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Type	PhD thesis
Title	Out of balance: implications of climate change for the ecological stoichiometry of harmful cyanobacteria
Author(s)	D.B. van de Waal
Faculty	FNWI: Institute for Biodiversity and Ecosystem Dynamics (IBED), FNWI: Institute for Biodiversity and Ecosystem Dynamics (IBED)
Year	2010

FULL BIBLIOGRAPHIC DETAILS:
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Chapter 5

Nitrogen pulse induces dynamic changes in amino acid composition and microcystin production of the harmful cyanobacterium *Planktothrix agardhii*

ABSTRACT - *Planktothrix agardhii* is a widespread harmful cyanobacterium of eutrophic waters, and has the ability to produce the hepatotoxins [Asp^3]microcystin-LR and [Asp^3]microcystin-RR. These two microcystin variants differ in their first variable amino acid position, which is occupied by either leucine (L) or arginine (R). Although microcystins are extensively investigated, little is known about the mechanisms that determine the production of different microcystin variants. We hypothesize that enhanced nitrogen availability will increase the intracellular content of the nitrogen-rich amino acid arginine, and thereby promote the production of the variant [Asp^3]microcystin-RR. To test this hypothesis, we transferred *P. agardhii* strain 126/3 from nitrogen-replete to nitrogen-deficient conditions, and after two weeks of growth under nitrogen deficiency we added a nitrate pulse. Upon nitrate addition, we found a rapid increase of the cellular N:C ratio and the amino acids aspartic acid and arginine, indicative for cyanophycin synthesis. This was followed by a more gradual increase of the total amino acid content. As expected, the [Asp^3]microcystin-RR variant increased strongly after the nitrate pulse, while the [Asp^3]microcystin-LR increased to a much lesser extent. We conclude that nitrogen enrichment affects the amino acid composition of harmful cyanobacteria, which, in turn, affects the production and composition of their microcystins.

This chapter is based on the manuscript: Dedmer B Van de Waal, Gonzalo Ferreruela, Linda Tonk, Ellen Van Donk, Jef Huisman, Petra M Visser, and Hans CP Matthijs. Nitrogen pulse induces dynamic changes in amino acid composition and microcystin production of the harmful cyanobacterium Planktothrix agardhii. *Submitted.*

5.1 Introduction

Aquatic ecosystems throughout the world have been enriched with nutrients derived from urban, industrial and agricultural activities (Vitousek *et al.* 1997; Galloway *et al*. 2004; Glibert *et al*. 2005). This anthropogenic eutrophication, in combination with global warming, benefits the proliferation of harmful cyanobacteria (Dokulil and Teubner 2000; Glibert *et al*. 2005; Jöhnk *et al.* 2008; Paerl and Huisman 2008), which have become an increasing nuisance in many freshwater lakes and brackish waters (Reynolds 1987; Carmichael 2001; Huisman *et al*. 2005). Dense cyanobacterial blooms can contain very high toxin concentrations, posing a major threat to birds, mammals and human health (Chorus and Bartram 1999; Carmichael 2001; Codd *et al*. 2005).

Several harmful cyanobacteria produce microcystins, a family of oligopeptides that can cause serious damage to the liver (Sivonen and Jones 1999; Carmichael 2001; Codd *et al*. 2005). Microcystins consist of seven amino acids, of which two amino acid positions are variable, whereas the other five positions are more conserved (Welker and Von Döhren 2006). In total, 89 microcystin variants have been described (Welker and Von Döhren 2006). These variants may differ in their toxicity (Sivonen and Jones 1999; Chen *et al.* 2006; Hoeger *et al.* 2007). Yet, little is known about the mechanisms that determine the production of different microcystin variants.

