Analyzing the Effects of Fertilizer Concentration on the Rate of Food Vacuole Formation in *Tetrahymena thermophila*

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Abstract

The unicellular ciliate *Tetrahymena thermophila* plays an important role in freshwater aquatic ecosystems, and through the use of phagocytosis, may be impacted by agricultural runoff. The purpose of this study was to determine the effects of varying concentrations of
fertilizer on the rate of food vacuole formation. Plant-Prod, a water soluble fertilizer, was diluted using *T. thermophila* nutrient media and combined with 2 mL of *T.thermophila* culture to create three replicates of three different treatments with a total volume of 4 ml. Treatment A was 20% of the base fertilizer solution, 0.8ml/L, Treatment B 5%, 0.2ml/L, and Treatment C was the control, containing no fertilizer. After 24 hours of incubation at 20 ° C, black dye was added to each test tube to allow for the identification of newly formed food vacuoles. 100 uL of sample was removed at 10 minutes, 150 minutes, and 210 minutes from each replicate, and placed with fixative in eppendorf tubes. The number of stained vacuoles in 5 *T.thermophila* cells were then counted using a compound microscope. We conducted a one-way ANOVA [$F(2, 132) = 4.12$, $p = 0.05$], and found that the presence of increased concentrations of fertilizer significantly increased the rate of food vacuole formation in treatment A (0.12 ± 0.02) and treatment B (0.08 ± 0.01) compared to treatment C (0.063 ± 0.01). The rate of food vacuole formation was positivelycorrelated with fertilizer concentration. The data did support the prediction that increasing the concentration of fertilizer would increase the rate of food vacuole formation.

Introduction

T.thermophila is a unicellular, ciliated protozoan that is common in freshwater environments. It's importance as a secondary producer, coupled with it's unique genetic traits, has made it a model organism for eco-toxicological evaluations and genetics (Coyne, 2011; Davoren & Fogarty, 2004). *T.thermophila* are capable of eating bacteria or other small organic matter through phagocytosis (illustrated in **Figure 1**); a fundamental process that

allows for the ingestion of large molecules to meet the organism's nutritional needs, as well as to dispose of waste and undigested material (Jacobs et al., 2006; Simon et al., 2008). Time and the availability of organic material are the two key factors involved in determining the rate of phagocytosis (Fok et al., 1988). *T. thermophila* is an ideal organism for observing phagocytosis due to their culturability and clearly visible food vacuoles. The formation of food vacuoles in *T. thermophila* is indicative of metabolism and organism health, and the presence of vacuoles has been shown to be essential for the multiplication of cells (Coyne, 2011).

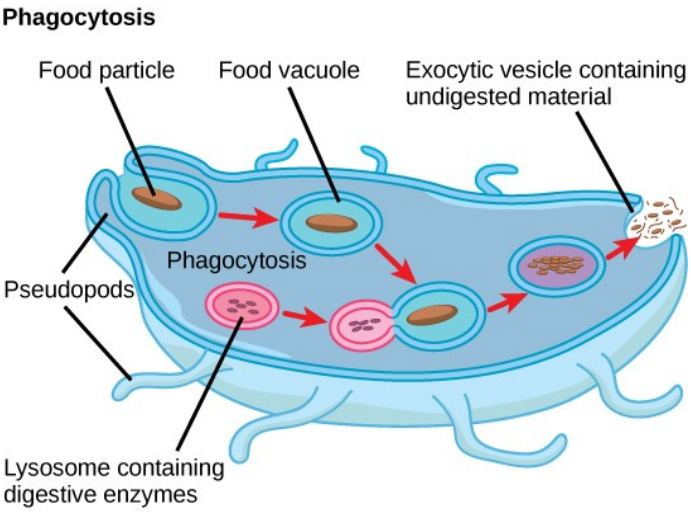

Figure 1: The stages of phagocytosis in *T.thermophila*. Showing the cilia moving food towards the oral groove, where the particle is engulfed, digested with lysosomal hydrolytic enzymes, and undigested materials are expelled

