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Effects of elevated CO_2 on dynamics of microcystin-producing and non-microcystin-producing strains during *Microcystis* blooms

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ABSTRACT

In an attempt to elucidate the effects of different CO_2 concentrations (270, 380, and 750 μL/L) on the competition of microcystin-producing (MC-producing) and non-MC-producing *Microcystis* strains during dense cyanobacteria blooms, an *in situ* simulation experiment was conducted in the Meiliang Bay of Lake Taihu in the summer of 2012. The abundance of total *Microcystis* and MC-producing *Microcystis* genotypes was quantified based on the 16S rDNA and *mcyD* gene using real-time PCR. The results showed that atmospheric CO_2 elevation would significantly decrease the pH value and increase the dissolved inorganic carbon (DIC) concentration. Changes in CO_2 concentration did not show significant influence on the abundance of total *Microcystis* population. However, CO_2 concentrations may be an important factor in determining the subpopulation structure of *Microcystis*. The enhancement of CO_2 concentrations could largely increase the competitive ability of non-MC-producing over MC-producing *Microcystis*, resulting in a higher proportion of non-MC-producing subpopulation in treatments using high CO_2 concentrations. Concurrently, MC concentration in water declined when CO_2 concentrations were elevated. Therefore, we concluded that the increase of CO_2 concentrations might decrease potential health risks of MC for human and animals in the future.

Introduction

In recent decades, harmful cyanobacterial blooms have occurred with increasing frequency and intensity in freshwater ecosystems worldwide (Paerl and Huisman, 2008). *Microcystis* is the most commonly reported bloom-forming cyanobacterial genus and can be classified as microcystin-producing (MC-producing) and non-MC-producing strains according to the presence or absence of microcystin synthetase genes (Fastner et al., 2001; Kaebernick and Neilan, 2001; Kurmayer et al., 2002). MC-producing strains release a wide variety of MCs that pose a health risk for both humans and animals (Chorus and Bartram, 1999; Carmichael, 2001). The waxing and waning of MC-producing and non-MC-producing *Microcystis* has been considered as the most important factor regulating MC concentrations in freshwater (Chorus and Bartram, 1999). Nutrient availability and environmental factors including light and temperature can influence the dynamics of MC-producing and non-MC-producing *Microcystis* genotypes and MC production (Briand et al., 2012; Davis et al., 2009; Kardinaal et al., 2007; Vézie et al., 2002; Yoshida et al., 2007).

The relentless combustion of fossil fuels has significantly increased the concentrations of atmospheric carbon dioxide, which have increased from the pre-industrial level of 270 μL/L to the present level of 380 μL/L, and it is predicted to double by the end of this century (Caldeira and Wickett, 2003; Solomon et al., 2007). Rising CO_2 concentrations can alter aquatic chemistry, and

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this is likely to have a profound effect on the ecophysiological characteristics and community structure of cyanobacteria (O'Neil et al., 2012; Qiu and Gao, 2002; Verschoor et al., 2013). During the outbreak of cyanobacterial blooms, the high photosynthetic activity of dense algae blooms can remove CO_2 from the surface water layer, thereby inducing high pH and carbon-limited growth conditions (Hein, 1997; Ibelings and Maberly, 1998; Talling, 1976). In contrast, the rising concentrations of dissolved CO_2 cause the pH to decrease and the concentration of dissolved inorganic carbon (DIC) to rise (Doney et al., 2009; O'Neil et al., 2012; Orr et al., 2005; Riebesell et al., 2007). Laboratory studies have revealed that an increase in the availability of inorganic carbon can alter microcystin production and *Microcystis* population, favoring the dominance of non-MC-producing cells over MC-producing cells (Jähnichen et al., 2007; Van de Waal et al., 2011). However, there is a lack of field studies investigating how CO_2 availability affects the competition of MC-producing and non-MC-producing strains in natural freshwater ecosystems.