Microcystins are produced stepwise, by non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS), which are large modular constructed enzymes in which each module is responsible for a cycle of polypeptide or polyketide chain elongation (Tillett *et al*. 2000; Börner and Dittmann 2005). The unique flexible binding pocket in the first module of the *McyB* enzyme enables incorporation of different amino acids at the two variable amino acid positions (Christiansen *et al.* 2003; Welker and Von Döhren 2006). In microcystin-LR, the first and second variable amino acid position are occupied by leucine (L) and arginine (R), while in microcystin-RR both positions are occupied by arginine (Sivonen and Jones 1999; Hesse and Kohl 2001). Recently, we showed that addition of leucine increased the microcystin-LR/RR ratio of the filamentous cyanobacterium *Planktothrix agardhii*, while addition of arginine reduced its microcystin-LR/RR ratio (Tonk *et al.* 2008; see also Chapter 4 in this thesis). Hence, the availability of different amino acids may determine which microcystin variants are produced.

Arginine contains four bound nitrogen atoms, whereas leucine contains only one nitrogen atom. Hence, microcystin-RR is a relatively nitrogen-rich microcystin variant. It is well known that the microcystin production of harmful cyanobacteria is favored by nitrogen enrichment (Long *et al*. 2001; Downing *et al*. 2005). Moreover, recent research showed that nitrogen enrichment specifically enhanced the production of the variant microcystin-RR (Van de Waal *et al.* 2009). Interestingly, many cyanobacteria can store excess nitrogen in the polypeptide cyanophycin, consisting of an aspartic acid backbone and arginine side groups (Allen 1984; Oppermann-Sanio and Steinbüchel 2002). Cyanophycin synthesis is

especially stimulated when nitrogen-deficient cyanobacteria are suddenly exposed to conditions of nitrogen excess (Allen 1984; Tapia *et al*. 1996; Oppermann-Sanio and Steinbüchel 2002; Maheswaran *et al*. 2006).

Pulsed nitrogen enrichment is a common phenomenon in many aquatic ecosystems. For instance, rain flushes nitrogen into lakes through enhanced surface run-off and discharge of upstream waters, storms may mix nitrogen-rich water from the hypolimnion into the surface water layers of stratified lakes, and fertilization of nearby agricultural fields may spill excess nitrogen into the surface waters. It seems likely that such nitrogen pulses will temporarily increase the intracellular availability of arginine to form the nitrogen-storage polymer cyanophycin in harmful cyanobacteria. Will these changes in amino acid composition be reflected in the cellular microcystin composition?

In this study, we investigate dynamic changes in amino acid composition and microcystin production after addition of a nitrate pulse to the harmful cyanobacterium *Planktothrix agardhii* strain 126/3. *P. agardhii* is a common microcystin-producing cyanobacterium that often proliferates in eutrophic shallow lakes (Briand *et al*. 2002; Janse *et al*. 2005; Kardinaal and Visser 2005b). We hypothesize that enhanced nitrogen availability will increase its intracellular amino acid content, and thereby promotes the production of microcystins. Moreover, in nitrogen-pulsed *P. agardhii* where arginine is readily available from cyanophycin, we expect a shift in microcystin composition towards microcystin-RR.

5.2 Materials and methods

Organism - The filamentous cyanobacterium *Planktothrix agardhii* strain 126/3 was provided by the Division of Microbiology, University of Helsinki. The microcystins produced by this *P. agardhii* strain are the demethylated variants [Asp^3]microcystin-LR and [Asp^3]microcystin-RR.

Experimental conditions - Three batch cultures of 400 mL were grown on a rotatory shaker in 2 L erlenmeyers at 21 ± 1 ºC. The cultures were supplied with O2 medium originally defined for *Oscillatoria* species (Van Liere and Mur 1978). Nitrogen was provided as nitrate (NO_3^-) at a concentration of 6 mM for nitrogen-rich conditions and 0.2 mM for nitrogen-deficient conditions, while all other nutrients were added in saturating concentrations. Light was supplied by white fluorescent tubes (Philips TL-M 40W/33RS) at an average incoming irradiance of 26 ± 1 µmol photons m^{-2} s^{-1}. Biomass was measured every two days by determining biovolume according to Tonk *et al.* (2008) using a Casy 1 TTC automated cell counter (Schärfe System GmbH) with a 150-µm capillary (Rohrlack and Utkilen 2007).

