<u>Effect of salinity on the growth of *Chlamydomonas reinhardtii*</u>

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Abstract

Over the years, human activities have led to increasingly saline conditions within freshwater ecosystems. This increase in salinity can have an impact on microorganisms including *Chlamydomonas reinhardtii.* It was hypothesized that increasingly saline conditions would lower growth rate and limit overall population size for this species of algae. A culture of *Chlamydomonas* at population equilibrium was diluted and subjected to three different conditions: ideal growth medium, 0 mM NaCl, 150 mM NaCl and 300 mM NaCl growth medium respectively. Three populations were developed in each condition for statistical significance. After 14 days, the cell density within each individual culture was counted using a hemocytometer, and data was analyzed using an ANOVA test. The results showed a negative correlation with increasing salinity and population size. However, this correlation was shown to be insignificant by way of statistical analysis. The lack of significance can be explained by many experimental errors stemming from a difficulty in homogenizing cell cultures for analysis. These errors in tandem with the statistical analysis results led to us being unable to validate or invalidate our initial hypothesis. Further research into the effect of increasingly saline conditions on *Chlamydomonas reinhardtii* is necessary to determine a significant relationship.

Introduction

Human activities, such as roadway de-icing, mining runoff, and agriculture, have increased the salinity of freshwater ecosystems (Kaushal S. et al., 2018). In Vancouver alone, an average of 5500 tonnes of road salt was used annually in the decade spanning from 1985-1995 (Birch R., 1998). The ensuing snowmelt, along with further rainwater, results in the transport of salt into the surrounding environment. While salinization affects species at all trophic levels of an ecosystem, the cascading effect originating from primary producers provides a strong motive for investigation into the salt tolerance of species at the base trophic level (Hintz W. et al., 2022).

One such example of a primary producer impacted by salinization is *Chlamydomonas spp.* which is a genus of green alga that inhabit a variety of soil and freshwater habitats. The model organism *C. reinhardtii* belongs to this genus and possesses several traits which lend itself well to laboratory study, such as a rapid doubling time of 8 hours when conditions are optimal (Sasso S. et al., 2018).

To characterize the impact of salinity on *C. reinhardtii*, populations of the algae will be exposed to high (300 mM) and medium (150 mM) saline treatments along with control of 0mM, then have their cell counts observed after an 11 day growth period. It is expected that a decrease in *C. reinhardtii* population growth will be observed with an increase in salinity. We predict that less cell concentration will be observed in high salinity population compared to medium salinity, and control will yield the largest cell concentration. The null hypothesis of the study is that we will see no difference between all the treatments. (Takouridis S. et al., 2015)

Methods

Making Treatments and Populations

A tube with a population of wildtype *C.reinhardt* grown in a growth medium for 2 weeks was obtained from our lab technician. Then, 3 samples from the growth medium were mixed with Potassium Iodide (fixative) to fix the cells in separate 500 ul plastic tubes, when the tube containing the *C.reinhardt* was opened it was flamed each time then before closing the tube was flamed again. Once all the cells were mixed with fixative, 10 uL samples were loaded onto a hemocytometer (Figure 1) where all cells were counted inside the 4 by 4 grid within the larger 5 by 5 grid until approximately 100

cells were obtained. The number of total boxes of cells was counted as well in order to apply in the cell concentration equation associated with the hemocytometer. The formula used divided the cell count by the number of boxes which contained approximately one hundred cells, then multiplied by a factor of 1.1 (which factors in the additional volume of the fixative into the solution) as well as multiplied by the 2.5 x 10^5 conversion factor used to determine the maximum number of cells in a 4x4 grid. Due to errors as described in the Discussion section later, the initial solution was diluted to a lower concentration than intended, using a low volume of initial population (3.63 mL) mixed with high amounts of growth media (92.63 mL). This resulted in a low initial population of "working culture," which was then divided into 9 separate populations, 3 per treatment.

