

# The effect of zinc chloride on the formation of food vacuoles in *Tetrahymena thermophila*

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## ABSTRACT

Heavy metals, such as zinc, are essential nutrients required by organisms for survival, but can be toxic at high concentrations. In this study, we tested the effects of increasing concentrations of zinc chloride on the formation of food vacuoles in *Tetrahymena thermophila*. We were also interested in seeing if increasing incubation times in zinc chloride affected food vacuole formation. These were the basis for our three hypotheses, which tested for the effect of zinc concentration, time, and zinc and time together, on the formation of food vacuoles. Our experiment involved introducing three different concentrations of zinc chloride ( $0 \text{ mg L}^{-1}$ ,  $50 \text{ mg L}^{-1}$ , and  $120 \text{ mg L}^{-1}$ ) to the nutrient medium of *T. thermophila*. We incubated for three different time intervals (20 minutes, 2 hours, and 26 hours) before adding India ink to stain the food vacuoles and fixing the *T. thermophila*. After the cells were fixed, we counted the number of black food vacuoles (those that have engulfed the India ink) for 10 cells in each treatment for 3 replicates each, resulting in nine averages for each permutation of  $[\text{ZnCl}_2]$  and time treatment. Finally, we conducted a two-way ANOVA analysis and found that our  $p$ -values for our  $[\text{ZnCl}_2]$ , time, and both factors considered together to be  $2.5 \times 10^{-3}$ ,  $2.6 \times 10^{-6}$ , and 0.31, respectively. We were able to reject our first two null hypotheses, providing support for our alternate hypotheses. Our results may be due to zinc's inhibitory effects on the formation of cilia as well as the physiological changes that starved *T. thermophila* undergo. However, the  $p$ -value was greater than 0.05 when both factors were considered together, so we failed to reject our final null hypothesis. This is most likely due to our low zinc concentration not being trace enough compared to successful experiments in the past (Nilsson 2003).

## INTRODUCTION

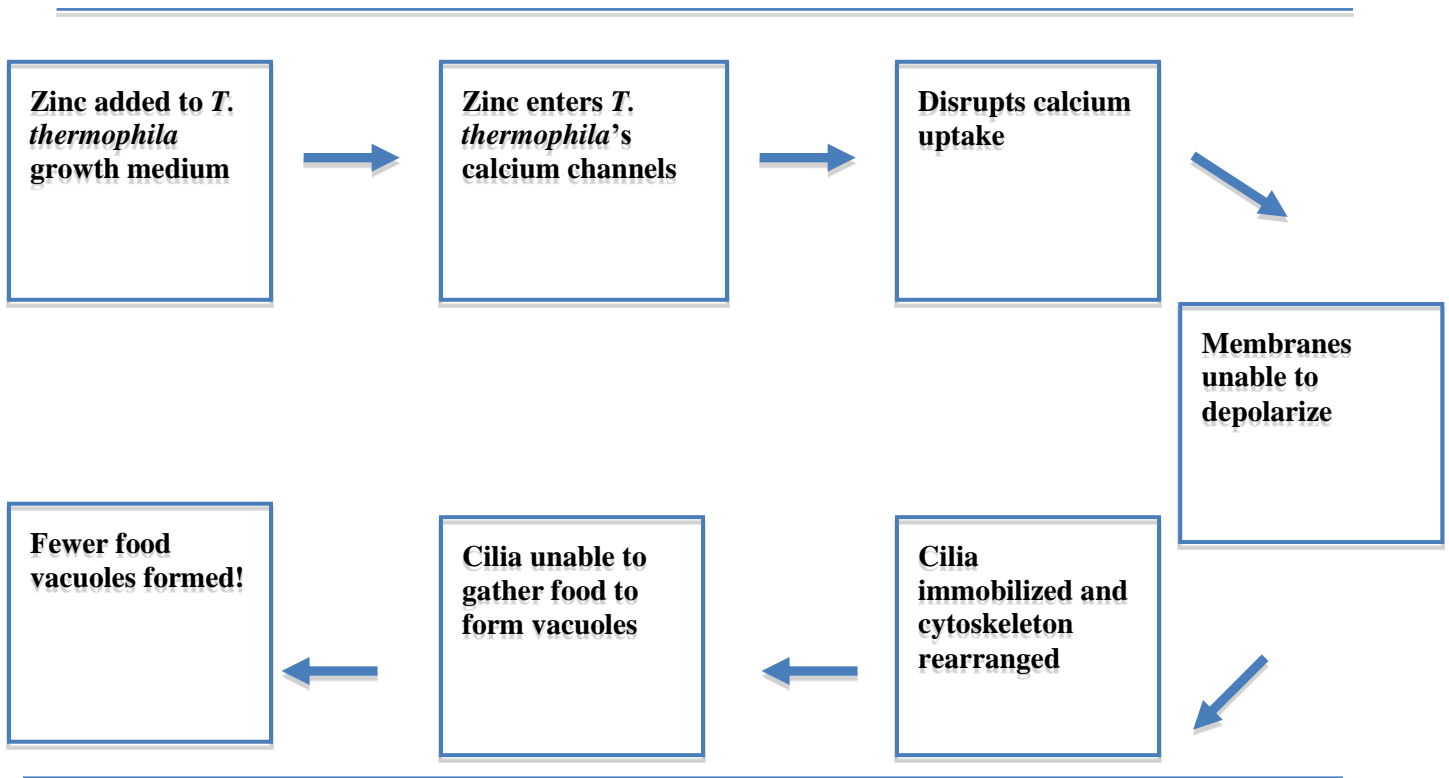
*Tetrahymena thermophila* are unicellular eukaryotic protozoans with numerous cilia (Collins and Gorovsky 2005). As aquatic organisms, *T. thermophila* are exposed to a variety of elements that enter aquatic environments from both natural and anthropogenic sources. Zinc is a heavy metal that is found in varying concentrations in the freshwaters inhabited by *T. thermophila* (K. Orians, Chemistry 301 professor, Pers. Comm.). Zinc is essential for living organisms in trace amounts and is necessary for various cell functions; however, excess amounts of zinc can have toxic effects on aquatic organisms including *T. thermophila* (Nilsson 2003). Excess zinc finds its way into aquatic environments through various sources, a primary one

