THE ROLE OF NITROGEN IN THE REGULATION OF MICROCYSTIN CONTENT IN *MICROCYSTIS AERUGINOSA*

TIMOTHY GRANT DOWNING

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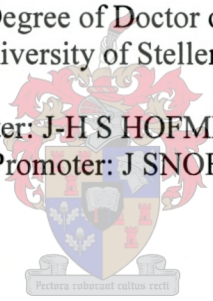

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Summary

Several genera of cyanobacteria produce a range of toxins. The increased rate of eutrophication of surface fresh waters due to anthropogenic inputs has resulted in more frequent and severe cyanobacterial bloom events. Such bloom events make impoundments unsuitable for recreational use and increase the cost of production of potable water due to the necessity for removal of toxins released from cells during the purification process. *Microcystis aeruginosa* is the major freshwater bloom-forming toxic cyanobacterium. Concentrations of the hepatotoxin, microcystin, are highly variable in blooms. Published literature on environmental conditions leading to increased microcystin production was often contradictory and in many cases did not consider all relevant parameters. However, environmental nitrogen and phosphorus, temperature and light, and growth rate were implicated in regulation of toxin content. The purpose of this work was therefore to investigate environmental factors (specifically nitrogen and phosphorus) and cellular activities (specifically carbon fixation and nitrogen uptake rates and growth rate) involved in the modulation of microcystin production in *M. aeruginosa* in order to clarify the role of these parameters, and in an attempt to identify regulatory mechanisms for microcystin production. Environmental nitrogen, phosphorus and growth rate were shown to co-modulate microcystin production in *M. aeruginosa*. Adequate phosphorus is required for photosynthetic carbon fixation. Phosphorus uptake by *M. aeruginosa* is strongly correlated with carbon fixation rate. Although microcystin content increased with increasing nitrogen:phosphorus ratios in culture medium, under phosphorus limitation microcystin content was lower irrespective of nitrogen concentrations. This observation and the requirements for fixed carbon for nitrogen assimilation therefore prompted investigation of the effects of cellular carbon fixation and nitrogen uptake in the modulation of microcystin production. Microcystin production was found to be enhanced when nitrogen uptake rate relative to carbon fixation rate was higher than that required for balanced growth. The cellular nitrogen:carbon ratio above which microcystin concentrations increased substantially, corresponded to the Redfield ratio for

balanced growth. Investigation of potential regulatory mechanisms involving the cyanobacterial nitrogen regulator, NtcA, yielded putative NtcA binding sites indicative of repression in the microcystin synthetase gene cluster. In culture, the polypeptide synthetase module gene, *mcyA*, and *ntcA* were inversely expressed as a function of carbon-fixation:nitrogen-uptake potential. However, no increase or decrease in microcystin production could be linked to either glutamine, glutamate or α-ketoglutarate, metabolites that are involved in regulation of *ntcA*. The role of NtcA in regulation of microcystin production could therefore not be confirmed. In conclusion, these data suggest that microcystin production is metabolically regulated by cellular C:N balance and specific growth rate. The primary importance of nitrogen and carbon was demonstrated by a simple model where only nitrogen uptake, carbon fixation and growth rate were used to predict microcystin levels. The model also explains results previously described in literature. Similarly, an artificial neural network model was used to show that the carbon fixation dependence on phosphorus allows accurate prediction of microcystin levels based on growth rate and environmental nitrogen and phosphorus.

Opsomming

Verskeie genera van sianobakterieë produseer 'n verskeidenheid van toksiene. Die toename in die tempo van eutrofikasie van varswater oppervlaktes as gevolg van antropogeniese insette veroorsaak al hoe meer en al hoe erger sianobakteriële infestasies. Dit veroorsaak probleme vir ontspanninggebruik van hierdie waters en verhoog die koste van produksie van drinkbare water as gevolg van die noodsaak om die toksiene wat deur die selle gedurende die suiweringsproses vrygelaat word te verwyder. *Microcystis aeruginosa* is die belangrikste varswater bloeisel-vormende toksiese sianobakterium. Die konsentrasie van die hepatotoksien mikrosistien is hoogs varieerbaar in sulke bloeisels. Gepubliseerde literatuur oor die omgewingskondisies wat lei na verhoogde mikrosistienproduksie is dikwels weersprekend en neem in vele gevalle nie al die relevante parameters in ag nie. Desnieteenstaande word omgewingstikstof, fosfor, temperatuur en lig, asook groeisnelheid, geïmpliseer in die regulering van toksieninhoud. Die doel van hierdie navorsing was dus om omgewingsfaktore (spesifiek stikstof en fosfor) en sellulêre aktiwiteite (spesifiek koolstoffiskering en die snelheid van stikstofopname en van groei) betrokke by die modulering van mikrosistienproduksie in *M. aeruginosa* te ondersoek in 'n poging om die rol van hierdie parameters te verstaan en om regulatoriese meganismes vir mikrosistienproduksie te identifiseer. In hierdie studie is aangetoon dat omgewingstikstof en fosfor sowel as groeisnelheid mikrosistienproduksie in *M. aeruginosa* ko-moduleer. Genoegsame fosfor word benodig vir fotosintetiese koolstoffiksering. Fosforopname deur *M. aeruginosa* korreleer sterk met die snelheid van koolstoffiksering. Alhoewel mikrosistieninhoud toegeneem het met 'n toename in die stikstof:fosfor verhouding in die kultuurmedium, was die mikrosistieninhoud onder kondisies van fosforlimitering laer ongeag die stikstofkonsentrasie. Hierdie waarneming, tesame met die noodsaak van gefikseerde koolstof vir stikstofassimilering, het gelei na 'n studie van die effekte van sellulêre koolstoffiksering and stikstofopname op die modulering van mikrosistienproduksie. Dit is gevind dat mikrosistienproduksie verhoog was wanneer die snelheid van stikstofopname relatief tot die snelheid van

koolstoffiksering hoër was as die waarde wat benodig word vir gebalanseerde groei. Die sellulêre stikstof:koolstof verhouding waarbo mikrosistienkonsentrasies beduidend verhoog is stem ooreen met die Redfield verhouding vir gebalanseerde groei. 'n Ondersoek na potensiële reguleringsmeganismes waarby die sianobakteriële stikstofreguleerder NtcA betrokke is het gelei na die ontdekking van moontlike NtcA bindingsetels; dit kan dui op die repressie van die mikrosistiensintetase geengroepering. Onder kultuurkondisies is gevind dat die geen vir die polipeptiedsintetase module, *mcyA*, en *ntcA* omgekeerd uitgedruk word as 'n funksie van koolstofopname:stikstofopname potensiale. Geen toename of afname in mikrosistienproduksie kon egter gekoppel word aan òf glutamien, òf glutamaat, òf α-ketoglutaraat nie, metaboliete wat betrokke is by die regulering van *ntcA*. Die rol van NtcA in die regulering van mikrosistienproduksie kon dus nie bevestig word nie. Die gevolgtrekking is dus gemaak dat mikrosistienproduksie metabolies gereguleer word deur die C:N balans en die spesifieke groeisnelheid. Die primêre belang van stikstof en koolstof is gedemonstreer deur 'n eenvoudige model waarin slegs stikstofopname, koolstoffiksering en groeisnelheid gebruik word om mikrosistienvlakke te voorspel. Die model verklaar ook resultate wat tevore in die literatuur beskryf is. Soortgelyk is 'n artifisiële neurale netwerkmodel gebruik om te toon dat die afhanklikheid van koolstoffiksering van fosfor akkurate voorspelling van mikrosistienvlakke gebaseer of groeisnelheid en omgewingstikstof en fosfor moontlik maak.

List of Abbreviations

Adda:	3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4,6-dienoic acid
ANOVA:	analysis of variance
BSA:	Bovine serum albumin
EGTA:	ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
ϕPSII:	quantum efficiency of photosystem II
GDH:	glutamate dehydrogenase
Gln:	glutamine
Glu:	glutamate
LD_{50}:	Dose resulting in death of 50% of target group
mcy:	microcystin synthetase gene
MCYST:	microcystin
MCYST–LR:	microcystin - leucine and arginine
MCYST–RR:	microcystin - arginine and arginine
MCYST–YM:	microcystin - tyrosine and methionine
$MCYST_{DW}$:	microcystin content per dry weight of cells (mass/biomass)
$MCYST_{Q}$:	microcystin cell quota (mass/cell)
$MCYST_{CC}$:	microcystin cellular concentration
MSX:	L-methionine-D,L-sulfoximine
μ:	specific growth rate
μ_{MAX}:	maximum specific growth rate
PAR:	photosyntehtically active radiation
PP1A:	protein phosphatase 1A
PP2A:	protein phosphatase 2A
PSI:	photosystem I
PSII:	photosystem II
PUFA:	polyunsaturated fatty acids
R_{MCYST}:	specific rate of microcystin production
RPA:	RN'ase protection assay
SS:	steady-state
Tris HCL:	2-Amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride

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"Everything should be made as simple as possible, but not simpler"

Albert Einstein

Chapter 1: Introduction

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 in this dissertation 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.

Thesis structure

Chapter 2 addresses 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.

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 in Chapter 3 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.

No definitive mechanism for the regulation of microcystin production by *Microcystis aeruginosa* is known. Work presented in chapters 2 and 3 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. 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. The results presented in Chapter 4 show 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.

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 and α-ketoglutarate are therefore indicators, if not direct regulators, of NtcA expression levels. Chapter 5 presents the results 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.

Data presented in Chapter 6 show 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.

Chapter 7 summarizes the results of the preceding chapters and presents a generalized model for environmental regulation of microcystin production in *M. aeruginosa*. The model is supported by unpublished data of photosynthetic efficiency, changes in distribution of cellular nitrogen between protein and microcystin as a function of carbon fixation or growth rates, and two simple models based on the environmental variables concluded to be of primary importance.

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.

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 (4). 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.

Microcystin

The microcystins are non-ribosomally synthesized (5) 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 (30). Figure 1.1 shows the generic structure for microcystins.

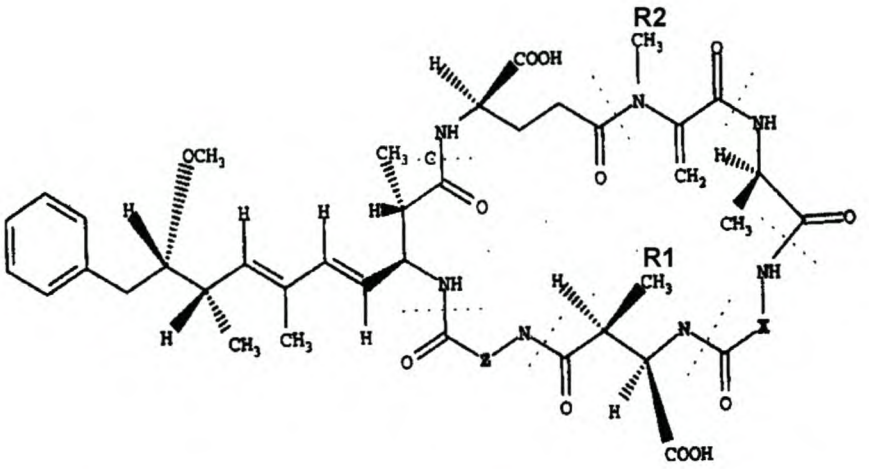

Figure 1.1 The generic structure for microcystins. Microcystin-LR has an L-leucine at position X and L-arginine at position Z (Ito *et al.*(10)). R1 and R2 indicate methyl groups of D-erythro-β-methylaspartic acid and N-methyldehydroalanine, respectively.

Over 60 variants (varying mostly in the L-amino acids) of microcystin (MCYST) have been identified (30).

Toxicity

Microcystins are potent hepatotoxins that are selectively taken up via a multispecific bile transport system. (6, 29). 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) (21). Toxicity is attributed to the inhibitory activity of microcystins on protein phosphatases 1 and 2A (PP1 and PP2A) (20) leading to hyperphosphorylation of the cellular cytoskeleton. The resultant sinusoidal collapse leads to severe intrahepatic heamorhaging and in severe cases, death (7). Protein phosphatase inhibition was demonstrated *in vitro* for MCYST-LR, MCYST-YR (tyrosine-arginine) and MCYST-RR (arginine-arginine) in mouse liver lysates (42) and *in vivo* for MCYST-LR and MCYST-YM (tyrosine-methionine) in liver homogenates of mice exposed to toxin (28). MCYST-RR, with an LD_{50} in mice of 600 μg/kg, is less toxic than MCYST-LR which has an LD_{50} 50 μg/kg (32). 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 (10). 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* (12) which form a multienzyme complex (33). 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 (12). 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 (22). 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 (23).

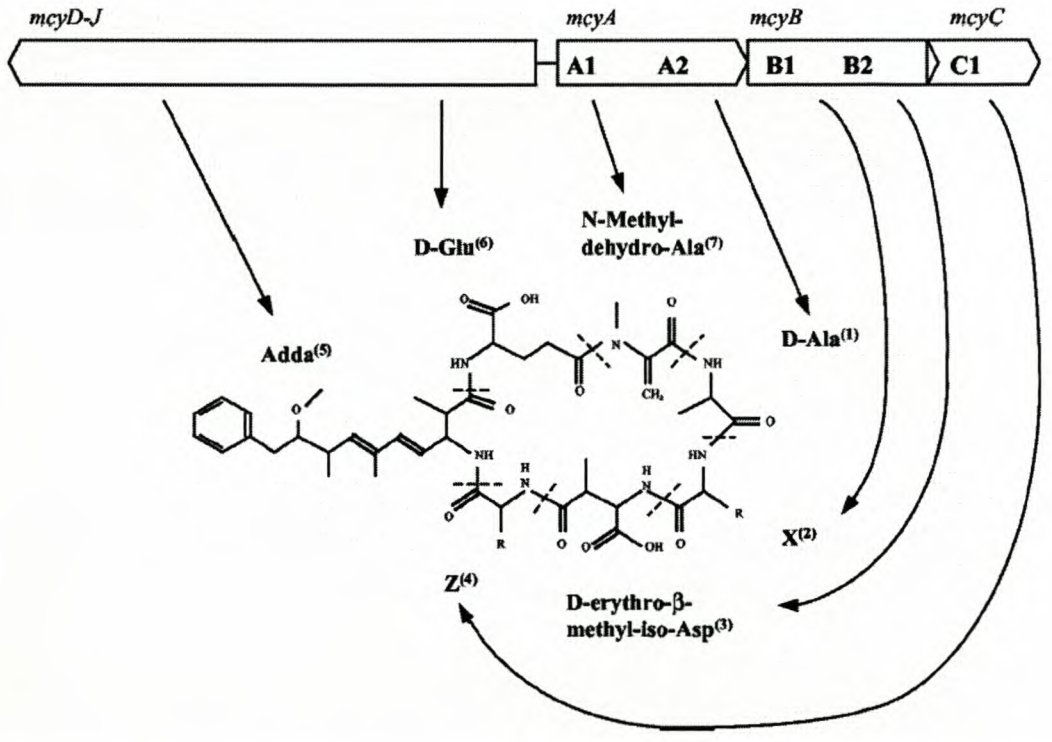

Figure 1.2 Role of *mcy* elements in biosynthesis of microcystin (Mikalsen *et al.* (22)). See text for details.

Polyketide synthesis domains in *mcyD, mcyE* and *mcyG* gene products are responsible for the fatty acid side chain of Adda (12). *mcyF* encodes a glutamate racemase (24). Putative tailoring functions have been assigned to *mcyI* and *mcyJ* (12). *mcyH* shows high identity to ABC transporter genes (27) 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.

Environmental modulation of microcystin

Toxin concentrations have been shown to be highly variable in natural environments (31, 36, 3). Research over the past two decades on modulation of MCYST production and cellular content has focused largely on the effects of specific growth rate (9, 18, 25, 26, 35, 38, 39, 40), growth phase (16), incident light intensity and quality (16, 18, 19, 34, 35, 38, 40, 41), macro-morphology or cellular arrangement and colony size (15,17), temperature (8, 9, 14, 35, 38, 41), availability and rates of uptake of nutrients, specifically nitrogen and phosphorus (16, 18, 25, 26, 34, 35, 38), and inorganic carbon availability and rates of carbon fixation (35). 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 (2). 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 mas 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 (1) 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 (8) 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 (38) cultured *M. aeruginosa* M228 under batch culture conditions in MA medium (9) 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 (35) for strain UV-006 cultured in modified BG11 (14). However, as for strain M228, this corresponded to an increase in growth rate for the same temperature difference. Wicks and Thiel (41) 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 (38) for temperatures between 25 and 32 °C, and by van der Westhuizen and Eloff (35) 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 (38). 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.* (39) 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 (9) 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 (26) 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.* (18) 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 (25). 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.* (40) 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 (35, 38). Toxicity in natural bloom samples also increased with increasing solar radiation and primary productivity (41). Watanabe and Oishi (38) reported a correlation between light intensity and toxicity, with LD_{50} values dropping from 36.9 mg/kg to 9.65 mg/kg 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 (34) 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 (34).

A subsequent study on *M. aeruginosa* PCC 7806 in continuous culture by Wiedner *et al.* (40) 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 (16, 18, 40), but it should also be noted that both increases (18) and decreases (19) 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 (38), van der Westhuizen and Eloff (35) nor Utkilen and Gjølme (34) measured corresponding variation in medium nutrients (specifically nitrogen and phosphorus). However, Orr and Jones (26) and Long *et al.* (18) 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.* (25) 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 (18) 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.* (16) 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.* (37) 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 were 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.* (15) 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.* (17) 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.

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* (13).

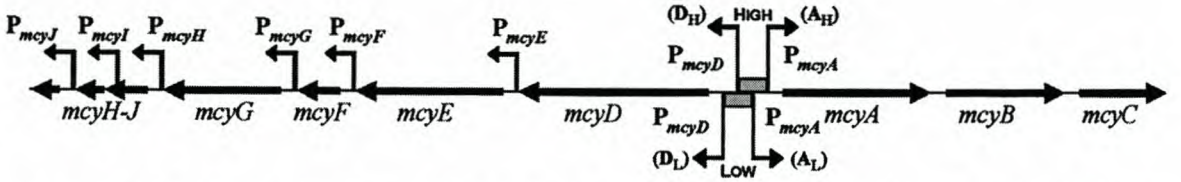

Figure 1.3 Transcriptional organization of the microcystin synthetase gene cluster *mcyABCDEFGHIJ*, showing putative promoters for *mcyEFGHIJ* and alternative promoters identified for *mcyA* and *mcyD*. D_H and A_H represent active promoters under high light and D_L and A_L under low light for *mcyD* and *mcyA* respectively. (Kaebernick *et al.* (13)).

Kaebernick *et al.* (11) 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 (13) 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 (11). 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 (40). 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.

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.

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Chapter 2: *Medium N:P ratios and specific growth rate co-modulate microcystin and protein content in *Microcystis aeruginosa* PCC7806 and *M. aeruginosa* UV027

Abstract

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 phosphorus atomic ratios. 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 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 dependent assimilation of nitrogen, and resulting total cellular protein levels, which may be further modulated by specific growth rate.

* Downing TG, Sember C, Gehringer MM, Leukes W (2005) *Microbial Ecology* 49:1-6

Introduction

Microcystis aeruginosa blooms are common and widespread in eutrophied freshwater impoundments. Many strains of this cyanobacterium produce potent hepatotoxins (3), the microcystins (MCYSTs), as a component of their diverse range of bioactive secondary metabolites. The soluble cyclic heptapeptide microcystins are hepatotoxic by virtue of their accumulation in the liver via multi-specific bile acid transporters (6) and inhibition of serine/threonine protein phosphatases 1 and 2A (15). An understanding of environmental conditions leading to increased toxicity would allow improved risk management for recreational exposure to, or consumption of, waters where *M. aeruginosa* blooms occur.

Despite the many studies that have been undertaken to determine the environmental factors governing microcystin production (10, 18, 21, 23, 24, 25, 29), no clear multi-parameter environmental regime has been identified that enhances microcystin production by *M. aeruginosa*. Both batch and continuous culture investigations on toxin production by strains of *M. aeruginosa* have focused on the effects of nitrogen (N) and phosphorus (P) concentrations (13, 14, 17), photon irradiance levels (8, 23, 25), pH and temperature (25). In most cases such studies have attempted to limit the environmental modulation to one specific factor.

Quantitative relationships between growth rate and MCYST content have been shown under N limitation (14) where the stated possibility that cellular MCYST quota is a function of N is based on the N quota control of specific growth rate (μ) (5). It follows that control of MCYST quota by μ as a function of P limitation should be observed if the modulating variable is μ and not specifically N. MCYST content under P limitation has however been shown to be higher at lower μ when expressed per dry weight (17). This was attributed to differences in dry weight obtained under P and N limiting conditions, since the MCYST production rate increased linearly with growth rate (17) in accordance with an earlier report on batch cultures of *M. aeruginosa* (18). Thus under either P or N limitation MCYST content appears to be determined

by μ reflecting the view that MCYST production is greatest under favorable growth conditions (20) and appears to be constitutively expressed (21).

Investigations in the absence of strict limitation of either N or P and batch culture toxin data have shown an increase in MCYST levels during exponential growth and a decline during stationary phase when measured per dry weight (28) or as cellular content (13). A substantial decrease in MCYST levels has been reported under reduced N conditions, but not under similarly reduced P conditions (27), and under reduced medium N and inorganic carbon (4). Lee *et al.* (13) also reported a slight negative correlation with total P content and a high correlation with total N content while Watanabe & Oishi (27) describe a slight increase with increasing P. Distinctly higher MCYST content was obtained by Lee *et al.* (13) at a total N:P atomic ratios of 16:1 and 50:1. Growth under these conditions in modified SW medium containing 104 μmol N l^{-1} and 6.5 μmol P l^{-1} yielded maximum MCYST content during exponential growth with a decline as the culture entered stationary phase. These data further suggest an optimal N:P ratio for MCYST production where maximal production occurs at such a ratio during exponential growth.

In an attempt to determine the relative significance of N:P ratio and growth rate on cellular MCYST content under non-limiting conditions, we therefore used batch culture with varying initial N:P ratios, and subsequent monitoring of the medium N:P ratio. Cellular MCYST content was determined under these conditions, for *M. aeruginosa* PCC7806 and UV027, prior to a decrease in μ as a result of any limitation.

Methods

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 $BG11_0$ (19) 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.

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) (11). Total cellular protein was measured at 550 nm using bicinchoninic acid as described by Smith *et al.* (22) using 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 (1, 26) 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 (20mM), 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 (2), and medium orthophosphate by the phosphomolybdate method (16).

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).

Results

Data presented are representative of conditions where individual nutrient concentrations exceeded the lower of either Km for specific uptake or Ks for growth for that nutrient, and where retardation of growth rate due to shading or other factors had not occurred. Data therefore represent conditions where 1 < N:P < 83 (unless otherwise stated) and µ > 0. N and P values reported are for the sample and not the original concentrations in the medium prior to inoculation. Correlation analysis of medium nutrient concentrations and ratios and cellular MCYST, protein and chlorophyll *a* content (Table 2.1) showed significant positive correlations between cellular MCYST, cellular protein, cellular chlorophyll *a* and µ. Correlations for all values of N:P (including N:P < 1) yielded significant positive correlations for cellular MCYST, cellular protein and cellular chlorophyll with medium N, and significant negative correlations of cellular MCYST and chlorophyll *a* with medium P, resulting in significant positive correlation of cellular MCYST with medium N:P ratio (data not shown). This indicated possible N limitation under these conditions and was

the basis for exclusion of this data from figures 2.1 and 2.2, and from table 2.1, where non-limiting conditions were analyzed.

Table 2.1 Summary of correlation coefficients between medium nutrient concentrations and ratios, cellular constituents and growth rates for *M. aeruginosa* UV027 and *M. aeruginosa* PCC7806.

	Variables[b]					
Parameters and Strains	N	P	N/P	Protein	Chl. *a*	μ
Cellular MCYST						
M. aeruginosa UV027	0.0972	0.1521	-0.0548	0.8112***	0.6265***	0.5029**
M. aeruginosa PCC7806	0.3006	0.1444	0.1236	0.8877***	0.8404***	0.7998***
Cellular Protein						
M. aeruginosa UV027	0.2526	0.2272	0.0386	----	0.6689***	0.4581**
M. aeruginosa PCC7806	0.3208	0.1830	0.1191	----	0.6711***	0.8145***
Cellular Chlorophyll *a*						
M. aeruginosa UV027	0.1885	0.0970	0.0392	0.6689***	----	0.0894
M. aeruginosa PCC7806	0.1814	0.0854	0.0697	0.6711***	----	0.6371***
Specific growth rate (μ)						
M. aeruginosa UV027	0.1419	0.1656	0.0584	0.4581**	0.0894	----
M. aeruginosa PCC7806	0.4025*	0.1433	0.2613	0.8145***	0.6371***	----

[a]N = 38 for *M. aeruginosa* UV027 and N = 36 for *M. aeruginosa* PCC7806. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$
[b]N: medium nitrogen, P: medium phosphorus, N/P: medium N:P atomic ratio, Protein: cellular protein content, Chl. *a*: cellular chlorophyll *a* content, μ: specific growth rate

Effects of N:P ratio

M aeruginosa strains PCC7806 and UV027 showed similar trends in MCYST content across the tested range of medium N:P atomic ratios. Both strains had elevated (95 percentile) MCYST content at medium N:P ratios of between 18 and 51 (Fig 2.1A.), with UV027 having maximum MCYST content (3.3 pg .cell^{-1}) at NP = 31.03 and PCC7806 having a maximum of 1.71 pg .cell^{-1} at N:P = 37.47.

