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SOURCE (OR PART OF THE FOLLOWING SOURCE):

Type	Dissertation
Title	Out of balance: implications of climate change for the ecological stoichiometry of harmful cyanobacteria
Author	D.B. van de Waal
Faculty	Faculty of Science
Year	2010
Pages	144
ISBN	978-94-61080-45-5

FULL BIBLIOGRAPHIC DETAILS:
http://dare.uva.nl/record/341589

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Chapter 4

Amino acid availability determines the composition of microcystin variants in the cyanobacterium *Planktothrix agardhii*

ABSTRACT - Cyanobacteria are capable of producing multiple microcystin variants simultaneously. The mechanisms that determine the composition of microcystin variants in cyanobacteria are still debated. [Asp^3]microcystin-RR contains arginine at the position where [Asp^3]microcystin-LR incorporates leucine. We cultured the filamentous cyanobacterium *Planktothrix agardhii* strain 126/3 with and without external addition of leucine and arginine. Addition of leucine to the growth medium resulted in a strong increase of the [Asp^3]microcystin-LR/RR ratio, while addition of arginine resulted in a decrease. This demonstrates that amino acid availability plays a role in the synthesis of different microcystin variants. Environmental changes affecting cell metabolism may cause differences in the intracellular availability of leucine and arginine, which can thus affect the production of microcystin variants. Because leucine contains one nitrogen atom while arginine contains four nitrogen atoms, we hypothesized that low nitrogen availability might shift the amino acid composition in favor of leucine, which might explain seasonal increases in the [Asp^3]microcystin-LR/RR ratio in natural populations. However, when a continuous culture of *P. agardhii* was shifted from nitrogen-saturated to a nitrogen-limited mineral medium, leucine and arginine concentrations decreased, but the leucine/arginine ratio did not change. Accordingly, while the total microcystin concentration of the cells decreased, we did not observe changes in the [Asp^3]microcystin-LR/RR ratio in response to nitrogen limitation.

This chapter is based on the paper: Linda Tonk, Dedmer B Van de Waal, Pieter Slot, Jef Huisman, Hans CP Matthijs, and Petra M Visser. 2008. Amino acid availability determines the ratio of microcystin variants in the cyanobacterium Planktothrix agardhii. *FEMS Microbiology Ecology 65: 383-390.*

4.1 Introduction

Cyanobacteria produce a wide variety of bioactive compounds, including hepatotoxic microcystins. Many cyanobacteria can produce multiple microcystin variants simultaneously. Currently, more than 70 microcystin variants have been described that differ considerably in their toxicity (Harada 1996; Sivonen and Jones 1999; Codd *et al*. 2005). Factors affecting the total microcystin production of cyanobacteria have been studied extensively, and include light intensity (Watanabe and Oishi 1985; Wiedner *et al*. 2003), temperature (Watanabe and Oishi 1985), nitrogen (Watanabe and Oishi 1985; Orr and Jones 1998; Long *et al*. 2001), phosphorus (Watanabe and Oishi 1985; Oh *et al*. 2000), iron (Utkilen and Gjølme 1995), and grazing (Jang *et al*. 2003).

Only a few studies have focused on the production of different microcystin variants and their relative abundances. These studies show that the cellular composition of microcystin variants may change in response to changing environmental conditions. For instance, the relative abundances of different microcystin variants are affected by temperature in *Anabaena* 90 (Rapala *et al*. 1997) and by light intensity and nutrient supply in *Microcystis aeruginosa* HUB 5-2-4 (Hesse and Kohl 2001). Recent experiments with the filamentous cyanobacterium *Planktothrix agardhii* have shown that the prevalence of the two microcystin variants [Asp^3]microcystin-LR and [Asp^3]microcystin-RR changed as a function of photon irradiance (Tonk *et al*. 2005). [Asp^3]microcystin-LR molecules contain leucine (L) at the position where [Asp^3]microcystin-RR contains arginine (R). An increased cellular content of [Asp^3]microcystin-LR in high light coincided with a decreased content of the RR variant, resulting in a fourfold increase in the [Asp^3]microcystin-LR/RR ratio (Tonk *et al*. 2005). Because mouse assays indicate that the microcystin-LR variant is four times more toxic than its RR counterpart, this change is of significance and may have ecological implications (Kotak *et al*. 1995; Harada 1996).

