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Article

Low environmental pH inhibits phagosome formation and motility of *Tetrahymena pyriformis*

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SUMMARY

***Tetrahymena pyriformis*, single-celled protozoans, populate ponds, lakes, and streams. As 2.57 million tons of carbon dioxide enter the atmosphere every second, Earth's bodies of water acidify rapidly, creating harmful habitats for organisms such as ciliates at the bottom of the food chain, like *T. pyriformis*. Investigating the ability of *T. pyriformis* to feed in acidic pHs presents a deeper understanding of the short-term ramifications of carbon dioxide emissions on freshwater ecological communities. In this experiment, we varied pH from 4.5 to 7.0 by diluting carbonated water. To observe *T. pyriformis* food vesicle formation, we counted the number of phagosomes located in iodine-fixed *T. pyriformis* after a 10-minute feeding period. We hypothesized that increased suspension acidity would reduce *T. pyriformis*' consumption of food vesicles due to inhibited motility functions and phagosome formation during phagocytosis. Our data suggests *T. pyriformis* best generate phagosomes within a pH range of 6.0 to 7.0. Data displayed a low average vesicle count of 0.60 ± 0.16 at a pH of 4.5 and a high average vesicle count of 3.73 ± 0.18 at a pH of 7.0. At a pH of 6.0, average vesicle count plateaued as its rate of increase slowed. We posit that as pH levels decrease, *T. pyriformis* lose feeding competence due to three probable mechanisms: increased membrane density, weakened myosin necessary for vesicle transport, and inhibition of ciliary movement –– all components necessary to initiate and complete phagocytosis.**

INTRODUCTION

Since the late 19th century, our industries, modes of transportation, agricultural operations, and residential needs have required the burning of fossil fuels, a process which releases harmful greenhouse gases into the atmosphere (1, 2). As global emissions have steadily increased due to population growth and its consequent demand, Earth's bodies of water have absorbed unexpected amounts of carbon dioxide, resulting in their acidification through the formation and subsequent dissociation of carbonic acid (3, 4). Even subtle changes in environmental conditions, such as water acidity, can disturb the balance of ecosystems (4). Investigating the potential consequences of acidification on aquatic organisms gives insight on the ecological changes human fossil fuel emissions can cause. Our study of *T. pyriformis* initiates this investigation, starting at the bottom of the food chain.

T. pyriformis are ciliated eukaryotes that inhabit fresh bodies of water, feeding on bacteria through the process of phagocytosis (5, 6). These unicellular paramecia function as model organisms due to their distinct, universal cellular functions such as their food vesicle maturation process (5). Using their cilia (**Figure 1A**), *T. pyriformis* pull bacterial particles into the base of their complex oral groove where they organize their food into fatty membrane vesicles, or phagosomes, using four ciliated membranelles (7). The phagosomes then enter the complex network of membrane trafficking pathways before exiting the organism through egestion (6). Membrane trafficking depends heavily on phospholipids residing in the cytoplasmic leaflet, called phosphoinositides, for vesicle recognition and transport (8, 9). In addition, vesicle transport from the oral apparatus requires the use of myosin motor proteins, composed of fibrous yet

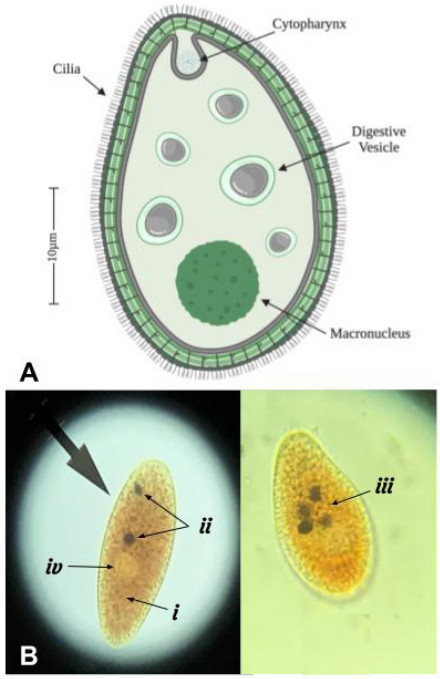

Figure 1: Cellular components of *Tetrahymena pyriformis*. A) Simplistic diagram of a *T. pyriformis* based on a camera lucida image of the organism (17, Abdullah, Shamall). Cellular contents of the organism are labeled. B) A microscopic view at 400X magnification of *T. pyriformis*, fixed with a 20% iodine solution (i, referring to the orange-brown coloration). The left image shows a *T. pyriformis* containing two India Ink food vesicles (ii) after feeding for 10 minutes in a 5.0 pH suspension, while the right image shows a *T. pyriformis* containing four India Ink food vesicles (iii) after feeding for 10 minutes in a 6.5 pH suspension. (iv) points towards the macronucleus of the organism. These photos were taken using an iPhone camera.

