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### 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<sub>2</sub> have been exhausted into the atmosphere. As a result, atmospheric CO<sub>2</sub> 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<sub>2</sub> will rise further, reaching ~750 ppm by the end of the 21<sup>st</sup> century (Solomon *et al.* 2007). This rapid increase in atmospheric CO<sub>2</sub> concentrations and other greenhouse gases is accompanied by global warming. Since 1850, average global temperatures have increased with 0.8C and are expected to increase with another 3 °C by the end of the 21<sup>st</sup> century (Solomon *et al.* 2007). These dramatic changes in atmospheric CO<sub>2</sub> 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<sub>2</sub> reacts with water forming carbonic acid (H<sub>2</sub>CO<sub>3</sub>). Carbonic acid rapidly dissociates into bicarbonate (HCO<sub>3</sub><sup>-</sup>) thereby releasing a proton (H<sup>+</sup>). At high pH, bicarbonate can dissociate further to carbonate (CO<sub>3</sub><sup>2-</sup>), releasing another proton. With higher atmospheric CO<sub>2</sub> levels, more CO<sub>2</sub> 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<sub>2</sub> in equilibrium with the atmosphere depends on the atmospheric CO<sub>2</sub> 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  $\text{CO}_2$  at low pH (Fig. 1.1). Rising atmospheric  $\text{CO}_2$  levels will thus increase the concentration of dissolved  $\text{CO}_2$ , but will decrease the carbonate concentration. Enhanced concentrations of dissolved  $\text{CO}_2$  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).

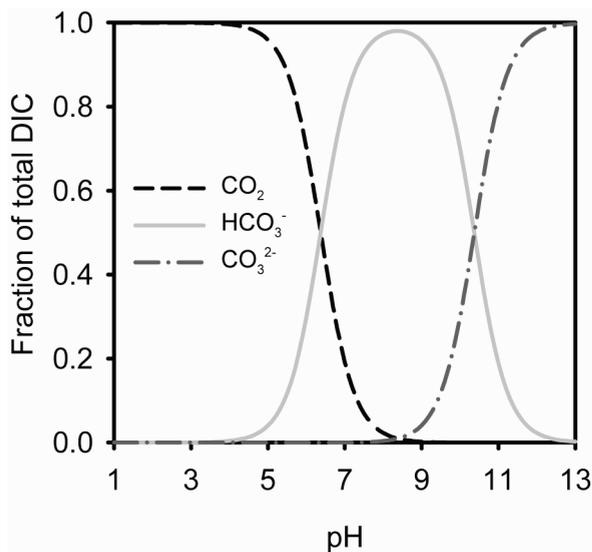


Figure 1.1. Relative contribution of the different carbon species to the total dissolved inorganic carbon (DIC) as a function of pH.

Global warming and rising atmospheric  $\text{CO}_2$  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  $\text{CO}_2$ . As a consequence, many lakes are supersaturated with  $\text{CO}_2$  (Sobek *et al.* 2005). Enhanced DOC input from terrestrial ecosystems may lead to an even further increase of the  $\text{CO}_2$  concentration in lakes.

Besides the vast amounts of CO<sub>2</sub> 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 20<sup>th</sup> 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<sub>2</sub> 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<sub>2</sub> 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,

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\* 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).

