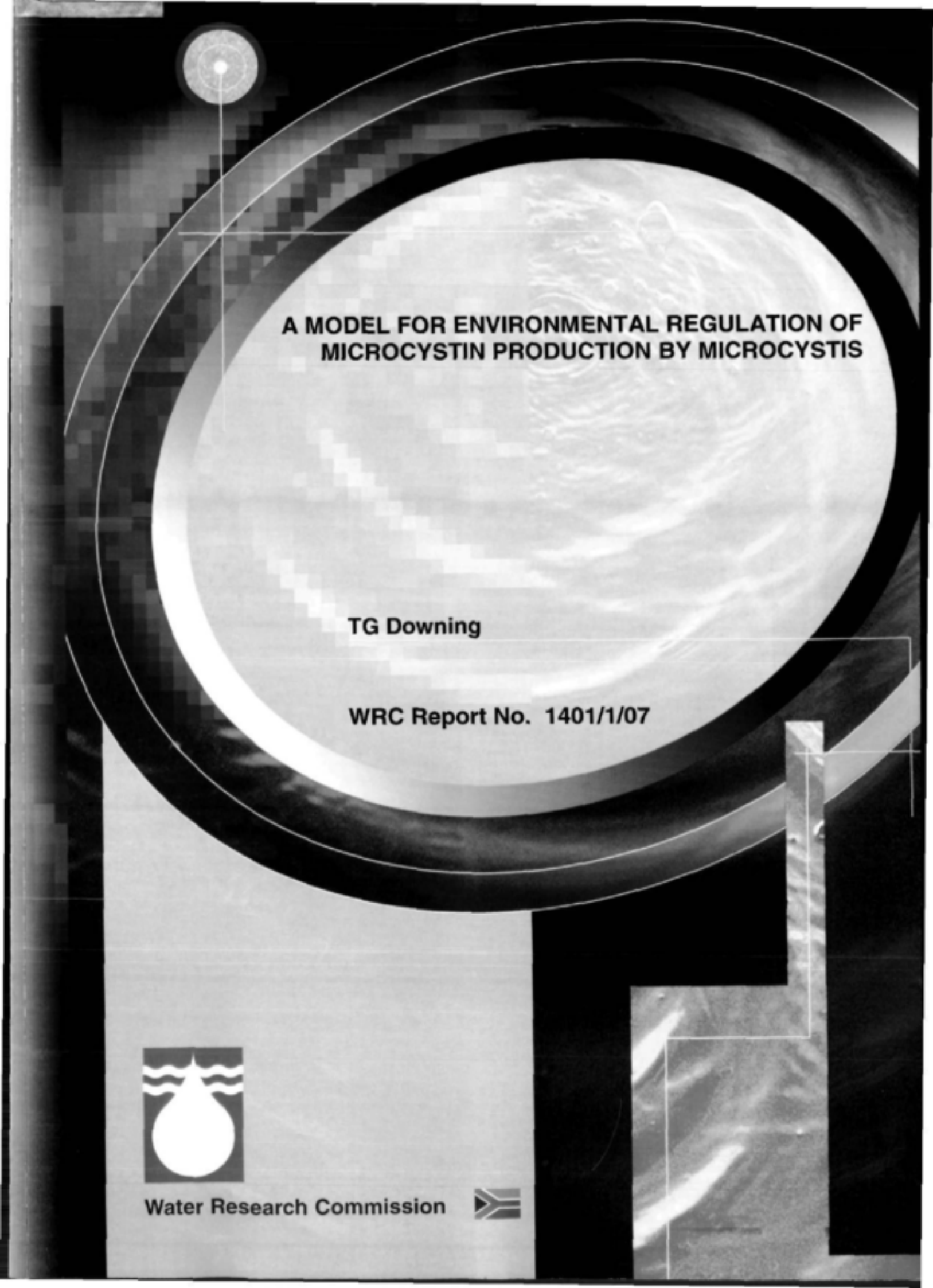

A MODEL FOR ENVIRONMENTAL REGULATION OF MICROCYSTIN PRODUCTION BY MICROCYSTIS

TG Downing

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Water Research Commission

A Model for Environmental Regulation of Microcystin Production by *Microcystis*

by

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Report to the Water Research Commission on the Project "Model for the prediction of toxic bloom events based on the cellular mechanisms of modulation of toxin production by nutritional environmental parameters in the cyanobacterial genus Microcyctis"

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EXECUTIVE SUMMARY

Background

Due to the ability of several genera of Cyanobacteria to produce a range of hepatotoxins and neurotoxins, these organisms have become particularly important and have received increased attention in recent years. The increased rate of eutrophication of surface fresh waters due to increased population densities, modern agricultural practices and industrial effluent has resulted in more frequent and severe cyanobacterial bloom events. Since many genera of freshwater cyanobacteria are capable of production of hepatotoxins, this increase in the frequency and severity of bloom events poses a problem for potable water supply in that classical treatment methods result in cell lysis and release of these toxins. An understanding of the environmental conditions that modulate toxin production would therefore be beneficial to the management of potable water supplies. Definition of the primary parameters and a model of the mechanism of modulation of toxin production would further facilitate management and treatment.

Hepatotoxin production in cyanobacteria has been shown to correlate with external stimuli such as light and nutrient concentrations and ratios although conflicting results have been reported. Modulation of microcystin production in *Microcystis aeruginosa* has been extensively studied in both batch and continuous culture. Positive correlations with medium nitrogen, medium phosphorous, light intensity, inorganic carbon availability and growth rate have been reported. Negative correlations have been reported between microcystin content and medium phosphorous. The only reported quantitative relationship between any variable and microcystin production was that of growth rate.

Objectives

The purpose of this work was therefore to investigate the environmental factors involved in the modulation of microcystin production in *M. aeruginosa*, with specific emphasis on the role of environmental nitrogen and the co-modulatory effects of environmental phosphorous. The primary objective of this work was to determine the modulatory role of environmental orthophosphate and nitrate levels on microcystin production by the dominant microcystin producing genus in South African freshwater impoundments and to develop a model to describe the cellular mechanisms by which these environmental parameters modulate microcystin content. The co-modulatory effect of environmental

phosphorous was investigated because of the role of cellular phosphorous in photosynthetic carbon fixation, and the resulting effects on cellular C:N ratios and on nitrogen assimilation. The modulatory effects of carbon fixation:nitrogen uptake and cellular C:N ratios were also investigated in the absence of variation of growth rate so as to relate environmental N:P ratios to cellular activities. In order to further clarify any modulatory effects of these environmental variables, potential regulatory mechanisms were investigated. Specifically, the potential role of NtcA (a cellular nitrogen regulator) and the levels of nitrogen assimilation products and carbon precursors were studied so as to determine the cellular status of these constituents under environmental conditions leading to increased microcystin production, thereby attempting to elucidate the potential mechanisms by which the relevant environmental factors enhanced microcystin production.

Results and Discussion

Specific growth rates and protein and microcystin content of *M. aeruginosa* PCC7806 and *M. aeruginosa* UV027 were determined under non-limiting batch culture conditions for a range of medium nitrogen and phosphorous atomic ratios (N:P). Both strains exhibited a similar optimal medium N:P ratio for increased cellular microcystin levels. Additionally, total cellular protein content and intracellular microcystin content were significantly correlated. Microcystin and protein content increased considerably as the maximum specific growth rate for the experimental conditions was reached. The correlation between cellular protein and microcystin content and their relative increase with increasing specific growth rate occurred within defined ranges of medium N:P ratios. This suggests a close association between microcystin production and N:P ratio dependant assimilation of nitrogen, and resulting total cellular protein levels, which may be further modulated by specific growth rate.

Microcystis aeruginosa PCC7806 was grown in continuous culture with varying medium nitrate concentrations and sampled at steady states for analysis of cell numbers, microcystin content, cellular N and P, residual medium nutrient concentrations and carbon fixation rates. Cellular microcystin quotas showed significant positive correlation with both nitrate uptake and cellular nitrogen content, and were negatively correlated with carbon fixation rates, phosphate uptake, and cellular phosphorous. Thus the ratios of nitrate uptake to phosphate uptake, cellular N to cellular P, and nitrate uptake to

carbon fixation were positively correlated to cellular microcystin. Microcystin quotas increased 10 fold between the lowest and highest steady state values. Contrary to what was previously reported, cellular microcystin content is therefore controlled to a significant extent by variables other than growth rate, with nitrogen being the most significant modulator. Batch culture in BG11 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 for a specific growth rate.

No definitive mechanism for the regulation of microcystin production by *Microcystis aeruginosa* is known. Work on modulation of microcystin content revealed a primary dependence on nitrogen and cellular nitrogen-uptake:carbon-fixation ratios which yield variations in the cellular carbon:nitrogen ratio that are in turn altered by growth and the stoichiometric requirements of growth for these cellular pools. NtcA is the cellular regulator of nitrogen metabolism in Cyanobacteria, regulating such metabolic activities as nitrate uptake, nitrate reduction, ammonium assimilation and carbon fixation rate. The potential for NtcA regulation of *mcy* expression was therefore investigated. Specifically, the regulatory region between *mcyABC* and *mcyDEFGH-J* was analysed for putative NtcA binding sites, and the relative *mcyA* and *ntcA* mRNA levels were determined under conditions known to alter cellular nitrate-uptake:carbon-fixation rates. A putative NtcA binding site was identified, overlapping the -10 region of the *mcyA* promoter and corresponding to motifs for known NtcA repressed genes. Incubation under high light yielded no detectable *mcyA* mRNA and an abundance of *ntcA* mRNA. In the absence of light and hence an increase in nitrate-uptake:carbon-fixation rate, *mcyA* and *ntcA* transcript levels were similar after 24 hours (with *ntcA* being reduced relative to high light incubation for 24 hours). After 48 hours in the absence of light, *mcyA* levels were further increased and *ntcA* transcript levels were further decreased, suggesting an inverse regulation of *ntcA* and *mcyA* and therefore a possible repression of *mcyA* by NtcA. The role of NtcA as a response regulator of cellular nitrogen status prompted investigation of the relationship between cellular nitrogen status and microcystin content as determined by cellular glutamine concentrations.

Microcystin content in *Microcystis aeruginosa* is co-modulated by cellular nitrogen:carbon ratio and growth rate. Cellular N:C ratio is a function of medium N:P

ratio and growth rate. Growth rate is in turn determined by medium N:P ratios and concentrations and photosynthetically active radiation. Cellular nitrogen status is therefore representative of all of these factors. Cellular glutamine is an indicator of cellular nitrogen status, and cellular glutamate, as the first product of the GS-GOGAT cycle, is an indication of nitrogen assimilation. In order to ascertain whether cellular nitrogen status was the primary modulator of microcystin production in *M. aeruginosa*, cultures were either starved of nitrogen or phosphorous, or treated with selective inhibitors of glutamate synthase and glutamine synthetase. The cellular glutamate, glutamine and microcystin concentrations were measured. Significant negative correlations between cellular concentrations and quotas of microcystin and glutamate were obtained for combined data. In control cultures cellular microcystin concentrations were however significantly negatively correlated with glutamine. This variation was partly attributed to growth rate variations in the different treatments. Inhibition of glutamate synthase resulted in positive correlations between glutamate and glutamine and microcystin, while inhibition of glutamine synthetase yielded a significant positive correlation between microcystin and glutamate. Variation in correlations obtained for different treatments suggests an additional regulatory mechanism besides the dependence on cellular nitrogen status.

Negative correlations between cellular microcystin and cellular glutamate under conditions with adequate photosynthetically active radiation on the one hand, and positive correlations between cellular microcystin and cellular glutamate under similar condition but with inhibition of nitrogen assimilation on the other, indicate a possible role for α-ketoglutarate in the regulation of microcystin production. This is supported by the putative NtcA binding site in a region of the *mcyA* promoter, indicative of repression, and the fact that α-ketoglutarate enhances NtcA binding. Cellular α-ketoglutarate and microcystin concentrations were therefore determined for cultures incubated (i) in the absence of light or nitrogen, (ii) with double the standard medium nitrogen, and (iii) in the presence of inhibitors of carbon fixation and glutamine synthesis, so as to induce variations in cellular α-ketoglutarate. Significant positive correlations between cellular microcystin and α-ketoglutarate concentrations were observed both in cultures maintained in the dark and in nitrogen starved cultures. A significant negative correlation occurred when carbon fixation was inhibited. These contradictory data and the absence of notably increased microcystin when α-ketoglutarate was reduced ten-fold because of

inhibition of carbon fixation suggest that α-ketoglutarate does not influence cellular microcystin concentrations. However, regulation of transcription and actual production of microcystin as a function of newly synthesized microcystin synthetase are necessarily temporally distinct, and depend on an adequate pool of nitrogen assimilation products for synthesis of the multi-enzyme complex. Such a pool requires an adequate supply of α-ketoglutarate as a precursor, complicating analysis and requiring high temporal resolution for clarity. Microcystin concentrations were increased under reduced carbon fixation as a result of limitation in inorganic carbon at steady state. A positive correlation between α-ketoglutarate and microcystin under carbon fixation inhibition during batch culture was, however, observed. This suggests the diversion of available carbon stores into carbon skeletons for nitrogen assimilation and subsequent microcystin production, indicating a possible role for microcystin in enhancement of carbon fixation. This is further supported by the cellular location of microcystin and a positive relationship between photosynthetic efficiency of photosystem II and cellular microcystin quota.

Microcystin production occurs where nitrogen assimilation exceeds the use of assimilation products for growth, where growth rate is determined primarily by carbon fixation rate (in turn dependant on phosphorous availability and photosynthetically active radiation), and nitrogen assimilation rate. The point at which microcystin production is relatively higher than dilution by growth is determined by the stoichiometry for growth and closely resembles the classical Redfield ratio. Reduction in photosynthetically active radiation, or inorganic carbon or phosphorous in the medium, results in an increase in cellular N:C ratio and production of microcystin. Modelling of microcystin production or cellular microcystin quotas in natural conditions is complicated by variations in PAR but is possible using biologically available environmental nitrogen and phosphorous, and monitoring specific growth rate. Based on these data, a general conceptual model is presented which adequately explains all apparent contradictions in published literature and is supported by evidence of uncoupling of growth related metabolic processes from cell division, a positive relationship between photosynthetic efficiency and microcystin, a simple equation relating cellular carbon:nitrogen fixation and uptake rates, and an artificial neural network model with medium nitrogen, medium phosphorous and growth rate as input nodes, that corresponds with measured data (r^2 = 0.8121). Additional models based on medium N:P and N and P uptake rates and the differences in stoichiometric requirements of these nutrients for growth and toxin production were

developed and validated against batch culture data. These models were reasonably accurate for real time prediction but suffered the same loss of accuracy for forecasting.

Conclusions

In conclusion, this work shows that the primary environmental modulators of MCYST production by *Microcystis* that are suitable for simple measurement, and use in predictive models, are environmental nitrogen and phosphorous and growth rate. However, given the complexity of the regulation of microcystin production and the strain variation in toxin production, it does not seem likely that a single general model will emerge that allows accurate long-term prediction of toxin levels unless accurate nutrient loading and PAR forecasts are possible. Despite this, reasonably accurate short term prediction does seem possible, as does a general prediction on whether toxin levels will increase or decrease over periods possibly as long as one month. Work currently in progress includes the validation and refinement of these types of models on fed batch cultures of various strains and communities. Artificial neural network models also appear to hold some promise and should be further investigated. This work clearly shows the primary environmental modulators that should constitute the input nodes of such models and therefore substantially adds to the current knowledge base on predictive modelling of microcystin production. Similarly, the models presented here are the first reported models of toxin production based on environmental variables and as such constitute a major advancement in both the understanding of the regulation of microcystin production and the approach to both structured and a-posteri modelling of toxin production by *M. aeruginosa*.

Recommendations

Future work should be aimed at refining the model by large scale validation in fed batch reactors and inclusion of additional environmental parameters. Ultimately the model should be validated on a suitable impoundment and refined for use on that specific water body so as to evaluate the predictive potential of microcystin modelling based on nutrient loading.

Knowledge Dissemination

Two articles have already been published and three more have been submitted for publication. Additionally, work emanating from this project was presented at the International Conference on Toxic Cyanobacteria in Bergen, Norway in 2004.

Articles

- DOWNING TG, SEMBER CS, GEHRINGER MM, LEUKES W (2005) Medium N:P ratios and specific growth rate comodulate microcystin and protein content in Microcystis aeruginosa PCC7806 and M. aeruginosa UV027. Microb Ecol. 49(3):468-73
- DOWNING TG, MEYER C, GEHRINGER MM, VAN DE VENTER M (2005) Microcystin content of Microcystis aeruginosa is modulated by nitrogen uptake rate relative to specific growth rate or carbon fixation rate. Environ Toxicol. 20(3):257-62.

