



# Diel Variation in Gene Expression of the CO<sub>2</sub>-Concentrating Mechanism during a Harmful Cyanobacterial Bloom

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Dense phytoplankton blooms in eutrophic waters often experience large daily fluctuations in environmental conditions. We investigated how this diel variation affects *in situ* gene expression of the CO<sub>2</sub>-concentrating mechanism (CCM) and other selected genes of the harmful cyanobacterium *Microcystis aeruginosa*. Photosynthetic activity of the cyanobacterial bloom depleted the dissolved CO<sub>2</sub> concentration, raised pH to 10, and caused large diel fluctuations in the bicarbonate and O<sub>2</sub> concentration. The *Microcystis* population consisted of three C<sub>i</sub> uptake genotypes that differed in the presence of the low-affinity and high-affinity bicarbonate uptake genes *bicA* and *sbtA*. Expression of the bicarbonate uptake genes *bicA*, *sbtA*, and *cmpA* (encoding a subunit of the high-affinity bicarbonate uptake system BCT1), the CCM transcriptional regulator gene *ccmR* and the photoprotection gene *flv4* increased at first daylight and was negatively correlated with the bicarbonate concentration. In contrast, genes of the two CO<sub>2</sub> uptake systems were constitutively expressed, whereas expression of the RuBisCO chaperone gene *rbcX*, the carboxysome gene *ccmM*, and the photoprotection gene *isiA* was highest at night and down-regulated during daytime. In total, our results show that the harmful cyanobacterium *Microcystis* is very responsive to the large diel variations in carbon and light availability often encountered in dense cyanobacterial blooms.

**Keywords:** carbon dioxide, climate change, CO<sub>2</sub>-concentrating mechanism, gene expression, harmful algal blooms, lakes, *Microcystis aeruginosa*

## INTRODUCTION

Harmful cyanobacterial blooms are a recurring problem in many eutrophic lakes worldwide (Chorus and Bartram, 1999; Huisman et al., 2005; Michalak et al., 2013). Cyanobacteria can produce a variety of hepatotoxins, gastrointestinal toxins, and neurotoxins causing liver, digestive and neurological disease in birds and mammals, including humans (Carmichael, 2001; Codd et al., 2005). This may lead to the closure of water bodies for recreational use, drinking or irrigation water, and aquaculture (Verspagen et al., 2006; Qin et al., 2010; Steffen et al., 2014). Cyanobacterial blooms are often favored by excessive nutrients and high temperatures (Jöhnk et al., 2008; Kosten et al., 2012), and it is foreseen that rising CO<sub>2</sub> concentrations and global warming will further

promote harmful cyanobacterial blooms (Paerl and Huisman, 2008; O'Neil et al., 2012; Verspagen et al., 2014; Visser et al., 2016).

Yet, effects of daily fluctuations in environmental conditions on harmful cyanobacterial blooms remain poorly investigated. The day–night cycle leads to large changes in the activity of phototrophic organisms. The high photosynthetic activity of cyanobacterial blooms during daytime can deplete the dissolved CO<sub>2</sub> (CO<sub>2</sub>(aq)) concentration, causing a concomitant increase in pH (Ibelings and Maberly, 1998; Balmer and Downing, 2011; Gu et al., 2011). Conversely, at night, cyanobacteria obtain their energy from respiration of the stored carbon compounds, releasing CO<sub>2</sub> back in the water column. Cyanobacteria adjust to this daily variation by changes in gene expression and the built-up or break-down of specific enzymes and cellular components (Kucho et al., 2005; Straub et al., 2011; Brauer et al., 2013). In particular, the daily excursions in the availability of both light and inorganic carbon (C<sub>i</sub>) imply that cyanobacteria may not only acclimate their light reactions of photosynthesis, but may also adjust their C<sub>i</sub> uptake machinery to the diel cycle.

Cyanobacteria have evolved a CO<sub>2</sub>-concentrating mechanism (CCM) to efficiently fix CO<sub>2</sub> at a wide range of C<sub>i</sub> conditions (Kaplan and Reinhold, 1999; Price et al., 2008; Raven et al., 2012; Burnap et al., 2015). The cyanobacterial CCM is based on the uptake of dissolved CO<sub>2</sub> and bicarbonate by different uptake systems and subsequent concentration of C<sub>i</sub> in specialized compartments, called carboxysomes, that also contain the CO<sub>2</sub> fixing enzyme RuBisCO. Five different C<sub>i</sub> uptake systems are known in cyanobacteria, three for bicarbonate uptake and two for CO<sub>2</sub> uptake (Price, 2011). The bicarbonate transporter BCT1 and the two CO<sub>2</sub> uptake systems are present in most freshwater cyanobacteria (Badger et al., 2006; Rae et al., 2011; Sandrini et al., 2014). BCT1 is directly ATP-dependent, and combines a high affinity with a low flux rate (Omata et al., 1999). The two redox-dependent CO<sub>2</sub> uptake systems, NDH-I<sub>3</sub> and NDH-I<sub>4</sub>, have contrasting properties: NDH-I<sub>3</sub> has a high affinity and low flux rate, while NDH-I<sub>4</sub> has a low affinity and high flux rate (Maeda et al., 2002; Price et al., 2002). The presence of the other two bicarbonate uptake systems, BicA and SbtA, varies among freshwater cyanobacteria (Badger et al., 2006; Rae et al., 2011; Sandrini et al., 2014). Both BicA and SbtA are sodium-dependent symporters, where SbtA usually has a high affinity but low flux rate, and BicA has a low affinity but high flux rate (Price et al., 2004; Du et al., 2014). We recently compared CCM gene sequences of 20 strains of the harmful cyanobacterium *Microcystis aeruginosa* (Kützing; 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, in *Microcystis*, three different C<sub>i</sub> uptake genotypes can be distinguished: *bicA* strains (with *bicA* but no *sbtA*), *sbtA* strains (with *sbtA* but no or incomplete *bicA*), and *bicA* + *sbtA* strains.

In laboratory studies, several cyanobacterial CCM genes were shown to be responsive to changes in both C<sub>i</sub> availability and light intensity (Hihara et al., 2001; Wang et al., 2004; Schwarz et al., 2011; Straub et al., 2011; Burnap et al., 2013; Sandrini et al., 2015a). However, whereas lab studies usually focus on a single

strain, cyanobacterial blooms often consist of multiple strains (Saker et al., 2005; Kardinaal et al., 2007). Recent work shows that different strains may respond differently to changes in CO<sub>2</sub> levels (Van de Waal et al., 2011; Sandrini et al., 2014, 2015b), which may complicate how daily fluctuations in C<sub>i</sub> availability affect gene expression and cellular physiology of cyanobacterial communities in lakes. Two lake studies used metatranscriptomics to study diel variation in cyanobacterial gene expression (Penn et al., 2014) or to compare gene expression between different locations (Steffen et al., 2015), yet detailed information on C<sub>i</sub> concentrations and differences in expression of, for example, the C<sub>i</sub> uptake genes were missing. Another interesting field study used RT-qPCR to investigate diel variation in the expression of CCM and other selected genes of a *Synechococcus* strain in the hot spring microbial mats from Yellowstone National Park (Jensen et al., 2011). However, detailed data about the C<sub>i</sub> conditions in the mats were not presented.

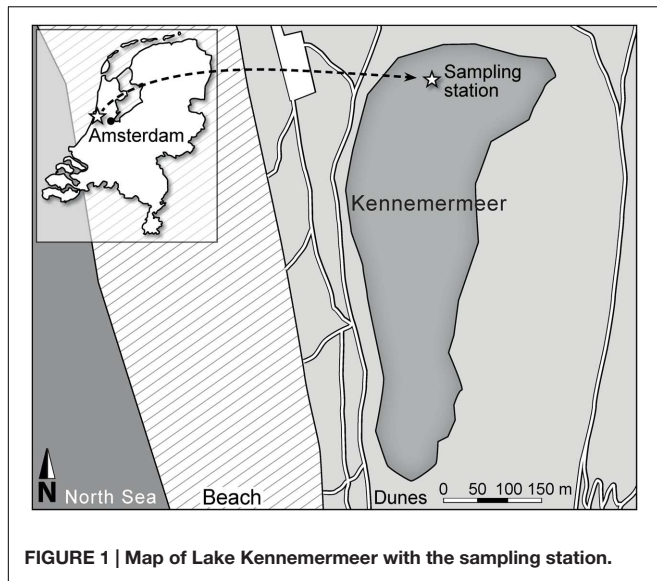
In this study, we investigate diel variation in gene expression patterns of *Microcystis* in a dense cyanobacterial bloom of a eutrophic lake. *Microcystis* is a ubiquitous harmful cyanobacterium that often threatens water quality by the production of dense blooms and hepatotoxins called microcystins (Carmichael, 2001; Huisman et al., 2005; Qin et al., 2010; Dittmann et al., 2013). We quantified expression of the CCM genes as well as a few other selected genes of *Microcystis* using RT-qPCR. Furthermore, we monitored diel changes in environmental conditions, to link observed gene expression patterns to fluctuations in C<sub>i</sub> and light availability. Our study provides a better understanding of the daily variations in the CCM during dense cyanobacterial blooms.

## RESULTS

### Community Composition

Changes in gene expression and environmental conditions were monitored in Lake Kennemermeer (**Figure 1**) for 24 h, from 13:00 to 13:00 the next day on July 17 and 18, 2013. At this time, the lake was dominated by cyanobacteria comprising >97% of the total phytoplankton biovolume. The three most abundant cyanobacterial groups included Pseudanabaenaceae (biovolume: 380 mm<sup>3</sup> L<sup>-1</sup>; cell counts: 10.9 × 10<sup>9</sup> cells L<sup>-1</sup>), *Anabaenopsis hungarica* (67 mm<sup>3</sup> L<sup>-1</sup>; 53 × 10<sup>7</sup> cells L<sup>-1</sup>) and small Chroococcales (40 mm<sup>3</sup> L<sup>-1</sup>; 24.6 × 10<sup>9</sup> cells L<sup>-1</sup>), whereas *Microcystis* spp. represented a smaller portion of the cyanobacterial community (1 mm<sup>3</sup> L<sup>-1</sup>; 70 × 10<sup>6</sup> cells L<sup>-1</sup>). We detected two morphotypes of *Microcystis*, known as *M. aeruginosa* and *M. flos-aquae* (Komárek and Anagnostidis, 1999). These two morphotypes differ in morphology of the *Microcystis* colonies, but belong to the same genetic species *M. aeruginosa* (Kützing) according to the Bacterial Code (Otsuka et al., 2001).

Quantification of the C<sub>i</sub> uptake genotypes of *Microcystis* was based on qPCR using a series of primers targeting the *bicA* and *sbtA* genes in the sampled gDNA. The results revealed that the total *Microcystis* population consisted of *bicA* strains (23 ± 1%), *sbtA* strains (15 ± 1%) and *bicA* + *sbtA* strains (62 ± 2%).



