The influence of light levels on carbon isotope fractionation by *Synechococcus* sp. PCC 7002

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1. Abstract

The biological fractionation of carbon isotopes (ε_P) is an important value used in the reconstruction of historical pCO_2 levels. This value is derived from the difference in the carbon isotope ratios between CO_2 in the environment and the biomass of living organisms. This study works to understand the ways in which ε_P is affected by different environmental conditions in *Synechococcus* sp. PCC 7002, a model cyanobacterial strain. ε_P is directly influenced by the ability of the cell to uptake carbon and, subsequently, by its ability to fix this carbon. Growth rate and CO_2 concentrations each lead to varying relationships with ε_P, due to their ability to regulate carbon fixation in the cell. Previously collected data on *Synechococcus* sp. PCC 7002 show that under high light concentrations there is a linear relationship between ε_P and CO_2 concentration. This work predicts the outcome of ε_P under low light conditions at identical CO_2 concentrations. If the driving factor for ε_P in low light conditions is CO_2 concentration we would expect to see larger ε_P values in cultures grown in air, and smaller values in cultures grown in high CO_2. Alternatively, if the controlling factor of ε_P is light availability, we would expect to see the same ε_P values across all CO_2 concentrations.

2. Introduction

Cyanobacteria are a class of single celled, photosynthetic bacteria constituting the largest and most diverse populations of photosynthetic prokaryotes[1]. Cyanobacteria are most abundant in marine systems and surface soils, but have also been reported to colonize unlikely environments, such as volcanic ash, desert sand, and rocks. Their capacity to live and survive in such an array of diverse environments can be attributed to their ability to withstand a wide range of salinity, alkalinity, pH, light levels, as well as temperatures[2].

Cyanobacteria have been responsible for major ecological and environmental changes over the course of Earth's history. The earliest Proterozoic Eon (2.5-2.0 billion years ago), was characterized by the formation and accumulation of atmospheric oxygen, which is now known as the Great Oxidation Event (GOE). The GOE, arguably the most significant environmental change in Earth's history, can be attributed to the accumulation of oxygen as a byproduct of cyanobacterial metabolism. It is because of cyanobacterial populations, and their subsequent release of oxygen, that life on earth was able to evolve to the complexity and diversity we see today[3].

Cyanobacteria are not just historically significant but continue to play a large ecological role today. Two cyanobacterial strains, *Synechococcus* and *Prochlorococcus,* make up the most abundant microorganismal population in the world's oceans[4]. It is estimated that 64% of photosynthesis that occurs within ocean systems can be attributed to cyanobacteria[5]. These bacteria play a significant role in the ocean food systems, particularly for grazing species and higher trophic levels[5]. Cyanobacteria additionally contribute to the supply of oxygen in Earth's atmosphere and also have a large environmental role in carbon and nitrogen fixation[6].

Carbon isotope fractionation is an important tool that can be used both to better understand the ecological history of cyanobacteria and to reconstruct environmental conditions across Earth's

history. The carbon isotopic composition found in sedimentary rock can be related back to the isotopic composition of biomass produced by ancient primary producers, as well as to the CO_2 concentration in past environments. Many organisms differentiate between the carbon isotopes used in the production of biomass. For example, biomass produced by phytoplankton has a lower $^{13}C/^{12}C$ ratio when compared to the same ratio in dissolved CO_2[7]. This isotopic offset, known as photosynthetic carbon isotopic fractionation (ε_P), is defined as the isotopic difference between the substrate for carbon fixation (dissolved CO_2) and resulting biomass:

$$(eq.\ 1) \qquad \varepsilon_p = \delta^{13}C_{CO_2} - \delta^{13}C_{biomass}$$

where δ notation indicates relative differences in $^{13}C/^{12}C$ ratios.

Photosynthetic carbon isotope fractionation, as measured by ε_P, is affected by a number of factors including carbon availability in the environment, temperature, species composition[8]. These factors determine how an underlying isotope effect associated with rubisco – the primary carbon fixing enzyme – is expressed. The dependence of ε_P values on CO_2 concentrations has been developed into a proxy for reconstructing past CO_2 concentrations[7,9]. However, interpreting these paleo-CO_2 records requires understanding how ε_P values depend on both CO_2 concentrations and other "confounding" factors, such as habitat and physiology. In this thesis, I explore how light levels impact ε_P values. This work will ultimately lead to a better understand of how differing environmental conditions may affect the use of ε_P values as a proxy.

In order to better understand the environmental changes that affect ε_P, our lab has been studying the response of different strains of cyanobacteria to varying environmental conditions. ε_P can be controlled by both growth rate (μ) or CO_2 concentration, and is therefore often plotted again $\mu/[CO_2]$(µmol/kg) (Figure 1). Previous experiments include growing *Synechococcus* sp. PCC 7002

at varying CO_2 conditions under excess light levels. In this experiment, ε_P values increased with decreasing CO_2 concentrations. In a complementary experiment conducted at much lower light levels, ε_P values depended on growth rate rather than CO_2 concentration. These two experiments suggest that under varying environmental conditions, different factors can control ε_P. Varying environment conditions also appear to lead to different ε_P relationships (i.e., different slopes; Figure 1). However, because the previous experiments were conducted at different CO_2 concentrations, their results cannot be directly compared. I have conducted a series of experiments aimed at addressing this discrepancy.

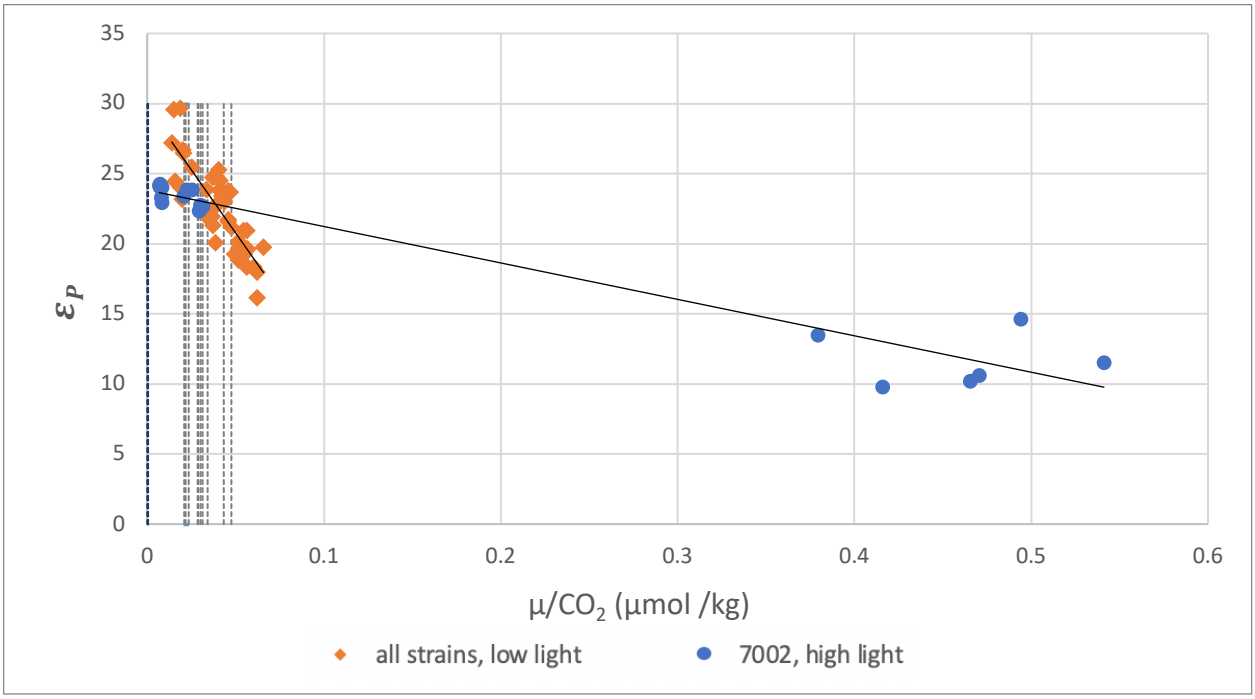


Figure 1. *Synechococcus* sp. PCC 7002 estimations of ε_P based on u/[CO_2] (μmol/kg). Dashed lines are representative of measured μ/[CO2](μmol/kg) for low light conditions at 37°C in A+ media.

