Effects of rising CO₂ on the harmful cyanobacterium Microcystis
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Chapter 1

General introduction
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Rising CO$_2$ concentrations

Atmospheric CO$_2$ concentrations are rising globally due to human activities since the industrial revolution. Today, the CO$_2$ concentration is ~400 ppm, while in 1800 the atmosphere contained only ~280 ppm CO$_2$ (Figure 1.1; Solomon et al., 2007).

![Figure 1.1. Atmospheric CO$_2$ concentration of the last 2000 years.](image)

Since 1958, the atmospheric CO$_2$ concentration is measured continuously at different places, including Mauna Loa, Hawaii. The data before 1958 is largely based on ice drillings from Antarctica and Greenland. This figure was adapted from Solomon et al. (2007), with permission from Cambridge University Press.

An overview of the global carbon cycle, in Figure 1.2, shows that annual human-induced CO$_2$ emissions are relatively small compared to respiration by and decomposition of plants, phytoplankton and other microorganisms. Yet, these emissions lead to a net annual increase in atmospheric CO$_2$ of ~4 ppm. Climate change studies predict that in the year 2100, the CO$_2$ concentration will reach 550-1,000 ppm CO$_2$ (Solomon et al., 2007).

CO$_2$ concentrations have been a lot higher in the past, reaching even 3,000 or 5,000 ppm, 150 and 375 million years ago respectively (Igamberdiev et al., 2006), yet the last 20 million years the CO$_2$ concentration appeared relatively stable between 140 and 320 ppm (Pearson and Palmer, 2000). A major influence on the atmospheric CO$_2$ concentration in the past has been the increased weathering (breakdown of rock minerals) by plants, during the Devonian period (~420-360 million years ago; Berner, 1998). Around this period, active C$_4$ fixation by plants made the CO$_2$ concentration drop from ~5,000 to ~500 ppm CO$_2$, while the O$_2$ concentration strongly increased from ~11 to ~22%. The active volcanos of the Siberian
traps (the term ‘trap’ was derived from the Swedish word for stairs, ‘trappa’, referring to the step-like hills of the landscape) during the End-Permian period (~252 million years ago), provide an example of a cause for a strong increase in atmospheric CO₂ in the past, when large amounts of coal were combusted (Ogden and Sleep, 2012), analogous to the human-induced CO₂ emissions from burning coal. The CO₂ increased from ~400 to 2200 ppm around the End-Permian period (Igamberdiev et al., 2006). The active Siberian traps led to the greatest mass extinction event in the Earth’s History, called the Great Dying (Clapham, 2013).

Figure 1.2. The global carbon cycle. The white numbers indicate stored carbon, the yellow numbers indicate natural fluxes, and the red numbers indicate carbon from human emissions in gigatons C per year. The values in parentheses (in gigatons C) are based on Houghton (2007). This figure was reproduced with permission from the United States Department of Energy (US DOE, 2008).

As a consequence of the current rise of the atmospheric concentrations of CO₂ and other greenhouse gases, such as methane, nitrous oxide and fluorocarbon compounds, global temperatures are increasing (Solomon et al., 2007). These trends have large effects on life on
our planet. My thesis focuses on the effects of rising CO$_2$ on cyanobacteria, and in particular highlights the adaptive mechanisms of the harmful cyanobacterium *Microcystis aeruginosa*.

**Chemistry of CO$_2$ in water**

When CO$_2$ dissolves in water, it also reacts with water to form carbonic acid (H$_2$CO$_3$). Carbonic acid is a weak acid, which can dissociate in bicarbonate (HCO$_3^-$), carbonate (CO$_3^{2-}$) and protons. The solubility of CO$_2$ in water increases with a higher partial pressure of CO$_2$. Therefore, elevated atmospheric CO$_2$ concentrations tend to increase the concentrations of dissolved inorganic carbon (DIC; CO$_2$, bicarbonate and carbonate) and lower the pH of aquatic ecosystems. The relative amounts of the inorganic carbon (C$_i$) species depend on the pH of the environment. At low pH there is mainly dissolved CO$_2$ (CO$_2$(aq)), at pH of about 8 there is mainly bicarbonate, and at high pH levels carbonate is the most dominant carbon species (Figure 1.3). Mineralization of dissolved organic carbon (DOC) from surrounding watersheds and sediments by bacteria adds to the C$_i$ supply of lakes.

