Rapid adaptation of harmful cyanobacteria to rising CO_2

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Rising atmospheric CO_2 concentrations are likely to affect many ecosystems worldwide. However, to what extent elevated CO_2 will induce evolutionary changes in photosynthetic organisms is still a major open question. 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 can develop toxic cyanobacterial blooms in many eutrophic lakes and reservoirs worldwide. *Microcystis* displays genetic variation in the C_i uptake systems BicA and SbtA, where BicA has a low affinity for bicarbonate but high flux rate, and SbtA has a high affinity but low flux rate. Our laboratory competition experiments show that *bicA* + *sbtA* genotypes were favored by natural selection at low CO_2 levels, but were partially replaced by the *bicA* genotype at elevated 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 strains with only the high-flux *bicA* gene when C_i concentrations increased later in the season. 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.

climate change | harmful algal blooms | *Microcystis aeruginosa* | microevolution | natural selection

Atmospheric CO_2 concentrations are predicted to double during this century (1, 2). 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 photosynthetic species to future CO_2 levels (3). Several recent laboratory studies have investigated the potential for evolutionary changes in response to rising CO_2 concentrations (4–9). For example, selection experiments with the green alga *Chlamydomonas reinhardtii* revealed that some cell lines grown at elevated CO_2 levels for 1,000 generations had obtained a reduced growth rate at ambient CO_2, presumably because mutations reduced the effectiveness of CO_2 acquisition (4, 5). Other laboratory studies argue that elevated atmospheric CO_2 fails to evoke specific evolutionary adaptation in phytoplankton species (7). Thus far, however, the specific genetic and molecular adaptations to rising CO_2 favored by natural selection are not well understood. Furthermore, adaptation to changing CO_2 conditions has rarely been investigated within complex species assemblages (9) and, to our knowledge, has never been reported from natural waters.

Cyanobacteria produce dense and often toxic blooms in many eutrophic lakes worldwide (10–12), and are likely to benefit from eutrophication and global warming (10, 13–15). Dense cyanobacterial blooms can deplete the dissolved CO_2 [CO_2(aq)] concentration (16–18), which provides an opportunity to study adaptation to changes in carbon availability. Cyanobacteria are often thought to be superior competitors when CO_2(aq) concentrations are depleted, because they use a very effective CO_2-concentrating mechanism (CCM) (19, 20). 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 (21). 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 (15, 21–23). 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 (21, 24). 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.

We recently compared CCM gene sequences of 20 strains of *Microcystis* (23), a ubiquitous cyanobacterium that can produce a potent family of hepatotoxins known as microcystins (25). The strains were very similar in their CCM gene composition, but 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 (23): *sbtA* strains (with *sbtA* but no or incomplete *bicA*), *bicA* strains (with *bicA* but no *sbtA*), and *bicA* + *sbtA* strains (Fig. 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 (23). Little is known, however, about the occurrence of these three C_i uptake genotypes in lakes, and how

Significance

Cyanobacterial blooms pose a major threat to the water quality of many eutrophic lakes and reservoirs. Cyanobacteria are thought to be very effective competitors when CO_2 levels are depleted during dense blooms. Their response to elevated CO_2 is less understood, however. We study competition among cyanobacteria, and find both laboratory and field evidence for natural selection of strains with different carbon uptake systems at different CO_2 levels. Our results demonstrate that changes in inorganic carbon availability act as an important selective factor in cyanobacterial communities and suggest that future harmful cyanobacterial blooms will have a genotype composition that differs from contemporary blooms and will be tuned to the high-CO_2 conditions.

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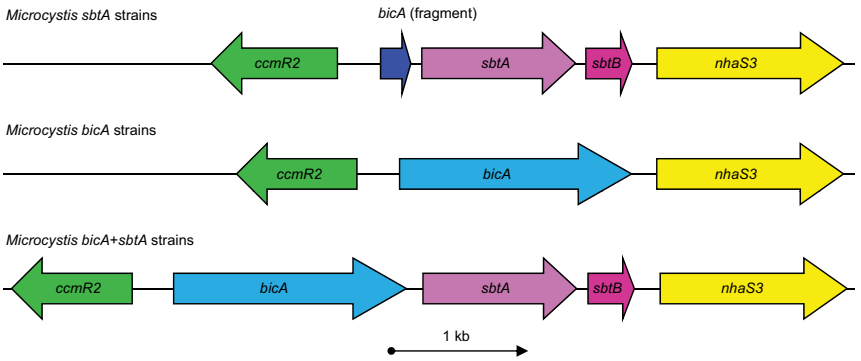