This experimental study here works to understand the way in which the ε_P value of *Synechococcus* sp. PCC 7002 is affected under low light conditions in varying concentrations of CO_2. This, in conjunction with previous observations, will allow more insight as to how

environmental conditions control the photosynthetic carbon isotopic record. Understanding of ε_P by cyanobacteria allows for its potential use as a proxy for pCO_2, both through Earth's history, and today.

3. Background

3.1 Photoautotrophy in Cyanobacteria

Presently, cyanobacteria are the only know prokaryotes capable of oxygenic photosynthesis[10]. Cyanobacterial life is sustained through two essential environmental aspects: ability to harvest light, and subsequent conversion of carbon dioxide into organic molecules. Cyanobacteria have the unique capability to occupy many niches, as long as there is light, carbon dioxide, and an electron carrier (water) present[11]. Their ability to capitalize on carbon dioxide and light for the production of biomass and a variety of natural products has become of recent interest in the scientific community, with rising levels of anthropogenic carbon dioxide and a rising necessity for biofuels.

Photosynthetic reactions occur in the thylakoid membrane of cyanobacterial cells. This intracellular membrane comprises much of the area of the cell within the chloroplast[12]. In eukaryotic photosynthetic organisms photosynthesis occurs in the chloroplast, a membrane bound organelle that contains thylakoids. Due to the prokaryotic nature of cyanobacteria, and their lack of membrane bound organelles, all photosynthetic reactions occur in a thylakoid membrane derived from the plasma membrane[13]. Within the thylakoid membrane of cyanobacteria there are two large complexes that complete the conversion of light energy into chemical energy: Photosystem II and Photosystem I. These complexes achieve this through the transfer of electrons across the membrane to create a proton gradient that is then used for the production chemical

energy. This chemical energy is then used for the conversion of inorganic carbon to organic carbon[14].

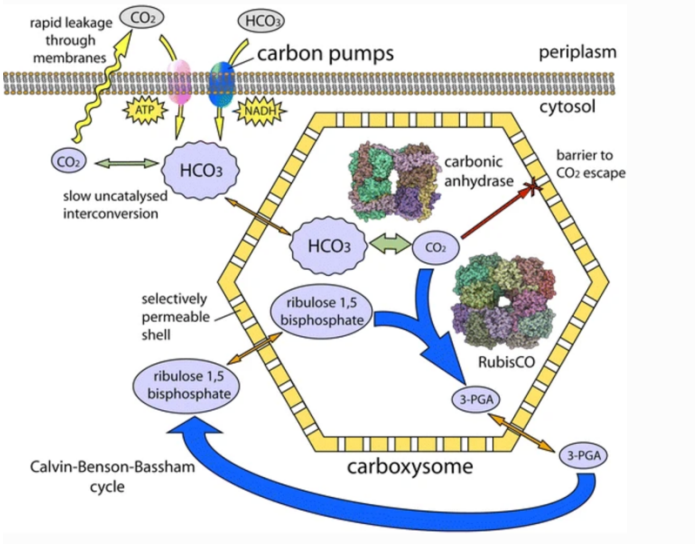
The absorption of photons in PSII allows for the excitation of the reaction center, thus allowing for the subsequent transfer of electrons, via a series of redox reactions, to PSI. The path of an electron through PSII not only allow for the transfer of the electron to PSI, but also for the formation of a proton gradient which is necessary for the generation of ATP. Electrons, now received at PSI, are then excited through the absorption of photons into the PSI reaction center, and another series of redox reactions occur, leading to the reduction of NADP+ to NADPH. This reaction is absolutely essential in photosynthetic systems as NADPH is a necessary electron donor for many metabolic processes. Each photon that is absorbed is absorbed through an antenna pigment, which is then funneled to the reaction center, and then used for the conversion of light into energy. These pigments, each with different absorbances, drive photosynthesis.

Synechococcus, the cyanobacterial species used for this experiment, employ the use of phycobilisomes, the predominant antenna complex in photosystem II (PSII). Light absorbed by the pigments within this photosystem ranges from 565nm – 655nm. One aspect that allows cyanobacteria to have such a wide range of environmental niches could be attributed to the array of different pigments throughout PSII that allow for the absorption of photons under many varying light conditions[4]. The exact pigments within the phycobilisome in PSII absorb specific wavelengths that will then lead to the excitation of an electron in the reaction center, the pigment in most reaction centers of cyanobacteria is chlorophyll a.

In order for photosynthetic organisms to sustain and support life, there needs to be a pathway that allows for the conversion of a carbon source into usable organic molecules. The energy that is harvested through the light reactions is funneled into another set of reactions, the

dark reactions, that allow for the fixation and reduction of carbon dioxide. While these reactions themselves are not directly dependent on the light itself, they are still reliant on the energy and electron carriers generated from PSII and PSI. Similar to C3 plants, cyanobacteria fix carbon dioxide through a process known as the Calvin-Benson-Bassham Cycle. This pathway relies on the key enzyme ribulose-1,5-biophospahate carboxylase (RuBisCO). This enzyme catalyzes the reaction between ribulose-1,5-biphosphate with carbon dioxide to create two molecules of glycerate 3-phosphate (3PGA), a necessary intermediate in the CBB pathway. What makes RuBisCO so significant in the carbon fixation pathway is its slow turnover rate and confused specificity for CO_2 leading for it to be the rate limiting enzyme in this pathway[15].

Due to the small and nonpolar nature of carbon dioxide, it rapidly diffuses out of the plasma membrane. In order to compensate for this and the inefficiency of RuBisCO, cyanobacteria evolved a process to capture and use carbon dioxide in ambient amounts, called the CO_2 Concentrating Mechanism (CCM). Within the bacteria there is a cytosolic inclusion that holds RuBisCO and carbonic anhydrase (CA), called a carboxysome, which as suggested by recent studies acts, as diffusional barrier for carbon dioxide. Carbon dioxide is actively pumped into the cytosol of the cell where it is converted to bicarbonate. Bicarbonate then enters through pores in the carboxysome, where it is converted back to carbon dioxide by CA. Now that the carbon dioxide is trapped, it can accumulate, allowing for sufficient amounts to bind and saturate RuBisCO. Upon ribulose-1,5-biphosphate binding RuBisCO and carbon dioxide complex, RuBisCO is able to generate 3-PGA, the next intermediate for the CCB cycle[16].



Figurer 2. Schematic of the CCM within cyanobacteria. CO_2 is actively pumped into the bacterial cell and converted to bicarbonates. Bicarbonate enters the carboxysome and is converted back into CO_2 by CA. Carbon dioxide is trapped, accumulates, and then used by RuBisCO, increasing efficiency[17].

3.2 *Carbon isotope fractionation*

Carbon has two stable isotopes ^{12}Cand ^{13}C, with one radioactive isotope, ^{14}C. The carbon isotope ^{14}C readily decays to ^{14}N and cannot be used for stable isotope analysis. ^{12}C is the most abundant form of carbon, composing 98.9% of all earth's carbon. ^{12}C is the lighter of the stable carbon isotopes with six neutrons, compared to the seven neutrons in ^{13}C. Many biological processes, including those of cyanobacteria, are selective for carbon isotopes and prefer to use ^{12}C as it is lighter and energetically more efficient to break ^{12}C-containing bonds. It is because of this selectivity that there is a difference in the ratios of carbon isotopes preset is biomass and present in the surrounding environment.

The fractionation of carbon isotopes in a sample can be understood from the following formula:

(eq. 2) $$\delta^{13}C_{sample} = \left[\frac{(\frac{C^{13}}{C^{12}})_{sample} - (\frac{C^{13}}{C^{12}})_{standard}}{(\frac{C^{13}}{C^{12}})_{standard}}\right] \times 1000$$

Two main processes that will influence biological isotope fractionation: the first being the import of inorganic carbon into the cell, the second being the fixation of dissolved carbon dioxide within the cell[18]. Our understanding of the photosynthetic carbon isotope fractionation (ε_P), can be understood in relation to carbon fixation by the following formula:

(*eq. 3*) $$\varepsilon_P = \varepsilon_f - f(\varepsilon_f - e_t)$$

$\varepsilon_f$is representative of the fractionation from the enzymatic conversion of inorganic carbon into fixed carbon, e_t is representative of fraction observed from the transport of carbon dioxide from the external environment to the carboxysome, and f indicates the ratio of carbon that is fixed versus removed from the carboxysome.

