Effect of varying pH adjusted media on the growth rate of *Chlamydomonas reinhardtii*

Gurjot Dhaliwal, Ted Lee, Divya Talwar, Owen Wang

Abstract

Changes in the pH of marine environments can have a major impact on the growth and survival of primary producers. Since the marine food chain is largely comprised of algae, and changes in lower trophic levels can cascade throughout higher trophic levels, it is important to enhance our understanding of how variations in pH affect algal growth. The objective of our study was to investigate the direct effect of pH on the growth rate of *Chlamydomonas reinhardtii* at pH 5, 6.7, and 8. *C. reinhardtii* was exposed to pH adjusted media over a set period of time and periodic cell counts were conducted using a hemocytometer and Zeiss Axiostar compound microscope. A one-way ANOVA test was used for statistical analysis, which depicted that growth rate was highest at the optimal pH of 6.7, lower at pH 8 and lowest at pH 5. The calculated p-value of 0.00036 ($p<0.01$) indicates that pH has an effect on growth rate of *C. reinhardtii*, as growth decreased significantly when exposed to pH conditions which deviated away from pH 6.7. Our findings suggest that environmental pH is a critical factor in achieving optimal growth for *C. reinhardtii*.

Introduction

The rising level of atmospheric carbon dioxide (CO_2), mainly driven by the burning of fossil fuels and deforestation, has been linked to ocean acidification and negative impacts on marine organisms. Increased uptake of atmospheric CO_2 by the ocean induces a reduction in pH, which may adversely influence the growth and survival of marine species (Brierley & Kingsford, 2009). A significant threat is posed on primary producers, which form the foundation of the ecological pyramid. In particular, a green algae named *Chlamydomonas reinhardtii* serves an integral role in the marine food web. Since most marine food chains begin from primary producers, changes in pH conditions can disturb the energy flow from algae to upper trophic levels (Guo et al., 2016), including the energy transferred to keystone species such as salmon (**Figure 1**).

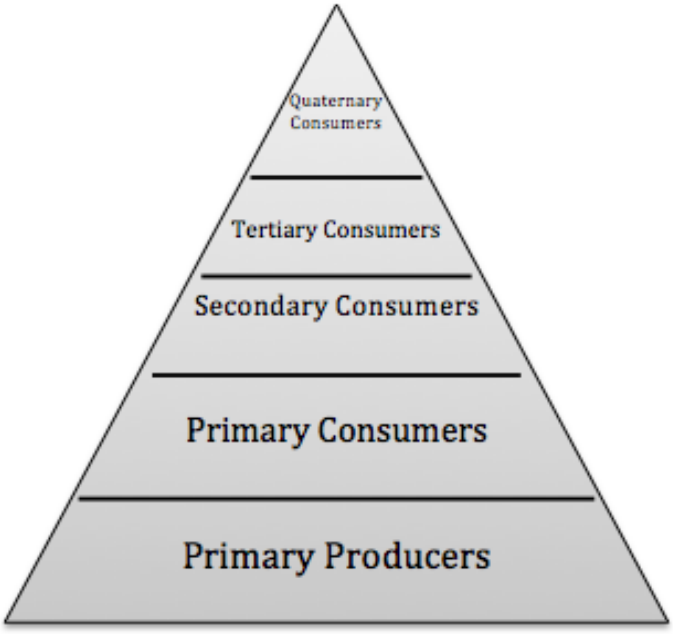

Figure 1. An ecological pyramid representing lower to upper trophic levels. Primary producers, including *C. reinhardtii*, form the foundation of the ecological pyramid while salmon are found in the upper trophic levels.

The objective of the study is to gain insight into the complex interactions between the growth of *C. reinhardtii* in varying pH conditions and the availability of energy for major keystone species, such as salmon. Yearly returns of spawning salmon contribute to the inflow of nutrients and organic matter, which in turn enhances the overall productivity of an ecosystem by providing a food source for organisms in higher trophic levels (Holtgrieve & Schindler, 2011). However, salmon depletion has become a growing concern over the past few years. Taking the marine food web into consideration, the problem behind declining rates of salmon returns may possibly be related to food scarcity beginning with lower trophic levels.

