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Determination of the *in situ* growth rate of *Microcystis* based on carbon and nitrogen stable isotope fractionation

Shucong Zhen and Wei Zhu

ABSTRACT

Stable isotope fractionation of carbon and nitrogen in algal cells can be affected by photosynthesis, temperature, nutrient and CO_2 concentrations, and cell size. As a consequence, carbon and nitrogen stable isotope techniques are not popular for determining algal growth rates. To counter these issues, this study used BG11 medium to cultivate *Microcystis* in the laboratory. First, the carbon and nitrogen stable isotope values of the culture medium and the algae are determined. Then, based on changes in isotope fractionation before and after cell division, a function ($\mu = 1.32(1 + x)^{-0.52}$) relating growth rate and stable isotope fractionation is established. By substituting stable isotope values from Taihu Lake water and *Microcystis* into this function, the growth rate of the *Microcystis* in Taihu Lake is calculated to be 0.64 d^{-1} in May and 0.12 d^{-1} in September, with an average growth rate of 0.42 d^{-1}. By incorporating most of the above-mentioned factors influencing isotope fractionation, this method can determine the growth rate of algae based directly on the stable isotope fractionation relationship, enabling simple and practical determination of algae growth rates.

Key words | algae growth rate, carbon and nitrogen stable isotopes, function fitting, *Microcystis*

Shucong Zhen
Wei Zhu (corresponding author)
College of Environment,
HoHAI University,
Nanjing,
Jiangsu 210018,
PR China
E-mail: *zscdxh@163.com*

Shucong Zhen
Yancheng Institute of Technology,
Yancheng,
Jiangsu 224051,
PR China

INTRODUCTION

In situ growth rate is a key metric used to measure algal growth and evaluate algal blooms. It is also used to explore the mechanisms of algal succession and elucidate algal bloom formation processes (Lewis & Diaz-Pulido 2017). Therefore, the determination of *in situ* algal growth rate plays an important role in studies of algal bloom. At present, common methods to determine *in situ* growth rate can be classified into two categories. The first includes calculations based on the physiological activity or schizogenesis characteristics of algae cells, such as the photosynthetic rate calculation method (Sutherland *et al.* 2015), the cell division frequency method (Bothwell & Kilroy 2011), the radioisotope method (Zhang & Mei 2015), the RNA/cell method (Nagai *et al.* 2011), and the black and white bottle method (Tortell *et al.* 2013). The second includes methods based on changes in the apparent biomass (or cell density), such as the *in situ* culture method (Mahdavi *et al.* 2015), the flow cytometry method (Faber *et al.* 2010), and the *in situ* enclosure or water column method (Mhlanga *et al.* 2014). Of these methods, the cell division frequency method is difficult to apply in practice (and has only been applied in *Microcystis* to date; Hlavová *et al.* 2015), the photosynthetic rate calculation is complicated (Shin *et al.* 2016), and the flow cytometry method cannot be applied in the field. In comparison, the *in situ* culture method cultivates algae cells in a container, after removing all predators, and determines the apparent growth rate of algae based on changes in the number of cells, biological volume, or other biomass indicators. This method has been widely used for determining the *in situ* growth rate of algae (Jazzar *et al.* 2015). This study aims to 1) determine the growth rate of *Microcystis* in Taihu Lake by applying the stable isotope and *in situ* culture techniques in the laboratory, and 2) evaluate the feasibility of using this method to determine the *in situ*

doi: 10.2166/ws.2017.172

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growth rate. The purpose of this study is to find a simple and feasible approach for determining algal growth rate using the stable isotope technique.

There are two methods to determine the plant growth rate (μ) based on carbon isotopes: the ^{14}C radioactive isotope method and the ^{13}C stable isotope method. The former uses radioactive ^{14}C to label chlorophyll, carotenoids, or proteins in phytoplankton to estimate μ. The chlorophyll labeling method, which is also known as the Redalje–Laws chlorophyll method after the scientists who first used it (Redalje & Laws 1981), is most commonly used. Other researchers have made subsequent improvements to this method, such as estimating μ by labeling carotenoids or proteins, but the principle remains the same. The Redalje–Laws chlorophyll labeling method is among the most widely accepted and commonly used methods for determining phytoplankton μ (Pei & Laws 2013, 2014). However, this method can underestimate μ due to decreasing C:Chl-a ratios in shade phytoplankton under intense light conditions, or, conversely, overestimate it due to increasing C:Chl-a ratios in sun phytoplankton under dark conditions (Mcgehee 2014). In view of these limitations, scholars have continued to modify the Redalje–Laws method. For example, Gieskes *et al.* estimated the μ of *diatoms* and *Cyanobacteria* by labeling and determining the isotopic ratios of various carotenoids (Gieskes & Kraay 1989). DiTullio and Laws calculated the value of μ by measuring radioactive absorption by protein components in phytoplankton cells (DiTullio & Laws 1986). Pei Shaofeng and Laws *et al.* studied diurnal cycle cultures for two representative marine algae species (*Isochrysis galbana* and *Chlorella kessleri*) with different respiratory patterns for carbon, and then used nitrogen as a limiting factor for growth rate control to examine the difference between primary productivity and actual productivity in the ocean; productivity was determined using the ^{14}C tracing technique (Pei *et al.* 2014). Xu *et al.* used the Redalje–Laws and carotenoid-labeling methods to calculate the growth rate of various phytoplankton species in the South China Sea, with results ranging from $0.07\ d^{-1}$ to $1.68\ d^{-1}$ (Xu & Huang 2010).

