TOXIN PRODUCTION BY CYANOBACTERIA

Report to the
Water Research Commission

on the project :

Scope and Dynamics of Toxins Produced by Cyanophytes in the Freshwaters of South Africa and the Implications for Human and Other Users

by

Johan U. Grobbelaar[1], Elsabe' Botes[1], J.A. van den Heever[1], Anna-M. Oberholster[2] and Paul J. Oberholster[2]

[1]Department of Plant Sciences, University of the Free State, Bloemfontein 9300
[2]Department of Genetics, University of Pretoria, Pretoria 0001

WRC Report No: 1029/1/04
ISBN No: 1-77005-191-0 **August 2004**

DISCLAIMER

This report emanated following research undertaken from a project funded by the South African Water Research Commission and is approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the WRC or the members of the steering committee, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Acknowledgements

Water Research Commission Steering Committee:

Dr MC Steynberg	Rand Water
Alt Dr H du Preez	
Dr P Kempster	Department of Water Affairs and Forestry
Ms C van Ginkel	Department of Water Affairs and Forestry
Mr M Graham	Umgeni Water
Dr GS Shephard	PROMEC
Dr F Carlsson	Environmentek, CSIR.
Dr WR Harding	Southern Waters
Mr R Moollan	Cape Town Metropolitan
Mr T Downing	UPE
Mrs JL Slabbert	Environmentek, CSIR.
Dr SA Mitchell	WRC

Project Leaders:

Prof JU Grobbelaar	University of the Free State
Prof A-M Oberholster	University of Pretoria

Research Staff and Students

Mr JA van den Heever	Research Assistant
Miss E Botes	MSc Student
Mr PJ Oberholster	MSc Student
Mr M Nthejane	Hons Student

A special word of thanks to Mrs APM Moolman from the WRC who also acted as chairperson for this project and the committee secretary Mrs CM Smit.

CONTENTS

1. INTRODUCTION

"It is completely unclear why certain strains of Microcystis aeruginosa *are able to produce toxins, whereas others are not".*

Meissner (1996)[*]

The worldwide occurrence of toxic cyanobacterial blooms in fresh and brackish eutrophic waters creates a problem for all life forms. Most water-based poisonings by cyanobacteria occur when heavy surface growths or scums accumulate near shorelines of lakes, ponds and reservoirs where animals have free access to high concentrations of these toxic cells. Deaths attributed to cyanobacterial toxins have been reported for man, animals, birds and fish.

Cyanobacterial blooms are ubiquitous, often associated with eutrophication and appear to be on the increase, also in South Africa. N, P and C are important nutrients for high growth rates and the ratios in the supply concentrations are often decisive in selecting for cyanobacterial dominance. These organisms are capable of scavenging their environments for resources and excessive or "luxury" uptake of nutrients allow them to survive extreme nutrient deficient conditions. Cyanobacteria flourish at high temperatures, neutral to alkaline conditions, high nutrient concentrations especially where the ratios of N:P are low and an adequate supply of iron is present. The buoyancy of certain species, due to especially the production of gas-vacuoles gives them a competitive advantage over other phytoplankton. All blooms of *Microcystis*, one of the most ubiquitous species, should be considered to be toxic and toxins are easily leached from the cells. They persist for long periods and are not easily degraded.

Microcystis aeruginosa is distributed worldwide that often form seasonal blooms, as is common in South Africa. This organism produces a vast number of peptides (microcystins), some of which are highly toxic. Microcystins consist of a seven-

[*] MEISSNER KE, DITTMANN E & BÖRNER T (1996) Toxic and non-toxic strains of the cyanobacterium *Microcystis aeruginosa* contain sequences homologous to peptide synthetase genes. FEMS Microbiology Letters **135**: 295-303.

membered peptide ring made up of five non-protein amino acids and two protein amino acids. More than 50 microcystins have been identified to date, most representing minor components of the total toxin complement of the cyanobacteria from which they were isolated. The most commonly occurring toxin is microcystin-LR, a cyclic heptapeptide hepatotoxin, where symptoms of exposure too, includes skin irritation, possible liver cancer as a result of cronic exposure, and even death. These toxins are inhibitors of serine/threonine protein phosphatase enzymes and are among the most potent tumour-promoting compounds. The mechanism of toxicity is exerted by the general inhibition of dephosphorylation of protein phosphatases 1 and 2A, leading to hyperphosphorylation in the cytosol.

