0.59	-0.33	0.70	0.68	-0.22	0.94	0.91	-0.21	-0.43	-0.42	-0.22	-0.12	-0.14	-0.23	-0.25	-0.23	-0.22	-0.26	-0.31	0.56	-0.51	-0.25	-0.17	-
10^(Si)	0.58	0.30	-0.30	-0.30	-0.17	-0.35	-0.30	0.07	0.08	0.10	0.50	0.54	0.47	-0.03	-0.06	-0.00	-0.16	0.67	0.63	-0.33	0.32	0.37	-0.11	-
DIN:DIP	-0.49	-0.39	0.36	0.36	-0.30	0.53	0.44	-0.30	-0.50	-0.51	-0.08	-0.24	-0.29	-0.29	-0.35	-0.32	-0.19	-0.28	-0.30	0.48	-0.80	-0.09	0.06	-
N:P	-0.49	-0.40	0.36	0.36	-0.30	0.54	0.44	-0.30	-0.50	-0.52	-0.08	-0.25	-0.29	-0.29	-0.35	-0.32	-0.19	-0.28	-0.30	0.48	-0.80	-0.08	0.07	-
K	-0.95	-0.28	0.59	0.58	0.01	0.67	0.58	-0.03	-0.14	-0.19	-0.68	-0.23	-0.18	-0.03	0.02	-0.02	-0.10	-0.43	-0.52	0.69	-0.35	-0.67	-0.49	
pH	-0.45	0.10	-0.17	-0.17	0.40	-0.17	-0.15	0.27	0.39	0.40	-0.67	-0.13	-0.00	0.29	0.37	0.30	0.05	-0.19	-0.26	-0.06	0.52	-0.64	-0.67	
TP	0.24	0.47	-0.50	-0.49	0.29	-0.46	-0.35	0.34	0.52	0.52	-0.30	0.57	0.61	0.35	0.42	0.42	0.14	0.11	0.05	-0.49	0.77	-0.34	-0.29	
TN	-0.94	-0.36	0.45	0.45	-0.06	0.63	0.56	-0.16	-0.28	-0.30	-0.66	-0.09	-0.07	-0.14	-0.13	-0.13	-0.30	-0.46	-0.55	0.61	-0.35	-0.71	-0.59	
flow	0.26	0.24	-0.39	-0.38	0.23	-0.47	-0.40	0.06	0.48	0.48	0.02	0.43	0.39	0.02	0.06	0.07	-0.17	0.23	0.22	-0.44	0.69	-0.08	-0.23	