being acid mine drainage (Sheoran and Sheoran 2005). It is important to understand the possible, detrimental effects of zinc when assessing the risk associated with a human-induced situation such as an acid mine leak.

The toxic effects of zinc include alteration of calcium uptake in *T. thermophila*, which in turn affects ciliary function (Figure 1) (Nilsson 2003). Cilia are crucial in numerous cell functions including phagocytosis, the process in which cells engulf food through the formation of a food vacuole (Collins 2012). Phagocytosis can be quantified by counting how many food vacuoles are formed in a certain time period (Bozzone 2000).

Our study aimed to observe the capacity of *T. thermophila* to form food vacuoles following incubation for three distinct time durations under increasing concentrations of zinc chloride, or  $[ZnCl_2]$ . We observed the effects of time and  $[ZnCl_2]$ , independently, as well as the combined effect of the two factors. Our first null hypothesis was that increasing  $[ZnCl_2]$  has no effect on food vacuole formation in *T. thermophila* (alternative hypothesis that increasing  $[ZnCl_2]$  has an effect). Our prediction was that increasing concentrations of  $[ZnCl_2]$  would have a negative effect on food vacuole formation (fewer vacuoles formed at higher concentrations). This prediction was based on the previously mentioned physiological effects of zinc. Our second null hypothesis was that increasing time has no effect on food vacuole formation in *T. thermophila* (alternate hypothesis that time has an effect). Three incubation times, 20 minutes, 2 hours and 26 hours, were used in order to test this hypothesis. We predicted that food vacuole formation would remain constant no matter how long *T. thermophila* were incubated. This was based on the cultures being kept in a closed system and thus, not being replenished with new nutrients and accumulating waste over time. Lastly, our third null hypothesis was that the effect of increasing  $[ZnCl_2]$  would not differ for increasing lengths of incubation time (alternative

hypothesis that  $[\text{ZnCl}_2]$  and time work in tandem to change the observed number of food vacuoles). Based on a similar past experiment by Nilsson (1981) studying the effects of copper on *Tetrahymena*, we predicted that we would observe an initial stimulatory effect of zinc. Nilsson (1981) found copper stimulated phagocytosis and thus, food vacuole formation within the first hour of exposure, decreasing with time- thereafter. We postulated that since zinc and copper share similar chemical properties, we would see a similar effect on food vacuole formation. We expected to observe more food vacuoles in the *T. thermophila* exposed to zinc for 20 minutes than in the *T. thermophila* exposed to unaltered medium for 20 minutes. For the other time intervals, we expected to see decreased vacuole formation with increasing  $[\text{ZnCl}_2]$ .