The kinetic isotope effects, represented by ε_f and e_t, describes the change in the rate of the reaction when one of the reactants atoms is replaced by its heavy isotope: here K_L designates the light isotope, and K_H designates the heavy isotope.

(*eq. 4*) $$KIE = \frac{K_L}{K_H}$$

RuBisCO, the rate limiting enzyme in the conversion of carbon dioxide to 3PGA, has a kinetic isotope effect of 22%. The kinetic isotope effect of rubisco allows us to set upper limits for ε_P through ε_f.

3.3 Previously collected data

In order to address some of these larger questions regarding the use of ε_P as a proxy for pCO2, a previous lab member (Kunmanee Bubphamanee) researched the response of four different strains of cyanobacteria under a set of different environmental conditions. The four strains of cyanobacterial cells she used were *Gloeomargarita lithophora, Synechococcus sp. PCC* 7002*,*

Synechococcussp. PCC 6312*,* and *Neosynechococcus sphagnicol*a in order to determine if the ε_P value in cyanobacteria is universal or strain-specific. From her research she found that ε_P, under light limiting conditions, is not strain-specific, nor is it dependent on carbon dioxide concentration, but rather that this value is dominantly controlled by growth rate.

Additional research previously conducted in our lab looked at the effects of high light conditions using *Synechococcus* sp. PCC 7002 in ε_P. Three experimental conditions were used: air, 1% CO_2, and 3% CO_2. It was determined that the ε_P for *Synechococcus* sp. PCC 7002 in air had an extremely low ε_P in comparison to the other carbon dioxide concentrations. Previous studies on *Synechococcus* sp. PCC 7002 in low light conditions had been done in BG11 media and at 30°C, so while this information provided useful in other ways, it could not be used to compare the ε_P values of *Synechococcus* sp. PCC 7002 in high light conditions. In order to bridge this gap in understanding this study works to collect information on the εP of *Synechococcus* sp. PCC 7002 growing under low light conditions, in A+ media, at 37°C, in both air and 3% CO_2.

This experimental study here works to understand the way in which the ε_P value of *Synechococcus* sp. PCC 7002 is affected under low light conditions in varying concentrations of CO_2. This, in conjunction with previous observations, will allow more insight as to how environmental conditions control the photosynthetic carbon isotopic record. Understanding of ε_P by cyanobacteria allows for its potential use as a proxy for pCO_2, both through Earth's history, and today.

4. Materials and Methods

4.1 *Experimental setup*

Cultures of *Synechococcus* sp. 7002 (*Synechococcus* 7002) were maintained in the Cameron Laboratory at the University of Colorado, Boulder. Cultures were grown in two

experimental conditions, 3% CO_2 and air. The cultures were acclimated four times, with each acclimation there were three biological replicates. This process of serial transfers was completed for both the high and low CO_2 conditions. The process of acclimation allowed for the *Synechococcus* 7002 to fully adapt to its environmental conditions, as well as limit the amount of biomass taken from the initial plate culture.

All cell cultures were grown in 125mL flasks, all autoclaved before use. Cultures were inoculated with 18% of the final cell density (cells ml^{-1}) in 25mL of A+ media and 25uL of vitamin B12.

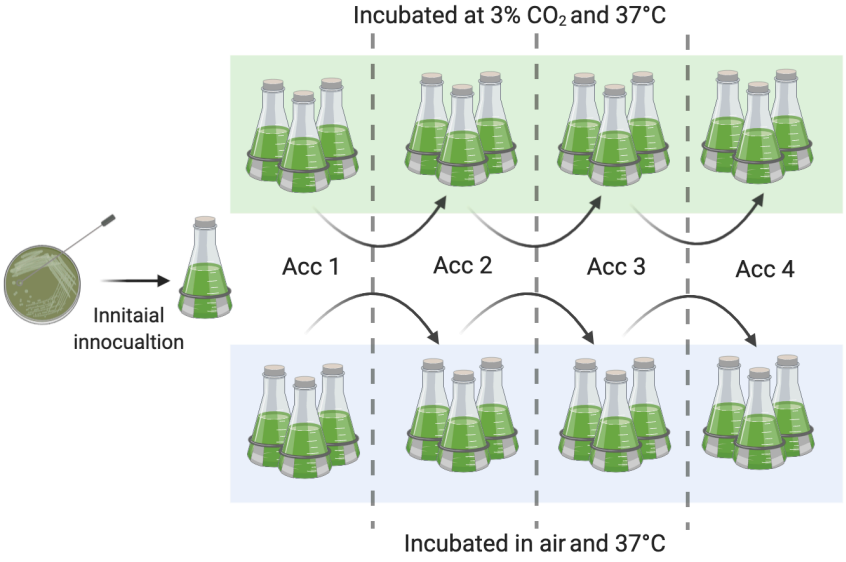


Figure 3. Schematic of experimental set up. Flasks highlighted in green were grown in 3% CO_2, flasks highlighted in blue were grown in air (0.04% CO_2). In total, there were four acclimations, each acclimation had three biological replicates.

Synechococcus 7002 was grown in 2 experimental conditions, 3% CO_2 and Air. The cultures were acclimated four times, with each acclimation there were three biological replicates. This process of serial transfers was completed for both the high and low CO_2 conditions. The process of acclimation allowed for the *Synechococcus* 7002 to fully adapt to its environmental conditions, as well as limit the amount of biomass taken from the initial plate culture.

Cyanobacterial cultures were grown in a Percival incubator, model AL-41L4 (Percival Scientific, Iowa, USA). The cultures were shaken at 200 rpm. The lighting capacities of the individual incubators, one for the air condition, one for the 3% CO_2 condition, varied slightly. In order to make the lighting consistent between conditions the lighting in the air incubator was set to 17% capacity and the lighting in the air incubator was set to 14% capacity. In addition, incubator stands were built to accommodate an opaque plexiglass panel that allowed us to further decrease the light levels. The sides and the front of the stand were wrapped in tinfoil to limit the amount of external light into the cultures.

The light levels at each position in the shaker placed in each individual incubator were measured to account for variability in different locations on the shaker. The concentration of light at each position in the shaker, in both the air and 3% CO_2 conditions, slightly varied. The position of each flask was maintained throughout its growth in the incubator. Light levels were measured prior to the first acclimation and then again after the completion of the fourth acclimation (Table 1).

3% CO_2 initial measurement		**3% CO_2 final measurement**	
Position	Light measurement (µmol)	Position	Light measurement (µmol)
1	12.21	1	9.78
2	11.67	2	9.19
3	12.43	3	10.14
Air initial measurement		**Air final measurement**	
Position	Light measurement (µmol)	Position	Light measurement (µmol)
1	9.95	1	10.17
2	10.39	2	9.55
3	10.84	3	11.02

Table 1. Light levels of cultures grown in 3% CO_2 and air

The growth of *Synechococcus* 7002 was monitored using optical density at 730 nm ($OD_{730\ nm}$) using a TECAN plate reader (TECAN, Mannedorf, Switzerland). 200uL aliquots of the culture solution were added to a 96 well plate. The OD measurements of the cultures were taken at 730nm, along with a blank solution containing A+ medium and B12 to subtract from the plate reading. OD readings were taken throughout the growth of each biological replicate, for each acclimation, and each condition.

The growth rate of *Synechococcus* sp. PCC 7002 was calculated using the following a calibration curve which relates optical density for *Synechococcus* sp. PCC 7002 at 37°C at 730nm to cell density (cells/mL).

