Effects of rising CO$_2$ on the harmful cyanobacterium Microcystis

Sandrini, G.

Publication date
2016

Document Version
Final published version

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 5

Rapid microevolutionary adaptation of harmful cyanobacteria to changes in CO$_2$ availability
Rapid microevolutionary adaptation of harmful cyanobacteria to changes in CO$_2$ availability

Giovanni Sandrini$^{1,†}$, Xing Ji$^{1,†}$, Jolanda M. H. Verspagen$^1$, Robert P. Tann$^1$, Pieter C. Slot$^1$, Veerle M. Luimstra$^{1,2}$, J. Merijn Schuurmans$^{1,3}$, Hans C. P. Matthijs$^1$, Jef Huisman$^1$

$^1$Department of Aquatic Microbiology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, The Netherlands
$^2$Wetsus, European Centre of Excellence for Sustainable Water Technology, Leeuwarden, The Netherlands
$^3$Department of Aquatic Ecology, Netherlands Institute of Ecology, Wageningen, The Netherlands

†These authors contributed equally to this work

Keywords: CO$_2$-concentrating mechanism; bicarbonate, climate change; harmful algal blooms; Microcystis aeruginosa; natural selection

This chapter is submitted for publication.
Abstract
Rising atmospheric CO$_2$ concentrations are likely to affect many ecosystems worldwide. However, it is still an open question to what extent elevated CO$_2$ will induce evolutionary changes in photosynthetic organisms (Collins and Bell, 2004; Raven et al., 2012; Low-Decarie et al., 2013). Here, we show rapid microevolutionary adaptation of a harmful cyanobacterium to changes in inorganic carbon (C$_i$) availability. We studied the cyanobacterium *Microcystis*, a notorious genus that is responsible for many toxic cyanobacterial blooms worldwide (Paerl and Huisman, 2008; Qin et al., 2010; Michalak et al., 2013). *Microcystis* displays genetic variation in the C$_i$ uptake systems BicA and SbtA (Sandrini et al., 2014), where BicA has a low affinity for bicarbonate but high flux rate, whereas SbtA has a high affinity but low flux rate (Price et al., 2004; Price, 2011; Sandrini et al., 2014). Laboratory competition experiments showed that the strain composition shifted from dominance by a *bicA+sbtA* strain at low CO$_2$ to coexistence by a *bicA* strain and another *bicA+sbtA* strain at high CO$_2$. Similarly, in a eutrophic lake, *bicA+sbtA* strains were dominant when C$_i$ concentrations were depleted during a dense cyanobacterial bloom, but were replaced by *bicA* strains when C$_i$ concentrations increased later in the season. Strains with only *sbtA* formed a minor component of the *Microcystis* population. Hence, our results provide both laboratory and field evidence that increasing carbon concentrations induce rapid adaptive changes in the genotype composition of harmful cyanobacterial blooms.

Introduction
Atmospheric CO$_2$ concentrations are predicted to double during this century. Species may adapt to elevated CO$_2$ by the sorting of existing genetic variation and the establishment of new beneficial mutations. These evolutionary processes can alter the physiological and ecological response of species to future CO$_2$ levels (Raven et al., 2012). Several recent laboratory studies have investigated the potential for evolutionary changes in response to rising CO$_2$ concentrations (Collins and Bell, 2004; Collins et al., 2006; Lohbeck et al., 2012; Low-Decarie et al., 2013; Scheinin et al., 2015). For instance, selection experiments with the green alga *Chlamydomonas reinhardtii* revealed that some cell lines grown at elevated CO$_2$ levels for 1,000 generations grew more slowly at ambient CO$_2$, presumably because mutations reduced the effectiveness of CO$_2$ acquisition (Collins and Bell, 2004; Collins et al., 2006). Other lab studies argue that elevated atmospheric CO$_2$ fails to evoke specific evolutionary adaptation in phytoplankton species (Low-Décarie et al., 2013). Thus far, however, the specific genetic and molecular changes underlying evolutionary responses to rising CO$_2$ are not well understood, evolutionary adaptation to changing CO$_2$ conditions has rarely been investigated within
complex species assemblages (Scheinin et al., 2015) and has never been reported from natural waters.

Dissolved CO$_2$ (CO$_2$(aq)) concentrations can be depleted by phytoplankton blooms in eutrophic lakes (Ibelings and Maberly, 1998; Balmer and Downing, 2011; Gu et al., 2011), which provides an opportunity to study adaptation to changes in carbon availability. Cyanobacteria use a CO$_2$-concentrating mechanism (CCM) to overcome low concentrations of CO$_2$(aq) in their environment. The cyanobacterial CCM is based on the uptake of CO$_2$ and bicarbonate, and subsequent accumulation of inorganic carbon (C$_i$) in specialized compartments, called carboxysomes, for CO$_2$ fixation by the enzyme RuBisCO (Price, 2011). Five C$_i$ uptake systems are known in cyanobacteria. Two CO$_2$ uptake systems and the ATP-dependent bicarbonate transporter BCT1 are present in most freshwater cyanobacteria (Rae et al., 2011; Sandrini et al., 2014). Two other bicarbonate uptake systems, BicA and SbtA, are less widespread. Both are sodium-dependent symporters, but BicA has a low affinity for bicarbonate and high flux rate, whereas SbtA has a high affinity and low flux rate (Price et al., 2004). Affinity refers here to the effectiveness of bicarbonate uptake at low bicarbonate concentrations, whereas the flux rate refers to the bicarbonate uptake rate at high bicarbonate concentrations.