Fig. 1. The three C_i uptake genotypes of *Microcystis*. The variable genomic regions contain the transcriptional regulator gene *ccmR2*, the sodium-dependent bicarbonate uptake genes *bicA* and *sbtA*, a posttranslation regulator gene of SbtA known as *sbtB*, and the sodium/proton antiporter gene *nhaS3*.

their relative frequencies may change in response to increasing C_i concentrations. 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 lake study.

Results

Competition Experiments. We ran competition experiments in three chemostats at low CO_2 (100 ppm) and three chemostats at high CO_2 (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) (*SI Appendix*, Table S1). Four strains produced the hepatotoxin microcystin, whereas PCC 7005 was the only nontoxic strain. In the low CO_2 chemostats, the total *Microcystis* biomass increased until a steady state was reached, at which the CO_2(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 (Fig. 2 *A* and *C*). The high CO_2 chemostats produced a 2.5-fold higher *Microcystis* biomass, with much higher steady-state CO_2(aq) and bicarbonate concentrations of 10 μmol·L^{-1} and 2,700 μmol·L^{-1}, respectively, and a lower pH of 8.8 (Fig. 2 *B* and *D*).

In the low CO_2 chemostats, the toxic *bicA* + *sbtA* strain PCC 7941 competitively replaced the two *sbtA* strains, the *bicA* strain, and the nontoxic *bicA* + *sbtA* strain, and comprised ∼90% of the total *Microcystis* population at steady state (Fig. 2*E*). In contrast, in the high CO_2 chemostats, the nontoxic *bicA* + *sbtA* strain PCC 7005 coexisted with the toxic *bicA* strain PCC 7806, with relative abundances of ∼60% and ∼30%, respectively, at steady state (Fig. 2*F*). Selection coefficients, calculated from the replacement rates of the strains and their generation times, ranged from 0.16 to 0.62 in the low CO_2 chemostats and from 0.08 to 0.20 in the high CO_2 chemostats (*SI Appendix*, Fig. S1 and Table S2).

Lake Study. Our field study was carried out in Lake Kennemermeer, a slightly brackish coastal dune lake in The Netherlands, in summer and autumn of 2013 (Fig. 3*A*). Water temperature was 20–23 °C in summer, and then gradually declined to 11 °C in early autumn (*SI Appendix*, Fig. S2). The lake contained high phytoplankton abundances, with particularly dense blooms in weeks 26–28 and week 36 (Fig. 3*B*). The phytoplankton community consisted largely of cyanobacteria (>95% of the total biovolume), including Pseudanabaenaceae, small Chroococcales, *Anabaenopsis hungarica* and *Microcystis* spp. The *Microcystis* population was dominated by potentially toxic genotypes containing the microcystin synthetase gene *mcyB* (*SI Appendix*, Fig. S2). Concentrations of *Microcystis* and the two dominant microcystin types, MC-LR and MC-RR, peaked in week 34 (Fig. 3*C*). The MC-LR concentration (up to 23.2 μg·L^{-1}) exceeded the provisional guideline for safe drinking water (1 μg·L^{-1}) of the World Health Organization and common guidelines for recreational waters (26), justifying closure of the lake for recreation.

In summer, CO_2(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 (Fig. 3*D*). When phytoplankton abundance decreased in autumn, the CO_2(aq) concentration increased to ∼15 μmol·L^{-1}, equilibrating with the atmospheric pCO_2 levels (390 ppm). CO_2 depletion during the summer bloom led to a strong increase in pH, with values up to ∼10 during the two peaks in phytoplankton abundance (Fig. 3*B*) (Pearson correlation of pH vs. log CO_2: $r = -0.98$, $n = 10$, $P < 0.001$). At pH between 8 and 10, bicarbonate is the dominant C_i species. Bicarbonate concentrations showed similar temporal dynamics as the CO_2(aq) concentration, with relatively low bicarbonate concentrations during the summer blooms and higher concentrations in autumn (Fig. 3*D*) [Pearson correlation of CO_2(aq) vs. bicarbonate: $r = 0.90$, $n = 10$, $P < 0.001$]. 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, because both transporters have half-saturation constants of 1–2 mmol·L^{-1} sodium (24, 27).