Japan and some other countries restrict the application of ^{14}C isotope methods due to the radioactivity of ^{14}C. As the ^{14}C and ^{13}C methods are consistent in principle and result, a number of scholars have turned to the stable isotope ^{13}C, which does not pose a radioactive hazard, to determine μ. Laws and Popp presented the first study on the use of the stable isotope ^{13}C to determine growth rate (Laws *et al.* 1995). In order to calculate μ, the relationships between μ, CO_2, and $\delta^{13}C(CO_2$ and plant) are established according to known photosynthetic processes. Parameters related to CO_2 may be affected by the coefficient describing CO_2 diffusion into the plant, the CO_2 fixation coefficient, the intracellular and extracellular CO_2 concentrations, and the cell volume or surface area, all of which are difficult to determine. Therefore, this method is impractical and unpopular, and has only been used in a limited body of research. Many scholars have explored the impacts of characteristics such as pH value, temperature, nutrient salt concentration, plant shape, plant size, and cell surface area on carbon and nitrogen fractionation. Laws and Popp determined the $\delta^{13}C$ (for both aqueous CO_2 and the phytoplankton), CO_2 concentrations, and μ of *Phaeodactylum tricornutum* under laboratory culture conditions, and established relationships between these factors; however, because it is difficult to measure the $\delta^{13}C$ of CO_2 in water, the practical applications of this method are limited (Laws *et al.* 1997). Popp formulated an equation relating stable carbon isotope fractionation, CO_2, and μ. Since this equation involves variables such as fixation, flow, and diffusion of CO_2 in a plant, calculating μ remains a challenge (Popp *et al.* 1998). In 2006, Popp proposed a method for estimating plant μ that, while similar to earlier methods, involves ketone labeling with ^{13}C instead of chlorophyll labeling with ^{14}C (Popp *et al.* 2006). Tanaka found that, for every change of $-1.7 \pm 0.2‰$ in $\Delta\delta^{13}C_{leaf\text{-}DIC}$, μ decreased by 1% according to a linear relationship between μ and $\delta^{13}C_{leaf\text{-}DIC}$. The greatest achievement of this study lies in its quantification of the relationship between μ and $\delta^{13}C_{leaf\text{-}DIC}$, which simplifies the determination of μ using the ^{13}C method. However, the simple linear fit constructed between $\Delta\delta^{13}C_{leaf\text{-}DIC}$ and μ had a correlation coefficient of only $r^2 = 0.39$, suggesting a low correlation (Tanaka *et al.* 2008). Therefore, the results obtained using this method may not reflect actual growth rates.

In summary, because ^{14}C is radioactive, its application has been restricted. In view of the consistency between ^{14}C and ^{13}C methods in principle and result, scholars have adopted the non-radioactive stable isotope ^{13}C to measure plant μ, but issues with this method include the large

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number of factors to be calculated, the complexity of the calculations, and the difficulty of quantifying growth rate. Carbon and nitrogen stable isotope fractionation relationships determined in previous works are used herein to calculate fractionation relationships before and after algal cell division using laboratory-cultured *Microcystis aeruginosa* at the logarithmic growth phase. Then, linear fits are used to obtain a function describing the relationship between growth rate and the carbon-nitrogen fractionation. This function is used to calculate the growth rate of *Microcystis* in different growth phases during an algal bloom in Taihu Lake. This method greatly simplifies the calculation and quantification processes, and can be used with field stable isotope measurements to rapidly calculate the growth rate of algae in various phases.

MATERIALS AND METHODS

Microcystis laboratory culture and counting

The algae samples were purchased from the Institute of Hydrobiology, Chinese Academy of Sciences (FACHB 469), and cultured in the BG11 medium. First, 150 mL of BG11 medium, which was previously filtered through a 0.45 μm membrane and sterilized under high temperature, was placed in a 250 mL Erlenmeyer flask. The initial algae density was 6.7×10^5 cells/L, culture temperature was 25 °C, light-dark ratio was 12:12 hours, and light intensity was 30 μmol·photos·m^{-2}·s^{-1}. In order to prevent the adhesion of *Microcystis* to the inner wall of the conical flask during the culture process, the conical flask was gently shaken three times a day.

To avoid the impact of cell population on counting accuracy, 10 mL of algae solution was extracted after thorough agitation and transferred into a 50 mL centrifuge tube. A water bath oscillator was used to sonicate the tube for 5 min below 100 °C at 120 rad·min^{-1} in order to separate the cell population into individual cells. Next, a blood cell counting chamber was used under a microscope (Olympus CX31, 400×) to count the cells; three replicates were performed. If the replicate error was below 10%, the average value was taken as the cell density of *Microcystis*; otherwise, the counting process was repeated until three groups of results with errors below 10% were achieved, after which the average value was used (Wang *et al.* 2015).

The growth rate is calculated using the equation $\mu = \ln (D_t/D_0)/t$, where μ is the growth rate, and D_t and D_0 are the cell density at time t and initial time, respectively.

Sampling points and frequency

Field sampling was conducted at Meiliang Bay in Taihu Lake. Taihu Lake, which is China's third largest freshwater lake, has suffered frequent large-scale *Microcystis* blooms, often spanning hundreds of square kilometers, in recent years. Eutrophication is severe in Meiliang Bay, which has been impacted by urbanization and agricultural production in the surrounding area, and *Microcystis* blooms occur yearly (Tang *et al.* 2016). Six samples in total were collected once per month from April to September. A map of the sampling locations is shown in Figure 1.