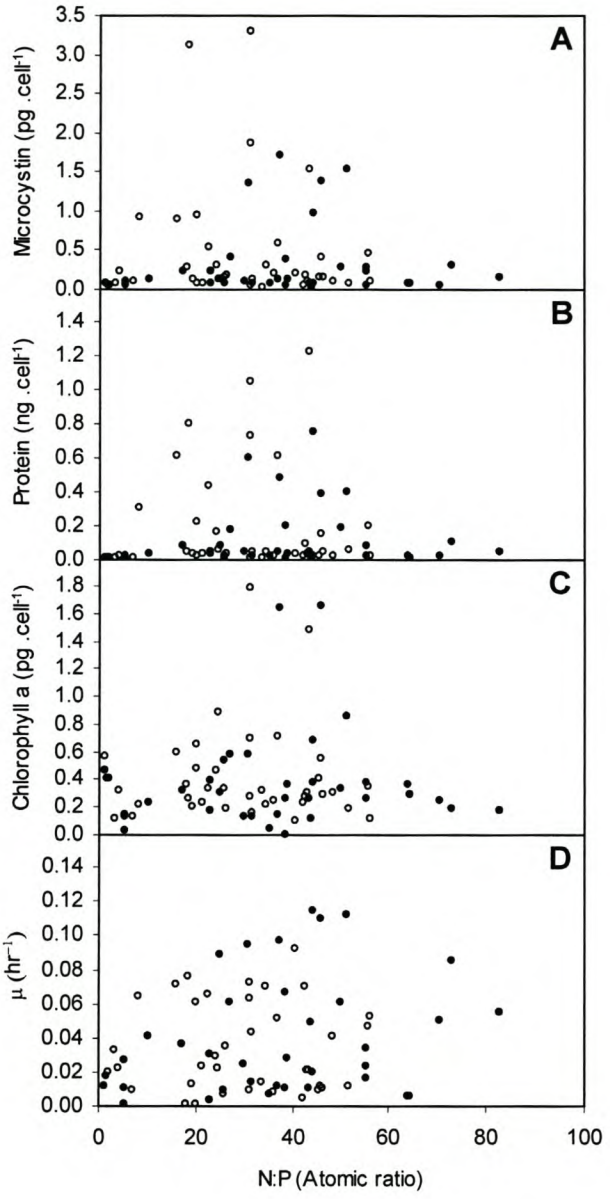

Figure 2.1. Cellular constituents [Microcystin pg .cell^{-1}(A), Total protein ng .cell^{-1}(B), Chlorophyll *a* pg .cell^{-1}(C), and μ .hr^{-1}(D)]of batch cultures of *M. aeruginosa* PCC7806(●) and *M. aeruginosa* UV027(○), for various medium N:P atomic ratios (1 < N:P < 83).

No nutrient limitation occurred for N:P ratios between 18 and 51, with 60 μM < P < 176 μM and 1.13 mM < N < 4.22 mM for UV027 cultures and 57 μM < P < 76 μM, and 0.98 mM < N < 3.95 mM for PCC7806 cultures. Nutrient concentrations remained non-limiting for higher N:P ratios (P > 46 μM and N > 2.6 mM for UV027 cultures, and P > 32 and N > 2.4 mM for PCC7806 cultures). Protein content showed similar trends to MCYST content for both strains. UV027 and PCC7806 had maximum protein content at N:P = 43.46

(1.23 ng .cell^{-1}) and N:P = 44.13 (0.75 ng .cell^{-1}) respectively (Fig 2.1B). The maximum protein content for each strain increased with increasing N:P ratio, to these values without nutrient limitation (as described above). Chlorophyll *a* content showed the same trend as both protein and MCYST content with highest values obtained at N:P ratios between 31 and 46 and no striking difference between strains (Fig 2.1C). UV027 did attain consistently higher maximum chlorophyll *a* levels at N:P ratios between 1 and 31. A range of μ values were obtained across the tested N:P ratio spectrum as was expected for varying N and P concentrations, with the maximum recorded μ increasing with increasing N:P ratio up to N:P = 44 for PCC7806 (μ=0.114 hr^{-1}) and N:P = 40 (μ=0.092 hr^{-1}) for UV027 (Fig 2.1D).

Effects of specific growth rate

MCYST, protein and chlorophyll *a* content showed increasing trends with increasing specific growth rate for both strains, and a dramatic increase as the maximum specific growth rate was approached. Increased cellular MCYST and protein content was achieved at μ > 0.09 .hr^{-1} for PCC7806 (95 percentile) and μ > 0.06 .hr^{-1} for UV027 (96 percentile) (Fig. 2.2A and 2.2B). Similar trends were observed for chlorophyll *a* in PCC7806, but the trend was less clear in UV027. The maximum recorded specific growth rate for UV027 (corresponding to P = 75.8 μM and N = 3.08 mM) did not correspond to the highest MCYST, protein or chlorophyll *a* content although this single anomalous result did not follow the observed trend which was similar to that for PCC7806.

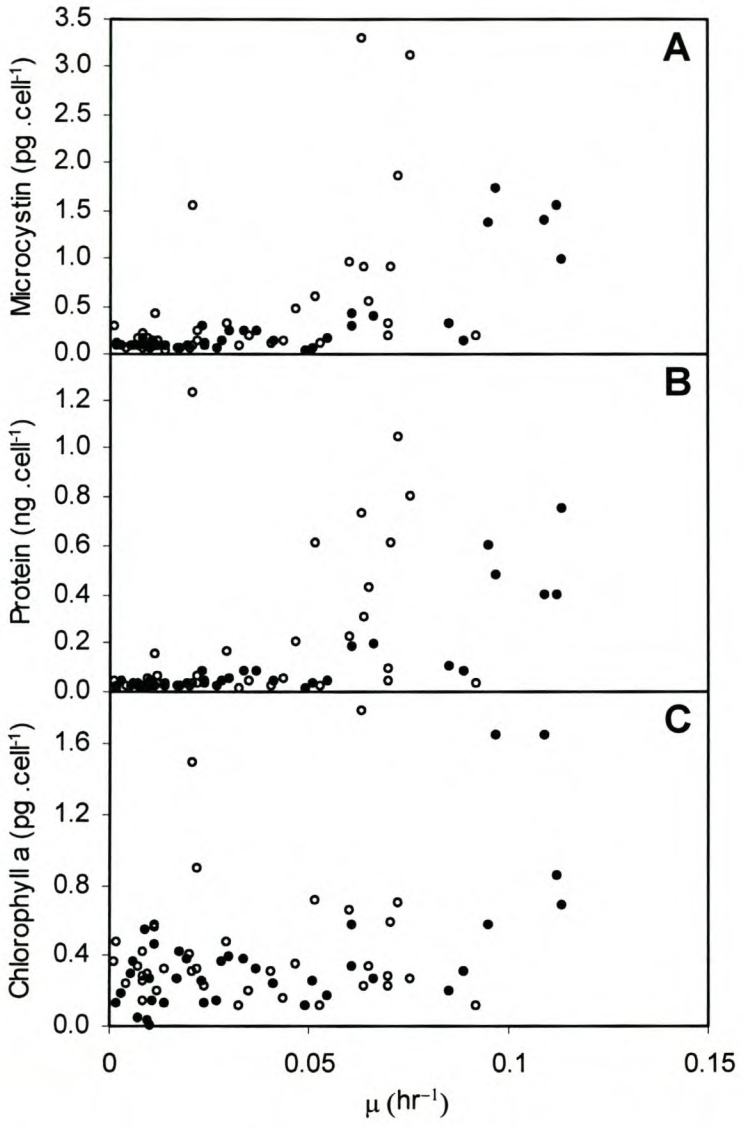

Figure 2.2. Cellular constituents [Microcystin pg .cell^{-1}(A), Total protein ng .cell^{-1}(B), and Chlorophyll *a* pg .cell^{-1}(C)] of *M. aruginosa* PCC7806(●) and *M. aruginosa* UV027(○), for specific growth rates recoded for batch culture in medium with various N:P atomic ratios (1 < N:P < 83).

Inclusion of data where N:P < 1 highlights the co-dependence of cellular MCYST on medium N:P ratio and growth rate. Normalized [normalized value = (recorded value – mean for category)/mean for category] MCYST levels plotted against normalized μ values for categorized N:P values shows this co-dependence (Fig. 2.3). For N:P <1 the highest MCYST levels were observed at relatively lower μ for both strains. UV027 exhibited an increase in MCYST with μ for categorized data representing 1 ≤ N:P < 10 (Fig 2.3B) while

PCC7806 had the opposite trend (Fig. 2.3A). For the category 10 ≤ N:P < 20 UV027 had a single higher value for MCYST content at a higher μ value. PCC7806 had intermediate values around the μ mean but insufficient data points to realize a trend, while UV027 showed increasing MCYST with increasing μ. PCC7806 had no observable trend although the highest MCYST value was obtained around μ_{mean} while UV027 had increased MCYST levels at higher μ values for 20 ≤ N:P < 30. For 30 ≤ N:P < 40 both strains showed increased MCYST levels with increasing μ while for 40 ≤ N:P < 50 PCC7806 also showed an increase in MCYST with increasing μ, although UV027 had a single high MCYST level at a normalized μ of –0.56.

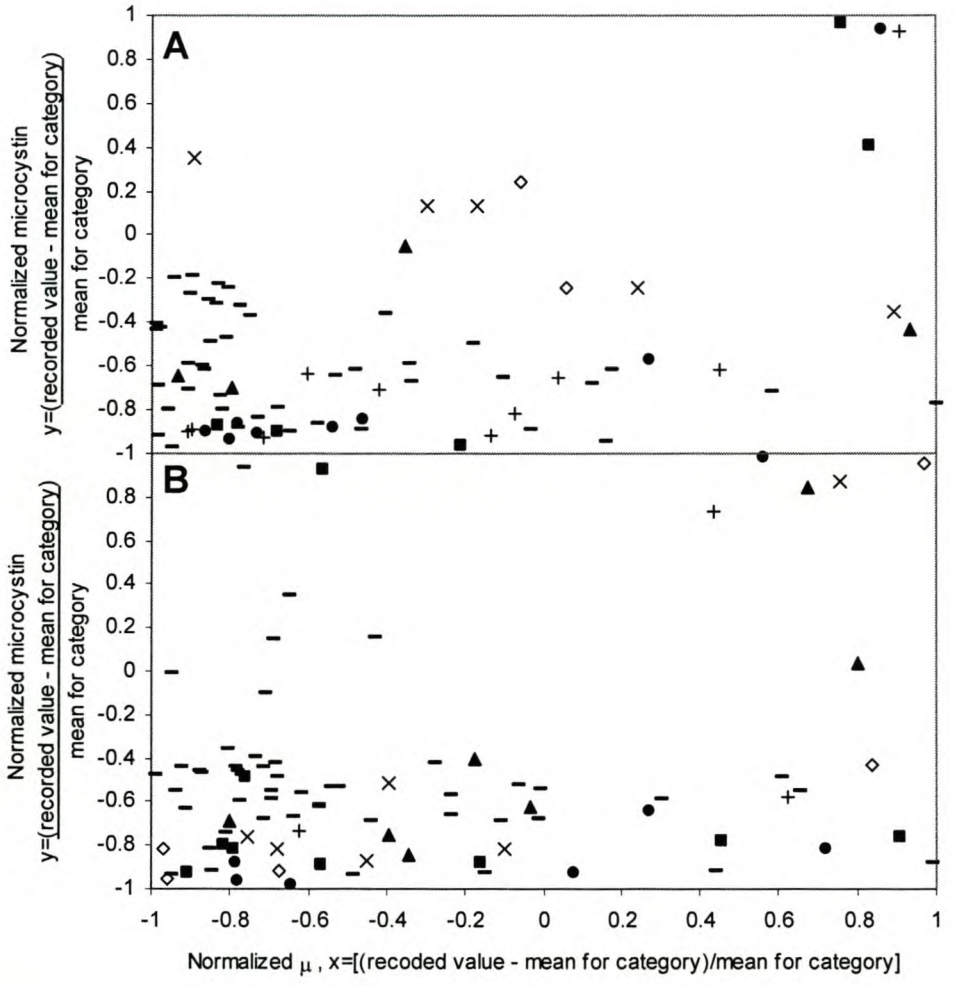

Figure 2.3. Normalized (actual value – mean/mean) MCYST content and μ for categories of N:P ratios where N:P < 1 (–), 1 ≤ N:P < 10 (x), 10 ≤ N:P < 20 (◇), 20 ≤ N:P < 30 (▲), 30 ≤ N:P < 40 (●) , 40 ≤ N:P < 50 (■), N:P ≥ 50 (+) for PCC.

Exclusion of this point yielded an increasing trend with μ. For N:P ≥ 50 PCC7806 exhibited a similar trend with a substantial increase in MCYST at higher μ values, while no such trend was observed with UV027.

Discussion

Quantification of MCYST by protein phosphatase inhibition is absolute only for strains producing a single microcystin variant. *M. aeruginosa* PCC7806 produces only microcystin LR and a small amount of D-Asp LR. The ratio of LR to D-Asp LR does not vary significantly and in all cases we have analyzed HPLC quantification has correlated to protein phosphatase inhibition using MCYST LR as a standard. UV027 on the other hand is known to produce several variants of the toxin and presumably (as has been shown for other strains producing more than one variant) the ratios may vary. However, the sum of the toxicity of the variants is highly correlated to the PPI test. Notwithstanding this, physiologically it would be preferable to have quantification of all variants, although with the number of samples concerned this was not practical. That the UV027 toxin data follows the same trend as the PCC7806 indicates that the variation in inhibition as a result of variation in ratios and resulting variant toxicity is less significant than the overall effect observed.

Cellular MCYST content correlated strongly with cellular protein content which was highest at medium N:P ratios between 18 and 51, irrespective of absolute nutrient concentrations. Higher specific growth rates were achieved within the same range of nutrient ratios irrespective of nutrient concentrations. These results confirm and expand on previous reports on MCYST levels as a function of N:P ratio, with maximum levels obtained for 18 < N:P < 51 compared to the increased MCYST levels obtained by Lee *et al.* (13) for *M. aeruginosa* UTEX 2388 at N:P = 16 and N:P = 50 at significantly lower nutrient concentrations (P fixed at 6.5 μM). This suggests a relative importance of N:P ratio over absolute nutrient concentration. Variations in measured cellular constituent levels, including chlorophyll *a*, at specific N:P

ratios were however observed across the tested range, indicating additional factors modulating protein and MCYST levels. Increased cellular MCYST and protein levels were observed at relatively higher specific growth rates, suggesting that the primary determining factor of MCYST and protein content at a particular N:P ratio is specific growth rate.

The absence of significant correlations between cellular constituents and growth rate and medium N (with the exception of specific growth rate of *M. aeruginosa* PCC7806 and medium N), P or N:P ratio was attributed to variations in absolute concentration of the second nutrient, the non-linear relationship between N:P ratio and the tested parameters, and most importantly the non-limiting nutrient concentrations.

It would therefore appear that the correlation between cellular protein and MCYST levels may result from the co-modulation of these variables by cellular nitrogen content as a function of N availability, or N assimilation rate, and specific growth rate. At increase μ without a corresponding increase in available N (or increase in assimilation rate) a resultant decrease in both cellular protein and MCYST content occurs, while decreasing growth rate under nutrient excess yields an increase in these variables. Clearly, however, an optimum medium N:P ratio exists for dynamic increases in cellular protein, and therefore MCYST content, concomitant with increased μ. It is tempting to speculate on the requirement for a specific N:P ratio being a function of P control of N assimilation (7) via control of C fixation (12) as has been shown for *Synechococcus sp.* PCC7942, which may also explain the observation that lower MCYST content is generally observed at lower light intensities and higher MCYST levels at higher light intensities (9).

Thus MCYST levels do increase with increasing μ, but only where nitrogen assimilation is sufficient to maintain relatively high protein content. Where maximum attainable μ was achieved for the experimental conditions but protein content was relatively higher, a corresponding increase in MCYST levels was observed. This may also contribute to the apparent contradictions

in published data (attributed by Sivonen *et al.* (21) to comparison of cellular MCYST and MCYST. dry weight^{-1}), where μ shows either a positive correlation to MCYST (14) or a reduction in MCYSTs with increasing μ (17). The higher MCYST content at low μ reported under P limitation (17) could therefore be attributed to reduced dilution of cellular N content by virtue of reduced growth. Similarly, the positive correlation between N and MCYST (13, 27) under N-limiting conditions could explain the reduction in MCYST content in stationary phase (28) where N limitation occurs. We therefore conclude that MCYSTs are produced as a function of cellular nitrogen status and that contradictions in published data are due to modulation of N content by factors controlling N uptake or assimilation, and dilution of cellular protein by growth.

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Chapter 3: †Microcystin content of *Microcystis aeruginosa* is modulated by nitrogen uptake rate relative to specific growth rate or carbon fixation rate

As described in the previous chapter

- Cellular microcystin levels increase with increasing growth rate only when nitrogen assimilation is sufficient to maintain relatively high protein content
- Where nitrogen uptake rate is greater than that required for growth, microcystin production increases
- Cellular microcystin content appears to be a function of cellular nitrogen status
- There is an apparent N:P ratio optimum for increased microcystin content
- Where N:P ratios were relatively high and growth was relatively low, microcystin levels were similarly reduced
- This suggests a role for phosphorous in promoting microcystin production

However

- Carbon fixation requires adequate phosphorus
- Nitrogen assimilation requires carbon skeletons

Therefore

- Reduced microcystin content at N:P ratios exceeding the optimum N:P ratio may indicate an insufficiency of phosphorus for carbon fixation to proceed at a rate sufficient to assimilate nitrogen to levels which result in elevated microcystin production
- This suggests that cellular carbon:nitrogen ratios may determine microcystin production rate
- This hypothesis is addressed in the following chapter

† 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 20:257-262

Abstract

Modulation of microcystin production 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. *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. 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 phosphorus. 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. Microcystin quotas increased 10 fold between the lowest and highest steady state values. 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.

Introduction

The environmental modulation of cellular concentrations of the heptapeptide hepatotoxin microcystin (MCYST) in *Microcystis aeruginosa*, and regulation of MCYST production by *M. aeruginosa*, remains an extensively studied but poorly understood subject. The effects of nitrogen (N), phosphorus (P), N:P ratio, light intensity and quality, iron, specific growth rate (μ), temperature and pH have been investigated to varying degrees in both batch and continuous culture.

Optimum temperature for toxin production during batch culture is below the optimal growth rate temperature (16). Watanabe and Oishi (17) reported a negative correlation of toxin content with μ as a function of temperature. Positive correlations between toxin content and μ as a function of light intensity for *M. aeruginosa* strains M228 and UV-006 were reported by Watanabe and Oishi (17) and van der Westhuizen and Eloff (16) respectively. Utkilen and Gjølme (15) and Wiedner et al. (19) found the same trends under continuous culture in strains CYA 228/1 and PCC7806 respectively, with toxin content increasing with μ and then decreasing at higher light intensities, which, in the case of PCC7806 were determined to be under photosynthetically active radiation (PAR) limiting conditions for positive correlation with growth rate and during PAR saturated conditions for negative correlation to growth rate. In batch culture of the axenic strain MASH01 MCYST concentrations correlated to initial medium nitrogen content and nitrogen removal from medium (11). Specific growth rate and final biomass also correlated with initial medium nitrogen leading the authors to conclude a linear relationship between specific MCYST production rate and specific cell division rate. In nitrogen-limited continuous culture Long et al. (8) showed the same linear relationship between MCYST production rate and μ, with MCYST cellular quota showing some variation as a function of cell volume changing with μ. The relationship was expressed as:

$$R_{MCYST} = \mu\left[\frac{\left(\mu \times Q_{MCYST_{max}}\right) - \left(\mu \times Q_{MCYST_{min}}\right)}{\mu_{max}}\right] + \left(\mu \times Q_{MCYST_{min}}\right)$$

where $Q_{MCYSTmin}$ and $Q_{MCYSTmax}$ are the minimum and maximum cell quotas respectively, μ is the specific growth rate, μ_{max} the maximum specific growth rate and R_{MCYST} the specific rate of MCYST production

In continuous culture with phosphorus limitation, Oh et al. (10) also noted a linear relationship between MCYST production rate and μ, and a corresponding decrease in MCYST measured per biomass dry weight.

Thus, MCYST production rate shows a linear relationship with growth rate irrespective of the limiting nutrient. However, since nitrate uptake is dependent on phosphorus (5) and carbon fixation (4) and nitrogen assimilation depends on carbon fixation in part via the availability of α-ketoglutarate (13), and generally since carbon fixation is dependent on phosphorus (7, 10), it follows that either phosphorus limitation, light limitation or inorganic carbon limitation will limit the nitrogen assimilation potential of the cell as part of the regulatory systems in place to maintain a C:N:P balance. This goes some way to explain the variation in MCYST content and production rate observed under various medium N:P ratios (7, 10, 3, 18) and the existence of strain specific optimum ratios. However, such balances are a requirement for growth and do not negate the potential for uptake beyond typical cellular ratios. To dissect the specific role of nitrogen it is therefore essential to modulate nitrogen availability and nitrogen uptake:carbon fixation rate ratios at a single growth rate and to evaluate the variation in MCYST content as a function of nitrogen uptake relative to growth rate in batch culture.

Methods

Culture and sampling

M. aeruginosa PCC7806 was obtained from Dr Neilan at UNSW (Sydney, Australia) and UV027 from Prof. J Grobbelaar at UFS (Bloemfontein, South

Africa). Axenic cultures of these strains were grown in triplicate 2000-ml bubble-lift vessels under constant illumination (51 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 0.22 μm) air at 150 ± 5 $ml.min^{-1}$. Modified $BG11_0$ (12) 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 with two strains, PCC7806 and UV027, in 1000 ml bubble lift vessels under identical temperature and light conditions with aeration adjusted for volume. Culture medium was BG11 (12). 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.

Sample analysis

Direct cell counts were performed in a haemocytometer (Neubauer). For batch culture the average growth rate was detemined for the period between each sampling time. Cellular MCYST content was measured as previously described (3). Medium nitrate was measured with Griess reagent after reduction with copper cadmium (1), and medium orthophosphate by the phosphomolybdate method (9). 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. Total cellular protein was measured at 550 nm using bicinchoninic acid as described by Smith et al. (14). Nutrient uptake rates were calculated as the product of medium nutrient minus residual nutrient and cell number over biomass.

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).

Results

Residual nutrient concentrations and *M. aeruginosa* cell numbers for each steady state are depicted in Figure 3.1. Residual chemostat nitrogen increased for each successive steady state (SS) relative to the previous SS. Cell number increased from SS-1 to SS-2 and then remained similar for subsequent SS's. Average cell radius showed a non-significant increase from SS-1 to SS-2 (2.8%) and from SS-2 to SS-3 (6.3%). From SS-3 to SS-4 there was a significant 23.7% increase and a further 18.1% increase from SS-4 to SS-5 (data not shown). Residual phosphorus was at no time limiting and ranged from 0.64 mM to 0.91 mM.

