Effects of rising CO_2 on the harmful cyanobacterium Microcystis

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Publication date
2016
Document Version
Final published version

Chapter 1

General introduction

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).

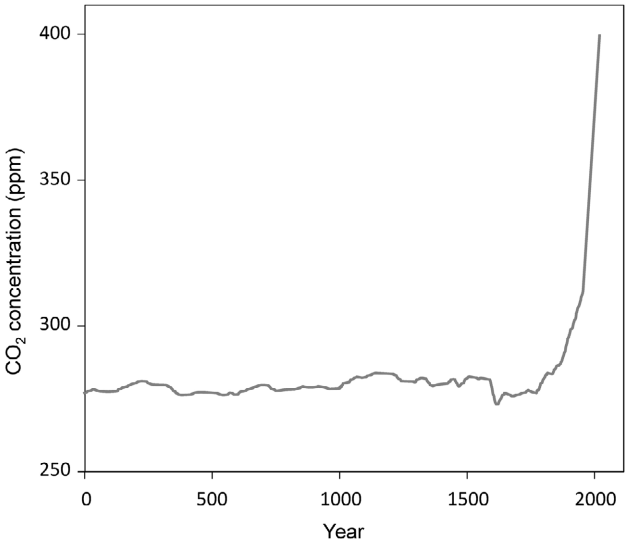

Figure 1.1. Atmospheric CO_2 concentration of the last 2000 years. Since 1958, the atmospheric CO_2 concentration is measured continuously at different places, including Mauna Loa, Hawaii. The data before 1958 is largely based on ice drillings from Antarctica and Greenland. This figure was adapted from 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_i 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 CO_2 in the past, when large amounts of coal were combusted (Ogden and Sleep, 2012), analogous to the human-induced CO_2 emissions from burning coal. The CO_2 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).

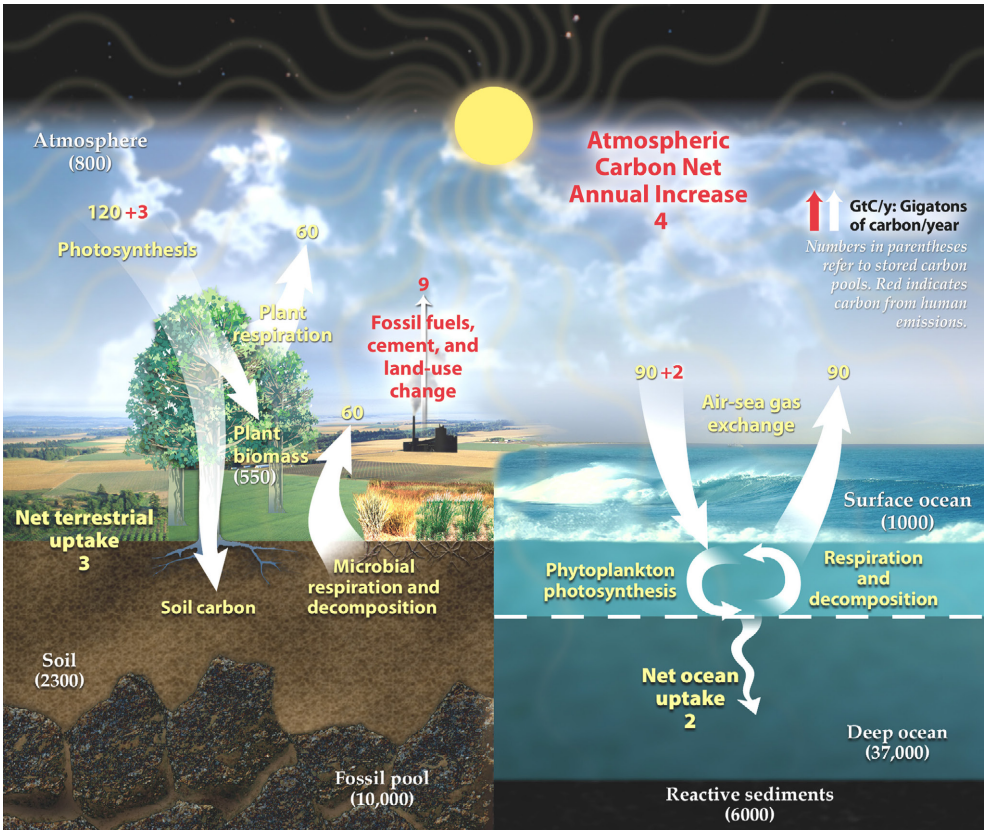

Figure 1.2. The global carbon cycle. The white numbers indicate stored carbon, the yellow numbers indicate natural fluxes, and the red numbers indicate carbon from human emissions in gigatons C per year. The values in parentheses (in gigatons C) are based on Houghton (2007). This figure was reproduced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

