The effect of iron concentration on the growth rate of *Chlamydomonas reinhardtii*

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This study addresses how the growth rate of *Chlamydomonas reinhardtii* is affected by the concentration of iron present in the growth medium. To investigate this effect, we grew cells of *Chlamydomonas reinhardtii* under zero, half, standard and double iron concentrations defined relative to the standard concentration of iron used in their standard growth medium (46.2 μM). Exponential growth was observed under all conditions throughout the 23 day measurement period apart from under zero iron conditions, where the growth was observed to plateau at approximately day 10 resulting in significantly lower cell abundance on day 23 compared to the three iron treatments. No significant differences were observed between cells grown under the half and standard iron concentrations, suggesting half (23.1 μM) of the standard concentration of iron is sufficient to support growth over this time scale. Increased iron concentration was shown to be stimulatory to growth, with the cells grown under the double iron concentrations (92.4 μM) showing significantly higher cell abundance compared to the cells grown under half iron concentrations by day 16, and significantly higher cell abundance than the cells grown under standard iron concentrations by the end of the measurement period on day 23. This result supports our alternate hypothesis that the growth rate of *Chlamydomonas reinhardtii* will increase with respect to increasing iron concentration present in growth medium.

Introduction

Although iron is abundant in the earth's crust, there is only a small amount available for use in biological functions, making it a limiting nutrient for life (Terauchi *et al*. 2010). In algae found in the ocean, a lack of free iron often limits photosynthesis (Glaesener *et al.* 2013). When iron is added to oceanic environments with naturally low amounts of iron, an algal bloom can often be observed (Terauchi *et al*. 2010). This observation suggests that iron plays a crucial role in the reproduction and growth rate of algae. Iron fertilization of areas of ocean with naturally low iron concentrations links iron to the carbon cycle through the stimulated growth of phytoplankton and therefore iron may have a significant effect on the concentrations of carbon dioxide in the atmosphere, related to glacial-interglacial cycles (Blain *et al.* 2007). The organism *Chlamydomonas reinhardtii* is a unicellular species of green algae that can be used to study this

relationship between iron concentration and algal blooms, as it can be grown in a laboratory under controlled conditions and displays a rapid rate of reproduction (Harris 2009).

Chlamydomonas reinhardtii has a well understood life cycle, providing researchers with an easy model to study cell behaviour, such as cell reproduction and growth (Harris 2001). In its resting stage, *C. reinhardtii* are normally found in two distinct haploid mating types designated m^+ and m^- (Harris 2001). During reproduction, *C. reinhardtii* displays two types of reproductive cycles: meiosis and mitosis. During meiosis the two mating types fuse together to produce a diploid zygote, which matures for a few days before dividing to produce a total of four haploid cells (Harris 2001). Mitosis of the haploid gametes usually occurs after meiosis and repeats every few days with a frequency of 2-3 rapid divisions in succession for every occurrence (Harris 2001).

Reproduction of *C. reinhardtii* relies on a circadian clock (Mittag *et al*. 2005). It has been shown that cell division peaks during the dark hours and light accumulation peaks during the light hours of a day (Mittag *et al*. 2005). In the presence of light, *C. reinhardtii* does not undergo reproduction to maximize its accumulation of light (Mittag *et al*. 2005). It is only in darkness that *C. reinhardtii* undergoes reproduction (Mittag *et al*. 2005). Interestingly, in the complete absence of light *C. reinhardtii* can continue reproducing constantly, provided that essential nutrients such as organic carbon are readily available (Spudich and Sager 1980).

In previous studies it was noted that *C. reinhardtii* often behaves differently in the presence of varying concentrations of iron in growth media (Glaesener *et al*. 2013). It was found that under optimal growth conditions as described by Harris (2009), *C. reinhardtii* displayed an increase in growth rate as the concentration of iron increased (Glaesener *et al*.

2013). This increase in growth rate however is not limitless. Studies in the uptake rate of iron by *C. reinhardtii* showed that saturation of the iron uptake pathways in the organism is possible, thus creating a limit on the growth rate of *C. reinhardtii* (Eckhardt and Buckhout 1998). In media with an excess concentration of iron, the organism has been shown to over-accumulate iron and display an inability to grow at high light intensities as a result (Glaesener *et al*. 2013). In lower iron concentrations, it was found that the expressions of iron uptake pathways were dramatically reduced and the expression of other nutrient uptake pathways increased (Glaesener *et al*. 2013).

