A Preliminary Study: Effect of low pH environments on the food vacuole formation rate of *Tetrahymena thermophila*

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Abstract

Tetrahymena thermophila are ciliates found in freshwater aquatic systems that fulfill their nutritional needs through the process of phagocytosis by food vacuole formation. This experiment aims to study the effects of decreasing pH on the metabolism of this microorganism over time by observing its food vacuole count and formation rate. *T. thermophila* were exposed to three treatment conditions of pH 5, pH 6, and pH 7 over a two-hour period. Samples were taken from each treatment every 10 minutes, and the average number of food vacuoles formed between 10 *Tetrahymena* cells was observed and recorded from every 10-minute time interval. Our research suggests that *T. thermophila* formed more food vacuoles and at a higher rate at pH 7, while pH 5 displayed dramatically less vacuole formation. Rate of food vacuole formation per minute was calculated following exposure to pH 5, pH 6, and pH 7 after two hours and was found to be 0.011, 0.036, and 0.046 vacuoles per minute respectively. Considering that the natural habitats of *Tetrahymena* are usually at pH levels of approximately 7, we can infer that this is the state in which this microorganism is most comfortable. This is likely why *T. thermophila* do not perform well at decreased pH levels, resulting in an inability to form as many food vacuoles.

Introduction

Tetrahymena thermophila are ciliates found in freshwater environments such as lakes, ponds, rivers and streams (Zheng et al., 2006). *Tetrahymena* obtain their nutrients via phagocytosis, a process in which they ingest whole microorganisms and degrade them to meet their nutritional needs (Jacobs et al., 2006). More specifically, *Tetrahymena's* ciliary activity brings food into their cytostome (cell mouth), where it is then carried to the site at which food vacuoles are made (Gonda et al., 2000). Essentially, vacuoles are carriers of food particles within the digestive systems of *Tetrahymena* (Jacobs et al., 2006). The food vacuoles are degraded by

digestive enzymes and the waste is then egested from the body (Sugita et al., 2009). It is clear that food vacuoles are an important part of *Tetrahymena*'s metabolism and, therefore, their overall growth and survival.

Tetrahymena are a food source for zooplankton, which, in turn, are a main food source for juvenile salmon (Eggers, 1978). *Tetrahymena* also play a role in the inactivation of bacteriophage viruses that infect and replicate within bacteria, causing the population to decrease (Pinheiro et al., 2007). This is important as bacterial organisms are a main food source for zooplankton and a decrease in their population would indirectly affect the salmon population as well (Pinheiro et al., 2007). Salmon are a keystone species; they play an important role in nutrient recycling, specifically of nitrogen, as they are caught and eaten by bears and wolves, which carry the carcasses away from rivers and streams and into areas with abundant plant biomass (Ben-David et al., 1998). The degradation of the salmon carcasses deposits nitrogen into the soil, which allows the plants to thrive (Ben-David et al., 1998). Plants, of course, provide the planet with oxygen that is crucial to our survival and remove carbon dioxide (CO_2) from the environment. It is for these reasons that a decrease in the *Tetrahymena* population could have very negative consequences for the entire ecosystem.

Very little research has been conducted on the effect of different pH levels on ciliate survival. However, research on phytoplankton has shown that ocean acidification (a decrease in water pH due to increased CO_2 in the atmosphere) has caused many species to become extinct or migrate from the low pH areas (Chu, 2015). Additionally, research on marine microbes has shown that as pH lowers due to an increase in CO_2, the microbes' abilities to perform nitrification (converting toxic ammonia into nitrate) lowered drastically (Huesemann et al.,

2002). The process, which is optimal at a pH of 8, was decreased by 50% at a pH of 7 and was completely inhibited at a pH of 6 (Huesemann et al., 2002). This decreased ability to perform nitrification made the environment more harmful to all species, as ammonia is very toxic in high concentrations (Liu et al., 2013).