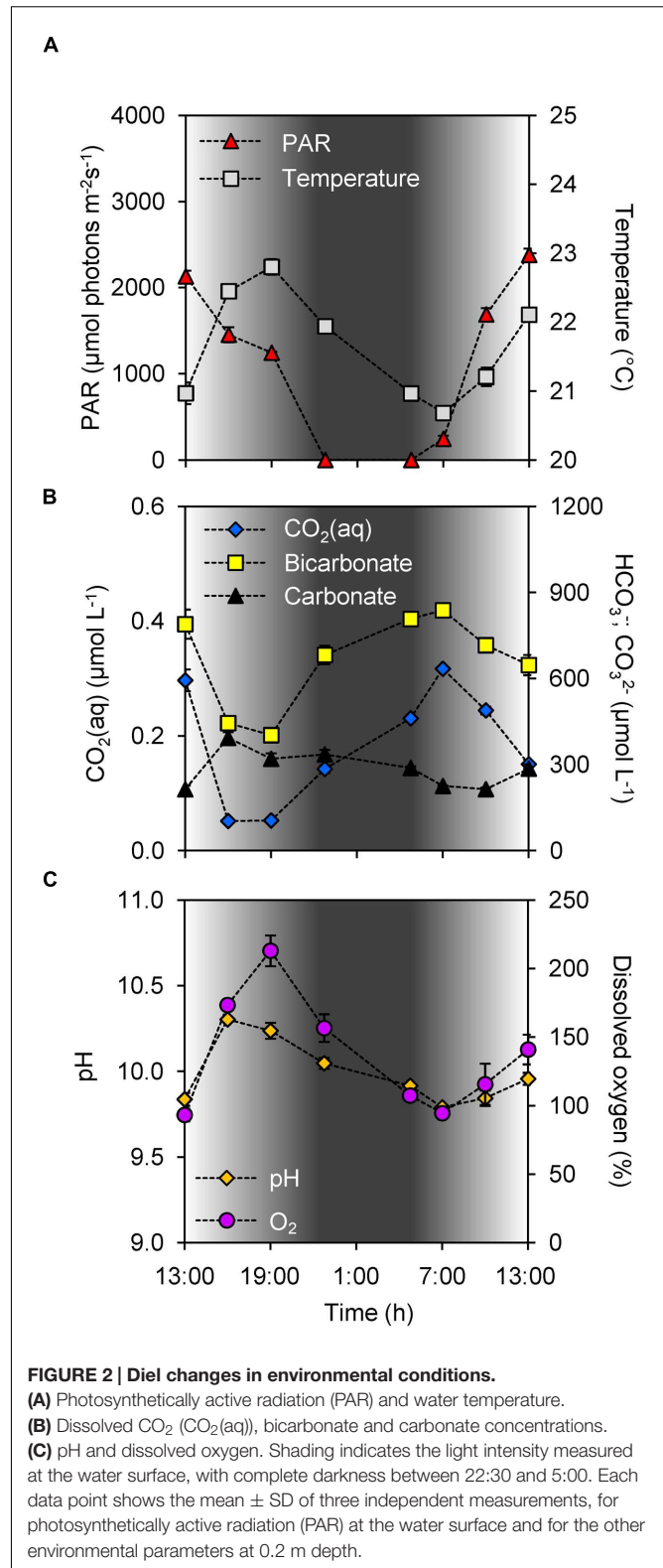
Hence, all three  $C_i$  uptake genotypes of *Microcystis* were present in the lake. Quantification of the microcystin synthetase gene *mcyB* showed that  $82 \pm 5\%$  of the *Microcystis* population was potentially toxic.

## Diel Variation in Environmental Conditions

During the study period it was sunny and dry at daytime, and the photosynthetically active radiation (PAR) increased above  $2,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  in the early afternoon (**Figure 2A**). Sunset was at 21:51, the night was dry with clear skies, with sunrise at 5:42. Water temperature was uniformly distributed over the depth of the water column, peaked at  $22.9^\circ\text{C}$  during early evening, and dropped to  $20.6^\circ\text{C}$  in the early morning of the next day (**Figure 2A**). The lake is only  $\sim 1$  m deep, and depth profiles of chlorophyll fluorescence indicated that the cyanobacterial community was uniformly distributed over the water column. We did not observe formation of scum layers at the water surface.

According to Henry's law, if the concentration of dissolved  $\text{CO}_2$  ( $\text{CO}_2(\text{aq})$ ) in the lake would be in equilibrium with an atmospheric partial pressure of 390 ppm, one would expect a  $\text{CO}_2(\text{aq})$  concentration of  $\sim 15 \mu\text{mol L}^{-1}$ . Instead, however, the  $\text{CO}_2(\text{aq})$  concentration was depleted to  $0.05 \mu\text{mol L}^{-1}$  (which is equivalent to a partial pressure of 1 ppm) during daytime, and increased during the night to a maximum of only  $0.32 \mu\text{mol L}^{-1}$  (8 ppm) at the early morning of the next day (**Figure 2B**). Hence, the lake was strongly  $\text{CO}_2$  undersaturated, even at night. Concomitant with the diel  $\text{CO}_2(\text{aq})$  fluctuations, the bicarbonate concentration showed large diel variation, from  $\sim 400 \mu\text{mol L}^{-1}$  in the early evening to  $\sim 850 \mu\text{mol L}^{-1}$  in the early morning of the next day (Pearson correlation of  $\text{CO}_2(\text{aq})$  vs. bicarbonate:  $\rho = 0.94$ ,  $n = 8$ ,  $p < 0.01$ ; **Figure 2B**).

$\text{CO}_2$  depletion by the cyanobacterial bloom led to an increase in pH with values up to 10.3 in the afternoon [Pearson



correlation of pH vs  $\log \text{CO}_2(\text{aq})$ :  $\rho = -0.99$ ,  $n = 8$ ,  $p < 0.001$ ; **Figures 2B,C**). The pH decreased  $< 0.2$  units over depth, indicating that this shallow lake was well mixed

with nearly uniform  $C_i$  concentrations over the depth of the lake.  $CO_2$  depletion was correlated with oxygen production by the bloom (Pearson correlation of  $CO_2(aq)$  vs. dissolved oxygen:  $\rho = -0.95$ ,  $n = 8$ ,  $p < 0.001$ ; **Figures 2B,C**), with dissolved oxygen peaking at  $\sim 200\%$  during the early evening. During most of the day the lake was supersaturated with oxygen, except for the early morning when it was slightly undersaturated. The sodium concentration in the lake was  $12.7 \pm 0.4 \text{ mmol L}^{-1}$ .

## Diel Variation in Gene Expression

Most of the investigated genes of *Microcystis* showed significant diel variation in gene expression (**Figure 3; Table 1**). Hierarchical cluster analysis revealed that the expression patterns of the studied genes could be grouped into four distinct clusters (**Figure 4**).

Expression of the genes in cluster 1 increased during daytime and decreased at night (**Figure 4**). This cluster comprised the three bicarbonate uptake genes *cmpA*, *bicA*, and *sbtA*, the transcriptional regulator gene *ccmR* and the flavodiiron protein gene *flv4*. Expression of *cmpA*, encoding for one of the subunits of the bicarbonate uptake system BCT1, showed the largest diel variation in this cluster. Its maximum expression during daytime was almost 360-fold higher ( $\log_2$  difference of 8.5) than its minimum expression at night (**Figure 3A**). Expression of *flv4* and *ccmR*, which is most likely a transcriptional regulator of the *cmpABCD* operon of *Microcystis* (Sandrini et al., 2015b), also showed large diel variation (**Figures 3C,F**). Expression of *bicA* and *sbtA*, encoding for the two sodium-dependent bicarbonate uptake systems, varied in tandem but at a lower amplitude than the other genes in this cluster (**Figure 3A**).

Cluster 2 consisted only of *ccmR2* (**Figure 4**), a transcriptional regulator of the *bicA-sbtA* operon of *Microcystis* (Sandrini et al., 2014). Similar to the genes in cluster 1, the expression of *ccmR2* increased during daytime and decreased at night, although at a lower amplitude than *ccmR* (**Figure 3C**). Its expression pattern differed from the genes in cluster 1 by a dip during the morning of the 2nd day.

Cluster 3 comprised the genes *chpX* and *chpY* (encoding the hydration subunits of the low-affinity and high-affinity  $CO_2$  uptake system, respectively), *ccaA* (encoding the carboxysomal carbonic anhydrase) and *mcyB* (encoding a microcystin synthetase; **Figure 4**). The cluster analysis suggests that these genes displayed a higher expression during the first daytime than during the second daytime period. However, diel variation of these genes was only minor and in most cases not significant, except for *mcyB* which decreased at night and increased briefly but significantly at first daylight of the 2nd day (**Figure 3; Table 1**).

Expression of the genes in cluster 4 was lowest during daytime and highest at night (**Figures 3 and 4**). This cluster comprised the CCM genes *rbcX* located in the RuBisCO operon and *ccmM* encoding for a carboxysomal shell protein, the gas vesicle protein gene *gvpC* and the gene *isiA* encoding for the iron-starvation induced protein.

## Comparison of Gene Expression Patterns with Environmental Conditions

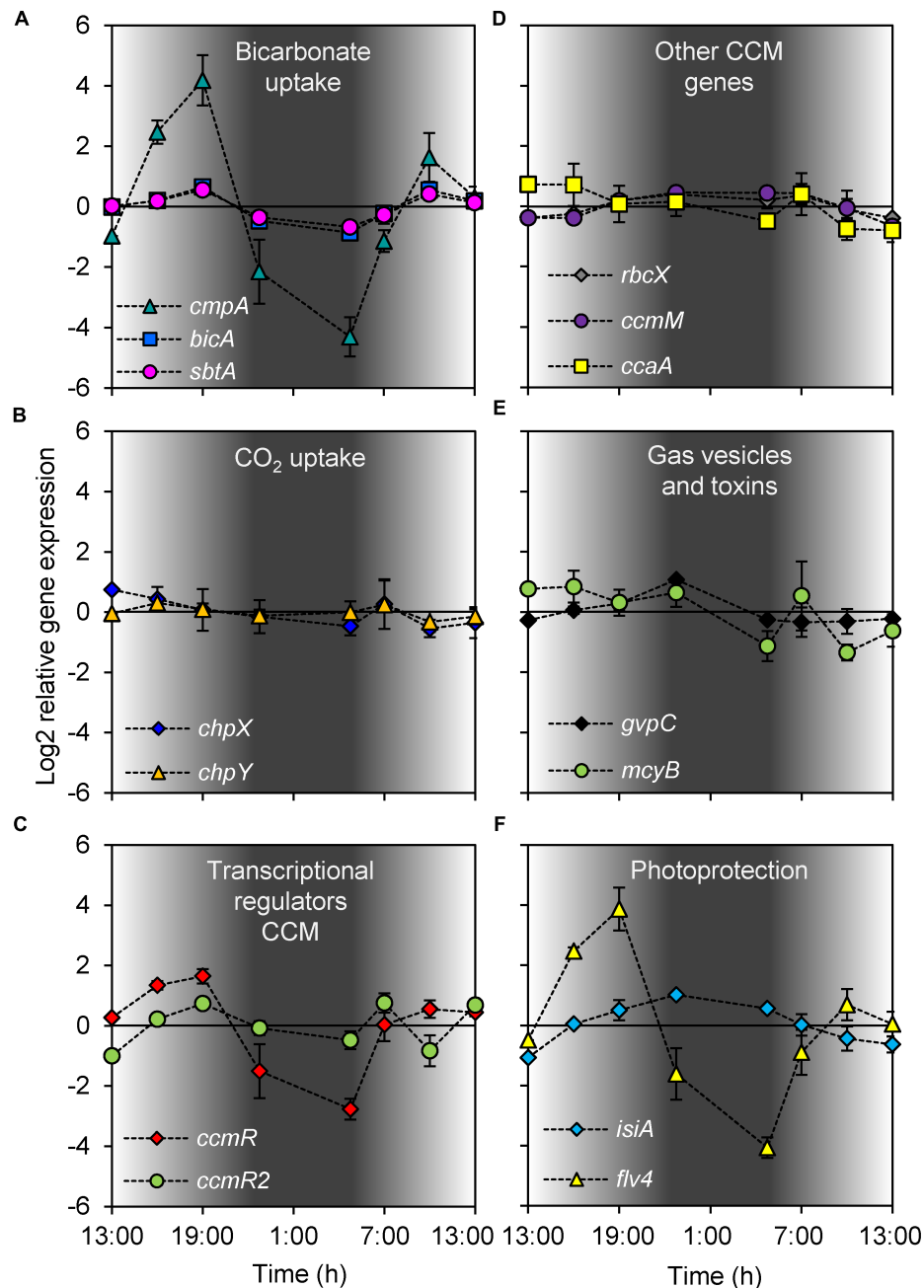
Several of the gene expression patterns were associated with diel changes in environmental conditions (**Table 2**). Correlations with the bicarbonate concentration and incident light intensity showed the most consistent response across the gene clusters. The expression of all five genes in cluster 1 showed a significant ( $p < 0.05$ ) or marginally significant ( $p < 0.10$ ) negative correlation with the bicarbonate concentration, whereas none of the other gene expression patterns were correlated with bicarbonate (**Figure 5; Table 2**). Of these five gene expression patterns, *cmpA* and *flv4* were strongly correlated with the bicarbonate concentration, whereas *bicA*, *sbtA*, and *ccmR* showed a weaker correlation.

