OUT OF BALANCE

Implications of climate change for the ecological stoichiometry of harmful cyanobacteria

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Chapter 1

Introduction

1.1 Global change

Since the start of the Industrial Revolution, vast amounts of CO_2 have been exhausted into the atmosphere. As a result, atmospheric CO_2 levels increased from 285 ppm in 1850 to today's 385 ppm, a level far exceeding the natural range of the past 650 000 years. Climate change scenarios predict that atmospheric CO_2 will rise further, reaching ~750 ppm by the end of the 21st century (Solomon *et al.* 2007). This rapid increase in atmospheric CO_2 concentrations and other greenhouse gases is accompanied by global warming. Since 1850, average global temperatures have increased with 0.8°C and are expected to increase with another 3 °C by the end of the 21st century (Solomon *et al.* 2007). These dramatic changes in atmospheric CO_2 levels and average global temperatures show remarkable effects on the Earth's climate. For instance, extreme rainfall events and storms become more frequent, glaciers are melting at unprecedented rates, and sea levels are rising (Solomon *et al*. 2007).

Atmospheric CO_2 reacts with water forming carbonic acid (H_2CO_3). Carbonic acid rapidly dissociates into bicarbonate (HCO_3^-) thereby releasing a proton (H^+). At high pH, bicarbonate can dissociate further to carbonate (CO_3^{2-}), releasing another proton. With higher atmospheric CO_2 levels, more CO_2 reacts with water, which will increase the proton concentration, and subsequently cause a drop in pH. This process is typically referred to as ocean acidification (Caldeira and Wickett 2003; Doney *et al*. 2009). A decrease of 0.3 pH units, from 8.1 to 7.8, as expected for the oceans in the year 2100 is equivalent to a doubling of the proton concentration. The concentration of dissolved CO_2 in equilibrium with the atmosphere depends on the atmospheric CO_2 level, but not on pH. However, concentrations of bicarbonate and carbonate do depend on pH. As a consequence, the dominant carbon species changes with decreasing pH; from carbonate at high pH, via

bicarbonate at an intermediate pH, to CO_2 at low pH (Fig. 1.1). Rising atmospheric CO_2 levels will thus increase the concentration of dissolved CO_2, but will decrease the carbonate concentration. Enhanced concentrations of dissolved CO_2 may promote photosynthesis in phytoplankton (Schippers *et al*. 2004; Rost *et al.* 2008). However, lower saturation of the water with carbonate ions may have severe consequences for calcifying organisms such as corals, foraminifers and coccolithophores, which depend on carbonate ions to form their calcium-carbonate skeletons (Orr *et al*. 2005; Hoegh-Guldberg *et al*. 2007; but see Iglesias-Rodriguez *et al*. 2008).

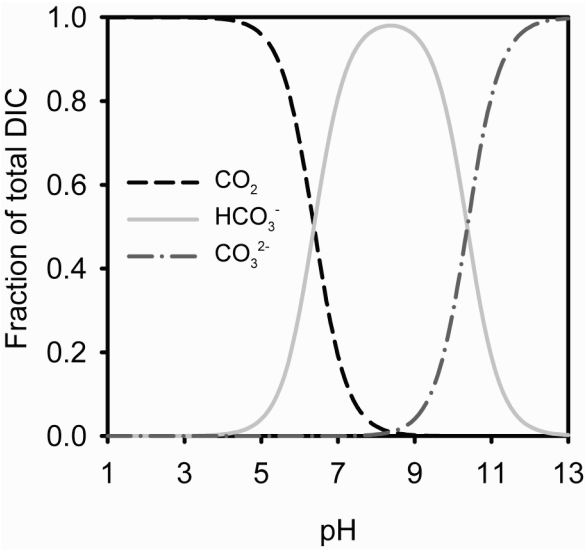

Figure 1.1. Relative contribution of the different carbon species to the total dissolved inorganic carbon (DIC) as a function of pH.

Global warming and rising atmospheric CO_2 levels may alter the input of dissolved organic carbon (DOC) into freshwater and coastal ecosystems. As a result of thawing permafrost at high latitudes, large amounts of trapped organic carbon may flow into ponds and lakes (Zimov *et al.* 2006). In addition, changes in plant community composition in peatlands may amplify the export of organic carbon from peatland catchments (Fenner *et al*. 2007). Enhanced rainfall events, predicted by climate scenarios (Solomon *et al*. 2007) may flush larger amounts of organic carbon from terrestrial ecosystems into lakes (Hinton *et al*. 1997). DOC serves as substrate for microbial respiration producing CO_2. As a consequence, many lakes are supersaturated with CO_2 (Sobek *et al.* 2005). Enhanced DOC input from terrestrial ecosystems may lead to an even further increase of the CO_2 concentration in lakes.

Besides the vast amounts of CO_2 that have been exhausted into the atmosphere, anthropogenic activities have also enriched many aquatic ecosystems with vast amounts of nutrients (Vitousek *et al*. 1997; Smith *et al*. 1999). In particular during the 20th century, high concentrations of nitrate and phosphate were used for agricultural purposes, which subsequently flushed into rivers, lakes and oceans (Harper 1992; Nixon 1995). For instance, the riverine nitrogen fluxes into the North Atlantic Ocean have increased up to 20 times as compared to pre-industrial times (Howarth *et al*. 1996). Even though water management measures have been taken to reduce the nutrient input into aquatic ecosystems, still many freshwater and coastal ecosystems contain high concentrations of nitrogen and phosphorus (Cloern 2001; Smith *et al.* 2006). Increasing amounts of nutrients in surface waters have a broad variety of effects on aquatic ecosystems, including oxygen depletion leading to hypoxia and associated fish kills (Rabalais *et al*. 2002) as well as an increasing frequency of harmful algal blooms (Huisman *et al*. 2005; Smith *et al.* 2006).

1.2 Ecological stoichiometry*

Changing resource conditions in surface waters as a result of rising CO_2 levels, global warming and eutrophication will have implications for the balance of carbon and nutrients in aquatic organisms. The field of ecological stoichiometry (Sterner and Elser 2002) looks at this balance of carbon and nutrients to describe the complex relationships between organisms and their environment. Using the same mass-balance approach routinely employed in chemistry, ecological stoichiometry provides insight into the feedbacks and constraints operating on carbon and nutrient fluxes in food webs. In autotrophic organisms, like phytoplankton and terrestrial plants, carbon and nutrients are taken up separately. As a result, CO_2 fixation and nutrient acquisition are relatively loosely coupled. This makes autotrophs flexible in their stoichiometry; their carbon:nutrient ratios can vary over large ranges, and partly reflect the resource availability in their environment. For example, when phosphorus is available in high concentrations, C:P ratios in phytoplankton cells are often relatively low. Conversely, when phosphorus is limiting but light and inorganic carbon are in ample supply, intracellular C:P ratios can become very high (Fig. 1.2).

Although the carbon:nutrient stoichiometry of autotrophs may vary widely, many heterotrophic organisms tend to keep their carbon:nutrient ratios within a narrow range (Fig. 1.2). This is known as homeostasis. Heterotrophs usually take up carbon and nutrients simultaneously, so their carbon and nutrient uptake reflects the carbon:nutrient ratio supplied by their food. However, the nutrient content in most heterotrophic organisms is relatively high compared to autotrophic organisms (Fig. 1.2). For many herbivores, the nutrient content of their food will thus be low relative to their own nutrient demands. Yet,

* *This paragraph is based on Panel 1 of Van de Waal* et al*. 2010.*

different species of heterotrophs may differ in their stoichiometric requirements. For instance, copepods typically have higher C:P ratios than cladocerans (Sterner and Elser 2002). Furthermore, stoichiometric requirements may vary with the developmental stage of heterotrophs. Larval and juvenile fish, for instance, often have higher C:P ratios than adult fish (Fig. 1.2).

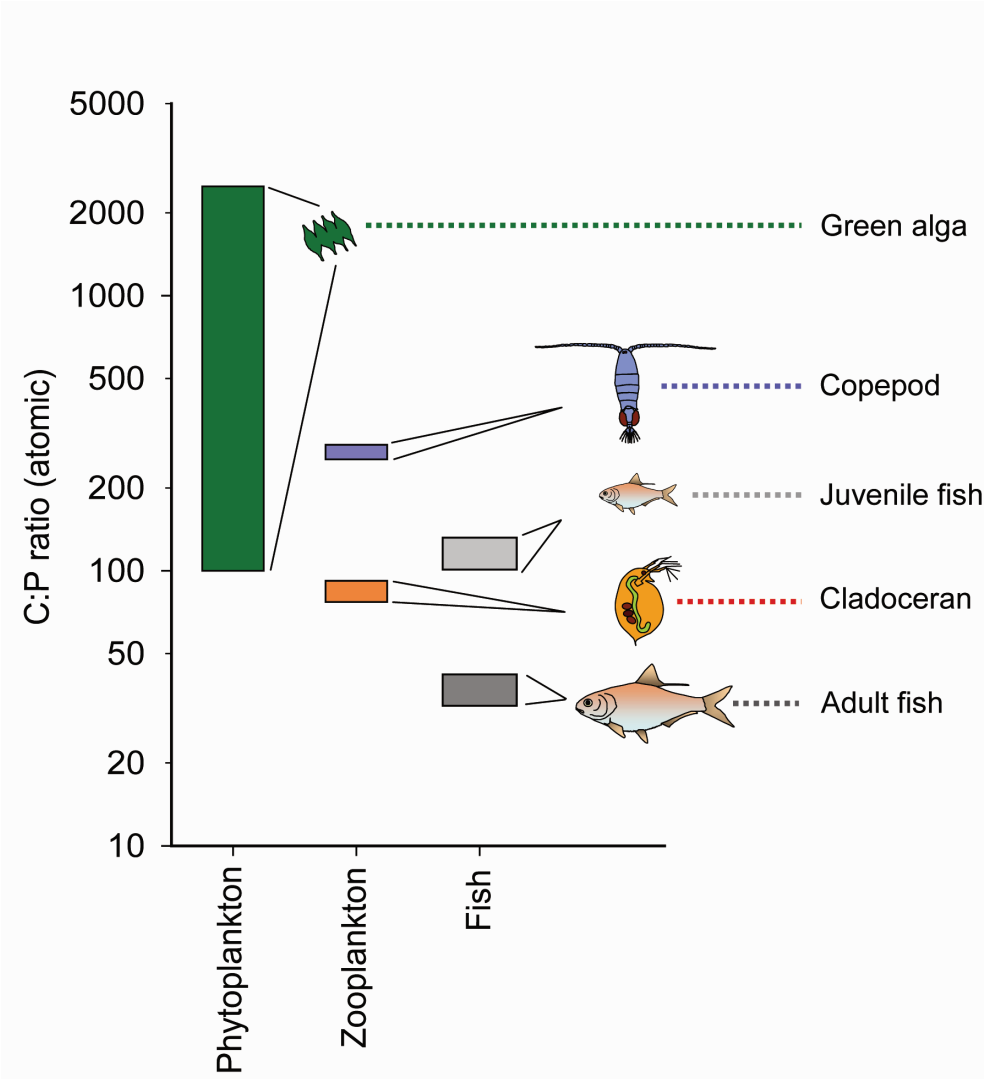

Figure 1.2. Phytoplankton display a wide range of relatively high C:P ratios, as shown here for the green alga *Scenedesmus obliquus* (data from Verschoor *et al*. 2004). Zooplankton and fish have rather narrow stoichiometric ranges (homeostasis). Copepods, such as *Eudiaptomus gracilis*, typically have higher C:P ratios than do cladocerans, such as *Daphnia galeata* (data from Vrede *et al.* 1999). The nutrient stoichiometry may also change during ontogenic development, as illustrated by the C:P ratio of juvenile and adult fish of the gizzard shad (*Dorosoma cepedianum*; data from Pilati and Vanni 2007).

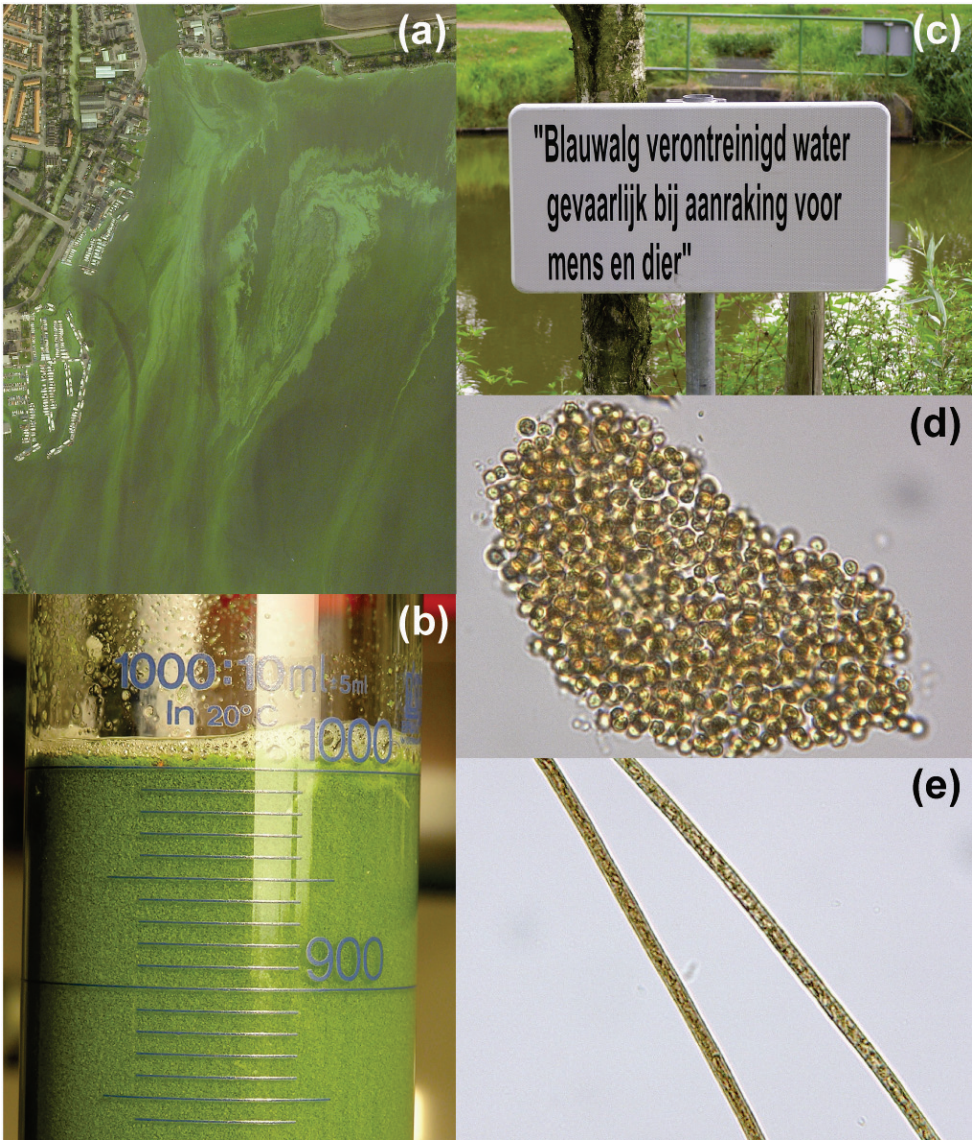

Figure 1.3. Harmful cyanobacteria. (a) Scum formation in lake Braassemermeer in September 2005. (b) Cyanobacterial scum in a measuring cylinder. (c) A signpost warning against harmful cyanobacteria: "Blue-green algae polluted water dangerous for humans and animals". (d,e) Microscopic photographs of the cyanobacteria (d) *Microcystis* sp. and (e) *Planktothrix* sp. Source: (a) Aerial photograph © Aerodata International Surveys; (d, e) Corrien AM Sigon, department of Aquatic Microbiology, UvA.

One important implication of the stoichiometric imbalance between autotrophs and their heterotrophic grazers is that grazers can become nutrient-limited. For instance, without stoichiometric considerations, one might think that an increased light supply, which

enhances primary production, will benefit heterotrophic grazers, because more food is being produced. However, stoichiometric theory predicts the opposite, because an increased light supply may suppress food quality (Sterner and Elser 2002). Indeed, laboratory and field experiments (Urabe *et al*. 2002, 2003) have confirmed that greater light availability increased phytoplankton abundance, but also the carbon:nutrient content of the phytoplankton, thus decreasing the quality of the zooplankton's food (Fig. 1.2). This change in phytoplankton stoichiometry can suppress the growth of nutrient-demanding zooplankton species (Sterner and Elser 2002; Urabe *et al*. 2002).

1.3 Harmful cyanobacteria

Besides affecting the elemental balance of phytoplankton, changes in resource conditions also have implications for phytoplankton abundance and community composition. The vast amounts of nutrients derived from terrestrial runoff and anthropogenic loading promote excessive growth of phytoplankton, and may shift the phytoplankton species composition towards harmful algal blooms (Smith *et al.* 2006). Among these harmful algal blooms are the cyanobacteria (blue-green algae), which are notorious for their toxin production (Huisman *et al*. 2005). Harmful cyanobacteria typically flourish in eutrophic lakes, especially during warm summers with high temperatures and photon irradiance (Jöhnk *et al.* 2008; Paerl and Huisman 2008). When vertical mixing is weak, during periods with low wind speed, buoyant cyanobacteria float to the water surface (Walsby *et al*. 1997; Huisman *et al*. 2004). Accumulation of cyanobacterial cells at the water surface may lead to the formation of dense surface blooms (Fig. 1.3a-c). Common bloom-forming cyanobacteria include colonial species of the *Microcystis* genus (Fig. 1.3d) and filamentous *Planktothrix* species (Fig. 1.3e), both capable of producing toxins known as microcystins. Microcystins are hepatotoxins, i.e., they primarily act on the liver (Sivonen and Jones 1999; Kuiper-Goodman *et al*. 1999). High microcystin concentrations pose a major threat to birds, mammals and human health, and make the water less suitable for drinking water, agricultural irrigation, fishing and recreational use (Chorus and Bartram 1999; Carmichael 2001; Huisman *et al*. 2005). In dense surface blooms, microcystin concentrations can reach values > 10 000 μg L^{-1} (Kardinaal and Visser 2005a), which is 500-fold higher than the guideline value for recreational waters adviced by the World Health Organization (Chorus and Bartram 1999).

Microcystins are small cyclic peptides consisting of seven amino acids, including two positions with a variable amino acid composition (Fig. 1.4). At present, at least 89 microcystin variants have been described (Welker and Von Döhren 2006). These microcystin variants may differ in their acute toxicity, which is estimated by LD_{50} assays on mice (the intraperitoneal dose lethal for 50% of the mouse population). A lower LD_{50} indicates a higher toxicity. For instance, microcystin-LR (LD_{50} = 33-73 μg kg^{-1}) and

microcystin-YR (LD_{50} = 70 µg kg^{-1}) are more toxic than microcystin-RR (LD_{50} = 310-630 µg kg^{-1}), while 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}) are quite similar (Sivonen and Jones 1999; Chen *et al*. 2006; Hoeger *et al*. 2007).

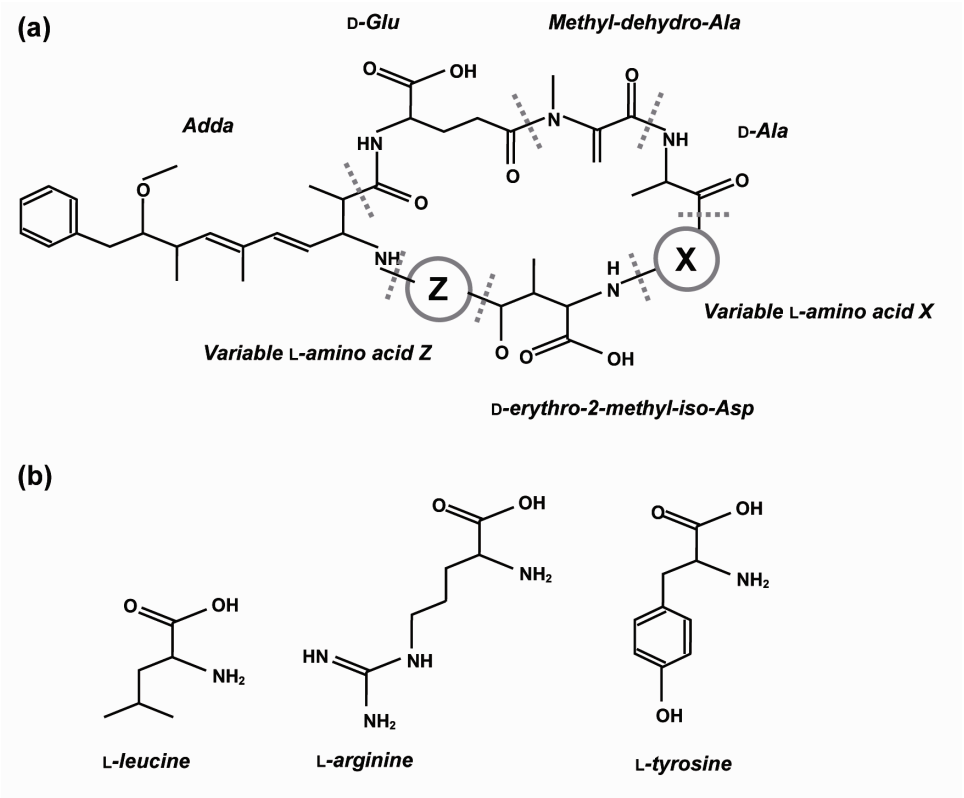

Figure 1.4. (a) General molecular structure of microcystins, which consist of seven amino acids including two variable positions (X and Z). (b) Position X is occupied by the amino acids L-leucine (L), L-arginine (R) or L-tyrosine (Y) in the common microcystin variants microcystin-LR, microcystin-RR, and microcystin-YR, respectively. Position Z is occupied by L-arginine in all three microcystin variants. Drawn after Welker and Von Döhren (2006).