Presentations

- DOWNING, TG, MEYER, C & VAN DE VENTER, M. Microcystin concentration in *M.aeruginosa* is modulated by nitrogen uptake rate relative to growth rate of carbon fixation. 6th International Conference on Toxic Cyanobacteria, Bergen, Norway.

Capacity building

PhD degree

- PhD, T.G Downing, THE ROLE OF NITROGEN IN THE REGULATION OF MICROCYSTIN CONTENT IN *MICROCYSTIS AERUGINOSA*

BSc (HONS)

- BSc (Hons), C Meyer, THE INFLUENCE OF NITRATE UPTAKE AT A CONSTANT GROWTH RATE, ON MICROCYSTIN CONTENT IN *M.AERUGINOSA* PCC 7806
- BSc (Hons) S Blundell, THE ROLE OF α-KETOGLUTARATE IN THE MODULATION OF MICROCYSTIN PRODUCTION WITHIN THE CYANOBACTERIUM MICROCYSTIS AERUGINOSA PCC 7806

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- Dr S du Plessis (University of the North West)
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- Mrs C van Ginkel (Department of Water Affairs and Forestry))
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1. INTRODUCTION

1.1 General Introduction

The cyanobacteria constitute one of the oldest life forms on earth. These oxygenic phototrophs contain both chlorophyll *a* and accessory pigments and include unicellular, colonial and filamentous types with individual cell sizes ranging from 2 to over 40 µm in diameter. The majority of known examples are planktonic in either freshwater or marine environments, or exist as symbionts with plants and fungi or in the benthos or soil. Cyanobacteria are widespread and common and inhabit a variety of environments from thermal springs to Antarctic lakes. Their ability to exist in such diverse habitats is a reflection of their ability as a group to fix nitrogen, adapt their light harvesting pigments, regulate buoyancy and exhibit cellular differentiation for the purpose of reproduction or dormancy. Because several genera of cyanobacteria produce a range of hepatotoxins and neurotoxins they are of particular importance and have received increased attention in recent years.

The increased rate of eutrophication of surface fresh waters due to increased population densities, modern agricultural practices and industrial effluent has resulted in more frequent and severe cyanobacterial bloom events. Since many genera of freshwater cyanobacteria are capable of producing toxins, this increase in the frequency and severity of bloom events poses a problem for the supply of potable water in that classical treatment methods result in cell lysis and release of these toxins. An understanding of the environmental conditions that modulate toxin production would therefore be beneficial for the management of potable water supplies.

Hepatotoxin production in cyanobacteria has been shown to correlate with external stimuli such as light and nutrient concentrations and ratios although conflicting results have been reported. Modulation of microcystin production in *Microcystis aeruginosa* has been extensively studied in both batch and continuous culture. Positive correlations with medium nitrogen, medium phosphorus, light intensity, inorganic carbon availability and growth rate have been reported. Negative correlations have been reported between microcystin content and medium phosphorus. The only reported quantitative relationship between any variable and microcystin production was that of growth rate.

The general purpose of the work described here was therefore to investigate the environmental factors involved in the modulation of microcystin production in *M. aeruginosa*, with specific emphasis on the role of environmental nitrogen and the co-modulatory effects of environmental phosphorus. The co-modulatory effect of environmental phosphorus was investigated because cellular phosphorus plays an important role in photosynthetic carbon fixation, which in turn affects cellular C:N ratios and nitrogen assimilation. The modulatory effects of carbon-fixation:nitrogen-uptake and cellular C:N ratios were also investigated at a constant growth rate to relate environmental N:P ratios to cellular activities. In order to clarify further any modulatory effects of these environmental variables, potential regulatory mechanisms were investigated. More specifically, the potential role of NtcA (a cellular nitrogen regulator) and the levels of nitrogen assimilation products and carbon precursors were studied to determine the cellular status of these constituents under environmental conditions that lead to increased microcystin production. In doing so an attempt was made to elucidate the potential mechanisms by which the relevant environmental factors enhanced microcystin production.

1.2 Report structure

A review of the relevant literature is followed by methods as used for each of the experimental sections. The methods and results sections are sub-divided into each section for each issue addressed and set of experiments used to answer the specific questions. Thus both methods and materials and the results are divided into sections for:

- **The effects of both environmental nitrogen and phosphorus concentrations and ratios on toxin content and specific growth rate of two strains of *Microcystis aeruginosa*.** Previous work on the role of these environmental variables in modulating microcystin content of *M. aeruginosa* was based largely on initial medium concentrations of the nutrients, or cellular N:P ratios. Published data on the effect of growth rate on toxin production does not always exclude retardation or stationary phase data, and in most cases no medium nitrogen and phosphorus data are reported. In the present study medium nutrients and cellular chlorophyll *a* and protein data were therefore recorded during unlimited growth in batch culture. Results indicated a co-modulatory effect of nitrogen and phosphorus on growth rate and microcystin content. Microcystin content was

further modulated by growth rate within given medium N:P ratios. These results suggested cellular carbon:nitrogen ratios as a potential modulator of microcystin content and were published in *Microbial Ecology* (2005) 49:1-6.

- **The relative roles of carbon fixation rate or growth rate and nitrogen uptake and assimilation on toxin production rate**. Based on the important role phosphorus plays in carbon fixation, medium nitrogen was varied in continuous culture at a single growth rate with excess phosphorus so as to exclude the co-modulatory role of growth rate on toxin content. Steady-state limitations in the data presented here were either nitrogen, inorganic carbon, or light. Microcystin content was shown to be reduced under nitrogen limitation and increased under carbon or light limitation. The ratio of cellular microcystin content to nitrogen uptake rate increased dramatically under carbon limitation. Data on microcystin content as a function of nitrogen uptake relative to growth rate in batch culture were also presented. Microcystin content was similarly increased where nitrogen uptake rate exceeded growth rate. These data, published in *Environmental toxicology* (2005) 20:257-262, suggested cellular nitrogen status or the regulation of nitrogen metabolism as the primary modulator of microcystin content.
- **A potential regulatory system for Microcystin.** No definitive mechanism for the regulation of microcystin production by *Microcystis aeruginosa* is known. Work presented here on the modulation of microcystin content revealed a primary dependence on nitrogen and cellular nitrogen-uptake:carbon-fixation ratios which yield variations in the cellular carbon:nitrogen ratio that are in turn altered by growth and the stoichiometric requirements of growth for these cellular pools. In the light of a possible link between regulation of microcystin production and cellular nitrogen metabolism regulation, the possibility of regulation of microcystin production by the cellular nitrogen metabolism regulator in cyanobacteria (NtcA) was investigated. This work revealed a putative NtcA binding site upstream of the *mcyABC* operon in a position suggesting repression by NtcA. Measurement of transcript levels revealed an inverse relationship between *ntcA* and *mcyA* mRNA levels as a function of light. These data supported the hypothesis that cellular nitrogen status may be the main determinant of microcystin production rate.
- **The role of cellular nitrogen status.** Glutamine is an indicator of cellular nitrogen status. Ammonium, α-ketoglutarate, carbamoyl phosphate and cyanate

have been implicated in regulation of NtcA expression. Glutamine, glutamate and α-ketoglutarate are therefore indicators, if not direct regulators, of NtcA expression levels. Results presented here are from of a series of experiments in which cellular glutamate, glutamine and microcystin were measured under a range of conditions, and with selected metabolic inhibitors (L-methionine-D,L-sulfoximine and azaserine), designed to vary cellular glutamate and glutamine. Glutamine, a generally accepted indicator of cellular nitrogen status, was not an indicator of microcystin content. Maximum microcystin production occurred at cellular glutamine:glutamate ratios of between 1 and 3, corresponding to cellular N:P ratios in the range previously reported. Growth rate further modified the effect of these metabolites on microcystin production. These data confirmed the co-modulatory effect of carbon and nitrogen, and in conjunction with the putative role for NtcA in regulation of microcystin production, suggested that α-ketoglutarate may play a role in enhancing repression of *mcy* transcription by NtcA.

- **Carbon skeletons and regulation of Microcystin**. Data presented in this section shows a strong negative correlation between cellular α-ketoglutarate and microcystin concentrations only in cultures where D,L-glyceraldehyde (a specific inhibitor of carbon fixation) was used. Strong positive correlations occurred for cultures grown in the absence of light or nitrogen. The lack of a general correlation between cellular α-ketoglutarate and microcystin concentrations was attributed to (i) the requirement for α-ketoglutarate to assimilate nitrogen, (ii) the variation in cellular glutamate:glutamine ratios that would occur as a result of increasing or decreasing α-ketoglutarate or ammonium, and (iii) the growth rate variations that did result, from the treatments used. Such correlations that did occur were thus based on α-ketoglutarate levels as a function of growth which in turn affects microcystin content.
- **Modelling microcystin production**. The final section presents a generalized model for environmental regulation of microcystin production in *M. aeruginosa*. The model is supported by unpublished data of photosynthetic efficiency, and the changes in distribution of cellular nitrogen between protein and microcystin as a function of carbon fixation or growth rates. These concepts are developed further to achieve the stated aim of the project and quantitative models are presented and discussed.

1.3 Review of Relevant Literature

Literature pertaining to the study organism, the structure and synthesis of microcystin and environmental regulation of transcription and microcystin content in *M. aeruginosa* is reviewed below.

1.3.1 Microcystis aeruginosa

M. aeruginosa is a non-nitrogen fixing, freshwater, planktonic, unicellular colonial cyanobacterium capable of producing microcystins (heptapeptide hepatotoxins). *Microcystis* is one of the primary bloom-forming toxic genera in eutrophied freshwater (Downing & van Ginkel, 2004). PCC 7806 is a non-colonial *M. aeruginosa* strain of approximately 3 µm in diameter. It produces microcystin-LR and D-*erythro*-β-methylaspartic microcystin-LR and has permanent, dispersed gas vacuoles.

1.3.2 Microcystin

The microcystins are non-ribosomally synthesized (Dittmann *et al.*, 1997) heptapeptides with the general structure cyclo [-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-] where Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid, Mdha is N-methyl dehydroalanine and X and Z are variable L-amino acids (Botes *et al.*, 1984, Sivonen & Jones, 1990). Figure 1.1 shows the generic structure for microcystins.

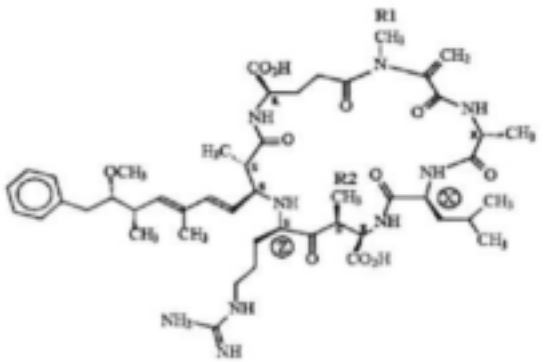

Figure 1.1. Microcystin-LR with an L-leucine at position X and L-arginine at position Z. Other variants differ at these positions. R1 and R2 indicate methyl groups of D-erythro-β-methylaspartic acid and N-methyldehydroalanine, respectively.

Over 60 variants (varying mostly in the L-amino acids) of microcystin (MCYST) have been identified (Sivonen & Jones, 1990).

Toxicity

Microcystins are potent hepatotoxins that are selectively taken up via a multispecific bile transport system (Eriksson *et al.*, 1990; Runnegar *et al.*, 1995). Non-hepatocyte cells are affected by exposure to MCYST at higher concentrations of toxin (100 μM MCYST-LR) than those required to elicit similar toxic effects in hepatocytes (0.8 μM MCYST-LR) (McDermott *et al.*, 1998). Toxicity is attributed to the inhibitory activity of microcystins on protein phosphatases 1 and 2A (PP1 and PP2A) (MacKintosh *et al.*, 1990) leading to hyperphosphorylation of the cellular cytoskeleton. The resultant sinusoidal collapse leads to severe intrahepatic heamorhaging and in severe cases, death (Falconer, 2001). Protein phosphatase inhibition was demonstrated *in vitro* for MCYST-LR, MCYST-YR (tyrosine-arginine) and MCYST-RR (arginine-arginine) in mouse liver lysates (Yoshizawa *et al.*, 1990) and *in vivo* for MCYST-LR and MCYST-YM (tyrosine-methionine) in liver homogenates of mice exposed to toxin (Runnegar *et al.*, 1993). MCYST-RR, with an LD_{50} in mice of 600 μg kg^{-1}, is less toxic than MCYST-LR which has an LD_{50} 50 μg kg^{-1} (Sivonen, 1996). MCYST-RR is twelve times less toxic than MCYST-LR when administered intratracheally to mice and yet both toxins inhibit PP1 and PP2A to a similar degree (Ito *et al.*, 2002). The lethality of a MCYST analogue therefore appears to depend on differences in uptake rates.

Synthesis

Synthesis of microcystins is directed by both polypeptide synthetase and polyketide synthase modules encoded by *mcyA-J* (Kaebernick & Neilan, 2001) which form a multienzyme complex (Tillet *et al.*, 2000). Figure 1.2 shows the organization and simplified biosynthetic model for the microcystin synthetase gene cluster.

The peptide synthetase employs the thio-template mechanism whereby individual sites of the multienzyme 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 & Neilan, 2001). The polypeptide synthetase genes *mcyA* and *mcyB* each contain two modules (A1 and A2, and B1 and B2), with variation in *mcyB1* modules resulting in various isoforms (Mikalsen *et al.*, 2003). A1 also encodes an N-methyltransferase activity. McyD contains two polyketide synthase modules, McyE contains an N-terminal polyketide synthase module and a peptide synthetase module at the C-terminus, while McyG contains a peptide

synthetase module at the N-terminus and a polyketide synthase at the C-terminus (Nishizawa *et al.*, 2000).

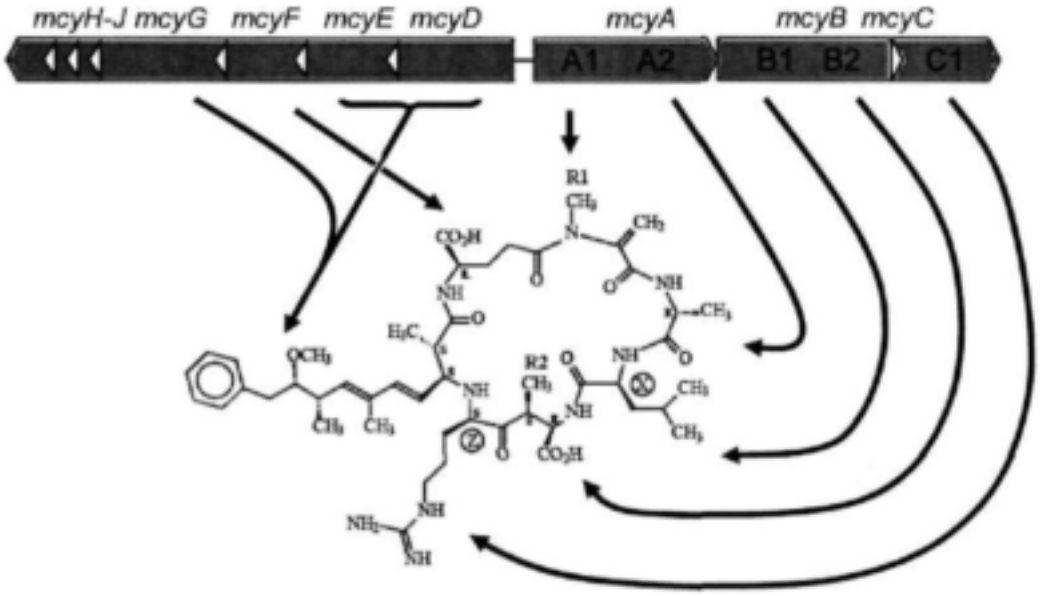

Figure 1.2. Role of *mcy* elements in biosynthesis of microcystin. See text for details.

Polyketide synthesis domains in *mcyD, mcyE* and *mcyG* gene products are responsible for the fatty acid side chain of Adda (Kaebernick & Neilan, 2001). *mcyF* encodes a glutamate racemase (Nishizawa *et al.*, 2001). Putative tailoring functions have been assigned to *mcyI* and *mcyJ* (Kaebernick & Neilan, 2001). *mcyH* shows high identity to ABC transporter genes (Pearson *et al.*, 2004) but no functional activity has been described. *mcyH* knockout mutants do not, however, produce MCYST; this suggests that it has a function other than toxin export.