To produce the treatments, a stock solution of 600 mM saline growth media was diluted with plain growth media to reach the desired concentration, and then had the derived cell population solutions added to each tube. The medium salinity treatment at 150 mM contained a mixture of 3.75 mL of 600 mM saline growth media, 3.75 mL of plain growth media, and 7.5 mL of the derived population group. The high salinity treatment at 300 mM contained a mixture of 7.5 mL of 600 mM saline growth media and 7.5 mL of the derived population group. The control treatment at 0 mM salinity contained a mixture of 7.5 mL of plain growth media and 7.5 mL of the derived population group.

Obtaining data

The cells were grown for 2 weeks under UV light and in an incubator set at 25 degrees celsius, with observations of cell growth being made every 1-3 days using a hemocytometer. The data was obtained by using the method to create fixed cells as described in the previous section “Making Treatments and Populations.” Although each population was vortexed until no large clumps were seen and was mixed again using the micropipettes to remove any smaller clumps before being added to the fixative. There were 2 pseudoreplicates per population, per treatment, for a total of 18 plastic tubes with fixed cells, each being counted using a hemocytometer using the equation previously used for creating “working culture.” In some cases, we counted all 400 boxes since cell populations were not high. This information was entered into Google Sheets using the formula from the above section. However, if cell count was lower as seen in the “High” salinity populations, the 5x5 grid and the 3x3 grid were used instead, meaning the constants multiplied in the formula changed from 2.5 x 10^5 to 1x10^4 or 1x10^3 for the cell concentration calculations.

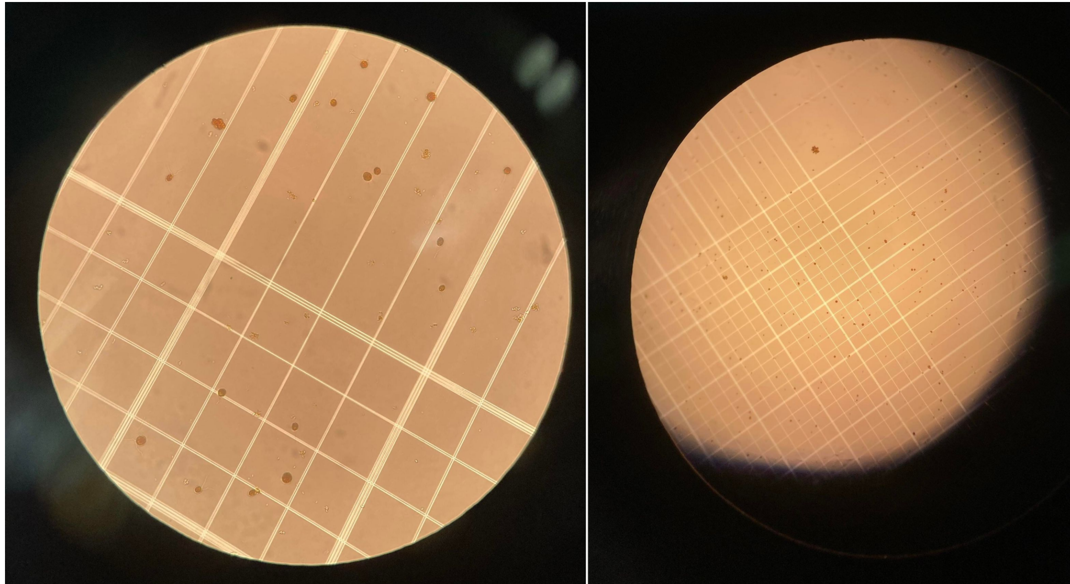

Figure 1: The appearance of *C.reinhardt* on a hemocytometer grid under the microscope on 40x magnification (left) and 10x magnification (right). Both images are from a sample of the diluted stock solution.

<u>Statistical Analysis</u>

Once data was obtained, an average was taken for each treatment population using the “Average” function in Google Sheets. Then, confidence intervals were calculated for each population in each treatment, with each standard deviation being measured using “ST.DEV” function and applying it to the confidence interval equation with an alpha value of 0.05 and n=2 for the pseudoreplicates; this was done using the “CONFIDENCE” function. A scatter chart was created on Google Sheets to compare the differences in each population through every treatment. Then for each treatment the population variance was compared using a Levene’s test, which consisted of comparing the pseudo replicates of each population to the average of all populations in a single treatment. Residuals were used for the comparisons, and one-way ANOVA analysis was performed on the residuals. This was repeated for each treatment to obtain 3

distinct p-values determining if cell concentration data was consistent in all 3 populations for each treatment.