As a consequence of the current rise of the atmospheric concentrations of CO_2 and other greenhouse gasses, 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_2CO_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.

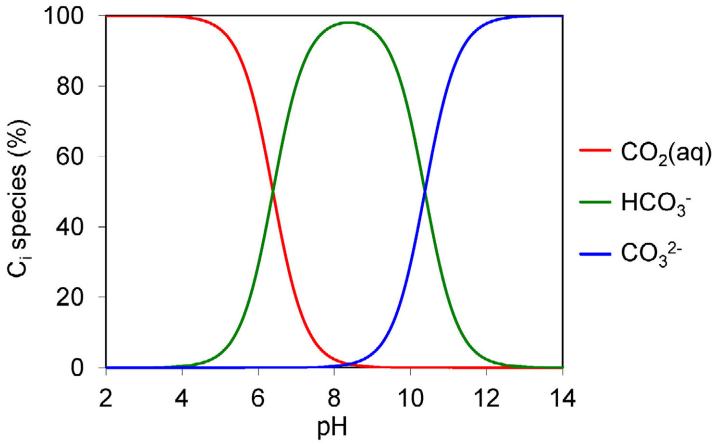

Figure 1.3. Relative abundances of the different inorganic carbon (C_i) species as a function of pH. Dissolved CO_2 (CO_2(aq)) is indicates in red, bicarbonate (HCO_3^-) in green and carbonate (CO_3^{2-}) in blue.

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

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_{CO2}) 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):

$$g_{CO2} = v\,(K_H\,pCO_2 - CO_2(aq)) \quad (1)$$

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 pCO_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 $g_{CO2} = v\,K_H\,pCO_2$. 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 (Schirrmeister *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.

