The effect of iron concentration on *Euglena gracilis* growth rate

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ABSTRACT

Euglena gracilis is a multi-flagellate microalgae found in most freshwater systems that forms the basis of the system's food web (Maier & Simenstad, 2009). Increased industrialization has led to more iron leaching into the water systems which can affect *E. gracilis* growth positively or negatively depending on the concentration (Olaveson, & Nalewajko, 2000). We tested how iron concentration would affect the growth rate of *E. gracilis*, hypothesizing that increasing the concentration would increase the growth rate. We grew *E. gracilis* samples in growth media with 3 different concentrations of iron added: 7.5 µM, 15µM, and 30 µM for two weeks. We calculated the number of cells in each flask after each day using a haemocytometer. After analyzing our data using a one way ANOVA test at a 95% confidence level, we obtained a P-value of 0.28 indicating that we do not have enough evidence to reject the null hypothesis which states that there is no difference in growth rate between iron concentrations. The growth rates did not show significant differences between iron concentrations. These findings offer a starting point for more comprehensive investigations into the effect iron concentrations have on how *E. gracilis* change morphologically and how their interactions with other primary producers change.

INTRODUCTION

Euglena gracilis is a fresh-water microalgae capable of autotrophic and heterotrophic feeding in the forms of photosynthesis, the use of organic carbon, and phagocytosis (Hasan, et. al., 2019). Being a primary producer in most fresh-water systems, *E. gracilis* forms the base of many food webs, specifically by being a food source for the riverine insects often preyed upon by juvenile salmon (Maier, et. al., 2009). As well, a 2009 study conducted by Skov, et. al. showed that salmon which ingested a particular □-glucan molecule produced from *E. gracilis* displayed an increased immune-response to enteric redmouth disease. Based on these aforementioned reasons, adequate levels of *E. gracilis* in fresh-water systems is necessary for successful salmon growth and fitness.

Iron is a common metal found in trace levels in most water systems as a result of soil, sediments, and groundwater (Maas, 2004). However, over the past few decades, heavy metal

concentrations, including iron, have been increasing steadily as a result of many anthropogenic factors such as mining, and industrial waste run-off (Lottermoser, 2007). *E. gracilis* lack cell walls and are therefore more susceptible to ingestion of iron than other microalgal species (Lee, et. al., 2015). Iron is necessary in *E. gracilis* as it regulates the expression of ascorbate peroxidase, an important protein that protects the cell from damage by preventing reactions of toxic H_2O_2 from occurring within the cell (Radtke, et. al., 1992). As well, iron depletion has been shown to arrest cell division in *E. gracilis* (Ishikawa, et. al., 1993).

E. gracilis growth follows an optimization curve, and while some iron is important to *E. gracilis* growth, too much can be extremely toxic, with 502 pmol/cell being the maximum concentration tolerated before cell growth decreases (Olaveson, et. al., 2000). As well, too much iron in water is unstable and often precipitates out, producing solids which decrease phosphate availability, and light penetration, two factors which can affect *E. gracilis'* ability to grow and divide (Olaveson, et. al., 2000). Lastly, *E. gracilis* growth in increased iron concentrations have been found to lead to morphological changes such as enlarged vacuoles which can inhibit chloroplast growth (Marsh Jr, Evans, & Matrone, 1963).

The purpose of our study is to investigate the change in growth rate of *E. gracilis*, in response to increased iron concentrations. Our null hypothesis states that there will be no significant difference in the mean growth rates of *E. gracilis* grown in different iron concentrations. Our alternative hypothesis is that there will be a significant difference in at least one growth rate of *E. gracilis* when grown in different iron concentrations. We predict that the growth rate of *E. gracilis* will increase as we increase iron concentration in our growth media. We hope the results of this study will help clarify the optimal environment to grow *E. gracilis*. This will aid in solidifying a strong base for the freshwater food web to

improve the sustainability of salmon feeding over time. Future studies can be conducted investigating how *E.gracilis* grown under these conditions interact and affect other primary producers in the freshwater food web.
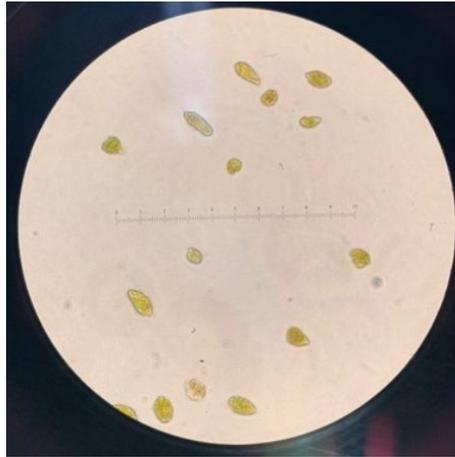
Figure 1. *E. gracilis* prepared during experiment, image taken using light microscope under 400X magnification