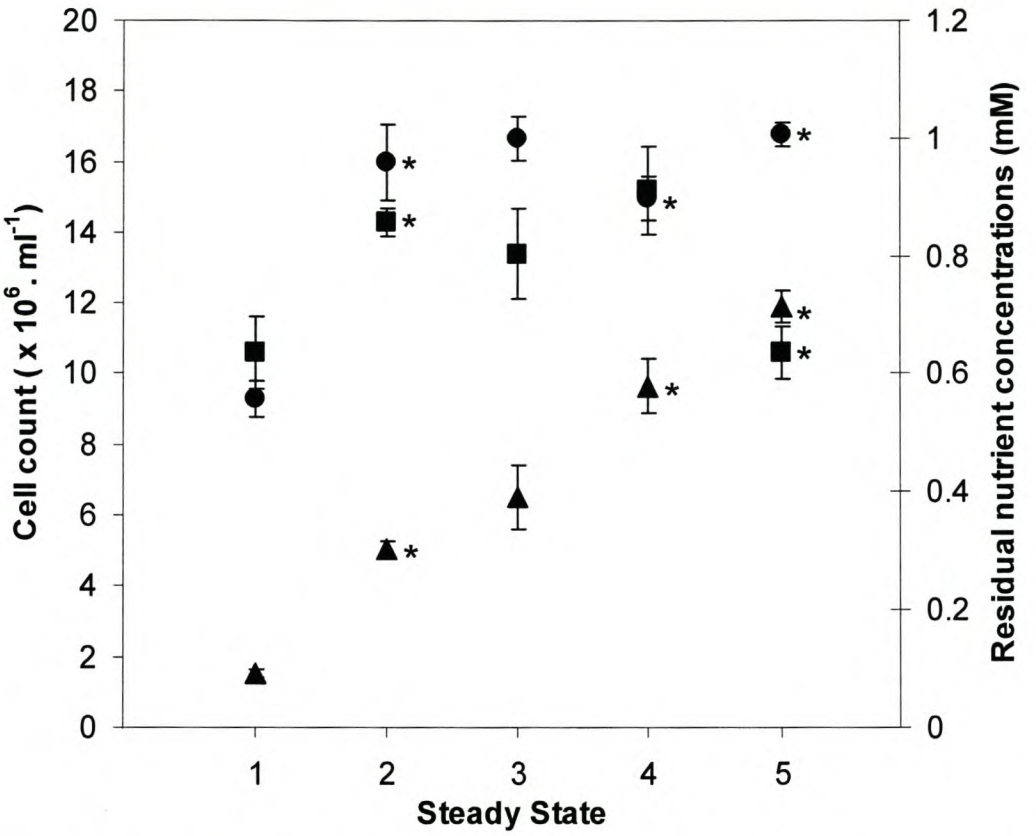

Figure 3.1 Residual nutrient concentrations and biomass for the five steady states. Cell number (●), residual nitrogen (▲), residual phosphorus (■). Error bars denote standard deviation (n = 3). Significant variance ($p<0.05$) from preceding SS is indicated with an *.

Figure 3.2 shows carbon fixation rate per volume chemostat and per cell. Total carbon fixation for SS-1, SS-2 and SS-3 were not significantly different. Total carbon fixation decreased significantly in SS-4 and decreased further in SS-5 indicating a reduction in the ability of the culture to fix carbon, but not a carbon limitation *per se*. Cellular carbon fixation rate decreased significantly from SS-1 to SS-2 as a function of increased biomass, decreased slightly but not significantly for SS-3 and then decreased significantly from SS-3 to SS-4 and from SS-4 to SS-5 further suggesting limitation in carbon fixation ability.

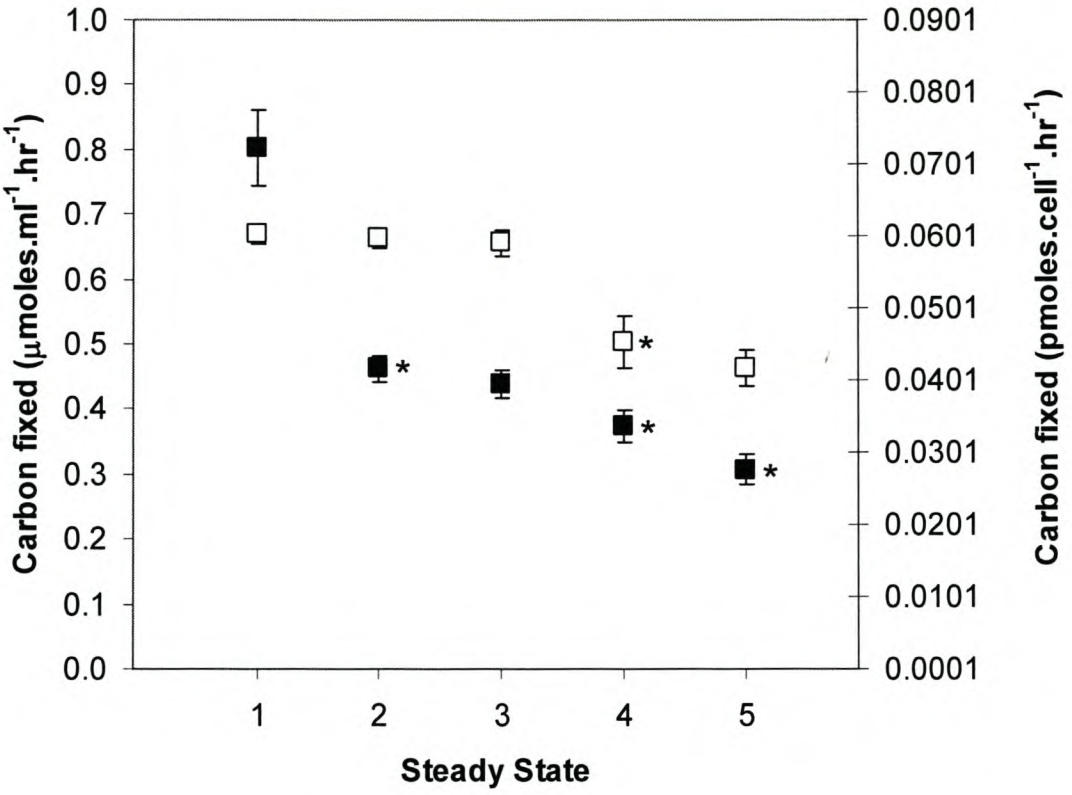

Figure 3.2 Carbon fixation rate per chemostat volume (□) and per cell (■). Error bars denote standard deviation (n = 3). Significant variance ($p<0.05$) from preceding SS is indicated with an *.

Figure 3.3 shows N and P uptake rates together with the carbon fixation rate and cellular MCYST. There was a substantial increase in MCYST when N uptake increased with a concomitant decrease in C fixation rate per cell, with significant increases in both MCYST content and N uptake rate between SS-1 and SS-2 and between SS-3 and SS-4, where significant decreases were observed for carbon fixation. Nitrogen uptake and MCYST content increased with increasing medium N while phosphorus uptake and carbon fixation were reduced. Nitrate uptake increased 117.4 fold between SS-1 and SS-2 and 2.46 fold between SS-2 and SS-5. Cellular nitrogen increased 1.27 fold between SS-1 and SS-2, and a further two fold between SS-2 and SS-5.

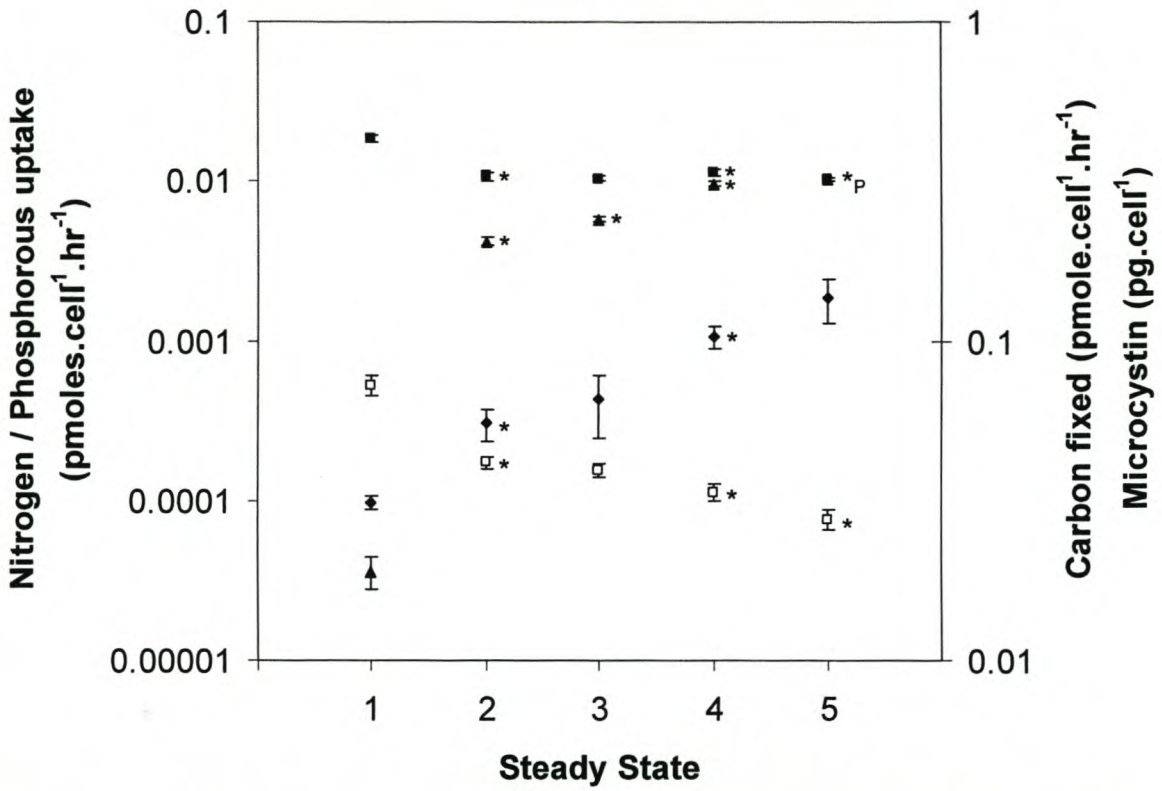

Figure 3.3 Nitrogen (▲) and phosphorus (■) uptake rates, carbon fixation rate (□) and cellular microcystin content (♦). Error bars denote standard deviation (n = 3). Significant variance ($p<0.05$) from preceding SS is indicated with an *. $*_P$ indicates significant variance for phosphate uptake.

Table 3.1 Summary of correlation coefficients between microcystin content and cellular nitrate and phosphate uptake rates and carbon fixation rates for cultures at steady state with μ = 0.01 hr-1.

Parameter	N uptake rate	P uptake rate	C fixation rate
Microcystin	0.8280 ($p<0.001$)	-0.4792 ($p=0.071$)	-0.7099 ($p=0.003$)
N uptake rate	--	-0.7517 ($p=0.001$)	-0.9192 ($p<0.001$)
P uptake rate	--	--	0.9381 ($p<0.001$)

Table 3.1 lists the correlations between MCYST and nutrient uptake rates and carbon fixation rates. Significant positive correlations were observed between N uptake and MCYST and between P uptake and carbon fixation rates. Significant negative correlations were observed between N uptake and carbon fixation rates, between N uptake and P uptake, and between MCYST content and carbon fixation rate. The negative correlations of MCYST to cellular P and P uptake are not significant. It should be noted that at steady state, for a given

growth rate, uptake rates and fixation rates are directly proportional to cellular content and therefore correlations with cellular N, P and C content are omitted.

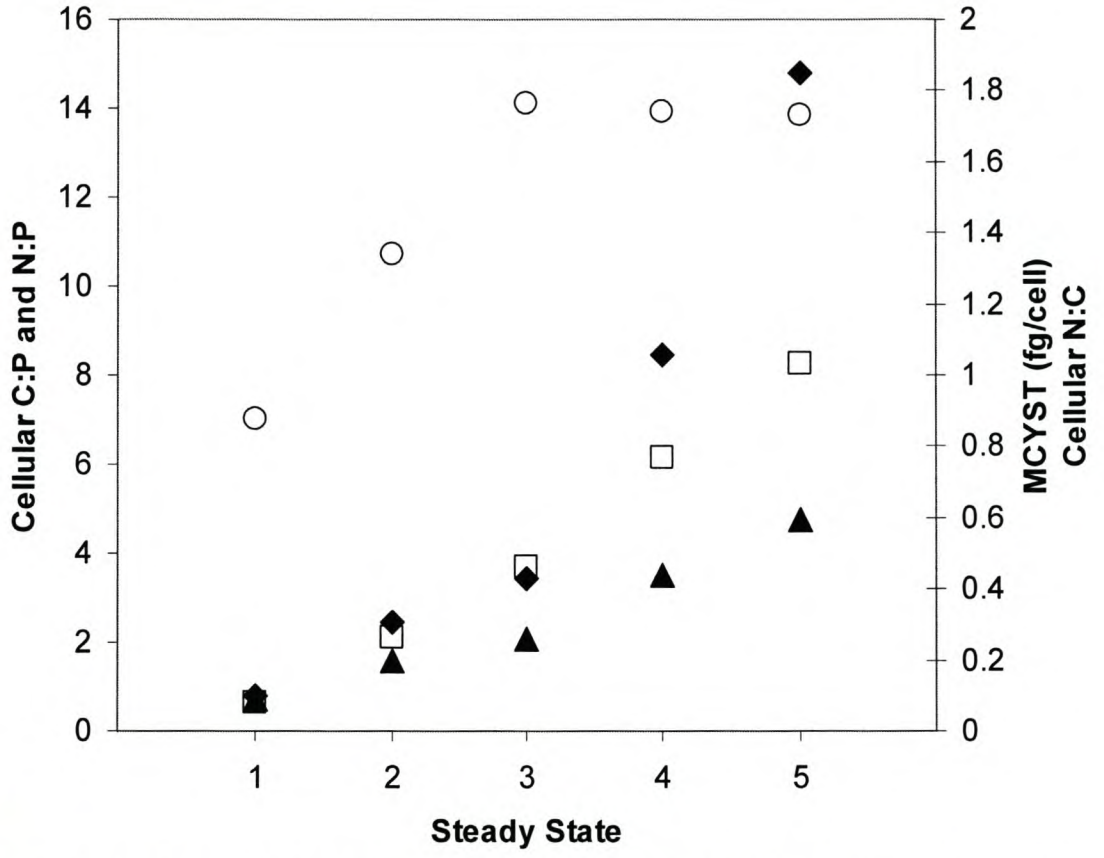

Figure 3.4 Atomic ratios of means of cellular constituents C:P (○), N:P (□), N:C(▲), and cellular microcystin content (♦).

Cellular atomic ratios clearly show the increase in MCYST when C:P reaches a maximum, due to reduction in carbon fixation rate, and N:P and N:C continue to increase (Fig. 3.4). Figure 3.5 shows the positive correlation between cellular N and cellular N:P ratio, and MCYST production. These relationships have been shown before for medium N:P ratios in this range. Figure 3.6 (inset) shows the increase in cellular MCYST with increasing N uptake as has been reported previously. However, the ratio of MCYST produced per nitrogen taken up is more useful in determining conditions for MCYST production as a function of N, and allows for the identification of the variable/s working in concert with N to modulate toxin production. Figure 3.6

therefore represents the slope of the inset as a function of changes in carbon fixation.

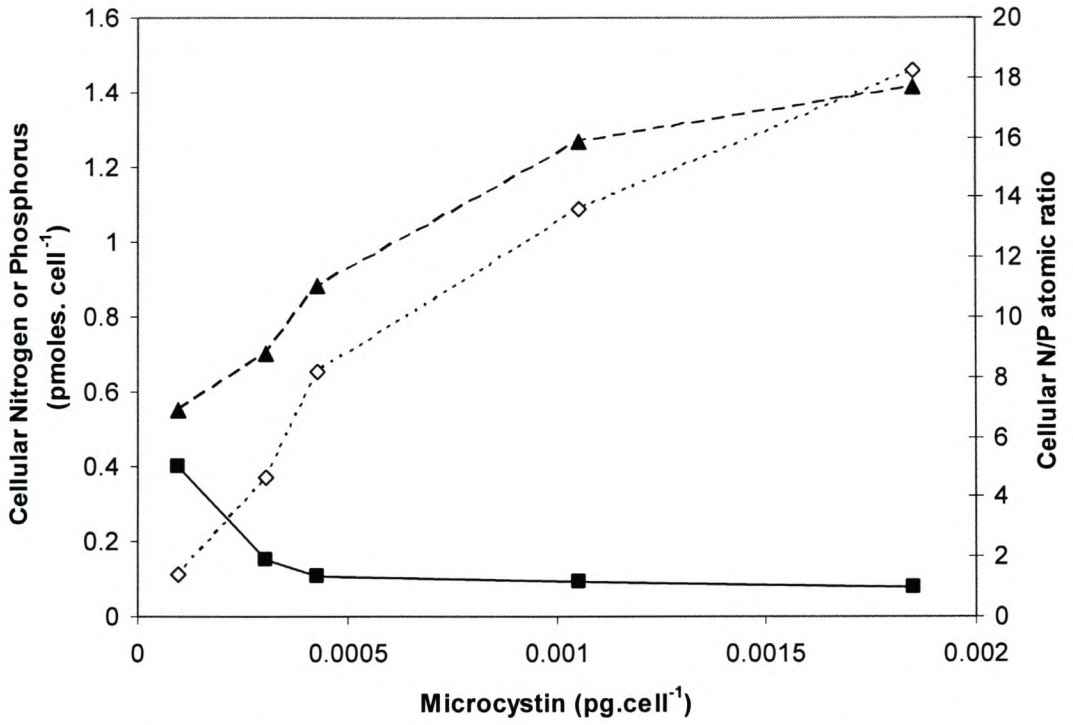

Figure 3.5 Cellular nitrogen (▲) and phosphorus (■) and cellular N:P atomic ratio (◊).

MCYST produced per N taken up increased considerably at reduced C fixation rates (Fig. 3.6) corresponding to maximum achieved C:P ratio. Thus, where nitrogen is accumulating in the cell, MCYST production increases. Batch culture data for two strains of *M. auruginosa* showed increased MCYST content when N uptake exceeded relative growth rate (Fig. 3.7) in accordance with continuous culture data.

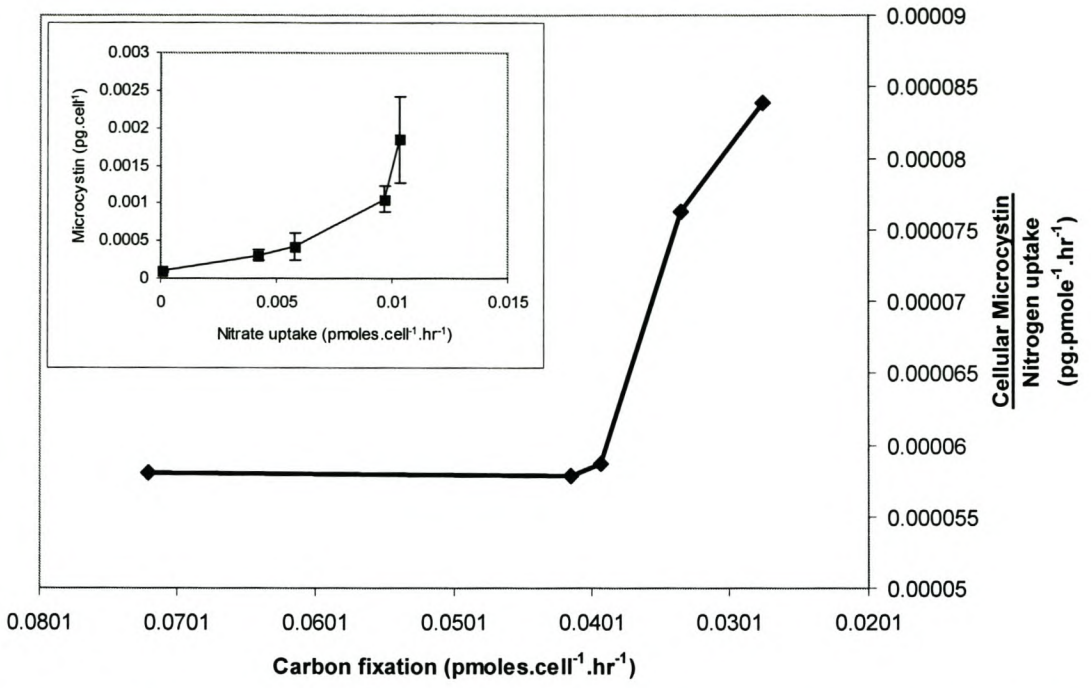

Figure 3.6 Cellular microcystin content/nitrogen uptake rate for measured carbon fixation rates. Inset shows MCYST as a function of N uptake rate.

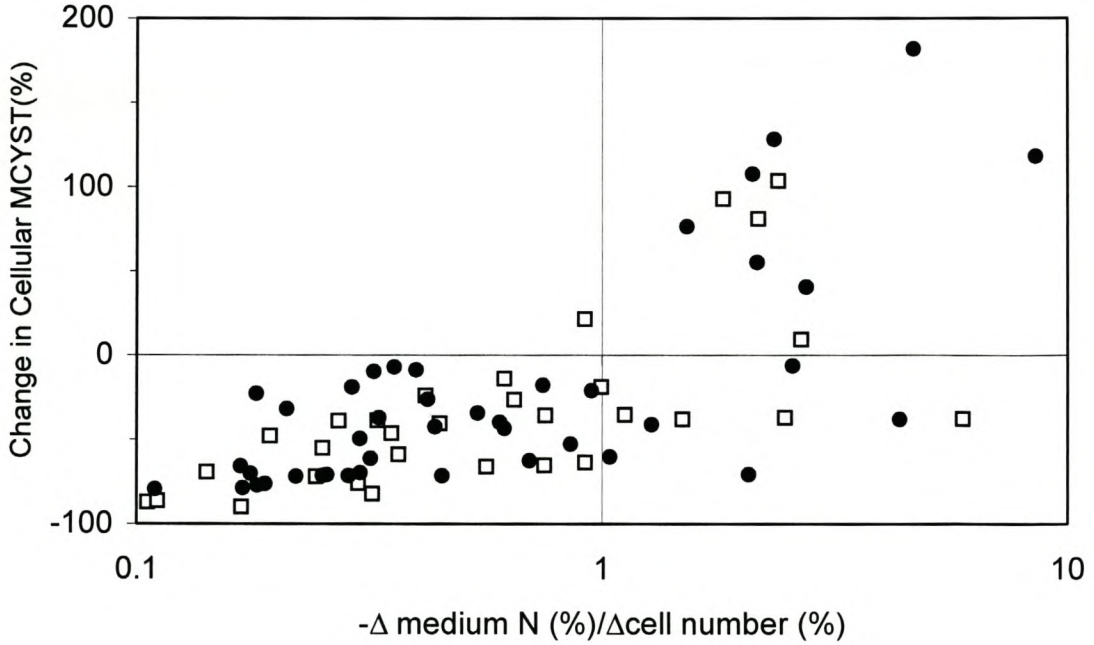

Figure 3.7 Change in cellular microcystin as a function of change in nitrogen uptake rate relative to cell division rate for strains UV027 (•) and PCC7806 (□).

Discussion

The reduced cell number in steady state one (SS-1) in Figure 3.1 was a result of N limitation (see also Fig. 3.3). With increasing medium N no further increase in cell numbers occurred between SS-2 and SS-5 indicating another limitation. Phosphate levels were, however, well above previously determined K_S values. SS-1, SS-2 and SS-3 show similar total carbon fixation and a reduction per cell due to increased biomass. Inorganic carbon limitation is indicated for SS-2 and SS-3. The reduction in total carbon fixed in SS-4 and SS-5 (Fig. 3.2) suggest another limitation other than C, N or P. Light limitation is suggested due to the increased cell size. MCYST content was therefore compared under N limitation, C limitation and reduced C fixation due to light limitation.

At steady state, cellular MCYST quota and MCYST production rate are equivalent since production rate is the product of cell quota and growth rate. Steady state cellular microcystin concentrations (and hence production rates) showed significant positive correlation with both nitrate uptake and cellular nitrogen content in accordance with previous publications (7) and reports on MCYST content based on original medium nitrogen (2, 11), and were negatively correlated with carbon fixation rates, phosphate uptake, and cellular phosphorus as previously reported (10). 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. It should be noted that the residual medium atomic N:P ratios in continuous culture were below reported optima of between 18 and 50 (7, 3) for MCYST production. Obtaining these residual concentration ratios would however require either phosphorus or carbon/light limitation, leading to increased MCYST production.

Cellular microcystin quotas increased ten fold between the lowest and highest steady state values whereas Long et al. (8) reported less than a three fold increase as a function of μ under nitrogen limitation. Cellular microcystin concentrations increased 4.43 fold despite the increase in cell volume. Batch culture in BG11 under similar conditions yielded increased microcystin when

nitrogen uptake exceeded relative growth rate, confirming the importance of nitrogen uptake in the modulation of microcystin content at a given μ. In the absence of nitrogen limitation therefore, and more specifically with phosphorus, carbon or light limitation, the linear relationship of MCYST production rate and μ is not valid. The increase in MCYST production rate reported by Long et al. (8) for the range of growth rates may therefore be attributed to the difference in maximum attainable specific growth rate (1.08 day^{-1}) relative to maximum attainable nitrogen uptake rate (not determined). Thus at increased μ nitrogen uptake is relatively higher and as μ_{max} is approached reduction in cellular MCYST is a function of approaching or exceeding maximum production rate relative to μ for the given medium nitrogen.