The genus *Microcystis* contains non-toxic and toxic strains and the toxicity is affected by various environmental factors such as water temperature, pH, intensity of solar radiation, dissolved oxygen and CO_2 availability. It has been reported that microcystin concentrations are usually higher under conditions, when the organisms are stressed by any of the above factors.

The molecular basis of toxin-production in *M. aeruginosa* was partially elucidated elsewhere and it was found that both toxin-producing and non toxin-producing strains of *M. aeruginosa* contained sequences that revealed a high degree of homology with several well-characterised peptide synthetases. In blotting experiments, a PCR fragment based on a portion of one of these peptide synthetases hybridised exclusively to restricted genomic DNA from toxin-producing strains indicating that this peptide synthetase was involved in toxin production.

2. AIMS OF THE PROJECT

The aims of the project were to:

- To acquire and maintain viable toxic and non-toxic cyanobacteria, specifically *Microcystis aeruginosa* strains.
- Determination of optimal growth conditions as well as those affecting maximal toxin production.
- Mass culturing of toxic cyanobacteria for use in tests as required.

- Elucidation of the genetic control of microcystin synthesis.
- Determination of possible correlations between the occurrence of *Microcystis* blooms and environmental conditions in natural systems.
- To attempt to develop a molecular screening tool for naturally occurring blooms of *M. aeruginosa* based on the presence or absence of the gene *mcyB*, which has previously been implicated in toxin production in *M. aeruginosa.*

3. METHODOLOGY, RESULTS AND DISCUSSION

3.1 Test material

Unrelated strains of *M. aeruginosa* were obtained from the Pasteur Institute, France; the National Institute for Environmental Studies, Japan; the Institute of Freshwater Ecology, UK; Universities of Pretoria and the Free State culture collections (Table 1). Based on conserved regions present in known sequences of the *mcyB* gene*,* four primer pairs were designed. The strains were maintained under standard conditions and the total genomic DNA was extracted from toxin-producing strains PCC 7813, UV 027 and non toxin-producing strain CCAP 1450/1.

3.2 DNA and HPLC analyses

PCR reactions were performed and the fragments generated with the various primer pairs were compared with expected fragment sizes. PCR products of the expected size were amplified in both toxin-producing strains with all four-primer pairs, signifying that these toxin-producing strains possessed a copy of the *mcyB* gene. It was also possible to generate PCR fragments with three primer pairs from the non toxin-producing strain CCAP 1450/1. These results indicated that this strain contained at least partial elements of *mcyB.*

Fragments amplified by PCR from toxin-producing strains were cloned into pGemT®-Easy (Promega) and sequenced. Basepair and translated amino acid alignment of

the assembled fragments showed a high degree of homology with previously deposited sequences of *mcyB* in the Genbank database.

A fragment amplified by PCR from strain PCC 7813 with primer pair Tox 7P/3M was randomly labelled and used as a probe to screen other strains of *M. aeruginosa* for the presence of *mcyB.* This probe hybridised to a fragment of the expected size in all toxin-producing strains as well as the non toxin-producing strain confirming PCR results that all strains contained this particular portion of *mcyB*.

Table 1 Different *Microcystis* strains used, their culture collection codes, collection organisation, nutrient medium, and known toxicity.

Culture ID	**Culture collection**	**Nutrient Medium**	**Toxicity**
PCC7806	Pasteur, France	BG-11	?
PCC7813	Pasteur, France	BG-11	?
PCC7820	Pasteur, France	BG-11	?
SAG14	Göttingen, Germany	BG-11	?
UV027	UFS, South Africa	BG-11	Toxic
CCAP1450/1	CCAP, UK	BG-11	Non-toxic
N88	NIES, Japan	BG-11	?
N89	NIES, Japan	MA	?
N90	NIES, Japan	MA	?
N91	NIES, Japan	MA	?
N99	NIES, Japan	MA	?
N101	NIES, Japan	MA	?
N299	NIES, Japan	MA	?
UP01	UP, South Africa	BG-11	?

