Effects of Salinity on the Population Growth of *C. reinhardtii*

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Abstract

Chlamydomonas reinhardtii's growth is affected by a variety of abiotic factors, including salinity. Salinity is an important abiotic stressor that can inhibit productivity and growth. This experiment aimed to test the effects of salinity on *C. reinhardtii*'s growth. Effects of salinity on *C. reinhardtii* are important to study as they are a vital source of nutrition for salmon, a keystone species with major influences on the animal kingdom. The null hypothesis was that greater salinity will result in no difference in cell growth rate of *C. reinhardtii.* The alternate hypothesis was that changes in salinity concentrations will result in differences in cell growth rate of *C. reinhardtii*. It was predicted that the 0 mM NaCl sample will have the highest rate of cell growth, and lowest growth rate would be observed at 150 mM NaCl. Samples of *C. reinhardtii* were grown in 0mM, 50mM, 100mM, and 150mM NaCl concentrations, and were counted over a period of 13 days. Mean rate of cell growth of the *C. reinhardtii* within the four salinity concentrations were determined. Numerical values of salinity concentrations were converted to categorical factors using R. These values were inputted into a one-way ANOVA test, followed by a Tukey HSD test to determine whether changes in mean cell growth rate were significant. Based on the Tukey HSD test, all comparisons yielded p-values <0.05, with exception of the 100mM and 150mM NaCl growth rates. Our data showed that the 0mM NaCl sample had the largest growth, while the 150mM sample had the smallest growth. This demonstrates that as salinity increased, the population growth rate of *C. reinhardtii* decreased. Therefore, we concluded that while *C. reinhardtii* can grow in saline conditions, growth is significantly restricted by increased salinity.

Introduction

Chlamydomonas reinhardtii is a unicellular green alga which is primarily found in soil and freshwater. It has multiple mitochondria, two flagella for mobility, and a chloroplast holding photosynthetic systems and important metabolic pathways (Nickelson & Kuck., 2000). *C. reinhardtii* have been studied extensively for decades and offers a powerful model for understanding biological processes. Furthermore, *C. reinhardtii* has a predictable sexual cycle, a photosynthetic system enabling for growth in both light and dark conditions and can adapt to differing light and nutrient conditions (Grossman et al., 2003). *C. reinhardtii* have high metabolic flexibility allowing it to live in many different environments and can survive

fluctuations in nutrient availability (Merchant et al., 2007). Therefore, *C. reinhardtii* is frequently used as a model system to test the effects of stressors such as salinity. In this experiment, we tested the effects of salinity on *C. reinhardtii's* growth rate.

Salinity is an important abiotic factor that measures the amount of salts dissolved in a medium. Salinity stress is a major abiotic stressor that inhibits *C. reinhardtii's* productivity, making it an important area of study (Boyer, 1982). For instance, photosynthetic rates are decreased, resulting in eventual cell death. Increased NaCl concentration in the surrounding environment causes osmotic stress, resulting in water loss from plant cells (Yokthongwattana et al., 2012).

How *C. reinhardtii* behaves in different salinities is important due to *C. reinhardtii* being a significant source of nutrition for salmon (Norambuena et al., 2015). Salmon are "keystone species", highlighting their importance for a sustainable food system (Helfield & Raiman, 2006). Keystone species have a disproportionately large effects on ecosystems relative to its abundance (Power et al., 1996). As salmon are a fundamental source of nutrition for land and water ecosystems and are linked to multiple trophic levels (Hyatt & Godbout, 1999), it is important to understand the effects of salinity on *C. reinhardtii*, as it may have an effect on salmon .

As we are living in a world that is becoming increasingly saline due to the anthropogenic effects of climate change, it is important to understand the effects of salinity on our ecosystem. Increasing temperatures due to climate change is expected to increase irrigation of farmland and water evaporation (Edwards, 2016), leading to increases in salinity in soil and waters (Utset & Borroto, 2001). Research into how *C. reinhardtii* behaves in

increasingly saline conditions may help us understand effects of salinity on organisms dependant on *C. reinhardtii*, such as salmon.