The differences between cellular nitrogen and nitrate uptake rate at the higher nitrate uptake rates were attributed to loss of reduced nitrate as a result of reduced cellular α-ketoglutarate. The resulting inability of the cells to assimilate ammonium would result in ammonium losses to the medium and subsequent losses from the medium due to aeration. The relatively small differences in cellular nitrogen as a function of medium nitrate, compared to differences in nitrate uptake rate at the different medium nitrate concentrations, and the significant correlation of MCYST to uptake rate suggest a greater importance of uptake rate relative to assimilation rate. This in turn indicates cellular ammonium and/or α-ketoglutarate concentrations as potential modulators of MCYST production.

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. Where nitrogen uptake exceeds the dilution of cellular nitrogen by growth, whether growth rate is at μ_{max} or is limited by temperature, inorganic carbon or phosphorus availability, cellular MCYST and MCYST production rate increase. This model explains the positive correlation of MCYST with growth under N limitation (11, 8), and the negative correlation with phosphorus availability (10) and with growth rate under PAR saturation (19) or at sub-optimal growth temperatures (16, 17) but

is complicated by the requirement of carbon fixation products and phosphorus for nitrogen assimilation as reflected by the existence of an optimum N:P ratio for toxin production (7, 10, 18, 3). Future quantitative relationships between MCYST production, or cellular MCYST content, must therefore be based on nutrient uptake, metabolite concentrations, and growth rate, necessitating a more structural approach to modelling MCYST modulation.

These data constitute a basis for investigations into the metabolites that correlate with increased MCYST production rates or cellular quotas, and suggest that nitrogen assimilation precursors or products may be involved in regulation of MCYST production. Such information might shed light on the nature of the mechanism of up-modulation of MCYST and the nitrogen species that play a role in this mechanism. An increase in nitrogen uptake relative to carbon fixation might imply a response to ammonium or accumulation of assimilation products and transamination products as a result of reduced α-ketoglutarate and hence a more direct correlation with cellular protein as has been reported (8,3). Dilution of cellular MCYST by growth, and hence reduced MCYST production relative to μ, does not offer sufficient evidence to speculate on which of these mechanisms might function but may be interpreted as positive modulation by ammonium or negative modulation by carbon fixation products since assimilation requires the presence of α-ketoglutarate and hence adequate carbon for growth. This can only be addressed by investigating the resulting cellular nitrogen species that predominate under carbon limitation.

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Chapter 4: Does NtcA play a role in the regulation of microcystin production in *Microcystis aeruginosa*?

As described in the previous chapter

- Microcystin production rate increases under conditions of nitrogen excess when:
  - Nitrogen uptake or assimilation exceeds use of assimilated nitrogen by growth related processes
  - Nitrogen accumulates due to carbon limitation of growth
- Microcystin production appears to be under control of cellular nitrogen status which is, in turn, controlled by
  - the availability of carbon for nitrogen assimilation
  - the use of assimilated nitrogen by growth

However

- Nitrogen metabolism in cyanobacteria is regulated by the protein NtcA
- NtcA activity is regulated by ammonium and α-ketoglutarate and microcystin production is regulated by cellular C:N balance
- Thus, both NtcA and microcystin production appear to have common regulators
- Under conditions where NtcA expression is repressed, microcystin production increases

Therefore

- Microcystin production may be regulated by NtcA at the level of transcription of the microcystin synthetase genes
- The following chapter addresses this hypothesis

Abstract

No definitive mechanism for the regulation of microcystin production by *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. 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, indicating an inverse regulation of *ntcA* and *mcyA*. These results suggest a possible repression of *mcyA* transcription by NtcA.

Introduction

No conclusive evidence relating to the mechanisms of regulation of microcystin (MCYST) exists. Furthermore, the function of the molecule is unknown. The fact that MCYST is a potent animal toxin may, in some part, be responsible for the lack of knowledge on function, as this ascribes a function, albeit anthropocentric, to MCYST. Despite this, several possible functions of MCYST have been suggested. Work regarding the regulation of MCYST production has also been extensively published but, as with function, no definitive mechanism of regulation has been identified.

Do proposed functions offer clues to regulation?

Proposed functions have largely been based on the toxicity of MCYST to plankton-grazers (20, 12) although more recent evidence suggests that factors other than MCYST may play a role in protection from grazing by Daphnia (18, 28). The ability of MCYST to chelate Fe^{2+} (9) and the apparent relationship between environmental iron and MCYST has also led to the suggestion that MCYST is an intracellular chelator which inactivates free Fe^{2+} (31) thus protecting cells from photo-oxidation. However, no such complexes have been identified and the increase in iron uptake under high irradiance levels (31) would relate to conditions with reduced MCYST (2). Iron may therefore however simply impose stress on the cells resulting in increased MCYST production (35). More recently a possible role as a communication molecule has been proposed based on a potential MCYST exporter (25). An alternative role for MCYST is that of a regulatory molecule for some cellular function, as is suggested by the cellular location of MCYST - predominantly thylakoids and nucleoid areas (29) - which may imply a function in regulation of photosynthesis. Hesse *et al.* (8) investigated the photosynthetic pigments of mutant strain of PCC 7806 with impaired MCYST production and found a 20% decrease in the content means of all pigments in the mutant strain, independent of light intensity. However, the light harvesting pigment phycocyanin was significantly increased at low light intensities in the mutant strain with a concomitant decrease in chlorophyll *a* relative to the wild type. This suggests a change in the PSI/PSII ratio typical of variations observed

during light adaptation in cyanobacteria (27). It should however be noted that Hesse *et al.* (8) did not consider the relative nitrogen uptake and cellular nitrogen status of the two strains under the variable light conditions. The absence of both MCYST and the peptide synthetase in the *mcy* knockout mutant would result in increased availability of cellular nitrogen. Such nitrogen would commonly be stored as either cyanophycin or, relevant to this case, phycocyanin thereby altering the PSI/PSII ratio. There remains, however, sufficient evidence to speculate on a role for MCYST in light adaptation. The association of MCYST with the thylakoid membranes is presumed to be due the insertion of the Adda (3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4,6-dienoic acid) moiety into the membrane (13). Hayakawa *et al.* (6) reported a change in fatty acid composition of *M. aeruginosa* during batch culture. Specifically the (18:3ω3 + 18:4ω3)/(18:2ω6 + 18:3ω6) ratio correlated with growth rate under culture conditions where maximum toxin content would be expected to correlate with growth rate. Similarly, significant increases in 18:4ω3 and 20:3ω6, and generally in poly-unsaturated fatty acids (PUFA) have been reported (and correlated with protein content) in batch culture of *M. aeruginosa* at 20 and 40 µmol m^{-2} s^{-1} compared to higher irradiance levels of 70 µmol m^{-2} s^{-1} (33). Protein content has previously been shown to correlate with MCYST content (3), again suggesting that these fatty acid changes correspond to levels of increased MCYST. MCYST content as a function of light intensity does follow a similar pattern (34) to that reported for PUFA (33), with production of MCYST increasing with PAR and reaching a maximum at approximately 80 µmol m^{-2} s^{-1}. Many green algae are known to change their fatty acid composition during growth in order to produce more cellular membrane without having to store more saturated fatty acids (21), whereas cyanobacteria in general (19) and *M. aeruginosa* specifically (26) do not. It would not be valid to draw any conclusions from this, but the structure of the Adda component of MCYST, inserted into thylakoid membranes, would effectively produce similar advantages to those offered by the changes expected in eukaryotic algae under similar conditions. In addition to this, *mcyABC* and *mcyDEFGHIJ* polycistronic transcripts contain two alternative transcription start sites which appear to be differentially expressed as a

function of light intensity (14) further suggesting a role for MCYST in a light-dependent process.

Were MCYST to be a regulatory molecule, MCYST production would have to respond to some physiological state or effector molecule and in turn be able to produce a response. Assigning a regulatory role to MCYST would require, initially, identification of regulators, and mechanisms for regulation of MCYST production so as to identify possible points for regulation by the molecule.

Nutrient control of toxin content

Nitrogen has been suggested as the primary modulator of MCYST content or cellular MCYST quota in *Microcystis aeruginosa*. Both the rate of nitrogen uptake relative to growth rate and the cellular protein content correlate with cellular MCYST content (3). The form of available nitrogen has, however, been shown to be significant in the control of MCYST production with amino acid components of MCYST actually inhibiting production, while urea and ammonia had little effect compared to nitrate (35). Specific growth rate and phosphorus have also been reported as modulators of MCYST production or cellular content. Where MCYST has been correlated with growth rate (15) this was under nitrogen limitation in continuous culture, or in batch culture where nitrogen became the limiting nutrient (24). In either case, correlations of MCYST content with growth rate, where growth rate is a function of nitrogen availability but not the limiting criterion, are not necessarily valid. Similarly, negative correlations with phosphorus concentrations in the medium may indicate enhanced MCYST production under reduced carbon fixation or growth (23), resulting in increased cellular nitrogen. The relationship between light intensity and MCYST content suggests a possible role for carbon fixation products in the regulation of MCYST production. However, this is complicated by the need for carbon skeletons for nitrogen assimilation but may nonetheless result in increased cellular nitrogen under these conditions.

The correlation of nitrogen uptake with MCYST content in *M. aeruginosa* PCC 7806 depends on the growth rate of the organism (3), indicating cellular nitrogen status as the modulator of MCYST production. For a given growth

rate, however, the carbon fixation rate relative to the nitrogen uptake rate determines the rate of MCYST production, with dramatically enhanced MCYST cell quotas per nitrate uptake rate occurring where carbon fixation was reduced either due to carbon or light limitation (2).

Nitrogen based regulation

Nitrogen-based regulation of MCYST production may be at a genetic or physiological level or regulation may occur at both levels. Any positive correlation with glutamate would suggest physiological regulation by virtue of the role of *mcyF* (glutamate racemase) (22). Higher cellular glutamate concentrations would result in increased D-glutamate for MCYST synthesis by existing polypeptide synthetase. Alternatively, increased cellular nitrogen may play a regulatory role in the transcription of the *mcy* operon.

NtcA is a global nitrogen homeostasis regulator in cyanobacteria (4) that functions as a transcriptional regulator when binding selectively to $GTAN_8TAC$ motifs (7) present upstream of regulated operons. NtcA functions as a transcriptional activator on several genes/operons involved in nitrogen uptake, assimilation or fixation in cyanobacteria, including *nirA, narB, ntcB, nrt, hetC, glnA, glnB* and *amt1* (17, 11) and functions as a transcriptional activator for *ntcA*. NtcA has been shown to function as a repressor of transcription as well, acting on *rbcL, gifA, gifB* (5) and *gor* (10) and thus activation of transcription of these genes occurs in the presence of adequate nitrogen, resulting in increased carbon fixation, reduced glutamine synthesis (*gifA* and *gifB* encode glutamine synthetase type I inhibitors) and increased levels of glutathione reductase. The mechanism by which NtcA recognizes the cellular N status is unknown (32) but inhibition of glutamine synthetase may indicate a response to glutamine, cyanate or α-ketoglutarate. Carbamoylphosphate and cyanate, have been shown to activate transcription of ammonium repressed genes, including the NtcA activated *nirA* and to de-repress the NtcA repressed *rbcL*. Thus these glutamine metabolites may be involved in regulation of NtcA expression (30) since glutamine concentration is an effective indicator of cellular C:N balance. It should however be noted that Suzuki *et al.* (30) also

showed enhanced *rbcL* transcription levels and reduced *nirA* levels with growth on ammonium. Enhanced *rbcL* transcription could still be attributed to increased glutamine (and hence potentially to NtcA) and repression of *nirA* by ammonium functions independently of the NtcA mechanism.

That NtcA functions not only in response to nitrogen depletion but also to increased cellular C:N is evidenced by the fact that α-ketoglutarate enhances binding of NtcA to appropriate DNA sequences (32). The increase in MCYST cell quota observed during carbon limitation and nitrogen excess (2) may, in turn, indicate de-repression of *mcyABC* during carbon limitation or activation of *mcyABC* during nitrogen excess.

The presence of potential NtcA binding sites upstream of either *mcyABC* or *mcyDEFG* may therefore indicate possible NtcA regulation of MCYST synthesis. MCYST production correlates with cellular nitrogen status and thus varies inversely with cellular α-ketoglutarate. NtcA levels would decrease under conditions of elevated cellular nitrogen status and enhancement of NtcA activity by binding by α-ketoglutarate would be reduced. Thus MCYST production should be negatively correlated with NtcA levels. However, whether this could be attributed to NtcA regulation of *mcyABC* would require evidence of altered transcription of *mcyABC*.

The following investigation was therefore intended to (a) confirm the role of nitrogen uptake in MCYST production and attempt to place a time frame on the production of MCYST relative to the uptake of nitrogen, (b) analyse the regulatory regions between the *mcyABC* operon and *mcyD* for potential NtcA binding sites, and (c) to determine if there was any relationship between the expression levels of *ntcA* and *mcyA* transcripts under conditions determined to enhance either MCYST production or the requirement for nitrogen.

Methods

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 1200ml 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 (3).

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 manufacturers instruction, gel-purified using the Qiagen RNeasy® Mini Kit as per manufacturers instructions, and purity and quantity determined spectrophotometrically at 260nm 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.

Results

Culture

Toxin levels increased following an increase in nitrogen uptake rates (at 2 or 3 days), with relatively higher toxin content occurring at higher medium nitrogen concentrations (Fig 4.1). The initial decrease in $MCYST_{DW}$ after 24 hours, although not significant, was attributed to decreased uptake of nitrogen and resumption of carbon fixation resulting in increased biomass without MCYST production. Decreases in $MCYST_{DW}$ levels subsequent to the $MCYST_{DW}$ peak were observed for all initial nitrate concentrations. Figure 4.2 shows the rates of ammonium uptake and the $MCYST_{DW}$ levels as a function of the increased nitrogen uptake. $MCYST_{DW}$ decreased throughout the incubation period.

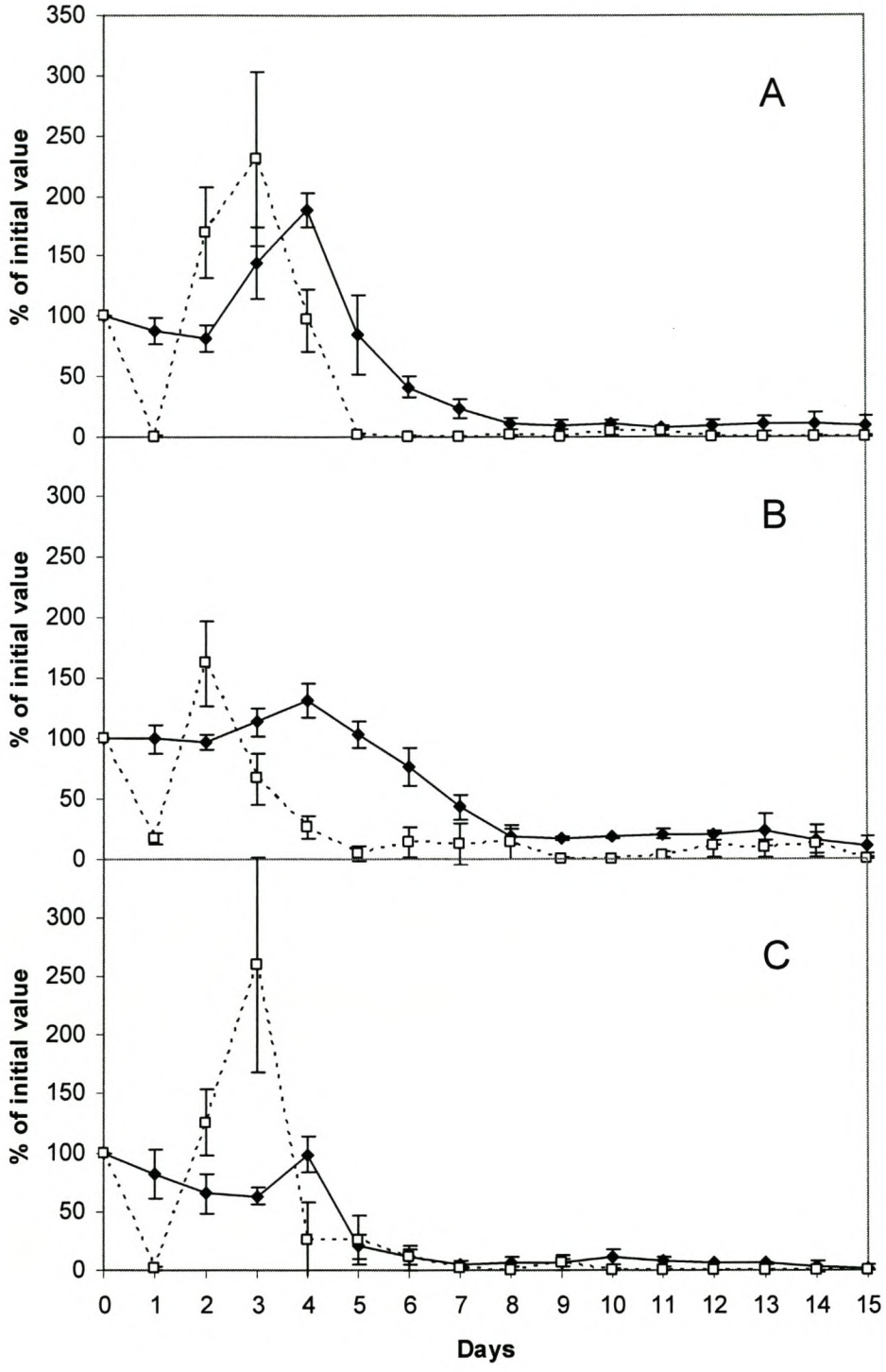

Figure 4.1 Percentage of initial values for nitrate uptake (□) and MCYST (♦) per dry weight of culture during batch culture on 2 mM (A), 1.5 mM (B) and 0.5 mM (C) initial nitrate and excess phosphate. Error bars denote standard deviation of triplicate samples.

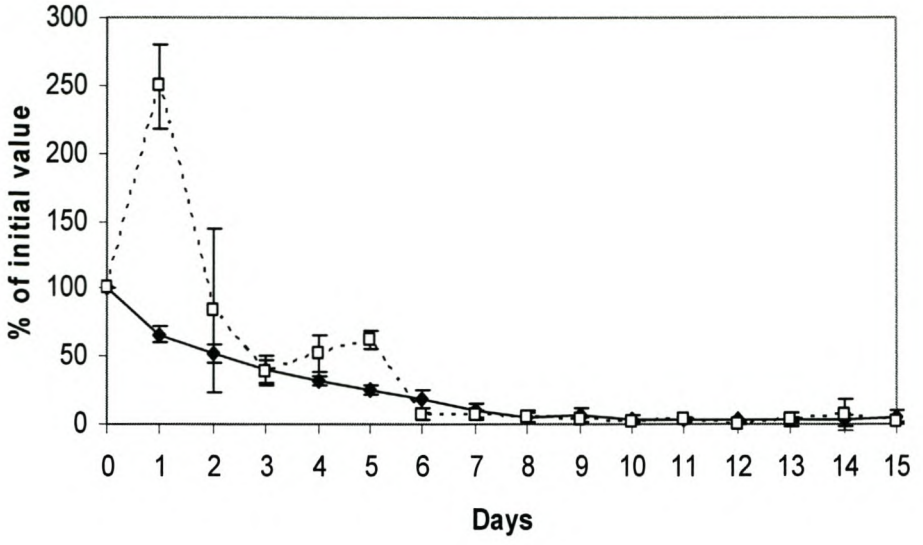

Figure 4.2 Ammonium uptake rate (□) and MCYST (♦) per dry weight as percentages of initial values during batch culture on 2 mM ammonia and excess phosphate. Error bars denote standard deviation of triplicate samples.

Analysis of mcyABC

mcyABC regulatory elements were analysed for potential NtcA binding sites. Figure 4.3 shows cyanobacterial genes reported to be either positively regulated by NtcA or repressed by NtcA, and published sequences of the region upstream of the *mcyABC* operon of three strains of *M. aeruginosa*. A putative NtcA binding site overlapping the -10 region upstream of the *mcyABC* operon was found to be conserved in all three strains. The pattern, $GATN_7TAC$, was similar to that reported as a repressor binding site for *gor* in PCC 7120 but retained the 3' TAC motif.

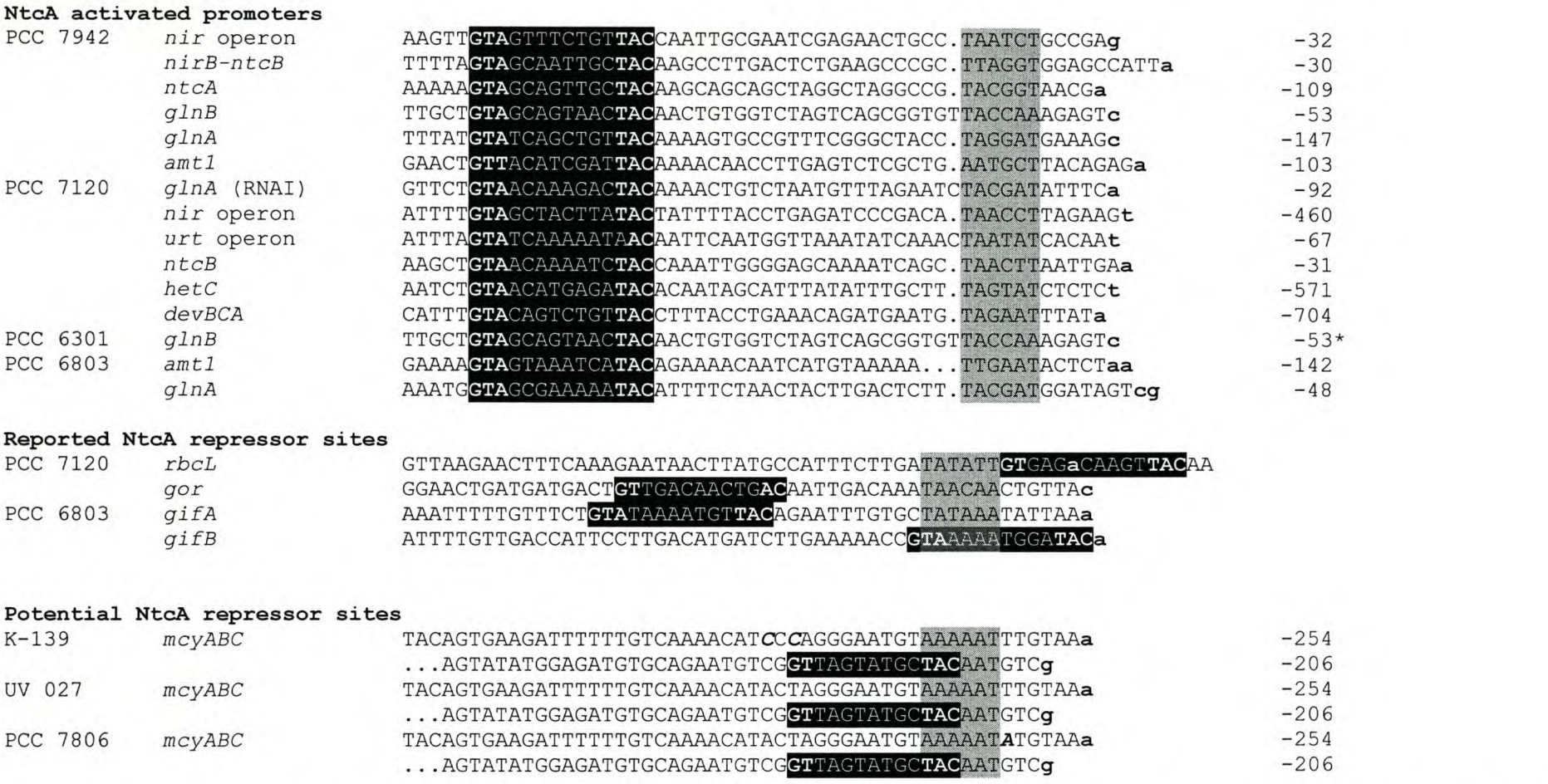

Figure 4.3 NtcA activated promoters, reported NtcA repressor sites, and putative NtcA binding sites upstream of the *mcyABC* of *Microcystis aeruginosa*. NtcA binding sites are shown with black boxes. Grey boxes indicate the -10 Pribnow box. Transcription start points are indicated by lowercase bold. * indicates a deduced ATG position based on sequence alignments.

RPA

Figure 4.4 shows the RPA results for 24 hours in light and for 24 and 48 hours in the dark. In high light *ntcA* > *mcyA* transcript levels. After 24 hours in the dark there is a decrease in *ntcA* and an increase in *mcyA* relative to the light samples of the same incubation period. After 48 hours the *ntcA* is further reduced while the *mcyA* transcript appears similar to the 24 hour sample, yielding *mcyA* > *ntcA*.