Variable	WST)	1/Kjel-N	NH4-N	2^(TAL)	TN:TP	1/PO4:NH4	1/PO4-P	DIN	2^(Ca)	2^(Cl)	DMS	EC	F	Mg	1/NO3_NO2	Na	2^(SO4)	10^(Si)	DIN:DIP	N:P	K	pH	TP	TN	flow
date	-0.19	-0.05	-0.08	-0.45	-0.51	-0.37	-0.17	-0.65	-0.51	-0.54	-0.64	-0.76	0.10	-0.72	0.24	-0.62	-0.59	0.58	-0.49	-0.49	-0.95	-0.45	0.24	-0.94	0.26
16S min	-0.11	-0.42	0.19	-0.22	-0.52	-0.17	-0.24	-0.55	-0.25	-0.38	-0.57	-0.56	0.16	-0.42	0.37	-0.67	-0.33	0.30	-0.39	-0.40	-0.28	0.10	0.47	-0.36	0.24
1/16Smax	-0.19	0.46	0.07	0.16	0.69	0.24	0.14	0.64	0.20	0.19	0.66	0.63	-0.18	0.52	-0.30	0.64	0.70	-0.30	0.36	0.36	0.59	-0.17	-0.50	0.45	-0.39
1/16S avg	-0.19	0.46	0.08	0.14	0.69	0.25	0.14	0.63	0.18	0.17	0.65	0.62	-0.17	0.51	-0.30	0.63	0.68	-0.30	0.36	0.36	0.58	-0.17	-0.49	0.45	-0.38
mcyB min	0.19	-0.42	-0.13	-0.15	-0.32	-0.23	-0.19	-0.41	-0.17	-0.25	-0.47	-0.39	-0.02	-0.33	0.18	-0.49	-0.22	-0.17	-0.30	-0.30	0.01	0.40	0.29	-0.06	0.23
1/mcyB max	-0.26	0.53	-0.12	0.79	0.63	0.16	0.27	0.78	0.82	0.69	0.83	0.83	-0.21	0.67	-0.34	0.63	0.94	-0.35	0.53	0.54	0.67	-0.17	-0.46	0.63	-0.47
1/mcyB avg	-0.24	0.47	-0.16	0.94	0.46	0.01	0.21	0.69	0.96	0.81	0.75	0.74	-0.21	0.58	-0.29	0.50	0.91	-0.30	0.44	0.44	0.58	-0.15	-0.35	0.56	-0.40
mcyE min	-0.08	-0.40	-0.00	-0.14	-0.35	-0.18	-0.19	-0.41	-0.16	-0.26	-0.44	-0.42	-0.02	-0.24	0.25	-0.51	-0.21	0.07	-0.30	-0.30	-0.03	0.27	0.34	-0.16	0.06
mcyE max	0.67	-0.59	0.27	-0.28	-0.61	-0.16	-0.38	-0.63	-0.33	-0.45	-0.70	-0.53	0.08	-0.48	0.23	-0.41	-0.43	0.08	-0.50	-0.50	-0.14	0.39	0.52	-0.28	0.48
mcyE avg	0.44	-0.61	0.14	-0.28	-0.61	-0.24	-0.35	-0.69	-0.32	-0.48	-0.75	-0.62	0.12	-0.57	0.37	-0.64	-0.42	0.10	-0.51	-0.52	-0.19	0.40	0.52	-0.30	0.48
1/NtcA min	-0.30	0.42	-0.11	-0.15	0.14	-0.14	0.06	-0.07	-0.17	-0.29	-0.06	-0.21	-0.25	-0.24	-0.31	-0.38	-0.22	0.50	-0.08	-0.08	-0.68	-0.67	-0.30	-0.66	0.02
Ntca max	-0.18	-0.31	0.85	-0.14	-0.33	0.20	-0.32	-0.23	-0.16	-0.17	-0.18	-0.23	0.53	-0.15	0.05	-0.19	-0.12	0.54	-0.24	-0.25	-0.23	-0.13	0.57	-0.09	0.43
Ntca avg	-0.16	-0.40	0.75	-0.16	-0.38	0.13	-0.34	-0.31	-0.18	-0.17	-0.28	-0.30	0.54	-0.20	0.18	-0.26	-0.14	0.47	-0.29	-0.29	-0.18	-0.00	0.61	-0.07	0.39
RbcL min	-0.06	-0.39	-0.07	-0.18	-0.36	-0.20	-0.18	-0.40	-0.18	-0.21	-0.43	-0.41	-0.00	-0.24	0.21	-0.47	-0.23	-0.03	-0.29	-0.29	-0.03	0.29	0.35	-0.14	0.02
RbcL max	0.11	-0.48	0.04	-0.17	-0.42	-0.19	-0.25	-0.46	-0.20	-0.21	-0.48	-0.44	0.04	-0.25	0.22	-0.41	-0.25	-0.06	-0.35	-0.35	0.02	0.37	0.42	-0.13	0.06
RbcL avg	0.00	-0.44	0.06	-0.16	-0.39	-0.17	-0.23	-0.43	-0.18	-0.21	-0.45	-0.42	0.06	-0.24	0.20	-0.44	-0.23	-0.00	-0.32	-0.32	-0.02	0.30	0.42	-0.13	0.07
MCN min	-0.13	-0.13	-0.23	-0.16	-0.29	-0.20	0.01	-0.30	-0.18	-0.26	-0.38	-0.33	-0.21	-0.18	0.03	-0.48	-0.22	-0.16	-0.19	-0.19	-0.10	0.05	0.14	-0.30	-0.17
MCN max	-0.17	-0.13	0.22	-0.18	-0.17	-0.11	-0.20	-0.36	-0.20	-0.32	-0.30	-0.43	0.15	-0.36	0.42	-0.43	-0.26	0.67	-0.28	-0.28	-0.43	-0.19	0.11	-0.46	0.23
MCN avg	-0.17	-0.06	0.15	-0.21	-0.16	-0.15	-0.17	-0.39	-0.24	-0.35	-0.34	-0.48	0.15	-0.43	0.41	-0.50	-0.31	0.63	-0.30	-0.30	-0.52	-0.26	0.05	-0.55	0.22
1/Chl a	-0.34	0.46	0.12	0.39	0.59	0.31	0.20	0.82	0.43	0.59	0.84	0.81	-0.32	0.83	-0.47	0.83	0.56	-0.33	0.48	0.48	0.69	-0.06	-0.49	0.61	-0.44
MD	0.54	-0.84	0.22	-0.41	-0.76	-0.44	-0.70	-0.92	-0.49	-0.39	-0.87	-0.86	0.45	-0.82	0.67	-0.60	-0.51	0.32	-0.80	-0.80	-0.35	0.52	0.77	-0.35	0.69
CO	-0.23	0.44	-0.31	-0.17	0.09	-0.22	0.11	-0.10	-0.19	-0.31	-0.12	-0.23	-0.39	-0.25	-0.31	-0.40	-0.25	0.37	-0.09	-0.08	-0.67	-0.64	-0.34	-0.71	-0.08
Mer_D	-0.24	0.53	-0.21	-0.11	-0.05	-0.03	0.37	-0.00	-0.13	-0.21	-0.10	-0.10	-0.10	-0.15	-0.20	-0.31	-0.17	-0.11	0.06	0.07	-0.49	-0.67	-0.29	-0.59	-0.23