Lake Taihu is the third largest freshwater lake in China, which is the primary drinking water source for 30 million residents in the Lake Basin and Shanghai (Ye et al., 2009). With a rapid economic development and excessive exploitation of the environment, Lake Taihu has become a hypertrophic lake, and MC-producing cyanobacteria blooms have been occurring annually during the summer over the past two decades. MC concentrations have exceeded the provisional guideline of 1 μg/L set by the World Health Organization (WHO) in some lake regions, *e.g.*, Meiliang Bay (Song et al., 2007; Xu et al., 2005). Therefore, it is urgent to understand how rising atmospheric CO_2 levels influence MC-producing and non-MC-producing *Microcystis* population and MC concentrations in Lake Taihu.

The aim of this study is to investigate the effects of elevated atmospheric CO_2 concentrations on the dynamics of MC-producing and non-MC-producing *Microcystis* strains during dense cyanobacteria blooms and to determine the competitive dominance of those two subpopulations at high and low CO_2 concentrations. To accomplish this, we performed an *in situ* mesocosm experiment and utilized real-time PCR to quantify the abundance of *Microcystis* genotypes and MC-producing *Microcystis* genotypes based on the 16S rDNA and *mcyD* genes. Our study verifies the results of laboratory experiments and predicts the response of toxic cyanobacterial bloom in Lake Taihu to future climate change.

1. Materials and methods

1.1. Experimental design

We collected lake water from Meiliang Bay. Lake water was passed through a 100 μm pore size nylon screen to remove large zooplankton and pumped into nine white 200 L plastic buckets. The buckets were placed in a pool at the Taihu Ecosystem Research Station (31°24′N and 120°13′E) to simulate the temperature and light of the Lake. The mesocosm experiment started from 16 August 2012 and lasted for 21 days.

We performed three treatments in this study using various CO_2 concentrations: pre-industrial concentration (270 μL/L), current concentration (380 μL/L), and future concentration (750 μL/L). The enriched CO_2 concentration was a mixture of ambient air and pure CO_2 that was automatically controlled by continuous CO_2-sensing and controlling systems equipped with a CO_2 chamber. This system varied the CO_2 concentrations by less than 5%. The low CO_2 concentration was obtained by pumping natural air through a CO_2 absorber (*i.e.*, a sodium carbonate solution). The air with three concentrations of CO_2 was released into the water just above the bottom of the mesocosm, where it was released through a pipe system equipped with a nozzle at its end at a rate of approximately 1.0 L/min. The CO_2 concentration of the air used to aerate the water and atmosphere was measured regularly using a CO_2 gas analyzer (Testo 535, Testo, Lenzkirch, Germany). Each treatment was triplicated. Nitrogen and phosphorus nutrients in plastic buckets and the ambient lake water were measured every two days. Phosphate, ammonium, and nitrate were added timely to ensure that the nutrient concentration between the plastic buckets and the ambient lake water body was nearly equal. Water samples were collected on days 0, 3, 6, 9, 12, 15, 18, 21 between 09:00 and 10:00 a.m.

1.2. pH and DIC concentration

The pH was measured with a pH meter (PHSJ-4A, Leici Ltd., Shanghai, China), DIC concentrations were measured by sampling filtered over GF/F glass fiber membrane filter (pore size, ~1.2 μm; Whatman, Maidstone, England, UK; *via* burning in the muffle furnace for 4 hr at 500°C), and DIC was analyzed by high temperature burning method with a TOC analyzer (Torch, Teledyne Tekmar, Ohio, USA).

1.3. Quantitative real-time PCR

Water samples were filtered onto GF/C filters and immediately stored at −80°C until extraction. Total DNA was extracted using the potassium xanthogenate sodium dodecyl sulfate method as described previously (Tillett and Neilan, 2000).