**Figure 1:** Model showing the effect of zinc on food vacuole formation in *T. thermophila* (Nilsson 2003)

## **METHODS**

### **Counting cell population density**

*T. thermophila* were grown in NEFF growth medium (0.25% Proteose Peptone (PP), 0.25% Yeast extract, 0.55% Glucose, 1.1mL FeCl<sub>3</sub>) and counted with a haemocytometer to ensure the population density would be high enough for the cells to survive the length of our experiment. We transferred 100 µL of stock *T. thermophila* to a microcentrifuge tube and fixed the cells with 10 µL glutaraldehyde. We then transferred 20 µL of the fixed cells onto the haemocytometer. Cells were counted in three squares of the same size on the haemocytometer grid, under a compound Axiostar microscope under 100X total magnification. Cell density was appropriate to proceed with the experiment based on a study by Nicolau et al. (1999).

### **Treatments**

Our study consisted of three different treatments: *T. thermophila* exposed to a low dose of ZnCl<sub>2</sub> [50 mg L<sup>-1</sup>], *T. thermophila* exposed to a high dose of ZnCl<sub>2</sub> [120 mg L<sup>-1</sup>], and a control treatment of *T. thermophila* in unaltered growth medium. To test the effects of increasing [ZnCl<sub>2</sub>] on *T. thermophila*, we referred to literature discussing the impact of various heavy metals on a similar organism, *Tetrahymena pyriformis*. Zinc has been shown to have an observable effect on these protozoans when doses ranging from 50 mg L<sup>-1</sup> to 300 mg L<sup>-1</sup> were administered (Nicolau et al. 1999). However, Nicolau et al. (1999) note that ZnCl<sub>2</sub> inhibits growth almost entirely above the 120 mg L<sup>-1</sup> dose. Therefore, to span the physiological range of *T. thermophila*, we chose 50 mg L<sup>-1</sup> [0.37 mM] ZnCl<sub>2</sub> for our low dosage and 120 mg L<sup>-1</sup> [0.88 mM] ZnCl<sub>2</sub> for our high dosage.

Research has shown that an initial stimulating effect occurs in *Tetrahymena* food vacuole formation from low copper administration within one hour of exposure at room temperature

(Nilsson 1981). We therefore decided to keep a constant room temperature (21 °C), over three different time intervals: 20 minutes, 2 hours and 26 hours. These time intervals were chosen based on our assumption that zinc would have a similar physiological effect on *T. thermophila* as copper did: within an hour (at the 20-minute mark), we should see an initial positive effect followed by a negative effect after one hour (at the 2 hour mark) (Nilsson 1981). In addition, we wanted to see what the effects of zinc would be after a longer exposure so we decided to extend the experiment for a 26-hour time interval. Each of the zinc treatments was measured at three different time intervals with three replicates each.

It is important to note that pH was not kept constant. The addition of zinc chloride may have increased the acidity of our solutions but we chose not to neutralize this slight change in acidity because it may have been too small to detect with our pH meter. However, we kept in mind that the change in pH could have an effect on our results, and was therefore, a possible source of error. Other factors such as oxygen levels, carbon dioxide levels and nutrient levels were changing as time elapsed due to the closed-system nature of our *T. thermophila* cultures.