METHODS

Treatments

We tested the population growth rate of *E. gracilis* at four different iron concentrations. A previous study of the role of iron in chloroplast formation in *E. gracilis* used a maximum, nonlethal concentration of 36μM Fe as treatment (Price, & Carell, 1964). Therefore, based on this study, we decided to use a maximum concentration 30μM Fe, and then smaller concentrations of 0μM, 7.5μM and15μM, where 0μM was the control group. A note should be made that these concentrations represent the amount of additional iron added to the media. There was a certain concentration of iron present in the growth media already that was unknown to us. We used iron in the form of $FeCl_3.6(H_2O)$ in solution with an initial concentration of $9.0 \times 10^{-5}M$, which was then diluted using *E. gracilis* growth media. Each of the treatments had four replicates with a total of 16 samples. The growth rate of each sample was measured over two weeks, with daily measurements.

Experimental Set-Up

The *E. gracilis* stock was previously cultured in specific growth media, in an Erlenmeyer flask, by Mindy Chow, the lab manager for BIOL 342 at the University of British Columbia. We started with a concentrated stock of *E.gracilis* which we diluted to $3x10^5$ cells/mL. To do the proper dilution we started by counting the number of cells in our initial stock. For this step, we used sterile techniques to fixate our cells. We pipetted 10μL of our fixative, potassium triiodide (IKI), into a 1.5mL Eppendorf tube, and also pipetted 100μL of our *E.gracilis* stock into the same Eppendorf tube and mixed well by slowly pipetting up and down.

After preparing the sample, we placed a Fisherbrand® micro-coverslip over the haemocytometer grid and pipetted 20μL of our sample under the coverslip. Then, we placed the haemocytometer under the stage of an Axio light compound microscope, viewing the slide with 100X total magnification. We counted 80 cells in the 1mm x 1mm grid, and we proceed to dilute our initial stock to $1.52x10^5 \; cells/mL$ to get our initial sample solution.

The *E. gracilis* sample solution was then divided into 16 different 25mL sterile Erlenmeyer flasks. Each Erlenmeyer flask was labeled with the group name, iron treatment concentration, and the replicate number. In each treatment flask, we added 5mL of the *E. gracilis* sample solution, and corresponding volumes of pure growth media and iron containing growth media, to achieve our desired concentrations in 15mL total volume, as shown in *Figure 2*.
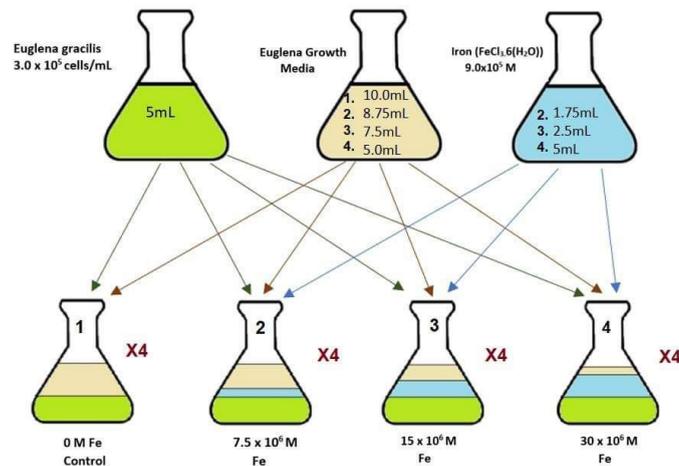
Figure 2. Experimental Design for preparation of treatment samples

Once the experimental set up was complete, we took samples from each of the flasks to check our initial cell count. As previously described, we prepared our samples by pipetting 10μL of our fixative, into a 1.5mL Eppendorf tube, and added 100μL from our sample into the same Eppendorf tube and mixed by pipetting up and down. Each tube was properly labeled with the treatment, replicate number, the date, and group name. Once completed, these samples were stored in the 4 °C fridge. The *E.gracilis* treatment groups were incubated at 25°C for two weeks.