Through our study we further explored the function of iron in the reproduction behavior of *C. reinhardtii*. We believe that by increasing the concentration of iron present in our cell media, the growth rate of our organisms will increase. Therefore our hypotheses are:

H_a: Growth rate of *Chlamydomonas reinhardtii* will increase with increasing iron concentration in the growth medium.

H_o: Growth rate of *Chlamydomonas reinhardtii* will decrease or remain constant with increasing iron concentration in the growth medium.

Methods

In order to observe the effect of iron on the growth rate of *C. reinhardtii*, we created four different culture media with different iron concentrations relative to the standard culture medium (iron concentration equals 46.2 µM), as listed in Table 1. We obtained the *C. reinhardtii* from the UBC Biology 342 lab, and used the CC-1690 – wild type mt+ 21gr strain. As this is an m+ strain, no meiosis occurs and all cell division occurs via mitosis.

Table 1: *Four treatment conditions.*

Treatment	Iron Concentration
Zero (Control) Treatment (0 x)	0 M
Half Iron Treatment (½ x)	23.1 µM
Standard Iron Treatment (1x)	46.2 µM
Double Iron Treatment (2x)	92.4 µM

To prepare the media for these treatments we used 300 ml (4 treatments x 4 replicates x 15ml per test tube) of a stock medium that had been prepared following the recipe listed in Table 4 in Appendix A, minus the iron component. We then added iron (obtained in the form of iron chloride hexahydrate (FeCl3-6 H20)) and sterile distilled water adding the volumes as listed in Table 2 to achieve our treatment concentrations.

Table 2*: Volumes of iron solution and sterile water added to each treatment to achieve desired concentration.*

Treatment =	0 x	½ x	1 x	2 x
Iron Chloride Hexahydrate Volume Added (µl)	0	18.75	37.5	75
Water Volume Added (µl)	75	56.25	37.5	0

We counted the initial number of *C. reinhardtii* cells and diluted the solution to a starting concentration of 10,000 cells/ml. We counted cells every two to four days over a ten day period using a haemocytometer; however this period was extended to 23 days. We stored our samples in an incubator at 17°C and counted our samples in the lab at the same time to ensure abiotic factors such as temperature and light intensity were kept constant. We set up four replicates per treatment.

Cell Extraction and Preparing Media

We received our *Chlamydomonas reinhardtii* cells in the standard growth media from the UBC Biology 342 lab (see Table 4 in Appendix A) and counted the initial cell concentration. We calculated the volume we would need to produce a final cell concentration of 10,000 cells/ml in the 300 ml of zero iron medium and found this to be 2.633 ml. We removed this volume and separated the cells from the standard media by using a centrifuge for 3 minutes at the highest speed (14,000 rpm) and pouring off the supernatant. We then re-suspended these cells in our 300ml of zero iron media in a new sterile beaker. We divided this solution between four flasks to create our four treatment solutions, giving us 75 ml of solution in each of the four flasks. Then, we labelled each flask with the different concentrations and added the correct volumes of iron solution and sterile water to produce the desired iron treatment concentrations as seen in Table 2. After thorough mixing, we transferred the contents of each flask to their four replicate tubes, with 15 ml in each. We then placed our test tubes on a shaker with a speed of 100 rpm in a room with a constant temperature of 17°C and following a light pattern of 14 hours of light (measured to be 850 LUX) and 10 hours of dark. This procedure can be seen in Figure 1.