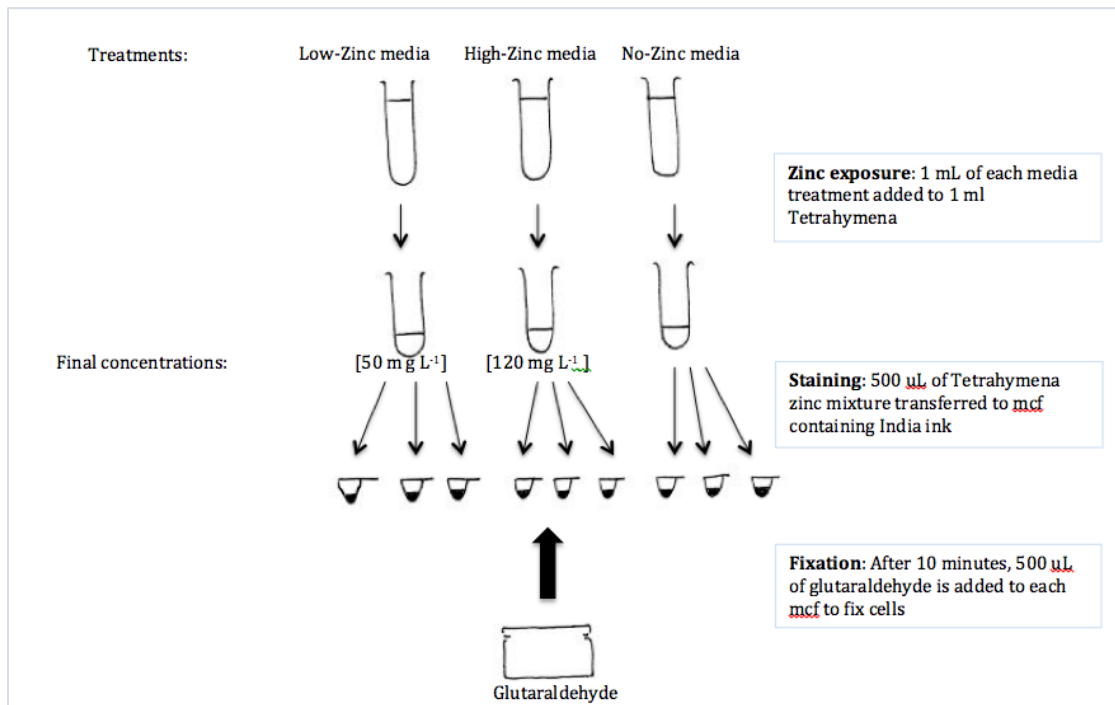
### **Dilutions**

The final [ZnCl<sub>2</sub>] of the media used were 120 mg L<sup>-1</sup>, 50 mg L<sup>-1</sup>, and 0 mg L<sup>-1</sup> for the high zinc, low zinc and control treatments, respectively.

### **Ink addition and fixation**

After 20 minutes of incubation from the initial exposure time, we transferred 500 µL of *T. thermophila* in zinc (or no zinc) media from each treatment test tube to a microcentrifuge tube containing 500 µL of 1% India ink. We repeated this three times for each zinc treatment to produce three replicates for each treatment (Figure 2). *T. thermophila* were incubated in the ink for 10 minutes before fixing, to allow enough time for ink to be engulfed in the vacuoles through

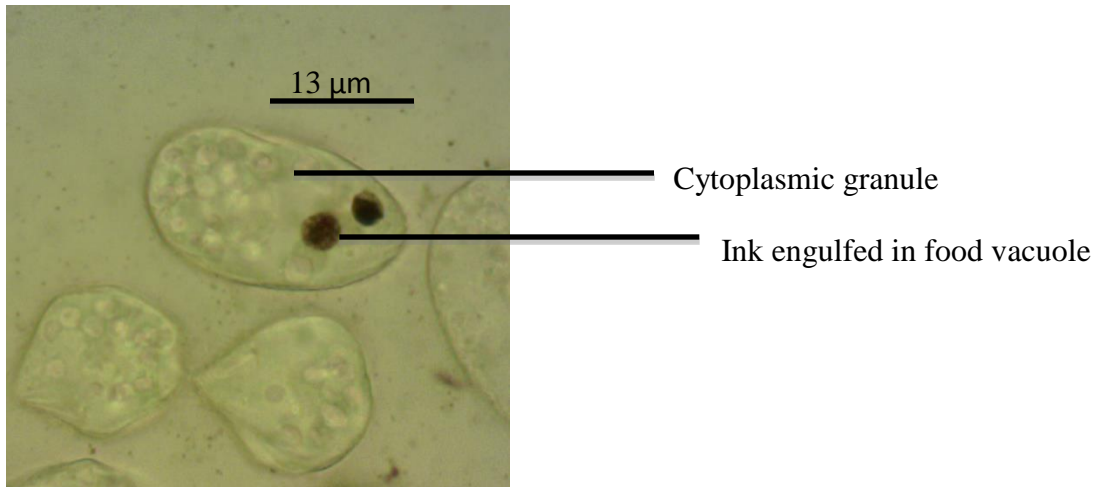
the process of phagocytosis. After 10 minutes, we added 500  $\mu\text{L}$  of glutaraldehyde fixative to the test tubes and pipetted three times to mix.