Carbon and nitrogen isotope analysis

Stable isotope analysis was performed for the experimental samples at the Isotope Analysis Laboratory at Hohai University, using an Integra 2 carbon and nitrogen isotope mass spectrometer. In order to ensure the accuracy of the results and the stability of the instruments, a standard sample was measured before testing experimental samples and again after every 10 samples. Each sample was tested in triplicate; the average of data points with relative errors within ±5% was used as the measured value of this sample. After preheating, 50 mL of culture medium was extracted and filtered through a 0.45 μm glass membrane. Then, 5 mL of alkaline

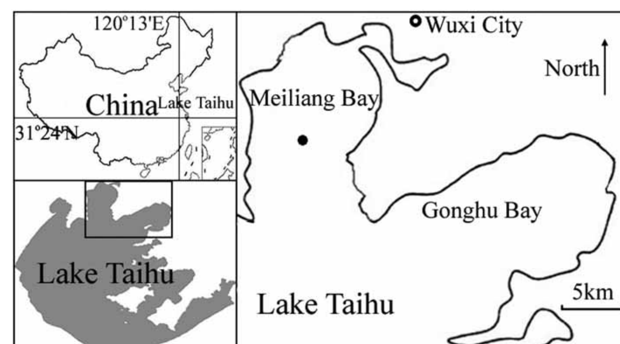

Figure 1 | Sampling locations.

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potassium persulfate solution was added to the solution. After digestion at 120 °C for 30 min, the solution was dried at 70 °C, and the resulting powder was collected, packaged in capsules, and analyzed to determine the stable nitrogen isotope content of the lake water. Excess $BaCl_2$ solution was added to a second sample after filtering. The sample was dried at 70 °C, and an appropriate amount of the residual solid was collected from the wall of the conical flask, encapsulated, and analyzed to determine the stable carbon isotope content. The glass membrane containing *Microcystis* residue from filtration was freeze-dried. Then, an appropriate amount of dry *Microcystis* was collected, encapsulated, and analyzed to determine the carbon and nitrogen stable isotope content of the given *Microcystis* sample.

The two-source linear model

In order to meet growth and reproduction requirements, *Microcystis* absorbs carbon- and nitrogen-containing nutrients from the culture medium during logarithmic propagation; *Microcystis* cells also inherit carbon and nitrogen nutrients from the parent body during proliferation. To evaluate the ratio of nutrients absorbed from the culture medium to those inherited from the parent body, a two-source mixed model is introduced. Taking the carbon stable isotope as an example, the equation is as follows:

$$K_{carbon} = 1 - \frac{\delta^{13}C_a - \delta^{13}C_{consumer} + \Delta\delta^{13}C}{\delta^{13}C_a - \delta^{13}C_b} \quad (1)$$

where K_{carbon} denotes the proportional contribution of food 'a' to the consumer; $\delta^{13}C_a$ and $\delta^{13}C_b$ represent the isotope ratios of foods 'a' and 'b,' respectively; $\Delta\delta^{13}C$ denotes the preconcentration of the carbon isotope (usually considered to be equal to 0‰); and $\delta^{13}C_{consumer}$ is the isotope ratio of the consumer.

RESULTS

The density and growth rate of laboratory-cultured *Microcystis aeruginosa*

The density and growth rate of *Microcystis aeruginosa* are shown in Figures 2 and 3, respectively. The algal density

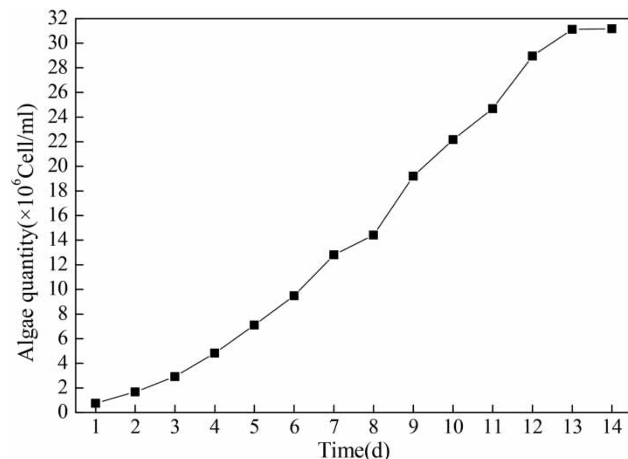

Figure 2 | Changes in the density of *Microcystis aeruginosa* over time.

reaches a maximum value on day 13 of culture, after which the density tends to stabilize (Figure 2). The growth rate decreases to 0.01 d^{-1} from an initial value of 0.79 d^{-1}.

Changes in total nitrogen and total inorganic carbon concentrations in culture medium and TN and total carbon in concentrations in *Microcystis aeruginosa*

Changes in total nitrogen (TN) and total inorganic carbon (DIC) concentrations in the culture medium are shown in Figure 4, while changes in TN and total carbon (TC) concentrations in *Microcystis aeruginosa* are shown in Figure 5. TN concentrations in the culture medium decrease gradually with increasing culture time, while DIC concentrations

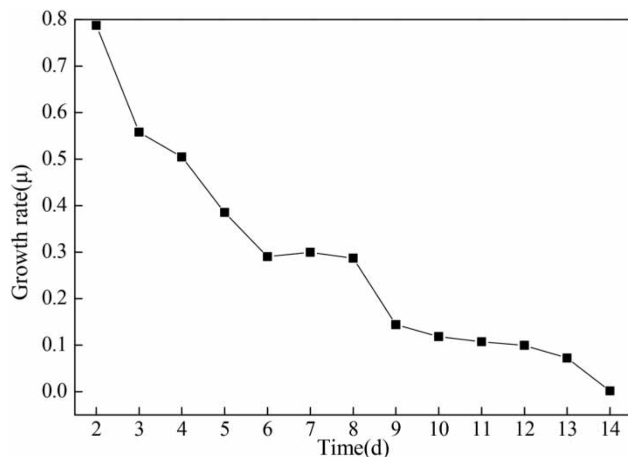

Figure 3 | The growth rate curve for *Microcystis aeruginosa*.