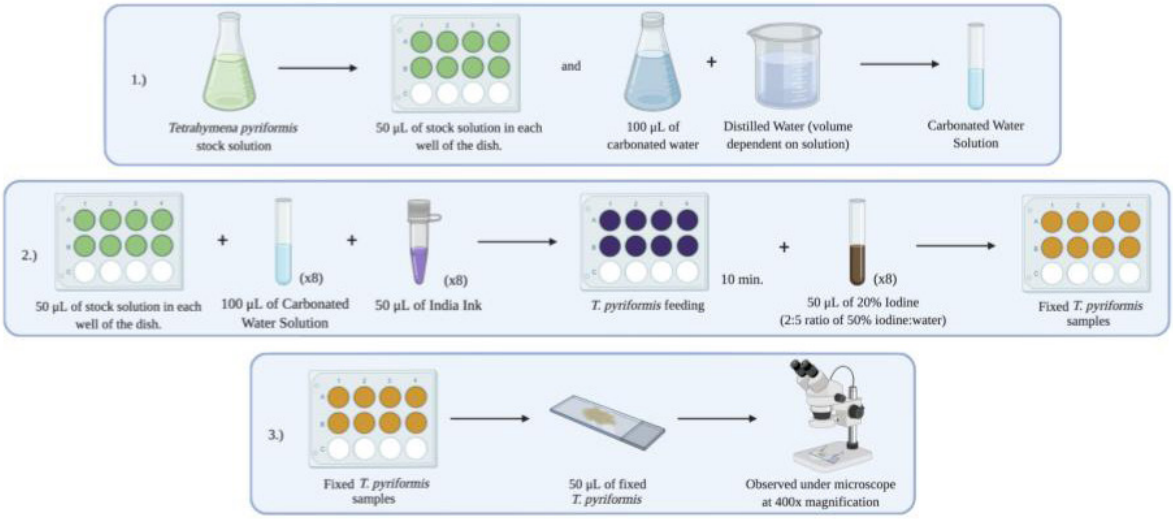

Figure 2: Overview of the stepwise experimental procedure for preparing the samples and the observing the fixed *T. pyriformis*. Step 1.) shows the configuration of the *T. pyriformis* solution, which includes the setup of the multi-well dish, as well as the preparation of the various carbonated water solutions used to alter the environmental acidity. Step 2.) incorporates and describes both the feeding and the fixing processes of the organism. Each of the 8 100 mL carbonated water solutions were separate treatments that varied in pH measurement. Lastly, Step 3.) depicts the sampling and data collection procedures.

dynamic actin molecules crucial for motility (9). All of the molecules work in concert to complete maturation; therefore, if any step is interrupted along the way because of extreme habitat conditions, phagocytosis may not be possible.

The goal of this experiment was to investigate the effect of varying environmental pH on *T. pyriformis'* ability to produce phagosomes containing 2% India Ink, an experimental proxy for food. Through simulating the acidification of freshwater environments due to excess CO_2 in the atmosphere, we aimed to discover optimal feeding pH for *T. pyriformis*. We hypothesized that as acidity increased, *T. pyriformis* would consume less, producing fewer food vesicles, which would implicate their inability to successfully complete phagocytosis. We propose that as environmental pH drops below the neutral freshwater surface conditions of a pH ranging from 7.5–8.5 (10), increased amounts of hydrogen ions (lower pH/acidic conditions) might negatively alter the molecular composition of cilia and membranes, inhibiting *T. pyriformis'* motor functions and vesicle formation processes. Our data uncovers that *T. pyriformis* consume the most food vesicles within an optimal pH range of 6.0–7.0. At any point below a pH of 6.0, *T. pyriformis* lose feeding competence, consuming fewer food vesicles. These results display the danger freshwater acidification poses to even small ciliated organisms, providing reasonable evidence to further research acidification's impact on other aquatic organisms and to uncover the urgency of limiting our fossil fuel emissions.