![Figure 1.3. Relative abundances of the different inorganic carbon (C$_i$) species as a function of pH. Dissolved CO$_2$ (CO$_2$(aq)) is indicated in red, bicarbonate (HCO$_3^-$) in green and carbonate (CO$_3^{2-}$) in blue.](image)

In fact, many lakes are oversaturated by CO$_2$ (as high as 10,000 ppm CO$_2$), while other lakes are undersaturated due to CO$_2$ uptake by phytoplankton blooms (down to 20 ppm CO$_2$) (Talling, 1976; Cole *et al.*, 1994; Sobek *et al.*, 2005). Lakes with dense phytoplankton blooms therefore show large changes in CO$_2$(aq) concentrations during a year. For example, Verspagen *et al.* (2014b) recorded changes in C$_i$ concentrations and pH of Lake Volkerak, a lake in the Netherlands that is often overwhelmed by dense phytoplankton blooms in summer and early autumn. They observed an increase in pH from 7.8 in winter to 9.3 during the
General introduction

summer bloom, accompanied by a decrease in CO$_2$(aq) from 55 to 2 µmol L$^{-1}$. In contrast, in equilibrium with the atmosphere one would expect a CO$_2$(aq) concentration of 13-27 µmol L$^{-1}$ depending on the temperature and salinity. During late autumn, winter and spring, the lake was supersaturated with CO$_2$ and lost carbon to the atmosphere, while in times with a dense phytoplankton bloom the lake was undersaturated and acted as a sink for atmospheric CO$_2$. In addition, the diel variation of pH and CO$_2$(aq) can also be strong in lakes with dense phytoplankton blooms. For example, during the summer bloom in Lake Esthwaite Water (UK) the pH sometimes increased up to 1.8 units during daytime and the diel variation in CO$_2$(aq) was up to -30 µmol L$^{-1}$ (Maberly, 1996).

Elevated atmospheric CO$_2$ concentrations will likely have the strongest effect on phytoplankton in lakes that experience CO$_2$ undersaturation. CO$_2$ undersaturation creates a gradient in partial pressure of CO$_2$ across the air-water interface, which leads to an influx of CO$_2$ from the atmosphere into the lake (Balmer & Downing, 2011). More specifically, the CO$_2$ influx ($g_{CO_2}$) is proportional to the difference between the expected concentration of dissolved CO$_2$ in water (CO$_2$(aq)) when in equilibrium with the atmosphere (calculated from Henry’s law) and the actual concentration of CO$_2$(aq) (Siegenthaler and Sarmiento; 1993; Cole et al., 2010):

$$g_{CO_2} = v \left( K_H p_{CO_2} - CO_2(aq) \right)$$

where $v$ is the gas transfer velocity (also known as piston velocity) across the air-water interface, $K_H$ is the solubility constant of CO$_2$ gas in water, and $p_{CO_2}$ is the partial pressure of CO$_2$ in the atmosphere. If it is assumed that a dense phytoplankton bloom has depleted the surface layer of CO$_2$(aq), this equation simplifies to $g_{CO_2} = v K_H p_{CO_2}$. Hence, during dense summer blooms, an increase in atmospheric pCO$_2$ will lead to a greater influx of CO$_2$. For instance, a doubling of the pCO$_2$ concentration from 400 to 800 ppm would lead to a two times greater influx of CO$_2$ into the lake during dense blooms.

Cyanobacteria

Cyanobacteria are Gram-negative bacteria that obtain their energy through oxygenic photosynthesis. They can have diverse morphologies, ranging from unicellular spheres or rods to large multicellular structures like filaments or colonies (Figure 1.4). Cyanobacteria can be found in a wide range of environments: in oceans, lakes, soils and on rocks, over a wide range of temperatures and light availability. They can be found in your backyard pond, in the Sahara desert and even in the Antarctic. Cyanobacteria are estimated to be responsible for ~15% of the current global net primary production (Garcia-Pichel et al., 2003; Field et al., 1998; Flombaum et al., 2013). They are also among the oldest organisms on the planet (Schopf, 1993) and it has been suggested that by producing oxygen through photosynthesis, they are
responsible for the Great Oxidation Event, ~2.4 billion years ago, dramatically changing life on Earth (Schirrmmeister et al., 2013). In addition, chloroplasts from plants are believed to originate from cyanobacterial endosymbiosis (McFadden, 2001). Cyanobacteria have not only been frontrunners in the oxygenation of the Earth atmosphere, they also contribute largely to the fixation of atmospheric nitrogen, that cannot be acquired independently by eukaryotes. The excretion of fixed nitrogen and carbon compounds into the environment caters many other organisms that rely directly on this supply.

Figure 1.4. Biodiversity of cyanobacteria. (A) Microcystis sp. (colony). (B) Planktothrix sp. (C) Aphanizomenon sp. (D) Nostoc sp. (E) Synechocystis sp. PCC 6803. (F) Synechococcus sp. BS5. (G) Anabaena sp. (H) Anabaena sp. (I) Anabaena sp. Photos: Giovanni Sandrini.