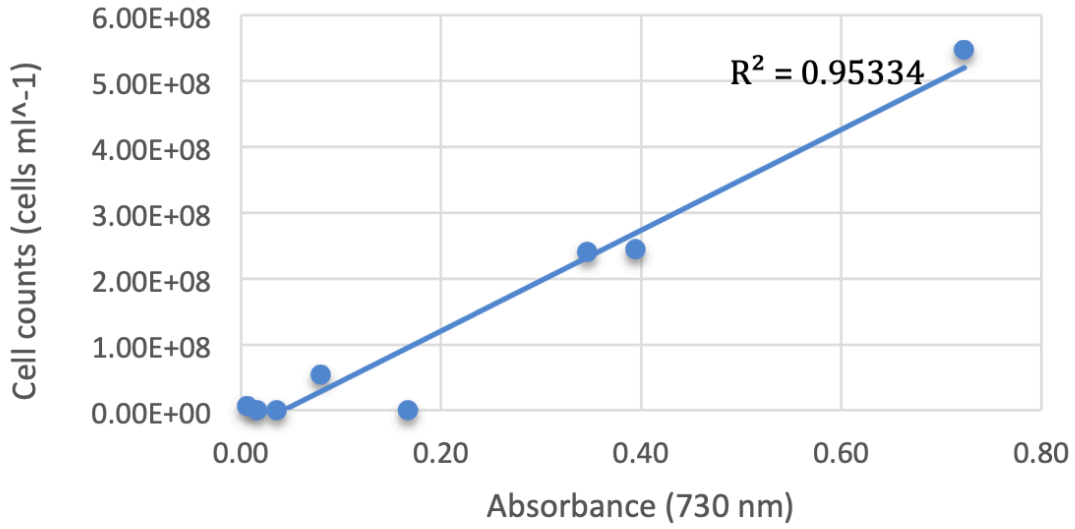


Figure 4. Calibration curve for determining cell density of *Synechococcus* sp. PCC 7002 at 37°C at an absorbance of 730nm.

(*eq. 5*) $$\rho = [(7.4 \times 10^8) \times OD_{730nm}] - 1.2 \times 10^7$$

The net growth rate of each experimental condition, and each biological replicate was calculated using only the initial and final cell density according to the following formula:

(*eq. 6*) $$\mu = \frac{((\ln(\rho final) - (\ln(\rho initial))}{time}$$

The initial cell density (ρ_{inital}) for each growth rate calculation was 2.5×10^7cells/mL, as this was the inoculated cell density for each culture.

Cyanobacterial cultures were harvested with OD_{730nm} between 0.16-0.23. This $OD730_{nm}$ range represent a threshold over which cyanobacterial cultures are no longer in exponential phase. The cells experience a lag phase early in the growth, followed by a log phase in which the cells experience an exponential division with time. The cultures were harvested in the log phase, before they reached the linear (self-shading) phase of growth.

In addition to the OD measurements collected at 730nm, absorbance readings for three additional wavelengths were collected at 435nm, 628nm, and 678nm. The absorption of 625nm corresponds to the maximum absorbance of phycocyanin, 435nm and 678nm correspond to the wavelengths absorbed by chlorophyll.

The pH of both the 3% CO_2 and air cultures were taken at the end of the fourth acclimation using. The pH of the 3% CO_2 was 6.7, and the pH of the cultures in air were 8. The partial pressure of CO_2 in 3% CO_2 was 0.02463 atm and the partial pressure of CO_2 in air was 0.0003284 atm. Using these two numbers as input values the total amount of CO_2 dissolved in each culture was calculated using the PHREEQC program developed by the US Geological Survey. The total calculated concentration of dissolved carbon in the was 571μmol/kg for the 3% CO2 cultures and 8μmol/kg for the air cultures.

Cultures, once they reached an ideal OD, were collected in sterile 15mL falcon tubes and frozen in a freezer (VWR Scientific, Pennsylvania, USA) at -70°C for analysis.

4.2 Measuring Carbon Isotope Content

We were planning on taking isotopic measurements of all cyanobacterial cultures prior to the shutdown of campus for COVID-19. I have included a description of how these measurements are taken.

Isotopic measurements will be taken in the Earth Systems Stable Isotope Lab at the University of Colorado, Boulder. Samples will be combusted in a Thermo Scientific Flash Elemental Analyzer, and resultant carbon dioxide will be analyzed with a Thermo Scientific Delta V Isotope Ratio Mass Spectrometer. Multiple standards will be used to ensure accuracy when measuring $\delta^{13}C_{sample}$.

Culture samples were thawed to room temperature from -70°C. Preparation of samples were conducted under sterile techniques. Samples were pippeted into microcentrifuge tubes. The volume of each sample was calculated so that the volume of the biomass added contained 35ug of carbon. Samples were then centrifuged for five minutes so that a pellet formed. The supernatant was decanted, and cells were then washed with 1mL of MilliQ water. Sample was then centrifuged, and the supernatant was decanted, leaving behind a pellet. 20uL of MilliQ water was added to pellet, the pellet and the water was then homogenized. 20uL of the homogenized sample was pippeted into pre-weighed capsules that were dried overnight in a 50°C oven. The weight of the biomass was then calculated by subtracting the mass of the empty capsule to the mass of the dried sample and capsule.

4.3 Calculation of dissolved CO_2 concentrations

In order to calculate the concentration of DIC species in both the cultures grown in air and 3% CO_2, the program PHREEQC (https://www.usgs.gov/software/phreeqc-version-3) was used. This program uses a detailed model to calculate DIC based upon measured conditions, including

pH, density, temperature, partial pressure of CO_2, as well as the concentration of minerals in the media. A detailed list of measured inputs can be seen in the appendix.

4.4 Computation of ε_P values

ε_P is the biological fractionation of stable carbon isotopes, and can be understood through the following formula:

(*eq. 1*) $$\varepsilon_P = \delta^{13}C_{CO_{2(aq)}} - \delta^{13}C_{biomass}$$

In order to calculate ε_P we must first calculate $\delta^{13}C_{CO_{2(aq)}}$. This can be done by initially finding the fractionation of dissolved carbon isotopes in aqueous media is temperature dependent, the following equation explains this relationship. T_C denotes the temperature of the condition in °C.

(*eq. 7*) $$\varepsilon_{CO_2\,(aq)-CO_2\,(g)} = 0.0049T_C - 1.31\%$$

From here the equation can be rewritten to express the value of the carbon isotope fractionation:

(*eq. 8*) $$\propto_{CO_2\,(aq)-CO_2\,(g)} = \frac{\propto_{CO_2\,(aq)-CO_2\,(g)}}{1000} + 1$$

The fractionation factor of aqueous carbon and atmospheric carbon can be understood as:

(*eq. 9*) $$\propto_{CO_2\,(aq)-CO_2\,(g)} = \frac{\delta^{13}C_{CO_{2(aq)}}+1000}{\delta^{13}C_{CO_{2(g)}}+1000}$$

In order to find the value of $\delta^{13}C_{CO_{2(aq)}}$ the previous two equations can be combined to the following:

(*eq. 10*) $$\delta^{13}C_{CO_{2(aq)}} = \left[\propto_{CO_2\,(aq)-CO_2\,(g)} \times \left(\delta^{13}C_{CO_{2(g)}} + 1000\right)\right] - 1000$$

From this calculation, and the measured $\delta^{13}C_{biomass}$values, the corresponding ε_P can be calculated.

5. Results

Synechococcus sp. PCC 7002, was grown under low light conditions (average of 10.0 μmol) in air (0.04% CO_2) and high CO_2 (3% CO_2). Under each environmental condition there were a total of four serial acclimations, and for each acclimation three biological replicates. The growth rate of each culture was monitored using OD at 730nm.

5.1 Pigment analysis

Within the thylakoid membrane of the cyanobacterial cell, where all photosynthetic reactions take place, there are a collection of pigments that exist to capture photons at different wavelengths and then funnel them to the reaction center where they will be used for the production of energy. Each of these pigments absorb a different wavelength of light. In order to better understand the photosynthetic reactions of *Synechococcus* sp. PCC 7002, measurements of the relative abundance pigments in each condition were taken. Absorbances were taken at 678nm, 625nm, and 435nm. The absorption of 625nm corresponds to the maximum absorbance of phycocyanin, 435nm and 678nm correspond to the wavelengths absorbed by chlorophyll.

The relative abundance of pigments was observed in air and 3% CO_2 conditions across each acclimation. The distribution of pigments across each acclimation, for each environmental condition, remained relatively uniform. When air conditions are compared to CO_2 conditions there is no significant change in abundance of pigment (figure 5). This data is consistent with what we would expect in different experimental carbon concentrations with the same light levels, as

pigment concentration is controlled by light. This verifies that the light conditions were consistent across varying light levels.