RESULTS

To determine whether our hypothesis that increased acidity would inhibit phagocytosis and prevent *T. pyriformis* from generating a baseline amount of food vesicles, we prepared seven different treatment groups of the varying acidities: 4.5, 5.0, 5.5, 6.0, 6.2, 6.5, and 7.0, which acted as a negative control, replicating a standard freshwater habitat pH. We created separate treatments by diluting carbonated water, which had a pH of 4.5, with differing amounts of distilled water. Carbonated water mimicked the conditions of a freshwater habitat after absorbing carbon dioxide, and each treatment simulated a more severely acidified environment. After quantifying each treatment's pH using paper pH strips, we mixed *T. pyriformis* and India Ink, a dark dye and proxy for food, into a treatment solution (**Figure 2**). After a 10 minute feeding period, we fixed the *T. pyriformis* with iodine and then, under 400x magnification, counted the number of India Ink food vesicles within 5 different *T. pyriformis* within a specific treatment (**Figure 2**); an increase or decrease in *T. pyriformis'* consumption of food signaled a loss in feeding competency resulting from changes in suspension conditions. The data collected from each *T. pyriformis* at this step represented a pseudo-trial, a replicate collected from within a single trial; yet, pseudoreplicates are not completely independent from one another and therefore cannot solely be used to assess statistical significance. As a result, we repeated these steps three times for each of the 7 treatments, obtaining a total of 15 replicates for every condition. A final treatment (pH = 7.0) without carbonation and without India Ink acted as a second negative control.

As pH increased, *T. pyriformis* formed more India Ink vesicles, consuming the largest mean number of food vesicles of 3.73 ± 0.18 (± standard error) at a pH of 7.0 (negative control), and the smallest mean number of food vesicles of 0.60 ± 0.16 at a pH of 4.5 (**Figure 3**). At a pH of 7.0 with no food available and no carbonation added (second negative control), no *T. pyriformis* held any food vesicles within their bodies. We observed no qualitative changes in morphology *T. pyriformis* across different pH suspensions (**Figure 1B**).

A one-way analysis of variance (ANOVA) performed on our dataset yielded significant variation among our tested conditions ($F(7, 112) = 58.41$, $p = 1.74 \times 10\text{-}34$). A post hoc Tukey's test revealed that mean food vesicle count increased with significance ($p < 0.001$) from 0.60 ± 0.16 to 2.07 ± 0.21 while suspension pH increased from 4.5 to 5.0; then, from a treatment pH of 5.0 to 5.5, mean vesicle count significantly increased ($p < 0.05$) again from 2.07 ± 0.21 to 2.87 ± 0.26 (**Table 1**). No statistical significance was identified between

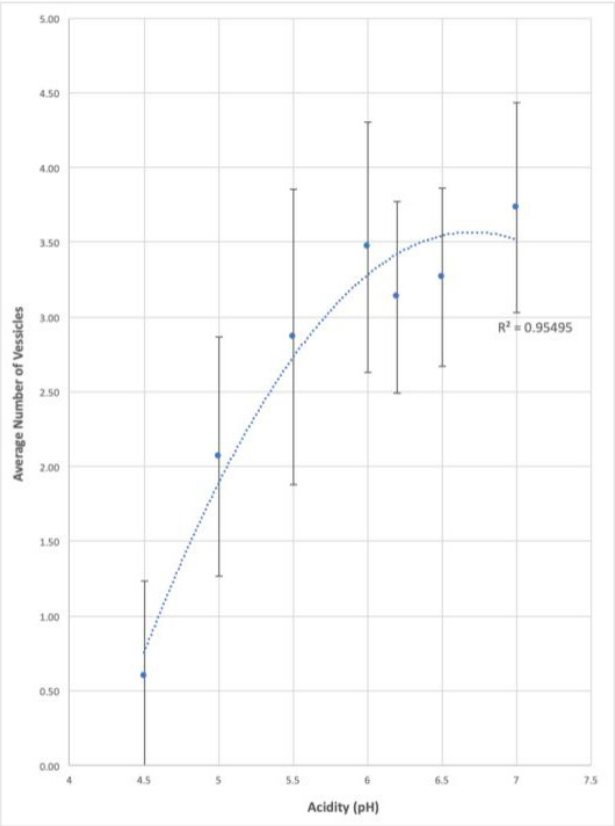

Figure 3: The mean value of India Ink vesicles counted within iodine-fixed *T. pyriformis* in variable pH environments. Means of food vesicles result from 15 replicates (n = 15) collected over 3 trials. Vesicles were counted (at 400X magnification) after *T. pyriformis* were fed India Ink for 10 minutes in various acidic concentrations of carbonated water (4.5 ≤ pH ≤ 7.0). Mean vesicle counts in treatments with a pH of 5.5 and below were statistically significant ($p \leq 0.05$), while data collected in a pH of 6.0 and higher was statistically similar ($p \geq 0.05$). When no carbonation and no India Ink were added to the environment, there were no vesicles observed in any *T. pyriformis* (not shown above). Error bars denote the calculated standard deviation (SD) of each run.

