Iron and Cobalt Limitation in Atlantic *Prochlorococcus* MIT9301

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I certify that I have read this thesis and that, in my opinion, it is satisfactory in scope and quality as a thesis for the degree of Bachelor of Science in Global Environmental Science.

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For my parents Annemarieke Jongsma and Gert van Dijken

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ABSTRACT

Prochlorococcus is an abundant marine microorganism playing a vital role in the Earth's carbon cycle and therefore climate. This organism is a cyanobacteria meaning it obtains energy through photosynthesis. A large portion of the ocean's phytoplankton populations are limited by low concentrations of trace metals such as iron and cobalt. Future climate change will likely alter ocean circulation and supply of trace metals to the surface ocean, and may impact *Prochlorococcus* populations, which can affect marine food webs. We only have information on iron and cobalt requirements for a few strains of *Prochlorococcus*. Therefore, we performed culturing experiments to understand the growth and decay of *Prochlorococcus* in a lab environment with changes in levels of iron and cobalt. Iron limitation on Atlantic *Prochlorococcus* did not have a significant impact on growth rates, while cobalt limitation experiments did significantly impact the growth of these phytoplankton when lowered. The growth rates resulting from these experiments can be used to model future changes in *Prochlorococcus* populations as the impacts of climate change become more prevalent.

Keywords: Prochlorococcus, trace metal, cobalt, iron, growth rate, limitation

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0 INTRODUCTION

1.1 Background

Prochlorococcus is an abundant genus of cyanobacterium that was discovered in 1988. This was done through a process called flow cytometry which is used to identify cell characteristics such as cell size and granularity (Partensky et al. 1999). *Prochlorococcus* is a cyanobacteria meaning they use sunlight to create energy in the form of sugars. In doing so they sequester atmospheric carbon dioxide and store it as biomass. These cyanobacteria have a large surface area for their mass making them very efficient primary producers. With such high carbon fixation rates, *Prochlorococcus* is extremely important when determining oligotrophic carbon sequestration (Hartmann et al. 2014). Cyanobacteria also play an important role in oceanic food webs. Because *Prochlorococcus* is an autotroph, they lie at the base of the food web and act as a major determining factor for the populations of higher trophic levels.

Prochlorococcus populations vary around the world but are primarily found between the latitudes of 40°S to 40°N (Partensky et al. 1999). Outside of these latitudes *Prochlorococcus* can still be found but, generally in much smaller concentrations (Figure 1). This suggests that *Prochlorococcus* favors warmer oligotrophic waters. The vast range of oceans where *Prochlorococcus* populations can thrive has contributed to its great genomic diversity (Kettler et al. 2007). For example, many of these populations have had major genetic changes over their evolutionary history that allow them to survive in high or low light at depths of about 100-200 meters (Partensky et al. 1999).

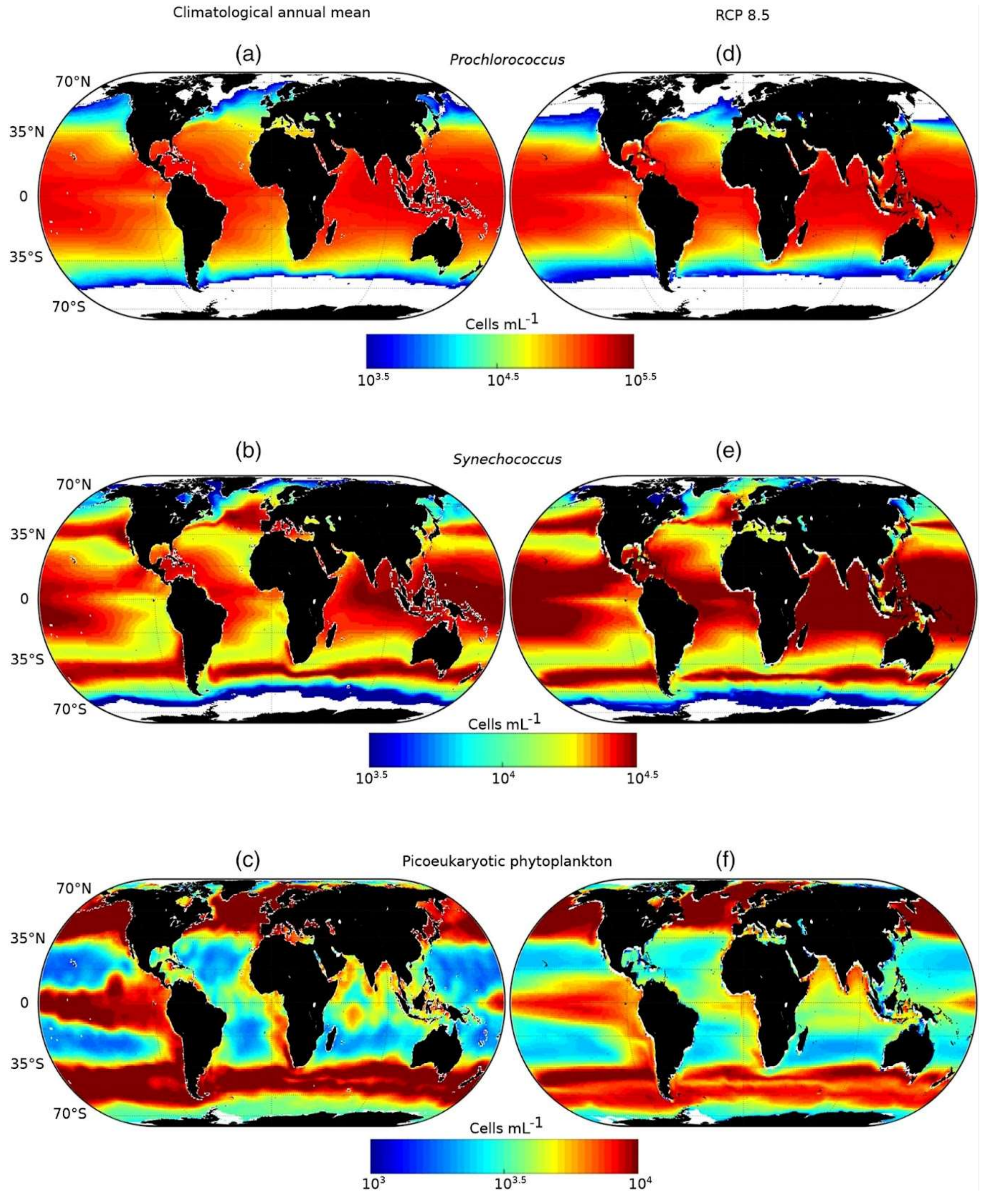

Figure 1. Map of *Prochlorococcus* population across world oceans (source: Visintini et al. 2021)

Across Earth's oceans, phytoplankton (including *Prochlorococcus*) are limited by different nutrients. Limiting nutrients in an environment determines the size a population can reach before there are not enough resources to sustain more growth. In the Pacific Ocean, phytoplankton are mostly limited by the low concentrations of iron, nitrogen, and in some regions cobalt (Figure 2). In the Atlantic Ocean phytoplankton populations are limited almost entirely by nitrogen and phosphorus within 40°S to 40°N. Higher latitudes of the Atlantic Ocean where *Prochlorococcus* are found have a variety of limiting trace metals. These metals include iron, cobalt, and zinc (Figure 2).

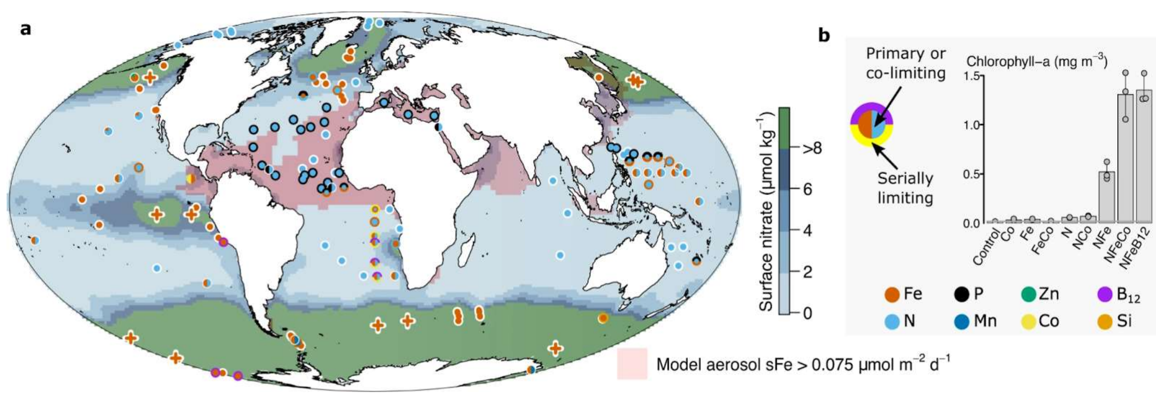

Figure 2. World map of ocean phytoplankton limiting nutrients (source: Browning et al. 2023)

1.2 Related Research

Since its discovery, *Prochlorococcus* has been studied in great detail. Because of its diversity there are many strains located all over the world's oceans. Out of these strains trace metal limitation experiments have been conducted on a handful. In the Pacific Ocean, trace metal limitation experiments have been carried out on strain MIT9215. This strain of *Prochlorococcus* was isolated in the Equatorial Pacific (Hawco et al. 2020). These limitation experiments investigated the iron and cobalt limitation of the specific strains of *Prochlorococcus*. Similar experiments on cobalt limitation in the Mediterranean Sea have been carried out to further understand these cyanobacteria (Saito et al. 2002) (Castruita et al. 2008).