1.3.3 Environmental modulation of microcystin

Toxin concentrations have been shown to be highly variable in natural environments (Codd & Bell, 1985; Sivonen *et al.*, 1995; Vézie *et al.*, 1998). Research over the past two decades on modulation of MCYST production and cellular content has focused largely on the effects of specific growth rate (Ichimura, 1979; Long *et al.*, 2001; Oh *et al.*, 2000, Orr & Jones, 1998; van der Westhuizen & Eloff, 1985; Watanabe & Oishi, 1985;

Watanabe *et al.*, 1989; Wiedner *et al.*, 2003), growth phase (Lee *et al.*, 2000), incident light intensity and quality (Lee *et al.*, 2000, Long *et al.*, 2001; Lyck, 2004; Utkilen & Gjølme, 1995; Van der Westhuizen & Eloff, 1985; Watanabe *et al.*, 1998; Wiedner *et al.*, 2003; Wicks & Thiel, 1990), macro-morphology or cellular arrangement and colony size (Kurmayer *et al.*, 2003; Leukes *et al.*, 2003), temperature (Gorham, 1964; Ichimura, 1979; Kruger & Eloff, 1978; Van der Westhuizen & Eloff, 1985; Watanabe & Oishi, 1985; Yoshizawa *et al.*, 1990), availability and rates of uptake of nutrients, specifically nitrogen and phosphorus (Lee *et al.*, 2000; Long *et al.*, 2001; Oh *et al.*, 2000; Orr & Jones, 1998; Utkilen & Gjølme, 1995; Van der Westhuizen & Eloff, 1985; Watanabe & Oishi, 1985), and inorganic carbon availability and rates of carbon fixation (Van der Westhuizen & Eloff, 1985). Despite this body of work, no consensus model for environmental modulation of MCYST has emerged. This is partly due to the development of more accurate quantitative methods for measuring MCYST which followed elucidation of the structure of the molecule (Botes *et al.*, 1984). This resulted in a change from expressing the total toxin content as LD_{50} of dry weight of cells, to MCYST mass/biomass or mass per cell number, or mass per cell also termed cell quota ($MCYST_Q$). Since variants of MCYST have variable LD_{50}'s, and changes in variant ratios occur as a function of environmental variables, much of the early data is not particularly useful. In addition to this problem, the use of protein phosphatase inhibition assays for microcystin quantification (Ash *et al.*, 1995) further complicates any quantification of the effects of environmental variables on MCYST content as variants yield different levels of inhibition and many strains produce several variants. The use of different strains has also resulted in occasionally contradictory results due to substantial variance in MCYST modulation responses between and within strains as a function of long term culture. The following sections describe the published effects of the abovementioned environmental parameters on toxin production.

Temperature

Gorham (1964) reported the highest toxicity values for *Microcystis* at 25°C with approximately 20% of the maximum toxicity (measured as LD_{50}) when cultured at 20°C and 50% when cultured at 30°C. Watanabe and Oishi (1985) cultured *M. aeruginosa* M228 under batch culture conditions in MA medium (Ichimura, 1979) at 18, 25 and 32°C. No significant difference in toxicity was observed between 18 and 25°C. A significant increase in LD_{50} (approximately 35%) was however observed between 25 and 32°C

corresponding to an increase in growth rate from 0.44 to 0.59 day^{-1}. In apparent contradiction of these results, a significant negative correlation between temperature and toxicity (measured as LD_{50}) between 16 and 20°C was reported by van der Westhuizen and Eloff (1985) for strain UV-006 cultured in modified BG11 (Kruger & Eloff, 1978). However, as for strain M228, this corresponded to an increase in growth rate for the same temperature difference. Wicks and Thiel (1990) reported that toxin content increased with temperature in natural bloom samples for temperatures between 15 and 25°C, but did not report growth rate data.

Growth rate

The increase in LD_{50} and corresponding increase in growth rate as a function of temperature described by Watanabe and Oishi (1985) for temperatures between 25 and 32°C, and by van der Westhuizen and Eloff (1985) for temperatures between 16 and 20°C indicates that toxicity decreases with increasing growth rate. However, from 20 to 36°C strain UV-006 showed a strong positive correlation between growth rate and toxicity, in contradiction of that reported for M228 (Watanabe and Oishi, 1985). Notable differences in culture conditions were light intensity (with UV-006 being exposed to 200 µmol photons m^{-2} s^{-1} while M228 was cultured under 30.1 µmol photons m^{-2} s^{-1}), and agitation/aeration by filtered air containing 0.5% CO_2 for culture of UV-006 and no agitation for M228. The apparent contradiction could therefore also be attributed to enhanced photosynthetic potential for UV-006. This suggests that carbon fixation rate may be responsible for increased toxin production at higher growth rates, while reduction in carbon fixation potential at higher growth rates would result in reduced available fixed carbon. Carbon fixation products may therefore play a role in MCYST production.

In a study of *M. aeruginosa* M228-12, and three variants in *M. viridis*, Watanabe *et al.* (46) measured toxin content per dry weight of cells ($MCYST_{DW}$) and changes in the cellular content of two MCYST variants over the duration of batch culture in MA medium (Ichimura, 1979) under 30.1 µmol photons m^{-2} s^{-1}. In both species, MCYST content correlated with growth rate. In *M. viridis*, MCYST-LR, MCYST-RR and MCYST-YR followed the same pattern, increasing during exponential growth of the culture and subsequently declining once the culture had reached stationary phase. In M228-12, MCYST-YR followed the same pattern but MCYST-LR declined throughout the 20 day duration of the culture. Metabolic processes related to cell growth can become

uncoupled from cell division as cells enter stationary phase, which may explain the decrease in $MCYST_{DW}$ during stationary phase. The absence of data on residual nutrient concentrations and in-flask photosynthetically active radiation (PAR) makes any conclusions drawn from these data questionable.

Orr and Jones (1998) performed batch culture experiments on *M. aeruginosa* MASH01 and a sub culture of this strain at 20°C and 30 μmol photons m^{-2} s^{-1} PAR with varying concentrations of initial nitrogen in the medium and measurement of residual nitrate-nitrogen throughout the 21 days of incubation. Under these conditions the specific rate of microcystin production (R_{MCYST}) varied directly with growth rate; this was also shown to hold true for other microcystin producing genera. However, growth rate and biomass yield also correlated with initial medium nitrogen concentrations, while MCYST concentrations in the culture were strongly correlated with initial medium nitrate and reduction in medium nitrogen. The general conclusion drawn from these data, that R_{MCYST} varies directly with cell division rate, is therefore not necessarily correct, and should rather be stated as 'MCYST production rate is directly proportional to cell division rate where cell division rate is a function of nitrogen uptake'. Long *et al.* (2001) adequately addressed this issue by showing that in nitrogen limited continuous culture of strain MAS01-A19, both $MCYST_Q$ and R_{MCYST} varied directly with growth rate. In addition to this, $MCYST_{DW}$ was also strongly correlated with growth rate. In phosphorus-limited continuous culture $MCYST_{DW}$ decreased with increasing growth rate, while R_{MCYST} increased with growth rate in *M. aeruginosa* UTEX2388 (Oh *et al.*, 2000). These data suggest that R_{MCYST} may be a function of growth rate irrespective of the growth-limiting factor and that the decrease in R_{MCYST} with increasing $MCYST_{DW}$ may be the result of uncoupling of cell division from growth related metabolic processes, specifically those related to nitrogen uptake and assimilation. Such uncoupling and subsequent effects on MCYST were further illustrated by Wiedner *et al.* (2003) who reported a strong correlation between growth rate and $MCYST_Q$ as a function of photon irradiance in continuous culture for light limited conditions, but not for light saturated conditions. Some parameters determine both growth rate and $MCYST_Q$. The correlation observed between growth rate and $MCYST_Q$ therefore depends on environmental conditions. Certain sets of environmental parameters result in increased growth rate and increased MCYST production, while other sets of conditions yield different rates of growth and MCYST production. Thus relating $MCYST_Q$ to growth rate alone is problematic.

Light

Enhanced toxicity was observed under conditions of increased growth rate (as a function of temperature) only at increased PAR and inorganic carbon availability (Van der Westhuizen & Eloff, 1985; Watanabe & Oishi, 1985). Toxicity in natural bloom samples also increased with increasing solar radiation and primary productivity (Yoshizawa *et al.*, 1990). Watanabe and Oishi (1985) reported a correlation between light intensity and toxicity, with LD_{50} values dropping from 36.9 mg kg^{-1} to 9.65 mg kg^{-1} when light intensity was increased from 7.53 to 30.1 μmol photons m^{-2} s^{-1}. This also corresponded to an increase in growth rate from 0.25 to 0.52 day^{-1}. A further increase from 30.1 to 75.3 μmol photons m^{-2} s^{-1} had no significant effect on toxicity under the conditions used. With agitation by filtered air at a rate of 72 l hr^{-1}, and thus excess inorganic carbon, in continuous culture at a growth rate of 0.013 hr^{-1} and with variation in light intensity from 10 to 45 μmol photons m^{-2} s^{-1}, Utkilen and Gjølme (1995) reported a strong correlation between $MCYST_{DW}$ and light intensity from 20 to 40 μmol photons m^{-2} s^{-1}, no significant change in $MCYST_{DW}$ from 40 to 55 μmol photons m^{-2} s^{-1}, and a significant decrease from 55 to 75 μmol photons m^{-2} s^{-1}. MCYST/protein was similarly correlated with $MCYST_{DW}$ up to 50 μmol photons m^{-2} s^{-1} but showed no further change up to 75 μmol photons m^{-2} s^{-1}. However, no indication of the limiting nutrient was given, making comparison with batch culture studies, where nutrient variations were measured, impossible. Natural samples obtained from a lake supported these data: $MCYST_{DW}$ decreased with depth as the light intensity decreased. Light quality was also shown to be significant, red light yielding the highest $MCYST_{DW}$ and the highest MCYST/total protein ratios (Utkilen and Gjølme, 1995).

A subsequent study on *M. aeruginosa* PCC 7806 in continuous culture by Wiedner *et al.* (2003) shed more light on the role of PAR in modulation of MCYST production. R_{MCYST} increased with increasing PAR from 0 to 100 μmol photons m^{-2} s^{-1}, and decreased when PAR was increased between 100 to 400 μmol photons m^{-2} s^{-1}, with the inflection point corresponding to the maximum achieved growth rate as a function of PAR intensity. $MCYST_Q$ increased directly with growth rate in conditions of PAR limitation, but decreased at values above 100 μmol photons m^{-2} s^{-1}; cellular concentrations of intracellular MCYST ($MCYST_{CC}$) followed the same trend but showed a more pronounced decrease, presumably due to increased cell size caused by uncoupling of growth related metabolic processes (specifically carbon fixation) and cell division.

Chlorophyll *a* content has also been shown to correlate with MCYST content (Lee *et al.*, 2000; Long *et al.*, 2001; Wiedner *et al.*, 2003), but it should also be noted that both increases (Long *et al.*, 2001) and decreases (Lyck, 2004) in chlorophyll *a* have been reported with increasing growth rate; this would suggest that the photosynthetic potential (and actual rates of carbon fixation under suitable conditions) relative to growth rate may be involved in MCYST modulation. That growth rate does not correlate with carbon fixation could be attributable to variation in another essential nutrient, implying a role for cellular ratios of carbon to some other nutrient as a potential modulator of MCYST production.

Nutrients

In their studies of temperature and light modulation of toxin production, neither Watanabe and Oishi (1985), nor van der Westhuizen and Eloff (1985) nor Utkilen and Gjølme (1995) measured corresponding variation in medium nutrients (specifically nitrogen and phosphorus). However, Orr and Jones (1998) and Long *et al.* (2001) showed, in batch and continuous culture respectively, that the relationship between growth rate and $MCYST_Q$ and R_{MCYST} was dependent on medium nitrogen, nitrogen uptake rate or nitrogen limitation. Oh *et al.* (2000) reported a weak positive correlation between cellular N:P atomic ratio and MCYST-LR and MCYST-RR, and weak negative correlation between carbon fixation rate and $MCYST_{DW}$ as a function of growth rate. The dependence of carbon fixation on phosphorus may explain this trend in a phosphorus limited chemostat. With nitrogen in excess, the strong correlation between R_{MCYST} and growth rate as reported for a nitrogen limited chemostat (Long *et al.*, 2001) thus occurs, further suggesting nitrogen as the primary modulator of MCYST production.

Investigation of initial medium N:P ratios and growth stage in batch culture by Lee *et al.* (2000) showed N:P ratios of 16:1 and 50:1 to yield maximum $MCYST_{DW}$. $MCYST_{DW}$ increased with total nitrogen, but the authors did not consider the effects of actual medium N:P ratios throughout the culture period. $MCYST_{DW}$ as a function of initial medium N:P ratios was also measured after seven days incubation where certain cultures were entering stationary phase while other cultures showed no signs of retardation. Where N limitation resulted in retardation, reduced MCYST was observed and attributed to low N:P ratio. Similarly at an N:P ratio of 100:1, MCYST was reduced

compared to values observed at 16:1 and 50:1; the authors concluded that MCYST content was highest as a direct result of these ratios. However, it could be that this merely indicates a possible P limitation which in turn would reduce carbon fixation and subsequent nitrogen assimilation. In addition to these problems in interpreting the data, the authors measured culture growth spectrophotometrically at 680 nm, thereby ignoring the effects that growth rate had on cell size and of limitation of a single nutrient on cell size and composition.

Vézie *et al.* (2002) reported a significant interactive effect of nitrogen and phosphorus on $MCYST_{CC}$ in *M. aeruginosa*. One strain tested appeared to show increased $MCYST_{CC}$ at reduced levels of both nitrogen and phosphorus and at very high levels of both nutrients, but reduced $MCYST_{CC}$ at reduced N and elevated P. Both strains had maximum $MCYST_{CC}$ at an atomic N:P ratio of approximately 500, but this data was not presented as a function of growth rate; in addition strains produced these maximum toxin levels at different stages of batch culture indicating that growth rate may have been different for each strain at the N:P ratio yielding maximum toxin. A second strain revealed a different pattern entirely with maximum toxin production at reduced P only (N:P from approximately 100 to 150). Growth responses of toxic strains did however follow the same pattern, with maximum growth occurring where both nutrients were elevated and increased growth occurring where either of the nutrients was elevated. Interestingly, non-toxic strains had completely different growth responses to these nutrients and nutrient ratios with one strain responding only to variation in N and the other having optimum concentrations of 0.14 to 0.29 mg l^{-1} P and 15.5 to 66 mg l^{-1} N.

Colony size

Kurmayer *et al.* (2003) reported a correlation between colony size and MCYST producing genotypes in natural bloom samples, with smaller colonies possessing less than larger colonies. This suggests a requirement by the cell for MCYST under reduced light conditions. Leukes *et al.* (2003) reported higher levels of MCYST (immunohistochemically detected) at regions of reduced light and increased nitrogen levels in a membrane photobioreactor, further suggesting a cellular function for MCYST under reduced light, or an increase in MCYST in conditions where photosynthetic rate is relatively lower than nitrogen uptake rate.

1.3.4 *Regulation of expression of mcy gene expression*

Figure 1.3 shows the transcriptional organization of the microcystin synthetase cluster. Transcription of two polycistronic operons (*mcyABC)* and *mcyDEFGHIJ* occurs from a central bi-directional promoter between *mcyA* and *mcyD* (Kaebernick *et al.*, 2002).

Figure 1.3. Transcriptional organization of the microcystin synthetase gene cluster *mcyABCDEFGHIJ*, showing putative promoters for *mcyEFGHIJ* and alternative promoters identified for *mcyA* and *mcyD* (Kaebernick *et al.*, 2002). Promoters are represented by black block arrows.

Kaebernick *et al.* (2000) showed that *mcyB* and *mcyD* were transcribed at similar rates under varying light conditions, with transcript levels increasing under medium light (31 μmol photons m^{-2} s^{-1}) relative to low light (16 μmol photons m^{-2} s^{-1}) and increasing dramatically when cultures were exposed to high light (68 μmol photons m^{-2} s^{-1}). The difference in transcription levels between high and low light corresponds to the subsequently reported (Kaebernick *et al.*, 2002) existence of alternative transcriptional start sites for both *mcyA* and *mcyD* at the same light intensities. Transcription levels have also been shown to be enhanced under red light but reduced under blue light or sodium stress (Kaebernick & Neilan, 2001). It is interesting to note that the change in transcription start points corresponds to the inflection point for the strong correlation between R_{MCYST} and growth rate under non-saturated PAR and the decrease in MCYST with further increased PAR (Wiedner *et al.*, 2003). In addition to this, the inflexion point corresponded to PAR levels yielding maximum growth rate where P or N were not limiting. This suggests that *mcy* gene transcription is increased where nutrient uptake or photosynthesis occurs at a rate that exceeds use of assimilated or fixed products by growth.