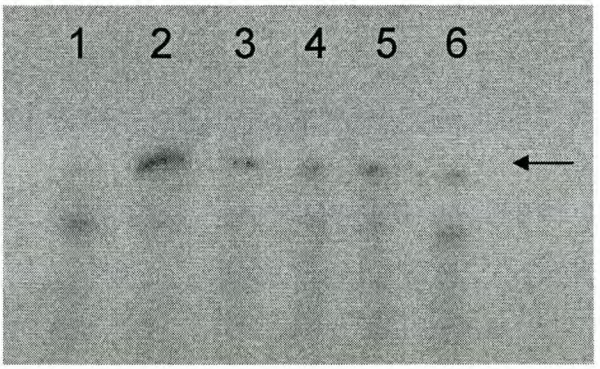

Figure 4.4 *mcyA* (1,3,5), *ntcA* (2,4,6). 1 and 2 are 24 hours incubation in high light (140 µmol photons m^{-2} s^{-1}). 3 and 4 are after 24 hours in dark. 5 and 6 are after 48 hours in dark. Arrow indicates position of the RPA products.

Discussion

Initial MCYST concentrations are representative of mid-log cultures in nitrogen excess that had been placed in the dark thereby limiting carbon fixation, and thus represent nitrogen excess - stationary phase conditions. A return to light and non-limiting nitrogen concentrations creates an initial increase in cellular nitrogen relative to carbon, followed by a reduction in cellular nitrogen by growth with a concomitant relative increase in carbon. The MCYST increase following an increase in nitrate uptake rate could therefore be directly attributed to an increase in cellular nitrogen status and the subsequent reduction in MCYST concentration per dry weight was attributed primarily to dilution by growth and to nitrogen limitation where uptake rates dropped significantly. It is also interesting to note that nitrogen-replete but

light-starved cultures retain relatively high MCYST levels as can be seen by comparing initial MCYST values with those later on the curve (Fig 4.1) where nitrogen becomes limiting or cellular nitrogen is diluted by growth due to excess carbon. The decrease in MCYST per dry weight in ammonia fed cultures was due to dilution by growth. No increase in MCYST was observed. Thus MCYST levels increased as a function of nitrate uptake, but peak levels were observed between 24 and 48 hours after nitrate uptake peaked. High levels were also observed in nitrogen-replete and light-starved (carbon-limited) cultures (initial values in Figures 4.1 and 4.2).

The putative NtcA binding site upstream of the *mcyABC* operon, and overlapping the -10 region, indicates possible negative regulation of MCYST production by NtcA. Similarly, the possible down-regulation of MCYST production by an increased cellular C:N ratio may be due to enhanced binding of NtcA by the increased α-ketoglutarate levels that occur under these conditions. RPA analysis supports this. Incubation under high light intensity resulted in relatively high *ntcA* transcript levels and relatively low *mcyA* transcript levels, consistent with the latter part of the curves in Figure 4.1. Incubation in the dark, where cellular C:N would decrease, yielded increased *mcyA* and decreased *ntcA* relative to the same period in light (Fig 4.4). Incubation for a longer period further decreased *ntcA* levels while *mcyA* levels appeared slightly higher. Thus with continuing lack of ability to fix carbon and adequate nitrogen, *ntcA* transcription would necessarily be lower. The increased *mcyA* RNA levels indicate regulation at a transcriptional level, suggesting a possible role for NtcA as a repressor of *mcyABC* expression with α-ketoglutarate as an enhancer of this repression. As previously reported (35) and shown in Figure 4.2, ammonia appears not to enhance MCYST production. It should be noted that NtcB (a LysR family protein) has been implicated in the nitrite activation of nitrate uptake and is required in addition to NtcA for activation of *nirA* (1). The absence of a response to ammonium uptake may be due to a similar mechanism operating during repression by NtcA.

It seems therefore feasible that MCYST production is regulated primarily by cellular C:N ratio, with increased uptake of nitrate under carbon fixation-limiting conditions giving rise to an increase in production. Insufficient evidence exists to confirm a role for MCYST as a regulatory molecule. If, however, it does have a regulatory function it follows that its function would be to restore C:N balance under C limitation or N excess and would therefore either prevent N uptake (unlikely since *M.aeruginosa* has the ability to store nitrogen) or enhance C-fixation. MCYST production may therefore be NtcA regulated in a similar fashion to *rbcL* or the apparent similarities in expression of *mcyABC* with NtcA regulated genes may be coincidental. Investigation of any correlation between either cellular glutamate or glutamine, or α-ketoglutarate and MCYST production may also assist in determining whether in fact NtcA plays any role in regulation of MCYST production.

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Chapter 5: Cellular nitrogen status is only one of the factors controlling microcystin content in *Microcystis aeruginosa* PCC7806

As described in the previous chapter

- The increase in MCYST cell quota observed during carbon limitation and nitrogen excess may indicate de-repression of *mcyABC* during carbon limitation or activation of *mcyABC* during nitrogen excess
- Microcystin production may therefore be NtcA regulated in a similar fashion to *rbcL*

However

- Carbamoylphosphate and cyanate have been shown to activate transcription of ammonium repressed genes, including the NtcA activated *nirA* and to de-repress the NtcA-repressed *rbcL*
- Thus these glutamine metabolites may be involved in regulation of NtcA expression

Therefore

- Glutamine and glutamate concentrations are an effective indicator of cellular C:N balance
    - High levels of glutamine relative to glutamate would indicate high cellular nitrogen, increased cellular ammonium and decreased cellular α-ketoglutarate
    - High levels of glutamate relative to glutamine would indicate reduced ammonium and potential excess of α-ketoglutarate
- Correlations between glutamine and microcystin cellular concentrations would support a role for NtcA in regulation of *mcy* expression
- The following chapter addresses this hypothesis

Abstract

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. 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 phosphorus, 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 levels 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. Cellular microcystin levels 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 the putative NtcA binding site on the *mcyA* promoter

region and relative expression levels of *ntcA* and *mcyA* described in Chapter 4.

Introduction

Microcystins (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 and X and Z are variable L-amino acids) are potent non-ribosomally synthesized heptapeptide hepatotoxins produced by several cyanobacterial genera including *Microcystis*. Synthesis is directed by both polypeptide synthetase and polyketide synthase modules encoded by *mcyA-J* (7). Investigations of environmental conditions conducive to maximum toxin production have yet to yield a definitive set of conditions that yield increased toxin production. This is in part due to the complexity imposed by the interplay between the major nutrients and light and the fact that conditions that have been shown to increase toxin production are generally the same that enhance growth.

Strong positive correlations have been observed between microcystin cellular quota ($MCYST_Q$) and initial medium nitrate concentrations (2, 15, 17, 21), nitrate uptake rate relative to growth (1), cellular protein content (2, 16) and total nitrogen (8) in batch culture of *Microcystis aeruginosa*. MCYST production rate has also been shown to correlate with nitrogen uptake rate and nitrogen content in continuous culture (1, 9). Optimum medium N:P ratios for MCYST production (2, 8, 14) of between 16 and 50 for *M. aeruginosa* UTEX 2388 (8) and 18 and 51 for *M. aeruginosa* PCC 7806 and UV 027 (2) have also been reported, with positive correlations between $MCYST_Q$ and N:P ratios up to these maxima. Vézie *et al.* (21) described substantial variation between strains in the response of both growth and $MCYST_Q$ to N:P ratio, with optimum ratios for $MCYST_Q$ of 458<N:P<664 for one strain and 237<N:P<753 for a second strain of *M. aeruginosa*. The significant interactive effect of nitrogen and phosphorus on growth and toxin production in *Microcystis* was also noted. In addition to this, $MCYST_Q$ increased with increased P under high N, but decreased under increased P at low N (21). Oh *et al.* (14) reported negative correlations between $MCYST_Q$ and medium phosphate, independent of N. Downing *et al.* (2) speculated that the correlation between N:P and MCYST was a function of P-control of carbon fixation and cellular carbon fluxes, which in turn affect the rate of nitrogen assimilation. Data relating the

C-fixation:N-uptake rate ratio to cellular MCYST content (1) appear to confirm this. At a constant growth rate, MCYST production as a function of nitrate uptake remained constant under carbon replete conditions with adequate photosynthetically active radiation (PAR) but increased dramatically under carbon limitation or increased shading (1). In batch culture where nitrate uptake rate exceeded growth rate, similar increases in $MCYST_Q$ were observed. MCYST production was therefore shown to be highly variable irrespective of growth rate, as previously reported (9). These data further support the hypothesis that MCYST production rate is determined by cellular nitrogen status or C:N ratio.

$MCYST_Q$ as a function of light has a defined maximum, with further increased light intensity causing a reduction in $MCYST_Q$ (19). This corresponds to the relationship seen between medium N:P ratio and MCYST quotas and has been attributed to the requirements for carbon skeletons for nitrogen assimilation (2). It may, however, also be partially due to the C:N:P ratio requirements for growth leading to increased biovolume (and hence reduced cellular nitrogen concentrations under high light) and to the inability to assimilate nitrogen at high N:P ratios where carbon is limiting. Similarly, the increased optical density of cultures under high N:P ratios may reduce photosynthesis due to culture self-shading. Positive correlations between MCYST and specific growth rate were established in nitrogen-limited continuous culture for various growth rates and medium nitrogen concentrations at 40 µmol m^{-2} s^{-1}[PAR] (9). Thus all cultures were nitrogen limited; at increased growth rates both light and carbon availability would be effectively reduced, thereby increasing the effective cellular N:C ratio with results similar to those recently reported (1). Reported relationships between MCYST and light (19), medium phosphorus (8, 14, 21) and specific growth rate (9) can therefore largely be explained in terms of nitrogen availability. Cellular nitrogen status has therefore been proposed as the primary modulator of $MCYST_Q$ in *M. aeruginosa* (1, 2).

Cyanobacteria commonly use nitrate, ammonium and urea, and dinitrogen in the case of nitrogen fixing species (3). Nitrate is taken up via an active

transport system and reduced sequentially by nitrate reductase and nitrite reductase to ammonium which is incorporated into carbon skeletons primarily via the glutamine synthetase-glutamate synthase cycle. α-Ketoglutarate is the carbon skeleton used to incorporate ammonium. Cyanobacteria lack an α-ketoglutarate dehydrogenase (5) and the function of $NADP^+$-isocitrate dehydrogenase is purely to provide α-ketoglutarate for nitrogen assimilation (6). Isocitrate dehydrogenase expression levels have been reported to be higher during nitrogen stress in the non-nitrogen fixing *Synechocystis* (12). At cellular ammonium concentrations below 1 mM, ammonium is assimilated by glutamine synthetase. At increased cellular ammonium levels glutamate dehydrogenase functions, due to its relatively high K_S for ammonium (11), thereby increasing the cellular glutamate (Glu) concentration relative to the cellular glutamine (Gln) concentration. Under elevated nitrogen status, glutamine synthetase may also be inhibited (4) thereby increasing Glu concentration relative to Gln concentration. Glu concentration may be significant in regulation of MCYST production as *mcyF* encodes a glutamate racemase for the production of D-Glu for incorporation into MCYST (7).

Cellular Gln concentration, and to a lesser extent cellular Glu concentration, is an indicator of nitrogen. The work presented in this chapter was therefore aimed at determining whether any relationship existed between cellular nitrogen status, specifically glutamine and glutamate concentrations, and $MCYST_{CC}$ and $MCYST_Q$ in *M. aeruginosa* PCC 7806.

Methods

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.

Culture

All axenic cultures were maintained in BG11 (16) at 25°C with 140 μmol photon m^{-2} s^{-1} provided by Triton Dayglo® fluorescent lamps in 1 litre bubble-lift vessels with aeration through a 0.22μm filter at a rate of 2 ml s^{-1} (± 0.5 ml s^{-1}). 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 $BG11_0$ (16) 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.

Analysis

Cell counts, biovolume, MCYST, protein, cellular N and P and carbon fixation rates were determined as previously described (1, 2). 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.

Statistical analysis

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

Results

Starvation experiment

Nitrogen starvation reduced growth to a much greater extent than phosphorus starvation (Fig 5.1). Figure 5.2 shows the cellular atomic N:P ratio, the cellular protein content, cellular L-glutamate and L-glutamine concentrations and MCYST cell quota ($MCYST_Q$) and cellular concentration.

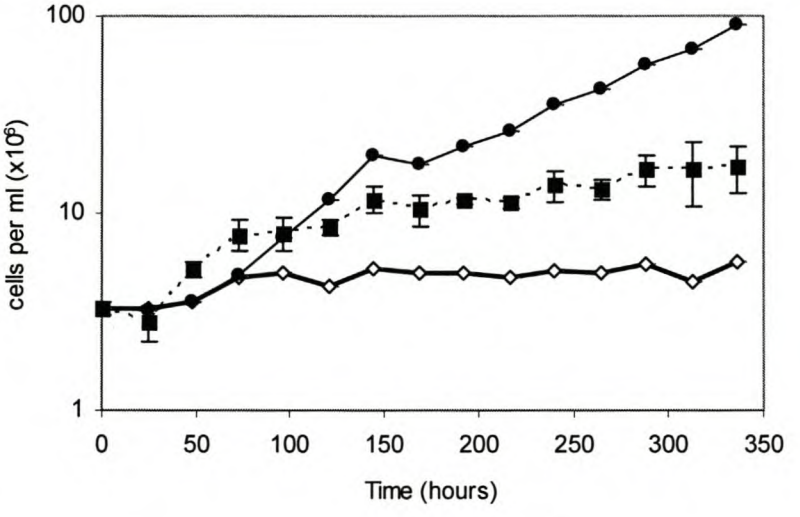

Figure 5.1 Cell numbers. Control (•), Nitrogen starved (◊), phosphate starved (■).

Cellular atomic N:P ratios were significantly higher in both control and phosphate starved cultures relative to nitrogen starved cultures (Fig 5.2A). Initial cellular N:P ratios in phosphate starved cultures were significantly higher than in control cultures but this difference disappeared within 100

hours and although phosphate starved cultures retained a higher N:P ratio this was generally not significant. Cellular protein content followed the same trend with phosphate starved and control cultures having a higher protein content (Fig 5.2D). Cellular glutamate levels were generally higher than control values in nitrogen starved cultures and lower than control values in phosphorus starved cultures (Fig 5.2B), while cellular glutamine concentrations were higher than control values in phosphorus starved cultures and lower than control values in nitrogen starved cultures (Fig 5.2C). $MCYST_Q$ and $MCYST_{CC}$ followed the same trend.

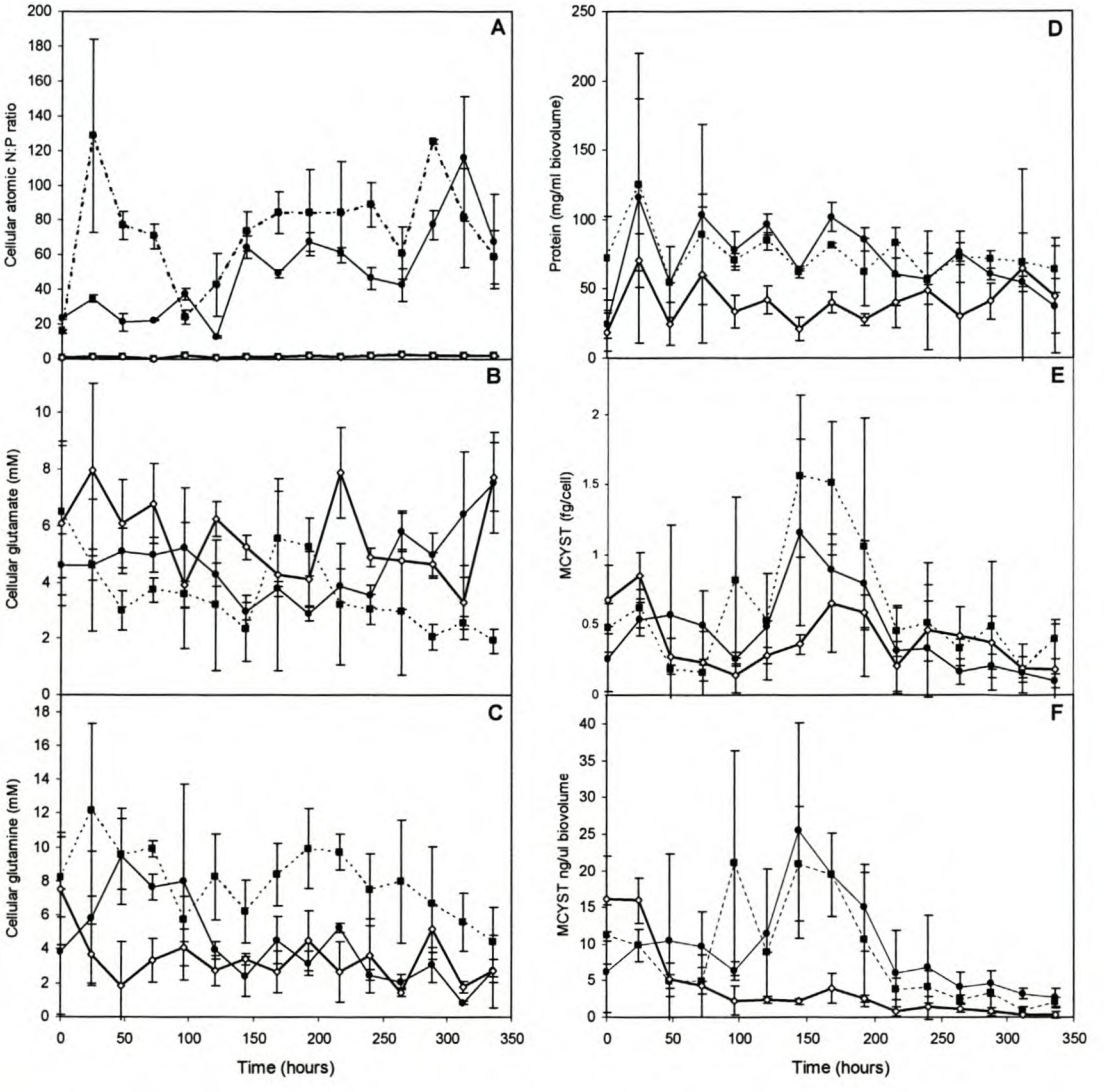

Figure 5.2 Cellular atomic N:P ratio (A), cellular glutamate concentration (B) cellular glutamine concentration (C) cellular protein (D) MCYST cell quota (E) and MCYST cellular concentration (F) for control (●), Nitrogen starved (◊), phosphate starved (■) cultures. Points are means and error bars denote standard deviation (n=3).

$MCYST_Q$ peaked between 100 and 200 hours for all treatments with phosphorus starved cultures having the highest values. Nitrogen starved cultures also appeared to exhibit an increase in $MCYST_Q$ at approximately 170 hours (Fig 5.2E). $MCYST_{CC}$ under nitrogen starvation was, however, not elevated at this time, and was significantly lower than either the control or phosphorus starved values (Fig 5.2F). Table 5.1 contains a summary of correlation coefficients for the relevant variables and $MCYST_Q$ and $MCYST_{CC}$. $MCYST_Q$ was negatively correlated with cellular glutamate concentrations. $MCYST_{CC}$ was only positively correlated with total cellular nitrogen and negatively correlated with cellular glutamate.

Table 5.1 Summary of product-moment correlation coefficients between cellular protein, nitrogen, L-glutamine, L-glutamate and microcystin cell quota and cellular concentration for *combined nitrogen and phosphorus starvation experiments and controls* (n=135).

Parameter	Cell nitrogen	Cell protein	Cell glutamine	Cell glutamate
$MCYST_Q$	0.0531	0.1265	0.1197	-0.2030*
$MCYST_{CC}$	0.2519**	0.0192	-0.1358	-0.2153*
Cell glutamate	-0.4813***	-0.2389*	-0.1541	
Cell glutamine	0.3532***	0.2091*		
Cell protein	0.2480**			

*P<0.05, ** p<0.01, *** p < 0.001

Correlation analysis of individual treatments revealed no significant correlation between any of the variables in nitrogen starved cultures although cellular Gln was slightly positively correlated with $MCYST_{CC}$ and cellular Glu had a weak negative correlation with $MCYST_{CC}$. Correlations for phosphate starved and control cultures are listed in Tables 5.2 and 5.3 respectively. The only significant correlations with MCYST were obtained in control cultures where $MCYST_Q$ was negatively correlated with cellular glutamate and $MCYST_{CC}$ was negatively correlated with cellular glutamine.

Table 5.2 Summary of product-moment correlation coefficients between cellular protein, nitrogen, L-glutamine, L-glutamate and microcystin cell quota and cellular concentration for *phosphorus starved cultures* (n=45)

Parameter	Cell nitrogen	Cell protein	Cell glutamine	Cell glutamate
$MCYST_Q$	-0.1067	-0.0165	0.1095	-0.0151
$MCYST_{CC}$	0.1297	-0.1208	-0.2454	-0.1114
Cell glutamate	-0.4430**	-0.1909	0.4993**	
Cell glutamine	-0.2691	0.0581		
Cell protein	-0.1009			

*P<0.05, ** p<0.01

Table 5.3 Summary of product-moment correlation coefficients between cellular protein, nitrogen, L-glutamine, L-glutamate and microcystin cell quota and cellular concentration for *control cultures* (n=45)

Parameter	Cell nitrogen	Cell protein	Cell glutamine	Cell glutamate
$MCYST_Q$	-0.0714	0.1080	0.1540	-0.4538**
$MCYST_{CC}$	0.0024	-0.1272	-0.4262**	-0.2818
Cell glutamate	-0.2312	-0.0069	0.0061	
Cell glutamine	0.1631	0.0736		
Cell protein	-0.5309**			

*P<0.05, ** p<0.01

The relationship between cellular Glu:Gln and cellular P and cellular N:P ratio are depicted in figure 5.3. Cellular Gln:Glu ratio was negatively correlated with P (-4.709, p<0.001) and positively correlated with N:P (0.5746, p<0.001). Figure 5.4 shows $MCYST_{CC}$ as a function of cellular Gln:Glu ratio. The highest $MCYST_{CC}$ (>15ng/µl biovolume) were obtained at cellular Gln:Glu ratios between 0.5 and 3, corresponding to cellular N:P ratios of between 40 and 80. No linear relationship between Gln:Glu ratio and $MCYST_{CC}$ was observed although an optimum Gln:Glu ratio of between 0.5 and 3 appeared to correspond with maximum $MCYST_{CC}$.

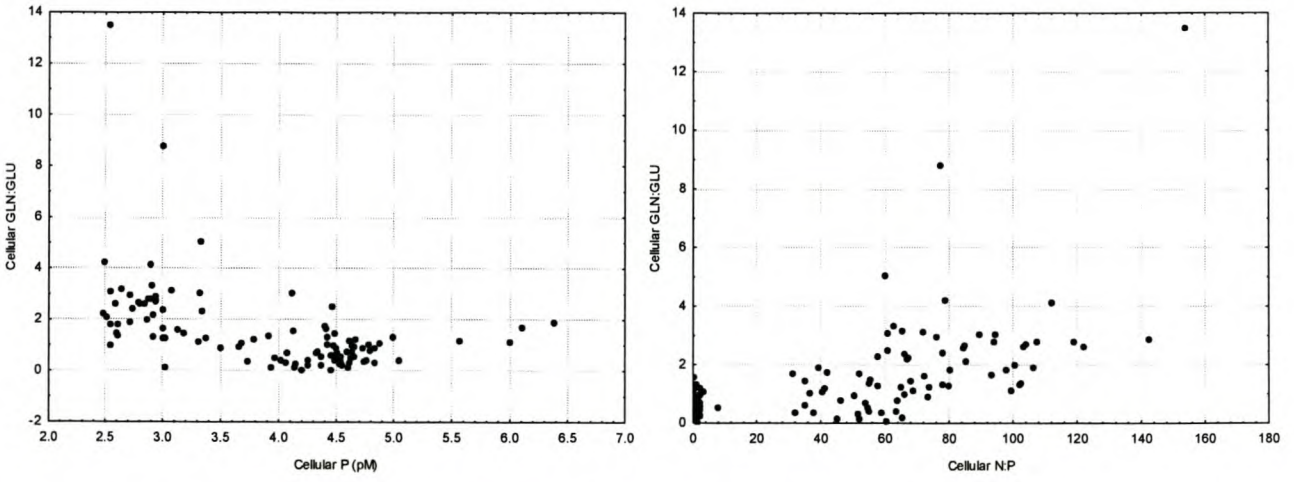

Figure 5.3 Cellular Gln:Glu ratios in relation to cellular P and cellular N:P ratio for nitrogen and phosphorus starved and un-starved cultures.