All three C_i uptake genotypes of *Microcystis* were present in the lake (Fig. 1 and *SI Appendix*, Tables S3 and S4), but their relative abundances changed during the study period (Fig. 4*A*). The *sbtA* strains represented ∼20% of the *Microcystis* population during the entire season (Fig. 4*B*). The *bicA* + *sbtA* strains dominated the *Microcystis* population in weeks 24–32, especially during the strong CO_2 depletion in weeks 28–29, but were largely replaced by *bicA* strains in late summer and autumn when C_i concentrations in the lake increased. Relative abundances of the *bicA* + *sbtA* strains were negatively correlated with the bicarbonate concentration (Fig. 4*C*) (Pearson correlation: $r = -0.84$, $n = 10$, $P < 0.01$), whereas those of the *bicA* strains were positively correlated with bicarbonate (Fig. 4*D*) (Pearson correlation: $r = 0.91$, $n = 10$, $P < 0.001$). Replacement of *bicA* + *sbtA* strains by *bicA* strains occurred in ∼2 months. With a generation time of 1.5–5.2 d (28), 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 (*SI Appendix*, Fig. S3 and Table S2).

Discussion

Our laboratory competition experiments and lake study show qualitatively similar changes in strain composition. In both systems, the *Microcystis* population was dominated by *bicA* + *sbtA* strains at low C_i levels that were (partly) replaced by *bicA* strains at high C_i levels (Figs. 2 and 4). Previous studies have shown that the high-affinity but low-flux bicarbonate uptake system SbtA is more effective at low bicarbonate concentrations, whereas the