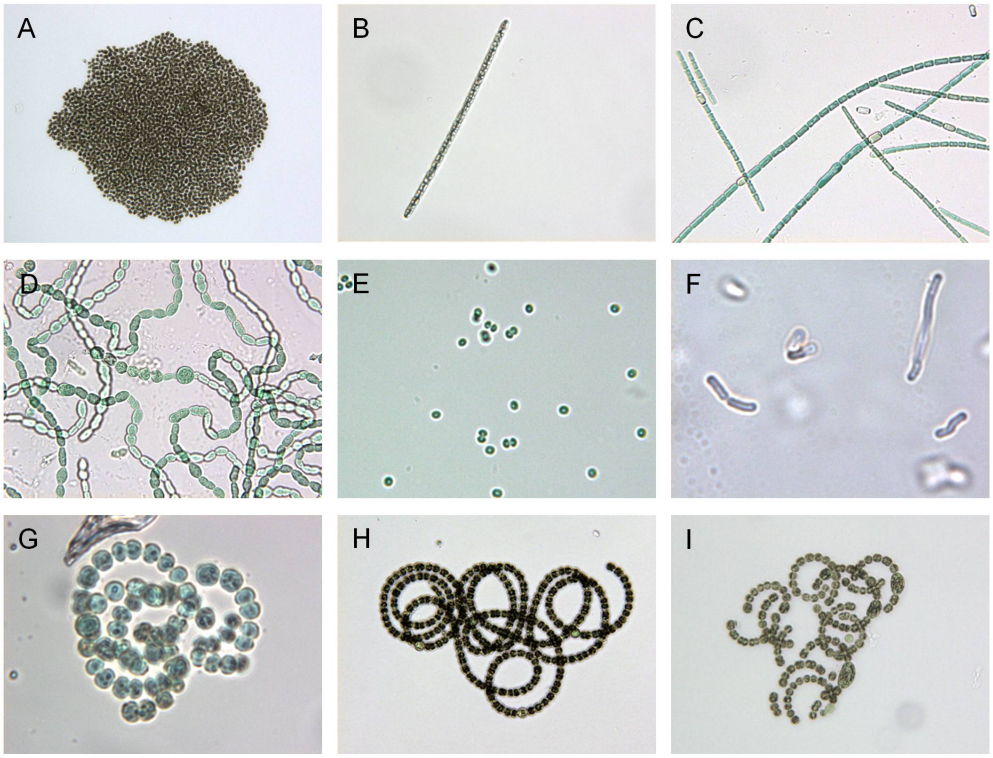

Figure 1.4. Biodiversity of cyanobacteria. (A) *Microcystis* sp. (colony). **(B)** *Planktothrix* sp. **(C)** *Aphanizomenon* sp. **(D)** *Nostoc* sp. **(E)** *Synechocystis* sp. PCC 6803. **(F)** *Synechococcus* sp. BS5. **(G)** *Anabaena* sp. **(H)** *Anabaena* sp. **(I)** *Anabaena* sp. *Photos: Giovanni 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 O_2, 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_6f 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 CO_2 fixation with the Calvin-Benson cycle (*i.e.*, the dark reactions).

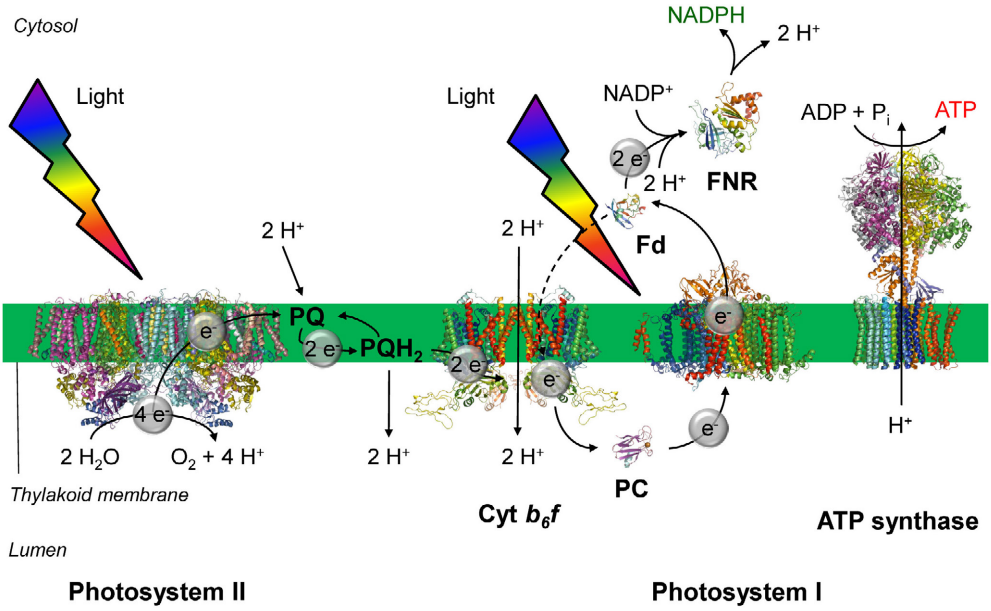

Figure 1.5. Light reactions of photosynthesis at the thylakoid membrane of cyanobacteria with the electron and proton flows indicated. The displayed structures are from *Thermosynechococcus elongatus* (PSII, PDB ID 4V62), *Nostoc* sp. PCC 7120 (cytochrome b_6f, PDB ID 4H44), *Synechococcus elongates* (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 PQH_2: 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 CO_2-concentrating mechanism of cyanobacteria

Phytoplankton, including cyanobacteria, use the enzyme RuBisCO for CO_2 fixation. Since RuBisCO has a low affinity for CO_2, and can also use O_2 as substrate in a process called photorespiration, many cyanobacteria evolved a CO_2-concentrating mechanism (CCM) (Kaplan and Reinhold, 1999; Raven *et al.*, 2008). As a first step, bicarbonate is taken up by specific uptake systems and CO_2 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 CO_2. RuBisCO, then surrounded by a high CO_2 concentration, incorporates CO_2 into the Calvin-Benson cycle, which uses energy from the light reactions (ATP and NADPH) and CO_2 to create organic compounds (**Figure 1.7**). The net reaction of photosynthesis (light-driven production of fixed carbon) for plants, algae and cyanobacteria is $6\ CO_2 + 6\ H_2O \rightarrow C_6H_{12}O_6 + 6\ O_2$.

