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**Out of balance: implications of climate change for the ecological stoichiometry of harmful cyanobacteria**

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# 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> in water, as a result of rising atmospheric CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> levels, phytoplankton may be promoted by higher CO<sub>2</sub> 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<sub>2</sub> depletion (Maberly 1996; Hein 1997; Ibelings and Maberly 1998). Uptake of large quantities of CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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).

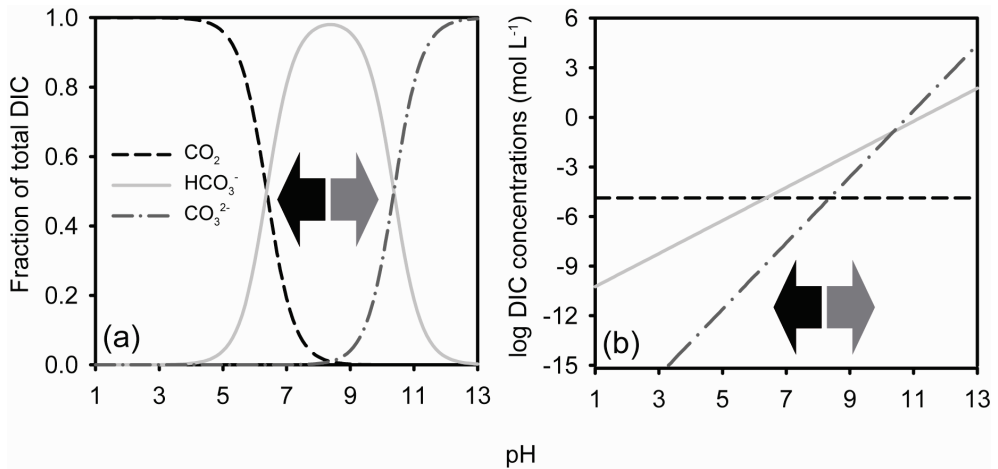


Figure 7.1. Inorganic carbon in aquatic ecosystems. (a) Relative composition of dissolved inorganic carbon (DIC) as a function of pH. (b) Absolute concentrations of dissolved inorganic carbon species as a function of pH assuming equilibrium with the atmosphere containing 380 ppm  $\text{CO}_2$ . The black arrows illustrate the effect of ocean acidification on carbon speciation (i.e., enhanced  $\text{CO}_2$  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  $\text{CO}_2$  and bicarbonate (Rost *et al.* 2003; Hansen *et al.* 2007; Trimbom *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  $\text{CO}_2$  will be promoted in the acidifying waters associated with rising  $\text{CO}_2$  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  $\text{CO}_2$  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<sub>2</sub> supply, whereas the nontoxic strain became dominant in a competition experiment with higher CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> and bicarbonate. Tilman (1982) developed a graphical approach using zero isoclines to assess the competitive abilities of species competing for two resources.

