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### Effects of rising CO<sub>2</sub> on the harmful cyanobacterium *Microcystis*

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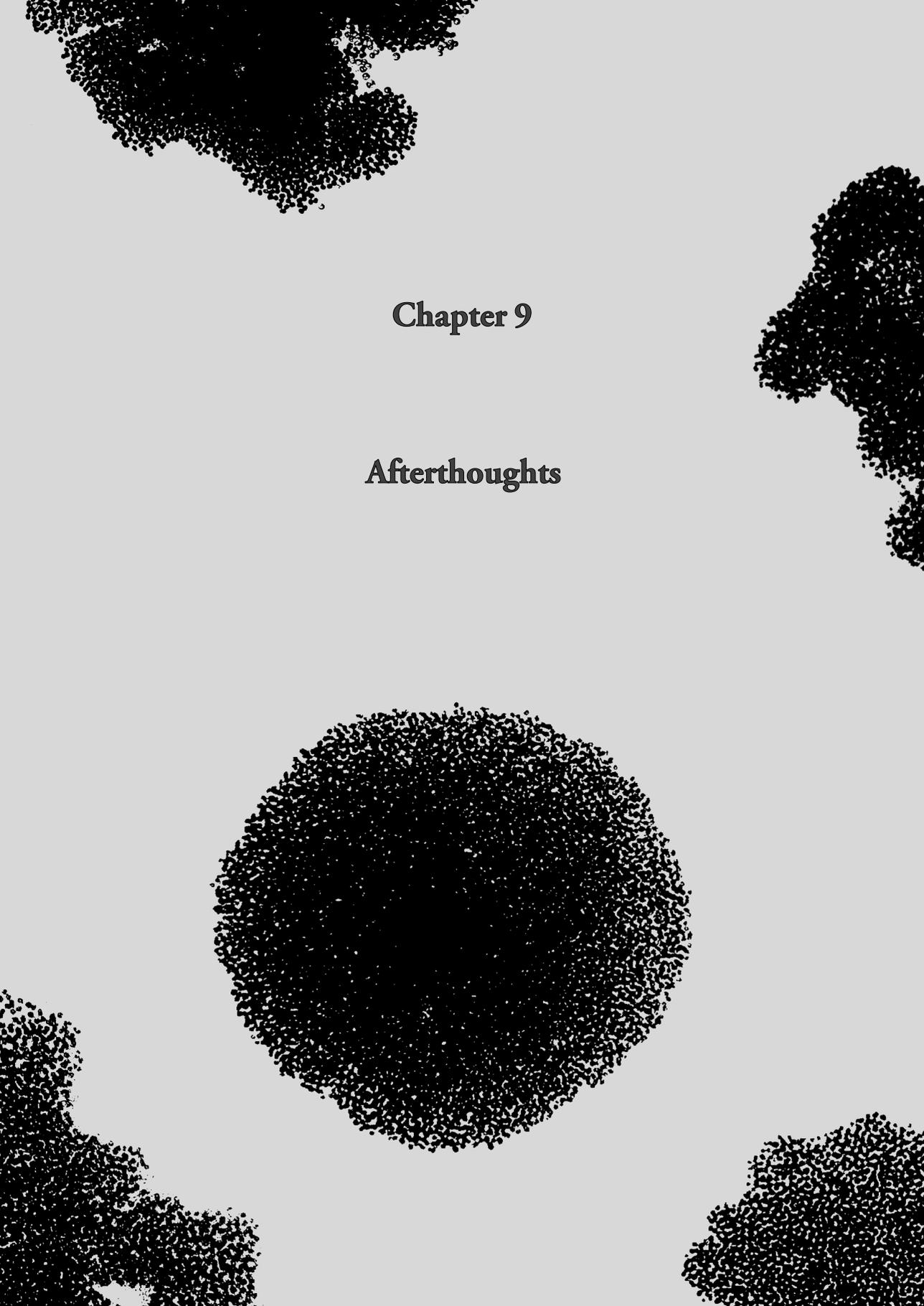
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**Chapter 9**

**Afterthoughts**

## Introduction

The goal of this thesis was to investigate effects of elevated CO<sub>2</sub> concentrations on harmful cyanobacteria. The ubiquitous cyanobacterium *Microcystis* was used as model organism. This cyanobacterium can develop dense blooms, causing problems in many lakes worldwide.

We found an intriguing genetic diversity of inorganic carbon uptake systems in *Microcystis*. Different strains have different C<sub>i</sub> uptake genes, and the strains also vary in their expression of these C<sub>i</sub> uptake genes. This genetic variation resulted in different growth responses to elevated CO<sub>2</sub>, and can cause microevolutionary adaptation of *Microcystis* blooms to the prevailing C<sub>i</sub> conditions. The current section puts these findings in perspective.

First, developments in techniques to study genotypes and gene expression will be discussed. Then, the genetic variability of the *Microcystis* CCM and the transcriptional regulation of *Microcystis* CCM genes will be evaluated. Next, the anticipated effects of climate change on *Microcystis* blooms and microcystin production are discussed. This is followed by an analysis of the foreseen effects of climate change on other harmful freshwater cyanobacteria, marine cyanobacteria and eukaryotic algae. Methods to effectively combat harmful cyanobacterial blooms are also appraised, and the chapter ends with the main conclusions of this thesis.

## Developments in techniques to study gene expression and genotypes

A wide variety of techniques have become available to study the genome and transcriptome of microorganisms. In this thesis, changes in gene expression were determined using microarrays (**Chapter 3**) and RT-qPCR (reverse transcription quantitative polymerase chain reaction; **Chapters 3, 4 and 6**).

Using RT-qPCR to study gene expression has several advantages. It is easier to apply than for instance microarrays, the data analysis is relatively straightforward, and the genes of interest can be studied in depth. In addition, the primers can be designed to match the sequences of many different strains or species. However, with this technique, one can only target a small number of genes at a time.

For laboratory studies of the entire transcriptome, microarrays and transcriptome sequencing ('RNA-seq') are the preferred choice. Microarrays were successfully applied in this thesis (**Chapter 3**) and previous studies with cyanobacteria (Wang *et al.*, 2004; Eisenhut *et al.*, 2007; Schwarz *et al.*, 2011; von Wobeser *et al.*, 2011; Krasikov *et al.*, 2012). In recent years, transcriptome sequencing has become more popular, because of increased sequencing capacity and much faster processing rates, longer reads, improved data analysis tools and reduced costs (Van Dijk *et al.*, 2014). The choice between microarrays and transcriptome sequencing will also depend on the expertise at hand and if one or many different organisms are to be studied.

For our fieldwork at Lake Kennemermeer (**Chapter 6**), microarrays and metatranscriptomics were not suitable to study the expression of *Microcystis* genes. A microarray is usually custom-made for a known strain. Because of the large variation in gene sequences and large number of strain-specific genes (e.g., Humbert *et al.*, 2013), microarray probes designed for a specific strain are often not suitable for other strains. Moreover, data analysis of metatranscriptome sequences becomes extremely complex for very diverse communities, information about transcripts is often obtained at a relative low resolution, and transcripts with relatively low abundance might be missed (Moran, 2009; Penn *et al.*, 2014). Therefore, we applied RT-qPCR for our lake study at Lake Kennemermeer, which enabled the quantification of gene expression in a complex mixture of different *Microcystis* strains embedded in a larger natural community.

We also used qPCR to quantify the genotype composition of *Microcystis* (**Chapter 5**). The relative abundances of five *Microcystis* strains were quantifiable in chemostat experiments, because each strain contained a different combination of genes (**Chapter 5**). In lake studies, detailed genetic information on individual strains is not available. However, the *bicA+sbtA* sequence of *Microcystis* can be used as ‘reference gene’ to calculate the ratios of strains with *bicA+sbtA* to strains with only *bicA* or only *sbtA*. Hence, the relative abundances of the *bicA*, *sbtA* and *bicA+sbtA* genotypes can be quantified (**Chapter 5**).

