1   **Effects of inorganic carbon and pH on growth kinetics of *Synechocystis* sp.**

2   **PCC 6803**

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12

Abstract

Growth pH and the concentration of bioavailable inorganic carbon (C_{ib}) are two essential factors controlling the rate of photoautotrophic growth of microalgae. We developed a novel experimental strategy to determine the effects of pH and C_{ib} independently by a pH-Stat and the use of NH_4NO_3 as the N source. We grew *Synechocystis* sp. PCC 6803 at pH values of 7.5, 8.5, and 9.5, with a stable inorganic carbon (C_i) concentration ranging from 0.08 to 3.3 mM, and a light intensity of $\sim 202 \mu E m^{-2} s^{-1}$. Among the pH values tested, pH 8.5 supported the highest maximum specific growth rate (μ_{max}), 2.4/day that was statistically larger than μ_{max} values at pH 7.5 and 9.5, about 2.0/day. Using the sum of dissolved CO_2 , carbonic acid, and bicarbonate (i.e., C_{ib}) to fit Monod kinetics, we found the half-maximum-rate concentrations (K_{Cib}) were in a narrow range: 86 μM for pH 8.5, 84 μM for pH 7.5, and 96 μM for pH 9.5, values that were statistically indistinguishable. All K_{Cib} values were small, indicating that *Synechocystis* has a high affinity for C_{ib} , attaining μ_{max} by ~ 1 mM. Analyzing the kinetics individually for each C_i species supports that bicarbonate best represented the rate of C_i uptake.

Keywords: Cyanobacteria, inorganic carbon, growth kinetics, pH, *Synechocystis*

1 Introduction

Inorganic carbon (C_i) is essential to microalgae that grow autotrophically, because carbon makes up ~50% mass of the biomass dry weight and embodies about 70% of the electrons generated by photosystem II [1,2]. The effects of the C_i concentration on growth kinetics have been studied extensively at the cellular and molecular levels (e.g. [3–5]) and in mass-culture contexts (e.g. [6–8]). The CO_2 -concentrating mechanism (CCM) enables microalgae to accumulate intracellular HCO_3^- up to 1000-fold higher than the HCO_3^- concentration in the growth medium [9]. That accumulation elevates dissolved CO_2 in the carboxysome, making carbon fixation by Rubisco more efficient [3].

Five C_i transporters, three for bicarbonate (HCO_3^-) and two for dissolved CO_2 , are located in the outer and thylakoid membranes, respectively [5,9,10]. The C_i transporters work in concert. Some have high flux, but relatively poor C_i affinity, while others have high affinity for C_i , but relatively low flux. Which transporters take the dominant role depends on the medium's C_i concentration and pH, which controls the C_i speciation among the bioavailable species ($CO_{2(aq)}$, H_2CO_3 , HCO_3^-) and non-bioavailable carbonate (CO_3^{2-}). For typical growth pHs of 7.5 to 9.5, HCO_3^- is the dominant species. However, intense photosynthesis and insufficient supply of C_i can lead to a higher pH, which partitions the inorganic carbon to CO_3^{2-} [11], which cannot be taken up by C_i transporters [9].

In addition to controlling C_i speciation, medium pH is well known to affect the functioning of proteins and the cell membrane [12,13], ATP/ADP pools [14,15], and growth kinetics [16,17]. Likewise, pH has a universal control over the speciation of certain nutrients, particularly phosphate, iron, and ammonium [18].

Despite intracellular C_i accumulation by CCM, mass-culture studies indicate that $C_i > 1$ to 2 mM may be required to prevent C_i limitation [19,20], especially since the growth pH usually increases during photosynthesis. Outdoor studies, such as by Moheimani (2013) [8] using *Tetraselmis suecica* CS-187 and *Chlorella* sp., indicated that C_i had to be maintained between 1 to 4 mM when the pH was from 7.0 to 7.5.

It is well recognized that C_i uptake during photosynthesis causes the pH to rise, since the medium's alkalinity is distributed into a smaller C_i concentration [16]. Nguyen and Rittmann (2015) [21] recognized that using either NO_3^- or NH_4^+ as the sole N source causes systematic changes in alkalinity: uptake of NO_3^- systematically increases the alkalinity, while uptake of NH_4^+ decreases the alkalinity. These systematic changes in alkalinity complicate the control of pH and C_i , since pH, C_i , and alkalinity are intrinsically linked [21,22].

Previous laboratory studies that have attempted to control the pH in microalgae cultures have used high-concentration buffers (20-50 mM) (e.g. [10,15,23,24]). Addition of a very high level of buffering is impractical for field application of microalgae, where the pH normally is controlled by sparging gaseous CO_2 [25,26]. In addition, a high buffer concentration adds salts that have their own adverse effects [27,28].