2^(WST)	1.00	-0.54	0.09	-0.16	-0.33	-0.13	-0.33	-0.41	-0.21	-0.18	-0.46	-0.26	0.22	-0.41	0.17	-0.05	-0.23	-0.33	-0.34	-0.34	0.12	0.63	0.35	0.14	0.60
1/Kjel-N	-0.54	1.00	-0.23	0.35	0.72	0.17	0.52	0.74	0.37	0.27	0.72	0.63	-0.46	0.52	-0.63	0.36	0.52	-0.22	0.52	0.52	0.10	-0.79	-0.85	-0.05	-0.62
NH4-N	0.09	-0.23	1.00	-0.23	-0.13	0.46	-0.22	-0.01	-0.22	-0.22	-0.02	0.03	0.47	0.05	-0.21	0.14	-0.11	0.34	-0.06	-0.07	-0.04	-0.06	0.41	0.13	0.50
2^(TAL)	-0.16	0.35	-0.23	1.00	0.21	-0.18	0.08	0.49	0.99	0.85	0.56	0.55	-0.20	0.39	-0.21	0.29	0.79	-0.20	0.25	0.26	0.42	-0.11	-0.18	0.40	-0.27
TN:TP	-0.33	0.72	-0.13	0.21	1.00	0.50	0.60	0.86	0.30	0.22	0.83	0.79	-0.31	0.72	-0.49	0.65	0.50	-0.37	0.75	0.76	0.48	-0.32	-0.88	0.46	-0.57
1/PO4:NH4	-0.13	0.17	0.46	-0.18	0.50	1.00	0.72	0.52	-0.04	-0.21	0.40	0.52	0.22	0.60	-0.39	0.41	-0.12	-0.12	0.80	0.79	0.20	-0.12	-0.25	0.48	-0.16
1/PO4-P	-0.33	0.52	-0.22	0.08	0.60	0.72	1.00	0.58	0.21	-0.04	0.45	0.53	-0.09	0.57	-0.35	0.22	-0.01	-0.33	0.91	0.91	0.09	-0.34	-0.60	0.26	-0.56
DIN	-0.41	0.74	-0.01	0.49	0.86	0.52	0.58	1.00	0.57	0.50	0.98	0.98	-0.34	0.92	-0.65	0.80	0.68	-0.40	0.80	0.80	0.61	-0.34	-0.73	0.60	-0.61
2^(Ca)	-0.21	0.37	-0.22	0.99	0.30	-0.04	0.21	0.57	1.00	0.83	0.62	0.63	-0.19	0.49	-0.24	0.35	0.78	-0.23	0.39	0.40	0.45	-0.11	-0.24	0.48	-0.32
2^(Cl)	-0.18	0.27	-0.22	0.85	0.22	-0.21	-0.04	0.50	0.83	1.00	0.61	0.54	-0.05	0.43	-0.05	0.54	0.75	-0.36	0.18	0.18	0.55	0.07	-0.16	0.51	-0.33
DMS	-0.46	0.72	-0.02	0.56	0.83	0.40	0.45	0.98	0.62	0.61	1.00	0.95	-0.29	0.88	-0.55	0.82	0.76	-0.32	0.71	0.71	0.62	-0.33	-0.68	0.61	-0.60
EC	-0.26	0.63	0.03	0.55	0.79	0.52	0.53	0.98	0.63	0.54	0.95	1.00	-0.31	0.92	-0.63	0.84	0.71	-0.45	0.78	0.78	0.70	-0.20	-0.63	0.71	-0.52
F	0.22	-0.46	0.47	-0.20	-0.31	0.22	-0.09	-0.34	-0.19	-0.05	-0.29	-0.31	1.00	-0.37	0.47	-0.14	-0.29	0.09	-0.10	-0.11	-0.23	0.21	0.55	0.12	0.51
Mg	-0.41	0.52	0.05	0.39	0.72	0.60	0.57	0.92	0.49	0.43	0.88	0.92	-0.37	1.00	-0.56	0.81	0.52	-0.34	0.80	0.80	0.69	-0.14	-0.56	0.67	-0.67
1/NO3_NO2	0.17	-0.63	-0.21	-0.21	-0.49	-0.39	-0.35	-0.65	-0.24	-0.05	-0.55	-0.63	0.47	-0.56	1.00	-0.37	-0.32	0.15	-0.46	-0.46	-0.15	0.51	0.47	-0.14	0.18
Na	-0.05	0.36	0.14	0.29	0.65	0.41	0.22	0.80	0.35	0.54	0.82	0.84	-0.14	0.81	-0.37	1.00	0.58	-0.49	0.53	0.53	0.81	0.11	-0.45	0.76	-0.43
2^(SO4)	-0.23	0.52	-0.11	0.79	0.50	-0.12	-0.01	0.68	0.78	0.75	0.76	0.71	-0.29	0.52	-0.32	0.58	1.00	-0.31	0.25	0.26	0.64	-0.18	-0.40	0.48	-0.38
10^(Si)	-0.33	-0.22	0.34	-0.20	-0.37	-0.12	-0.33	-0.40	-0.23	-0.36	-0.32	-0.45	0.09	-0.34	0.15	-0.49	-0.31	1.00	-0.34	-0.35	-0.64	-0.30	0.41	-0.50	0.35
DIN:DIP	-0.34	0.52	-0.06	0.25	0.75	0.80	0.91	0.80	0.39	0.18	0.71	0.78	-0.10	0.80	-0.46	0.53	0.25	-0.34	1.00	1.00	0.37	-0.24	-0.61	0.57	-0.56
N:P	-0.34	0.52	-0.07	0.26	0.76	0.79	0.91	0.80	0.40	0.18	0.71	0.78	-0.11	0.80	-0.46	0.53	0.26	-0.35	1.00	1.00	0.38	-0.24	-0.61	0.57	-0.56
K	0.12	0.10	-0.04	0.42	0.48	0.20	0.09	0.61	0.45	0.55	0.62	0.70	-0.23	0.69	-0.15	0.81	0.64	-0.64	0.37	0.38	1.00	0.44	-0.31	0.83	-0.40
pH	0.63	-0.79	-0.06	-0.11	-0.32	-0.12	-0.34	-0.34	-0.11	0.07	-0.33	-0.20	0.21	-0.14	0.51	0.11	-0.18	-0.30	-0.24	-0.24	0.44	1.00	0.45	0.47	0.29
TP	0.35	-0.85	0.41	-0.18	-0.88	-0.25	-0.60	-0.73	-0.24	-0.16	-0.68	-0.63	0.55	-0.56	0.47	-0.45	-0.40	0.41	-0.61	-0.61	-0.31	0.45	1.00	-0.13	0.68
TN	0.14	-0.05	0.13	0.40	0.46	0.48	0.26	0.60	0.48	0.51	0.61	0.71	0.12	0.67	-0.14	0.76	0.48	-0.50	0.57	0.57	0.83	0.47	-0.13	1.00	-0.15
flow	0.60	-0.62	0.50	-0.27	-0.57	-0.16	-0.56	-0.61	-0.32	-0.33	-0.60	-0.52	0.51	-0.67	0.18	-0.43	-0.38	0.35	-0.56	-0.56	-0.40	0.29	0.68	-0.15	1.00