When the cultures in nitrogen-replete conditions reached the exponential growth phase, samples were taken during three consecutive days. After the third day of sampling, cultures were pooled and centrifuged at 3500 *g* for 20 minutes. The supernatant was removed and pellets were washed twice with nitrogen-deficient O2 mineral medium containing 0.2 mM of nitrate. Washed cells were redistributed in three 2 L erlenmeyers to continue growth under nitrogen-deficient conditions until a stationary phase was reached, and subsequently samples were taken for three consecutive days. Nitrogen deficiency was detected by the decrease of the nitrogen-rich pigment phycocyanin (Allen 1984). Estimates of phycocyanin (PC) and chlorophyll-a (chl-a) were based on their absorbance at wavelengths of 627 nm and 438 nm, respectively, using an Aminco DW-2000 double-beam spectrophotometer (Olis Inc.). After this period of nitrogen-limited growth, a pulse of nitrate was added to the cultures to reach the initial concentration of 6 mM nitrate at once. Culture growth resumed and samples were taken daily until the end of the exponential growth phase was reached. All samples were analyzed as described below.

Microcystin analysis - For determination of the intracellular microcystin contents, aliquots of the culture suspension were filtrated over a Whatman GF/C filter (pore size ~1.2 µm) in triplicate. The filters were freeze-dried and intracellular microcystins were extracted in three rounds with 75% MeOH according to Fastner *et al*. (1998) with an additional grinding step using 0.5 mm beads and a Mini Beadbeater (BioSpec Products Inc.). Dried extracts were dissolved in 50% MeOH prior to analysis by high performance liquid chromatography (HPLC) with photodiode array detection (Kontron Instruments Ltd.). Separation of the different microcystin structural variants was done by a LiChrospher 100 ODS 5 µm LiChorCART 250-4 cartridge system (Merck) and using a 30 to 70% acetonitrile gradient in milli-Q water with 0.05% trifluoroacetic acid at a flow rate of 1 mL min^{-1}. The different microcystin variants were identified by their characteristic UV-spectra and quantified using a microcystin-LR and microcystin-RR gravimetrical standard provided by the University of Dundee. Extracellular microcystin concentrations were below the detection limit of the HPLC (2.5 ng of microcystin) and were considered negligible, as they typically comprise less than 3% of the total microcystin concentration (Long *et al*. 2001; Wiedner *et al*. 2003; Tonk *et al*. 2005).

Analysis of carbon and nitrogen content - The cellular carbon and nitrogen contents were estimated in aliquots of the culture suspension, in triplicate. To collapse the gas vesicles, samples were pressurized at 10 bar and samples were centrifuged at 2000 *g* for 15 minutes. The supernatant was removed and the pellet was re-suspended in milli-Q water, transferred into eppendorf tubes and centrifuged for 5 minutes at 15 000 *g*. Then, the supernatant was removed and pellets were freeze-dried for dry weight determination. The carbon and nitrogen content of homogenised freeze-dried cell powder was analysed using a Vario EL Elemental Analyzer (Elementar Analysensysteme GmbH).

Amino acid analysis - An aliquot of culture material was hydrolyzed with 30% HCl at 110 °C for 12 hours. Subsequently, the extract was vaporized to dryness under vacuum at 40 °C, and a borate buffer was added to maintain a constant pH of 9.8. After derivatization with *o*-phthaldialdehyde and *N*-isobutyrylcysteine as in Fitznar *et al*. (1999), amino acids were analyzed on reversed phase HPLC. The Waters Alliance 2690 separation module (Waters Corporation) was equipped with a Nova-Pak C18 3.9 x 150 mm column (Waters Corporation) with an Alltech Allsphere ODS-1 guard column (Alltech Associates) and a Waters fluorescence detector 474 (Waters Corporation). Amino acid concentrations in the extract were calculated based on a series of standard amino acid solutions (Sigma-Aldrich).