In this study, we systematically evaluated the independent effects of the concentration of bioavailable carbon (C_{ib} , which is the sum of dissolved CO_2 , H_2CO_3 , and HCO_3^-) and pH on the growth kinetics of cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*). We designed a novel approach to achieve simultaneously fixed, but independent values of pH and C_i . The pH was fixed by adding CO_2 gas automatically using a pH-Stat. The alkalinity and C_i concentrations were held constant by combining ammonium and nitrate as the N source so that the alkalinity and C_i did not change with photosynthetic growth [21].

73   We independently and systematically tested pH values of 7.5, 8.5, and 9.5 and C_i
74   concentration between 0.08 mM and 3.3 mM, which are typical in natural water [29] and for
75   other studies on C_i growth kinetics [8,20]. Therefore, we were able to separate the effects of
76   growth pH and C_i on the growth kinetics of *Synechocystis*. We applied Monod kinetics to
77   interpret the results through the maximum specific growth rate ($\mu_{\max}$) and the half-maximum-rate
78   concentration for C_{ib} (K_{cib}), as well as to identify which C_i species best represented the rate of C_i
79   uptake.

2 Materials and Methods

2.1 Inoculum preparation

We obtained wild-type *Synechocystis* as pure colonies on agar plates provided by the laboratory of Dr. Willem F. J. Vermaas, School of Life Sciences, Arizona State University (Tempe, AZ, U.S.). Colonies were transferred to BG-11 liquid medium [30] and incubated for 3-5 days at 30°C and $\sim 80 \mu\text{E m}^{-2} \text{s}^{-1}$ to grow sufficient biomass for subsequent experiments. We centrifuged the inoculum twice, each 18,000g for 5 minutes (model 5810R, Eppendorf, NY, U.S.), and then washed and suspended the pellet in a modified BG-11 medium prepared for the upcoming experiments.

2.2 Growth media

We modified the standard BG-11 medium to independently evaluate effects of pH and C_i on growth of *Synechocystis*. First, we included stocks #3, 4, 5, 6, 7, and 9, and doubled the amount of stock #2 to prepared 1 L of liquid medium [31]. The medium was steam-sterilized by a liquid cycle (121°C and 15 minutes) in a Tabletop Sterilizer (SterileMax, Thermo Scientific, IA, U.S.). We let the medium cool overnight before adding stock #1 to achieve a final concentration of 2 mM. Before each experiment, we added one-time sodium bicarbonate (NaHCO_3 , 0.4M) to a desired concentration (0.08 to 3.4 mM). To maintain a stable total alkalinity, we prepared separate stocks of ammonium nitrate (NH_4NO_3 , 0.5N) and titrated these stock to pH=7.5, 8.5, or 9.5 and added NH_4NO_3 stepwise in the beginning and after each sampling except the last one of each run. The final concentration of NH_4NO_3 varied from 0.25 to 0.5 mN, depending on the expected growth rate and the biomass produced between two sampling points. Finally, we titrated the whole solution with 0.5 M NaOH to achieve the experimental pH (7.5, 8.5, or 9.5).

Adding NH_4NO_3 stepwise eliminated any effects from the possible preferential uptake of NH_4^+ over NO_3^- . Adding NaHCO_3 at the experimental concentration helped establish a stable alkalinity and the desired C_i concentration from the beginning of the experiment.

2.3 Experimental setup

We used a 1.5-L photobioreactor (PBR) equipped with a pH-Stat as described in [21]. The pH-Stat automatically maintained a set pH value by adding pure CO_2 gas when the pH reading exceeded the set point (i.e., 7.5, 8.5, or 9.5). The PBR was continually mixed by a stir bar at 200 rpm (VWR, U.S.), and the culture temperature was maintained at 30°C by $3\times 12\text{-W}$ air fans (Minebea-Matsushita Motor Corp., Japan). The total incident light intensity was $324\ \mu\text{E m}^{-2}\text{ s}^{-1}$, provided by two T5 fluorescent bulbs (Hydrofarm, U.S.). **Figure S1** in Supplementary Information shows the layout of the PBR and light source.

Each batch experiment (with a set pH of 7.5, 8.5, or 9.5) lasted up to 24 hours, and samples were taken at 4-h to 8-h intervals. For sampling, we withdrew 24 mL of liquid using a syringe inserted through a tube that reached midway into the culture. Optical density (OD) was measured immediately by a spectrometer (described below), and the remainder of the sample was placed in a 50-ml conical plastic tube (VWR, U.S.) and stored at 4°C until assayed for C_i .

The starting concentrations of N and P were designed to be sufficient for biomass synthesis and avoided N or P limitation. Using stoichiometry, P comprises about 1.3% of dry weight [1]. After production of biomass to 0.5 OD ($\sim 130\text{ mg/L}$), P in medium was about 8.0 mg/L. For N, we also measured the medium's N content periodically and always found at least 28 mgN/L using Dimethylphenol method (TNT836, Hach, CO, U.S.).

2.4 Light Intensity (LI)

The incident light intensity was measured by a U1252 micro-ammeter (Agilent, U.S.) equipped with a LI-190 PAR sensor (LI-COR, U.S.). We took measurements at 32 points inside the PBR. We then applied the Beer-Lambert law [1,32] to calculate the average internal LI (AILI) inside the PBR. Details of the LI measurements and calculation AILI are provided in Supplementary Information.