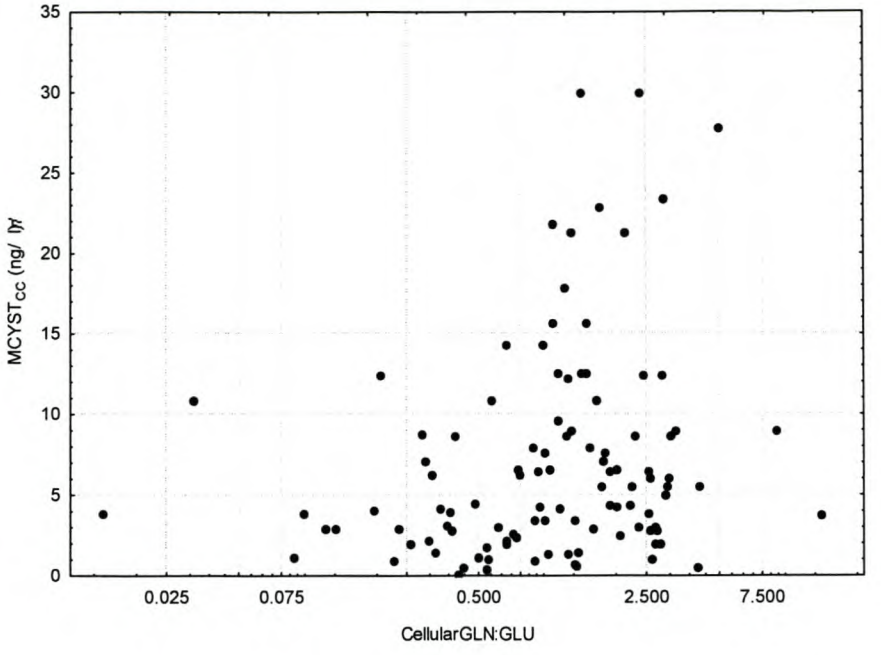

Figure 5.4 Cellular MCYST concentrations as a function of cellular Gln:Glu ratio for starved and un-starved cultures.

Cellular Glu correlated positively with carbon fixation rate (0.3789, $p<0.001$). No significant correlation was observed between Gln and carbon fixation rate. Figure 5.5 shows the relationship between carbon fixation, cellular N:P ratio and cellular Glu and Gln concentrations. Gln concentrations depended primarily on elevated cellular N:P ratios (or cellular N) whereas Glu concentrations depended primarily on carbon fixation rate within the optimal N:P range. Gln production depended on Glu and available reduced nitrogen and was increased under conditions where Glu was elevated and adequate nitrogen was available, or under excess nitrogen. $MCYST_{CC}$ values were highest within the optimal N:P ratio range where the cellular Gln:Glu ratio was between 1 and 3. Higher Gln:Glu ratios did not correspond with increased $MCYST_{CC}$. Thus $MCYST_{CC}$ appeared to depend on both cellular Glu and cellular Gln. These data suggest cellular nitrogen in excess of that required for growth as the modulator for MCYST production. Figure 5.7 shows the cellular Glu and Gln concentrations as a function of average growth rate for each of the treatments. In P starved cultures Glu and Gln were positively correlated while in N starved and control cultures no correlation was observed.

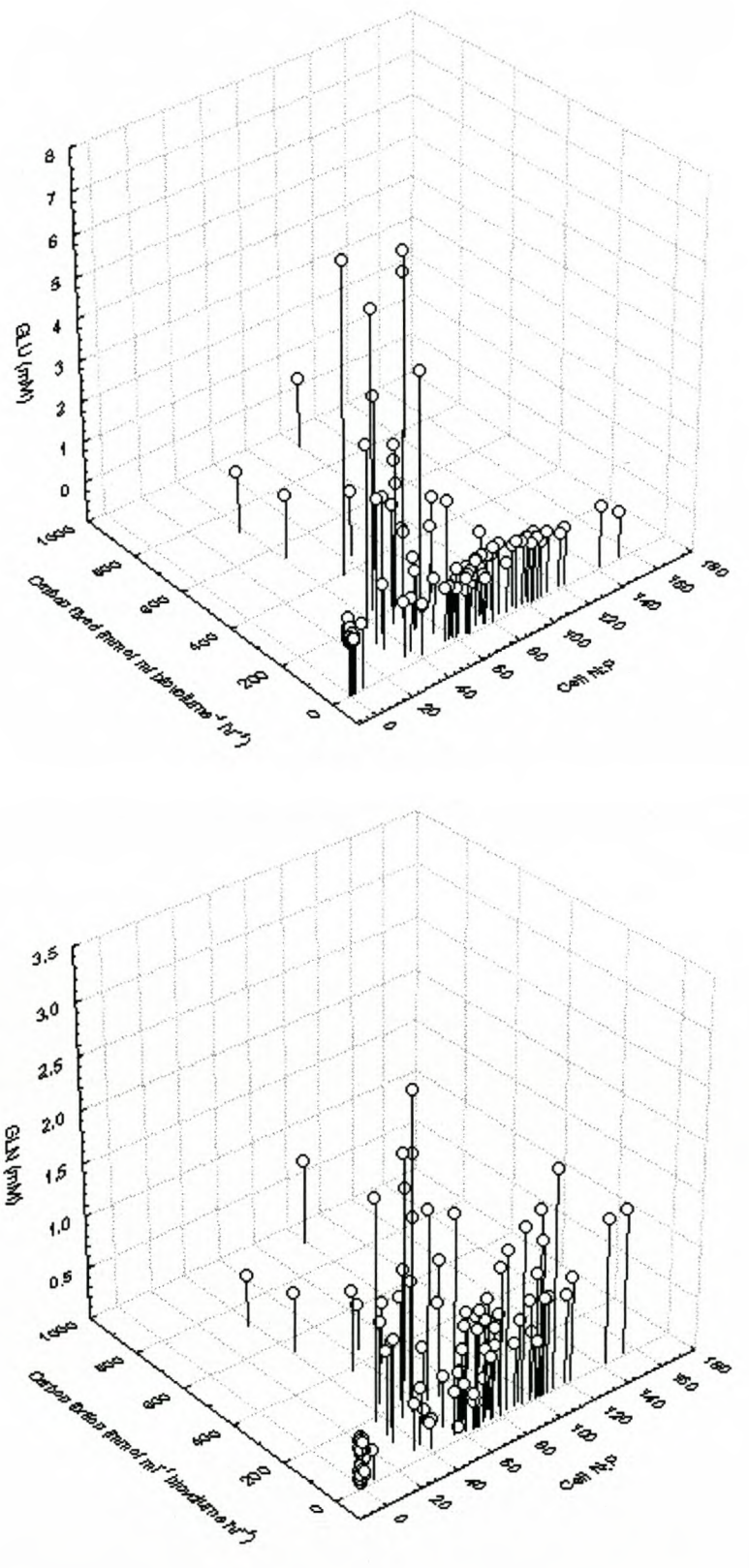

Figure 5.5 Relationship between carbon fixation rate, cellular N:P ratio and cellular L-glutamate and cellular L-glutamine. Data from starved and starvation control cultures.

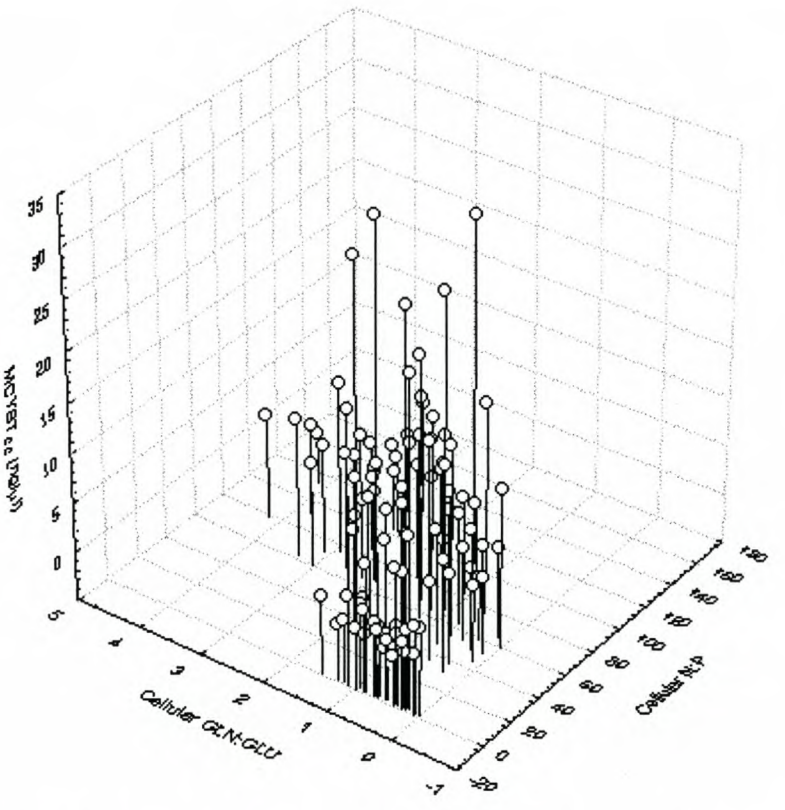

Figure 5.6 Relationship between cellular microcystin concentrations and cellular Gln:Glu and N:P ratios.

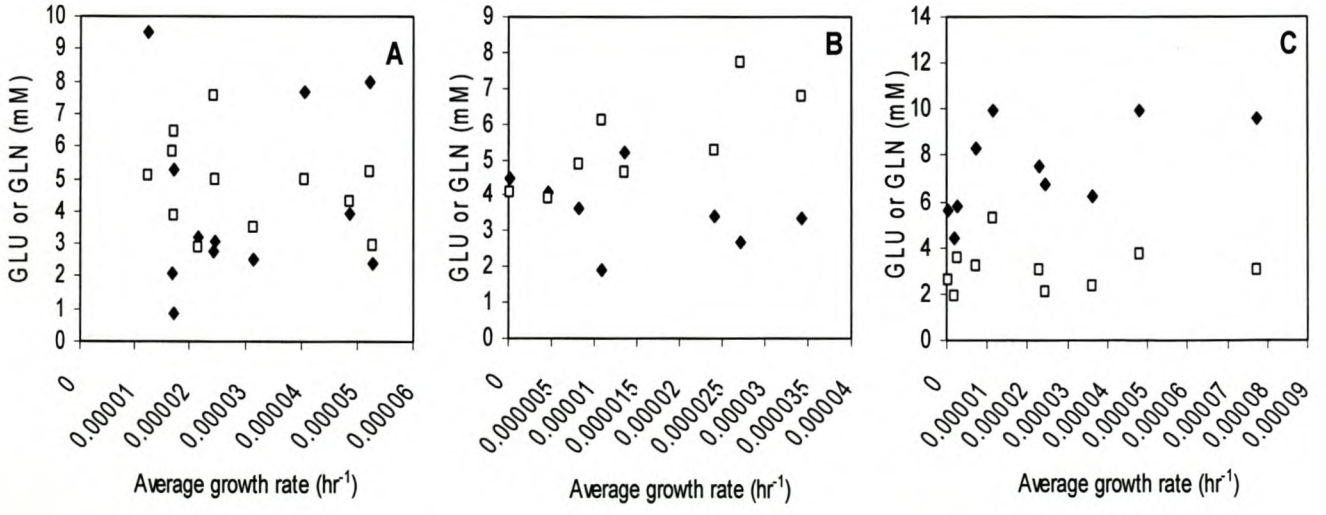

Figure 5.7 Cellular glutamate (□) and glutamine (♦) for control (A), nitrogen starved (B) and phosphorus starved (C) cultures as a function of average growth rate. Data are averages of triplicate values. Error bars omitted for clarity.

The relationship between $MCYST_{CC}$ and average growth rate for each treatment is shown in Figure 5.8. Under nitrogen starvation conditions cellular Glu concentrations were higher than cellular Gln concentrations at all but the lowest observed positive average growth rates. Under phosphorus starvation Gln concentrations were higher at all observed positive growth rates. In starvation control cultures no consistent trend in the relationship was observed (Fig 5.7). In cultures with elevated Gln relative to Glu, higher $MCYST_{CC}$ values were obtained at lower growth rates.

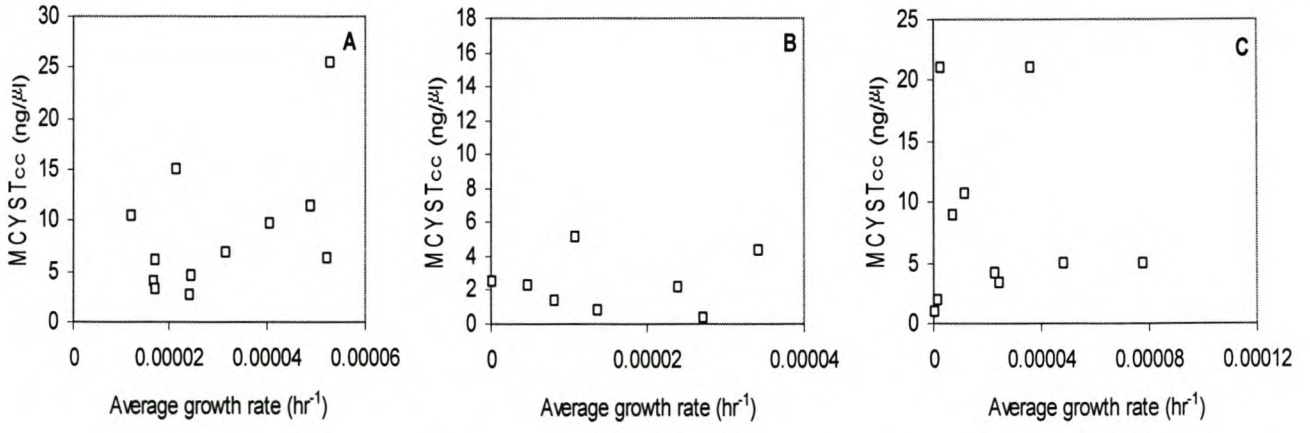

Figure 5.8 $MCYST_{CC}$ for control (A), nitrogen starved (B) and phosphorus starved (C) cultures as a function of average growth rate. Data are averages of triplicate values. Error bars omitted for clarity.

The relationship between $MCYST_{CC}$ and $MCYST_Q$ is shown in figure 5.9. At elevated $MCYST_Q$, $MCYST_{CC}$ increased. Thus cellular MCYST concentrations are variable and $MCYST_Q$ is not a function of cell size.

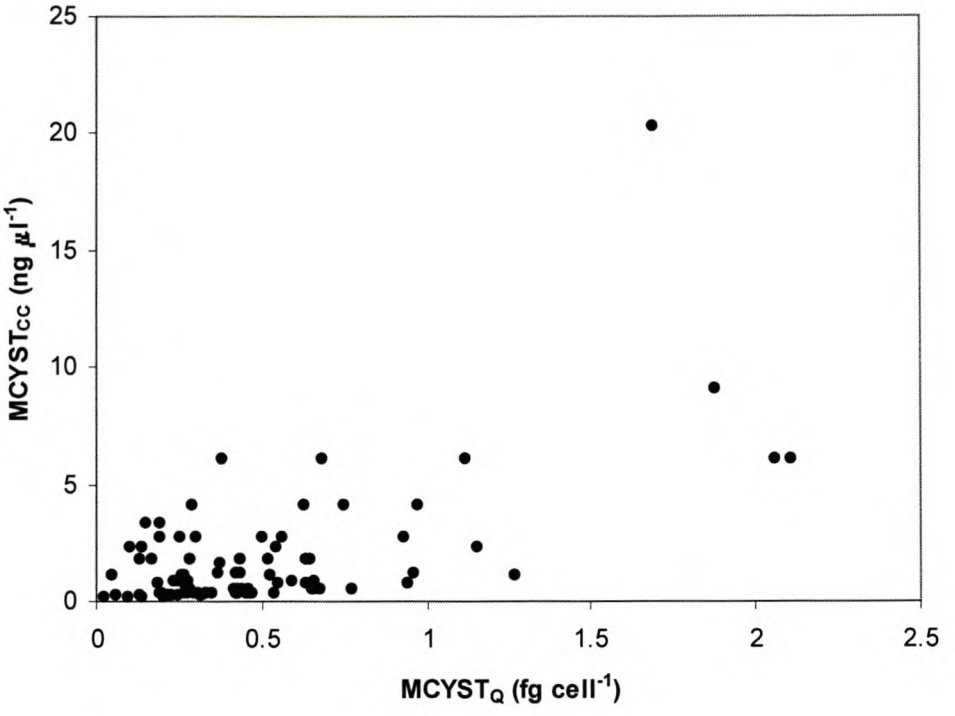

Figure 5.9 Relationship between cellular microcystin concentration and microcystin cellular quota.

Inhibition experiment

Figure 5.10 shows the cellular MCYST, Glu and Gln concentrations for the control and MSX and azaserine-treated cultures. L-glutamate levels were significantly reduced in azaserine-treated cultures compared to the control and MSX-treated cultures. In MSX-treated cultures L-glutamate was also significantly reduced compared to the control at 54 hours. MSX treatment resulted in a significant decrease in L-glutamine levels relative to both control and azaserine-treated cultures, but only after 10 hours. $MCYST_{CC}$ was significantly higher in control cultures compared to both azaserine and MSX-treated cultures after 54 hours. In azaserine-treated cultures $MCYST_{CC}$ appeared to follow the same pattern as Gln, whereas in MSX-treated cultures $MCYST_{CC}$ appeared to follow the same pattern as Glu.

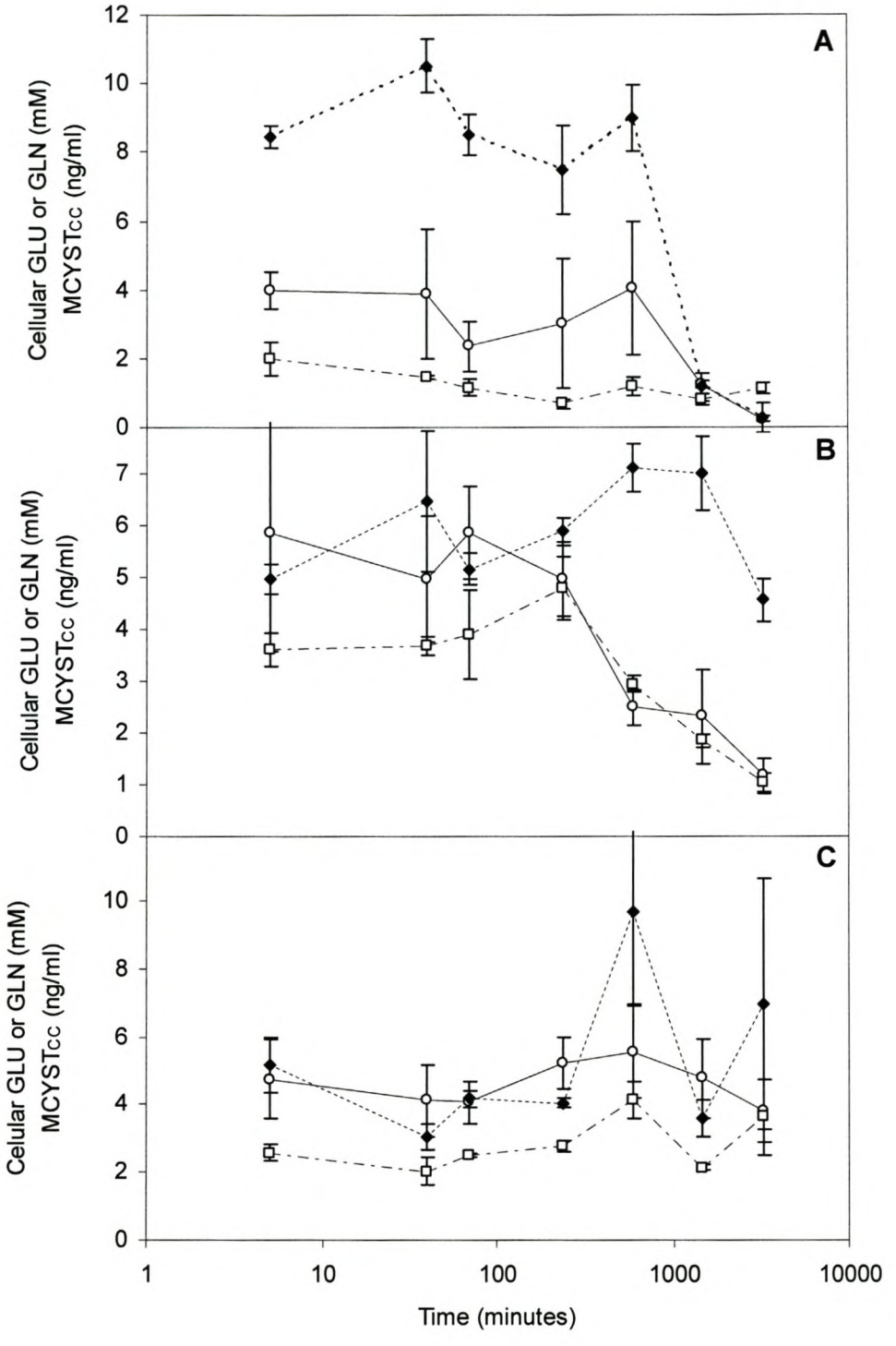

Figure 5.10 Cellular microcystin (○), L-glutamate (□) and L-glutamine(♦) concentrations in cultures exposed to azaserine (A), MSX (B) and control cultures (C). Points are means and error bars denote standard deviation (n=3).

Table 5.4 summarizes the correlation coefficients for $MCYST_{CC}$, Glu and Gln for all data in azaserine and MSX treatments and controls. In the absence of

nutrient limitation, but with manipulation of cellular Glu and Gln, the only correlation was between $MCYST_{CC}$ and Glu.

Table 5.4 Summary of correlation coefficients between cellular MCYST, Glu and Gln for cultures treated with inhibitors (n=63).

Parameter	Cell glutamine	Cell Glutamate
$MCYST_{CC}$	0.2160 (p=0.089)	0.5946 (p<0.001)
Cell glutamate	0.1595 (p=0.212)	

A significant positive correlation was observed between cellular glutamate and $MCYST_{CC}$. Due to variations in the trends observed for different treatments, individual treatments were subjected to correlation analysis. Table 5.5 shows the results of this analysis.

Table 5.5 Summary of correlation coefficients between cellular MCYST, Glu and Gln for each treatment (n=21)

Treatment	Control		Azaserine		MSX	
Parameter	Glu	Gln	Glu	Gln	Glu	Gln
$MCYST_{CC}$	-0.0150	0.0328	0.4333*	0.7307***	0.7638***	-0.1901
Gln	0.8755***		0.7307***		0.0340	

*P<0.05, *** p < 0.001

With the exception of the MSX-treated culture where Gln production was inhibited leading to an insignificant negative correlation, Glu and Gln cellular concentrations were strongly positively correlated. Average growth rates for the duration of the experiment were 0.0065 hr^{-1} for the azaserine-treated cultures, 0.0043 hr^{-1} for MSX-treated cultures and 0.102 hr^{-1} for control cultures. Similarly, only the control culture showed a significant increase in total biovolume, increasing from 0.461 µl ml $culture^{-1}$ to 1.583 µl ml $culture^{-1}$ over 54 hours. For the same period MSX-treated culture total biovolume increased by only 38.6% while the culture total biovolume of azaserine-treated cultures decreased by 5.1%. The lower Glu and Gln concentrations in control cultures were therefore in part due to dilution by growth. Similarly, the strong positive correlations between $MCYST_{CC}$ and Gln in azaserine-treated cultures and between $MCYST_{CC}$ and Glu in MSX-treated cultures may have been due to accumulation of both the substrate for the inhibited enzyme and MCYST due to inhibition of growth.

Discussion

$MCYST_{CC}$ was not negatively correlated with cellular Glu in any of the individually analysed 350 hr culture treatments. Combined starvation and starvation control data yielded a strong negative correlation between $MCYST_{CC}$ and cellular Glu suggesting one of the varied culture conditions or associated effects as a co-modulator of both Glu and $MCYST_{CC}$.

Cellular Gln:Glu ratios depended on cellular P with decreasing Gln:Glu at increased P, while increased cellular N:P ratios increased cellular Gln:Glu ratios (Fig 5.3). The peak cellular Glu:Gln ratio observed at intermediate cellular phosphorus levels may be related to the previously reported (2, 8, 14) peak production of MCYST as a function of medium N:P ratio, suggesting either Gln:Glu ratios or Gln as the primary modulator of MCYST synthesis. This is further supported by the increased MCYST production as Gln:Glu increases, although no significant positive correlations were observed between $MCYST_{CC}$ and cellular Gln concentrations. The highest $MCYST_{CC}$ values were obtained at Gln:Glu ratios between 1 and 3 (Fig 5.4), corresponding to N:P ratios of between 40 and 80 (see also Figure 5.6). Cellular Glu increased with increasing carbon fixation rate within the optimal N:P ratio range. Cellular Gln increased with increasing N:P at low carbon fixation rates but achieved maximum values under conditions yielding maximum Glu. However, since Gln production requires adequate reduced nitrogen and Glu, it follows that correlations with Gln would not necessarily yield useful results. Gln did not correlate negatively with $MCYST_{CC}$ in nitrogen replete treatments either. Thus both cellular Glu and Gln were elevated under conditions previously shown (2) to yield increased $MCYST_{Q}$ (Fig 5.5), while $MCYST_{CC}$ increased under adequate Glu and increased Gln:Glu, or in conditions where nitrogen was in excess of that required to yield sufficient Glu for growth.