Previous studies have shown that increasing salinity results in lower rates of *C. reinhardtii* cell growth and photosynthesis. Salinity stressors have a direct and indirect effect on *C. reinhardtii's* growth (Neale & Mellis, 1989). Salinity lowers light-saturated photosynthetic rates (direct-effect) and inhibits recovery of photodamaged cells (indirect-effect) (Neale & Mellis, 1989). Research has shown that 200mM NaCl is the maximum salinity *C. reinhardtii* can survive in. (León & Galván, 1994). *C. reinhardtii* is typically found in freshwaters, so its lack of survivability in saline waters is as expected. (Nickelson & Kuck., 2000).

To examine the effects of increasing salinity on *C. reinhardtii*'s growth rate, the growth rate of *C. reinhardtii* was tested over the course of two weeks under varying concentrations of NaCl. The null hypothesis was that greater salinity will result in no difference in cell growth rate of *C. reinhardtii.* The alternate hypothesis was that changes in salinity concentrations will result in differences in cell growth rate of *C. reinhardtii*. Given previous research, we predicted that the 0 mM NaCl sample will have the highest rate of cell growth, and lowest growth rate would be observed at 150 mM NaCl.

Methods

Preparation

Prior to the experiment, *C. reinhardtii* was cultured in media within a flask by a TA. The culture was mixed thoroughly once the culture was received. Using sterile technique, 100μL was pipetted and placed into an eppendorf tube that contained 10μL of the previously pipetted potassium triiodide (IKI), which was the fixative. The solution was mixed by

resuspension with a micropipette and 10μL of the mixture was transferred onto a haemocytometer (Perez, 2006). The cells on the haemocytometer were counted using a compound microscope, allowing the calculation of cells/ml in the solution with a sample dilution factor of 1.1 and square dilution factor (Fig 2) (Perez, 2006). Then using the same media used to culture the organism, the *C. reinhardtii* was diluted to $5.0x10^5$ cells/ml.

Within each labelled test tube, varying volumes of the media and provided NaCl solution of known concentration were pipetted to obtain NaCl concentrations of 0mM, 50mM, 100mM and 150mM in a final volume of 10ml. The volume of NaCl solution and media added resulted in volumes of 5ml. Then 5ml of the diluted *C. reinhardtii* solution was added into the test tubes of varying concentrations to obtain a total volume of 10ml in each tube, marking the start time of the growth curve. Therefore, the initial concentration of the organism was $2.5x10^5$ cells/ml. 4 replicates were prepared for each NaCl concentration, one replicate being an extra for unforeseen scenarios, resulting in a total of 16 test tubes.

Fixation and Counting

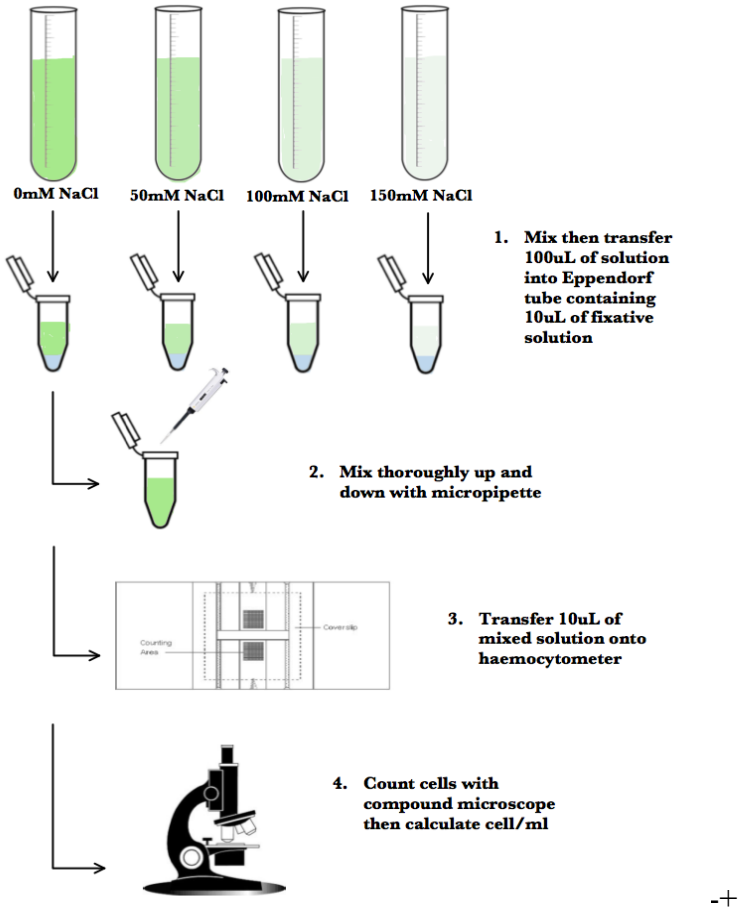

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Figure 1. **Method for fixation and counting of cells**. Steps 2-4 completed for all eppendorf tubes of varying concentrations. 3 replicates for each NaCl concentration, a total of 12 test tubes for whole process.