1.3 Significance of Study

This cyanobacterium is particularly important because of their high rates of primary production (8 Gt C y^{-1}) which equates to about 8.5% of total ocean net primary production (Flobaum et al. 2013). As stated, before there has been much research into different strains of *Prochlorococcus* around the world and their limiting factors, but little is known about Atlantic *Prochlorococcus* specifically MIT9301 and how it reacts to different nutrient limitations. With increased stressors on the Earth's climate, the growth of these vital organisms will change. Because these cyanobacteria are a key part of global carbon sequestration it is important to understand them further.

2.0 METHODS

2.1 Summary of *Prochlorococcus* Limitation Experiments

Lab experiments on Cyanobacterial *Prochlorococcus* cells were conducted in the University of Hawaii's Center for Microbial Oceanography: Research and Education (CMORE). This includes culturing of the cells, transferring, and growth measurements. More project preparations were conducted in a cleanroom environment in the Hawaii Institute of Geophysics (HIG) in the Hawco Lab, which ranges from sterilization of equipment to creating stock solutions used in the *Prochlorococcus* media. Preparations for this culturing experiment began in Fall 2022 and concluded in Spring 2024. The strain of *Prochlorococcus* used in these experiments was MIT9301, which originates in the Atlantic Ocean. An Atlantic strain of *Prochlorococcus* was chosen because of the high levels of trace metals due to dust deposition originating from the Sahara Desert.

2.2 *Prochlorococcus* Media

All *Prochlorococcus* cultures were acclimated to Pro99 over several transfers (Table 1). Pro99 media has a high density of trace metals and leads to relatively fast growth rates in *Prochlorococcus* cells (Hartmann et al. 2014). High densities of iron and cobalt made this media ideal for limitation experiments. This media used a seawater base, with several trace metals and nutrients added. Seawater for this media was taken at Station Aloha (22°75.0 N, -158°00.0) on a Hawaii Ocean Time-series (HOT) research expedition. The seawater was sterilized by reaching a boil in a polycarbonate bottle. The sterilization process was used to eliminate any biological activity before adding inorganic nutrients and later to house the cells.

To continue the media, there are a number of additions required for cell growth in the cultures. Ammonium and phosphate are added at 800µM and 50µM respectively. These nutrients were first chelated to minimize the risk of trace metal contamination and dissolved into nutrient stocks, which could then be pipetted into the seawater base (Hartmann et al. 2014). High concentrations of ammonium and phosphate were used to ensure that the culture growth rates will not be restricted by lack of these nutrients

The Pro99 media needs a specific concentration of metals in order to promote *Prochlorococcus* cell growth. Following the Pro99 recipe Iron, cobalt, magnesium, zinc, sodium molybdate, nickel, selenium, and EDTA are dissolved into the seawater base (Moore et al. 2007). Metal stock solutions were made to create separate seawater solutions with the trace metals dissolved in them. When creating an Iron stock solution, 0.5mL of 10% Hydrochloric acid (HCl) was added to the stock to assist the dissolving process into the seawater. All nutrients at their respective concentrations were then pipetted into the seawater base at their respective concentrations. Pipetting nutrients into the sterile seawater base was done in a clean lab under a laminar flow hood, to ensure there were no airborne trace metal contamination or airborne cells added into the media.

Following the completion of the nutrient and trace metal stock solutions the separate media for limitation experiments could be made. For the iron limitation experiments there were five separate medias varying in iron concentrations (Table 1). The first media has no added iron in it to use as a baseline for how the *Prochlorococcus* cells react to complete iron limitation. The next three media are on a log scale with Pro99 as the maximum. The second lowest concentration of iron in a media was 0.0351μM iron. The next media was raised in iron concentration with the second media having an iron concentration of 0.117μM, a high iron concentration at 0.351μM, and the highest concentration of iron in the Pro99 media of 1.17μM. The cobalt limitation experiments included 4 concentrations of cobalt (Table 1). These concentrations are as follows: 10 nM, 1 nM, 0.1 nM, and a media with 0 nM cobalt.

Table 1. Concentration of macronutrients and trace metals used in *Prochlorococcus* media in iron and cobalt limitation experiments (µM)

Trace Metal Concentrations in Seawater Stock Solutions	PRO99	Low Iron	Medium Iron	High Iron	Low Cobalt	Medium Cobalt	High Cobalt
Macronutrients:							
NH_4Cl	800	800	800	800	800	800	800
$NaH_2PO_4 \cdot H_20$	50	50	50	50	50	50	50
Trace Metals:							
Na2EDTA·2H20	1.17	1.17	1.17	1.17	1.17	1.17	1.17
$FeCl_3 \cdot 6H_2O$	1.17	0.0351	0.117	0.351	0.351	0.351	0.351
$MnCl_2 \cdot 4H_20$	0.09	0.09	0.09	0.09	0.09	0.09	0.09
ZnSO4·7H20	0.008	0.008	0.008	0.008	0.008	0.008	0.008
$CoCl_2 \cdot 6H_20$	0.005	0.005	0.005	0.005	0.0001	0.001	0.01
Na2MoO4·2H20	0.003	0.003	0.003	0.003	0.003	0.003	0.003
Na_2SeO_3	0.01	0.01	0.01	0.01	0.01	0.01	0.01
$NiCl_2 \cdot 6H_20$	0.01	0.01	0.01	0.01	0.01	0.01	0.01

2.3 Bottle cleaning

Before culturing could begin in the lab, all culture containers followed a strict acid washing procedure to remove all trace metal contamination in the clean lab, in HIG. The equipment was first soaked in 1% Citranox detergent (Alconox) for 24 hours, then rinsed multiple times with milli-Q water. The equipment was then submerged in a bath containing 10% hydrochloric acid for one week. Bottles were then rinsed again several times in Milli-Q water to remove all the hydrochloric acid. After this period of time, the 30mL polycarbonate bottles which would be used for culturing were first sterilized with milli Q water and then the seawater media could be added.

2.4 Cell Transfer and Growth

Cells were transferred into 30mL tubes (7 replicates per treatment) by pipetting 500μM of cells into them inside a laminar flow bench at CMORE Hale. To prevent carbon dioxide limitation of the cultures, the caps of the 30mL tubes were slightly unscrewed to allow for gas exchange. Depending on the density of cells transferred, growth would become apparent in 1-5 days and then would grow exponentially from there on. This period with little growth is called the acclimation phase where the *Prochlorococcus* cells adapt to the new media. After the acclimation phase, the *Prochlorococcus* cultures generally had a growth period called the exponential phase which could last up to 10 days. At the peak of the culture's growth, it was the optimal time to transfer cells to a new 30ml tube (to ensure the highest chance of growth in new media). When transferring cells, it was best to pipette 250-500µl for optimal growth and acclimation. Once the peak was reached the cell population would either collapse or have a stationary phase depending on how high the population density was and amount of available nutrients (Figure 7). The stationary phase means that the growth has stopped but the cell populations find a steady state. Single cultures could live for about 7-20+ days in a single 30mL tube without being transferred. Cells in these experiments were grown in a controlled environment (Percival Intellus Control System). In the climate-controlled environment there was a day and night cycle, as well as temperature control. These cultures were held in racks and spaced apart, and their individual positions were randomized every 24 hours to reduce error from minor differences in light (Figure 3).

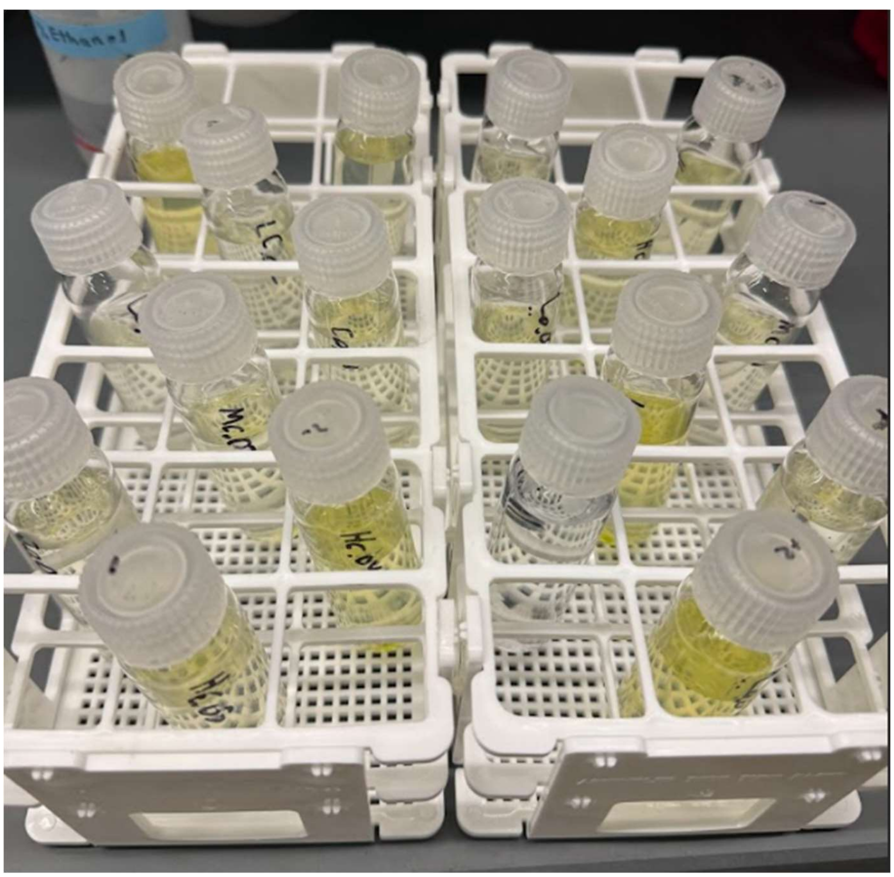

Figure 3. Varying levels of *Prochlorococcus* cell populations during cobalt limitation experiments.