Sequences in Fasta format

Part of the 16S rDNA gene of Microcystis aeruginosa PCC 7806

AGCTTTCGTCCYTGRGTGTCAGATACAGCCCAGTAGCACGCTTTCGCCACCGATGTTCTT
CCCAATCTCTACGCATTTCACCGCTACACTGGGAATTCCTGCTACCCCTACTGCTCTCTAG
TCTGCCAGTTTCCACCGCCTTTAGGTCGTTAAGCAACCTGATTTGACGGCAGACTTGGCT
GACCACCTGCGGACGCTTTACGCCCAATAATTCCGGATAACGCTTGCCTCCCCCGTATTA
CCGCGGCTGCTGGCACGGAGTTAGCCGAGGCTGATTCCTCAAGTACCGTCAGAACTTCTT
CCTTGAGAAAAGAGGTTTACAATCCAAAGACCTTCCTCCCTCACGCGGCGTTGCTCCGTC
AGGCTTTCGCCCATTGCGGAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTG
TCTCAGTCCCAGTGTGGCTGCTCATCCTCTCAGACCAGCTACTGATCGTCGCCTTGGTAG
GCYCTTACCCCACCAACTAGCTAATCAGACGCAAGCTCTTCTCCAGGCCAATTAGGTTTC
ACCTYGCGGCACATCGGGTATTAGCAGTCGTTTCCAACTGTTGTCCCCGTCCTGAAGTTA
GATTCTTACGCGTTACTCACCCGTCCGCCACTAGAATCCGAAGATTCCCGTTCGACTTGC
ATGTGTTAGG

Part of the 16S rDNA gene of Microcystis aeruginosa CCAP 1450/1

CYTAGCTTWCGTCCCTGAGTGTCAGATACAGCCCAGTAGCACGCTTTCGCCACCG
ATGTTCTTCCCAATCTCTACGCATTTCACCGCTACACTGGGAATTCCTGCTACCCC
TACTGCTCTCTAGTCTGCCAGTTTCCACCGCCTTTAGGTCGTTAAGCAACCTGATT
TGACGGCAGACTTGGCTGACCACCTGCGGACGCTTTACGCCCAATAATTCCGGAT
AACGCTTGCCTCCCCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGAGGCTG
ATTCCTCAAGTACCGTCAGAACTTCTTCCTTGAGAAAAGAGGTTTACAATCCAAA
GACCTTCCTCCCTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAAA
TTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGC
TGCTCATCCTCTCAGACCAGCTACTGATCGTCGCCTTGGTAGGCTCTTACCCCACC
AACTAGCTAATCAGACGCAAGCTCTTCTTCAGGCCAATTAGTTTTCACCTTGCGG
CACATCGGGTATTAGCAGTCGTTTCCAACTGTTGTCCCCGTCCTGAAGTTAGATT
CTTACGCGTTACTCACCCGTCCGCCACTAGAATCCGAAGATTCCCGTTCGACTTG
CATGTGTTAGGMACGCCGCCASCGTTCATCCTGAGCCAGGATTCAAACTCTTGGT

Part of the 16S rDNA gene of Microcystis UV027

AGTGGCGGACGGGTGAGTAACGCGTGGGAATCTGCCCTCAGGAGGGGGATAACG
GCCGGAAACGGCCGCTAATACCCCATATGCCGAGAGGTGAAATGAATTTCGCCT
GAGGATGAGCCCGCGTCTGATTAGCTAGTTGGTGTGGTAATGGCGCACCAAGGC
TTCGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACG

GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGCAAGC
CTGACGGAGCAACGCCGCGTGAGGGACGAAGGCCTCTGGGCTGTAAACCTCTTT
TCTCAAGGAAGAAGACATGACGGTACTTGAGGAATAAGCCACGGCTAATTCCGT
GCCAGCAGCCGCGGTAATACGGGAGTGGCAAGCGTTATCCGGAATTATTGGGCG
TAAAGCGTCCGCAGGCGGCCCAGCAAGTCTGTTGTTAAATAGTGGAGCTTAACTC
CATCCAGGCAATGGAAACTGCTGGGCTAGAGTGTGGTAGGGGCAGAGGGAATTC
CCGGTGTAGCGGTGAAATGCGTAGATATCGGGAAGAACACCAGTGGCGAAGGCG
CTCTGCTGGGCCATAACTGACGCTCATGGACGAAAGCCAGGGGAGCGAAAGGGA
TTAGATACCCCTGTAGTCCTGGGCCGTAAACGATGAACACTAGGTGTCGGGGGA
ATCGACCCCCTCGGTGTCGTAGCCAACGCGTTAAGTGTTCCGCCTGGGGAGTACG
CACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGT
ATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAGGGTTTGACATCCTGCG
AATCCCTTGGAAACTTGGGAGTGCCTTCGGGAGCGCAGTGACAGGTGGTGCATG
GCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCACGTCTTTAGTTGCCAGCATTCAGTTGGGCACTCTAGAGAGACCGCCGGTGAT
AAAACCGGAGGAAGGTGTGGATGACGTCAAGTCATCATGCCCCTTACATCCTGG
GCTACACACGTACTACAATGCTACGGACAAAGGGCAGCAAACTCGCGAGAGCTA
GCAAATCCCATAAACCGTGGCTCAGTTCAGATCGTAGGCTGCAACTCGCCTACGT
GAAGGAGGAATCGCTAGTAATCGCAGGTCAGCATACTGCGGTGAATACGTTCCC
GGGCCTTGTACACCACCGCCCGTCACACCAT

Part of the 16S rDNA gene of Microcystis vd Bijl

ACACATGCAGTCGAACGGAGTTCTTCGGAACTTAGTGGCGGACGGGTGAGTAAC
ACGTGAGAACCTACCTTCAGAATGGGGACAACAGTTGGGAAACGACTGCTAATA
CCCAATGTGCCGAAAGGTGAAAGATTTATCGTCTGAAGATGGGCTCGCGTCTGAT
TAGCTAGATGGTGGGGTAAGAGCCTACCATGGCAACGATCAGTAGCTGGTCTGA
GAGGATGAGCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGACGGAGCAATACCGCGTG
AGGGAGGAAGGTCCTTGGATTGTAAACCTCTTTTATCAGGGAAGAAGTTCTGACG
GTACCTGATGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACTR
MGGAGGMTGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGTCCGTAGGTGGT
TATGCAAGTCTGCCGTTAAAGAATGGAGCTTAACTCCATAGGAGCGGTGGAAAC
TGCAAGACTAGAGTACAGTAGGGGTAGCAGGAATTCCCAGTGTAGCGGTGAAAT
GCGTAGATATTGGGAAGAACATCGGTGGCGAAAGCGTGCTACTGGGCTGAAACT

