Effect of India Ink Concentration on the Vacuole Formation of *Tetrahymena pyriformis*
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This paper was written as an independent research project under the guidance of Dr. Sarah Beth Mallory

**Abstract**

The increase of India ink concentration can result in increased vacuole formation per cell of *Tetrahymena pyriformis*. An increase in concentration of India ink may lead to an increase in vacuole formation because more particles are readily available for the *Tetrahymena* to ingest. The objective of this study was to evaluate the vacuole formation at various concentrations of India ink. Previous studies suggest that as the India ink concentration increases, vacuole formation decreases. Clinically reproduced *Tetrahymena* were transferred to prepared tubes of *Tetrahymena* medium and left out at room temperature for 48 hours. In order to investigate the effects of India ink concentration, some *Tetrahymena* were exposed to a mixture that was approximately 1% India ink, while others were treated with an India ink concentration of 5%. Five samples from each concentration were placed in microcentrifuge tubes. The feeding organisms were injected with a paralyzing substance at scheduled time intervals. Slides were prepared from each treated tube and examined in order to quantitate an average vacuole formation per cell. The slides from the 1% ink displayed a higher vacuole formation than the slides from the 5% ink. These results suggest that although there is an increase in India ink concentration, other factors may play a role in the lower concentration producing a higher rate of vacuole formation.

**Effect of India Ink Concentration on the Vacuole Formation of *Tetrahymena pyriformis***

*Tetrahymena pyriformis* is a ciliated, unicellular, freshwater protozoan. Its physiological structure is teardrop-shaped with a length of about 50 µm. *Tetrahymena* species are non-pathogenic and are very common in aquatic habitats. Having a short reproduction time and the ability to grow to high cell density in inexpensive media allow *Tetrahymena* to be ideal organisms to study. *Tetrahymena* are used extensively in genetic research because of the ease at which they are grown and manipulated. Although these *Tetrahymena* were grown in a bacteria-free medium, they are capable of eating bacteria and other small organic debris through a process called phagocytosis. Phagocytosis is an endocytotic process in which the organism brings its food into its mouthparts, then ingests it by forming a vacuole to translocate the food throughout the cell. For this research, India ink was used as the food for the *Tetrahymena* in order to recognize and tally the number of food vacuoles that form.

**Method**

*Preparation of Specimen*

Two samples of *Tetrahymena pyriformis* were grown 48 hours prior to the experiment. To do this procedure, samples of *Tetrahymena pyriformis* and *Tetrahymena* medium were acquired from Carolina Biological Supply Company. Aseptic technique was performed while transferring the *Tetrahymena* from the sample to the medium. The aseptic technique insures that the *Tetrahymena* are not being contaminated by atmospheric
components such as fumes, dust particles, and other bacteria. To insure sterile conditions, the entire transfer process of *Tetrahymena* was performed in a sanitized Nuaire® Biological Safety Cabinet, which allows continuous airflow resulting in sterile conditions. During the transfer, sterile pipettes were used, which means no other living organisms or other potentially contaminating particles were present within or on the surface of the pipette. First, the lid of the tube containing the *Tetrahymena* was removed and the mouth of the tube was quickly passed through the flame of a Master Glow™ burner. The pipette used to remove the *Tetrahymena* was also quickly passed through the flame and a pipette was used to withdraw a 1ml sample of the *Tetrahymena*. After the sample was obtained, the mouth of the tube was then passed through the flame again before replacing the lid to insure sterile conditions for future samples. The lid of the tube containing the *Tetrahymena* medium was then removed and the mouth of the tube was passed through the flame of the burner. The 1ml sample acquired from the first tube was placed into the tube containing the medium. The mouth of the tube containing the medium was then quickly passed through the flame, and the lid was replaced. This process was repeated to produce two freshly grown samples of *Tetrahymena* for the experiment. The *Tetrahymena* were then allowed to grow in the medium for forty eight hours prior to the experiment.