The objective of our research is to determine how the decrease in pH of freshwater systems will affect the rate of food vacuole formation in *Tetrahymena thermophila*. Our null hypothesis is that there will be no difference in food vacuole formation rate between the different pH conditions and our alternate hypothesis is that there will be a difference between one or more of the experimental pH treatments. We predict that the highest rate of food vacuole formation will occur at a pH of 7, because subcellular organelles will likely be negatively affected by lower pH levels. Furthermore, nutrient availability in the environment will likely decrease as well, due to the inability of microorganisms, such as *Tetrahymena thermophila*, to assist in nitrification at decreasing pH levels (Huesemann et al., 2002).

Methods and Materials

Preparation of Experiment

The study organism for this experiment was *Tetrahymena thermophila*, prepared and grown in a neutral medium and stored in a 250 mL Erlenmeyer flask. As is seen in Figure 1, this experiment tested the influence of three different pH levels (pH 5, 6, and 7) on food vacuole formation rate by *T. thermophila* over a two-hour period. Over the course of two hours, we gathered one sample at every ten-minute interval from each pH treatment. Collection began at the zero-minute mark, resulting in a total of 39 samples.

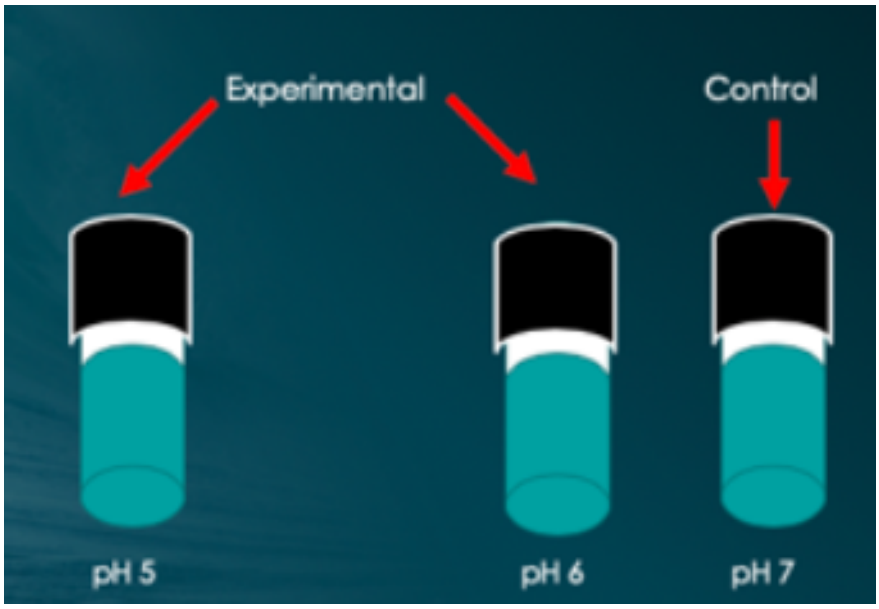

Figure 1. An illustration of each experimental treatment in 50 mL falcon tubes post-centrifugation containing the *T. thermophila* pellets with the added pH mediums.

After thoroughly swirling the flask to ensure proper mixing of the stock culture, we pipetted 25 mL into each of the three 50 mL falcon tubes. In order to separate the *Tetrahymena* from its medium, the tubes were centrifuged, and we decanted the media into a waste beaker. The three experimental mediums were adjusted to pH 5, pH 6 and pH 7 and were all treated with a buffer in order to keep the pH levels constant. We added all 25 mL of each medium to the labelled falcon tubes containing the pellets of *T. thermophila,* as seen in Figure 1.

During centrifugation, we prepared 39 Eppendorf tubes with 10 uL of glutaraldehyde in order to fix the *Tetrahymena* at each exact time interval during sample collection. We labelled every Eppendorf tube with the time interval the sample was taken at and placed them in three Eppendorf tube trays coloured differently depending on the pH treatment.