The real-time PCR assay was used to quantify the 16S rDNA and *mcyD* gene regions. The 16S rDNA gene was amplified using the 184F (5′-GCCGCRAGGTGAAAMCTAA-3′) and 431R (5′-AAT CCAAARACCTTCCTCCC-3′) primers (Neilan et al., 1997), and the *mcyD* gene was amplified using the F2 (5′-GGTTCGCCTG GTCAAAGTAA-3′) and R2 (5′-CCTCGCTAAAGAAGGGTTGA-3′) primers (Kaebernick et al., 2000). The *Microcystis* 16S rDNA gene, which is specific to the *Microcystis* genus, was used to quantify the abundance of the total *Microcystis* population. The *mcyD* gene, found within the microcystin synthetase gene operon, only appears in toxic strains of *Microcystis* (Tillett et al., 2000), enabling the quantification of toxic *Microcystis* population.

External standards used to determine 16S rDNA and *mcyD* gene copy numbers were prepared using genomic DNA of *Microcystis aeruginosa* strain PCC7806 obtained from the FACHB-Collection (Freshwater Algal Culture Collection of Institute of Hydrobiology, China). Cells from a known volume of the *M. aeruginosa* PCC 7806 culture were filtered through GF/C filters, and the DNA extraction was as described above. The DNA concentration and purity were determined by a spectrophotometer at 260 and 280 nm. The copy numbers of two genes above were calculated by Vaitomaa et al. (2003). A 10-fold dilution series of the DNAs was prepared and amplified with the 16S rDNA and *mcyD* gene real-time PCR assays.

The real-time PCR was performed with the *Mastercycler realplex* 4 system (Eppendorf, Hamburg, Germany) using 25 μL of a reaction mixture, containing 12.5 μL of SYBR Premix EX Taq™ (TaKaRa, Kusatsu, Japan), 10 μmol of each primer, 10.5 μL of distilled water, and 1 μL of the template DNA. Amplification was performed as follows: The first step was an initial preheating for

2 min at 95°C for 16S rDNA and *mcyD*, and the initial preheating step was followed by 40 cycles: 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. The melting temperature for the real-time PCR products was determined using the manufacturer's software. All of the samples were amplified in triplicate.

1.4. MC analysis

For intracellular MC analysis, 200 mL of the water samples was filtered in triplicate using GF/C filters (pore size, ~1.2 μm; Whatman, Maidstone, England, UK). The filters were lyophilized and extracted with 5% (V/V) acetic acid solution followed by 80% (V/V) aqueous methanol (Barco et al., 2005), with an additional step for grinding of the filters using in a Fast Prep-24 automated homogenizer (MP Biomedicals, Santa Ana, USA) with 0.5 mm silica beads. After centrifugation (9500 r/min, 10 min), the supernatants were pooled and diluted with distilled water. The distilled supernatants were concentrated using solid phase extraction cartridges (C18, 0.5 g), eluted with 100% (0.1% TFA) methanol. Blown dry using nitrogen at 40°C, the residue was then re-suspended in 150 μL of 50% aqueous methanol prior to HPLC analysis.

MCs were analyzed using high performance liquid chromatography with photodiode array detection (Agilent 1200, Agilent, Palo Alto, CA, USA) equipped with an ODS column (Agilent Eclipse XDB-C18, 5 μm, 4.6 mm × 150 mm), using a gradient of 30 to 70% (V/V) acetonitrile (with 0.05% (V/V) trifluoroacetic acid) at a flow rate of 1 mL/min. MCs were identified using their characteristic UV spectra. Total MC concentrations were quantified as the sum of all MC peaks using MC-LR, -RR, and -YR standards (Sigma, München, Germany).

1.5. Statistical analysis

All experiments were performed in triplicate. The data were expressed as the mean values ± standard deviation (SD). Statistical analysis of data was performed using SPSS 16.0 for Windows (SPSS Inc. Chicago, USA). Statistical significance of the data was tested with one-way analysis of variance (ANOVA), with the significance level set at 0.05.