*Preparation of Ink*

To prepare the ink dilutions for the experiment, two 3 ml samples of de-ionized water, obtained from the LaGrange College chemistry lab was placed into a sterile tube. De-ionized water contains no additives which are found in tap water, which could be harmful to the *Tetrahymena*. Also, 2 fl. oz. of Speedball® Super Black® India Ink at a 10% concentration was acquired. To dilute the ink to the 1% and 5% dilutions needed for the experiment, calculations were performed to determine how much 10% ink should be added to 3ml of de-ionized water by the following formulas:

$$C_1V_1 = C_2V_2 \quad 10 \times V_1 = 5 \times (3\text{ml})$$

$$10 \times V_1 = 15 \quad V_1 = 1.5\text{ml}$$

$$C_1V_1 = C_2V_2 \quad 10 \times V_1 = 1 \times (3\text{ml})$$

$$10 \times V_1 = 3 \quad V_1 = 0.3\text{ml}$$

After performing calculations, it was determined that 1.5 ml of 10% ink should be added to 3ml of de-ionized water to create the 5% ink solution. It was also determined that 300µl of 10% ink should be added to 3ml of water to create a 1% ink solution. The calculated amounts of 10% ink were added to the two 3ml samples of de-ionized water accordingly and set aside. A complete list of materials used can be found in Appendix A.

*Treatment with Ink*

A bag of sterile 1.5ml Fisherbrand® microcentrifuge tubes were obtained for test samples. Six microcentrifuge tubes were taken from the bag. One of the tubes was for the *Tetrahymena* sample, and the five remaining tubes were for the samples of *Tetrahymena* withdrawn at various time intervals. A 300µl sample of the *Tetrahymena*, which were grown over a 48 hour time span, was placed into one of the microcentrifuge tubes and set aside. The five remaining tubes were labeled as follows: 1%/ 0 min, 1%/ 2min, 1%/ 5min,
A 2µl sample of Lugol’s, which is an Iodine solution that causes the Tetrahymena to die, was added to the five labeled tubes. Since Tetrahymena move at such rapid rates through the ink, it is difficult to accurately observe vacuole formation without adding Lugol’s. A 300µl sample of 1% ink was withdrawn using a micropipette and placed into the microcentrifuge tube containing 300 µl of Tetrahymena. Immediately a sample was extracted and placed into the test tube labeled 1%/0min containing 2µl of Lugol’s, and the timer began. At time intervals of two minutes, five minutes, ten minutes, and twenty minutes, samples of Tetrahymena were removed from the microcentrifuge tube which contained 300µl of Tetrahymena and 300µl of 1% ink and placed into the microcentrifuge tubes labeled accordingly. This process was then repeated for a second run with the 1% ink. The process of distributing the 1% ink was also performed for the 5% ink and also replicated for a second run.

**Preparation of Slides**

Samples were withdrawn from the microcentrifuge tubes that were treated with Lugol’s. One drop, approximately 2µl, from each tube was placed onto a different microscope slide and a cover slip was positioned appropriately. The slides were then placed under a microscope for observation.

**Statistical Data Analysis**

Tetrahymena which have formed vacuoles of India ink will be noticeable because of the presence of black spheres within the cell. This is measured in different samples by counting the vacuoles per cell and calculating the average at each time interval for both concentrations of India ink. Because Tetrahymena move at such a rapid rate, they must be exterminated by Lugol’s solution in order to efficiently count the vacuole content of each cell. A sample of 30 specimens was counted from each slide in order to insure statistical fairness and valid distribution. In this experiment, the bulk of the testing dealt with timing rather than a trial run of a new chemical, for example. From the 30 specimens examined for each ink concentration at every time interval, an average count of the vacuoles that formed was calculated.

**Results**

Although the procedure for both ink concentrations was kept the same, the results were noticeably different between the two concentrations. The completed data charts for all runs can be found in Appendix B; however for ease of interpretation, Appendix C shows a summary table of the experiment and the average values for each run performed. During the first run of the 1% concentration, the averages increased by 1-1.5 vacuoles for every increase in time. The second run was very similar with a slightly larger increase between 2 and 5 minutes and the times following. Throughout the first run for the 5% concentration, the averages increased by 0.1-0.9 vacuoles with each increase of time. The second run produced larger counts overall with an increase of 0.5-2.7 more vacuoles than the first run for every time interval. Comparing the two ink concentrations, the time the cells were exposed to the ink is the most influencing factor. For the 0 and 2 minute intervals, the 5% concentration produced more vacuoles per cell than the 1% concentration with an average difference of 0.08 and 0.11 vacuoles, respectively. The 1% concentration produced more vacuoles per cell over time than the 5% concentration, with an average difference of 1.22, 0.84, and 0.79 for the 5, 10, and 20 minute intervals respectively. Overall the 1% concentration showed more vacuoles per cell than the 5% concentration.
Discussion