1.3.5 *Summary*

Under conditions that reduce growth rate but not necessarily carbon fixation or the uptake of nutrients, accumulation of assimilated nitrogen may lead to enhanced MCYST production, suggesting the following as the primary modulators: nitrogen availability or uptake and assimilation rate, and carbon fixation rate or the availability of phosphorus

and adequate PAR. The effects of these variables include changes in growth rate which in turn further modifies MCYST content and production rate. Thus the primary effectors of MCYST content in *M. aeruginosa* appear to be nitrogen assimilation and carbon fixation rates. However, both light intensity and quality, and phosphorus availability affect carbon fixation rate. Nitrogen, carbon and phosphorus are the primary determinants of growth. The relative abundance of cellular C, N and P therefore regulates both growth rate and MCYST production and is in turn a function of environmental levels. Published data is limited to cellular N:P ratio and initial medium N:P ratio in laboratory culture, and data where growth rate was not defined at each N:P ratio. Thus the effect of environmentally available nitrogen and phosphorus and their ratios and the relationship between these variables should be investigated under conditions where neither nutrient is limiting and where growth rate is variable. Similarly, carbon fixation rate relative to nitrogen uptake rate (and associated cellular C:N ratio) should be investigated in the absence of variation in growth rate. Variations in concentrations of carbon fixation and nitrogen assimilation metabolites should also be investigated to determine whether such environmental changes are reflected in cellular metabolism. These types of investigations should further our understanding of the environmental modulation of MCYST and provide evidence for regulatory mechanisms and, possibly, evidence to support a function for cellular MCYST.

2. METHODS AND MATERIALS

2.1 The Effect of Environmental N:P Ratio on Microcystin Content

2.1.1 Organisms and culture conditions

M. aeruginosa PCC7806 was obtained from Dr Neilan at UNSW (Sydney, Australia) and *M. aeruginosa* UV027 was provided by Prof. J Grobelaar at UFS (Bloemfontein, South Africa). Cells were grown in 1000-ml bubble-lift vessels under constant illumination (140 ± 5 µmol of photons m^{-2} s^{-1}) using Triton Dayglo® fluorescent lamps at 23 ± 0.5°C with agitation supplied by filtered (2 x Cameo acetate 0.22µm) air at 2 ± 0.5 ml s^{-1}. Culture medium was modified $BG11_0$ containing $NaNO_3$ and K_2HPO_4 at 1.18 and 0.067 mM, 3.53 and 0.111 mM, 3.53 and 0.067 mM, 14.1 and 0.175 mM, 17.7 and 0.211 mM, 5.89 and 0.67 mM, 17.7 and 0.175 mM, 21.2 and 0.175 mM, 17.7 and 0.140 mM and 3.53 and 0.022 mM yielding initial medium N:P ratios of 17.61, 31.80, 52.69, 80.57, 83.89, 87.91, 101.14, 121.14, 126.43 and 160.45 respectively. A single vessel per treatment was inoculated with 50 ml of late log phase cultures (OD_{740} = 1.2 in BG11) of each strain, and vessels were sampled in triplicate according to the sampling regime.

2.1.2 Sampling and analysis.

Growth was monitored daily by measuring optical density at 740 nm and chlorophyll *a*. Samples were taken for analysis at regular intervals depending on growth rate and medium composition, and cell numbers, chlorophyll *a*, protein content, MCYST and medium N and P were measured for samples where $\mu > 0$. Direct cell counts were performed in a haemocytometer (Neubauer). Specific growth rate was calculated from cell counts for the period between samplings therefore representing the mean μ for that period. Chlorophyll *a* was measured spectrophotometrically after extraction with boiling ethanol (90% v/v). Total cellular protein was measured at 550 nm using bicinchoninic acid with bovine serum albumin (BSA) as a standard. Cellular MCYST content was measured for 5 ml samples pelleted (3000g x 5 minutes) and snap frozen in liquid nitrogen before freeze drying for 24 hours and extraction in 5 ml 70% methanol (Ash *et al.*, 1995) with sonication (Bandlin Sovorex NK51) for 16 hours. The resulting extract was dried (Savant SC100) and resuspended in 100 µl 50mM Tris HCL pH7.5 containing 0.1mM EGTA, 0.1% (v/v) β-mercaptoethanol and 0.03% (v/v) Brij-35. Quantification was performed in triplicate on 100 µl of appropriately diluted extracted samples by protein

phosphatase inhibition with 0.5U protein phosphatase 1 catalytic subunit (α isoform, rabbit recombinant in *Escherichia coli*, Sigma) resuspended in 50 µl of 50 mM Tris HCL (pH 7.5) 0.1 mM EGTA, 0.1% (v/v) β-mercaptoethanol and 1mg/ml BSA, 100 µl of 0.033 mM *p*-nitrophenol phosphate in 50 mM Tris HCL (pH 7.5) 0.1mM EGTA, 0.1% (v/v) β-mercaptoethanol, and 50 µl aqueous manganous chloride (20 mM), in a total volume of 0.3 ml. Enzyme activity was determined by the amount of *p*-nitrophenol released in 30 minutes (measured at 410 nm in a Metrohm 665-Dorsimat with Multiskan MS Labsystems software), and the MCYST quantified off a standard curve (r^2=0.999) of %inhibition constructed with MCYST-LR standard (Sigma). Medium nitrate was measured with Griess reagent after reduction with copper cadmium, and medium orthophosphate by the phosphomolybdate method.

2.1.3 Statistical analysis

Pearson product-moment correlation coefficients were calculated between the growth parameters and medium nutrient concentrations and ratios using Statistica for windows release 4.3 (Osiris Technical systems).

2.2 The Role of Cellular N:C Balance and Assimilation/Fixation Rates

2.2.1 Culture and sampling

Axenic cultures of the same two strains were grown in triplicate 2000-ml bubble-lift vessels under constant illumination at 51 µmol of photons $m^{-2}.s^{-1}$ using Triton Dayglo® fluorescent lamps, and with all other conditions as previously described. Modified $BG11_0$ containing a constant phosphate concentration of 0.195 mM and varying nitrate (0.125, 7, 10.0, 15.0 and 18 mM) was supplied at a flow rate yielding a dilution rate of 0.01 hr^{-1}. Culture purity was monitored by microscopy and periodic plating on a variety of media. Steady state was determined by monitoring cell numbers, optical density at 740 nm and medium nutrient concentrations. Where no statistical variance occurred in these variables with time, steady state was considered to have been reached. Batch cultures were performed as described above but with light conditions with aeration adjusted for volume. Five vessels per strain were inoculated with 50 ml of late log phase cultures (OD_{740} = 1.2 in modified $BG11_0$ containing 1.18 mM $NaNO_3$ and 0.067 mM K_2HPO_4) of each strain, and vessels were sampled every three days for 33 days.

2.2.2 *Sample analysis*

Direct cell counts and growth rate were determined for the period between each sampling time as described above. Cellular MCYST content, total cellular protein, medium nitrate and orthophosphate were measured as described above. Carbon fixation rates were determined by incubation of 1 ml of the sampled culture at the same temperature and light intensity as the original culture, at each sampling time, with 0.5 pCi ^{14}C bicarbonate (specific activity of 50 $mCi.mM^{-1}$) for 60 minutes, removal of unfixed carbon by centrifugation and acidification of the pellet with 100 µl 5N sulfuric acid followed by vigorous mixing and exposure to partial vacuum for 30 minutes. Subsequent scintillation counting was performed in 3 ml scintillation cocktail (Ultima Gold XR, Packard) in a Packard Tricarb 2300 TR after an initial two hour dark incubation. Cellular nitrogen and total cellular phosphorus were determined by Kjehdahl treatment and by the Lachat method respectively, on washed filtered cells. Cellular carbon was calculated as per standard chemostat theory as the product of the carbon fixation rate and generation time at steady state. Nutrient uptake rates were calculated as the product of medium nutrient minus residual nutrient and cell number over biomass.

2.2.3 *Statistical analysis*

Pearson product-moment correlation coefficients calculated between the nutrient uptake rates and microcystin content, and ANOVA with post hoc analysis of means using Newman-Keuls test ($p = 0.05$) were performed on steady state values using Statistica for windows release 4.3 (Osiris Technical systems).

2.3 Regulatory Elements: Does NtcA Regulate Microcystin Production?

2.3.1 *Culture*

Cells from a mid-log culture of *M.* aeruginosa PCC7806 were collected by centrifugation (3000g x 10 minutes), re-suspended in starvation medium ($BG11_0$ lacking any phosphorus), re-centrifuged and the resulting pellet re-suspended in starvation medium to the original culture volume. This culture was incubated in the dark to prevent photo-bleaching. After incubation for five days the cells were collected and washed as before and re-suspended in 1200 ml of $BG11_0$ supplemented with either 2 mM (high nitrogen), 1.5 mM (medium nitrogen) or 0.5 mM (low nitrogen) nitrate or 2 mM ammonium. Each of the four cultures was further divided into triplicate flasks and incubated under cool white

fluorescent light at 40 µmol m^2 s^{-1}. Nitrate and ammonium uptake rates and MCYST content were measured per culture dry weight one hour after re-suspension in the different media and then daily for two weeks, as previously described.

2.3.2 Ribonuclease Protection Assay (RPA)

Probe sequences were amplified using primers designed based on consensus regions of published cyanobacterial *ntcA* genes. The forward primer had the sequence (5'-CAGTGTTTTTGGGGTGYT-3') and the reverse primer sequence was (5'-GTTTCAATCATCATTTCCGT-3'). Primers were designed for the *mcyA* gene with the forward primer sequence (5'-TTATTCCAAGTTGCTCCCCA-3') and reverse primer (5'-GGAAATACTGCACAACCGAG-3'). Amplification products were verified on 1% agarose gel, visualised using ethidium bromide and a BioRad Gel Doc 2000 transilluminator, and recorded using the Quantity One 4.1R computer program (BioRad). Amplification products were cloned into pGEM®-T Easy (Promega) and sequenced to determine the orientation for RNA runoff. Probes were produced using the Ambion Maxiscript Sp6/T7 *in vitro* transcription kit as per manufacturer's instruction, gel-purified using the Qiagen RNeasy® Mini Kit as per manufacturer's instructions, and purity and quantity determined spectrophotometrically at 260 nm and 280 nm.

All cultures were maintained at 25°C. A stationary phase culture (BG11) was diluted with fresh BG11 to an OD_{740} of 0.65 and split it into 6 identical 250 ml flasks containing 55 ml of the culture. The OD_{740} was monitored until the cultures were growing exponentially. Three of the flasks were incubated in the dark and three in cool white fluorescent light at approximately 140 µmol m^2 s^{-1}. 20 ml samples were removed at 24 and 48 hours, centrifuged at 5000g at 4°C for 10 minutes and the resulting pellets frozen on dry ice before storage at -40°C.

RNA was extracted by extensive grinding of pellets in liquid nitrogen before grinding in 1 ml TRIZOL. The ground material was collected in powdered form and stored on ice. RNA was purified by precipitation and the triplicate RNA extracts pooled for RPA as per manufacturers instructions (Ambion RPA III) unless otherwise stated, using 10 µg purified RNA per lane and 2.5 µg purified transcript and hybridising at 55°C overnight. The product was digested with five times the manufacturers recommended RNase

concentration, and separated on 10% polyacrylamide gel containing 8 M urea at constant current (25 mA) for two hours. X-ray film was exposed to the gel for 72 hours before developing.

2.4 Cellular Nitrogen Status and Microcystin Production

2.4.1 Metabolic inhibitors

In order to induce variations in Glu and Gln concentrations, cultures were starved of either nitrogen or phosphorus or treated with L-methionine-D,L-sulfoximine (MSX) (an inhibitor of glutamine synthetase (EC 6.3.1.2)) or azaserine (an inhibitor of glutamate synthase (EC 1.4.7.1)) so as to alter cellular glutamine and glutamate levels. In so doing, the potential effects of nitrogen or phosphorus availability on the relationship between $MCYST_{CC}$ and $MCYST_{Q}$ and indicators of cellular nitrogen status were eliminated.

2.4.2 Culture

All axenic cultures were maintained in BG11 as previously described. Cultures for either starvation or inhibition experiments were prepared by inoculating 20% (v/v) stationary phase ($0.9<OD_{740}<1$) into fresh BG11, grown to mid log phase ($OD_{740}=0.6$) before use as inoculum (20%) in fresh BG11. Once this culture had reached $OD_{740}=0.6$, cells were harvested by centrifugation at 4°C (3000g x 10 minutes) and re-suspended in the original volume of either BG11 for the starvation control culture or $BG11_0$ containing either 0.04 g K_2HPO_4 for nitrogen starvation or 1.5 g $NaNO_3$ for phosphorus starvation. Re-suspended cultures were divided into triplicate 1200 ml vessels, each containing 1000 ml culture, for each treatment. Cultures for the inhibition experiment were re-suspended in the original volume of BG11 for the control and either BG11 containing 1 mM MSX or 100 µM azaserine. Re-suspended cultures were divided into three vessels for each treatment, each containing 100 ml. Experimental culture conditions were as for culture maintenance. Starvation cultures were sampled after re-suspension in the different media and then again daily for two weeks. Inhibition cultures were sampled five minutes after addition of the inhibitors and then at 40 min, 70 min, 4 hours, 10 hours, 24 hours and 54 hours.

2.4.3 Analysis

Cell counts, biovolume, MCYST, protein, cellular N and P and carbon fixation rates were determined as previously described. L-glutamate was quantified using the Amplex Red®

glutamic acid/glutamate oxidase assay (Molecular Probes). Extracts for analysis were prepared by filtration of 5 ml culture (Whatman GC 0.5 mm glass fibre filters). The filters were snap frozen in liquid nitrogen and ground in a mortar and pestle before addition of 0.5 ml MeOH and sonication (Bandlin Sovorex NK51) for two hours. Extracts were centrifuged at 3000 g for 5 minutes and the supernatant was vacuum dried (Savant SC100). The extract was re-suspended in 0.1 M Tris/HCl buffer (pH 5). The resulting extract was diluted in the same buffer before quantification of L-glutamate, as per manufacturers instructions, off a standard curve (r^2=0.9933) prepared with L-glutamate (Sigma) in the range 0 to 10 µM. L-glutamine was quantified using the identical protocol after dilution as above of the extract and addition of 0.1 U glutaminase (Sigma) and subtraction of L-glutamate concentrations. The standard curve (r^2 = 0.9995) for L-glutamine was prepared using L-glutamine in the range 0 to 20 µM.

2.4.4 Statistical analysis

Statistical analyses were performed using Statistica® 7 from StatSoft Inc.

2.5 Carbon Skeletons: C:N Balance and Modulation of the Nitrogen Effect

2.5.1 Culture

Axenic *M. aeruginosa* PCC 7806 was maintained on BG11. A single batch culture of 2200 ml was grown to mid-log phase (OD_{740} = 0.82), collected by centrifugation at 4°C (3000g x 10 minutes) and re-suspended in appropriate medium for each treatment before splitting into triplicate 250 ml flasks each containing 120 ml culture. For experimental conditions with no nitrogen and double standard nitrogen re-suspension was in $BG11_0$ and BG11 containing twice the standard nitrate concentration respectively. All other cultures were re-suspended in BG11. Cultures to be incubated in the absence of PAR were wrapped in foil. D,L-glyceraldehyde and L-methionine-D,L-sulfoximine (MSX), inhibitors of RubisCo and Glutamine synthetase respectively, were added to final concentrations of 0.1 mM and 1 mM respectively to appropriate flasks at time 0. All flasks were incubated at 24°C and under 80 µmol photons m^{-2} s^{-1} and samples were taken at 5, 30, 120 and 270 minutes for analysis.

2.5.2 Analysis

Cellular MCYST was extracted with methanol from lyophilised samples as previously described (Downing *et al.*, 2005b) and quantified using ELISA (Abraxis) off a standard curve (r^2 = 0.9859). Cells were collected by centrifugation from 2 ml of each sample and the resulting pellet re-suspended in 1 ml 0.3 M $HClO_4$ (4°C) and kept on ice for 15 minutes before centrifugation to remove cell debris. 0.5 ml of the resulting supernatant was neutralized by the addition of 0.1 ml 2 M K_2CO_3 and the precipitate removed by centrifugation. The α-ketoglutarate in the resulting supernatant was detected by monitoring NAD reduction in the presence of glutamate dehydrogenase and quantified off a standard curve constructed for this purpose (r^2 = 0.9973). Cell counts and total biovolumes were determined as previously described and used to calculate final cellular concentrations of MCYST and α-ketoglutarate. Statistical analysis was performed using Statistica 7 ® (Statsoft).

2.6 Development of Quantitative Models

Identification of the primary modulators of microcystin production allowed the development of a conceptual model and subsequent quantitative models based on accumulated data. Kinetic models were based on the conceptual model and tested against data from batch and continuous culture experiments. Artificial neural network models are trained on a subset of available data which include both input and output values. Training a neural network using a gradient descent algorithm in which the mean square error between the network's output and the desired output is minimized, creates a function which is minimized iteratively by back-propagating the error from the output nodes to the input nodes. Once this error is less than a specified threshold value, the network has converged and is considered to be trained. By sequentially reducing the number of different input variables, while retaining an error below the threshold value, and validating this model on additional data, primary effectors were identified.