5.3 Results

At the onset of the experiment, the cyanobacterial cells were characterized by a high phycocyanin to chlorophyll-a ratio (PC:Chl-a ratio) and high cellular nitrogen to carbon ratio (N:C ratio), both indicative for nitrogen-replete conditions (Fig. 5.1). After cells were transferred to nitrate depleted conditions, cultures reached a nitrogen-limited stationary phase in 10 days (Fig. 5.1a). Nitrogen limitation became evident from the gradual decreasing PC:Chl-a ratios (Fig. 5.1b) and cellular N:C ratios (Fig. 5.1c). After addition of nitrogen as a nitrate pulse, both the PC:Chl-a ratio and cellular N:C ratio returned to values measured at the start of the experiment within 10-15 days.

Likewise, the total amino acid content increased gradually to values measured at the initial nitrogen-replete conditions, within 13 days after the nitrate pulse (Fig. 5.2a). Interestingly, both L-arginine and L-aspartic acid increased more rapidly than other amino acids and reached initial values within one and two days after the nitrate pulse, respectively (Fig. 5.2b,c). This resulted in a transient increase of the relative contents of L-arginine and L-aspartic acid (Fig. 5.3). The relative L-arginine content increased from ~5% of the total amino acids to more than 10% (Fig. 5.3a), while L-aspartic acid increased from ~10% of the total amino acids to nearly 18% (Fig. 5.3b). An increase of L-arginine and L-aspartic acid is indicative of cyanophycin production (Allen 1984; Oppermann-Sanio and Steinbüchel 2002; Maheswaran *et al*. 2006). L-Leucine and other amino acids showed a more gradual increase, comparable to the pattern observed in the total amino acid content (Fig. 5.2d; Table 5.1). N:C ratios of the total amino acid pool increased after the nitrate pulse and decreased to initial conditions at the end of the experiment (Table 5.1).

Table 5.1. Average cellular quantities of the analyzed amino acids at four different stages of the experiment: the nitrogen-rich start (day 0), the nitrogen-limited cells just before the nitrate pulse (day 16), one day after the nitrate pulse (day 17), and the last day of the experiment (day 31). The second column indicates the N:C ratio of each amino acid. The last row shows the overall N:C ratio of the cellular amino acid pool at the four different stages of the experiment.

Amino acid (AA)	N:C ratio (molar)*	Average content (µg mm^{-3})			
		Start (day 0)	Before N-pulse (day 16)	After N-pulse (day 17)	End (day 31)
D-Alanine	1:3	0.07	0.13	0.16	0.29
L-Alanine	1:3	4.11	1.32	1.54	4.43
L-Arginine	4:6	3.45	0.97	3.24	3.64
D-Aspartic acid	1:4	0.32	0.09	0.19	0.26
L-Aspartic acid	1:4	4.98	1.96	3.96	5.49
D-Glutamic acid	1:5	0.53	0.25	0.28	0.54
L-Glutamic acid	1:5	5.96	2.05	2.47	6.34
L-Glycine	1:2	2.76	0.96	1.12	3.03
L-Histidine	3:6	0.85	0.16	0.20	0.48
L-Isoleucine	1:6	2.62	0.91	1.07	2.89
L-Leucine	1:6	4.68	1.53	1.80	5.07
L-Lysine	2:6	2.54	0.75	0.89	2.59
L-Methionine	1:5	0.91	0.10	0.09	0.59
L-Phenylalanine	1:9	2.28	0.84	0.97	2.54
D-Phenylalanine	1:9	0.04	0.01	0.01	0.03
L-Serine	1:3	2.65	0.81	0.94	2.72
L-Threonine	1:4	2.92	1.04	1.20	3.15
L-Tyrosine	1:9	2.62	0.76	0.77	2.79
L-Valine	1:5	3.02	1.02	1.20	3.22
Total AA		*47.3*	*15.7*	*22.1*	*50.1*
Total N:C ratio AA (molar)		*0.26*	*0.26*	*0.29*	*0.26*