All oxygenic phototrophs use light from the sun as energy source. For this purpose, the energy present in the photosynthetic active radiation (PAR; from blue 400 nm to far-red 700 nm) region of the sunlight spectrum is absorbed by photosynthetic pigments. For example, the pigment chlorophyll, that can be found in both cyanobacteria and plants, is bound to functional light trapping units in multiple protein complexes that are called photosystems.
The photosynthesis reactions can be divided into two processes, the light-dependent and light-independent reactions (the latter are also called dark reactions). In the light-dependent reactions, the energy from sunlight is used to split the electron donor water into O₂, protons and electrons (Figure 1.5). Excitation of electrons leads to charge separation over the electrically insulating thylakoid membrane. Like in a battery, this voltage span can be used for electron flow. The excited electrons pass through a range of carriers - from Photosystem II (PSII) via plastoquinone and cytochrome \( b_{ef} \) to Photosystem I (PSI) - that use the energy to translocate protons over the thylakoid membrane, which creates a proton gradient that installs a proton-motive force (Campbell et al., 1998; Figure 1.5). The latter is used to drive an ATP-synthetase enzyme by which the energy gained from photons is stored in the energy-rich biomolecule ATP. At PSI the electrons are re-energized, which is used for the production of NADPH. ATP and NADPH are both molecules that are used for virtually all metabolic processes in cells, including CO₂ fixation with the Calvin-Benson cycle (i.e., the dark reactions).

Figure 1.5. Light reactions of photosynthesis at the thylakoid membrane of cyanobacteria with the electron and proton flows indicated. The displayed structures are from *Thermosynechococcus elongatus* (PSII, PDB ID 4V62), *Nostoc* sp. PCC 7120 (cytochrome \( b_{ef} \), PDB ID 4H44), *Synechococcus elongates* (PSI, PDB ID 1JB0 and Fd: ferredoxin, PDB ID 2JCO), *Anabaena* sp. PCC 7119 (FNR: ferredoxin-NADP⁺ reductase, PDB ID 1QUE), *Synechocystis* sp. PCC 6803 (PC: plastocyanin, PDB ID 1JXD) and *E. coli* (ATP synthase, PDB IDs 1C17 and 1JNV). PQ: plastoquinone and PQH₂: plastoquinol. Solid lines indicate electron (\( e^- \)) and proton (H⁺) transfer and the dashed line shows PSI-driven cyclic electron flow. The figure was adapted from Kurisu et al. (2003) and Yeremenko (2004), with permission from the American Association for the Advancement of Science and Nataliya Yeremenko.
The CO₂-concentrating mechanism of cyanobacteria

Phytoplankton, including cyanobacteria, use the enzyme RuBisCO for CO₂ fixation. Since RuBisCO has a low affinity for CO₂, and can also use O₂ as substrate in a process called photorespiration, many cyanobacteria evolved a CO₂-concentrating mechanism (CCM) (Kaplan and Reinhold, 1999; Raven et al., 2008). As a first step, bicarbonate is taken up by specific uptake systems and CO₂ diffusing into the cell is converted to bicarbonate by specific protein complexes. The accumulated bicarbonate then diffuses from the cytoplasm into specialized compartments, called carboxysomes (Figure 1.6; Price, 2011). In the carboxysomes, carbonic anhydrases convert bicarbonate to CO₂. RuBisCO, then surrounded by a high CO₂ concentration, incorporates CO₂ into the Calvin-Benson cycle, which uses energy from the light reactions (ATP and NADPH) and CO₂ to create organic compounds (Figure 1.7). The net reaction of photosynthesis (light-driven production of fixed carbon) for plants, algae and cyanobacteria is 6 CO₂ + 6 H₂O → C₆H₁₂O₆ + 6 O₂.

Five cyanobacterial Ci uptake systems are known so far, three for bicarbonate uptake and two for conversion of CO₂ to bicarbonate (Price, 2011; Figure 1.6). The Ci uptake systems have different characteristics and are dependent on different co-factors, such as sodium, calcium, ATP or NADPH. The diverse set of Ci uptake systems allows cyanobacteria to respond effectively to variations in Ci availability (Burnap et al., 2015). Yet, not all five Ci uptake systems are present in each cyanobacterium (Badger et al., 2006; Rae et al., 2011). Indeed, several studies have indicated strong dependence on CO₂ uptake for some cyanobacteria (Woodger et al., 2005; Rae et al., 2011; Verspagen et al., 2014b), whereas other studies have indicated that bicarbonate uptake is the predominant form of Ci uptake in cyanobacteria at both low and high CO₂ conditions (Sültemeyer et al., 1995; Benschop et al., 2003; Rae et al., 2011; Hopkinson et al., 2014; Eichner et al., 2015).

The first bicarbonate uptake system, BCT1, has a relative high affinity for bicarbonate ($K_{0.5} = 10-15 \text{ µmol L}^{-1}$ bicarbonate) but low flux rate (Omata et al., 1999; Omata et al., 2002). BCT1 is directly ATP-dependent, uses Ca²⁺ as a co-factor and is encoded by the cmpABCD operon, which shares high similarity with the nitrate transporter operon nrtABCD (Omata et al., 1999; Koropatkin et al., 2007). The gene cmpA encodes for the substrate-binding periplasmic segment, cmpB encodes for the membrane integral component, while cmpC and cmpD encode for cytoplasmic domains of the transporter BCT1 (Maeda et al., 2000; Omata et al., 2002).