Phagocytic ciliates such as *T. thermophila* are important in aquatic ecosystems due to their ability to transfer nutrient material through coastal food webs, particularly as they may be the dominant group of micro zooplankton in temperate coastal waters (Pierce & Turner, 1992). Zooplankton abundance has been shown to be under the influence of a salmon-imitated trophic cascade, as zooplankton are an important component of the juvenile salmon's diet during their maturation in streams (Estes, 2014). Our investigation into the impacts of fertilizer on *T. thermophila* is relevant due to the expansion of agriculture and the resulting increase in fertilizer runoff into streams, which is a significant problem for aquatic ecosystems. Streams that drain agricultural lands have concentrations of nitrate and phosphate nine times greater than streams that drain forested areas (Binkley *et al.,* 1998).

The objective of this experiment was to determine what the effects of varying concentrations of plant fertilizer are on the rate of food vacuole formation in *Tetrahymena thermophila*. The hypothesis for this experiment was that at increasing concentrations of fertilizer, the rate of food vacuole formation would increase. If this hypothesis is correct and we follow the given protocol, we should see an increase in the rate of food vacuole formation in *T.thermophila* when exposed to a higher concentration of fertilizer. This hypothesis is based on knowledge of the role that limiting nutrients, particularly nitrogen and phosphorus, play in organism growth. Nitrogen is necessary for the formation of amino acids and vitamins, and is involved in the production of carbohydrates, while phosphorus is essential for cell division (Uchida, 2000). The provision of nitrogen and phosphorus has shown to significantly

increase ciliate abundance, implying that they are limiting factors in growth and replication (Wickham *et al.,* 2014). We can infer that nitrogen, phosphorus, and other elements provided in the fertilizer may also be limiting nutrients for food vacuole formation, thus we would see an increase when they are provided.

Methods

Subjects

The composition of the fertilizer was: 20% nitrogen (N), 20% phosphoric acid (H3PO4),

20% soluble potassium (K), 0.05% chelated copper (Cu), 0.02% boron (B), 0.10% chelated iron

(Fe), and 0.05% chelated manganese (Mn).

Protocol Design

The experiment was conducted on two consecutive days; experimental preparation was conducted on the first day. Prior to commencing the experiment, stock solutions of fertilizer and *T. thermophila* were prepared. The fertilizer "All-purpose plant-prod" was prepared according to the given instructions, substituting water with SSP medium, to produce a stock fertilizer solution with an initial concentration of 4 mL/L, to which a value of 100% was ascribed. A stock solution of cultured *T. thermophila* was also used, and before *T. thermophila* was added to the test tubes, the flask was mixed well to verify an appropriate distribution of the organism to each of the test tubes. Eppendorf tubes, microscope slides and the nine test tubes used for the three treatments, with three replicates each, were labeled. The treatment solutions were prepared, each containing the appropriate amount of fertilizer and *T. thermophila.* Treatment A contained a fertilizer concentration of 0.8 mL/L and 0.2 mL/L

which corresponded to 20% of the initial concentration, treatment B contained a fertilizer concentration of 0.2 mL/L which corresponded to 5% of the initial fertilizer concentration, and the control contained no fertilizer. For treatment A replicates, 2 mL of *T. thermophila* culture was transferred into a test tube, followed by 2 mL of the 40% 4 mL/L fertilizer solution. For treatment B replicates, 2 mL of *T. thermophila* culture was transferred into a test tube, followed by 0.5 mL of the 40% 4 mL/L fertilizer solution. For the control, Treatment C, 2 mL of *T. thermophila* culture was transferred and 2 mL of standard media was transferred. All the test tubes were then placed in an incubator at 20ºC overnight.