** N:C ratios are given as actual number of nitrogen and carbon atoms.*

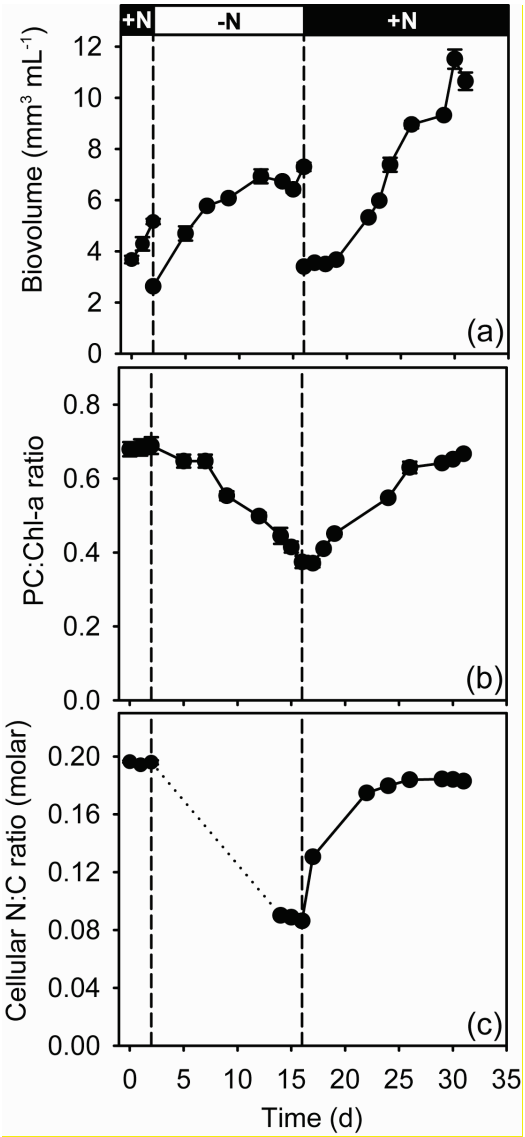

Figure 5.1. Changes in (a) biovolume, (b) phycocyanin to chlorophyll-a (PC:Chl-a) ratio and (c) cellular nitrogen to carbon (N:C) ratio during the experiment. Top bar indicates nitrogen-rich (black bar, +N) or nitrogen-deficient (white bar, -N) conditions. Error bars indicate the standard error of the mean (n = 3).

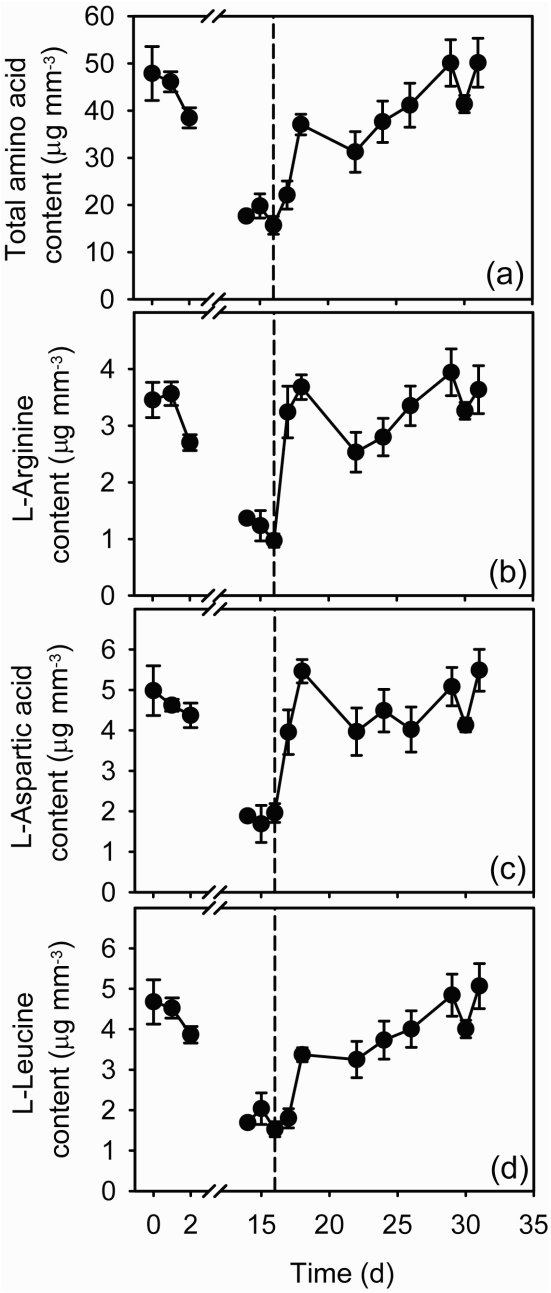

Figure 5.2. Changes in (a) total cellular amino acid content, (b) cellular L-arginine content, (c) cellular L-aspartic acid content and (d) cellular L-leucine content during the experiment. Error bars indicate the standard error of the mean (n = 3). The timing of the nitrate pulse is indicated by the vertical dashed line.