This method works for *Microcystis*, because *bicA* and *sbtA* are located on the same operon. The method cannot be used to quantify different  $C_i$  uptake genotypes in other cyanobacteria, because in all other cyanobacteria sequenced so far the *bicA* and *sbtA* gene are located at separate locations in the genome. In addition, while *bicA* or *sbtA* are present in all *Microcystis* strains analyzed so far, both genes are absent in some of the *Planktothrix* and *Anabaena* strains (which were therefore called ‘minimalist genotypes’; **Chapter 7**). As a consequence, relative abundances of the *bicA* gene and *sbtA* gene can be quantified, but relative abundances of the *bicA* genotype, *sbtA* genotype, *bicA+sbtA* genotype and minimalist genotype cannot be quantified in blooms of *Planktothrix*, *Anabaena* and other cyanobacteria. For example, suppose that 75% of the cells in a *Planktothrix* bloom contain the *bicA* gene and 60% the *sbtA* gene, then it is still not known which percentage of these cells contains both *bicA* and *sbtA*, and which percentage contains only *bicA* or only *sbtA*.

Alternatively, amplicon sequencing can be used to quantify different cyanobacterial strains (Shokralla *et al.*, 2012; Poretsky *et al.*, 2014). Amplicon sequencing is nowadays often replacing Denaturing Gradient Gel Electrophoresis (DGGE), which was introduced in microbial ecology by Muyzer *et al.* (1993). In amplicon sequencing, PCR is used to create numerous copies of part of a gene, that are subsequently sequenced. The PCR is based on specific primers that can bind to regions of genes that are strongly conserved among a

microbial community (Klindworth *et al.*, 2012). To reduce costs, short nucleotide sequences (tags) can be ligated to the DNA fragments or included in the primer design (Binladen *et al.*, 2007; Meyer *et al.*, 2007), giving samples a unique barcode and allowing multiple samples to be sequenced simultaneously. Often, amplicon sequencing is based on sequencing of the 16S rRNA gene. However, this is complicated by the fact that bacterial genomes often have multiple copies of this gene, and the different copies are not always 100% identical in their DNA sequence (Větrovský and Baldrian, 2013). Alternatively, the internal transcribed spacer (ITS) region, located between the 16S rRNA and 23S rRNA, can be used in amplicon sequencing, because it shows more variation among different strains compared to the 16S rRNA gene. Yet, multiple ITS regions are also present in the genomes of many bacteria, including cyanobacteria.

Both amplicon sequencing and DGGE are not suitable to detect the different *Microcystis* C<sub>1</sub> uptake genotypes in lakes, because strains differ not only in the sequences of *bicaA* and *sbtA*, but also in the presence of these genes. Yet, amplicon sequencing of other genes in the *Microcystis* genome can be used for example to determine relative abundances of defined strains in laboratory competition experiments, such as the competition experiments described in **Chapter 5**. For *Microcystis*, *ccmR2* could be a suitable gene to determine relative strain abundances using amplicon sequencing, since the gene is already sequenced for many strains, only one copy of the gene is present in *Microcystis* genomes, the gene is present in all *Microcystis* strains but not in the other cyanobacteria investigated so far, and the gene shows considerable variation between strains.

Fluorescence *in situ* hybridization (FISH) has also proven to be a successful method to study the genetic composition and gene expression of laboratory and field samples. An advantage of this technique is that information about genes can be combined with information about morphology. Application of FISH techniques to cyanobacteria requires strong fluorescence signals using bright probes, to overcome the strong autofluorescence of cyanobacteria. Yet, 'normal FISH', that is typically used on 16S rRNA, cannot be applied to mRNA or genomic DNA sequences, because of the low copy numbers and hence low fluorescence signal. Alternatively, RING-FISH (recognition of individual genes by a long network of connected probes) has been successfully used on *Microcystis* communities, to quantify *mcyD* mRNA and to quantify toxic and non-toxic genotypes (Dziallas and Grossart, 2011; Dziallas *et al.*, 2011). However, applying this method with new untested probes could prove to be difficult and time-consuming, and combining different probes for different targets seems problematic. CARD-FISH, in which the signal is amplified with horseradish peroxidase, has been used successfully as well to detect toxic *Microcystis* cells (Metcalf *et al.*, 2009). Furthermore, Stellaris FISH, that uses multiple fluorescently labelled oligonucleotides with

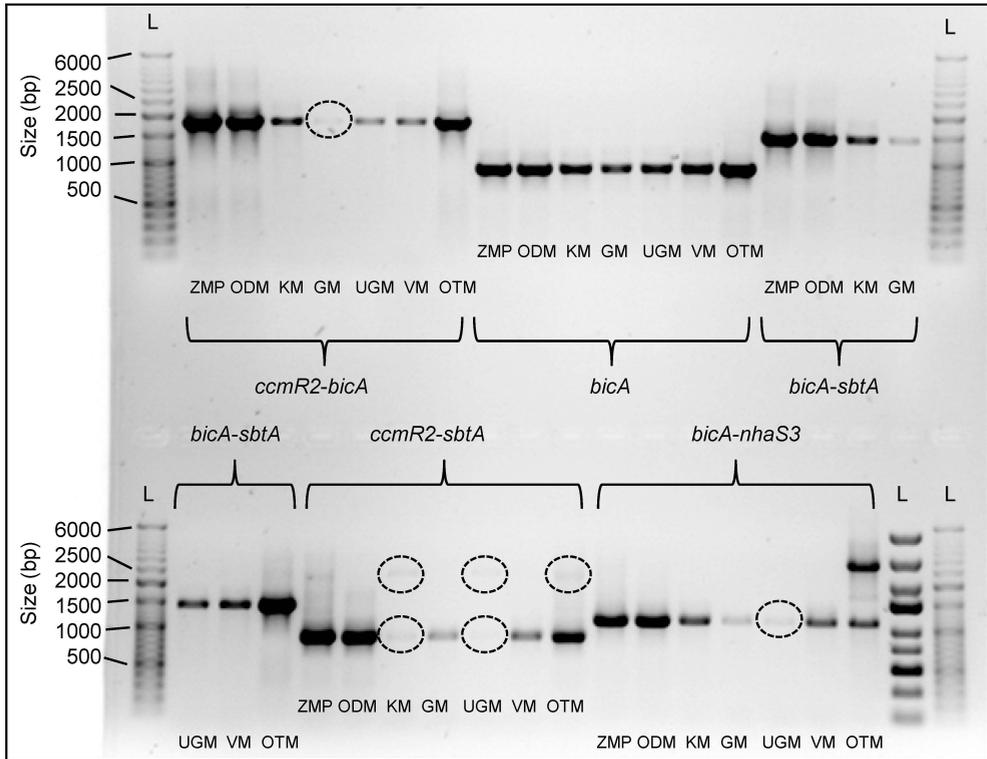
different sequences that bind collectively along the same target to produce a strong signal, might be used to detect specific mRNA or genes in cyanobacterial cells. The fluorescence signal in FISH could also be enhanced by quantum dots (Qdots; Smith and Nie, 2004). These Qdots are fluorescent semiconductor nanoparticles that provide long-term photostability. Although, it might prove difficult to get the relatively large Qdot molecules (2-10 nm) into cyanobacterial cells without destroying the cells completely, they have already been successfully used to detect cyanobacterial proteins (Orcutt *et al.*, 2009).

In conclusion, the ideal technique to study genetic diversity and gene expression will depend largely on the type of experiment. In our experience, qPCR is a powerful method to target specific genes at low concentrations and we expect it will continue to be a popular method in the upcoming years, until (meta)genome and (meta)transcriptome sequencing provide adequate resolution to obtain data about all relevant genes for an affordable price.

### **Genetic variability of the CCM of *Microcystis***

Results of this thesis show that the genomes of *Microcystis* strains differ in the presence or absence of the sodium-dependent bicarbonate uptake genes *bicA* and *sbtA* (**Chapter 2**). We distinguished three different  $C_i$  uptake genotypes in *Microcystis*: *bicA* strains, *sbtA* strains and *bicA+sbtA* strains. In addition to the 20 *Microcystis* strains of **Chapter 2**, we now also analyzed the more recently sequenced genomes of *Microcystis* SPC777 (Fiore *et al.* 2013), TAIHU98 (Yang *et al.*, 2013), NIES-44 (Okano *et al.*, 2015), NIES-2549 (Yamaguchi *et al.*, 2015) and FACHB-1757 (Yi and Rui, unpublished; GenBank sequence CP011339.1), and analyzed strains NIES-1099, PCC 7820 and SAG1785 using PCR with specific primers targeting the CCM genes. These analyses revealed two additional *bicA* strains (FACHB1757 and SAG1785), five *sbtA* strains (NIES-44, NIES-1099, PCC 7820, SPC777 and TAIHU98), and one *bicA+sbtA* strain (NIES-2549).