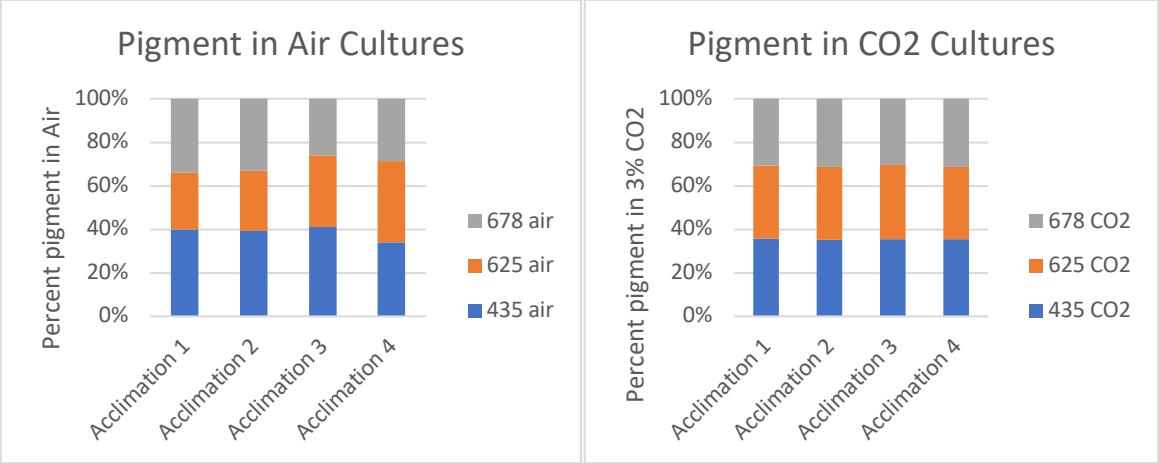


Figure 5. Relative abundance of pigments absorbing light at three different wavelengths in cultures grown in air (left) and 3% CO_2 (right) in low light conditions across acclimations

When air conditions are compared to CO_2 conditions there is no significant change in abundance of pigment. This data is consistent with what we would expect in different experimental carbon concentrations with the same light levels, as pigment concentration is controlled by light. This verifies that the light conditions were consistent across varying light levels.

5.2 Growth rate analysis

Growth was monitored for each biological replicate across four transfers in each condition (Figure 6 and Figure 7). Cultures were transferred and harvested at the end of log phase ($OD_{730\ nm}$ of 0.16-0.22) in an attempt to normalize the growth rates across the acclimations.

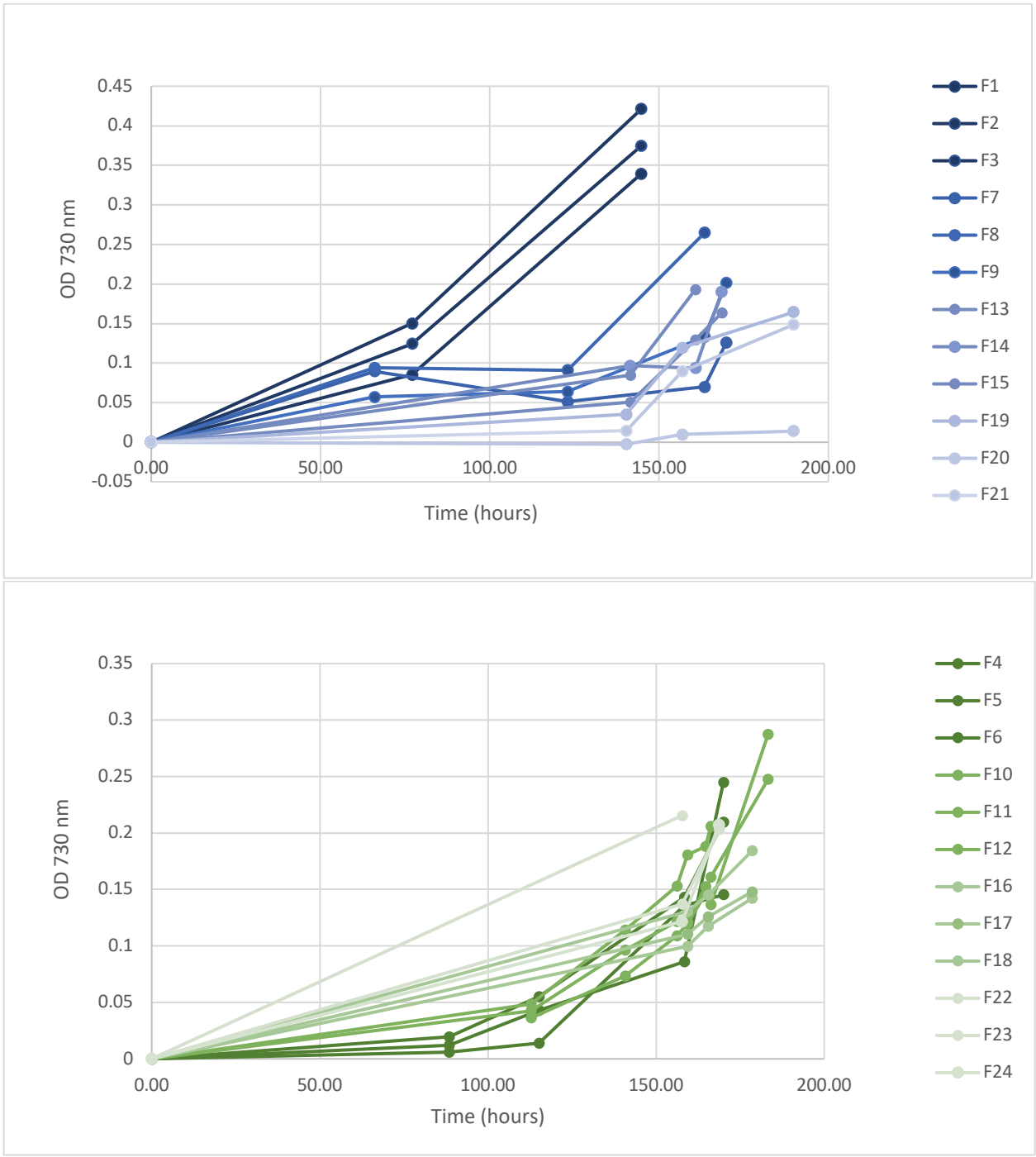


Figure 7. *Synechococcus* sp. PCC 7002 growth curve cultured in 3% CO_2. Acclimation 1 includes F4-6 (dark green), acclimation 2 includes F10-12 (medium green), acclimation 3 includes F16-18 (light green), and acclimation 4 includes F22-F24 (lightest green).

Cells in both air and 3% CO_2 both reached log phase, represented in an OD_{730nm} between 0.16 and 0.22, in a similar amount of time. In the growth curves of the cultures grown in air,

subsequent acclimations appear to be slowing down (i.e., taking longer to reach the final OD). However, this growth pattern is not seen in the cultures grown in 3% CO_2, as the acclimations growth curves have more overlap.

The value for growth rate calculated and used is the net growth rate, defined by the final and initial cell density (eq. 6). This was used instead of the maximum growth rate, in order to calculate growth rates as they correspond to the total amount of carbon fixed over the course of the culture. The net growth rates of each condition between acclimations 1 and 2 are more variable, while the growth rate for acclimations 3 and 4 in each condition are similar (Figure 8). This suggests that the cultures were successfully acclimated to air and 3% CO_2 conditions.