Cyanobacteria produce dense and often toxic blooms in many eutrophic lakes worldwide (Qin et al., 2010; Michalak et al., 2013; Verspagen et al., 2014b), and are predicted to expand with eutrophication and global warming (Paerl and Huisman, 2008; O’Neil et al., 2012; Verspagen et al., 2014b). We recently compared CCM gene sequences of 20 strains of the ubiquitous cyanobacterium Microcystis (Sandrini et al., 2014). Interestingly, some strains lacked the high-flux bicarbonate uptake gene bicA, whereas others lacked the high-affinity bicarbonate uptake gene sbtA. Hence, three different C$_i$ uptake genotypes can be distinguished: sbtA strains (with sbtA but no or incomplete bicA), bicA strains (with bicA but no sbtA), and bicA+sbtA strains (Figure 5.1). The three genotypes produce different phenotypes. Laboratory experiments showed that the growth rate of the sbtA genotype is reduced at high C$_i$ levels, the bicA genotype has reduced growth at low C$_i$ levels, whereas the bicA+sbtA genotype maintains a constant growth rate across a wide range of C$_i$ levels (Sandrini et al., 2014). Here, we test the potential for adaptive microevolution of Microcystis in response to elevated CO$_2$, by investigating changes in the relative frequencies of the different C$_i$ uptake genotypes in laboratory competition experiments and a field study.
Rapid microevolutionary adaptation of Microcystis

Figure 5.1. The three C₄ uptake genotypes of Microcystis. The variable genomic regions (Sandrini et al., 2014) contain the transcriptional regulator gene ccmR2, the sodium-dependent bicarbonate uptake genes bicA and sbtA, a post-translation regulator gene of SbtA known as sbtB, and the sodium/proton antiporter gene nhaS3.

Results

We ran competition experiments in three chemostats at low CO₂ (100 ppm) and three chemostats at high CO₂ (1,000 ppm) levels. Each chemostat was inoculated with five Microcystis strains, including two sbtA strains (CCAP 1450/10 and HUB 5-2-4), one bicA strain (PCC 7806) and two bicA+sbtA strains (PCC 7005 and PCC 7941) (Table S5.1, Supplementary Information). Four strains produced the hepatotoxin microcystin, whereas PCC 7005 was the only non-toxic strain. In the low CO₂ chemostats, the total Microcystis biomass increased until a steady state was reached, at which the CO₂(aq) and bicarbonate concentration were depleted to 1.5×10⁻⁴ µmol L⁻¹ and 15 µmol L⁻¹, respectively, and pH increased to 11.2 (Figure 5.2A,C). The high CO₂ chemostats produced a 2.5-fold higher Microcystis biomass, with much higher steady-state CO₂(aq) and bicarbonate concentrations of 10 µmol L⁻¹ and 2700 µmol L⁻¹, respectively, and a lower pH of 8.8 (Figure 5.2B,D).

In the low CO₂ chemostats, the toxic bicA+sbtA strain PCC 7941 competitively replaced the two sbtA strains, the bicA strain and the non-toxic bicA+sbtA strain, and comprised ~90% of the total Microcystis population at steady state (Figure 5.2E). In contrast, in the high CO₂ chemostats, the non-toxic bicA+sbtA strain PCC 7005 and the toxic bicA strain PCC 7806 coexisted, with relative abundances of ~60% and ~30%, respectively, at steady state (Figure 5.2F). Selection coefficients, calculated from the replacement rates of the strains and their generation times, ranged from 0.16 to 0.62 in the low CO₂ chemostats and from 0.08 to 0.20 in the high CO₂ chemostats (Figure S5.1 and Table S5.2, Supplementary Information).
Figure 5.2. Laboratory competition experiments with five *Microcystis* strains at low and high CO$_2$ levels. Left panels show low CO$_2$ chemostats and right panels high CO$_2$ chemostats. (A,B) *Microcystis* biomass (expressed as biovolume) and the light intensity $I_{out}$ transmitted through the chemostats. (C,D) Concentrations of dissolved CO$_2$ (CO$_2$(aq)) and bicarbonate, and pH. (E,F) Relative abundances of the five *Microcystis* strains. The data points show the mean values (±1 s.d.) of three replicated chemostat experiments.
Rapid microevolutionary adaptation of *Microcystis*

Our field study was carried out in Lake Kennemermeer, the Netherlands, in summer and autumn of 2013 (Figure 5.2A). Water temperature was 20-23°C in summer, and then gradually declined to 11°C in early autumn (Figure S5.2, Supplementary Information). The lake contained high phytoplankton abundances, with particularly dense blooms in weeks 26-28 and week 36 (Figure 5.3B). Total phytoplankton biovolume consisted for >95% of cyanobacteria, including Pseudoanabaenaceae, small Chroococcales, *Anabaenopsis hungarica* and *Microcystis* spp. (Figure S5.2, Supplementary Information). *Microcystis* can produce a potent family of hepatotoxins, known as microcystins (Codd *et al.*, 2005). The *Microcystis* population was dominated by potentially toxic genotypes containing the microcystin synthetase gene *mcyB* (Figure S5.2, Supplementary Information). Concentrations of *Microcystis* and the two dominant microcystin types, MC-LR and MC-RR, peaked in week 34 (Figure 5.3C). The MC-LR concentration (up to 23.2 µg L⁻¹) exceeded the provisional guideline for safe drinking water (1 µg L⁻¹) by the World Health Organization as well as common guidelines for recreational waters (Ibelings *et al.*, 2014), justifying closure of the lake for recreation.

In summer, CO₂(aq) concentrations in the lake were reduced below 3.5 µmol L⁻¹ and were strongly depleted to <0.4 µmol L⁻¹ in weeks 28-30 and week 36 (Figure 5.3D). When phytoplankton abundance decreased in autumn, the CO₂(aq) concentration increased to ~15 µmol L⁻¹, equilibrating with the atmospheric pCO₂ levels (390 ppm). CO₂ depletion during the summer bloom led to a strong increase in pH, with values up to ~10 during the two peaks in phytoplankton abundance (Figure 5.3B; Pearson correlation of pH vs log CO₂: ρ = -0.98, n = 10, p < 0.001). At pH between 8 and 10, bicarbonate is the dominant Cᵢ species. Bicarbonate concentrations showed similar temporal dynamics as the CO₂(aq) concentration (Figure 5.3D; Pearson correlation of CO₂(aq) vs bicarbonate: ρ = 0.90, n = 10, p < 0.001). The sodium concentration in the lake (measured in week 29) was 12.7±0.4 mmol L⁻¹, which allows maximum or near-maximum activity of the sodium-dependent bicarbonate transporters BicA and SbtA, since both transporters have half-saturation constants of 1-2 mmol L⁻¹ sodium (Price *et al.*, 2004; Du *et al.*, 2014).