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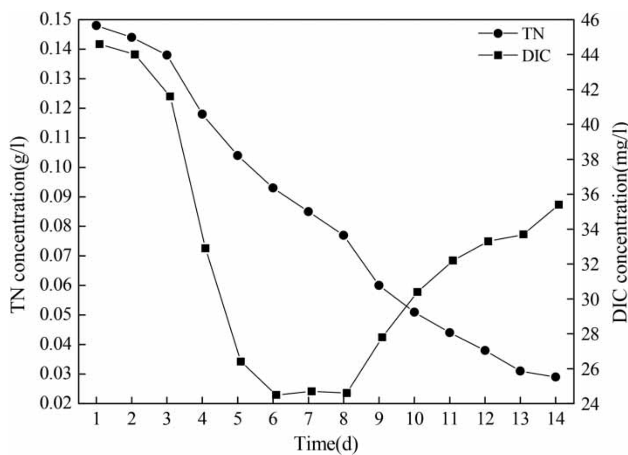

Figure 4 | Changes in the TN and DIC concentrations in the culture medium with culture time.

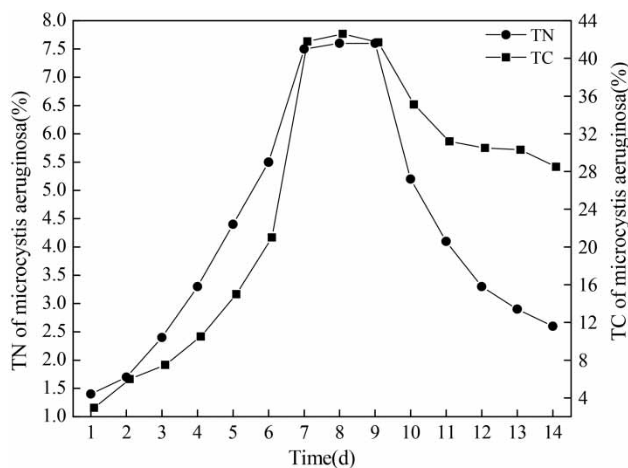

Figure 5 | Changes in the TN and TC contents of *Microcystis aeruginosa* with culture time.

decrease gradually at first and then begin to increase. DIC concentrations reach a minimum between 6–8 d of culture time, during which the growth rate is maintained at ~0.3 d^{-1} (Figure 3). With increasing culture time, the TN and TC contents of *Microcystis aeruginosa* first increase and then decrease. They both stabilize at their maximum values during the 7–9 d interval.

Changes in δ^{13}C and δ^{15}N in *Microcystis aeruginosa* and the culture medium

δ^{13}C values for *Microcystis aeruginosa* and the culture medium decrease from −24.03‰ and −17.44‰ to −25.79‰ and −24.60‰, respectively, with increasing culture time (Figures 6 and 7). δ^{15}N values for both substances first increase, then decrease, with increasing culture time. The δ^{15}N value for *Microcystis aeruginosa* reaches a maximum of 11.28‰ between 6–7 d of culture time, while the δ^{15}N value for the culture medium reaches a maximum of 16.36‰ at 11 d. Changes in the *Microcystis aeruginosa* δ^{15}N occur prior to those in the culture medium.

Carbon and nitrogen isotope fractionation relationships in laboratory-cultured *Microcystis aeruginosa*

Microcystis aeruginosa proliferate rapidly in laboratory culture environments due to favorable growth conditions including high temperature, ample light, and plentiful

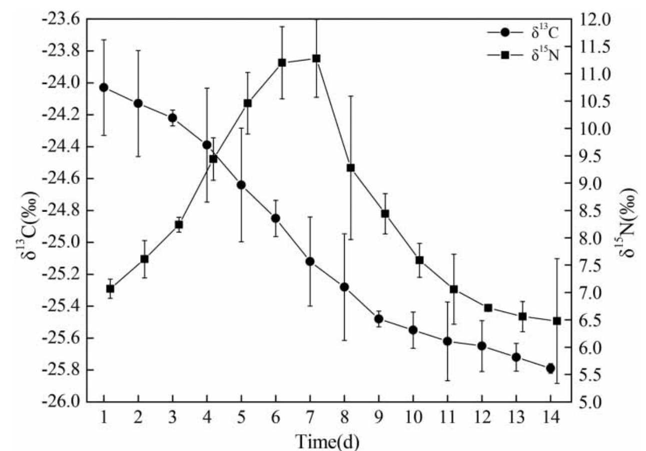

Figure 6 | Changes in δ^{13}C and δ^{15}N in *Microcystis aeruginosa* with culture time.

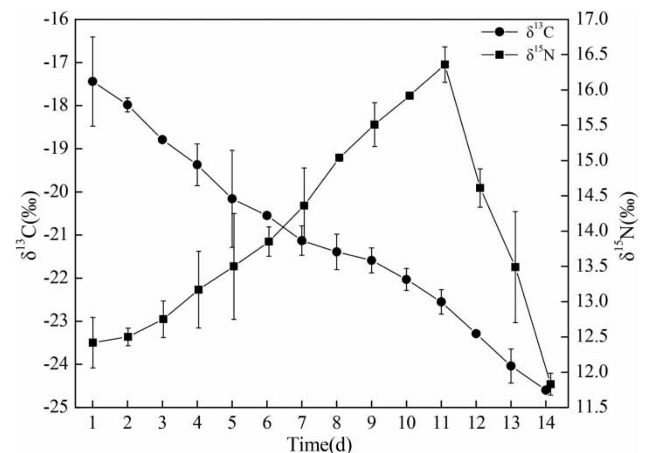

Figure 7 | Changes in δ^{13}C and δ^{15}N in the culture medium with culture time.

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nutrients. During the proliferation process, newly generated *Microcystis aeruginosa* inherit some of their carbon and nitrogen nutrients from their parent body and absorb more from the external environment to maintain their growth. Carbon and nitrogen fractionation relationships for *Microcystis aeruginosa* before and after proliferation are shown in Table 1.