**Figure 2:** Procedure: measuring  $\text{ZnCl}_2$  effects on food vacuole formation for one time interval.

### Counting food vacuoles

We transferred 100  $\mu\text{L}$  of fixed cells from each microcentrifuge tube onto a slide and observed using an Axiostar compound microscope. We observed the cells under 400X total magnification, and collectively decided as a group what we identified as a food vacuole (Figure 3). We counted food vacuoles in 10 cells per replicate and used the average number of food vacuoles per cell. The food vacuoles were coloured a distinctive black colour by the uptake of ink and should not be confused with cytoplasmic granules that form as a result of the attempt to detoxify the addition of zinc (Nilsson 2003). The ink addition, fixation and food vacuole count were repeated for the remaining replicates at the 2-hour interval and the 26- hour interval.



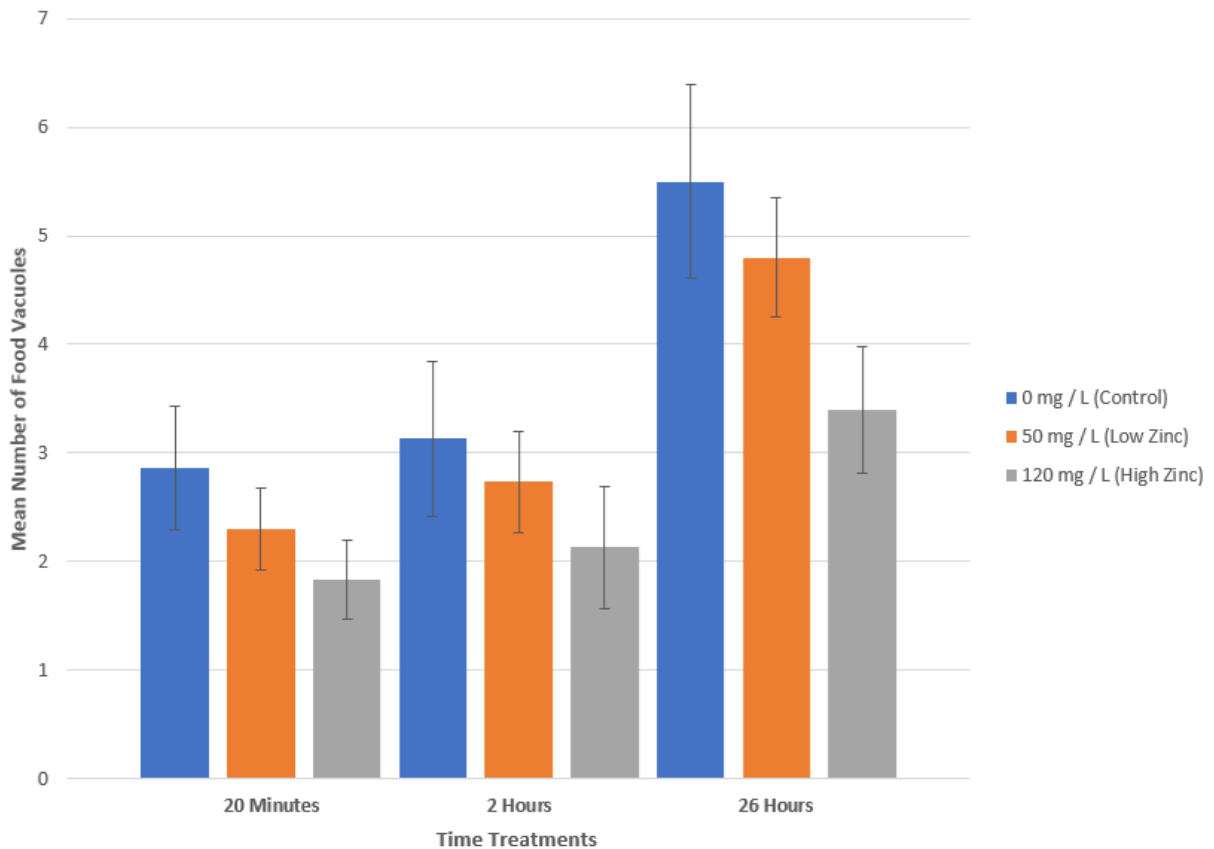
**Figure 3:** Two food vacuoles stained with 1% India ink in *Tetrahymena thermophila*. Observed under 400X total magnification.