2.5 Analyses

OD was measured by a UV-Vis BioSpec-mini spectrometer at 730 nm (Shimadzu Corp., Japan). To ensure a reliable reading, a sample was diluted with particle-free deionized water to obtain an OD less than 0.7. The OD reading was converted to biomass dry weight using an empirical conversion factor of 254 mg dry weight biomass/L per unit of OD [33].

Growth pH was measured using the pH electrode supplied with the pH-Stat system (Neptune System, CA, U.S.). The pH electrode was calibrated regularly using two-point calibration (7.01 and 10.0, 25°C) using certified standard pH solutions (Thermo, MA, U.S.). The glass membrane was cleaned using an electrode cleaner solution (Mettler-Toledo AG, Switzerland) to reduce organics binding on the glass surface. pH readings were automatically recorded every 5 minutes using the software from the pH-Stat system.

C_i was analyzed using a TOC analyzer with an auto-sampling tray (Shimadzu Corp., Japan). A sample was filtered through a 0.20-μm polyvinylidene fluoride (PVDF) syringe filter (Whatman GPX, VWR, U.S.), and the filtrate was collected in glass Falcon tubes and sealed immediately by Parafilm M® to prevent any carbon or water loss. To analyze C_i, a 50-μL aliquot was acidified in an Inorganic Carbon chamber that contained 25% H₃PO₄ by weight and having

pH ~ 0.3. All C_i species were then converted to CO_2 that was sparged by ultra-zero air and quantified by a non-dispersive infrared (NDIR) gas analyzer (Shimadzu Corp., Japan). The C_i concentration was determined from a calibration curve established using a mixture having a 50/50 molar ratio of sodium carbonate and sodium bicarbonate (Nacalai Tesque, Inc., Japan). The instrument can inject each sample up to 20 times. We used 5 to 10 injections so that the standard deviation for all readings was less than 2% of the mean concentration. Acidified deionized water (18.2 M Ω , pH = 2-3) vials were included at the beginning, the end, and between every 4-6 samples to assure sufficient cleaning and quality control.

2.6 Kinetic modeling

We used Monod (1949) kinetics to represent the effect of each species of C_i on the specific growth rate:

$$\mu = \mu_{\max} \frac{C_x}{K_{C_x} + C_x} \quad (1)$$

where: μ is the experimental specific growth rate (1/day); $\mu_{\max}$ is the maximum specific growth rate (1/day); C_x is the mM concentration of either the concentration of C_{ib} (the sum of $H_2CO_3^*$ and HCO_3^-), $H_2CO_3^*$, HCO_3^- , or CO_3^{2-} ; and K_C is the C_x concentration giving one-half the maximum specific growth rate (mM). We calculated the experimental μ by Rittmann and McCarty (2001) [34]:

$$\mu = \frac{1}{OD} \frac{dOD}{dt} = \frac{\ln \left(\frac{OD_{t_2}}{OD_{t_1}} \right)}{t_2 - t_1} \quad (2)$$

where: OD_{t_2} and OD_{t_1} are the optical biomass densities at times t_2 and t_1 (day).

163 2.7 Computing C_i speciation

164 Henry's law (Eqn. 3) and gas-liquid mass-transfer kinetics govern the transfer between gaseous
 165 and dissolved CO_2 , in which $C_{\text{CO}_2(\text{aq})}$ is concentration of dissolved CO_2 in water (mole/L),
 166 $P_{\text{CO}_2(\text{gas})}$ is partial pressure of CO_2 in gas phase, $P_{\text{CO}_2(\text{gas})} = 1.0$ bar with pure CO_2 in open
 167 atmosphere, and K_H is Henry's constant for CO_2 , which is 3.0×10^{-2} mole L^{-1} bar $^{-1}$ at 30°C .

$$C_{\text{CO}_2(\text{aq})} = P_{\text{CO}_2(\text{gas})} \times K_H \quad (3)$$

168 $\text{CO}_{2(\text{aq})}$ is hydrated to carbonic acid (H_2CO_3), and the two species normally are grouped together
 169 as H_2CO_3^* (Eqns. 4 and 5) [22]:

$$[\text{H}_2\text{CO}_3^*] = [\text{H}_2\text{CO}_3] + [\text{CO}_{2(\text{aq})}] \quad (5)$$

170 H_2CO_3^* dissociates to two more basic species, bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}), that
 171 are in equilibrium with H_2CO_3^* according to two well-known acid-base reactions and their acid-
 172 dissociation constants ($K_{a,1}$ and $K_{a,2}$, value extrapolated for 30°C) ([22]).

173 C_i (mM) is the sum of the three species: $C_i = [\text{H}_2\text{CO}_3^*] + [\text{HCO}_3^-] + [\text{CO}_3^{2-}]$, while $C_{\text{ib}} =$
 174 $[\text{H}_2\text{CO}_3^*] + [\text{HCO}_3^-]$.