Eppendorf tubes were labelled and 10µL of fixative was pipetted into each tube. As illustrated in Figure 1, 100µL from each replicate test tube of *C. reinhardtii* for all salinity concentrations were pipetted into the designated eppendorf tubes. Prior to pipetting the sample, the test tubes were mixed by resuspension using a micropipette. Once placed into the eppendorf tube, the solution was mixed again to evenly distribute the fixative (Fig.1) and all were stored in a refrigerator for future counting. This was repeated in intervals of 2-3 days for 13 days.

To determine the cell concentrations of the fixed samples, 10µL of the solutions in the stored eppendorf tubes were pipetted and transferred to a haemocytometer. Using an Axiostar compound microscope with a 10x objective lense, the number of cells distributed were counted. Once counted, the cell concentration of each tube was calculated using a sample dilution factor of 1.1 and varying haemocytometer square dilution factors that altered based on the size of the square counted, as illustrated in Figure 2 (Perez, 2006). The view as seen through the microscope can be seen in Figure 3.

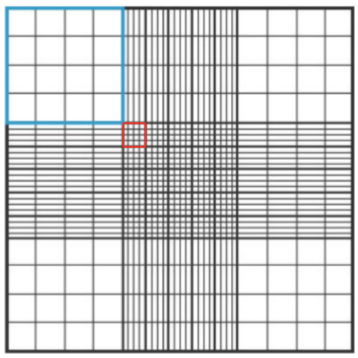

Figure 2 . Haemocytometer grid as seen under microscope. Red square required dilution factor $2.5x10^5$. Blue square required dilution factor of $1x10^4$. Whole grid required dilution factor of $1x10^3$.

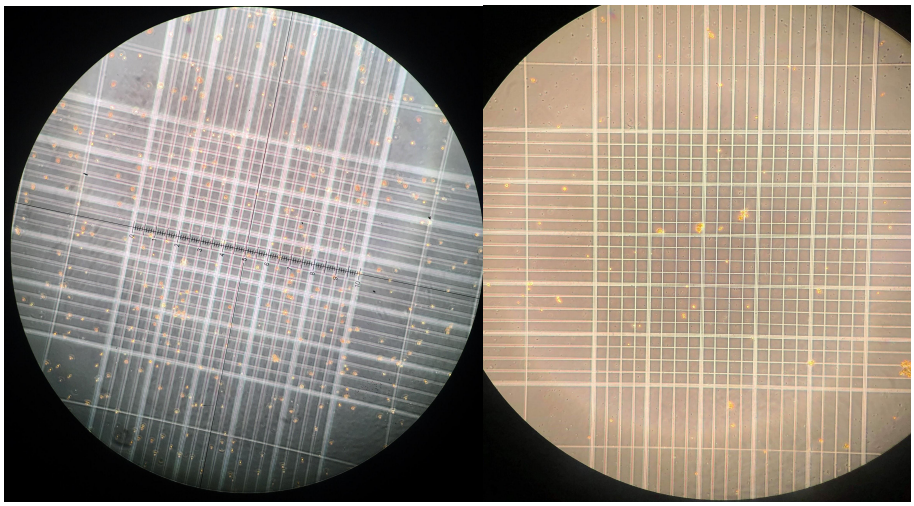

Figure 3. Cell Population of 0mM and 150mM NaCl concentrations placed on a hemocytometer. *C. reinhardtii* are seen as the glowing circular cells. Left is the view of the 0mM NaCl concentration. Right is the view of the 150mM NaCl concentration.

Data Analysis and Statistics

To analyze the data, the values recorded for each test tube were plotted on cell concentration vs. time scatter plots on Excel, yielding a total of 12 graphs. The best fit line of each plot was graphed and the slope was recorded to obtain the cell growth rate of the organism. Then for each salinity concentration, the rates obtained were averaged for mean cell growth rate, a sample size of 3 for each treatment. The standard deviations and 95% intervals for each average were also calculated through use of R commander. These values were all plotted on a rate of cell growth vs. salinity concentration scatter plot with the 95% confidence intervals graphed as error bars.