The experiment proper, which focused on the vacuole formation of *T. thermophila*, was conducted on the second day. Four Zeiss Axiostar compound microscopes were set up. 10 μL of Glutaraldehyde fixative was added to each eppendorf tube. 1 mL of black dye was added to each test tube, and mixed by swirling each test tube for 5 seconds, then the timer was started. After 10 minutes, 100 μL of the culture from each of test tubes were added to corresponding Eppendorf tubes, as set up in **Figure 2**. This was repeated at 150 minutes and 210 minutes. Then, 25 μL from the Eppendorf tube was transferred to correspondingly labeled microscope slide. The number vacuoles with any amount of dye in them were counted using a handheld counter.

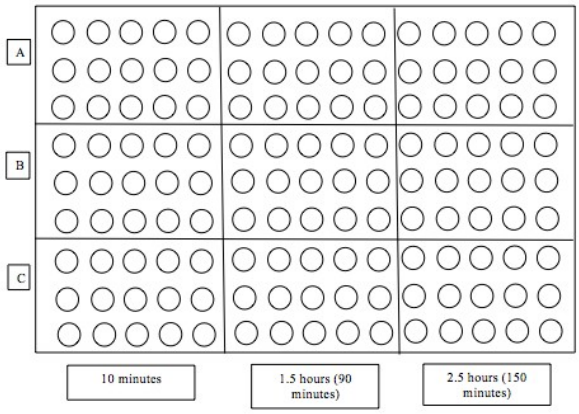

Figure 2: Shows the Eppendorf labeling system. A_a (1- 10), A_b (1-10), A_c (1-10). The same was done for B and C. This was used for the three times tested.

If no vacuoles were observed in the cell, a count of 0 was given. Results were recorded onto prepared tables. **Figure 3** shows an example of *T. thermophila* and black-stained vacuoles. In order to test whether the means of the three treatments have any significant differences, a one- way ANOVA test was used at a 95% confidence interval.

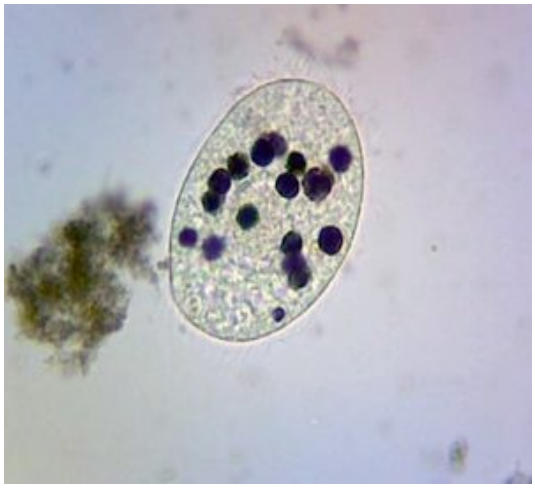

Figure 3: Observed *T. thermophila* showing black-stained food vacuoles. The photo was taken using a mobile phone camera in conjunction with a Zeiss Axiostar compound microscope at 400x magnification.

Data Analysis & Statistics

Data Analysis

The number of vacuoles obtained per cell were classified by treatment and time. The data was arranged into treatments once the average rate of vacuole formation per minute was calculated. Using Microsoft excel, the information required for the analysis of variance such as the mean average rates of each treatment, was calculated. The graphs were made by plotting the average rate of vacuole formation on the y-axis, and treatment on the x-axis. A bar graph was used to represent any trends in the results or significant differences through the use of error bars. The error bars represent 95% confidence intervals. If these confidence intervals overlap or touch, we can determine that there is not a statistically significant difference, and that we fail to reject the null hypothesis. If they do not overlap, then it can be concluded that there is a

statistically significant difference, and we reject the null hypothesis. This significant difference is indicated by the use of letters on the graph; letters different between bars signifies a significant difference.