The molecular mechanisms that direct changes in the pattern of synthesis and the composition of microcystin variants have not yet been resolved (Rapala *et al*. 1997; Tonk *et al*. 2005). Although microcystins are oligopeptides, their synthesis does not involve the ribosomal protein synthesis machinery. Instead, an elaborate chain of enzymes functions as synthetase for microcystins (Dittmann *et al*. 1997; Nishizawa *et al*. 1999, 2000; Tillett *et al*. 2000). The enzymatic sites of the microcystin synthetase complex are arranged in a modular way (Dittmann *et al*. 1997; Marahiel *et al*. 1997). The order of the modules, together with the number and type of catalytic domains present determines the structure of the resulting polyketide product (Tillett *et al*. 2000). The first module of the *McyB* enzyme of *P. agardhii* contains a flexible binding pocket that facilitates the incorporation of a variety of different amino acids at the variable X position within the microcystin structure (Christiansen *et al*. 2003); to the best of our knowledge, this is a unique feature not found in other non-ribosomal peptide synthetase (NRPS) systems. Questions remain about the nature

of the ecophysiological factors that actually give rise to the differences in the relative abundance of microcystin variants within a single cyanobacterium.

Several mechanisms have been proposed that might steer the relative abundances of microcystin variants at the level of synthesis (Tonk *et al*. 2005). A conformational change of the substrate-binding pocket at the first module of the *McyB* enzyme could lead to a change in the substrate specificity of the module. Alternatively, changing environmental conditions (e.g. light, nutrients, temperature) could lead to changes in the amino acid composition available for incorporation during microcystin synthesis.

Here, we hypothesize that the relative availability of different amino acids directs the specificity of microcystin synthesis. Cyanobacteria possess mechanisms for the direct uptake of amino acids such as leucine and arginine from their environment (Herrero and Flores 1990; Kamjunke and Jahnichen 2000). Incorporation of leucine into *M. aeruginosa* cells is known to increase with increasing leucine concentration in the medium (Kamjunke and Jahnichen 2000). Hence, our first hypothesis is that addition of leucine will increase the [Asp^3]microcystin-LR/RR ratio, while addition of arginine will decrease the [Asp^3]microcystin-LR/RR ratio.

From an ecological perspective, it is of interest that arginine is a very nitrogen-rich amino acid, because it has four nitrogen atoms instead of the one nitrogen atom present in most other amino acids including leucine. During nitrogen limitation, the synthesis of arginine likely decreases to a minimum due to the low availability of nitrogen for amino acid synthesis. Thus, our second hypothesis is that nitrogen limitation will increase the [Asp^3]microcystin-LR/RR ratio.

In this study, we tested the first hypothesis that amino acid availability determines the composition of microcystin variants, by adding the amino acids leucine and arginine to the growth medium of *P. agardhii*. Because earlier studies had shown that the [Asp^3]microcystin-LR/RR ratio depends on the light conditions (Tonk *et al*. 2005), we performed these addition experiments at both low and high light intensities. To investigate the second hypothesis, we examined whether a shift from nitrogen saturation to nitrogen limitation in a chemostat would change the prevalence of available amino acids in *P. agardhii* cells and would thereby change the [Asp^3]microcystin-LR/RR ratio.

4.2 Materials and methods

Organism - *Planktothrix agardhii* strain 126/3 was provided by the Division of Microbiology, University of Helsinki. The strain was grown in a nutrient-rich mineral medium originally defined for growth of freshwater *Oscillatoria* species and known as O2 medium (Van Liere and Mur 1978). The O2 medium has a nitrate concentration of 6 mM, and all other nutrients are also provided in saturating concentrations, thus preventing nutrient deficiencies during growth.

Semi-continuous turbidostats - *Planktothrix agardhii* was grown in semi-continuous cultures, using a turbidostat approach. The OD_{750} nm was kept constant between 0.1 and 0.2 cm^{-1} by diluting the culture with O2 medium once every 2 days. Flat culture vessels were used with a working volume of 400 mL and constant aeration with compressed filter-sterilized air (0.2 µm membrane, Millipore) to ensure homogeneous mixing and to provide sufficient amounts of CO_2. Temperature was maintained at 22 ºC (± 1ºC) by leading the compressed air used for aeration through a temperature-controlled water bath (Colora, thermocryostat). In addition, a ventilator was used to diffuse the heat emitted by the light source. Light was provided by white fluorescent tubes (Philips PL-L 24W/840/4P) directed toward the front surface of the culture vessel. Incident irradiance (I_{in}) and outgoing irradiance (I_{out}) were measured using a LI-COR LI-250 quantum photometer at seven points on the front surface and back surface of the culture vessel, respectively. The average photon irradiance in the culture vessel was calculated according to the following equation (Huisman *et al*. 2002):

$$I_{\text{avg}} = \frac{I_{\text{in}} - I_{\text{out}}}{\ln I_{\text{in}} - \ln I_{\text{out}}} \tag{4.1}$$