3. RESULTS AND DISCUSSION

3.1 Environmental and Physiological Modulation of Microcystin Production

In order to attempt any modelling of toxin production by *Microcystis aeruginosa*, it was necessary to first determine the primary environmental modulators and physiological regulators of toxin production. This section describes the results obtained for environmental and physiological control of Microcystin production and is followed by the results of modelling attempts using the data generated here.

3.1.1 Medium N:P ratios and microcystin modulation

Hepatotoxin production in cyanobacteria has been shown to correlate to external stimuli such as light and nutrient concentrations and ratios although conflicting results have been reported. Specific growth rates and protein and microcystin content of *M. aeruginosa* PCC7806 and *M. aeruginosa* UV027 were determined under non-limiting batch culture conditions for a range of medium nitrogen and phosphorous atomic ratios.

Irrespective of absolute nutrient concentrations, $MCYST_Q$ was substantially increased in *M. aeruginosa* PCC 7806 and UV 027 at medium N:P ratios between 18 and 50 (5); these N:P ratios correspond to those between 16 and 50 described as optimum for $MCYST_{DW}$ (Lee *et al.*, 2000). However, unlike the data presented by Lee *et al.* (2000), these data do not include conditions where limitation of either nutrient occurred. Thus the reduced $MCYST_{DW}$ at lower and higher N:P ratios (Lee *et al.*, 2000) was not a function of retardation due to limitation of either nutrient and holds true irrespective of absolute concentrations of nutrients. In addition to this, the positive relationship between cellular N:P ratio and $MCYST_Q$ was shown to hold true for cellular N:P ratios between 1 and 18 (Downing *et al.*, 2005b). Vezie *et al.* (2002) also reported a significant interactive effect between N and P and MCYST production but did not present this data as a function of growth rate and showed strain variation in terms of optimal N:P ratios and MCYST content. Data published by Downing *et al.* (2005a) therefore clarifies the relationship between N:P, growth rate and $MCYST_Q$. Specifically, medium N:P ratios effect MCYST production in the following manner:

a) Increased N:P yields increased MCYST production if the uptake/assimilation of N is relatively higher than growth rate.
b) At high N:P ratios P is the growth limiting nutrient

c) Assimilated nitrogen levels depend on adequate carbon skeletons
d) Cellular P and N are reduced as a function of growth
e) Carbon availability for N assimilation requires adequate PAR and cellular P

These observations explain the range of optimum N:P ratios for $MCYST_Q$ and suggest that MCYST content (as $MCYST_{DW}$ or $MCYST_Q$) would be linearly related to growth rate under N-limited growth in accordance with data presented by Long *et al.* (2001) and Orr and Jones (1998). They also suggest that phosphorous limitation-induced reduction in carbon fixation, or light limitation due to low incident light or self-shading at increased biomass, would produce increases in MCYST as a result of increased cellular N:C ratios.

Additionally, total cellular protein content and intracellular microcystin content were significantly correlated to each other (r^2 = 0.81, $p<0.001$). Microcystin and protein content increased considerably as the maximum specific growth rate for the experimental conditions was reached. The significant correlation of cellular protein and microcystin content and their relative increase with increasing specific growth rate, within defined ranges of medium N:P ratios, suggests a close association between microcystin production and N:P ratio dependant assimilation of nitrogen, and resulting total cellular protein levels, which may be further modulated by specific growth rate.

3.1.2 Carbon fixation

Modulation of microcystin production has been extensively studied in both batch and continuous culture. Positive correlations with medium nitrogen, medium phosphorous, light intensity, inorganic carbon availability and growth rate have been reported. Negative correlations have been reported between microcystin content and medium phosphorous. The only reported quantitative relationship between any variable and microcystin production was that of growth rate. *Microcystis aeruginosa* PCC7806 was therefore cultured under continuous culture conditions in a bubble-lift reactor at a growth rate of 0.01 hr^{-1} in modified BG11 (constant phosphate concentration of 0.195 mM and varying nitrate from 0.125 mM to 18 mM) and sampled at steady states for analysis of cell numbers, microcystin content, cellular N and P, residual medium nutrient concentrations and carbon fixation rates.

The importance of the carbon fixation rate relative to the nitrogen uptake rate (and the related cellular N:C ratios that result) in modulating $MCYST_{CC}$ was determined (Downing

et al., 2005b). Experimental conditions yielding nitrogen-limited cultures and carbon-limited cultures revealed a significant increase in R_{MCYST} under carbon limitation and a significant decrease under nitrogen limitation at the same growth rate (Downing *et al.*, 2005b), corresponding to matching changes in cellular N:C ratios. Microcystin quotas increased 10 fold between the lowest and highest steady state values.

Cellular microcystin quotas showed significant positive correlation with both nitrate uptake and cellular nitrogen content, and were negatively correlated with carbon fixation rates, phosphate uptake, and cellular phosphorous. Thus the ratio of nitrate uptake to phosphate uptake, cellular N to cellular P, and nitrate uptake to carbon fixation were positively correlated to cellular microcystin.

Cellular C:N ratios remain constant for all specific growth rates where maximum carbon fixation rate or nitrogen uptake (or assimilation rate) is not exceeded. Similarly, cellular C:N ratios remain constant if either carbon fixation rate or nitrogen uptake (or assimilation rate) is not limited unless the maximum achievable rates differ from the stoichiometry required for growth. In the event of increased N uptake relative to carbon fixation, the distribution of carbon to nitrogen assimilation will increase. Any variation from the stoichiometry required for growth will be enhanced as a function of variation in growth rate. This was illustrated (Downing *et al.*, 2005b) where, for a single growth rate, increased $MCYST_{CC}$/nitrogen uptake was observed at reduced carbon fixation rates. The dependence of $MCYST_Q$ on nitrogen uptake and carbon fixation is illustrated by the following simple equation which describes the $MCYST_Q$ as a function of its rate of production relative to specific growth rate. R_{MCYST} is modified by the actual ratio of nitrogen-uptake/carbon-fixation divided by the ratio of maximum achievable rates. The minimum cell quota is defined by the product of the maximum microcystin production rate and the minimum value for nitrogen-uptake/carbon-fixation divided by the ratio of maximum achievable rates.

$$\tilde{M}CYST_Q = \frac{\bar{R}_{MCYST\ \max}}{\mu}\left(\frac{\phi}{\phi_{\max}}\right) + \bar{R}_{MCYST\ \max}\left(\frac{\phi_{\min}}{\phi_{\max}}\right)$$

where : $\phi = \bar{R}_{N\ uptake} \Big/ \bar{R}_{C\ fixation}$, $\tilde{M}CYST_Q$ = cellular MCYST quota

μ = specific growth rate,

and $\bar{R}_{MCYST\ \max}$ = maximum MCYST production rate

Chemostat data from Downing *et al.* (2005b) yielded the results shown in Figure 3.1.

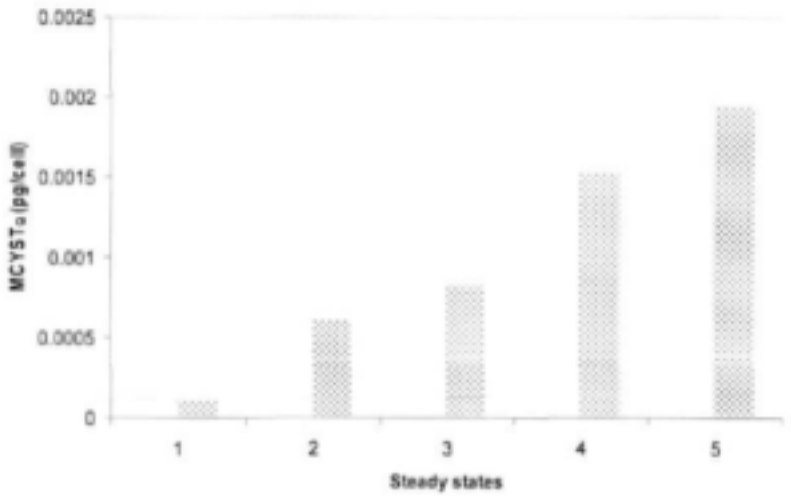

Figure 3.1. Modelled (shaded bars) and experimental (open bars) data comparison. Data from Downing et al. (2005b)

That carbon fixation depends on PAR remains a problem for modelling MCYST production, although the dependence of carbon fixation on P (Downing *et al.*, 2005b) and the dependence on fixed carbon for growth allows the use of environmental biologically available nitrogen and phosphorous, as well as growth as parameters, since growth rate incorporates carbon fixation rate as a function of PAR.

Cellular microcystin content is therefore controlled to a significant extent by variables other than growth rate, as was previously reported, with nitrogen being the most significant modulator. Batch culture in BG11 under identical conditions yielded increased

microcystin when nitrogen uptake exceeded relative growth rate, confirming the importance of nitrogen uptake in the modulation of microcystin content for a specific growth rate.

3.1.3 Specific growth rate

The positive relationship between growth rate and $MCYST_Q$ for two strains of *M. aeruginosa* was described by Downing *et al.* (2005a), in accordance with the linear relationship between R_{MCYST} and growth rate in phosphorous-limited continuous culture reported by Oh *et al.* (2000). In nitrogen-limited continuous culture the same relationship between growth rate and R_{MCYST} was observed by Long *et al.* (2001), leading to the conclusion that growth rate was the primary modulator of R_{MCYST} where growth rate was a function of cellular N quota (Long *et al.*, 2001)). The apparent contradiction that arises from a similar relationship occurring under phosphorous limitation, and the implication that growth rate is the primary modulator irrespective of limiting nutrient, is a result of increased cellular N:P ratios that occurred in phosphorous limited chemostats at higher growth rate (Oh *et al.*, 2000). Results presented by Downing *et al.* (2005a) clarify this. In the absence of N or P limitation induced reduction in growth rate, $MCYST_Q$ is a function of medium N:P ratio (which corresponds to cellular N:P ratio at non-limiting levels) and growth rate. Thus at low N:P levels (below 20), higher $MCYST_Q$ is observed at lower growth rates, while at higher N:P ratios increased $MCYST_Q$ was observed at relatively higher growth rates leading to the conclusion that the relationship between nitrogen assimilation and growth rate determines the rate of MCYST production (Downing *et al.*, 2005b). This is further illustrated by the positive relationship between $MCYST_{CC}$ and growth rate under non-limiting conditions, the absence of a noticeable relationship in nitrogen-limited cultures and a negative relationship in phosphorous-limited cultures. The uncoupling of nitrogen assimilation and growth related metabolism from cell division is illustrated in Figure 3.2 which shows the cellular protein and $MCYST_Q$ as a function of nitrogen uptake/phosphorous uptake (data from Downing *et al.*, 2005a). The increase in $MCYST_Q$ that occurs at elevated uptake ratios of N:P corresponds to a decrease in cellular protein which correlates with growth rate (Downing *et al.*, 2005a).

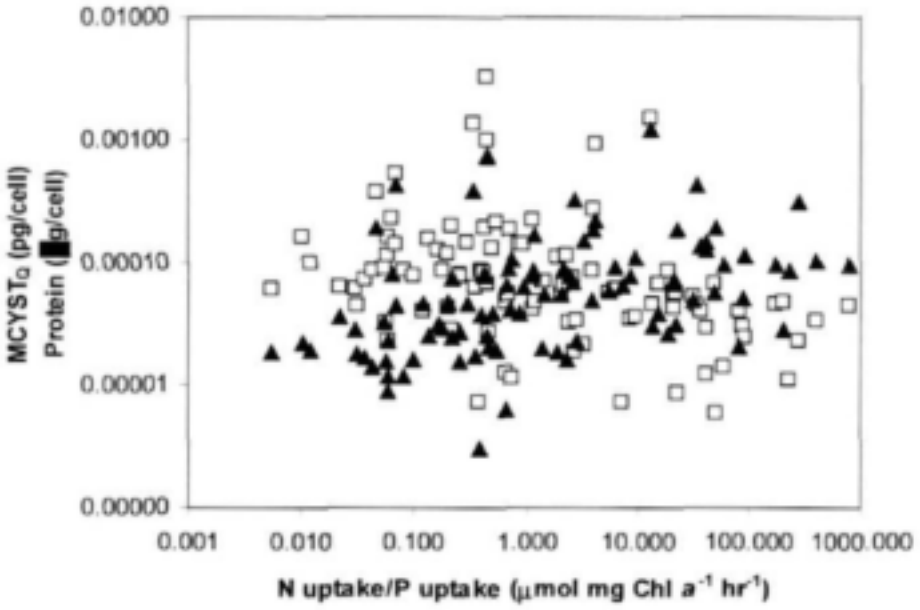

Figure 3.2. MCYST$_Q$ (□) and cellular protein (■) as a function of nitrogen uptake/phosphorous uptake during unlimited growth of *M.aeruginosa* PCC 7806 and UV 027.

Specific growth rate is therefore not the primary determinant of MCYST content in *M. aeruginosa*, but is a significant in terms of dilution of cellular assimilated nitrogen by growth and thus a reduction in available assimilated nitrogen for MCYST production. Where available nitrogen is taken up in excess of that required for growth, MCYST$_Q$ is elevated.

3.1.4 Transcriptional regulation of microcystin production

MCYST$_{CC}$ and cellular protein content are strongly correlated during unlimited growth at growth rates less than the maximum attainable growth rate (Downing *et al.*, 2005a) suggesting constitutive MCYST production. *mcy* transcript levels have, however, been show to vary as a function of light (Kaebernick *et al.*, 2000). No definitive mechanism for the regulation of microcystin production in *Microcystis aeruginosa* exists. Work on modulation of microcystin content has revealed a primary dependence on medium nitrogen and nitrogen-uptake:carbon-fixation ratios which yield cellular carbon:nitrogen ratio variations that are in turn altered by growth and the stoichiometric requirements for growth of these cellular pools. NtcA is the regulator of nitrogen metabolism in

cyanobacteria, regulating such metabolic activities as nitrate uptake, nitrate reduction, ammonium assimilation and carbon fixation rate. The possibility of NtcA regulation of *mcy* expression was therefore investigated. Specifically, the regulatory region between *mcyABC* and *mcyDEFGH-J* was analysed for potential NtcA binding sites, and the relative *mcyA* and *ntcA* mRNA levels were determined under conditions known to alter cellular nitrate-uptake:carbon-fixation rates. This document constitutes the first report of the putative NtcA binding site within the regulatory elements of the *mcy* operon and relationship between *mcy* and *ntcA* transcript levels in *Microcystis*. The putative NtcA binding site was identified overlapping the -10 region of the *mcyA* promoter and corresponding to motifs for known NtcA repressed genes. These results suggest a possible repression of *mcyA* transcription by NtcA.

In the absence of light, *mcyA* transcript levels increased relative to those of *ntcA* whereas high light effectively reduced *mcyA* transcript levels and increased those of *ntcA*. The increase in NtcA expression was expected, since high light would increase carbon fixation in the presence of adequate inorganic carbon and hence allow for increased nitrogen assimilation, requiring increased expression of NtcA up-regulated genes. The reduction (below detection sensitivity) of *mcyA* mRNA under high light conditions suggests that either MCYST has a function in enhancing carbon fixation (not required under the conditions of the experiment), or that NtcA does repress *mcy* transcription and may be enhanced by increased α-ketoglutarate.

The absence of a significant negative correlation between α-ketoglutarate and $MCYST_{CC}$ in all cultures except those where carbon fixation was inhibited may be interpreted as evidence for the putative NtcA binding site upstream of *mcyA* being non-functional. However, it is more likely that the increased cellular N:C ratio that resulted from carbon fixation limitation yielded increased Glu and Gln resulting in the increased $MCYST_{CC}$. The significant positive correlation between $MCYST_{CC}$ and α-ketoglutarate observed in cultures in the absence of light similarly suggests that NtcA is not involved in regulation. However, the temporal delay in synthesis of the multi-enzyme polypeptide-polyketide microcystin synthetase may be responsible. Such a delay was observed by Downing *et al.* (2005a), where increased $MCYST_{DW}$ was observed 24 hours after increased nitrate uptake. In addition to this, the potential function of MCYST as an up-regulator of carbon fixation would necessarily produce a positive correlation between α-ketoglutarate and

$MCYST_{CC}$, except where carbon fixation was inhibited, in which case a strong negative correlation would be expected as observed in D,L-glyceraldehyde treated cultures as the cell attempted to further increase carbon fixation rate.