A second probe generated from strain PCC 7813 with primer pair Tox 1P/1M representing the fragment of *mcyB* not amplified by PCR in strain CCAP 1450/1 was

synthesised. This probe hybridised to a fragment of the expected size in all toxin-producing strains and the non toxin-producing strain. Hybridisation of this probe to *Pvu*II digested DNA from CCAP 1450/1 indicated that there was enough target DNA in the CCAP 1450/1 genome for the Tox 1P/1M/PCC 7813 probe to hybridise to, hinting at the possibility that this strain also possessed a complete copy of the gene.

Amplified fragment length polymorphism (AFLP) was used to determine the genetic relationships of the geographically unrelated strains. A total of 909 bands were amplified from the eight primer combinations, of which 665 was informative, 207 non-informative and 37 monomorphic, with an average of 83.12 polymorphic bands per primer combination. Definite groupings were obtained, that confirmed the value of AFLP analyses for the identification of genetic diversity and population structures of *M. aeruginosa*.

Based on conserved motifs present in known sequences of *mcyB* four primer pairs were designed and used to identify strains with toxicity or not. Analysing the strains and using the insertions/deletions (indels) to discriminate between *M. aeruginosa* and *M. wesenbergii* in raw water samples it confirmed the value of PCR assays as an indicator of toxicity and taxonomical characteristics.

Crude aqueous cell extracts made from all strains, were investigated and analysed by HPLC for the presence of microcystin-LR. Microcystin-LR was detected in all toxin-producing strains as well as the 'non toxin-producing' strain CCAP 1450/1.

3.3 Photosynthetic characteristics

The thirteen different *Microcystis* strains varied more than 3-fold in their photosynthetic characteristics. Large variations were also seen in the microcystin YR and LR contents. The relatively low I_k values (onset of light saturated photosynthesis) and high α^B rates (photosynthetic efficiencies) indicated highly efficient usage of low light intensities, making them suitable organisms for turbid environments. UV027, a known highly toxic *Microcystis* strain did not contain the highest total microcystin content. The highest toxin contents (YR + LR) were found in

the strains SAG14, N 88, 89, 91, 99 and 101. Neither the photosynthetic potential, nor a measure such as the Chl *a* content appeared to give an indication of the potential toxicity of a particular strain.

Microcystis is essentially a photoautotrophic organism, but may also be mixotrophic. This implies that production of the secondary metabolites, such as microcystin has to be derived from primary photochemistry. Using chlorophyll fluorescence it was found that the different strains showed marked variations in both the primary photochemical characteristics, such as photon absorption per reaction centre and the electron transport per reaction centre, as well as oxygen liberation at different light intensities. The results also demonstrated acclimation to differing conditions, indicating the adaptability of *Microcystis*. There was some indication that photosynthetic performance may imply higher toxicity and vice versa.

Many factors and combinations of factors influence bloom formation of cyanobacteria and the only conclusions that can be made from this study are that there is a high probability that cyanobacteria may form blooms, when eutrophic conditions are present, water temperature is high and water pH's are alkaline. Water temperature appeared to be the most important factor influencing bloom development in a eutrophic pond and little growth was seen at temperatures below 18 ^{o}C. Once blooms develop toxin measurements are the only means of determining the presence or not of these secondary metabolites and PCR assays should be used.

4. RESEARCH NEEDS

Essentially all the research objectives were resolved and the results clearly indicated the extreme complexity of toxin production by cyanobacteria. The following research needs were identified.

