Medium N:P Ratios and Specific Growth Rate Comodulate Microcystin and Protein Content in *Microcystis aeruginosa* PCC7806 and *M. aeruginosa* UV027

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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 nonlimiting batch culture conditions for a range of medium nitrogen and phosphorous 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, suggest 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.

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 multispecific 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, 23–25, 29], no clear multiparameter 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

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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, whereas Watanabe and 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 nonlimiting 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 $\pm$ 5 μmol of photons m^{-2} s^{-1}) using Triton Dayglo fluorescent lamps at 23 $\pm$ 0.5°C with agitation supplied by filtered (2$\times$ Cameo acetate 0.22 μm) air at 2 $\pm$ 0.5 mL s^{-1}. Culture medium was modified BG11$_0$ [19] containing NaNO$_3$ and K$_2$HPO$_4$ 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 μ > 0. Direct cell counts were performed in a hemocytometer (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 (3000 *g* for 5 min) and snap frozen in liquid nitrogen before freeze drying for 24 h and extraction in 5 mL, 70% methanol [1, 26] with sonication (Bandlin Sovorex NK51) for 16 h. The resulting extract was dried (Savant SC100) and resuspended in 100 μL 50 mM Tris HCl pH 7.5 containing 0.1 mM 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 0.5 U 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) 0.1 mM EGTA, 0.1% (v/v) β-mercaptoethanol, and 50 μl aqueous manganous chloride (20 mM), in a total volume of 0.3 mL. Enzyme activity was determined by the amount of *p*-nitrophenol released in 30 min (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 K_m for specific uptake or K_s for growth for that nutrient, and where retardation of growth rate due to

Table 1. Summary of correlation coefficients between medium nutrient concentrations and ratios, cellular constituents, and growth rates for *M. aeruginosa* UV027 and *M. aeruginosa* PCC7806

Parameters and strains[a]	Variables[b]					
	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.

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, proteins and chlorophyll *a* content (Table 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 Figs. 1 and 2, and from Table 1, where nonlimiting conditions were analyzed.

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 (95th percentile) MCYST content at medium N:P ratios of between 18 and 51 (Fig. 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. 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 nonlimiting 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. 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. 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 h^{-1}) and N:P = 40 (μ = 0.092 h^{-1}) for UV027 (Fig. 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 h^{-1} for PCC7806 (95th percentile) and μ $>$ 0.06 h^{-1} for UV027 (96th percentile) (Fig. 2A). Similar trends were observed for chlorophyll *a* in PCC7806, but the trend was less in 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.

Inclusion of data where N:P < 1 highlights the codependence of cellular MCYST on medium N:P ratio and
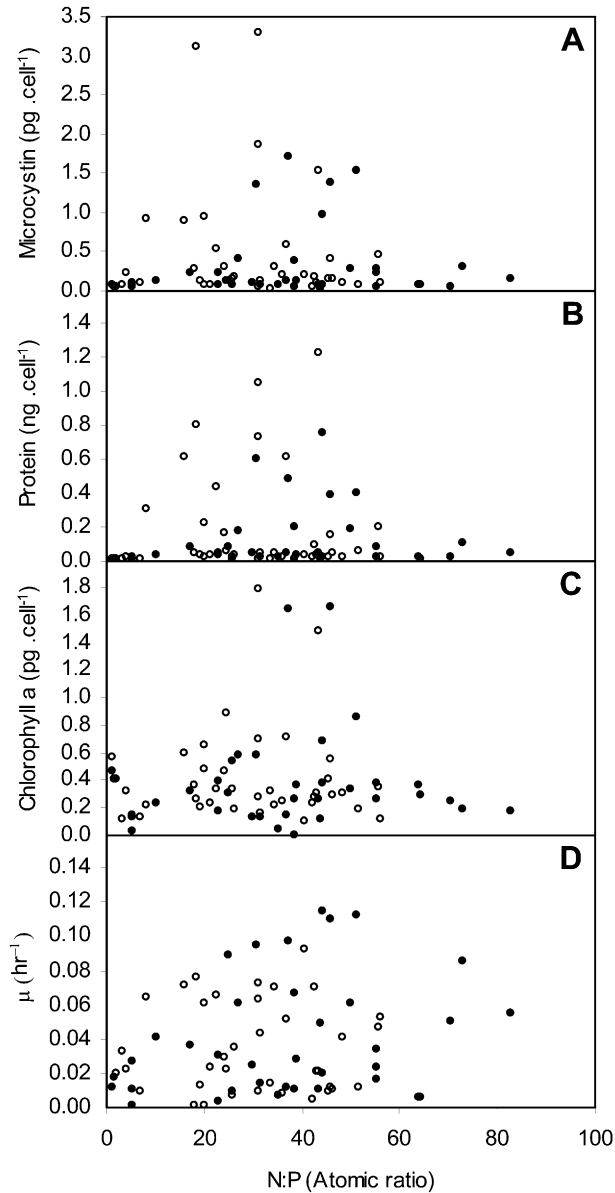
Figure 1. Cellular constituents [microcystin pg cell^{-1} (A), total protein ng pg. cell^{-1} (B), chlorophyll a pg. cell^{-1} (C), and μ h^{-1} (D)] of batch cultures of *M. aeruginosa* PCC7806 (●) and *M. aeruginosa* UV027 (○), for various medium N:P atomic ratios (1 < N:P < 83).