Based on literature, *C. reinhardtii* is unable to grow when exposed to extreme acidic conditions, at pH 2 (Lustigman et al., 1995). Studies have demonstrated that electron transfer reactions which are catalyzed by cytochrome b6f complex are dependent on pH (Finazzi, 2002). Low pH conditions can delay such reactions and impact the overall energy production capacity,

and ultimately limit survival in acidic conditions (Finazzi, 2002). According to a study conducted by Messerli et al. (2005), the optimal pH range for *C. reinhardtii* growth was found to be between 5.5 and 8.5. While many studies focus on the effect of low pH conditions on algae, the other end of the spectrum, high pH, is not well understood.

In our research, we exposed *C. reinhardtii* to control pH 6.7 and treatment pH 5 and 8, and consequently measured the growth rate in each condition. Our proposed null hypothesis was that deviation from the optimum pH of 6.7, either above or below, would not increase or affect the growth rate of *C. reinhardtii*. Taking previous literature into consideration, we alternatively hypothesized that deviation from the optimum pH of 6.7, either above or below, would decrease the growth rate of *C. reinhardtii.* We predicted that the population size of *C. reinhardtii* has the potential to decline when exposed to suboptimal pH conditions.

Methods and Procedure

Preparation of samples

C. reinhardtii culture was grown in maintenance medium at pH 6.7 and incubated at 25℃. The pH adjusted media was prepared for each treatment level at pH 5, 6.7 and 8. An initial cell concentration of 2.5 x 10^5 cells mL^{-1} was needed for each treatment level. In order to do this, the stock culture was thoroughly mixed and 25 mL was divided into three falcon tubes, labelled pH 5, 6.7 and 8. To first separate the mixture containing *C. reinhardtii* cells and pH 6.7 media, the falcon tubes were centrifuged and the separated media was removed with a pipette. Using a micropipette, 100 µl of cell culture was transferred into an Eppendorf tube containing 10 µl of Lugol's iodine fixative (IKI). After the mixture was thoroughly resuspended using a

micropipette, 10 µl was transferred to a hemocytometer and covered with a coverslip. The hemocytometer was placed under the Zeiss Axiostar microscope and the initial cell count was recorded for each falcon tube containing *C. reinhardtii* cells. To obtain a final concentration of 2.5 x 10^5 cells mL^{-1} per treatment level, the volume of pH adjusted media required to dilute each individual treatment level was calculated. Once found, the specific volume of pH adjusted media was pipetted into the individual falcon tubes and vortexed. The procedure was repeated for each treatment level, until all three falcon tubes contained a cell concentration of 2.5 x 10^5 cells mL^{-1} in the correctly adjusted media (**Figure 3**).

Preparation of replicates

To prepare each replicate, 10 mL test tubes were labelled according to pH level (5, 6.7, 8) and replicate letter (A, B, C). Each treatment level contained three replicates. Using sterile techniques, 8 mL of the cell culture suspended in pH adjusted media was transferred to each of the nine test tubes. For instance, 10 mL of pH 6.7 cell culture was transferred into the correctly labeled test tube “6.7A”. Subsequently, each prepared replicate was resuspended and placed into the test tube rack. For the duration of the experiment, the replicates for each pH level were

incubated at 25°C and allowed to grow for twelve days.