We applied a 12 h light:12 h dark cycle, with an incident irradiance of I_{in} = 26 ± 0.1 µmol m^{-2} s^{-1} (I_{avg} = 21 ± 1 µmol m^{-2} s^{-1}) for the low-light cultures and I_{in} = 104 ± 0.5 µmol m^{-2} s^{-1} for the high-light cultures (I_{avg} = 84 ± 4 µmol m^{-2} s^{-1}). Experiments were run in triplicate. After 2 weeks of acclimation to the imposed light conditions, the triplicates were split: in the first experiment 10 mM L-leucine was added to the culture vessel, in the second experiment 10 mM L-arginine was added to the culture vessel, while the third experiment served as a control. For the amino acids a final concentration of 10 mM was chosen to exclude nitrogen deficiency during growth.

Chemostat experiment - *Planktothrix agardhii* was grown in continuous culture using a flat culture vessel with a working volume of 1.85 L in combination with constant aeration of filtered and moistened air to ensure homogeneous mixing and to provide sufficient amounts of inorganic carbon (Matthijs *et al*. 1996; Huisman *et al*. 2002). We used a chemostat approach in which the dilution rate was fixed at D = 0.34 day^{-1}. Temperature was kept constant at 21 ºC (± 1 ºC) by means of a transparent water jacket, connected to a Colora thermocryostat that was placed between the light source and the culture vessel. The incident irradiance was I_{in} = 38 ± 4 µmol m^{-2} s^{-1}. The average irradiance was calculated according to Eq. 4.1, using 10 points on the front surface and back surface of the culture vessel. A 12-h light:12-h dark cycle was used. After reaching a steady state, the nitrogen-saturated O2 medium (6 mM nitrate) was replaced by nitrogen-limited O2 medium with a nitrate concentration of 200 µM. The changes in culture properties were recorded until full nitrogen depletion of the cells was reached.

Sampling - Samples were taken from the semi-continuous turbidostats at Day 0 (before addition of leucine and arginine) and at Days 1, 3 and 5 (after addition of leucine and arginine). The chemostat experiment was sampled during the nitrogen-saturated steady state (Days 1-5), and every other day during the transient state caused by the onset of nitrogen limitation (Days 7-23). Samples were always taken 1 h after the light was switched on. Aliquots of all samples were analyzed in triplicate for intracellular microcystin content, biovolume, and C/N concentration.

Microcystin analysis - For intracellular microcystin analysis, 10 mL of the culture suspension was filtered in triplicate using Whatman GF/C filters (pore size ~1.2 µm). Filters were freeze dried and stored at -20 ºC. Microcystin was extracted in 75% MeOH (three extraction rounds) according to Fastner *et al*. (1998) with an extra step for grinding of the filters in a Mini Beadbeater (Biospec products) with 0.5 mm silica beads (Tonk *et al*. 2005). Dried extracts were stored at -20 ºC and dissolved in 50 % MeOH for analysis of microcystin using HPLC with photodiode array detection (Kontron Instruments). The extracts were separated using a LiChrospher 100 ODS 5 µm LiChorCART 250-4 cartridge system (Merck) and a 30-70 % acetonitrile gradient in water with 0.05 % trifluoroacetic acid at a flow rate of 1 mL min^{-1}. The different microcystin variants were identified based on their characteristic UV-spectra and quantified by means of a microcystin (MC)-LR gravimetrical standard provided by the University of Dundee. Extracellular microcystin concentrations were below the detection limit of the HPLC.

Biovolume - Biovolumes of *P. agardhii* filaments were measured using an automated cell counter (Casy 1 TTC, Schärfe System) with a 150-µm capillary (Rohrlack and Utkilen 2007). Samples prepared for automated cell counting were diluted in an electrolyte solution. The automated cell counter monitored the conductivity of the electrolyte solution flowing through an aperture in the capillary. Changes in conductivity were proportional to the volumes of the suspended filaments. The specific growth rate, μ, was calculated according to the following equation:

$$\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} + D \tag{4.2}$$

where x_1 and x_2 represent the total biovolumes of the cultures at times t_1 and t_2, respectively, and D represents the dilution rate of the culture.