Analysis of the *Microcystis* community in Lake Kennemermeer showed that all three  $C_i$  uptake genotypes co-occurred in the same lake (**Chapters 5 and 6**). Additional screening of other Dutch lakes (Gooimeer, Oosterduinse Meer, Ottermeer, Uitgeestermeer, Valkenburgse Meer and Zoetermeerse Plas) using PCR and specific primers revealed that each of the three *Microcystis*  $C_i$  uptake genotypes were present in all seven lakes (**Figure 9.1**). This genetic variation in  $C_i$  uptake genes gives *Microcystis* communities the ability to adapt their genotype composition to changes in  $C_i$  conditions (**Chapters 4 and 5**).



**Figure 9.1.** PCR-based detection of the three different *Microcystis*  $C_i$  uptake genotype in samples from seven Dutch lakes. Samples with gDNA from the lakes were exposed to different primers using PCR and the PCR products were subsequently analyzed using gel electrophoresis. The PCR approach targets different regions of the *ccmR2-bicA-sbtA-nhaS3* operon, as described in the Supplementary Information of Chapter 5. L indicates marker lanes. The dashed circles indicate PCR products that are present but hardly visible. The tested samples are from lakes that contained *Microcystis* and were sampled during summer/autumn 2013. ZMP: Zoetermeerse Plas; ODM: Oosterduinse Meer; KM: Kennemermeer; GM: Gooimeer; UGM: Uitgeerstermeer; VM: Valkenburgse Meer; OTM: Ottermeer.

In contrast to the bicarbonate uptake genes *bicA* and *sbtA*, the *cmpABCD* operon encoding for the bicarbonate transporter BCT1 was present in all studied *Microcystis* strains. So, why was this operon not lost in any of the strains? Low sodium concentrations in a lake could make the sodium-dependent bicarbonate transporters BicA and SbtA ineffective, and then BCT1 would be the only suitable uptake system for bicarbonate uptake. Yet, many lakes have sufficiently high sodium concentrations ( $> 1.7 \text{ mmol L}^{-1}$ ) to allow BicA and SbtA to operate above half of their maximum activity (Parker *et al.*, 1997; Price *et al.*, 2004; Du *et al.*, 2014; **Chapter 5**). Alternatively, the directly ATP-dependent bicarbonate transporter BCT1 could be very effective at high light but low  $C_i$  conditions, when plentiful ATP supply via linear and cyclic photophosphorylation is provided, and *cmpABCD* was therefore not lost in

*Microcystis* strains. This would be especially relevant for *Microcystis*, because it often develops surface dwelling blooms that are exposed to high light, and provide shading for other phytoplankton (Ibelings and Maberly 1996; Huisman *et al.*, 2004). Indeed, our field study in Lake Kennemermeer shows that the *cmpA* gene was highly expressed during daytime (**Chapter 6**). There are, however, other cyanobacteria without the *cmpABCD* operon, but with *bicA* and *sbtA*, such as the marine cyanobacterium *Synechococcus* PCC 7002 (Rae *et al.*, 2011).

Genes encoding the two CO<sub>2</sub> uptake systems were present in all strains. Furthermore, there was no variation among the strains in the presence of carboxysome and RuBisCO genes, except for the presence of an additional carboxysomal carbonic anhydrase gene (*ccaA2*) in some of the strains.

The observed genetic diversity in the C<sub>i</sub> uptake systems of *Microcystis* (**Chapter 2**) may be just a snapshot in time. Horizontal gene transfer and recombination events are very common among bacteria. In this way, in response to rising CO<sub>2</sub>, future cyanobacteria could acquire genes for low-affinity C<sub>i</sub> uptake from other cyanobacteria, and may lose genes for high-affinity C<sub>i</sub> uptake. Interestingly, like many other bacteria also for *Microcystis* pili structures were identified that connect cells. These retractable pili structures likely facilitate DNA uptake from the environment, and *Microcystis* might therefore alter its gene pool relatively easily (Nakasugi and Neilan, 2005; Nakasugi *et al.*, 2007).

This thesis provides the first example of a cyanobacterium in which the bicarbonate uptake genes *bicA* and *sbtA* can be found in one and the same operon (**Chapter 2**). Current and future research is focusing on the integration of efficient cyanobacterial CCM genes, including *bicA* and *sbtA*, in C<sub>3</sub> plants to increase the photosynthesis and productivity of various crops (Price *et al.*, 2011a; Price *et al.*, 2013; McGrath and Long, 2014; Pengelly *et al.*, 2014). Recently, the more efficient RuBisCO of a *Synechococcus* strain was successfully integrated in tobacco plants (Lin *et al.*, 2014; Price and Howitt 2014) and *bicA* was integrated in tobacco chloroplasts (Pengelly *et al.*, 2014). Models indicate that the incorporation of bicarbonate uptake systems is a key step that may particularly increase the photosynthetic rate of C<sub>3</sub> plants (McGrath and Long, 2014). *Microcystis* strains with the combination of *bicA* and *sbtA* in one operon might be an interesting donor of these genes for plants, to introduce bicarbonate uptake over a wide range of bicarbonate concentrations.

## Transcriptional regulation of the C<sub>i</sub> uptake genes

*Microcystis* strains display considerable variation in the transcriptional regulation of their C<sub>i</sub> uptake genes. Most *Microcystis* strains downregulated *bicA* and *sbtA* at elevated CO<sub>2</sub> (**Chapters 3 and 4**), and the lake data also showed downregulation of *bicA* and *sbtA* during the increase in C<sub>i</sub> availability at night (**Chapter 6**). However, strain PCC 7005 displayed constitutive high expression of the *bicA-sbtA* operon and strain NIVA-CYA 140, with a transposon insert in complete *bicA*, showed constitutive low expression of the operon. Expression of the *cmpABCD* operon encoding for the BCT1 bicarbonate transporter was reduced at elevated CO<sub>2</sub> in all strains, and showed large diel fluctuations in response to the daily changes in C<sub>i</sub> availability in the lake data (**Chapter 6**). In contrast, expression of the CO<sub>2</sub> uptake genes of *Microcystis* hardly changed in response to elevated CO<sub>2</sub> (**Chapters 3, 4 and 6**). In this respect, *Microcystis* differs from *Synechocystis* and *Synechococcus* strains, in which genes encoding the high-affinity CO<sub>2</sub> uptake system NDH-I<sub>3</sub> are strongly upregulated at low CO<sub>2</sub> levels (Woodger *et al.*, 2003; Wang *et al.*, 2004; Woodger *et al.*, 2005; Eisenhut *et al.*, 2007, Schwarz *et al.*, 2011). Possibly, *Microcystis* attempts to secure imported C<sub>i</sub> as effectively as possible, by a continuous usage of both the high- and low-affinity CO<sub>2</sub> uptake systems to intercept CO<sub>2</sub> leaking from the carboxysomes, because it is often exposed to C<sub>i</sub> limitation in its natural environment. Furthermore, expression of the carboxysome and RuBisCO genes and of carbon assimilation genes in pathways downstream of C<sub>i</sub> fixation hardly changed when *Microcystis* was exposed to elevated CO<sub>2</sub> (**Chapters 3 and 4**). Hence, from the results in this thesis, it is evident that transcriptional regulation of the CCM genes of *Microcystis* occurs mainly at the bicarbonate uptake genes, although transcriptional regulation of *bicA* and *sbtA* varies among *Microcystis* strains (**Chapter 3 and 4**).