### Data Analysis

Since our experiment investigated two factors ([ZnCl<sub>2</sub>] and time), a two-way ANOVA test was used to analyze our data and to determine whether or not the effects of time and zinc concentration were significant ( $p$  less than or equal to 0.05) and related.

### RESULTS

We calculated the mean number of food vacuoles for each of the time intervals and their respective [ZnCl<sub>2</sub>] treatments, resulting in nine different averages. Figure 4 shows that as the [ZnCl<sub>2</sub>] increases, there is an overall decrease in the mean number of food vacuoles. This trend is apparent for all three different incubation times. Similarly, as incubation time increases, there is an overall increase in the mean number of food vacuoles. The error bars for our control and high zinc treatments do not overlap for any of the three incubation times, indicating that these concentrations may be contributing to the statistically significant differences observed. For the other cases, though, we see at least one instance of overlapping.



**Figure 4.** Mean number of food vacuoles of *T. thermophila* in three different treatments of  $\text{ZnCl}_2$  at concentrations of  $0 \text{ mg L}^{-1}$ ,  $50 \text{ mg L}^{-1}$ , and  $120 \text{ mg L}^{-1}$  at 20 minutes, 2 hours, and 26 hours of incubation time. Error bars represent the 95% confidence intervals. Each bar represents the average of  $n = 3$  replicates.

To investigate the statistical significance of these data, we conducted a two-way ANOVA and calculated  $p$ -values of  $2.5 \times 10^{-3}$  for our first hypothesis (the  $[\text{ZnCl}_2]$  factor),  $2.6 \times 10^{-6}$  for our second hypothesis (the time factor), and 0.31 for our third hypothesis (the combined  $[\text{ZnCl}_2]$  and time factors). For the individual  $[\text{ZnCl}_2]$  and time factors, the calculated  $p$ -values were less than the critical  $p$ -value of 0.05 and the  $p$ -value for our combined  $[\text{ZnCl}_2]$  and time null hypothesis was greater than the critical  $p$ -value of 0.05.

## DISCUSSION

With a  $p$ -value of 0.0025, we were able to reject  $H_{01}$  and provide support for the alternate hypothesis that increasing  $[\text{ZnCl}_2]$  does have an effect on food vacuole formation. Similarly,



since the  $p$ -value for the second set of hypotheses was 0.0000026, we can reject the null hypothesis and provide support for the alternate hypothesis, which states that increasing time of incubation in  $\text{ZnCl}_2$  does have an effect on food vacuole formation. Lastly, with a  $p$ -value 0.31, we fail to reject the null hypothesis, which states that the effect of increasing  $[\text{ZnCl}_2]$  on food vacuole formation is the same for different incubation times.

In the case of the effect of increasing  $[\text{ZnCl}_2]$  on food vacuole formation, we predicted that an increase in  $[\text{ZnCl}_2]$  would decrease the food vacuole count at both  $50 \text{ mg L}^{-1} \text{ ZnCl}_2$  and  $120 \text{ mg L}^{-1} \text{ ZnCl}_2$ . We based this prediction on Nilsson's (2003) study on the phagocytic capabilities of *Tetrahymena pyriformis* with exposure to zinc. Although Nilsson (2003) focused on *T. pyriformis*, it is reasonable to assume that *T. thermophila* will respond in a similar manner as they belong to the same genus. In our experiment and in a part of Nilsson's experiment (2003), there was a definite decrease in food vacuole formation with increasing [zinc], supporting our initial prediction. We ascribed this observation to the toxic effects of zinc on *T. thermophila*'s physiology. In trace amounts, zinc is an essential nutrient, which is a component of over 300 enzymes (Nilsson 2003). However, increasing concentrations of zinc above trace amounts have been shown to disrupt calcium metabolism in *T. thermophila* (Nilsson 2003).  $\text{Ca}^{2+}$  ions are essential for the proper functioning of the *T. thermophila* ciliary system (Machemer 1988, cited in Nilsson 2003). According to Nilsson (2003), it is likely that zinc ions enter through calcium channels, changing the intracellular calcium concentrations and preventing *Tetrahymena* from depolarizing membranes. If the membrane is unable to depolarize, ciliary movement will be effectively immobilized (Nilsson 2003). Ciliary movement is essential in bringing food particles into the feeding apparatus of *T. thermophila* (Collins 2012) and may explain the lack of food vacuoles formed with increased  $[\text{ZnCl}_2]$  as seen in Figure 4. In addition to affecting ciliary