The other two bicarbonate uptake systems are called BicA and SbtA. BicA has a low to low affinity ($K_{0.5} = 70-350 \text{ µmol L}^{-1}$ bicarbonate) but high flux rate, whereas SbtA usually has a high affinity ($K_{0.5} \leq 5 \text{ µmol L}^{-1}$ bicarbonate) but low flux rate (Price et al., 2004; Du et al., 2014). Both transporters are sodium-dependent symporters (Shibata et al., 2002; Price et
General introduction

BicA is encoded by a single gene, *bicA*, and shares distant homology to plant sulfate transporters, and is therefore often incorrectly annotated. SbtA is also encoded by a single gene, *sbtA*, and frequently another gene, *sbtB*, is found downstream. Although SbtB is not essential for SbtA functioning (Price, 2011), recently it was shown that SbtB functions as a post-translational regulator of SbtA, presumably inactivating SbtA during darkness (Du et al., 2014). Several freshwater cyanobacteria also hold SbtA and BicA homologues, called SbtA2 and BicA2 (Rae et al., 2011), but the function of these proteins remains unknown, and no evidence has been published that they are involved in C\textsubscript{i} uptake.

All three bicarbonate uptake systems, BCT1, BicA and SbtA, are located in the plasma membrane (Price, 2011). Additionally, bicarbonate porins can be present in the outer membrane of cyanobacteria to assist in C\textsubscript{i} uptake, by transporting bicarbonate through the outer membrane into the periplasmic space (Figure 1.6).

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**Figure 1.6. Overview of the cyanobacterial CO\textsubscript{2}-concentrating mechanism.** Up to five different C\textsubscript{i} uptake systems can be found in cyanobacteria. These C\textsubscript{i} uptake systems have different affinities and flux rates, and are dependent on different co-factors. In carboxysomes, accumulated bicarbonate is converted to CO\textsubscript{2} via carbonic anhydrase (CA) enzymes, surrounding RuBisCO (PDB ID 1RBL) by a high CO\textsubscript{2} concentration. RuBisCO fixes CO\textsubscript{2} by the production of 3-phosphoglycerate (3PG). However, RuBisCO can also use O\textsubscript{2} as substrate, in a process called photorespiration, which produces toxic 2-phosphoglycerate (2PG). CO\textsubscript{2} leakage from the carboxysomes can be partly intercepted by the CO\textsubscript{2} uptake systems (shown by the dashed lines). This figure was based on Badger et al. (2006), with permission from Oxford University Press.
Figure 1.7. The Calvin-Benson cycle. The cycle is used to convert CO₂ into more complex molecules such as carbohydrates and starch. These reactions are also called the dark reactions. The number of carbon atoms in the molecules is indicated in blue.

The two CO₂ uptake systems consist of modified plastoquinone oxidoreductase NADPH dehydrogenase (NDH-I) respiratory complexes. The CO₂ uptake systems convert CO₂ into bicarbonate to trap the C₅ inside the cell. NADPH functions as the electron donor for conversion of CO₂ into bicarbonate (Price et al., 2002; Figure 1.6). While NDH-I₃ has a relatively high affinity (Kₐ₅ = 1-2 µmol L⁻¹ CO₂) but low flux rate, NDH-I₄ has a lower affinity (Kₐ₅ = 10-15 µmol L⁻¹ CO₂) but higher flux rate (Maeda et al., 2002; Price et al., 2002). NDH-I₃ is positioned in the thylakoid membranes, and NDH-I₄ is situated in the thylakoid membranes as well or in the plasma membrane (Price, 2011). The positioning of the CO₂ uptake systems also allows for interception of CO₂ leakage from the carboxysomes (Price et al., 2002; Figure 1.6).

Although the CCMs of several model cyanobacteria, including Synechocystis PCC 6803, Synechococcus PCC 7002 and Synechococcus PCC 7942, have been studied in detail (Price et al., 2004; Wang et al., 2004; Woodger et al., 2007; Schwarz et al., 2011; Burnap et al., 2015), the functioning of the CCMs of harmful cyanobacteria like Microcystis remains an open
question. Moreover, it is not clear how much variation exists between different strains of the same species, and which properties of the CCM will benefit strains at elevated CO₂. Additionally, it has been found that microcystins can bind to RuBisCO (Zilliges et al., 2011) and that rising CO₂ concentrations can affect the relative abundances of microcystin-producing versus non-microcystin-producing strains (Van de Waal et al., 2011). It is not known, however, whether there is a relationship between the toxicity of cyanobacterial blooms and the layout of the CCM.

**Harmful cyanobacteria and toxins**

Nutrient enrichment of lakes caused by the intensification of agricultural, industrial and urban activities has led to eutrophication, an environmental status that is characterized by a high primary production and dense phytoplankton blooms (Smith, 1998). In many eutrophied waters, cyanobacteria dominate the phytoplankton community. Cyanobacterial blooms lead to various economical and ecological problems in many lakes (Huisman et al., 2005). These blooms can suppress the growth of aquatic macrophytes and invertebrates. Nighttime oxygen depletion induced by blooms can lead to mass mortality of fish. In addition, subtropical cyanobacterial blooms can harbor the pathogenic bacterium *Vibrio cholera*, that can hide in the mucilaginous sheath of cyanobacteria and can cause cholera infection (Islam et al., 1990; Chaturvedi et al., 2015). Moreover, several cyanobacterial species are capable of producing toxins, that are dangerous to birds and mammals, including livestock, dogs and humans. Well-known toxin-producing cyanobacterial genera include *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Microcystis*, *Nodularia*, *Nostoc*, and *Planktothrix*.