GACACTGAGGGACGAAAGCTAGGGTAGCGAAAGGGATTAGATACCCCTGTAGTC
CTAGCCGTAAACGATGGATACTAGGCGTGGCTTGTATCGACCCGAGCCGTGCCG
AAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTC
AAAGGAATTGACGGGGGCCGCACAGCGTGGAGTATGTGGTTAATTCGATGCACG
CGAAGAACCTTACCAAGGCTTGAMATCCCTGGAATYCTG

Part of the 16S rDNA gene of Microcystis Loch Vaal

AGTGGCGGACGGGTGAGTAACGCGTGGGAATCTGCCCTCAGGAGGGGGATAACG
GCCGGAAACGGCCGCTAATACCCCATATGCCGAGAGGTGAAATGAATTTCGCCT
GAGGATGAGCCCGCGTCTGATTAGCTAGTTGGTGTGGTAATGGCGCACCAAGGC
TTCGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGCAAGC
CTGACGGAGCAACGCCGCGTGAGGGACGAAGGCCTCTGGGCTGTAAACCTCTTT
TCTCAAGGAAGAAGACATGACGGTACTTGAGGAATAAGCCACGGCTAATTCCGT
GCCAGCAGCCGCGGTAATACGGGAGTGGCAAGCGTTATCCGGAATTATTGGGCG
TAAAGCGTCCGCAGGCGGCCCAGCAAGTCTGTTGTTAAATAGTGGAGCTTAACTC
CATCCAGGCAATGGAAACTGCTGGGCTAGAGTGTGGTAGGGGCAGAGGGAATTC
CCGGTGTAGCGGTGAAATGCGTAGATATCGGGAAGAACACCAGTGGCGAAGGCG
CTCTGCTGGGCCATAACTGACGCTCATGGACGAAAGCCAGGGGAGCGAAAGGGA
TTAGATACCCCTGTAGTCCTGGCCGTAAACGATGAACACTAGGTGTCGGGGGAA
TCGACCCCCTCGGTGTCGTAGCCAACGCGTTAAGTGTTCCGCCTGGGGAGTACGC
ACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGT
ATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAGGGTTTGACATCCTGCG
AATCCCTTGGAAACTTGGGAGTGCCTTCGGGAGCGCAGTGACAGGTGGTGCATG
GCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCACGTCTTTAGTTGCCAGCATTCAGTTGGGCACTCTAGAGAGACCGCCGGTGAT
AAACCGGAGGAAGGTGTGGATGACGTCAAGTCATCATGCCCCTTACATCCTGGG
CTACACACGTACTACAATGCTACGGACAAAGGGCAGCAAACTCGCGAGAGCTAG
CAAATCCCATAAACCGTGGCTCAGTTCAGATCGTAGGCTGCAACTCGCCTACGTG
AAGGAGGAATCGCTAGTAATCGCAGGTCAGCATACTGCGGTGAATACGTTCCCG
GGCCTTGTACACACCGCCCGTCACACCATGGAAGTTGGCCATGCCCGAAGTCGTT
ACTCCAACCCGTAAGGGAGGAGGACGCCGAAGGTGGGGCTGATGACTGGGGTG
AAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCTCCTAACAG

GGAGACAAAACTGATTGTGATGTCTGAGCGCTATGCGGATGAAATCTGAGTAGC
A

Part of the 16S rDNA gene of Microcystis (Boer)

TGAGTGTCAGATACAGCCCAGTAGCACGCTTTCGCCACCGATGTTCTTCCCAATC
TCTACGCATTTCACCGCTACACTGGGAATTCCTGCTACCCCTACTGCTCTCTAGTC
TGCCAGTTTCCACCGCCTTTAGGTCGTTAAGCAACCTGATTTGACGGCAGACTTG
GCTGACCACCTGCGGACGCTTTACGCCCAATAATTCCGGATAACGCTTGCCTCCC
CCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGAGGCTGATTCCTCAAGTACC
GTCAGAACTTCTTCCTTGAGAAAAGAGGTTTACAATCCAAAGACCTTCCTCCCTC
ACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAAAATTCCCCACTGCTGC
CTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGCTCATCCTCTCA
GACCAGCTACTGATCGTCGCCTTGGTAGGCTCTTACCCCACCAACTAGCTAATCA
GACGCAAGCTCTTCTTCAGGCCAATTAGGTTTCACCTTGCGGCACATCGGGTATT
AGCAGTCGTTTCCAACTGTTGTCCCCGTCCTGAAGTTAGATTCTTACGCGTTACTC
ACCCGTCCGCCACTAGAATCCGAAGATTCCCGTTCGACT

Part of the 16S rDNA gene of Spirulina (Gert)

TAGTGGCGGACGGGTGAGTAACGCGTGAGGATCTGCCCTTAGGATGGGGACAAC
CACTGGAAACGGTGGCTAATACCCAATGTGCCGAGAGGTGAAACATTTATGGCC
TGAGGATGAGCTCGCGTCTGATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGG
CGACGATCAGTAGCTGGTCTAAGAGGATGATCAGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGCAAG
CCTGACGGAGCAACGCCGCGTGAGGGAGGAAGGCCTTAGGGTTGTAAACCTCTT
TTCTCTGGGAAGAAGATCTGACGGTACCAGAGGAATAAGCCTCGGCTAACTCCG
TGCCAGCAGCCGCGGTAAGACGGAGGAGGCAAGCGTTATCCGGAATTATTGGGC
GTAAAGCGTCCGCAGGCGGCTTTTTAAGTCTGTTGTCAAAGGTCACAGCTCAACT
GTGGATCGGCAATGGAAACTGGAGAGCTTGAGTGTGGTAGGGGTAGAGGGAATT
CCCGGTGTAGCGGTGAAATGCGTAGATATCGGGAAGAACACCAGTGGCGAAGGC
GCTCTACTGGGCCACAACTGACGCTGAGGGACGAAAGCTAGGGGAGCGAAAGG
GATTAGATACCCCTGTAGTCCTAGCTGTAAACGATGGATACTAGGTGTTGCGCGT
ATCGACCCGTGCAGTACCGTAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTAC
GCACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA
GGATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTCG

CGAATCTTTCAGGATGAGAGAGTGCCTTCGGGAGCGCGAACACAGGTGGTGCAT
GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTCGTTTTTAGTTGCCATCATTCAGTTGGGCACTCTAGAGAGACTGCCGTGGA
CAACACGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTACGTCTTGG
GCTACACACGTCCTACAATGCTACGGACAGAGGGCAGCGAGCCAGCGATGGTAA
GCAAATCCCATAAACCGTAGCTCAGTTCAGATTGCAGGCTGCAACTCGCCTGCAT
GAAGGCGGAATCGCTAGTAATCGCAGGTCAGCATACTGCGGTGAATACGTTCCC
GGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGCCACGCCCGAAGTCG
TTACTCTAACCGTTCGCGGAGGAGGGCGCCGAAGGCAGG

Part of the 16S rDNA gene of Spirulina (BFN)

GTCGAACGGGCTCTTCGGAGCTAGTGGCGGACGGGTGAGTAACGCGTGAGAATC
TGGCTCCCGGTCGGGGACAACAGAGGGAAACTTCTGCTAATCCCGGATGAGCCG
AAAGGTAAAAGATTTATCGCCGGGAGATGAGCTCGCGTCTGATTAGCTAGTTGGT
GAGGTAAAGGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TTTTCCGCAATGGGCGCAAGCCTGACGGAGCAAGACCGCGTGGGGGAGGAAGGC
TCTTGGGTTGTAAACCCCTTTTCTCAAGGAAGAACACAATGACGGTACTTGAGGA
ATAAGCCTCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGAGGCAAGC
GTTATCCGGAATGATTGGGCGTAAAGCGTCCGTAGGTGGCAGTTCAAGTCTGCTG
TCAAAGACAGTAGCTCAACTACTGAAAGGCAGTGGAAACTGAACAGCTAGAGTA
CGGTAGGGGCAGAGGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATCGGGA
AGAACACCGGTGGCGAAAGCGCTCTGCTGGGCCGTAACTGACACTGAGGGACGA
AAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGAT
GGAAACTAGGTGTAGCCTGTATCGACCCGGGCTGTGCCGAAGCTAACGCGTTAA
GTTTCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCT
TACCAGGGCTTGACATGTCCGGAATCTTGGTGAAGCCGAGAGTGCCTTCGGGAG
CCGGAACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTCGTCCTTAGTTGCCATCATTCAGTTGGGCAC
TTTAGGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCA
TCATGCCCCTTACGTCCTGGGCTACACACGTACTACAATGGGGGGGACAAAGGG
TAGCCAAGACGCGAGTCTGAGCCAATCCCGTAAACCTCTCCTCAGTTCAGATTGC
AGGCTGCAACTCGCCTGCATGAAGGAGGAATCGCTAGTAATCGCAGGTCAGCAT

ACTGCGGTGAATCCGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGAAG
TTAGCCACGCCCGAAGTCGTTACTCTAACCGTTCGCGGAGGAGGATGCCGAAGG
CAGGGCTGATGACTGGGGTGAAGTCGTAACAAGGTA

Part of the 16S rDNA gene of Arthruspira

TAGTGGCGGACGGGTGAGTAACACGTGAGAATCTGGCTCCCGGTCGGGGACAAC
AGAGGGAAACTTCTGCTAATCCCGGATGAGCCGAAAGGTAAAAGATTTATCGCC
GGGAGATGAGCTCGCGTCTGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGG
CGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGCAAG
CCTGACGGAGCAAGACCGCGTGGGGGAGGAAGGCTCTTGGGTTGTAAACCCCTT
TTCTCAAGGAAGAACACAATGACGGTACTTGAGGAATAAGCCTCGGCTAACTCC
GTGCCAGCAGCCGCGGTAATACGGAGGAGGCAAGCGTTATCCGGAATGATTGGG
CGTAAAGCGTCCGTAGGTGGCAGTTCAAGTCTGCTGTCAAAGACAGTAGCTCAA
CTACTGAAAGGCAGTGGAAACTGAACAGCTAGAGTACGGTAGGGGCAGAGGGA
ATTCCCGGTGTAGCGGTGAAATGCGTAGATATCGGGAAGAACACCGGTGGCGAA
AGCGCTCTGCTGGGCCGTAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAAT
GGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGAAACTAGGTGTAGCC
TGTATCGACCCGGGCTGTGCCGAAGCTAACGCGTTAAGTTTCCCGCCTGGGGAGT
ACGCACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG
GAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAGGGCTTGACATGT
CCGGAATCTTGGTGAAGCCGAGAGTGCCTTCGGGAGCCGGAACACAGGTGGTGC
ATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGC
AACCCTCGTCCTTAGTTGCCATCATTCAGTTGGGCACTTTAGGGAGACTGCCGGT
GACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTACGTCCT
GGGCTACACACGTACTACAATGGGGGGGACAAAGGGTAGCCAAGACGCGAGTCT
GAGCCAATCCCGTAAACCTCTCCTCAGTTCAGATTGCAGGCTGCAACTCGCCTGC
ATGAAGGAGGAATCGCTAGTAATCGCAGGTCAGCATACTGCGGTGAATCCGTTC
CCGGGCCTTGTACACACCGCCCGTCACACCATGGAAGTTAGCCACGCCCGAAGT
CGT-ACTCTAACCG-TTCG-CGGAGGAG