To statistically analyze our data, the mean cell growth rate of each salinity were inputted into R Commander to perform a one-way ANOVA test. A one-way ANOVA was selected as our data only had one independent factor and the entire dataset was divided into four groups, which prevented usage of other tests such as the t-test. The result of one-way ANOVA would indicate whether any of the mean cell growth rates of each treatment were statistically different from each other or not.

Once the one-way ANOVA was confirmed to be significant with $p<0.05$, a Tukey HSD test was performed to determine specifically which means were significantly different from each other. To perform TukeyHSD, “agricolea” was installed into R Commander which analyzed the ANOVA model and produce multiple p-values and 0.95 confidence intervals. A total of six comparisons were made within the Tukey HSD, obtaining a p-value for each comparison.

Results

Post data collection, calculations were done prior to making graphs and performing a one-way ANOVA Test. Time data was converted to number of hours for interpretation in R Commander.

Formula: days * (24hours / 1 day) + (minute / 60 seconds) = # of hours

Example of calculation: (7days * 24hours) + (1minute/60seconds) = 168.02hrs

After changing the variables and calculating the mean cell growth rates, the graph of figure 4 was created.

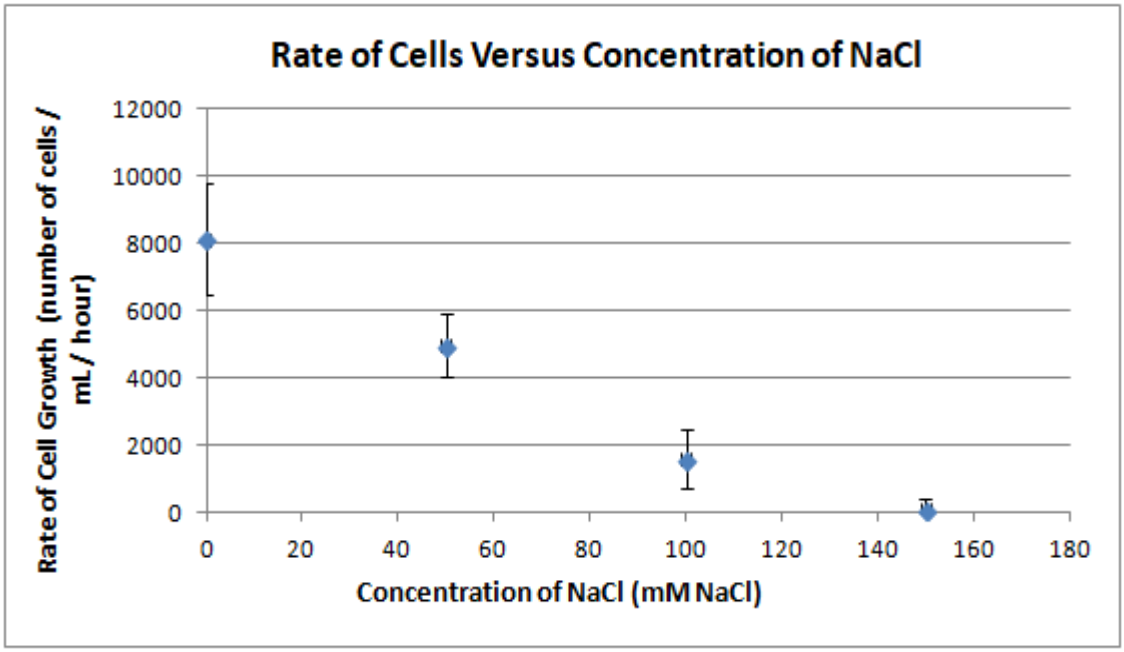

Figure 4. **Scatterplots of rate of cell growth of *C. reinhardtii reinhardtii* versus different concentrations of NaCl media, sampled from October 26th, 2018 to November 9th, 2018.** The sample size(n) for this graph is 12, and each point represents the average of cell growth rates of the three test tubes. Error bars represent the 95% confidence intervals.