All three Cᵢ uptake genotypes of *Microcystis* were present in the lake (Figure 5.1; Tables S5.3 and S5.4, Supplementary Information), but their relative abundances changed during the study period (Figure 5.4A). The sbtA strains represented ~20% of the *Microcystis* population during the entire season (Figure 5.4B). The bicA+sbtA strains dominated the *Microcystis* population in weeks 24-32, especially during the strong CO₂ depletion in weeks 28-29, but were largely replaced by bicA strains in late summer and autumn when Cᵢ concentrations in the lake increased. Relative abundances of the bicA+sbtA strains were negatively correlated with the bicarbonate concentration (Figure 5.4C; Pearson correlation: ρ =
Chapter 5

-0.84, n = 10, p < 0.01), whereas those of the bicA strains were positively correlated with bicarbonate (Figure 5.4D; Pearson correlation: \( \rho = 0.91, n = 10, p < 0.001 \)). Replacement of bicA+sbtA strains by bicA strains occurred in ~2 months. With a generation time of 1.5-5.2 days (Wilson et al., 2006), this corresponds to 12-42 generations and a selection coefficient of 0.06-0.19 per generation, which is comparable to the selection coefficients in the chemostats (Figure S5.3, Table S5.2, Supplementary Information).

Figure 5.3. Phytoplankton biomass and inorganic carbon speciation in Lake Kennemermeer. (A) Map of Lake Kennemermeer with the sampling station. (B) Total phytoplankton biomass (expressed as biovolume) and pH. (C) Microcystis biomass (expressed as biovolume) and concentrations of the two most abundant microcystin variants (MC-LR and MC-RR). (D) Concentrations of CO\(_2\) (aq), bicarbonate and carbonate. Data points represent the mean (±1 s.d.) of three replicate measurements; error bars that are not visible do not exceed the size of the symbols.
Rapid microevolutionary adaptation of Microcystis

Discussion

Our laboratory competition experiments and field study both show major changes in strain composition, with dominance by bicA+sbtA strains at low C\textsubscript{\textsc{i}} conditions that were (partly) replaced by bicA strains at high C\textsubscript{\textsc{i}} conditions (Figures 5.2 and 5.4). Previous studies have shown that the high-affinity but low-flux enzyme SbtA is more effective at low C\textsubscript{\textsc{i}} conditions, whereas the low-affinity but high-flux enzyme BicA is more effective at high C\textsubscript{\textsc{i}} conditions (Price, 2011; Sandrini et al., 2014, 2015a). Hence, the trade-off between affinity and flux rate offers a likely explanation for the observed shift in strain composition. The same trade-off can
also explain the reversal in competitive dominance in earlier competition experiments (Van de Waal et al., 2011; Sandrini et al., 2014). We note that, in *Microcystis*, *bicA* and *sbtA* are located on the same operon, and hence are co-transcribed in *bicA+sbtA* strains (Sandrini et al., 2014). Superfluous production of the SbtA enzyme or its post-transcriptional down-regulation, for example by SbtB (Du et al., 2014), will be costly when SbtA cannot be efficiently used, which will disfavour *bicA+sbtA* strains at high bicarbonate concentrations. Hence, our results provide laboratory and field evidence demonstrating that *bicA* strains have a strong selective advantage at high C₄ availability.

At a broader level, our results show that natural selection enables adaptive changes of cyanobacteria in response to variation in resource availability. In our case, these adaptive changes are most likely caused by sorting of existing genetic variation rather than by de novo mutations. This implies that at the time scale of cyanobacterial bloom development, the traits (in this case, the C₄ uptake kinetics) of cyanobacterial species may change due to a reshuffling of the relative abundances of different genotypes within the species. Hence, predictions of harmful algal bloom development cannot be made assuming that the species traits remain stable. Instead, an eco-evolutionary approach will be required (Hairston et al., 2005; Buckling et al., 2009; Schoener, 2011), in which traits evolve in response to changes in environmental conditions that are at least partly induced by the phytoplankton blooms themselves.

In conclusion, our study shows that changes in C₄ availability act as an important selective factor in cyanobacterial communities. These changes may cause a seasonal succession of different C₄ uptake genotypes during bloom development. At longer time scales, rising atmospheric CO₂ levels create a steeper pCO₂ gradient across the air-water interface during dense summer blooms, which will lead to a higher influx of atmospheric CO₂ into lakes (Balmer and Downing, 2011; Cole et al., 2010). Rising atmospheric CO₂ levels may therefore result in less intense CO₂ depletion during blooms (Verspagen et al., 2014b), and a later onset and shorter duration of CO₂-depleted conditions during the summer period. Our results suggest that high-flux C₄ uptake genotypes will become relatively more abundant when CO₂ concentrations increase. Hence, future harmful algal blooms will most likely have a genetic composition that differs from contemporary blooms and will be tuned to the high-CO₂ conditions.

**Materials and Methods**

**Competition experiments**

Competition experiments were conducted in CO₂-controlled chemostats designed specifically for phytoplankton studies (Van de Waal et al., 2011; Sandrini et al., 2015a). The chemostats consisted of flat culture vessels with an optical path length of 5 cm and an effective working
Rapid microevolutionary adaptation of Microcystis

The chemostats were illuminated from one side at a constant incident irradiance of $I_{in} = 40 \mu \text{mol photons m}^{-2} \text{s}^{-1}$ using white fluorescent tubes (Philips Master TL-D 90 De Luxe 18 W/965, Philips Lighting, Eindhoven, the Netherlands). The chemostats were maintained at a constant temperature of 25°C and aerated with different CO$_2$ concentrations (Sandrini et al., 2015a; 100 or 1,000 ppm pCO$_2$) at a flow rate of 30 L h$^{-1}$. The chemostats were run at a dilution rate of 0.2 day$^{-1}$ and the Microcystis strains were grown in a nutrient-rich mineral medium (Sandrini et al., 2015a) without (bi)carbonate salts.