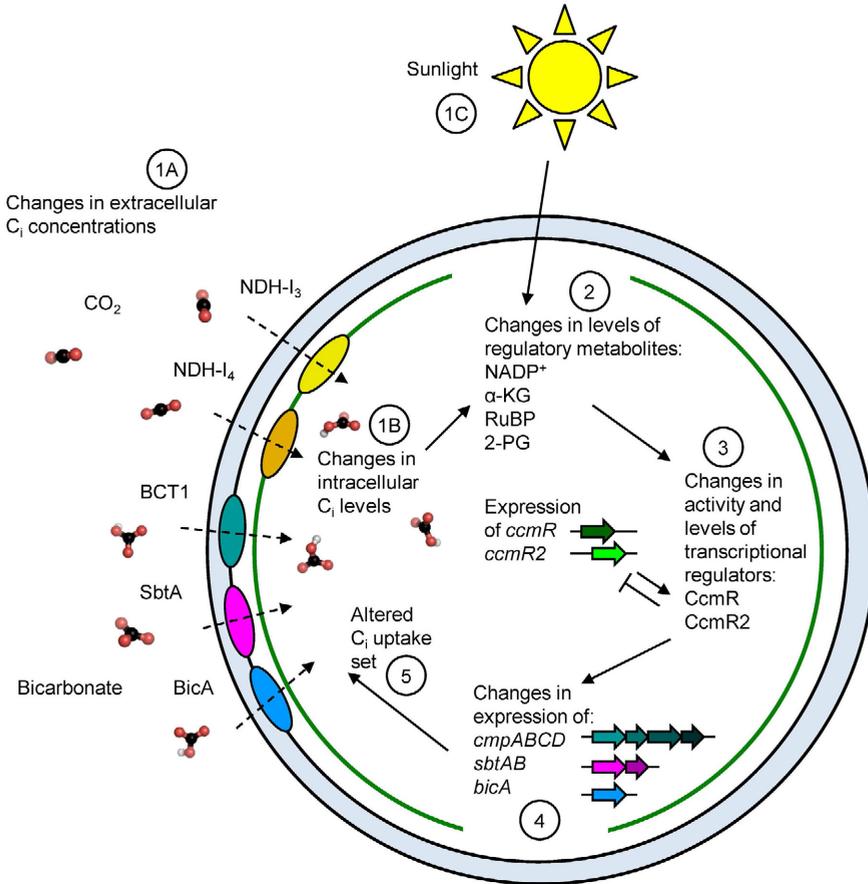
The thesis has identified a new cyanobacterial CCM transcriptional regulator gene, which was called *ccmR2* (**Chapter 2**), in addition to the previously discovered CCM repressor gene *ccmR* (Figge *et al.*, 2001) and activator gene *cmpR* (Omata *et al.*, 2001). The gene *ccmR2* is found upstream of the *bicA-sbtA* operon in *Microcystis* and therefore most likely regulates the expression of *bicA* and *sbtA* in this organism. In other cyanobacteria, *ccmR2* has not been detected so far, and CcmR regulates the expression of *bicA* or *sbtA*, and also the expression of high-affinity CO<sub>2</sub> uptake genes (Wang *et al.*, 2004; Woodger *et al.*, 2007). *Microcystis* lacks the common activator CmpR of the *cmpABCD* operon (Omata *et al.*, 2001). Instead, in *Microcystis*, CcmR likely regulates the expression of *cmpABCD*, because expression of *ccmR* and *cmpA* varied in concert in both our laboratory experiments and lake study (**Chapter 4 and 6**).

The CCM transcriptional regulators are regulated in turn by intracellular concentrations of metabolites whose concentrations depend on the dissolved C<sub>i</sub> levels (**Figure 9.2**). Recently, it was shown that  $\alpha$ -ketoglutarate ( $\alpha$ -KG) and NADP<sup>+</sup> act as co-repressors of

CcmR in *Synechocystis* PCC 6803 (Daley *et al.*, 2012; Burnap *et al.*, 2015). In addition, ribulose-1,5-bisphosphate (RuBP) and 2-phosphoglycerate (2PG) were identified as co-activators of CmpR in *Synechocystis* PCC 6803 (Daley *et al.*, 2012; Burnap *et al.*, 2015).  $\alpha$ -KG is an import metabolite that is used to supply carbon skeletons for nitrogen assimilation, forming a link between C and N metabolism. NADP<sup>+</sup> is a metabolite that can be reduced to NADPH at photosystem I or alternatively NADPH can be oxidized to NADP<sup>+</sup> by the Calvin-Benson cycle. RuBP is a metabolite of the Calvin-Benson cycle which can be converted to 3-phosphoglycerate (3PG) by carboxylation when RuBisCO utilizes CO<sub>2</sub> as substrate, or alternatively the oxidation of RuBP leads to the formation of 2PG when RuBisCO utilizes O<sub>2</sub> as substrate. For example, a shift from high C<sub>i</sub> conditions to C<sub>i</sub>-limitation would increase the NADPH/NADP<sup>+</sup> ratio and lead to relatively lower NADP<sup>+</sup> levels. Furthermore, a shift to C<sub>i</sub>-limitation would increase the 2PG and decrease the  $\alpha$ -KG levels by increased oxygenation of RuBP and decreasing  $\alpha$ -KG formation levels respectively. Because RuBP concentrations probably do not increase during C<sub>i</sub> limitation, 2PG is likely the primary metabolite responsible for activation of CmpR and indirectly the *cmpABCD* operon in *Synechocystis* PCC 6803. For *Microcystis* it is not yet known if CcmR and CcmR2 act as repressor or activator, and which metabolites regulate the activity of CcmR and CcmR2, but NADP<sup>+</sup>, RuBP,  $\alpha$ -KG and 2PG are probable candidates (**Figure 9.2**).

It is currently still difficult to construct *Microcystis* mutants, in contrast to for example *Synechocystis* PCC 6803, because of relatively high intracellular and extracellular nuclease activity and poor growth on agar plates of *Microcystis* (Dittmann *et al.*, 1997). This hampers study of the transcriptional regulators in *Microcystis* in more depth. Yet, in several *Microcystis* strains, changes in expression of the *cmpA* and *bicAlsbtA* genes did not occur concomitantly (**Chapter 4**), suggesting that *ccmR* and *ccmR2* are regulated by different metabolites. Possibly there is also variation between strains in the CCM regulatory metabolites they respond to, considering the different responses in CCM gene expression to elevated CO<sub>2</sub> of, for example, strains PCC 7005 and HUB 5-3 (**Chapter 4**).

In addition to these regulatory metabolites, small RNAs might also affect the transcription of CCM genes, and possibly other enzymes influence the gene products at the post-transcriptional or post-translational level (Burnap *et al.*, 2015).



**Figure 9.2. Proposed regulatory mechanisms of the  $C_i$  uptake systems in *Microcystis*.** Changes in extracellular  $C_i$  availability (1A) can lead to altered intracellular  $C_i$  levels (1B). This, together with light availability (1C) triggers changes in the levels of metabolites, such as NADP<sup>+</sup>, α-ketoglutarate (α-KG), ribulose-1,5-bisphosphate (RuBP) and 2-phosphoglycerate (2PG) (2). These metabolites can alter the activity of the transcriptional regulators CcmR and CcmR2 by allosteric modulation (3). In addition, CcmR and CcmR2 can suppress the expression of *ccmR* and *ccmR2*. Changes in activity and levels of CcmR and CcmR2 lead to changes in expression of the bicarbonate uptake genes *cmpABCD*, *sbtA* and *bicA* (4). Altered expression of the bicarbonate uptake genes will lead to an altered set of  $C_i$  uptake systems (5). The two CO<sub>2</sub> uptake systems NDH-I<sub>3</sub> and NDH-I<sub>4</sub> are expressed constitutively in *Microcystis*.

