The Effect of Lowered pH on the Rate of Phagocytosis in *Tetrahymena thermophila*

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Abstract

With global warming acidifying oceanic and freshwater environments, the effects of pH have a significant impact on aquatic organisms (Heino et al., 2009). Salmon, a keystone species, rely heavily on ciliated protozoans as a food source (Orias et al., 2011; Pauly et al. 1996). The purpose of this experiment was to test the effects of pH on phagocytosis rate of *Tetrahymena thermophila.* This was done by exposing cells to ink-stained, pH-adjusted media and counting the number of ink-stained phagocytized vacuoles in five cells from three replicates of each treatment condition every 10 minutes during a two-hour feeding period. The mean rates determined were: 0.057 vpm at pH 5, 0.074 vpm at pH 6, and 0.074 vpm at pH 7. Using a one-way ANOVA, we obtained a test statistic (F) of 0.073 (Df =2) and a p-value of 0.930 suggesting that the mean rate of phagocytosis does not differ between pH treatments (pH 5, pH 6, pH 7). Based on these results, we do not have sufficient evidence to support our alternative hypothesis that pH does have an effect on the rate of phagocytosis of *T. thermophila.*

Introduction

Tetrahymena spp. are ciliated Protozoans that inhabit freshwater environments worldwide and play an important role in supporting the life cycle of salmon (Orias et al., 2011; Pauly et al., 1996). However, due to climate change, the livelihoods of these unicellular organisms is being threatened, thus resulting in potential large-scale effects to the salmon population that depend on them (Lee, 1942).

Globally, climate change has resulted in accelerated acidification of freshwater streams (Heino et al., 2009). Lower pH environments have decreased buffer capacity, thus lowering the ability of freshwater streams to resist changes in pH as they become more acidic (Schindler, 1988). Our investigation was inspired by this trend of freshwater ecosystem acidification as well as the potential impacts of changes in pH to the health of the Fraser River. Freshwater ecosystems can support life ranging from pH 6.5 to 9 and *T. thermophila* specifically are globally distributed in freshwater ecosystems of pHs ranging from 7-7.6

(Orias et al., 2011; Hanley, 2012; Alabaster and Lloyd, 1980). As of November 13, 2018, the acidity of the Fraser River at the Main Arm water quality buoy was pH 7.55 (Environment and Climate Change Canada, 2018). Therefore, the pH level of the Fraser River is not yet of concern, however we hope that our research provides insight on the potential effects of decreasing pH.

Johannessen and Macdonald (2009) claim that the pH of the deep basins of the Strait of Georgia is likely declining and may harm organisms. In addition, until recently, *Neocalanus plumchrus* has been the most abundant zooplankton in the Strait of Georgia but its biomass has sharply declined along with the total zooplankton population (El-Sabaawi et al., 2009). This is concerning as zooplankton which are closely related to *Tetrahymena* spp., are a major food source for larval fish, and therefore similar impacts could be expected (Pauly et al., 1996). Lowered culture levels in areas where salmon rely heavily for feeding could potentially reduce the survival and reproduction rates of salmon.

The goal of this study was to determine how acidity affects the ability of *T. thermophila* to form phagocytic food vacuoles. The phagocytic process involves the intake of food particles facilitated by the sweeping of ciliary membranelles into the oral groove (Grønlien et al., 2002; Nilsson, 1972). Our null hypothesis is that pH has no effect on the rate of phagocytosis, whereas our alternative hypothesis is that pH does have an effect on the rate of phagocytosis. Acidic environments below pH 5 have been shown to inactivate ciliary activity in *Paramecium* spp., a similar unicellular ciliate to *Tetrahymena* spp., which also feeds by the phagocytic mechanism (Lee, 1942). We believe that this may suggest a similar relationship could be at play in *T. thermophila* in response to decreasing pH; therefore, we predicted that the number of vacuoles formed per minute will decrease as pH decreases.

