Chlamydomonas reinhardtii and salinity: the effects of NaCl on population growth.

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Abstract

Salinity is an important abiotic variable prevalent in aquatic habitats and can have an effect on population growth, including that of algae. The effect of salinity on the population growth of *Chlamydomonas reinhardtii* was investigated using NaCl. The null hypothesis assumes that as salt concentration increases the growth of *C.reinhardtii* will remain the same as initial cell concentration. One alternative hypothesis is that as salinity increases the growth of the organism decreases. Lastly, our other alternative hypothesis states that as salinity increases so will the growth of the organism. We grew *C. reinhardtii* under four different NaCl concentrations (0μM, 100μM, 150μM, and 200 μM), taking samples six times non-consecutively over a period of two weeks. At the end of the two weeks, we used a haemocytometer to count cells. For our analysis, we performed a two-way ANOVA on the cell count data after two weeks to determine whether the differences in growth due to increased salinity was significant. we found that the effect of salinity on growth is significantly different in our control group which experienced exponential growth ($p= 6.373 \times 10^{-14,}$ at 0.05 significance level). For 100μM concentration, some significant growth was observed for specific treatments while for both 150μM and 200μM concentrations, no significant growth occurred. The data suggests that *C. reinhardtii* can grow in very little salt concentration, but exponential growth only occurs in the absence of NaCl. The implications of the experiment are discussed.

Introduction

Plants, algae, and one-celled organisms are often used as models to unfold basic biological processes as it is common practice in biological sciences to build upon simple models. *Chlamydomonas reinhardtii* is a unicellular photoautotrophic green microalga that uses flagella to swim. It can survive in many different environments from fresh water to damp soil and even the sea since it has evolutionary roots in aquatic and terrestrial environments. Furthermore, it can reproduce asexually or sexually, depending on environmental constraints. Because of its adaptability it is often used as a model organism for the study of the effects of certain stress factors at a cellular and molecular level, especially salinity, since high salt concentrations in the environment is directly responded to by individual cells (Yokthongwattana et al., 2012).

Salinity of an aquatic environment is one of the important abiotic factors that controls biological processes in organisms. More specifically, salinity stress is a major restricting factor in plant productivity and photosynthesis (Boyer, 1982 as cited in Yokthongwattana et al., 2012). Furthermore, excessive salinity results in osmotic stress leading to water loss, disruption of organismal internal salt concentrations and toxicity for plants and microalgae (Munns et al., 2006 as cited in Yokthongwattana et al., 2012). However, salts, in ambient concentrations allows for the preservation of concentration gradients and metabolic processes in microalgae and plants, since virtually all aspects of physiology

and metabolism for these organisms is affected by osmolarity (Perrineau et al., 2014). Naturally then, it is favourable for an organism to adapt to fluctuating salinity in the environment to maintain its internal functioning.

There have been many significant studies conducted on *Chlamydomonas* in relation to salinity which display its usefulness as a model organism. Takouridis and collogues (2015) increased the tolerance of freshwater *Chlamydomonas* to increasing salt concentrations (NaCl) using random mutagenesis for the first successful display of genome shuffling with microalgae. They introduced random mutations in the organism through UV radiation and then allowed the organism to grow in culture either sexually or asexually. They found that the resulting progeny, from both types of reproduction, were more tolerant of increased salinity such that the sexual line could grow in up to 700μM NaCl when previously only 300μM was tolerable. This is potentially useful in the production of biofuels since it is favorable for algal species being considered for commercial production to be resistant to environmental stressors.

This study aims to add to the discussion of the effects of salt concentrations on algal species. Since salt concentrations in aquatic environments are subject to external factors such as global warming, rainfall and run-off, insight into how algae can adapt to function even in increased salinity provides a basis for further research on more complex animals, such as salmon.