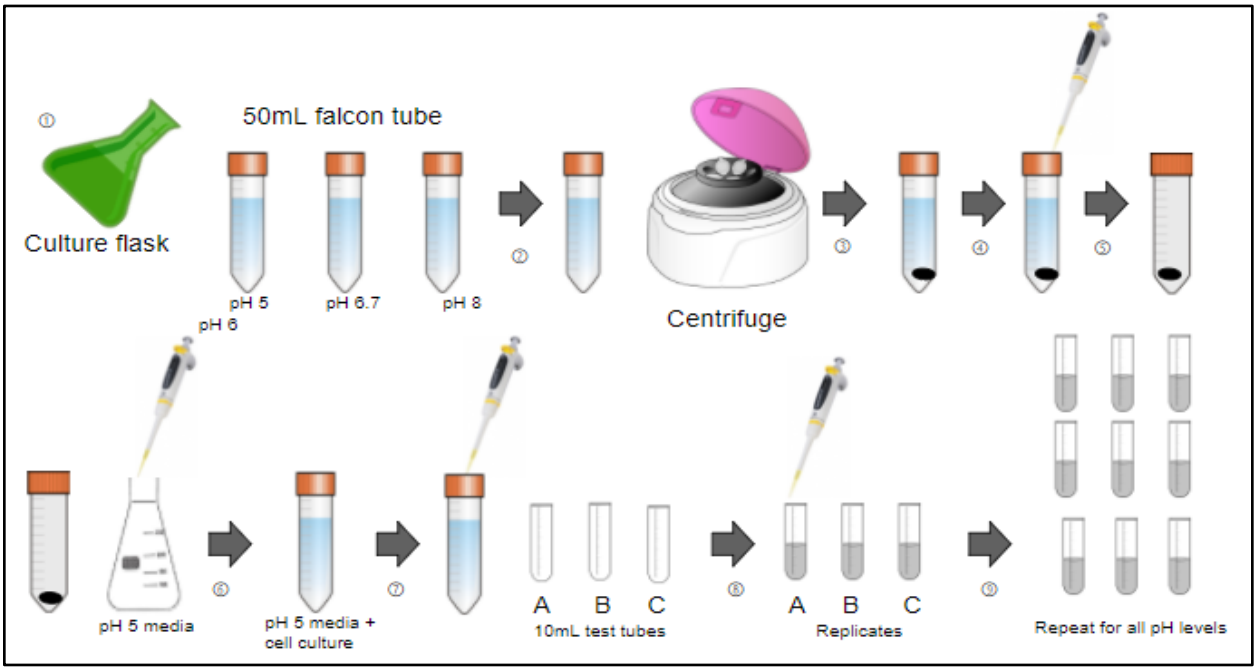

Figure 3. Flowchart illustrating the procedural steps taken to prepare each replicate per treatment level.

Counting cells

To prevent further growth of cells, samples were fixed on day one, three, five, eight, ten and twelve per treatment level. To fix cells, 10 µl of fixative (IKI) and 100 µl of culture were pipetted into properly labelled Eppendorf tubes and resuspended with a vortex. Cell counts were conducted on day five and twelve. After obtaining the samples from the incubator, each test tube was vortexed thoroughly and observations regarding their appearance were recorded. To conduct cell counts, 10 µl of each fixed sample was transferred onto a hemocytometer and placed under the microscope to count. To ensure accuracy, a tally counter was employed for each cell count. This was performed for each treatment level and replicate. Cell counts per treatment level were recorded and the cell concentration was calculated using appropriate dilution factors.

Furthermore, the average cell count per treatment level was calculated. To do this, the number of cells per square were calculated with the appropriate conversion factors and the number of cells per day were extrapolated.

Statistical Analysis

In order to determine whether the individual average growth is statistically different than each treatment level, a one-way ANOVA test was performed. In addition, growth curves were generated for each replicate within a treatment level over the twelve day period.

Results

It was found that in the control condition (pH 6.7), the number of *C. reinhardtii* grown was highest over the twelve day period. The pH 8 treatment was the second highest and the pH 5 treatment is the last, in terms of number of *C. reinhardtii* cells grown. We did a one-way ANOVA test for the daily growth rate within three replicates (A, B, C) and between three different pH treatments (5, 6.7, and 8). Statistical analysis showed that the Fcrit = 5.14 is smaller than the F value = 39.16. The p-value is 0.00036. Based on the analysis, it suggests that the results are statistically significant. This can be shown graphically in **Figure 5.** The growth curves for control (in green) are significantly higher than the growth curves for the pH 5 treatment (in orange). Likewise, the growth curves for pH 6.7 are above the growth curve for pH 8 (in blue), showing that growth rate for the control is higher than the pH 8 treatment. The average of the daily growth rate at three treatment levels are shown in comparison as bar graphs in **Figure 6**. The control had an average daily growth rate of 216445 ± 29624 cells/mL/day (mean ± 95%

confidence interval), while the pH 5 treatment had 80009 ± 7537 cells/mL/day, and the pH 8 treatment had 161862 ± 12222 cells/mL/day.