Thus NtcA may be involved in regulation of MCYST production under conditions where carbon fixation is environmentally reduced. This is supported by the inverse relationship between *mcyA* and *ntcA* transcript levels observed when cultures were maintained in the absence of light, and by the retention of increased MCYST levels under these conditions (Downing *et al.*, 2005a). Clarification of the role of NtcA in MCYST regulation would however require a high temporal resolution analysis of both $MCYST_Q$ and $MCYST_{CC}$, NtcA cellular concentrations and *mcy* and *ntcA* mRNA levels, as well as confirmation of NtcA binding to the putative regulatory element.

3.1.5 Nitrogen assimilation metabolites and MCYST modulation

Microcystin content in *Microcystis aeruginosa* is modulated by cellular nitrogen:carbon ratio, a function of medium N:P ratio and growth rate which is in turn determined by medium N:P ratios and concentrations as well as photosynthetically active radiation (Downing *et al.*, 2005a; Downing *et al.*, 2005b). Cellular nitrogen status is representative of these factors. Cellular glutamine is an indicator of cellular nitrogen status, and cellular glutamate, as the first product of the GS-GOGAT cycle, is representative of assimilation. In order to ascertain whether cellular nitrogen status was the primary modulator of microcystin production in *M. aeruginosa*, cultures were either starved of nitrogen or phosphorous, or treated with selective inhibitors of glutamate synthase and glutamine synthetase after which the cellular glutamate, glutamine and microcystin concentrations measured. Negative correlations between cellular concentrations and quotas of microcystin and glutamate were obtained for combined data. However, in control cultures cellular microcystin concentrations were negatively correlated with glutamine. This variation was partly attributed to growth rate variations in the different treatments. Inhibition of glutamate synthase resulted in positive correlations between glutamate and glutamine and microcystin with all concentrations decreasing over the incubation period. Inhibition of glutamine synthetase yielded a positive correlation between microcystin and glutamate, with both metabolites decreasing over the incubation period, and no significant change in glutamine concentrations.

The relative dependence of MCYST production on cellular Glu and Gln was illustrated by selective inhibition of either glutamine synthase or glutamate synthetase. Where glutamate synthase was inhibited, a stronger and more significant correlation between $MCYST_{CC}$ and Gln was observed although $MCYST_{CC}$ was lower. This indicates a greater requirement for Glu, which was demonstrated by inhibition of glutamine synthetase yielding higher $MCYST_{CC}$ and a significant correlation with Glu.

Cellular Glu levels were highest at carbon fixation rates between 100 and 400 pmole ml biovolume^{-1} hr^{-1} and cellular N:P ratios between 40 and 60. Cellular Gln levels were highest at similar carbon fixation and cellular N:P ratios but were also elevated linearly with cellular N:P ratio at carbon fixation rates below 100 pmole ml biovolume^{-1} hr^{-1}. Cellular Gln:Glu ratio therefore decreased as a function of increasing cellular P and increased as a function of cellular N:P ratio. Cellular Glu was notably increased relative to cellular Gln at higher growth rates in nitrogen-starved cultures, whereas cellular Gln concentrations were higher than cellular Glu concentrations at all measured growth rates for phosphorous-starved cultures. $MCYST_{CC}$ and $MCYST_{Q}$ were significantly negatively correlated with cellular Glu and $MCYST_{CC}$ was highest at cellular Gln:Glu ratios between 1 and 3, corresponding to N:P ratios of between 60 and 100; these values were notably higher than those previously recorded. However, the N:P dependant increase in cellular Gln at low carbon fixation rates, corresponding to reduced levels of Glu, suggests Glu as the primary metabolite affecter of MCYST production. The negative correlation between $MCYST_{CC}$ and Glu was therefore due to racemization of L-Glu and inclusion into MCYST. Thus where Glu accumulated due to an insufficiency of additional amino acids for completion of MCYST, the negative relationship resulted. Similarly, where sufficient additional amino acids were available, MCYST was produced, driving the racemization by depletion of D-Glu. Verification of this concept would however require free amino acid analysis of cultures with varying $MCYST_{CC}$. It should, however, be noted that glutamate serves as a direct precursor to the MCYST components, aspartate [E.C.2.6.1.1], alanine [E.C.2.6.1.2] and leucine [E.C.2.6.1.6] whereas the requirement for the Gln is via its product, carbamoyl phosphate which is required, in addition to Glu, for arginine synthesis and subsequent inclusion into MCYST. Carbons C2 and C3 of Adda are derived from phenylalanine and methyl carbon groups on positions 2, 6, 8, and of Adda are derived from methionine (Moore, 1999). Thus in general for MCYST-LR, the required initial Gln:Glu ratio is substantially lower than the optimum ratio observed.

In addition to the stoichiometric requirements for MCYST production, P_{II}, a phosphoprotein that signals the carbon/nitrogen status of the cells, forms a tight complex with the key enzyme of the arginine biosynthetic pathway, *N*-acetylglutamate (NAG) kinase when un-phosphorylated, as occurs in the absence of α-ketoglutarate (Romero *et al.*, 1985). In complex with P_{II}, the catalytic activity of NAG kinase is strongly enhanced (Heinrich *et al.*, 2004), thereby increasing cellular Gln at the expense of cellular Glu. In addition to this, arginine is glucogenic, resulting in a further reduction in Gln under carbon limitation.

Evidence for this proposed amino acid composition-based modulation includes the more rapid increase in MCYST-RR than MCYST-LR under excess nitrogen and the subsequent greater and more rapid decline in MCYST-RR relative to MCYST-LR on retardation due to nitrogen depletion reported by Watanabe *et al.* (1989). Similarly Lee *et al.* (2000) showed a significant increase in MCYST-RR at N:P atomic ratios of between 16 and 50, whereas MCYST-LR increased but to a lesser degree at these N:P ratios; Oh *et al.* (2000) showed similar increases in MCYST-LR and MCYST-RR at N:P ratios from 14 to 24.

The requirement for α-ketoglutarate in nitrogen assimilation complicates analysis of any potential role in MCYST modulation. Under adequate nitrogen, increased α-ketoglutarate would be expected to correlate with MCYST as was the case in cultures incubated in the absence of PAR. However, when carbon fixation was inhibited a negative correlation was observed, while in the absence of medium nitrogen the correlation was positive. These seemingly contradictory results may be attributed to α-ketoglutarate enhancement of NtcA binding, but in the absence of conclusive evidence this remains speculation.

Cellular Gln:Glu ratios are therefore determined by available N:P ratio by virtue of P regulation of carbon fixation and the resulting changes in the ratio of α-ketoglutarate and unassimilated cellular nitrogen. Since Glu is almost directly available for MCYST synthesis, the balance between Glu and Gln determines R_{MCYST}. At elevated Glu, Gln and arginine products are partitioned between growth and MCYST production, thereby reducing R_{MCYST}. This further explains the decrease in MCYST observed at saturating PAR (Wiedner *et al.*, 2003; Wicks & Thiel, 1990).

Cellular microcystin levels therefore increased generally with cellular glutamine:glutamate ratios but were highest at ratios between 1 and 3, corresponding to previously reported optimal medium N:P ratios and carbon-fixation:nitrate-uptake ratios. This, and the variation in correlations obtained for different treatments suggests an additional regulatory mechanism besides the dependence on cellular nitrogen status. The reduction in cellular glutamate due to inhibition of glutamate synthase would result in increased α-ketoglutarate levels, suggesting a possible role for α-ketoglutarate in the regulation of microcystin production. This is supported by a putative NtcA binding site in a region of the *mcyA* promoter, in a position which is indicative of repression, and the fact that α-ketoglutarate enhances NtcA binding. Cellular α-ketoglutarate and microcystin concentrations were therefore determined for cultures incubated under conditions that induce variations in cellular α-ketoglutarate: (i) in the absence of light or nitrogen (ii) with double standard medium nitrogen, and (iii) in the presence of inhibitors of carbon fixation and glutamine synthesis. Significant positive correlations between cellular microcystin and α-ketoglutarate concentrations were observed both in cultures maintained in the dark and nitrogen starved cultures. Microcystin and α-ketoglutarate were negatively correlated when carbon fixation was inhibited. These contradictory data and the absence of notably increased microcystin when α-ketoglutarate was reduced ten-fold due to inhibition of carbon fixation suggests that α-ketoglutarate does not influence cellular microcystin concentrations. However, regulation of transcription and actual production of microcystin as a function of newly synthesized microcystin synthetase are necessarily temporally distinct and dependant on an adequate pool of nitrogen assimilation products for synthesis of the multi-enzyme complex. Such a pool requires adequate α-ketoglutarate as a precursor, complicating analysis and requiring high temporal resolution for clarity

3.2 Modelling Microcystin Production

The relatively simple model, based on carbon fixation rate and nitrogen uptake rate, described above, requires fairly accurate measurements of both cellular nitrogen uptake and carbon fixation rate. Such a model is therefore unsuitable for practical application. In order to develop a model based on environmental variables, it was first necessary to develop a conceptual model linking the cellular variables to environmental variables based on data described above.

3.2.1 A general model

Figure 3.3 shows a generalized conceptual model for the modulation of MCYST production in *Microcystis* based on the data presented above and in the cited literature. The model makes the initial general assumption that MCYST production is constitutive and that cellular carbon, nitrogen and phosphorous pools are equally available for growth and MCYST production. Pools represent available nutrients as opposed to total cellular content. Increases in cellular N:C pool ratios would occur where cellular ammonium concentrations were such that distribution of α-ketoglutarate to nitrogen assimilation would be increased. Similarly, increased carbon fixation in cells with reduced ammonium would result in accumulation of α-ketoglutarate and subsequent re-distribution of carbon pool elements to growth, thereby resulting in preferential use of nitrogen pool elements for growth and a reduction in MCYST production. The N:C ratio of MCYST-LR is 0.205 ($C_{49}H_{74}N_{10}O_{12}$), while generally the N:C ratio of *Microcystis* under unlimited growth is approximately 0.15 based on the Redfield ratio of C:N:P = 106:16:1 (Redfield *et al.*, 1963). Thus where cellular nitrogen and carbon pools are available in an N:C ratio below 0.15, growth rate generally corresponds to R_{MCYST} and $MCYST_Q$ is fairly constant. Where the pool N:C ratio exceeds 0.2 R_{MCYST} exceeds growth rate and $MCYST_Q$ is relatively higher. This is reflected in data published by Downing *et al.* (2005a), where a substantial increase in MCYST/protein (where protein represents distribution of nitrogen to growth) occurred at carbon fixation rates below 0.04 pmoles $cell^{-1}$ hr^{-1}.

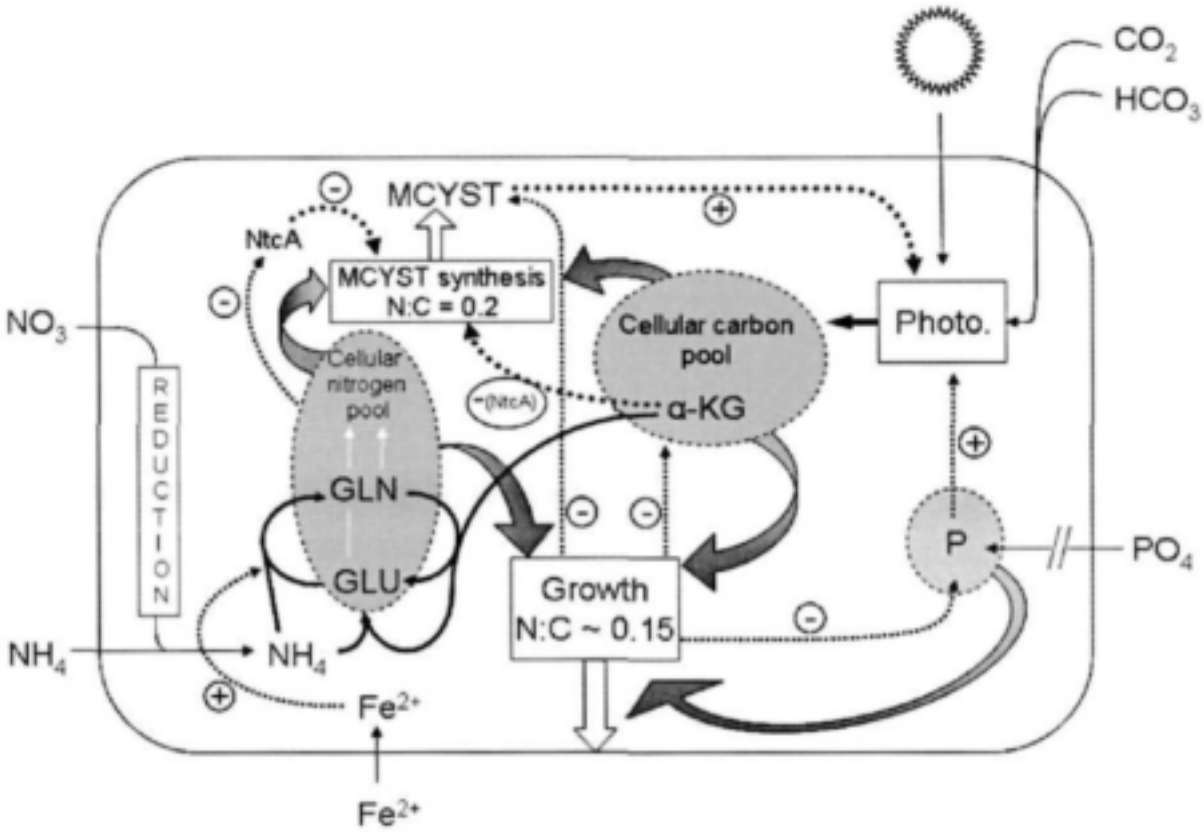

Figure 3.3. A generalized conceptual model for the modulation of microcystin production in Microcystis. Internalisation of nutrients and assimilatory metabolism is depicted with solid black arrows. Grey areas indicate cellular pools. Effects of components of the model on reactions is depicted with dashed lines. Speculative effects are represented by bold dotted arrows. Block arrows indicate use of the cellular pools for the processes indicated. White arrows represent transamination reactions. See text for details. Note: The generalized model ignores energy requirements.

At carbon fixation rates higher than this, the cellular N:C ratio was less than 0.15, while at carbon fixation rates below this value cellular N:C ratios ranged from 0.2 to 0.58, corresponding to a significant increase in $MCYST_Q$ at the same growth rate (Downing *et al.*, 2005b). The distribution of carbon and nitrogen pools on the basis of composition of MCYST and cellular C:N ratio therefore appears to be the determining factor in modulation of R_{MCYST} indicating a purely metabolic regulation of R_{MCYST}. Consideration of putative NtcA regulation of *mcy* expression reveals that under reduced pool N:C ratios, increased α-ketoglutarate and increased expression of NtcA would repress *mcy* expression enhancing the effect of pool N:C ratio. Where the pool N:C ratio was higher, NtcA levels would be reduced as would α-ketoglutarate levels, resulting in increased

mcy expression and increased R_{MCYST}. If this regulatory mechanism is valid, the result would be to limit the production of the polypeptide-polyketide synthetase and MCYST under conditions where insufficient nitrogen was available and sufficient carbon fixation was occurring.

$MCYST_Q$ is, however, further affected by growth rate due to dilution by growth. This effect will be enhanced if growth rate exceeds R_{MCYST}, with continual reduction in $MCYST_Q$ with each generation where pool N:C ratios are below 0.15. This model, however, allows for a continual minimum R_{MCYST} since the nitrogen pool is equally accessible to the multienzyme microcystin synthetase and growth related processes. In addition to this, D-GLU is the first amino acid added to Adda (Kaebernick & Neilan, 2001). Thus with a functional *mcyF* product, racemization of the first ammonium assimilation product would yield substrate for MCYST production. NtcA based repression would decrease because of reduction in absolute concentrations of α-ketoglutarate, thereby allowing a positive R_{MCYST} and retention of minimum $MCYST_Q$.

The optimal medium N:P ratio for maximum $MCYST_Q$ can be explained in terms of the model in that carbon fixation and P uptake are strongly correlated in the presence of adequate PAR (Downing *et al.*, 2005b) and cellular N:P corresponds to medium N:P. Thus at N:P ratios below 18, which corresponds to the Redfield ratio (as does the cellular N:P ratio of 16 reported by Lee *et al.* (2000)) $MCYST_Q$ is reduced (Downing *et al.*, 2005a). At N:P ratios between 18 and 50, which exceeds the Redfield ratio, maximum $MCYST_Q$ is observed. At N:P ratios exceeding 50 $MCYST_Q$ is reduced. The apparent contradiction with the model at N:P ratios exceeding 50 is inadequately explained by the increased growth rate observed at these N:P ratios (Downing *et al.*, 2005a), suggesting a further down-regulation of MCYST synthesis at these ratios. Data presented here did not include these levels and the effects on and of cellular C:N ratio could therefore not be determined. The effects of increased cellular ammonium would, however, include a depletion of glutamate, resulting in a reduction in available D-glutamate for incorporation into MCYST. This is supported by the higher correlation between Glu and $MCYST_{CC}$ than Gln and $MCYST_{CC}$, specifically the strong positive correlation observed in MSX-treated cultures, and by the reduction in $MCYST_{CC}$ at higher growth rates in phosphorous-starved cultures. The reduction in $MCYST_{CC}$ was also noted at maximum attainable Gln:Glu ratios.