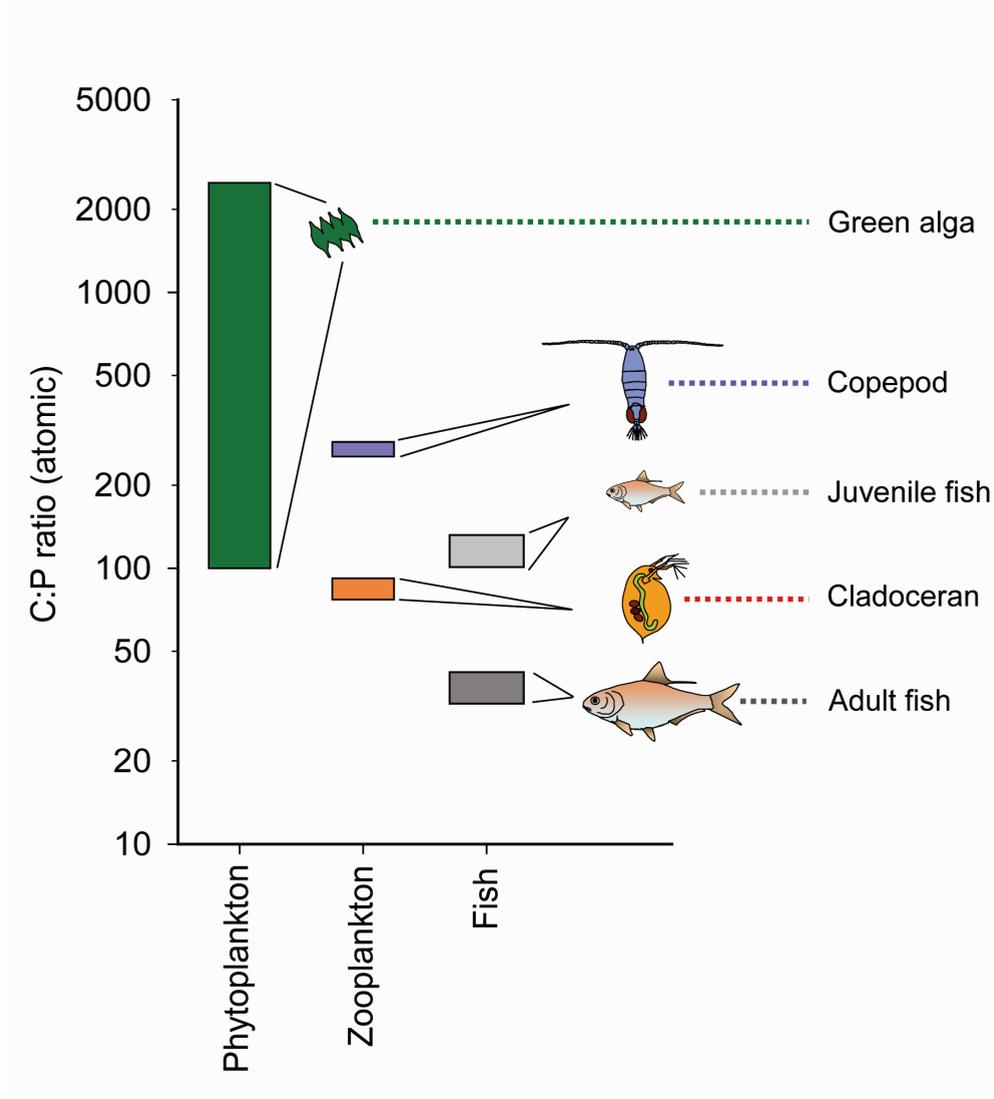


Figure 1.2. Phytoplankton display a wide range of relatively high C:P ratios, as shown here for the green alga *Scenedesmus obliquus* (data from Verschoor *et al.* 2004). Zooplankton and fish have rather narrow stoichiometric ranges (homeostasis). Copepods, such as *Eudiaptomus gracilis*, typically have 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).