Microcystis strains CCAP 1450/10, HUB 5-2-4, PCC 7806, PCC 7005 and PCC 7941 (Table S5.1, Supplementary Information) were pre-cultured in monoculture chemostats at 400 ppm CO$_2$. Subsequently, six chemostats were each inoculated with the five pre-cultured strains mixed at equal initial abundances and a total initial Microcystis biovolume of ~160 mm$^3$ L$^{-1}$. Three chemostats were exposed to 100 ppm pCO$_2$ (‘low CO$_2$’) and the three other chemostats to 1,000 ppm pCO$_2$ (‘high CO$_2$’). The chemostats were run for a total of 175 days and were sampled 1-3 times per week for further analysis. Cell numbers and biovolumes, light conditions and pH were measured as described before (Sandrini et al., 2015a).

Lake study
Lake Kennemermeer (52°27’18.5”N, 4°33’48.6”E) is located north-west of Amsterdam, the Netherlands, close to the North Sea (Figure 5.2A). The lake is not an official swimming location, because of yearly recurrent problems caused by dense harmful cyanobacterial blooms. The lake has a maximum depth of ~1 m and a surface area of ~0.1 km$^2$. The lake is well mixed by wind throughout the year.

From June to October 2013, we sampled the lake two to three times per month, always at 10:30 AM, at a fixed location at the north side using a small boat. Aliquots of lake water (three replicates of 5 L each) were collected 0.2 m below the surface and processed immediately on land. A Hydrolab Surveyor and Datasonde 4a (OTT Hydromet, Loveland, CO, USA) measured temperature and pH at 0.2 m depth. We averaged the three replicate measurements at each time point, and used these averages as the data points in our analysis. Phytoplankton cells were preserved with Lugol’s iodine for microscopic analysis (see Supplementary Methods for details).

Chemical analyses
Dissolved inorganic carbon and sodium concentrations were measured in filtered supernatant of the chemostat and lake samples. Microcystins were extracted from filtered cells with 75% MeOH and analysed by HPLC (Van de Waal et al., 2011). See Supplementary Methods for details.
Analysis of genotype composition
Genomic DNA (gDNA) was isolated from chemostat samples and lake samples filtered on-site, using spin column DNA extraction kits (see Supplementary Methods for details).

We first investigated whether all three *Microcystis* Ci uptake genotypes were present in purified gDNA lake samples, using PCR reactions with the GoTaq® Hot Start Polymerase kit (Promega Corporation, Madison, WI, USA) according to the supplier’s instructions (see Supplementary Methods). The *Microcystis*-specific primers used for this purpose are listed in Table S5.3, Supplementary Information.

Subsequently, qPCR reactions were applied to purified gDNA to quantify the relative abundances of the different genotypes in the chemostat and lake samples (see Supplementary Methods). For this purpose, we designed gene-specific primers to target the 16S rRNA gene, *rbcX*, *bicA*, *sbtA*, *bicA+sbtA*, *mcyB* and *isiA* (Table S5.3, Supplementary Information), using gDNA from axenic laboratory strains as reference samples. LinRegPCR software (version 2012.3) was used for baseline correction, calculation of Cq values and calculation of the amplification efficiency of individual runs (Table S5.3, Supplementary Information). Relative ratios of the numbers of gene copies were calculated according to the comparative C_T method (Livak and Schmittgen, 2001). The qPCR analysis was validated using defined mixtures of isolated *Microcystis* strains (Figure S5.4, Supplementary Information). Since our field data set was limited to one lake sampled during one summer, we did not attempt to separate the statistical effects of many environmental variables on the genotype assemblage. Instead, we applied simple correlation analyses to describe relationships between the relative frequencies of the Ci uptake genotypes and the Ci concentration. The rate of replacement was estimated from the slope of the linear regression of ln(x1/x2) versus time (Dykhuizen and Hartl, 1983), where x1 and x2 are the relative frequencies of two genotypes. The selection coefficient was calculated by scaling the replacement rate to the generation time. The generation time in the chemostats was calculated as \( t_d = \ln(2)/\mu \), where \( \mu \) is the growth rate of the total *Microcystis* population (taking into account the dilution rate).

Acknowledgements
We thank Cathleen Broersma for assistance with the laboratory competition experiments, Bas van Beusekom and Hélène Thuret for support with lake sampling, Leo Hoitinga for assistance with the DIC measurements, Dennis Jakupovic for assistance with the gDNA extractions, and Jan van Arkel for drawing the map of Lake Kennemermeer. This research was supported by the Division of Earth and Life Sciences (ALW) of the Netherlands Organization for Scientific Research (NWO). VML acknowledges support by Wetsus. JMS acknowledges support by the BE-BASIC Foundation.
Author Contributions

GS, XJ, JMHV, HCPM and JH designed the study. GS developed the methods for the genotype analysis. XJ and JMHV performed the laboratory competition experiments and analysed the chemostat samples. GS, RPT and PS performed the fieldwork, assisted by JMS and HCPM. GS analysed most field samples, and VML performed the microscopy. GS, XJ, HCPM and JH analysed the data and wrote the manuscript, and all authors commented on the final version.
Supplementary Information

Contents
This section contains the following data:

I. Supplementary Methods
II. Supplementary Figures
III. Supplementary Tables
I. Supplementary Methods

Cell counts of lake samples
Lugol’s iodine was added to fresh lake samples (1:100 v/v of a 5% solution) to preserve phytoplankton cells for microscopy. The samples were stored at 4°C until identification. Phytoplankton was identified to genus level, and if possible to the species level, and counted according to the Utermöhl-method adjusted to the European standard protocol NEN-EN 15204 using a Lyca DM IRB inverted light microscope (Lyca Microsystems BV, Rijswijk, the Netherlands). Biovolume was estimated from cellular dimensions and geometry (Hillebrand et al., 1999). Individual Microcystis cells were counted after disintegrating the colonies with KOH (Kardinaal et al., 2007).

