The effect of salinity on the number of food vacuoles formed over time in *Tetrahymena thermophila*

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Abstract

Many organisms require a specific salinity in their environment to grow and reproduce and changes to the salinity concentration can have drastic effects on their survival. The ciliate *Tetrahymena thermophila* is typically found in freshwater environments, thus, the objective of our experiment was to determine if salinity affects the number of food vacuoles present over time. We hypothesized that higher salinity will negatively affect the number of food vacuoles formed over time in *T.thermophila. T.thermophila* was placed in 0 mM, 5 mM and 100 mM NaCl solutions and vacuole number was observed 20, 40 and 60 minutes into the treatments. We found that the 0mM treatment group exhibited positive growth over time and the higher salinity treatment groups exhibited no significant positive vacuole formation over time. *T.thermophila* did not exhibit any vacuole growth in 100mM salinity treatment at any time point, indicating this salinity is above their upper tolerance for vacuole formation. This significant difference in food vacuole numbers allows us to reject the null hypothesis that higher salinity does not affect the number of food vacuoles formed over time in *T. thermophila*.

Introduction

Tetrahymena thermophila is a unicellular eukaryote about 30x50µm in size and is normally found in temperate freshwater environments (Collins & Gorovsky, 2005; Collins, 2012). It has become a model organism for both molecular and cellular biology, as it grows quickly and can be used to study a variety of cellular processes (Bozzone, 2000), therefore, it lends itself to experimental manipulations. To provide the cell with nutrients, *T.thermophila* uses phagocytosis, which is a specialized type of endocytosis (Gray et al., 2012) for ingesting particles in the extracellular environment larger than 0.5µm in diameter (Jacobs et al, 2006). A specialized feature involved in this process is the oral apparatus which is found on the cell's surface and whose role is to gather food and act as the sole site of food vacuole formation (Samaranayke et al, 2011). After formation, the food vacuole will transport the ingested particulate material into the cell and, once inside, the newly formed vacuole will fuse with other

smaller heterogenous vesicles that contain hydrolytic enzymes (Guerrier et al. 2017). This then allows for the internalized particulates in the food vacuoles to be digested (Guerrier et al., 2017).

A unique characteristic of *T.thermophila* is that it is relatively easy to stain and view the food vacuoles under a compound light microscope. Because of this, the food vacuoles lend themselves to experimental manipulations and observations. Previously, it has been shown that abiotic factors such as temperature, light, and pH can affect the growth and number of food vacuoles present in *T.thermophila* (Rasmussen, 1973). However, it appears that the effect of salinity remains understudied. Therefore, in hopes of contributing to the current knowledge gap, we are interested in understanding the effect of salinity on the number of food vacuoles formed over time. Some research into the effect of salinity on food vacuole formation has been done by Ayre et al (2017) and Carnegie et al (2020) where both papers found a significant difference between their high salinity group and the control. We wish to expand on the current literature through our experiment, and further test the salinity limits of *T.thermophila*, where our chosen concentrations (5mM of the low salinity group and 100mM for the high salinity group) have, not to our knowledge, been tested. We hypothesize that salinity will affect the number of food vacuoles formed over time in *T.thermophila*. Therefore, we predict that the number of food vacuoles will be highest in the control environment as it will most accurately represent their natural habitat (Collins, 2012, p 287). We also predict that the number of food vacuoles will increase with time as the cells can engulf more food particles over time (Carpenter-Boesch et al., 2016).

Methods

To prepare for the experiment, we began by labeling 9 test tubes that we would use to apply the treatments (5 mM NaCl and 100 mM NaCl) and control (0 mM NaCl), as well as 63 Eppendorf tubes to use for sampling (3 trials for each treatment/control, with samples collected at 7 different time intervals). We then pipetted 20 uL of fixative ahead of time into all the Eppendorf tubes inside the fume hood, in order to make sampling quicker and more efficient once the experiment began. To set up the treatments, we pipetted different amounts of standard (0 mM NaCl) and special (200 mM NaCl) media to the test tubes in order to obtain different salinities (Figure 1).