Analysis of carbon and nitrogen content - For determination of the intracellular carbon and nitrogen content, 10 mL of the culture suspension was concentrated over a 0.45 µm HA membrane filter (Millipore) in triplicate. The residue on the filters was collected into 2 mL Eppendorf tubes; these were centrifuged (5 min, 12 000 *g*), pellets were stored at -20 ºC and

subsequently freeze-dried. Carbon and nitrogen content was quantitatively analyzed in 70 mg of the freeze-dried cell powder using a Vario EL Elemental Analyzer (Elementar Analysensysteme GmbH).

Amino acid analysis - The continuous culture was sampled at Day 8 (at the onset of nitrogen limitation) and at Days 22-29 (during nitrogen limitation), for analysis of the total amino acid composition of the cells. Samples, containing 40 mL of culture material, were centrifuged and freeze dried. In order to split the proteins into constituting amino acids, ~8 mg of each freeze-dried sample was hydrolyzed in 6 M HCl for 24 h at 105-110 ºC. Separation of the amino acids was carried out using an amino acid analyzer (Biochrom Alpha II Plus), which separated the amino acids by differential binding to a column filled with a weak cation exchanger bound to a solid matrix; subsequent elution was based on citric acid to which an increasing amount of lithium hydroxide was added. Detection and quantification of the amino acids was based on postcolumn derivatization with Ninhydrin at 570 nm (primary amines) or 440 nm (secondary amines).

Data analysis - Independent sample *t*-tests were performed, using SPSS version 11, to detect significant differences in microcystin contents between two treatments (i.e. low vs. high light).

4.3 Results

Effects of light intensity in semi-continuous turbidostats - The specific growth rates of *P. agardhii* grown in nutrient-saturated mineral medium were μ = 0.25 day^{-1} (SD = 0.06; n = 3) and μ = 0.37 day^{-1} (SD = 0.11; n = 3) for low-light and high-light conditions, respectively. In the control cultures, the average [Asp^3]microcystin-RR content was significantly lower at high light intensity than at low light intensity (Fig. 4.1a,b; comparison of the means using *t*-test: $t_6 = 6.0$, $P < 0.001$). Conversely, the average [Asp^3]microcystin-LR content was significantly higher under high light intensity than that under low light intensity (Fig. 4.1a,b; *t*-test: $t_6 = 5.9$, $P < 0.001$). As a consequence, the [Asp^3]microcystin-LR/RR ratio was much higher at high-light than at low-light conditions (Fig. 4.2a,b).

Addition of amino acids - In cultures supplied with leucine, the decline of [Asp^3]microcystin-RR and the associated increase of [Asp^3]microcystin-LR were evident under both light conditions (Fig. 4.1c,d). Accordingly, the [Asp^3]microcystin-LR/RR ratio showed a strong increase after leucine addition (Fig. 4.2c,d). In cultures supplied with arginine, the [Asp^3]microcystin-RR content remained approximately the same as in the control, while the [Asp^3]microcystin-LR content was reduced (Fig. 4.1e,f). Hence, the [Asp^3]microcystin-LR/RR ratio was reduced after arginine addition (Fig. 4.2e,f). Before the

actual experiments shown here, we ran a small pilot experiment. The pilot experiment showed essentially the same results (data not shown), indicating the robustness of our findings.