Data Collection

Daily, 16 samples were fixated from our treatments and stored in 1.5mL Eppendorf tubes in a 4°C fridge. Fixation was performed using 100μL of the treatment solution and 10μL of the fixative. Cell count was performed in the following days, using a haemocytometer and an Axio light compound microscope. The fixated samples were mixed before its addition to the haemocytometer and were placed under the stage of the light microscope, as shown in *Figure 3*. We counted each replicate three times, and then use Equation 1 (below) to calculate the final cell count per mL.

$$\text{Cell count } \frac{cells}{mL} = Num. \text{ of cells } * \text{ Haemocytometer Dilution factor } * \text{ Correction factor fixative}$$

Equation 1. Cell Count
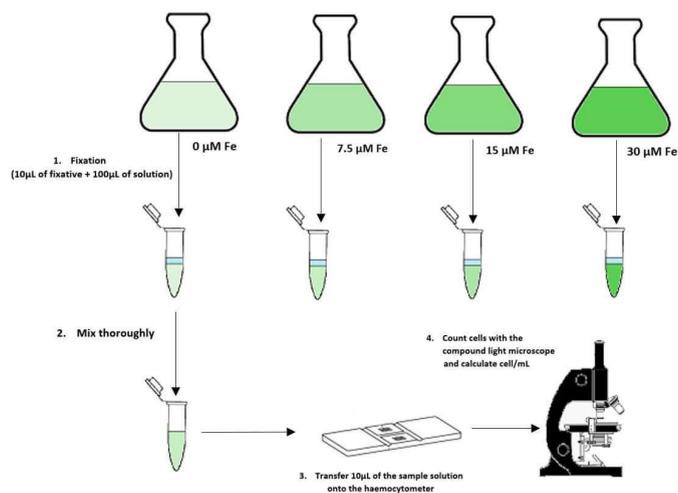
Figure 3. Cell Fixing/Counting Design

We used the haemocytometer grid to record the number of squares that we used for each count, and the number of cells counted per square.
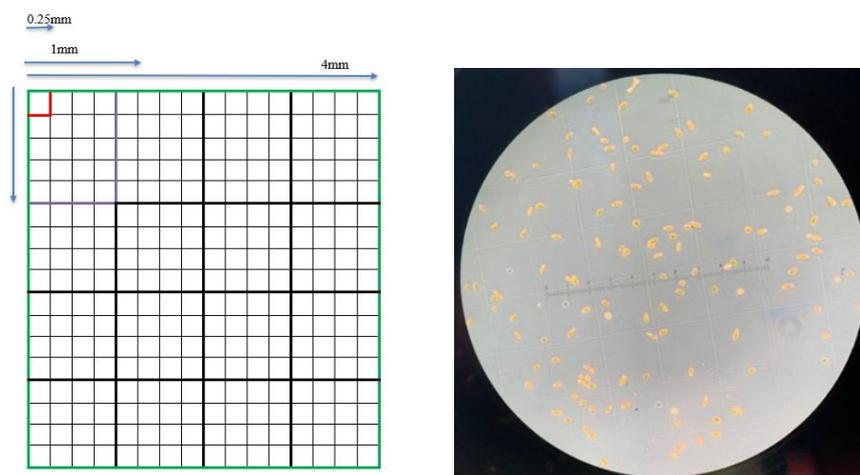
Figure 3. *Euglena gracilis* haemocytometer grid and view of grid under light microscope at 100X magnification

Data Analysis

After collecting all the data, we analyzed our results by calculating the final cell counts for each of the samples. Then, we calculated the average cell count for each of the concentrations using the four replicates. Finally, we constructed a growth curve for each of our treatments and performed a one-way ANOVA test.