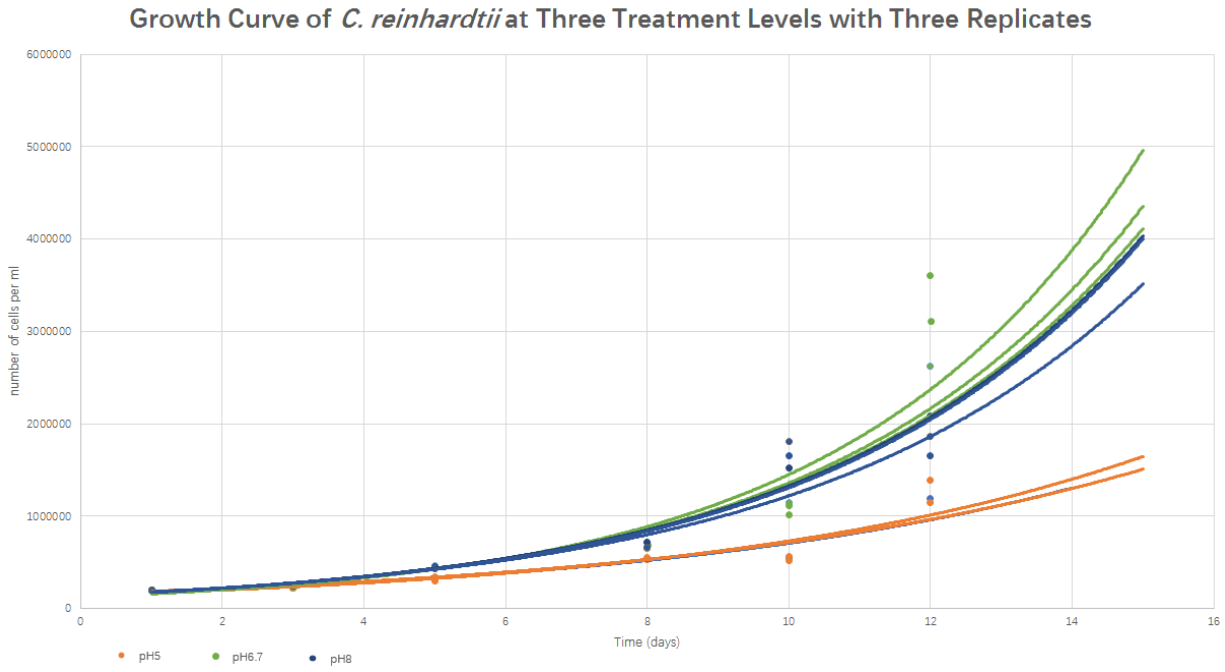

Figure 5. The growth curve of *C. reinhardtii* at pH level of 5, 6.7, and 8 with three replicates over a twelve day scheme (three day forecast makes it fifteen days). The solid line represents the best fit line (exponential curve) of the data points with x representing days and y representing the number of cells per ml.