175 If C_i and pH are known, concentrations of H_2CO_3^* , HCO_3^- , and CO_3^{2-} can be computed by Eqns.
 176 8, 9, and 10 [22]:

$$[\text{H}_2\text{CO}_3^*] = C_i \frac{[\text{H}^+]^2}{[\text{H}^+]^2 + K_{a,1}[\text{H}^+] + K_{a,1}K_{a,2}} \quad (8)$$

$$[\text{HCO}_3^-] = C_i \frac{K_{a,1}[\text{H}^+]}{[\text{H}^+]^2 + K_{a,1}[\text{H}^+] + K_{a,1}K_{a,2}} \quad (9)$$

$$[\text{CO}_3^{2-}] = C_i \frac{K_{a,1}K_{a,2}}{[\text{H}^+]^2 + K_{a,1}[\text{H}^+] + K_{a,1}K_{a,2}} \quad (10)$$

177 2.8 Statistical analyses

178 We used the software package Prism (version 6, GraphPad Software, Inc., CA, US) to fit the μ
 179 data to the Monod model (Eqn.(1)). **Table 1** (in Results and Discussion) summarizes the key
 180 outputs for μ fittings using the Michaelis-Menten routine in Prism, since the Michaelis-Menten
 181 and Monod models are identical mathematical representations. In **Table 1**, numbers following $\pm$
 182 represents one standard deviation. In **Figure 1** (in Results and Discussion), the C_i concentration
 183 was averaged from two to four sampling points, and the numbers followed by $\pm$ represent one
 184 standard deviation. With a Gaussian distribution, the range of the mean value and two standard
 185 deviations presents the 95% probability that a measurement falls within this range. We pairwise
 186 compared the fitted K_{Ci} and $\mu_{\max}$ values, with their 95% confident levels, for the three pHs to
 187 determine if the values were statistically different.

3 Results and Discussion

3.1 Monod-kinetics for C_{ib}

We carried out experiments with a range of C_i concentrations from 0.08 to 3.3 mM and pH values of 7.5, 8.5, and 9.5. The AILI was $202 \mu\text{E m}^{-2} \text{s}^{-1}$ in all cases. **Figure 1**, which presents the biomass concentrations for the three pH values, shows that *Synechocystis* grew well with the stable C_i values shown in the legends. The cultures also exhibited a healthy green color throughout all experiments. The divergence of growth curves with pH = 8.5 (**Figure 1**) is an artifact of different starting biomass density and C_i concentrations that resulted in different μ .

Figure 1: Biomass growth for *Synechocystis* grown at fixed pH values of 7.5, 8.5, or 9.5 using NH_4NO_3 as the N source and with the average C_i (mM) concentrations shown in the legends (averages with the 95% confidence levels).

Figure 2 presents the measured specific growth rates of *Synechocystis* (symbols) and the curves for the Monod-model fitting using C_{ib} as the modeled rate-limiting species. The top rows of **Table 1** summarize the best-fit Monod parameters when C_{ib} was the rate-limiting species. *Synechocystis* had the fastest growth rate at pH 8.5: μ_{max} was 2.4/day for pH 8.5, compared to ~2.0/day for pH 7.5 and 9.5. The K_C for total C_{ib} had a small range, from 85 to 96 μM , with the lowest value at pH 9.5 and highest for 7.5. Our K_{Cib} values are similar to those of Badger and Andrews (1982) [35], who estimated the photosynthesis rate of the cyanobacterium *Synechococcus* sp. using buffers to control pH and measuring the kinetics of oxygen evolution. The fitted K_{Cib} value with their 95% confidence level were statistically indistinguishable for all pairwise comparisons. In contrast, the μ_{max} value at pH = 8.5 was statistically larger than μ_{max} value at pH = 7.5 or 9.5.

Figure 2: Measured specific growth rate and Monod best-fit curves using C_{ib} (the sum of $H_2CO_3^*$ and HCO_3^-) for the fixed pH values of 7.5, 8.5, and 9.5. The best-fit values of μ_{max} and K_{Cib} , along with R^2 values, are given in the top rows of **Table 1**.

Table 1: Best-fit Monod parameters for *Synechocystis* sp. PCC 6803 with three pH values and a common AILI of 202 $\mu\text{E m}^{-2} \text{s}^{-1}$.