all sequential treatment pairings with pHs of 5.5 and higher (**Table 1**). However, a comparison between pH treatments of 5.5 and 7.0 with mean vesicle counts of 2.87 ± 0.26 and 3.73 ± 0.18, respectively, also yielded statistical difference ($p < 0.025$, **Table 1**). The data set fits the trend of a quadratic polynomial curve with an r2 value of 0.955. Additionally, p-values calculated using two-tailed T-tests between different sets of pseudo-trials within each run were all greater than 0.05, showing statistical similarity, with the exception of two different trials from testing in a pH of 6.0 ($p < 0.0004$).

DISCUSSION

Our data supports the hypothesis that a lower environmental pH decreases the amount of visible food vesicles within *T. pyriformis* after a 10-minute feeding period. However, the data collected also revealed a new element to this trend: as pH increased from acidic to neutral, food vesicle count increased at a decreasing rate. Mean food vesicle count plateaued at pHs higher than 5.5 (**Figure 3**), when data points no longer were statistically different from one another ($p > 0.05$, **Table 1**). But

Treatment Pairs	Tukey HSD p-value
pH 4.5 v. pH 5.0	0.00100
pH 5.0 v. pH 5.5	0.04727
pH 5.5 v. pH 6.0	0.28596
pH 6.0 v. pH 6.2	0.89999
pH 6.2 v. pH 6.5	0.89999
pH 6.5 v. pH 7.0 (no carb)	0.59689
pH 5.5 v. pH 7.0 (no carb)	0.02235

Table 1: Results of a post-hoc Tukey's Test indicating statistical significance between treatments groups. Calculated Tukey HSD p-values are listed for treatment group comparisons labeled in the leftmost column. Green coloration denotes statistical significance ($p \leq 0.05$) while red coloration signals statistical similarity ($p \geq 0.05$).

mean vesicle counts between pH treatments of 5.5 and 7.0 remained statistically different ($p < 0.025$), demonstrating that the optimal feeding environment of *T. pyriformis* lies within a pH range of 6.0 to 7.0. The most probable explanation for this phenomenon is acidity's direct effect on *T. pyriformis'* process of phagocytosis. As the hydrogen ion concentration of their suspension increases, *T. pyriformis'* cell membrane thickness and structure can change rapidly and drastically. Studies investigating other membranes such as phosphatidylcholine bilayers, which occur naturally in eggs, reveal that acidic environments cause decreased head repulsions in phospholipids around a pH of 5.5, increasing interfacial tension and density of the bilayer within nanoseconds (11, 12). Bilayers containing phospholipids with amphiphilic heads occurring in eukaryotic cells, such as *T. pyriformis*, may react similarly to protonation caused by high hydrogen ion concentrations. Increased membrane density could directly inhibit the function of phosphoinositides, which would prevent phagosome transport and organization. Phosphoinositides are essential elements in endosome dynamics, as well as various cell signaling pathways, necessary for regulating the movement of food vesicles throughout phagocytosis (9). Without functional phosphoinositides, food vesicles may not successfully enter membrane trafficking pathways or even reach their respective destinations after phagocytosis, potentially explaining why fewer vesicles were observed in lower pH environments. Though we saw no visible alterations in food vesicle morphology, even subtle changes in membrane density may have hindered phosphoinositide function. Finally, phagosomes rely on actin-based myosin motors to move from the oral apparatus into the cell's interior; yet, acidic environments with a pH of lower than 6.5 can weaken myosin's average force by 20% (13). Even if weakened myosin can still transport phagosomes, they must do so at a slower rate, a discovery which may explain the drastic drop in vesicle count seen in pHs lower than 6.5 (**Figure 3**).

Distilled H_2O (µL)	Carbonated H_2O (µL)	Resulting pH
0	100	≈ 4.5
20	100	≈ 5.0
40	100	≈ 5.5
60	100	≈ 6.0
80	100	≈ 6.2
100	100	≈ 6.5
100	0	≈ 7.0

Table 2: Ratios of distilled water to carbonated water to obtain 6 distinct carbonated water solution pHs. Micropipettes were used to control amounts of each component added into the mixture. Acidity values were measured using paper pH strips.