To set up the experiment, sterile heating was performed on three labelled 6 mL test tubes before we pipetted 1 mL of black dye medium to each tube. To initiate the start of the experiment, we pipetted 5 mL of the pH 5 *Tetrahymena* medium into its designated test tube, resuspending the mixture to allow for thorough mixing of solutions. We immediately began data collection for this treatment by pipetting a 100 uL sample into the Eppendorf tube containing glutaraldehyde and labelled as the time interval "zero minutes." We set one of the timers to ten minutes in order to accurately keep track of sample collection. The samples were taken from the top of the test tubes to ensure collection of active cells, as the ones that collect at the bottom of the tube may be dead. While waiting for the next ten-minute interval to collect a sample for pH 5, we conducted the same procedure for pH 6 and then for pH 7. Three separate timers ran, one for each treatment, with a few minutes differing between them in order to ensure that the samples were collected as closely as possible to the intended time interval for each condition. A simple breakdown of the sample collection and preparation is illustrated in Figure 2.

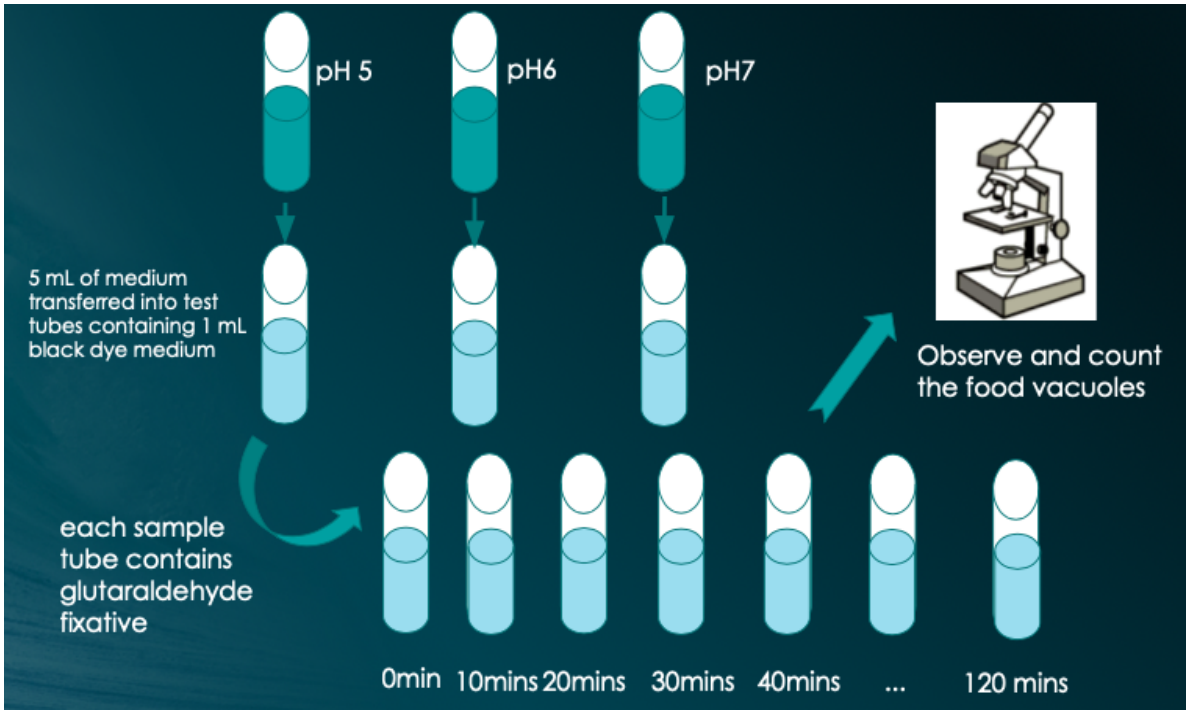

Figure 2. A flowchart illustrating the basic steps taken in preparation of the experimental test tubes as well as the time-point data collection and observations that were taken of each sample from each experimental treatment.