Expression of *bicA*, *sbtA*, and *ccmR* (cluster 1) showed a weak but significant positive correlation with the incident light intensity, whereas expression of *rbcX*, *ccmM*, and *isiA* (cluster 4) showed a significant negative correlation with the incident light intensity (**Figure 6; Table 2**). Furthermore, the expression of several genes (*cmpA*, *bicA*, *ccmR*, *ccmR2*, *mcyB*, and *flv4*) increased significantly from 4:45 to 7:00 AM, i.e., at first daylight (**Figure 3; Table 1**) before the  $C_i$  concentration was depleted by the bloom (**Figure 2B**).

## DISCUSSION

### Inorganic Carbon Dynamics

Most lakes worldwide are supersaturated with  $CO_2$  in the absence of phytoplankton blooms, because of, e.g., mineralization of organic carbon produced in the lake and received from the surrounding watershed (Cole et al., 1994; Sobek et al., 2005; Lazzarino et al., 2009). For instance, in a comparative study across almost 5,000 lakes, Sobek et al. (2005) found a mean  $pCO_2$  of 1,287 ppm [equivalent to a  $CO_2(aq)$  concentration of  $\sim 50 \mu\text{mol L}^{-1}$  according to Henry's law]. However, as our lake study illustrates, dense phytoplankton blooms in eutrophic lakes can deplete the  $CO_2(aq)$  concentration and increase pH, and bring about substantial diel variation in the concentrations of dissolved inorganic carbon (DIC) and oxygen. During daytime, the  $CO_2(aq)$  concentration was reduced to only  $0.05 \mu\text{mol L}^{-1}$  (1 ppm) and pH increased above 10. At night, the  $CO_2(aq)$  concentration increased, but the lake remained severely undersaturated. Similar observations have been made in other lakes with dense phytoplankton blooms. The photosynthetic activity of dense blooms can go up to  $12.5\text{--}50 \mu\text{mol C L}^{-1} \text{ h}^{-1}$  (Hein, 1997), which is sufficient to deplete the  $CO_2(aq)$  concentration in lakes within a few hours (Talling, 1976; Maberly, 1996). Balmer and Downing (2011) reported several eutrophic lakes in which the  $CO_2(aq)$  concentration was drawn down to less than  $0.1 \mu\text{mol L}^{-1}$  during daytime, comparable to the low concentrations measured in our study. One limitation of our study is that we investigated only a single 24-h period, but other studies monitored the  $CO_2$  dynamics in lakes over longer time spans. Maberly (1996) monitored the inorganic carbon concentrations of Lake Esthwaite Water



**FIGURE 3 | Diel changes in gene expression. (A)** Bicarbonate uptake genes *cmpA*, *bicA*, and *sbtA*. **(B)** CO<sub>2</sub> uptake genes *chpX* and *chpY*. **(C)** CCM transcriptional regulator genes *ccmR* and *ccmR2*. **(D)** RuBisCO chaperone gene *rbcX*, carboxysome gene *ccmM* and carbonic anhydrase gene *ccaA*. **(E)** Gas vesicle gene *gvpC* and microcystin gene *mcyB*. **(F)** Photoprotection genes *isiA* and *flv4*. Gene expression was quantified as log<sub>2</sub> ratios of the expression at the given time point relative to the mean expression over the 24-h period. Shading indicates the light intensity measured at the water surface, with complete darkness between 22:30 and 5:00. Each data point shows the mean  $\pm$  SD of three independent lake samples. Significant differences in gene expression between time points are reported in **Table 1**.

(UK) for an entire year, and found large diel fluctuations in the CO<sub>2</sub>(aq) concentration and pH (sometimes up to 1.8 units) throughout the summer. During dense phytoplankton blooms, CO<sub>2</sub>(aq) concentrations in Lake Esthwaite remained undersaturated with respect to the atmospheric CO<sub>2</sub> level for

several consecutive weeks. Verspagen et al. (2014) monitored Lake Volkerak (The Netherlands) on a biweekly basis for two consecutive years, during winter, the lake was supersaturated with CO<sub>2</sub>. In summer and early fall, however, Lake Volkerak was covered by dense blooms of the cyanobacterium *Microcystis*,

**TABLE 1 | Testing for significant differences in gene expression between time points.**

Gene	df <sub>1</sub> , df <sub>2</sub>	F	p	Time points							
				13:00 (day 1)	16:00 (day 1)	19:00 (day 1)	22:45 (day 1)	4:45 (day 2)	7:00 (day 2)	10:00 (day 2)	13:00 (day 2)
<i>cmpA</i>	7, 16	53.808	0.000	bc	ef	f	b	a	bc	de	cd
<i>bicA</i>	7, 16	34.031	0.000	cd	de	f	ab	a	bc	ef	cde
<i>sbtA</i>	7, 16	23.886	0.000	bcd	de	e	ab	a	abc	de	cd
<i>chpX</i>	7, 16	1.620	0.200	a	a	a	a	a	a	a	a
<i>chpY</i>	7, 16	0.990	0.473	a	a	a	a	a	a	a	a
<i>ccmR</i>	7, 16	36.693	0.000	cd	de	e	b	a	c	cde	cd
<i>ccmR2</i>	7, 16	22.693	0.000	a	bc	c	b	ab	c	a	c
<i>rbcX</i>	7, 16	7.987	0.000	a	ab	bc	c	bc	c	abc	a
<i>ccmM</i>	7, 16	7.940	0.000	ab	ab	bc	c	c	c	abc	a
<i>ccaA</i>	7, 16	4.870	0.004	b	b	ab	ab	ab	ab	a	a
<i>gvpC</i>	7, 16	10.850	0.000	a	a	a	b	a	a	a	a
<i>mcyB</i>	7, 16	7.317	0.001	c	c	bc	c	ab	c	a	abc
<i>flv4</i>	7, 16	60.401	0.000	bcd	e	e	b	a	bc	d	cd
<i>isiA</i>	7, 16	22.980	0.000	a	bc	cd	d	cd	bc	ab	ab

This table tests differences in gene expression between time points, for the data shown in **Figure 3**. The first four columns in the table indicate the genes, the degrees of freedom (df<sub>1</sub> and df<sub>2</sub>), the value of the F-statistic (F), and the corresponding probability (p) of a one-way analysis of variance. The subsequent columns indicate significant differences in gene expression between time points based on Tukey's HSD post hoc test ( $\alpha = 0.05$ ). Time points that do not share the same letter are significantly different from each other.

and the lake became undersaturated with CO<sub>2</sub> for several months.

Depletion of the CO<sub>2</sub>(aq) concentration by dense phytoplankton blooms creates a pCO<sub>2</sub> gradient across the air-water interface, which implies that these lakes act as a sink for atmospheric CO<sub>2</sub> (Balmer and Downing, 2011). More specifically, the CO<sub>2</sub> influx (gCO<sub>2</sub>) from the atmosphere into a lake can be estimated from the difference between the expected concentration of CO<sub>2</sub>(aq) in equilibrium with the atmosphere (according to Henry's law) and the measured concentration of CO<sub>2</sub>(aq) (Siegenthaler and Sarmiento, 1993; Cole et al., 2010):

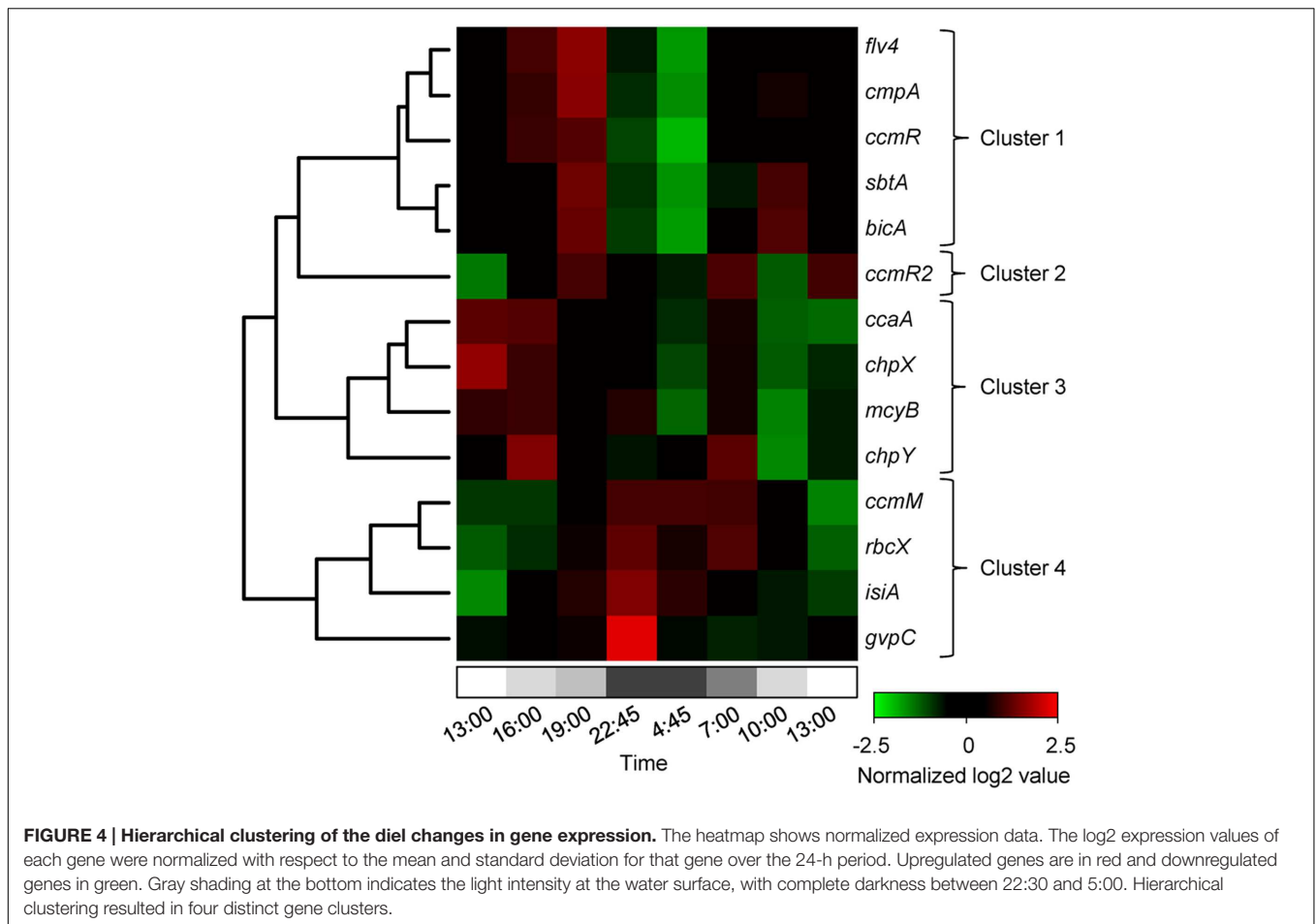
$$g_{CO_2} = \nu(K_H pCO_2 - CO_2(aq)) \quad (1)$$

where  $\nu$  is the gas transfer velocity across the air-water interface,  $K_H$  is the solubility constant of CO<sub>2</sub> gas in water ( $K_H = 0.0375 \text{ mol L}^{-1} \text{ atm}^{-1}$  at 21.5°C; Weiss, 1974), and pCO<sub>2</sub> is the partial pressure of CO<sub>2</sub> in the atmosphere. Assuming a typical gas transfer velocity of  $\nu = 0.02 \text{ m h}^{-1}$  (Crusius and Wanninkhof, 2003; Cole et al., 2010), an atmosphere with a pCO<sub>2</sub> of 390 ppm and an average CO<sub>2</sub>(aq) of 0.2  $\mu\text{mol L}^{-1}$  (**Figure 2B**), the daily CO<sub>2</sub> influx into Lake Kennemermeer would amount to  $\sim 7 \text{ mmol m}^{-2} \text{ d}^{-1}$ . This is a substantial CO<sub>2</sub> influx, which will fuel further development of the cyanobacterial bloom. Moreover, the above calculation indicates that a doubling of the atmospheric CO<sub>2</sub> concentration to 780 ppm will cause a near-doubling of the CO<sub>2</sub> influx into the lake to  $\sim 14 \text{ mmol m}^{-2} \text{ d}^{-1}$ , if we assume that dense cyanobacterial blooms still deplete the dissolved CO<sub>2</sub> concentration. These results are in line with model predictions that rising atmospheric CO<sub>2</sub> concentrations will stimulate cyanobacterial blooms in eutrophic lakes (Schippers et al., 2004; Verspagen et al., 2014; Visser et al., 2016).