In this study, the growth of *Chlamydomonas* was monitored over a period of two weeks under various concentrations of NaCl to investigate the effect of increased salt concentrations on freshwater *C.reinhardtii* growth. The null hypothesis is that as NaCl concentration increases, the growth of *C. reinhardtii* will remain the same as the initial cell concentration (H_0). Our Alternative hypothesis is that as salt concentration (NaCl) increases the growth rate of *Chlamydomonas reinhardtii* will decrease since no gene shuffling is going to be conducted to aid tolerance (H_1). Further still, our other alternative hypothesis is that as salt concentration increases so will the growth of *C. reinhardtii* (H_2).

Methods and Materials

Algae culture

This freshwater *Chlamydomonas reinhardtii* strain was provided by Celeste Leander from The University of British Columbia. The cells were cultured under growth media provided by Mindy Chow (UBC), incubated at 25.7 °C with an initial pH of 6.5. We conducted initial cell counts using a

haemocytometer from a provided stock solution of the organism. Media was chosen for the growth of *C. reinhardtii* to have no NaCl to be able to better measure NaCl concentrations in each treatment.

Methodology

There were in total four salt treatments (0µM, 100µM, 150µM, and 200µM) and three replicates for each treatment (see figure 1 below). Using sterile pipettes and micropipettes, we placed in each test tube: sterile 400mM NaCl, growth media and equal amounts of our organism resulting in a final volume of 10 ml. Ideally, we wanted $2x10^5$ cells/ml in each test tube and equal concentration (5ml) of cells in all test tubes. To achieve this, we did initial cell counts using a Hemocytometer which gave an initial cell count of $8.58x10^5$ cells/ml. Then using serial dilutions, we changed the amount of NaCl and media for each treatment while keeping the amount (ml) of organism from stock solution in each test tube constant (see figure 1 below).

C. reinhardtii was grown in each test tube for two weeks in an incubator at a temperature of 25.7°C and we took samples six times (non-consecutively) in two weeks. We used sterile technique to micropipette 100µL of sample from test tubes in an Eppendorf tube and fixed with 10µL IKI fixative which renders *C. reinhardtii* immotile making cell counting easier. We conducted cell counts when all samples were collected. We vortexed samples, to resuspend the dead cells at the bottom, in each Eppendorf tube for ten seconds and placed them on a haemocytometer slide using a micro-pipette to count cells.

Analyses

To determine whether there were significant differences in population growth between different concentrations of NaCl over a period of two weeks we analyzed the data using Two-way ANOVA statistical testing (see hypotheses above).

	Amount of stock solution organism (ml)	Amount of media (ml)	Amount of 400 mM NaCl (ml)
0µM NaCl	2.331	7.669	0.000
100 µM NaCl	2.331	5.169	2.500
150 µM NaCl	2.331	3.919	3.750
200µM NaCl	2.331	2.669	5.000

Figure 1. Contents of each test tube of specific NaCl concentration. This was repeated three times to give three replicates with four salt concentrations treatments in each replicate.

Results

To determine if there was a change in growth due to salinity change, we graphed the growth curve of Chlamydomonas for the different [NaCl] of 0 µM (treatment 1), 100 µM (treatment 2), 150 µM (treatment 3) and 200 µM (treatment4).

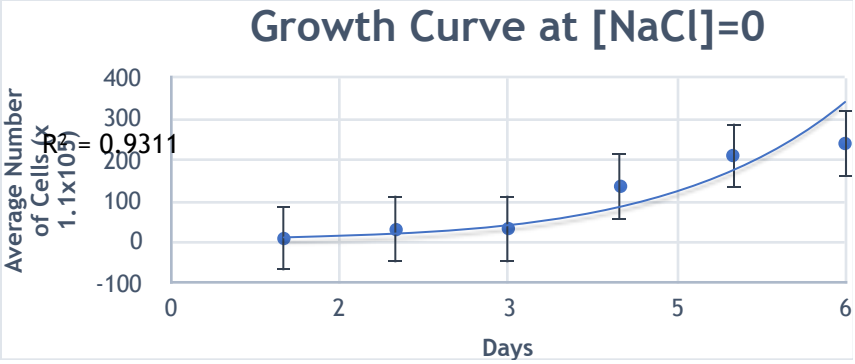

Figure 2) The graph shows the average number of Chlamydomonas reinhardtii cells over time at a NaCl concentration of 0 µM. The error bars indicate 95% confidence interval. The regression line is exponential.