By looking at the graph, the decrease of cell growth rate due to concentration of NaCl was visible, but further statistical analysis was performed in order to confirm the differences (Figure 4). The one-way ANOVA test was utilized to view the relationships between the cell

growth rate and concentrations. Since the p-value was 0.0000265 ($p<0.05$), the null hypotheses were rejected, and relationships between the cell growth rate and NaCl concentrations were confirmed. For further analysis of how different salinities affect cell concentrations, Tukey HSD was performed. The test resulted in six different p-values, which were 0.0144721 for 0-50mM NaCl, 0.0001298 for 0-100mM NaCl, 0.0000285 for 0-150mM NaCl, 0.0098475 for 50-100mM NaCl, 0.0010026 for 50-150mM NaCl and 0.2826761 for 100-150mM NaCl. Within the six comparisons, only the comparison between the 100mM and 150mM NaCl groups had a p-value greater than 0.05. Therefore the null hypothesis was rejected for all comparisons between the groups except the comparison of 100-150mM NaCl. Consequently, the results proved the alternate hypothesis that changes in salinity concentrations will result in differences in cell growth rate of *C. reinhardtii*, despite the 100-150mM NaCl p-value.

Discussion

Due to the effects of salinity on *C. reinhardtii*, we hypothesized that changes in salinity concentrations will have an effect of the cell growth rate of the *C. reinhardtii*.. A scatter plot displaying rate graph was created to show the different rates *C. reinhardtii* grew at varying salinities. The results from the one-way ANOVA test allowed us to perform a Tukey HSD test, which showed that cell growth rate between all four salinity concentrations were significantly different except between the groups 100mM and 150mM NaCl. Although insignificantly different from the 100mM NaCl group, the 150mM group was still observed as the lowest cell growth rate. This matched with our prediction that the highest observed population will be when salinity is at 0mM, and the lowest observed population will be when salinity is at 150mM. The null hypothesis was rejected, favoring the alternate hypothesis.

Previous studies investigated the effect of salinity on the growth of *C. reinhardtii.* (Fan & Zheng, 2017). Heterotrophic cultures were made coupling with salt and light stress, the cells were measured over a twenty-day period (Fan & Zheng, 2017). It was concluded that NaCl treatments generally suppressed cell growth and the high concentrations of NaCl dose causes permanent damage with high rates of cell death, as high as 45% (Fan & Zheng, 2017). This suggests high salinity is detrimental to the survival and reproduction of *C. reinhardtii.* It is evident that as salinity increases, the amount of cells significant decreased.

From the experiment, we have noticed that there were significant loss of cells when *C. reinhardtii* was initially put into the varying salinities. This is because *C. reinhardtii* requires time to adapt to the differing salinities. Acclimatization occurs when *C. reinhardtii* are put into mediums of different salinities. Initially, population count will likely decrease due to the inability to acclimatize, however, after several hours, cell growth will rise as the surviving cells adapt (León, 1999). *C. reinhardtii* grows slowly and is strongly dependent on light conditions (Oldenhof, Zachleder, & Van Den Ende, 2006), it is not able to grow much at the beginning of our experiment, leading to low results. Although our group began the experiment early in the project period to let the organism grow, it is crucial that future studies on the growth of *C. reinhardtii* to have a longer experimental time.

It was shown from the TukeyHSD test that there was no significant difference between salinities of 100mM and 150mM. This phenomenon was also shown in another study where they noticed that cells lost their green pigmentation and became yellowish under 100-200 mM NaCl conditions, whereas cells grown in 0-50 mM NaCl retained their dark-green pigmentation (Fan & Zheng, 2017). Based on this study, it was likely that the cells growing under 100mM and 150mM salinities within our experiment were both unhealthy, resulting in little difference in growth and thus lead to no significance difference between the

two groups from the TukeyHSD test. This further confirmed that *C. reinhardtii* grows well under low salinity and their population becomes depleted once salinity reaches over 100mM.

Ambiguity in the visual observation of the cells caused a large degree of variation existed in the number of cells that is counted from the hemocytometer, which may have been a possible cause of variation within the data. Although it was made sure that each person counted the cell samples in a staggered fashion to eliminate biased counting, the technique is subjective to each person's interpretation. Data demonstrates that even multiple counts of the same sample by different users reveal the imprecise nature of the tool and it takes a significant amount of time to count (Hsiung et al., 2013). Future studies should use an automated cell counter which decreases cell count time, as well as remove human subjectivity (Hsiung et al., 2013).