Owing to the high pH induced by the cyanobacterial bloom, bicarbonate and carbonate were the most abundant inorganic carbon species in Lake Kennemermeer, whereas the CO<sub>2</sub>(aq) concentration was more than three orders of magnitude lower. The amplitude of the daily variation in CO<sub>2</sub>(aq) concentration was less than 0.3  $\mu\text{mol L}^{-1}$ , whereas the bicarbonate concentration varied more than 400  $\mu\text{mol L}^{-1}$  during the 24-h period. We note that *Microcystis* often forms colonies. Colony morphology may affect gas transfer inside the colony, and cells deep within the colony may experience other CO<sub>2</sub> and bicarbonate concentrations than cells at the outer edge of the colony. Nevertheless, given the low CO<sub>2</sub>(aq) concentration in the ambient lake water, it seems most likely that colonial cyanobacteria in these dense blooms will be strongly dependent on bicarbonate uptake to cover the high C demands of their photosynthetic activity, and may adapt to the large diel fluctuations in bicarbonate availability by adjusting the production of their different bicarbonate uptake systems.

## Co-occurrence of C<sub>i</sub> Uptake Genotypes

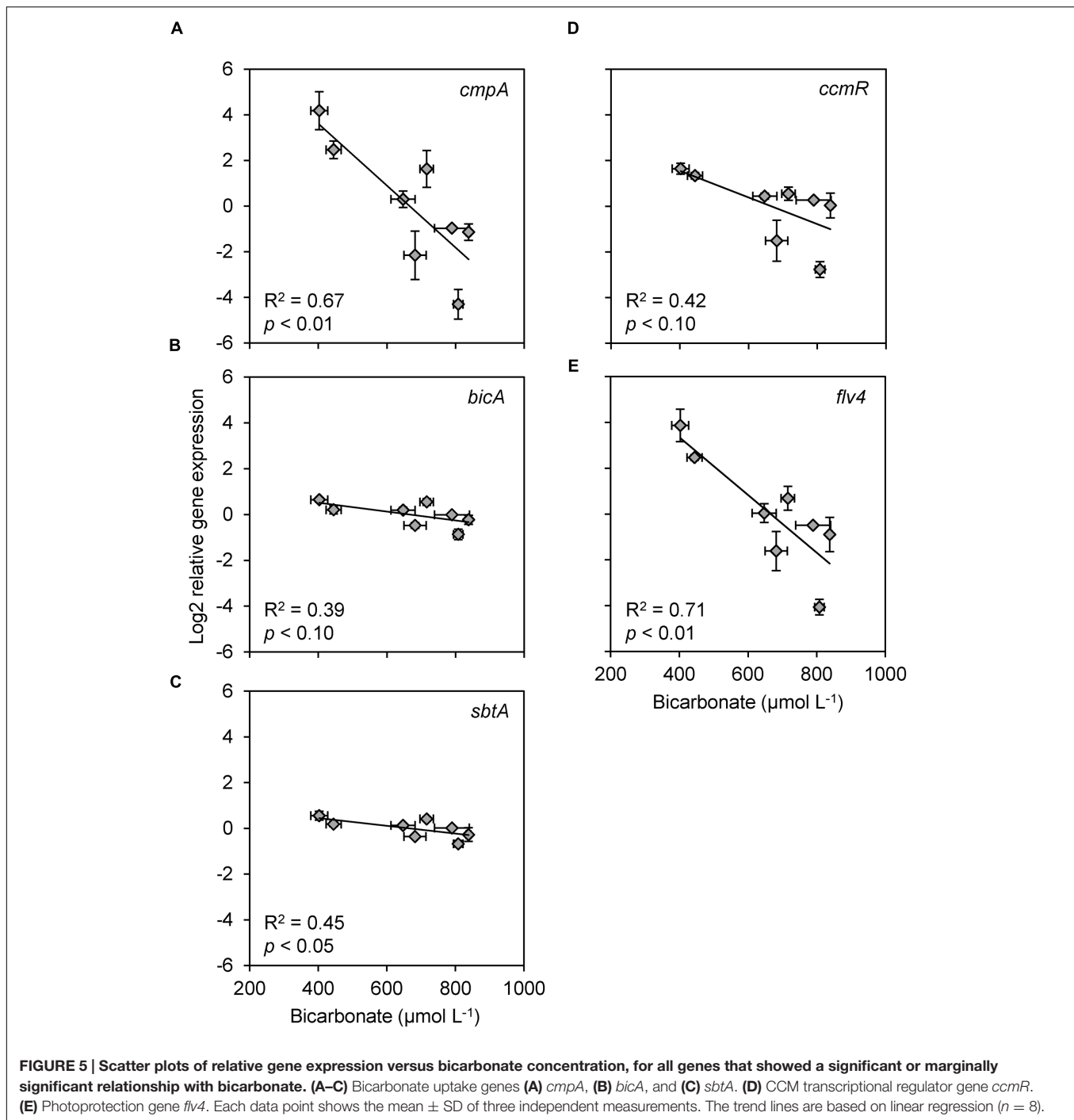
The phytoplankton bloom in Lake Kennemermeer consisted of a mixture of cyanobacterial species, including Pseudanabaenaceae, *A. hungarica*, small Chroococcales and *Microcystis* spp. For most of these species, genome sequences are not available, and we could not develop suitable primers to target their CCM genes. Therefore, we restricted our analysis to *Microcystis*, for which extensive sequence information is available (Kaneko et al., 2007; Frangeul et al., 2008; Humbert et al., 2013; Sandrini et al., 2014). For the interpretation of the results, however, it is important to realize that *Microcystis* does not grow in isolation but competes for C<sub>i</sub> and other potentially limiting resources with other species in the lake. Changes in the relative abundances of C<sub>i</sub> uptake



**TABLE 2 | Pearson correlation coefficients between gene expression patterns and environmental variables ( $n = 8$ ).**

Gene	CO <sub>2</sub> (aq)	Bicarbonate <sup>§</sup>	pH	Temperature	Light <sup>§</sup>	Dissolved oxygen
<b>Cluster 1:</b>						
<i>cmpA</i>	ns	-0.82**	ns	0.69*	ns	0.68*
<i>bicA</i>	ns	-0.62 <sup>#</sup>	ns	ns	0.69*	ns
<i>sbtA</i>	ns	-0.67*	ns	ns	0.70*	ns
<i>ccmR</i>	ns	-0.65 <sup>#</sup>	ns	ns	0.67*	ns
<i>flv4</i>	-0.62 <sup>#</sup>	-0.84**	ns	0.72*	ns	0.70*
<b>Cluster 2:</b>						
<i>ccmR2</i>	ns	ns	ns	ns	ns	ns
<b>Cluster 3:</b>						
<i>chpX</i>	ns	ns	ns	ns	ns	ns
<i>chpY</i>	ns	ns	ns	ns	ns	ns
<i>ccaA</i>	ns	ns	ns	ns	ns	ns
<i>mcyB</i>	ns	ns	ns	ns	ns	ns
<b>Cluster 4:</b>						
<i>rbcX</i>	ns	ns	ns	ns	-0.92**	ns
<i>ccmM</i>	ns	ns	ns	ns	-0.94**	ns
<i>isiA</i>	ns	ns	ns	ns	-0.83**	ns
<i>gvpC</i>	ns	ns	ns	ns	ns	ns

<sup>#</sup> $p < 0.10$  (two-tailed), \* $p < 0.05$  (two-tailed), \*\* $p < 0.01$  (two-tailed); ns, not significant ( $p > 0.10$ ). <sup>§</sup> Scatterplots of gene expression vs. bicarbonate concentration and incident light intensity are shown in **Figures 5 and 6**.



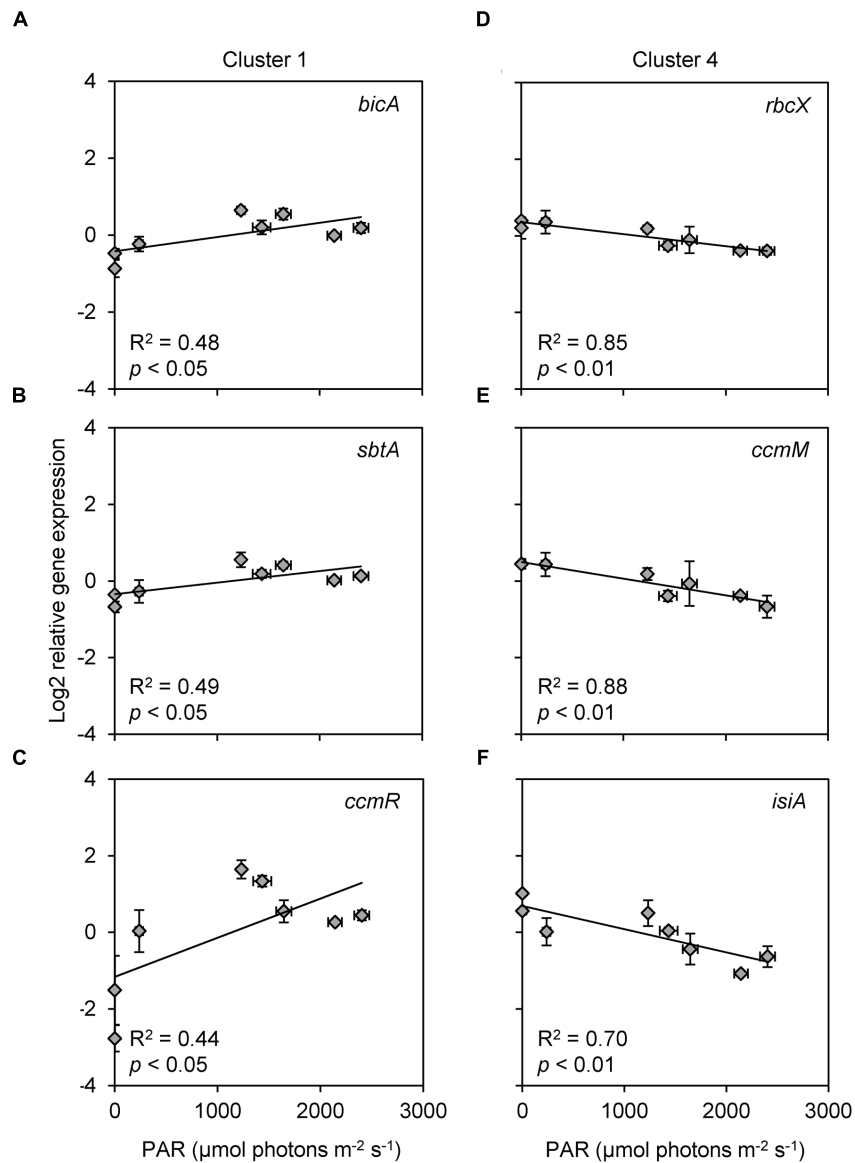
genotypes and CCM gene expression of *Microcystis* are related to  $C_i$  drawdown and  $CO_2$  production by the entire bloom community.

Earlier we reported that *Microcystis* isolates from Lake Pehlitzsee (Germany) and Lake Volkerak (The Netherlands) contained both *sbtA* and *bicA* + *sbtA* strains, suggesting that these two  $C_i$  uptake genotypes may coexist (Sandrini et al., 2014). Our current results show that each of the three  $C_i$  uptake genotypes of *Microcystis* were present in Lake Kennemermeer

with relative abundances of  $\geq 15\%$ , demonstrating that all three genotypes can co-occur in the same lake.