2.5 Harvesting Culture Biomass

The *Prochlorococcus* cultures were harvested in order to measure the amount of trace elements in each treatment group. This creates valuable information to compare trace metal uptake in each treatment. When cultures reached their carrying capacity in the 30mL tube they were then ready to be filtered. The peak was determined by looking at the exponential growth curve of the individual *Prochlorococcus* cultures. When the growth rate began to slow down the peak could be estimated, and the filtering process could take place. For the purpose of this experiment, cultures were run through acid-washed filters (0.45 µm) to measure the metal uptake of these cells. The filters were placed in the blue and white cylinders in the upper portion of figure 4 after a rinse with Milli Q water. The cultures were then poured into the top piece of the cylinder above the filter. Using an air compressor to create a vacuum, the water is pushed through the filter leaving the trace metals and biomass on the filter. These filters were then stored using plastic tweezers to transfer them into a 15ml tube, which was then refrigerated until digestion.

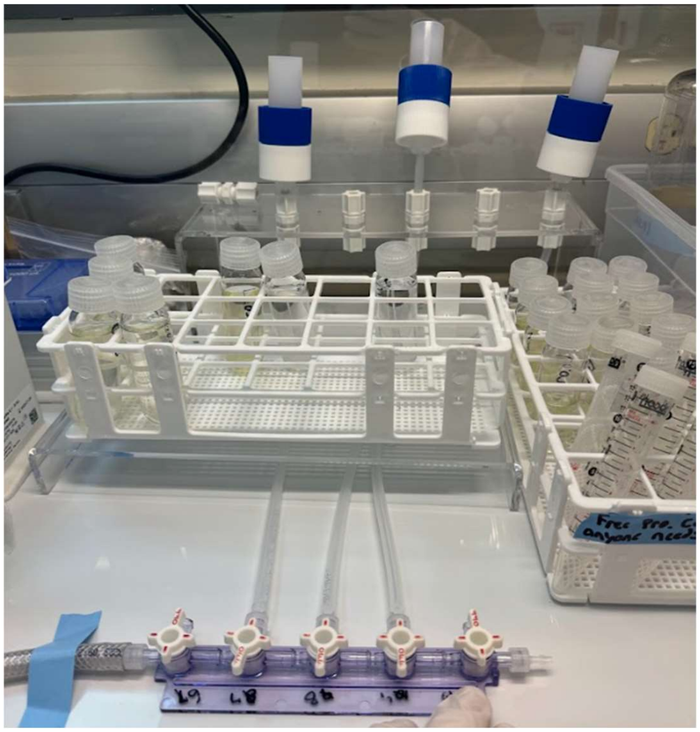

Figure 4. Configuration used in the filtration process of the *Prochlorococcus* cells. This setup is located in the CMORE Model Systems Lab inside of a laminar flow chamber.

The digestion process in this experiment was conducted in the clean lab in the Hawaii Institute of Geophysics building. To begin this process the filters were placed in individual capsules with 2 ml of 50% nitric acid. They were then put on a hot plate for a minimum of 3 hours at a temperature of 120°C (with caps on). Because the filters were being heated at such a high temperature, the cells on the filters would burst and the trace metals dissolved into the nitric acid. The filters were then rinsed with Mili Q to ensure all the trace metals were dissolved in the nitric acid solution. After this was completed, the capsules were placed in an evaporation chamber overnight at 110°C with the caps off (Figure 5). What was left in the capsules was a film of trace metals with possible yellow spots if there was a high density of biomass on the filter. 3 ml of 0.1 M nitric acid was then pipetted into the evaporated capsules. This change of nitric acid concentration was necessary for the sample to run correctly through the mass spectrometer.

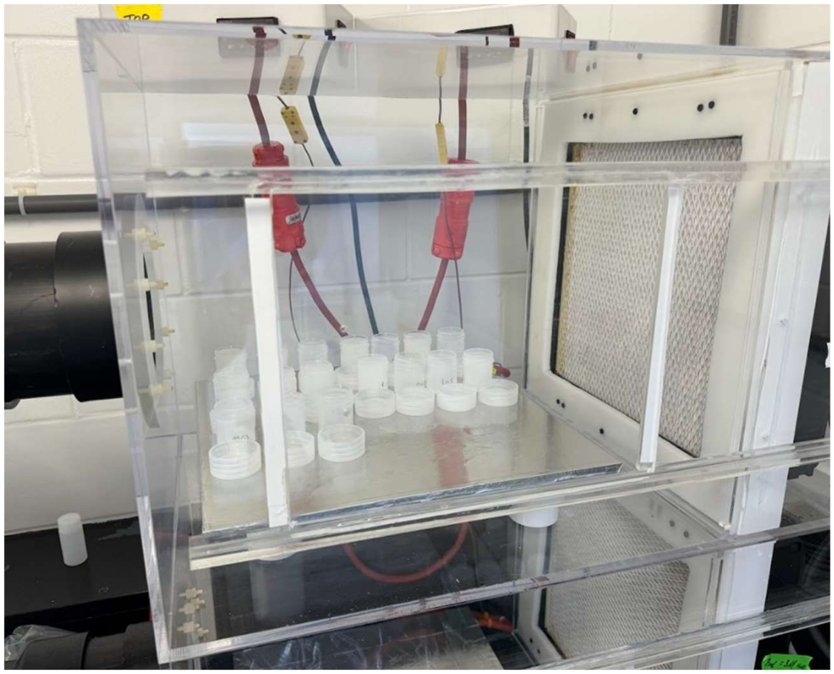

Figure 5. Evaporation chamber in the Hawaii Institute of Geophysics clean lab

The mass spectrometer used in this experiment was an ICP-MS (Thermo Fisher Scientific iCAP TQ ICP-MS). An ICP-MS is an inductively coupled plasma mass spectrometer. This machine was used to analyze the number of certain elements in counts per second. These values would then be converted into parts per billion (ppb) by calibration with standards. To account for differences in volume and biomass, the values of each trace metal (ppb) were taken for each trace metal filter and normalized by phosphorus to get metal-phosphorus ratios. These ratios made the results of each filter easier to analyze and compare to one another. When we used the ICP-MS there was often instrumental drift over the course of the measurements. Generally over time the ICP-MS would give less counts per second. To account for this drift, the element Indium was used as an internal standard. Indium is not found in any of these media and has little to no chance of entering from contamination. Because of this, the Indium counts could be used to measure the percent decrease in counts per second, and the data for all of the metals could be adjusted accordingly.

2.6 Growth Rate Analysis

A growth rate analysis was used to measure the speed at which cultures grow. This information was used to compare limitations between different concentrations of trace metals. Measuring the growth rate (Day^{-1}) of the *Prochlorococcus* cultures was conducted daily by measuring the chlorophyll content using a fluorometer (Turner Design) in the CMORE model systems lab (Figure 6). The fluorometer was calibrated using a Turner Design TD-700 fluorometer solid standard for red light. The fluorescence of the cultures was measured through a measurement of light from the fluorometer and was used as a proxy for biomass. After a 10-minute warm up period for the fluorometer, the cultures were placed into the machine. Blue light (440-460 nm) was emitted from the fluorometer into the culture tube. Chlorophyll in the culture would then fluoresce light in the red range (685 nm). This red light was measured by the fluorometer giving a reading in fsu (fluorescent signal units). The fluorescence value correlates to chlorophyll and therefore population density.

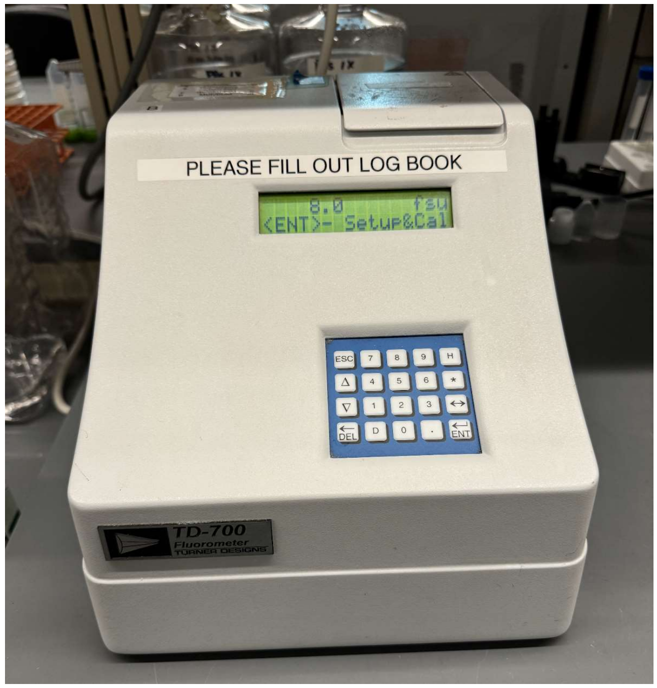

Figure 6. TD-700 fluorometer in CMORE model systems lab used to measure fluorescence of all cultures.