- It was not possible to conclude with any measure of certainty that mcyB was involved in toxin production in M. aeruginosa strains investigated in this study, as no proper negative control was available. This should be established.
- More non toxin-producing strains should be obtained and very importantly, these strains should be monitored for toxin production on a regular basis, before and during molecular investigations. PCR fragments obtained with primer pairs Tox 3P/2M, Tox 7P/3M and Tox 10P/4M from CCAP 1450/1 should be cloned and sequenced and new primers designed to elucidate the exact nature of the anomaly present in strain CCAP 1450/1 in the region represented by primer pair Tox 1P/1M.
- The best molecular screening tool is to investigate the possible presence of a molecular activation/inactivation mechanism involved in the transcription of the microcystin synthetase gene cluster. Transcriptional levels of the microcystin synthetase gene cluster have recently been linked to light quality. This phenomenon should be further investigated to verify conclusively that an increase in transcriptional levels of the gene cluster correlates to higher levels of toxin production. If this can be verified, a molecular screening tool based on the presence, expression and functionality of the gene/genes involved in regulation, in conjunction with the same parameters for all the genes in the gene cluster could be developed.
- The environmental factors influencing toxin production, either directly or indirectly via regulating mechanisms, should further be identified and studied extensively so that this knowledge can be applied to preventing, controlling or at least understanding naturally occurring *M. aeruginosa* blooms.
- The variability and dynamics of microcystin production within strains of *Microcystis* needs to be further investigated to determine the scope and thresholds of toxin production.

- PCR assays should be applied to natural occurring blooms and a long-term data base should be established for future reference purposes.
- The role of phototrophy and mixotrophy in affecting growth and toxin production should be clarified.
- The variation and acclimation in photosynthetic responses and performance needs further investigation. The coupling between stress, secondary metabolite production and toxicity requires elucidation.
- The fact that microcystin can easily leach from *Microcystis* cells poses further important questions, e.g.; how much of the variation seen between strains is due to release of toxins from healthy growing cells, since excreted organics are common among phytoplankton, what is the role of the excreted toxins in the aquatic environment, and has toxicity been measured extra-cellular from so-called non-toxic strains?

Excluded in the above are health related aspects of the toxins and consequences for water treatment and supply.

Important is that this research continues and a specialised working group under the direction of the WRC should regularly meet to discuss developments and disseminates information where and if required. Because it is not possible to predict toxicity it is important to establish a microcystin alarm and analyses facility, where potential toxic samples could be assayed and water users warned timeously if necessary. The results clearly point to PCR assays as the desired method and this responsibility should be with DWAF.

5. PROJECT OUTPUTS

5.1 Publications in refereed journals

Grobbelaar JU and JA van den Heever (2002) Variations in toxicity and growth characteristics of various *Microcystis* strains. Verh. Internat. Verein . Limnol. 28: 717 - 720.

Oberholster PJ, Botha A-M and JU Grobbelaar (2004) *Microcystis aeruginosa*: source of toxic microcystins in drinking water. African J. Biotech. 3: 159-168.

5.2 Conference contributions

Grobbelaar JU (2001) Variations in the toxicity of of several *Microcystis* strains. South African Association of Botanists, 27th Annual Congress, Rand Afrikaans University, January.

Grobbelaar JU and JA van der Heever (2001). Variations in toxicity and growth characteristics of a number of *Microcystis* strains, 28th SIL Congress, Melbourne, Australia, February.

Oberholster PJ, Botha A-M and JU Grobbelaar (2003) *Microcystis aeruginosa* strain identification using molecular techniques. 5th Asia-Pacific Conference on Algal Biotechnology, Qindao, China, October.

6. CAPACITY BUILDING

MSc Degrees:

Botes E (2002) Molecular characterisation of toxin-producing and non toxin-producing strains of *Microcystis aeruginosa*. M.Sc., UFS, 87 pp.

Oberholster PJ (2004) Assessing genetic diversity and identification of *Microcystis aeruginosa* strains through AFLP and PCR-RFLP analyses. M.Sc., UFS, 114 pp.

Hons Degree:

Nthejane M (2003) Influence of some environmental factors on *Microcystis* growth and microcystin-LR production in a eutrophic pond. Department of Plant Sciences, University of the Free State, 20 pp.