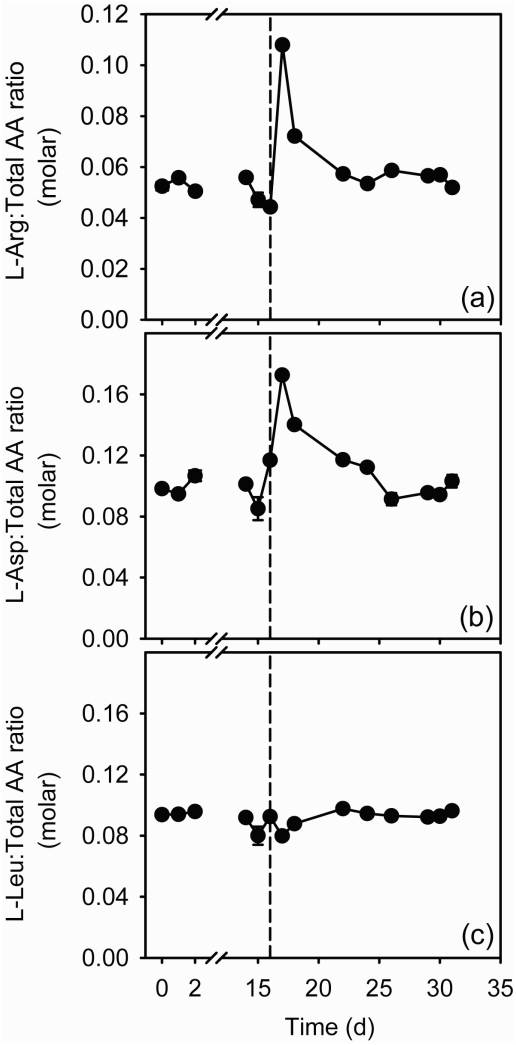

Figure 5.3. Changes in (a) the relative contribution of L-arginine to the total amino acid pool, expressed as the L-Arg:Total AA ratio, and (b) the relative contribution of L-aspartic acid to the total amino acid pool, expressed as the L-Asp:Total AA ratio. Error bars indicate the standard error of the mean (n = 3). The timing of the nitrate pulse is indicated by the vertical dashed line.

The total cellular microcystin content was strongly correlated with the cellular amino acid content (Fig. 5.4; Pearson product-moment correlation: $r = 0.958$, $n = 9$, $P < 0.0001$). The amount of amino acids allocated to microcystins was negligible and remained below 0.5% of the total amino acid pool. Under nitrogen-limited conditions, both [Asp^3]microcystin-RR and [Asp^3]microcystin-LR content were lower compared to the initial nitrogen-rich condition. After the nitrate pulse, [Asp^3]microcystin-RR increased to values measured at the initial nitrogen-replete conditions (Fig. 5.5a). However, the [Asp^3]microcystin-LR

content increased only slightly and remained far below the concentration measured at the initial nitrogen-replete conditions (Fig. 5.5b).

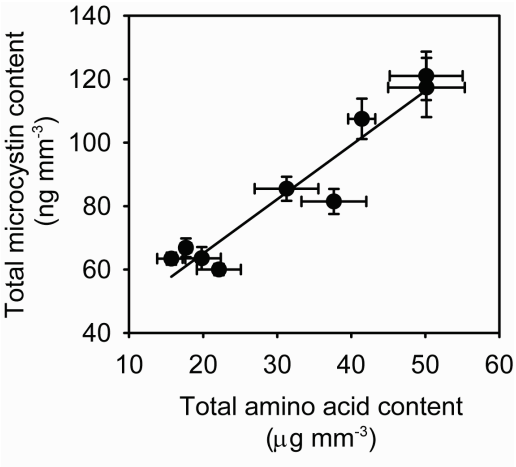

Figure 5.4. The total cellular microcystin content in relation to the total cellular amino acid content. Error bars indicate the standard error of the mean (n = 3). The solid line shows the linear correlation.