This method was carried out every 24 hours over the growth period. Because the *Prochlorococcus* cells grow exponentially, they were linearized by taking the natural log of the fluorescence values (Figure 7). For each culture, the growth rate was obtained by taking the natural log of 3 consecutive days of fluorescence values. The largest 3-day slope in the growth cycle of each culture was interpreted as the exponential growth rate. The average and standard deviation were calculated from the 3-day exponential growth rate in the individual bottles.

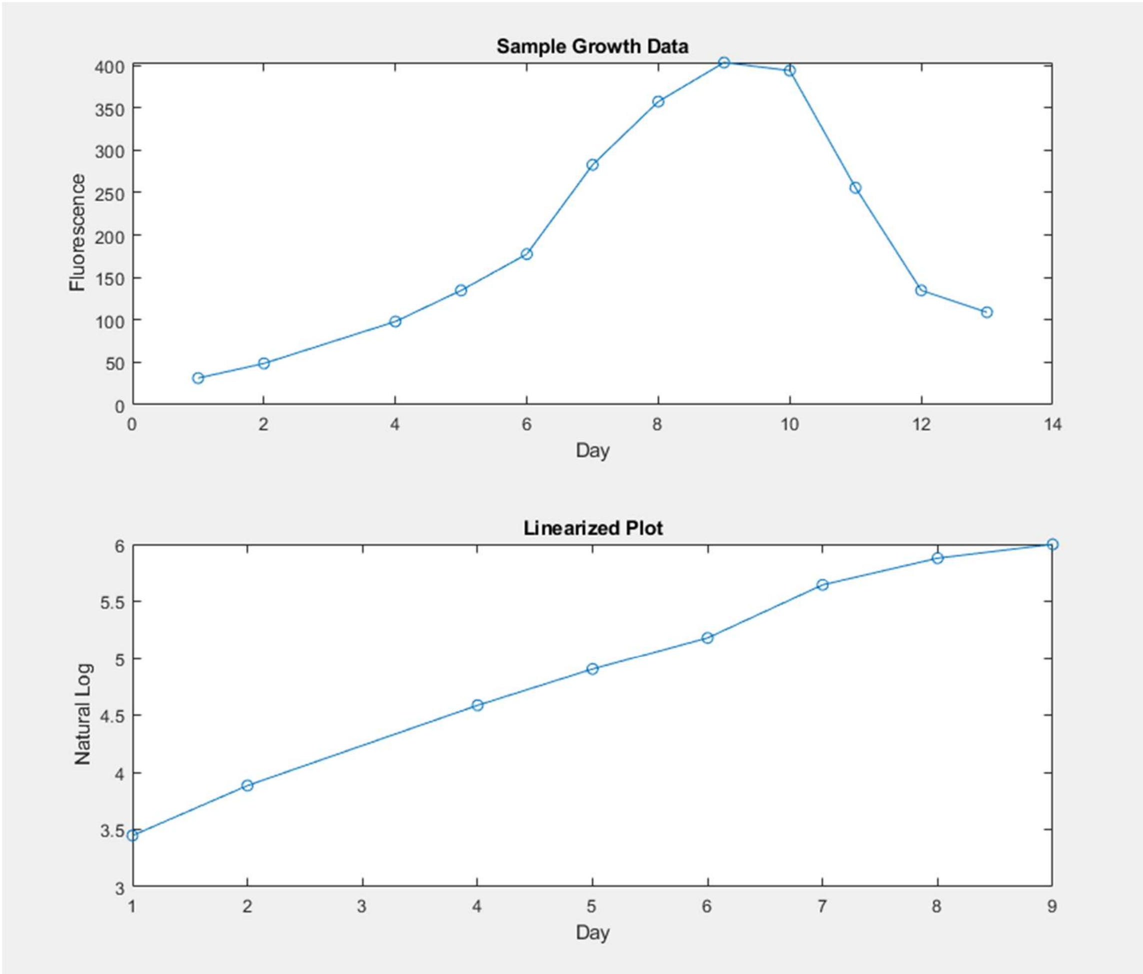

Figure 7. Sample data of an expected *Prochlorococcus* growth and decay curve before (Top) and after linearization (Bottom).

3.0 RESULTS

3.1 Iron Limitation Experiments

3.1.1 Initial Iron Limitation Experiment

We hypothesized that *Prochlorococcus* MIT9301 will show signs of growth limitation at higher iron and cobalt concentrations compared to other strains due to its Atlantic origin. To test this hypothesis an initial Iron limitation experiment was conducted with two different media: one with 1 μM of iron added and one with no iron at all. In this initial experiment it was apparent that iron limitation was occurring (Figure 8).

The cultures which contained the 1 µM iron had a peak fluorescence of 415.0 fsu and an average peak of 354.8 fsu (Figure 8). The cells cultured in the media with no iron added had a fluorescence peak of 279.6 fsu and an average peak height of 206.1 fsu. These Cultures peaked after a growth period of 10 days.

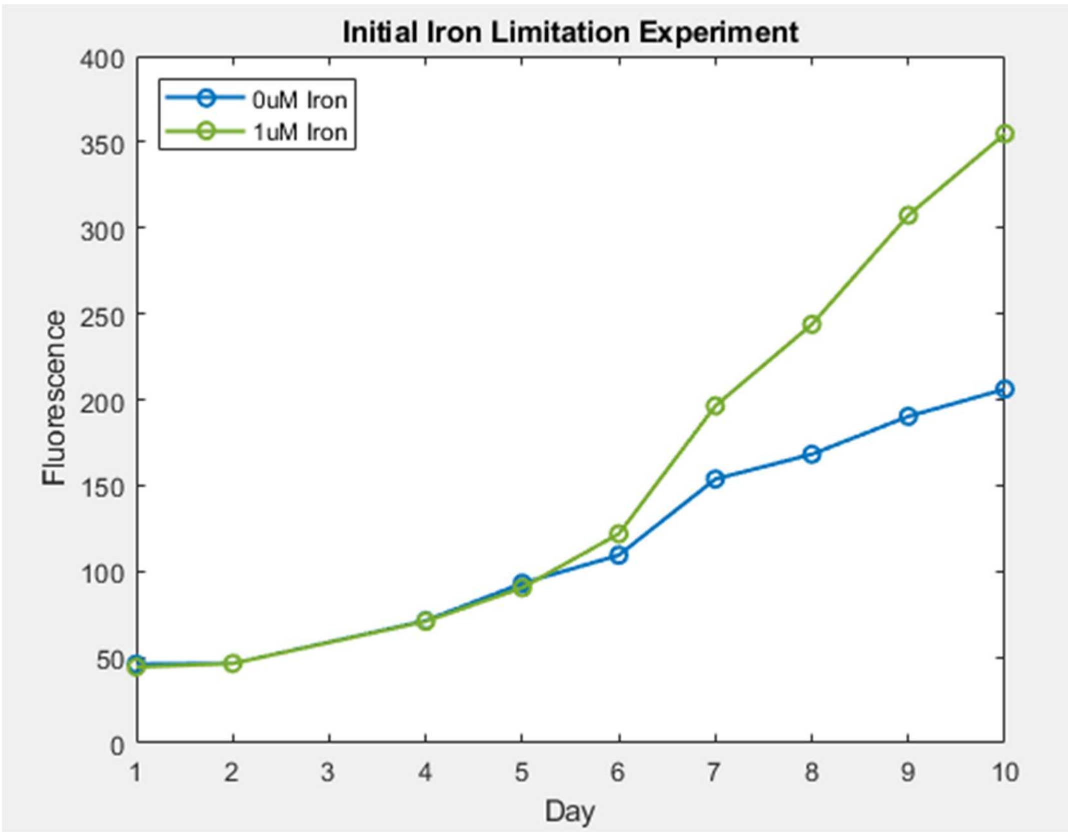

Figure 8. Initial iron limitation experiment between cultures in a 0 µM added media (blue) and a 1 µM media (green). The fluorescence values above correlate to the average fluorescence between replicates of each treatment group.

The natural log of the growth curve was taken, and the growth rates were calculated (Figure 9). For the cultures containing 0 µM added iron, the maximum growth rate was 0.245±0.050 (Day^{-1}). For the cultures with 1 µM of iron the maximum growth rate was 0.393±0.0272 (Day^{-1}). These values verified iron limitation in the MIT9301 cultures. The growth rates of these two concentrations when compared have a p-value of 0.01 meaning there is significance (Tukey HSD test).