Data Collection

After collection of all 39 samples, we began microscopic analysis on the fixated cells. We used a pipette to transfer 25 uL of fixated cells from the bottom of the Eppendorf tubes onto microscope slides for vacuole counting. We randomly selected ten *Tetrahymena* cells for observation from each sample based on the following characteristics: the *Tetrahymena* must be free-floating, not grouped with other cells, and it must clearly have an intact membrane with at least one dark vacuole present. We observed the cells under a compound microscope at 400X total magnification.

We had to complete the entire experiment in one day to ensure that experimental factors were the same for all treatments and to avoid degradation of the cells and the dye.

Statistical Analysis

We presented the data in a line graph comparing the mean number of food vacuoles formed from 10 cells at each time interval for the three different pH levels. We calculated the vacuole formation rate for each pH level and illustrated them in a bar graph. We observed both of the graphs in order to see if there was a trend present so that suggestions and assumptions may be made about the data. Statistical analysis could not be conducted on the data as we only collected one sample at each time interval from each of the three treatments.

Results

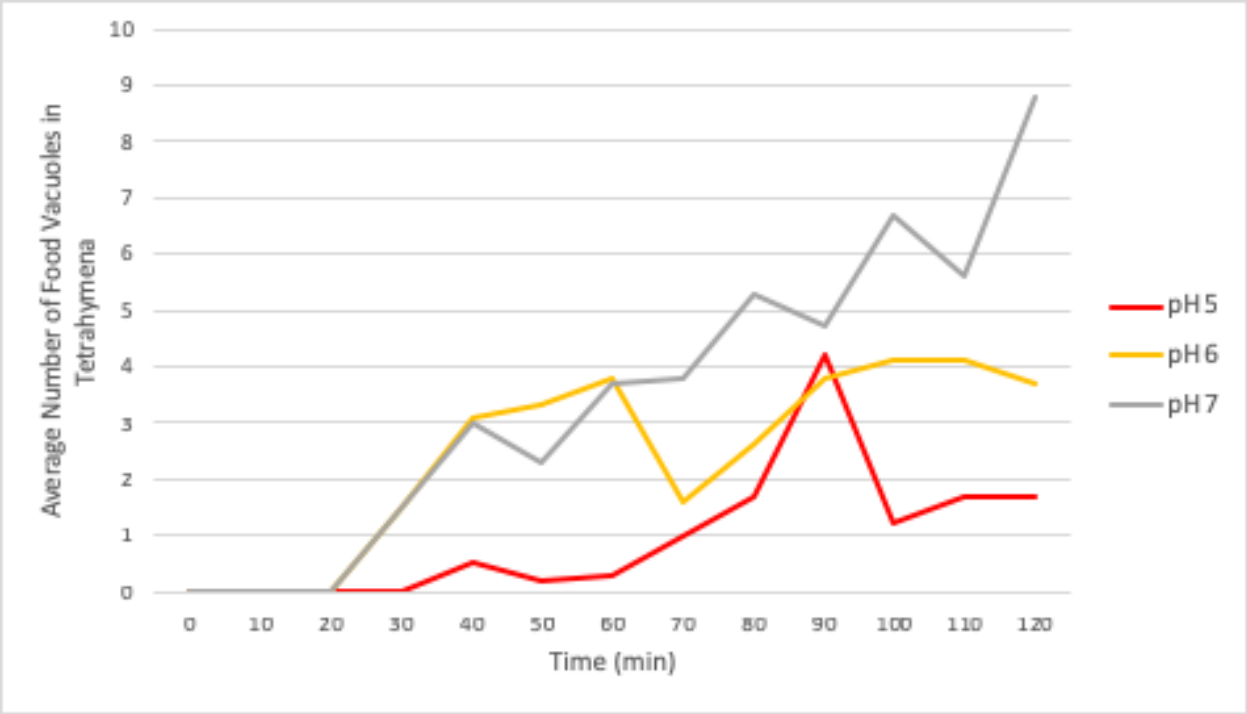

Figure 3. A line graph comparing the mean number of food vacuoles formed in *T. thermophila*, calculated from 10 pseudo-replicates, at three different pH levels over the course of two hours. Each line represents the growth or decline in formation of food vacuoles exhibited in the three different pH media.