*Microcystis* is a ubiquitous harmful cyanobacterium that can be found in fresh and brackish water (Verspagen et al., 2006; Qin et al., 2010; Michalak et al., 2013). The cells are spherical shaped, reaching 2-8 µm in diameter, and are usually structured into colonies (Figure 1.4). The cells can produce gas vesicles and during summer, when conditions are most favorable, the genus can form surface blooms consisting of dense scum layers (Figure 1.8A). Many different colonial morphologies of *Microcystis* are known (Komárek and Komárková, 2002), and originally different *Microcystis* morphologies were considered different species. Yet, Otsuka et al. (2001) suggested that the different morphospecies of *Microcystis* should be united into one species, *Microcystis aeruginosa*, because of a lack of molecular relatedness of the different morphospecies and overlapping morphologies. In this thesis, the nomenclature of Otsuka et al. (2001) is followed that describes all morphospecies of *Microcystis* under the common name *M. aeruginosa*.

*Microcystis* can produce a potent family of hepatotoxins known as microcystins (MacKintosh et al., 1990; Nishiwaki-Matsushima et al., 1992; Codd et al., 2005). Microcystins
can cause severe liver damage, as well as skin rashes, stomach pains, diarrhea and eye irritation (Chorus et al., 2000). Even situations have been reported where people have died from exposure to these hepatotoxins (Jochimsen et al., 1998). Over 80 microcystin variants are known, with microcystin-LR (MC-LR) being the most abundant and one of the most toxic versions (Figure 1.8B; Zurawell et al., 2005). The World Health Organization advises a provisional guideline for MC-LR in drinking water (based on MC-LR) of 1 µg L⁻¹, while most guidelines for recreational waters suggest a limit of 20 µg L⁻¹ (Ibelings et al., 2014). Microcystins are not synthesized directly by ribosomes, but rather indirectly by synthetases encoded by two large operons (mcyABC and mcyDEFGHIF; Tillett et al., 2000). Microcystins typically remain inside the cell, until the cell is lysed, after which microcystins are released in the environment. Varying half-lives have been reported for microcystins in water, ranging from days to weeks, depending on, for example, the presence of bacteria that can break down microcystins (Chorus and Bartram, 1999). Although the role of most cyanobacterial toxins remains a puzzle, recently microcystins were associated with protection against oxidative stress in Microcystis, for example caused by high light (Zilliges et al., 2011; Meissner et al., 2013). It was shown that microcystins can actually bind to specific cellular proteins like RuBisCO, protecting them from degradation (Zilliges et al., 2011).

![Figure 1.8. Dense Microcystis bloom and the molecular structure of the toxin it typically produces. (A) Microcystis bloom in Lake Volkerak, the Netherlands. (B) Molecular structure of microcystin-LR (PDB ID 1LCM; Trogan et al., 1996).](image)

**Harmful cyanobacteria and climate change**

Fossil fuel combustion boosts the CO₂ concentration in the atmosphere, which increases global temperatures and can raise the C₄ pool in aquatic ecosystems (Figure 1.9). Many cyanobacteria benefit from higher temperatures with increased growth rates (Paerl and Huisman, 2008; Paerl and Huisman, 2009; O’Neil et al., 2012; Carey et al., 2012). Increased global temperatures will
also affect the weather patterns. Longer drought periods can increase the residence times of harmful blooms (Paerl and Huisman, 2009; Paerl and Paul, 2012), while more intense future storms can enhance the nutrient loads (N and P) in lakes (Jeppesen et al., 2009; 2011) (Figure 1.9). Furthermore, summer droughts in combination with rising sea levels may lead to local increases in salinity levels. Several harmful cyanobacteria, including *Microcystis*, are considered to be quite salt tolerant (Tonk et al., 2007; Paerl and Huisman, 2009; Paerl and Paul, 2012), and can be found in both fresh and brackish water (Lehman et al., 2005; Kardinaal et al., 2007), suggesting they can relatively easily withstand increasing salinities.