In a companion study, we monitored the population dynamics of the  $C_i$  uptake genotypes of *Microcystis* in Lake Kennemermeer from June to October 2013 (Sandrini, 2016). The results showed that *bicA* + *sbtA* strains dominated during the dense summer bloom, whereas *sbtA* strains and *bicA* strains were present at lower abundances, as reported here. However, *bicA* + *sbtA* strains were replaced by *bicA* strains when  $C_i$  concentrations increased





**FIGURE 6 | Scatter plots of relative gene expression versus light intensity, for all genes that showed a significant or marginally significant relationship with light. (A,B)** Bicarbonate uptake genes **(A)** *bicA* and **(B)** *sbtA*. **(C)** CCM transcriptional regulator gene *ccmR*. **(D)** RuBisCO chaperone gene *rbcX*. **(E)** Carboxysomal gene *ccmM*. **(F)** Photoprotection gene *isiA*. Light intensity is indicated as PAR at the water surface. Each data point shows the mean  $\pm$  SD of three independent measurements. The trend lines are based on linear regression ( $n = 8$ ).

during the demise of the cyanobacterial bloom in autumn. The trade-off between affinity and flux rate of the bicarbonate uptake systems provides a parsimonious explanation for these results. The SbtA enzyme has a high affinity for bicarbonate but low flux rate, whereas the BicA enzyme has a low affinity but high flux rate (Price, 2011). The dominance of *bicA* + *sbtA* strains, containing both bicarbonate uptake systems, indicates that strains that can cope with strong diel fluctuations of the bicarbonate concentration may have a selective advantage during the summer bloom. Indeed, laboratory experiments showed that *bicA* + *sbtA* strains can grow well across a wide range of  $C_i$  concentrations (Sandrini et al., 2014). The subsequent

replacement of *bicA* + *sbtA* strains by *bicA* strains indicates that the high-affinity uptake system SbtA becomes superfluous when the  $C_i$  availability increases in autumn, providing a selective advantage for strains with only the high-flux uptake system BicA at high  $C_i$  levels.

### Expression of $C_i$ Uptake Genes

In contrast to most laboratory studies, our gene expression data reflect the average gene expression over the entire *Microcystis* population rather than the expression of a single strain. Nevertheless, our lake study shows distinct diel patterns of gene expression. In cyanobacteria, the expression of many genes is

under the control of the circadian clock genes *kaiA*, *kaiB*, and *kaiC* (Ishiura et al., 1998; Iwasaki et al., 2002), and it is difficult to disentangle the role of environmental variation versus an internal circadian clock based on field observations alone. However, *kai*-mutants of the cyanobacterium *Synechococcus* PCC 7002 have shown that several CCM genes are *kai*-independent cycling genes (Ito et al., 2009). Therefore, it is plausible that the CCM genes of *Microcystis* are *kai*-independent as well. Our field data show that diel changes in the expression of the  $C_i$  uptake genes were associated with diel variation in  $C_i$  and/or light availability. This is in agreement with many laboratory experiments, which have demonstrated that the expression of cyanobacterial CCM genes can be strongly affected by changes in  $C_i$  and light availability (Hihara et al., 2001; McGinn et al., 2003; Woodger et al., 2003; Wang et al., 2004; Schwarz et al., 2011; Burnap et al., 2013; Sandrini et al., 2015a). We will therefore focus our interpretation of the expression data on diel variation in these environmental factors.

The expression of *cmpA*, encoding for a subunit of the high-affinity bicarbonate transporter BCT1, was negatively correlated with the bicarbonate concentration in the lake (**Figure 5A**). This pattern is in agreement with laboratory studies of *Microcystis* and other cyanobacteria, where *cmpA* expression was also strongly down-regulated at elevated  $C_i$  conditions (Sandrini et al., 2015a,b), and up-regulated under  $C_i$ -limiting conditions (Woodger et al., 2003; Wang et al., 2004; Schwarz et al., 2011). Bicarbonate concentrations in the lake decreased at daytime and increased at night, which likely caused the high *cmpA* expression in the late afternoon and a low *cmpA* expression at the end of the night. The large amplitude of the diel changes in *cmpA* expression suggests that the BCT1 enzyme is largely degraded at night and resynthesized each day. Expression of *cmpA* was not significantly correlated with light availability. However, bicarbonate uptake by BCT1 is ATP-dependent and ATP synthesis is driven by the light reactions of photosynthesis, which may explain the increase in *cmpA* expression at first daylight when the bicarbonate concentration was still high (**Figures 2B** and **3A**). This explanation is also in agreement with previous laboratory studies under  $C_i$ -limited conditions, which showed increased *cmpA* expression at elevated light levels in several other cyanobacteria (Woodger et al., 2003; McGinn et al., 2004). Yet, ATP itself is probably not a regulator of the *cmpABCD* operon (Burnap et al., 2015). More likely, regulatory metabolites such as NADP<sup>+</sup>,  $\alpha$ -ketoglutarate ( $\alpha$ -KG), 2-phosphoglycolate (2PG) and ribulose-1,5-bisphosphate (RuBP), of which the levels can be affected by both light and  $C_i$  levels, control the activity of the CCM transcriptional regulators that in turn regulate the expression of the  $C_i$  uptake genes.

Expression of the bicarbonate uptake genes *bicA* and *sbtA* was also negatively correlated with the bicarbonate concentration (**Figures 5B,C**). In addition, their expression was positively correlated with the incident light intensity (**Figures 6A,B**). The expression of *bicA* and *sbtA* fluctuated in tandem (**Figure 3A**), likely because the *Microcystis* population was dominated by *bicA* + *sbtA* strains, in which both genes are located on the same operon (Sandrini et al., 2014). However, the diel variation in

expression of *bicA* and *sbtA* had a much smaller amplitude than *cmpA*. The sodium concentration in the lake ( $12.7 \pm 0.4$  mmol L<sup>-1</sup>) allowed near-maximum activity of the sodium-dependent bicarbonate uptake systems BicA and SbtA, since both uptake systems have half-saturation constants of 1–2 mmol L<sup>-1</sup> sodium (Price et al., 2004; Du et al., 2014). Therefore, the sodium concentration is unlikely to limit the amplitude of *bicA* and *sbtA* expression. Laboratory studies have shown that the response of *bicA* and *sbtA* expression to changes in  $C_i$  availability varies among *Microcystis* strains (Sandrini et al., 2015b). In some strains, *bicA* and *sbtA* expression is strongly down-regulated at elevated  $C_i$  conditions, whereas in other strains these genes are constitutively expressed. Hence, whether the observed low-amplitude variation in our field data reflects a generic pattern for many *Microcystis* blooms or a specific pattern for the *Microcystis* populations in our lake study remains to be investigated. Price et al. (2013) suggested that BicA and SbtA are simply inactivated by darkness and remain safeguarded for renewed activity at dawn, which may explain the low amplitude variation in gene expression, although the exact mechanisms of inactivation and re-activation remain to be revealed. Indeed, recently it was shown that SbtB is involved in the inhibition of SbtA during darkness (Du et al., 2014).

The *chpX* and *chpY* genes, encoding hydration subunits of the low-affinity and high-affinity CO<sub>2</sub> uptake system, respectively, were constitutively expressed in our lake study (**Figure 3B**). Expression of these genes was not correlated with diel variation in CO<sub>2</sub>(aq) concentration or light intensity (**Table 2**). A possible reason could be that the diel variation in CO<sub>2</sub>(aq) concentrations was too low (from 0.05 to 0.32  $\mu$ mol L<sup>-1</sup>) to affect expression of the CO<sub>2</sub> uptake genes. However, both CO<sub>2</sub> uptake genes were also constitutively expressed in laboratory studies with axenic *Microcystis* strains exposed to a much larger increase in CO<sub>2</sub>(aq) concentration (Sandrini et al., 2015a,b). In other cyanobacterial species, constitutive expression was established for the low-affinity CO<sub>2</sub> uptake gene *chpX* whereas expression of the high-affinity CO<sub>2</sub> uptake gene *chpY* was induced at low  $C_i$  levels (Woodger et al., 2003; Wang et al., 2004; Eisenhut et al., 2007; Schwarz et al., 2011). Furthermore, Jensen et al. (2011) found upregulation of both *chpY* and *chpX* during daytime for a *Synechococcus* mat. Hence, the CO<sub>2</sub> uptake genes of different species respond differently to changing environmental conditions.

Expression of the CCM transcriptional regulator gene *ccmR* was negatively correlated with the bicarbonate concentration, similar to the bicarbonate uptake genes (**Figure 5D**). *Microcystis* lacks the transcriptional regulator gene *cmpR*, which regulates expression of the *cmpABCD* operon in some other cyanobacteria (Omata et al., 2001). Instead, CcmR most likely regulates expression of the *cmpABCD* operon in *Microcystis* (Sandrini et al., 2014, 2015b), which explains the similar gene expression patterns of *cmpA* and *ccmR* in our study. The *ccmR2* gene is located directly upstream of the *bicA/sbtA* operon, and appears to be involved in the transcription of this operon (Sandrini et al., 2014). This gene formed a separate cluster in our analysis (**Figure 4**), probably because of a single deviant data point at 10:00 AM. Omitting this single data point, *ccmR2* showed similar

diel variation in expression as the bicarbonate uptake genes and *ccmR* (Figures 3A,C).

## Diel Changes in Expression of Other Genes

The flavodiiron protein gene *flv4*, which is in one operon with *flv2*, was assigned to the same cluster as the bicarbonate uptake genes and the regulator gene *ccmR* (Figure 4). Flavodiiron proteins are stress proteins known to be involved in the acclimation to low  $C_i$  conditions (Zhang et al., 2009; Allahverdiyeva et al., 2011; Bersanini et al., 2014; Sandrini et al., 2015a), and can also be up-regulated by high light (Zhang et al., 2009). Similarly, in our study *flv4* expression was increased at first daylight and reached high levels when  $C_i$  was depleted in the afternoon (Figure 3F). In contrast, Straub et al. (2011) found no significant diel variation in the expression of *flv2* and *flv4* in laboratory experiments with the axenic *Microcystis* strain PCC 7806, probably because their experiments were sparged with 1%  $CO_2$  and hence  $C_i$  concentrations were not limiting in their study. Both genes, *flv2* and *flv4*, were shown to be involved in photoprotection of the PSII complex (Zhang et al., 2009). Hence, our study indicates that Flv2/4 proteins are highly active in daytime photoprotection of cyanobacterial blooms.

Interestingly, the iron-stress chlorophyll-binding protein IsiA is also involved in photoprotection (Havaux et al., 2005), but the expression pattern of *isiA* was very different from *flv4* (Figure 3F). Expression of *isiA* was high at night but low during daytime (Figure 3F). In addition to its photoprotective function, IsiA is also involved in the formation of PSI antennae enhancing the light harvesting ability and in the storage of free chlorophyll molecules (Yeremenko et al., 2004; Havaux et al., 2005). Possibly, the turnover of photosystems and presence of free chlorophylls at night might stimulate *isiA* expression for temporary chlorophyll storage.

Expression of the *rbcX*, *ccmM*, *ccaA*, *gvpC*, and *mcyB* genes showed only minor diel variation. The RuBisCO gene *rbcX* and carboxysomal gene *ccmM* were negatively correlated with light intensity (Figures 6D,E), which could indicate that cells slightly increase the RuBisCO and carboxysome numbers at night to prepare for cell division, although it was previously shown that *Microcystis* cell division occurs both during daytime and at night (Yamamoto and Tsukada, 2009). Alternatively, the negative correlation might reflect an afternoon photosynthesis dip due to photoinhibition (Ibelings and Maberly, 1998; Wu et al., 2011) when the incident light intensity exceeded  $1,500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  (Figure 2A). Expression of the other three genes (*ccaA*, *gvpC*, and *mcyB*) did not reveal a significant correlation with any of the environmental variables investigated (Table 2).

## CONCLUSION

Cyanobacteria are often assumed to be favored at low  $C_i$  and high pH conditions, because of the presence of an effective CCM (e.g., Shapiro, 1997). However, recent laboratory studies revealed considerable genetic and phenotypic diversity in the CCM of the ubiquitous harmful cyanobacterium *Microcystis*

(Sandrini et al., 2014, 2015b). Some strains perform well at low  $C_i$  levels, whereas other strains are much better competitors under high  $C_i$  conditions, suggesting that it might be difficult to foretell how natural mixtures of different *Microcystis* strains will respond to changes in  $C_i$  availability. Yet, our lake study showed consistent patterns in gene expression. The bicarbonate concentration in the lake showed large diel fluctuations, which may explain the predominance of *bica* + *sbtA* strains and the concurrent diel fluctuations in expression of the bicarbonate uptake genes and their regulator genes. Hence, we conclude that the genetic and phenotypic versatility of the CCM of *Microcystis* enables a remarkably flexible but coherent response to the large diel fluctuations in  $C_i$  conditions often encountered in dense blooms.