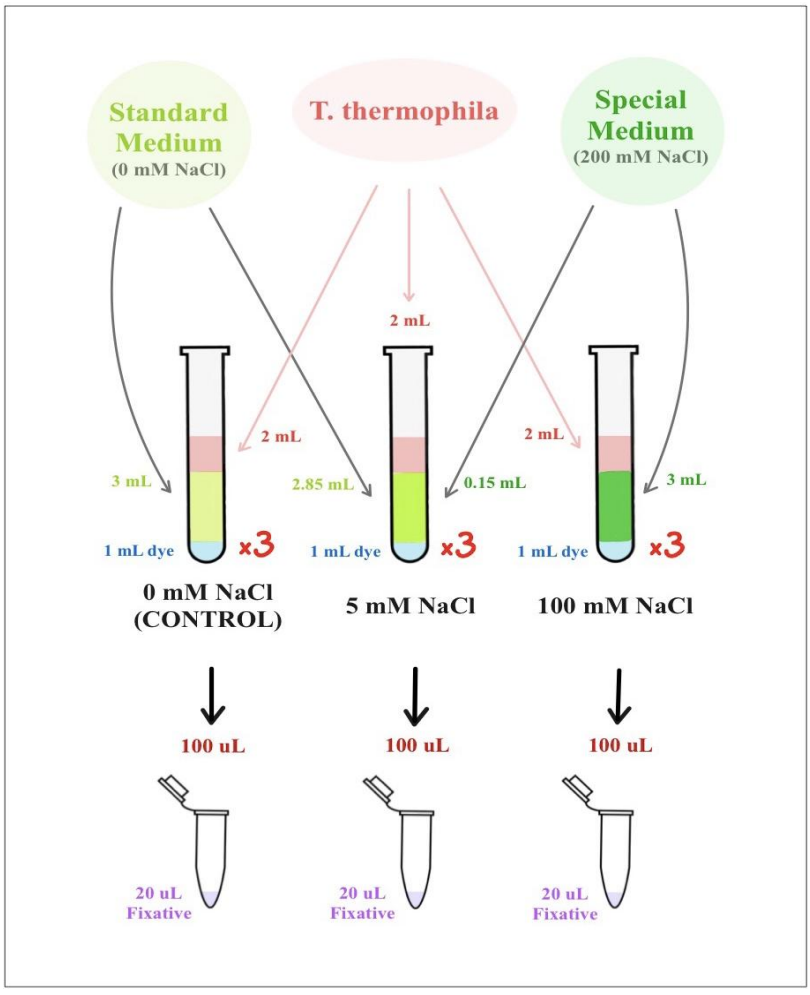

Figure 1. Diagram of experimental treatment set-up.

The values in the figure above were calculated beforehand by setting the final volume of the sample solutions to be 6 mL: 3 mL of medium, 2 mL of *T.thermophila,* and 1 mL of dye. For the control test tubes, we added 3 mL of standard medium. For the 5 mM NaCl treatment test tubes, we added 2.85 mL of standard medium and 0.15 mL of special medium. For the 100 mM NaCl treatment test tubes, we added 3.0 mL of a special medium. In each test tube, the solution was resuspended after both media were added. Then, 2 mL of *T.thermophila* was pipetted into each test tube, followed by resuspension once again. As soon as *T.thermophila* was added and resuspended, we began a timer and collected 100 uL samples from each test tube and deposited them into the corresponding Eppendorf tubes – these initial samples represented time = 0. After 10 minutes, another round of 100 uL samples was collected in the same manner, representing *time* = 10 minutes. We repeated this process every 10 minutes for a total of 1 hour, and altogether this gave us 3 replicates each of the 7 time point samples for each treatment/control.

Once the sampling was finished, we created wet mount slides with 25~50 uL of each sample (the volume used to create the slides was not constant as we started with 25 uL but noticed it was too small and readjusted until we stuck with 30uL). Due to the lack of time, however, we chose to only observe samples from t = 20 minutes, t = 40 minutes, and t = 60 minutes. The slides were placed under the compound microscope (models: Axiostar plus #1236, 1240, 1266 and 1238) at 400X magnification to count the number of dyed vacuoles present in 5 different *T.thermophila* cells. In order to keep consistency, all slides were observed from the upper left to upper right, then lower right to lower left, and so on. Only the first 5 cells observed in this order were counted (Figure 2) and teared or destroyed cells were not counted (Figure 3).

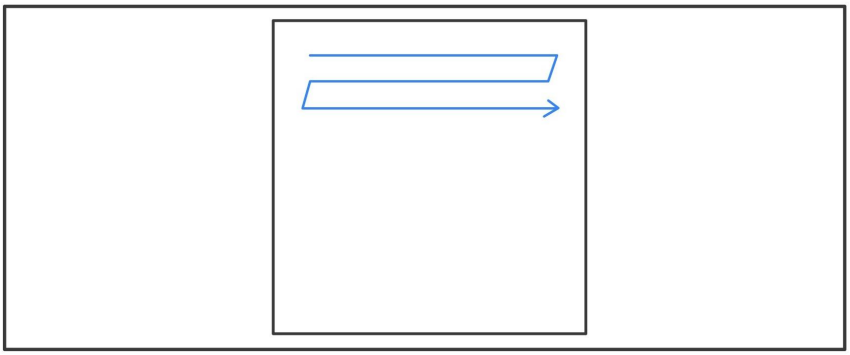

Figure 2. Direction of observation for each slide, until 5 cells were reached.