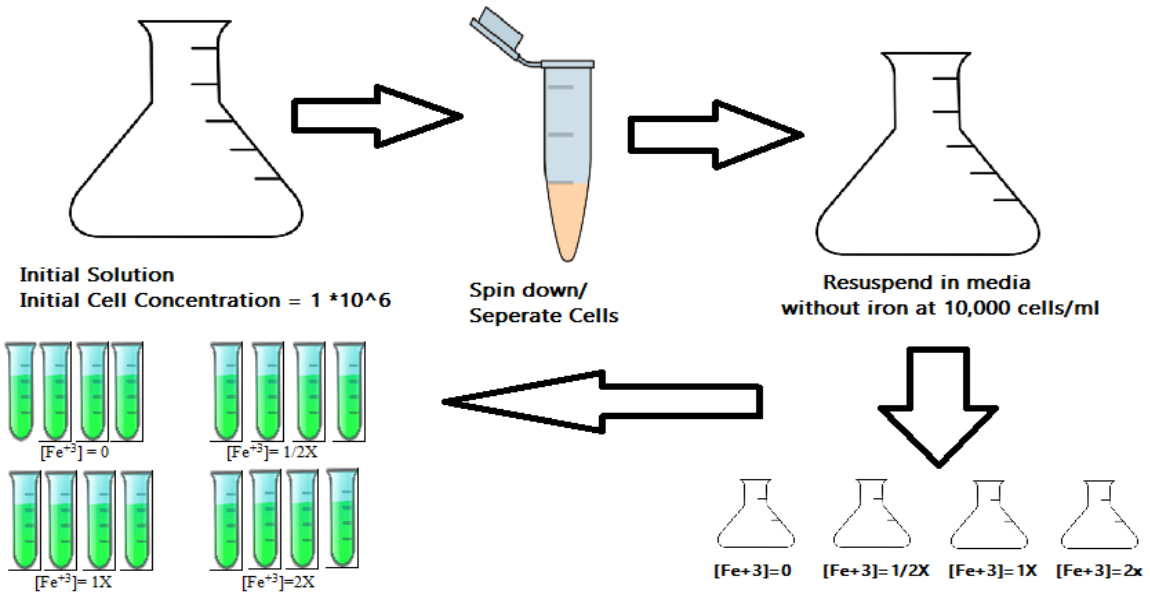

***Figure 1**: Schematic diagram of the experimental procedure.*

Cell Counts

We mixed each test tube well and removed 100 µl of solution from each tube to microcentrifuge (mcf) tubes. We then added 10 µl of IKI fixative to each mcf tube and mixed it with the solution using a pipette. We placed a glass coverslip over the grid region of the haemocytometer and pipetted 10 µl of sample into the haemocytometer chamber. We counted the number of *C. reinhardtii* cells present on the haemocytometer by observing in compound microscopes. When counting the cells, we were careful to ignore small, irregularly shaped objects as these were most likely cell fragments or other debris. Using our cell count data, we then calculated the mean, standard deviation and 95% confidence intervals for each treatment. We plotted mean cell abundance (in mean number of cells/ml) against the concentration of iron to clearly see the relationship between the iron concentration and the abundance of cells.

Results

Exponential growth was observed in all treatments over the 23 day measurement period with the exception of the control (0 iron) treatment. This can be seen as the mean number of *Chlamydomonas reinhardtii* cells per ml observed shown in Table 3.

Table 3: *Mean number of cells per ml and 95% confidence intervals for each of the 4 treatments.*

	Day =	6	8	10	14	16	20	23
0 x	Mean	306	11,306	32,083	43,389	36,972	97,778	38,806
	95% CI	599	8,320	9,142	21,615	24,043	58,573	23,682
1/2 x	Mean	1,528	2,750	17,111	21,083	24,139	154,917	206,250
	95% CI	1,147	1,147	4,482	6,366	9,349	43,533	38,133
1 x	Mean	917	3,667	23,833	26,278	44,611	111,833	168,056
	95% CI	599	2,587	7,086	18,046	17,508	42,792	8,384
2 x	Mean	917	3,056	17,111	17,417	113,361	210,528	267,972
	95% CI	1,147	1,546	978	10,505	61,250	98,088	42,080

The control initially demonstrated the fastest exponential growth pattern but no significant differences were found between the mean cell abundance observed from day 10 through day 23. Figure 2 shows the three days on which significant differences were found between treatments. The faster initial growth of the control can be observed in day 10, where the control was found to have a significantly higher mean cell abundance than both the half and double iron treatments. After day 10, no significant difference was found in the mean cell abundances observed of the control treatment and by day 23, the three iron treatments had all outgrown the control with significantly higher mean cell abundances observed.