Analysis comparing all populations in each treatment was done as well. The average of all populations in each treatment was taken along with the confidence intervals with a new “n” of 3 (accounting for how many populations in each treatment). Another scatter plot was made to compare the differences of each treatment type. To further analyze the differences, ANOVA was done using the XL miner analysis toolpak extension on Google Sheets to determine any significant differences amongst treatments.

Results

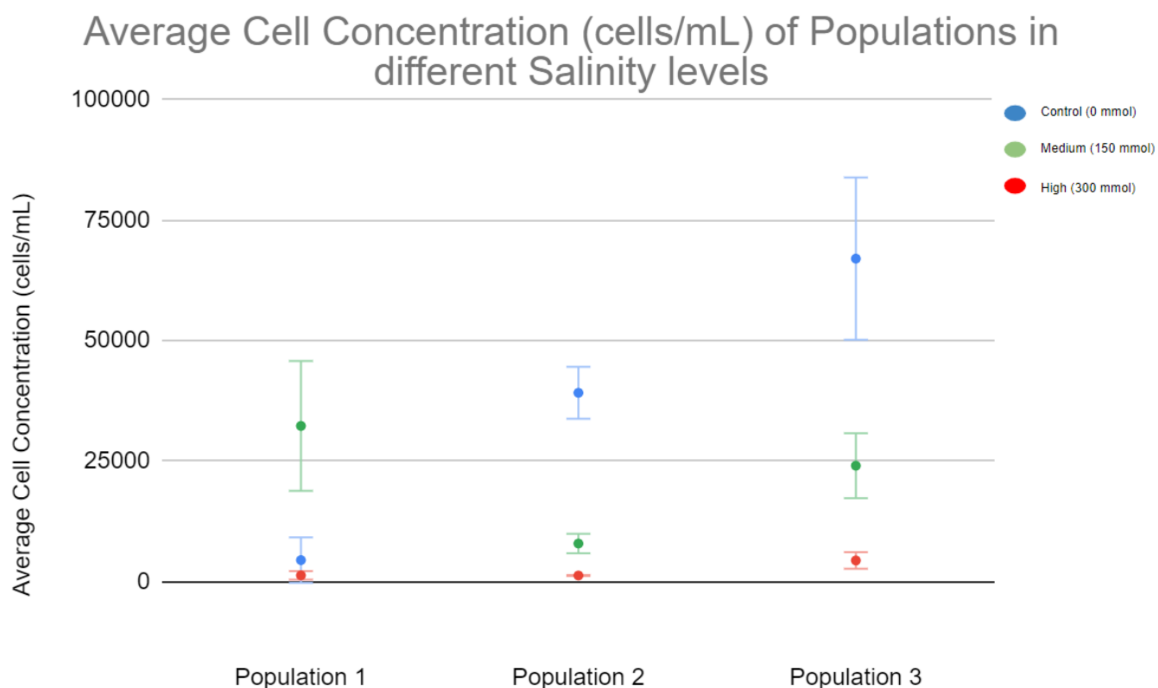

Figure 2: Data points are an average of 2 pseudoreplicates measuring cell concentration per milliliter of each population. The error bars represent 95% confidence intervals. There were 3 populations grown and measured per treatment type as indicated by the legend.