Statistical fitting between μ and the carbon fractionation ratio

As shown in Table 1, the carbon isotope exhibits continuous changes in fractionation with increasing culture time, but the nitrogen isotope does not. Thus, the fitting process is conducted for μ and the carbon fractionation relationship, and the results are shown in Figure 8.

The relationship between μ and carbon fractionation takes the form of the power function $\mu = 1.32(1+x)^{-0.52}$, where μ is the growth rate and x is the carbon fractionation ratio of the offspring inherited from the parent.

$\delta^{13}C$ values for lake water and *Microcystis* from Meiliang Bay in Taihu Lake

Carbon isotope data for lake water and *Microcystis* collected between April and September are shown in Table 2.

Calculation of the *Microcystis* growth rate in Taihu Lake

Substituting the carbon isotope values from Table 2 into the fitting function shown in Figure 8 yields the growth rate of *Microcystis* in Taihu Lake between April and September. The results of this calculation are shown in Table 3. The growth rate of *Microcystis* in Taihu Lake reached a maximum of 0.64 d^{-1} in May and a minimum of 0.12 d^{-1} in September. The average growth rate is 0.42 d^{-1}.

DISCUSSION

The natural growth rate of lake algae

This study determined the *in situ* growth rate of laboratory-cultured *Microcystis aeruginosa*, and the carbon and nitrogen stable isotope content of the culture medium and the algae. The carbon fractionation ratio inherited from the *Microcystis* parent was calculated using the carbon isotope content of the culture medium and *Microcystis*. Then, the functional relationship between the *in situ* growth rate and the carbon fractionation ratio inherited from the parent was obtained by statistical analysis. The latter was calculated for the period between April and September using carbon isotope values from water and *Microcystis* collected at Meiliang Bay in Taihu Lake. The results indicate that the growth rate of the *Microcystis* at Meiliang Bay ranged from 0.12 d^{-1} to 0.64 d^{-1} and that it varied over time, reaching the maximum value in May and the minimum value in September.

Since the growth of *Microcystis* is affected by water quality, water flow, predation, competition, and other factors

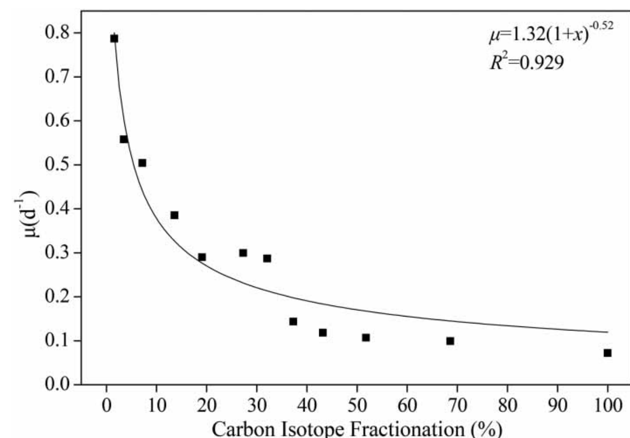

Figure 8 | A statistical fit of the relationship between carbon fractionation and growth rate for laboratory-cultured *Microcystis*.

Table 1 | Carbon and nitrogen fractionation from the parent body for laboratory-cultured *Microcystis*

Cultured Time(d)	2	3	4	5	6	7	8	9	10	11	12	13	14
Carbon Fractionation (%)	1.6	3.5	7.2	13.6	19.1	27.3	32.1	37.3	43.2	51.8	68.6	99.8	*
Nitrogen Fractionation (%)	–	–	–	–	–	–	–	–	–	0.1	4.4	7.4	11.0

Note: 1. * indicates >100; 2. – indicates <0.

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Table 2 | Carbon isotope values for lake water and *Microcystis* from Meiliang Bay in Taihu Lake

Time	April	May	June	July	August	September
Lake Water $\delta^{13}C$ (‰)	−14.48	−16.86	−17.30	−17.43	−17.83	−22.69
Microcystis $\delta^{13}C$ (‰)	−22.70	−22.88	−22.85	−23.15	−23.33	−20.52

Table 3 | Growth rates of *Microcystis* in Taihu Lake

Time	April	May	June	July	August	September
Fractionation (%)	–	3.0	4.1	7.9	11.5	100
μ (d^{-1})	–	0.64	0.57	0.42	0.36	0.12