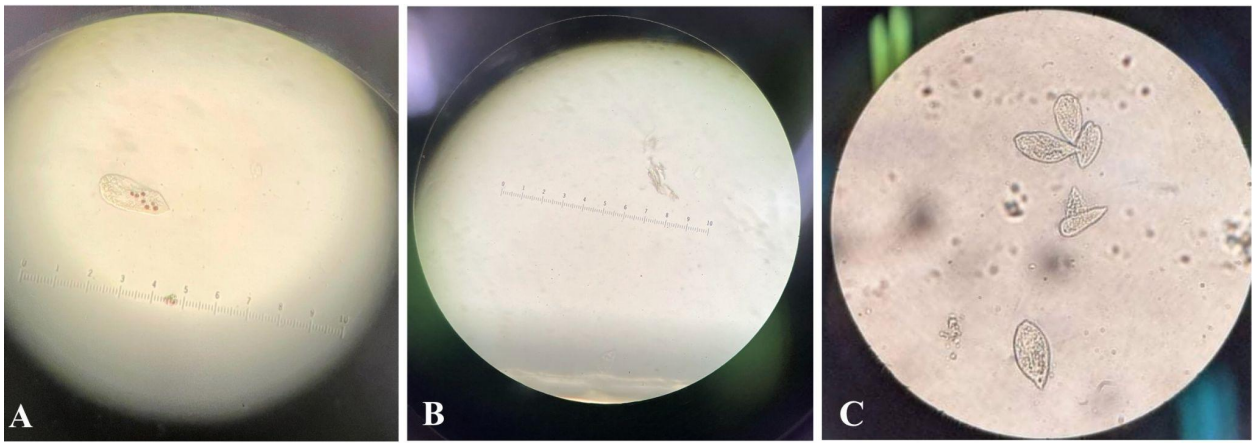

Figure 3. A - Example image of normal T.thermophila cell with dyed vacuoles in the compound microscope, under 400X magnification. B - Example image of destroyed T.thermophila cell in the compound microscope, under 400X magnification. C - Example image of T.thermophila cells with no vacuoles formed in the compound microscope, under 400X magnification.

Using our raw data of vacuole counts, we then plotted a graph of the average number of vacuoles formed at $t = 20$, $t = 40$ and $t = 60$ shown in Figure 4. We then performed a statistical test using a two way ANOVA, as well as a Tukey HSD to determine which treatment groups showed significant difference in their results.

Results

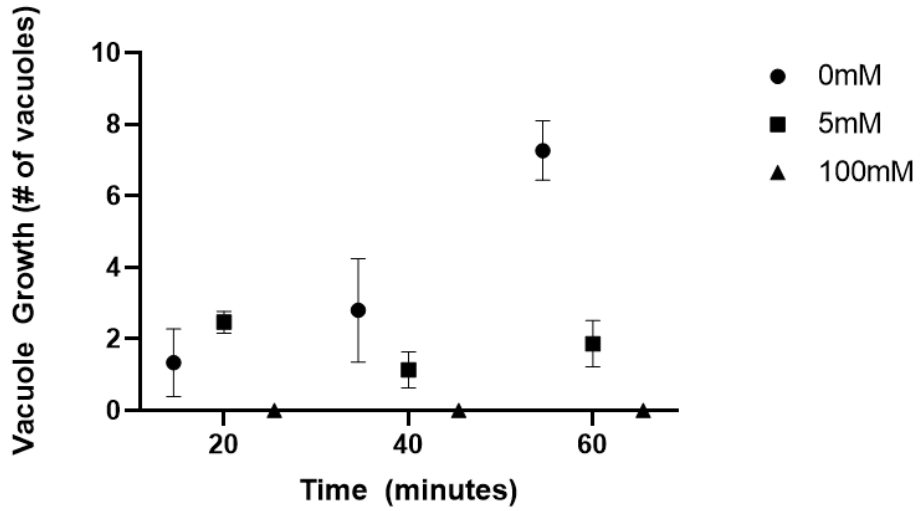

Figure 4. Data shows mean and s.d. of vacuole growth in *Tetrahymena thermophila* in varying salinity mediums: 0mM (circle, n=3), 5mM (square, n=3), 100mM (triangle, n=3). Upper and lower whiskers represent s.d.. (p=<0.0001).

Each vacuole growth data point of a replicate at a certain time point was averaged from the first 5 specimen observations from the same fixed sample (n=5). Data points measured at time=0 were excluded.

Table 1. A sample calculation for the 5mM salinity group replicate #1 at t=40 to get average vacuole growth of the salinity treatment replicates at each time interval.