Monod-parameter value	Growth pH		
	7.5	8.5	9.5
μ_{max} , 1/day	2.03 $\pm$ 0.05	2.37 $\pm$ 0.04	2.00 $\pm$ 0.07
K_{Cib} , μM	86 $\pm$ 12	85 $\pm$ 7	96 $\pm$ 17
R^2 (C_{ib})	0.891	0.971	0.878
$K_{\text{H}_2\text{CO}_3^*}$, μM	5.1 $\pm$ 0.7	0.52 $\pm$ 0.04	0.06 $\pm$ 0.01
R^2 (H_2CO_3^*)	0.892	0.972	0.878
$K_{\text{HCO}_3^-}$, μM	81 $\pm$ 11	83 $\pm$ 7	96 $\pm$ 17
R^2 (HCO_3^-)	0.891	0.971	0.878
$K_{\text{CO}_3^{2-}}$, μM	0.13 $\pm$ 0.02	1.3 $\pm$ 0.1	15 $\pm$ 3
R^2 (CO_3^{2-})	0.891	0.971	0.877

Figure 3 compiles the measured μ values for all pH values (7.5, 8.5, and 9.5) as a function of C_{ib} . The rapid increase of μ up to μ_{max} confirms that *Synechocystis* has a high affinity to C_{ib} : It achieved more than 90% of its maximum growth rate with $C_{\text{ib}} > 0.5 \text{ mM}$ (6 mgC/L) and approached its maximum growth rate with $C_{\text{ib}} > 1.0 \text{ mM}$ (12 mgC/L). Natural waters typically contain C_{ib} from 0.6 to 4.3 mM [29], values that saturate the rate of the inorganic carbon uptake by *Synechocystis*. A *Synechocystis*-based PBR should be able to attain a high growth rate with a modest C_{ib} concentration ($\geq 1.0 \text{ mM}$) with a pH in the range of 7.5 to 9.5. However, if a PBR with a high biomass-production rate has an inadequate CO_2 -supply rate, intense photosynthesis can cause the pH to reach as high as 11, which shifts the speciation of C_{i}

towards non-bioavailable CO_3^{2-} [11]. In such a high-pH situation, a high-productivity PBR would need to maintain a very high C_i concentration, achieved by using a very high alkalinity, to avoid C_{ib} limitation and to achieve a maximum production rate.

Figure 3: Measured specific growth rate and Monod best-fit curves using bioavailable inorganic carbon (C_{ib}) with pH= 7.5, 8.5, and 9.5. The parameter values for the Monod model were best fit to the composite data.

3.2 Kinetics for H_2CO_3^* , HCO_3^- , and CO_3^{2-}

We evaluated each C_i species for its ability to fit the measured specific growth rate to the Monod-kinetic model. **Figure 4** shows the experimental data and model fits, and the best-fit values of $\mu_{\max}$ and K_C are included in the bottom rows of **Table 1**. Each species was able to give a good fit to the experimental value of μ ; this includes CO_3^{2-} , which is not available for uptake by *Synechocystis* [5,36].

While each species could give a good fit to the experimental data, the K_C values had very different patterns that shed light on what species best represents C_i uptake. Using HCO_3^- as the modeled species (**Table 1** and the middle row of **Figure 4**), K_C had a narrow range, from 81 μM

to 96 μM of C. In contrast, the K_C value for H_2CO_3^* (**Table 1** and the top row of **Figure 4**) varied by about two orders of magnitude, from 0.06 μM (pH 9.5) to 5 μM (pH 7.5). This change in K_C corresponds to the change in H_2CO_3^* concentration with pH. CO_3^{2-} (**Table 1** and the bottom row of **Figure 4**) had a similarly wide range of K_C , but with the opposite pattern, from 0.13 μM (pH 7.5) to 15 μM (pH 9.5). Similar to the trend with H_2CO_3^* , the K_C value for CO_3^{2-} correlated with the pH-dependent change in concentration.

Three pieces of information support that HCO_3^- best represented the rate of C_i uptake for our experiments: HCO_3^- was the dominant species, only K_C for HCO_3^- had little variability with pH, and CO_3^{2-} is not taken up by *Synechocystis*. HCO_3^- was the dominant inorganic-carbon species, comprising 86% (pH 9.5), 94% (pH=7.5), or 97% (pH 8.5) of C_i . The wide ranging and opposite trends for H_2CO_3^* and CO_3^{2-} reflect that their concentrations, which were small compared to HCO_3^- , were tied to the HCO_3^- concentration via the pH, as shown in Eqns. (8), (9), and (10). That non-bioavailable CO_3^{2-} could give a good fit strongly reinforces that a K_C value that changes in proportion to pH is evidence that the C_i species was not rate controlling.

Figure 4: Measured specific growth rate and Monod best-fit curves using H_2CO_3^* (top row), HCO_3^- (middle row), and CO_3^{2-} (bottom row) for the fixed pH values of 7.5, 8.5, and 9.5. The best-fit values of $\mu_{\max}$ and K_C , along with R^2 values, are given in **Table 1**.

3.3 Independent control pH and C_i

Beyond quantifying the separate effects of C_{ib} and pH on the growth kinetics of *Synechocystis*, our approach provides a general tool that overcomes the drawbacks of previous attempts to evaluate effects of pH and C_i . Delivering CO_2 automatically based on a pH-Stat function offers a superior tool to control pH. Using a high buffer intensity to maintain pH is impractical at large scale and can introduce side effects from salts build up [28,37,38]. Buffers have limited pH working ranges that depend on acid-association constant(s) (K_a) of each acid/base pair. A working pH must be close to the acid/base pair's pK_a if the buffer is to have strong buffering capability, but it is not feasible to find a biologically acceptable acid/base pair for all pH values of interest. This buffer-intensity limitation is overcome by a CO_2 -based pH-Stat, which directly controls the pH based on feedback from a pH probe. Because of its advantages, controlling pH by automated CO_2 addition is the most practical technique for large-scale PBRs [8,39].