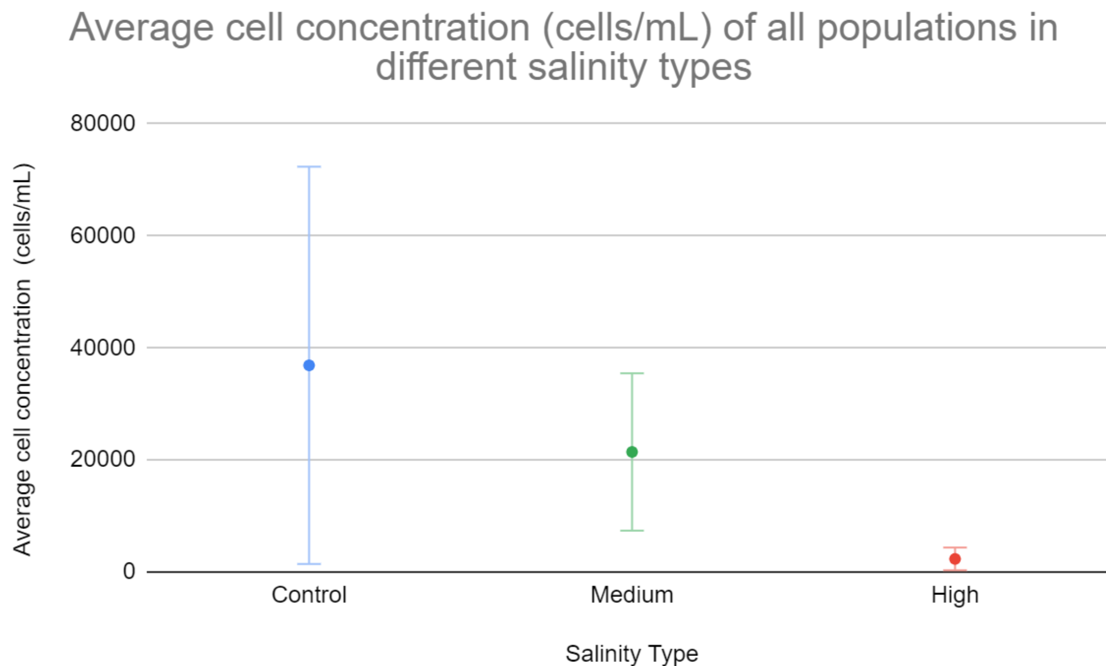

Figure 3: This graph shows a comparison of the populations that belong to each treatment: Control (0 mM saline solution), Medium (150mM saline solution) and High (300 mM saline solution). Each point represents the average cell concentration of all populations in each treatment; each population was an average of 2 pseudoreplicates. The error bars represent 95% confidence intervals.

Comparisons were made between populations as seen in figure 2. This shows the differences among the populations in the treatments of Control, Medium and High. For the control data, we found that none of the confidence intervals overlap; population 3 has the highest average cell concentration compared to populations 1 and 2, and population 2 had higher cell concentration than population 1. In the medium salinity treatment, overlapping confidence interval bars occur between populations 1 and 3, and both averages are significantly higher cell concentrations than population 2. The High salinity data shows overlapping of error bars between all 3 populations. A Levene's test

determined that medium and high salinity had p-values greater than 0.05, being 0.35 and 0.44 respectively, while the control was slightly below 0.05 as p-value was 0.049.

The graph in figure 3 represents the differences amongst the average cell concentration across all 3 treatments. Similar to figure 2, error bars represent the confidence intervals, which are compared to see whether overlap occurs. Figure 3 shows overlap between the control and medium salinity treatment, and between the control and high salinity treatment, although no overlap was seen between the medium salinity and high salinity treatments. An ANOVA test was done on the average of all pseudoreplicates of each population for the respective treatments. The test showed a P-value of 0.177, compared to alpha value of 0.05, results show $p>0.05$. The F-value found was to be 2.37 which was lower than F-crit value of 5.14.

Discussion

Data

Based on previous literature about the effects of high salt stress on eukaryotic green algae, such as a reduction in cell division rate, size, motility, and photosynthetic activity (Shetty et al., 2019; Bazzani et al., 2021), we predicted that increasingly saline conditions would reduce growth rate and limit population size. Our results appear to be consistent with this literature and our hypothesis, as we observed a substantial decrease in population size in the highest salinity treatment of 300 mM NaCl compared to the control on average (Figure 3).

In terms of statistical tests, a Levene's test was done to evaluate the equality of variances across populations in each treatment. In medium and high salinity treatments,

p-values of greater than 0.05 were calculated, meaning that we fail to reject the null hypothesis that population variances are equal However, in the control, $p<0.05$, meaning that we reject the null hypothesis and there is a significant difference in variance compared amongst populations.

