The Effect of Salinity on the Growth Rate of *Tetrahymena thermophila*

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ABSTRACT

The objective of our study was to determine the effect of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) on *Tetrahymena thermophila. T. thermophila* is a common ciliated protozoan in freshwater and is an important primary producer for many ecosystems. This study measured the growth rate of *T. thermophila* in 0mM, 2mM, 20mM and 200mM of $CaCl_2 \cdot 2H_2O$ (n=3). Cell concentration was measured in each test tube when first adding the organisms and then after 2, 4, 7, 9, 25 and 28 hours to find the overall growth rate. The results of this experiment showed that the 0mM concentration of salt had the highest average growth rate of 1259 cells/mL/h, followed by 2mM with a growth rate of 1164 cells/mL/h and then 20mM with a growth rate of 758.2 cells/mL/h. The 200mM concentration of salt had a negative growth rate of -186.1 cells/mL/h, with the cell concentration decreasing over time. Using ANOVA and Tukey's post hoc statistical analysis, it was revealed that the 200mM concentration was significantly different than the other three concentrations of 0mM (p=0.0001), 2mM (p=0.0002), and 20mM (p=0.0023) respectively. The other three concentrations were not significantly different from each other.

INTRODUCTION

Tetrahymena thermophila is a ciliated protozoan that is commonly used in research (Ayre et al. 1). *T. thermophila* can be grown in various environments like bacterized peptone or skim-based media and has a rapid doubling time of around two hours in its optimal condition (Cassidy and Donna 13). When it is searching for its food source, it shows transformation of its body shape such as growing of its posterior cilium and more coordinated propulsion. To obtain nutrients, this organism engulfs food particles through its oral cavity and make a membrane-bound food vacuole, which is also known as a phagosome, in order to assist the digestion (Collins and Gorovsky 1). The natural habitat of *T. thermophila* is almost every freshwater environment (Elliot 80). It reproduces exclusively by binary fission, and it exchanges its genetic material with other *T. thermophila* via conjugation (Orias et al. 579).

T. thermophila has a close relationship with salmon. Salmon live in freshwater during the juvenile stage and spawning stage of their life, which is also a habitat for wild *T. thermophila* (Bardonnet and Baglinière 497). In the same environment, the zooplankton serves its role as a

food source to salmon (Eggers 1114). At the same time, it prevents pathogens from infecting salmon and other animals higher in the food chain by engulfing the infected bacteria by phagocytosis (Pinheiro et al. 643). While the organism has beneficial effects, it can also be harmful to salmon. Based on a study by Ferguson (191), this protozoon can cause cranial ulceration in young salmon. It may also activate a Chum Salmon Reovirus, which has cytopathic effects according to Pinheiro (694). In addition, the organism can invade skin, muscles and internal organs of the salmon and lead to fatal diseases (Matthews 632). Providing both beneficial and harmful effects, *T. thermophila* can have various impacts on salmon.

Salinity is an extremely important abiotic factor that affects all organisms living in water. As salts occur naturally within soils and water, the associated cations are responsible for water salinization and can influence the chemical processes within aquatic organisms (El-Ashry et al. 48). The salinity level may alter naturally due to evaporation and precipitation of water since it can change the total volume of water. (Yadav et al. 667) However, human activity can also impact the salinity of water; irrigation for agriculture can cause salinity pollution and can increase the concentration of salt in both water and soil (El-Ashry et al. 48). Sodium chloride (NaCl) is one of the most common salts in the ocean (Taylor and Kuwairi 492) and many studies have looked at the relationship between NaCl and *T. thermophila*. Around 20mM of NaCl slows down the food vacuole formation, which affects the overall metabolism and the growth rate of *T. thermophila* (Juren et al. 7). However, the effect of the calcium cation on *T. thermophila* has not been well researched.