Five cyanobacterial C_i uptake systems are known so far, three for bicarbonate uptake and two for conversion of CO_2 to bicarbonate (Price, 2011; **Figure 1.6**). The C_i uptake systems have different characteristics and are dependent on different co-factors, such as sodium, calcium, ATP or NADPH. The diverse set of C_i uptake systems allows cyanobacteria to respond effectively to variations in C_i availability (Burnap *et al.*, 2015). Yet, not all five C_i uptake systems are present in each cyanobacterium (Badger *et al.*, 2006; Rae *et al.*, 2011). Indeed, several studies have indicated strong dependence on CO_2 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 C_i uptake in cyanobacteria at both low and high CO_2 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 µmol L^{-1} bicarbonate) but low flux rate (Omata *et al.*, 1999; Omata *et al.*, 2002). BCT1 is directly ATP-dependent, uses Ca^{2+} 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 µmol L^{-1} bicarbonate) but high flux rate, whereas SbtA usually has a high affinity ($K_{0.5} \leq 5$ 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*

al., 2004). 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_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_i uptake, by transporting bicarbonate through the outer membrane into the periplasmic space (**Figure 1.6**).

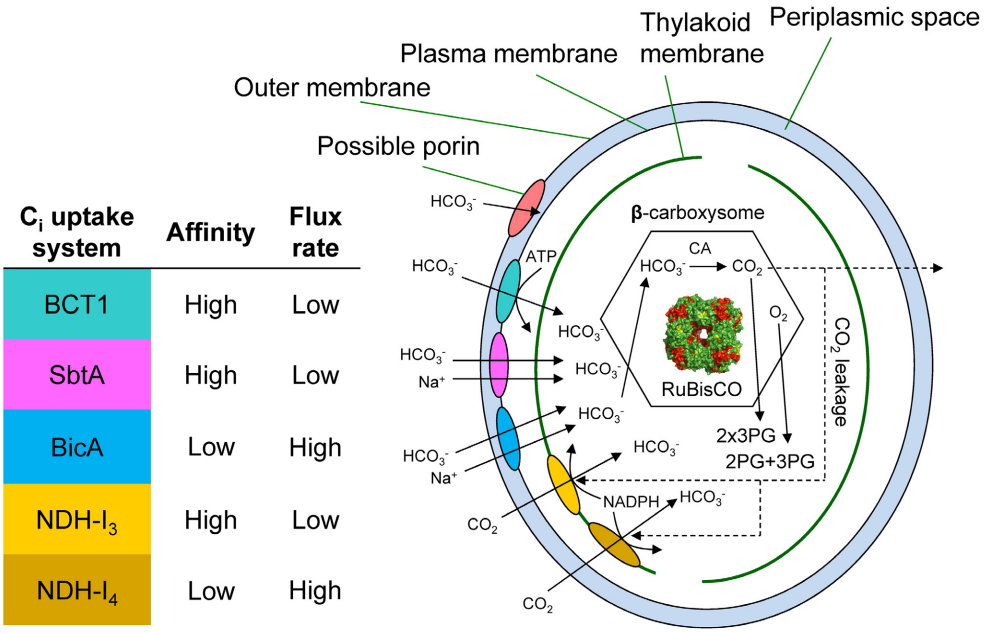

Figure 1.6. Overview of the cyanobacterial CO_2-concentrating mechanism. Up to five different C_i uptake systems can be found in cyanobacteria. These C_i uptake systems have different affinities and flux rates, and are dependent on different co-factors. In carboxysomes, accumulated bicarbonate is converted to CO_2 via carbonic anhydrase (CA) enzymes, surrounding RuBisCO (PDB ID 1RBL) by a high CO_2 concentration. RuBisCO fixes CO_2 by the production of 3-phosphoglycerate (3PG). However, RuBisCO can also use O_2 as substrate, in a process called photorespiration, which produces toxic 2-phosphoglycerate (2PG). CO_2 leakage from the carboxysomes can be partly intercepted by the CO_2 uptake systems (shown by the dashed lines). This figure was based on Badger *et al.* (2006), with permission from Oxford University Press.