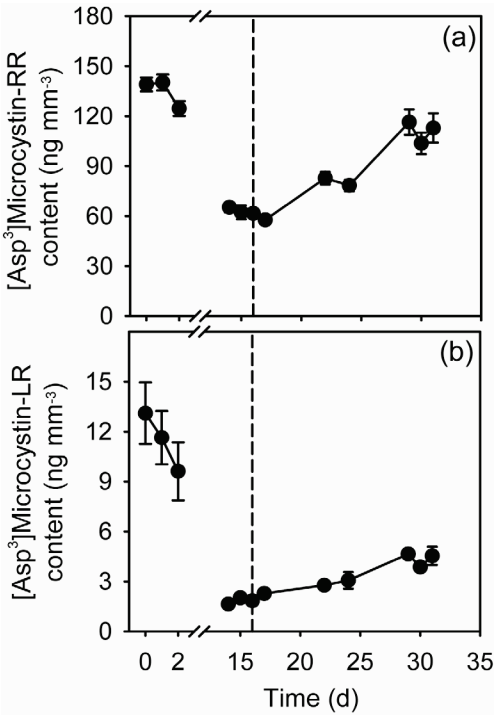

Figure 5.5. Changes in (a) cellular [Asp^3]microcystin-RR content, and (b) cellular [Asp^3]microcystin-LR content during the experiment. Error bars indicate the standard error of the mean (n = 3). The timing of the nitrate pulse is indicated by the vertical dashed line.

5.4 Discussion

To the best of our knowledge, this is the first study that shows concomitant changes in the intracellular amino acid content and the total microcystin content of harmful cyanobacteria. Moreover, extending earlier work by Tonk *et al*. (2008), we provide evidence that the relative contents of different microcystin variants are affected by the intracellular amino acid composition.

Addition of a nitrate pulse to nitrogen-deficient *P. agardhii* cells resulted in a rapid increase of the cellular N:C ratio (Fig. 5.1c) and the relative contents of both L-arginine and L-aspartic acid (Fig. 5.3). This is indicative of nitrogen storage in the polypeptide cyanophycin (Allen and Hutchison 1980; Allen 1984; Mackerras *et al.* 1990). Subsequently, cells resumed growth (Fig. 5.1a) and the relative L-arginine and L-aspartic acid content declined (Fig. 5.3), accompanied by a gradual increase of the other amino acids (Fig. 5.2a) and the nitrogen-rich pigment phycocyanin (Fig. 5.1b). This indicates that part of the nitrogen temporarily stored in cyanophycin is invested in growth and reallocated into other nitrogen-rich compounds. More specifically, hydrolysis of cyanophycin provides the cell with relatively high availability of L-arginine, which can be incorporated in the L-arginine-rich variant [Asp^3]microcystin-RR. This explains the strong increase in the [Asp^3]microcystin-RR content after the nitrate pulse. Notably, [Asp^3]microcystin-LR contents remains low and does not recover to its initial values (Fig. 5.5). These results demonstrate that nitrogen enrichment can change the intracellular amino acid composition, in particular the content of L-arginine, which plays an important role in the regulation of different microcystin variants.

Shifts in the intracellular amino acid composition may also offer an explanation for changes in the microcystin composition of other harmful cyanobacterial species. A recent laboratory study showed that nitrate addition resulted in increased cellular N:C ratios in the harmful cyanobacterium *Microcystis aeruginosa* HUB 5-2-4 (Van de Waal *et al*. 2009). This increase in the N:C ratio was accompanied by an increased microcystin content, particularly of the nitrogen-rich microcystin-RR variant. Moreover, a survey of several *Microcystis*-dominated lakes showed that the relative microcystin-RR content increased with the seston N:C ratio in these lakes (Van de Waal *et al*. 2009). It seems likely, given our current results with *Planktothrix*, that the enhanced production of microcystin-RR with increasing cellular N:C ratios in *Microcystis* is mediated by changes in the L-arginine content as well.