## MATERIALS AND METHODS

### Study Area and Sampling

Lake Kennemermeer ( $52^{\circ}27'18.5''\text{N}$ ,  $4^{\circ}33'48.6''\text{E}$ ) is located north-west of Amsterdam, The Netherlands, near the North Sea coast (Figure 1). The man-made lake has been used as bathing water, but nowadays it is no longer in use as recreational lake because of yearly recurrent problems with harmful cyanobacterial blooms. The lake has a maximum depth of  $\sim 1$  m, and surface area of  $\sim 0.1 \text{ km}^2$ . The shallow lake is well mixed by wind throughout the year.

During a dense summer bloom in July 2013, we monitored diel variation in gene expression and environmental conditions. The blooming area spanned the entire lake and the phytoplankton biomass peaked two times that year, from end of June till the 3rd week of July and in the 1st week of September. A small boat was used to sample the north side of the lake (Figure 1) at 13:00, 16:00, 19:00, and 22:45 of July 17, and at 4:45, 7:00, 10:00, and 13:00 of July 18. At each time point, the incident light intensity PAR just above the water surface, and vertical profiles of light intensity, water temperature, pH, dissolved oxygen and chlorophyll fluorescence were measured in triplicate at depth intervals of 0.1 m using a Hydrolab Surveyor with a Datasonde 4a (OTT Hydromet, Loveland, CO, USA). Furthermore, three independent water samples of 5 L each were collected at 0.2 m depth using plastic 10 L tanks. The samples were processed immediately for further analyses.

### Cell Counts

Lugol's iodine was added to 40 mL aliquots of fresh lake samples (1:100 v/v of a 5% solution) to preserve phytoplankton cells for microscopy. The samples were stored at  $4^{\circ}\text{C}$  until identification. Phytoplankton was 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). Phytoplankton was identified to the genus level, and if possible to the species level. 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

For analysis of the DIC and sodium concentration, lake samples were filtered on site using 1.2  $\mu\text{m}$  pore size 47 mm GF/C filters (Whatman GmbH, Dassel, Germany) followed by 0.45  $\mu\text{m}$  pore size 47 mm polyethersulfone membrane filters (Sartorius AG, Goettingen, Germany). The filtrate was transferred to sterile plastic urine analysis tubes (VF-109SURI; Terumo Europe N.V., Leuven, Belgium) which were filled completely (with an inserted needle to release all air), and stored at 4°C until further analysis. A TOC-V<sub>CPH</sub> TOC analyzer (Shimadzu, Kyoto, Japan) was used to determine the DIC concentration, with 3–5 technical replicates per sample. Concentrations of dissolved CO<sub>2</sub> (CO<sub>2</sub>(aq)), bicarbonate and carbonate were calculated from DIC and the pH and temperature of the lake (Stumm and Morgan, 1996). Sodium ion concentrations were measured using an Optima 8000 ICP-OES Spectrometer (Perkin Elmer, Waltham, MA, USA).

## RNA Extraction

For RNA extraction, lake samples were filtered on-site with large 90 mm GF/C filters (Whatman GmbH, Dassel, Germany; 1.2  $\mu\text{m}$  pore size) to concentrate phytoplankton biomass. Next, the 90 mm filters were dissected into several pieces using sterile surgical blades, which were transferred to 2 mL tubes that were filled with 1 mL TRIzol (Thermo Fisher Scientific, Waltham, MA, USA). The tubes were shaken vigorously, transported to the lab in a CX 100 Dry Shipper (Taylor Wharton, Theodore, AL, USA), and stored at –80°C until further analysis. RNA was extracted using 0.5 mm bashing beads (Zymo Research, Orange, CA, USA) to facilitate cell disruption, and purified according to Sandrini et al. (2015a) with the Direct-Zol™ RNA MiniPrep kit (Zymo Research, Orange, CA, USA) including in-column DNase I digestion. RNA concentrations were quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). All RNA samples had  $A_{260}/A_{280}$  and  $A_{260}/A_{230}$  values above 1.8.

## Primer Development

Primers were developed to study expression of the *Microcystis* genes *bicA*, *sbtA*, *cmpA*, *chpX*, *chpY*, *ccmR*, *ccmR2*, *rbcX*, *ccmM*, *ccaA*, *mcyB*, *gvpC*, *isiA*, and *flv4*, using 16S rRNA as ‘reference gene.’ The complete list of primers is shown in Supplementary Table S1. The primers were designed to match the sequences of *Microcystis* strains NIES-843, PCC 7005, PCC 7941, PCC 7806, PCC 9432, PCC 9443, PCC 9701, PCC 9717, PCC 9806, PCC 9807, PCC 9808, PCC 9809, and T1-4. The *bicA*, *sbtA* and *ccmR2* primers were also based on sequences of strains CCAP 1450/10, CCAP 1450/11, HUB 5-2-4, HUB 5-3, NICA-CYA 140, V145, and V163 (Sandrini et al., 2014). The primer design made use of IDT SciTools (Owczarzy et al., 2008) to develop primers with a similar melting temperature and GC content, such that all primers could be used under the same PCR conditions. We also used IDT SciTools to avoid undesirable secondary structures such as hairpins, self-dimers, or heterodimers. Furthermore, we performed primer-BLAST searches to assess the specificity of the primers (Ye et al., 2012). In this way, we ensured that the primers

matched the gene sequences of *Microcystis*, but always included several mismatches with gene sequences of other cyanobacteria. The primers were tested on gDNA (obtained as described below) of 11 different *Microcystis* strains to ensure one target PCR product was formed. These PCR reactions 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  $\mu\text{mol L}^{-1}$  primers and 10 ng gDNA in a total reaction volume of 25  $\mu\text{L}$ . Other reaction components were added as instructed by the supplier. Gel electrophoresis showed that only the targeted gDNA sequences were amplified (no by-products were detected). As a negative control, the primers were tested on gDNA of *Anabaena circinalis* CCAP 1403/18, *Aphanizomenon flos-aqua* CCAP 1401/7, and *Planktothrix agardhii* CCAP 1460/1, which did not result in PCR amplification.

## RT-qPCR Gene Expression Analysis

To quantify gene expression, cDNA was synthesized by reverse transcription of the RNA samples with Superscript III (Thermo Fisher Scientific, Waltham, MA, USA) according to Sandrini et al. (2015a). Subsequently, the qPCR Maxima® SYBR Green Master Mix (2x; Thermo Fisher Scientific, Waltham, MA, USA) was applied on the cDNA samples according to Sandrini et al. (2015a), 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  $\mu\text{mol L}^{-1}$  primers and 1  $\mu\text{L}$  of 10 times diluted cDNA from the RT reaction in a total reaction volume of 25  $\mu\text{L}$ . Other reaction components were added as instructed by the supplier. ROX solution was used to correct for any well-to-well variation. Melting curve analysis was performed on all measured samples to rule out non-specific PCR amplification. The melting curves confirmed that only one PCR product was amplified in each run.

Amplification efficiencies of individual runs ( $E$ ) were calculated with LinRegPCR (version 2012.3; Ramakers et al., 2003; Ruijter et al., 2009) and were between 1.8 and 2.0 (Supplementary Table S1). Application of the primer sets to gDNA from six different *Microcystis* strains showed that the primer sets amplify the target genes from the different genotypes with similar efficiencies (see Supplementary Table S1 in Sandrini et al., 2015b). Time point 0 (13:00 of the 1st day) was used as ‘reference sample,’ and 16S rRNA was used as ‘reference gene.’ Each RT-qPCR plate contained a reference sample and samples with primers targeting 16S rRNA to correct for plate effects (differences in absolute fluorescence signal between individual plates). The data were baseline corrected using LinRegPCR, and the same software was used to calculate quantification cycle ( $C_q$ ) values. LinRegPCR did not detect samples without amplification, without a plateau, with a baseline error or noise error, or with deviating amplification efficiencies. Negative control samples

did not show significant amplification. Gene expression was quantified as the log<sub>2</sub> ratio of the expression at a given time point relative to the mean expression over the 24-h period using the comparative C<sub>T</sub> method (Livak and Schmittgen, 2001). To determine if gene expression varied significantly between time points, one-way analysis of variance (ANOVA) was used with *post hoc* comparison of the means based on Tukey's HSD test ( $\alpha = 0.05$ ) using SPSS version 20.0.

Hierarchical clustering was applied to compare the expression patterns of the studied genes. For each gene, time series of the expression values were normalized by the transformation  $(x - \mu)/\sigma$ , where  $x$  is the original data point,  $\mu$  is the mean of the time series, and  $\sigma$  is its standard deviation. Thus, all genes obtained normalized expression patterns with mean 0 and standard deviation 1. Hierarchical clustering and heatmap representation of the normalized gene expression data was done using the *hclust* and *heatmap.2* functions of the *gplots* package in R version 3.0.2. We used the complete linkage clustering method for hierarchical clustering (Everitt et al., 2011).

## Quantification of *Microcystis* Genotypes

To quantify the relative abundances of different *Microcystis* genotypes, we applied qPCR on purified gDNA (Supplementary Table S1). Lake samples were filtered on-site over 1.2  $\mu\text{m}$  pore size 25 mm GF/C filters (Whatman GmbH, Dassel, Germany), and loaded filters were stored at  $-20^{\circ}\text{C}$ . Subsequently, gDNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research, Orange, CA, USA) and further purified using the DNA Clean and Concentrator™-25 kit (Zymo Research) according to the supplier's instructions. The gDNA samples were analyzed using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), which resulted in A<sub>260</sub>/A<sub>280</sub> values above 1.8 for all samples.

The Maxima® SYBR Green Master Mix (2x) kit (Thermo Fisher Scientific, Waltham, MA, USA) was applied to the 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, and the reaction settings and melting curve analysis were the same as in the RT-qPCR section (see above). The reactions contained 0.3  $\mu\text{mol L}^{-1}$  primers and 10 ng gDNA from lake samples in a total reaction volume of 25  $\mu\text{L}$ . For data analysis, the LinRegPCR software tool (version 2012.3; Ramakers et al., 2003; Ruijter et al., 2009) and the comparative C<sub>T</sub> method (Livak and Schmittgen, 2001) were used.

To determine the relative abundances of the different C<sub>i</sub> uptake genotypes, we used the *bicA* gene (primers bicA-F1 and

bicA-R1; Supplementary Table S1) and the *sbtA* gene (primers sbtA-F1 and sbtA-R1; Supplementary Table S1) as 'target genes.' The *bicA + sbtA* gene of the *bicA + sbtA* strains (primers bicA-F2 and sbtA-R2; Supplementary Table S1) served as 'reference gene.' Purified gDNA of the axenic laboratory strains PCC 7005 and PCC 7941 (both *bicA + sbtA* strains; Sandrini et al., 2014) served as 'reference samples,' to calculate the relative ratios of (1) the *bicA* gene versus the *bicA + sbtA* gene, and (2) the *sbtA* gene versus the *bicA + sbtA* gene. We note that the *bicA* gene is present in both *bicA* strains and *bicA + sbtA* strains, and similarly the *sbtA* gene is present in both *sbtA* strains and *bicA + sbtA* strains. Hence, the relative abundances of the different C<sub>i</sub> uptake genotypes can be calculated from the above two ratios based on the assumption that the sum of the *bicA* strains, *sbtA* strains and *bicA + sbtA* strains equals 100%.

To determine the relative abundance of potentially toxic genotypes, we used *mcyB* (primers mcyB-F and mcyB-R; Supplementary Table S1) 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.'