This model therefore solves the apparent contradictions in literature and explains the following published results:

- *The increased MCYST at sub-optimal growth temperatures* (Watanabe & Oishi, 1985)
- The increased LD_{50} of cultures where increased temperatures resulted in increased growth rate (Watanabe & Oishi, 1985)
- Negative correlation between MCYST and growth rate (Van der Westhuizen & Eloff, 1985)
- Variation in relative concentrations of toxin variants (Lee *et al.*, 2000; Watanabe *et al.*, 1989)
- R_{MCYST} correlation with initial medium nitrogen (Orr & Jones, 1998)
- The negative relationship between carbon fixation rate and $MCYST_{DW}$ (Downing *et al.*, 2005b; Oh *et al.*, 2000)
- The effect of cellular C:N ratio on R_{MCYST} (Downing *et al.*, 2005b)
- The relationship between $MCYST_Q$ and the uncoupling of cell division and growth related metabolic processes (Wiedner *et al.*, 2003)
- The direct relationship between R_{MCYST} and growth rate in nitrogen limited chemostats (Long *et al.*, 2001)
- Positive correlations with non-saturating PAR under adequate nitrogen conditions and decrease in MCYST at saturating PAR Wiedner *et al.*, 2003)
- Optimum N:P ratio for elevated $MCYST_Q$ (Downing *et al.*, 2005a; Lee *et al.*, 2000)
- Distribution of MCYST producing genotypes in natural planktonic colonies (Kurmayer *et al.*, 2003)
- Distribution of MCYST in *Microcystis* hollow fibre photobioreactor cultures (Leukes *et al.*, 2003)
- The positive relationship between iron and MCYST (Utkilen & Gjølme, 1995) by virtue of the predominance of ferredoxin dependant GOGAT found in cyanobacteria (Okuhara *et al.*,1999).
- Variation in *mcy* transcript levels as a function of light (Kaebernick *et al.*, 2000).
- The presence of alternative *mcy* transcription start points for high and low light (Kaebernick & Neilan, 2001)
- The correlation between cellular carbohydrate and $MCYST_Q$ under non-limiting conditions (Lyck, 2004)

Additionally, the model illustrates that:

- The putative transcriptional regulation conforms to metabolic data.
- $MCYST_Q$ as a function of PAR relates to μ_{MAX} as a function of PAR

3.2.2 The function of microcystin

The generalized model for modulation of MCYST, substantiated by published data on MCYST modulation and that presented in this work, suggests several possible functions for MCYST. MCYST may function as a nitrogen storage molecule, although this seems unlikely given the ability of *Microcystis* to produce cyanophycin. Sufficient evidence to implicate MCYST in intercellular signalling is lacking. The increased $MCYST_{CC}$ under

reduced carbon fixation as a result of limitation in inorganic carbon at steady state (Downing *et al.*, 2005b), but positive correlation between α-ketoglutarate and MCYST under carbon fixation inhibition during batch culture, suggests diversion of available carbon stores into carbon skeletons for nitrogen assimilation and subsequent MCYST production. This indicates a possible role for MCYST in enhancement of carbon fixation that is supported by the cellular location of MCYST and unpublished data presented in Figure 3.4 which shows a positive relationship between photosynthetic efficiency of photosystem II (ϕPSII) and $MCYST_Q$.

Although chlorophyll *a* was observed to show a strong positive correlation with $MCYST_Q$ as has been noted previously (Lyck, 2004), the relationship represented in Figure 3.3 was independent of chlorophyll *a* cell quotas and specific growth rate, indicating a direct association between photosynthetic efficiency and $MCYST_Q$ thus adding support to the hypothesis that MCYST may be involved in enhancement of photosynthetic activity.

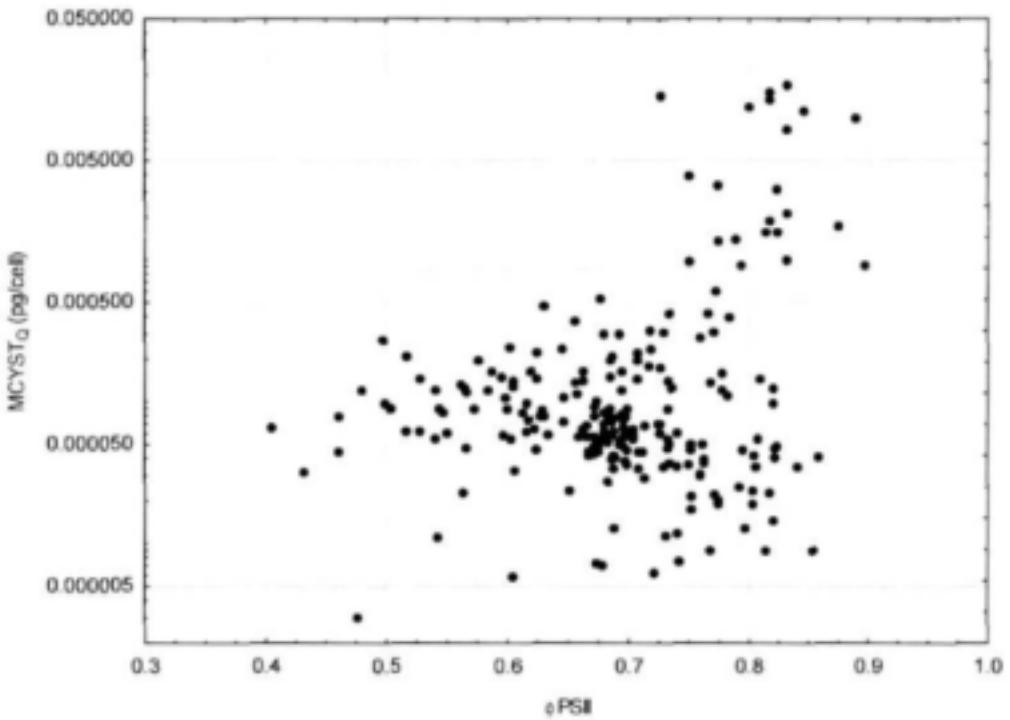

Figure 3.4. Relationship between ϕPSII and $MCYST_Q$. Data from Downing *et al.* (Downing *et al.*, 2005a)

3.2.3 Quantitative Models

The production of MCYST by *M. aeruginosa* is modulated primarily by nitrogen availability and potential for assimilation of nitrogen. Nitrogen assimilation potential is a function of carbon fixation rate and cellular energy status which in turn is largely determined by phosphorous availability, while available assimilated nitrogen use is distributed between housekeeping, growth, nitrogen storage and the production of MCYST and the MCYST synthetase multi-enzyme complex. Growth is a function of appropriate balance between cellular C, N and P (for a given temperature). Since carbon fixation rate has been shown to depend significantly on P, whereas growth rate depends on other parameters, notably temperature, the primary environmental modulators of MCYST are biologically available nitrogen and phosphorous, and those factors besides nutrition and light that determine growth rate. That these are the primary modulators is adequately demonstrated by artificial neural network modelling using error back-propagation.

Artificial neural network models are trained on a subset of available data which include both input and output values. Training a neural network using a gradient descent algorithm in which the mean square error between the network's output and the desired output is minimized, creates a function which is minimized iteratively by 'backpropagating' the error from the output nodes to the input nodes. Once this error is less than a specified threshold value, the network has converged and is considered to be trained. By sequentially reducing the number of different input variables, while retaining an error below the threshold value, and validating this model on additional data, primary effectors can be identified. Figure 3.5 shows the results of a trained network using only medium N, medium P and growth rate as the input nodes and the back-propagation algorithm.

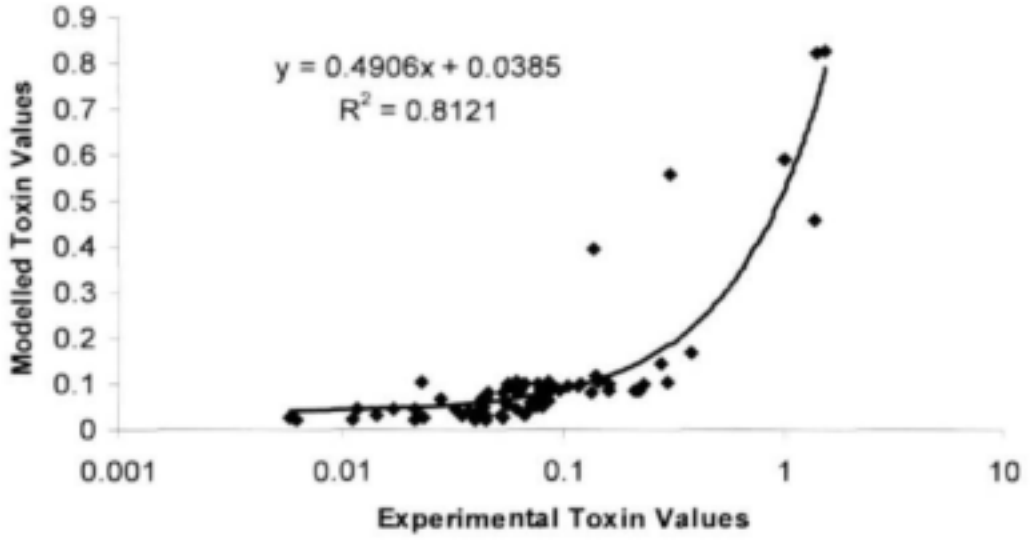

Figure 3.5. Modelled and experimental data comparison for a neural network model using only medium nitrogen and phosphorous and specific growth rate as input nodes. The network was trained on a random subset of data (Downing *et al.*, 2005a) and verified on the remaining data. Values represent $MCYST_Q$.

The effect of PAR on MCYST production, and the effects of temperature, was circumvented by inclusion of growth rate as an input node, since diurnal and local weather variations make modelling in natural environments more problematic. The inclusion of P is necessary as this is required for carbon fixation and hence nitrogen assimilation, independent of growth. R_{MCYST} relative to growth related metabolism is determined largely by the differences in stoichiometric requirements for carbon and nitrogen and the resulting variations in concentrations of different amino acids.

MCYST production is therefore increased when nitrogen assimilation exceeds the use of assimilation products by growth, where growth rate is determined primarily by carbon fixation rate (in turn dependant on phosphorous availability and PAR) and nitrogen assimilation rate. Reduction in PAR, inorganic carbon or phosphorous results in an increase in cellular N:C ratio and production of MCYST. Modelling of MCYST production or $MCYST_Q$ in natural conditions is complicated by variations in PAR but may be possible using biologically available environmental nitrogen and phosphorous, and monitoring specific growth rate.

The development and application of artificial neural network models appears to offer a simple modelling solution. However, the predictive accuracy of such models is limited

even under controlled growth conditions as shown in Figure 3.6 where the network was trained on output data delayed 48 hours from input data.

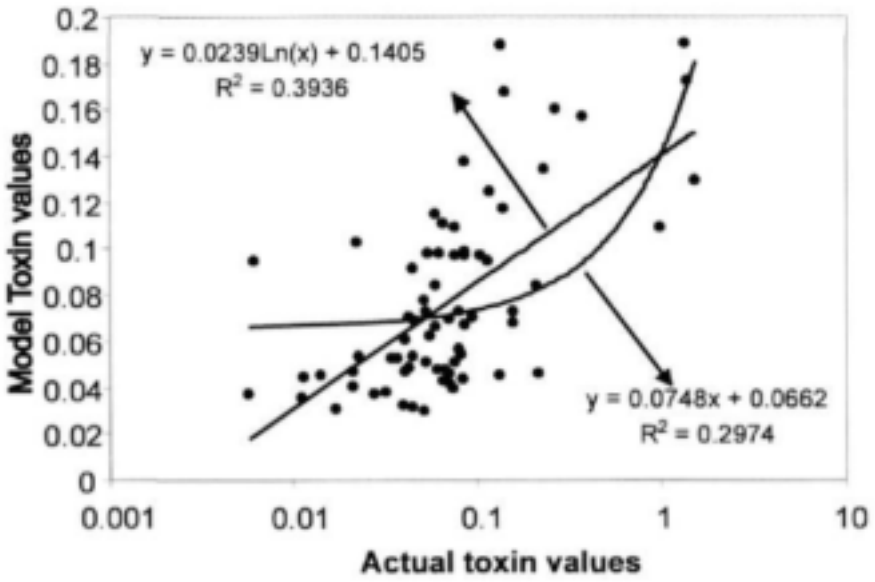

Figure 3.6. Modelled and experimental data comparison for a neural network model using only medium nitrogen and phosphorous and specific growth rate as input nodes. The network was trained on a random subset of data (Downing *et al.*, 2005a) with output data delayed 48 hours from input data, and verified on the remaining data.

Application of neural network models for prediction of MCYST based solely on environmental N, P and growth rate of *Microcystis* therefore appear to lack the necessary accuracy. However, inclusion of more input variables will probably increase the predictive accuracy, which would in turn require more extensive data collection to maintain any increased accuracy that may result.

More accurate and simpler models are possible with measurement of carbon fixation as shown in figure 3.1. However, the accuracy of field measurements and the need for sophisticated equipment and specialized training makes such an approach unfeasible. Similarly, temporal variations in PAR make this an unsuitable parameter for generating a predictive model. That nitrogen appears to be the primary modulator of microcystin production, and that carbon fixation is directly dependant on phosphorous, makes these suitable parameters for modelling. Variations in PAR and other parameters that influence growth related metabolism and actual cell division rate can be accommodated if growth

rate is used as a modelling parameter. Thus, using the simple equation for $MCYST_Q$ based on carbon fixation rate and nitrogen uptake rate, and incorporating growth rate (as shown in Fig 3.1), a simple model that requires relatively straightforward analyses to be performed to obtain the necessary values, is possible. Such a model is shown below. Previously published data (Downing *et al.*, 2005a) was used to assess the model. The results are shown in figure 3.7.

$$MCYST_Q = \frac{R_{MCYST\ \max}}{\mu}\left(\frac{\Psi}{\Psi_{\max}}\right) + R_{MCYST\ \max}\left(\frac{\Psi_{\min}}{\Psi_{\max}}\right)$$

$$\text{where : } \Psi = {}^{R_{N\ uptake}}\Big/_{R_{P\ uptake}}, \quad MCYST_Q = \text{cellular MCYST quota}$$

$$\mu = \text{specific growth rate,}$$

$$\text{and } R_{MCYST\ \max} = \text{maximum MCYST production rate}$$

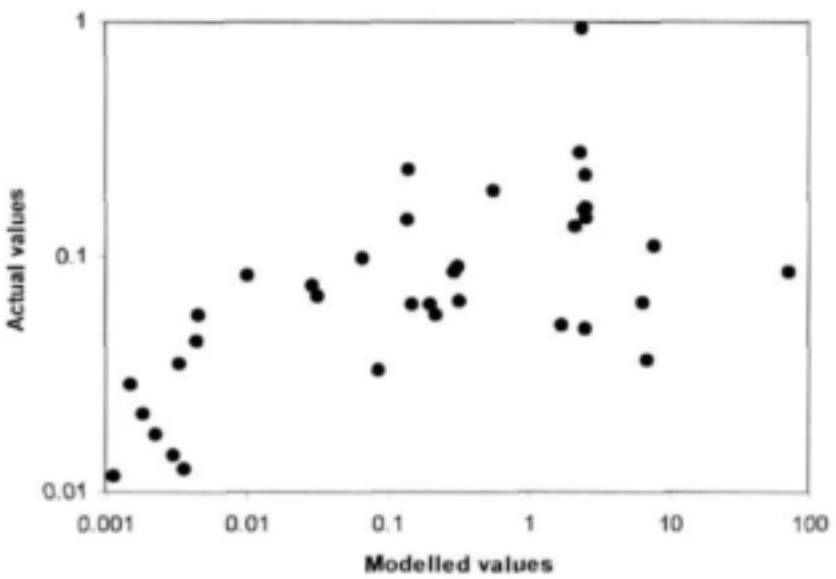

Figure 3.7. Modelled and experimental data comparison for a model using nitrogen and phosphorous uptake rates and specific growth rate as variables. Values for modelling were $MCYST_Q$ (pg cell^{-1}). Data from Downing *et al.* (2005a). The model lacks a maximum MCYST content function and modelled values therefore have no maximum limit.

Application of this model to real situations would however require measurement of nutrient uptake rates. In order to determine these, nutrient loadings would need to be

measured and calculations of nutrient levels based on these loadings and impoundment volume would be required. Additionally, these calculations could not adequately take into account additional abiotic factors affecting nutrient levels. Thus any model for general use should be based on actual environmental nutrient levels which can be estimated from potential nutrient loadings.