Microcystin variants differ in their nitrogen:carbon stoichiometry. For instance, the variable position X of microcystin-LR, microcystin-YR and microcystin-RR is occupied by leucine, tyrosine, and arginine, respectively (Fig. 1.4). The amount of nitrogen in these amino acids differs. Arginine contains four nitrogen atoms, while leucine and tyrosine each contain only one nitrogen atom. As a consequence, the molar N:C ratio of microcystin-RR (N:C = 0.27) is higher than that of microcystin-LR and microcystin-YR (N:C = 0.20 and 0.19, respectively).

1.4 This thesis

The principle aim of this thesis is to determine how climate-driven changes in resource conditions may alter the stoichiometry and toxin production of harmful cyanobacteria. The work presented in this thesis consists of a comprehensive literature review, laboratory experiments, fieldwork, and the development of a new model. The outline of this thesis is as follows:

In **Chapter 2**, we describe the current state of knowledge on the potential effects of climate change on the ecological stoichiometry of aquatic ecosystems (Van de Waal *et al*. 2010). This chapter is inspired by a scientific symposium supported by the Royal Netherlands Academy of Arts and Sciences (KNAW), entitled 'Climate-driven changes in the ecological stoichiometry of aquatic ecosystems' (CLIMAQS; Amsterdam, 2007). Rising atmospheric CO_2 levels enrich aquatic ecosystems with carbon that becomes available for phytoplankton growth. Global warming strengthens the thermal stratification of aquatic ecosystems, which suppresses the supply of nutrients from deep water layers into the surface layer. An enhanced carbon availability, but reduced nutrient availability, will shift the carbon:nutrient balance of phytoplankton towards higher values. Such high phytoplankton carbon:nutrient ratios, however, are of low nutritional value for zooplankton grazers. Hence, climate-driven changes in the phytoplankton stoichiometry may cascade throughout the entire aquatic food web.

A major question is whether changes in carbon and nutrient availability will affect the cellular stoichiometry and microcystin composition in harmful cyanobacteria. In **Chapter 3**, we tested whether the carbon-nutrient balance hypothesis, a well established theory to describe secondary metabolite production in terrestrial plants, also applies to toxin production in harmful cyanobacteria (Van de Waal *et al*. 2009). More specifically, we determined the effects of carbon, nitrogen, and light limitation on the nitrogen:carbon stoichiometry and microcystin production of the harmful cyanobacterium *Microcystis aeruginosa*. Our laboratory experiments show that production of the nitrogen-rich microcystin variant microcystin-RR is indeed promoted by high cyanobacterial nitrogen:carbon stoichiometry, consistent with the carbon-nutrient balance hypothesis. These results were largely in agreement with a lake survey where the microcystin-RR content increased with the nitrogen:carbon stoichiometry of the lake seston.

Thus, the nitrogen:carbon stoichiometry of harmful cyanobacteria influences its microcystin composition. But what are the underlying physiological mechanisms for this relationship? Because microcystins consist of amino acids, we investigated in **Chapter 4** whether amino acids supplied in the growth medium will affect the microcystin composition (Tonk *et al*. 2008). Addition of leucine resulted in a strong increase of the microcystin-LR:microcystin-RR ratio, while addition of arginine resulted in a decrease of this ratio. This demonstrates that amino acid availability plays a role in the synthesis of

different microcystin variants. Thus, resource conditions that alter the amino acid composition of the cells may induce changes in the microcystin composition as well. We investigate this hypothesis further in **Chapter 5**, where we grew *Planktothrix agardhii* under nitrogen-depleted conditions and gave cells a sudden nitrate pulse. Upon nitrate addition, we found a rapid increase of the cellular nitrogen:carbon 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 nitrogen-rich microcystin-RR variant (which contains two arginine molecules) increased strongly after the nitrate pulse, while microcystin-LR increased to a much lesser extent. This demonstrates that the effect of the nitrogen:carbon stoichiometry on microcystin synthesis and composition is mediated by amino acids.

So far, we studied the effect of changing carbon and nutrient availability on the ecological stoichiometry and toxin production of harmful cyanobacteria. As a next step, we investigated how changes in these resources may alter the outcome of competition between harmful cyanobacteria. Resource competition theory has extensively addressed competition for nutrients and light. Yet, competition for inorganic carbon has not been resolved. Therefore, in **Chapter 6** we developed a new model that describes phytoplankton competition for carbon. We performed monoculture and competition experiments in chemostats with a toxic and a nontoxic *Microcystis aeruginosa* strain under carbon-limited conditions. In addition, we tested the model on earlier experiments by Kardinaal *et al*. (2007b), who used the same two strains but grown under light-limited conditions. The model could qualitatively and quantitatively predict the outcome of competition. The low CO_2 concentrations in the carbon-limited chemostats were accompanied by high pH, leading to dominance of the toxic strain. Thus, the toxic strain was either a better competitor for CO_2 or it could tolerate a higher pH than the nontoxic strain. At low light conditions, in combination with high CO_2 concentrations, the nontoxic strain became dominant. These results show that changes in carbon and light availability may result in a complete reversal of the outcome of competition between toxic and nontoxic strains of harmful cyanobacteria.

In **Chapter 7**, I will wrap up the results presented in this thesis. The overall conclusion is that the toxin composition of harmful cyanobacteria is sensitive to changes in inorganic carbon and nitrogen availability. In addition, the competitive dominance of toxic versus nontoxic strains may shift with changes in CO_2 availability. Climate change is likely to alter the carbon and nitrogen availability in many aquatic ecosystems, and may thereby affect not only the elemental balance and species composition of phytoplankton communities, but also the nature of the toxins that they can produce.

Chapter 2

Climate-driven changes in the ecological stoichiometry of aquatic ecosystems

ABSTRACT - Advances in ecological stoichiometry, a rapidly expanding research field investigating the elemental composition of organisms and their environment, shed new light on the impacts of climate change on freshwater and marine ecosystems. Current changes in the Earth's climate alter the availability of carbon and nutrients in lakes and oceans. In particular, CO_2 concentrations will rise to unprecedented levels by the end of this century, while global warming will enhance stratification of aquatic ecosystems and may thereby diminish the supply of nutrients into the surface layer. These processes enrich phytoplankton with carbon, but suppress nutrient availability. Phytoplankton with a high carbon:nutrient content provide poor food quality for most zooplankton species, which may shift the species composition of zooplankton and higher trophic levels to less nutrient-demanding species. As a consequence, climate-driven changes in plankton stoichiometry may alter the structure and functioning of entire aquatic food webs.

This chapter is based on the paper: Dedmer B Van de Waal, Antonie M Verschoor, Jolanda MH Verspagen, Ellen Van Donk, and Jef Huisman. 2010. Climate-driven changes in the ecological stoichiometry of aquatic ecosystems. Frontiers in Ecology and the Environment 8: 145-152

2.1 Out of balance

In 1934, Alfred C Redfield reported that the ratios between the elements carbon, nitrogen. and phosphorus (C, N, and P, respectively) in marine phytoplankton were remarkably constant (Redfield 1934). His famous C:N:P ratio of 106:16:1 (by atom) has become known as the "Redfield ratio" (Falkowski and Davis 2004). Redfield further noted that the N:P ratios of phytoplankton resembled the nitrate:phosphate ratio found in the deep waters of the oceans (Redfield 1934). Thus, the elemental composition of marine plankton reflected that of their environment, and vice versa. However, even the seemingly constant pelagic environment is currently affected by changes in the Earth's atmosphere. The 1934 atmosphere contained ~300 parts per million (ppm) of carbon dioxide (CO_2), which has risen to the present-day value of 385 ppm, a level that by far exceeds that of the natural range of the past 650 000 years. Atmospheric CO_2 levels are expected to rise further, to ~750 ppm by the year 2100 (Solomon *et al*. 2007). The rapid increase in atmospheric CO_2 and other greenhouse gases is accompanied by global warming. Average global temperatures have risen by 0.6 °C since 1934, and an additional 3 °C increase is expected to occur over the course of the 21st century (Solomon *et al*. 2007). These changes in global climate will affect many chemical and physical processes in aquatic ecosystems, with possible implications for the elemental composition of plankton communities.

Ecological stoichiometry is a rapidly expanding research field investigating how the elemental composition of organisms affects ecological processes (Sterner and Elser 2002). Inspired by Redfield and the recent advances in ecological stoichiometry, this review explores the potential impacts of climate change on the carbon and nutrient availability in aquatic ecosystems, its consequences for the C:N:P stoichiometry of plankton communities, and its implications for the structure of aquatic food webs.

2.2 Rising CO_2 and ocean acidification

The current rise in atmospheric CO_2 levels is having a major impact on the carbon chemistry of the oceans (Doney *et al*. 2009). In fact, it is estimated that almost 50% of the anthropogenic CO_2 input into the atmosphere since the Industrial Revolution has been absorbed by the oceans (Sabine *et al*. 2004). Compared to the large pool of bicarbonate (HCO_3^-), dissolved CO_2 constitutes only a minor fraction of the total concentration of dissolved inorganic carbon (DIC) in the oceans. Yet, rising concentrations of atmospheric CO_2 increase the concentration of dissolved CO_2 in ocean surface waters. Dissolved CO_2 reacts with water to form carbonic acid (H_2CO_3), which rapidly dissociates into HCO_3^-, releasing a proton and thereby reducing pH levels. This phenomenon has become known as "ocean acidification" (Caldeira and Wickett 2003; Doney *et al.* 2009). Since the industrial

revolution, ocean acidification has decreased the pH of ocean surface waters by ~0.1 units, to an average surface pH of 8.1 (Orr *et al*. 2005). At atmospheric CO_2 levels of 750 ppm – expected by the year 2100 – the pH of surface seawater will have dropped by an additional 0.3 units, to a pH of ~7.8 (Orr *et al*. 2005). This is equivalent to a doubling of the proton concentration. The projected concentration of dissolved CO_2 at that point will be outside the natural range of the past 20 million years (Solomon *et al*. 2007).

Ocean acidification causes a decrease in the concentration of carbonate ions (Caldeira and Wickett 2003; Solomon *et al*. 2007). This increases the solubility of calcium carbonate ($CaCO_3$) in the oceans. Cold waters are less saturated with respect to $CaCO_3$ than are warmer waters; high-latitude ecosystems are therefore likely to be the first to suffer from ocean acidification. Model simulations project that Southern Ocean surface waters will begin to become undersaturated with respect to aragonite, a metastable form of $CaCO_3$, by about the year 2050 (Orr *et al*. 2005; Doney *et al*. 2009). By 2100, this undersaturation could extend throughout the entire Southern Ocean and into the subarctic Pacific Ocean. When water becomes undersaturated with aragonite, key marine organisms, such as corals, foraminifers, and coccolithophores, have difficulty in forming their $CaCO_3$ skeletons (Orr *et al*. 2005; Hoegh-Guldberg *et al*. 2008). Laboratory experiments with coccolithophores exposed to elevated CO_2 yielded malformed and smaller coccoliths (Riebesell *et al*. 2000), although effects varied depending on the strain and growth conditions (Iglesias-Rodriguez *et al*. 2008). Many other calcifying organisms, including pteropods, mollusks, crustaceans, and sea urchins, will also be negatively affected by ocean acidification (Orr *et al*. 2005; Doney *et al.* 2009).

2.3 Carbon supersaturation in freshwater ecosystems

In contrast to open ocean waters, many freshwater ecosystems, estuaries, and some coastal environments receive substantial amounts of carbon from terrestrial ecosystems (Sobek *et al*. 2005). This occurs mainly in the form of dissolved organic carbon (DOC). Bacterial activity mineralizes the available DOC into CO_2. As a result, the CO_2 concentration of lakes is usually not in equilibrium with the atmosphere, but is related to the concentration of DOC (Fig. 2.1). In fact, CO_2 concentrations of 1000-3000 ppm are quite common in lakes. On a global basis, inland waters receive up to 1.9 petagram carbon $year^{-1}$ (equal to 1.9×10^{15} g C $year^{-1}$) from the surrounding watershed, of which about half is ultimately flushed into the oceans (Cole *et al*. 2007). A fraction of the carbon received by inland waters is buried in sediments, whereas an estimated 0.8 petagram carbon $year^{-1}$ is processed by aquatic biota, where it is finally respired to CO_2 (Cole *et al*. 2007; although the exact values are still under debate; LJ Tranvik pers. comm.). Accordingly, most inland waters are supersaturated with CO_2, and thus serve as net sources of CO_2 to the atmosphere (Sobek *et al*. 2005; Cole *et al*. 2007).

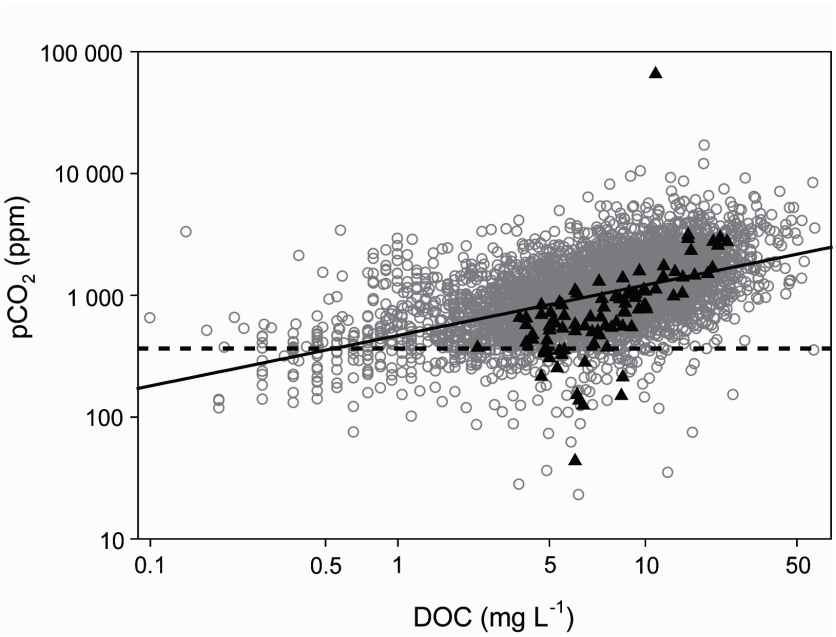

Figure 2.1. Carbon dioxide concentration (pCO_2) against the concentration of dissolved organic carbon (DOC) in 4555 globally distributed lakes. Each point represents one individual lake. Circles denote lakes with a single measurement. Triangles represent mean values for lakes with multiple measurements. The solid line shows linear regression for all data points ($\log[pCO_2] = 2.67 + 0.414 \times \log[DOC]$; $r^2 = 0.26$; $n = 4555$; $P < 0.0001$). The horizontal dashed line indicates the present atmospheric CO_2 level of 385 ppm. (From Sobek *et al.* 2005. Reproduced with permission from the American Geophysical Union. © 2005 American Geophysical Union).

Global warming and rising atmospheric CO_2 levels may alter the input of DOC into freshwater ecosystems, although the magnitude and direction of these changes will vary regionally. At high latitudes, rising temperatures will thaw large areas covered by permafrost in Siberia, Canada, and Alaska, releasing massive amounts of trapped organic carbon that may flow into ponds and lakes (Zimov *et al*. 2006). Climate scenarios also predict more extreme rainfall events and more frequent storms in many parts of the world (Solomon *et al*. 2007), which may result in increased runoff of DOC from terrestrial ecosystems into lakes (Hinton *et al*. 1997). Conversely, DOC concentrations in the Experimental Lakes Area in Canada decreased by 15-25% during two decades of climatic warming, as a result of reduced streamflow caused by drought (Schindler *et al*. 1997). Rising atmospheric CO_2 concentrations lead to increased stomatal closure of terrestrial vegetation. This reduces plant transpiration, and has been estimated to increase global runoff by 6% (Betts *et al*. 2007). In peatlands, elevated CO_2 induces shifts in plant species composition, which could amplify DOC export from peatland catchments (Fenner *et al*. 2007). Other processes also affect the DOC input into freshwater ecosystems, however. For

instance, increased DOC concentrations in lakes in Europe and North America have been explained by a reduction in atmospheric acid deposition (Monteith *et al*. 2007). All in all, the effects of climate change on DOC concentrations in lakes will greatly depend on the local setting (e.g. local meteorology, catchment hydrology, land use, vegetation, and water retention times). Yet, the processes described above suggest that DOC concentrations may increase in many lakes.

2.4 Global warming and stratification

Rising temperatures influence the physical structure of aquatic ecosystems. In particular, warming of the upper layers of lakes and oceans enhances the density difference between the surface mixed layer and the deeper waters beneath. All else being equal, this increased density difference will strengthen the vertical stratification of both lakes (Jöhnk *et al*. 2008; Peeters *et al*. 2008) and oceans (Sarmiento *et al*. 2004; Doney 2006). Stratification suppresses vertical mixing across the density gradient, with contrasting effects on nutrient and light availability for phytoplankton growth. On the one hand, stratification reduces the nutrient influx from deep, nutrient-rich waters into the surface mixed layer, thus limiting the availability of nutrients for phytoplankton growth (Behrenfeld *et al*. 2006; Huisman *et al*. 2006). On the other hand, stratification keeps phytoplankton populations in the surface mixed layer, thereby improving light conditions for phytoplankton growth (Huisman *et al*. 1999; Berger *et al.* 2007).

Many waters in the tropics and subtropics are permanently stratified. Nutrient concentrations in the surface mixed layer of these waters are strongly depleted (Karl *et al*. 1997; Huisman *et al.* 2006). As a result of severe nutrient limitation, permanently stratified oceans in the tropics and subtropics are characterized by extremely low primary production (Fig. 2.2a, b). Climate-ocean models predict that, by the year 2050, the ocean area covered by permanent stratification will have expanded by 4.0% and 9.4% in the Northern and Southern hemispheres, respectively (Sarmiento *et al*. 2004), thereby reducing overall ocean productivity (Behrenfeld *et al.* 2006). These predictions are surpassed by recent observations, which indicate a much faster expansion of the ocean's least productive waters over the past 9 years (Fig. 2.2c–e; Polovina *et al*. 2008).

In the temperate zone and at high latitudes, waters are not permanently stratified, and deep mixing during winter and/or spring provides nutrients into the surface layer. In these regions, phytoplankton growth is often light-limited in winter, due to short day lengths as well as deep vertical mixing. Climate warming causes an earlier onset of stratification in spring, which brings the phytoplankton cells in the well-lit surface layer while nutrients are not yet depleted, and thereby favors their growth. This leads to an earlier spring bloom and a substantially longer growing season in the temperate zone (Winder and Schindler 2004; Peeters *et al*. 2008).

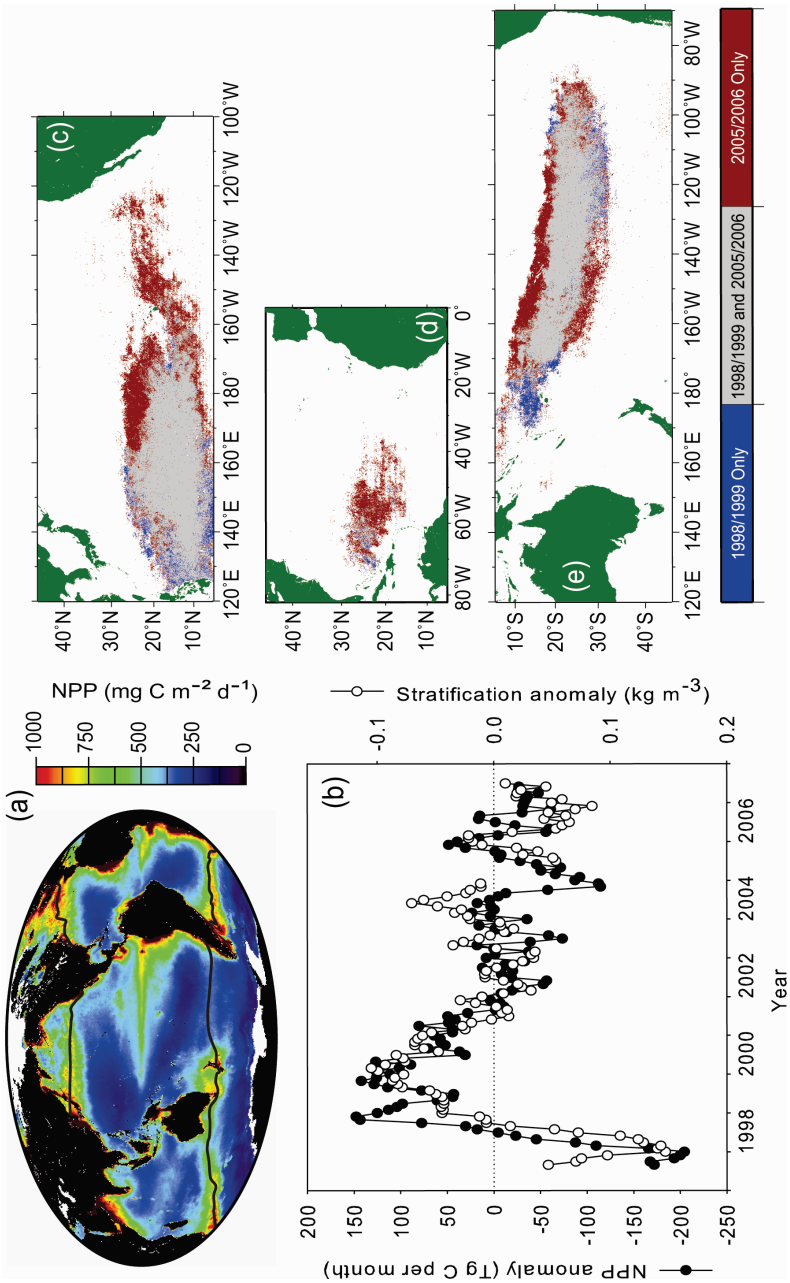

Figure 2.2. Relationships between stratification and primary production. (a) Global distribution of annual net primary production (NPP), with low values (blue) in permanently stratified waters of subtropical and tropical oceans. (b) Changes in ocean stratification (open symbols) are well correlated with NPP anomalies (closed symbols) in the subtropical and tropical oceans (Pearson product-moment correlation: $r^2 = 0.73$; $n = 112$; $P < 0.001$). Expansion of waters with very low net primary production during the past decade, for (c) North Pacific Ocean, (d) North Atlantic Ocean, and (e) South Pacific Ocean. Grey areas had very low chlorophyll concentrations in both 1998/1999 and 2005/2006, blue areas only in 1998/1999, and red areas only in 2005/2006. ([a] and [b] from Behrenfeld *et al.* 2006; adapted with permission from Macmillan Publishers Ltd: Nature, © 2006. [c–e] from Polovina *et al.* 2008; reproduced with permission from the American Geophysical Union. Published 2008 American Geophysical Union).