## AUTHOR CONTRIBUTIONS

GS, HM, and JH designed the study. GS, RT, and HM performed the fieldwork, assisted by JMS and SB. GS analyzed most field samples. GS, RT, HM, and JH analyzed the data. GS, HM, and JH wrote the manuscript, and all authors commented on the final version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.00551>

## REFERENCES

- Allahverdiyeva, Y., Ermakova, M., Eisenhut, M., Zhang, P., Richaud, P., Hagemann, M., et al. (2011). Interplay between flavodiiron proteins and photorespiration in *Synechocystis* sp. PCC 6803. *J. Biol. Chem.* 286, 24007–24014. doi: 10.1074/jbc.M111.223289
- Badger, M. R., Price, G. D., Long, B. M., and Woodger, F. J. (2006). The environmental plasticity and ecological genomics of the cyanobacterial CO<sub>2</sub> concentrating mechanism. *J. Exp. Bot.* 57, 249–265. doi: 10.1093/jxb/eri286
- Balmer, M. B., and Downing, J. A. (2011). Carbon dioxide concentrations in eutrophic lakes: undersaturation implies atmospheric uptake. *Inland Waters* 1, 125–132. doi: 10.5268/IW-4.1.614
- Bersanini, L., Battchikova, N., Jokel, M., Rehman, A., Vass, I., Allahverdiyeva, Y., et al. (2014). Flavodiiron protein Flv2/Flv4-related photoprotective mechanism dissipates excitation pressure of PSII in cooperation with phycobilisomes in cyanobacteria. *Plant Physiol.* 164, 805–818. doi: 10.1104/pp.113.231969
- Brauer, V. S., Stomp, M., Rosso, C., Van Beusekom, S. A. M., Emmerich, B., Stal, L. J., et al. (2013). Low temperature delays timing and enhances the cost of

- nitrogen fixation in the unicellular cyanobacterium *Cyanothece*. *ISME J.* 7, 2105–2115. doi: 10.1038/ismej.2013.103
- Burnap, R. L., Hagemann, M., and Kaplan, A. (2015). Regulation of CO<sub>2</sub> concentrating mechanism in cyanobacteria. *Life (Basel)* 5, 348–371. doi: 10.3390/life5010348
- Burnap, R. L., Nambudiri, R., and Holland, S. (2013). Regulation of the carbon-concentrating mechanism in the cyanobacterium *Synechocystis* sp. PCC6803 in response to changing light intensity and inorganic carbon availability. *Photosynth. Res.* 118, 115–124. doi: 10.1007/s11120-013-9912-4
- Carmichael, W. W. (2001). Health effects of toxin-producing cyanobacteria: “The CyanoHABs.” *Hum. Ecol. Risk Assess.* 7, 1393–1407. doi: 10.1080/20018091095087
- Chorus, I., and Bartram, J. (1999). *Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management*. London: E & FN Spon.
- Codd, G. A., Morrison, L. F., and Metcalf, J. S. (2005). Cyanobacterial toxins: risk management for health protection. *Toxicol. Appl. Pharmacol.* 203, 264–272. doi: 10.1016/j.taap.2004.02.016
- Cole, J. J., Bade, D. L., Bastviken, D., Pace, M. L., and Van de Bogert, M. (2010). Multiple approaches to estimating air-water gas exchange in small lakes. *Limnol. Oceanogr. Methods* 8, 285–293. doi: 10.4319/lom.2010.8.285
- Cole, J. J., Caraco, N. F., Kling, G. W., and Kratz, T. K. (1994). Carbon dioxide supersaturation in the surface waters of lakes. *Science* 265, 1568–1570. doi: 10.1126/science.265.5178.1568
- Crusius, J., and Wanninkhof, R. (2003). Gas transfer velocities measured at low wind speed over a lake. *Limnol. Oceanogr.* 48, 1010–1017. doi: 10.4319/lo.2003.48.3.1010
- Dittmann, E., Fewer, D. P., and Neilan, B. A. (2013). Cyanobacterial toxins: biosynthetic routes and evolutionary roots. *FEMS Microbiol. Rev.* 37, 23–43. doi: 10.1111/j.1574-6976.2012.12000.x
- Du, J., Förster, B., Rourke, L., Howitt, S. M., and Price, G. D. (2014). Characterisation of cyanobacterial bicarbonate transporters in *E. coli* shows that SbtA homologs are functional in this heterologous expression system. *PLoS ONE* 9:e115905. doi: 10.1371/journal.pone.0115905
- Eisenhut, M., Aguirre von Wobeser, E., Jonas, L., Schubert, H., Ibelings, B. W., Bauwe, H., et al. (2007). Long-term response toward inorganic carbon limitation in wild type and glycolate turnover mutants of the cyanobacterium *Synechocystis* sp. strain PCC 6803. *Plant Physiol.* 144, 1946–1959. doi: 10.1104/pp.107.103341
- Everitt, B. S., Landau, S., Leese, M., and Stahl, D. (2011). *Cluster Analysis, Hierarchical Clustering*, 5 Edn. Chichester: John Wiley & Sons, 71–110.
- Franguel, L., Quillardet, P., Castets, A. M., Humbert, J. F., Matthijs, H. C. P., Cortez, D., et al. (2008). Highly plastic genome of *Microcystis aeruginosa* PCC 7806, a ubiquitous toxic freshwater cyanobacterium. *BMC Genomics* 9:274. doi: 10.1186/1471-2164-9-274
- Gu, B., Schelske, C. L., and Coveney, M. F. (2011). Low carbon dioxide partial pressure in a productive subtropical lake. *Aquat. Sci.* 73, 317–330. doi: 10.1007/s00027-010-0179-y
- Havaux, M., Guedeney, G., Hagemann, M., Yermenko, N., Matthijs, H. C. P., and Jeanjean, R. (2005). The chlorophyll-binding protein IsiA is inducible by high light and protects the cyanobacterium *Synechocystis* PCC6803 from photooxidative stress. *FEBS Lett.* 579, 2289–2293. doi: 10.1016/j.febslet.2005.03.021
- Hein, M. (1997). Inorganic carbon limitation of photosynthesis in lake phytoplankton. *Freshw. Biol.* 37, 545–552. doi: 10.1046/j.1365-2427.1997.00180.x
- Hihara, Y., Kamei, A., Kanehisa, M., Kaplan, A., and Ikeuchi, M. (2001). DNA microarray analysis of cyanobacterial gene expression during acclimation to high light. *Plant Cell* 13, 793–806. doi: 10.1105/tpc.13.4.793
- Hillebrand, H., Dürselen, C. D., Kirschtel, D., Pollinger, U., and Zohary, T. (1999). Biovolume calculation for pelagic and benthic microalgae. *J. Phycol.* 35, 403–424. doi: 10.1046/j.1529-8817.1999.3520403.x
- Huisman, J., Matthijs, H. C. P., and Visser, P. M. (2005). *Harmful Cyanobacteria*. Berlin: Springer.
- Humbert, J. F., Barbe, V., Latifi, A., Gugger, M., Calteau, A., Coursin, T., et al. (2013). A tribute to disorder in the genome of the bloom-forming freshwater cyanobacterium *Microcystis aeruginosa*. *PLoS ONE* 8:e70747. doi: 10.1371/journal.pone.0070747
- Ibelings, B. W., and Maberly, S. C. (1998). Photoinhibition and the availability of inorganic carbon restrict photosynthesis by surface blooms of cyanobacteria. *Limnol. Oceanogr.* 43, 408–419. doi: 10.4319/lo.1998.43.3.0408
- Ishiura, M., Kutsuna, S., Aoki, S., Iwasaki, H., Andersson, C. R., Tanabe, A., et al. (1998). Expression of a gene cluster kaiABC as a circadian feedback process in cyanobacteria. *Science* 281, 1519–1523. doi: 10.1126/science.281.5382.1519
- Ito, H., Mutsuda, M., Murayama, Y., Tomita, J., Hosokawa, N., Terauchi, K., et al. (2009). Cyanobacterial daily life with Kai-based circadian and diurnal genome-wide transcriptional control in *Synechococcus elongatus*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 14168–14173. doi: 10.1073/pnas.0902587106
- Iwasaki, H., Nishiwaki, T., Kitayama, Y., Nakajima, M., and Kondo, T. (2002). KaiA-stimulated KaiC phosphorylation in circadian timing loops in cyanobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 99, 15788–15793. doi: 10.1073/pnas.222467299
- Jensen, S. I., Steunou, A.-S., Bhaya, D., Kühl, M., and Grossman, A. R. (2011). In situ dynamics of O<sub>2</sub>, pH and cyanobacterial transcripts associated with CCM, photosynthesis and detoxification of ROS. *ISME J.* 5, 317–328. doi: 10.1038/ismej.2010.131
- Jöhnk, K. D., Huisman, J., Sharples, J., Sommeijer, B., Visser, P. M., and Stroom, J. M. (2008). Summer heatwaves promote blooms of harmful cyanobacteria. *Global Change Biol.* 14, 495–512. doi: 10.1111/j.1365-2486.2007.01510.x
- Kaneko, T., Nakajima, N., Okamoto, S., Suzuki, I., Tanabe, Y., Tamaoki, M., et al. (2007). Complete genomic structure of the bloom-forming toxic cyanobacterium *Microcystis aeruginosa* NIES-843. *DNA Res.* 14, 247–256. doi: 10.1093/dnares/dsm026
- Kaplan, A., and Reinhold, L. (1999). CO<sub>2</sub> concentrating mechanisms in photosynthetic microorganisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50, 539–570. doi: 10.1146/annurev.arplant.50.1.539
- Kardinaal, W. E. A., Janse, I., Kamst-Van Agterveld, M., Meima, M., Snoek, J., Mur, L. R., et al. (2007). *Microcystis* genotype succession in relation to microcystin concentrations in freshwater lakes. *Aquat. Microb. Ecol.* 48, 1–12. doi: 10.3354/ame048001
- Komárek, J., and Anagnostidis, K. (1999). *Cyanoprokaryota. 1. Teil: Chroococcales*. Jena: Gustav Fischer Verlag, 225–236.
- Kosten, S., Huszar, V. L., Bécares, E., Costa, L. S., Donk, E., Hansson, L. A., et al. (2012). Warmer climates boost cyanobacterial dominance in shallow lakes. *Global Change Biol.* 18, 118–126. doi: 10.1111/j.1365-2486.2011.02488.x
- Kucho, K. I., Okamoto, K., Tsuchiya, Y., Nomura, S., Nango, M., Kanehisa, M., et al. (2005). Global analysis of circadian expression in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *J. Bacteriol.* 187, 2190–2199. doi: 10.1128/JB.187.6.2190-2199.2005
- Lazzarino, J. K., Bachmann, R. W., Hoyer, M. V., and Canfield, D. E. Jr. (2009). Carbon dioxide supersaturation in Florida lakes. *Hydrobiologia* 627, 169–180.
- Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-ΔΔC<sub>T</sub></sup> method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262
- Maberly, S. C. (1996). Diel, episodic and seasonal changes in pH and concentrations of inorganic carbon in a productive lake. *Freshw. Biol.* 35, 579–598. doi: 10.1111/j.1365-2427.1996.tb01770.x
- Maeda, S., Badger, M. R., and Price, G. D. (2002). Novel gene products associated with NdhD3/D4-containing NDH-1 complexes are involved in photosynthetic CO<sub>2</sub> hydration in the cyanobacterium, *Synechococcus* sp. PCC7942. *Mol. Microbiol.* 43, 425–435. doi: 10.1046/j.1365-2958.2002.02753.x
- McGinn, P. J., Price, G. D., and Badger, M. R. (2004). High light enhances the expression of low-CO<sub>2</sub>-inducible transcripts involved in the CO<sub>2</sub>-concentrating mechanism in *Synechocystis* sp. PCC6803. *Plant Cell Environ.* 27, 615–626. doi: 10.1111/j.1365-3040.2004.01175.x
- McGinn, P. J., Price, G. D., Maleszka, R., and Badger, M. R. (2003). Inorganic carbon limitation and light control the expression of transcripts related to the CO<sub>2</sub>-concentrating mechanism in the cyanobacterium *Synechocystis* sp. strain PCC6803. *Plant Physiol.* 132, 218–229. doi: 10.1104/pp.019349
- Michalak, A. M., Anderson, E. J., Beletsky, D., Boland, S., Bosch, N. S., Bridgeman, T. B., et al. (2013). Record-setting algal bloom in Lake Erie caused by agricultural and meteorological trends consistent with expected future conditions. *Proc. Natl. Acad. Sci. U.S.A.* 110, 6448–6452. doi: 10.1073/pnas.1216006110