The original intention of this work was to produce a structured model for MCYST production which reflected the regulatory mechanisms. However, growth rate has been shown to be a significant modulator of MCYST content. Structured models for growth are highly complex and those attempted to date do not include significant variables such as PAR and temperature. Application of a model including such variables would of necessity require high temporal resolution monitoring of these variables and although such a process could be automated, the cost implications are high. However, a semi structured model which incorporates nutrient uptake and partitioning of assimilated nutrients between MCYST production and growth is an alternative. Modelling of growth based only on nitrogen and phosphorous is in itself a complex and not particularly accurate.

Artificial neural network models suggest that medium N, P and growth rate are sufficient to predict toxin levels. Exclusion of nutrient effects on growth, and measuring biovolume or cell numbers allows a semi-structured model including nutrient uptake. Such an approach would limit errors in calculation of nutrients taken up, since the uptake rates are explicit in the model as a function of environmental concentrations. Thus losses to abiotic processes are not introduced. Such a model is illustrated in figure 3.8.

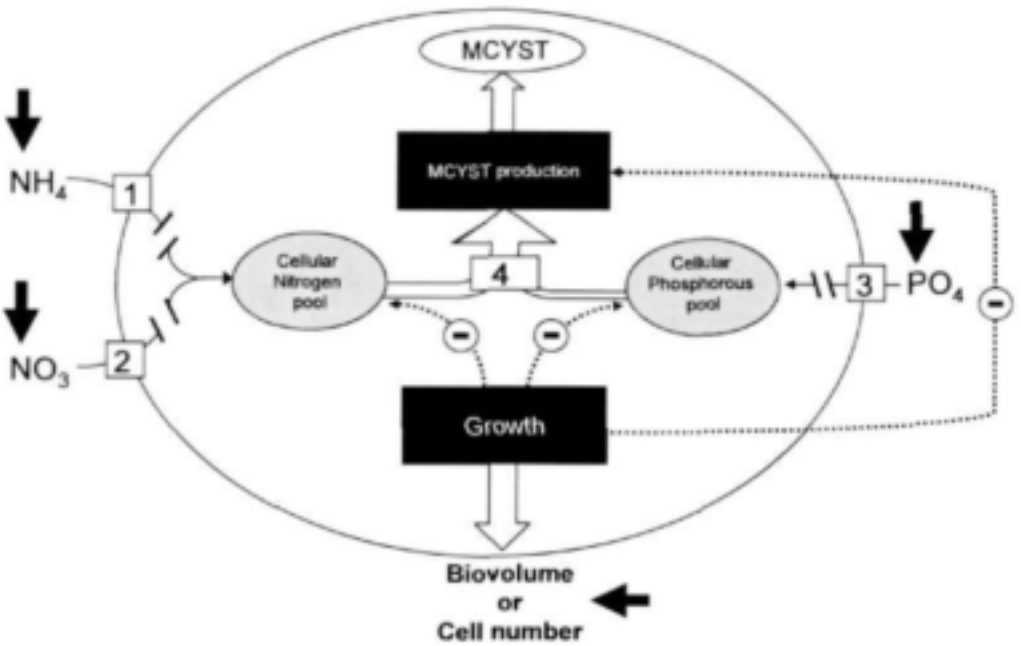

Figure 3.8. Modelled reactions are depicted by numbered blocks. Additionally, the dotted lines represent dilution by growth which is also incorporated into the model. Black block arrows indicate the variables to be measured. Reaction 4 is based on the ratio of available N and P and proceeds at a faster rate when the N:P ratio exceeds the stoichiometric requirements for growth.

In its simplest form, this model may take the form of that for which results are depicted in figure 3.7 where ψ would be replaced by the product of the rate equations for uptake of nutrients and their respective nutrients. Thus the need for numerical approximation of a set of differential equations is not required. Inclusion of a function in which the stiochiometry for MCYST production determines the rate of production based on cellular nutrient pools, yields an equation where the controlling cellular factors determine the MCYST production rate in a more structured fashion. The inclusion of the term where the ratio of uptake rates relative to the optimum ratio for growth, allows the removal of the term R_{MCYST} x $(\Omega_{min}/\Omega_{max})$, the function of which was to define a minimum cell quota. In the following model, simple monod kinetics are applied to substrate internalisation and toxin production is based on mass action.

$$MCYST\ \ volume^{-1} = \frac{R_{MCYST\ \max}}{\mu}\left(\frac{\Omega}{\Omega_{opt}}\right) \times population\ \ volume^{-1}$$

$$\text{where}\ :\ \Omega = \frac{\left(\frac{V_{NO_3\max}\cdot[NO_3]}{K_{m\,NO_3}+[NO_3]} + \frac{V_{NH_4\max}\cdot[NH_4]}{K_{m\,NH_4}+[NH_4]}\right)}{\frac{V_{PO_4\max}\cdot[PO_4]}{K_{m\,PO_4}+[PO_4]}}$$

μ = specific growth rate,

and $R_{MCYST\ \max}$ = maximum MCYST production rate

Figure 3.9 shows the results of this model compared to actual data for *M. aeruginosa* PCC 7806 (Downing *et al.* (2005a)).

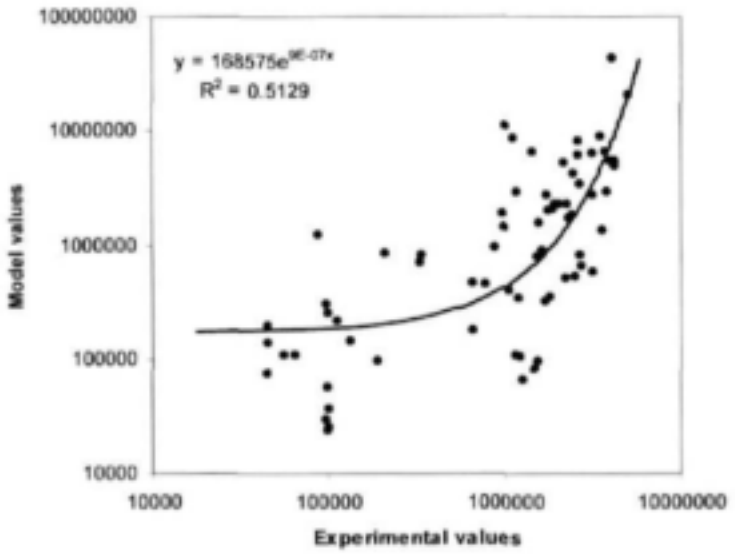

Figure 3.9. Comparison of modelled and experimental data for *M. aeruginosa* PCC 7806 (Downing *et al.* (2005a)). Model was fitted using the following constants. K_m and V_{max} values as previously determined, R_{MCYST} as previously determined (Downing *et al.* (2005b)). Values are per volume.

Figure 3.10 shows the results from data for *M. aeruginosa* UV027. The improved fit obtained with $y = 0.038x^{1.1799}$ $R^2 = 0.372$ compared to the exponential fit highlights strain differences, but may have been partly due to the use of protein phosphatase inhibition to measure MCYST since different variants have slightly different inhibitory activity against the enzyme and UV 027 produces a number of variants. In addition to this, differences in production rates of different variants have been reported under varying environmental conditions (Redfield *et al.*, 1963, Vezie *et al.*, 2002).

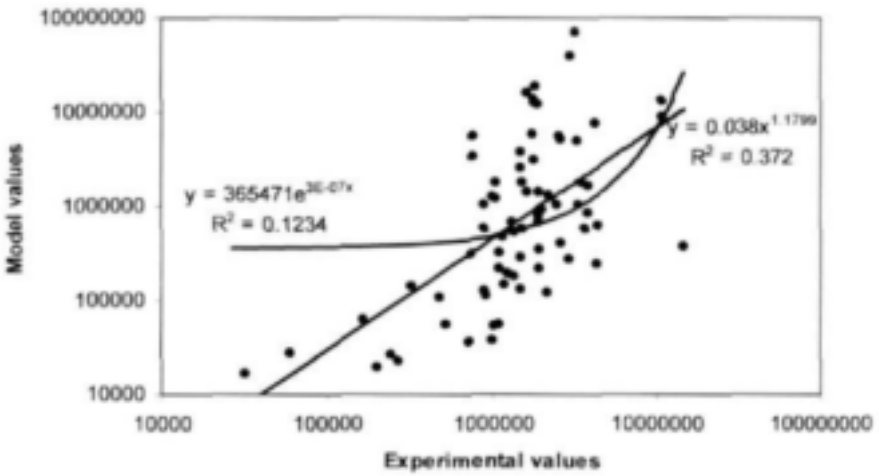

Figure 3.10. Comparison of modelled and experimental data for *M. aeruginosa* UV 027 (Downing *et al.* (2005a)). Model was fitted using the following constants. K_m and V_{max} values as previously determined, R_{MCYST} as previously determined (Downing *et al.* (2005b)). Values are per volume.

The validity of the model for combined data (PCC 7806 and UV 027) is shown in figure 3.11.

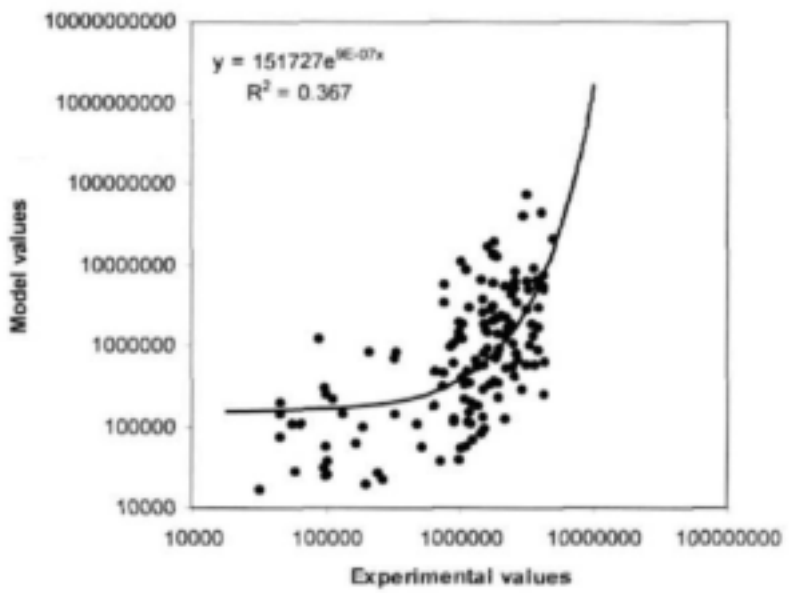

Figure 3.11. Comparison of modelled and experimental data for *M. aeruginosa* UV 027 and PCC 7806 (Downing *et al.* (2005a)). Model was fitted using the following constants. K_m and V_{max} values as previously determined, R_{MCYST} as previously determined (Downing *et al.* (2005b6)). Values are per volume.

Figures 3.12 and 3.13 show the comparison of modelled and experimental values for PCC 7806 using nutrient and growth rate data from batch culture under various nutrient regimes (Downing *et al.*, 2005a) with MCYST values modelled on 46 hr old and 69 hr old nutrient and growth rate data.

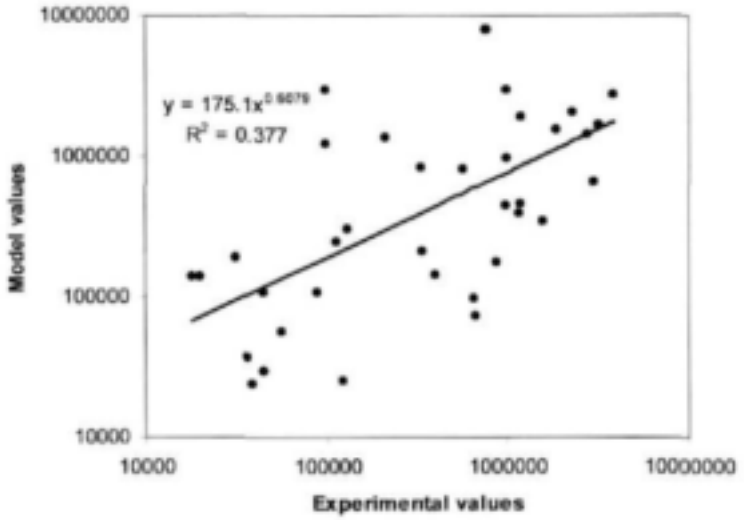

Figure 3.12. Comparison of modelled and experimental data for *M. aeruginosa* PCC 7806 (Downing et al. (2005a)) with 46 hr toxin level prediction. Model was fitted using the following constants. K_m and V_{max} values as previously determined, R_{MCYST} as previously determined (Downing *et al.* (2005b)). Values are per volume.

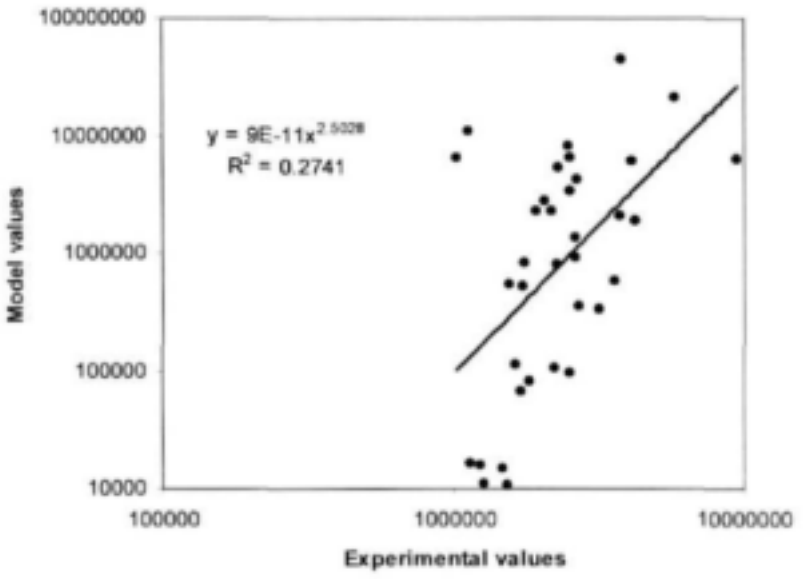

Figure 3.13. Comparison of modelled and experimental data for *M. aeruginosa* PCC 7806 (Downing et al. (2005a)) with 69 hr toxin level prediction. Model was fitted using the following constants. K_m and V_{max} values as previously determined, R_{MCYST} as previously determined (Downing *et al.* (2005b)). Values are per volume.

As can be seen from these figures, the predictive accuracy decreases with increasing time. Additionally the prediction is skewed, with lower model values lower than experimental and higher model values substantially higher than experimental values. This is relatively simple to correct but such a correction would constitute loss of biological meaning of the model. Such coefficient corrections would additionally be impoundment and population structure specific and as such could be implemented on a case by case application of such a model. Increased accuracy of MCYST level prediction would also result if nutrient loading could be forecast.

4. CONCLUSIONS

In conclusion, this work shows that the primary environmental modulators of MCYST production by *Microcystis* that are suitable for simple measurement, and use in predictive models, are environmental nitrogen and phosphorous and growth rate. However, given the complexity of the regulation of microcystin production and the strain variation in toxin production, it does not seem likely that a single general model will emerge that allows accurate long-term prediction of toxin levels unless accurate nutrient loading and PAR forecasts are possible. Despite this, reasonably accurate short term prediction does seem possible, as does a general prediction on whether toxin levels will increase or decrease over periods possibly as long as one month. Work currently in progress includes the validation and refinement of these types of models on fed batch cultures of various strains and communities. Artificial neural network models also appear to hold some promise and should be further investigated. This work clearly shows the primary environmental modulators that should constitute the input nodes of such models and therefore substantially adds to the current knowledge base on predictive modelling of microcystin production. Similarly, the models presented here are the first reported models of toxin production based on environmental variables and as such constitute a major advancement in both the understanding of the regulation of microcystin production and the approach to both structured and a-posteri modelling of toxin production by *M. aeruginosa*.

4.1 Applicability and possible applications of research results

The models presented here constitute the basis for developing applied toxicity models. Application and refinement of these models should allow practical application once the necessary field testing and refinement have occurred.

4.2 Recommendations for future research

The fundamental questions relating to microcystin function should be addressed so as to fully understand the biology underpinning the conceptual model developed and presented here. The absence of adequate fundamental research hampers the development of applicable models, processes and systems for applied water management. The models developed here constitute a starting point and should be further refined and modified for continuous or semi-continuous data input and tested on suitable reservoirs.

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The strategy proposed here is based on comparing and contrasting the current directions and emerging issues in international cyanobacterial research with identified South African needs. From this analysis a suite of key research issues have been formulated..l

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