2. Results

2.1. Chemical environment

Rising concentrations of dissolved CO_2 led to a decline in pH, the elevated CO_2 treatments had a lower pH value than the control treatments by 0.37 ($p < 0.01$), and pH in the high CO_2 treatments was significantly lower than in the low CO_2 treatments by 0.59 ($p < 0.01$) (Fig. 1a). In contrast, rising CO_2 caused an increase in DIC concentration. The average levels of DIC were 13.08 mg/L under 270 μL/L of CO_2 concentration, and 15.76 mg/L under 750 μL/L of CO_2 concentration. Significant differences were observed for DIC concentration among the three treatments ($p < 0.05$) (Fig. 1b).

2.2. Standard curve for real-time PCR

Standard curves were established by conducting serial dilutions of genomic DNA extracted from *M. aeruginosa* PCC 7806 culture. From the standard curves for the 16S rDNA and *mcyD* gene, a highly significant linear curve was observed between the threshold value and the log value of the template DNA copy numbers. The real time PCR data show that the range of 16S rDNA copy numbers was from 5.78×10^1 to 5.78×10^7 copies in the reaction mixture, and the detection range of *mcyD* copy numbers was from 2.06×10^1 to 2.06×10^7 (Fig. 2). The melting temperature of the 16S rDNA and *mcyD* real-time PCR products showed a peak at approximately 89.2 and 84.5°C, respectively, corresponding to the melting temperature of the standard strain, *M. aeruginosa* PCC 7806 (data not shown), which demonstrated the reliability of the real-time PCR amplification.

2.3. Variations in the abundance of total *Microcystis* population at different CO_2 treatments

The abundance of total *Microcystis* population increased in three CO_2 treatments during the experiment. The initial growth of the *Microcystis* population was slow on days 0–6 but then increased rapidly on day 9 until it reached steady growth on days 12–21. The total *Microcystis* abundance varied from 6.52×10^6 to 2.09×10^7 copies/mL (270 μL/L), from 6.54×10^6 to 2.15×10^7 copies/mL (380 μL/L), from 6.53×10^6 to 2.43×10^7 copies/mL (750 μL/L), respectively. No significant difference was found between three CO_2 treatments ($p > 0.05$) (Fig. 3).

2.4. Changes in the proportions of MC-producing and non-MC-producing *Microcystis*

In the low CO_2 treatments, the initial proportion of MC-producing *Microcystis* was 45%, and this proportion rapidly increased from 53% to 63% from day 6 onwards. Thus, MC-producing *Microcystis* became predominant by the end of experiment, leading to the higher MC-producing *Microcystis* abundance. In the control treatments, the proportion of MC-producing *Microcystis* remained stable at approximately 55% from day 6 until the end of the experiment. In the high CO_2 treatments, the proportion of MC-producing *Microcystis* increased to 52% on day 6 and subsequently decreased to less than 30% within 15 days, whereas the proportion of non-MC-producing *Microcystis* increased from 48% to 71% in the same period (Fig. 4), resulting in the lower MC-producing *Microcystis* abundance. A significant difference was observed in the abundance of MC-producing *Microcystis* at different CO_2 treatments ($p < 0.05$).

2.5. MC production

In the initial six days, the intracellular MC concentration increased rapidly, ranging from 4.21 to 8.91 μg/L, and it was not significantly different among the three CO_2 treatments ($p > 0.05$). From day 9 onwards, the MC concentration in the all treatments was stable. However, the intracellular MC concentration in the high CO_2 treatments was significantly lower than that in another two treatments, especially that in the low CO_2 treatments ($p < 0.05$) (Fig. 5). For all CO_2 treatments, MC concentrations were positively correlated