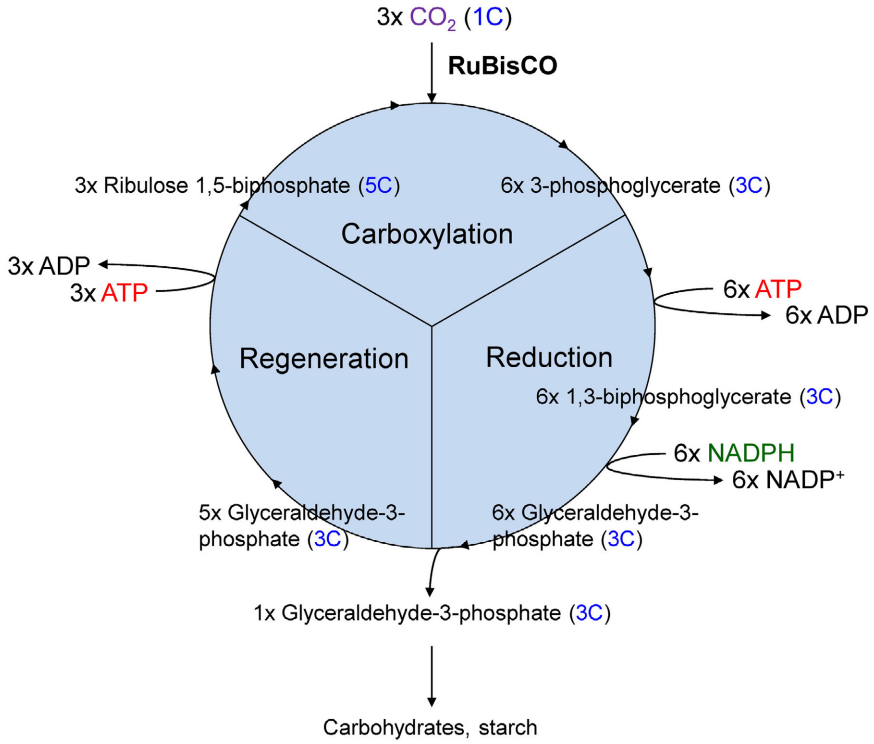

Figure 1.7. The Calvin-Benson cycle. The cycle is used to convert CO_2 into more complex molecules such as carbohydrates and starch. These reactions are also called the dark reactions. The number of carbon atoms in the molecules is indicated in blue.

The two CO_2 uptake systems consist of modified plastoquinone oxidoreductase NADPH dehydrogenase (NDH-I) respiratory complexes. The CO_2 uptake systems convert CO_2 into bicarbonate to trap the C_i inside the cell. NADPH functions as the electron donor for conversion of CO_2 into bicarbonate (Price *et al.*, 2002; **Figure 1.6**). While NDH-I_3 has a relatively high affinity ($K_{0.5}$ = 1-2 µmol L^{-1} CO_2) but low flux rate, NDH-I_4 has a lower affinity ($K_{0.5}$ = 10-15 µmol L^{-1} CO_2) but higher flux rate (Maeda *et al.*, 2002; Price *et al.*, 2002). NDH-I_3 is positioned in the thylakoid membranes, and NDH-I_4 is situated in the thylakoid membranes as well or in the plasma membrane (Price, 2011). The positioning of the CO_2 uptake systems also allows for interception of CO_2 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 CO_2. Additionally, it has been found that microcystins can bind to RuBisCO (Zilliges *et al.*, 2011) and that rising CO_2 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 lifestock, 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 advices 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 *mcyDEFGHIJ*; 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).