Dissolved inorganic carbon and sodium
Chemostat samples for dissolved inorganic carbon (DIC) measurements were centrifuged for 15 minutes at 4,000 g and 4°C, and subsequently the supernatant was filtered using a 47 mm GF/C filter (Whatman, Maidstone, UK) followed by a 0.45 µm pore size 47 mm diameter polyethersulfone membrane filter (Sartorius AG, Göttingen, Germany). Lake samples for analysis of DIC and sodium concentrations were filtered on-site using the same filtration procedure. The filtrates of chemostat and lake samples were transferred to sterile plastic urine analysis tubes (VF-109SURI; Terumo Europe N.V., Leuven, Belgium), which were filled completely and stored at 4°C until further analysis. DIC (3-5 technical replicates per sample) was measured with a TOC-VCPH TOC analyzer (Shimadzu, Kyoto, Japan). Concentrations of CO₂(aq), bicarbonate and carbonate were calculated from DIC, pH and temperature (Stumm and Morgan, 1996). Sodium concentrations were measured using an Optima 8000 ICP-OES Spectrometer (Perkin Elmer, Waltham, MA, USA).

Toxin analysis
For microcystin analysis, lake samples were filtered on-site over 1.2 µm pore size 25 mm diameter GF/C filters (Whatman). The loaded filters were stored at -20°C and subsequently freeze-dried. Microcystins were extracted with 75% MeOH and analyzed by HPLC as described previously (Van de Waal et al., 2011), using a Shimadzu LC-20AD HPLC system with a SPD-M20A photodiode array detector (Shimadzu, Kyoto, Japan). The two largest microcystin peaks of the lake samples matched with the retention times and UV-spectra of MC-LR and MC-RR standards kindly provided by the University of Dundee.
DNA isolation

Chemostat samples for gDNA isolation were centrifuged for 15 min at 4,000 g and 4°C. Subsequently, gDNA was isolated from the pellets using the DNeasy Blood & Tissue kit (Qiagen GmbH, Hilden, Germany) according to the supplier’s instructions. Lake samples were filtered on-site over 1.2 µm pore size 25 mm diameter GF/C filters (Whatman), and loaded filters were stored at -20°C. Subsequently, gDNA from lake samples was extracted using the ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research, Orange, CA, USA) according to the supplier’s instructions. Both the chemostat and the lake gDNA samples were further purified using the DNA Clean & Concentrator™-25 kit (Zymo Research) according to the supplier’s instructions. The quality of the gDNA in the chemostat and lake samples was assessed using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), which resulted in $A_{260}/A_{280}$ values above 1.8 for all samples. The gDNA concentrations were quantified using the Nanodrop 1000 spectrophotometer for the lake samples and a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) for the chemostat samples.

Primer development and PCR reactions

To detect the presence of different Microcystis C$_i$ uptake genotypes, we designed primers targeting bicA encoding for a low-affinity but high-flux bicarbonate uptake system, sbtA encoding for a high-affinity but low-flux bicarbonate uptake system, and the combined presence of bicA+sbtA (Table S5.1, Supplementary Information). We also designed primers targeting sbtB (a gene often linked with sbtA and encoding for a post-translational regulator of SbtA; Du et al., 2014), the sodium/proton antiporter gene nhaS3 and the LysR-family transcriptional regulator ccmR2. In Microcystis, the bicA, sbtA, sbtB and nhaS3 genes are all located on the same operon, and ccmR2 is the transcriptional regulator located upstream (Sandrini et al., 2014; Figure 5.1 of the main text). Furthermore, to detect the presence of toxic (microcystin-producing) genotypes, we included primers targeting the mcyB gene involved in microcystin synthesis. We developed primers targeting the iron stress induced protein gene isiA, to distinguish strain CCAP 1450/10 from the other strains (Table S5.1, Supplementary Information). Finally, we also developed primers targeting the 16S rDNA genes and the RuBisCO chaperone gene rbcX, to quantify the overall Microcystis population.

The primers were designed to match sequences of 13 Microcystis strains for which the full genome was sequenced, including Microcystis NIES-843 (Kaneko et al., 2007), Microcystis PCC 7806 (Frangeul et al., 2008), Microcystis PCC 7005 (Sandrini et al., 2014) and ten Microcystis strains recently sequenced (Humbert et al., 2013). The primers targeting the ccmR2, bicA, sbtA, sbtB and nhaS3 genes also matched sequences of seven additional strains for which only ccmR2 and the bicA-sbtAB-nhaS3 operon were sequenced (Sandrini et al., 2014); hence,
these primers are based on 20 *Microcystis* strains in total. Sequences of several other (non-*Microcystis*) cyanobacteria were used to ensure that the primer design included several mismatches with these other sequences. Furthermore, discrimination between *Microcystis* and other cyanobacteria is aided by the observation that *Microcystis* is thus far the only known cyanobacterial genus with *bicA* and *sbtA* located next to each other in the genome in one operon (Sandrini *et al.*, 2014). Other cyanobacteria with both *bicA* and *sbtA* have these genes placed in separate locations in the genome. **Table S5.3, Supplementary Information** provides an overview of all primers used in this study.