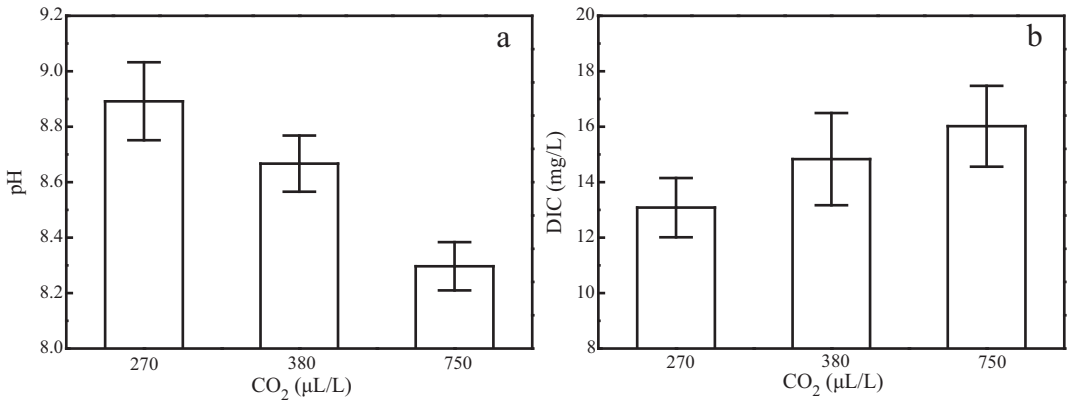

Fig. 1 – pH (a) and dissolved inorganic carbon (DIC) concentration (b) at different CO_2 concentrations. The data represent the means of all measurements during the experiment ± standard deviation (n = 21).

with MC-producing *Microcystis* abundance (r = 0.85, $p < 0.01$, data not shown).

3. Discussion

In this study, to investigate competition for dissolved CO_2 between MC-producing and non-MC-producing *Microcystis* during dense cyanobacteria blooms, we examined their dynamic changes in chemical environment, MC production, and the proportions of MC-producing and non-MC-producing genotypes. The results suggested that MC-producing *Microcystis* could outcompete non-MC-producing ones at low CO_2 concentration, whereas non-MC-producing *Microcystis* became dominant at high CO_2 concentration.

Rising concentration of atmospheric CO_2 can change the carbon chemical environment. In our experiment, the low CO_2 concentrations might be insufficient to compensate for the high photosynthetic rates of the dense algal blooms, which cause dissolved DIC to be depleted and the pH to rise. In contrast, carbon chemistry was reversed at elevated CO_2 concentrations. Doubling of CO_2 concentration could enhance CO_2 dissolution and lower the pH values (Fig. 1). Previous laboratory and field studies reported similar results showing that dense phytoplankton blooms could lead to CO_2 depletion accompanied by an increase of pH (Ibelings and Maberly, 1998; Maberly, 1996; Talling, 1976), and elevated CO_2 concentrations depressed the pH in freshwater (Verschoor et al., 2013).

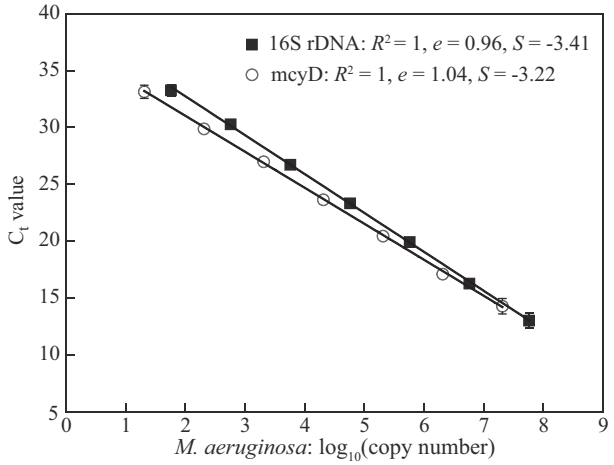

Fig. 2 – Standard curves obtained by the 16S rDNA gene and mcyD real-time PCR assays with the *Microcystis aeruginosa* strain PCC7806 as a function of gene copy numbers. Each data point shows the threshold cycle (C_t) of standard DNA samples performed in triplicate. Amplification efficiency (e, %) was calculated as follows: $e = 10^{-1/S} - 1$, where S is the slope. Error bars represent the standard deviations.