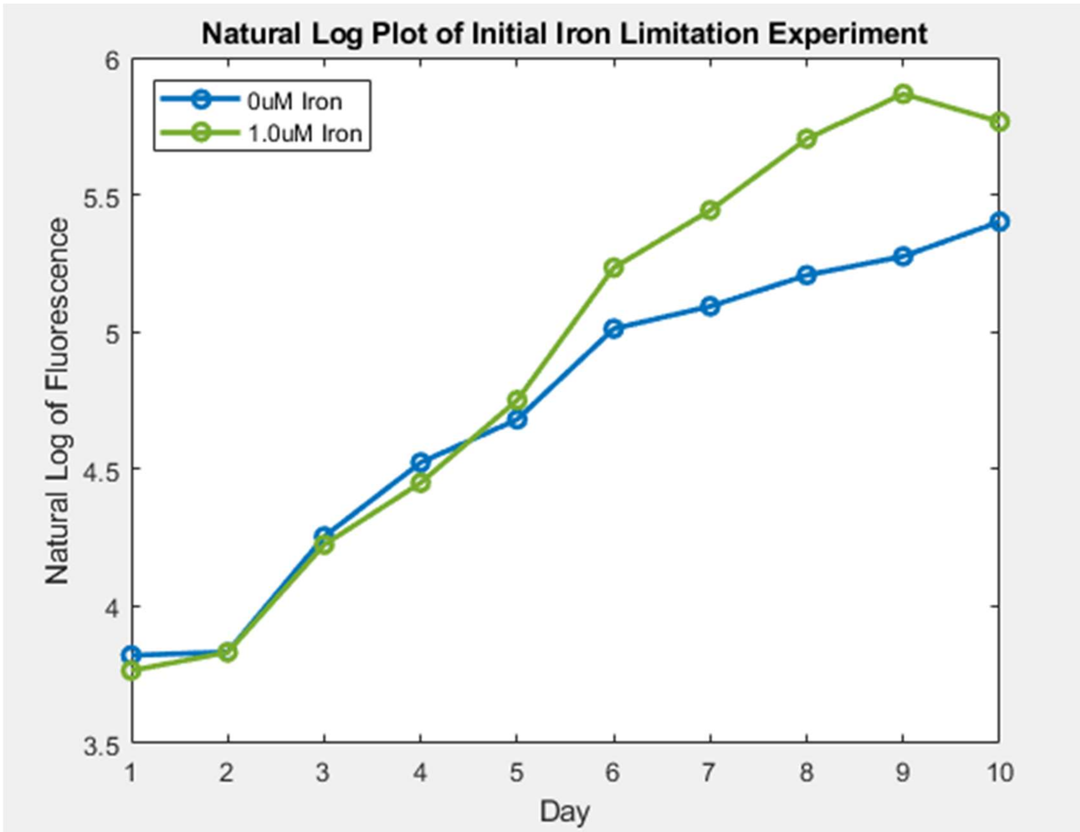

Figure 9. Linearized plot of the initial iron limitation experiment, blue line is 0 μM iron concentration media, and the green line is 1 μM iron concentration.

3.1.2 Large Scale Iron Limitation Experiment

With the confirmation of iron limitation, larger scale iron limitation experiments were performed with five different concentrations of iron (Table 1). The MIT9301 cultures grew for a total of 11 days before reaching their peak except for the 0.117 μM media which peaked at 12 days. The fluorescence values obtained in this experiment varied between treatments. The cultures with no iron added to the media showed a maximum fluorescence of 383.2 fsu and an average fluorescence peak of 332.3 fsu. In the 0.0351μM iron media there was a fluorescence peak of 494.4 fsu and an average peak of

434.73 fsu (Figure 10ab). The next 0.117 μM iron cultures exhibited a peak fluorescence of 624.5 fsu and had an average peak of 426.23 fsu (Figure 10ab). In the cultures with higher iron concentration media (0.351 μM) there was a maximum fluorescence of 541.5 fsu and an average peak of 435.67 fsu (Figure 10ab). In the cultures with the highest molarity of iron (1.17 μM) there was a fluorescence peak of 608.4 fsu and an average peak of 346.2 fsu (Figure 10ab). In this experiment, there was a high deviation in cultures growth within the treatment groups (Figure 10a). Over the course of this experiment, a high mortality rate was also observed in these cultures. The only concentration that did not experience a culture without growth was in the 0 μM iron concentration. The 0.0351 μM iron concentration had 1 culture with no growth, in the 0.117 μM iron media there were 3 cultures that experienced no growth. In the higher concentrations of 0.351 μM, and 1.17 μM there was a respective mortality of 3 cultures and 1 culture (Figure 10a).

After natural log transformation, the slope of these growth rates could be analyzed (Figure 11). Growth rates in this experiment averaged 0.360±0.073 Day^{-1} for the 0 μM iron cultures, 0.422±0.096 Day^{-1} for the 0.0351μM iron cultures, 0.381±0.124 Day^{-1} in the 0.117 μM iron cultures, 0.352±0.075 Day^{-1} in the 0.351μM iron cultures, and 0.384±0.075 Day^{-1} in the 1.17 μM iron cultures. Cultures that did not grow were not included in the growth rate calculations. Looking at the natural log values of the growth curve we see lines that are not linear, meaning the individual cultures in each treatment group had high variation which can be seen in figure 10a (Figure 10d).

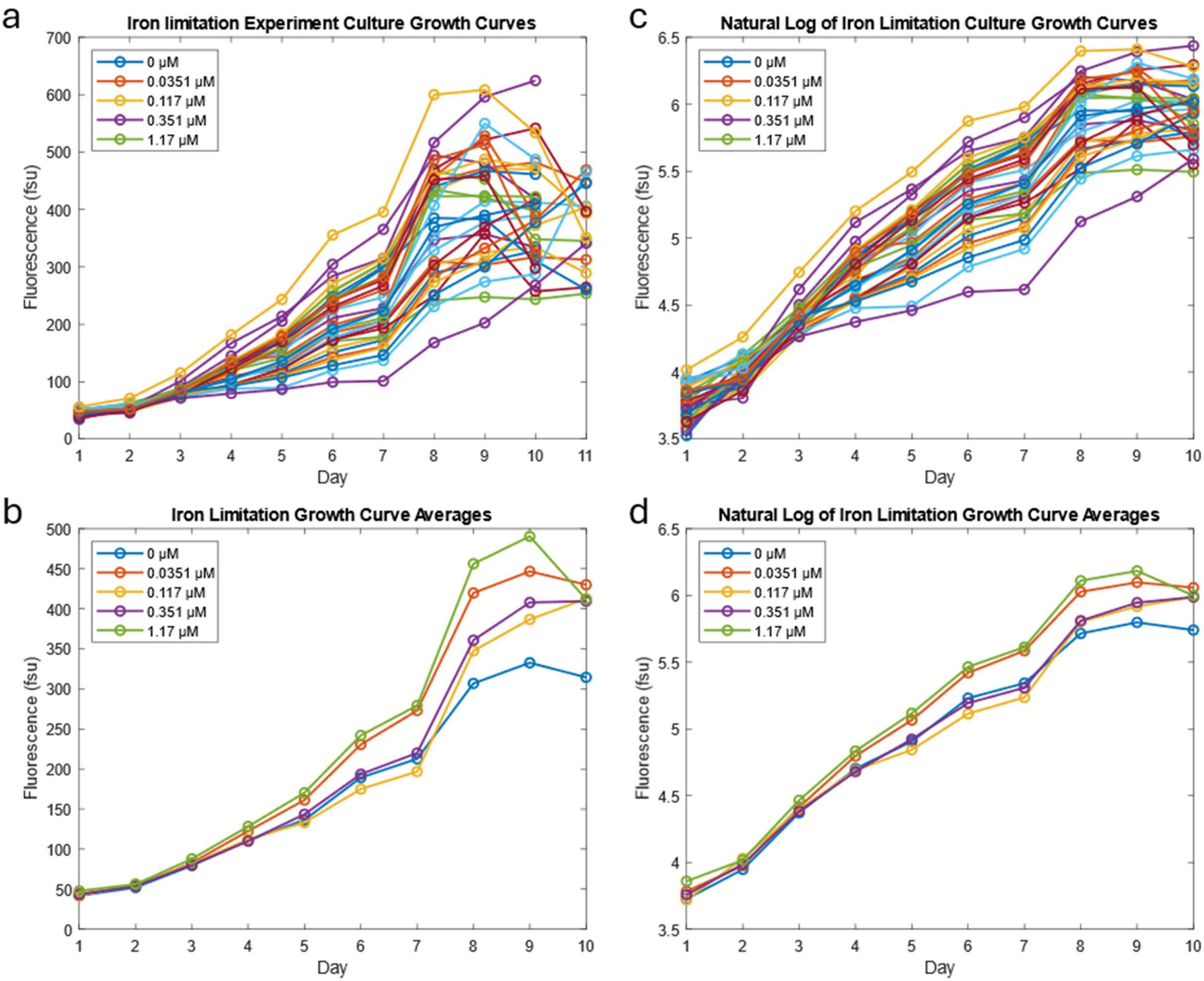

Figure 10. Growth curves for individual cultures under different iron concentrations (a), averages of each treatment group (b). Natural log of the individual cultures under different iron concentrations (c), and natural log of treatment averages (d).