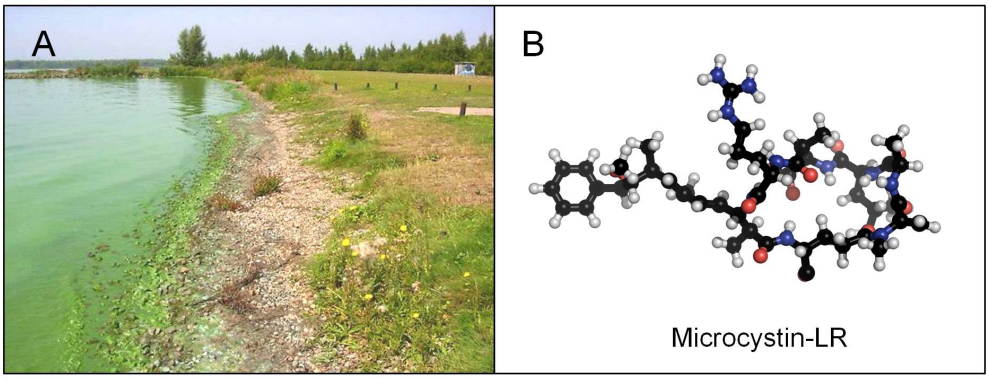

Figure 1.8. Dense *Microcystis* bloom and the molecular structure of the toxin it typically produces. (A) *Microcystis* bloom in Lake Volkerak, the Netherlands. **(B)** Molecular structure of microcystin-LR (PDB ID 1LCM; Trogen *et al.*, 1996).

Harmful cyanobacteria and climate change

Fossil fuel combustion boosts the CO_2 concentration in the atmosphere, which increases global temperatures and can raise the C_i 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 non-toxic 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 CO_2 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 CO_2(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 CO_2(aq) concentrations (Shapiro, 1997). They ensure their dominance by reducing CO_2 concentrations to very low levels available only to themselves. This reasoning suggests that cyanobacteria might become less dominant at elevated CO_2 levels. However, other studies have suggested that cyanobacterial blooms in eutrophic and hypertrophic lakes will likely intensify in response to rising CO_2, 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 CO_2, whereas non-toxic strains dominated at elevated CO_2 (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 CO_2 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 CO_2 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 CO_2 requires further study.