motion, calcium metabolism also controls the cytoskeleton of *Tetrahymena* and therefore its phagocytic abilities, as cytoskeleton rearrangements are responsible for food vacuole formation (Bennett and Weeds 1986). This piece of evidence further confirms that zinc's toxic effect on calcium metabolism can decrease food vacuole formation.

Nilsson's (2003) results differed in that at low zinc concentrations (32.69 mg L<sup>-1</sup> and 49.04 mg L<sup>-1</sup>) there was elevated food vacuole formation. To explain why we did not observe an elevated rate of food vacuole formation at our low concentration of 50 mg L<sup>-1</sup> [ZnCl<sub>2</sub>] we need to appreciate how the harmful effects of zinc can be protected by the presence of organic matter (Nicolau et al. 1999). Heavy metal cations such as Zn<sup>2+</sup> can bind to the negative charges on the surface of organic matter and precipitate out of the *T. thermophila* medium solution (Nicolau et al. 1999). With part of the introduced zinc ions bound to organic matter, there will be less zinc available to harm the organism, in this case *T. thermophila* (Nicolau et al. 1999). Relating this to the differing results, it is important to note that *T. thermophila* in our experiment were cultured in 0.25% PP whereas Nilsson's (2003) cultures were grown in 1-2% PP. It could be possible that since our cultures had diluted organic matter, the *T. thermophila* in our experiment were exposed to higher concentrations of zinc and hence, rather than seeing a positive effect, we observed a harmful effect. On the other hand, since Nilsson's (2003) cultures were grown in a more concentrated medium at 1-2% PP, some free Zn<sup>2+</sup> could have bound with the organic matter, protecting the *T. pyriformis* from the harmful effects. Instead, it may have provided a concentration of Zn<sup>2+</sup> that is in trace amounts and therefore, stimulatory.

Four different individuals counted the food vacuoles and so, it is entirely possible that different definitions of what a food vacuole looks like could have affected our results. For example, someone counting the 0.37 mM samples could have greatly underestimated the food

vacuole count. In this case, we could have received skewed results that prevented us from observing a higher food vacuole count at low  $\text{ZnCl}_2$  concentrations. In particular, we think it is important to mention that regular intracellular handling and detoxification of zinc ions is mediated by the formation of small cytoplasmic granules (Nilsson 1989, cited in Nilsson 2003). This introduces experimental error because the granules look similar to the food vacuoles we were counting, often presenting a challenge to distinguish between the two. To minimize this error, as a group we used the Dino-lite software to analyze a food vacuole and distinguish between the two (Figure 3). We noticed that the cytoplasmic granules were often a bit smaller in circumference and lighter in colour than the food vacuoles. Another source of error that may have accounted for the difference in results is that we did not account for the decrease in pH that occurs when you add zinc chloride into the medium. In other words, we did not measure the pH of the cultures before or after the addition of the  $\text{ZnCl}_2$ . Nilsson (2003) added NaOH to the *T. pyriformis* cultures in order to neutralize the increased acidity that results from the addition of zinc. It could be that the increased acidity in our experiment contributed to the overall toxicity, decreasing food vacuole formation. Perhaps, if we had neutralized the acid as Nilsson (2003) did, we would have observed higher food vacuole formation. Further studies could examine how pH changes affect food vacuole formation in *T. thermophila*.