in the natural environment, *Microcystis* cells propagate relatively slowly. As the effects of the factors mentioned above are difficult to quantify, it is challenging to obtain the actual net growth rate of *Microcystis* in field conditions. In September 1998, Tsujimura (2003) established five sampling points in Nagahama Bay in Lake Biwa, Japan, between the shore and center of the lake, determining that the near-shore growth rates of *Microcystis aeruginosa* and *Microcystis wesenbergii* were 0.36 d^{-1} and 0.49 d^{-1}, respectively. Yamamoto measured the *in situ* growth rate of three types of algae in a small eutrophic pond in Japan between July and October 2005 using the frequency of dividing cells (FDC) method (Yamamoto & Tsukada 2009). In that study, the growth rate of *Microcystis aeruginosa* ranged from 0.15 d^{-1} to 0.38 d^{-1}, the growth rate of *Microcystis viridis* ranged from 0.14 d^{-1} to 0.63 d^{-1}, and the growth rate of *Microcystis wesenbergii* ranged from 0.18 d^{-1} to 0.61 d^{-1}. Cao *et al.* measured the *in situ* growth rate at Meiliang Bay in Taihu Lake between March and June 2005 using the *in situ* water column culture method (Cao *et al.* 2006), finding that the *in situ* growth rate of *Microcystis* at Meiliang Bay ranged from 0.008 d^{-1} to 0.18 d^{-1}. Wu *et al.* measured the *in situ* growth rate of *Microcystis* at Meiliang Bay in August 2007 using the FDC method (Wu & Kong 2008) and a modified formula based on indoor culture conditions. They calculated the growth rates at four sampling points between the bay and the lake center to be 0.23, 0.19, 0.37, and 0.26 d^{-1}. The Meiliang Bay growth rates determined in this study are similar to those measured by Tsujimura, Yamamoto, and Wu *et al.* In particular, the growth rate of 0.37 d^{-1} (the third sampling point) in Wu *et al.* is extremely similar to the growth rates determined herein, and these similarities support the credibility of results obtained using the method presented here. The results of this study are inconsistent with those of Cao *et al.*; however, their results also differ from the studies concerning natural water bodies presented in the discussion above. The low growth rate reported in Cao *et al.* may derive from the time and method of measurement. As the *Microcystis* in Taihu Lake are just beginning to recover (i.e., are still in the adaption phase) in March, it is difficult to measure growth rate at this time. In addition, the *in situ* water column culture method isolates the interior water column from the external environment, hindering the exchange of matter. When algal propagation begins, this isolation leads to nutrient deficiencies inside the main body, which in turn affects algal growth and reproduction. As a result, the measured growth rate may be lower than the natural growth rate.

The relationship between carbon and nitrogen isotope fractionation and the growth rate

During the logarithmic growth phase, *Microcystis aeruginosa* requires a large amount of CO_2 for photosynthesis in order to maintain a high growth rate. However, the only external carbon source in the culture medium is CO_2 dissolved from air, of which the concentration is quite low. In addition, algal propagation further decreases the carbon concentration in the culture medium, causing lower-than-normal dissolved CO_2 concentrations. As a result, *Microcystis aeruginosa* respiration is enhanced and the organic matter originally produced by photosynthesis decomposes into CO_2, which is released into the medium. Thus, carbon isotope fractionation is accelerated, leading to the loss of ^{13}C in the algae cells; in other words, the $\delta^{13}C$ value of *Microcystis aeruginosa* becomes more negative (Figure 5). These results support the conclusions of Popp *et al.* (1998).

During the process of converting absorbed carbon and nitrogen into organic matter, plants will make preferential use of light isotopes (such as ^{14}N), leaving heavy isotopes (such as ^{15}N) behind (Hungate *et al.* 2015). During the logarithmic growth phase, *Microcystis aeruginosa* requires large amounts of carbon and nitrogen nutrients to maintain rapid growth and propagation. The lack of external nitrogen sources means there is a shortage of nitrogen nutrients in the culture medium, and with decreasing nitrogen availability and exhaustion of the light isotope, the rate of utilization of the heavy nitrogen isotope gradually increases. This process leads to increasing $\delta^{15}N$ values in both the algae and the culture medium. During algal growth and reproduction processes, some cells will decay and release nitrogen. Thus, the initially insufficient nitrogen concentration in the culture medium will recover to a certain degree, and as a result the $\delta^{15}N$ values of the algae and the culture medium gradually decrease. The $\delta^{15}N$ values of the algae and the culture medium will reach a maximum value when *Microcystis aeruginosa* is in the logarithmic growth phase. Although *Microcystis aeruginosa* decay releases nitrogen into the culture medium continuously, gradual increases in algae cell nitrogen absorption lead to decreasing nitrogen concentrations in the culture medium, often below the initial concentration (Figure 6). While the $\delta^{15}N$ value for *Microcystis aeruginosa* reaches a maximum during the logarithmic growth phase, the $\delta^{15}N$ value of the culture medium minimizes during the stabilized phase, indicating that extensive algal proliferation can lead to decreasing $\delta^{15}N$. This is consistent with the findings of Peterson *et al.* (2001), which suggest that *Cyanobacteria* blooms can reduce the $\delta^{15}N$ value of water. Algal growth and decay control the process of nitrogen isotope absorption from, and release into, the culture medium; thus, the $\delta^{15}N$ value of the culture medium changes drastically during the culture process. Therefore, it is impossible to use laboratory culture data, such as $\delta^{15}N$ and μ, to establish a functional relationship for *Microcystis aeruginosa.*

The feasibility of using the stable isotope fractionation fitting method to determine the growth rate

During algal growth, algal propagation, or changes in the external environment, the scale of stable isotope fractionation can be affected by a variety of factors including photosynthesis, plant size, temperature, nutrient concentration, pH value that effect on CO_2 or HCO_3^- concentration, and dissolved oxygen. When the pH value is relatively low, the CO_2 concentration in the water is high. During the photosynthesis process, there are sufficient C sources to be used by the algae. In accordance with the rules of isotope utilization, *Microcystis* will give priority to the use of light isotopes, resulting in the decrease of ^{13}C content in *Microcystis* and the increase of ^{13}C content in the water (culture medium). If the pH value is relatively high, the C sources in the water body are mainly CO_3^{2-}, and the photosynthesis of algae will be restricted. As a consequence, the available C sources will be reduced (Wu *et al.* 2012), while ^{13}C will accumulate in the algae. Therefore, the variation in the pH value will lead to the changes of C fractionation and affect the growth rate of algae. Unreasonably low content of dissolved oxygen will lead to algae death, while unreasonably high content of dissolved oxygen will inhibit algae photosynthesis. Both conditions can change the growth rate of algae. A variety of external conditions, such as ultrasonic treatment and algaecide, can lead to algae death directly, resulting in the changes of algae growth rate (Emery *et al.* 2015). Both the changes of the photosynthesis of algae itself and the changes of environmental factors, including temperature, nutrient concentration and pH value, as well as some external factors (ultrasonic treatment and algaecide) may lead to the variation in algae growth rate, and in turn change the C fractionation. Because of the reasons above, all these factors must be carefully considered, the stable isotope technique for determining algal growth rates is not as simple or convenient as the radioactive isotope technique. These difficulties have contributed to the slow application of the stable isotope technique in this field. In the method used here, the process of calculating the algal growth rate is similar to the determination of TN and TP via spectrophotometry; the functional relationship obtained is equivalent to the standard curve, while the field data are equivalent to the absorbance. Therefore, the method described in this paper is theoretically feasible. It incorporates various factors that affect stable isotope fractionation into the fitting function and avoids a number of interferences, greatly simplifying the determination of algae growth rate using the stable

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isotope technique. The application of this method therefore becomes feasible.