Over the course of this experiment there were quite a few glitches that had to be overcome. The main problems developed in the beginning with calculating the appropriate amounts of specimen, India ink, and Lugol’s to use. After examining some of the first slides, it was clear to see that too little of the specimen and too much of the Lugol’s was used. There were no specimens present on the slides because apparently the few that existed were completely disintegrated by the debilitating Lugol’s substance. After finally settling on a suitable quantity for all of the substances used, the experiment was able to continue as planned. The procedure itself did not take a great amount of time; it was gathering the data from the slides that was the most time-consuming. After analyzing the data, the hypothesis was proven correct in that a decrease of vacuole formation will occur with an increase in India ink concentration. This is contrary to the general belief that if the food concentration is increased, the organism will produce more vacuoles.

Notes


3*Tetrahymena pyriformis*. http://cancerweb.ncl.ac.uk/cgi-bin/omd?tetrahymena+pyriformis


**Appendix A** – Detailed List of Materials

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
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<tr>
<td>24 Fisherbrand® Micro centrifuge Tubes</td>
<td>Carolina Biological Supply Company</td>
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<tr>
<td>1.5mL Graduated Tube with Flat Cap</td>
<td>Proteose Peptone Medium</td>
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<td>Model No. 02-682-550</td>
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<td>2 50mL Fisherbrand® Disposable Sterile Centrifuge Tubes</td>
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<td>Model No. 05-539-7</td>
<td>Carolina Biological Supply Company</td>
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<td>1 box Great Glove Disposable Latex Gloves</td>
<td>Model No. 336-584-0381</td>
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<td>1 Speedball® Super Black® India Ink</td>
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<tr>
<td>Model No. 3338</td>
<td>1 15 mL Protoslo®</td>
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<td>3 tubes prepared <em>Tetrahymena</em> Medium</td>
<td>Carolina Biological Supply Company</td>
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<tr>
<td>Carolina Biological Supply Company Model No. 13-2315</td>
<td>Model No. 88-5141</td>
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<td>1 tube <em>Tetrahymena pyriformis</em></td>
<td>1 box Tip One 0.1-10µl Extended</td>
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<tr>
<td>Model No. U121917K-3628</td>
<td>Length Natural Tips</td>
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<tr>
<td>USA Scientific, Inc.</td>
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<td>Model No. 1110-3800</td>
<td>1 Eppendorff® T.I.P.S Box 50-1000µl</td>
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1 Eppendorff® T.I.P.S Box 2-200µl
Model No. U122-807N-1634

1 Eppendorff® Micropipette 0.5-10µl
1 Eppendorff® Micropipette 50-200µl
1 Eppendorff® Micropipette 100-1000µl
1 Test Tube Rack

1 MasterGlow® burner
1 NuAire® Biological Safety Cabinet
Class II Type A/B3
Model No. NV-425-600

1 Swift M3500-D microscope

1 box microscope slides
1 box microscope cover slips
Appendix B – Completed Data Charts
Appendix C – Summary Table of Data

<table>
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<tr>
<th>Time (min)</th>
<th>1% ink Run 1</th>
<th>1% ink Run 2</th>
<th>Average</th>
<th>5% ink Run 1</th>
<th>5% ink Run 2</th>
<th>Average</th>
<th>Diff. in Ave.</th>
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<td>6.00</td>
<td>4.65</td>
<td>0.79</td>
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</table>
Appendix D – Graphs Displaying Data

*Figure 1.* Comparison of the two runs performed with the 1% concentration of India ink.

![1% Concentration: Comparison of Run 1 and Run 2](image)

*Figure 2.* Comparison of the two runs performed with the 5% concentration of India ink.

![5% Concentration: Comparison of Run 1 and Run 2](image)
Figure 3. Comparison of both concentrations at the different time intervals.

![Comparison of Ink Concentrations](image-url)