High temperatures and also the intrusion of saline water promote vertical stratification of lakes (Paerl and Huisman, 2009). In contrast to eukaryotic algae, harmful cyanobacteria like *Microcystis* can produce gas vesicles, which provide the cells with buoyancy and enable them to dominate the surface layer. Cyanobacteria can regulate their vertical migration by accumulating carbohydrates as ballast and synthesizing more gas vesicles to stimulate buoyancy (Visser et al., 1995). Hence, buoyant cyanobacteria are foreseen to benefit from increased vertical stratification of lakes. Rising temperatures also decrease the viscosity of water, and therefore decrease the resistance against the upward migration of buoyant cyanobacteria. Surface blooms can optimally use the available light, while casting shade over non-buoyant phytoplankton present deeper in the water column (Figure 1.9). Moreover, it was suggested that rising temperature stimulate especially toxic *Microcystis* strains compared to nontoxic strains (Davis et al., 2009). Several studies have investigated to what extent cyanobacterial dominance can be explained by high nutrient levels (N and P) and high temperature (Wagner and Adrian 2009; Rigosi et al., 2014). The relative importance of these two factors and their interaction appeared to be dependent on the trophic state of the lake and differed between cyanobacterial species. For example, *Anabaena* appeared more responsive to enhanced nutrient levels, whereas *Microcystis* appeared more responsive to rising temperature (Rigosi et al., 2014).

Effects of rising CO₂ concentrations on cyanobacteria are less well understood than effects of elevated temperatures (Paerl and Huisman, 2009; O’Neil et al., 2012). Although *Microcystis* blooms can occur in waters with a pH of ~6 (Oberholser et al., 2010), they are usually found in alkaline waters with a pH of 7-10 (Millie et al., 2009; Liu et al., 2011; Verspagen et al., 2014b), with relatively low CO₂(aq) and relatively high bicarbonate concentrations (Figure 1.3). It has been argued that, owing to their advanced CCMs, cyanobacteria are especially effective at low CO₂(aq) concentrations (Shapiro, 1997). They ensure their dominance by reducing CO₂ concentrations to very low levels available only to themselves. This reasoning suggests that cyanobacteria might become less dominant at elevated CO₂ levels. However, other studies have suggested that cyanobacterial blooms in eutrophic and hypertrophic lakes will likely intensify in response to rising CO₂, by (partially) alleviating these
blooms from carbon limitation (Verspagen et al., 2014b). Laboratory experiments with different Microcystis strains showed that toxic strains were competitively dominant at low CO₂, whereas non-toxic strains dominated at elevated CO₂ (Van de Waal et al., 2011), but the molecular-physiological mechanisms underlying this reversal in competitive dominance remained unclear. Furthermore, laboratory studies have suggested that elevated CO₂ can cause changes in the community structure of freshwater phytoplankton (Low-Décarie et al., 2015), but failed to detect an evolutionary response when phytoplankton species were grown at elevated CO₂ for hundreds of generations (Low-Décarie et al., 2013). From the available literature, it is therefore evident that the specific genetic and physiological traits that determine the response of cyanobacteria to elevated CO₂ requires further study.

Figure 1.9. Figure illustrating effects of climate change on harmful cyanobacterial blooms. The figure was based on Paerl and Huisman (2009), with permission from John Wiley and Sons.
Defeating harmful cyanobacterial blooms

As described above, the nuisance by harmful cyanobacteria can be very problematic. Numerous methods to eliminate cyanobacteria have therefore been studied and tested.

One of the most important methods is nutrient reduction, which mainly focuses on the reduction of N and P to decrease or prevent cyanobacterial blooms (Anderson et al., 2002; Conley et al., 2009). Nutrient reduction can be obtained for example by limiting the use of fertilizers and improved wastewater treatments. However, often it takes years before a reduction in nutrient loading has an effect.

Another recent method to combat cyanobacterial blooms makes use of the high sensitivity of cyanobacteria to H\textsubscript{2}O\textsubscript{2}, because of the absence of the plant-type Mehler reaction in cyanobacteria, and its replacement by a Mehler-like reaction that produces no reactive oxygen species or H\textsubscript{2}O\textsubscript{2} (Helman et al., 2005). Lake treatments have demonstrated that cyanobacteria can be selectively killed with H\textsubscript{2}O\textsubscript{2} within a few hours, whereas other phytoplankton, zooplankton, fish and water plants remain unaffected (Matthijs et al., 2012). The added H\textsubscript{2}O\textsubscript{2} decomposes into water and O\textsubscript{2} within a few days, and hence leaves no long-term chemical traces in the environment.

An alternative promising method that uses no addition of chemicals into the water, is artificial mixing of the water column by air plumes introduced from the sediment (Visser et al., 2015). Deep vertical mixing prevents the formation of surface blooms by buoyant cyanobacteria like Microcystis, which eliminates their competitive advantage with respect to other phytoplankton species (Visser et al., 1996; Huisman et al., 2004). Yet, implementation and running costs for artificial mixing are quite high, and the method cannot be applied in shallow lakes.

Many other methods have been suggested to combat harmful algal blooms, but several of these approaches including the widespread use of Cu\textsuperscript{2+} are detrimental to the ecosystem (Jančula and Maršálek, 2011). Precipitation of phosphate with metal cations (e.g., Fe\textsuperscript{3+}) and subsequent sedimentation has found increased application, including variants in which the phosphate is bound by metal cations that are present in mineral clay (Lürling and Faassen, 2011). However, microbial processes in anaerobic sediments can reduce the insoluble iron(III)phosphate into soluble iron(II)salts, returning the phosphate into the lake water.