Methods

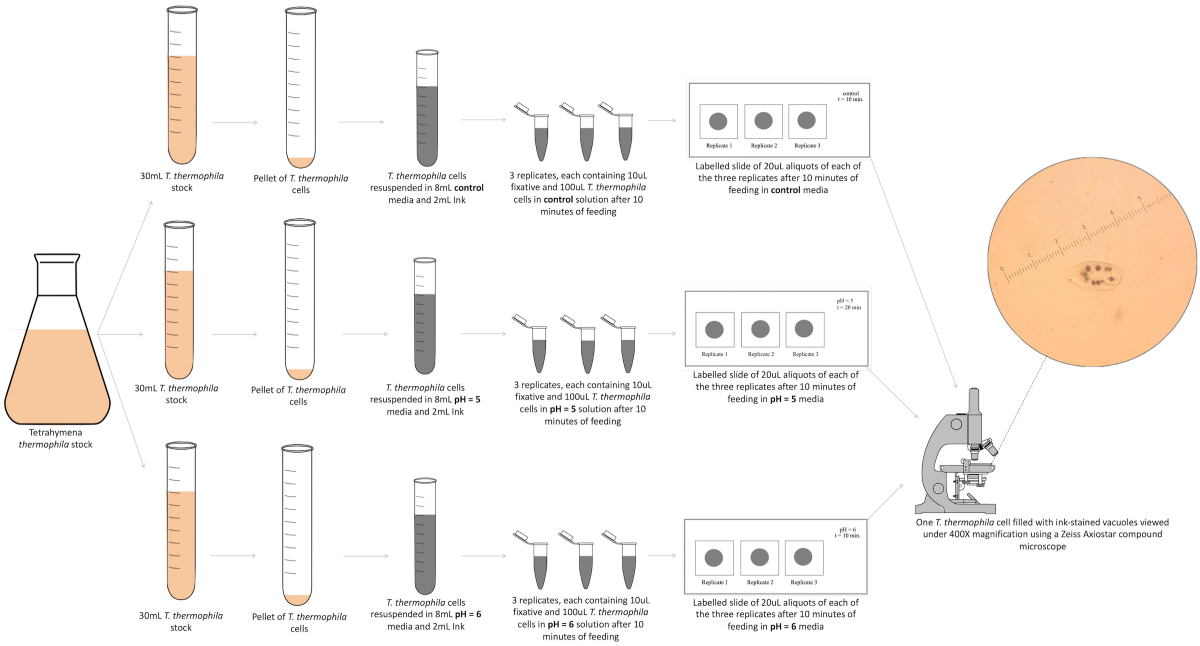

Figure 1. **Overview of experimental procedure outlining sample generation and cell observation**. All cells were viewed at 400X total magnification using a Zeiss Axiostar compound microscope. N = 3.

We swirled the *T. thermophila* stock solution and pipetted 30mL aliquots into three 50mL falcon tubes. We then centrifuged the samples at full speed for 20 minutes to compact the cells into a pellet. At this point, we removed the supernatant and resuspended the cells in 30mL of pH-adjusted *T. thermophila* media: pH = 5, and pH = 6, control (pH = 7). The acidic treatments of pH = 5 and pH = 6 were chosen to be consistent with Peng et al.'s 2012 study that found cell viability decreased significantly at acidities lower than pH = 5; therefore, in the chosen treatments we should not see absence of vacuoles due to cell death. We created three replicates by dividing the 30mL pH-adjusted stocks into three 8mL aliquots. To these test tubes, we added 2mL of pH-adjusted India ink. Every 10 minutes for a 2-hour period, we pipetted three 100µL aliquots from the surface of each test tube into three microcentrifuge tubes containing 10µL of glutaraldehyde fixative to kill cells and preserve their structures for

observation. Further, we chose to pipette from the surface of the test tubes since our lab technician, Mindy Chow, informed us that fully functional T. *thermophila* cells tend to swim to the surface of the growth medium. We had 3 replicates that corresponded to our three treatment conditions (pH = 5, and pH = 6, control), with a sample taken from each replicate every 10 minutes post-introduction to India Ink for a 2-hour time period.

We prepared slides for vacuole counting by pipetting 20µL from each sample as seen in Figure 1. Again, according to Mindy Chow, it was recommended that we pipette from the bottom of the microcentrifuge tubes as dead cells tend to sink. This process was repeated for subsequent samples under all treatment conditions. We placed coverslips on the respective 20µL aliquots and used petroleum jelly to seal the edges of the coverslip to maintain slide viability and preventing desiccation. We then counted the number of ink-stained vacuoles present in 5 cells under 400X total magnification using a Zeiss Axiostar microscope. Selection bias was minimized by counting cells in the upper right quadrant of the slide first, and then moving clockwise from this point if not enough cells were found. Only cells containing vacuoles were included as to ensure a functioning phagocytic mechanism.