During a lecture given by Lekhi, it was stated that calcium is generally the most concentrated cation in freshwater systems and the calcium can enter the water system through various sources, such as erosion of limestone and dissociation of calcium carbonate ($CaCO_3$)

shells of some aquatic organisms under acidic conditions. And some previous literature has studied the relationship between this ion and *T. thermophila*, as in the study by Joglar-Ramirez and Renaud on the regulatory function of calcium in the regeneration of cilia in *T. thermophila* (413-417). However, the effects of calcium on the growth rate of *T. thermophila* have yet been examined. The initial experimental design of our study during development considered $CaCO_3$ as the salt compound, as it is a commonly occurring natural salt. But due to its striking insolubility in pure water (Joglar-Ramirez 415), $CaCl_2 \cdot 2H_2O$ was chosen instead.

The purpose of this experiment is to observe the impact of $CaCl_2 \cdot 2H_2O$, a calcium salt, on the growth rate of *T. thermophila*. Our null hypothesis is that $CaCl_2 \cdot 2H_2O$ would not have a significant effect on the growth curve of the organism and our alternate hypothesis is that the calcium salt will have a significant effect on the growth rate. Our prediction to the question was that the calcium salt would have a significant impact on the organism's growth curve because sodium chloride, a common salt, is known to affect the growth rate of *T. thermophila*. In addition, the natural habitat of the organism is freshwater so unusually high salinity caused by the calcium salt would have a negative influence on the organism's reproduction.

METHODS

Prior to the experiment, *T. thermophila* was cultured in the SSP medium which consists of 2% Proteose Peptone, 0.1% Yeast Extract, 0.2% Glucose and 33 µM $FeCl_3$ (Cornell University 1). The culture was mixed thoroughly using micropipette before transferring the solution, since most of the organisms settle down in the tube when left untouched for a period (Sanders 12). Using sterile technique, 10µL of the 3% Glutaraldehyde (fixative) and 100µL of the culture was pipetted into an Eppendorf tube, which was then mixed by resuspension with a micropipette. 20µL of the solution from the Eppendorf tube was transferred into Fuchs Rosenthal

hemocytometer, and the cells were counted using compound microscope and the dilution factor of 1.1. The concentration of cells in the working stock was around 1.58 x 10^4 cells/mL. Since the ideal concentration of *T. thermophila* before the growth curve is 2.0 x 10^4 cells/mL (Cassidy and Donna 9), dilution of the culture was not necessary.

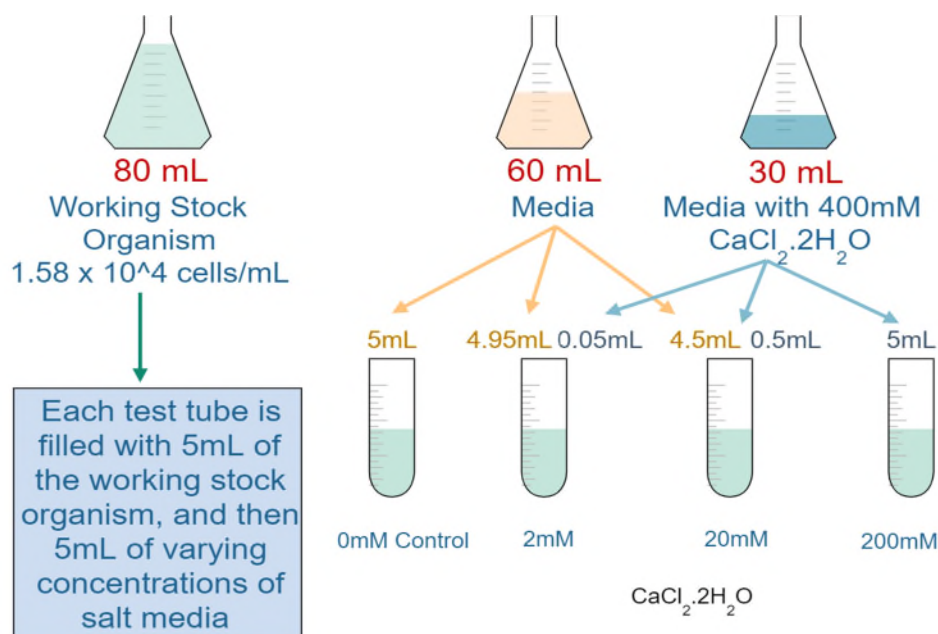

Figure 1. Steps for preparing the different concentration media for the *T. thermophila* growth curve.