As can be seen in Figure 3, the number of food vacuoles formed in *T. thermophila* was highest at pH 7, while pH 6 was not far behind. The condition of pH 5 appears to have relatively

less counts of vacuoles compared to the other two treatments, especially up to the 60-minute mark. Vacuole formation began at the 20-minute mark for both pH 6 and pH 7, while it was delayed until the 30-minute mark for pH 5. The trend of vacuole formation between pH 6 and pH 7 does not appear to differ very much until the 60-minute mark is reached and formation falters for pH 6. It is also notable to mention that both pH 5 and pH 6 experienced a decreasing trend in vacuoles present per *Tetrahymena* by the end of the 2-hour period, while pH 7 continued to grow overall.

Apart from the spike at 90 minutes of 4.2 vacuoles per *Tetrahymena*, the levels of food vacuole formation appear to be drastically different between pH 5 and pH 7 when analyzing Figure 3. We expect this spike at pH 5 to be an anomaly as it appears to be uncharacteristically high compared to the rest of the data. Furthermore, there appears to be a longer lag phase in vacuole formation for the pH 5 population of *T. thermophila* compared to the other two pH conditions.

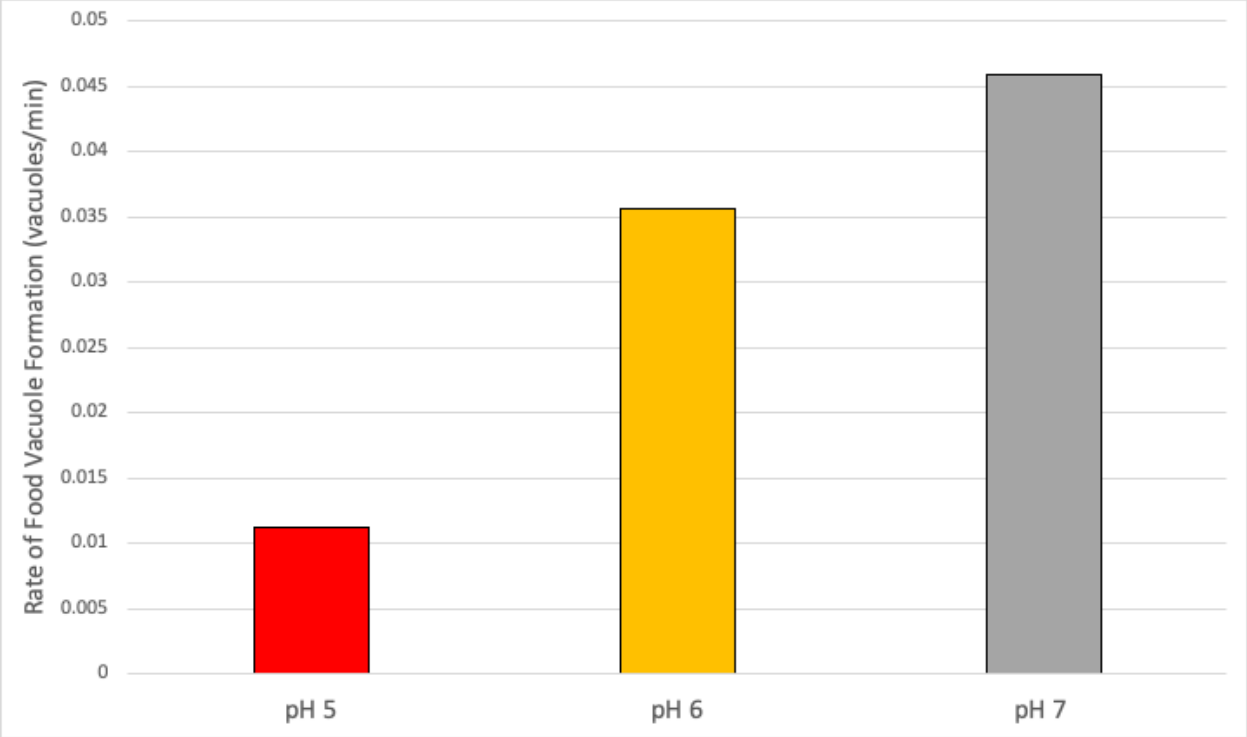

Figure 4. Bar graph comparing the rate of food vacuole formation per minute in *T. thermophila* exhibited at each pH level treatment.