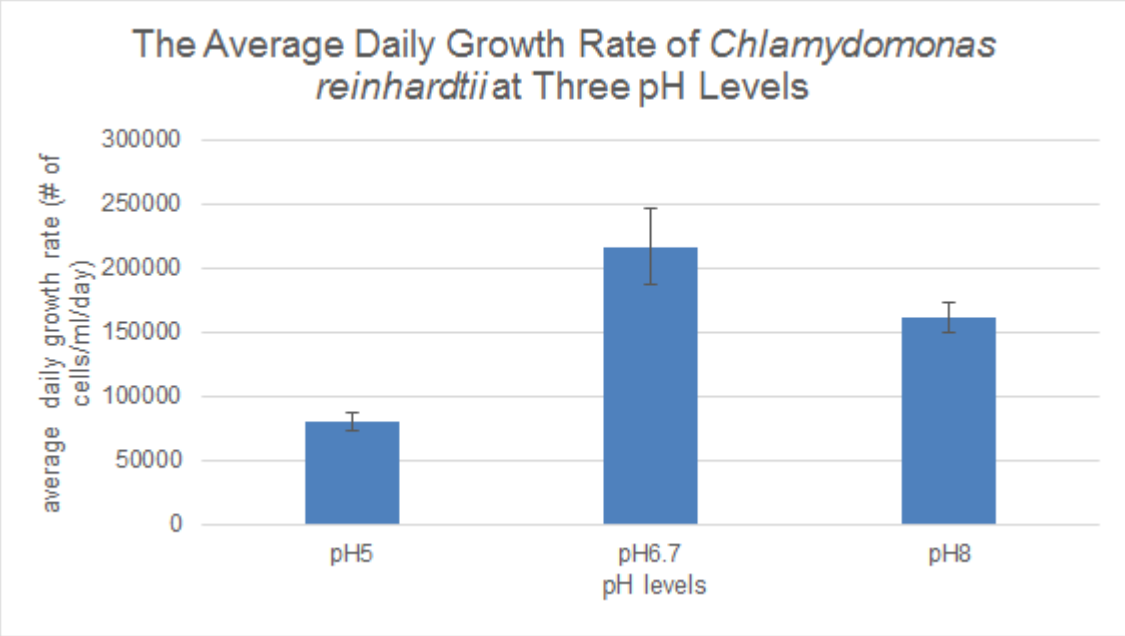

Figure 6. The average daily growth rate of *C. reinhardtii* at different pH levels. The bars represent the mean (± 95% confidence intervals) daily growth rate in cells/mL/day over 12 days at pH levels of 5 (n = 3), 6.7 (n = 3), and 8 (n = 3).

Sample calculations to demonstrate how the average daily growth rates were obtained are shown below.

For the pH 5 treatment:

N (number of replicates) = 3;
Sum (sum of the numbers of cells in treatment) = 77067+88574+74388= 240029;
Average = Sum / N = 240029 / 3=80010
Variance = ($\sum$ (Number of cells - Average)^2) / (N-1) = ((77067-80010)^2 + (88574-80010)^2 + (74388-80010)^2) / (3-1) = 56805114
Standard deviation = (Variance)$^{0.5}$ = (56805114)$^{0.5}$ = 7537

Discussion

The objective of this study was to determine how pH affects the growth rate of *C. reinhardtii*, with growth rate defined as number of cells per milliliter measured over time. Our experiments showed that the growth rate of *C. reinhardtii* is greatest at pH 6.7 (the optimum pH),

and decreases with deviations 2 pH levels above or below the optimum pH. This was determined for *C. reinhardtii* as this was approximately the pH condition of the habitat from which the organism was isolated. Statistical analysis revealed that the growth rate at pH 6.7 is significantly greater than at pH 5 and pH 8. Our prediction was that deviations from the optimum pH of 6.7 would contribute to a lesser growth response of *C. reinhardtii*. This was made on the biological basis that in algae, extreme pH conditions influence photosynthesis and growth (Gensemer et al., 1993).

One of the most important factors in algal growth is pH since it determines the solubility and availability of CO_2 and essential nutrients, as well as having a significant impact on algal metabolism (Juneja et. al, 2013). From a larger perspective, the overall structure of an ecosystem is adversely impacted when suboptimal pH conditions limit the growth of *C. reinhardtii*, meaning decreased energy availability due to insufficient productivity translates into reduced energy flow from lower to higher trophic levels (Guo et al., 2016). Our results were consistent with our prediction, indicating that pH had significant influence on the growth of *C. reinhardtii*. This allows us to reject our null hypothesis that deviation from the optimum pH of 6.7 would not increase or affect the growth rate of *C. reinhardtii*, and support our alternative hypothesis that deviation from the optimum pH of 6.7 would decrease the growth rate of *C. reinhardtii.*