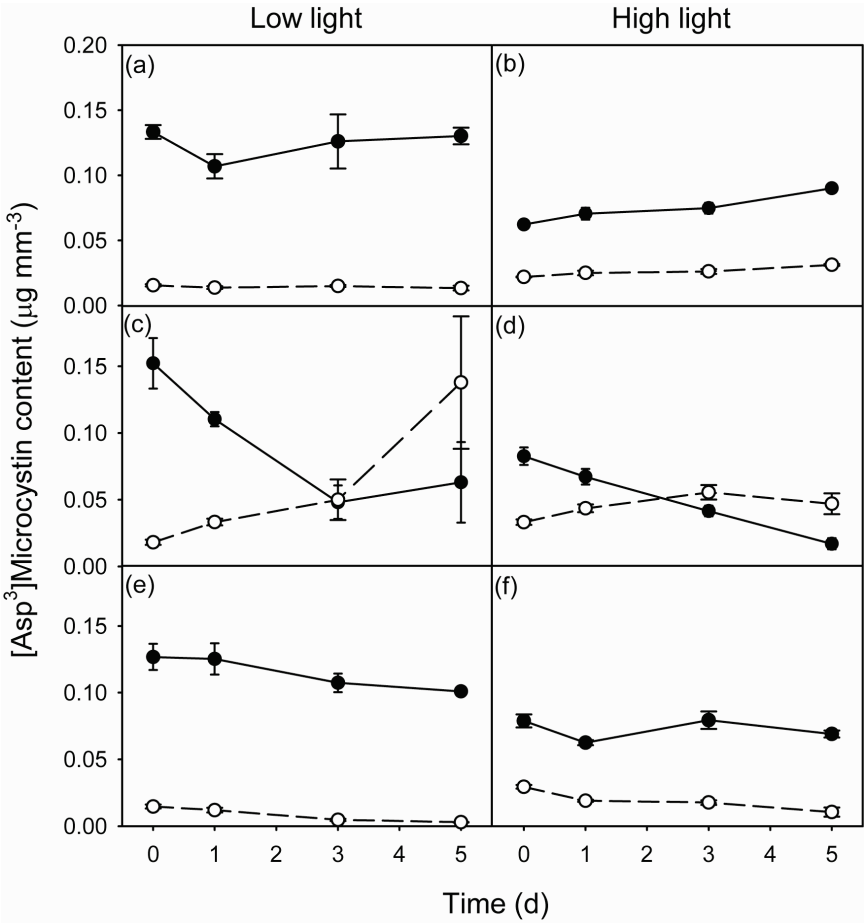

Figure 4.1. [Asp^3]microcystin-RR (closed circles) and [Asp^3]microcystin-LR (open circles) contents of *Planktothrix agardhii* grown in semi-continuous turbidostats: (a,b) with nutrient-saturated medium, (c,d) after addition of leucine, and (e,f) after addition of arginine. Panels on the left show results at low light intensity (I_{avg} = 21 µmol m^{-2} s^{-1}), while panels on the right show results at high light intensity (I_{avg} = 84 µmol m^{-2} s^{-1}). Error bars denote SD (n = 3).