Vacuole count	3+2+1+0+0	6 vacuoles
Average vacuoles	6 vacuoles / 5	**1.2 vacuoles**

Using a 2-way ANOVA test we found that p=<0.0001, this suggested that one or more treatments are significantly different. We then used a Tukey Multiple Comparisons test to find the differences of each replicate at each time point. As shown in figure 4, the 0mM treatment group showed a significant trend (p=<0.05) of vacuole growth points increasing over time. The 5mM treatment group showed insignificant differences at each time point, instead the data

showed a plateaued vacuole count over time. The 100mM group consistently had 0 vacuole growth over time and no significant difference over time.

Overall, at time=20 the 5mM treatment group exhibited 2.467 more vacuoles on average than the 100mM treatment group with significance (p=0.0012). At time=40, the 0mM group exhibited 1.667 (p=0.0241) and 2.800 (p=0.0003) more vacuoles on average than the 5mM and 100mM group, respectively. At time=60, all groups showed a significant difference in vacuole growth. The 0mM group showed 5.400 (p=<0.0001) and 7.267 (p=<0.0001) more vacuoles on average than the 5mM and 100mM group. The 5mM group exhibited 1.867 more vacuoles on average than the 100mM group (p=0.0115). Thus, we rejected the null hypothesis that vacuole growth does not negatively differ in *T. thermophila* under higher salinity treatments.

Discussion

The purpose of this study is to measure the impact of NaCl on the growth of food vacuoles in *T.thermophila*. Based on the ANOVA test and the Tukey HSD test, we are able to reject the null hypothesis that salinity does not negatively affect the number of food vacuoles present in T.thermophila over time. This is because our results found that as we observed treatment groups with higher salinities compared to the control, we found less vacuole growth on average. The number of food vacuoles formed only increased in the control group but showed no significant growth in 5mM NaCl groups and remained at 0 count in the 100 mM NaCl groups. Ultimately finding that our highest mM salinity group completely prevented vacuole formation.

T.thermophila are generally found in its optimal growth environment which is freshwater where the salinity level is 0 ppt (Collins & Gorovsky, 2005; Collins, 2012). In accordance with this, we predicted that the higher the salinity level is, less food vacuoles will form as it will be

harder for *T.thermophila* to grow. In a previous study, Ayre et al (2018) conducted an experiment testing food vacuole formation, using a high salinity group of 200mM NaCl. The results demonstrated that the cells did not grow in this concentration and cell death occurred. Another study was done by Carnegie et al (2020) with a high salinity group of 50mM NaCl and it was shown that *T.thermophila* were still able to grow and form food vacuole in this concentration. The results of our study can provide important knowledge for future studies because we found that 100mM salinity treatment prevents food vacuole formation, and thus is likely a newly found upper tolerance level for the organism.

During the study, there are several sources of uncertainty and variation that we encountered that could have affected our results. First, inconsistency in cell identification could have occurred as even though a standard food vacuole counting procedure was determined beforehand, the counting was done by three group members and some selection bias may have occurred. Moreover, the visualization of the cells was different on some microscopes. Therefore, in order to reduce the possibility of selection bias, preparing more replicates of each sample for each treatment are recommended to generate more data (Lee et al., 2020). Another possible solution to reduce selection bias is for one person to do the counting with the same microscope. Furthermore, because the counting procedure involved cell staining and fixation of the samples, debris of the staining dye was present on the slide and caused confusion in the counting process, which may have affected our results. It is recommended that cells that exhibited 0 vacuoles should have been excluded in the data. This is because the T.thermophila with 0 vacuoles could be a result of dying in the beginning instead of the salinity treatment. Although careful calculations were carried out, there could have been food vacuoles that were miscounted. In addition to that, another source of variation in our experimental data could be caused by the time

difference of vacuoles counting. On the first day of the experiment, after all samples in each time point were fixated, the vacuole counting process started. However, another counting session was held on a second day. Overnight, the visualization of the cell was worse than the first day of counting. Consequently, counting the vacuoles became more confusing and difficult.

For further development of similar studies in the future, a few changes in experimental design are recommended. More replicates of each experiment condition are suggested in order to provide clearer results. Another adjustment is to void the measurements at time 0, as this data will not provide influential enough results since no growth is possible to be recorded at this time. In order to generate more accurate results, dead cells with 0 vacuoles should be omitted from the calculation with the exception of when every specimen in the sample has 0 vacuoles at every time point calculation.

Conclusion

Overall, we reject the null hypothesis that the number of vacuoles formed over time in *Tetrahymena thermophila* are affected in an environment with salinity concentration higher than 0mM. Our prediction that higher salinity would result in lower food vacuole formation is supported by the higher growth rate in the 0mM group in comparison to the 5mM and 100mM treatment group. Our results helped us understand that *T.thermophila* tolerance towards salinity is highly sensitive to changes in salinity and that the upper tolerance levels lie under 100mM salinity.

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