Our count data results were likely directly influenced by human error and variability among group members. Although our method for fixing cells was consistent and controlled, counting with the hemocytometer introduced much more chance for error. All group members were involved in the counting process, but everyone has different perception and potential different classifications of what to count as a cell. Despite this, a guide on how to use to

hemocytometer was rigorously followed by all group members to minimize bias. If discrepancy or uncertainty in counting emerged, more than one group member would recount those particular cells. Our controlled methods may have contributed to our significant results obtained (p-value of 0.00036, which is less than 0.01).

Our finding that the growth rate of *C. reinhardtii* is greatest at pH 6.7, and decreases with deviations from this pH, is consistent with the results of similar studies. Ali et. al (2016) did an experiment on a similar species *Chlamydomonas noctigama*, testing the effect of pH on growth. Their results showed that the highest growth (4.052 μg mL^{-1}) was found at pH 6.5, and lower at pH 5, 5.5, 6, 7, 7.5, and 8 (Ali et. al 2016). In a second study done in 2013, a similar experiment was conducted on *Chlamydomonas applanta* to observe the tolerance of pH of the organism. Results showed that exponential growth occurred for up to five days at pH 5.4 and 8.4, but maximum growth was achieved at pH 7.4 (Juneja et. al 2013). Compared to both these findings, we see that our findings were comparable to theirs, strengthening the confidence in our results.

One limitation of this study is that we only measured three different pH treatment levels and used three replicates per treatment, due to the limited time we had in the laboratory. In addition, the experiment was run over the course of only two weeks, which is a relatively short time period to run an experiment. If this experiment were repeated, it should be carried out over a longer period of time. Also, it should have more incremental pH levels (perhaps 5, 5.5, 6, 6.5, 7, 7.5, and 8), to have more treatments for comparison. Finally, there should be more replicates per treatment, to strengthen the confidence in our findings and ensure that our results are not due to chance.

Conclusion

We found that the growth rate of *C. reinhardtii* was greatest at its optimum pH of 6.7 and decreased with deviations above or below this pH. Results were statistically significant and were consistent with literature, supporting our alternative hypothesis. Future experiments should also be conducted to strengthen the confidence in our results, as this work has significant implications on organisms in higher trophic levels, such as salmon.

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Appendix

Data Table for analysis

	Treatment (slope of the curve, so the numbers are the growth rate)		
Replicate Number	pH5	pH6.7	pH8
1	77067	213808	170131
2	88574	188227	147824
3	74388	247299	167632

Results Table

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance		SD
pH5	3	240029	80009.6667	56805114.3		7536.92
pH6.7	3	649334	216444.667	877589304		29624.1
pH8	3	485587	161862.333	149367352		12221.6

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2.8294E+10	2	1.4147E+10	39.1602658	0.00036	5.14325
Within Groups	2167523542	6	361253924			
Total	3.0461E+10	8				

Raw Data

Day	pH 6.7	ph 5	pH 8
1			
A	2.00E+05	200000	200000
B	200000	200000	200000
C	200000	200000	200000
Average	2.00E+05	200000	200000
3			
A	237600	227333.3333	250800
B	248600	220000	242000
C	231000	240166.6667	239800
Average	239066.6667	229166.6667	244200
5			
A	462000	316250	454666.6667
B	469333.3333	305250	447333.3333
C	443666.6667	346500	432666.6667
Average	458333.3333	322666.6667	444888.8889
8			
A	687500	539000	715000
B	715000	528000	665500
C	654500	555500	660000
Average	685666.6667	540833.3333	680166.6667
10			
A	1025000	550000	1519736.842
B	1145833.333	522500	1650000
C	1111956.522	566500	1804687.5
Average	1094263.285	546333.3333	1658141.447
12			
A	3116666.667	1188000	2090000
B	2625000	1388095.238	1650000
C	3609375	1145833.333	1870000
Average	3117013.889	1240642.857	1870000