(*eq. 6*) $$\mu = \frac{((\ln(\rho final) - (\ln(\rho initial))}{time}$$

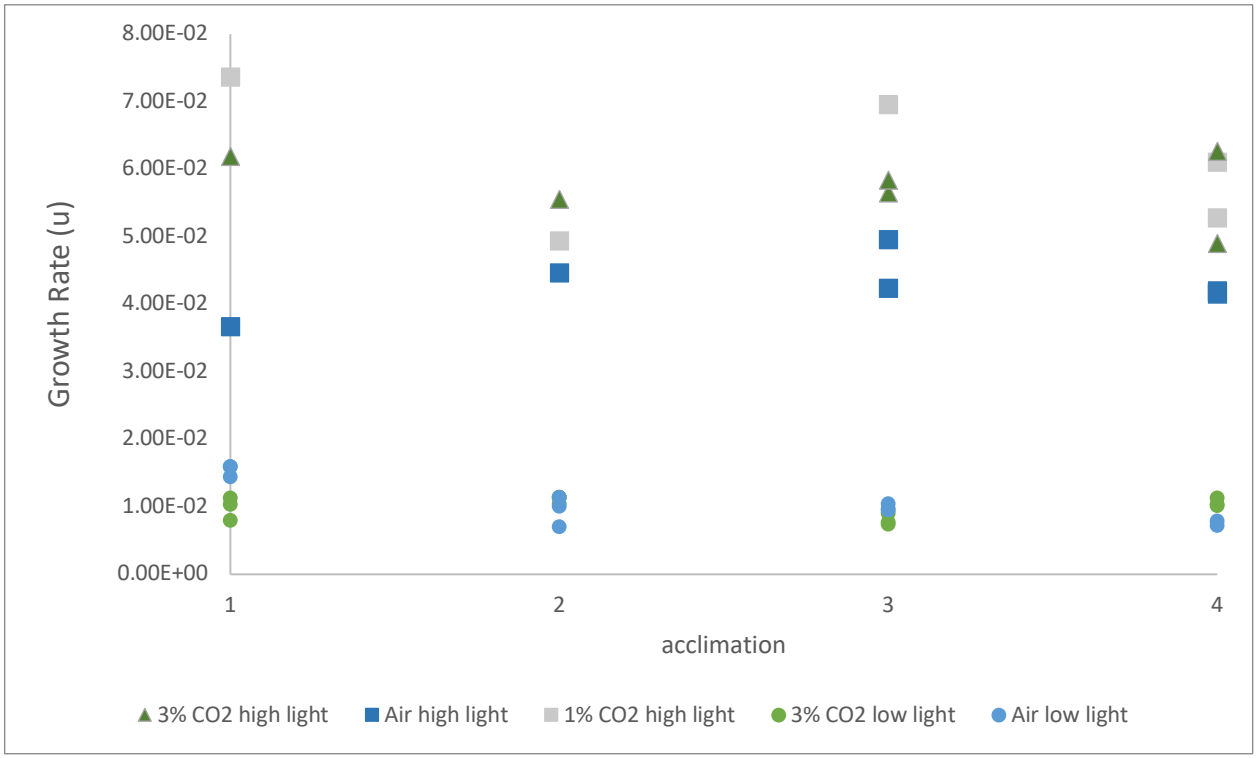


Figure 8. *Synechococcus* sp. PCC 7002 growth rates as a function of acclimation. All blue points are representative of air (0.04% CO_2) conditions, all green points are representative of 3% CO_2 conditions, grey points are representative of 1% CO_2 conditions. The plots on this graph include both low light and high light conditions.

In order to better understand if *Synechococcus* sp. PCC 7002 growth was light or carbon limited, the growth rate calculated for each flask was plotted as a function of the percentage of CO_2 in the headspace (Figure 9). We compared growth rate data for the current set of experiments to growth rates of *Synechococcus* 7002 grown under both low light and high light conditions. In general, the low light conditions have much lower growth rates relative to the high light conditions (Figure 9).

Under low light conditions, growth rates for *Synechococcus* sp. PCC 7002 did not increase with increasing CO_2 concentration (Figure 9). It appears that *Synechococcus* 7002 is unable to capitalize on the increased availability of carbon dioxide due to the limited energy supplied by light. This indicates a light limiting environment in which the cell lacks the energy to fix increased concentrations of CO_2. This relationship is seen for both experiments conducted with A+ media at 37°C and BG11 media at 30°C, even though each experimental condition had different growth rates.

Under high light conditions there is an overall increase in growth rate with increasing CO_2 concentrations. This indicates that under high light conditions the factor limiting increased growth is concentration of carbon. The cell has abundant energy for the fixation of carbon but cannot increase in growth rate unless this carbon is available. However, upon increasing carbon concentration in high light conditions from 1% to 3% there is no longer increased growth (Figure 9). This may represent the maximum growth capacity for *Synechococcus* sp. PCC 7002 or that some other factor has become limiting.

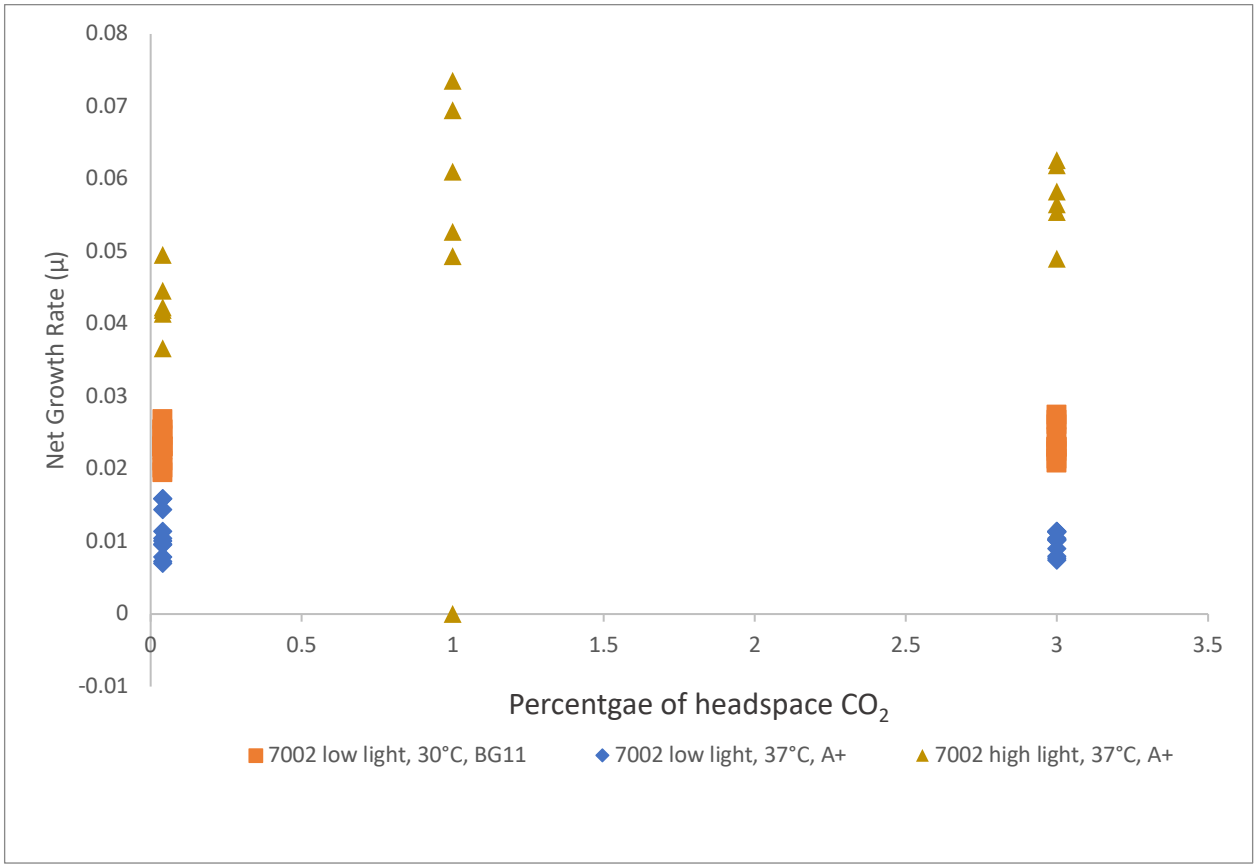


Figure 9. *Synechococcus* sp. PCC 7002 growth rates as a function of percentage of CO_2 in both low and high light.

6. Discussion

Phenomenological models for ε_P relationships, as touched on previously[18], suggests that ε_P depends on the import of inorganic carbon into the cell, and the fixation of inorganic carbon. ε_P is thought to be proportional to the proportion of incoming CO_2 that is fixed into biomass versus removed from cell (f. In carbon limiting conditions f will be large; the cell has sufficient energy to convert a large proportion of CO_2 into organic molecules. The $f$can also be related to growth rate. At slower growth rates, for example in conditions that are light limited, f may be expected to be smaller as carbon fixation by the cell cannot keep up with the supply of CO_2. A larger f will result in a smaller ε_P, and a smaller f will result in a larger ε_P. The limiting factors of

cyanobacterial cell growth, in this study, light and carbon concentration, will have a direct impact on the ability of the cell to intake carbon, and in turn, fix carbon into organic molecules. These environmental conditions influence the factors that directly control ε_P, indicating changes in environment may influence ε_P.