In this study, the BG11 culture medium was used for the *in situ* laboratory culture of *Microcystis*. The composition of this culture medium differs from that of Taihu Lake water; thus, the functional fitting curve obtained in this study may not accurately reflect the relationship between algal isotope content and growth rate in Taihu Lake. However, the main objectives of this study were to 1) examine the feasibility of the stable isotope technique for the determination of growth rate, and 2) determine whether this method can be popularized. Therefore, the BG11 medium was not replaced by Taihu Lake water. At the same time, the method described in this paper does not apply to extreme environmental conditions, such as the water body with discharge of untreated sewage and the calculation of growth rate after algal removal treatment. Hence, the use of this method is subject to certain restrictions. Future studies may improve the laboratory culture process to obtain the actual functional relationship between isotopic content and growth rate, thereby enabling the accurate determination of algal growth rates in natural environments.

CONCLUSIONS

1) Carbon and nitrogen fractionation differs among *Microcystis cyanobacteria*. It is possible to establish a functional relationship between carbon fractionation and growth rate, but not between nitrogen fractionation and growth rate.
2) During the algal proliferation period, when the carbon fractionation of the offspring inherited from the parent is between 0–100%, the algae undergo a period of logarithmic growth. The relationship between carbon fractionation (x) and growth rate (μ) in the logarithmic growth phase is $\mu = 1.32(1 + x)^{-0.52}$.
3) Considering the regularity of the isotope fractionation in laboratory-cultured *Microcystis cyanobacteria*, the functional relationship between algal growth rate and isotope fractionation can be used to calculate *Microcystis* growth rates in Taihu Lake. Calculated growth rates are $0.64\ d^{-1}$ in May and $0.12\ d^{-1}$ in September, with an average growth rate of $0.42\ d^{-1}$.

ACKNOWLEDGEMENTS

This paper was supported by Japan contributed to scientific research cause Basic C(15K00630) and the National Science and Technology Major Project of the Ministry of Science and Technology of China (2013ZX07113001).

REFERENCES

Bothwell, M. L. & Kilroy, C. 2011 Phosphorus limitation of the freshwater benthic diatom *Didymosphenia geminata* determined by the frequency of dividing cells. *Freshwater Biology* **56** (3), 565–578.

Cao, H., Kong, F., Tan, X., Yang, Z., Zhang, M. & Xing, P. 2006 Comparison of recruitment from sediments with pelagic growth of *cyanobacteria* in Lake Taihu, China. *Lake Sci.* **18** (6), 585–589.

Ditullio, G. R. & Laws, E. A. 1986 Diel periodicity of nitrogen and carbon assimilation in five species of marine phytoplankton: accuracy of methodology for predicting N-assimilation rates and N/C composition ratios. *Marine Ecology Progress* **32** (2–3), 123–132.

Emery, C., Close, L. & Wormington, J. 2015 Eutrophication and its effect on algae growth and dissolved oxygen. *Journal of Introductory Biology Investigations* **2** (4), 222–237.

Faber, M. J., Smith, L. M. J., Boermans, H. J., Stephenson, G. R., Thompson, D. G. & Solomon, K. R. 2010 Cryopreservation of fluorescent marker-labeled algae (*selenastrum capricornutum*) for toxicity testing using flow cytometry. *Environmental Toxicology & Chemistry* **16** (5), 1059–1067.

Gieskes, W. W. C. & Kraay, G. W. 1989 Estimating the carbon-specific growth rate of the major algal species groups in eastern Indonesian waters by ^{14}C labeling of taxon-specific carotenoids. *Deep Sea Research Part A Oceanographic Research Papers* **36** (8), 1127–1139.

Hlavová, M., Vítová, M. & Bišová, K. 2015 Synchronization of green algae by light and dark regimes for cell cycle and cell division studies. *Methods in Molecular Biology* **1370**, 3–16.

Hungate, B. A., Mau, R. L., Schwartz, E., Caporaso, J. G., Dijkstra, P. & Van, G. N. 2015 Quantitative microbial ecology through stable isotope probing. *Applied & Environmental Microbiology* **81** (21), 7570–7581.

Jazzar, S., Quesada-Medina, J., Olivares-Carrillo, P., Marzouki, M. N., Fernández-Sevilla, J. M. & Molina-Grima, E. 2015 A whole biodiesel conversion process combining isolation, cultivation and in situ, supercritical methanol transesterification of native microalgae. *Bioresource Technology* **190**, 281–288.

Laws, E. A., Popp, B. N. & Bidigare, R. R. 1995 Dependence of phytoplankton carbon isotopic composition on growth rate and $[CO_2]$aq: theoretical considerations and experimental results. *Geochimica Et Cosmochimica Acta* **59** (6), 1131–1138.

Uncorrected Proof

Laws, E. A., Bidigare, R. R. & Popp, B. N. 1997 Effect of growth rate and CO_2 concentration on carbon isotopic fractionation by the marine diatom *Phaeodactylum tricornutum*. *Limnology and Oceanography* **42** (7), 1552–1560.