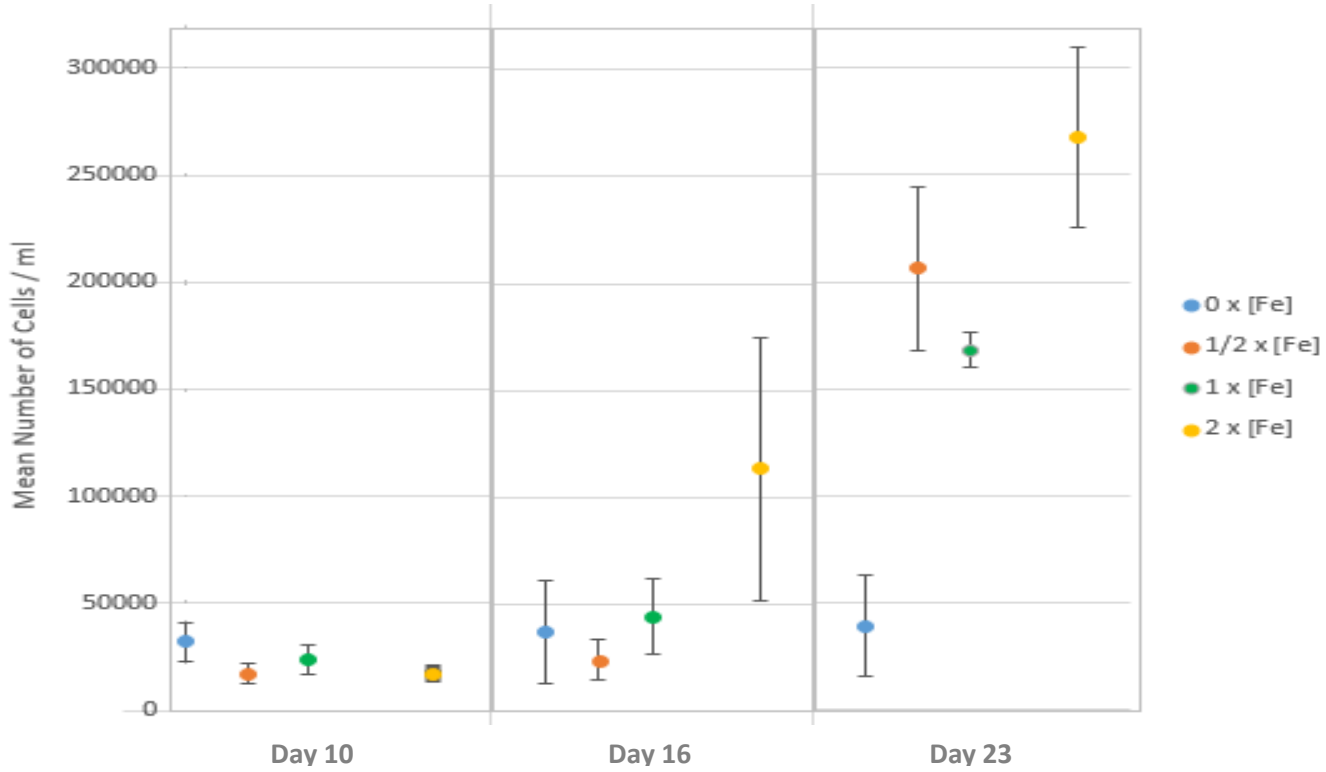

Figure 2: *Mean number of cells per ml for each of the four treatments at days 10, 16 and 23 Error bars represent 95% confidence intervals. Number of replicates for each treatment was four.*

No significant difference was found between the half and standard iron treatments at any point over the measurement period.

The double iron treatment was slow to grow initially, but showed the greatest total growth overall. By day 16 this treatment was observed to have the highest cell abundance of 113,361 ± 61,250 cells/ml, and despite the high variance in this measurement, it was found to be significantly higher than the half iron treatment. By day 23, the double iron treatment had increased even further to a cell abundance of 267,972 ± 42,080 cells/ml and was found to be significantly higher than both the control and standard iron treatments. There was no significance between the half and double iron treatments on day 23 as both were found to have

high variance (95% confidence intervals of ±38,133 and ±42,080 respectively) leading to the results overlapping.