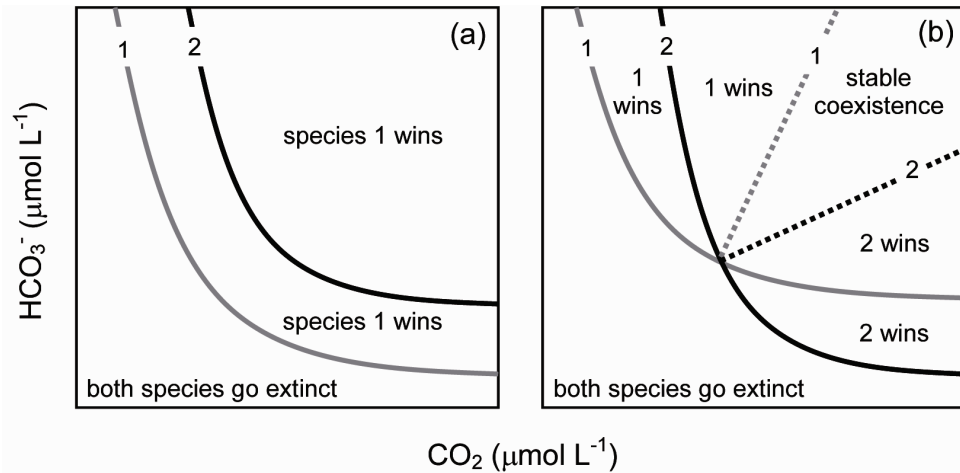


Figure 7.2. Graphical representation of the outcome of competition for bicarbonate and  $\text{CO}_2$  between two phytoplankton species. The solid lines represent the zero isoclines of the species. The dotted lines represent the slopes of the consumption vectors (i.e. the rate at which bicarbonate is taken up relative to the uptake of  $\text{CO}_2$ ) of the species. In each region of the graphs, the outcome of competition is indicated for the combination of bicarbonate and  $\text{CO}_2$  supply falling into that region. Graph (a) illustrates the scenario where species 1 is the better competitor for both bicarbonate and  $\text{CO}_2$ . There is no trade-off between competitive abilities for bicarbonate and  $\text{CO}_2$ . 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  $\text{CO}_2$ , whereas species 2 is the better competitor for bicarbonate. Hence, there is a trade-off between the competitive ability for bicarbonate and  $\text{CO}_2$ . If the better  $\text{CO}_2$  competitor (species 1) consumes relatively more bicarbonate, and the better bicarbonate competitor (species 2) consumes relatively more  $\text{CO}_2$ , 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  $\text{CO}_2$  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  $\text{CO}_2$  and bicarbonate, the zero isoclines of the competing species do not intersect (Fig. 7.2a). That is, the species that can deplete  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  and bicarbonate. In this case, the zero isoclines intersect (Fig. 7.2b). With increasing  $\text{CO}_2$ , the species interactions will gradually shift from competition for  $\text{CO}_2$  to competition for bicarbonate. Thus, the species composition will shift from dominance of superior  $\text{CO}_2$  competitors at low  $\text{CO}_2$  and high bicarbonate conditions towards dominance of superior bicarbonate competitors at high  $\text{CO}_2$  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<sub>2</sub> 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 (Stern 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<sub>2</sub> and light availability will decrease the phytoplankton nutrient:carbon stoichiometry (Stern 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<sub>2</sub>. 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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.



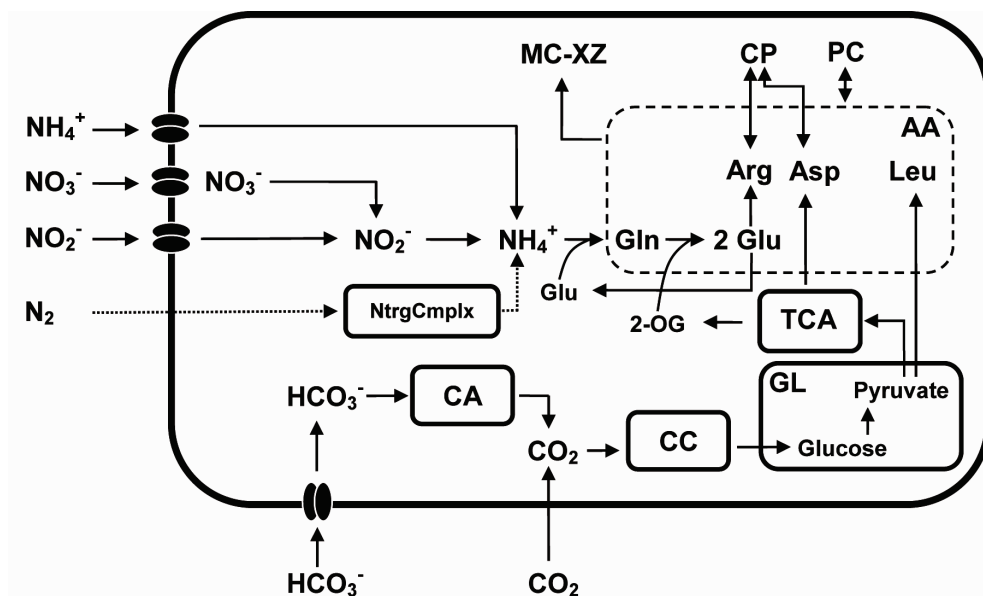


Figure 7.3. Schematic diagram of inorganic carbon assimilation and inorganic nitrogen assimilation in microcystin-producing cyanobacteria.  $\text{CO}_2$  diffuses through the cell wall, or is actively taken up as bicarbonate through  $\text{C}_i$  transporters.  $\text{CO}_2$  is further assimilated 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  $\text{N}_2$ , 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.

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## 7.5 Cyanobacterial stoichiometry and zooplankton grazing

Rising CO<sub>2</sub> levels may shift resource limitation patterns in phytoplankton communities. In particular, higher atmospheric CO<sub>2</sub> 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<sub>2</sub> 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 (Lüring 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<sub>2</sub> 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.

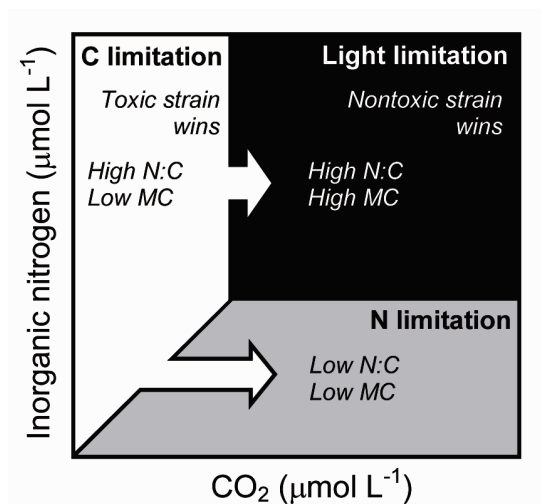


Figure 7.4. A summary of some of the key findings in this thesis. Low  $\text{CO}_2$  and high nitrogen availability will lead to carbon limitation, high  $\text{CO}_2$  and low nitrogen availability will lead to nitrogen limitation, while high  $\text{CO}_2$  and high nitrogen availability will lead 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  $\text{CO}_2$  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  $\text{CO}_2$  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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