In order to gain further insight into the formation of food vacuoles in *Tetrahymena* under these pH conditions, a bar graph was created to compare the rates of formation per minute, as can be seen in Figure 4. The rate of formation in vacuoles per minute was calculated at each ten-minute time interval and the mean of this data was found to represent the overall rate for each treatment. In Figure 4, there is a very obvious trend of increasing vacuole formation rate from pH 5 to pH 7. However, it cannot be said with certainty that there is a significant difference between the pH levels as statistical analysis cannot be conducted when no sample replicates were collected. Although, pH 5 does differ very dramatically in rate compared to the other two treatments and it can be assumed that decreasing pH does have a negative effect on the rate of vacuole formation in *T. thermophila.*

Qualitative Observations

For pH 5 and pH 6, as time increased, it became more difficult to find *Tetrahymena* with food vacuoles. There were many cells present, but the majority of the cells did not have any vacuoles inside of them.

Additionally, the vacuoles were very difficult to identify. It took a long time to finally deduce what a vacuole looked like under the microscope. In Figure 5 below is a photo of a *Tetrahymena* with clear black-dyed vacuoles present, matching the guidelines set earlier for what a living *Tetrahymena* with vacuoles looks like.

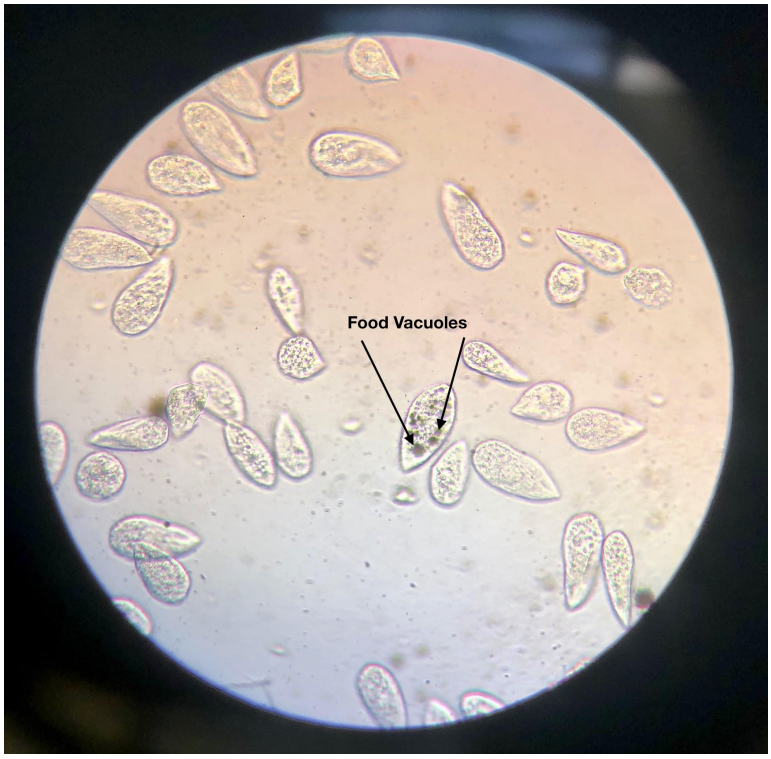

Figure 5. This is a photo of a *T. thermophila* with vacuoles. It is clear that the surrounding cells do not have vacuoles present as none of them contain the black-dyed circles that the indicated cell does.