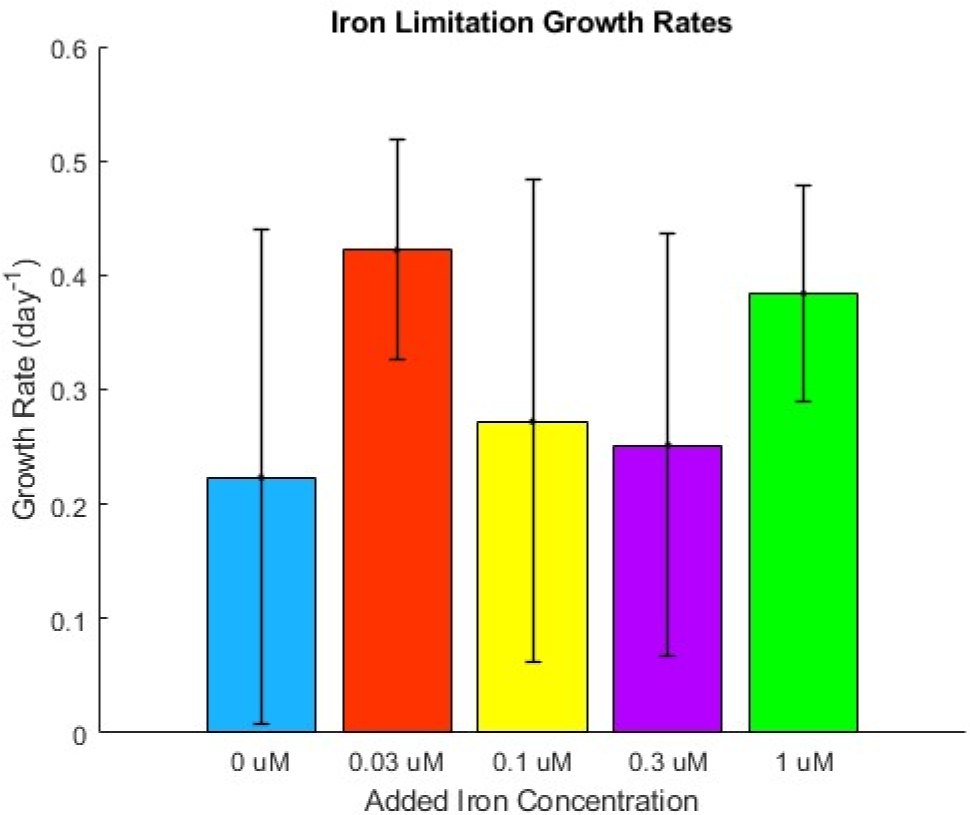

Figure 11. Average growth rate and error between iron treatment groups

To find the significance of these iron limitation experiments, we used the ANOVA (analysis of variance) test to analyze the variance of the results. The highest 3-day growth rate for each culture was used in this test. In the case of the iron limitation experiments, there was a p-value of 0.7 and an F statistic of 0.55. This is higher than our alpha of 0.05, meaning that none of the concentrations had a significant difference in growth rate when comparing them all to one another (Figure 11). This may be due to differences in light across the cultures: this experiment had a higher number of cultures per treatment and therefore there was less space between them. This may have led to uneven lighting leading to more random growth rates with changes in light every day. A factor that may have also led to these results is iron contamination within the cultures. Iron is more easily spread through aerosols and could therefore be transmitted into the cultures more easily than cobalt for example.

3.2 Cobalt Limitation Experiment

3.2.1 *Prochlorococcus* Growth Under Cobalt Limitation

Similarly to iron limitation, we expected that an Atlantic Strain of *Prochlorococcus* would exhibit high levels of limitation when decreasing the concentration of inorganic cobalt. The cobalt limitation experiments consisted of 4 concentrations of cobalt. Looking at the average fluorescence of all the cultures in each media type, cobalt limitation of growth rates is clear. The highest average biomass densities were 428.26 fsu (10 nM), 242.65 fsu (1 nM), 177.74 fsu (0.1nM), and 175.43 fsu (0 nM) (Figure 12b). The highest fluorescence observed in this experiment for each treatment was 621.9 fsu (10 nM), 304.3 fsu (1 nM), 286.5 fsu (0.1nM), and 289.7 fsu (0 nM) (Figure 12a). In this experiment there was a low mortality rate with only one of the 1nM Cobalt cultures failing to grow. Looking at the linearized plots of the growth rates we can see the exponential phase when the plotted lines are linear (Figure 12cd).

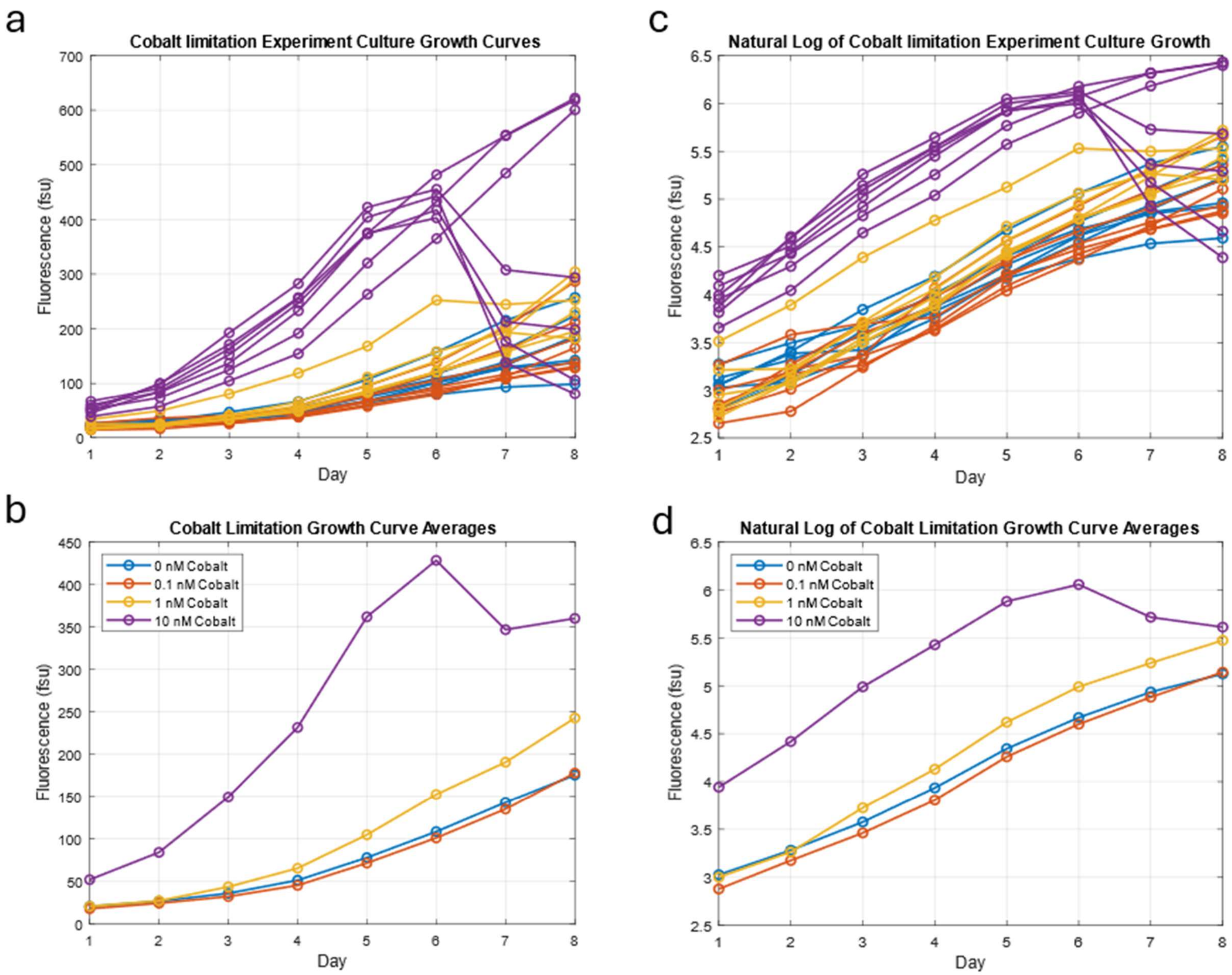

Figure 12. Growth curves for individual cultures under different cobalt concentrations (a), and averages of each treatment group (b). Natural log of the individual cultures under different cobalt concentrations (c), and natural log of treatment averages (d).

In this experiment the high cobalt media (10nM) experienced a divide on day 6 where half of the cultures peaked and declined rapidly, and the other half continued their growth up to their peaks (Figure 12c). While this did not affect the exponential growth rate calculations it is interesting to point out. The 10 nM cultures that declined after day 6 had higher growth rates the 3 days before their decline than the other half of cultures which kept on growing. This means that they may have had unsustainable growth which inhibited the possibility of further growth. In the 0.1 nM cobalt cultures there was one culture with high relative growth (Figure 13). While this culture was not determined to be a significant outlier, it did increase the average growth rate of the 0.1 nM treatment group. Including this culture there is an average growth rate of 0.44 Day^{-1} but, when that culture is excluded from growth rate calculations the average growth rate is 0.42 Day^{-1}.