Figure 2. Cellular constituents [microcystin pg cell^{-1} (A), total protein ng cell^{-1} (B), and chlorophyll a pg·cell^{-1} (C)] of *M. aeruginosa* PCC7806(●) and *M. aeruginosa* UV027(○), for specific growth rates recorded for batch culture in medium with various N:P atomic ratios (1 < N:P < 83).

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 show this codependence (Fig. 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. 3B) while PCC7806 had the opposite trend (Fig. 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
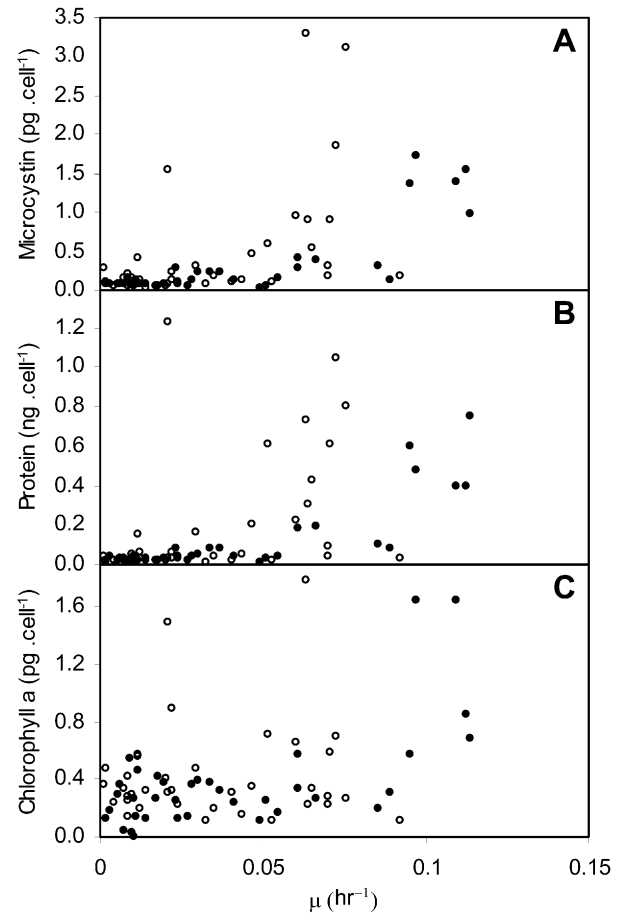
UV027 showed increasing MCYST with increasing μ. PCC7806 had no observable trend, although the highest MCYST value was obtained around μ_{mean} while UV027 had increased MCYST levels at higher μ values for 20 ≤ N:P < 30. For 30 < N:P < 40 both strains showed increased MCYST levels with increasing μ while 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. 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, whereas 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
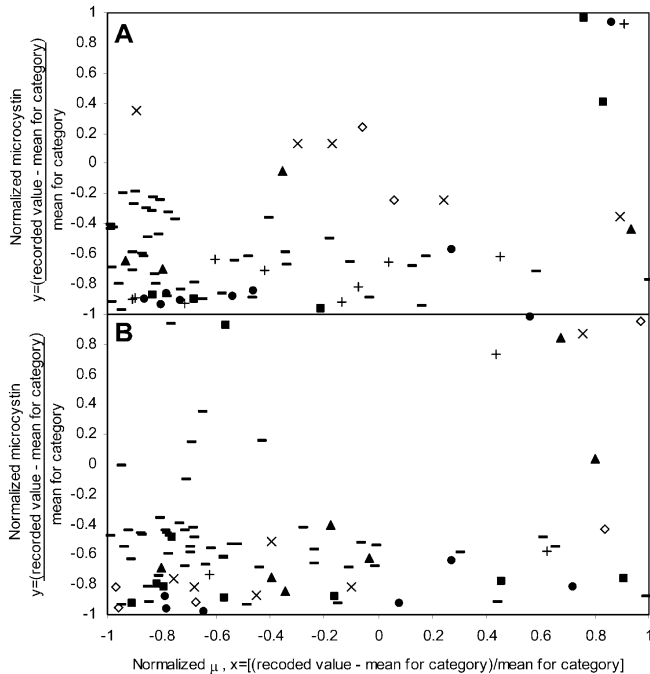
Figure 3. Normalized (actual value − mean/mean) MCYST content and μ for categories of N:P ratios where N:P < 1 (−), 1 ≤ N:P < 10 (×), 10 ≤ N:P < 20 (◇), 20 ≤ N:P < 30 (▲), 30 ≤ N:P < 40 (●), 40 ≤ N:P < 50 (■), N:P ≥ 50 (+) for PCC7806 (A) and UV027 (B).

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 nonlinear relationship between N:P ratio and the tested parameters, and most importantly the nonlimiting nutrient concentrations.

It would therefore appear that the correlation between cellular protein and MCYST levels may result from the comodulation of these variables by cellular nitrogen content as a function of N availability, or N assimilation rate, and specific growth rate. At increased 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 and Jones [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.

Acknowledgments

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