We then calculated the average rate of vacuole formation for each treatment condition and performed a one-way ANOVA in order to infer if a significant difference existed in the mean rates of vacuole formation between the three treatments using a confidence level of 95% (alpha = 0.05).

Results

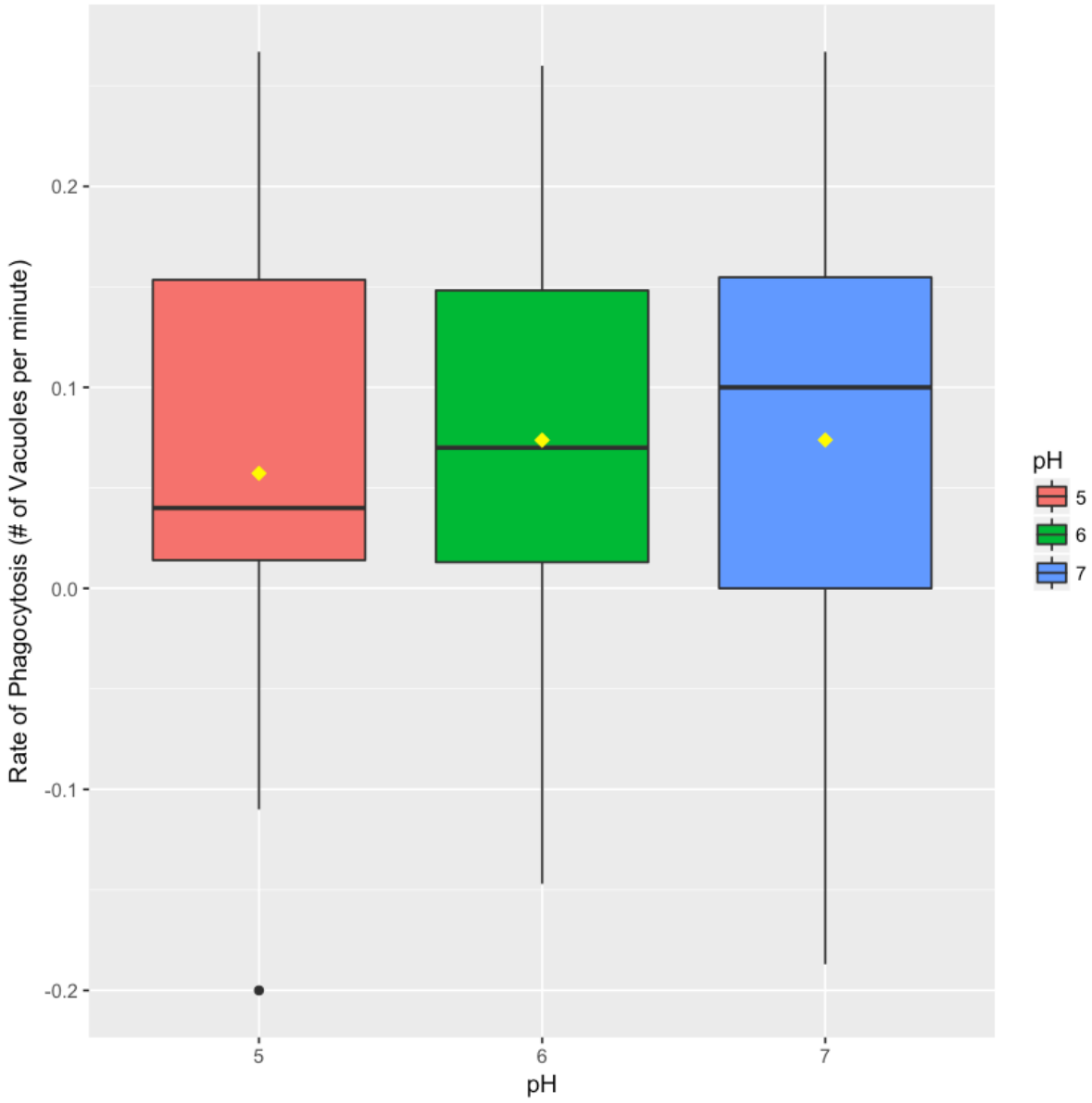

Figure 2: **Box and whisker plot of the mean rate of phagocytosis (number of vacuoles formed per minute) in *T. thermophila* in treatments of pH 5, 6, and a control pH of ~7.**
The thick bar in the middle of each boxplot is the median. The yellow diamonds indicate the mean. The upper and lower bounds of the box extend from the first to the third quartile. The vertical lines (whiskers) extend outward of the box, stopping at the smallest and largest non-extreme values. The plot reveals one outlier in the pH 5 condition. Test statistic (F) is calculated to be 0.073 under 2 degrees of freedom and its associated p-value is 0.930 | N = 3.