2.5 Changes in phytoplankton stoichiometry

The elemental composition of phytoplankton can vary widely, and often reflects the resource availability in their environment (Sterner and Elser 2002). Climate-driven changes in carbon, light, and nutrient availability are therefore likely to affect phytoplankton stoichiometry. In dense phytoplankton blooms, the photosynthetic demand for inorganic carbon can be very high, and this can deplete inorganic carbon to limiting concentrations (Ibelings and Maberly 1998). Rising CO_2 levels may alleviate these dense blooms from carbon limitation, especially in low-alkalinity freshwater ecosystems. In marine ecosystems, however, DIC is dominated by vast amounts of HCO_3^-.

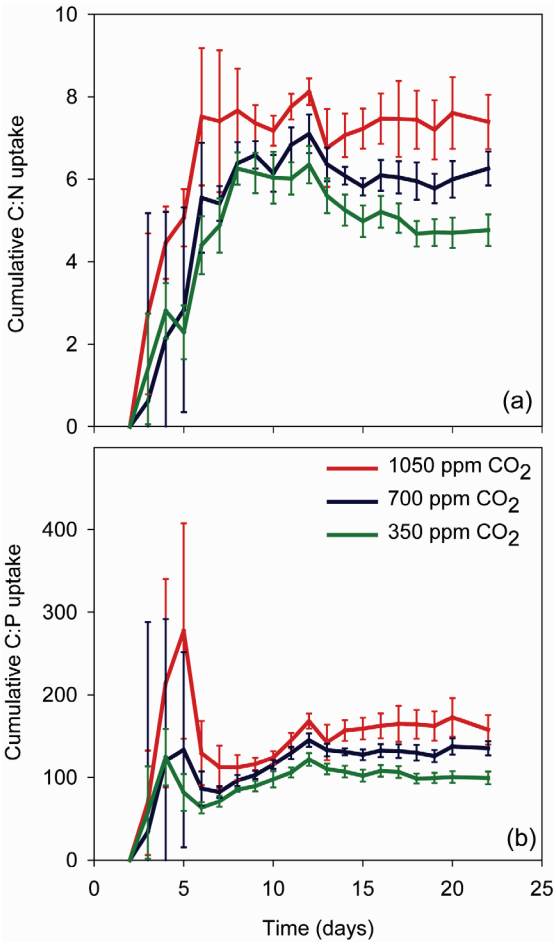

Figure 2.3. Carbon:nutrient uptake ratios during a marine CO_2 enrichment study. The graphs illustrate (a) C uptake relative to N uptake (C:N), and (b) C uptake relative to P uptake (C:P), both integrated over the duration of the experiment from day 2 onwards. The treatments consisted of 350, 700, and 1050 ppm CO_2, represented by green, blue, and red lines, respectively. Error bars indicate one standard deviation. (From Bellerby *et al*. 2007).

Many phytoplankton species can take up both dissolved CO_2 and HCO_3^- (Kaplan and Reinhold 1999; Martin and Tortell 2008). Inorganic carbon is therefore rarely a limiting resource in marine ecosystems. However, large, species-specific differences exist in the relative uptake rates of HCO_3^- versus CO_2 (Martin and Tortell 2008). This interspecific variability will likely have important implications for the phytoplankton species composition in response to rising CO_2 concentrations.

In a recent, large-scale mesocosm experiment in a Norwegian fjord, an increase of the CO_2 partial pressure to 1050 ppm resulted in 39% more uptake of inorganic carbon by the natural plankton community (Bellerby *et al*. 2007; Riebesell *et al*. 2007). Since nutrient uptake remained largely unaffected in this experiment, this resulted in enhanced uptake of carbon relative to nitrogen and phosphorus (Fig. 2.3). Furthermore, recent laboratory experiments have shown that increased concentrations of CO_2 stimulated both nitrogen and carbon fixation in a marine nitrogen-fixing cyanobacterium (Hutchins *et al*. 2007; Levitan *et al*. 2007). For these nitrogen-fixing organisms, C:N ratios did not change with increasing CO_2 availability, because their cellular nitrogen and carbon contents increased in parallel. However, phosphorus uptake was not stimulated, so that their C:P and N:P ratios increased markedly at higher CO_2 levels (Hutchins *et al*. 2007; Levitan *et al*. 2007).

Changes in stratification patterns are likely to affect phytoplankton stoichiometry in two ways. First, a stronger stratification reduces the nutrient supply from deep waters below. Second, a stronger stratification keeps the phytoplankton in the well-lit surface layer. Phytoplankton exposed to these nutrient-limited but light-saturated conditions typically show increased cellular carbon:nutrient ratios (Sterner and Elser 2002). Moreover, a reduced nitrogen supply and increased light availability promote the competitive success of nitrogen-fixing cyanobacteria (Agawin *et al*. 2007). The proliferation of these organisms may force phosphorus concentrations in surface waters to limiting values. This mechanism seems to apply to the subtropical Pacific Ocean, where the presence of nitrogen-fixing organisms yields an overall increase in cellular N:P ratios, and shifts the entire ecosystem towards phosphorus limitation (Karl *et al*. 1997). This suggests that, with climate-driven warming and the expansion of ocean stratification, cellular carbon:nutrient ratios – and in particular C:P ratios – are likely to increase in substantial parts of the world's oceans.

Thus, both rising atmospheric CO_2 levels and enhanced stratification by global warming may shift phytoplankton stoichiometry in a similar direction, causing an increased carbon:nutrient ratio of the phytoplankton community and associated changes in phytoplankton species composition toward species that can proliferate under high CO_2/low nutrient conditions.

2.6 Implications for zooplankton and higher trophic levels

The elemental composition of phytoplankton communities affects the cellular concentrations of proteins, fatty acids, and various other phytoplankton constituents important for zooplankton growth (Sterner and Elser 2002; Jensen and Verschoor 2004). The carbon:nutrient ratio of phytoplankton is therefore a major determinant of their quality as food for zooplankton (see also Chapter 1 in this thesis). Zooplankton can become nutrient limited if the nutrient content of their food is low compared to their nutrient demands. Several zooplankton species are capable of compensating for the reduced nutrient content of their food through compensatory feeding (i.e. by increasing their feeding rates). Nevertheless, the costs of compensatory feeding and processing of surplus carbon restricts this strategy to a limited range of carbon:nutrient ratios. Indeed, laboratory experiments have shown that growth rates of herbivorous zooplankton species, such as the cladoceran *Daphnia* and the rotifer *Brachionus*, are reduced when feeding on phytoplankton with high C:P ratios (Sterner and Elser 2002; Urabe *et al*. 2003; Jensen and Verschoor 2004).

As an example, laboratory experiments performed by Urabe *et al.* (2003) showed that elevation of the CO_2 concentration from 360 to 1500 ppm resulted not only in an increased phytoplankton biomass (Fig. 2.4a), but also in an increased C:P ratio (Fig. 2.4b). This reduction in food quality suppressed the growth rate of the cladoceran *Daphnia* at elevated CO_2 (Fig. 2.4c). Similar results were obtained by Urabe and Waki (2009), although the latter study also shows that this adverse effect of rising CO_2 on *Daphnia* growth can be mitigated by mixed algal diets. Other zooplankton species with lower phosphorus requirements, like copepods, may be able to sustain their growth when feeding on phytoplankton with high C:P ratios. Accordingly, if growth rates of high phosphorus-demanding species, such as cladocerans, are reduced, low phosphorus-demanding species, such as copepods, might gain a competitive advantage. This may cause shifts in zooplankton species composition in response to climate-driven changes in phytoplankton stoichiometry.

An elemental imbalance between phytoplankton and zooplankton may also affect the recycling efficiency of different elements (Sterner and Elser 2002). For instance, zooplankton feeding on phytoplankton with a high N:P ratio show an increased release of nitrogen as compared to phosphorus (Elser and Urabe 1999). Similar relationships apply for carbon:nutrient ratios, as zooplankton excrete the excess carbon obtained in their food (Hessen and Anderson 2008). Consequently, a climate-driven increase in phytoplankton carbon:nutrient ratios may enhance carbon release by zooplankton, whereas nutrients are retained. This positive feedback will further increase DOC concentrations and, subsequently, the CO_2 availability for phytoplankton. Since nutrient limitation continues or is enhanced, due to reduced nutrient recycling by herbivores, this leads to even higher carbon:nutrient ratios in the phytoplankton.

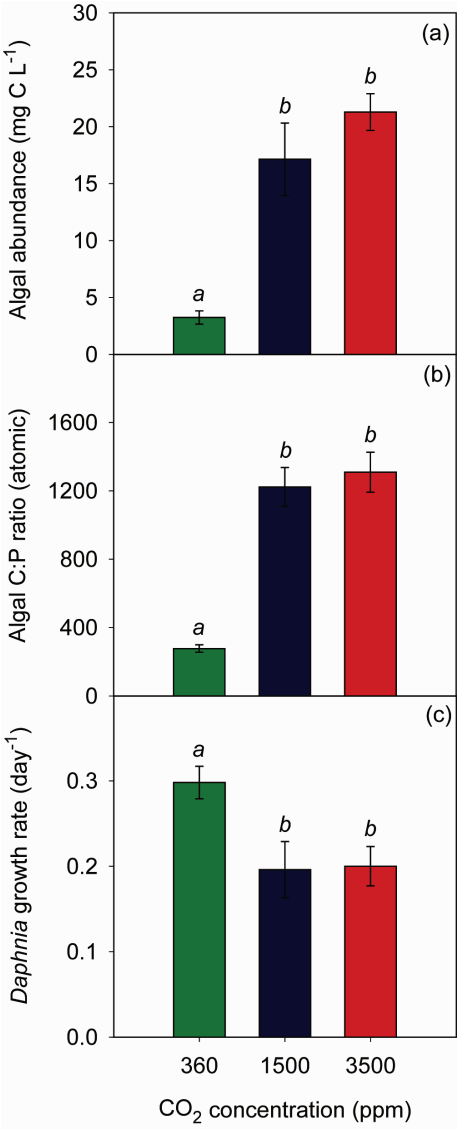

Figure 2.4. Effects of CO_2 enrichment in a laboratory experiment. (a) Biomass of the green alga *Scenedesmus*, (b) the corresponding algal C:P stoichiometry, and (c) specific growth rates of *Daphnia* when fed with these green algae. The treatments consisted of 360, 1500, and 3500 ppm CO_2, represented by green, blue, and red bars, respectively. Error bars indicate the standard error of the mean. Bars with different letters were significantly different ($P < 0.05$, Tukey's pairwise comparison). (From Urabe *et al*. 2003; adapted with permission from Blackwell Publishing Ltd: Global Change Biology).

The potential increase in carbon:nutrient ratios of phytoplankton and the subsequent shift in zooplankton species composition may cascade up through higher trophic levels. During the past decades, shifts in large zooplankton and fish communities have been observed in many seas and oceans. For instance, recent studies indicate that krill in the Southern Ocean is gradually being replaced by salp species (Atkinson *et al.* 2004), and jellyfish numbers seem to be on the rise worldwide (Purcell 2005). Furthermore, fish size distributions are shifting towards smaller fish in various marine ecosystems (Pauly *et al.* 2005). Although these changes are primarily attributed to fisheries, climate change may also be playing a role (Pauly *et al*. 2005; Purcell 2005). Interestingly, salp seems to have a low body phosphorus demand compared to copepods and krill, whereas jellyfish and small fish have a lower body phosphorus demand than larger fish (Ikeda and Mitchell 1982; Malej *et al*. 1993; Sterner and Elser 2002). Furthermore, there are indications that the growth rates of some phosphorus-rich fish species, particularly of herbivorous fish growing on high C:P food, can become phosphorus limited (Hood *et al*. 2005). A climate-driven decrease in the phosphorus content of plankton may therefore exacerbate the impacts that fisheries have on the species composition of higher trophic levels in marine pelagic food webs.

2.7 Caveats and conclusions

Climate change can have many impacts on aquatic ecosystems. It may favor the development of harmful algal blooms (Paerl and Huisman 2008), cause ocean acidification (Caldeira and Wickett 2003), and affect the biogeographical distribution of many species (Hays *et al*. 2005). Current knowledge about the impacts of climate change on plankton stoichiometry is still limited. Although we have argued that climate change is likely to increase the carbon:nutrient stoichiometry of plankton communities, several processes may impede or completely reverse this pattern. For instance, as a result of global warming, air temperatures are increasing to a greater extent than ocean water temperatures, causing more frequent storms (Solomon *et al.* 2007). Storms can prevent or suppress stratification, and the resultant vertical mixing of the water column may enhance the flux of nutrients from deeper waters into surface layers. Furthermore, stoichiometric effects of climate change may be mitigated or even counteracted by eutrophication, through anthropogenic nitrogen and phosphorus loading. These processes provide more nutrients for phytoplankton growth, especially near the more densely human populated regions of our planet, and may thereby lead to local or regional trends in plankton stoichiometry that deviate from our expected pattern.

Moreover, the response of species, communities, and ecosystems to changing environmental conditions can be highly complex, with many non-linear interactions and surprising feedbacks (Scheffer *et al*. 2001; Benincà *et al*. 2008). Many planktonic organisms are also quite flexible in their stoichiometry, and have considerable potential for

physiological acclimation and evolutionary adaptation to changing carbon and nutrient conditions (Sterner and Elser 2002). It may therefore be difficult to predict long-term changes in the ecological stoichiometry of aquatic ecosystems, based on only a few climatic drivers.

Nevertheless, by and large, the current state of knowledge indicates that rising atmospheric CO_2 levels will increase CO_2 availability for biological production, while global warming will tend to strengthen vertical stratification of aquatic ecosystems, thus reducing nutrient availability in the surface mixed layer. It seems plausible that the combination of rising CO_2 availability and reduced nutrient levels will result in higher phytoplankton carbon:nutrient ratios. These alterations in phytoplankton stoichiometry are likely to suppress zooplankton growth, shift zooplankton species composition to less nutrient-demanding species, and thereby affect the entire aquatic food web. If we were to follow in the footsteps of Redfield (1934) and measure plankton stoichiometry at the end of the 21st century, we might discover a different Redfield ratio, one reflecting a carbon-rich world.

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Chapter 3

The ecological stoichiometry of toxins produced by harmful cyanobacteria: an experimental test of the carbon-nutrient balance hypothesis

ABSTRACT - The elemental composition of primary producers reflects the availability of light, carbon, and nutrients in their environment. According to the carbon-nutrient balance hypothesis, this has implications for the production of secondary metabolites. To test this hypothesis, we investigated a family of toxins, known as microcystins, produced by harmful cyanobacteria. The strain *Microcystis aeruginosa* HUB 5-2-4, which produces several microcystin variants of different N:C stoichiometry, was cultured in chemostats supplied with various combinations of nitrate and CO_2. Excess supply of both nitrogen and carbon yielded high cellular N:C ratios accompanied by high cellular contents of total microcystin and the nitrogen-rich variant microcystin-RR. Comparable patterns were found in *Microcystis*-dominated lakes, where the relative microcystin-RR content increased with the seston N:C ratio. In total, our results are largely consistent with the carbon-nutrient balance hypothesis, and warn that a combination of rising CO_2 and nitrogen enrichment will affect the microcystin composition of harmful cyanobacteria.

This chapter is based on the paper: Dedmer B Van de Waal, Jolanda MH Verspagen, Miquel Lürling, Ellen Van Donk, Petra M Visser, and Jef Huisman. 2009. The ecological stoichiometry of toxins produced by harmful cyanobacteria: an experimental test of the carbon-nutrient balance hypothesis. Ecology Letters 12: 1326-1335.

3.1 Introduction

Primary producers link the living with the nonliving world through the conversion of light energy, CO_2 and inorganic nutrients into biomass. The relative availability of these inorganic resources is a major determinant of the elemental stoichiometry of primary producers, and affects their production of organic compounds such as fatty acids, proteins and nucleic acids (Sterner and Elser 2002; Klausmeier *et al*. 2004; Moe *et al*. 2005). The relative availability of carbon and nutrients may also affect the production of secondary metabolites, which often play a role as toxins in anti-herbivore defence. For instance, according to the carbon-nutrient balance hypothesis (Bryant *et al.* 1983; see also Stamp 2003), nutrient limitation will favour carbon-based metabolites such as phenols, whereas nitrogen-rich metabolites such as alkaloids are favoured in fertile ecosystems. Eutrophication and the global rise of atmospheric CO_2 concentrations are currently enriching many ecosystems with vast loads of carbon and nutrients. This could alter the carbon-nutrient balance of primary producers (Van de Waal *et al*. 2010) and their production of secondary metabolites (Bezemer and Jones 1998; Reich *et al*. 2006).

Harmful cyanobacteria are notorious toxin producers, which proliferate in many eutrophic waters (Reynolds 1987; Huisman *et al*. 2005). During weak vertical wind mixing, buoyant cyanobacteria float to the water surface (Walsby *et al*. 1997; Huisman *et al*. 2004; Jöhnk *et al*. 2008). Accumulation of cyanobacterial cells at the water surface leads to the formation of dense surface blooms. High toxin concentrations in dense surface blooms pose a major threat to birds, mammals and human health, and make the water less suitable for drinking water, agricultural irrigation, fishing, and recreational use (Chorus and Bartram 1999; Carmichael 2001; Paerl and Huisman 2008). Dense cyanobacterial blooms can strip surface waters from dissolved inorganic carbon, depleting the carbon availability for photosynthesis to limiting levels (Ibelings and Maberly 1998). However, the current rise in atmospheric CO_2 concentrations (Solomon *et al*. 2007) may counter this process by enriching surface waters with carbon dioxide, and could thereby shift dense surface blooms from carbon limitation to nutrient and/or light limitation.

Several harmful cyanobacteria produce a family of toxins known as microcystins. Microcystins are nonribosomal peptides that may cause serious damage to the liver (Sivonen and Jones 1999; Carmichael 2001; Huisman *et al*. 2005). Nitrogen is an important constituent of microcystins, and nitrogen availability is known to affect the microcystin production of isolated strains (Long *et al*. 2001; Downing *et al*. 2005). Microcystins consist of seven amino acids, including two positions with a variable amino acid composition. At present, at least 89 different microcystin variants have been described (Welker and Von Döhren 2006). The composition of microcystin variants differs among cyanobacterial strains, and also depends on the intracellular availability of different amino acids that can occupy the variable positions (Tonk *et al*. 2008). Common microcystin variants include microcystin-LR, microcystin-RR, and microcystin-YR. These three variants are identical

except at the first variable amino acid position, which is occupied by leucine (L), arginine (R) or tyrosine (Y), respectively (Sivonen and Jones 1999; Hesse and Kohl 2001). The amount of nitrogen in these amino acids differs. Arginine contains four nitrogen atoms while leucine and tyrosine each contain only one nitrogen atom. As a consequence, the molar N:C ratio of microcystin-RR (N:C = 0.27) is higher than that of microcystin-LR and microcystin-YR (N:C = 0.20 and N:C = 0.19, respectively). Hence, could enhanced nitrogen loading and rising CO_2 levels affect the carbon to nutrient stoichiometry of harmful cyanobacteria? And, if so, could this affect their production and composition of microcystins?

In this paper, we test the hypothesis that changes in the relative availability of nitrogen and carbon dioxide will affect the cellular N:C stoichiometry, total microcystin content, and microcystin composition of cyanobacteria. To investigate this hypothesis, we have grown the freshwater cyanobacterium *Microcystis aeruginosa* HUB 5-2-4 in chemostats at different concentrations of dissolved inorganic nitrogen and CO_2. In addition, we also analyzed the microcystin composition and seston stoichiometry of several *Microcystis*-dominated lakes to investigate whether our laboratory findings are consistent with lake observations. Our results show that an increase in nitrogen availability can shift the microcystin composition towards the nitrogen-richest variant microcystin-RR. Moreover, our results demonstrate that the toxin composition of harmful cyanobacteria is especially affected by rising CO_2 concentrations in nitrogen-rich waters, which may present a likely future scenario for many eutrophic lakes.