The amount of base generated by photosynthesis with NO_3^- as the N source increases alkalinity and C_i , which means that pH and C_i cannot be controlled independently in the typical situation (see [20,21,40–42]). Likewise, using NH_4^+ as the N source causes a systematic decline in alkalinity and C_i . We demonstrated that using NH_4NO_3 as the N source, when combined with a pH-Stat, stabilized the alkalinity of the growth medium so that we could study C_i -limited kinetics independently from the effects of pH [21]. Exploiting this advantage, we were able to manipulate the C_i concentration independently of growth pH and identify which C_i species best represented C_i -uptake kinetics.

4 Conclusion

Combining a pH-Stat PBR with NH_4NO_3 as the N source enabled us to examine the effects of pH and the concentration inorganic carbon (C_i) independently. For the cyanobacterium *Synechocystis* sp. PCC 6803, pH 8.5 supported the highest maximum specific growth rate, 2.4/day for an average internal light intensity of $\sim 202 \mu\text{E m}^{-2} \text{s}^{-1}$; the rates were about 2.0/day for pH 7.5 and pH 9.5. Using the sum of the three bioavailable species (i.e., C_{ib}) to fit Monod kinetics, the half-maximum-rate concentrations (K_{Cib}) were in a narrow range: 86 μM for pH 8.5, 84 μM for pH 7.5, and 96 μM for pH 9.5. All K_{Cib} values are small, indicating that *Synechocystis* has a high affinity for C_{ib} , since the specific growth rate approaches its maximum value by $\sim 1 \text{ mM}$ (12 mgC/L). Comparing the range of the 95% confidence level pairwise, μ_{max} at pH 8.5 was statistically greater than μ_{max} at pH 7.5 or pH 9.5, while the K_{Cib} value were statistically identical. Analyzing the kinetics individually for each of the inorganic-carbon species shows that bicarbonate had a relatively constant range of K_{Ci} and represented the rate of inorganic-carbon uptake more accurately than dissolved CO_2 or total dissolved inorganic carbon. Maintaining $\text{C}_{ib} \geq 1 \text{ mM}$ should give the maximum growth rate for *Synechocystis*, and a more intensive supply of CO_2 to increase C_i does not offer added benefit. This study offers means to independently evaluate the external pH and C_{ib} on growth kinetics of other cyanobacteria and eukaryotic microalgae.

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References

- [1] H.W. Kim, R. Vannela, C. Zhou, C. Harto, B.E. Rittmann, Photoautotrophic nutrient utilization and limitation during semi-continuous growth of *Synechocystis* sp. PCC 6803, *Biotechnol. Bioeng.* 106 (2010) 553–563. doi:10.1002/bit.22724.
- [2] D.C. Ducat, J.C. Way, P.A. Silver, Engineering cyanobacteria to generate high-value products, *Trends Biotechnol.* 29 (2011) 95–103. doi:10.1016/j.tibtech.2010.12.003.
- [3] M.R. Badger, A. Gallagher, Adaptation of photosynthetic CO₂ and HCO₃⁻ accumulation by the cyanobacterium *Synechococcus* PCC 6301 to growth at different inorganic carbon, *Funct. Plant Biol.* (1987) 189–201. doi:10.1071/PP9870189.
- [4] M.R. Badger, CO₂ concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution, *J. Exp. Bot.* 54 (2003) 609–622. doi:10.1093/jxb/erg076.
- [5] G.D. Price, M.R. Badger, F.J. Woodger, B.M. Long, Advances in understanding the cyanobacterial CO₂-concentrating-mechanism (CCM): functional components, C_i transporters, diversity, genetic regulation and prospects for engineering into plants, *J. Exp. Bot.* 59 (2008) 1441–1461. doi:10.1093/jxb/erm112.
- [6] J. Goldman, E. Carpenter, A kinetic approach to the effect of temperature on algal growth, *Limnol. Oceanogr.* 19 (1974) 756–766.
https://www.aslo.org/lo/toc/vol_19/issue_5/0756.pdf (accessed December 1, 2015).
- [7] Y. Azov, Effect of pH on inorganic carbon uptake in algal cultures, *Appl. Environ. Microbiol.* 43 (1982) 1300–1306. <http://aem.asm.org/content/43/6/1300.short> (accessed December 1, 2015).
- [8] N.R. Moheimani, Inorganic carbon and pH effect on growth and lipid productivity of *Tetraselmis suecica* and *Chlorella* sp. (Chlorophyta) grown outdoors in bag photobioreactors, *J. Appl. Phycol.* 25 (2013) 387–398. doi:10.1007/s10811-012-9873-6.
- [9] G.D. Price, Inorganic carbon transporters of the cyanobacterial CO₂ concentrating mechanism, *Photosynth. Res.* 109 (2011) 47–57. doi:10.1007/s11120-010-9608-y.
- [10] M. Shibata, H. Ohkawa, H. Katoh, Two CO₂ uptake systems in cyanobacteria: four systems for inorganic carbon acquisition in *Synechocystis* sp. strain PCC 6803, *Funct. Plant Biol.* 29 (2002) 123–129. doi:10.1071/PP01188.
- [11] H.W. Kim, R. Vannela, C. Zhou, B.E. Rittmann, Nutrient acquisition and limitation for the photoautotrophic growth of *Synechocystis* sp. PCC 6803 as a renewable biomass source., *Biotechnol. Bioeng.* 108 (2011) 277–85. doi:10.1002/bit.22928.
- [12] E. Fourest, B. Volesky, Contribution of sulfonate groups and alginate to heavy metal biosorption by the dry biomass of *Sargassum fluitans*, *Environ. Sci. Technol.* 30 (1996) 277–282. doi:10.1021/es950315s.