The mean rate of phagocytosis at pH 5 is 0.057 vacuoles per minute (vpm) with a 95% confidence interval ranging from -0.029 vpm to 0.143 vpm. The standard deviation for the pH 5 condition is 0.135 vpm. The mean rate of phagocytosis at pH 6 is 0.074 vpm with a

95% confidence interval ranging from 0.001 to 0.146 vpm. The standard deviation for the pH 6 condition is 0.114 vpm. The mean rate of phagocytosis at the control treatment of pH 7 is 0.074 vpm with a 95% confidence interval ranging from -0.001 to 0.148 vpm. The standard deviation for the pH 7 condition is 0.117 vpm. There were 3 replicates for each treatment and twelve 10-minute time intervals. The sets of data obtained from all 3 pH conditions were normally distributed with equal variance. With this information, we performed a parametric, one-way ANOVA using RStudio. We calculated the test-statistic, F, to be 0.073 (Df =2) and the p-value to be 0.930.

Figure 2 summarizes the percentiles of phagocytosis rate in all 3 treatments. Based on the samples collected in pH 5, the first quartile is 0.014 and the third quartile is 0.154 vpm. For the data sampled in pH 6, the first quartile is 0.013 and the third quartile is 0.148 vpm. Lastly, for pH 7, the first quartile is 0.00 and the third quartile is 0.155 vpm. As depicted in Figure 2, the median for the treatment of pH 5 is 0.040 vpm, median for pH 6 is 0.070 vpm (close proximity to the mean), and median for pH 7 is 0.100 vpm.

Discussion

Tetrahymena spp. internalize food particles via phagocytosis by beating their oral ciliary membranelles to sweep in food into the oral groove (Grønlien et al., 2002; Nilsson, 1972). In this experiment, we use the rate of vacuole formation as a proxy to measure the rate of phagocytosis. The average rate of phagocytosis was lowest at pH 5 and the same at pH 6 and 7. The results of this study showed that the differences of media pH levels at pH 5, 6, and 7 do not have a significant effect on the rate of phagocytosis of *T. thermophila*. Statistical analyses of our data resulted in a p-value of 0.930, which is greater than the alpha value of 0.05. Therefore, we are unable to reject our null hypothesis that pH has an effect on the rate

of phagocytosis of *T. thermophila*. Conversely, we are unable to support our alternate hypothesis that pH does affect the rate of phagocytosis. We predicted that similar to *Paramecium* spp., a decrease in pH would result in the inactivation of ciliary membranelles found at the oral groove (Lee, 1942). However, we did not obtain significant differences for the rate of phagocytosis between varying pH treatments and consequently, no such conclusion can be made.

The optimal pH for *Tetrahymena gleii* to grow was determined to be pH 7.25-7.30, with minor variations between pH 5.65-8.40 having little changes to the growth rate (Prescott, 1958). The researcher conducted a growth curve assay by measuring the time it took to produce a new generation of cells (Prescott, 1958). Population growth at pH 5.3 was observed but at a slow rate of a 7-hour generation time, which is a drastic difference compared to the pH 5.65 generation time of 3 hours (Prescott, 1958). The generation time Prescott (1958) determined at the optimal pH was approximately 2.5 hours and at pH 6 was between 2.5 to 3 hours. Therefore, the insignificance of our results may have been due to the experimental time of exposure to the treatments of pH 5, 6 and 7. Since nutrient uptake via phagocytosis results in growth (Elliot, 1973), and growth can be measured in generation time, it can be inferred from Prescott's (1958) study that a longer experimental duration is needed to observe any significant effects of pH on the phagocytosis rate of *Tetrahymena* spp. Our experiment was conducted over a 2-hour time period based on instructor recommendation to modify the *Measuring Phagocytosis with Tetrahymena in 1% India ink* protocol by Giannini and Severson (2015). Even with the extension of the time period, this was perhaps an insufficient amount of time to yield a significant difference in the rate of phagocytosis across the treatments.