3.2 Materials and methods

Experimental set-up - The cyanobacterium *Microcystis aeruginosa* HUB 5-2-4 was provided by the Humboldt University of Berlin, Germany. The predominant microcystin variants produced by this strain are microcystin-LR, microcystin-RR, and microcystin-YR (Hesse and Kohl 2001). This *Microcystis* strain was cultured as single cells in laboratory chemostats with flat culture vessels specifically designed for phytoplankton studies (Matthijs *et al.* 1996; Huisman *et al.* 2002). The chemostat cultures were unialgal but not axenic. Regular microscopic inspection confirmed that population densities of heterotrophic bacteria remained low (i.e., well below 1% of the total biomass). The chemostats had an optical path length ('mixing depth') of 5 cm, and a working volume of 1.7 L. They were maintained at a constant temperature of 23 ± 1 ºC using a metal cooling finger connected to a Colora thermocryostat, and at a constant incident irradiance (I_{in}) of 50 ± 1 µmol photons m^{-2} s^{-1} supplied by white fluorescent tubes (Philips PL-L 24W/840/4P; Philips Lighting). The chemostats were aerated with sterilised (0.2 µm Millex-FG Vent Filter, Millipore) and moistened N_2 gas enriched with different CO_2 concentrations to a final gas flow of 25 L hr^{-1} using Brooks Mass Flow Controllers (Brooks Instrument).

Previous studies have found a strong correlation between cellular microcystin contents and the growth rate of *Microcystis* (Orr and Jones 1998; Long *et al*. 2001). To avoid confounding effects of differences in growth rate, we therefore ran all our experiments at the same dilution rate of 0.15 d^{-1}. At steady state, the specific growth rate of *Microcystis* will equal the dilution rate of the chemostat, and hence the specific growth rate will be the same in all experiments irrespective of the imposed experimental treatment. The mineral medium consisted of different concentrations of $NaNO_3$ (Table 3.1), 220 µM K_2HPO_4, 400 µM $MgSO_4$, 180 µM $CaCl_2$, 500 µM $NaHCO_3$, 22 µM $FeCl_2$, 14 µM Na_2EDTA, 44 µM H_3BO_3, 9 µM $MnCl_2$, 0.8 µM $ZnSO_4$, 0.0016 µM $(NH_4)6Mo_7O_{24}$, 0.3 µM $CuSO_4$ and 0.3 µM $Co(NO_3)_2$.

Experimental treatments - Ten chemostats were supplied with different concentrations of $NaNO_3$ in the mineral medium and different concentrations of CO_2 in the gas flow (Table 3.1). Nitrate concentrations in our mineral medium were one or two orders of magnitude higher than the nitrate concentrations typically found in eutrophic lakes. This might suggest that the applicability of our chemostat experiments is limited. Under light-limited conditions, however, phytoplankton population density scales inversely with mixed-layer depth (Huisman 1999; Diehl *et al*. 2002). More specifically, a given light supply per unit area can support a given primary production per unit area. This primary production, which is typically distributed over several meters depth in lakes, is now compressed into our chemostats of only 5 cm depth. This scaling rule results in very high population densities in light-limited laboratory chemostats (Huisman *et al*. 2002). To sustain these high population densities, while avoiding nitrogen limitation, requires mineral media with high nitrogen concentrations. Similarly, the high primary production of laboratory chemostats also requires a sufficient supply of CO_2. This was achieved by a high gas flow rate through our chemostats, such that we could maintain the CO_2 concentrations within the natural range. Dissolved CO_2 concentrations may vary from < 10 ppm in lakes with dense phytoplankton blooms (Maberly 1996) to > 5000 ppm in lakes with high concentrations of dissolved organic carbon (Sobek *et al*. 2005).

We expected that chemostats supplied with low $NaNO_3$ concentrations will become nitrogen limited, chemostats supplied with low CO_2 concentrations will become carbon limited, while chemostats supplied with high inputs of both $NaNO_3$ and CO_2 will become light limited. The limiting resource was assessed post-hoc, by measurements of the residual concentrations of nitrate and dissolved inorganic carbon, the phycocyanin to chlorophyll-a ratio, pH, and light penetration through the chemostat vessels (I_{out}). The pigment phycocyanin is a nitrogen-rich compound that is very sensitive to nitrogen availability (Allen 1984), and hence can be used as a good indicator of nitrogen limitation. Depletion of CO_2 leads to a high pH, which can be used as a good indicator of carbon

Table 3.1. Overview of the experimental treatments and steady-state characteristics. Depending on the concentration of $NaNO_3$ in the mineral medium and CO_2 in the gas flow, the chemostats became either nitrogen-, carbon-, or light-limited. Superscript letters indicate significant differences between the steady-state characteristics of these three different resource limitations (based on one-way ANOVA and post-hoc comparison of the means).

Chemostat	Treatment			Steady-state characteristics						
	$NaNO_3$ (µM)	CO_2 (ppm)	Limitation	Residual nitrate (µM)	PC:Chl-a ratio*	DIC_{av} (µM)†	pH	I_{out} (µmol m^{-2} s^{-1})‡	Population density ($\times 10^9$ cells L^{-1})	Cell diameter (µm)
1	200	400	N	3.4^a	0.13^a	1133^a	7.7^a	20.3^a	10.2^a	3.9^a
2	200	800	N	1.2^a	0.11^a	745^a	7.5^a	17.3^a	11.5^a	4.0^a
3	400	100	N	4.5^a	0.10^a	902^a	9.2^a	17.9^a	12.9^a	3.6^a
4	400	800	N	8.0^a	0.11^a	1952^a	8.4^a	19.3^a	13.1^a	3.5^a
5	400	50	C	75.8^b	0.18^b	688^b	10.0^b	20.4^a	2.6^b	4.5^b
6	12000	50	C	9355^b	0.20^b	212^b	10.7^b	15.9^a	4.5^b	4.4^b
7	12000	100	C	9236^b	0.25^b	318^b	10.7^b	9.9^a	6.9^b	4.5^b
8	12000	400	Light	8852^b	0.31^c	2512^a	8.5^a	4.8^b	13.3^a	4.3^{ab}
9	12000	2800	Light	9567^b	0.30^c	2172^a	7.5^a	5.6^b	16.6^a	4.0^{ab}
10	12000	2800	Light	8191^b	0.36^c	4168^a	8.5^a	3.5^b	21.7^a	3.9^{ab}

** PC:Chl-a ratio = phycocyanin to chlorophyll-a ratio.*

† DIC_{av} = dissolved inorganic carbon available for photosynthesis (sum of dissolved carbon dioxide and bicarbonate).

‡ I_{out} = light penetration through the chemostat vessel

limitation in our experiments. Depletion of the light flux through the chemostat vessels (i.e., a low I_{out}) is indicative of light limitation (Huisman 1999; Huisman *et al.* 2002).

Measurements - At steady state, the chemostats were sampled and several variables were measured every other day for a period of ten days. The incident irradiance (I_{in}) and the irradiance penetrating through the chemostat vessel (I_{out}) were measured with a LI-COR LI-250 quantum photometer (LI-COR Biosciences) at 10 randomly chosen positions on the front and back surface of the chemostat vessel, respectively. The pH was measured with a SCHOTT pH meter (SCHOTT AG). Absorbances of chlorophyll-a (chl-a) and phycocyanin (PC) were measured in culture suspensions at wavelengths of 438 nm and 627 nm, respectively, using an Aminco DW-2000 double-beam spectrophotometer (SLM Instruments Inc.). Biovolumes and cell concentrations were determined in triplicate using a Casy 1 TTC cell counter with a 60 µm capillary (Schärfe System GmbH).

Intracellular C and N content were sampled in triplicate. Samples were pressurized at 10 bar to collapse the gas vesicles of *Microcystis* and subsequently centrifuged for 15 min at 2000 *g*. After discarding the supernatant, the pellet was resuspended in demineralised water, and centrifuged for 5 min at 15 000 *g*. The supernatant was discarded, pellets were stored at 20 ºC and subsequently freeze dried and weighed to determine dry weight. The C and N content of homogenised freeze-dried cell powder was analysed using a Vario EL Elemental Analyzer (Elementar Analysensysteme GmbH).

Residual nitrate concentrations and dissolved inorganic carbon concentrations in the chemostats were determined by sampling 15 mL of culture suspension, which was immediately filtered over 0.45 µm membrane filters (Whatman). Nitrate concentrations were analyzed using a Skalar SA 400 autoanalyzer (Skalar Analytical). The dissolved inorganic carbon concentration (DIC) was analyzed by a Model 700 TOC Analyzer (OI Corporation). Cyanobacteria use carbon dioxide and bicarbonate for carbon fixation, but not the carbonate ion. We therefore define available DIC (DIC_{av}) as the sum of the carbon dioxide and bicarbonate concentration, which was calculated from total DIC and pH (Stumm and Morgan 1996).

Intracellular microcystin contents were determined in triplicate by sampling 5-20 mL of culture suspension, which was immediately filtered using Whatman GF/C filters (pore size ~1.2 µm). Filters were frozen at -20 °C and subsequently freeze-dried. Microcystins were extracted in three rounds with 75% MeOH according to Fastner *et al*. (1998), with an additional step for grinding of the filters using a Mini Beadbeater (BioSpec Products) with 0.5 mm silica beads (Tonk *et al*. 2005). Dried extracts were stored at -20 °C and dissolved in 50% MeOH for microcystin analysis using high performance liquid chromatography (HPLC) with photodiode array detection (Kontron Instruments, Watford, UK). The different microcystin variants were separated using a LiChrospher 100 ODS 5 µm LiChorCART 250-4 cartridge system (Merck) and a 30 to 70% acetonitrile gradient in milli-Q water with 0.05% trifluoroacetic acid at a flow rate of 1 mL min^{-1}. Identification of

the different microcystin variants was based on their characteristic UV-spectra (Lawton *et al*. 1994), and quantified using gravimetrical standards of microcystin-LR, microcystin-RR (both provided by the University of Dundee) and microcystin-YR (Sigma-Aldrich). Since *Microcystis* changed its cell size in response to the experimental treatments (Table 3.1), we expressed intracellular microcystin contents per unit of cellular biovolume. Extracellular microcystin concentrations were considered negligible, as they always comprised less than 3% of the total microcystin concentrations in the chemostat experiments.

Lake survey - In August 2007, 19 water samples were collected from 12 freshwater lakes in The Netherlands in which the phytoplankton community was dominated by *Microcystis* spp. (see Table A1 in Appendix 1). From each lake, 10 L was sampled from ~1 m depth during the late morning. Additionally, we also took samples near the water surface (at ~5 cm depth) in those lakes that showed dense surface blooms. Microcystin concentrations and seston C and N contents were analyzed as described above. *Microcystis* abundances were quantified by inverted light microscopy, after disintegration of the colonies into single cells according to Kardinaal *et al.* (2007a).

Statistical analysis - In total, we obtained time series of 5 consecutive steady-state measurements for each chemostat experiment. These were time-averaged, to obtain a single value of each measured variable in each chemostat. Based on these data, we clustered the ten chemostat experiments into three groups (nitrogen limited, carbon limited, light limited). Significant differences between the three groups were tested using one-way ANOVA, followed by post-hoc comparison of the means using Tukey's HSD (Sokal and Rohlf 1995). Variables were log-transformed if this improved the homogeneity of variances, as evaluated by Levene's test.
The experimental data suggested that the relationships between microcystin contents and cellular N:C ratio were nonlinear. We investigated these relationships by fitting the data to a three-parameter exponential model:

$$y = y_0 + a\mathrm{e}^{bx} \tag{3.1}$$

where x is the cellular N:C ratio, y is the cellular microcystin content, and y_0 can be interpreted as the 'baseline' microcystin content. This is a nonlinear model, because the baseline y_0 prevents transformation of the model to a linear form. Parameter estimation was based on minimization of the residual sum of squares using the iterative procedure of nonlinear regression (SPSS version 16.0). Parameter estimates were validated by checking convergence of the iteration for different initial conditions. We tested the three-parameter model of Eq. 3.1 against a two-parameter exponential model without baseline microcystin

content using Schwarz's (1978) Bayesian Information Criterion (BIC). In all cases, this showed that the three-parameter model gave the "better fit" (i.e., a lower BIC value).

Lake data were analyzed by testing for associations between the cellular microcystin content and N:C ratio of lake seston. Because the cellular microcystin content and relative microcystin-RR content of lake seston were not normally distributed (as evaluated by the Shapiro-Wilk test), the associations were investigated using Spearman's rank correlation test (Sokal and Rohlf 1995).

3.3 Results

Resource limitation - All chemostat experiments reached a steady state within ~40 days. The steady-state characteristics enabled clustering of the chemostat experiments into three groups according to their resource limitation (Table 3.1). At steady state, nitrogen-limited chemostats had significantly lower residual nitrate concentrations (ANOVA on log-transformed nitrate data, $F_{2,7}$ = 25.3, P = 0.001) and phycocyanin:chlorophyll-a ratios (ANOVA, $F_{2,7}$ = 60.1, P < 0.001) than carbon-limited and light-limited chemostats. Carbon-limited chemostats had significantly lower population densities (ANOVA, $F_{2,7}$ = 16.5, P = 0.002), significantly lower concentrations of available DIC (ANOVA on log-transformed DIC_{av} data, $F_{2,7}$ = 15.3, P = 0.003) and significantly higher pH (ANOVA, $F_{2,7}$ = 13.8, P = 0.004) than nitrogen-limited and light-limited chemostats. Light-limited chemostats had high concentrations of residual nitrate and available DIC but significantly lower light penetration through the chemostat vessels (ANOVA, $F_{2,7}$ = 19.6, P = 0.001) than nitrogen-limited and carbon-limited chemostats. Cell diameter also varied between the treatments. Cells in carbon-limited chemostats were significantly larger (ANOVA, $F_{2,7}$ = 12.9, P = 0.005) than cells in nitrogen-limited chemostats, while the cells in light-limited chemostats were of intermediate size (Table 3.1).

N:C stoichiometry - The cellular N:C ratio varied between treatments, and increased with the ratio of dissolved inorganic nitrogen to available DIC in the chemostat vessel (Fig. 3.1a; linear regression: R^2 = 0.82, n = 10, P < 0.001). Differences in cellular N:C ratio were not reflected in the cellular carbon content (Fig. 3.1b; linear regression: R^2 = 0.05, n = 10, P = 0.55). As a consequence, there was a strong relationship between the cellular nitrogen content and the N:C ratio of the cells (Fig. 3.1c; linear regression: R^2 = 0.97, n = 10, P < 0.0001).

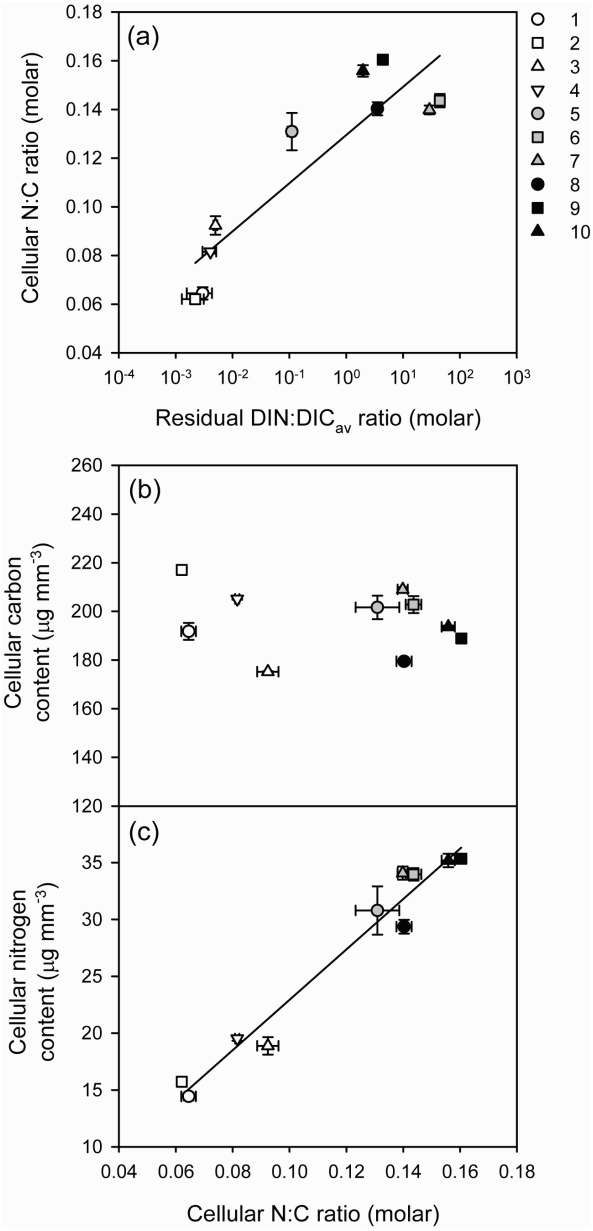

Figure 3.1. (a) Cellular N:C ratio as a function of the ratio of dissolved inorganic nitrogen to available dissolved inorganic carbon (DIN:DIC$_{av}$ ratio) in the chemostat vessel. (b) Cellular carbon content and (c) cellular nitrogen content in relation to the cellular N:C ratio. Each data point represents a steady-state chemostat, grown under nitrogen limitation (white symbols), carbon limitation (grey symbols), or light limitation (black symbols). Error bars indicate the standard error of the mean (n = 5). Numbers correspond to the chemostats enlisted in Table 3.1. Significant regressions are shown by solid lines.

Microcystin composition - Microcystin-LR and microcystin-RR were the predominant microcystin variants in the chemostat experiments, together accounting for more than 80% of the total cellular microcystin in all treatments. The microcystin-LR content did not change over the measured range of cellular N:C ratios (Fig. 3.2a; nonlinear regression: y_0 = 0.31 µg mm^{-3}, R^2 = 0.01). In contrast, both microcystin-RR and microcystin-YR increased with the cellular N:C ratio (Fig. 3.2b,c; nonlinear regression for microcystin-RR: y_0 = 0.060 µg mm^{-3}, R^2 = 0.90; nonlinear regression for microcystin-YR: y_0 = 0.062 µg mm^{-3}, R^2 = 0.66). This pattern is reflected in the total cellular microcystin content, which also increased with the cellular N:C ratio (Fig. 3.2d; nonlinear regression: y_0 = 0.43 µg mm^{-3}, R^2 = 0.62). The relative contribution of microcystin-RR to the total cellular microcystin content increased with the cellular N:C ratio (Fig. 3.3c; nonlinear regression: y_0 = 13.28, R^2 = 0.81).

Lake survey - Microcystin concentrations in the sampled lakes varied over more than three orders of magnitude, and were strongly related to *Microcystis* abundance (Fig. 3.3a; linear regression: R^2 = 0.85, n = 19, P < 0.001). The cellular microcystin content did not correlate with the seston N:C ratio (Fig. 3.3b; Spearman's rank correlation: ρ = -0.126, n = 19, P = 0.303). In many lakes, microcystin-LR was the most abundant microcystin variant, and in some lakes it was even the only microcystin variant that could be detected (Appendix 1). Microcystin-RR and microcystin-YR were found in many lakes as well, ranging from 0 to ~50% of the total microcystin concentration in the lake seston. Other microcystin variants always contributed less than 5% of the total microcystin concentration. The lake samples showed a remarkably similar range in relative microcystin-RR contents and seston N:C ratios as the chemostat experiments (Fig. 3.3c). More specifically, the relative microcystin-RR content in the lakes showed a significant positive correlation with the seston N:C ratio when all lake samples were included in the analysis (Spearman's rank correlation: ρ = 0.557, n = 19, P = 0.007). When lake samples without microcystin-RR were excluded, the positive correlation was on the edge of significance (Spearman's rank correlation: ρ = 0.418, n = 16, P = 0.054). The three lake samples without microcystin-RR all had low seston N:C ratios (Fig. 3.3c).

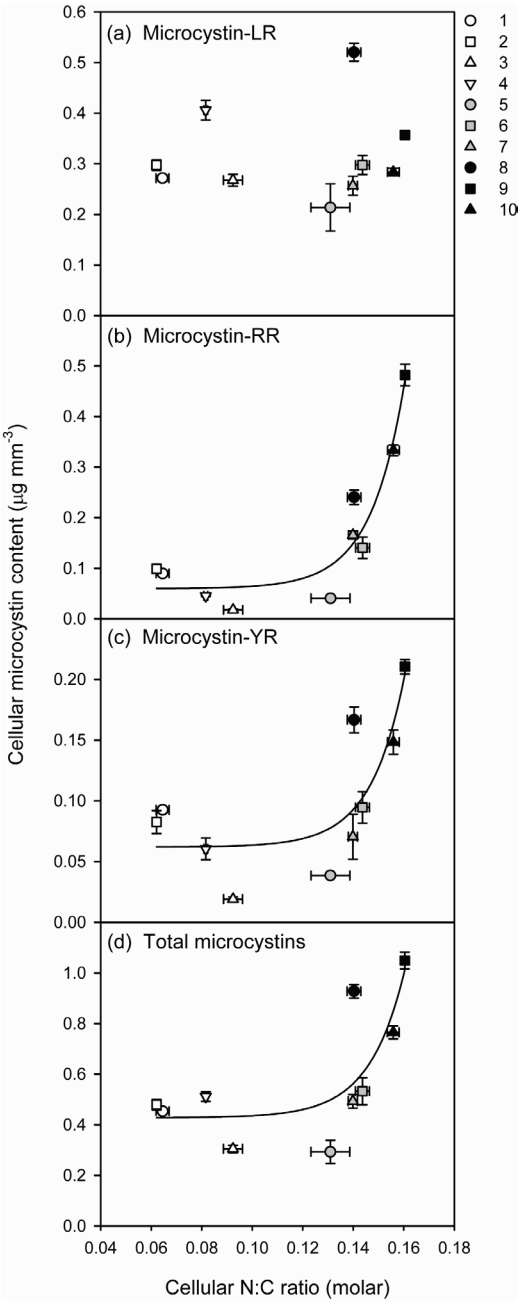

Figure 3.2. Cellular contents of (a) microcystin-LR, (b) microcystin-RR, (c) microcystin-YR, and (d) total microcystins in relation to the cellular N:C ratio. Each data point represents a steady-state chemostat, grown under nitrogen limitation (white symbols), carbon limitation (grey symbols), or light limitation (black symbols). Error bars indicate the standard error of the mean (n = 5). Numbers correspond to the chemostats enlisted in Table 3.1. Solid lines show the fit of the three-parameter exponential model.