C. reinhardtii is a green algae which forms the base of the aquatic food chain, acting as a major resource of food for salmon (Norambuena et al., 2015). It is proven that small amounts of algae in salmon diets results in positive effects, such as growth performance, feed utilization efficiency, etc. (Norambuena et al., 2015). Therefore, knowing how ocean salinity affects the growth of *C. reinhardtii* is crucial to knowing the growth of salmon and rest of the ecosystem since it all depends on one another. Studies about ways to prevent the oceans from exceeding salinity levels should be done in the future since C.reinhardtii is known to grow up to a maximum salinity of 200mM NaCl (León & Galván, 1994).

Conclusion

In our experiment, greatest cell growth was observed for *C. reinhardtii* in salinity concentration of 0mM while the lowest was observed in 150mM, as coinciding with our prediction. Through the use of one-way ANOVA and Tukey HSD test, we determined

whether the decreases were significant enough to be statistically different. As a result, we obtained p-values smaller than 0.05 for all comparisons except the comparison of the 100mM NaCl and 150mM NaCl treatment groups. Therefore, we rejected the null hypothesis and supported the alternate hypothesis. This also allowed us to conclude that there was a significant interaction between salinity concentrations and cell growth. Based on this, we understand that salinity of the waters in our ecosystem must remain at low levels in order to allow the *C. reinhardtii* to thrive and continue to provide plentiful food sources for the keystone species, salmon.

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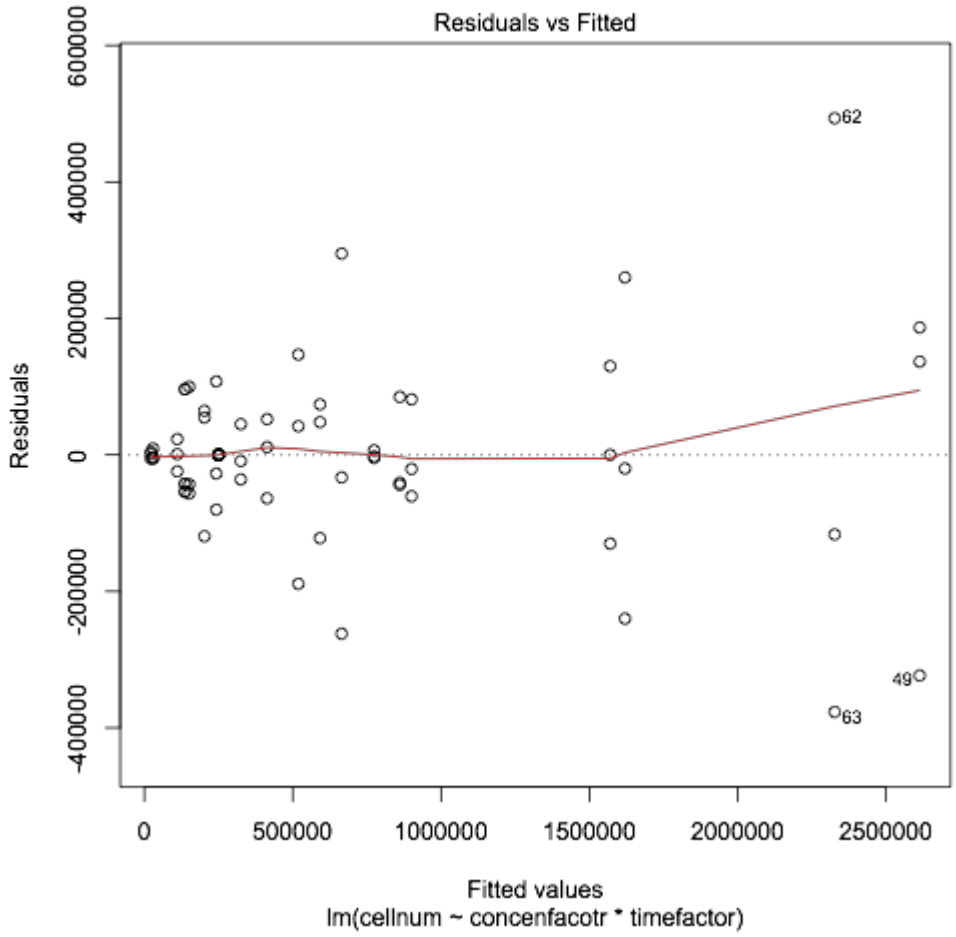
Appendix