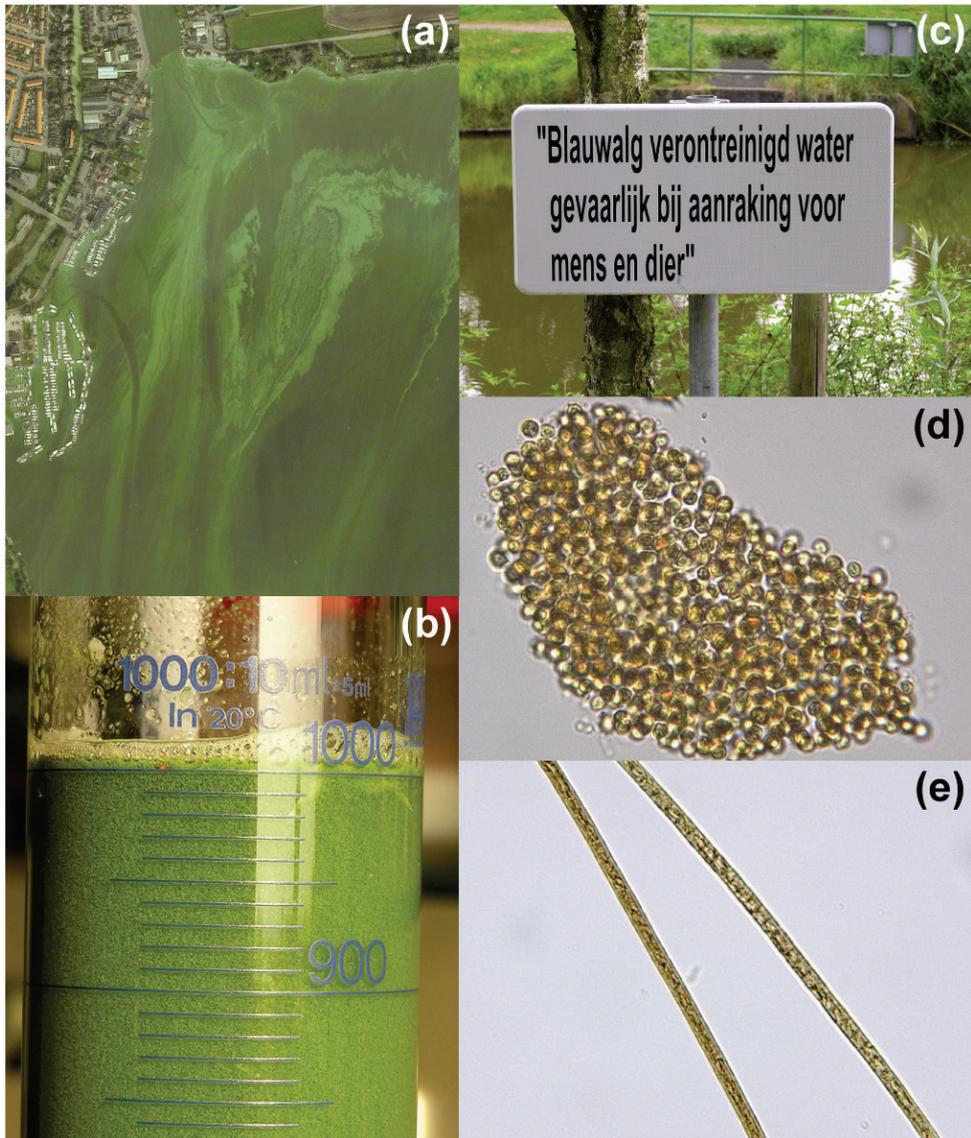


Figure 1.3. Harmful cyanobacteria. (a) Scum formation in lake Braassemermeer in September 2005. (b) Cyanobacterial scum in a measuring cylinder. (c) A signpost warning against harmful cyanobacteria: “Blue-green algae polluted water dangerous for humans and animals”. (d,e) Microscopic photographs of the 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  $> 10\,000\ \mu\text{g L}^{-1}$  (Kardinaal and Visser 2005a), which is 500-fold higher than the guideline value for recreational waters advised 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<sub>50</sub> assays on mice (the intraperitoneal dose lethal for 50% of the mouse population). A lower LD<sub>50</sub> indicates a higher toxicity. For instance, microcystin-LR (LD<sub>50</sub> = 33-73  $\mu\text{g kg}^{-1}$ ) and

microcystin-YR ( $LD_{50} = 70 \mu\text{g kg}^{-1}$ ) are more toxic than microcystin-RR ( $LD_{50} = 310\text{-}630 \mu\text{g kg}^{-1}$ ), while the toxicity of the demethylated variants [Asp<sup>3</sup>]microcystin-LR ( $LD_{50} = 160\text{-}300 \mu\text{g kg}^{-1}$ ) and [Asp<sup>3</sup>]microcystin-RR ( $LD_{50} = 250\text{-}360 \mu\text{g kg}^{-1}$ ) are quite similar (Sivonen and Jones 1999; Chen *et al.* 2006; Hoeger *et al.* 2007).

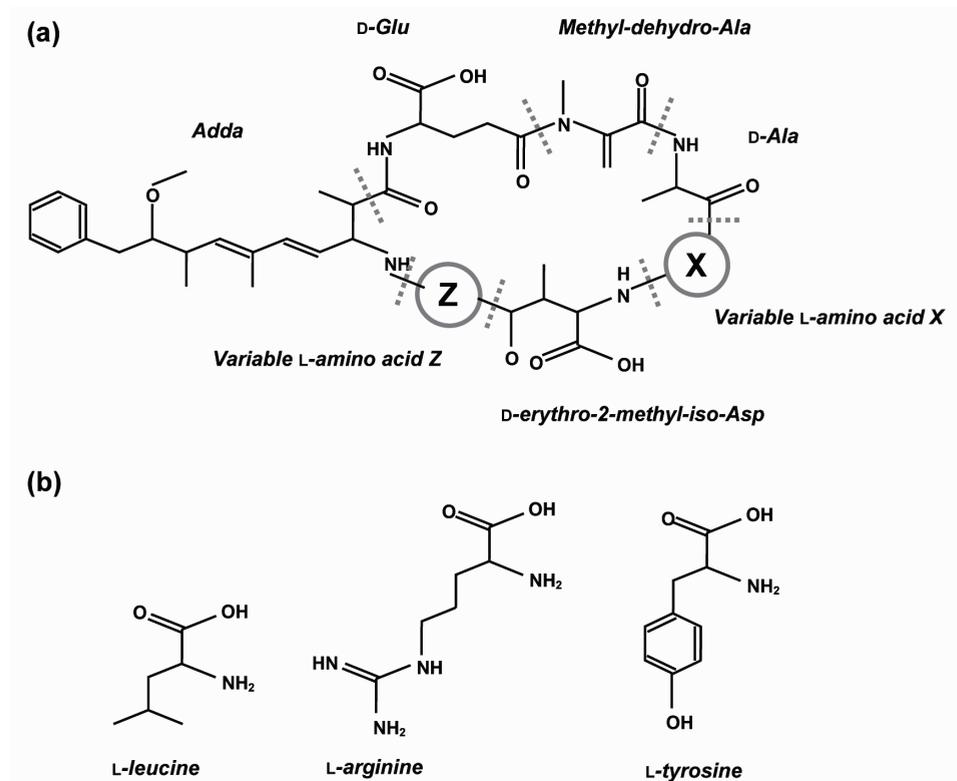


Figure 1.4. (a) General molecular structure of microcystins, which consist of seven amino acids including two variable positions (X and Z). (b) Position X is occupied by the amino acids L-leucine (L), L-arginine (R) or L-tyrosine (Y) in the common microcystin variants microcystin-LR, microcystin-RR, 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> or it could tolerate a higher pH than the nontoxic strain. At low light conditions, in combination with high CO<sub>2</sub> 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<sub>2</sub> 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.