- Omata, T., Gohta, S., Takahashi, Y., Harano, Y., and Maeda, S. (2001). Involvement of a CbbR homolog in low CO<sub>2</sub>-induced activation of the bicarbonate transporter operon in cyanobacteria. *J. Bacteriol.* 183, 1891–1898. doi: 10.1128/JB.183.6.1891-1898.2001
- Omata, T., Price, G. D., Badger, M. R., Okamura, M., Gohta, S., and Ogawa, T. (1999). Identification of an ATP-binding cassette transporter involved in bicarbonate uptake in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *Proc. Natl. Acad. Sci. U.S.A.* 96, 13571–13576. doi: 10.1073/pnas.96.23.13571
- O'Neil, J. M., Davis, T. W., Burford, M. A., and Gobler, C. J. (2012). The rise of harmful cyanobacteria blooms: the potential roles of eutrophication and climate change. *Harmful Algae* 14, 313–334. doi: 10.1016/j.hal.2011.10.027
- Otsuka, S., Suda, S., Shibata, S., Oyaizu, H., Matsumoto, S., and Watanabe, M. M. (2001). A proposal for the unification of five species of the cyanobacterial genus *Microcystis* Kützinger ex Lemmermann 1907 under the rules of the Bacteriological Code. *Int. J. Syst. Evol. Microbiol.* 51, 873–879. doi: 10.1099/00207713-51-3-873
- Owczarzy, R., Tataurov, A. V., Wu, Y., Manthey, J. A., McQuisten, K. A., Almabrazi, H. G., et al. (2008). IDT SciTools: a suite for analysis and design of nucleic acid oligomers. *Nucleic Acids Res.* 36, W163–W169. doi: 10.1093/nar/gkn198
- Paerl, H. W., and Huisman, J. (2008). Blooms like it hot. *Science* 320, 57–58. doi: 10.1126/science.1155398
- Penn, K., Wang, J., Fernando, S., and Thompson, J. (2014). Secondary metabolite gene expression and interplay of bacterial functions in a tropical freshwater cyanobacterial bloom. *ISME J.* 8, 1866–1878. doi: 10.1038/ismej.2014.27
- Price, G. D. (2011). Inorganic carbon transporters of the cyanobacterial CO<sub>2</sub> concentrating mechanism. *Photosynth. Res.* 109, 47–57. doi: 10.1007/s11120-010-9608-y
- Price, G. D., Badger, M. R., Woodger, F. J., and Long, B. M. (2008). Advances in understanding the cyanobacterial CO<sub>2</sub>-concentrating-mechanism (CCM): functional components, Ci transporters, diversity, genetic regulation and prospects for engineering into plants. *J. Exp. Bot.* 59, 1441–1461. doi: 10.1093/jxb/erm112
- Price, G. D., Maeda, S., Omata, T., and Badger, M. R. (2002). Modes of active inorganic carbon uptake in the cyanobacterium, *Synechococcus* sp. PCC7942. *Funct. Plant Biol.* 29, 131–149. doi: 10.1071/PP01229
- Price, G. D., Pengelly, J. J., Forster, B., Du, J., Whitney, S. M., von Caemmerer, S., et al. (2013). The cyanobacterial CCM as a source of genes for improving photosynthetic CO<sub>2</sub> fixation in crop species. *J. Exp. Bot.* 64, 753–768. doi: 10.1093/jxb/ers257
- Price, G. D., Woodger, F. J., Badger, M. R., Howitt, S. M., and Tucker, L. (2004). Identification of a SulP-type bicarbonate transporter in marine cyanobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 101, 18228–18233. doi: 10.1073/pnas.0405211101
- Qin, B., Zhu, G., Gao, G., Zhang, Y., Li, W., Paerl, H. W., et al. (2010). A drinking water crisis in Lake Taihu, China: linkage to climatic variability and lake management. *Environ. Manag.* 45, 105–112. doi: 10.1007/s00267-009-9393-6
- Rae, B. D., Forster, B., Badger, M. R., and Price, G. D. (2011). The CO<sub>2</sub>-concentrating mechanism of *Synechococcus* WH5701 is composed of native and horizontally-acquired components. *Photosynth. Res.* 109, 59–72. doi: 10.1007/s11120-011-9641-5
- Ramakers, C., Ruijter, J. M., Deprez, R. H., and Moorman, A. F. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* 339, 62–66. doi: 10.1016/S0304-3940(02)01423-4
- Raven, J. A., Giordano, M., Beardall, J., and Maberly, S. C. (2012). Algal evolution in relation to atmospheric CO<sub>2</sub>: carboxylases, carbon-concentrating mechanisms and carbon oxidation cycles. *Phil. Trans. R. Soc. B* 367, 493–507. doi: 10.1098/rstb.2011.0212
- Ruijter, J. M., Ramakers, C., Hoogaars, W. M., Karlen, Y., Bakker, O., van den Hoff, M. J., et al. (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res.* 37, e45. doi: 10.1093/nar/gkp045
- Saker, M. L., Fastner, J., Dittmann, E., Christiansen, G., and Vasconcelos, V. M. (2005). Variation between strains of the cyanobacterium *Microcystis aeruginosa* isolated from a Portuguese river. *J. Appl. Microbiol.* 99, 749–757. doi: 10.1111/j.1365-2672.2005.02687.x
- Sandrini, G. (2016). *Effects of Rising CO<sub>2</sub> on the Harmful Cyanobacterium Microcystis*. Ph.D. thesis, University of Amsterdam, Amsterdam.
- Sandrini, G., Cunsolo, S., Schuurmans, J. M., Matthijs, H. C. P., and Huisman, J. (2015a). Changes in gene expression, cell physiology and toxicity of the harmful cyanobacterium *Microcystis aeruginosa* at elevated CO<sub>2</sub>. *Front. Microbiol.* 6:401. doi: 10.3389/fmicb.2015.00401
- Sandrini, G., Jakupovic, D., Matthijs, H. C. P., and Huisman, J. (2015b). Strains of the harmful cyanobacterium *Microcystis aeruginosa* differ in gene expression and activity of inorganic carbon uptake systems at elevated CO<sub>2</sub> levels. *Appl. Environ. Microbiol.* 81, 7730–7739. doi: 10.1128/AEM.02295-15
- Sandrini, G., Matthijs, H. C. P., Verspagen, J. M. H., Muijzer, G., and Huisman, J. (2014). Genetic diversity of inorganic carbon uptake systems causes variation in CO<sub>2</sub> response of the cyanobacterium *Microcystis*. *ISME J.* 8, 589–600. doi: 10.1038/ismej.2013.179
- Schippers, P., Lürling, M., and Scheffer, M. (2004). Increase of atmospheric CO<sub>2</sub> promotes phytoplankton productivity. *Ecol. Lett.* 7, 446–451. doi: 10.1111/j.1461-0248.2004.00597.x
- Schwarz, D., Nodop, A., Hüge, J., Purfürst, S., Forchhammer, K., Michel, K. P., et al. (2011). Metabolic and transcriptomic phenotyping of inorganic carbon acclimation in the cyanobacterium *Synechococcus elongatus* PCC 7942. *Plant Physiol.* 155, 1640–1655. doi: 10.1104/pp.110.170225
- Shapiro, J. (1997). The role of carbon dioxide in the initiation and maintenance of blue-green dominance in lakes. *Freshw. Biol.* 37, 307–323. doi: 10.1046/j.1365-2427.1997.00164.x
- Siegenthaler, U., and Sarmiento, J. L. (1993). Atmospheric carbon dioxide and the ocean. *Nature* 365, 119–125. doi: 10.1038/365119a0
- Sobek, S., Tranvik, L. J., and Cole, J. J. (2005). Temperature independence of carbon dioxide supersaturation in global lakes. *Global Biogeochem. Cycles* 19:GB2003. doi: 10.1029/2004GB002264
- Steffen, M. M., Belisle, B. S., Watson, S. B., Boyer, G. L., Bourbonniere, R. A., and Wilhelm, S. W. (2015). Metatranscriptomic evidence for co-occurring top-down and bottom-up controls on toxic cyanobacterial communities. *Appl. Environ. Microbiol.* 81, 3268–3276. doi: 10.1128/AEM.04101-14
- Steffen, M. M., Belisle, B. S., Watson, S. B., Boyer, G. L., and Wilhelm, S. W. (2014). Status, causes and controls of cyanobacterial blooms in Lake Erie. *J. Great Lakes Res.* 40, 215–225. doi: 10.1016/j.jglr.2013.12.012
- Straub, C., Quillardet, P., Vergalli, J., De Marsac, N. T., and Humbert, J. F. (2011). A day in the life of *Microcystis aeruginosa* strain PCC 7806 as revealed by a transcriptomic analysis. *PLoS ONE* 6:e16208. doi: 10.1371/journal.pone.0016208
- Stumm, W., and Morgan, J. J. (1996). *Aquatic Chemistry: Chemical Equilibria and Rates in Natural Waters*. New York, NY: Wiley-Interscience.
- Talling, J. F. (1976). The depletion of carbon dioxide from lake water by phytoplankton. *J. Ecol.* 64, 79–121. doi: 10.2307/2258685
- Van de Waal, D. B., Verspagen, J. M. H., Finke, J. F., Vournazou, V., Immers, A. K., Kardinaal, W. E. A., et al. (2011). Reversal in competitive dominance of a toxic versus non-toxic cyanobacterium in response to rising CO<sub>2</sub>. *ISME J.* 5, 1438–1450. doi: 10.1038/ismej.2011.28
- Verspagen, J. M. H., Passarge, J., Jöhnk, K. D., Visser, P. M., Peperzak, L., Boers, P., et al. (2006). Water management strategies against toxic *Microcystis* blooms in the Dutch delta. *Ecol. Appl.* 16, 313–327. doi: 10.1890/04-1953
- Verspagen, J. M. H., Van de Waal, D. B., Finke, J. F., Visser, P. M., Van Donk, E., and Huisman, J. (2014). Rising CO<sub>2</sub> levels will intensify phytoplankton blooms in eutrophic and hypertrophic lakes. *PLoS ONE* 9:e104325. doi: 10.1371/journal.pone.0104325
- Visser, P. M., Verspagen, J. M. H., Sandrini, G., Stal, L. J., Matthijs, H. C. P., Davis, T. W., et al. (2016). How rising CO<sub>2</sub> and global warming may stimulate harmful cyanobacterial blooms. *Harmful Algae* (in press).
- Wang, H. L., Postier, B. L., and Burnap, R. L. (2004). Alterations in global patterns of gene expression in *Synechocystis* sp. PCC 6803 in response to inorganic carbon limitation and the inactivation of ndhR, a LysR family regulator. *J. Biol. Chem.* 279, 5739–5751. doi: 10.1074/jbc.M311336200
- Weiss, R. (1974). Carbon dioxide in water and seawater: the solubility of a non-ideal gas. *Mar. Chem.* 2, 203–215. doi: 10.1016/0304-4203(74)90015-2
- Woodger, F. J., Badger, M. R., and Price, G. D. (2003). Inorganic carbon limitation induces transcripts encoding components of the CO<sub>2</sub>-concentrating mechanism in *Synechococcus* sp. PCC7942 through a redox-independent pathway. *Plant Physiol.* 133, 2069–2080. doi: 10.1104/pp.103.029728

- Wu, X., Kong, F., and Zhang, M. (2011). Photoinhibition of colonial and unicellular *Microcystis* cells in a summer bloom in Lake Taihu. *Limnology* 12, 55–61. doi: 10.1007/s10201-010-0321-5
- Yamamoto, Y., and Tsukada, H. (2009). Measurement of in situ specific growth rates of *Microcystis* (cyanobacteria) from the frequency of dividing cells. *J. Phycol.* 45, 1003–1009. doi: 10.1111/j.1529-8817.2009.00723.x
- Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, L., Rozen, S., and Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13:134. doi: 10.1186/1471-2105-13-134
- Yeremenko, N., Kouril, R., Ihalainen, J. A., D'Haene, S., Van Oosterwijk, N., Andrizhiyevskaya, E. G., et al. (2004). Supramolecular organization and dual function of the IsiA chlorophyll-binding protein in cyanobacteria. *Biochemistry* 43, 10308–10313. doi: 10.1021/bi048772l
- Zhang, P., Allahverdiyeva, Y., Eisenhut, M., and Aro, E. M. (2009). Flavodiiron proteins in oxygenic photosynthetic organisms: photoprotection of photosystem II by Flv2 and Flv4 in *Synechocystis* sp. PCC 6803. *PLoS ONE* 4:e5331. doi: 10.1371/journal.pone.0005331
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