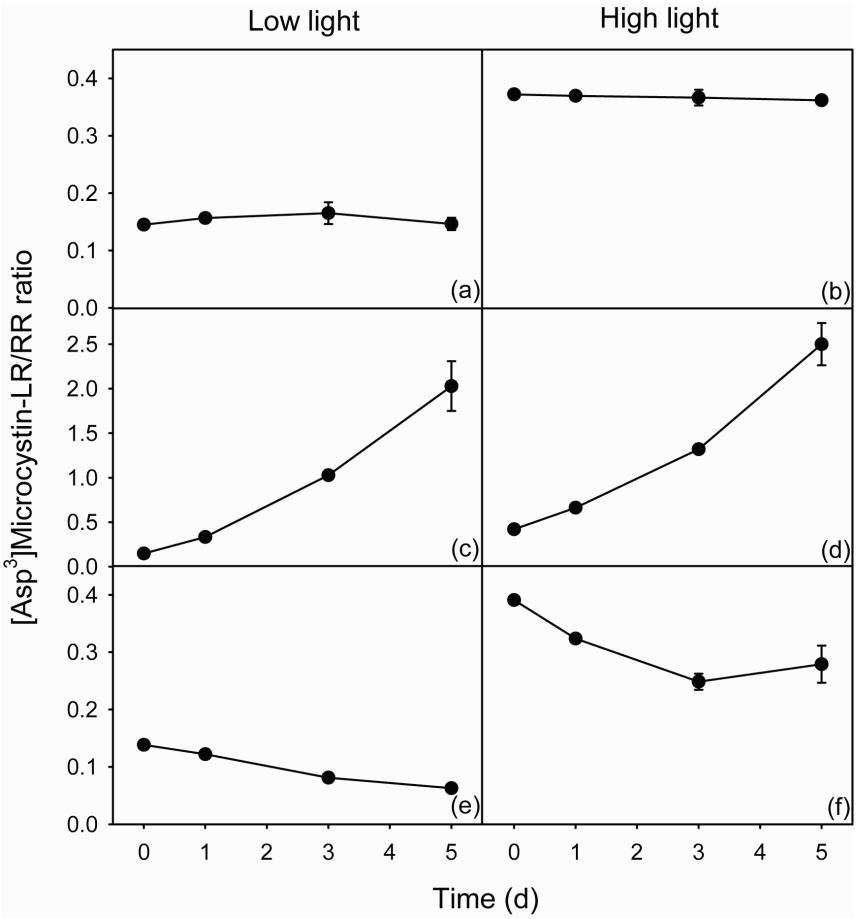

Figure 4.2. [Asp^3]microcystin-LR/RR ratios of *Planktothrix agardhii* grown in semi-continuous turbidostats: (a,b) with nutrient-saturated medium, (c,d) after addition of leucine and (e,f) after addition of arginine. Panels on the left show results at low light intensity (I_{avg} = 21 µmol m^{-2} s^{-1}), while panels on the right show results at high light intensity (I_{avg} = 84 µmol m^{-2} s^{-1}). Error bars denote SD (n = 3).

Chemostat experiment - In this experiment, the nitrogen-saturated medium of a continuous culture containing *P. agardhii* grown at a relatively low light intensity (I_{in} = 38 ± 4 µmol m^{-2} s^{-1}) was replaced by nitrogen-limited medium at Day 5, thereby slowly decreasing the nitrogen availability in the culture vessel from Day 5 onwards. The dilution rate of the chemostat was maintained at D = 0.34 day^{-1} throughout the experiment. The [Asp^3]microcystin-RR content, [Asp^3]microcystin-LR content, nitrogen content in the cells and the average light intensity in the culture were judged to be stable during the initial steady state (Days 1-5, Fig. 4.3). When provided with a nitrogen-limited medium, the biovolume started to decrease from the default level of ~1.0 mm^3 mL^{-1} during the first 5

days to 0.4 mm^3 mL^{-1} on Day 24 (Fig. 4.3a). The average light intensity in the culture increased (Fig. 4.3c) as a result of both a lower population density and a lower pigment content of the remaining cells under nitrogen-limited conditions. A decrease in the cellular nitrogen content was observed from Day 10 onwards (Fig. 4.3b).

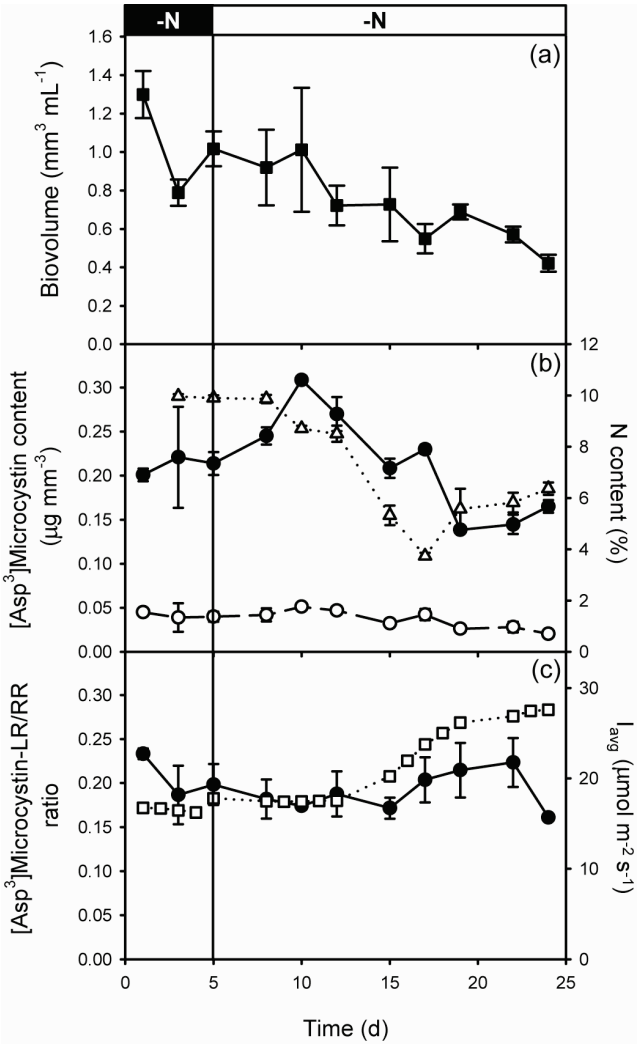

Figure 4.3. Time course of changes in selected properties of *Planktothrix agardhii* grown in continuous culture. (a) Biovolume (closed squares). (b) [Asp^3]microcystin-RR content (closed circles), [Asp^3]microcystin-LR content (open circles) and nitrogen content (open triangles), where the nitrogen content is expressed as percentage of the dry weight. (c) [Asp^3]microcystin-LR/RR ratio (closed circles) and depth-averaged light intensity in the culture (open squares). Error bars indicate SD (n = 3). The black bar (+N) indicates the use of nitrogen saturated-medium, and the white bar (-N) denotes the use of nitrogen-limited medium.