- 336 [13] J.T. Matheickal, Q. Yu, G.M. Woodburn, Biosorption of cadmium (II) from aqueous
337 solutions by pre-treated biomass of marine alga *Durvillaea potatorum*, Water Res. 33
338 (1999) 335–342. doi:10.1016/S0043-1354(98)00237-1.
- 339 [14] E. Padan, S. Schuldiner, Energy transduction in the photosynthetic membranes of the
340 cyanobacterium (blue-green alga) *Plectonema boryanum*, J. Biol. Chem. 253 (1978)
341 3281–3286. <http://www.jbc.org/content/253/9/3281.short> (accessed December 1, 2015).
- 342 [15] R. Emerson, The quantum yield of photosynthesis, Annu. Rev. Plant Physiol. 9 (1958) 1–
343 24. doi:10.1146/annurev.pp.09.060158.000245.
- 344 [16] J.C. Goldman, Y. Azov, C.B. Riley, M.R. Dennett, The effect of pH in intensive
345 microalgal cultures. I. Biomass regulation, J. Exp. Mar. Bio. Ecol. 57 (1982) 1–13.
346 doi:10.1016/0022-0981(82)90140-X.
- 347 [17] A. Mayo, Effects of temperature and pH on the kinetic growth of unialga *Chlorella*
348 *vulgaris* cultures containing bacteria, Water Environ. Res. 69 (1997) 64–72.
349 <http://www.jstor.org/stable/25044843> (accessed December 1, 2015).
- 350 [18] Y. Azov, J.C. Goldman, Free ammonia inhibition of algal photosynthesis in intensive
351 cultures., Appl. Environ. Microbiol. 43 (1982) 735–739.
352 <http://aem.asm.org/content/43/4/735.short>.
- 353 [19] A. Miller, D. Turpin, D. Canvin, Growth and photosynthesis of the cyanobacterium
354 *Synechococcus leopoliensis* in HCO_3^- -limited chemostats, Plant Physiol. 75 (1984) 1064–
355 1070. doi:10.1104/pp.75.4.1064.
- 356 [20] D.R. Clark, K.J. Flynn, The relationship between the dissolved inorganic carbon
357 concentration and growth rate in marine phytoplankton, Proc. Biol. Sci. 267 (2000) 953–
358 959. doi:10.1098/rspb.2000.1096.
- 359 [21] B.T. Nguyen, B.E. Rittmann, Predicting dissolved inorganic carbon in photoautotrophic
360 microalgae culture via the nitrogen source, Environ. Sci. Technol. 49 (2015) 9826–9831.
361 doi:10.1021/acs.est.5b01727.
- 362 [22] V. Snoeyink, D. Jenkins, Water chemistry, John Wiley, 1980.
- 363 [23] Y. Azov, Effect of pH on inorganic carbon uptake in algal cultures inorganic carbon
364 uptake in algal cultures, Appl. Environ. Microbiol. 43 (1982) 1300.
365 <http://aem.asm.org/content/43/6/1300>.
- 366 [24] M. Lopo, A. Montagud, E. Navarro, I. Cunha, A. Zille, P.F. de Córdoba, P. Moradas-
367 Ferreira, P. Tamagnini, J.F. Urchueguía, Experimental and modeling analysis of
368 *Synechocystis* sp. PCC 6803 growth, J. Mol. Microbiol. Biotechnol. 22 (2012) 71–82.
369 doi:10.1159/000336850.
- 370 [25] O. Pulz, Photobioreactors: production systems for phototrophic microorganisms, Appl.
371 Microbiol. Biotechnol. 57 (2001) 287–293. doi:10.1007/s002530100702.