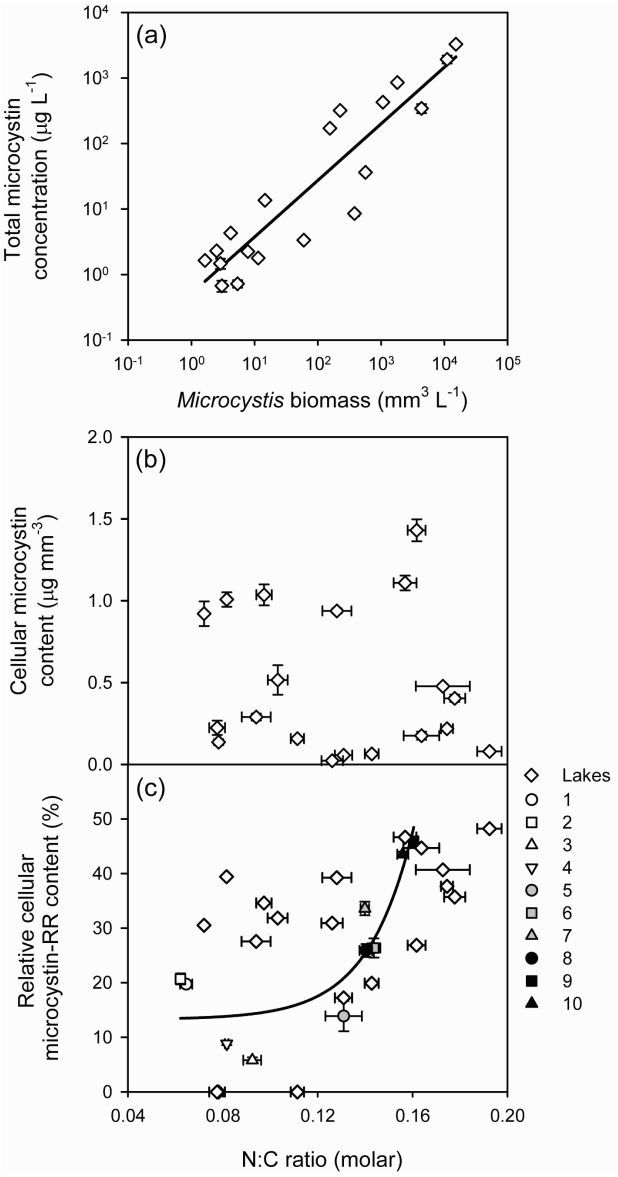

Figure 3.3. (a) Total microcystin concentration in several lakes as a function of *Microcystis* biomass. Each data point represents a different lake sample. The solid line is based on linear regression. (b) The cellular microcystin content of *Microcystis* in relation to the seston N:C ratio measured in the lake samples. (c) Relative contribution of microcystin-RR to the total microcystin content plotted as a function of the N:C ratio. Data points in (c) represent lake samples (open diamonds) as well as steady-state chemostat experiments (other symbols). The solid line shows the fit of the three-parameter exponential model to the chemostat data. The chemostats were grown under nitrogen limitation (white symbols), carbon limitation (grey symbols), or light limitation (black symbols). Numbers correspond to the chemostats enlisted in Table 3.1. Error bars indicate the standard error of the mean (n = 3 for the lake data, n = 5 for the chemostat experiments). The lake data are tabulated in Table A1 of Appendix 1.

3.4 Discussion

Our results show that the environmental availability of inorganic nitrogen and inorganic carbon affects the cellular N:C stoichiometry and microcystin composition of harmful cyanobacteria. The amount of nitrogen invested in microcystins was only a small fraction, less than 1%, of the total amount of nitrogen in the cells. Yet, the total microcystin content varied 4-fold across the experimental treatments, consistent with earlier studies that showed a similar range of variation (Long *et al.* 2001; Wiedner *et al*. 2003; Kardinaal and Visser 2005b). The microcystin composition responded even stronger. In particular, the cellular content of the variant microcystin-RR showed a 50-fold variation across the different chemostat experiments. Apparently, environmental growth conditions have a much larger impact on the production of individual microcystin variants than on the total microcystin production.

To what extent are our results consistent with the carbon-nutrient balance hypothesis (Bryant *et al.* 1983; Stamp 2003) and the more general theory of ecological stoichiometry (Sterner and Elser 2002)? The carbon-nutrient balance hypothesis inspired much research comparing carbon-based versus nitrogen-based secondary metabolites of primary producers, but also sparked a heated debate (Hamilton *et al*. 2001; Koricheva 2002; Stamp 2003). For instance, it has been pointed out that nitrogen-rich alkaloids are produced by a different enzymatic machinery, and may be even more costly in terms of carbon investments than many carbon-based phenols (Hamilton *et al*. 2001). In other words, it was argued that studies comparing the carbon-nutrient stoichiometry of different secondary metabolites compared apples with oranges. In our study, we could circumvent this important criticism on the carbon-nutrient balance hypothesis. Microcystins constitute a family of closely related toxins that are all synthesized nonribosomally by the same enzymatic machinery (Tillett *et al*. 2000; Welker and Von Döhren 2006). This large enzyme complex can incorporate a variety of different amino acids in the microcystin molecule, thus producing microcystin variants of different carbon-nutrient stoichiometry (Tonk *et al*. 2008).

Our results show that the microcystin composition of *Microcystis aeruginosa* is indeed sensitive to the availability of nitrogen and carbon. According to the carbon-nutrient balance hypothesis, one would expect a strong response of the nitrogen-rich variant microcystin-RR to changes in nitrogen and carbon availability. However, one would expect a less clear response, or perhaps no response at all, of microcystin variants that contain less nitrogen. As expected, at low nitrogen availability, cells were characterized by low cellular N:C ratios accompanied by low contents of the nitrogen-rich variant microcystin-RR (Fig. 3.2b). Conversely, excess supply of both nitrogen and carbon resulted in light-limited conditions with high cellular N:C ratios and high contents of microcystin-RR. Microcystin production seems to involve a fixed and a flexible component. The model fits indicate a fixed baseline production of each microcystin variant. On top of this baseline, cells produce

additional microcystin-YR and microcystin-RR at high nitrogen and carbon availability (Fig. 3.2). This combination of a fixed plus flexible component in microcystin production is in good agreement with the carbon-nutrient balance hypothesis, which predicts that the surplus of nitrogen and carbon is allocated to secondary metabolites (Stamp 2003). Interpretation of the microcystin composition in terms of nitrogen and carbon availability also offers a plausible explanation for earlier results of Tonk *et al.* (2005), who studied the microcystin composition of the filamentous cyanobacterium *Planktothrix agardhii* in relation to light availability. High light intensities enhance photosynthesis, which typically lead to low cellular N:C ratios (Sterner and Elser 2002). Indeed, the nitrogen-rich microcystin-RR variant decreased twofold, whereas the less nitrogen-rich microcystin-LR variant increased threefold with increasing light intensity (Tonk *et al*. 2005). All in all, these findings show that the carbon-nutrient balance hypothesis offers a suitable explanation for differences in microcystin composition under nitrogen-limited versus light-limited conditions.

Interestingly, however, we obtained different results under carbon-limited conditions. Carbon limitation yielded high intracellular N:C ratios, but not an increased microcystin-YR and microcystin-RR content (Fig. 3.2). The pronounced difference in microcystin production between carbon-limited and light-limited conditions was not predicted by the carbon-nutrient balance hypothesis. These observations might be explained by the coupling between nitrogen and carbon metabolism of cyanobacteria. For instance, it is known that nitrogen uptake rates of cyanobacteria are partly suppressed under carbon limitation (Tandeau de Marsac *et al*. 2001; Forchhammer 2004). This will restrict the intracellular availability of nitrogen for microcystin production under carbon-limited conditions. In contrast, both inorganic nitrogen and carbon are in excess under light-limited conditions, and their accumulation in the cells may enhance the production of microcystins. It is also known that several amino acids, including the amino acid arginine incorporated in microcystin-RR, can serve as a source of both nitrogen and carbon (Lu 2006; Commichau *et al.* 2006). Under carbon-limited conditions, cells might therefore utilize arginine as a carbon source and restrict the availability of this amino acid for microcystin-RR synthesis. Further research on the regulation of microcystin production will be needed to fully address this intriguing contrast between carbon-limited versus light-limited conditions.

To investigate whether the laboratory results would be consistent with lake observations, we measured concentrations of different microcystin variants in *Microcystis*-dominated lakes. This revealed that differences in *Microcystis* abundance caused substantial variation in the total microcystin concentrations in these lakes, consistent with findings of earlier studies (e.g., Kardinaal *et al*. 2007a). Whereas our chemostat experiments used only a single *Microcystis* strain, lakes often contain a mixture of different *Microcystis* genotypes producing different microcystin variants (Kardinaal *et al*. 2007a; Briand *et al*. 2009). Indeed, as anticipated, the relation between microcystin composition and seston N:C ratio was less clear in the lakes than in the chemostat experiments. Yet, the relative microcystin-

RR contents and N:C ratios in the lake samples spanned a similar range as in the chemostat experiments. Moreover, the lake samples showed a positive correlation between the relative microcystin-RR content and seston N:C ratio (Fig. 3.3c). These patterns indicate that, in essence, the lake data on the microcystin composition are consistent with our laboratory findings.

Microcystins are toxic for many invertebrates, birds, and mammals, including humans (Chorus and Bartram 1999; Carmichael 2001; Huisman *et al*. 2005). However, individual microcystin variants differ in their toxicity (Sivonen and Jones 1999). The toxicity of microcystins for mammals has been estimated by LD_{50} assays on mice. A lower LD_{50} value (the dose lethal for 50% of the mouse population) indicates a higher toxicity. In our experiment, microcystin-LR is the most toxic microcystin variant (LD_{50} = 50 µg kg^{-1}), closely followed by microcystin-YR (LD_{50} = 70 µg kg^{-1}), while microcystin-RR is the least toxic for mice (LD_{50} = 600 µg kg^{-1}; Sivonen and Jones 1999). We found that the cellular content of microcystin-LR was not affected by the treatments, while the cellular contents of microcystin-YR and microcystin-RR were highest under light-limited conditions. However, given that microcystin-RR is much less toxic than the other two microcystin variants, the marked increase in microcystin-RR content under light-limited conditions will probably yield only a small increase in the total toxicity of *Microcystis* cells.

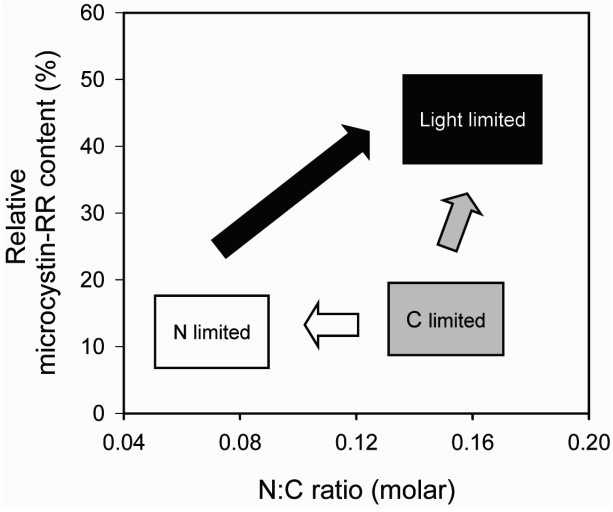

Figure 3.4 Schematic diagram of the relative microcystin-RR content and cellular N:C ratio, under nitrogen-, carbon- and light-limited conditions. Arrows indicate the predicted effects of rising CO_2 levels. The white arrow shows that a shift from carbon to nitrogen limitation will reduce the cellular N:C ratio, but will hardly affect the cellular microcystin composition. The grey arrow shows that a shift from carbon to light limitation will hardly affect the cellular N:C ratio, but will shift the microcystin composition towards microcystin-RR. The black arrow shows that a shift from nitrogen to light limitation, in waters enriched with nitrogen and CO_2, will increase both the cellular N:C ratio and cellular microcystin-RR content.

Several studies report that rising levels of atmospheric CO_2 increase the intracellular carbon to nitrogen ratio of phytoplankton and terrestrial plants (Reich *et al*. 2006; Urabe and Waki 2009; Van de Waal *et al.* 2010). According to our study, the impact of rising CO_2 on the carbon-nitrogen stoichiometry and microcystin composition of *Microcystis* will depend on the limiting resource (Fig. 3.4). Rising atmospheric CO_2 concentrations may alleviate dense surface blooms of *Microcystis* from carbon limitation (Ibelings and Maberly 1998). In nitrogen-poor waters, elevated CO_2 may therefore shift cyanobacterial blooms from carbon limitation towards nitrogen limitation. Our results show that this shift will reduce the N:C ratio of cyanobacterial cells, but has relatively little effect on their microcystin composition (Fig. 3.4). In contrast, in nitrogen-rich waters, elevated CO_2 will induce light-limited conditions. This will shift the microcystin composition of cells to higher concentrations of the nitrogen-rich variant microcystin-RR (Fig. 3.4). Thus, our findings warn that rising levels of atmospheric CO_2 in eutrophic waters will not only tend to favor cyanobacterial growth, but will also affect the ecological stoichiometry of the toxins they produce.

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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 $\mu = 0.25$ day^{-1} (SD = 0.06; $n = 3$) and $\mu = 0.37$ 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.

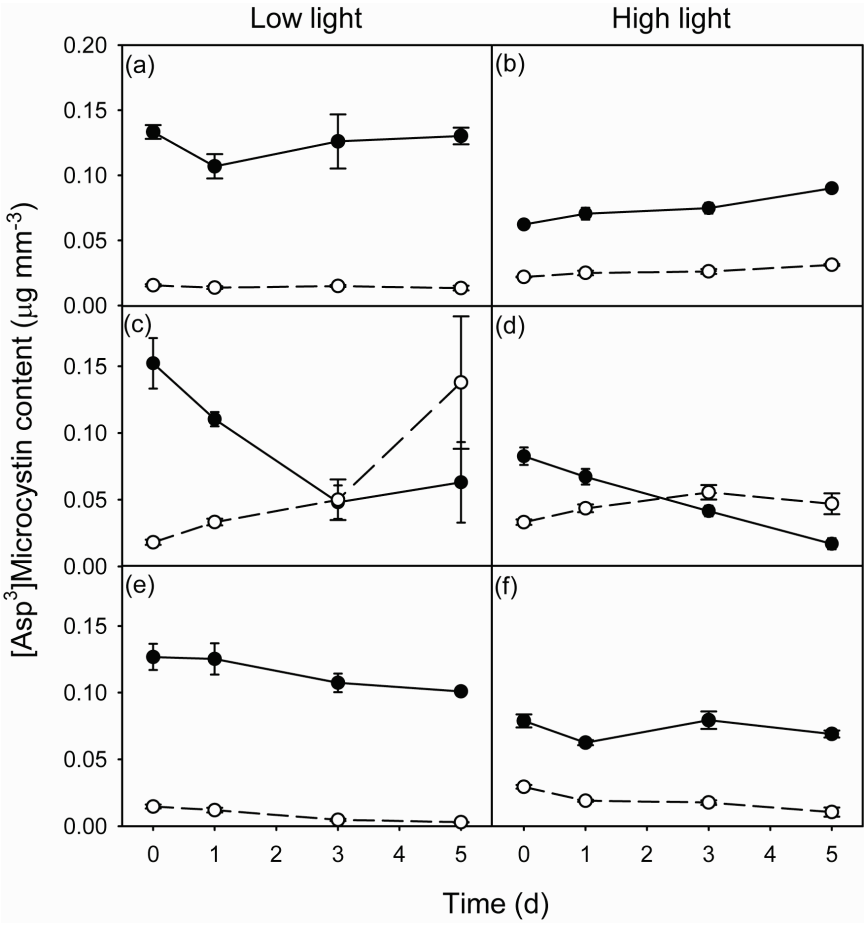

Figure 4.1. [Asp^3]microcystin-RR (closed circles) and [Asp^3]microcystin-LR (open circles) contents of *Planktothrix agardhii* grown in semi-continuous turbidostats: (a,b) with nutrient-saturated medium, (c,d) after addition of leucine, and (e,f) after addition of arginine. Panels on the left show results 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).

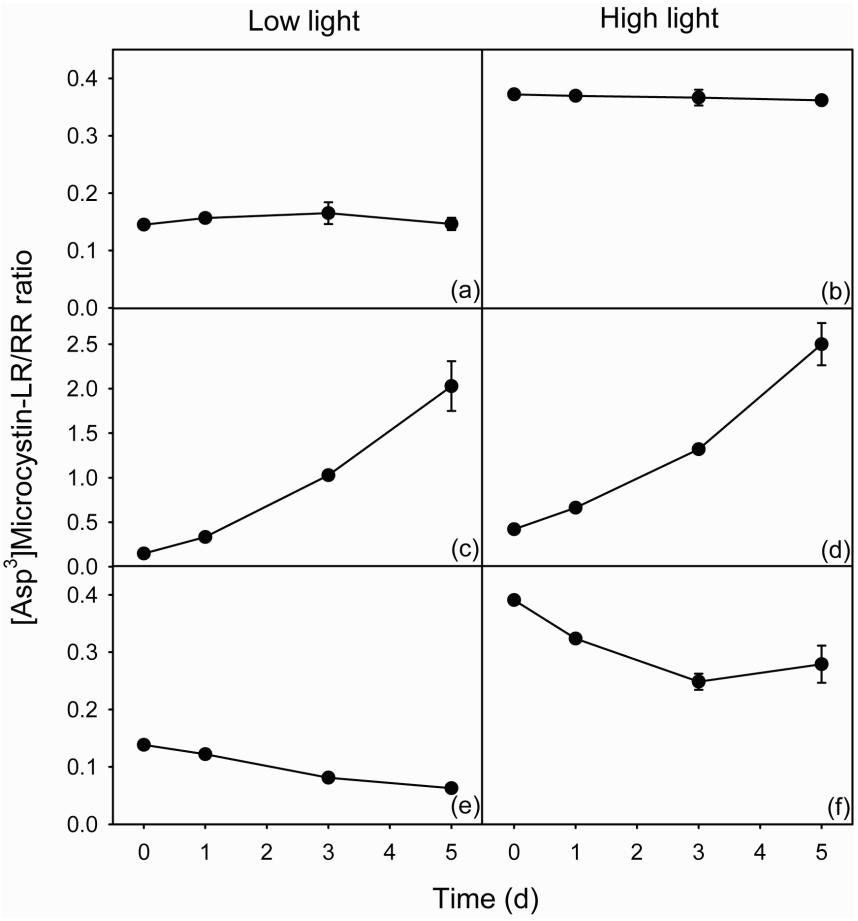

Figure 4.2. [Asp^3]microcystin-LR/RR ratios of *Planktothrix agardhii* grown in semi-continuous turbidostats: (a,b) with nutrient-saturated medium, (c,d) after addition of leucine and (e,f) after addition of arginine. Panels on the left show results at low light intensity (I_{avg} = 21 µmol m^{-2} s^{-1}),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

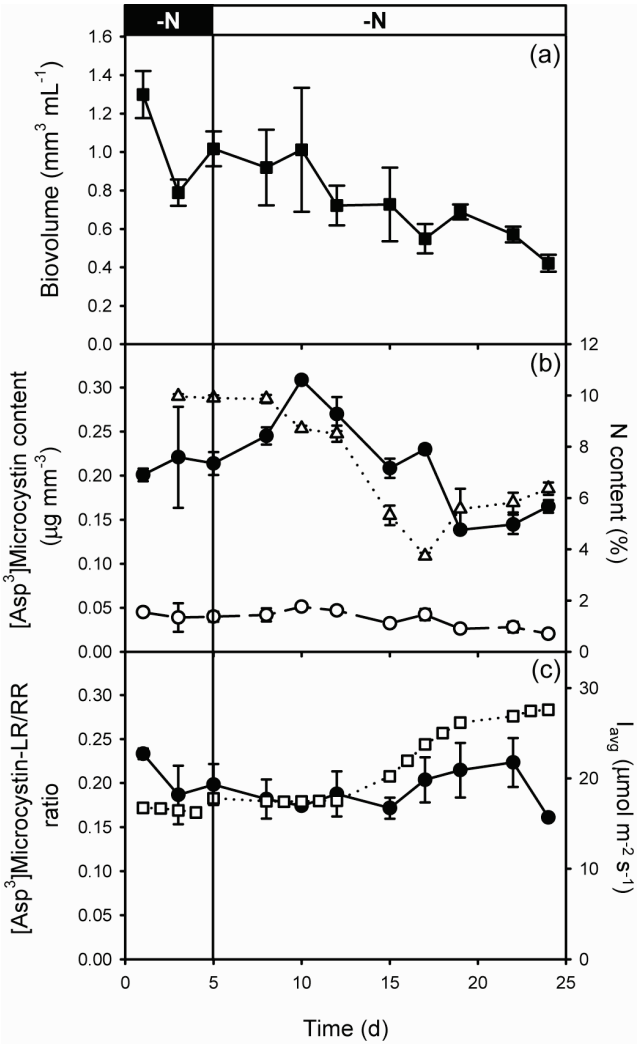

Figure 4.3. Time course of changes in selected properties of *Planktothrix agardhii* grown in continuous culture. (a) Biovolume (closed squares). (b) [Asp^3]microcystin-RR content (closed circles), [Asp^3]microcystin-LR content (open circles) and nitrogen content (open triangles), where the nitrogen 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.