In the control treatment, with no addition of NaCl, we observed exponential growth during the two weeks. The exponential regression model has a R^2 value of 0.9311. The number of cells increased from day 3 (33 x 10^5 cells/ml/day) to day 5 (210 x 10^5 cells/ml/day) and grew to a maximum number of 250 x 10^5 cells/ml/day. The size of the error bars represents the 95% confidence interval. There was a significant increase, as visually represented by the 95% confidence bars not overlapping, in the number of cells from the first 3 measurements to days 5 and 6.

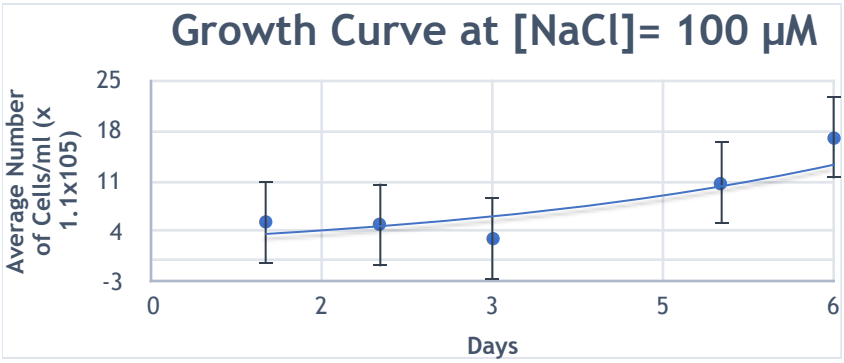

Figure 3) The graph shows the average number of Chlamydomonas reinhardi cells over time at a NaCl concentration of 100 µM. The error bars indicate 95% confidence interval. The regression line is exponential.

Figure 3 shows a regression line value of 0.6154 and that the only significant difference, as suggested by the 95% confidence interval curves, occurred between the first 3 days and day 6. Due to test tube for replicate 2 spilling accidently, data for the 4th day of this treatment was omitted.

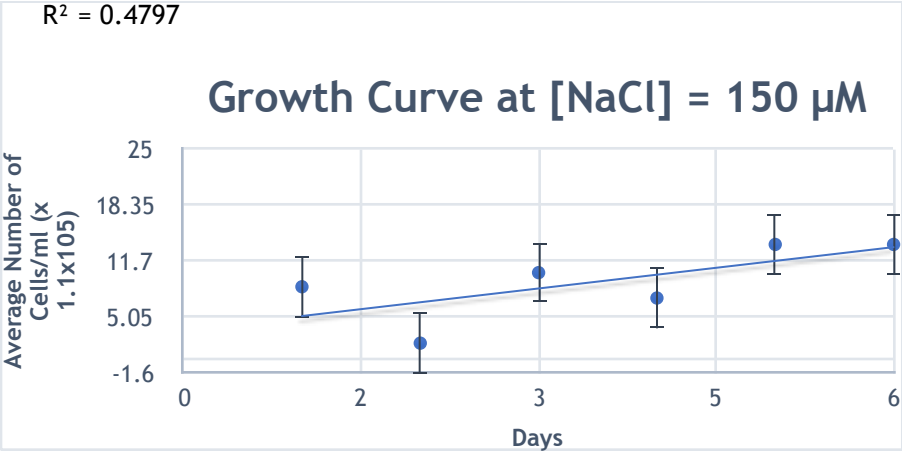

*Figure 4) The graph shows the average number of Chlamydomonas reinhardtii cells over time at a NaCl concentration of 150 µM. Measurements were done over 2 weeks on 6 different days. The error bars indicate 95% confidence interval. The best regression line is linear.*Figure 4 shows that *Chlamydomonas reinhardtii* did not experience any significant growth under 150µM from the first count to the last.