Sample Calculations*:*

Cell Abundance: $\frac{Cell\ Count \times Dilution\ Factor}{Volume\ of\ Haemocytometer\ (ml)} = \frac{1 \times 1.1}{9 \times 10^{-4}} = 1222\ \text{cells/ml}$

Mean Cell Concentration: $\frac{\sum Cell\ Abundances}{Number\ of\ Replicates}$

Standard Deviation: $\sqrt{\frac{\sum_{i=1}^{n}(x - mean\ x)^2}{Number\ of\ Replicates - 1}}$

95% Confidence Interval: $\frac{1.96 \times Standard\ Deviation}{\sqrt{Number\ of\ Replicates}}$

Discussion

Results show that increasing the concentration of iron stimulated the growth rate of *C. reinhardtii*. The mean cell abundance in the 2x treatment was observed to be significantly higher than the abundance in the 1/2x treatment on day 16 and was then found to be significantly higher than the 1x treatment on day 23. Based on these results, we can support our alternate hypothesis that growth rate of *C. reinhardtii* will increase with respect to increasing iron concentration in growth medium and reject our null hypothesis. This is consistent with Glaesener *et al.* (2013) who concluded that *C. reinhardtii* cells display an increase in cell growth as the iron concentration in the medium increases. The concentration of iron used in our highest treatment (2x treatment) does not appear to have reached the excess amount that would limit growth of *C. reinhardtii* as described by Eckhardt and Buckhout (1998).

This finding supports the idea that introducing iron into an ocean environment that is naturally limited by its iron concentration will stimulate growth and potentially result in an algal bloom. This links the effect of iron concentration on ocean productivity to carbon cycle through the uptake of carbon from the atmosphere via photosynthesis as suggested by Blain *et al.* (2007).

No significant differences were observed between the cell abundances of the 1/2x and 1x treatments at any point over the 23 day period. This suggests that half of the standard iron concentration is sufficient to maintain normal cell growth of *C. reinhardtii* over this time period. If the time frame were extended, we expect that the 1/2x treatment would plateau before the 1x treatment as the iron concentration becomes limiting to growth and after this point, a significant difference would be observed between the cell abundances in these two treatments.

These combined results were consistent with Long's study (2008), which found that under lower light intensity (2700 LUX, which is comparable to our study, as our organisms were grown under a lower light intensity, 870 LUX) *C. reinhardtii* cells displayed highest viability when grown in medium with an iron concentration of 100 μM. Our results are consistent with this finding as our 2x treatment, which had 92.4 μM iron concentration, showed greatest growth amongst all the treatments. Furthermore, cells' viability when grown in 20 μM iron medium was similar to cells' viability when grown in 50 μM iron medium (Long 2008), just as we found no significant differences in cell growth between our 1/2x and 1x treatments, which had concentrations of 23.1 μM and 46.2 μM of iron respectively.

Iron is essential for manufacturing proteins that are involved in the electron transport chain in the membrane of both the mitochondria and chloroplasts and is necessary to carry out

respiration and photosynthesis (Glaesener *et al.* 2013). It is also an important cofactor in many essential metabolic reactions (Glaesener *et al.* 2013). Based on this, we would expect to observe the slowest growth rate in the zero iron concentration (0x) treatment. Interestingly, we observed that cells initially grew fastest in the medium of the 0x treatment up to day ten, but the growth rate then plateaued and no significant changes to cell abundances were observed between day 10 and day 23. There are two possible explanations for the faster initial growth of the cells in our 0x treatment. Firstly, there may have been trace amounts of iron remaining in the medium of the 0x treatment due to incomplete disposal of the supernatant during cell separation. Secondly, the cells may have already taken up the iron from the original medium before being transferred to the iron-free medium. The growth rate eventually plateaued suggesting that the cells had used up both the trace amount of iron in the medium, and their internal stores. However this doesn't explain why the growth rate was faster in the zero treatment, as the cell growth rate would still be expected to be lower in this treatment than in the other three treatments. We believe this may have occurred due to time requirements for *C. reinhardtii* to adjust to a new environment. All treatments were initially stressed when transferred to a zero iron medium. The three iron treatments were then stressed again when iron was added to achieve the treatment conditions studied. As the zero iron treatment was only stressed once (as opposed to twice for the iron treatments), the length of its lag phase to adapt to the new conditions may have been shorter.