PCR reactions, to test the developed primers, were done with the GoTaq® Hot Start Polymerase kit (Promega Corporation, Madison, WI, USA) according to the supplier’s instructions. After an initial denaturation of 2 min at 95°C, 35 cycles were used that consisted of a denaturation step at 95°C for 45 s, an annealing temperature step at 60°C for 30 s and an extension step at 72°C for 3 min. Subsequently, a final extension step at 72°C was used for 5 min. The reactions contained 0.3 µmol L⁻¹ primers and 10 ng gDNA in a total reaction volume of 25 µL. Other reaction components were added as instructed by the supplier. The developed primers were tested with gDNA of various *Microcystis* laboratory strains (CCAP 1450/10, CCAP 1450/11, HUB 5-3, HUB 5-2-4, NIES-843, NIVA-CYA 140, PCC 7005, PCC 7806, PCC 7941, V145 and V163) using PCR and gel electrophoresis, which confirmed that only the targeted gDNA sequences were amplified (no by-products were detected). As negative control, the primers were tested on gDNA of several ubiquitous freshwater cyanobacterial species, *Anabaena circinalis* CCAP 1403/18, *Aphanizomenon flos-aquae* CCAP 1401/7 and *Planktothrix agardhii* CCAP 1460/1, which did not result in amplified PCR products.

Next, PCR reactions and gel electrophoresis were performed to detect the different *Microcystis C₄* uptake genotypes in purified gDNA from Lake Kennemermeer samples. Again the GoTaq® Hot Start Polymerase kit (Promega Corporation) was used, and gDNA of five *Microcystis* laboratory strains was investigated for comparison. All three *Microcystis C₄* uptake genotypes, *bicA* strains (no *sbtA*), *sbtA* strains (no *bicA*) and *bicA+sbtA* strains, were detected in all lake samples (**Table S5.4, Supplementary Information**). PCR-products of strain NIVA-CYA 140, which has a transposon insert in *bicA* (Sandrini *et al.*, 2014), were not detected in Lake Kennemermeer. This indicates that strains with such a transposon insert in *bicA* were not present in the lake.

**Quantification of C₄ uptake genotypes with qPCR**
Relative abundances of the different genotypes of *Microcystis* were quantified using qPCR, by applying the Maxima® SYBR Green Master Mix (2x) kit (Thermo Fisher Scientific, Waltham, MA, USA) to purified gDNA according to the supplier’s instructions in an ABI 7500 Real-
Time PCR system (Applied Biosystems, Foster City, CA, USA). The two-step cycling protocol was used, with a denaturation temperature of 95°C (15 s) and a combined annealing/extension temperature of 60°C (60 s) during 40 cycles. The reactions contained 0.3 µmol L⁻¹ primers, with 1 ng gDNA when using chemostat samples and 10 ng gDNA when using lake samples, in a total reaction volume of 25 µL. Other reaction components were added as instructed by the supplier. ROX solution was used to correct for any well-to-well variation and melting curve analysis was performed on all measured samples to rule out non-specific qPCR products. Each qPCR plate contained reference gDNA samples and samples with primers targeting reference genes (see below) to overcome plate effects. The LinRegPCR software tool (version 2012.3; Ramakers et al., 2003; Ruijter et al., 2009) was used for baseline correction, calculation of quantification cycle (Cq) values using linear regression, and calculation of the amplification efficiency of each individual run (Table S5.3, Supplementary Information). LinRegPCR did not detect samples without amplification or a plateau (except for negative controls), or samples with a baseline error or noise error, or with deviating amplification efficiencies. To calculate relative ratios between the numbers of gene copies, the comparative C_T method was used (Livak and Schmittgen, 2001).

To determine the relative abundance of toxic genotypes, we used the microcystin synthetase gene mcyB (primers mcyB-F and mcyB-R) as ‘target gene’ and the RuBiSCO chaperone gene rbcX (primers rbcX-F and rbcX-R) present in all Microcystis strains as ‘reference gene’. Purified gDNA of the axenic toxic strains PCC 7806 and PCC 7941 was used as ‘reference samples’.

To determine the relative abundances of the five Microcystis strains in the chemostat experiments, we used the bicA, sbtA, bicA+sbtA, mcyB and isiA genes as ‘target genes’ to distinguish the different strains (Table S5.1, Supplementary Information). We used the 16S
rRNA gene (primers 16S-F and 16S-R) to quantify the overall *Microcystis* population ('reference gene'). Purified gDNA of monocultures of strain PCC 7941 (which contained all five target genes) served as 'reference samples' to calculate the relative abundances of strains with and without *bicA*, *sbtA*, *bicA+sbtA*, *mcyB* and *isiA*, assuming that the sum of the five strains equals 100%.

**Validation of qPCR method**

To validate the developed method, exponentially growing cultures of the five laboratory strains of *Microcystis* were mixed with equal cell numbers (20% each when mixing five strains, and 50% each when mixing two strains). Cell numbers were counted using a Casy 1 TTC cell counter with a 60 µm capillary (Schärfe System GmbH, Reutlingen, Germany).

The gDNA of the mixtures was extracted and used for relative quantification of the three defined *Microcystis* C₄ uptake genotypes using the qPCR approach described above. Non-mixed gDNA of the monocultures of strains PCC 7005, PCC 7806 and PCC 7941 was used as reference samples for the calculations.

In all our validation experiments, the relative abundances of the different genotypes measured by qPCR were very similar to the expected relative abundances ([Figure S5.4, Supplementary Information](#)).
II. Supplementary Figures

Figure S5.1. Replacement rates of the C\textsubscript{i} uptake genotypes in the chemostat experiments. (A) Replacement rate of the bic\textsubscript{A} strain by bic\textsubscript{A}+sbt\textsubscript{A} strains in the low CO\textsubscript{2} chemostats. (B) Replacement rate of the sbt\textsubscript{A} strains by bic\textsubscript{A}+sbt\textsubscript{A} strains in the low CO\textsubscript{2} chemostats. (C) Replacement rate of strain PCC 7005 by strain PCC 7941 in the low CO\textsubscript{2} chemostats. (D) Replacement rate of sbt\textsubscript{A} strains by bic\textsubscript{A} and bic\textsubscript{A}+sbt\textsubscript{A} strains in the high CO\textsubscript{2} chemostats; note the biphasic pattern. (E) Replacement rate of strain PCC 7941 by strain PCC 7005 in the high CO\textsubscript{2} chemostats. The rate of replacement was calculated from the slope of the linear regression of ln(genotype 1/genotype 2) versus time. The data points show the mean values of three replicated chemostat experiments.
Rapid microevolutionary adaptation of Microcystis