The effects of atmospheric elevated CO_2 concentrations on growth of bloom-forming cyanobacteria remain controversial. Laboratory study has shown that the growth rate of *M. aeruginosa* increases by 52%–77% under doubling of CO_2 concentrations (Qiu and Gao, 2002). A recent model also indicated that the abundance of marine phytoplankton may increase by as much as 40% between current CO_2 concentration and 700 μL/L CO_2 (Schippers et al., 2004). As observed in our experiments, the abundance of total *Microcystis* quantified by 16S rDNA gene was not significantly different among three CO_2 concentrations (Fig. 3). This suggests that the abundance of *Microcystis* population was not sensitive to elevated CO_2 concentrations in summer. Likewise, several studies have indicated that the growth and photosynthetic rate of phytoplankton do not respond significantly to rising atmospheric CO_2 (Goldman, 1999; Tortell et al., 2000, 2002). This inconsistency can be explained by the synergistic effects of nutrient availability and environmental factors on algal growth in natural water ecosystems (Boyd and Hutchins, 2012). In fact, the growth of *Microcystis* in Meiliang Bay of Lake Taihu is largely limited by the availability of nitrogen and phosphorus in summer (Xu et al., 2010). Under this nutrient-depleted condition, the effect of elevated CO_2 may not be so significant on the *Microcystis* growth.

Interestingly, our results showed that the rise in atmospheric CO_2 caused a shift in the dominant subpopulations of *Microcystis* community from MC-producing strains to non-MC-producing strains (Fig. 4). The rise in atmospheric CO_2 could increase the concentration of DIC (CO_2, H_2CO_3, HCO_3^-, CO_3^{2-}) in water. Although many phytoplankton species can utilize both dissolved CO_2 and HCO_3^-

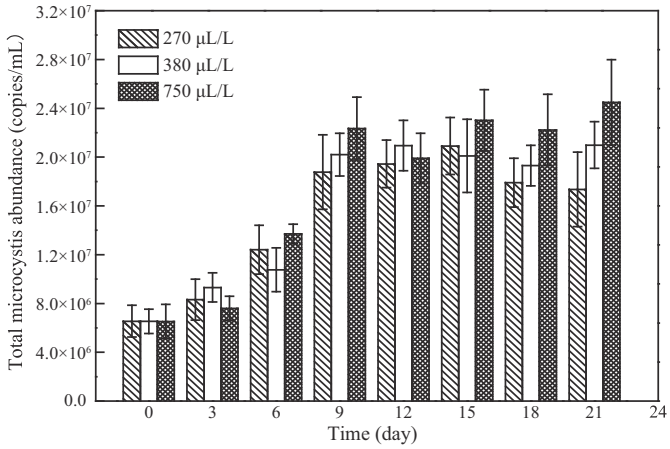

Fig. 3 – Changes in the abundance of total *Microcystis* population at different CO_2 concentrations. Error bars represent the standard deviations in triplicate.

as carbon source (Kaplan and Reinhold, 1999; Price et al., 2008), the affinity of phytoplankton for HCO_3^- is much lower than for CO_2 (Kranz et al., 2009; Rost et al., 2003). The uptake ability of CO_2 might be one factor in determining which species has a competitive advantage when the atmospheric CO_2 concentration rises. Van de Waal et al. (2011) conducted monoculture experiments to compare the fitness of a toxic and non-toxic strain of *M. aeruginosa* cultured under carbon-limited conditions; they found that the lower half-saturation constants for CO_2 and HCO_3^- of the toxic strain allowed it to outcompete the non-toxic ones at low CO_2 levels. Our results based on an *in situ* mesocosm experiment were consistent with the results from this laboratory study.