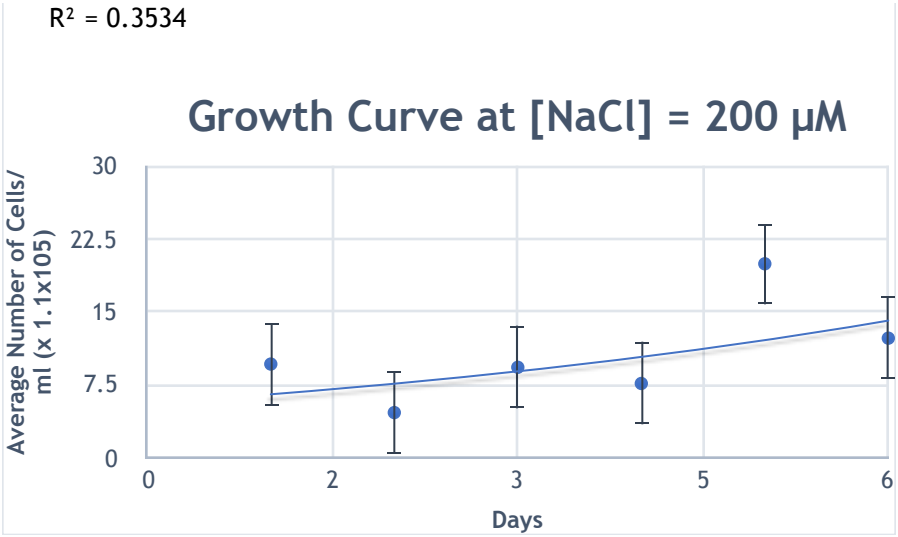

Figure 5) The graph shows the average number of Chlamydomonas reinhardi cells over time at a NaCl concentration of 200 µM. Measurements were done over 2 weeks on 6 different days. The error bars indicate 95% confidence interval. The regression line is exponential.

Figure 5 shows that *Chlamydomonas reinhardi* did not have a significant growth in cell count under 200μM over the course of the experiment.

We performed statistical analysis using two-way ANOVA to determine whether there is a significant difference in growth of *Chlamydomonas* between any of our treatments at a of 0.05 significance level. The analysis considers both the effect of time and salinity on the growth of the organism. We obtained a P-value of 6.373×10^{-14} for the effect of salinity on growth which indicates that the growth in at least one of the samples is significantly different than the others at indicated significance level which, in this case, happens to be our control group with no addition of NaCl.

The statistical test also considers time as another factor in determining whether our results are significant, for which we obtained a P-value of 2.842×10^{-6}. This indicates that the number of cells counted on different days are significantly different at 0.05 significance level. Finally, we obtained a P-value of 1.17×10^{-7} for the interaction of the two sources of variation (salinity and time), that is the effect of time at different salinity concentrations on the growth of the organisms. Since the P-value is much smaller than 0.05, our results are statistically significant.

Figure 6 below is a combination of the growth curves under the various concentrations of NaCl. The figure more clearly confirms the results of our statistical analysis. It is evident that at NaCl concentration of 0 μM (treatment 1), the growth rate is much greater than the rest. While Chlamydomonas cells grew over 200×10^5 counts in treatment 1 by day 6, they remained below 20×10^5 counts for the treatments with 100, 150 and 200 μM over the same period of time.