Statistical Analysis

H_0: The mean values are not significantly different

H_A: The mean values are significantly different

Results

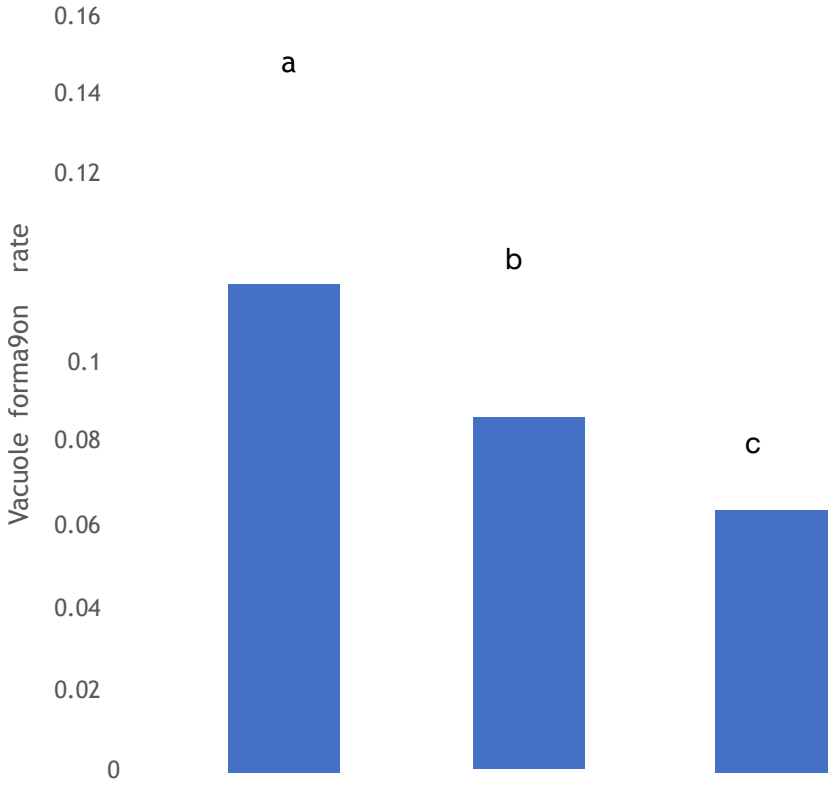

Figure 4: The average rate of food vacuole formation in *Tetrahymena thermophila* at varying concentrations of fertilizer. N= 9

The bars represent the average rate of food vacuole formation across three treatments, A = 20% fertilizer concentration, B = 5% fertilizer concentration and C = 0% fertilizer concentration. Error bars represent 95% confidence intervals. Letters a – c represent statistically significant differences. Testing was performed in the same environmental conditions at room temperature.

Results

The results from this experiment show that the mean rate of vacuole formation increased in the presence of increasing concentrations of fertilizer. Refer to **Figure 4.** Comparing the mean rate of vacuole formation and confidence intervals between treatments can support this trend. Treatment A has a mean rate of formation of 0.12 ± 0.02, while treatment B has a mean rate of formation of 0.08 ± 0.01, and treatment C has a mean rate of formation of 0.063 ± 0.01.

The mean rate of formation between these three treatments was significantly different, as seen by the absence of overlap in the confidence intervals. An analysis of variance was performed to compare the means of the groups and a significant result was obtained. The F-ratio value obtained, 4.14 is higher than the critical value F (1), 2, 132 = 3.08 and therefore the p-value is higher than 0.05. Thus, increasing the concentration of fertilizer to 5% or 20% significantly increased the rate of food vacuole formation.

Discussion

This study aimed to test the hypothesis that the rate of food vacuole formation in *T. Termophila* increases with an increased concentration of fertilizer. It was predicted that we would see an increase in the rate of food vacuole formation with increased fertilizer concentration, as it contains limiting nutrients such as nitrogen and phosphorus, which have positive effects on cellular growth and function. The results were consistent with what we predicted. In **Figure 4.,** there is insufficient overlap in the confidence intervals of the mean rate of formation, thus we can reject the null hypothesis and consider the mean values to significantly different. The hypothesis that increasing the concentration of fertilizer would increase the rate of food vacuole formation in *T. Termophila* was supported by the data.