This phenomenological model provides a predictive framework for ε_P values as a function of growth rate and CO_2 (Figure 13). Values of ε_P are expected to increase with increasing CO_2 concentrations (Figure 13A). As f values increase (the proportion of fixed CO_2 vs. the excess CO_2 that is remove from the cell), ε_P values will be smaller. However, ε_P relationships are typically plotted against μ/CO_2, so we've plotted ε_P versus $1/CO_2$ to show the similar directionality of the slopes for ε_P versus $1/CO_2$ and ε_P versus growth rate (μ) (Figure 13B-C). Values of ε_P are additionally expected to decrease with increasing growth rates. Cells growing faster, have less of an ability to discriminate between ^{12}C and ^{13}C, leading to a smaller ε_P value. Cells that have slower growth rates do not need to fix carbon as readily, allowing for a larger ε_P .

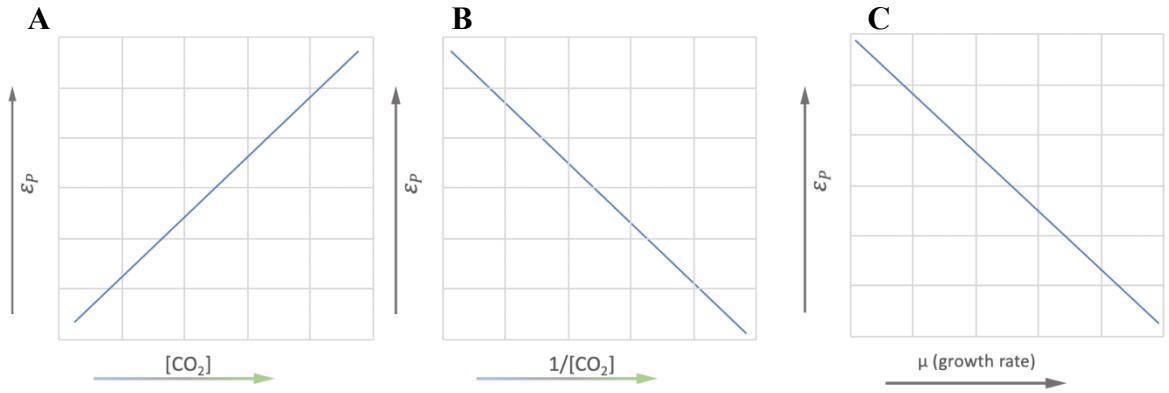


Figure 13. Estimations of ε_P based on limiting conditions. **A and B)** carbon limited **C)** light limited.

In order to separate these cofounding factors influencing ε_P, the individual influence of growth rate and CO_2 concentrations need to be considered. Using previously collected data of ε_P values *Synechococcus* sp. PCC 7002 under high light and low light conditions in varying CO_2

concentrations, we can use the above framework to hypothesize where the ε_P values for my data may fall.

We know from previously collected data that ε_P under light limited conditions is controlled by growth rate rather than CO_2 concentration (figure 14; orange dataset). When ε_P is plotted as a function of growth rate we see a liner change in ε_P as growth rate increase for *Synechococcus* sp. PCC 7002 under low light in air (Figure 14; orange dataset). In contrast, this linear relationship is not mirrored in data where we know conditions are carbon limited (represented in figure 14 as grey, yellow, and blue data points). Under carbon limiting conditions, the opposite is true. Plotting the relationship between ε_P and CO_2 concentration under high light conditions reveals that as CO_2 concentrations increase so does ε_P (Figure 15) .

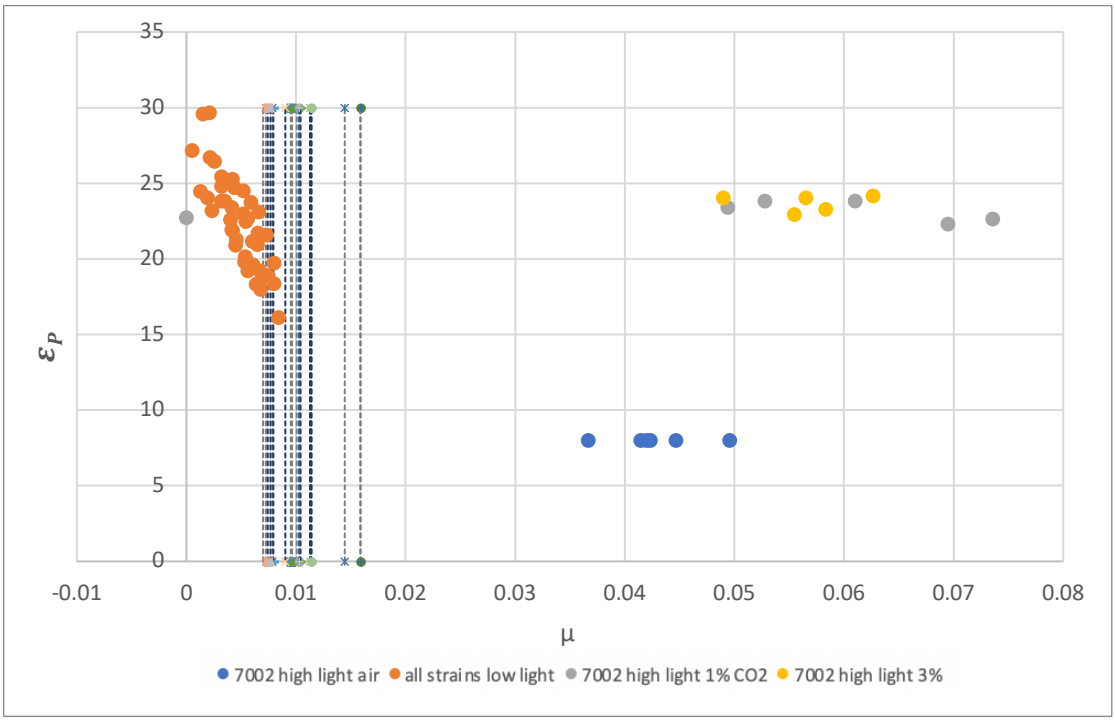


Figure 14. *Synechococcus* sp. PCC 7002 ε_P vs growth rate, orange data represents previous data under low light, blue, yellow, and grey represent high light data. Dashed lines indicate growth rates under low light conditions in air and 3% CO_2.

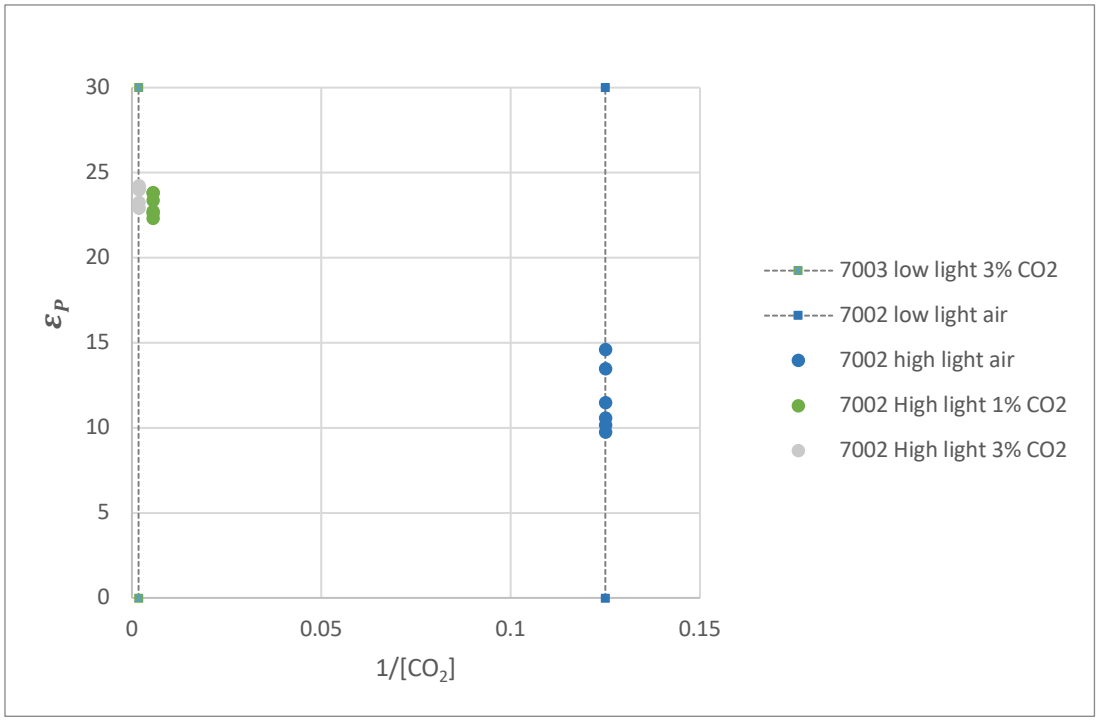


Figure 15. *Synechococcus* sp. PCC 7002 ε_P vs. 1/[CO2] in high light conditions, in air, 1% CO_2, and 3% CO_2, with vertical lines representing the 1/[CO_2] values measured for low light conditions in air and 3% CO_2.