In total, there were 4 different treatment groups for $CaCl_2{\cdot}2H_2O$: 2mM, 20mM and 200mM with one control which did not contain $CaCl_2{\cdot}2H_2O$. For each treatment, there were 3 replicates in order to minimize outliers. As illustrated by Figure 1, varying volumes of the media and 400mM of $CaCl_2{\cdot}2H_2O$ solution was pipetted into the test tubes, resulting in total volume of 5 mL with varying concentrations of salt within tube. 5 mL of working stock organism was then added to each of the test tubes resulting in the total volume of 10 mL and obtaining $CaCl_2{\cdot}2H_2O$ concentrations of 0mM, 2mM, 20mM and 200mM. Therefore, the initial concentration of the organism in every test tube was 7.9 x 10^3 cells/mL, marking the start time for the growth curve.

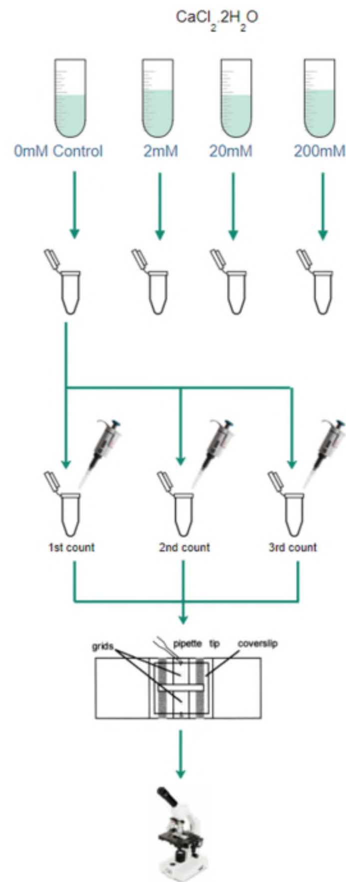

Figure 2. Steps for counting the number of cells in each treatment during various time intervals.

The test tubes were incubated for 28 hours at 30 °C as it is the optimum temperature for the growth of *T. thermophila* (Nagel and Wunderlich 151). After each time interval of 2, 4, 7, 9, 25 and 28 hours, 100µL of the solution was pipetted from the test tube into Eppendorf tubes, containing 10µL of the fixative, as illustrated by Figure 2. Then, 20µL of the solution was transferred into hemocytometer and Axiostar compound microscope with a 10x objective lens was used to count the number of cells. There were three separate counts from each Eppendorf tube to get the average number of cells. Once counted, the cell concentration of each time tube was calculated using a sample dilution factor of 1.1 and varying hemocytometer square dilution factors that altered based on the size of the square counted (Perez 6).

After transforming the cell counts to concentrations of cells/mL, the growth rate for each tube was calculated. This was done by taking the average count of cell concentration in each tube and plotting the values over time. The x-axis was transformed to $log_{10}[x]$ so that an exponential growth curve could be seen for each test tube. The slope of the two points which most accurately represented exponential growth was used as the growth rate for each tube. To statistically analyze the data, mean cell growth rate for each test tube was inputted in GraphPad Prism 8 to perform one-way ANOVA test between each concentration group. The result of the one-way ANOVA would indicate whether there was a significant relationship between salt concentration and growth rate or not.

For the post hoc analysis, a Tukey Multiple Comparison test was also used using GraphPad Prism 8 to analyze the mean growth rates between each group. This test was used to give us better insight on which specific groups were statistically different from each other. Tukey Multiple Comparisons test compared the concentration of 0mM vs 2mM, 0mM vs 20mM, 0mM vs 200mM, 2mM vs 20mM, 2mM vs 200mM and 20mM vs 200mM totaling in 6 different comparison and resulting in 6 different P values, and 95% confidence interval on the difference between the groups.