Time (hour)	cell growth in 0mM NaCl (#cell/mL)	cell growth in 50mM NaCl (#cell/mL)	cell growth in100mM NaCl (#cell/mL)	cell growth in150mM NaCl (#cell/mL)
0	250000	250000	250000	250000
68.85	592000	151200	29100	22100
119.53	774333.3	519000	202366.7	136866.7
168.82	900666.7	861000	325000	136866.7
235.47	2613333.3	1570000	413666.7	110933.3
287.28	2326666.7	1620000	665000	242333.3

Output

```
> summary(AnovaModel.1)
             Df    Sum Sq  Mean Sq F value    Pr(>F)
concenfactor  3 115161568 38387189   43.63 0.0000265 ***
Residuals     8   7038397   879800
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
        mean        sd data:n
0   8126.337 1474.8812      3
50  4982.377  819.4355      3
100 1613.660  774.5890      3
150  122.580  269.1860      3
```

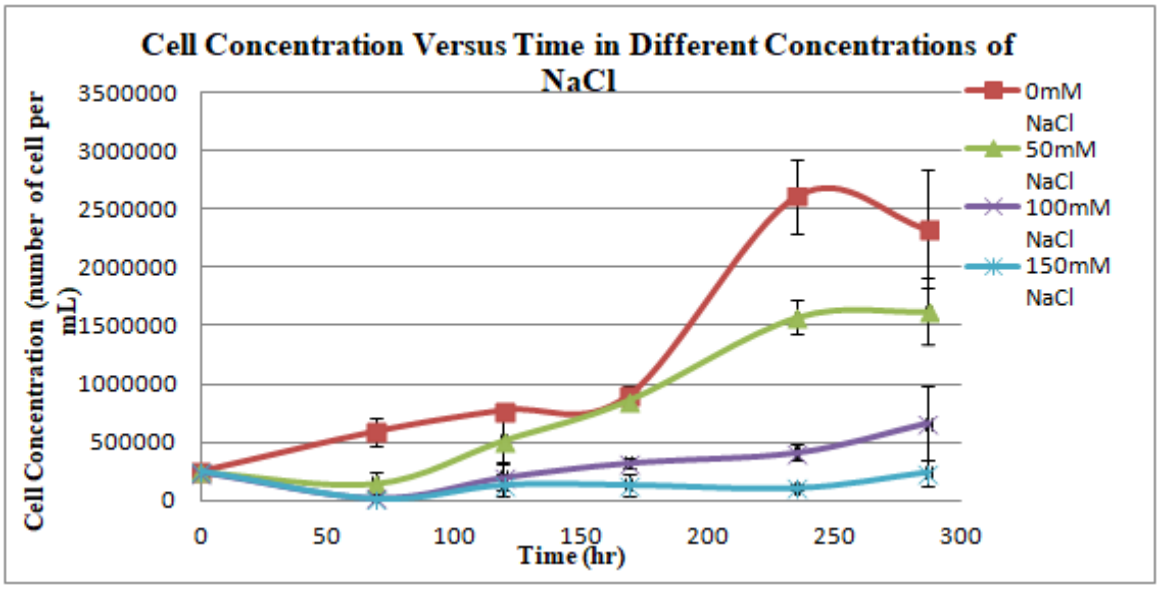
Confidence interval for 0mM NaCl	Confidence interval for 50mM NaCl	Confidence interval for 100mM NaCl	Confidence interval for 150mM NaCl
1668.9543	927.26152	876.5139	304.607

Tukey multiple comparisons of means
95% family-wise confidence level
factor levels have been ordered

Fit: aov(formula = cellrate ~ concenfactor, data = cellrate)

$concenfactor

	diff	lwr	upr	p adj
100-150	1491.080	-961.4556	3943.616	0.2826761
50-150	4859.797	2407.2611	7312.332	0.0010026
0-150	8003.757	5551.2211	10456.292	0.0000285
50-100	3368.717	916.1811	5821.252	0.0098475
0-100	6512.677	4060.1411	8965.212	0.0001298
0-50	3143.960	691.4244	5596.496	0.0144721


Cell Concentration Versus Time in Different Concentrations of NaCl
Cell Concentration (number of cell per mL)
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
0
50
100
150
200
250
300
Time (hr)
0mM NaCl
50mM NaCl
100mM NaCl
150mM NaCl