Lewis, B. & Diaz-Pulido, G. 2017 Suitability of three fluorochrome markers for obtaining in situ, growth rates of coralline algae. *Journal of Experimental Marine Biology & Ecology* **490**, 64–73.

Mahdavi, H., Prasad, V., Liu, Y. & Ulrich, A. C. 2015 In situ biodegradation of naphthenic acids in oil sands tailings pond water using indigenous algae-bacteria consortium. *Bioresource Technology* **187**, 97–105.

Mcgehee, A. 2014 Effects of selective grazing by microzooplankton on phytoplankton composition in a subtropical estuary. *American Journal of Cardiology* **72** (2), 223–225.

Mhlanga, L., Mhlanga, W. & Tendaupenyu, P. 2014 Response of phytoplankton assemblages isolated for short periods of time in a hyper-eutrophic reservoir (Lake Chivero, Zimbabwe). *Water S A.* **40** (40), 157–164.

Nagai, T., Tomioka, N., Kawasaki, T., Imai, A. & Matsushige, K. 2011 In-situ growth rate of *Microcystis*, spp. and their growth-limiting factors: use of cellular RNA content. *Limnology* **12** (3), 235–243.

Pei, S. & Laws, E. A. 2013 Does the ^{14}C method estimate net photosynthesis? Implications from batch and continuous culture studies of marine phytoplankton. *Deep Sea Research Part I Oceanographic Research Papers* **82**, 1–9.

Pei, S. & Laws, E. A. 2014 Does the ^{14}C method estimate net photosynthesis? II. Implications from cyclostat studies of marine phytoplankton. *Deep Sea Research Part I Oceanographic Research Papers* **91**, 94–100.

Pei, S. F., Laws, E. A. & Ye, S. Y. 2014 Study on the discrepancy in applying ^{14}C tracer technique to measure marine primary productivity. *Marine Sciences* **38** (12), 149–156.

Peterson, B. J., Wollheim, W. M. & Mulholland, P. J. 2001 Control of nitrogen export from watersheds by headwater streams. *Science* **292** (5514), 86–90.

Popp, B. N., Laws, E. A. & Bidigare, R. R. 1998 Effect of phytoplankton cell geometry on carbon isotopic fractionation. *Geochimica Et Cosmochimica Acta* **62** (1), 69–77.

Popp, B. N., Bidigare, R. R. & Deschenes, B. 2006 A new method for estimating growth rates of alkenone-producing haptophytes. *Limnology and Oceanography: Methods* 114–129.

Redalje, D. G. & Laws, E. A. 1981 A new method for estimating phytoplankton growth rates and carbon biomass. *Marine Biology* **62** (1), 73–79.

Shin, W. S., Lee, H., Sung, M. G., Hwang, K. T., Jung, M. G. & Kwon, J. H. 2016 Enrichment as a screening method for a high-growth-rate microalgal strain under continuous cultivation system. *Biotechnology and Bioprocess Engineering* **21** (2), 268–273.

Sutherland, D. L., Montemezzani, V., Howardwilliams, C., Turnbull, M. H., Broady, P. A. & Craggs, R. J. 2015 Modifying the high rate algal pond light environment and its effects on light absorption and photosynthesis. *Water Research* **70**, 86–96.

Tanaka, Y., Miyajima, T. & Yamada, K. 2008 Specific growth rate as a determinant of the carbon isotope composition of the temperate seagrass Zostera marina. *Aquatic Botany* **89** (3), 331–336.

Tang, C., Li, Y. & Acharya, K. 2016 Modeling the effects of external nutrient reductions on algal blooms in hyper-eutrophic Lake Taihu, China. *Ecological Engineering* **94**, 164–173.

Tortell, P. D., Mills, M. M., Payne, C. D., Maldonado, M. T., Chierici, M. & Fransson, A. *et al.* 2013 Inorganic c utilization and c isotope fractionation by pelagic and sea ice algal assemblages along the antarctic continental shelf. *Marine Ecology – Progress Series* **483** (5), 47–66.

Tsujimura, S. 2003 Application of the frequency of dividing cells technique to estimate the in situ growth rate of *Microcystis* (*Cyanobacteria*). *Freshwater Biology* **48** (11), 2009–2024.

Wang, C., Wu, X., Tian, C., Li, Q., Tian, Y. & Feng, B. 2015 A quantitative protocol for rapid analysis of cell density and size distribution of pelagic and benthic *Microcystis*, colonies by flowcam. *Journal of Applied Phycology* **27** (2), 1–10.

Wu, X.-d. & Kong, F.-x. 2008 The determination of in situ growth rates of the bloomed *Microcystis* in Meiliang Bay, Lake Taihu. *China Environmental Science* **28** (6), 552–555.

Wu, Y., Ma, X. & Li, Y. 2012 Impact assessment and tolerable threshold value of CO_2 leakage from geological storage on agro-ecosystem. *Transactions of the Chinese Society of Agricultural Engineering* **28** (2), 196–205.

Xu, S. L. & Huang, B. Q. 2010 Photopigment radiolabelling as a tool for determining group-specific phytoplankton photosynthesis rate and growth rate. *Journal of Applied Oceanography* **29** (4), 478–487.

Yamamoto, Y. & Tsukada, H. 2009 Measurement of in situ specific growth rates of *Microcystis* (*Cyanobacteria*) from the frequency of dividing cells. *Journal of Phycology* **45** (5), 1003.

Zhang, X.-f. & Mei, X.-y. 2015 Effects of benthic algae on release of soluble reactive phosphorus from sediments: a radioisotope tracing study. *Water Science and Engineering* **8** (2), 127–131.

First received 31 March 2017; accepted in revised form 3 August 2017. Available online 18 August 2017