RESULTS

For each of the four salt concentration treatments, there were three replicates. The ANOVA test returned an F statistic of $F_{(3, 8)} = 30.58$ and a p-value of <0.0001. This test concluded that there was a significant effect of salt concentration on growth rate of *T. thermophila*. Figure 3 shows the average growth rate of *T. thermophila* in 0mM, 2mM, 20mM, and 200mM concentrations of $CaCl_2 \cdot 2H_2O$ to be M= 1259 cells/mL/h, 95% CI [473.0, 2046], M= 1164 cells/mL/h, 95% CI [528.0, 1800], M= 758.2 cells/mL/h, 95% CI [653.0, 863.4], M= -

186.1 cells/mL/h, 95% CI [-331.8, -40.52] respectively. This shows that the mean growth rate at each concentration on average decreased as the concentration of $CaCl_2 \cdot 2H_2O$ increased. 0mM had the highest mean, 2mM with the next highest, 20mM with the next highest, and 200mM with the lowest growth rate. The 200mM concentration was shown to have a negative growth rate, while the other concentrations had a positive growth rate (Figure 3). A post hoc Tukey Multiple Comparison test compared each mean to every other mean to determine if they were significant or not. This test found that the 200mM concentration was significantly different from the other concentrations of 0mM (p=0.0001), 2mM (p=0.0002), and 20mM (p=0.0023), though the other concentrations were not significantly different from each other.

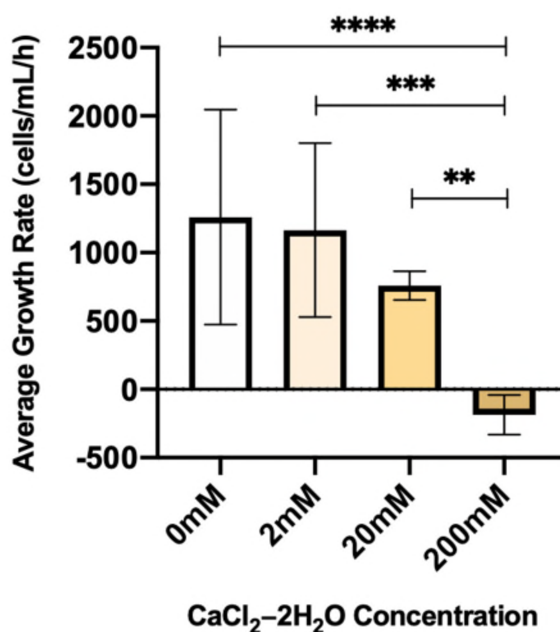

Figure 3. The mean growth rates of *T. thermophila* grown in the four salt concentrations (n=3) with bars representing the 95% confidence interval. An ANOVA test on the data resulted in a $F_{(3, 8)} = 30.58$ and a p-value of <0.0001. Significance between the means from a Tukey Multiple Comparison Test are shown as >0.05 = ns, ≤0.05=*, ≤0.01=**, ≤0.001 = ***, ≤0.0001 = ****.

DISCUSSION

The purpose of this study was to measure the impact of $CaCl_2 \cdot 2H_2O$ on the growth rate of *T. thermophila*. A one-way ANOVA test revealed that we were able to reject the null hypothesis,

indicating that $CaCl_2 \cdot 2H_2O$ concentration had a significant effect on the growth rate of *T. thermophila.* A Tukey post hoc analysis revealed that only the 200mM concentration was significantly different than the other concentrations of $CaCl_2 \cdot 2H_2O$.