At Day 17, cells started to appear pale; a common phenomenon in nitrogen-starved cyanobacteria caused by the breakdown of phycobilisomes and chlorophyll-a. The [Asp^3]microcystin-RR content showed an initial increase, but then also started to decrease from Day 10 onwards (Fig. 4.3b). The initial increase in [Asp^3]microcystin-RR, from Days 5 to 10, might be the consequence of the release of nitrogen stocks from phycobilin and cyanophycin degradation. During the transition to nitrogen limitation, both the [Asp^3]microcystin-RR and the [Asp^3]microcystin-LR content decreased, while the ratio of the two microcystin variants remained constant (Fig. 4.3c). As a consequence, the expected shift toward [Asp^3]microcystin-LR under nitrogen-limiting conditions was not found.

Total amino acid analysis was performed on samples taken from the chemostat at Day 8 (when the cellular nitrogen content was still high) and at Days 22-29 (during nitrogen limitation). The expected increase of the L/R ratio in response to nitrogen limitation was not observed, and likewise the expected increase in the [Asp^3]microcystin-LR/RR ratio was not found. In fact, all of the amino acids, leucine, arginine, and aspartic acid, decreased proportionally under nitrogen-limited conditions, such that the cells maintained a constant L/R ratio and, thus, a constant [Asp^3]microcystin-LR/RR ratio (Table 4.1).

Table 4.1. Amino acid content and microcystin content of *P. agardhii* grown in the chemostat experiment[a]

	N-saturated	**N-limited**
Amino acid content (μg mg^{-1})		
Aspartic acid	73.8	53.5
Leucine	67.7	45.9
Arginine	41.8	30.7
L/R ratio[b]	1.62	1.50
D/R ratio[b]	1.77	1.74
Microcystin content (μg mm^{-3})		
Microcystin-LR	0.042	0.025
Microcystin-RR	0.245	0.149
Microcystin-LR/RR ratio	0.171	0.167

[a] *N-saturated samples were taken on Day 8, and N-limited samples on Days 22-29.*

[b] *D = aspartic acid; L = leucine; R = arginine.*

4.4 Discussion

In this study, we provided evidence that amino acid availability determines the relative composition of microcystin variants in cyanobacteria, shown here for the filamentous cyanobacterium *P. agardhii* strain 126/3. Addition of leucine resulted in an increase in the [Asp^3]microcystin-LR/RR ratio (Fig. 4.2c,d), while addition of arginine resulted in a decrease of this ratio (Fig. 4.2e,f). Remarkably, while addition of leucine resulted in a higher [Asp^3]microcystin-LR content (Fig. 4.1c,d), addition of arginine did not yield a higher [Asp^3]microcystin-RR content (Fig. 4.1e,f) compared with the control. Instead, addition of arginine suppressed the [Asp^3]microcystin-LR content substantially and thereby lowered the [Asp^3]microcystin-LR/RR ratio. Arginine can be taken up by several cyanobacteria, but has also been judged to be inhibitory or even nonpermissive in quite a few cases (Montesinos *et al*. 1997; Stephan *et al*. 2000). A comprehensive overview of metabolic pathways for L-arginine in a range of cyanobacteria has been published recently, in which differences between species have become apparent (Schriek *et al*. 2007). Although details for *P. agardhii* are not yet available, the observations of strong side-effects of L-arginine in other cyanobacteria might explain why addition of arginine did not increase the [Asp^3]microcystin-RR content. We conclude from these amino acid addition experiments that the availability of amino acids during microcystin polyketide synthesis is a major determinant of the composition of microcystin variants in cyanobacteria. Nevertheless, our findings do not rule out other mechanisms that could control the synthesis of different microcystin variants. For instance, in addition to the role of amino acid availability reported here, conformational changes in synthetase enzymes could also contribute to changes in the production of different microcystin variants.