However, low pH might not hinder only the process of phagocytosis; in environments with pHs lower than 5.0, *T. pyriformis'* cilia can become completely inactivated (14). High hydrogen ion concentrations slow the mechanical activity of cilia in all paramecia, causing an exponential drop in cilia's mechanical speed starting at a pH of around 5.5 (15). Not only does this inhibition of ciliary mechanics prevent *T. pyriformis* from reaching food, but it also prevents them from sweeping food into their oral groove at a normal rate. The critical role of ciliary mechanics in *T. pyriformis'* movement and food consumption offers two explanations for the decreased phagosome production observed below a pH of 5.5.

Ciliary mechanics change in basic solutions as well, solidifying our prediction of a trendline projection that peaks around a pH of 6.5, after which the mean vesicle count would decrease again. At pH solutions above a pH of 7.0, the speed of paramecium's ciliary movement slows, eventually becoming completely deactivated at a pH of 9.5 (15). Other researchers have observed a significant decrease in food uptake of paramecium in basic solutions (14). Based on this information, we predict a decrease in food vesicle count within *T. pyriformis* in more basic solutions, an outcome supporting the idea that *T. pyriformis* feed best in an optimal pH range of 6.5 to 7.0.

A few confounding variables existed in the context of this experiment. Primarily, the size and membrane composition of each food vesicle was inconsistent, meaning *T. pyriformis* with a few large vesicles could have consumed the same quantity of food as ones with many smaller vesicles. Calculating food vesicle area in ImageJ could prove a more accurate method of quantifying food intake. Quickly measuring carbonated water with pipettes generated another source of uncertainty. Bubbles of carbonation would often enter the pipette tip, causing ambiguity in our measurements. To produce more precise results, runs could have been staggered farther apart to eliminate any rush while pipetting. A third source of uncertainty was the determination of pH measurements of each solution using pH strips, since the color of the strips was compared to a limited color scale. The chart increases in whole number increments, so when the color of the strip seemed to lie between two hues, it was difficult to determine the decimal of the pH. Instead, using an electronic pH meter may have increased precision because of its ability to detect minute changes in acidity.

In the future, we could initiate feeding periods after *T. pyriformis* have acclimated to their respective pH suspensions for longer periods of time, to investigate whether *T. pyriformis* can adapt to more acidic environments, better simulating the long-term process of fresh-water acidification. Additionally, studying phagocytosis or other bodily processes in different organisms may further solidify our claims and reveal more consequences of freshwater acidification. We could also collect samples from freshwater ponds, lakes, or streams to study the changing pH levels of our local habitats and how the *T. pyriformis* that reside in them are coping with their environment's varying conditions.

METHODS

Variables and Control Groups

By carbonating water, acidic freshwater environments were replicated by lowering water pH through the reactions: $H_20 + CO_2 \rightarrow H_2CO_3 \rightarrow H^+ + HCO_3^-$. Adding different concentrations of carbonated water to the *T. pyriformis* suspension varied environmental acidity and consequently lowered the overall pH to 4.5, 5.0, 5.5, 6.0, 6.2, and 6.5 respectively. For these six conditions, three trials each consisting of five pseudo-trials were conducted to determine the amount of India Ink vesicles within random *T. pyriformis* in solution. In addition to the six pH solutions listed above, two other control groups were included in the experiment. The first negative control was the original *T. pyriformis* solution with food added but no pH change, and the second negative control group was the same *T. pyriformis* solution with no pH change or food. The first control group served as a baseline for the other tests, since the natural pH that *T. pyriformis* exist in is an average freshwater pH of 7.0 (10). The second control group was necessary to confirm that without food, the *T. pyriformis* would hold no food vesicles.

Configuration of *T. pyriformis* Solutions

T. pyriformis solution (50 µL) was added into 8 separate wells of a 12-well dish. The carbonic acid concentrations, or proportionate mixtures of CO_2 and distilled H_2O, were combined in a separate multi-well dish. The carbonic acid was prepared by placing a bottle of distilled water in a SodaStream® and pressing the carbonation button for 10 seconds, as suggested by the instructions. The pH of the purely carbonated water was around 4.5; this was established as the minimum bounds of the IV. To achieve the other less carbonated solutions, the original carbonated water solution was diluted with distilled water to raise solution pH. Preliminary testing demonstrated that adding 20 µL increments of distilled water to 100 µL of carbonated water would increase the pH by roughly 0.5 per addition (**Table 2**).