We established that a $CaCl_2 \cdot 2H_2O$ concentration above 0mM have an adverse effect on the organism and as the concentration increased, the mean growth rate of *T. thermophila* also decreased. Although the rates were different, cells proliferated and the organism still grew over time in 0mM, 2mM, and 20mM solutions (Figure. 3). In 2mM solution, the growth rate was slower than that of the control but was faster than the 20mM solution. A drastic change in this pattern was observed in the 200mM, where there was a definite negative growth rate, as illustrated by Figure 3. This suggests that 20mM is a rough threshold at which the organism could still withstand salt stress with some disruption in growth, but at 200mM cells most likely underwent apoptosis or lysis rather than replicating during the monitored period.

Based on a toxicity test conducted in past literature by Gilron et al., it was determined that 20.5mM of NaCl in solution was toxic enough to cause abnormalities and have adverse effects on the organism and they report on behavioral responses that can provide early indication of sublethal effects (1814-1815). Along with our experimental data, this could be rendering that with increased presence of salt, *T. thermophila* will start to die due to the hypertonic environment resulting in crenated cells. This decreased cytosol volume would then strain or inhibit cellular processes required to grow and hinder normal physiological responses. This can be supported by Allen and Naitoh who recognized that at high levels of salt, the contractile vacuoles of various protozoa would disappear, and cellular mechanisms would be inhibited (351). With these combined observations, we suspect that *T. thermophila* have optimum functionality in isotonic conditions with low salt concentration and are subjected to cell damage

or death in solution with extremely high salinity. According to Lekhi, calcium is not the only ion that contributes to salinity and this study can serve as a reference or foundation for future studies that may examine other contributing factors that correlate to a true freshwater system.

During the study, a major concern for errors or discrepancy in the data was from inconsistency in fixation and cell count. Although a standard procedure for cell counting was arranged between the four student experimenters, everyone had difficulty depicting cells at high salt concentrations (e.g. 200mM) due to all the grains of salt present on the slide. This caused further problems of accurately focusing the microscope to view and count the cells, as the large salt particles elevated the cover slip resulting in a false level of cross-sectional view of the specimen. Although best efforts were made to only focus on the cells, there could have been cells that were unobserved and missed.

Another source of variation in our data could be a result of insufficient time given for *T. thermophila* to grow, given that the organism was subjected to a pessimal environment. There could have been other contributing factors, but the increased salt stress could have deterred the average doubling time of less than two hours (Cassidy-Hanley 240), causing them to grow and replicate less. A way to eliminate this may have been to gather more data beyond 28 hours to fully consider the possibility of lagged growth due to the increased stress levels.

For further development or future studies, it is recommended that the cell cultures are established and incubated one to two days prior to the main experimental day while monitoring growth every two hours. This will allow for the experimenter to observe the approximate growth rate of *T. thermophila* in the given conditions and see if the doubling time is different from the literature value. It will further ensure enough growth is occurring and more data could be gathered in the log phase for better sample size. Ideally, the total counts for test and control

should exceed 100 cells per replicate in order to ensure an estimate of cell density that is statistically precise. It is also recommended that further studies incorporate salt concentrations around 20mM and in between 20mM and 200mM to be more conclusive about the exact maximum threshold for salt tolerance for *T. thermophila*. Also, the experimental design should be further developed to establish the salt particle sizes and filter the salts, especially at extreme concentrations, without losing cells prior to cell count to eliminate discrepancy in cell count due to unclear field of view.

CONCLUSION

In conclusion, with F statistic of $F_{(3, 8)} = 30.58$ and a p-value of <0.0001, we can reject the null hypothesis and conclude that it is probable that there is a significant difference between the growth rate of *T. thermophila* in different concentration of $CaCl_2 \cdot 2H_2O$. Our prediction of the lower growth rate of *T. thermophila* with increasing concentration of $CaCl_2 \cdot 2H_2O$ was supported by the data. Based on these data, we believe that the concentration of the $CaCl_2 \cdot 2H_2O$ should be regulated in our environment. *T. thermophila* are a food source for zooplankton, which juvenile salmon consume, and it is suspected that any changes in the population and growth rate of *T. thermophila* will ultimately impact the population of our keystone species, salmon; hence, factors in the environment affecting growth of *T. thermophila* requires consideration.

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