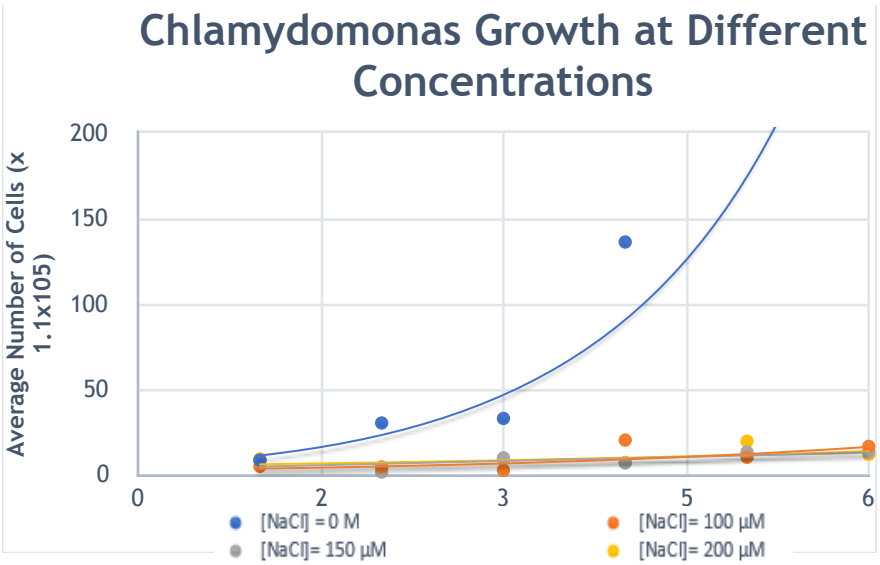

Figure 6) The graph shows the average number of Chlamydomonas reinhardtii cells over time at different NaCl concentrations of 0, 100, 150, and 200 µM. Measurements were done over 2 weeks on 6 different days. The average cell counts are multiplied by the dilution factor 1.1x10E5 to account for the dilutions. Error bars are not shown to enhance clarity.

Figure 7 and figure 8 below are pictures from our experiment. Figure 7 depicts the three replicates with 4 treatments of NaCl. The decline in the characteristic green hue of C. reinhardtii is clearly visible from the control treatments (starting from the left) to 200µM (Continuing to the right of the control). Figure 8 is a side by side comparison of the slides used to count cells of a control and 200µM treatment.

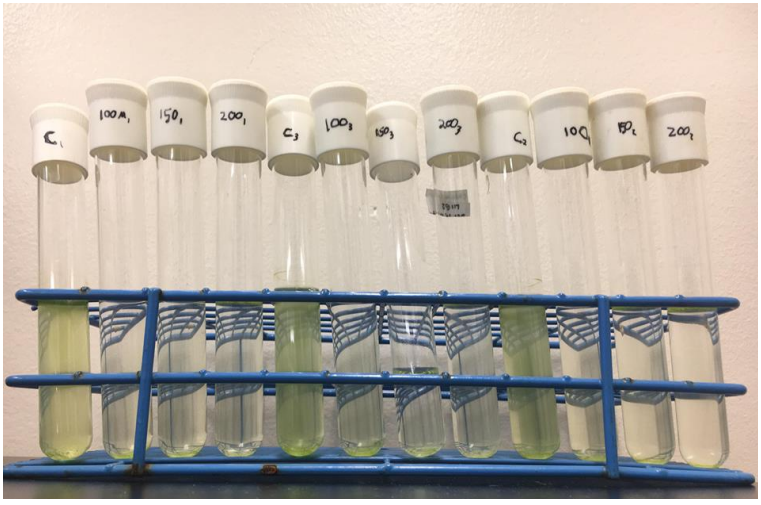

Figure 7. The test tubes photographed on the last day of sample collection. Three replicates are shown here, C stands for control and subscripts represent replicates.