Speculations on the function of microcystins are multiple, and include physiological functions such as metal ion chelators (Utkilen and Gjølme 1995) or intraspecific signaling molecules (Dittmann *et al*. 2001), as well as protection against predators such as zooplankton (Koski *et al*. 1999; Rohrlack *et al*. 1999, 2001). It has been suggested that cyanobacterial toxins also play a role in the competition between cyanobacteria and other phototrophic organisms (Keating 1977; Gross 2003), but recent studies have questioned whether microcystins have significant allelopathic effects (Babica *et al*. 2006; Kardinaal *et al*. 2007b). In analogy with the strategy that bacteria use for synthesis of antibiotics by a similar nonribosomal mechanism, variation in microcystin composition may broaden the effectiveness of action. Assembling different microcystin variants may thus be a strategy of cyanobacteria to sustain continued production of microcystins under different environmental conditions.

Now that we have observed that the availability of amino acids is indeed important for the production of the two microcystin variants, the question remains as to what factor initiates changes in the free amino acid pool. We hypothesized that nitrogen limitation can decrease the relative availability of the nitrogen-rich amino acid arginine in the free amino

acid pool. Subsequently, according to our findings on leucine and arginine addition, we expected that the decrease in arginine relative to leucine would favor [Asp^3]microcystin-LR synthesis over [Asp^3]microcystin-RR synthesis. However, when the nitrogen-saturated medium was replaced by the nitrogen-limited medium in our chemostat experiment, the anticipated suppression of arginine content and increase in [Asp^3]microcystin-LR/RR ratio were not observed (Table 4.1; Fig. 4.3c). Instead, nitrogen limitation induced an overall decrease of the cellular amino acid content and microcystin content (Table 4.1; Fig. 4.3b).

Our experiments did show a significantly higher [Asp^3]microcystin-LR/RR ratio at high light than at low light (Fig. 4.2a,b). The same response to light intensity was also found in previous experiments with *P. agardhii* (Tonk *et al*. 2005), which demonstrates that patterns of microcystin composition can be reproduced in different experiments. Discrepancies between the absolute values in microcystin contents reported by Tonk *et al*. (2005) and the microcystin contents reported in this article probably stem from different methods used for biovolume determination. In Tonk *et al*. (2005), biovolume was determined by means of microscope countings on lugol fixed samples, while in the present study we determined biovolumes of fresh samples using a Casy cell counter. The established strong response of the [Asp^3]microcystin-LR/RR ratio to light made us question why this ratio failed to respond to nitrogen limitation. It might be that in our experiment, an alternative nitrogen source compensated for nitrogen depletion. For instance, cyanobacteria might reallocate nitrogen obtained from the breakdown of cyanophycin or the degradation of phycocyanin pigments to microcystin production. During nitrogen excess, cyanobacteria can store the arginine surplus in cyanophycin, a nitrogen-rich polypeptide consisting of arginine and aspartic acid (Oppermann-Sanio and Steinbüchel 2002). In rapidly growing cyanobacteria that are in a physiological balance with their environment, cyanophycin is present only in small amounts (Allen 1984). During periods of nitrogen deficiency, however, arginine stored in cyanophycin can play an important role in the balance between carbon and nitrogen metabolism of cyanobacteria (Maheswaran *et al*. 2006). Thus, nitrogen storage in cyanophycin might provide a buffer against changes in external nitrogen availability, which might explain the absence of the expected shift in the [Asp^3]microcystin-LR/RR ratio in response to nitrogen limitation.

In conclusion, we have demonstrated that the relative availability of different amino acids is an important factor in the nonribosomal synthesis of different microcystin variants, and thereby affects the composition of microcystin variants in cyanobacteria. However, nitrogen limitation induced neither obvious changes in the relative availability of the amino acids leucine and arginine nor changes in microcystin variant composition.

Acknowledgements - The research of LT, DBvdW, JH, and PMV was supported by the Earth and Life Sciences Foundation (ALW), which is subsidized by the Netherlands Organization for Scientific Research (NWO). In addition, LT and PMV were supported by

a European Union grant within the program PEPCY. We thank Kaarina Sivonen of the University of Helsinki for providing the *P. agardhii* strain used in this study, Geoff Codd and coworkers of the University of Dundee for providing the MC-LR gravimetrical standard and the reviewers for their helpful comments on the manuscript.