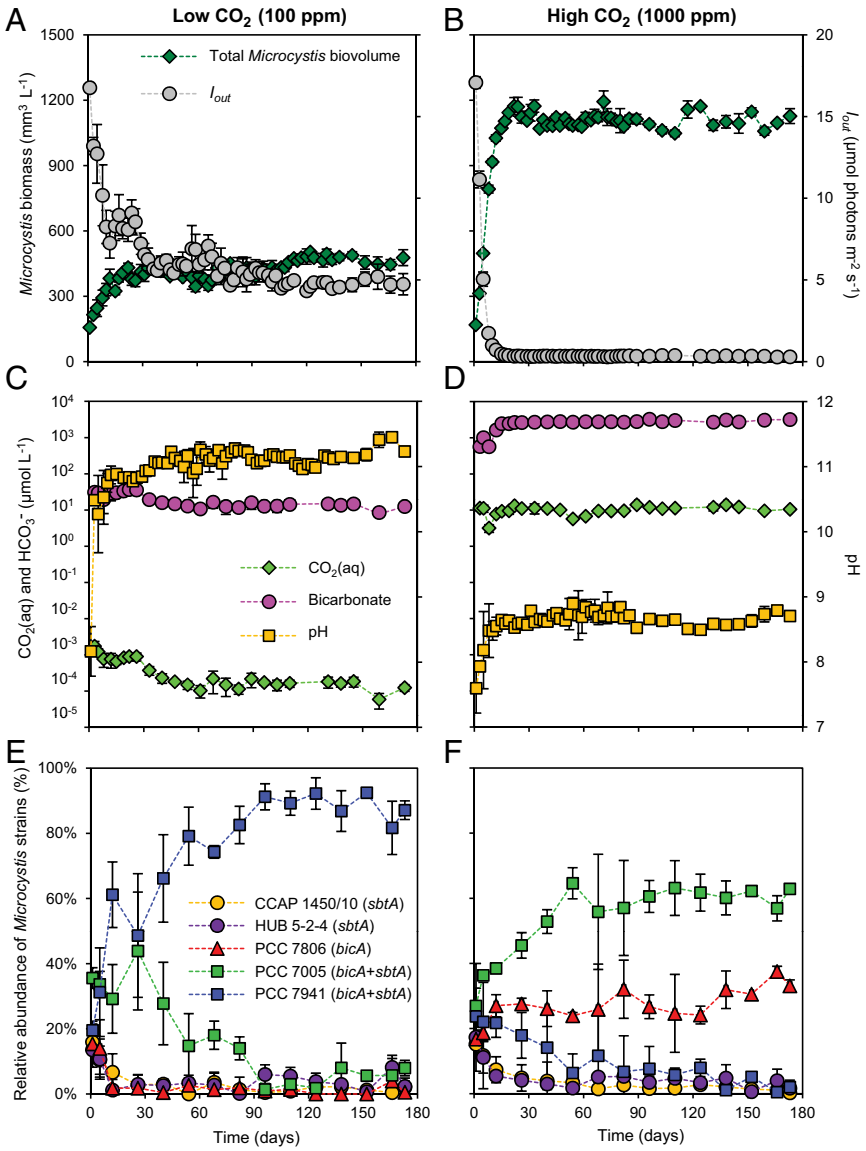

Fig. 2. Laboratory competition experiments with five *Microcystis* strains at low and high CO_2 levels. (*Left*) Low CO_2 chemostats. (*Right*) High CO_2 chemostats. (*A* and *B*) *Microcystis* biomass (expressed as biovolume) and the light intensity I_{out} transmitted through the chemostats. (*C* and *D*) Concentrations of dissolved CO_2 [CO_2(aq)] and bicarbonate, and pH. (*E* and *F*) Relative abundances of the five *Microcystis* strains. The data points show the mean values (±SD) of three replicated chemostat experiments.

low-affinity but high-flux enzyme BicA is more effective at high bicarbonate concentrations (23, 24). Hence, the trade-off between affinity and flux rate offers a likely explanation for the observed shift in strain composition. In particular, with increasing CO_2 and bicarbonate concentrations, high-affinity bicarbonate uptake systems are no longer needed. We note that in *bicA* + *sbtA* strains of *Microcystis*, *bicA* and *sbtA* are located on the same operon and hence are cotranscribed (23). Superfluous transcription of *sbtA* or posttranscriptional down-regulation of the SbtA enzyme, for example by SbtB (27), will be costly when SbtA cannot be efficiently used, which will disfavor *bicA* + *sbtA* strains at high bicarbonate concentrations. Our results thus provide both laboratory and field evidence demonstrating that *bicA* strains; that is, strains with low-affinity but high-flux bicarbonate uptake systems, have a selective advantage at high C_i availability.

In addition to bicarbonate uptake systems, cyanobacteria also deploy two intracellular CO_2 "uptake" systems, which convert CO_2 passively diffusing into the cell into bicarbonate (21, 29, 30). In all *Microcystis* strains investigated so far, both CO_2 uptake systems were present (23) and the genes encoding these two CO_2 uptake systems were constitutively expressed (31–33). A possible explanation might be that the sustained activity of both intracellular CO_2 uptake systems helps to maintain a low CO_2(aq) concentration in the cytoplasm, thus maximizing the diffusive influx of CO_2. Bloom-forming cyanobacteria like *Microcystis* usually occur in mildly to highly alkaline waters (pH > 7.5), where bicarbonate concentrations are much higher than the CO_2(aq) concentration (Fig. 3*D*). Laboratory studies have shown that the high-affinity bicarbonate uptake system BCT ceases its activity, whereas the BicA enzyme remains active at elevated CO_2 levels (31, 32), and that bicarbonate still accounts for most of the C_i uptake even at elevated CO_2 levels (34–36). Hence, in alkaline waters, adaptation of the bicarbonate uptake systems to changes in C_i availability is indeed likely to have major fitness consequences.