Part of the 16S rDNA gene of Oscillatoria simplicissima (Planktothrix pseudagarhii)

AGTGGGGACGGGTGAGTAACACGTAAGAACCTGCCTCTTGGACGGGGACAACAG
TTGGAAACGGCTGCTAATCCCGGATAAGCCGAAAGGTAAAAGATTTATCGCCAA
GAGAGGGGCTTGCGTCTGATTAGCTAGTTGGTAGGGTAAGAGCCTACCAAGGCG
ACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCC
TGACGGAGCAAGACCGCGTGGGGGAGGAAGGTTCTTGGATTGTCAACCCCTTTT
CTCAGGGAAGAAGAAAGTGACGGTACCTGAGGAATCAGCATCGGCTAACTCCGT
GCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCG
TAAAGAGTCCGTAGGTGGCTGATCAAGTCTGCTGTTAAAGAGCGAGGCTTAACTT
CGTCAAAGCAGTGGAAACTGAAGAGCTAGAGTGTAGTAGGGGCAGAGGGAATT
CCTGGTGTAGCGGTGAAATGCGTAGAGATCAGGAAGAACACCGGTGGCGAAAGC
GCTCTGCTGGGCTACAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGG
ATTAGATACCCCAGTAGTCCTAGCGGTAAACGATGGAAACTAGGTGTGGCTGTA
TCGACCCGGGCCGTGCCGGAGCTAACGCGTTAAGTTTCCCGCCTGGGGAGTACG
CAACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA
GTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAGGACTTGACATTTCG
GGAATCCCTTTGAAAGGAGGGAGTGCCGAAAGGAGCCCGAAAACAGGTGCTGC
ATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGC
AACCCTCGTCGTTAGTTGCCATCATTAAGTTGGGAACTCTAGCGAGACTGCCGGT
GACAAACCGGAGGAAGGTGAGGATGACGTCAAGTCAGCATGGCCCTTACGTCCT
GGGCGACACACGTACTACAATGCTAAGGACAGAGAGCAGCCAACCCGCGAGGG
TGAGCGAACCTCTTAAACCTTGGCACAGTTCAGATTGCTCTCTGCAACTCGAGAG
CATGAAGGAGGAATCGCTAGTAATCGCAGGTCAGCATACTGCGGTGAATCCGTT
CCCGGGCCTTGTACACACCGCCCGTCACACCATGGAAGTGAGCCACGCCCGAA-
GTCATTACTCTAACCGAAAGGAGGAG

Part of the 16S rDNA gene of Planktothrix agardhii NIVA CYA 126/8

AGTGGCGGACGGGTGAGTAACACGTAAGAACCTGCCTCTAGGACGGGGACAACA
GTTGGAAACGACTGCTAAACCCGGATGAGCCGAAAGGTAAAAGATTAATCGCCT
AGAGAGGGGCTTGCGTCTGATTAGCTAGTTGGTGAGGTAAGAGCCCACCAAGGC
GACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGC
CTGACGGAGCAAGACCGCGTGGGGGAGGAAGGTTCTTGGATTGTCAACCCCTTT

TCTCAGGGAAGAACACAATGACGGTACCTGAGGAAAAAGCATCGGCTAACTCCG
TGCCAGCAGCCGCGGTAATACGGGGGATGCAAGCGTTATCCGGAATGATTGGGC
GTAAAGAGTCCGTAGGTAGTCATCCAAGTCTGCTGTTAAAGAGCGAGGCTTAAC
CTCGTAAAGGCAGTGGAAACTGGAAGACTAGAGTGTAGTAGGGGCAGAGGGAA
TTCCTGGTGTAGCGGTGAAATGCGTAGAGATCAGGAAGAACACCGGTGGCGAAG
GCGCTCTGCTGGGCTATAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATG
GGATTAGATACCCCAGTAGTCCTAGCGGTAAACGATGGAAACTAGGTGTGGCCT
GTATCGACCCGGGCCGTGCCGAAGCAAACGCGTTAAGTTTCCCGCCTGGGGAGT
ACGCACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG
GAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAGGACTTGACATCT
CTGGAATCTCCTTGAAAGGGGAGAGTGCCGTAAGGAACCAGAAGACAGGTGCTG
CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTCGTCGTTAGTTGCCATCATTAAGTTGGGAACTCTAGCGAGACTGCCGG
TGACAAACCGGAGGAAGGTGAGGATGACGTCAAGTCAGCATGGCCCTTACGTCC
TGGGCGACACACGTACTACAATGCGAGGGACACAGAGCAGCCAACCCGCGAGG
GAGAGCGAATCTCACAAACCCTGGCACAGTTCAGATTGCAGGCTGCAACTCGCC
TGCATGAAGGAGGAATCGCTAGTAATCGCAGGTCAGCATACTGCGGTGAATCCG
TTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGAAGTGAGCCACGCCCGA
AGTCATTACTCTAACCCTTTCGGGGGAGGAGGGTGCCGAAGGCGGGGTTGATGA
CTGGGGTGAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGTGGCTGGATCACCT
CCTTTTAGGGAGACCTACTTTTGA

Part of the 16S rDNA gene of Planktothrix (NIVA CYA 153/1)

AGTGGCGGACGGGTGAGTAACACGTAAGAACCTGCCTCTTGGACGGGGACAACA
GTTGGAAACGGCTGCTAATCCCGGATAAGCCGAAAGGTAAAAGATTTATCGCCA
AGAGAGGGGCTTGCGTCTGATTAGCTAGTTGGTAGGGTAAGAGCCTACCAAGGC
GACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGC
CTGACGGAGCAAGACCGCGTGGGGGAGGAAGGTTCTTGGATTGTCAACCCCTTT
TCTCAGGGAAGAAGAAAGTGACGGTACCTGAGGAATCAGCATCGGCTAACTCCG
TGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGC
GTAAAGAGTCCGTAGGTGGCTGATCAAGTCTGCTGTTAAAGAGCGAGGCTTAAC
TTCGTCAAAGCAGTGGAAACTGAAGAGCTAGAGTGTAGTAGGGGCAGAGGGAAT
TCCTGGTGTAGCGGTGAAATGCGTAGAGATCAGGAAGAACACCGGTGGCGAAAG

CGCTCTGCTGGGCTACAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGG
GATTAGATACCCCAGTAGTCCTAGCGGTAAACGATGGAAACTAGGTGTGGCCTG
TATCGACCCGGGCCGTGCCGGAGCTAACGCGTTAAGTTTCCCGCCTGGGGAGTAC
GCACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA
GTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAGGACTTGACATTTCG
GGAATCCCTTTGAAAGGAGGGAGTGCCGAAAGGAGCCCGAAAACAGGTGCTGC
ATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGC
AACCCTCGTCGTTAGTTGCCATCATTAAGTTGGGAACTCTAGCGAGACTGCCGGT
GACAAACCGGAGGAAGGTGAGGATGACGTCAAGTCAGCATGGCCCTTACGTCCT
GGGCGACACACGTACTACAATGCTAAGGACAGAGAGCAGCCAACCCGCGAGGG
TGAGCGAACCTCTTAAACCTTGGCACAGTTCAGATTGCTCTCTGCAACTCGAGAG
CATGAAGGAGGAATCGCTAGTAATCGCAGGTCAGCATACTGCGGTGAATCCGTT
CCCGGGCCTTGTACACACCGCCCGTCACACCATGGAAGTGAGCCACGCCCGAAG
TCATTACTCTAACCGAAAGGAGGAGGGTGCCGAAGGCAGGGCTGATGACTGGGG
TGAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGTGGCTGGATCACCTCCTTTTC
AGGGAGACCTACTTTTGGAGAAGTGCCAATCCCAACAAAAAATAGCACTCCCAG
AAGTCATCCCAGGTC

NtcA gene sequencing

Part of the NtcA gene of M. aeruginosa CCAP 1450/1

GGTTTATTTTTTGCTGAAAGGAGCCGTTAAGTTATCGCGAGTYTATGAAGCGGGA
GAGGAAATTACCGTGGCTCTACTGCGGGAAAATAGCGTCTTTGGTGTGCTATCCT
TACTGACTGGTCAGCGATCGGATCGTTTTTATCATGCCGTGGCTTTTACTCCGGTA
GAATTACTCTCGGCCCCGATCGATCAGGTAGAAAGATCCCTTCGCAATAATCCCG
ATTTATCAATGTTGATGCTGCAGGGTTTGTCCTCGCGGATTCTGCAAACGGAAAT
GATGATCGAAACCTTGGCTCACCGGGATATGGGTTCACGTTTGGTCAGTTTTTTA
TTAATTCTCTGTCGTGATTTKGGGGTTCCCACCACCG

Part of the NtcA gene of M. aeruginosa PCC 7806

AGTTATCTCGGGTTTACGARRCCGGRGAAGAGATTACCGTCGCTCTGTTGAGGGA
GAACAGTGTTTTTGGGGTGTTATCATTAGTTACTGGGCAACGTTCTGACCGTTTTT
ACCACGCAGTGGCTTTYACCCCGGTGGAGTTGCTGTCTGCCCCCATTGAACAGGT
AGAACAGGCTCTSAAGGAGCATCCCGACCTGTCATTGTTAATGTTGCAGGGCCTT
TCTTCCCGAATTCTCCAGACGGAAATGATGATTGAAACCCTAGCCCACCGGGATA

TGGGTTCTCGTTTAGTTAGTTTTTTGCTGATTCTCTGTCGAGATTTTGGSGTACCTG
CCCCGGACGGCATTCGC

Part of the NtcA gene of Microcystis(vd Bijl)

TATCTAGGGTTTACGAAGCCGGGGAAGAAATTACCGTCGCTCTGTTGAGGGAGA
ACAGTGTTTTTGGGGTGTTATCATTAGTTACCGGGCAACGTTCTGACCGTTTTTAC
CACGCAGTGGCCTTTACCCCGGTGGAGTTACTGTCTGCCCCCATTGAACAGGTGG
AACAGGCTCTCAAGGAGCATCCCGACCTGTCATTGTTRATGTTACAGGGCCTTTC
TTCCCGMATTCTCCAGACGGAAATGATGATTGAAACCCTAGCCCACCGGGATAT
GGGTTCTCGTTTRGTTAGTTTTTTGCTGATTCTCTGTCGAGATTT

RbcL gene sequencing

Part of the RbcL gene of Microcystis aeruginosa PCC 7806

CTGCGTTGGCGCGATCGTTTCCTCTTTGTCCAAGAGGCGATCGTTAAWTCCCAAG
CARAAMCCAACGAAGTTAARGGACATTACTTAAACGTAMCCGCTCCTACCTGCR
AGCARATGATGCAACGGGCTGAATTCGCCGCCRAAATCAAAACCCCCATCATCA
TGCACGACTACCTCACCGGKGGTTTCACCGCTAACACCACCCKGGCRAAATTCTG
CCGCGACAAAGGGTTACKGCTCCACATTCACCGGGCAAKGCACGCGGTTATCGA
CCGTCARAAAAATCAKGGTATCCACTTCCGCGTTTTASCTAAGKGCTTACGTCTC
TCTGGTGGTGATCACCTGCACTCGGRAACCGTTGTCGGTAAACTCRAAGGTGAAC
GCGRAATCACRATGGGTTTTGTTGACCTGATGCGKGAARACTATGTARAARAARA
TCGCGYTCGKGGWATTTTCTTCACCCAARACTACSCTTCCTTACCCGGGGTAATG
CCGGTTGCTTCCGG