The difference in uptake ability of CO_2 between MC-producing and non-MC-producing *Microcystis* might be attributable to genetic diversity in the CCM gene. Cyanobacteria possess efficient CO_2-concentrating mechanisms (CCMs) that enable them to grow well at low CO_2 concentrations (Giordano et al., 2005; Raven et al., 2012). *bicA* and *sbtA* are two bicarbonate transporter genes in bicarbonate uptake systems (Price et al., 2004; Shibata et al., 2002). A recent study showed that genetic variation in inorganic carbon uptake systems provides *Microcystis* with the potential for microevolutionary adaptation to changing CO_2 conditions. According to that result, strains with *sbtA* were a superior competitor at low CO_2 concentrations, whereas strains with both *bicA* and *sbtA* were dominant at high CO_2 concentrations (Sandrini et al., 2013). This may explain our finding of the shift in competitive dominance from MC-producing *Microcystis* with only *sbtA* at low CO_2 concentrations towards non-MC-producing *Microcystis* with *bicA* and *sbtA* at high CO_2 concentrations.

The superior competitive ability of MC-producing *Microcystis* under low CO_2 concentrations can be attributed to the ecological function of MC. The MC analysis in our study showed that dominance of MC-producing *Microcystis* at low CO_2 concentrations leads to a higher MC concentration (Fig. 5). MC is a secondary metabolite, and it can reduce the RUBISCO level and CO_2 consumption to increase intracellular inorganic carbon accumulation under conditions of C-limitations (Gerbersdorf, 2006; Jähnichen et al., 2001). The function of MC under a carbon-limited environment was further confirmed by the experiment comparing the wild type strain *M. aeruginosa* PCC 7806 with its *mcyB* mutant strain, which showed that the MC-producing wild type had a strong selective advantage over the mutant strain at low CO_2 levels (Van de Waal et al., 2011).

In addition, the dominance of non-MC-producing *Microcystis* over MC-producing *Microcystis* at rising CO_2 concentrations may associate with the energy costs and benefits of producing MC. At low CO_2 concentrations, the benefits of producing MC under growth-limiting conditions, outweigh its cost, thus leading to the predominance of microcystin-producing strains. At high CO_2 concentrations, the cost of producing microcystin under optimum growth condition, might outweigh the benefits, which induced the counter-selection of microcystin-producing strains. The similar results were reported by Briand et al. (2008), who found that the microcystin-producing strains of *Planktothrix agardhii* exhibited better fitness than non-microcystin-producing strains under growth-limiting conditions; in contrast, the non-microcystin-producing strains showed greater fitness under environment condition favorable for growth. In the further study, They using the microcystin-producing *M. aeruginosa* PCC 7806 strain (WT) and its non-microcystin-producing mutant (MT) in co-culture experiments under different growth conditions, the result demonstrated that the effective competitors of these two strains under optimum growth conditions were attributable to the cost of producing microcystins by microcystin-producing cells (Briand et al. 2012).

4. Conclusions

As an important factor of climate change, the atmospheric CO_2 elevation may influence the proliferation and community composition of harmful algal blooms. In this study, we investigate the effects of elevated atmospheric CO_2 concentrations on the dynamics of MC-producing and non-MC-producing *Microcystis* strains during dense cyanobacteria blooms based on real-time PCR method. The results suggest that rising CO_2 availability can lead to a turnover in outcome of the competition between MC-producing and non-MC-producing strains of