RESULTS

The cells were collected over a two week period until they reached the stationary phase. The initial cell count was approximately $3x10^{-4}$ instead of the expected $5x10^{-4}$, however, since it was consistent between all replicates, we chose to continue and adjust our subsequent calculations. Our samples were very close to the growth media's color with very little green, likely due to our samples being heavily diluted. The lag phase was observed from day 0 to approximately day 2 where little growth occur. And the logarithmic growth was observed from day 2 to day 8. All of the replicates grew at almost the exact same rate for the first 3 days, on day 6 two samples of 30μM seemed to grow much more than the others, however, during the next day the other replicates caught up. We would be more sure of these data points if we had collected data over the weekend. Logarithmic growth was observed for 6 days and we calculated the growth rate based on these days.
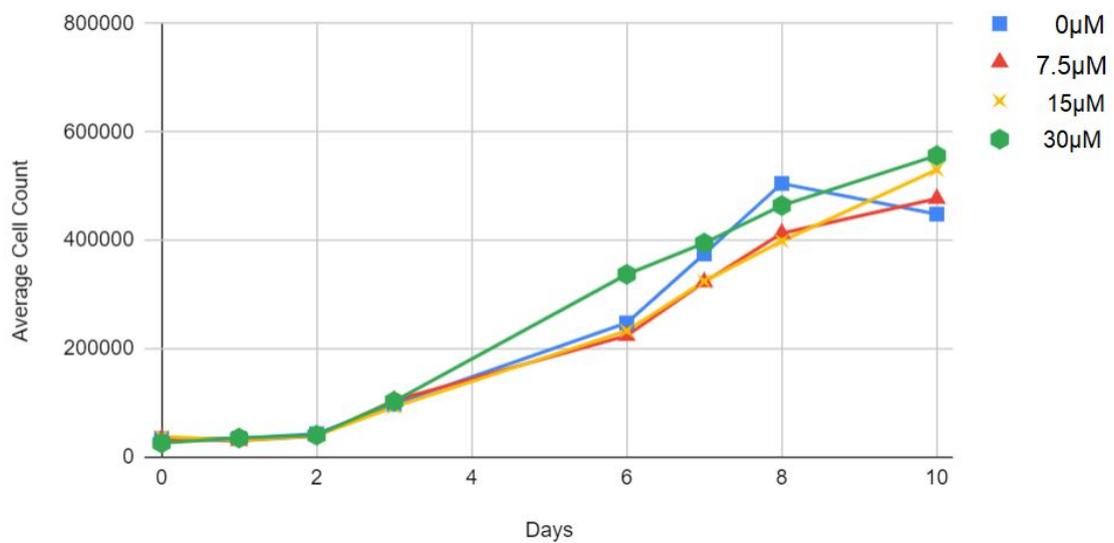
Figure 4. Growth Curve of *E. gracilis* in Iron over time, triangles are 0μM, squares are 7.5μM, x's are 15μM, and hexagons are 30μM

After collecting all the data, we performed a one-way ANOVA. This method was chosen because we have one treatment variable with 4 categories of 0μM Fe, 7.5μM Fe, 15μM and 30 μM Fe. We constructed a growth curve for each replicate, as seen in Figure 4. The growth rate was calculated using the final concentration and initial concentration, as well as the number of days it was grown for. The ANOVA was performed on a Microsoft Excel spreadsheet with 4 categories and 4 replicates in each. The p-values evaluated at a 95% confidence level were 0.289 for 6 days and 0.397 for 5 days. Based on these values, we do not have enough evidence to reject the null hypothesis.

Based on the results of the ANOVA, we found the growth rate was not significantly different among the different treatment samples. As growth rate can be calculated at any point of the growth curve, the 5 day curve also showed no significant difference between populations.
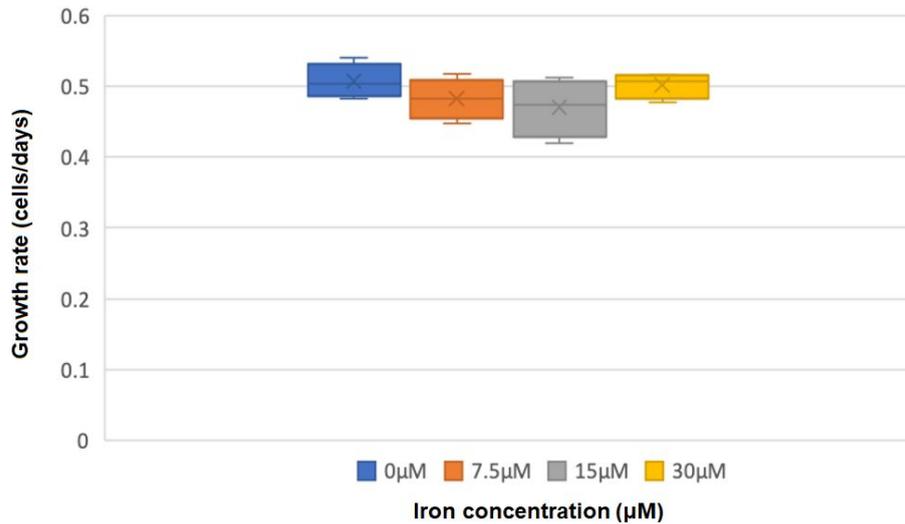
Figure 5. The average growth rate of *Euglena* in Iron media after 6 days, the bars overlap at all around a growth rate of 0.5.

DISCUSSION

The analysis of the results showed that there is no significant difference between the growth rates of *E.gracilis* at different iron concentrations. The results from the one-way ANOVA test showed a p-value of 0.289. At a 5% significance level, we do not have enough evidence to reject the null hypothesis, therefore, we can conclude there was not a significant difference in the growth rate of the different groups, meaning that iron concentration did not significantly affect the growth rate of *E.gracilis*.