Quantitatively, *bicA* strains responded more strongly to increasing C_i concentrations in the lake than in the chemostat

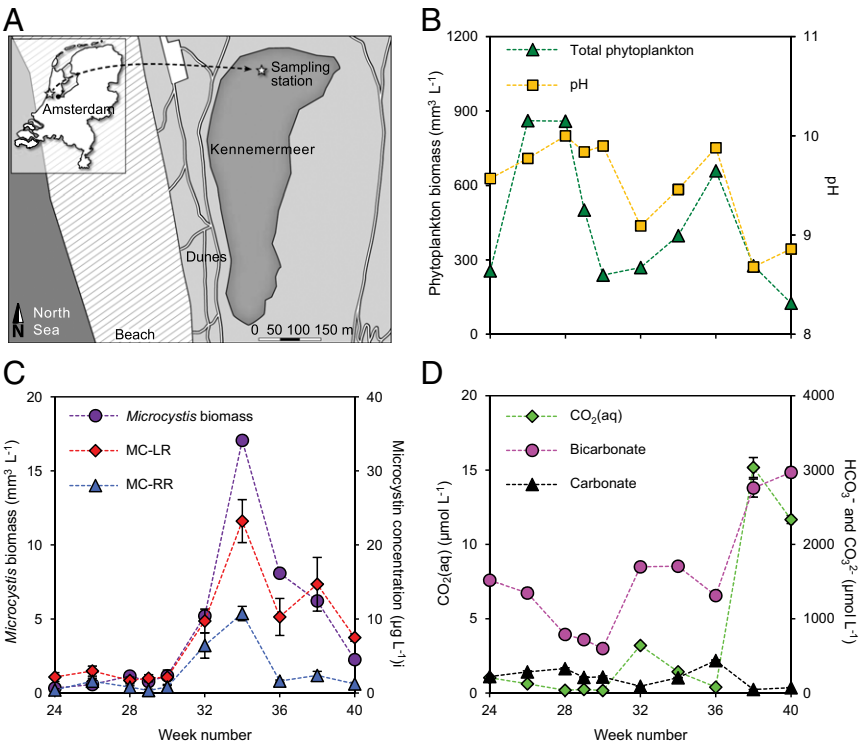

Fig. 3. Phytoplankton biomass and inorganic carbon speciation in Lake Kennemermeer. (*A*) Map of Lake Kennemermeer with the sampling station. (*B*) Total phytoplankton biomass (expressed as biovolume) and pH. (*C*) *Microcystis* biomass (expressed as biovolume) and concentrations of the two most abundant microcystin variants (MC-LR and MC-RR). (*D*) Concentrations of CO_2(aq), bicarbonate, and carbonate. Data points represent the mean (±SD) of three replicate measurements; error bars that are not visible do not exceed the size of the symbols.

experiments, whereas *sbtA* strains persisted in the lake but were competitively excluded from the chemostats. These dissimilarities might be attributed to differences in environmental conditions between lakes and laboratory experiments. For example, the chemostat experiments used continuous illumination, whereas *Microcystis* in Lake Kennemermeer experienced large daily fluctuations in both light conditions and inorganic carbon concentrations, which induced diel variation in expression of the bicarbonate uptake genes and several other CCM genes (33). Furthermore, the chemostats applied homogeneous mixing to single-celled *Microcystis* populations, whereas *Microcystis* often develops multicellular colonies migrating vertically in stagnant lakes (37). *Microcystis* is also known to survive prolonged burial in lake sediments, after which it can be resuspended in the water column (38). Both spatiotemporal heterogeneity and reseeding from the sediments tend to promote diversity, and may explain the observed co-occurrence of all three C_i uptake genotypes in the lake.

Some of our results may also be due to further variation among *Microcystis* strains other than their C_i uptake genes (39, 40). For instance, the strains in our selection experiments differed in the production of microcystin. In addition to its toxicity to humans and mammals, microcystin can also bind to cyanobacterial proteins such as the RuBisCO enzyme to offer protection against oxidative stress (41). Carbon-limited conditions are likely to induce more oxidative stress than elevated CO_2 concentrations, which may explain why the microcystin-producing *bicA* + *sbtA* strain PCC 7941 performed better at 100 ppm CO_2, whereas the nonmicrocystin-producing *bicA* + *sbtA* strain PCC 7005 performed better at 1,000 ppm (Fig. 2). Our lake study, however, did not show a relation between the relative abundance of microcystin-producing strains and the C_i concentrations (*SI Appendix*, Fig. S2*C*), indicating that selection among the different C_i uptake genotypes played a much larger role in the adaptation to changing C_i concentrations than genetic variation in microcystin production.