## Effects of climate change on *Microcystis* blooms

Most of the available literature suggests that especially freshwater cyanobacteria like *Microcystis* will benefit from elevated CO<sub>2</sub> concentrations and rising temperatures, and also from increasing N and P loads associated with climate change (Davis *et al.*, 2009; Paerl and Huisman, 2009; O'Neil *et al.*, 2012; Verspagen *et al.*, 2014b; **Chapter 7**). Indeed, this thesis shows that *Microcystis* strongly increases its biomass when relieved from C<sub>i</sub> limitation (**Chapter 3**), which is in agreement with recent model predictions and chemostat experiments (Verspagen *et al.* 2014b). It is foreseen that rising CO<sub>2</sub> levels will particularly stimulate cyanobacterial blooms in nutrient-rich waters, but less in oligotrophic waters (Verspagen *et al.*, 2014a). The effects of reduced pH values as a consequence of elevated CO<sub>2</sub> concentrations are expected to be small on freshwater cyanobacteria, since they are already accustomed to large fluctuations in pH (Maberly, 1996; Verspagen *et al.*, 2014b; **Chapter 5**).

Freshwater cyanobacteria are often assumed to be especially effective at high pH and low CO<sub>2</sub> conditions, because of the presence of an effective CCM (Shapiro, 1997). Yet, this thesis shows that the CCM differs among strains of *Microcystis* (**Chapter 2**), and varies even more among different cyanobacterial genera (**Chapter 4**). These genetic differences can lead to phenotypic variation in the CO<sub>2</sub> response of *Microcystis* strains (**Chapters 2 and 4**). Hence, it is now clear that cyanobacteria have different optimal C<sub>i</sub> conditions. At low CO<sub>2</sub> concentrations, all *Microcystis* strains rely strongly on bicarbonate uptake. However, the strains use different sets of bicarbonate uptake systems (**Chapters 2 and 4; Table 9.1**), and hence some strains perform better at low bicarbonate concentrations whereas others perform better at high bicarbonate concentrations (**Chapter 5**). At high CO<sub>2</sub> conditions, *sbtA* strains rely mainly on CO<sub>2</sub> uptake, whereas *bicA* and *bicA+sbtA* strains combine CO<sub>2</sub> uptake with bicarbonate uptake (**Chapter 3 and 4; Table 9.1**). The strong dependence on CO<sub>2</sub>(aq) of *sbtA* strains at high CO<sub>2</sub> conditions is in agreement with the study of Verspagen *et al.* (2014b), which showed that the growth rates of strains HUB 5-2-4 and NIVA-CYA 140 were far below their maximum when grown at high bicarbonate, but low CO<sub>2</sub>(aq) concentrations.

The experiments and lake data in this thesis (**Chapters 2-5**) show that *bicA* strains have an advantage at elevated CO<sub>2</sub> conditions. Strains with BicA will benefit especially in environments with high bicarbonate concentrations combined with sodium concentrations >5 mmol L<sup>-1</sup>. The bicarbonate concentration depends on pH and alkalinity, and on the influx of atmospheric CO<sub>2</sub> and mineralization of dissolved organic carbon. Hence, changes in any of these factors can affect the genotype composition of cyanobacterial blooms.

**Table 9.1. Variation in activity of C<sub>i</sub> transporters between *Microcystis* strains at low versus high CO<sub>2</sub> conditions.**

<i>Microcystis</i> strain	C <sub>i</sub> transporters active	
	Low CO <sub>2</sub> conditions	High CO <sub>2</sub> conditions <sup>a</sup>
PCC 7806 ( <i>bicA</i> strain)	CO <sub>2</sub> uptake systems + BCT1 + BicA	CO <sub>2</sub> uptake systems + BicA (↓)
HUB 5-3 ( <i>bicA</i> + <i>sbtA</i> strain)	CO <sub>2</sub> uptake systems + BCT1 + SbtA + BicA	CO <sub>2</sub> uptake systems + SbtA (↓) + BicA (↓)
PCC 7005 ( <i>bicA</i> + <i>sbtA</i> strain)	CO <sub>2</sub> uptake systems + BCT1 + SbtA + BicA	CO <sub>2</sub> uptake systems + SbtA + BicA
NIES-843 + CCAP 1450/10 ( <i>sbtA</i> strain)	CO <sub>2</sub> uptake systems + BCT1 + SbtA	CO <sub>2</sub> uptake systems
NIVA-CYA 140 (inactive <i>bicA-sbtA</i> operon)	CO <sub>2</sub> uptake systems + BCT1	CO <sub>2</sub> uptake systems

<sup>a</sup>A downward arrow indicates that transcription of the gene is downregulated, but that the O<sub>2</sub> optode experiments in Chapters 3 and 4 indicate that the C<sub>i</sub> transporter is still active.

Several previous studies showed that in other cyanobacteria, bicarbonate uptake is also highly active at elevated CO<sub>2</sub> levels (Yu *et al.*, 1994b; Sültemeyer *et al.*, 1995; Benschop *et al.*, 2003; Hopkinson *et al.*, 2014; Eichner *et al.*, 2015). These studies used a membrane inlet mass spectrometer (MIMS) to measure C<sub>i</sub> fluxes according to the method introduced by Badger *et al.* (1994). In cells acclimated to either low or high CO<sub>2</sub> conditions, bicarbonate uptake was predominant at saturating CO<sub>2</sub> and bicarbonate concentrations, at least in strains with the bicarbonate uptake system BicA (**Table 9.2**). These experiment signify the importance of bicarbonate uptake for cyanobacteria containing *bicA*, and can explain the selective advantage of *bicA* strains and to a certain extent *bicA+sbtA* strains at high C<sub>i</sub> concentrations in **Chapter 5**. Future experiments with a MIMS are needed to interrogate the exact CO<sub>2</sub> and bicarbonate uptake rates in *Microcystis* strains grown at ambient and elevated CO<sub>2</sub> concentrations.

**Table 9.2. Contribution of bicarbonate uptake to total C<sub>i</sub> uptake in cyanobacteria provided with saturating CO<sub>2</sub> and bicarbonate concentrations.**

Cyanobacterial strain	C <sub>i</sub> uptake systems	% bicarbonate uptake of total C <sub>i</sub> uptake <sup>a</sup>		Reference
		Low-CO <sub>2</sub> acclimated cells (≤380 ppm)	High-CO <sub>2</sub> acclimated cells (≥ 900 ppm)	
<i>Synechocystis</i> PCC 6803	BCT1, BicA, SbtA, NDH-I <sub>3</sub> , NDH-I <sub>4</sub>	66%	56%	Benschop <i>et al.</i> , 2003
<i>Synechococcus</i> PCC 7002	BicA, SbtA, NDH-I <sub>3</sub> , NDH-I <sub>4</sub>	75%	75%	Sültemeyer <i>et al.</i> , 1995
<i>Synechococcus</i> PCC 7942	BCT1, SbtA, NDH-I <sub>3</sub> , NDH-I <sub>4</sub>	50%	∓ <sup>b</sup>	Yu <i>et al.</i> , 1994a,b; Woodger <i>et al.</i> , 2005; Price <i>et al.</i> , 2008
<i>Trichodesmium erythraeum</i> IMS101	BicA, NDH-I <sub>4</sub>	80%	80%	Kranz <i>et al.</i> , 2009, 2010; Eichner <i>et al.</i> , 2015
<i>Prochlorococcus</i> MED4	BicA2, SbtA2	100%	100%	Hopkinson <i>et al.</i> , 2014

<sup>a</sup>Total C<sub>i</sub> uptake = V<sub>max</sub>(CO<sub>2</sub> uptake) + V<sub>max</sub>(bicarbonate uptake).

<sup>b</sup>In studies of Yu *et al.* (1994a,b), bicarbonate uptake was responsible for 60% of the C<sub>i</sub> uptake in high-CO<sub>2</sub> acclimated cells of *Synechococcus* PCC 7942. However, Price *et al.* (2008) suggested predominance of CO<sub>2</sub> uptake in high-CO<sub>2</sub> acclimated cells of *Synechococcus* PCC 7942 based on the data of Woodger *et al.* (2005).

Climate change may also increase the salinity of some lakes, because of rising sea levels and more intense droughts (Paerl and Huisman, 2009; Paerl and Paul, 2012). Salinization strengthens vertical density stratification, which benefits buoyant cyanobacteria. Furthermore, several studies showed that *Microcystis* can occur in brackish waters, and hence can be quite salt tolerant (Orr *et al.*, 2004; Tonk *et al.*, 2007; Moisaner *et al.*, 2009). However, the results of this thesis indicate that salt tolerance is also one of those traits that varies considerably among *Microcystis* strains. **Chapter 8** shows that although some *Microcystis* strains, such as PCC 7806, are relatively salt tolerant, many *Microcystis* strains lack effective mechanisms to tolerate increasing salinities. Consequently, nearshore freshwater lakes with cyanobacterial blooms that experience increasing salinities due to climate change will likely see major changes in strain composition.