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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 paper: 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. *FEMS Microbiology Ecology, doi:10.1111/j.1574-6941.2010.00958.x.*

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.*

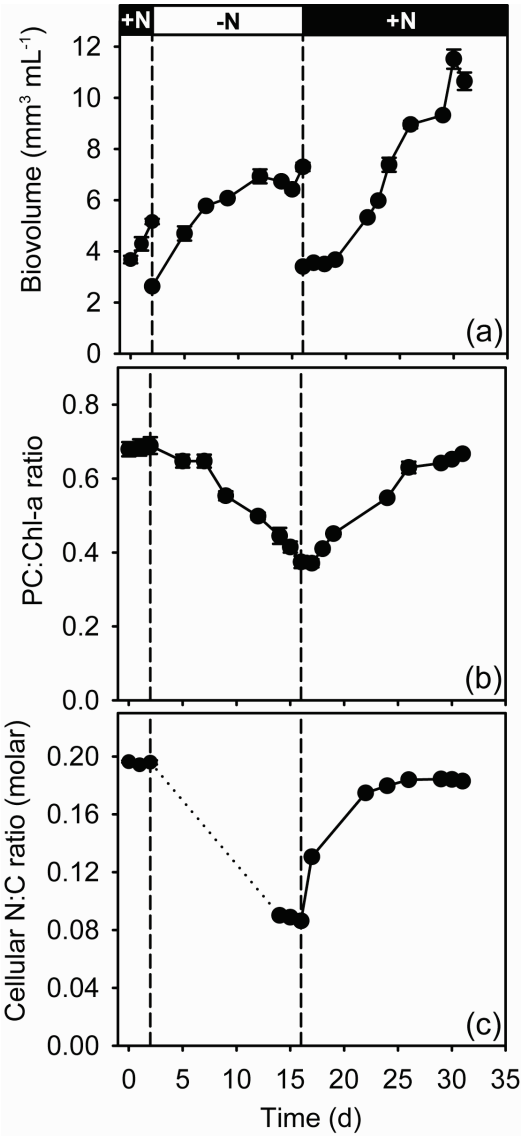

Figure 5.1. Changes in (a) biovolume, (b) phycocyanin to chlorophyll-a (PC:Chl-a) ratio and (c) cellular nitrogen to carbon (N:C) ratio during the experiment. Top bar indicates nitrogen-rich (black bar, +N) or nitrogen-deficient (white bar, -N) conditions. Error bars indicate the standard error of the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

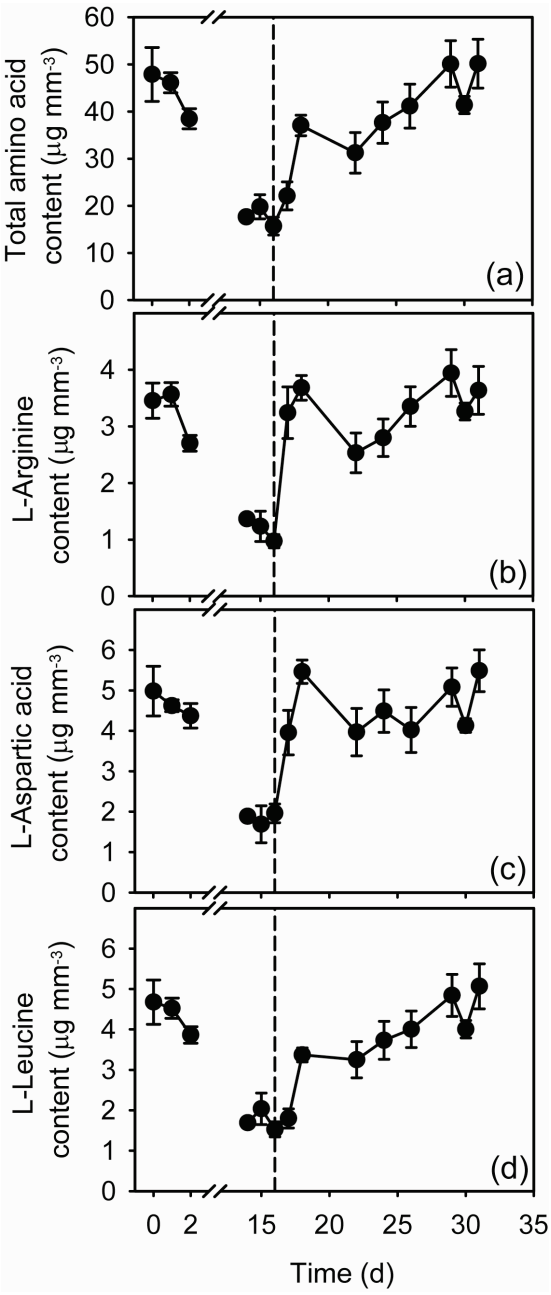

Figure 5.2. Changes in (a) total cellular amino acid content, (b) cellular L-arginine content, (c) cellular L-aspartic acid content and (d) cellular L-leucine content during the experiment. Error bars indicate the standard error of the mean (n = 3). The timing of the nitrate pulse is indicated by the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

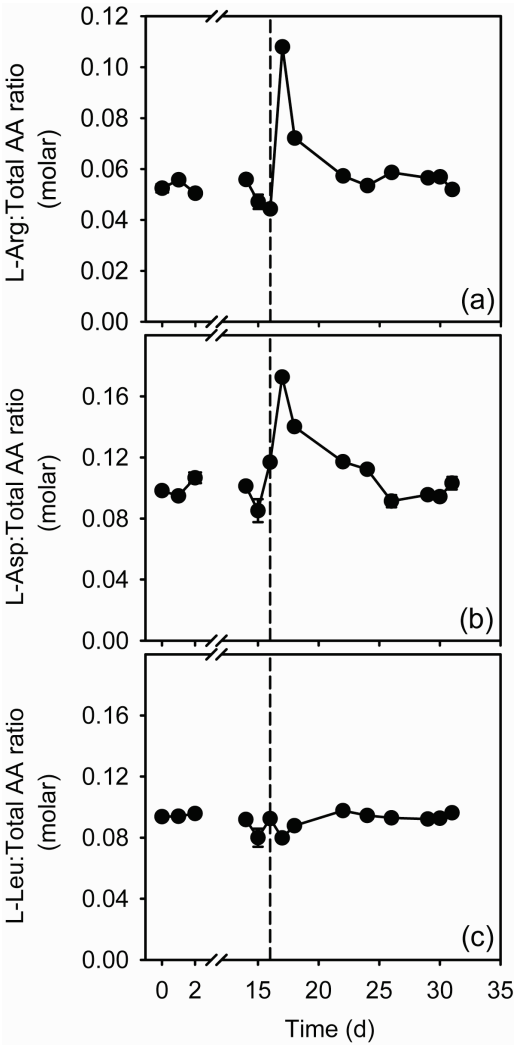

Figure 5.3. Changes in (a) the relative contribution of L-arginine to the total amino acid pool, expressed as the L-Arg:Total AA ratio, and (b) the relative contribution of L-aspartic acid to the total amino acid pool, expressed as the L-Asp:Total AA ratio. Error bars indicate the standard error of the 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).

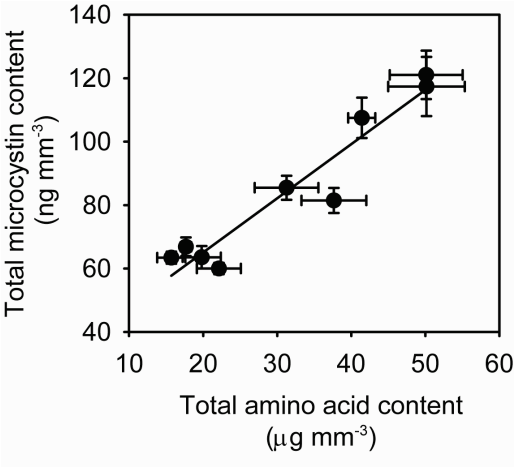

Figure 5.4. The total cellular microcystin content in relation to the total cellular amino acid content. Error bars indicate the standard error of the mean (n = 3). The solid line shows the linear correlation.

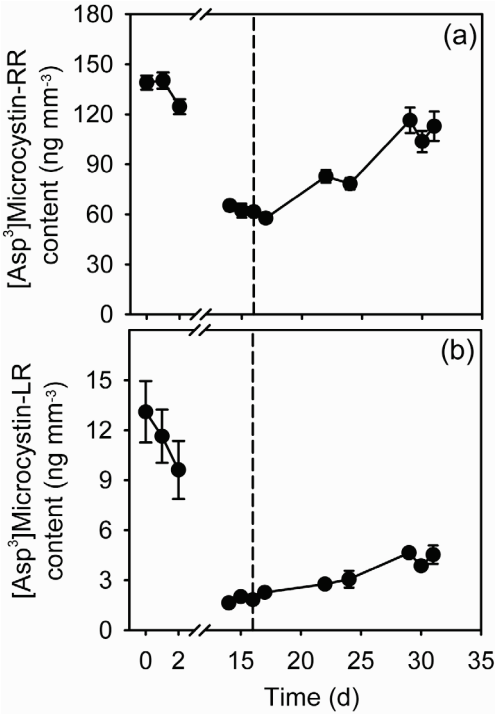

Figure 5.5. Changes in (a) cellular [Asp^3]microcystin-RR content, and (b) cellular [Asp^3]microcystin-LR content during the experiment. Error bars indicate the standard error of the mean (n = 3). The timing of the nitrate pulse is indicated by the vertical dashed line.

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.

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Chapter 6

Competition for CO_2 between phytoplankton species: theory and experiments

ABSTRACT - Global atmospheric CO_2 levels are rising and will affect many processes in aquatic ecosystems. Resource competition theory has extensively addressed competition for nutrients and light. Yet, competition for inorganic carbon has not been resolved. We developed a model to investigate phytoplankton competition for inorganic carbon. The model describes dynamic changes in carbon dioxide, carbon speciation, light intensity, alkalinity and pH. We tested the model predictions using monoculture and competition experiments in chemostats with a toxic and nontoxic strain of the cyanobacterium *Microcystis aeruginosa*. In monoculture experiments with low CO_2 supply, the increasing cyanobacterial biomass depleted the concentration of dissolved CO_2, leading to a high pH. The toxic strain reduced the CO_2 concentration to lower values and raised pH to higher values than the nontoxic strain. Conversely, the nontoxic strain performed better at low light intensities. As predicted, the toxic strain became dominant in a competition experiment with low CO_2 supply, whereas the nontoxic strain became dominant in a competition experiment with higher CO_2 supply but low light availability. The model captured the observed reversal in competitive dominance, and was also quantitatively in good agreement with the population dynamics during the competition experiments. Our results demonstrate theoretically and experimentally that rising CO_2 levels can alter the community composition and toxicity of harmful algal blooms.

This chapter is based on the manuscript: Dedmer B Van de Waal, Jolanda MH Verspagen, Jan F Finke, Vasiliki Vournazou, W Edwin A Kardinaal, Linda Tonk, Sven Becker, Ellen Van Donk, Petra M Visser, and Jef Huisman. Competition for CO_2 between phytoplankton species: theory and experiments. Embargo until publication.

Chapter 7

Afterthoughts

7.1 Introduction

The principle aim of this thesis is to determine how climate-driven changes in resource availability affect the ecological stoichiometry and toxin production of harmful cyanobacteria. To this aim, we first reviewed the existing literature to explore potential effects of climate change on carbon and nutrient availability in aquatic ecosystems (Chapters 1-2). Thereafter, we investigated the impacts of CO_2 and nitrogen availability on the carbon:nitrogen stoichiometry and toxin composition of harmful cyanobacteria (Chapter 3). This was extended with a more detailed study, where we investigated how changes in nitrogen availability affect the intracellular amino acid composition, which in turn is a major determinant of the microcystin composition of harmful cyanobacteria (Chapters 4-5). Finally, we developed new theory to describe how changes in CO_2 availability affect the competition between different phytoplankton species, and tested this new theory in competition experiments with a toxic and non-toxic cyanobacterial strain (Chapter 6).

In this chapter, I will discuss effects of rising CO_2 levels and eutrophication on inorganic carbon availability and pH in aquatic ecosystems, and their implications for competitive interactions between phytoplankton species. Thereafter, I will describe physiological mechanisms linking carbon and nitrogen assimilation to the synthesis of amino acids and microcystins. Then, I will discuss the potential implications of changes in the carbon:nitrogen stoichiometry of cyanobacteria for zooplankton species feeding on these cyanobacteria. I will conclude the chapter with a brief summary of the key findings presented in this thesis.

7.2 Changes in carbon availability and pH

Enhanced dissolution of CO_2 in water, as a result of rising atmospheric CO_2 levels, causes a drop in pH (Fig. 7.1). This process is typically referred to as ocean acidification (Caldeira and Wickett 2003; Doney *et al.* 2009; Van de Waal *et al*. 2010; see also Chapter 1 in this thesis), although this general phenomenon is certainly not restricted to oceans only. Higher concentrations of dissolved CO_2 may promote photosynthesis in some phytoplankton species (Schippers *et al*. 2004; Rost *et al*. 2008), while lower pH can have negative consequences for calcifying phytoplankton species, such as coccolithophores, although the pH sensitivity varies greatly between species (Riebesell *et al*. 2000; Langer *et al*. 2006; Zondervan 2007). Thus, with rising CO_2 levels, phytoplankton may be promoted by higher CO_2 availability, but can be negatively affected by lower pH.

Anthropogenic eutrophication may have the opposite effect. Eutrophication may cause excessive growth of phytoplankton in freshwater lakes and coastal waters (Carpenter *et al*. 1998; Cloern 2001; Glibert *et al*. 2005). These phytoplankton blooms typically have a high inorganic carbon demand, which may ultimately lead to CO_2 depletion (Maberly 1996; Hein 1997; Ibelings and Maberly 1998). Uptake of large quantities of CO_2 by phytoplankton photosynthesis leads to elevation of pH in both freshwater lakes (Talling 1976; Maberly 1996; Hein 1997) and coastal waters (Macedo *et al*. 2001; Hansen 2002; Engel *et al*. 2005). A high pH may have direct implications for phytoplankton growth (Elzenga *et al*. 2000; Hansen 2002; Hansen *et al*. 2007), and a pH > 10 sometimes observed in dense phytoplankton blooms approaches or even exceeds the pH tolerance of many phytoplankton species (Goldman *et al*. 1982; Hansen 2002). Changes in pH also shift the speciation of dissolved inorganic carbon (DIC), from CO_2 at low pH (pH < 6), to bicarbonate at intermediate pH (7.5 < pH < 9.5), and carbonate at high pH (pH > 10.5). Thus, although CO_2 becomes limiting during a phytoplankton bloom, bicarbonate may still be available in excess (Fig. 7.1). Many phytoplankton species can utilize bicarbonate to cover at least part of their carbon requirements (Kaplan and Reinhold 1999; Rost *et al*. 2003; Martin and Tortell 2008). Hence, in dense phytoplankton blooms, some species may become CO_2 limited and the rising pH may exceed their tolerance limit. However, other phytoplankton species may flourish at a high pH while covering their carbon demands by a high bicarbonate affinity.

This brief overview illustrates that ocean acidification and dense phytoplankton blooms resulting from anthropogenic eutrophication have opposite effects on the pH and carbon speciation in aquatic ecosystems (Fig. 7.1).

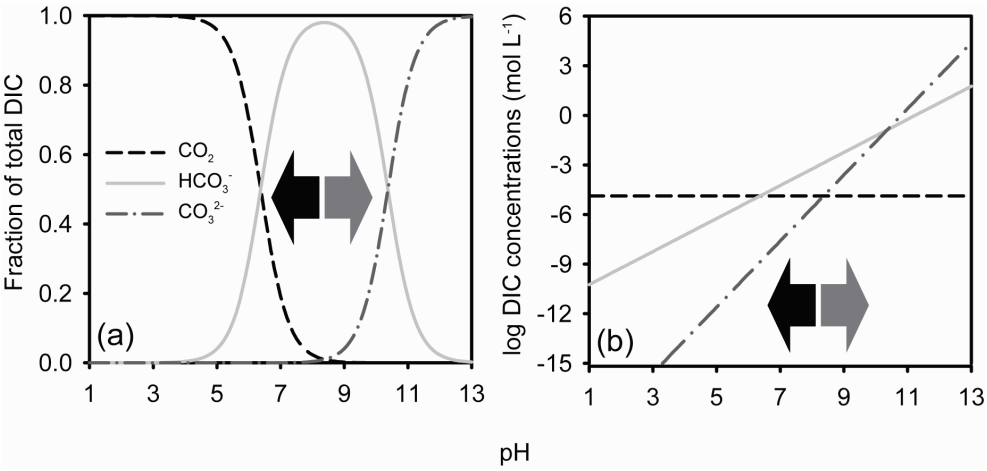

Figure 7.1. Inorganic carbon in aquatic ecosystems. (a) Relative composition of dissolved inorganic carbon (DIC) as a function of pH. (b) Absolute concentrations of dissolved inorganic carbon species as a function of pH assuming equilibrium with the atmosphere containing 380 ppm CO_2. The black arrows illustrate the effect of ocean acidification on carbon speciation (i.e., enhanced CO_2 availability and lower pH). The grey arrows illustrate the effect of dense phytoplankton blooms on carbon speciation (i.e., enhanced bicarbonate availability and higher pH).

7.3 Competition for inorganic carbon

Phytoplankton species differ in their affinities for CO_2 and bicarbonate (Rost *et al*. 2003; Hansen *et al*. 2007; Trimborn *et al*. 2008). As indicated above, phytoplankton species also vary in their tolerance to pH, which can become either too low (Riebesell *et al*. 2000; Langer *et al*. 2006; Zondervan 2007), or too high (Elzenga *et al*. 2000; Hansen 2002; Hansen *et al*. 2007). Therefore, changes in carbon availability and pH are likely to affect the phytoplankton community structure. However, only a few studies have investigated how changes in carbon availability affect phytoplankton communities (Pedersen and Hansen 2003; Engel *et al*. 2005; Feng *et al*. 2009). It seems conceivable that phytoplankton species which can tolerate a reduction in pH and have a high affinity for CO_2 will be promoted in the acidifying waters associated with rising CO_2 levels. In contrast, phytoplankton species that can tolerate high pH and have a high affinity for bicarbonate will likely be promoted in dense phytoplankton blooms of eutrophic waters (Fig. 7.1).

In chapter 6, we describe a new model to investigate competition for inorganic carbon among phytoplankton species. We tested the model predictions using monoculture and competition experiments in chemostats with a toxic and nontoxic strain of the cyanobacterium *Microcystis aeruginosa*. The monoculture experiments showed that the toxic strain depleted the dissolved CO_2 concentration to lower levels than the nontoxic

strain. Conversely, the nontoxic strain performed better at low light intensities (Kardinaal *et al*. 2007b). As predicted by the model, the toxic strain became dominant in a competition experiment with low CO_2 supply, whereas the nontoxic strain became dominant in a competition experiment with higher CO_2 supply but low light availability. These results provide the first experimental demonstration that changes in the relative availability of inorganic carbon and light can lead to a reversal in the outcome of phytoplankton competition.

Depletion of CO_2 in the carbon-limited chemostat experiments was accompanied by an increase in pH (Chapter 6). The pH reached higher values in monocultures of the toxic strain than of the nontoxic strain. The toxic strain also won the competition. Therefore, although we explained our competition experiments in terms of depletion of inorganic carbon, the outcome of our competition experiments might also be explained by differences in pH tolerance between the two strains. Thus, instead of comparing the R* values for CO_2 to predict the outcome of competition according to traditional resource competition theory (Tilman 1982), our results could also be predicted based on species-specific pH* values. That is, the species with highest pH* value may win the competition. *Microcystis* strains can tolerate very high pH values, however. Some strains even tolerate pH > 11.5 (Bañares-España *et al*. 2006). This matches preliminary experiments in batch culture, which indicated that variation in pH had little effect on the specific growth rates of our two *Microcystis* strains over the entire pH range covered by our experiments (Verspagen JMH, unpublished results). Thus, for our competition experiment, an explanation of the outcome of competition in terms of pH tolerance seems unlikely.