Although the means are relatively similar in all 3 treatments, the median seems to increase as pH increases (becomes more neutral). Unlike the median, the mean is much more sensitive to extreme measurements which is indicated by the black dot denoting the one outlier (-0.2 vpm in pH 5) in Figure 2. Furthermore, we would like to acknowledge the negative rates observed on the data plot represented by whiskers below 0 vpm and the pH 5 outlier at -0.2 vpm. This may have been due to a different set of cells fixed at each time point, and by random chance those cells had less vacuoles observed than the previous interval. To improve on this experiment, we recommend using a live tracking method to follow the same set of cells over the entire time frame.

Future experiments with a longer time frame could better elucidate the effects of pH on *T. thermophila'*s rate of phagocytosis. Based on Prescott's 1958 experiment, we recommend increasing the intervals of at least 12 time points to 20 minutes for a total duration of 4 hours or extending the time frame as deemed feasible. Moreover, by increasing the number of replicates, a significant difference in the rate of phagocytosis may be observed

Limitations of this experiment may have also factored into the results. A clear criteria of visual characteristics were established in order to count vacuoles (dark, clearly defined, and did not fade when adjusting the focus). However, five individuals contributed to the counting process which may have introduced uncertainty. Moreover, samples were fixed and slides were prepared for counting in one work period. However, some samples were observed to have an insufficient number of cells or an insufficient number of cells containing vacuoles. A second work period of counting and slide preparation was necessary using the fixed samples from the first work period to collect data. At this time, the cells had settled to the bottom of the microcentrifuge tubes which made it easier to locate cells with vacuoles.

Conclusion

Results of this experiment showed that there was no statistical difference in the rate of phagocytosis between the pH treatments of 5, 6, and 7 (control). Contrary to our prediction, the rate did not decrease significantly as pH decreased. Therefore, we are unable to provide evidence to reject the null hypothesis that pH does not have an effect on the rate of phagocytosis. Since pH may have effects on other parts of the ecosystem that we did not study such as alternative food sources, the results cannot be extrapolated to salmon feeding or survivability.

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Appendix

Table A1: **Raw data for pH 5.**
Counted the first five cells with vacuoles in each replicate (A, B, and C) using a Zeiss Axiostar compound light microscope at 400X magnification.

Time	A (pH 5) → # of vacuoles	B (pH 5) → # of vacuoles	C (pH 5) → # of vacuoles
10 minutes	2, 1, 1, 1, 1	3, 1, 4, 5, 2	1, 1, 1, 1, 1
20 minutes	1, 2, 4, 2, 6	1, 4, 2, 1, 2	2, 3, 1, 1, 2
30 minutes	3, 1, 2, 3, 2	4, 3, 2, 4, 3	2, 2, 2, 3, 3
40 minutes	2, 2, 2, 2, 1	3, 5, 3, 1, 1	4, 2, 3, 3, 3
50 minutes	4, 5, 4, 6, 1	7, 6, 7, 6, 7	3, 2, 6, 7, 6
60 minutes	4, 6, 9, 5, 5	5, 4, 3, 9, 5	4, 3, 8, 6, 5
70 minutes	2, 5, 4, 2, 11	6, 10, 5, 7, 5	8, 5, 8, 8, 2
80 minutes	6, 8, 5, 11, 8	13, 8, 9, 6, 10	8, 10, 8, 8, 6
90 minutes	10, 9, 9, 8, 5	10, 6, 11, 9, 9	7, 13, 6, 9, 9
100 minutes	4, 8, 7, 9, 7	13, 7, 3, 6, 11	7, 8, 4, 9, 8
110 minutes	8, 11, 9, 11, 6	7, 9, 7, 12, 11	8, 4, 9, 7, 14
120 minutes	10, 7, 8, 6, 5	9, 5, 6, 8, 7	12, 7, 6, 1, 6

Table A2: **Raw data for pH 6.**
Counted the first five cells with vacuoles in each replicate (A, B, and C) using a Zeiss Axiostar compound light microscope at 400X magnification.