Based on previous investigations, iron is essential for growth and chlorophyll production in *E.gracilis* (Price and Carell, 1964). Although the mechanism through which iron affects *E. gracilis* is unknown, a significant change in the iron content of water can be highly detrimental for these organisms. A reduction in aquatic primary producers, such as *E.gracilis*, could result in bottom-up effects on invertebrate grazers (Macneale, et al 2010). Juvenile salmon feed on invertebrate prey in freshwater and estuaries. (Macneale, et al.

2010). Therefore any abrupt change in the iron content of aquatic systems can have a bottom-up effect on the freshwater food web and negatively impact salmon.

After an in-depth analysis our methods and results, we found three potential explanations for the lack of significance in our results. The first one related to the iron content in the *E. gracilis* media provided for the experiment, the second one related to the iron concentrations used for the experiment and the third one related to the fixation and count of *E.gracilis* from the treatment groups.

One of the possible explanations for the lack of significance in our results is that iron was provided within the media in which *E.gracilis* cells were grown. As the media used to dilute our sample was a mixture of many compounds such as sodium acetate, tryptone, $CaCl_2 \cdot 2H_2O$, yeast extract, and beef extract, we think that traces of iron might be present in the *E. gracilis* media before we treated them with iron. In previous studies of the effect of iron in *E. gracilis* chloroplasts formation, an analysis of the iron media content was performed prior to treatment application, where media that contributed with more than 0.2μg of iron per liter was purified (Price and Carell, 1964). The purification, in this case, serves as a way to control for the initial iron content in each treatment (Price and Carell, 1964).

Assuming that iron was already present in the solution, *E.gracilis* had enough iron to grow at normal rates even without the iron treatments provided, and therefore no difference was captured for any of the iron groups. Therefore, for future studies, a purification of the media prior to treatment would be essential to characterize the effect of iron in *E. gracilis* growth.

Similarly, the concentrations of iron that we used for the experiment were different by a factor of 2, which may have not been enough to induce a significant change in the growth rate of *E.gracilis*. As we did not want to kill the organisms, we used an iron concentration of

30μM which is lower than the 36μM proposed by previous studies (Price and Carell, 1964). Therefore, the applied iron concentrations could have not influenced as much the growth rate of *E. gracilis*. For future studies, different concentrations should be tested, potentially iron concentrations over 36μM and lower than 7.5μM, to see if there is any significant difference in the growth rates.

Sources of Error and Variations

This study presented some errors in some of the sample preparation steps, the initial *E.gracilis* stock dilution calculation, and the sample cell count. One of the sources of error or variations was the technique used to fixate the sample each day. The technique used for fixating the solution was the same for all the samples, however, the mixing might have been different, as different people fixated the samples each day. Mixing is an important step to ensure a correct cell suspension and a representative amount of cell per uL being measured, therefore, a different mixing technique may have affected the samples collected on different days.

Another source of variation within the experiment was the initial dilution of the *E.gracilis* stock. After we counted the number of cells in our day 0 sample, which was our initial sample, we realized that the cell count per mL was so low that we were only able to count up to 90 cells within the whole hemocytometer.

Another source of error in our experimental procedure was the lack of consistency when preparing the sample to be measured for cell count. Again, the mixing was an important factor that played a huge role while counting the number of cells. Each person prepared their coverslip, hemocytometer, and the sample to be counted. Mixing in this step was very important to ensure cell resuspension and a more accurate measurement of the number of cells in the given sample. However, each person may have mixed differently while pipetting

the 20 uL of the treatment solution from our 110uL fixated sample in the hemocytometer. This could have introduced a source of error in the cell count and the final growth rate calculation.

Another issue was that most of the logarithmic growth happened on the weekend. We missed, crucial days that could have greatly impacted the significance of our data. Even though we measured many data points we could've only extracted growth curve data from 2 days worth of samples. We did have the day to day change of the early logarithmic growth. If we had more data we could have more proof that 30μM samples were growing faster.

For future studies, we would minimize the source of errors related to the mixing technique by creating a more detailed protocol of how the samples are fixed and prepared for the cell count. Also, is very important to ensure that the correct dilution calculations are done for the initial experimental setup. This could be achieved by properly counting the cells in the initial fixated sample to calculate the correct initial concentration of the *E.gracilis* stock.

CONCLUSION

After analyzing our results we can conclude that different iron concentrations applied to *E.gracilis* media does not significantly affect their growth rate. However, as our results were highly dependent on the concentration of iron in *E.gracilis* media, future studies need to be performed to understand the exact role and mechanism that iron plays in growth. Iron is an essential species in the growth of *E. gracilis*. This study is significant in helping us better understand how iron acts as an essential abiotic factor in the growth of E. gracilis, which is an important primary producer in the freshwater ecosystem.

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