Although we did not find an effect of pH on competition in our experiments, dense cyanobacterial blooms may raise the pH to such an extent that it does exceed the pH tolerance of other phytoplankton species such as green algae and diatoms (Shapiro 1990; Caraco and Miller 1998; Hansen 2002). Indeed, some studies indicate that the phytoplankton species with highest pH tolerance (i.e., highest pH*) wins the competition (Goldman *et al*. 1982; Hansen 2002). However, because pH and the availability of CO_2 and bicarbonate are strongly correlated, it remains difficult to determine to what extent phytoplankton is competing for inorganic carbon, and to what extent the competition is driven by differences in pH tolerance of the competing species. More detailed studies on pH tolerance and controlled competition experiments for inorganic carbon should be done to further elucidate these intriguing interactions between inorganic carbon availability, pH and phytoplankton competition.

As already indicated, many phytoplankton species can utilize both CO_2 and bicarbonate as carbon source (Kaplan and Reinhold 1999; Rost *et al*. 2003; Martin and Tortell 2008). Therefore, competition for inorganic carbon involves two resources: CO_2 and bicarbonate. Tilman (1982) developed a graphical approach using zero isoclines to assess the competitive abilities of species competing for two resources.

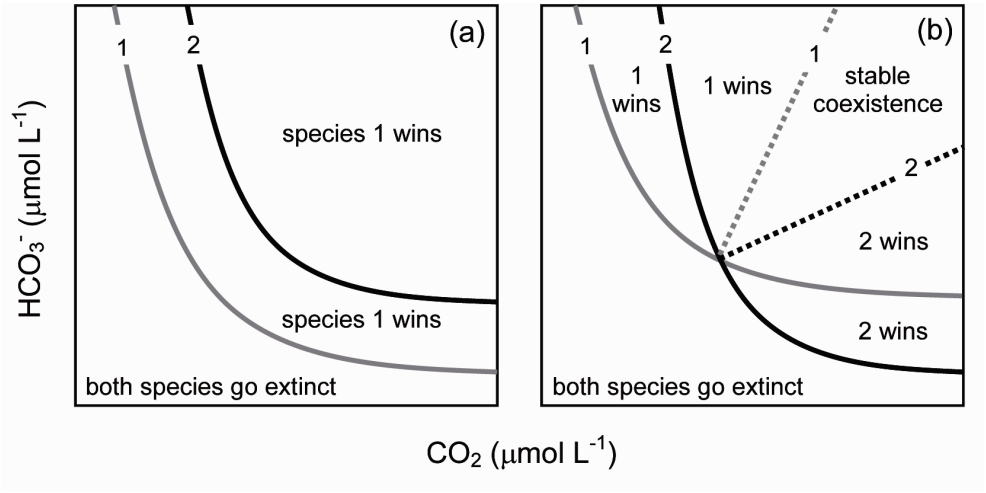

Figure 7.2. Graphical representation of the outcome of competition for bicarbonate and CO_2 between two phytoplankton species. The solid lines represent the zero isoclines of the species. The dotted lines represent the slopes of the consumption vectors (i.e. the rate at which bicarbonate is taken up relative to the uptake of CO_2) of the species. In each region of the graphs, the outcome of competition is indicated for the combination of bicarbonate and CO_2 supply falling into that region. Graph (a) illustrates the scenario where species 1 is the better competitor for both bicarbonate and CO_2. There is no trade-off between competitive abilities for bicarbonate and CO_2. Here, competition will lead to competitive exclusion of species 2. Species 1 will win. Graph (b) illustrates the scenario where species 1 is the better competitor for CO_2, whereas species 2 is the better competitor for bicarbonate. Hence, there is a trade-off between the competitive ability for bicarbonate and CO_2. If the better CO_2 competitor (species 1) consumes relatively more bicarbonate, and the better bicarbonate competitor (species 2) consumes relatively more CO_2, then there will be an intermediate region of stable coexistence as indicated in the graph. Modified after Tilman (1982) and Passarge *et al*. (2006).

The zero isoclines are plotted in a resource plane, with CO_2 concentrations on the x-axis and bicarbonate concentrations on the y-axis (Fig. 7.2). If there is no trade-off between competitive ability for CO_2 and bicarbonate, the zero isoclines of the competing species do not intersect (Fig. 7.2a). That is, the species that can deplete CO_2 to the lowest value can also deplete bicarbonate to the lowest value, and is therefore predicted to win the competition for inorganic carbon. For instance, in our chemostat experiments, the toxic strain could deplete both CO_2 and bicarbonate to lower levels than its nontoxic competitor, and indeed won the competition for inorganic carbon (Chapter 6). It is conceivable, however, that other phytoplankton species may exhibit a trade-off between competitive abilities for CO_2 and bicarbonate. In this case, the zero isoclines intersect (Fig. 7.2b). With increasing CO_2, the species interactions will gradually shift from competition for CO_2 to competition for bicarbonate. Thus, the species composition will shift from dominance of superior CO_2 competitors at low CO_2 and high bicarbonate conditions towards dominance of superior bicarbonate competitors at high CO_2 and low bicarbonate conditions (Fig. 7.2b). There might even be an intermediate region with stable coexistence of the two species, depending on the configuration of the consumption vectors. These results illustrate that, if

there is a trade-off as described in Fig. 7.2b, a change in CO_2 and bicarbonate availability in aquatic ecosystems will alter the phytoplankton species composition.

7.4 The C and N of cyanobacterial nuisance

According to the carbon-nutrient balance hypothesis (CNBH), the relative availability of light, carbon and nutrients in the environment has implications for the production of secondary metabolites (Bryant *et al*. 1983; Stamp 2003). More specifically, a higher nitrogen:carbon ratio will promote nitrogen-based secondary metabolites, whereas a lower nitrogen:carbon ratio will promote carbon-based secondary metabolites. The CNBH can be regarded as a specific precursor of the more general theory of ecological stoichiometry (Sterner and Elser 2002). This theory postulates that the elemental composition of primary producers reflects the resource availability in their environment. In other words, a higher nutrient availability will increase the phytoplankton nutrient:carbon stoichiometry, whereas a higher CO_2 and light availability will decrease the phytoplankton nutrient:carbon stoichiometry (Sterner and Elser 2002).

To test whether the CNBH is applicable to secondary metabolite production in harmful cyanobacteria, Chapter 3 investigated the impact of the cellular nitrogen:carbon (N:C) ratio of a harmful cyanobacterium on its microcystin composition (Van de Waal *et al*. 2009). The strain *Microcystis aeruginosa* HUB 5-2-4, which produces several microcystin variants of different N:C stoichiometry, was cultured in chemostats supplied with various combinations of nitrate and CO_2. A low nitrate supply resulted in low cellular N:C ratios and a low content of the nitrogen-rich variant microcystin-RR. Excess supply of both nitrate and CO_2 yielded high cellular N:C ratios accompanied by high cellular contents of total microcystin, and in particular that of the nitrogen-rich variant microcystin-RR. Comparable patterns were found in *Microcystis*-dominated lakes, where the relative microcystin-RR content increased with the seston N:C ratio. These results illustrate that the CNBH indeed provides a useful theoretical framework to understand and predict the microcystin composition of harmful cyanobacteria (Van de Waal *et al*. 2009).

Therefore, we studied the carbon:nutrient stoichiometry of microcystins in further detail. Microcystins consist of seven amino acids. Two of these amino acid positions are variable, whereas the other five positions are more conserved (Welker and Von Döhren 2006; see also Chapter 1 in this thesis). 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). In Chapter 4, we showed that addition of leucine to the growth medium resulted in a strong increase of the microcystin-LR/RR ratio in *Planktothrix agardhi*, while addition of arginine resulted in a decrease of this ratio (Tonk *et al*. 2008). Arginine contains four bound nitrogen atoms, whereas leucine contains only one nitrogen atom. We therefore hypothesized that

the increase of the microcystin-RR content in response to a high nitrogen supply, as reported in Chapter 3, was mediated by an increase in the intracellular availability of arginine.

In Chapter 5, we demonstrated that the amino acid content of *Planktothrix agardhii* increased with nitrogen availability. Furthermore, the amino acid composition showed a transient increase towards arginine and aspartic acid, which is indicative for the nitrogen storage compound cyanophycin. Interestingly, the cellular microcystin content followed the increase in cellular amino acid content. Moreover, in line with our hypothesis, the microcystin composition shifted towards relatively more of the arginine-based microcystin-RR in response to the transient increase of cellular arginine. Thus, environmental factors influencing the cellular amino acid composition have consequences for the microcystin composition as well.

To clarify this physiological mechanism, I propose here a scheme of nitrogen and carbon assimilation in microcystin-producing cyanobacteria based on known physiological pathways and the results of our experiments (Fig. 7.3). Inorganic carbon is taken up as CO_2 and bicarbonate, and assimilated via the Calvin cycle to form low-molecular sugars such as glucose. During a process known as glycolysis, glucose is subsequently converted to pyruvate, which can be used for amino acid synthesis. Inorganic nitrogen is taken up as nitrate, nitrite or ammonium through permeases, or as N_2 via nitrogen fixation, and reduced to ammonium. Cellular ammonium is subsequently incorporated into carbon skeletons through the glutamine synthetase-glutamate synthase pathway. Nitrogen is then distributed from glutamine or glutamate to other amino acids like the nitrogen-rich arginine (Flores and Herrero 2005). For the incorporation of nitrogen into glutamate, 2-oxoglutarate is used, which is derived from pyruvate obtained by glycolysis (Vázquez-Bermúdez *et al*. 2000). To produce the polypeptide cyanophycin, cells incorporate arginine into a backbone of aspartic acid, which is also derived from pyruvate (Fig. 7.3).

Accordingly, synthesis of amino acids tightly involves cellular nitrogen and carbon metabolism. More specifically, under conditions of nitrogen excess, there is a high demand for 2-oxoglutarate to serve as carbon skeleton for the glutamine synthetase-glutamate synthase pathway. This drain on 2-oxoglutarate may deplete the pyruvate availability for leucine synthesis (Fig. 7.3). Hence, nitrogen excess may result in enhanced arginine, but reduced leucine contents. Conversely, when nitrogen is depleted, but sufficient carbon and light is available, cellular leucine levels are likely to increase relative to arginine. Hence, changes in resource conditions that alter nitrogen or carbon assimilation will likely affect amino acid synthesis, which may have implications for the production of different microcystin variants.

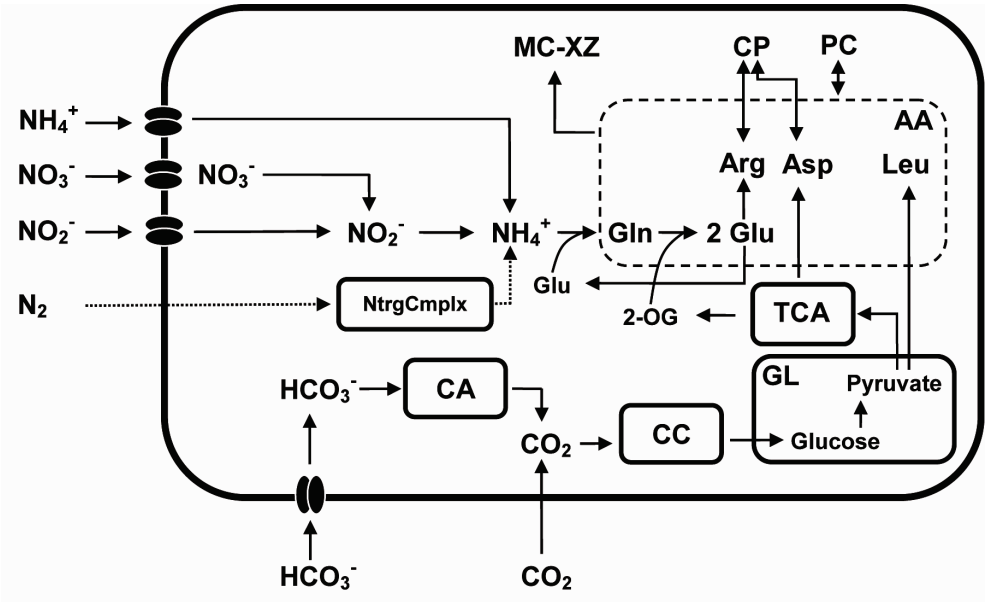

Figure 7.3. Schematic diagram of inorganic carbon assimilation and inorganic nitrogen assimilation in microcystin-producing cyanobacteria. CO_2 diffuses through the cell wall, or is actively taken up as bicarbonate through C_i transporters. CO_2 is further assimilated via the Calvin-cycle which provides carbon skeletons for amino acid synthesis. Nitrogen is taken up as nitrate, nitrite or ammonium by permeases and subsequently reduced to ammonium. Some microcystin-producing cyanobacteria (e.g., *Anabaena* spp.) have the ability to fix atmospheric N_2, which is also converted to ammonium. Cellular ammonium is subsequently incorporated into carbon skeletons through the glutamine synthetase–glutamate synthase pathway. From the cellular amino acid pool (dashed box), different cellular compounds can be produced including the pigment phycocyanin, the polypeptide cyanophycin and the different microcystin variants. NtrgCmplx, nitrogenase complex; Gln, glutamine; Glu, glutamate; CA, carbonic anhydrase; CC, Calvin cycle; GL, glycolysis; TCA, tricarboxylic acid cycle; 2-OG, 2-oxoglutarate; AA, total amino acid pool; Arg, arginine; Asp, aspartic acid; Leu, leucine; CP, cyanophycin; PC, phycocyanin; MC-XZ, microcystin variants with variable amino acid position X and Z. Modified after Flores and Herrero (2005).

Cells supplied with low nitrogen availability but with ample carbon and light may enhance their cellular concentrations of leucine, and thereby lead to enhanced microcystin-LR production relative to microcystin-RR. Conversely, cells supplied with high nitrogen availability but low carbon and light will likely enhance their cellular concentrations of arginine, and thereby favor the production of microcystin-RR relative to microcystin-LR. This mechanism may explain our findings described in Chapter 3 (for *Microcystis aeruginosa*) and Chapters 4 and 5 (for *Planktothrix agardhii*). However, more species should be tested at a wider range of carbon, nitrogen and light conditions to fully elucidate the intriguing relationship between carbon and nitrogen metabolism, cellular N:C stoichiometry, amino acid synthesis, and the microcystin composition of harmful cyanobacteria.

7.5 Cyanobacterial stoichiometry and zooplankton grazing

Rising CO_2 levels may shift resource limitation patterns in phytoplankton communities. In particular, higher atmospheric CO_2 concentrations may alleviate surface blooms of buoyant cyanobacteria from carbon limitation (Ibelings and Maberly 1998). In nitrogen-poor waters, this would shift cyanobacterial blooms from carbon limitation to nitrogen limitation (Fig. 7.4). Conversely, in nitrogen-rich waters elevated CO_2 concentrations may induce light-limited conditions. According to our results, such changes in resource limitation will affect the cellular N:C stoichiometry and microcystin production of cyanobacteria (Fig. 7.4).

Changes in the nutritional value and toxicity of cyanobacteria will in turn have implications for zooplankton. The nutrient content of many zooplankton species is relatively high compared to that of phytoplankton. Hence, phytoplankton with a low nutrient content provide poor food quality for zooplankton (Sterner and Elser 2002; Urabe *et al*. 2003; Van de Waal *et al*. 2010). Furthermore, the growth and reproduction of many zooplankton species is suppressed by microcystins (Demott *et al*. 1991; Rohrlack *et al*. 2001; Trubetskova and Haney 2006), although some zooplankton species seem less affected than others (Lürling 2003; Kim *et al*. 2006; Semyalo *et al*. 2009; Wilken *et al*. 2010). According to our results, a shift from carbon to nitrogen limitation will result in lower cellular N:C ratios of cyanobacteria, reducing their nutritional value for zooplankton (Fig. 7.4). A shift from carbon to light limitation will result in higher cellular microcystin contents of cyanobacteria, increasing their toxicity. Thus, our results suggest that alleviation of carbon limitation by rising CO_2 levels will reduce the quality of cyanobacteria as food for zooplankton, either through a reduced nutritional value or through enhanced toxicity of the cyanobacterial cells.

However, changes in resource limitation may also have consequences for the relative abundances of toxic versus nontoxic strains. For instance, in our experiments, a toxic *Microcystis* strain won the competition for inorganic carbon, whereas a nontoxic strain won the competition for light (Chapter 6; Kardinaal *et al*. 2007b). We do not know to what extent this reversal in competitive dominance depended on the specific strains used in our competition experiments or is a more general phenomenon. More strains should be investigated before firm conclusions can be drawn on the competitive relationships between toxic and nontoxic strains. Yet, this finding illustrates that a shift from carbon limitation to light limitation might cause a shift in dominance from toxic strains towards nontoxic strains. If so, this shift in strain composition would counteract the predicted increase in the toxicity of toxic strains (Fig. 7.4).

To what extent zooplankton will respond to these changes in their food is not clear. Experiments with zooplankton grazing on mixtures of toxic and nontoxic cyanobacterial strains could further elucidate the implications of changes in carbon and nitrogen availability for the quality of cyanobacteria as food for zooplankton.

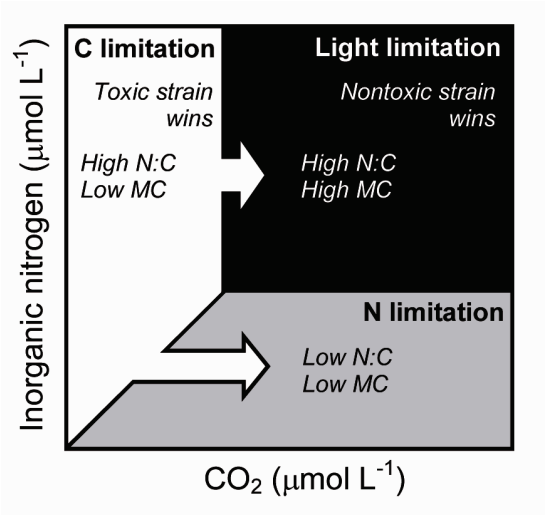

Figure 7.4. A summary of some of the key findings in this thesis. Low CO_2 and high nitrogen availability will lead to carbon limitation, high CO_2 and low nitrogen availability will lead to nitrogen limitation, while high CO_2 and high nitrogen availability will lead to light limitation. According to our results, changes in resource limitation will affect the cellular N:C stoichiometry and cellular microcystin content (MC) of cyanobacteria. In addition, the competitive relationship between toxic and nontoxic strains might depend on the limiting resource, although more strains should be investigated to draw firm conclusions. White arrows illustrate the shift in resource limitation as a result of rising atmospheric CO_2 levels. Based on Chapter 3 (Van de Waal *et al*. 2009) and Chapter 6.

7.6 Conclusions

The work in this thesis demonstrates that the toxin composition of harmful cyanobacteria is sensitive to changes in carbon and nitrogen availability. In addition, the outcome of competition between toxic versus nontoxic strains may shift with changes in CO_2 availability. Climate change is likely to alter the carbon and nitrogen availability in many aquatic ecosystems, and thereby affects the elemental balance and species composition of phytoplankton communities as well as the nature of the toxins that they produce.

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Appendix 1

Lake data

We measured *Microcystis* biomass, microcystin composition and seston N:C ratios in 12 *Microcystis*-dominated lakes in The Netherlands. Samples were taken from the open water (at 1 m depth) and, if present, from surface blooms (at 5 cm depth), resulting in a total of 19 lake samples. The data are presented in Table A1 below. In addition to the relationships reported in Fig. 3 of the main text, we note here that the biomass, microcystin concentration and seston N:C ratio were higher in surface blooms than in the open water.

Table A1. *Microcystis* biomass, microcystin composition and seston N:C ratios measured in the lake samples.

Lake sample	Lake	Sample position*	*Microcystis* biomass ($mm^3 L^{-1}$)	Total MC ($\mu g L^{-1}$)	MC-LR ($\mu g L^{-1}$)	MC-RR ($\mu g L^{-1}$)	MC-YR ($\mu g L^{-1}$)	Seston N:C ratio (molar)
1	Braassemermeer	W	2.88	1.49	1.0	0.49	0	0.103
2	Braassemermeer	S	566	36.3	17.2	7.2	11.9	0.143
3	Eemmeer	W	59.8	3.38	1.4	0.58	1.4	0.131
4	Eemmeer	S	15202	3310	1737	1247	326	0.174
5	Gooimeer-Almere 1	W	7.8	2.26	0.92	0.62	0.72	0.094
6	Gooimeer Almere 1	S	4356	346	179	167	0	0.192
7	Gooimeer-Almere 2	W	3.0	0.68	0.68	0	0	0.078
8	Gooimeer-Almere 2	S	11039	1940	1073	867	0	0.164
9	Gooimeer-Huizen	W	380	8.6	5.9	2.7	0	0.126
10	Nijkerkernauw	S	1063	429	276	153	0	0.178
11	Noord Aa	W	14.6	13.7	7.0	5.4	1.3	0.128
12	Noord Aa	S	155	171.3	91.4	79.9	0	0.157
13	't Joppe	W	4.2	4.34	2.6	1.5	0.24	0.097
14	't Joppe	S	1800	858.4	415	349	94.4	0.173
15	Vlietlanden	W	2.5	2.3	1.6	0.70	0	0.072
16	Vlietlanden	S	224	320.7	210	86.3	24.4	0.162
17	Westeinderplassen	W	5.4	0.73	0.73	0	0	0.078
18	Wijde Aa	W	11.4	1.79	0.85	0	0.94	0.111
19	Zegerplas	W	1.6	1.65	0.79	0.65	0.21	0.082

**W = sample from open water; S = sample from surface bloom.*

Appendix 2

Full description of the model

Embargo until publication.

Appendix 3

Parameter values

Embargo until publication.

Appendix 4

Drawing the zero isoclines

Embargo until publication.