Discussion

From Figures 3 and 4, it is apparent that there is an obvious trend of increasing vacuole formation rates from pH 5 to pH 7. However, it cannot be concluded with any certainty how pH affects food vacuole formation rates in *Tetrahymena* as statistical analysis could not be conducted.

The observation that a higher rate of vacuole formation occurred at pH 7 can be explained by Weisse and Staddler's (2006) study that found that subcellular organelle function is affected by high proton concentrations (low pH). The high concentration of protons negatively affects the transport processes driven by concentration gradients in the cells and reduces the bioavailability of nutrients in the medium that can be used by the organisms (Weisse et al., 2006). Accordingly, with less nutrients available in the environment, it is expected that less food vacuoles will be formed at lower pH levels. Furthermore, particulate matter promotes the formation of food vacuoles (Rasmussen et al., 1970) and the chemical properties of particulate materials are negatively affected by pH (Dispirito et al. 1983). Therefore, in the pH 5 medium, *Tetrahymena* may not have been able to absorb as many food particles as was possible at pH levels of 6 or 7 due to the variation in quantity of particulates.

From the data obtained in Weisse's study (2017), it can be inferred that the trend of global water acidification will negatively affect the well-being of *T. thermophila* as lower pH conditions yield lower food vacuole formation rates. This inference coincides with a similar study conducted by Lee (1942), which found that low pH negatively affected food vacuole formation in *P. multimicronucleatum*, another ciliate like *Tetrahymena.* As cilia play a key role in collecting

food particles and forming food vacuoles, it is very possible that low pH could also hinder cilia movement and result in the decrease of vacuole formation rates (Wloga et al., 2012).

Figure 3 shows that that the average number of food vacuoles barely seemed to increase over the two-hour experimental period at the pH 5 condition. A previous experiment conducted by Schlenk (1994) revealed that increasing or decreasing pH could negatively affect the physiological reactions of *Tetrahymena* over a certain period of time and compromise their survival rates. Since energy is being used to maintain stable cellular pH under acidic conditions and to express the appropriate physiological reactions, the energy put into forming food vacuoles will likely decrease (Weisse et al., 2006). Therefore, the change in priority of expenditure of energy is likely to result in fewer vacuoles present at lower pH levels.

Looking again at Figure 3, we see that the trend between the pH 6 and pH 7 conditions were quite similar for the first 60 minutes of experimentation. However, after that point, vacuole formation of *T. thermophila* in the pH 6 medium decreased for 10 minutes before slowly increasing back to approximately the same maximum number of vacuoles observed at 60 minutes. Including some information from the aforementioned Weisse and Staddler (2006) study, we can extrapolate an explanation for this phenomenon seen in Figure 3. As we know, increases in proton concentration can negatively affect nutrient availability for organisms (Weiss et al,. 2017). Therefore, we predict that in the pH 6 condition at the 60-minute mark, all available nutrients had been absorbed and the downward spike was caused by the death of multiple *T. thermophila* who had no nutrients to feed off of. Additionally, we predict that this death of some organisms resulted in a release of nutrients that may account for the increase in formation after the spike from 60 to 70 minutes.

To improve the accuracy of this study and its results, several improvements could be implemented into future experimentation. The first source of error that came into our study was that some samples appeared to have a lower concentration of *T. thermophila* to count 10 readily. A solution for this would be to centrifuge all samples in order to displace *T. thermophila* to the bottom of their respective Eppendorf tubes. From there, the additional note to sample from the bottom of the tube when micro-pipetting would ensure that as many organisms are being sampled as possible.