Our results add to the growing literature showing that plankton species are capable of rapid evolutionary adaptation to changing conditions; for example, in response to rising CO_2 levels (6, 8, 9), increasing temperature (42, 43), and changes in predation pressure (44, 45). A key advance of the present work is that the adaptive changes could be linked to specific genetic and molecular traits, which enabled monitoring of natural selection not only in confined laboratory experiments but also in lakes. In our study, and several previous studies (6, 42–45), the 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 C_i 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 based on the assumption that species traits remain constant. Instead, an ecoevolutionary approach will be required (46–48), in which traits evolve in response to changes in environmental conditions that, in the case of CO_2 depletion, are at least partly induced by the phytoplankton blooms themselves.

In conclusion, our study shows that changes in C_i availability act as an important selective factor in cyanobacterial communities. Some strains perform better at low C_i concentrations, whereas other strains are better competitors at high C_i levels, causing a succession of different C_i uptake genotypes during bloom development. Models and laboratory experiments predict that rising atmospheric CO_2 levels will lead to higher CO_2(aq) and bicarbonate concentrations, and a later onset and shorter duration of CO_2-depleted conditions during dense summer blooms (14, 15). Our results suggest that this increased C_i availability will favor low-affinity but high-flux bicarbonate uptake genotypes. Hence, future harmful

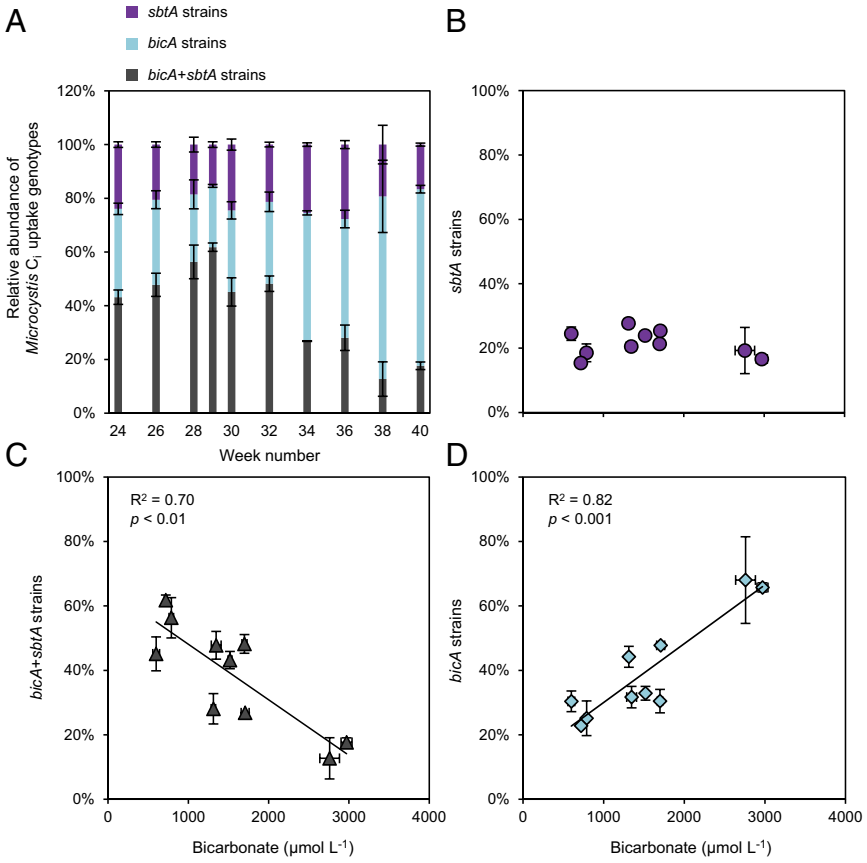

Fig. 4. C_i uptake genotypes of *Microcystis* in the lake study. (*A*) Relative abundances of the three C_i uptake genotypes (*sbtA* strains, *bicA* strains, and *bicA* + *sbtA* strains). (*B–D*) Relation between the relative abundances of the C_i uptake genotypes and the bicarbonate concentration. The data points show the mean (±SD) of three replicate measurements. The trend lines are based on linear regression (n = 10).

cyanobacterial blooms will most likely have a genotype composition that differs from contemporary blooms and will be adapted to the new conditions in a high-CO_2 world.