Figure S5.2. Temperature and cyanobacterial composition of Lake Kennemermeer. (A) Water temperature. (B) Biomass (expressed as biovolume) of the dominant cyanobacteria in summer and autumn of 2013, including Pseudanabaenaceae (Pseudanabaena and Planktolyngbya), small Chroococcales (mainly Cyanonephron and Cyanodictyon), Anabaenopsis hungarica, and Microcystis spp. (M. aeruginosa and M. flos-aquae). Early in the season, Microcystis spp. also includes some Woronichinia pusilla. (C) Relative abundance of toxic Microcystis genotypes, based on the mcyB gene for microcystin production. (D) Total microcystin concentration increases with the Microcystis biomass (expressed as biovolume) (Pearson correlation of log microcystin concentration vs log Microcystis biomass: $\rho = 0.92$, $n = 10$, $p < 0.001$). The trend line is based on linear regression. Error bars represent one standard deviation ($n = 3$).
Figure S5.3. The replacement rate of \textit{bicA+sbtA} strains by \textit{bicA} strains in the lake study. The rate of replacement was calculated from the slope of the linear regression of $\ln(\text{bicA strains/} \text{bicA+sbtA} \text{ strains})$ versus time, using the data points of week 29-38 (blue markers). The data points are the mean of three replicate measurements. Regression statistics: $y = 0.0368x - 8.2066$ ($R^2 = 0.89$, $n = 6$, $p < 0.01$).
Rapid microevolutionary adaptation of Microcystis

Figure S5.4. Validation of the qPCR method quantifying the relative abundances of different *Microcystis* genotypes. (A) Mixture of CCAP 1450/10 (*sbtA* strain) and PCC 7806 (*bicA* strain), with 50% of cell numbers each. (B) Mixture of HUB 5-2-4 (*sbtA* strain) and PCC 7941 (*bicA+sbtA* strain), with 50% of cell numbers each. (C,D) Mixture of CCAP 1450/10, HUB 5-2-4, PCC 7005, PCC 7806 and PCC 7941, with 20% of cell numbers each. Quantification of the different genotypes was based on, (A-C), 16S rRNA gene as reference gene and, (D), *bicA+sbtA* as reference gene. Error bars represent one standard deviation obtained from four replicate qPCR measurements.
Chapter 5

III. Supplementary Tables

Table S5.1. Properties of the five *Microcystis* strains in the competition experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin'</th>
<th>C&lt;sub&gt;i&lt;/sub&gt; uptake genotype</th>
<th>Genes present&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Microcystin production</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCAP 1450/10 GBR</td>
<td>sbtA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HUB 5-2-4 DEU</td>
<td>sbtA</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>PCC 7806 NLD</td>
<td>bicA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PCC 7005 USA</td>
<td>bicA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PCC 7941 CAN</td>
<td>bicA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup>The origins of the strains are indicated with three-letter ISO codes of the different countries.  
<sup>b</sup>The plus sign (+) or dash (−) indicates the presence or absence of a specific gene or microcystin production.

Table S5.2. Selection coefficients calculated for the chemostat experiments and lake study.

<table>
<thead>
<tr>
<th>Strain replacement</th>
<th>Replacement rate (day&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Generation time (days)</th>
<th>Selection coefficient (per generation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low CO&lt;sub&gt;2&lt;/sub&gt; chemostats:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bicA by bicA+sbtA strains</td>
<td>0.252</td>
<td>2.5</td>
<td>0.62</td>
</tr>
<tr>
<td>sbtA by bicA+sbtA strains</td>
<td>0.168</td>
<td>2.5</td>
<td>0.41</td>
</tr>
<tr>
<td>PCC 7005 by PCC 7941</td>
<td>0.047</td>
<td>3.5</td>
<td>0.16</td>
</tr>
</tbody>
</table>

High CO<sub>2</sub> chemostats:  
bicA by bicA+sbtA and bicA strains (first 12 days) | 0.108 | 1.9 | 0.20 |
bicA by bicA+sbtA and bicA strains (next 40 days) | 0.024 | 3.5 | 0.08 |
PCC 7941 by PCC 7005                             | 0.036 | 3.5 | 0.13 |