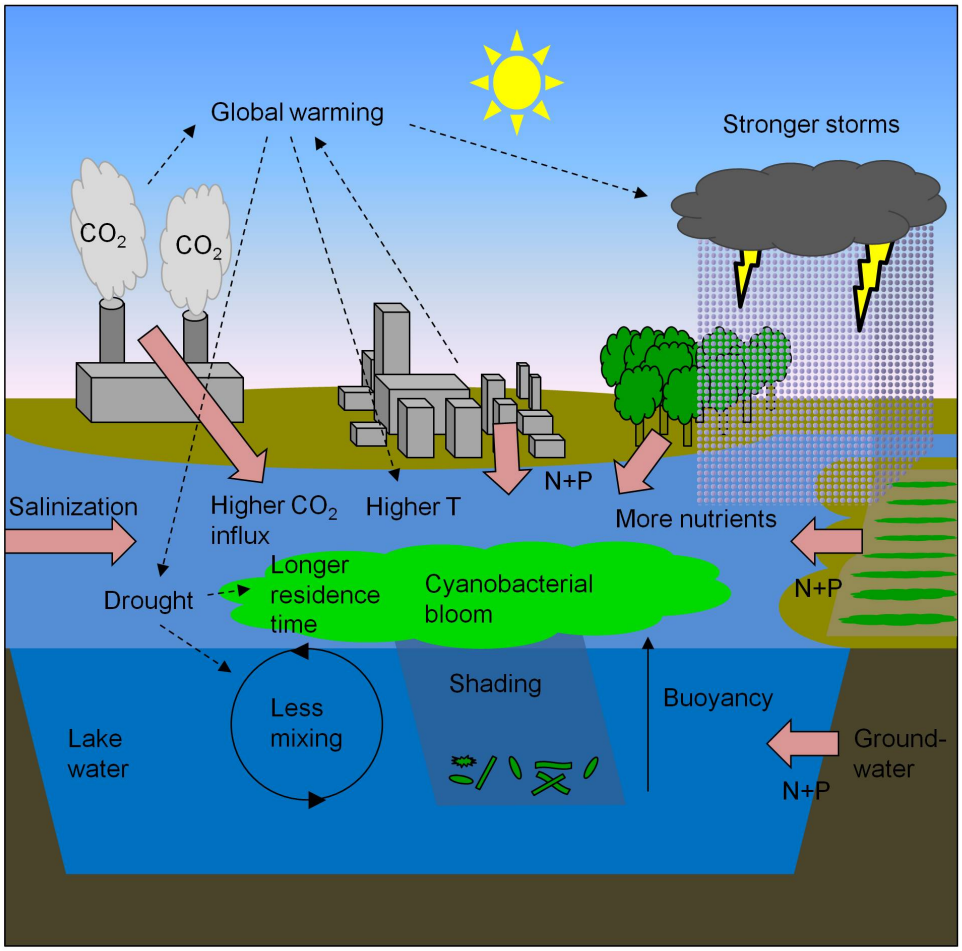

Figure 1.9. Figure illustrating effects of climate change on harmful cyanobacterial blooms. The figure was based on Paerl and Huisman (2009), with permission from John Wiley and Sons.

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_2O_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_2O_2 (Helman *et al.*, 2005). Lake treatments have demonstrated that cyanobacteria can be selectively killed with H_2O_2 within a few hours, whereas other phytoplankton, zooplankton, fish and water plants remain unaffected (Matthijs *et al.*, 2012). The added H_2O_2 decomposes into water and O_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^{2+} are detrimental to the ecosystem (Jančula and Maršálek, 2011). Precipitation of phosphate with metal cations (*e.g.*, Fe^{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^{-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**).

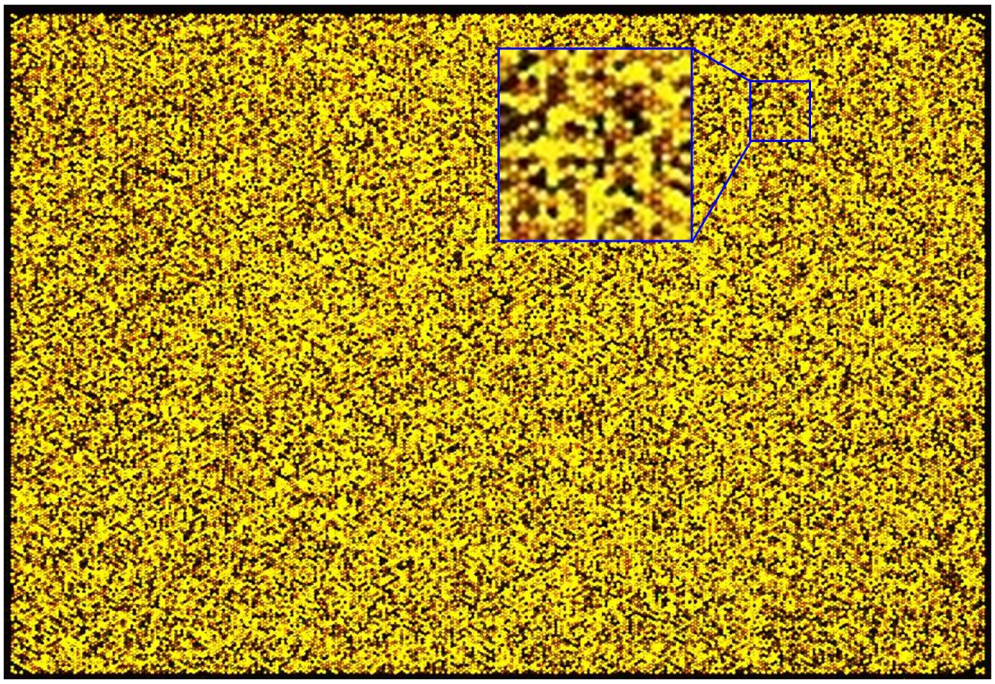

Figure 1.10. Example of an Agilent microarray scan. Green spots indicate down-regulated genes, red spots indicate up-regulated genes, and yellow spots indicate genes that do not change expression. The inset displays a magnification of a small section of the scan. The scan is from one of the *Microcystis* PCC 7806 microarrays that will be dissussed in more detail in Chapter 3. The predominance of yellow spots illustrates that many genes did not change expression in response to elevated CO_2. *Photo: Giovanni Sandrini*.

This thesis

In this thesis I investigate effects of elevated CO_2 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 CO_2? Will their blooms become denser and more toxic, and will rising CO_2 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 CO_2 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 CO_2 availability in a cyanobacteria-dominated lake (**Figure 1.11**).