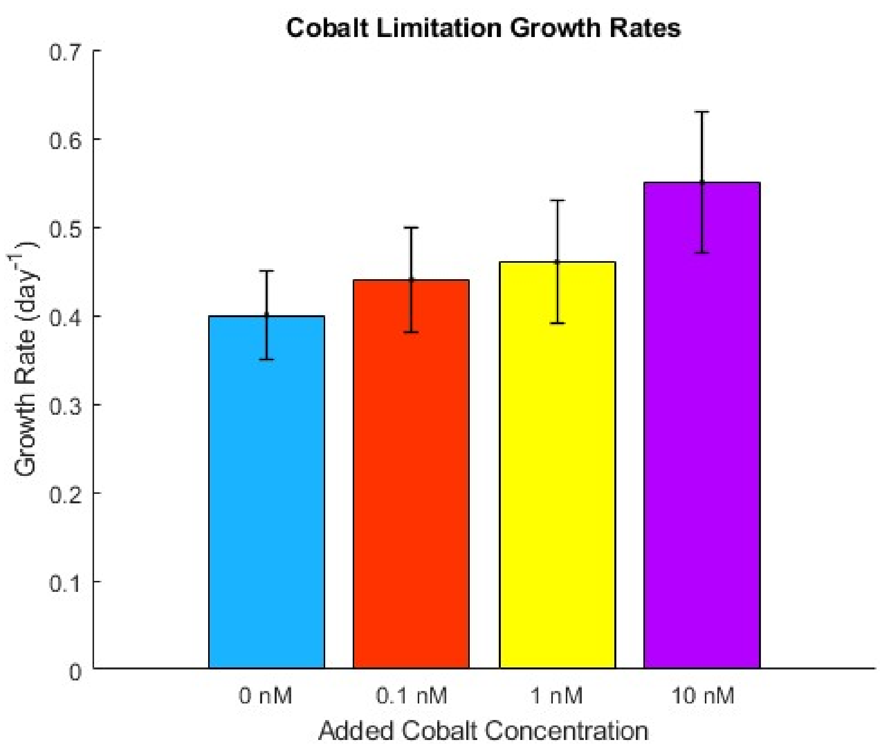

Figure 13. Average growth rate and error between cobalt treatment groups.

When calculating the ANOVA test on the growth rates of these experiments, we calculate a F statistic of 8.95 and a corresponding p-value of 0.0004. Because the p-value is less than 0.05 it suggests that one or more of the treatments have a significantly different growth rate from one another. To find which growth rates have significant or insignificant results we used the post-hoc Tukey HSD test. The result of this test concluded that the 10nM cobalt concentration growth rates were significantly greater than the 0nM, 0.1nM, and 1nM cobalt concentration values (Figure 13). The respective p-values of these tests were 0.001, 0.008, and 0.037. The three lower cobalt concentration growth rates were concluded to be not significant when compared to each other. What this means is that there was significant limitation between the 10-1nM cobalt range. When there is 10nM cobalt in the cultures the growth rate averaged at 0.55 Day^{-1}. When you get below this limitation point all the growth rates are decreased by over 17% at 0.46 (1 nM), 0.44 (0.1 nM), and 0.40 (0 nM).

Cultures run through the ICP-MS fluctuated in their iron to phosphorus ratios (Fe:mol P). In the cultures with less biomass the iron to phosphorus ratio fluctuated between 15,000-25,000 μmol Fe:mol P (Table 2). The average iron to phosphorus ratio in the lower biomass group (0-1nM treatments) was 22,180 μmol Fe:mol P. Cultures with a higher fluorescence peak ranged between 8,900-25,000 μmol Fe:mol P (Table 2). But in these high biomass cultures (10nM treatments) they were generally on the lower end averaging at a ratio of 15,036 μmol Fe:mol P.

3.2.2 Cobalt Limitation Experiments Cell Digestion

Treatment Type	Mn:P Ratio	Fe:P Ratio	Co:P Ratio	N:P Ratio	Cu:P Ratio	Zn:P Ratio	Cd:P Ratio
0 nM Cobalt Culture	1579.02	15991.78	5.46	111.14	1099.85	1565.93	0.56
0 nM Cobalt Culture	1697.40	15732.30	4.24	95.09	1023.37	1148.82	0.30
0 nM Cobalt Culture	1348.41	25133.67	8.17	237.91	4183.96	4506.53	1.35
0 nM Cobalt Culture	1406.39	41310.36	67.03	362.40	5464.85	5758.04	1.65
0.1 nM Cobalt Culture	1970.59	25585.63	5.25	97.93	1391.29	3258.76	0.24
1 nM Cobalt Culture	1992.62	16207.72	3.20	67.97	788.36	996.60	0.16
1 nM Cobalt Culture	1376.85	15297.45	5.15	100.50	1066.34	1542.58	0.30
10 nM Cobalt Culture	1100.15	24223.30	55.46	36.14	1007.64	628.71	0.54
10 nM Cobalt Culture	655.21	8980.81	26.69	112.39	108.07	1516.10	0.18
10 nM Cobalt Culture	1224.12	10472.67	34.01	27.14	474.96	452.17	0.29

Table 2. Metal to phosphorus ratios (mmol/mol) observed in cobalt limitation experiment cell harvesting.

Trace metal to phosphorus ratios were calculated using the biomass harvesting procedure in section 2.5. In these results there is one anomaly in row 4 of table 2. This row of data is the result of a digest 0 nM cobalt culture. This culture demonstrates the highest levels of almost every metal that was tested for. In a culture with 0nM added cobalt, the cobalt should be especially low, meaning there was likely metal contamination in the harvesting process. Because of this the results for that filter will be voided in further calculations and discussion. When this filter is excluded, we can see that the high cobalt cultures take in much higher amounts of cobalt compared to the lower cobalt cultures (Table 2).

4.0 DISCUSSION

4.1 Oceanic Concentrations of Trace Metals

Prochlorococcus strain MIT9301 was isolated in the Sargasso Sea at 34° 10.1'N 66° 18.2'W and 90m deep (Chisholm Lab). Most of the Northern Atlantic Ocean has nitrogen as its limiting nutrient (Figure 2). The Atlantic Ocean has varying concentrations of trace metals due to deposition from the Sahara Desert. Strain MIT9215 was isolated in the Equatorial Pacific at 0°00'N 140°00'W. The Pacific Ocean is extremely low in nutrients including trace metals. MED4 was isolated in the Mediterranean (43°12'N 6°52'E) where there is high metal content due to its close proximity to land.

The dissolved iron concentrations in the isolation locations of each strain vary. In the Sargasso Sea, ocean iron concentrations range from about 0.3-1.7 nM (Figure 14). Equatorial Pacific concentrations of dissolved iron are around 0.1-0.15 nM, while Station

Aloha dissolved iron concentrations range from 0.04-0.12 nM (Figure 14). Looking at the Mediterranean Sea dissolved iron concentrations are generally around 1.5-2.0 nM (Figure 14). The dissolved iron concentration was observed to be the highest in the Mediterranean Sea, then iron concentrations decrease in the Atlantic Ocean, and then are lowest in the Pacific Ocean.

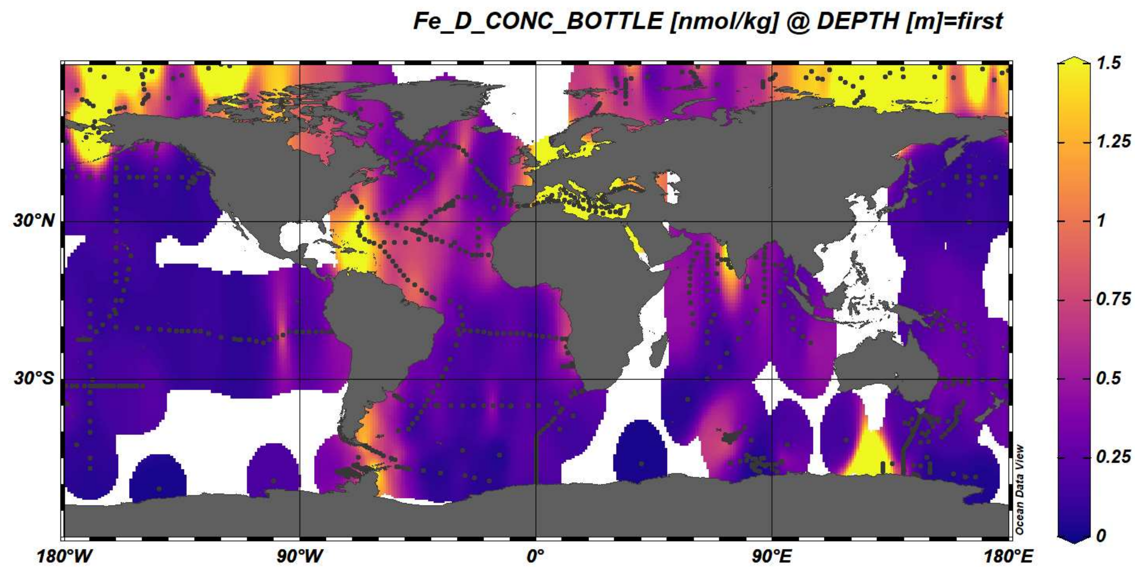

Figure 14. Global ocean concentrations of dissolved iron (nM). This data was obtained from a variety of GEOTRACES cruises. GEOTRACES is an international program that studies large scale trace elements throughout the world's oceans.