- 372 [26] I. Fernández, F.G. Acién, J.M. Fernández, J.L. Guzmán, J.J. Magán, M. Berenguel,
373 Dynamic model of microalgal production in tubular photobioreactors, *Bioresour. Technol.*
374 126 (2012) 172–181. doi:10.1016/j.biortech.2012.08.087.
- 375 [27] R. Jeanjean, H.C.P. Matthijs, B. Onana, M. Havaux, F. Joset, Exposure of the
376 cyanobacterium *Synechocystis* PCC 6803 to salt stress induces concerted changes in
377 respiration and photosynthesis, *Plant Cell Physiol.* 34 (1993) 1073–1079.
- 378 [28] C. Yeesang, B. Cheirsilp, Effect of nitrogen, salt, and iron content in the growth medium
379 and light intensity on lipid production by microalgae isolated from freshwater sources in
380 Thailand, *Bioresour. Technol.* 102 (2011) 3034–3040. doi:10.1016/j.biortech.2010.10.013.
- 381 [29] N. Assayag, K. Rivé, M. Ader, D. Jézéquel, P. Agrinier, Improved method for isotopic
382 and quantitative analysis of dissolved inorganic carbon in natural water samples., *Rapid*
383 *Commun. Mass Spectrom.* 20 (2006) 2243–2251. doi:10.1002/rcm.2585.
- 384 [30] R. Rippka, J. Deruelles, J.B. Waterbury, M. Herdman, R.Y. Stanier, Generic assignments,
385 strain histories and properties of pure cultures of cyanobacteria, *J. Gen. Microbiol.* 111
386 (1979) 1–61. doi:10.1099/00221287-111-1-1.
- 387 [31] UTEX, BG-11 Medium, (2009). <http://utex.org/products/bg-11-medium> (accessed January
388 12, 2016).
- 389 [32] I.S. Suh, S.B. Lee, A light distribution model for an internally radiating photobioreactor.,
390 *Biotechnol. Bioeng.* 82 (2003) 180–189. doi:10.1002/bit.10558.
- 391 [33] H.W. Kim, R. Vannela, B.E. Rittmann, Responses of *Synechocystis* sp. PCC 6803 to total
392 dissolved solids in long-term continuous operation of a photobioreactor, *Bioresour.*
393 *Technol.* 128 (2013) 378–84. doi:10.1016/j.biortech.2012.10.046.
- 394 [34] B.E. Rittmann, P.L. McCarty, *Environmental biotechnology: principles and applications*,
395 Tata McGraw-Hill Education, 2001.
- 396 [35] M.R. Badger, T.J. Andrews, Photosynthesis and inorganic carbon usage by the marine
397 cyanobacterium, *Synechococcus* sp., *Plant Physiol.* 70 (1982) 517–523.
398 doi:10.1104/pp.70.2.517.
- 399 [36] M. Giordano, J. Beardall, J.A. Raven, CO₂ concentrating mechanisms in algae:
400 mechanisms, environmental modulation, and evolution, *Annu. Rev. Plant Biol.* 56 (2005)
401 99–131. doi:10.1146/annurev.arplant.56.032604.144052.
- 402 [37] K.A. Bérubé, J.D. Dodge, T.W. Ford, Effects of chronic salt stress on the ultrastructure of
403 *Dunaliella bioculata* (Chlorophyta, Volvocales): mechanisms of response and recovery,
404 *Eur. J. Phycol.* 34 (1999) 117–123. doi:10.1017/S0967026299002085.
- 405 [38] M. Takagi, Karseno, T. Yoshida, Effect of salt concentration on intracellular accumulation
406 of lipids and triacylglyceride in marine microalgae *Dunaliella* cells., *J. Biosci. Bioeng.*
407 101 (2006) 223–226. doi:10.1263/jbb.101.223.

- 408 [39] N.R. Moheimani, M.A. Borowitzka, Increased CO₂ and the effect of pH on growth and
 409 calcification of *Pleurochrysis carterae* and *Emiliania huxleyi* (Haptophyta) in
 410 semicontinuous cultures, Appl. Microbiol. Biotechnol. 90 (2011) 1399–1407.
 411 doi:10.1007/s00253-011-3174-x.
- 412 [40] W.P. Mayo, T.G. Williams, D.G. Birch, D.H. Turpin, Photosynthetic adaptation by
 413 *Synechococcus leopoliensis* in response to exogenous dissolved inorganic carbon, Plant
 414 Physiol. 80 (1986) 1038–1040. doi:10.1104/pp.80.4.1038.
- 415 [41] S. Wangwibulkit, C. Limsuwan, N. Chuchird, Effects of salinity and pH on the growth of
 416 blue-green algae, *Oscillatoria* sp. and *Microcystis* sp., isolated from pacific white shrimp
 417 (*Litopenaeus vannamei*), 2008. [http://www.fish.ku.ac.th/pdf/Fishery Bulletin no 32-1-](http://www.fish.ku.ac.th/pdf/Fishery%20Bulletin%20no%2032-1-1.pdf)
 418 1.pdf (accessed January 12, 2015).
- 419 [42] Y. Kezhen, J. Gilmour, D., B. Zimmerman, W., Effects of CO₂ and pH on growth of the
 420 microalga *Dunaliella salina*, J. Microb. Biochem. Technol. 06 (2014) 167–173.
 421 doi:10.4172/1948-5948.1000138.