Another limitation that was faced was the difficulty of reaching a consensus as to what was or was not considered a food vacuole. Having four different experimenters with different conceptions of what a vacuole looked like introduced inconsistency in counting and may have introduced error into the study. Although guidelines were created defining what a proper vacuole looks like, it is advised that for future studies one experimenter is tasked with the job of counting and observing the vacuoles to allow for consistency across all treatments and replications. Additionally, the experimenter who is chosen should observe the samples blindly, meaning that they do not know which experimental treatment they are observing, in order to decrease experimenter bias.

Although the limitations above did affect the study, the largest limitation faced when conducting this experiment was time. As the dye used for the experiment was prone to degradation, analysis of all samples had to be done in the same sitting as the preparation and the collection of the samples. Due to time constraints, it was impossible to obtain all anticipated data points, resulting in the use of pseudo-replicates rather than actual sample replicates. Furthermore, because statistical analysis cannot be performed with pseudo-replicates, it is difficult to analyze

data objectively. For future studies, researchers should try and obtain a dye that does not degrade and will allow for better preservation which would allow for later analysis.

It is important to complete a thorough future investigation into how global water acidification may affect microorganisms such as *Tetrahymena* who hold crucial places in their ecosystems. Pinheiro et al. (2007) found that *T. thermophila* have an important role in the aquatic ecosystem as they prevent bacteriophages from negatively influencing the size and diversity of bacterial populations. With consideration that bacteria are a food source to zooplankton and zooplankton are an important food source to juvenile salmon, we can predict that if bacterial diversity and availability decreases as a result of a decrease in *Tetrahymena*, the livelihood of salmon, a keystone species, will be affected as well (Eggers, 1978). Furthermore, *Tetrahymena* assist in the process of nitrification in aquatic ecosystems that convert toxic ammonia to nitrate, a nutrient. Without the removal of toxic substances from the environment, this may pose a threat to many microorganisms that exist at the bottom of the food chain and would ultimately affect all consecutive levels as well.

Conclusion

We are not able to comment on the null hypothesis of this study as statistical analysis of the data could not be conducted. However, the results of this research suggest that *Tetrahymena thermophila* experiences decreased food vacuole formation at decreased pH levels. This was illustrated in Figures 3 and 4 by the dramatic decrease in vacuole count and formation rate at pH 5 compared to pH 6 and pH 7. In future studies, concrete results can be achieved with the replication of samples and the use of a dye that will not degrade and will allow for experimentation and analysis over multiple days. Statistical analysis methods will be enabled

with more replicates of samples and more precise results can be obtained objectively. With water conditions acidifying globally, this may compromise the survival of *Tetrahymena* and many other similar organisms. If *T. thermophila* are not able to fulfill their nutritional needs necessary for growth and health through optimal food vacuole formation, the well-being of many organisms will be affected, even those existing at higher trophic levels, such as salmon.

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Appendix

Average Number of Vacuoles per *Tetrahymena thermophila*					
pH 5		**pH 6**		**pH7**	
Time (min)	**# of Vacuoles (/ 10)**	**Time (min)**	**# of Vacuoles (/ 10)**	**Time (min)**	**# of Vacuoles (/10)**
0	0	**0**	0	**0**	0
10	0	**10**	0	**10**	0
20	0	**20**	0	**20**	0
30	0	**30**	1.5	**30**	1.5
40	0.5	**40**	3.1	**40**	3
50	0.2	**50**	3.3	**50**	2.3
60	0.3	**60**	3.8	**60**	3.7
70	1	**70**	1.6	**70**	3.8
80	1.7	**80**	2.6	**80**	5.3
90	4.2	**90**	3.8	**90**	4.7
100	1.2	**100**	4.1	**100**	6.7
110	1.7	**110**	4.1	**110**	5.6
120	1.7	**120**	3.7	**120**	8.8