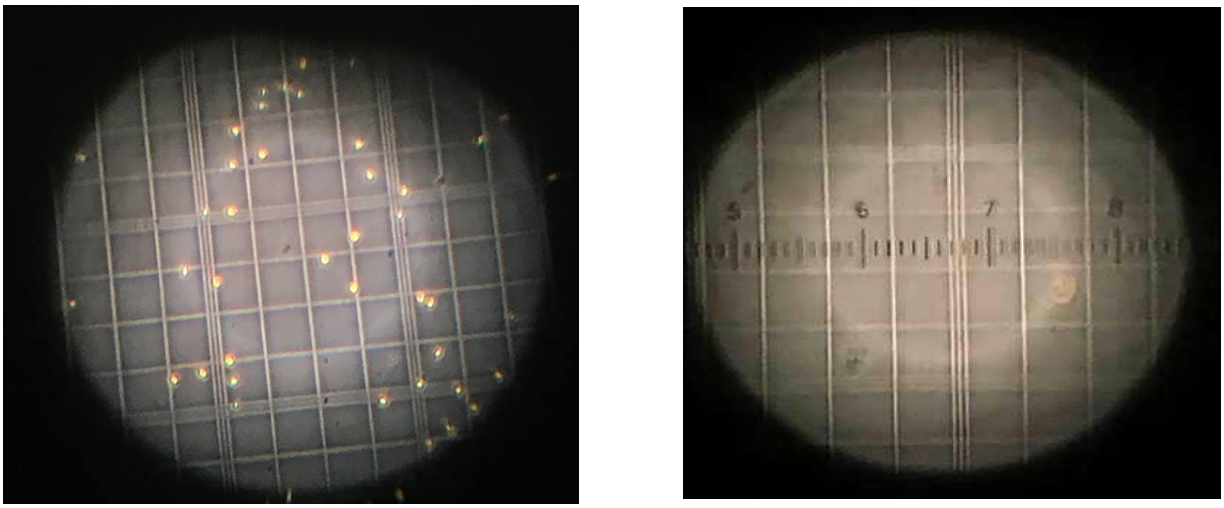

Figure 8. A haemocytometer photograph on the last day of sample collection. The green dots are C. reinhardi cells. The picture on the left is from a control test tube and the picture on the right is from a 200μM treatment test tube.

Discussion

Water salinity is a key abiotic factor in determining the quality of organismal life in aquatic environments. Further, algae are also a significant component of aquatic ecosystem as they form the foundation for the aquatic food chain, and are vital for other organisms as a food source (Norambuena et al., 2015). Because algae is connected to all aquatic life and since human activity near aquatic habitats will inevitably affect these organisms, it is important to determine the threshold of external factors such as salinity on algae to better understand overall health of an aquatic ecosystem.

In this study, we measured the effect of different concentrations of NaCl on the growth of *Chlamydomonas reinhardtii* to identify how changes in salinity may affect their presence in an ecosystem. Through the use of hemocytometers to perform cell counts, we found that *Chlamydomonas reinhardtii* is incredibly sensitive to even the smallest increase in salinity. As shown in our results, any treatment the included some NaCl prevented the *C. reinhardtii* from having any significant increase in population. Since the P-value we obtained for the interaction between salinity and time is smaller than our alpha level of 0.05 we have evidence to reject our null hypothesis that stated that salinity would have no effect on the growth rate of *C. reinhardtii*. Instead we have evidence to support our alternative hypothesis that as salt concentration increases the organismal growth of *C. reinhardtii* decreases (H_1).

Past research has shown that *Chlamydomonas* is capable of developing tolerance to NaCl up to 200μM (Perrineau et al., 2014). However, at 150μM NaCl there are morphological changes such as resorption of flagellum, reduced cell size and cell growth (Neelam and Subramanyam, 2013 as cited in Perrineau et al., 2014). This contrasts with our findings as the 150μM NaCl treatment shows no significant growth over the course of the experiment, though we did not look at morphological changes in detail. Similarly, another study also found that sexually reproducing *C. reinhardtii* could tolerate up to 300μM (Takouridis et al. 2015) NaCl, a vast difference from the results produced by this experiment. It was noted, however, that even though we did not measure the cell size of cells in different treatments, cell size did appear to be smaller in the treatments with salinity higher than 100uM, which is in agreement with past research (Neelam and Subramanyam, 2013 as cited in Perrineau et al., 2014). Contrary to our experiment, most literature used TAP as their medium for cell growth, whereas our study used a provided media. This may have had unforeseen effects on *C. reinhardtii* as some of the other salts in the solution could have also had inhibitory effects on its growth.