C. reinhardtii also have a unique ability in that they can carry out both photosynthesis and respiration depending on their environment (Harris 2001). When most *C. reinhardtii* face iron depleting stresses (trace amount of iron in environment), they respond by distributing iron

from their chloroplasts to their mitochondria so as to maintain respiration and attempt to ensure cell survival (Glaesener *et al.* 2013). This may have induced increased respiration in the 0x treatment, and therefore increased initial growth while the three treatments were adapting to their new environment.

Sources of Variation/Error

There was high variation in our results and this can be explained by a number of factors. The growth trends of our organisms in our three iron treatments still appear to be following an exponential growth trend at day 23 and therefore are still in log phase growth. Using a higher starting abundance of cells and allowing the cell populations time to plateau and reach stationary phase could decrease variance and increase the chances of observing significant differences between our treatments.

Data from days prior to day 6 was discarded due to very low cell counts and the initial cell abundances being too low to possibly provide significant differences between treatments. When cell abundances are so low, there are only one or two cells present on the haemocytometer which makes it difficult to make comparisons. The initial cell abundances were much lower than intended as initial cell abundance in the zero iron solution of 10,000 cells/ml was not achieved. We attribute this to either an error occurring in the cell separation step, with not all cells being re-suspended, or some cells being destroyed by centrifugation. Also, as discussed earlier, the process of transferring the cells into the new treatment concentrations may have stressed the cells and resulted in some cell death.

Other errors may have contributed to the large variations in our results. For example, as we transferred solutions from container to container (either in the initial setup or during each

cell count), insufficient mixing may have occurred, which would result in the cell abundance in the transferred volume not being identical to the source solution. No initial cell counts were performed because as already mentioned, too few cells would be present on the haemocytometer to provide an accurate measure of cell abundance, and therefore we had to assume that each treatment would contain equal initial cell abundances as they came from the same source solution. As a result, there is a chance that each treatment may not have contained exactly the same amount of cells initially, which could lead to significantly more cells in the long run due to the exponential growth pattern of *C. reinhardtii*. Also, inaccurately pipetting at any stage may have contributed to the variations in our calculated concentrations, either of the initial iron solutions or of the cell abundances during any of our cell counts. The process of performing the cell counts themselves may have also contributed errors; for example, one researcher may have incorrectly identified cell fragments and debris as *C. reinhardtii* cells. This would increase total cell counts for the replicates that they counted and would result in a larger confidence interval for that treatment as this would differ to replicates counted by another researcher. We tried to limit the possible effects of this potential error by ensuring that a number of researchers performed cell counts on the same treatment and therefore, any differences in counting would be averaged out. However, a combination of these errors could contribute to our large confidence intervals and reduce the chances of finding significant differences between treatments.

Conclusion

Chlamydomonas reinhardtii demonstrated exponential cell growth under all of the four iron concentration treatments ranging from 0 µM to 92.4 µM over the 23 day interval with the exception of the zero iron treatment, which showed no growth after day 10. The concentration of iron has been shown to have a positive effect on cell growth, with the cell abundance increasing the most in the double iron treatment followed by the standard and half iron concentrations showing equal growth, and the zero iron concentration showing the least growth. Therefore as the results follow the hypothesized trend, we can support our alternate hypothesis, which states the growth rate of *Chlamydomonas reinhardtii* with respect to increasing iron concentration in growth medium will increase, and we can reject our null hypothesis.

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Appendix A

Table 4: Standard Chlamydomonas cell culture media used in UBC Biology Department. Based on a modified version of Sager and Granick's medium http://www.chlamy.org/SG.html.

Compound	Stock Solution (g/L)	Use For Culture (ml/L)
KH_2PO_4 -$7H_2O$	20.0	5.0
K_2HPO_4	26.0	5.0
$FeCl_3$	12.5	1.0
$MgSO_4 – 7H_2O$	60.0	5.0
$CaCl_2$	95.0	0.5
Na_3citrate – $2H_2O$	100.0	1.0
NH_4NO_3	120.0	2.5
Trace Metals:		
H_3BO_3	4.0	1.0
$ZnSO_4 – 7H_2O$	4.0	
$MnSO_4 – 4H_2O$	1.6	
$COCl_2 – 6H_2O$	0.8	
$CuSO_4$	0.2	
NH_4Moltbdate	0.8	