For our second hypothesis, we predicted that with more time in  $\text{ZnCl}_2$ , independent of concentration, food vacuole formation would not change. We made this prediction because these *T. thermophila* cultures were in a closed system and all other factors were kept constant. However, we did observe an increase in food vacuoles with increasing time, in control cultures (with no zinc) across all three times. This may be attributed to what actually occurs over time within a closed-system *T. thermophila* culture. With increasing time, the cells enter a starvation

state as food levels become scarcer than at a previous time. Starving induces physiological changes such that *T. thermophila* develop larger cilia with more coordinated propulsion through the medium (Collins and Gorovsky 2005). Levy and Elliot (1968) reasoned that these physiological changes occur in order to survive in a low nutrient environment (Levy and Elliott 1968, cited in Chapman and Dunlop 1981). When the cells are introduced to the nutrient-rich ink medium for 10 minutes, it may be that this faster propulsion allows the *T. thermophila* to graze more efficiently for food particles, forming more food vacuoles with time. In addition, since *T. thermophila* bring food into the feeding apparatus with its cilia (Collins 2012), larger cilia may allow more efficient feeding and in turn, produce more food vacuoles with increasing time. Nilsson (1976) also found that exposing starved cells to a nutrient-rich medium would stimulate rapid food vacuole formation (Nilsson 1976, cited in Chapman and Dunlop 1981).

Based on Nilsson's (1981) study on copper exposure to *Tetrahymena pyriformis*, we predicted that there would be an initial stimulatory effect of  $[ZnCl_2]$  on food vacuole formation, slowly reverting back to a negative response as time elapsed. The initial positive trend of food vacuole formation in Nilsson's (1981) experiment can be attributed to the fact that *T. pyriformis* treated with copper have higher nutritional requirements (Nilsson 1981). More specifically, *Tetrahymena* cells treated with copper need 10-20% more nutrients compared to untreated cells (Nilsson 1981). This observation may explain why there are more food vacuoles formed with copper exposure, but it doesn't explain why this increase in food vacuoles is short lived, reverting back to a harmful effect as time elapses. To understand this, we need to appreciate what occurs in the vacuolar membrane of *T. pyriformis* cells (Nilsson 1981). Essentially, if a cell needs more nutrients, they will feed more and produce more food vacuoles as previously mentioned but as the cell makes more food vacuoles, it uses twice as much of the vacuolar

membrane compared to the untreated cells (Nilsson 1981). It is important to keep in mind that there is a limited amount of vacuolar membrane that *T. pyriformis* cells can use to produce food vacuoles, and so the supply will eventually be exhausted (Nilsson 1981). As time progresses, when the cells cannot meet this membrane demand, one will observe decreased food vacuole formation (Nilsson 1981). In fact, according to Nilsson (1981), it takes about 2-3 hours before the *T. pyriformis* can recycle their membranes and recover from the stress of the copper.

In our study, it is clear from the two-way ANOVA test that time and zinc concentrations do not interact to affect food vacuole formation in *T. thermophila*. More specifically, there was no initial stimulatory effect of  $[ZnCl_2]$  as there was in Nilsson's (1981) copper exposure study. We can attribute this to the physiological effect that zinc has compared to copper. As previously discussed, zinc interferes with the cilia and the cytoskeleton, decreasing food vacuole formation (Nilsson 2003). The stress of zinc on *T. thermophila* directly impairs the mechanism that produces food vacuoles (Nilsson 2003). On the other hand, copper interferes with how the vacuolar membrane is used, stimulating food vacuole formation until the available membrane is exhausted (Nilsson 1981). Zinc's effect on *T. thermophila* is harmful at 20-minute exposure, 2-hour exposure and 26-hour exposure (Figure 4). Zinc's effect is only stimulatory if presented in concentrations small enough to be rendered trace (Nilsson 2003). From this, we can postulate that unless at trace zinc concentrations, at any length of exposure, we will observe a harmful effect that results in a decrease in food vacuole formation.

## **CONCLUSION**

In conclusion, our results showed a significant decrease in the mean number of food vacuoles with increasing  $[ZnCl_2]$  in medium. We also observed an increased mean number of

food vacuoles with longer incubation time in  $ZnCl_2$ , independent of  $[ZnCl_2]$ . We did not find any relationship in the effects of these factors when they are considered together.

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