A one-way ANOVA test was also performed, in which the calculated p-value was greater than the alpha of 0.05, and the F-critical value was greater than the calculated F-value, meaning that we failed to reject the null hypothesis. As a result, our lack of statistical significance between treatments prevents us from making any meaningful conclusions about the effect of salinity on the population growth of *C. reinhardtii*.

Despite the lack of statistical significance in our data, previous studies testing the effects of salinity on *C. reinhardtii* were consistent with the literature. A study conducted by Takouridis et al. (2015) testing the improvement of salt tolerance through selective breeding found that *C. reinhardtii* grew relatively well in typical freshwater conditions up until 200 mM NaCl, at which growth was significantly reduced and continued to reduce with increasing salinity.

Additionally, studies done on the same topic using methodology that closely resembled ours also found a similar, statistically significant trend when compared to the literature. Chen et al. (2018) and Atif et al. (2017) both compared the growth of *C. reinhardtii* in varying concentrations of salt (0 mM, 50 mM, 100 mM, 150 mM and 0μM, 100μM, 150μM, and 200 μM NaCl respectively) via hemocytometer counts over a two week time course and found that increasing salinity levels decreased growth rate.

Sources of Error

Due to a calculation error, the initial concentration of our stock-diluted sample was lower than intended, so the initial growth rate of *Chlamydomonas* was very slow in the first week. As a result, this made algae counting in the first week redundant as there were few, and in many cases, no countable cells in our samples when viewed under a microscope, causing us to require a longer span of time for meaningful growth observation. This error greatly increased the lag phase of our population growth and thus hindered our ability to observe the plateau phase and the entire sigmoid growth curve in our populations within our two week data collection period.

One unforeseen circumstance was that as time progressed and the algae population increased, much of the algae growth began to float to the surface and accumulate after incubation, forming a layer that was difficult to disperse. Our initial attempts to solve this issue included using a large pipette to draw up and resuspend the top layer into solution as well as vortexing the entire tube at a low speed, but both solutions yielded negligible results as the surface algae layer persisted. In fact, these efforts contributed to sample loss, as the surface algae began to stick to the inside and outside of the pipette tip when taken out of solution, as well as the sides of the sample tube itself after vortexing (Figure 3). This sample loss decreased the amount of algae present in solution after each attempt, contributing to error via the reduction of cells and thus, a decrease in growth rate.

(A) **(B)**

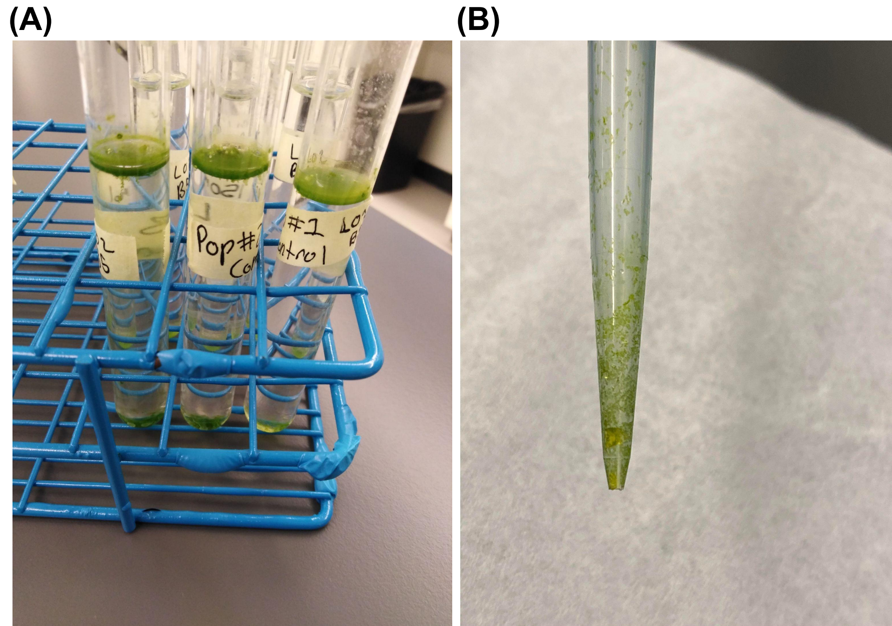

Figure 3: The figures show photographs taken over the duration of experiments. **(A)** shows photographs taken on Day 14 (March 21, 2022) of the experiments showing algae populations accumulating on the top of the growing media. All populations seen in this photograph are representing the Control. **(B)** Photograph shows that when the pipette tip has ejected its extracted media, algae sticks to the tip of the pipette. Image taken on Day 3 (March 10, 2022) of the algae growing period.