Time	A (pH 6) → # of vacuoles	B (pH 6) → # of vacuoles	C (pH 6) → # of vacuoles
10 minutes	1, 2, 2, 1, 1	1, 1, 1, 1, 2	1, 1, 3, 1, 1
20 minutes	2, 1, 3, 3, 2	1, 2, 3, 2, 1	4, 3, 2, 5, 4
30 minutes	2, 4, 3, 1, 4	3, 2, 3, 2, 1	4, 4, 3, 2, 2
40 minutes	4, 5, 4, 6, 5	5, 5, 2, 5, 3	2, 5, 4, 7, 6
50 minutes	11, 3, 2, 3, 4)	2, 5, 7, 5, 1	2, 6, 5, 9, 5
60 minutes	7, 7, 5, 6, 4	7, 5, 11, 11, 3	4, 5, 3, 5, 8
70 minutes	8, 6, 5, 4, 7	7, 6, 3, 5, 8	5, 2, 9, 4, 6
80 minutes	8, 1, 10, 7, 11	7, 6, 4, 6, 9	10, 10, 12, 5, 5
90 minutes	10, 6, 10, 8, 9	8, 8, 9, 8, 6	8, 4, 2, 10, 7
100 minutes	2, 5, 8, 3, 5	5, 10, 5, 6, 8	2, 3, 7, 13, 9
110 minutes	10, 7, 8, 4, 9	12, 7, 9, 7, 7	10, 12, 8, 11, 9
120 minutes	7, 9, 9, 12, 10	10, 13, 5, 4, 11	7, 9, 8, 6, 13

Table A3: **Raw data for pH 7.**
Counted the first five cells with vacuoles in each replicate (A, B, and C) using a Zeiss Axiostar compound light microscope at 400X magnification.

Time	A (pH 7) → # of vacuoles	B (pH 7) → # of vacuoles	C (pH 7) → # of vacuoles
10 minutes	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0
20 minutes	1, 2, 2, 2, 1	3, 1, 1, 2, 3	3, 1, 2, 1, 1
30 minutes	5, 6, 1, 1, 2	3, 2, 3, 6, 3	5, 5, 3, 2, 3
40 minutes	5, 1, 2, 1, 6	3, 1, 6, 8, 4	3, 1, 3, 1, 5
50 minutes	3, 7, 3, 3, 5	6, 5, 4, 4, 7	5, 5, 5, 3, 8
60 minutes	6, 10, 8, 3, 8	5, 6, 8, 10, 10	6, 9, 11, 8, 5
70 minutes	4, 7, 6, 5, 4	6, 4, 6, 7, 5	6, 9, 7, 5, 4
80 minutes	8, 5, 6, 3, 8	4, 12, 3, 8, 9	9, 7, 4, 6, 8
90 minutes	7, 8, 8, 9, 11	3, 8, 4, 4, 8	9, 7, 5, 6, 2
100 minutes	11, 9, 10, 4, 6	2, 6, 8, 8, 7	11, 5, 6, 11, 3
110 minutes	3, 6, 8, 6, 6	15, 9, 10, 7, 7	5, 11, 4, 8, 12
120 minutes	10, 11, 7, 7, 2	8, 12, 11, 7, 13	8, 11, 8, 9, 9

Table A4: **Statistical ANOVA table generated using RStudio (Jupyter Notebook).**

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
pH	2	0.002189056	0.001094528	0.0729034	0.9298399
Residuals	33	0.495442167	0.015013399	NA	NA

$$\frac{Cell\ 1 + Cell\ 2 + Cell\ 3 + Cell\ 4 + Cell\ 5}{5} = Average\ number\ of\ vacuoles\ per\ cell\ (n\ interval \mid z\ replicate)$$

Figure A1. Equation for calculating the average number of vacuoles formed in 5 cells of replicate z after n minutes of feeding.

$$\frac{average\ \#\ of\ vacuoles\ (2nd\ interval) - average\ \#\ of\ vacuoles\ (1st\ interval)}{10\ minutes} = Rate\ (vacuoles\ per\ minute)$$

Figure A2. Equation for calculating the average number of vacuoles formed between three samples of the same treatment condition (pH = 5, 6, or control) after n minutes of feeding.

$$\frac{Replicate\ A + \ Replicate\ B\ + \ Replicate\ C}{3} = Average\ number\ of\ vacuoles\ (n\ interval)$$

Figure A3. Equation for calculating the average rate of vacuole formation between two adjacent 10-minute feeding time intervals.