Growth rate was reduced in phosphorus-starved cultures relative to control cultures and further reduced in nitrogen-starved cultures. A significant negative correlation between $MCYST_{CC}$ and cellular Gln was observed in control cultures. In nitrogen starved cultures there was a weak positive

correlation while in phosphorus starved cultures a weak negative correlation was obtained. Thus, in rapidly growing cultures Gln was significantly negatively correlated with $MCYST_{CC}$ while in the slower growing phosphorus starved cultures the correlation remained negative but not significant. In cultures with highly reduced growth rates (nitrogen starved) the correlation was positive but not significant. Correlations between $MCYST_{CC}$ and Glu for each treatment were insignificant but the most rapidly growing culture had the highest negative correlation, while phosphorus starved cultures had the lowest. These trends appear to indicate no dependence of MCYST production on cellular Gln or Glu.

It is useful to consider a few alternative explanations for the negative correlations between $MCYST_{CC}$ and Glu and Gln (Table 5.1). The negative correlations may be a function of depletion of cellular Gln or Glu due to growth and concomitant production of MCYST to maintain cellular concentrations, and hence constitutive MCYST production. This corresponds with the increase in $MCYST_{CC}$ with increasing growth rate observed in control cultures (Fig 5.8). Alternatively, the negative correlations between assimilated nitrogen in the form of these amino acids may indicate repression of MCYST production under nitrogen replete conditions. This is, however, not the case, as has been previously shown (2, 1). As a second alternative, depletion of either or both of these amino acids may represent α-ketoglutarate limitation as this is required for nitrogen assimilation. This is suggested by the decrease in cellular Glu and increase in Gln (Fig 5.7) in phosphorus starved cultures relative to control cultures at increased growth rates, and decreased $MCYST_{CC}$ (Fig 5.8) at these increased growth rates. This, in turn, suggests a requirement for Glu for MCYST production and a rapid utilization of Glu for MCYST production in order to yield the observed negative correlations. In nitrogen starved cultures $MCYST_{CC}$ was consistently lower than in the other treatments.

In nutrient-replete conditions but with inhibition of either glutamate synthase or glutamine synthetase, cellular Glu was significantly correlated with $MCYST_{CC}$. However, as with starvation cultures, analysis of individual treatments was

more meaningful. No correlation between either Glu or Gln and $MCYST_{CC}$ was observed in control cultures presumably due to dilution by growth. In MSX-inhibited cultures $MCYST_{CC}$ was strongly correlated with Glu, which accumulated due to glutamine synthetase inhibition. In azaserine-treated cultures both cellular Glu and Gln were significantly positively correlated with $MCYST_{CC}$, with Gln having a stronger correlation. This correlation was attributed to the reduced growth rate and decrease in biovolume and resulting large increase in cellular Gln concentrations.

Thus with reduced growth due to either phosphorus limitation or with inhibition of glutamate synthase, increased $MCYST_{CC}$ was observed at lower growth rates within the growth rates achieved due to phosphorus limitation. Azaserine similarly reduced growth. This produced a significant correlation with Gln as Gln accumulated because of reduction in carbon fixation rate under adequate nitrogen in phosphorus starved cultures or because of inhibition of glutamate synthase resulting in accumulation of Gln. This apparent correlation with Gln may indicate a role for Gln in the regulation of MCYST production but is more likely due to continued production of MCYST at a given rate but no increase in biovolume (Fig 5.2F). The relatively higher $MCYST_{CC}$ observed in MSX-treated cells was attributed to the function of *mcyF* , a glutamate racemase (13) responsible for the production of D-Glu for incorporation into MCYST. Under excess ammonium, active GDH would also result in transiently decreased Gln:Glu ratios and subsequent increases in D-glutamate.

Cellular Gln, recognised as a nitrogen status indicator, does not in itself provide an indication of $MCYST_{CC}$ since adequate cellular Glu is also required for MCYST production. This suggests a requirement for sufficient carbon fixation for Glu production to serve either as a precursor to D-glutamate or as a requirement for growth. In either case, growth is intimately linked to MCYST production (as concluded by Long *et al.* (9)) but with substantial variation as a function of nitrogen (as suggested by Downing *et al.* (1)). $MCYST_{CC}$ in *M. aeruginosa* PCC 7806 is therefore determined by the relationship between cellular nitrogen status and growth rate with $MCYST_{CC}$ increasing as a

function of reduced growth, under nitrogen-replete or nitrogen-excess conditions. Cellular nitrogen status is primarily a function of available nitrogen concentration and type, orthophosphate availability and PAR. Development of a model for prediction of MCYST content from environmental variables would therefore require the incorporation of availability of, and the effects of, these variables on growth and MCYST production. In its simplest form such a model would consider excess environmental nitrogen as the primary modulator of MCYST production, although this would be complicated by the type of available nitrogen due to the effects of ammonium compared to those of nitrate on toxin production (20). It would therefore appear that despite the primary role of nitrogen, no definitive model for environmental regulation of MCYST production by *Microcystis* is likely to emerge until detailed mechanisms of regulation and accurate models for growth that include all these interdependent variables are available.

Despite the lack of correlation between cellular nitrogen status and $MCYST_{CC}$, the possibility of indirect nitrogen regulation of MCYST production by changes in cellular α-ketoglutarate concentrations remains. Increased cellular Gln and Glu with reduced growth rate would result from reduction in carbon fixation and hence reduction in α-ketoglutarate. α-Ketoglutarate has been shown to enhance the binding of NtcA, a cellular nitrogen-metabolism regulator that may play a role in the regulation of MCYST production. A putative NtcA binding site has been identified upstream of the *mcyABC* operon, in a position suggesting that NtcA may repress *mcyABC* transcription (Chapter 4). Similarly, correlations between $MCYST_{CC}$ and cellular glutamate under nitrogen excess may result from de-repression of *mcy* by reduction in α-ketoglutarate as a result of activity of glutamate dehydrogenase under excess cellular ammonium.

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Chapter 6: Cellular α-ketoglutarate and microcystin production in *Microcystis aeruginosa*

As described in the previous chapter

- Microcystin cellular concentrations increase with increasing glutamine:glutamate ratios in accordance with results obtained for microcystin as a function of cellular C:N balance
- There is no consistent relationship between microcystin and glutamine or glutamate that can be explained in terms of regulation of *mcy* transcription
- Ammonium does not induce microcystin synthesis but does repress NtcA expression
- There is therefore no indication of a regulatory mechanism based on glutamate or glutamine

However

- NtcA functions not only in response to nitrogen depletion but also to increased cellular C:N - α-ketoglutarate enhances binding of NtcA to appropriate DNA sequences
- Negative correlations between microcystin and glutamate may indicate α-ketoglutarate regulation of NtcA activity and hence the possibility of NtcA regulation of *mcy* expression remains

Therefore

- The possibility exists that α-ketoglutarate may be involved in regulation of microcystin production via NtcA control of *mcy* expression
- The following chapter addresses this hypothesis

Abstract

Under conditions with adequate photosynthetically active radiation cellular microcystin and cellular glutamate are negatively correlated. Positive correlations between cellular microcystin and cellular glutamate occur under similar condition but with inhibition of nitrogen assimilation. This indicates 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 dependent 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.

Introduction

Cellular microcystin concentration ($MCYST_{CC}$) in *Microcystis aeruginosa* does not generally correlate significantly with cellular nitrogen status (Chapter 5) although increased $MCYST_{CC}$ is observed at increased N uptake (2) and increased cellular N:C ratios (1). Where cellular glutamine (Gln) accumulates due to α-ketoglutarate limitation, either as a function of phosphorus limitation or reduction in photosynthetically active radiation (PAR), $MCYST_{CC}$ did, however, correlate positively with cellular Gln. Similarly, with increased cellular Gln or glutamate (Glu), but under conditions yielding reduced growth rate, $MCYST_{CC}$ correlated with these amino acid indicators of cellular nitrogen status. Where growth rate was reduced due to nitrogen (N) limitation, no such correlations were observed. Negative correlations between $MCYST_{CC}$ and Gln in uninhibited, non nutrient-limited cultures were similarly attributable to production of MCYST at a rate corresponding to growth rate, and thus depletion of Gln. This was supported by data showing elevated $MCYST_{CC}$ at increased growth rate and a significant negative correlation between MCYST cell quota ($MCYST_Q$) and cellular Glu; this suggests a reduction in the rate of increase in biovolume as a result of reduced α-ketoglutarate and hence reduced Glu production (Chapter 5).

Correlations between $MCYST_{CC}$ and cellular Glu or Gln therefore appear to occur as a function of reduced growth rate (1, 2) or as a result of reduced cellular α-ketoglutarate due to reduction in carbon fixation rate relative to growth rate. The latter may occur either because of carbon limitation (1), reduced PAR (1), or due to P limitation. In all cases where significant positive correlations were observed between either Glu or Gln and $MCYST_{CC}$, these correlations could be attributed either to the accumulation of the pertinent amino acid due to inhibition of the relevant enzyme, or due to reduction in growth rate as a function of P limitation. In accordance with these data, $MCYST_{CC}$ is increased at elevated cellular Gln:Glu ratios, with peak levels being achieved at ratios of between 1 and 3. This corresponds to optimum N:P ratios for maximal MCYST production (2). That both N:P and Gln:Glu ratios follow the same trend supports the suggestion that α-ketoglutarate may

play a role in the regulation of MCYST production since α-ketoglutarate would effectively be reduced concomitantly with increases in these ratios. Similarly, increased cellular Glu as a result of increased cellular ammonium would effectively reduce cellular α-ketoglutarate because of activation of glutamate dehydrogenase. This, in turn, would result in de-repression of *mcy* and a positive correlation between Glu and $MCYST_{CC}$, which further implicates α-ketoglutarate as a potential regulator of MCYST production.

Analysis of the polypeptide synthetase gene cluster (*mcy*A-C) promoter region suggests NtcA as a potential repressor for *mcyA-C* (Chapter 4). NtcA expression is up-regulated under nitrogen limitation, and binding of NtcA to appropriate promoter regions is enhanced by α-ketoglutarate. Transcription rates of *ntcA* and *mcyA* have been shown to be inversely related with enhanced *mcyA* transcription under dark, nitrogen replete conditions (Chapter 4). Thus, were NtcA to function as a repressor of *mcyA* and therefore a repressor of MCYST production, $MCYST_{CC}$ would be expected to show a negative correlation with α-ketoglutarate. The presence or strength of any correlation would however, as with Glu and Gln correlations with $MCYST_{CC}$, potentially be influenced by growth rate.

The aim of this work was therefore to elucidate any relationship that may exist between cellular α-ketoglutarate and $MCYST_{CC}$ under conditions devised to yield reduced cellular nitrogen, reduced nitrogen assimilation and reduced carbon fixation, within a period of time sufficiently small to limit the effects of growth rate.

Methods

Culture

Axenic *M. aeruginosa* PCC 7806 was maintained on BG11 (8). 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 120ml 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.

Analysis

Cellular MCYST was extracted with methanol from lyophilised samples as previously described (2) and quantified using ELISA (Abraxis) off a standard curve (r^2 = 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 as described by Muro-Pastor *et al.* (6) and quantified off a standard curve constructed for this purpose (r^2 = 0.9973). Cell counts and total biovolumes were determined as previously described (1) and used to calculate final cellular concentrations of MCYST and α-ketoglutarate. Statistical analysis was performed using Statistica 7 ® (Statsoft).

Results

Figure 6.1 shows the cellular α-ketoglutarate and $MCYST_{CC}$ for all treatments over the 270 minute incubation. Control cultures showed a steady decline in α-ketoglutarate over the period, with a large significant increase in $MCYST_{CC}$ at 120 minutes and subsequent return to lower levels after 270 minutes. Cultures maintained in the absence of PAR showed the same trend as control cultures in α-ketoglutarate levels and $MCYST_{CC}$ levels and values were not significantly different from control values with the exception of the initial (five minutes) α-ketoglutarate average value which was 35% lower. Inhibition of

carbon fixation resulted in declining α-ketoglutarate levels for the first 120 minutes followed by a significant increase at 270 minutes relative to both control and dark cultures (Fig 6.1 C). α-Ketoglutarate levels in MSX-treated cultures were similar to control values while no significant difference from the control in $MCYST_{CC}$ levels was observed except at 120 minutes where $MCYST_{CC}$ was approximately 30% of the control value (Fig 6.1 D).

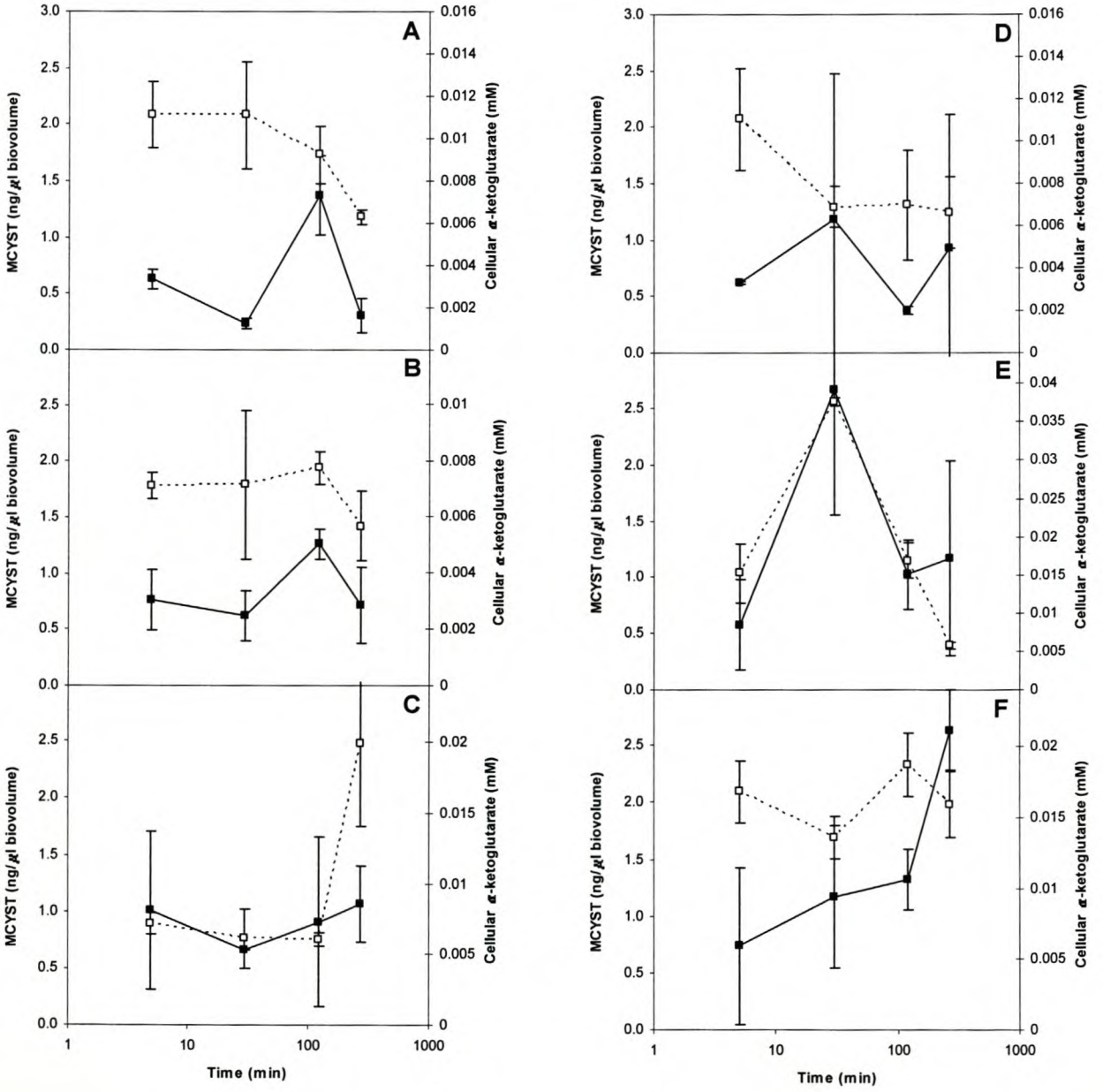

Figure 6.1 Cellular α-ketoglutarate (□) and microcystin (■) concentrations in control (A), dark (B), D,L-Glyceraldehyde (C), MSX (D), nitrogen starved (E) and double medium nitrogen (F) cultures.

Significant increases in $MCYST_{CC}$ were observed at 30 minutes for the culture containing no nitrogen in the medium; these increases were accompanied by similar increases in α-ketoglutarate with values for both molecules being more than double that of any other treatment at this time. For 120 minutes and 270 minutes both molecules returned to levels comparable to control values. Cultures exposed to double the standard nitrogen had significantly elevated α-ketoglutarate levels relative to the control at all but the 30 minute point and $MCYST_{CC}$ levels increased steadily over the 270 minutes with the large significant increase at 270 minutes.

Figure 6.2 shows the relationship between cellular α-ketoglutarate and $MCYST_{CC}$ for individual data points and table 1 is a summary of correlation coefficients between cellular α-ketoglutarate and $MCYST_{CC}$ for all cases and for each individual treatment. Significant positive correlations were observed for cultures incubated in the absence of PAR or medium nitrogen. A significant negative correlation was observed in D,L-Glyceraldehyde-treated cultures. Analysis of all data points revealed a significant positive correlation between $MCYST_{CC}$ and α-ketoglutarate.

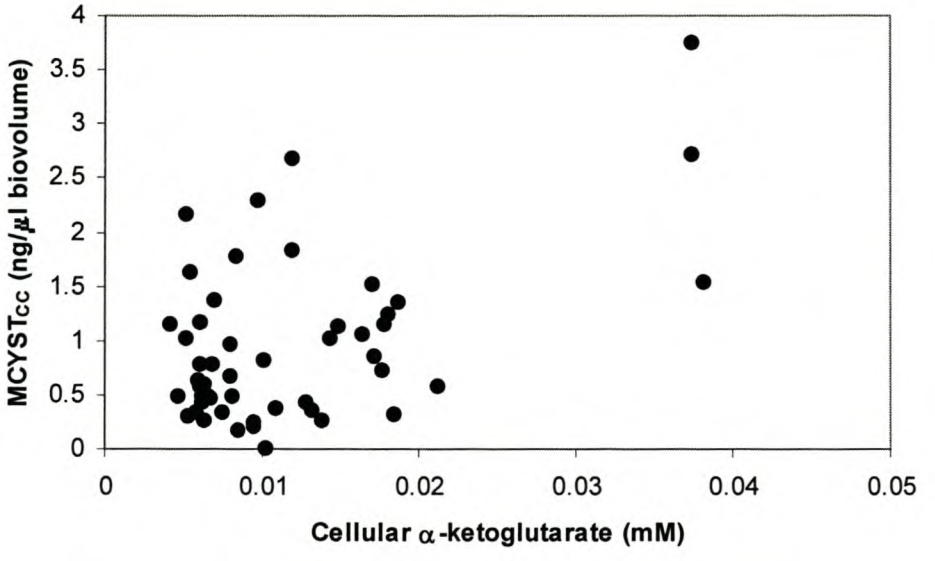

Figure 6.2 Relationship between cellular α-ketoglutarate and microcystin concentrations.

Table 6.1 Summary of correlation coefficients between cellular α-ketoglutarate and microcystin concentrations for all data (n = 72) and each treatment (n = 12) separately.

All cases	Control	Dark	MSX	DL-G	No nitrogen	2 x nitrogen
0.4353	-0.0775	0.5249	-.0691	-0.5652	0.6273	0.2864
$p < 0.05$	NS*	$p < 0.05$	NS	$p < 0.05$	$p < 0.05$	NS

*NS, $p > 0.05$

Discussion

A positive correlation between $MCYST_{CC}$ and α-ketoglutarate does not necessarily imply positive regulation, but may in fact suggest a role for MCYST in enhancement of carbon fixation. Thus a decrease in α-ketoglutarate would result in enhanced *mcy* transcription but the presence of elevated $MCYST_{CC}$ would result in increased carbon fixation and a return to lower rates of *mcy* expression. The inverse relationship observed between *ntcA* and *mcyA* transcript levels (Chapter 3) would therefore result from carbon limitation and not nitrogen excess per se. This is in accordance with data obtained under carbon limitation and nitrogen excess at a constant growth rate (1).

However, MCYST values were not significantly reduced in MSX-treated cultures relative to the control. The initial result of inhibition of glutamine synthetase would be an accumulation of Glu and a resulting increase in cellular α-ketoglutarate. The absence in significant reduction in MCYST levels may therefore have been due to existing enzyme complexes and increased Glu availability for incorporation into toxin, and may not have been the result of an absence of repression of *mcy* translation products.

It would therefore appear that the positive correlation between α-ketoglutarate and $MCYST_{CC}$ is due to enhanced nitrogen assimilation potential, or increased cellular Glu due to reduced nitrogen availability in the presence of excess α-ketoglutarate, and the subsequent production of D-Glu for incorporation into MCYST. The relationship between nitrogen availability and $MCYST_{CC}$ would therefore be controlled by α-ketoglutarate concentrations in such a way that excess nitrogen would result in increased MCYST production

if adequate α-ketoglutarate were available to yield increased Glu concentrations (as observed in Figure 6.1F). Alternatively, since α-ketoglutarate is a primary regulator of the NrtABC nitrite/nitrate transporter (3, 5), the positive correlation may result from the requirement for α-ketoglutarate for nitrate uptake, in turn leading to adequate Glu for racemization (7) and inclusion into MCYST. Similarly, elevated NtcA levels would result in repression of *gifA* and *gifB*, resulting in increased glutamine synthetase activity (4) and subsequent reduction in α-ketoglutarate as a function of increased nitrogen assimilation, increased Glu and therefore increased $MCYST_{CC}$, leading to a positive correlation between $MCYST_{CC}$ and α-ketoglutarate.

There is at present insufficient evidence to implicate α-ketoglutarate in the regulation of MCYST production. The complex control of carbon fixation and nitrogen uptake and assimilation, and the capacity for storage of carbon and nitrogen compounds by *M. aeruginosa* further complicate the investigation of both physiological and molecular regulatory mechanisms for MCYST. The possibility exists that increased α-ketoglutarate is a result of increased $MCYST_{CC}$, rather than α-ketoglutarate being involved in regulation of MCYST production; the reason is that a positive correlation between $MCYST_{CC}$ and cellular Glu, as occurs at elevated Glu levels (Chapter 5), would result in depletion of α-ketoglutarate under nitrogen excess. Investigations into comparative photosynthetic activity and energy distribution to carbon fixation for MCYST producing and non-MCYST producing strains may shed light on any role for MCYST in enhancement of carbon fixation.

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Chapter 7: Final discussion & conclusions

As described in the previous chapter

- The negative correlation between microcystin and α-ketoglutarate observed when carbon fixation was inhibited was not observed in cultures grown in the dark or in the presence of excess nitrogen
- In the absence of nitrogen, a positive correlation was observed between microcystin and α-ketoglutarate

Thus

- There exists no evidence for NtcA regulation of *mcy* expression on the basis of α-ketoglutarate control of NtcA

Therefore

- Microcystin content in *M. aeruginosa* appears to be metabolically regulated based primarily on the distribution of amino acids between growth related processes and microcystin production, subject to the potential of the cell to assimilate nitrogen at a greater rate than is used by growth
- The following chapter concludes this thesis

Introduction

The work described in this thesis was conducted (i) to elucidate the role of environmental N:P ratios in the modulation of MCYST content of *M. aeruginosa*, (ii) to determine what changes in primary cellular metabolism resulted from changes in environmental N:P ratios, and (iii) to investigate potential regulatory mechanisms for MCYST production related to these metabolic changes. As a result of data obtained in investigating medium N:P ratio effects on $MCYST_Q$, protein levels, chlorophyll *a* levels and growth rate, the relative effects of carbon fixation rate and medium nitrogen availability were studied in continuous culture. Data obtained in these experiments suggested nitrogen as the primary modulator of MCYST production; growth rate and carbon fixation rate being secondary modulators, with carbon fixation being intimately linked to nitrogen assimilation. NtcA is a global nitrogen metabolism regulator in cyanobacteria; its activity is also subject to α-ketoglutarate and hence to carbon fixation rate relative to growth rate and cellular ammonium concentrations. Relative transcript levels of *mcyA* and *ntcA* were therefore determined for conditions that yield either increased nitrogen uptake relative to carbon fixation or increased carbon fixation relative to nitrogen uptake. Cellular glutamate, glutamine and α-ketoglutarate levels were also investigated under conditions determined to alter cellular levels of these metabolites, and the relationship between levels of these metabolites and cellular MCYST was recorded. The results obtained in relation to previously published data are discussed below.