For cultures grown in excess light, ε_P values depend on CO_2 concentration (Figure 15). For cultures grown under light limitation, ε_P values depend on growth rate (Figure 14). The vertical lines in both figure 14 and figure 15 represent the data I collected for *Synechococcus* sp. PCC 7002 under low light conditions in 37°C. In figure 15 these lines fall directly in line with data collected in high light conditions, illustrating the matching CO_2 conditions. However, in figure 14 the slow growth rate under light limiting conditions is similar to the previous low light data. Each of these separate data sets would predict different ε_P values for my data. Using the ε_P relationships between these previous datasets, we can hypothesize how ε_P values from the current experimental will compare (Figure 16).

We can use the phenomenological framework in Figure 13 to predict ε_P relationships for the current experiments. For the current light limited experiments, we know that the growth rate is slower than the growth rate for the same cultures grown at high light (Figure 14, vertical lines). Therefore, for a given CO_2 condition, we would predict that our ε_P values for cultures grown at low light would be greater than the previous cultures grown at high light due to the slower growth rates (Figure 16, Stars A and B). This prediction assumes that CO_2 concentrations are primarily controlling ε_P values, with a secondary influence of growth rate.

However, we know from the previous light-limited dataset that under these conditions the primary control on ε_P values appears to be growth rate. Data from the current low light experiments support this idea: growth rates do not increase with increase CO_2 concentrations. So under this model we would predict that ε_P values for *Synechococcus* sp. PCC 7002 under low light conditions to be growth rate controlled, leading to the same ε_P values for all CO_2 conditions. This is represented by the relationship between stars B and C in Figure 16.

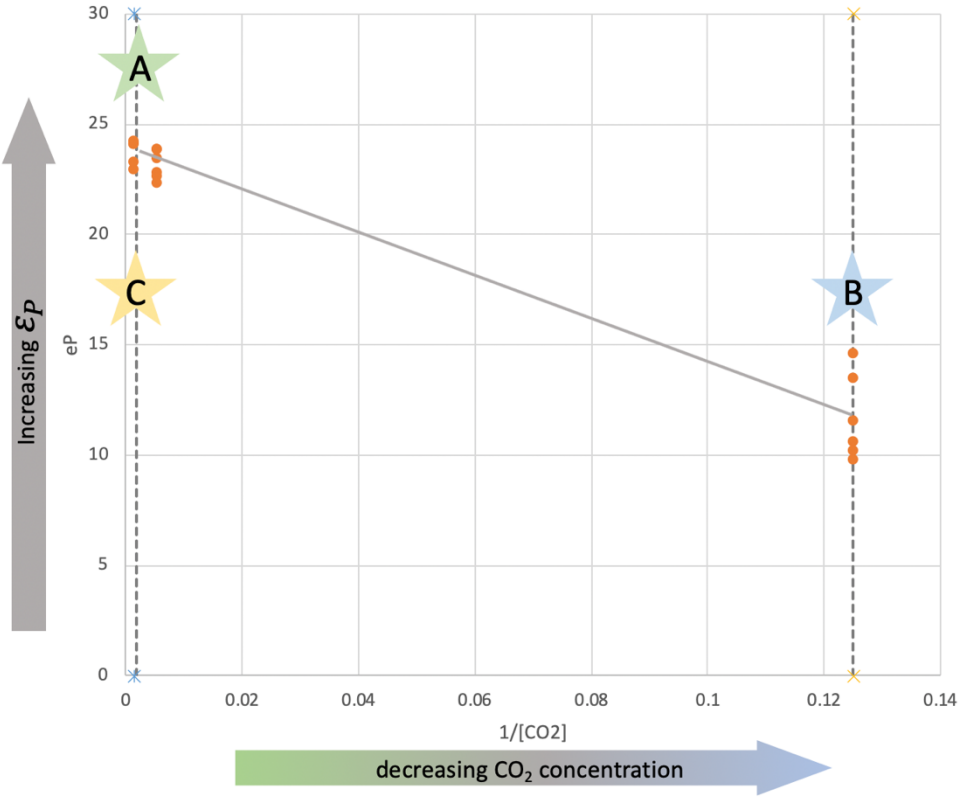


Figure 16. Prediction for where data collected for *Synechococcus* sp. PCC 7002 under low light conditions in varying CO_2 concentration may fall on plot of ε_P vs. 1/[CO_2]. If ε_P values are controlled by CO_2 concentration, then we would predict a dynamic that looks like stars A and B. If ε_P is controlled by light concentration, then we would predict ε_P values that look like stars C and B.

Because different environmental conditions lead to different ε_P relationships, a proxy based on ε_P cannot be uniformly applied across varying environments. We need to understand how individual factors (such as growth rate and CO_2 concentration) affect εP. Based on the growth rate results here, we predict that εP will behave differently under light-limited versus carbon-limited conditions. Thus the value of ε_P under one experimental condition can be drastically different from the ε_P under another set of conditions. When considering the use of ε_P as a proxy for pCO_2, it is important to consider the conditions that influenced ε_P. It is not enough to use this ε_P across all conditions, especially when we know light availability plays a large role carbon

isotope fractionation. If you blindly measure ε_P without considering these environmental conditions, your approximation of pCO_2 could be inaccurate; if carbon was abundant in light limiting conditions, your ε_P value would not be indicative of high CO_2 concentrations but rather represent carbon isotope fractionation under insufficient energy.

7. Conclusion

The use of ε_P as a proxy for pCO_2 is a valuable tool for understanding previous concentrations of CO_2 in the environment, yet ε_P values behave differently among different environmental conditions. Under high light concentrations *Synechococcus* sp. PCC 7002 exhibits carbon limited growth, and under low light conditions exhibits light limited growth. When the high light conditions is plotted as ε_P vs $1/[CO_2]$ it is clear that the ε_P values from the cultures are dependent on CO_2 concentration. Previous data collected under low light conditions suggests that ε_P is not dependent on CO_2 concentrations, as the same ε_P value but rather light availability. With these relationships in mind, we can predict where ε_P values may fall under low light conditions in varying CO_2 concentrations. If the ε_P value under these experimental conditions is controlled by CO_2, then we would predict ε_P values that are larger in air and smaller in high CO_2. However, if we predict the controlling factor for ε_P under light limited conditions is light controlled, then we would ε_P values to be the same across all CO_2 concentrations.

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10. Appendix

Appendix A:

A+ media recipe

Compound	Molecular Weight (g/mol)	For 1L Stock Solution	For 1L Media Solution
NaCl	58.44		18 g
$MgSO_4$ $7H_2O$	246.47		5 g
Na_2EDTA $2H_2O$	327.24	3 g	10 mL
KCl	74.55	60 g	10 mL
$CaCl_2$ $2H_2O$	147.02	37 g	10 mL
$NaNO_3$	84.99	100 g	10 mL
KH_2PO_4	228.22	5 g	10 mL
Tris HCl	121.14	100 g	10 mL
Trace Minerals			10 mL

After all stock solutions have been added and media has been autoclaved add 1 mL of 1000X Vitamin B12

100x Trace Mineral Stock Solution

Compound	Molecular Weight (g/mol)	Final concentration
H_3BO_3	61.83	55.5 mM
$ZnCl_2$	136.28	230 μM
MoO_3	143.94	21 μM
Ferric ammonium citrate	261.98	300 μM
$MnCl_2$	125.84	2.2 mM
$CuSO_4$	154.61	1.2 μM
$CoCl_2$	125.83	5 μM

Appendix B:

Calculations for dissolved inorganic carbon were obtained through PHREEQC, a program developed by the USGS (https://www.usgs.gov/software/phreeqc-version-3)