## Increasing toxicity of *Microcystis* with rising CO<sub>2</sub>?

The genetic variation in C<sub>i</sub> uptake genes was not linked to the presence or absence of microcystin synthetase genes (**Chapter 2**). However, elevated CO<sub>2</sub> concentrations led to a large increase in the intracellular microcystin content in *Microcystis* PCC 7806 (**Chapter 3**) and earlier in *Microcystis* HUB 5-2-4 (Van de Waal *et al.*, 2009). It is known that several environmental factors influence the synthesis of microcystins, including *e.g.* light intensity, temperature and the availability of nitrogen, phosphorus, sulphur and iron (**Figure 9.3**; Long *et al.*, 2001; Vezie *et al.*, 2002; Wiedner *et al.*, 2003; Sevilla *et al.*, 2008; Van de Waal *et al.*, 2009; Long, 2010; Neilan *et al.*, 2013). Recent studies connected the production of microcystins by *Microcystis* to protection against oxidative stress (Zilliges *et al.*, 2011; Kaplan *et al.*, 2012; Meissner *et al.*, 2013). In our experiments (**Chapter 3**) and the experiments of Van de Waal *et al.* (2009), elevated pCO<sub>2</sub> released the cells from photo-oxidative stress, by increasing the C<sub>i</sub> concentration in the mineral medium while reducing the light availability through shading from the denser cyanobacterial population (Murata *et al.*, 2007; Latifi *et al.*, 2009).

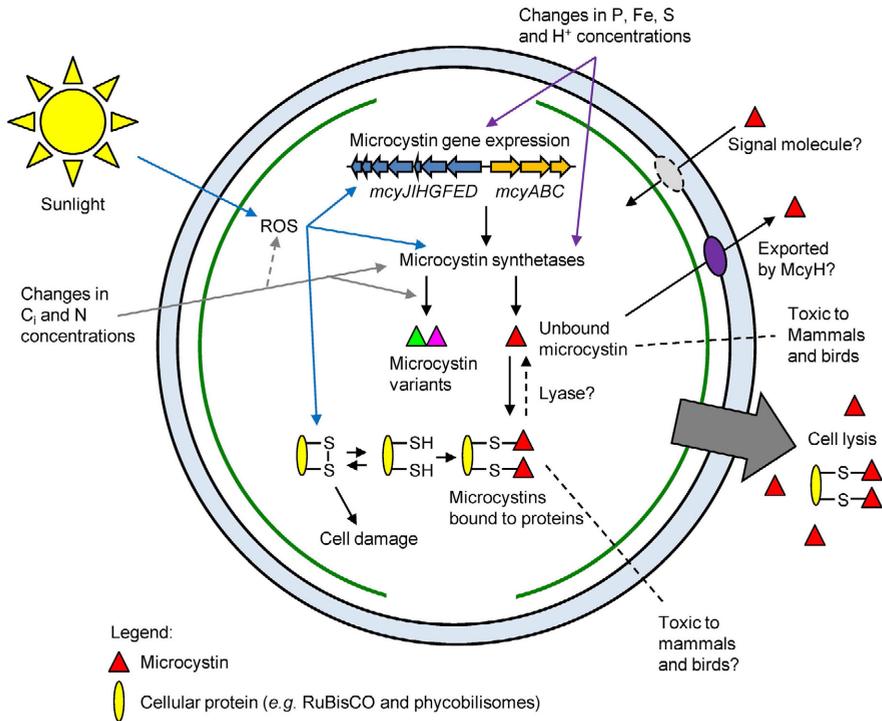
As in most other studies, only the methanol-extractable microcystin was quantified, which does not include microcystin molecules that are covalently bound to protein (Meissner *et al.*, 2013). Hence, potential bond breakage between protein and bound microcystin during the transition from a C<sub>i</sub>-limited to a light-limited state could be a plausible explanation for the observed increase in methanol-extractable microcystins at elevated CO<sub>2</sub> levels, although the formation of the thioether bond between microcystin and a cysteine residue is regarded irreversible (Runnegar *et al.*, 1995). Yet, possibly there is a not yet disclosed enzymatic microcystin-protein lyase present in *Microcystis* that is able to cleave the protein-microcystin covalent bond (**Figure 9.3**). This lyase would free microcystin in times of abundant nutrient supply and growth opportunities that lower oxidative stress, such that microcystin can fill in its well-established role as a toxin. However, such a bond-cleaving enzyme has not yet been found in *Microcystis*. Meissner *et al.* (2013) suggest it is more likely that proteins with bound microcystins are degraded and resynthesized. From this perspective, cells with a higher methanol-extractable microcystin content at elevated CO<sub>2</sub> may have synthesized microcystins *de novo*, in response to the decreased pH and increased C<sub>i</sub> levels.

Microcystins bound to proteins via a thioether bond were suggested to be much less toxic for mammals, including humans (Meissner *et al.*, 2013). Yet, hydrolysis of microcystins by peptidases in the stomach is inadequate and it is likely that a significant amount of microcystin passes the intestinal barrier and is absorbed (Chorus and Bartram, 1999). Peptides with attached microcystin are then actively taken up into hepatocytes and concentrated in the liver (Runnegar *et al.*, 1981). Since the human liver contains enzymes that can actually cleave

thioether bonds (cystathionases; EC. 4.4.1.1), it might be that bound microcystins do play an important role in the toxicity (**Figure 9.3**). This should be investigated in more detail, as the possible risks of protein-bound microcystin are currently not accounted for in water safety regulations.

*Microcystis* blooms often consist of a mixture of microcystin-producing ('toxic') and non-microcystin-producing ('non-toxic') strains (Kurmayer *et al.*, 2002; Kardinaal *et al.*, 2007; Davis *et al.*, 2009). Laboratory competition experiments by Van de Waal *et al.* (2011) and in this thesis (**Chapter 5**) found dominance of toxic strains at low CO<sub>2</sub> conditions, but dominance of non-toxic strains at high CO<sub>2</sub> conditions. These changes in strain composition were explained by the trade-off between affinity and flux rate of their C<sub>i</sub> uptake systems, because the strains differed not only in toxicity but also in their C<sub>i</sub> uptake genes. Yet, the possibility that microcystin production also plays a role cannot be excluded. Microcystin production may offer protection against oxidative stress (Zilliges *et al.*, 2011), which can be induced by *e.g.* C<sub>i</sub>-limited conditions, suggesting that toxic strains might have a competitive advantage at low CO<sub>2</sub> levels whereas non-toxic strains may increase in relative abundance at elevated CO<sub>2</sub> levels. However, in our field study at Lake Kennemermeer (**Chapter 5**), the abundance of toxic strains remained at ~72% despite large changes in the C<sub>i</sub> concentrations.

At present, it is not known why cyanobacteria produce more than 80 different microcystin variants. Possibly, since cyanobacteria fight a war with other plankton as well, different microcystin variations might require different defense mechanisms by other plankton. Another possible explanation for the large diversity of microcystin variants is that toxin synthesis relies on non-ribosomal peptide synthase enzymes, that may be less rigid in amino acid selection than ribosomes would be. This also further complicates any prediction about dominant future toxin variants. Currently, microcystin-LR is one of the most common but also most toxic variants (Zurawell *et al.*, 2005). However, laboratory experiments by Van de Waal *et al.* (2009) indicate that, under nitrogen-rich conditions, elevated CO<sub>2</sub> levels may lead to an increased formation of the less toxic microcystin-RR. This finding might be understood from our results. **Chapter 3** of this thesis shows that rising CO<sub>2</sub> induces glutamine synthetase (*glnV*). Moreover, rising CO<sub>2</sub> levels may also stimulate carbon fixation rates, providing the carbon skeletons (*e.g.*, 2-oxoglutarate) for the conversion of glutamine to glutamate. Glutamate is a direct precursor of the amino acid arginine. In microcystin-RR, the first and second variable amino acid position are both occupied by arginine (R). Indeed, addition of arginine (R) is known to stimulate the production of microcystin-RR (Tonk *et al.*, 2008). Hence, the results of **Chapter 3** provide a physiological explanation for the observation by Van de Waal *et al.* (2009) that elevated CO<sub>2</sub> levels may lead to an increased production of microcystin-RR in strains capable of producing this variant.