Ultimately, to mitigate this problem, we mixed the test tubes by inverting them upside down a few times to disperse the surface layer. Any remaining clumps of algae floating around in solution were then broken up by pipetting the solution in and out continuously until no more clumps were visible. Then during extraction, we would pipette such that the tip was in the middle of the solution to obtain a more consistent sample. A similar issue was also observed in which a minor amount of algae would accumulate on the bottom of the test tube, but was easily dispersed via mixing with a pipette.

For future studies, a wider range of temperatures and at smaller salinity increments could be tested to gain a better understanding of how growth rate and population size changes on a smaller scale. Additionally, future studies could also test

for how decreasing salinity affects population growth in C. reinhardtii, as much of the literature focuses on high salt stress and salinity tolerance. Utilization of more replicates over a longer time course compared to our experiment will also increase the statistical power of these experiments as well as allow the population to reach its carrying capacity, both of which were limited in our study due to time and errors.

Conclusion

The results of this experiment showed a slightly negative correlation between *Chlamydomonas reinhardtii* population growth and increasing salinity conditions which was predicted by our initial hypothesis. However, significant sources of error from surface algae growth, clustering of cells, and sampling errors contributed to a lack of statistical significance with the results. Thus no final conclusion can be drawn about how increasingly saline conditions affect the organism *Chlamydomonas reinhardtii*.

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Bibliography

- Atif, M., Lima, J., Mirahmadi, M., & Wong, B. (2017). *Chlamydomonas reinhardtii and salinity: The effects of NaCl on population growth.* The Expedition. Retrieved March 27, 2022, from https://ojs.library.ubc.ca/index.php/expedition/article/view/190211
- Birch, R. (1998, March 9). Administrative Report by the General Manager of Engineering Services . Vancouver, British Columbia; City of Vancouver.
- Chen, P., Ibrahimi, M., Jiang, J., & Yan, C. (2018). *Effects of salinity on the population growth of C. reinhardtii*. The Expedition. Retrieved March 27, 2022, from https://ojs.library.ubc.ca/index.php/expedition/article/view/191443
- Hintz, W. D., Arnott, S. E., Symons, C. C., Greco, D. A., McClymont, A., Brentrup, J. A., Cañedo-Argüelles, M., Derry, A. M., Downing, A. L., Gray, D. K., Melles, S. J., Relyea, R. A., Rusak, J. A., Searle, C. L., Astorg, L., Baker, H. K., Beisner, B. E., Cottingham, K. L., Ersoy, Z., … Weyhenmeyer, G. A. (2022). Current Water Quality Guidelines across North America and Europe do not protect lakes from Salinization. *Proceedings of the National Academy of Sciences*, *119*(9). https://doi.org/10.1073/pnas.2115033119
- Kaushal, S. S., Likens, G. E., Pace, M. L., Utz, R. M., Haq, S., Gorman, J., & Grese, M. (2018). Freshwater salinization syndrome on a continental scale. *Proceedings of the National Academy of Sciences*, *115*(4). https://doi.org/10.1073/pnas.1711234115

- Sasso, S., Stibor, H., Mittag, M., & Grossman, A. R. (2018). From molecular manipulation of domesticated Chlamydomonas reinhardtii to survival in nature. *ELife*, *7*. https://doi.org/10.7554/elife.39233
- Takouridis, S. J., Tribe, D. E., Gras, S. L., & Martin, G. J. O. (2015). The selective breeding of the freshwater microalga Chlamydomonas reinhardtii for growth in salinity. *Bioresource Technology*, *184*, 18–22. https://doi.org/10.1016/j.biortech.2014.10.120