Medium N:P ratios and microcystin modulation

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 (Fig 2.1); these N:P ratios correspond to those between 16 and 50 described as optimum for $MCYST_{DW}$ (9). However, unlike the data presented by Lee *et al.* (9), these data do not include conditions where limitation of either nutrient occurred. Thus the reduced $MCYST_{DW}$ at lower and higher N:P ratios (9) 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 (Fig 3.5). Vezie *et al.* (21) 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. The data presented in Chapter 2 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 confirm 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.* (11) and Orr and Jones (16). They also suggest that phosphorus 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.

Carbon fixation

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 (Chapter 3). Experimental conditions yielding nitrogen-limited cultures and carbon-limited cultures (Fig 3.2) revealed a significant increase in the microcystin production rate (R_{MCYST}) under carbon limitation and a significant decrease under nitrogen limitation at the same growth rate (Fig 3.1), corresponding to matching changes in cellular N:C ratios.

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 in Figure 3.6 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 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.

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

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

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

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

Chemostat data from Chapter 3 yields the results shown in Figure 7.1

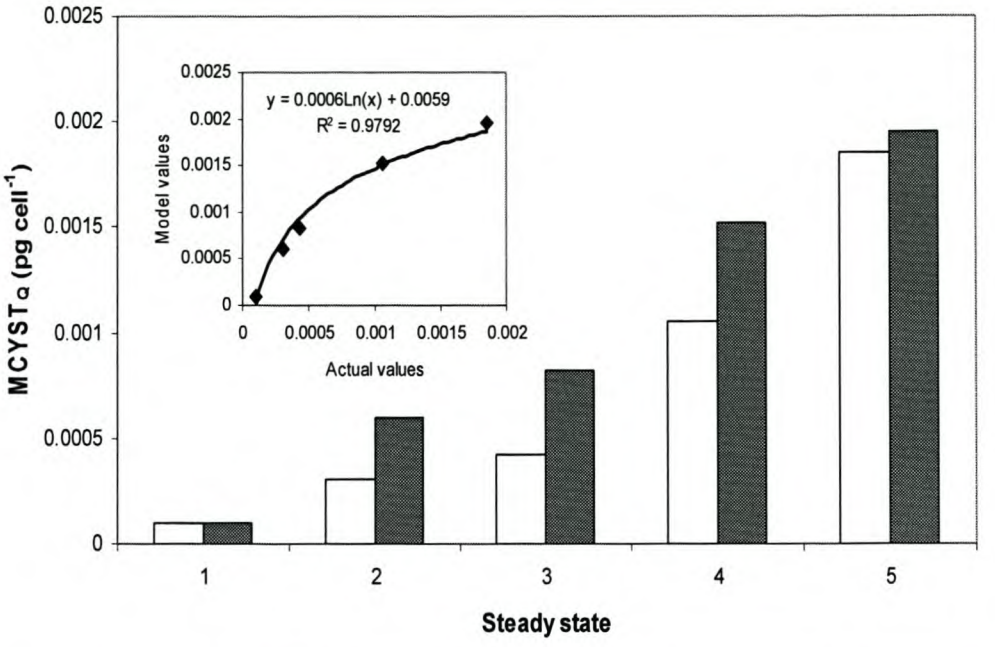

Figure 7.1 Modelled (shaded bars) and experimental (open bars) data comparison. Data presented in Chapter 3 was used. Inset shows relationship between modelled and experimental values.

That carbon fixation depends on PAR remains a problem for modelling MCYST production, although the dependence of carbon fixation on P (Table 3.1) and the dependence on fixed carbon for growth allows the use of environmental biologically available nitrogen and phosphorus, as well as growth as parameters, since growth rate incorporates carbon fixation rate as a function of PAR.

Specific growth rate

Figure 2.2A shows the positive relationship between growth rate and $MCYST_Q$ for two strains of *M. aeruginosa*, in accordance with the linear relationship between R_{MCYST} and growth rate in phosphorus-limited continuous culture reported by Oh *et al.* (14). In nitrogen-limited continuous culture the same relationship between growth rate and R_{MCYST} was observed by Long *et al.* (11), leading to the conclusion that growth rate was the primary modulator of R_{MCYST} where growth rate was a function of cellular N quota (11). The apparent contradiction that arises from a similar relationship occurring under phosphorus 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 phosphorus limited chemostats at higher growth rate (14). Results presented in Figure 2.3 of this document 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 (Fig 3.7). 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 phosphorus-limited cultures (Fig 5.8). The uncoupling of nitrogen assimilation and growth related metabolism from cell division is illustrated in Figure 7.2 which shows the cellular protein and $MCYST_Q$ as a function of nitrogen uptake/phosphorus uptake (data from Chapter 1). 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 (Table 2.1).

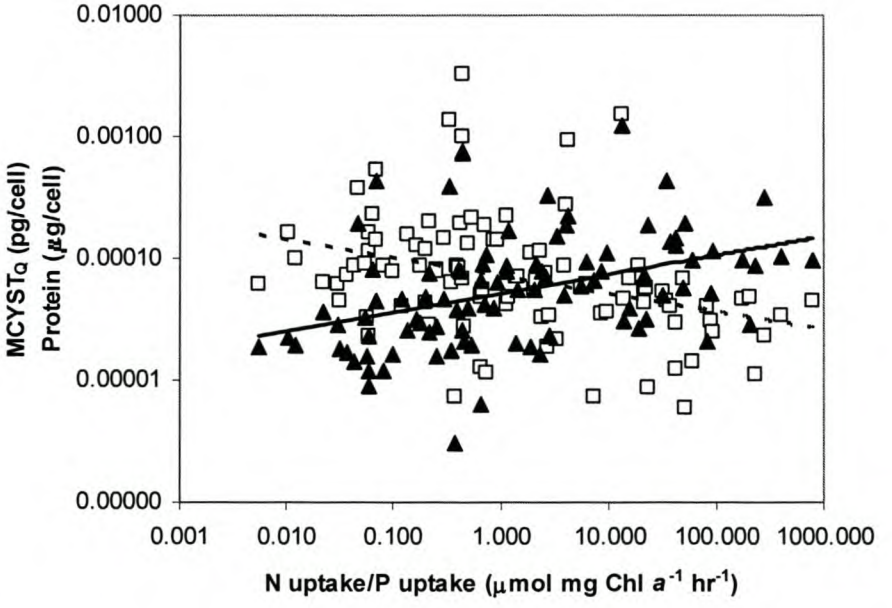

Figure 7.2 $MCYST_Q$ (▲) and cellular protein (□) as a function of nitrogen uptake/phosphorus uptake during unlimited growth of *M.aeruginosa* PCC 7806 and UV 027. Regressions for protein (dashed line) $y = 7x10^{-5}x^{-0.1475}$, $r^2 = 0.1276$ and $MCYST_Q$ (solid line) $y = 5x10^{-05}x^{0.1561}$, $r^2 = 0.1593$ are included to show trends.

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.

Transcriptional regulation of microcystin production

$MCYST_{CC}$ and cellular protein content are strongly correlated during unlimited growth (Table 2.1) suggesting constitutive MCYST production. *mcy* transcript levels have, however, been show to vary as a function of light (5). Chapter 4 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*. 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* (Fig 4.4). 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 (Table 6.1) 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 $MCYST_{CC}$ (as described in Table 5.4 and depicted in Figure 5.4). The significant positive correlation between $MCYST_{CC}$ and α-ketoglutarate observed in cultures in the absence of

light (Table 6.1) 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 is depicted in Figure 4.1, 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 (See Table 6.1) 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 (Fig 4.4) observed when cultures were maintained in the absence of light, and by the retention of increased MCYST levels under these conditions (Fig 4.1). 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.

Nitrogen assimilation metabolites and MCYST modulation

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 (Table 5.5) although $MCYST_{CC}$ was lower (Fig 5.10). 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} (Fig 5.5). 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 phosphorus-starved cultures (Fig 5.7). $MCYST_{CC}$ and $MCYST_{Q}$ were significantly negatively correlated with cellular Glu (Table 5.1) and $MCYST_{CC}$ was highest at cellular Gln:Glu ratios between 1 and 3 (Fig 5.4), corresponding to N:P ratios of between 60 and 100; these values were notably higher than those previously recorded. However, the N:P dependent increase in cellular Gln at low carbon fixation rates, corresponding to reduced levels of Glu (Fig 5.5), suggests Glu as the primary metabolite affecter of MCYST production. The negative correlation between $MCYST_{CC}$ and Glu was therefore possibly 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 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 (13). Thus in general for MCYST-LR, the required initial Gln:Glu ratio is substantially lower than the optimum ratio observed in Figure 5.4.

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 (18). In complex with P_{II}, the catalytic activity of

NAG kinase is strongly enhanced (4), 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.* (23). Similarly Lee *et al.* (9) 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.* (14) 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 (Table 6.1). These seemingly contradictory results may be attributed to α-ketoglutarate enhancement of NtcA binding, but in the absence of conclusive evidence this remains speculation.

The above discussion suggests that cellular Gln:Glu ratios are a function of 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 (24, 25).

A general model

Figure 7.3 shows a generalized conceptual model for the modulation of MCYST production in *Microcystis* based on the data presented in Chapters 2 through 6 and the cited literature. The model makes the initial general assumption that MCYST production is constitutive and that cellular carbon, nitrogen and phosphorus 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 (17). 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 figure 3.6 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}.

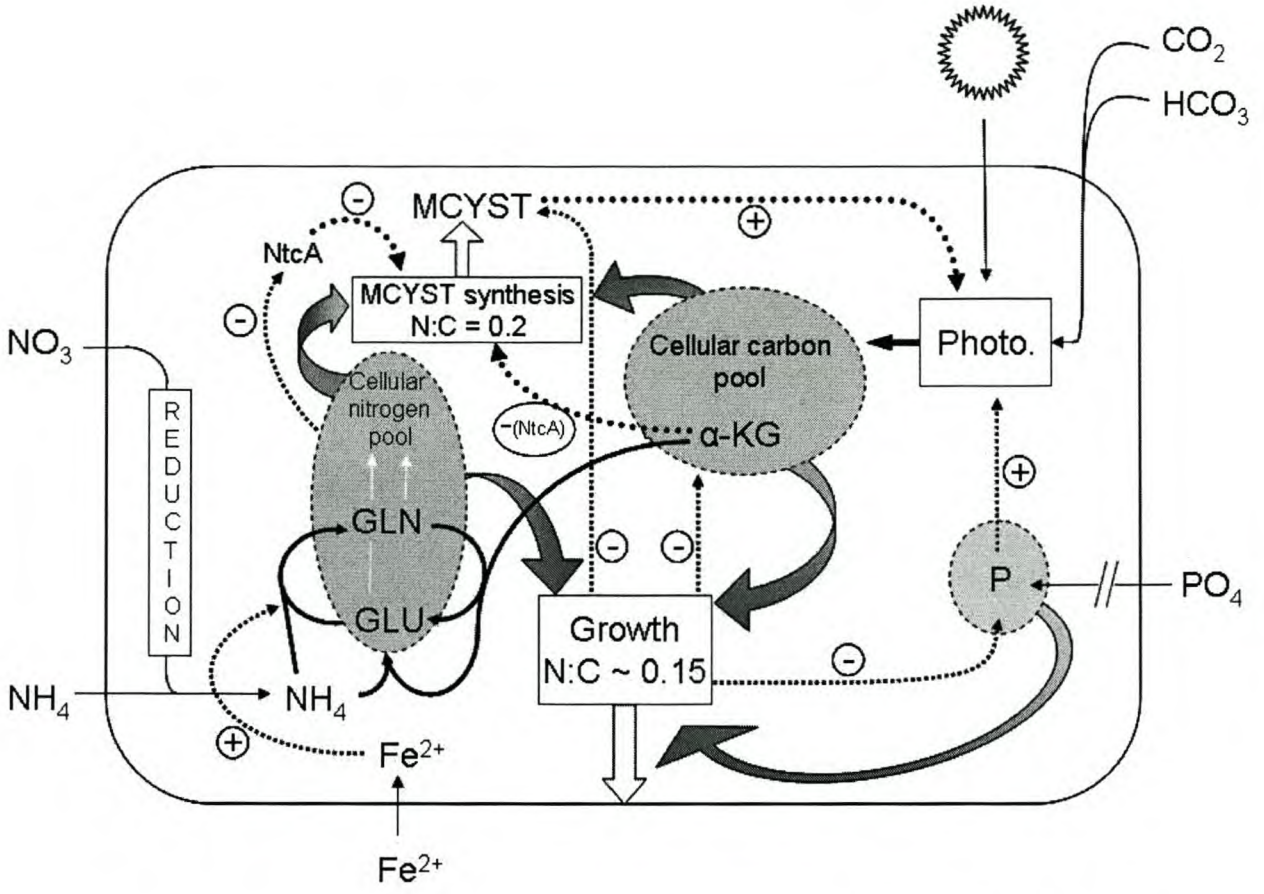

Figure 7.3 A generalized conceptual model for the modulation of microcystin production in Microcystis. Internalisation of nutrients and assimilatory metabolism is depicted with solid black arrows. Grey areas indicate cellular pools. Effects of components of the model on reactions is depicted with dashed 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 (Fig 3.4). The distribution of carbon and nitrogen pools on the basis of composition of MCYST and cellular C:N ratio therefore appears the 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 (Chapter 4) 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 (6). 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 (Table 3.1) 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.* (9)) $MCYST_Q$ is reduced (Fig 2.1A). 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 (Fig 2.1D and 2.2A), suggesting a further down-regulation of MCYST synthesis at these ratios. Data presented in Chapter 3 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 $MCYST_{CC}$ (Table 5.4), specifically the strong positive correlation observed in MSX-treated

cultures (Table 5.5), and by the reduction in $MCYST_{CC}$ at higher growth rates in phosphorus-starved cultures (Fig 5.8). The reduction in $MCYST_{CC}$ was also noted at maximum attainable Gln:Glu ratios (Fig 5.4).

This model therefore solves the apparent contradictions in literature discussed in Chapter 1 and explains the following published results:

- The increased MCYST at sub-optimal growth temperatures (22).
- The increased LD_{50} of cultures where increased temperatures resulted in increased growth rate (22)
- Negative correlation between MCYST and growth rate (20)

The model explains these data in that growth limitation in non-optimal temperatures would not necessarily impede nitrogen assimilation. Cellular nitrogen would thus accumulate resulting in increased MCYST production.

- The relationship between $MCYST_Q$ and the uncoupling of cell division and growth related metabolic processes (24)

The model shows that where cell division is reduced but nitrogen and carbon metabolism continue, MCYST production is increased primarily due to availability of nitrogen assimilation products.

- R_{MCYST} correlation with initial medium nitrogen (16)
- The direct relationship between R_{MCYST} and growth rate in nitrogen limited chemostats (11)

The model shows that MCYST production is essentially a function of available nitrogen. The rate of production of MCYST during exponential growth would be proportional to the initial available nitrogen since nitrogen assimilation at a rate exceeding the requirement for growth would result in more assimilated nitrogen for MCYST production. Conversely, the model shows that under nitrogen limitation, the assimilated nitrogen pool would be partitioned between growth and MCYST production according to the relative rates of these processes. Thus MCYST production would correlate to growth rate.

- Distribution of MCYST producing genotypes in natural planktonic colonies (8)
- Distribution of MCYST in *Microcystis* hollow fibre photobioreactor cultures (10)
- The effect of cellular C:N ratio on R_{MCYST} (2)
- The negative relationship between carbon fixation rate and $MCYST_{DW}$ (2, 14)
- The correlation between cellular carbohydrate and $MCYST_Q$ under non-limiting conditions (12)

The model predicts that under conditions of increased nitrogen availability relative to carbon fixation potential (or PAR intensity), increased MCYST would result due to altered cellular C:N ratios. Under unlimited growth conditions, cellular carbohydrate is proportional to nitrogen assimilation and hence correlates with $MCYST_Q$.

- The positive relationship between iron and MCYST (19) by virtue of the predominance of ferredoxin dependent GOGAT found in cyanobacteria (15).

The model shows the dependence of MCYST production on nitrogen fixation. Since ferredoxin dependent GOGAT is predominant in cyanobacteria, adequate iron is necessary for MCYST production.

- $MCYST_Q$ as a function of PAR relates to μ_{MAX} as a function of PAR
- Positive correlations between MCYST and PAR under non-saturating PAR when adequate nitrogen is present (24)

The model predicts that where growth rate cannot increase as a function of PAR (μ_{MAX}) and PAR continues to increase, $MCYST_Q$ will increase if nitrogen assimilation rate increases because of available carbon skeletons.

- Optimum medium N:P ratio for elevated $MCYST_Q$ (1, 9)

The model highlights the dependence of nitrogen assimilation on carbon skeletons. Carbon fixation is shown to be dependent on phosphorus. Data for medium N:P ratios (1, 9) were based on phosphorus limitation at the highest N:P ratios. Thus nitrogen assimilation would be reduced at the highest reported medium N:P ratios, limiting MCYST production relative to growth.

That no such optimum is observed for nitrate uptake rate (see Fig 3.6) confirms this.

- Variation in *mcy* transcript levels as a function of light (5).
- The presence of alternative *mcy* transcription start points for high and low light (6)
- A decrease in MCYST at saturating PAR (24)

Although not explicit in the model, the variation in *mcy* transcript levels and the presence of alternative, light dependent transcription start points can, be accommodated. The basis for variation in MCYST production is cellular C:N balance which is partly a function of light. Under low light, and hence increased cellular N:C ratios, increased *mcy* transcription occurs while at saturating PAR, the cellular N:C ratios are reduced. No data are available for the efficiency of transcription from different transcriptional start points. Additionally, the model illustrates that he putative transcriptional regulation by NtcA conforms to metabolic data.

The model does not directly address the following:

- Variation in relative concentrations of toxin variants (9, 23)

Insufficient data are available to include this in the model. It is possible that the changes in ratios of MCYST variants is as a function of environmental parameters is due to availability of appropriate amino acids. Or subtle expression differences due to sequence variation in the *mcy* genes.

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 (Fig 3.3), but positive correlation between α-ketoglutarate and MCYST under carbon

fixation inhibition during batch culture (Table 6.1), 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 7.4 which shows an 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 (12), the relationship represented in Figure 7.3 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.

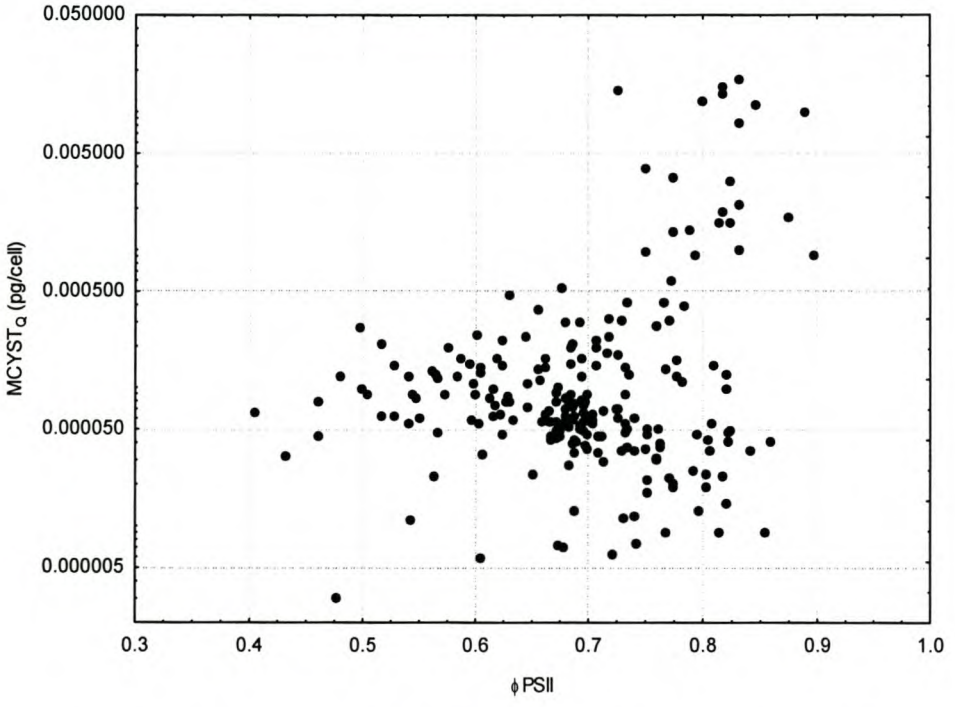

Figure 7.4 Relationship between ϕPSII and $MCYST_Q$ (Data from Chapter 2)

Conclusions and future work

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 phosphorus 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 phosphorus, 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 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 can be identified. Figure 7.5 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.

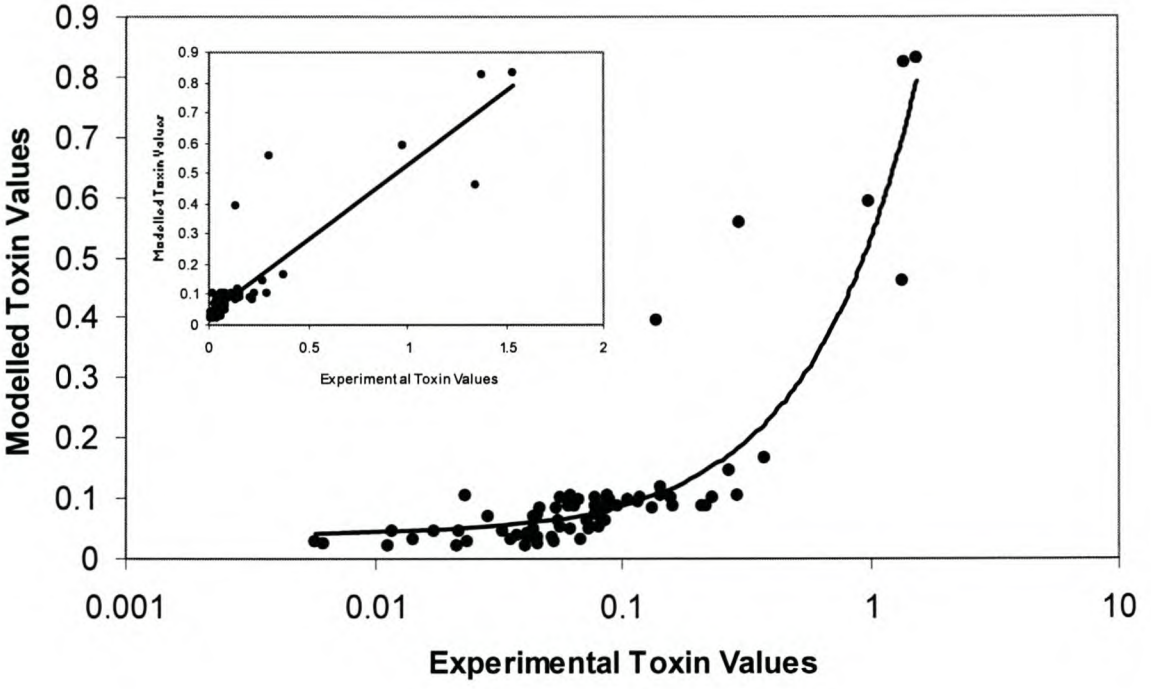

Figure 7.5 Modelled and experimental data comparison for a neural network model using only medium nitrogen and phosphorus and specific growth rate as input nodes. The network was trained on a random subset of data presented in Chapter 1 and verified on the remaining data. Regression: y=0.4906x + 0.0385. Inset shows this relationship with linear scales.

The effect of PAR on MCYST production, and the effects of temperature, were 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.

In conclusion, MCYST production occurs where nitrogen assimilation exceeds the use of assimilation products by growth, where growth rate is determined primarily by carbon fixation rate (in turn dependent on phosphorus availability and PAR), and nitrogen assimilation rate. Reduction in PAR, inorganic carbon or phosphorus 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 is possible using biologically available environmental nitrogen and phosphorus, and monitoring specific growth rate.

Work currently in progress includes comparison of L-Glu and D-Glu concentrations and ratios in relation to MCYST, the expansion of artificial neural network models to conditions more closely resembling natural water bodies, construction of a minimal set of equations to predict MCYST levels that encompasses nutrient loading and resulting residual nutrient concentrations in natural impoundments, and investigation of the role of MCYST as a potential enhancer of carbon fixation.

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