Lake study:  
bicA vs sbtA by bicA strains | 0.037 | 1.5-5.2 | 0.06-0.19 |

<sup>a</sup>Replacement rates were estimated from the slope of the linear regression of ln(x<sub>1</sub>/x<sub>2</sub>) versus time, where x<sub>1</sub> and x<sub>2</sub> are the relative frequencies of two genotypes (Figures S5.1 and S5.3, Supplementary Information).  
<sup>b</sup>Generation times were calculated as t<sub>d</sub> = ln(2)/µ, where µ is the growth rate of the total *Microcystis* population (including the dilution rate).  
<sup>c</sup>Selection coefficients were calculated as products of the replacement rates and generation times.
Table S5.3. Overview of *Microcystis* primer pairs used for PCR analyses.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5'→3' (length)</th>
<th>Target gene(s)</th>
<th>Accession no. (Genbank)</th>
<th>Expected product size (bp)</th>
<th>Amplification efficiency (E) ± SD(^b)</th>
<th>Normal PCR (1) or qPCR (2)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>bicA-F1</td>
<td>CCTTGGCGATCATC TCTCTG (20)</td>
<td>bicA</td>
<td>IPF_4911 AM778949.1</td>
<td>902/2123</td>
<td>nd</td>
<td>1</td>
<td>This study</td>
</tr>
<tr>
<td>bicA-R1</td>
<td>AGGGACATCGCTCA AGTOCA (20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sandrini et al., 2015a</td>
</tr>
<tr>
<td>bicA-F1</td>
<td>CTTTGGCGATCATC TCTCTG (20)</td>
<td>bicA+sbtA</td>
<td>IPF_4911 AM778949.1</td>
<td></td>
<td>1443</td>
<td>nd</td>
<td>This study</td>
</tr>
<tr>
<td>sbtA-R1</td>
<td>GTTGGAAACTGCAA TGAGGA (20)</td>
<td>ccmR2+sbtA</td>
<td>MAE_62070 AP009552.1</td>
<td>843/2231/2351</td>
<td>nd</td>
<td>1</td>
<td>This study</td>
</tr>
<tr>
<td>sbtA-R2</td>
<td>CAAGCTAACGGTCG CATCAT (20)</td>
<td>bicA+nhaS3</td>
<td>IPF_4911 AM778949.1</td>
<td></td>
<td>1109/2789</td>
<td>nd</td>
<td>Sandrini et al., 2015a</td>
</tr>
<tr>
<td>nhaS3-R</td>
<td>GTCTGAGTAGTAC CAGATCTG (24)</td>
<td>sbtA+SbtB</td>
<td>MAE_62090 AP009552.1</td>
<td>1335</td>
<td>nd</td>
<td>1</td>
<td>This study</td>
</tr>
<tr>
<td>bicA-F2</td>
<td>CCTTGGCGATCATC TCTCTG (20)</td>
<td>bicA</td>
<td>IPF_4911 AM778949.1</td>
<td></td>
<td>132</td>
<td>1.89±0.02</td>
<td>Sandrini et al., 2015a</td>
</tr>
<tr>
<td>bicA-R1</td>
<td>AGGGACATCGCTCA AGTOCA (20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sandrini et al., 2015a</td>
</tr>
<tr>
<td>sbtA-R2</td>
<td>AGTTGGATGCGG GATGG (19)</td>
<td>sbtA</td>
<td>MAE_62090 AP009552.1</td>
<td>143</td>
<td>1.88±0.02</td>
<td>2</td>
<td>Sandrini et al., 2015c</td>
</tr>
<tr>
<td>sbtB-R</td>
<td>GTGCGAGGGAAGTC TCCCTTC (22)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sandrini et al., 2015c</td>
</tr>
<tr>
<td>bicA-F3</td>
<td>TCAAGCCCATCCT CACCA (19)</td>
<td>bicA+sbtA</td>
<td>IPF_4911 AM778949.1</td>
<td></td>
<td>264</td>
<td>1.86±0.02</td>
<td>This study</td>
</tr>
<tr>
<td>sbtA-R3</td>
<td>GCCGCAATCAGAA GGCA (20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sandrini et al., 2015c</td>
</tr>
<tr>
<td>rbcX-F</td>
<td>CGGATGATGAGGG TAAAGAACA (23)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sandrini et al., 2015c</td>
</tr>
<tr>
<td>rbcX-R</td>
<td>ATCCGATGATGC GGTCAG (22)</td>
<td>rbcX</td>
<td>IPF_2531 AM77933.1</td>
<td>157</td>
<td>1.88±0.02</td>
<td>2</td>
<td>Sandrini et al., 2015c</td>
</tr>
<tr>
<td>mcyB-F</td>
<td>ATCCCATGCTGAG GACGTT (20)</td>
<td>mcyB</td>
<td>IPF_375 AM778952.1</td>
<td>163</td>
<td>1.87±0.02</td>
<td>2</td>
<td>Sandrini et al., 2014</td>
</tr>
<tr>
<td>mcyB-R</td>
<td>AGATGTCGGCAGG ATTCA (20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sandrini et al., 2014</td>
</tr>
<tr>
<td>isiA-F</td>
<td>CTTTGGCTTGTGGA GTGGCAG (22)</td>
<td>isiA</td>
<td>IPF_5322 AM778872.1</td>
<td>117</td>
<td>1.89±0.01</td>
<td>2</td>
<td>This study</td>
</tr>
<tr>
<td>isiA-R</td>
<td>GTGAAATAAGGCT CCTGCTC (21)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>This study</td>
</tr>
</tbody>
</table>

\(^a^\)The locus tags are from *Microcystis* PCC 7806 (IPF) and *Microcystis* NIES-843 (MAE).

\(^b^\)The amplification efficiency \(E\) was based on 50-65 amplification curves for each of the primer sets. nd: not determined.
Chapter 5

Table S5.4. Results of PCR reactions on gDNA from lake samples.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Expected PCR product size (bp)</th>
<th>PCC 7806</th>
<th>NIES-843</th>
<th>CCAP 1450/10</th>
<th>NIVA-CYA 140</th>
<th>PCC 7005</th>
<th>Samples from Lake Kennemermeer</th>
</tr>
</thead>
<tbody>
<tr>
<td>bicA-F1 &amp; bicA-R1</td>
<td>bicA</td>
<td>902/2123</td>
<td>902</td>
<td>-</td>
<td>-</td>
<td>2123</td>
<td>902 902</td>
</tr>
<tr>
<td>bicA-F1 &amp; sbtA-R1</td>
<td>bicA+sbtA</td>
<td>1443</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1443</td>
<td>1443 1443</td>
</tr>
<tr>
<td>ccmR2-F1 &amp; sbtA-R1</td>
<td>ccmR2+sbtA</td>
<td>843/2231/2351/3572</td>
<td>-</td>
<td>843</td>
<td>2231</td>
<td>-</td>
<td>2351 843+2351</td>
</tr>
<tr>
<td>bicA-R2 &amp; nhaS3-R</td>
<td>bicA+nhaS3</td>
<td>1109/2789</td>
<td>1109</td>
<td>-</td>
<td>-</td>
<td>2789</td>
<td>2789 1109</td>
</tr>
<tr>
<td>sbtA-F1 &amp; sbtB-R</td>
<td>sbtA+sbtB</td>
<td>1335</td>
<td>-</td>
<td>1335</td>
<td>1335</td>
<td>1335</td>
<td>1335 1335</td>
</tr>
</tbody>
</table>

The PCR reactions on gDNA were used to investigate the presence of the different C\textsubscript{i} uptake genotypes in lake samples (last column). For comparison, five isolated Microcystis strains were used as reference. A dash ‘−’ indicates that no PCR product was detected because the gene was absent in that strain.