Feeding the *T. pyriformis*

After the concentrations were made and the pHs were quantified prior to each trial using pH strips (**Table 2**), the acidic dilutions and food proxy were simultaneously added to the *T. pyriformis* solutions. First, 100 µL of the 4.5 pH solution and 50 µL of 2% India Ink were added to 50 µL of *T. pyriformis* in the first dish well, for a 1:1 ratio of 2% India Ink to *T. pyriformis*. These values, along with the amount of carbonated solution added, were controlled every single trial

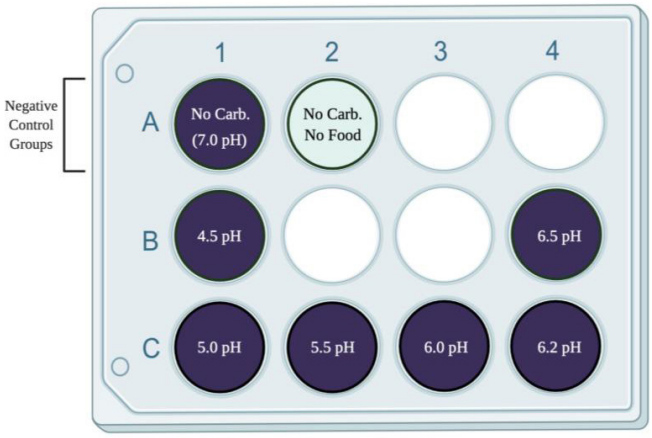

Figure 4: A multi-well dish containing all solutions observed under the microscope during testing. Each solution, with the exception of the two negative control groups, contains 50 µL of *T. pyriformis*, 50 µL of 2% India Ink, and 100 µL of specific carbonated water dilutions, which were all added simultaneously. The first negative control group (well A1) contains 50 µL of *T. pyriformis* and 50 µL of 2% India Ink, whereas the second negative control group (well A2) contains only 50 µL of *T. pyriformis*.

and run to prevent any other variables from influencing our results. Exactly 30 seconds later, a sufficient window of time to refill micropipettes, 100 µL of the 5.0 pH solution and 50 µL of 2% India Ink were added to the 50 µL of *T. pyriformis* in the second well of the dish. This process was continued for the rest of the remaining pH solutions at 30 second intervals (**Figure 3**). In the penultimate well of the dish, 50 µL of 2% India Ink was mixed with 100 µL of distilled water and combined with the 50 µL of *T. pyriformis* solution. In the last well of the dish, the 50 µL of *T. pyriformis* solution was unaltered; it was expected that this *T. pyriformis* solution would confirm that without food, *T. pyriformis* held no food vesicles (**Figure 4**). They were given exactly 10 minutes to feed.

Preparation and Administration of Fixing Solution

During the 10-minute waiting period, the dilute iodine solution used to fix the *T. pyriformis* was prepared. This dilution was generated by mixing 200 µL of 50% iodine with 500 µL of distilled water to create 700 µL of diluted 20% iodine. The 20% solution was used because the 50% iodine made the *T. pyriformis* too dark to inspect under the microscope, an observation confirmed by preliminary testing. After the 10-minute feeding, 50 µL of the 20% iodine solution was added to the first well containing the 4.5 pH solution to fix the *T. pyriformis*, allowing them to be easily observed under a microscope. Next, after 30 seconds, 50 µL of the iodine dilution was added to the second well containing the 5.0 pH solution. This same fixing procedure was repeated for all solutions in the other wells of the dishes again at 30 second intervals, including both control groups (**Figure 4**). After this process was completed, 50 µL of solution from each run was added to a slide and labeled. (**Figure 2**).

Data Collection

These slides were then inspected under a microscope at 400X magnification, and the number of large, dark food vesicles (**Figure 1B**) within five random *T. pyriformis* for each of the eight slides was counted and recorded. Occasionally, microscope focus was adjusted when looking at a single *T. pyriformis* to observe all of its bodily contents, as vesicles can lay on different focus planes within their bodies. After collecting the vesicle data for five random *T. pyriformis*, the first trial was concluded. The second and third trials, each with five pseudo-trials, were commenced in the same manner as the first. Once all data was collected, mean, SD, and standard error calculations were completed. Additionally, an ANOVA and post hoc Tukey's Test were performed in Excel to assess statistical variance among our dataset. In **Figure 2**, SD calculations were used to produce error bars, and Excel was employed to produce a best fit polynomial trend to the data.

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