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Summary

All life on Earth consists of a set of chemical elements such as carbon (C), nitrogen (N) and phosphorus (P). Primary producers stand at the very base of aquatic and terrestrial food webs due to their ability to transform inorganic elements into organic compounds such as carbohydrates and proteins. These organic compounds provide the food source for animals, transferring carbon, nitrogen, phosphorus and other elements to higher levels in the food web. The field of 'Ecological Stoichiometry' studies the balance of these chemical elements to describe the complex relationships between organisms and their environment.

In this thesis, I address the following three main questions:

1) What are the implications of climate-driven changes in resource availability on the ecological stoichiometry of aquatic ecosystems (Chapter 2)?
2) How will changes in resource availability alter the nitrogen:carbon stoichiometry and toxin production of harmful cyanobacteria (Chapter 3-5)?
3) What are the implications of rising CO_2 concentrations for the competitive interactions between phytoplankton species (Chapter 6)?

To address the first question, we performed a detailed literature review (Chapter 2). Increasing global temperatures tend to strengthen the thermal stratification of aquatic ecosystems, suppressing vertical mixing and thereby reducing the upward flux of nutrients into the surface layer. Rising CO_2 concentrations but reduced nutrient availability will decrease the nutrient:carbon stoichiometry of phytoplankton. Phytoplankton with a low nutrient:carbon content provide poor-quality food for most zooplankton species, which may shift the species composition of zooplankton and higher trophic levels to less nutrient-demanding species. As a consequence, climate-driven changes in plankton stoichiometry may alter the structure and functioning of entire aquatic food webs.

To address the second question, we performed a series of laboratory experiments with the cyanobacterium *Microcystis aeruginosa* producing the hepatotoxin microcystin (Chapter 3). *Microcystis* increased its cellular nitrogen:carbon ratio under excess CO_2 and nitrate supply. Its high nitrogen:carbon ratio was accompanied by high cellular contents of total microcystin, and in particular the nitrogen-rich variant microcystin-RR. Conversely, under nitrogen-limited conditions, *Microcystis* had a low cellular nitrogen:carbon ratio and low cellular content of microcystin-RR. Comparable patterns were found in *Microcystis*-dominated lakes, where the relative microcystin-RR content increased with the seston nitrogen:carbon ratio. To the best of my knowledge, this is the first time that the carbon-

nutrient balance hypothesis, originally developed to describe secondary metabolite production in terrestrial plants has been applied to toxic cyanobacteria.

Hence, the cellular nitrogen:carbon stoichiometry influences the toxin production and toxin composition of harmful cyanobacteria. But what are the underlying physiological mechanisms? Because microcystins consist of amino acids, we investigated whether amino acids supplied in the growth medium affected the microcystin composition in the harmful cyanobacterium *Planktothrix agardhii* (Chapter 4). Addition of leucine resulted in a strong increase of the microcystin-LR/microcystin-RR ratio, while addition of arginine resulted in a decrease of this ratio. In addition, we grew the same *P. agardhii* strain under nitrogen-depleted conditions and added a nitrate pulse (Chapter 5). This caused a rapid increase of the cellular nitrogen:carbon stoichiometry, which was accompanied by a transient increase in the amino acids aspartic acid and arginine, indicative for cyanophycin synthesis, and by a more gradual increase of the total amino acid content. As expected, the nitrogen-rich microcystin-RR variant (which contains two arginine molecules) increased strongly after the nitrate pulse, while microcystin-LR increased to a much lesser extent (Chapter 5). These results show that the effect of nitrogen:carbon stoichiometry on microcystin production and composition in harmful cyanobacteria is mediated by their amino acid synthesis.

To address the third question, we developed a new model that describes phytoplankton competition for inorganic carbon. We performed monoculture and competition experiments in chemostats with a toxic and a nontoxic *Microcystis aeruginosa* strain under carbon-limited conditions. In addition, we tested our model on earlier experiments (Kardinaal *et al*. 2007b) in which the same two strains were used, but grown under light-limited conditions. The model could qualitatively and quantitatively predict the outcome of competition. The low CO_2 concentration in the carbon-limited chemostats led to dominance of the toxic strain. In contrast, the high CO_2 but low light conditions in the light-limited chemostat led to dominance of the nontoxic strain. Thus, the toxic strain was a better competitor for CO_2, whereas the nontoxic strain was a better competitor for light. These results show both theoretically and experimentally that changes in CO_2 and light availability may result in a complete reversal of the outcome of competition in harmful algal blooms.

The work in this thesis demonstrates that the toxin composition of harmful cyanobacteria is sensitive to changes in inorganic carbon and nitrogen availability. In addition, the competitive dominance of toxic versus nontoxic strains can shift with changes in CO_2 availability. Rising CO_2 concentrations and associated global warming are likely to alter the carbon and nitrogen availability in many aquatic ecosystems, and may thereby affect the elemental stoichiometry and species composition of phytoplankton communities as well as the nature of the toxins that they produce.

Samenvatting

Al het leven op Aarde bestaat uit een aantal chemische elementen, zoals koolstof (C), stikstof (N) en fosfor (P). Primaire producenten staan aan de basis van het voedselweb in zowel aquatische als terrestrische ecosystemen, omdat ze anorganische elementen omzetten in organische stoffen zoals koolhydraten en eiwitten. Deze organische stoffen vormen een voedselbron voor vele dieren, die op hun beurt de opgenomen koolstof, stikstof en fosfor doorgeven aan de hogere niveaus in het voedselweb. Het onderzoeksveld 'Ecologische Stoichiometrie' bestudeert de balans tussen deze chemische elementen om de complexe relaties van organismen met hun omgeving te beschrijven.

In dit proefschrift worden de volgende hoofdvragen gesteld:

1) Wat is het effect van klimaatverandering op de beschikbaarheid van koolstof, nutriënten en licht in het water, en hoe beïnvloedt dit de ecologische stoichiometrie van aquatische ecosystemen (Hoofdstuk 2)?
2) Hoe beïnvloeden veranderingen in de beschikbaarheid van CO_2, nutriënten en licht de stikstof:koolstof stoichiometrie en toxine productie in giftige cyanobacteriën (blauwalgen) (Hoofdstuk 3-5)?
3) Wat zijn de gevolgen van toenemende CO_2 concentraties voor de concurrentie tussen fytoplanktonsoorten (Hoofdstuk 6)?

Voor het beantwoorden van de eerste vraag hebben we een uitgebreid literatuuronderzoek uitgevoerd (Hoofdstuk 2). Een hogere temperatuur zorgt voor een sterkere stratificatie (gelaagdheid) van aquatische systemen, waardoor er minder verticale menging kan optreden en de toestroom van nutriënten naar de bovenlaag van het water afneemt. Stijgende CO_2 concentraties gecombineerd met een afname van de nutriëntenbeschikbaarheid zal de nutriënt:koolstof ratio van fytoplankton verlagen. Fytoplankton met een lage nutriënt:koolstof ratio is van een slechte voedselkwaliteit voor de meeste zoöplanktonsoorten. Daardoor kan de soortensamenstelling van het zoöplankton alsmede de organismen op hogere niveaus in het voedselweb verschuiven naar soorten die minder nutriënten nodig hebben. Dus klimaat-gerelateerde veranderingen in de ecologische stoichiometrie van het fytoplankton kunnen de samenstelling en het functioneren van het totale aquatische ecosysteem beïnvloeden.

Voor het beantwoorden van de tweede vraag hebben we laboratorium experimenten uitgevoerd met de cyanobacterie *Microcystis aeruginosa*, die de gifstof microcystine produceert (Hoofdstuk 3). Bij een hoge beschikbaarheid van CO_2 en nitraat gaat de

stikstof:koolstof ratio van *Microcystis* cellen omhoog. Daarnaast bevatten deze cellen ook een hoog gehalte aan microcystines, met name van de stikstofrijke variant microcystine-RR. Echter, onder stikstof-gelimiteerde condities hebben de cellen van *Microcystis* juist een lage stikstof:koolstof ratio en een laag gehalte microcystine-RR. We vonden een vergelijkbare relatie in een aantal Nederlandse meren waarin *Microcystis* dominant is. Het relatieve gehalte microcystine-RR in deze meren nam toe met de stikstof:koolstof ratio van het seston.
Deze bevindingen komen overeen met de zogenaamde koolstof-nutriëntbalans hypothese, die oorspronkelijk is ontwikkeld om de samenstelling van secondaire metabolieten in terrestrische planten te verklaren. Voor zover ons bekend is dit de eerste keer dat deze hypothese gebruikt is voor het verklaren van de chemische samenstelling van gifstoffen in cyanobacteriën.

Dus de stikstof:koolstof stoichiometrie beïnvloedt de productie en samenstelling van gifstoffen in cyanobacteriën. Maar wat is het onderliggende fysiologische mechanisme hiervoor? Omdat microcystines bestaan uit aminozuren hebben we eerst gekeken of toevoeging van aminozuren invloed heeft op de productie van verschillende microcystine-varianten in de giftige cyanobacterie *Planktothrix agardhii* (Hoofdstuk 4). Na toevoeging van leucine aan het groeimedium nam de ratio van microcystine-LR/microcystine-RR in de cellen toe, terwijl na toevoeging van arginine deze ratio juist afnam. Daarna hebben we in een vervolgexperiment *P. agardhii* gekweekt onder stikstof-arme condities. Op het moment dat de cellen stikstofgelimiteerd waren, hebben we een nitraatpuls toegevoegd (Hoofdstuk 5). Hierdoor nam de stikstof:koolstof ratio van de cellen snel toe. Dit ging gepaard met een tijdelijke verhoging van de aminozuren asparaginezuur en arginine, wat wijst op de productie van cyanophycine, een polymeer voor de opslag van stikstof. Ook de totale hoeveelheid aminozuren in de cellen steeg geleidelijk. Zoals verwacht nam de concentratie van de stikstofrijke microcystine-RR variant (met twee arginine moleculen) sterk toe na de nitraatpuls, terwijl de concentratie van de microcystine-LR variant veel minder steeg.
Deze resultaten laten zien dat de invloed van de stikstof:koolstof stoichiometrie op de samenstelling van microcystines in giftige cyanobacteriën kan worden verklaard aan de hand van de aminozuren die worden aangemaakt.

Voor het beantwoorden van de derde vraag hebben we een nieuw model ontwikkeld. Dit beschrijft de concurrentie om anorganische koolstof tussen fytoplanktonsoorten. Om het model te testen hebben we concurrentie experimenten uitgevoerd met een giftige en niet-giftige *Microcystis aeruginosa* stam onder koolstofgelimiteerde condities. Daarnaast hebben we het model getest met eerder uitgevoerde experimenten (Kardinaal *et al*. 2007b), waarbij dezelfde twee stammen groeiden onder lichtgelimiteerde omstandigheden. Het model kon de resultaten van beide concurrentie experimenten zowel kwalitatief als kwantitatief goed voorspellen. De lage CO_2 concentratie in het koolstof-gelimiteerde

experiment leidde tot dominantie van de giftige stam. Echter, de hoge CO_2 concentratie maar lage licht beschikbaarheid in het lichtgelimiteerde experiment leidde tot dominantie van de niet-giftige stam. Dus de giftige stam was sterker in de concurrentie om CO_2 , terwijl de niet-giftige stam sterker was in de concurrentie om licht. Deze resultaten tonen aan dat veranderingen in de beschikbaarheid van CO_2 en licht kunnen leiden tot een complete ommekeer van de uitkomst van de concurrentiestrijd tussen giftige en niet-giftige cyanobacteriën.

Het werk in dit proefschrift laat zien dat de chemische samenstelling van gifstoffen in cyanobacteriën gevoelig is voor veranderingen in de beschikbaarheid van anorganisch koolstof en stikstof. Daarnaast kunnen de dominantie verhoudingen tussen giftige en niet-giftige stammen verschuiven door veranderingen in de beschikbaarheid van CO_2. Het is waarschijnlijk dat de toenemende CO_2 concentraties en daarmee gerelateerde opwarming van onze aarde de beschikbaarheid van koolstof en stikstof in veel aquatische ecosystemen zal beïnvloeden. Dit zal gevolgen hebben voor de stoichiometrie en soortensamenstelling van het fytoplankton, alsmede voor de gifstoffen die zij produceren.

Gearfetting

Al it libben op ierde bestiet út in tal gemyske eleminten, lykas koalstof (C), stikstof (N) en fosfor (P). Primêre produsinten steane oan de basis fan it fiedselweb, sawol yn akwatyske as terrestryske ekosystemen, om't se anorganyske eleminten omsette yn organyske stoffen lykas koalhydraten en aaiwiten. Dy organyske stoffen binne yngrediïnten fan it iten fan in protte bisten, dy't op harren beurt de koalstof, stikstof en fosfor dy't se opnimme trochjouwe oan de hegere nivo's yn it fiedselweb. It ûndersyksfjild 'Ekologyske Stoichiometry' bestudearret de balâns tusken dy gemyske eleminten om de komplekse relaasjes fan organismen mei harren omjouwing te beskriuwen.

Yn dit proefskrift wurde de neifolgjende haadfragen steld:

1) Wat is it effekt fan klimaatferoaring op de beskikberens fan koalstof, nutriïnten en ljocht yn it wetter en hoe beynfloedet dat de ekologyske stoichiometry fan akwatyske ekosystemen (Haadstik 2)?
2) Hoe beynfloedzje feroarings yn de beskikberens fan CO_2, nutriïnten en ljocht de stikstof:koalstof stoichiometry en toksineproduksje yn giftige syanobaktearjes (blau-algen) (Haadstik 3-5)?
3) Wat binne de gefolgen fan tanimmende CO_2-konsintraasjes foar de konkurrinsje tusken fytoplanktonsoarten (Haadstik 6)?

Foar it beäntwurdzjen fan de earste fraach hawwe wy in wiidweidich literatuerûndersyk dien (Haadstik 2). In hegere temperatuer soarget foar in sterkere stratifikaasje (yndieling yn ferskillende lagen) fan akwatyske systemen. Dêrtroch kin in fertikaal mingen him minder foardwaan en nimt de tastream fan nutriïnten nei de boppelaach fan it wetter ôf. Gruttere CO_2-konsintraasjes yn kombinaasje mei in ôfnimmen fan de beskikberens fan nutriïnten sille de nutriïnt:koalstof ratio fan fytoplankton ferleegje. De kwaliteit fan fytoplankton mei in lege nutriïnt:koalstof ratio as iten foar de measte zoöplanktonsoarten is min. Dêrtroch kin de gearstalling fan de soarten zoöplankton en ek fan de organismen op hegere nivo's yn it fiedselweb ferskowe nei soarten dy't minder nutriïnten nedich hawwe. Klimaat-relatearre feroarings yn de ekologyske stoichiometry fan it fytoplankton kinne de gearstalling en it funksjonearjen fan it hiele akwatyske ekosysteem sadwaande beynfloedzje.

Foar it beäntwurdzjen fan de twadde fraach hawwe wy laboratoariumeksperiminten dien mei de syanobaktearje *Microcystis aeruginosa*, dy't de gifstof mikrosystine produsearret (Haadstik 3). Wannear't der in protte CO_2 en nitraat foarhannen is, giet de stikstof:koalstof ratio fan *Microcystis*-sellen omheech. Dêrnjonken hawwe dy sellen ek in heech gehalte oan

mikrosystines, benammen fan de stikstofrike fariant mikrosystine-RR. As der lykwols net safolle stikstof oanwêzich is, hawwe de *Microcystis*-sellen just in lege stikstof:koalstof ratio en in leech gehalte oan mikrosystine-RR. Wy fûnen in ferlykbere relaasje yn in tal Nederlânske marren dêr't *Microcystis* dominant is. It relative gehalte oan mikrosystine-RR yn dy marren naam ta mei de stikstof:koalstof ratio fan it seston.
Dy befinings komme oerien mei de saneamde koalstof-nutriïntbalâns hypoteze, dy't oarspronklik ûntwikkele is om de gearstalling fan sekundêre metaboliten yn terrestryske planten te ferklearjen. Foarsafier't ús bekend is, is dit de earste kear dat dy hypoteze brûkt is foar it ferklearjen fan de gemyske gearstalling fan gifstoffen yn syanobaktearjes.

De stikstof:koalstof stoichiometry beynfloedet sadwaande de produksje en gearstalling fan gifstoffen yn syanobaktearjes. Mar wat is it ûnderlizzende fysiologyske meganisme dêrfoar? Om't mikrosystines besteane út aminosoeren, hawwe wy earst besjoen oft it tafoegjen fan aminosoeren ynfloed hat op de produksje fan ferskillende mikrosystinefarianten yn de giftige syanobaktearje *Planktothrix agardhii* (Haadstik 4). Nei it tafoegjen fan leusine oan it groeimedium naam de ratio fan mikrosystine-LR/mikrosystine-RR yn de sellen ta, wylst nei it tafoegjen fan arginine dy ratio krekt ôfnaam. Dêrnei hawwe wy yn in ferfolcheksperimint *P. agardhii* kweekt mei minder stikstof. Op it stuit dat der minder stikstof yn de sellen siet, hawwe wy in nitraatpuls tafoege (Haadstik 5). Dêrtroch naam de stikstof:koalstof ratio fan de sellen rap ta. Tagelyk waard in tydlike ferheging fan de aminosoeren asparaginesoer en arginine konstatearre, wat wiist op de produksje fan syanofysine, in polymear foar de opslach fan stikstof. Der waarden njonkenlytsen ek mear aminosoeren yn de sellen oanmakke. Sa as ferwachte naam de konsintraasje fan de stikstofrike mikrosystine-RR fariant (mei twa arginine molekulen) fiks ta nei de nitraatpuls, wylst de konsintraasje fan de mikrosystine-LR fariant gâns minder tanaam.
Dy resultaten litte sjen dat de ynfloed fan de stikstof:koalstof stoichiometry op de gearstalling fan mikrosystines yn giftige syanobaktearjes ferklearre wurde kin oan 'e hân fan de aminosoeren dy't oanmakke wurde.

Foar it beäntwurdzjen fan de tredde fraach hawwe wy in nij model ûntwikkele. Dat beskriuwt de konkurrinsje om anorganyske koalstof tusken fytoplanktonsoarten. Om it model te testen hawwe wy konkurrinsje-eksperiminten dien mei in giftige en in net-giftige *Microcystis aeruginosa*-stam ûnder koalstoflimitearre kondysjes. Dêrnjonken hawwe wy it model test mei earder útfierde eksperiminten (Kardinaal *et al*. 2007b), wêrby't deselde twa stammen groeiden yn in situaasje mei minder ljocht. It model koe de resultaten fan beide konkurrinsje- eksperiminten sawol kwalitatyf as kwantitatyf goed foarsizze. De lege CO_2-konsintraasje yn it koalstof-limitearre eksperimint late ta dominânsje fan de giftige stam. Lykwols, de hege CO_2-konsintraasje, mar lege beskikberens fan ljocht yn it eksperimint mei minder ljocht late ta dominânsje fan de net-giftige stam. Dat de giftige stam wie sterker

yn de konkurrinsjestriid om de CO_2, wylst de net-giftige stam sterker wie yn de konkurrinsjestriid om it ljocht. Dy resultaten litte sjen dat feroarings yn de beskikberens fan CO_2 en ljocht liede kinne ta in folsleine omkear fan de útkomst fan de konkurrinsjestriid tusken giftige en net-giftige syanobaktearjes.

It wurk yn dit proefskrift lit sjen dat de gemyske gearstalling fan gifstoffen yn syanobaktearjes gefoelich is foar feroarings yn de beskikberens fan anorganyske koalstof en stikstof. Dêrnjonken kinne de dominânsjeferhâldings tusken giftige en net-giftige stammen ferskowe troch feroarings yn de beskikberens fan CO_2. It hat wol eigenskip dat de tanimmende CO_2-konsintraasjes en yn ferbân dêrmei de opwaarming fan ús ierde de beskikberens fan koalstof en stikstof yn in soad akwatyske ekosystemen beynfloedzje sille. Dat sil gefolgen hawwe foar de stoichiometry en de soartegearstalling fan it fytoplankton en ek foar de gifstoffen dy't sy produsearje.

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Curriculum vitae

Dedmer B. van de Waal was born on the 8th of January 1982 in Wijnjewoude, the Netherlands. After following secondary school at the CSG Liudger in Drachten he studied Environmental Sciences at the Van Hall Institute in Leeuwarden, where he obtained his Bachelor Hons degree in 2003. Thereafter, he continued with an MSc program in Hydrology and Water Quality Management at Wageningen University, where he graduated in 2005. During his education he performed research projects at the University of Udine (Italy), the Royal Netherlands Institute for Sea Research, Wageningen University, the Netherlands Institute of Ecology, and the University of Amsterdam. In these projects he got acquainted with various aspects of phytoplankton ecology. This included colony formation of marine haptophytes, competition between green algae and cyanobacteria, cyanobacterial mortality by cyanophages, and nitrogen fixation in marine cyanobacteria.

Upon graduation, Dedmer started the PhD research described in this thesis. The work was conducted at the Laboratory of Aquatic Microbiology of the University of Amsterdam, in collaboration with the Department of Aquatic Ecology of the Netherlands Institute of Ecology, under supervision of Prof. dr. Jef Huisman (UvA) and Prof. dr. Ellen van Donk (NIOO). The research resulted in several publications and this thesis.

As of December 2009, Dedmer is a Postdoctoral Research Associate at the Alfred Wegener Institute for Polar and Marine Research in Bremerhaven, Germany, in the ERC Research Group of Dr. Björn Rost.

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