The isolation locations of each strain also vary in their concentrations of dissolved cobalt. The Sargasso Sea had ocean cobalt concentrations ranging from about 30-70 pM (Figure 15). In the Equatorial Pacific, concentrations of dissolved cobalt are around 3-30 pM, while Station Aloha dissolved cobalt concentrations range from 6-16 pM (Figure 15). Looking at the Mediterranean Sea dissolved cobalt concentrations are generally around 126-154 pM (Figure 15). The highest dissolved cobalt concentrations out of the strain isolation points were found in the Mediterranean Sea, then cobalt concentrations decrease in the Atlantic Ocean, and are lowest in the Pacific Ocean.

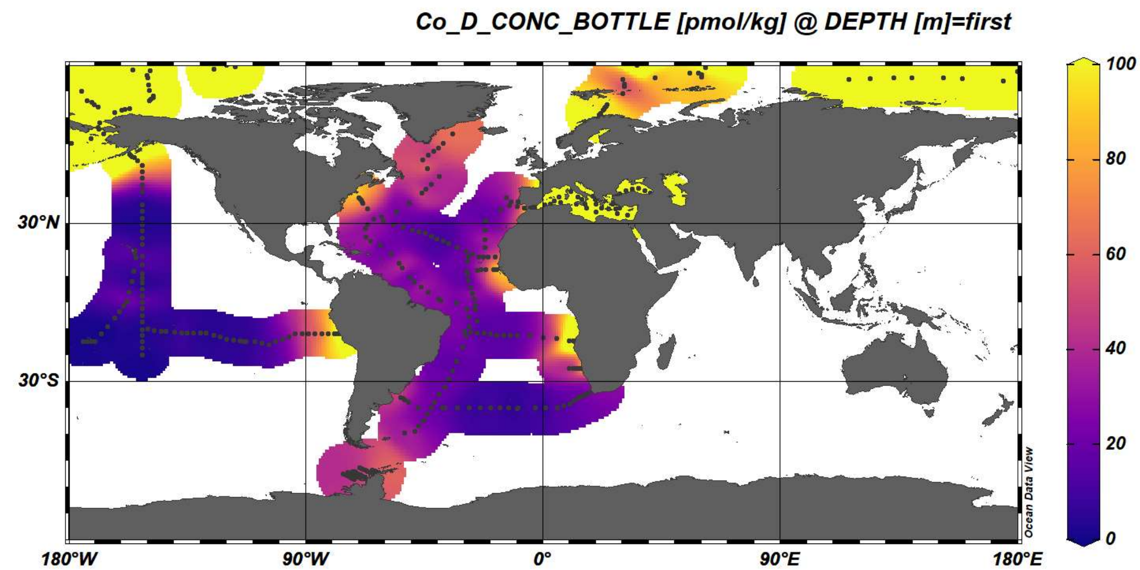

Figure 15. Global ocean concentrations of dissolved cobalt (pM). Data acquired from GEOTRACES database.

4.2 Growth Rates of *Prochlorococcus* Strains Under Iron and Cobalt Limitation

4.2.1 Pacific *Prochlorococcus*

In the Pacific Ocean the concentration of trace metals is much lower than in the Atlantic Ocean. This leads to an interesting comparison between strains and how they have adapted to their environment. We can compare these differences by analyzing the growth rates of MIT9215, originating from the equatorial Pacific (0°00'N 140°00'W) on the surface, and MIT9301, isolated from Sargasso Sea (34° 10.1'N 66° 18.2'W) at 90m deep, respectively (Chisholm Lab) (Hawco et al. 2018). Both MIT9215 and MIT9301 are high-light adapted strains of *Prochlorococcus* which is the most abundant ecotype. High light adapted strains such as MIT9215 are isolated at a depth of 90m. While this is a deep isolation depth, they still have high concentrations from the surface to almost 150m deep, where their concentrations decline rapidly (Malmstrom et al. 2010). In these comparisons bioavailable concentrations of trace metals were calculated based on EDTA complexed into the media. This EDTA binds to dissolved metals, making lower concentrations available for cellular uptake. In the experiment carried out by Hawco et al. the growth rates of MIT9215 decreased significantly when inorganic cobalt concentrations were lowered to 3pM (Hawco et al. 2018). Significant inorganic cobalt limitation for strain MIT9301 began when inorganic cobalt concentrations decreased from 15 to 1.5 pM (10 to 1 nM added cobalt cultures). This means that in both the Pacific and Atlantic high light *Prochlorococcus* there were similar cobalt concentrations where growth was limited.

The trace metal to phosphorus ratios Atlantic of MIT9215 were also measured in the limitation experiments conducted by Hawco et al. in 2018. In these experiments the Co:P ratios ranged from 72 mmol/mol in the high inorganic cobalt cultures (15.5pM) to 4 mmol/mol in the cultures with the lowest inorganic cobalt. In our experiments conducted on MIT9301, the high cobalt cultures (15.5pM) had an average Co:mol P ratio of 38.72 (Table 2). In the low cobalt cultures (0-1.5pM) the average Co: mol P ratio was 5.25 (Table 2). When looking at the Fe:mol P ratios when strain MIT9215 was not under iron limitation there was a ratio of 3.98 mmol/mol while in MIT9301 there was Fe:P ratio of about 14.56 mmol/mol.

Overall MIT9301 an Atlantic strain became limited at a similar cobalt concentration as Pacific *Prochlorococcus*. A possible explanation for this is that the adaptation for low cobalt levels most likely evolved in *Prochlorococcus* before these strains split off into their separate ocean basins. Looking at iron uptake at high iron concentrations in the *Prochlorococcus* cells, we found a lower uptake of iron in Pacific *Prochlorococcus* compared to its Atlantic counterpart. This is possibly due to the Atlantic Ocean being the isolation location of MIT9301, where there are higher concentrations of trace metals than in the Pacific. Pacific *Prochlorococcus* (MIT9215) over large geological timescales has most likely adapted to take in less iron because of its environmental scarcity. This Pacific strain could have evolved to use a reduced number of proteins containing iron to lower their minimal iron requirements (Cunningham et al. 2017). In comparison to Atlantic *Prochlorococcus* where adaptation to low iron concentrations is not needed, so they could keep these proteins containing iron.

4.2.2 Mediterranean *Prochlorococcus*

MED4 is a Mediterranean strain of *Prochlorococcus* first isolated off the southern coast of France. Iron uptake experiments on this strain of *Prochlorococcus* have determined a growth rate of 0.41 day^{-1} in cultures with 0.3 μM iron concentrations, 0.36 day^{-1} in 0.06 μM cultures (Castruita et al. 2008). In the MIT9301 the growth rates were calculated to be 0.38 day^{-1} in 0.351 μM iron cultures, and 0.35 day^{-1} in 0.117 μM iron cultures. These comparisons do not show any major differences when it comes to iron limitation in a Mediterranean strain compared to an Atlantic strain. When looking at the metal to phosphorus ratios we can see that in MED4 the iron to phosphorus ratios were between 6,970-26,400 μmol/mol. In the MIT9301 cultures the Fe:mol P ratios stayed between 8,980-25,585 μmol/mol. These values give evidence towards similar iron uptake between the two strains.

There have also been cobalt limitation experiments on *Prochlorococcus* strain MED4 by Saito et al. in 2002. In these experiments they saw that the growth rates of the Mediterranean *Prochlorococcus* decrease once the cobalt concentration was lowered below 1000 pM (1 nM) (Saito et al. 2002). This is similar to our experiments on Atlantic *Prochlorococcus*. In our experiments on MIT9301 we found that the growth rates also decrease significantly when lowered to 1 nM. This means that MED4 likely has a similar cobalt tolerance to MIT9301 giving evidence towards *Prochlorococcus* evolution for low cobalt concentrations happening earlier on (before the strains adapted to their individual basins) than for low iron concentrations.

5.0 CONCLUSION

Prochlorococcus is a cyanobacterium with global importance, not only for carbon sequestration but also, in its role in ecosystems in almost every major body of oligotrophic water. They are able to sustain growth in heavily nutrient limited environments and have evolved immensely to account for changes in different bodies of water and their respective limiting nutrients. In the Pacific Ocean strains of *Prochlorococcus* such as MIT9215 have adapted to reduce trace metal needs to account for the iron throughout the Pacific Ocean. This is evident when comparing trace metal limitation experiments conducted on Atlantic *Prochlorococcus* strain MIT9301. Iron uptake in Atlantic MIT9301 was shown to be higher than in the Pacific isolated strain MIT9215. In the cobalt experiments conducted on MIT9301 we found significant limitation when cultures cobalt concentrations were reduced from 10nM to 1nM, which was similar to limitation shown in Pacific and Mediterranean *Prochlorococcus*. Mediterranean *Prochlorococcus* MED4, also was found to have similar iron to phosphorus ratios and can withstand similar concentrations of cobalt providing a similar adaptation to high trace metals. Research on *Prochlorococcus* is key to analyzing the resilience these cyanobacteria will have to changes in nutrient availability and changing trace metal concentrations. Moving forward it will be important to conduct more research and modeling regarding limitations on oligotrophic *Prochlorococcus* to predict and mitigate possible impacts on marine ecology and oceanic biogeochemical cycles.

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