Furthermore, the addition of KCl to specifically combat Microcystis blooms has been suggested, given the high sensitivity of Microcystis to potassium ions (Parker et al., 1997; Shukla and Rai, 2007). Experiments have shown that addition of low concentrations of potassium ions (1-5 mmol L\textsuperscript{-1}) specifically inhibits the growth of Microcystis, while for example duckweed is not affected by these increased potassium ion concentrations (Parker et al., 1997). Yet, Tonk et al. (2007) showed that some Microcystis strains are quite salt tolerant, and
potassium ions plays an important role in acclimation to salt stress (Wood, 1999). Therefore, the sensitivity of *Microcystis* to potassium ions clearly requires further study.

**Molecular approaches to study cyanobacteria**

Genes carry the genetic information required for the growth and functioning of organisms. Nowadays, many genomes of a wide range of cyanobacteria have been sequenced. This greatly facilitated in-depth study of the genetics and diversity of cyanobacteria as well as their potential physiological and ecological traits. While the genome of the model cyanobacterium *Synechocystis* PCC 6803 was sequenced already in 1996 (Kaneko *et al.*, 1996), the genomes of harmful cyanobacteria like *Microcystis* were sequenced more recently. After the sequencing of *Microcystis* NIES-843 (Kaneko *et al.*, 2007) and *Microcystis* PCC 7806 (Frangeul *et al.*, 2008), several more *Microcystis* genomes followed (Humbert *et al.*, 2013; Fiore *et al.*, 2013; Yang *et al.*, 2013; Okano *et al.*, 2015; Yamaguchi *et al.*, 2015). I have used these genome sequences to search for genes encoding different components of the cyanobacterial CCM in a wide variety of *Microcystis* strains. Furthermore, the work of this thesis includes the sequencing of the genome of *Microcystis* PCC 7005 (*Chapter 2*).

The available genome information also allows study of the expression of genes (i.e., the transcriptome). Gene expression data provide information of what the cell is doing at a specific time-point or how it responds to certain environmental changes. For studies of a small number of specific genes, application of the reverse transcription quantitative polymerase chain reaction (RT-qPCR) is the default choice. In this technique, primers are designed to amplify specific fragments of cDNA (reverse-transcribed RNA) and the amplification is monitored real-time via the detection of a fluorescent DNA-binding dye or fluorescently-labeled sequence specific probes. The analysis compares expression of the target gene versus a reference gene and treated samples versus control samples, and determines the number of cycles at which the fluorescence exceeds the threshold. In this way, one can calculate the relative increase or decrease of a gene transcript in the treated samples (Livak and Smittgen, 2001). To analyze the complete transcriptome of cyanobacteria, containing thousands of genes, RT-qPCR would be too demanding, and instead microarrays have been extensively used (Wang *et al.*, 2004; Yeremenko, 2004, Eisenhut *et al.*, 2007, Schwarz *et al.*, 2011; Krasikov, 2012). On a microarray, thousands of specific DNA probes across the entire genome are attached to a solid surface, forming spots. The microarray is hybridized with cDNA obtained from the transcriptomes of treatment versus control samples and labeled with different fluorophores (Cy3 or Cy5). After washing off nonspecific-bound sequences, probe-target hybridization is detected and quantified by a microarray scanner (*Figure 1.10*). Relative intensities of each fluorophore are then used in the data analysis to identify up- and down-regulated genes.
Alternatively, RNA sequencing is increasingly used nowadays to analyze transcriptomes, since sequencing is becoming cheaper, faster and generates more data of higher quality (Van Dijk et al., 2014). In this thesis, RT-qPCR and microarrays are used to analyze the gene expression of Microcystis strains (Chapters 3, 4 and 6).

Figure 1.10. Example of an Agilent microarray scan. Green spots indicate down-regulated genes, red spots indicate up-regulated genes, and yellow spots indicate genes that do not change expression. The inset displays a magnification of a small section of the scan. The scan is from one of the Microcystis PCC 7806 microarrays that will be discussed in more detail in Chapter 3. The predominance of yellow spots illustrates that many genes did not change expression in response to elevated CO₂. Photo: Giovanni Sandrini.

This thesis
In this thesis I investigate effects of elevated CO₂ on harmful cyanobacteria, with particular emphasis on Microcystis. I want to address several questions. For instance, are the CCMs of harmful cyanobacteria very different or similar to the cyanobacteria that have been investigated so far? Is there genetic and phenotypic variation among the CCMs of harmful cyanobacteria, e.g., among different Microcystis strains? How is the expression of their CCM genes regulated? Do harmful cyanobacteria, like Microcystis, benefit from rising CO₂? Will their blooms become denser and more toxic, and will rising CO₂ affect the genetic composition of these blooms?
The work presented in this thesis applies a variety of different techniques (Figure 1.11). The research questions are tackled with bioinformatics and molecular techniques (e.g., sequencing, RT-qPCR, microarrays) to investigate the presence and expression of CCM genes, in combination with culture experiments (e.g., batch and chemostat experiments) to determine the CCM activity of Microcystis at different CO₂ levels. The laboratory results are compared with field data on the genetic and phenotypic variation of the CCM of Microcystis during daily and seasonal changes in CO₂ availability in a cyanobacteria-dominated lake (Figure 1.11).