The nitrogen to carbon stoichiometry of cyanobacteria is also affected by light availability (Sterner *et al.* 1997; Sterner and Elser 2002). Therefore, it is likely that the microcystin production and composition of harmful cyanobacteria responds to changes in light conditions as well. Increasing light availability enhanced the total microcystin production of *Microcystis* PCC 7806 under light-limited conditions, but the total microcystin production decreased under light-saturating conditions (Wiedner *et al.* 2003).

When light is limiting, an increased light intensity will enhance the photosynthetic rate. The additional reducing power produced by photosynthesis can be invested in nitrate reduction, and the resultant ammonium can be used for amino acid synthesis (Flores *et al*. 2005; Flores and Herrero 2005). These amino acids, in turn, favor the synthesis of microcystins. However, when light saturation is reached, high photosynthetic rates are likely to reduce the N:C ratio of the cells (Sterner and Elser 2002). Low cellular N:C ratios may subsequently suppress amino acid synthesis, and this would explain the decrease in total microcystin production at high light intensities observed by Wiedner *et al*. (2003). A reduction in cellular N:C ratios in response to increasing light intensities may also affect the amino acid composition, and suppress the intracellular availability of L-arginine. This offers a plausible explanation for observations of Tonk *et al.* (2005), who reported a shift from [Asp^3]microcystin-RR towards [Asp^3]microcystin-LR as predominant microcystin variant in *P. agardhii* with increasing light intensity.

Changes in microcystin composition may have consequences for the toxicity of cyanobacterial cells. Microcystin variants differ in their acute toxicity, which is estimated by LD_{50} assays on mice. A lower LD_{50} (the intraperitoneal dose lethal for 50% of the mouse population) indicates a higher toxicity. Although microcystin-LR (LD_{50}=33-73 µg kg^{-1}) is more toxic than microcystin-RR (LD_{50}=310-630 µg kg^{-1}), the toxicity of the demethylated variants [Asp^3]microcystin-LR (LD_{50}=160-300 µg kg^{-1}) and [Asp^3]microcystin-RR (LD_{50}=250-360 µg kg^{-1}) is more comparable (Sivonen and Jones 1999; Chen *et al*. 2006; Hoeger *et al*. 2007). Therefore, the toxicity of the *P. agardhii* strain used in our study is mainly determined by the total microcystin content, and increases with nitrogen availability.

Nitrogen is an important limiting nutrient for phytoplankton growth in aquatic ecosystems (Elser *et al*. 2007). Many lakes experience strong variability in nitrogen availability, from replete conditions in winter and early spring to limiting concentrations in summer as a result of high primary production (Phlips *et al.* 1997; McCarthy *et al*. 2007). Eutrophication from agricultural sources and urban development has enriched many aquatic ecosystems with nitrogen (Vitousek *et al*. 1997), shifting the elemental balance of phytoplankton towards higher N:C ratios (Sterner and Elser 2002). Conversely, to counter negative effects of eutrophication, management measures have recently resulted in re-oligotrophication and reduced nitrogen loading of several lakes in Europe and North America (Anderson *et al.* 2005; Weyhenmeyer *et al.* 2007). Current changes in the global climate are also likely to alter the carbon and nutrient availability in many aquatic ecosystems, with consequences for the elemental stoichiometry of phytoplankton species (Van de Waal *et al.* 2010). Our results show that these dynamic changes in nitrogen availability modify the nitrogen to carbon stoichiometry and amino acid composition of harmful cyanobacteria, and thereby affect the production and composition of their microcystin variants.

Acknowledgements - The research of DBvdW, LT, JH and PMV was supported by the Earth and Life Sciences Foundation (ALW), 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 Prof. Kaarina Sivonen of the University of Helsinki for providing the *Planktothrix agardhii* strain 126/3 and Prof. Geoff Codd of the University of Dundee for providing the microcystin gravimetrical standards.