**Figure 9.3. Overview of the possible roles of microcystins in *Microcystis*.** Changes in C<sub>i</sub> and N concentrations can affect the intracellular availability of different amino acids, which can lead to altered synthesis rates of microcystins and the production of different microcystins variants. Environmental conditions like P, Fe, S, H<sup>+</sup> concentrations can affect the expression and synthesis of microcystins. Sunlight can lead to the formation of reactive oxygen species (ROS) in the cell which can modify proteins by creating sulfur bridges at cysteine residues of proteins and can cause cell damage. Microcystins can bind to cysteine residues of specific proteins, including RuBisCO and phycobilisomes, protecting them from degradation. ROS can also trigger increased expression of the two *mcy* operons and increased microcystin synthesis by the microcystin synthetases. It is currently unknown if the binding of microcystins to specific proteins is reversible and how toxic protein-bound microcystins are to mammals and birds. Unbound microcystins are possibly partly exported by McyH outside the cell, where microcystins might act as a signal molecule between *Microcystis* cells. This figure was adapted from Kaplan *et al.* (2012), with permission from Frontiers.

In conclusion, the toxicity of *Microcystis* blooms is expected to increase with rising CO<sub>2</sub>, because of increased toxin production of the cells and an increased *Microcystis* cell concentration during blooms. Furthermore, elevated CO<sub>2</sub> may potentially increase the buoyancy of the cells (**Chapter 3**), which can lead to a further accumulation of *Microcystis* cells in the surface layer. This might be counteracted by possible shifts from toxic to non-toxic strains, but convincing evidence for this hypothesis is lacking. Furthermore, there are indications that the ubiquitous variant microcystin-LR might be replaced to some extent by the

less toxic variant microcystin-RR. For future research it would be valuable to further investigate the possible changes in microcystin variants, the role and toxicity of bound versus unbound microcystins, and the production of other polyketides of *Microcystis*.

## Effects of rising CO<sub>2</sub> on other harmful freshwater cyanobacteria

**Chapter 7** showed that intraspecific variation in the presence of the *bicA* and *sbtA* genes is not unique for *Microcystis*. Similar variation can also be found in other harmful cyanobacteria such as *Anabaena*, *Aphanizomenon* and *Planktothrix*. Moreover, some *Anabaena*, *Aphanizomenon* and *Planktothrix* strains even lack both *bicA* and *sbtA* genes, and hence form a fourth C<sub>i</sub> uptake genotype, whereas in *Microcystis* either *bicA* or *sbtA* was always present so far. The genes encoding the two CO<sub>2</sub> uptake systems and the BCT1 bicarbonate transporter were found in all strains of *Anabaena*, *Aphanizomenon* and *Planktothrix*, similar to *Microcystis*. However, only the CCM transcriptional regulator gene *ccmR* was detected in the *Anabaena*, *Aphanizomenon*, and *Planktothrix* strains, but not the transcriptional regulator gene *ccmR2* found so far only in *Microcystis* (Sandrini *et al.*, 2014) and neither the gene *cmpR* found in some other cyanobacteria (Omata *et al.*, 2001). Presumably, the genetic diversity of C<sub>i</sub> uptake systems in *Anabaena*, *Aphanizomenon*, and *Planktothrix* produces a similar phenotypic variation as in *Microcystis*, with a selective advantage for strains with *sbtA* at very low CO<sub>2</sub> conditions and a selective advantage for strains with *bicA* at high CO<sub>2</sub> conditions. The ability of *Anabaena*, *Aphanizomenon*, and *Planktothrix* strains to synthesize microcystins was not linked to the absence or presence of the various C<sub>i</sub> uptake systems, as was shown before for *Microcystis* (Sandrini *et al.*, 2014).

Often *Microcystis* co-exists with *Anabaena*, *Aphanizomenon*, and *Planktothrix* in the same lake. However, *Aphanizomenon* and *Anabaena* are able to fix nitrogen using heterocysts, while the other two genera cannot. Therefore, *Anabaena* and *Aphanizomenon* are often found in nitrogen-limited lakes or earlier in the season when low concentrations of nitrate and ammonium can limit phytoplankton growth. Then, later during the season, other cyanobacterial genera, like *Microcystis* and *Planktothrix*, can take over, which are strong competitors when enough bound nitrogen is available (Barica *et al.*, 1980). Furthermore, all four genera can produce gas vesicles, and hence can float upwards to dominate the surface layer (Walsby, 1994; Huisman *et al.*, 2004). *Planktothrix agardhii* is typically found in shallow lakes, whereas *Microcystis* is often found in deep lakes with a high stability due to temperature stratification during summer (Tonk *et al.*, 2005; Kardinaal *et al.*, 2007). *Microcystis* also thrives well in shallow lakes (Chen *et al.*, 2003; Verspagen *et al.*, 2006) and other *Planktothrix* species such as *P. rubescens* have their niche in deep lakes (Jacquet *et al.*, 2005; Cuyper *et al.*, 2011). Other factors, like phosphate, iron, temperature, salinity and grazing zooplankton, also

influence the dominance of different cyanobacterial species (Paerl *et al.*, 2001; Huisman *et al.*, 2005; Paerl and Huisman, 2009). The preference for different lake conditions indicates that, despite rising CO<sub>2</sub> concentrations, this diversity of cyanobacterial genera will likely persist. However, within each genus, the relative abundances of the different C<sub>i</sub> uptake genotypes and, hence, the genetic composition of harmful cyanobacterial blooms, will likely be altered in response to elevated CO<sub>2</sub>.

## Effects of rising CO<sub>2</sub> on marine cyanobacteria

While freshwater systems can show large fluctuations in C<sub>i</sub> and pH (Maberly, 1996; Sobek *et al.*, 2005; Verspagen *et al.*, 2014b; **Chapter 5**), marine systems typically show much smaller fluctuations in C<sub>i</sub> and pH. Freshwater cyanobacteria, including *Microcystis*, mostly contain  $\beta$ -type carboxysomes. In contrast, most marine cyanobacteria contain an  $\alpha$ -type carboxysome (Rae *et al.*, 2011). The  $\alpha$ - and  $\beta$ -type carboxysome are formed by different gene products, but are functionally highly similar (Whitehead *et al.*, 2014). *Synechococcus* and *Prochlorococcus* are the two main cyanobacterial genera found in the oceans (Garcia-Pichel *et al.*, 2003).

It was previously shown that *Synechococcus* strains can have either an  $\alpha$ - or  $\beta$ -type carboxysome, and that there is a large diversity in the set of C<sub>i</sub> uptake systems present in the different strains of this genus (Rae *et al.*, 2011). Interestingly, most  $\alpha$ -type marine *Synechococcus* strains of the open ocean lack all three high-affinity C<sub>i</sub> uptake systems SbtA, BCT1 and NDH-I<sub>3</sub>, in marked contrast to the  $\beta$ -type freshwater strains. The lack of high-affinity C<sub>i</sub> uptake systems in oceanic cyanobacteria is in agreement with the C<sub>i</sub> availability in the oligotrophic oceans, where the CO<sub>2</sub>(aq) concentration is seldom depleted. Coastal *Synechococcus* strains can have a more elaborate set of C<sub>i</sub> uptake systems, which they seem to have acquired from freshwater cyanobacteria through horizontal gene transfer and which enables them to deal more effectively with fluctuations in C<sub>i</sub> availability (Rae *et al.*, 2011).