The outline of the thesis is the following:

In Chapter 2, the CCM diversity among various Microcystis strains is investigated. After initial screening of two published Microcystis genomes, the CCMs of 18 additional strains are analyzed using molecular techniques and bioinformatics tools, and the entire genome of strain PCC 7005 is sequenced de novo. The results show that, with respect to CCM attributes,
the main difference between strains appears to be the presence or absence of the bicarbonate transporter genes *bicA* and *sbtA*. The genetic variation is situated in one operon of the genome, and enables a distinction between three C\textsubscript{i} uptake genotypes: *bicA* strains, *sbtA* strains, and *bicA+sbtA* strains. Subsequently, I designed a new high-throughput culture system (Figure 1.11B) to investigate whether this genetic variation causes phenotypic variation in CO\textsubscript{2} response.

In Chapter 3, the specific cell response of *Microcystis* strain PCC 7806 to elevated CO\textsubscript{2} is investigated using chemostats. Changes in gene expression (using microarrays) and several physiological parameters (e.g., biomass, carbohydrate storage, microcystin production) are monitored after the CO\textsubscript{2} concentration is increased from 200 to 1450 ppm CO\textsubscript{2}. The microarray study shows that this strain downregulated only a surprisingly small number of genes at elevated CO\textsubscript{2} (Figure 1.10), which might be an effective strategy for a species experiencing strongly fluctuating C\textsubscript{i} concentrations.

In Chapter 4, the gene expression response of *Microcystis* to elevated CO\textsubscript{2} is investigated in more detail, by comparing the response of six strains representing the three different C\textsubscript{i} uptake genotypes. In parallel to changes in gene expression, the C\textsubscript{i} uptake activity of the different strains under a variety of conditions is also reported. The results show that CCM gene regulation and activity differs among *Microcystis* strains. The observed genetic and phenotypic variation in CCM responses may offer an important template for natural selection.

Chapter 5 studies the competition between different *Microcystis* C\textsubscript{i} uptake genotypes using laboratory chemostats and a lake study. The laboratory competition experiments investigate which C\textsubscript{i} uptake genotypes become dominant at low versus high CO\textsubscript{2} concentrations. The field study was performed at Lake Kennemermeer, during summer and autumn of 2013. The lake displays strong seasonal fluctuations in C\textsubscript{i} concentration and pH as a consequence of the development of cyanobacterial blooms. The lake study is used to investigate whether different *Microcystis* C\textsubscript{i} uptake genotypes may co-occur in the same lake and whether their relative abundances are affected by seasonal changes in C\textsubscript{i} availability.

In Chapter 6, the 24-hour expression patterns of CCM and other selected genes of *Microcystis* are investigated in Lake Kennemermeer. The lake shows large daily fluctuations in bicarbonate concentrations, pH and dissolved oxygen as a consequence of the high photosynthetic activity of the cyanobacterial bloom. The 24-hour data will reveal which genes display major differences in expression between day and night, and which genes show minor or no fluctuations. Moreover, this study attempts to unravel the environmental signals that drive the expression of the CCM genes.

Chapter 7 reviews the current understanding of the effects of rising CO\textsubscript{2} on harmful cyanobacteria. First, the chapter discusses whether rising atmospheric CO\textsubscript{2} concentrations will
Chapter 1

intensify the development of cyanobacterial blooms. Next, the CCM diversity of *Microcystis* is compared to that of other harmful cyanobacteria, and it is discussed how genetic and phenotypic variation in the CCM can direct changes in cyanobacterial communities. Subsequently, it is addressed how nutrient availability may modify effects of elevated CO₂ on cyanobacterial growth, and how rising CO₂ may affect nitrogen fixation and cyanobacterial toxin production. The chapter ends with an outlook to the future research needs in this field.

Chapter 8 investigates the idea of Parker *et al.* (1997) that potassium ions can be used as an effective method to combat harmful *Microcystis* blooms. In this chapter, several *Microcystis* strains with a freshwater or brackish water origin are exposed to elevated concentrations of potassium and other salts, and the growth rates are compared. Furthermore, the presence of genes involved in salt acclimation are compared among the different *Microcystis* strains. The results indicate that potassium treatments will not always be effective against *Microcystis* blooms, and have implications for the composition of mineral media used in laboratory cultures of cyanobacteria.

In Chapter 9, the results from the thesis are put in perspective. First, developments in techniques to study genotypes and gene expression will be discussed. Then, the genetic variability and transcriptional regulation of *Microcystis* CCM genes will be evaluated. Next, the anticipated effects of climate change on *Microcystis* blooms and microcystin production are discussed, followed by an analysis of the effects on other cyanobacteria and eukaryotic algae. Methods to effectively combat harmful cyanobacterial blooms are also discussed. The chapter ends with the main conclusions of this thesis.