Materials and Methods

Competition Experiments. Competition experiments were conducted in CO_2-controlled chemostats designed specifically for phytoplankton studies (14, 31, 49). The chemostats consisted of flat culture vessels with an optical path length of 5 cm and an effective working volume of 1.8 L. The chemostats were illuminated from one side at a constant incident irradiance of I_{in} = 40 µmol photons·m^{-2}·s^{-1} using white fluorescent tubes (Philips Master TL-D 90 De Luxe 18 W/965; Philips Lighting). The chemostats were maintained at a constant temperature of 25 °C, using a nutrient-rich mineral medium without (bi)carbonate salts, and aerated with small gas bubbles containing either 100 or 1,000 ppm CO_2 at a flow rate of 30 L·h^{-1} (31). The chemostats were run at a dilution rate of 0.2 d^{-1}.

Microcystis strains CCAP 1450/10, HUB 5-2-4, PCC 7806, PCC 7005, and PCC 7941 (*SI Appendix*, Table S1) were precultured in monoculture chemostats at 400 ppm CO_2. Subsequently, six chemostats were each inoculated with the five precultured 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 d and were sampled one to three times per week for further analysis. Cell numbers, biovolumes, light conditions, and pH were measured as described before (31).

Lake Study. Lake Kennemermeer (52°27′18.5″N, 4°33′48.6″E) is a shallow coastal dune lake located northwest of Amsterdam, The Netherlands, close to the North Sea (Fig. 2*A*). 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) measured temperature and pH at 0.2-m depth. Phytoplankton cells were preserved with Lugol's iodine for microscopic analysis (*SI Appendix, SI Materials and Methods*).

Chemical Analyses. Dissolved inorganic carbon (DIC) and sodium concentrations were measured in filtered supernatant of the chemostat and lake samples. CO_2(aq), bicarbonate, and carbonate concentrations were calculated from DIC, pH, and temperature (*SI Appendix*, Table S5). Microcystins were extracted from filtered cells with 75% MeOH and analyzed by HPLC (49). See *SI Appendix, SI Materials and Methods* for details.

Analysis of Genotype Composition. Genomic DNA (gDNA) was isolated from chemostat samples and lake samples that were filtered on-site, using spin column DNA extraction kits (*SI Appendix, SI Materials and Methods*).

We first investigated whether all three *Microcystis* C_i uptake genotypes were present in purified gDNA lake samples, using PCR reactions with the GoTaq Hot Start Polymerase kit (Promega) according to the supplier's instructions (*SI Appendix, SI Materials and Methods*). The *Microcystis*-specific primers used for this purpose are listed in *SI Appendix*, Table S3.

Subsequently, qPCR reactions were applied to purified gDNA to quantify the relative abundances of the different genotypes in the chemostat and lake samples (*SI Appendix, SI Materials and Methods*). For this purpose, we designed gene-specific primers to target the 16S rRNA gene, *rbcX*, *bicA*, *sbtA*, *bicA* + *sbtA*, *mcyB*, and *isiA* (*SI Appendix*, Table S3), using gDNA from axenic laboratory strains as reference samples. LinRegPCR software (v2012.3) was used for baseline correction, calculation of Cq values, and calculation of the amplification efficiency of individual runs (*SI Appendix*, Table S3). Relative ratios of the numbers of gene copies were calculated according to the comparative C_T method (50). The qPCR analysis was validated using defined mixtures of isolated *Microcystis* strains (*SI Appendix*, Fig. S4). Because our field data set was limited to one lake sampled during one summer, we did not

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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 C_i uptake genotypes and the C_i concentration. The rate of replacement was estimated from the slope of the linear regression of $\ln(x_1/x_2)$ versus time (51, 52), where x_1 and x_2 are the relative frequencies of two genotypes. The selection coefficient was calculated by scaling the replacement rate to the generation time (51). The generation time in the chemostats was calculated as $t_d = \ln(2)/\mu$, where μ is the growth rate of the total *Microcystis* population (taking into account the dilution rate).

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