*Prochlorococcus* is a ubiquitous cyanobacterium in the oceanic subtropical gyres, and known as an oligotrophic specialist with its streamlined genome (Partensky and Garczarek, 2010). In *Prochlorococcus*, the known C<sub>i</sub> uptake genes of cyanobacteria are lacking, despite the presence of an  $\alpha$ -type carboxysome in this genus (Rae *et al.*, 2011; Roberts *et al.*, 2012). Yet, Palinska *et al.* (2002) showed that *Prochlorococcus* is capable of bicarbonate uptake and it was found that *Prochlorococcus* strains do contain genes that encode for homologues resembling BicA and SbtA, named BicA2 and SbtA2 (Hopkinson *et al.*, 2014; Gaudana *et al.*, 2015). Similar to *Microcystis* (**Chapter 2**), it is possible that *Prochlorococcus* also has two different bicarbonate transporter genes (*bicA2* and *sbtA2*) in one operon (Gaudana *et al.*, 2015). However, several BicA and SbtA homologues did not appear to be actual bicarbonate transporters (Rae *et al.*, 2011). For example, *sbtA2* of *Microcystis* (locus tag *IPF\_6431* in strain

PCC 7806) most likely does not encode for a bicarbonate transporter, because SbtA2 shares only ~24% sequence homology with SbtA, and expression of *sbtA2* was slightly increased in response to elevated CO<sub>2</sub> instead of lowered like the actual bicarbonate uptake genes (**Chapter 3**). Furthermore, the bicarbonate transporter BicA also shares substantial homology with sulfate transporters (Price *et al.*, 2004). Hopkinson *et al.* (2014) did not see a strong change in expression of the two candidate bicarbonate uptake genes *bicA2* and *sbtA2* of *Prochlorococcus* in response to changing CO<sub>2</sub> conditions, although it is possible that the genes are expressed constitutively, as was observed in this thesis for *Microcystis* PCC 7005 (**Chapter 4**). Remarkably, active CO<sub>2</sub> uptake systems appear to be lacking in *Prochlorococcus* (**Table 9.2**; Hopkinson *et al.*, 2014).

Fu *et al.* (2007) investigated the effect of increased temperatures and CO<sub>2</sub> concentrations on the marine cyanobacteria *Synechococcus* sp. CCMP1334 containing BicA, BicA2 and NDH-I<sub>4</sub>, and *Prochlorococcus* CCMP1986 containing BicA2 and SbtA2. The results showed that the *Synechococcus* strain profited strongly from increased temperatures and CO<sub>2</sub> levels, but the *Prochlorococcus* strain did not. This implies that climate change could influence the dominance relationships among *Synechococcus* and *Prochlorococcus* in the oceans.

Effects of rising CO<sub>2</sub> have also been investigated for marine diazotrophic (nitrogen-fixing) cyanobacteria. The responses varied among species and strains, as reviewed in **Chapter 7**. *Trichodesmium erythraeum* IMS101 possesses the high-flux C<sub>i</sub> uptake systems BicA and NDH-I<sub>4</sub> (**Table 9.2**; Kranz *et al.*, 2011). Therefore, in line with expectation, nitrogen fixation rates of *Trichodesmium erythraeum* IMS101 strongly increase with elevated CO<sub>2</sub> (Hutchins *et al.*, 2007; Levitan *et al.*, 2007; Kranz *et al.*, 2010; Garcia *et al.*, 2011). However, Hutchins *et al.* (2013) found large intraspecific variation in the response of nitrogen fixation rates to elevated CO<sub>2</sub> among different strains of *Trichodesmium* and *Crocospaera*. This indicates that these strains may differ in the presence and/or expression of their C<sub>i</sub> uptake systems, similar to the variation among *Microcystis* strains described in this thesis.

The findings reviewed in this section imply that rising CO<sub>2</sub> concentrations could also constitute an important selective force shaping the genetic diversity and community composition of marine cyanobacteria. Hence, selection experiments are recommended to get a better idea of which marine cyanobacteria are favored at elevated CO<sub>2</sub>.

## Effects of rising CO<sub>2</sub> on eukaryotic algae

Eukaryotic algae can also employ a CCM, yet it operates differently from the CCM typically found in cyanobacteria. In *Chlamydomonas reinhardtii*, the CCM is based on a pH gradient that is set up across the chloroplast thylakoid membrane (Moroney and Ynalvez 2007; Moroney *et al.*, 2011). In light, the chloroplast stroma has a pH of ~8 and the thylakoid lumen

a pH of 4-5, at which bicarbonate is the dominant  $C_i$  species in the chloroplast stroma, while  $CO_2$  is the dominant  $C_i$  species in the thylakoid lumen. Bicarbonate transported into the thylakoid lumen is thus converted to  $CO_2$ , and in this way increases the local  $CO_2$  concentration in the thylakoid lumen as well as in the adjacent pyrenoid in which RuBisCO is located. Several carbonic anhydrases and bicarbonate transporters are involved in this CCM (Spalding, 2008), yet much less is known about the CCM genes and proteins of eukaryotic algae compared to cyanobacteria.

An interesting selection experiment revealed that when *C. reinhardtii* was exposed to elevated  $CO_2$  for 1,000 generations, some cell lines lost the ability to induce high-affinity  $CO_2$  uptake (Collins and Bell 2004; Collins *et al.*, 2006). This was assigned to mutations in CCM genes. This firstly shows that *Chlamydomonas*, similar to cyanobacteria, likely has a set of high-affinity and low-affinity  $C_i$  uptake genes, and secondly that eukaryotic algae can evolve in response to elevated  $CO_2$ . In contrast to these results, Low-Décarie *et al.* (2013) indicated that they found no evidence for irreversible evolutionary change in response to elevated  $CO_2$  in long-term evolution experiments with seven axenic freshwater phytoplankton species (including five eukaryotic species and two cyanobacteria). A possible explanation for the lack of evolutionary change in their experiments is that Low-Décarie *et al.* (2013) worked with axenic strains, and hence did not introduce genetic variation in their experiments. Their results differ markedly from our laboratory competition experiments and field study at Lake Kennemermeer (Chapter 5), which showed that elevated  $CO_2$  can rapidly alter the genetic composition of *Microcystis* populations, by selecting for *bicA* strains without the high-affinity bicarbonate uptake gene *sbtA*.

## Combating harmful blooms

Eutrophication and climate change are foreseen to intensify harmful cyanobacterial blooms. To counter the increasing dominance of harmful cyanobacteria like *Microcystis*, eutrophication of lakes should be halted or reversed by nutrient reduction measures and other effective treatments against harmful cyanobacterial blooms. Examples of successful lake treatments include hydrogen peroxide addition ( $H_2O_2$ ; Matthijs *et al.*, 2012) and artificial mixing of deep lakes (Visser *et al.*, 1996; Huisman *et al.*, 2004). Potassium addition has also been suggested, because earlier reports indicated that *Microcystis* can be very sensitive to potassium (Parker *et al.*, 1997). However, the results of Chapter 8 show that potassium sensitivity varies among *Microcystis* strains. In particular, salt-tolerant *Microcystis* strains are also tolerant of high potassium concentrations. Hence, the application of potassium to combat *Microcystis* blooms might be successful in freshwater lakes on the short-term, but in due time salt-tolerant

*Microcystis* strains and other (potentially harmful) cyanobacterial species are likely to succeed the suppressed *Microcystis* strains (**Chapter 8**).

## Conclusions

This thesis has contributed to a better understanding of how harmful cyanobacteria deal with changes in  $C_i$  concentrations in their natural habitat. The results demonstrate that harmful cyanobacterial blooms are well-adapted to large diel and seasonal fluctuations in  $C_i$  concentrations, and show how cyanobacteria subtly adjust their cells at the molecular and physiological level to changes in  $C_i$  availability. Harmful cyanobacteria are therefore likely to benefit strongly from increasing  $CO_2$  levels in eutrophic lakes. In particular, elevated  $CO_2$  levels will favor the dominance of cyanobacterial strains with the low-affinity but high-flux  $C_i$  uptake gene *bicA*.

Beyond the cyanobacteria, one of the key lessons of this work is that future climate change studies should keep in mind the large genetic and physiological variation within species. It would be interesting and valuable to further elucidate the (diversity of) CCMs in other species, to assess how rising  $CO_2$  concentrations may affect the species composition of our planet in the coming decades.