The main limitation of our experiment is the small number of treatments considered. The difference in salinity between the control group and the first treatment is double the difference seen between the other treatments. When taking into account that our findings show that 100uM is the highest salinity that *Chlamydomonas reinhardtii* can tolerate while still growing within this time frame, observing the change in growth at lower salinities would provide some insight into its sensitivity. In addition, our data shows particularly high standard deviations and error margins, which suggest that our methodology should be improved to reduce variations and increase precision in its execution. One way this could be improved is to reorganize researchers who are performing the data collection, specifically cell counts, in a way to reduce variance due to different ways of counting. Additionally, a test tube from replicate 2 for the 100μM NaCl was dropped accidently on the second last day of data collection and half contents were lost. However, this would not have major effects on concentration.

Further research could investigate other factors that correlate with cell growth. For example, our study produced visual confirmation that higher salinity samples did not have the characteristic green hue of *C. reinhardtii*. Recording and comparing the differences in light absorbency using spectroscopy methods could provide insight on how salinity affects the production of chlorophyll in *C. reinhardtii*.

Conclusion

Our study found that increasing salinity does have inhibitory effects on the growth of *Chlamydomonas reinhardtii*, though at a much lower concentration than we initially anticipated. Our findings reject our null hypothesis that salinity has no effect on the growth of *Chlamydomonas reinhardtii* and support our alternative hypothesis that salinity has an inhibitory effect on its growth. This study has shown that even small changes in salinity in aquatic ecosystems have the ability to cause serious damage to its microbiome. Thus, policy makers and members of the general public should be more aware of the effects of human activity on aquatic ecosystems and increase the effort to reduce any effect we might have on ecosystems near factories, farms, or sewage.

Acknowledgements

We would like to first thank The University of British Columbia for giving us the opportunity to take Biology 342 and providing us with the laboratory space and equipment to conduct this experiment. Next, they would like to thank Professor Celeste Leander for her assistance in forming the procedure for this experiment and for providing feedback during various parts of the experiment. Additionally, they would like to thank Mindy Chow for preparing stock solutions of the organism, preparing the growth media and for her overall help in equipment management and calculations. Finally, we would like to thank Megan Fass for her general feedback on this experiment and anonymous readers for their review of our experiment.

References

Norambuena F, Hermon K, Skrzypczyk V, Emery JA, Sharon Y, Beard A, et al. (2015) Algae in Fish Feed: Performances and Fatty Acid Metabolism in Juvenile Atlantic Salmon. *PLOS one, 4,* 1-18.

Perrineau, M.M., Zelzion, E., Gross, J., Price, D.C., Boyd, J., Bhattacharya, D. (2014). Evolution of salt tolerance in a laboratory reared population of *Chlamydomonas reinhardtii*. *Environmental Microbiology, 16,* 1755-1766.

Takouridis, J.S., Tribe, D.E., Gras, S. L., Martin, G.O. (2015). The selective breeding of the freshwater microalga *Chlamydomonas reinhardtii* for growth in salinity. *Bioresource Technology, 184*, 18-22

Yokthongwattana, C., Mahong, B., Roytrakul, S., Phaonaklop, N., Narangajavana, J. (2012). Proteomic analysis of salinity-stressed Chlamydomonas reinhardtii revealed differential suppression and induction of a large number of important housekeeping proteins. *An international Journal of Plant Biology, 235,* 649-659.

Appendix

Raw data

	Days																	
[NaCl] (M)	1									2					5			6
Trials	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
0		2750000	2200000	2640000	1320000	6160000	2750000	2530000	5720000	1408000	2794000	2970000	1826000	3432000	1716000	2046000	2915000	2981000
100	660000	440000	660000	99000	440000	220000	880000	110000	0	407000	187000	880000	154000	55000	143000	176000	176000	209000
150	1100000	440000	1320000	0	220000	440000	330000	1320000	1760000	132000	66000	440000	66000	176000	209000	187000	110000	154000
200	12100000	11000000	8800000	88000	550000	110000	1650000	660000	7700000	99000	110000	440000	209000	198000	253000	209000	110000	88000