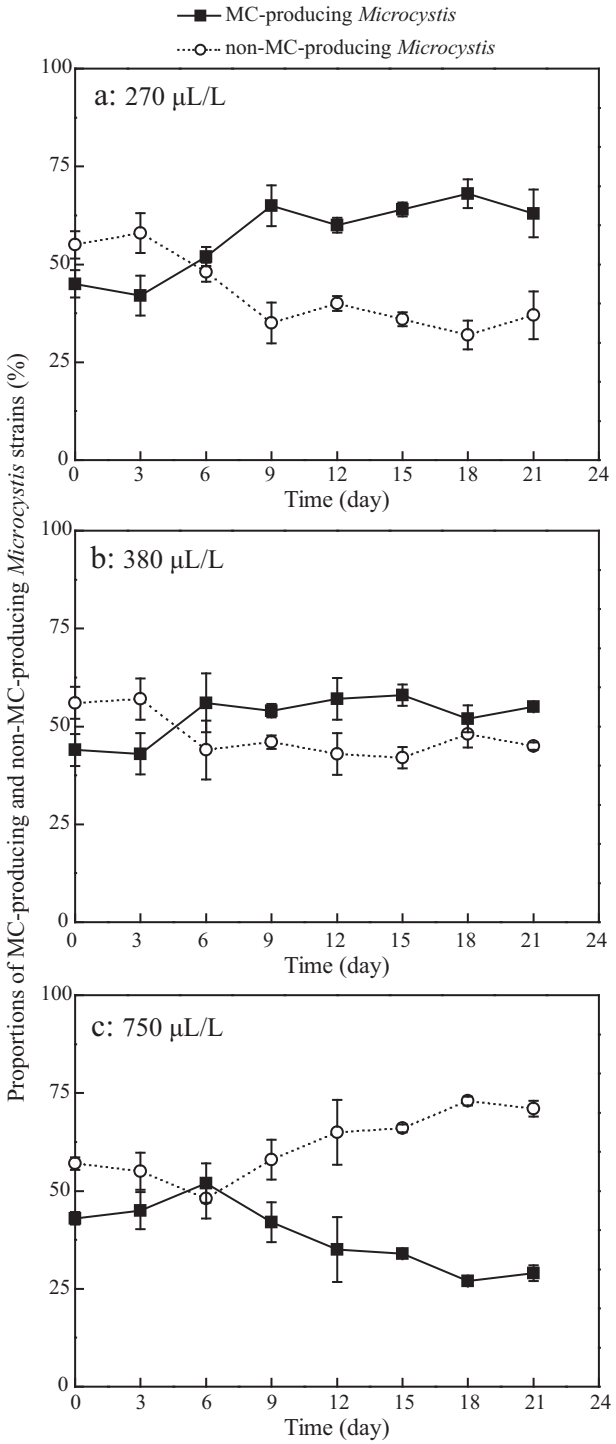

Fig. 4 – Time-course of the proportions of microcystin-producing (MC-producing) and non-MC-producing *Microcystis* strains at different CO_2 concentrations. Error bars represent the standard deviations in triplicate.

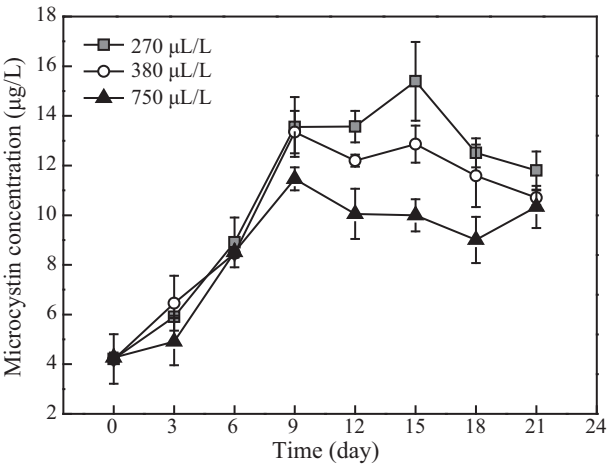

Fig. 5 – Variations in microcystin concentration at different CO_2 concentrations. Error bars represent the standard deviations in triplicate.

Microcystis. Rising atmospheric CO_2 concentrations cause non-MC-producing strains to outcompete MC-producing strains, thereby reducing MC concentrations, whereas the reverse is true under low CO_2 concentrations. The variation of MC concentrations at high and low CO_2 concentrations suggest that differing CO_2 availability may shift the MC genotype composition in the total *Microcystis* population, which in turn can change the MC levels in the water column. These results highlight the need for future field research to obtain a better understanding of the interactions between CO_2 availability, the competitive success of *Microcystis*, and MC dynamics. It is important for monitoring and predicting the potential health risks associated with MC levels that are changing in response to climate change.

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