The rates of vacuole formation vary between the times of exposure to the black dye, which may be attributed to different factors. When the cells were fixated after being exposed to the dye for 10 minutes, most of the vacuoles observed had not been stained yet; unstained vacuoles were formed before the addition of the dye at the onset of the experiment. Contrary to this short time of exposure, cells that had been exposed to the dye for 210 minutes showed

a high number of stained vacuoles. After 210 minutes, the metabolism of *T. termophila* had reached saturation, reaching the maximum number of vacuoles the organism can produce (Nilson, 1977). The rate of formation was disproportionate at 10 minutes and plateaus at 210 minutes. The average rate of vacuole formation per minute for each treatment was used for the one-way ANOVA test and the results were significant. There was a significant difference in the average rate of formation between the high fertilizer concentration, low fertilizer concentration and control. Therefore, we are able to reject the null hypothesis. Our results show our second important finding which is the trend observed: As fertilizer concentration increases, the rate of vacuole formation for *T. Termophila* increases. This type of trend is consistent with previous studies in *T. thermophila* that have shown that increased nitrogen sources tend to result in higher cell density (Hofmann & Cleffmann,1981).

The accuracy of the experiment can be improved by looking at shorter window times in exposure duration of the cells to the dye since as it was mentioned previously, there can be an exposure time bias between the times of exposure used. Shorter windows of time results in a smaller dt, and with enough data points perhaps an instantaneous velocity could be interpolated. A larger sample size would also produce more uniform results, and would grant more power when subject to statistical analysis. Sources of error in our experiment include human error; a baseline of the image of dyed vacuole was established but there was variation in counts due to subjective perception of the viewer. Other sources of error include equipment error, as four different compound microscopes were used during the counting process. Even though an effort was made to standardize settings between the microscopes, each microscope still had unique optical traits.

In order to check the validity of our results, a two-way ANOVA was conducted utilizing the same set of raw data. The two-way ANOVA was conducted to see if there was anysignificant difference between the These results corroborate with our one-way ANOVA analysis, as it is logical that a significant difference in vacuole counts as a function of time and fertilizer concentration would correspond to a significant difference in the rate of vacuole formation over time due to fertilizer concentration.

Our experiment could be improved upon by increasing the difference in concentration between the high and low fertilizer concentration treatments. Exaggerating the difference in concentrations could produce more pronounced results. For further studies, the effects of natural fertilizers could be tested. The trend observed in our experiment of how a higher nitrogen concentration results in an increase in the average rate of vacuole formation in *T. thermophila* is important and should be furthered studied. Further experiments should be carried out with different fertilizers, since some trace elements that are part of fertilizers have been seen to be harmful to ciliates (Dayeh *et al,* 2005). Fertilizer run-off could have harmful effects on the salmon ecosystem. An increase in the metabolism of *T. termophila* could have similar effects to those of algal blooms such as a decrease in oxygen availability. Research on the effects of fertilizer on the ciliate is of utmost importance to ensure healthy salmon ecosystems in the present and for the future

Conclusion

We observed a statistically significant increase in the rate of food vacuole formation in the presence of increased concentrations of fertilizer. This was consistent with what we

predicted; the rate of food vacuole formation in *T. thermophila* increases with the concentration of fertilizer in nutrient media, leading us to reject the H_0 and provide support for the H_A. This experiment provided effective insight into the influence of fertilizer and limiting nutrients on the formation of food vacuoles and phagocytosis in Tetrahymena thermophila, a ciliate with significant roles in aquatic ecosystems. This research reflects the need to better monitor agricultural run off and invest in further studies to determine the cascade effects of increasing *T. thermophila* populations.

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