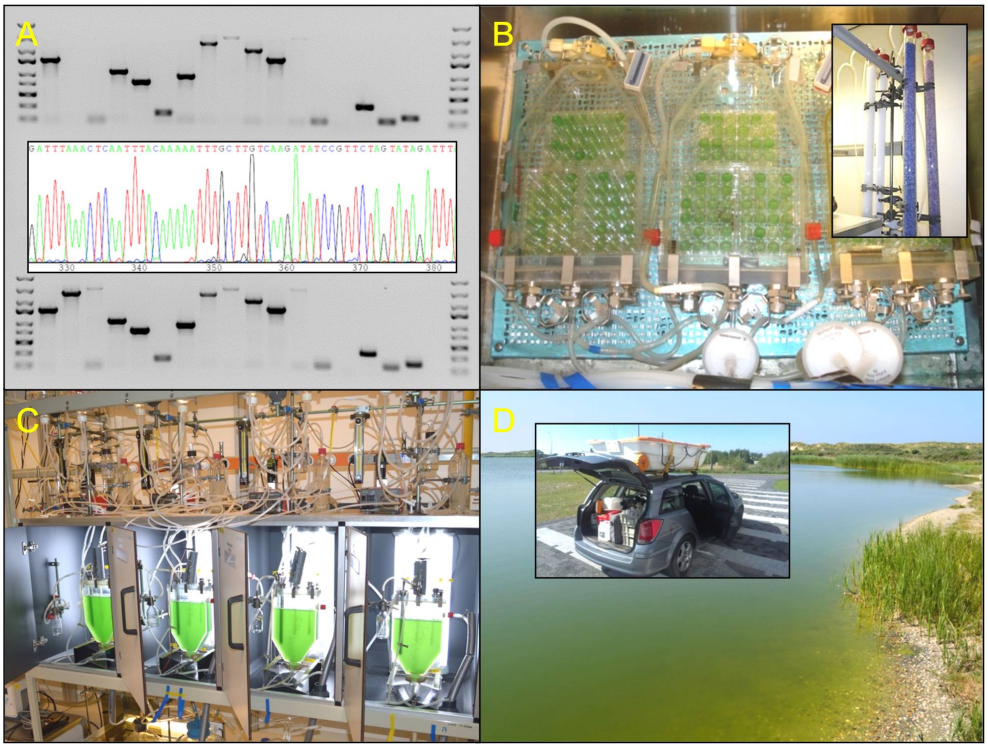

Figure 1.11. Overview of the diversity of techniques applied in this thesis. (A) Genetic analysis using PCR and sequencing. **(B)** High-throughput batch culturing at various CO_2 concentrations (inset: glas columns with NaOH and silica pellets to scrub CO_2 from the air). **(C)** Controlled chemostat experiments. **(D)** Fieldwork at Lake Kennemermeer (inset: sampling equipment and boat). *Photos: Giovanni Sandrini.*

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_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_2 response.

In **Chapter 3**, the specific cell response of *Microcystis* strain PCC 7806 to elevated CO_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_2 concentration is increased from 200 to 1450 ppm CO_2. The microarray study shows that this strain downregulated only a surprisingly small number of genes at elevated CO_2 (**Figure 1.10**), which might be an effective strategy for a species experiencing strongly fluctuating C_i concentrations.

In **Chapter 4**, the gene expression response of *Microcystis* to elevated CO_2 is investigated in more detail, by comparing the response of six strains representing the three different C_i uptake genotypes. In parallel to changes in gene expression, the C_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_i uptake genotypes using laboratory chemostats and a lake study. The laboratory competition experiments investigate which C_i uptake genotypes become dominant at low versus high CO_2 concentrations. The field study was performed at Lake Kennemermeer, during summer and autumn of 2013. The lake displays strong seasonal fluctuations in C_i concentration and pH as a consequence of the development of cyanobacterial blooms. The lake study is used to investigate whether different *Microcystis* C_i uptake genotypes may co-occur in the same lake and whether their relative abundances are affected by seasonal changes in C_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_2 on harmful cyanobacteria. First, the chapter discusses whether rising atmospheric CO_2 concentrations will

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 CO_2 on cyanobacterial growth, and how rising CO_2 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.