The effects of increasing iron chloride concentrations on the population growth of *Tetrahymena thermophila*

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Abstract

This study examined the effect of different iron concentrations on the population size of *Tetrahymena thermophila.* Iron is an essential nutrient for these organisms and contributes to population growth. Three treatments were prepared, using a standard control medium used in *T. thermophila* studies containing a small portion of iron, 2.012 M and 4.011M of iron in a standard growth medium. Over a period of 25 hours, samples were collected and population numbers were counted at one-hour intervals. It was found that in all three treatments populations increased in size relatively constantly over the first 10-hour period, with little difference among them. After analysis of the data collected, we found the results were not statistically significant and we were unable to draw any conclusions about the effect of iron on population size.

Introduction

Tetrahymena thermophila is a microorganism that lives in freshwater ecosystems, such as ponds. Iron is a common element found in freshwater and also plays an important role in the metabolic processes of cells, particularly in oxygen-transporting proteins (Vuori 1995). Increased leaching of iron into freshwater environments due to draining of arable lands and forests (Vuori 1995) has led to higher iron concentrations in these ecosystems. Certain levels of iron are necessary for growth in freshwater aquatic communities, but there is a limit to which iron concentrations can be sustained until they have toxic ecological effects. The benchmarks are approximately 0.31 mg/L and 1.74 mg/L (Linton *et al*. 2007). The objective of our experiment is to see how increasing iron concentrations affect the population growth of *T. thermophila* and thus indicate how

changing iron concentrations affect the overall health of freshwater ecosystems. The role of *T. thermophila* in freshwater ecosystems can also be inferred by our experiment. If our organism consumes iron as a nutritional requirement, it is taking iron out of the ecosystem and possibly allowing other organisms to grow. This can give us an idea of the complex biological relationships that occur in freshwater environments such as ponds. Another objective of our experiment was to see how *T. thermophila* populations adapt to increased iron concentrations over time. Rasmussen *et al.* (1984) determined that iron-starved organisms would cease to multiply in iron concentrations over 10 um, but the populations are able to adapt to higher concentrations over time. Iron is a nutritional requirement of many microorganisms and is a constituent of enzymes such as cytochromes, which are responsible for the generation of ATP via electron transport and catalase, which catalyzes the decomposition of hydrogen peroxide to water and oxygen (Vuori 1995). Thus our alternative hypothesis is that increasing the concentration of $FeCl_3$ will increase the population size of *T. thermophila*. This is also supported by Wang *et al.* (2000) as their study indicates that exposure to elements such as iron increased population growth in *Tetrahymena shanghaiensis*, a species closely related to our study species. At a certain point, it is expected that iron concentrations that are too high will result in a decrease in growth (Shug *et al.* 1969). Conversely, our null hypothesis states that increasing the concentration of $FeCl_3$ will decrease or have no effect on the population size of *T. thermophila*.

Methods

In the experiment, we incubated *T. thermophila* culture in three different concentrations of iron chloride. A growth medium, with 0.024μM, was used as the

growth medium for the *T. thermophilia* cells, and the cell count of *T. thermophilia* 3950 cells per mL. Increased iron levels were obtained by using an 8 μM concentration of iron chloride. In the first treatment, the level of iron was determined by the amount in the media, giving a concentration of .024μM of iron chloride. This was achieved by pipetting (we used sterile technique for all transfers throughout the experiment) 1.1 mL of *T. thermophilia* cells into a large 6mL test tube with another 1.1 mL of media. The second treatment was created with an iron chloride concentration of 2.0117μM. This was obtained, by pipetting 0.55mL of 8 μM/mL-iron chloride into a large 6mL test tube with 0.55mL of medium and 1.1mL of cells. In the third treatment, 1.1 mL of 8μMof iron chloride was pipetted with 1.1 mL of cells into a six mL test tube. This then gave the final concentration of 4.011μM of iron chloride for the third treatment of *T. thermophilia*. With each different iron concentration, three replicates were made for each, resulting in nine samples in total. Each sample was treated individually, with no cross over among replicates of the same iron concentration. Figure 1 shows how the samples were initially set up with 6mL test tubes, and how they were set in the incubator.

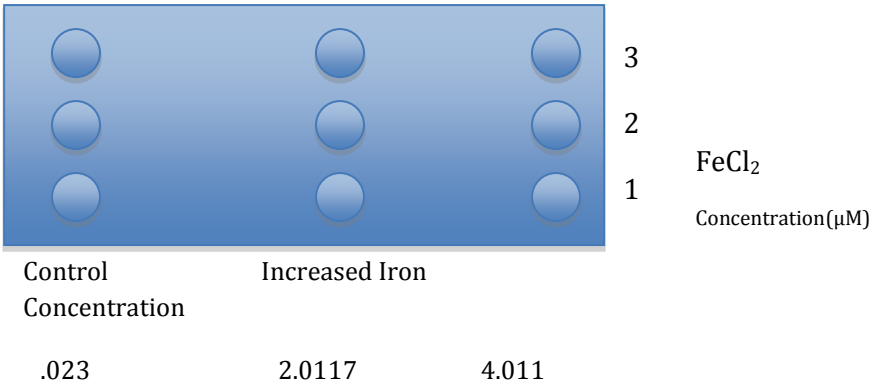

Figure 1: The layout of the experiment, where there are three treatments and three replicates for each.

Once all nine samples were set, the test tubes were then incubated in a 30-degree Celsius incubator on a non-moving rack. The test tubes were incubated for an hour before we took the first sample. The temperature of the incubator fluctuated between 29-30 degree Celsius, and this temperature resulted in a three to four hour doubling time of the population. After the first hour, a 100μL sample was taken from each test tube, and deposited into 2mL centrifuge test tubes. All nine samples were taken, and placed into a test tube and ten microliters of fixative, 3% glutaraldehyde was then added and the solution was mixed. After fixing all nine samples, the large 6mL test tubes with the remaining media were returned to the incubator and left for another hour. Working with the first fixed sample, a 15μL sample of the fixed *T. thermophilia* was taken from the centrifuge tube and pipetted onto a clean haemocytometer. We counted the cells within the whole larger square of the haemocytometer; we did not include the cells on the borders of a square. Once we had a number for the whole large square, we multiplied that number by $3.125*10^2$, and then by 1.1mL, constants for using a haemocytometer and correcting for the addition of fixative to get the total number of cells. We continued the process of taking samples out of the incubator, fixing and then counting for ten hours. We recorded how the total number of cells changed over time, and within the three different levels of iron chloride concentrations. We calculated 95% confidence to analyze the data. Also, we took the means of the three replicates for each sample to get the most meaningful points, leaving only the three varying iron concentration samples on graph.

Results

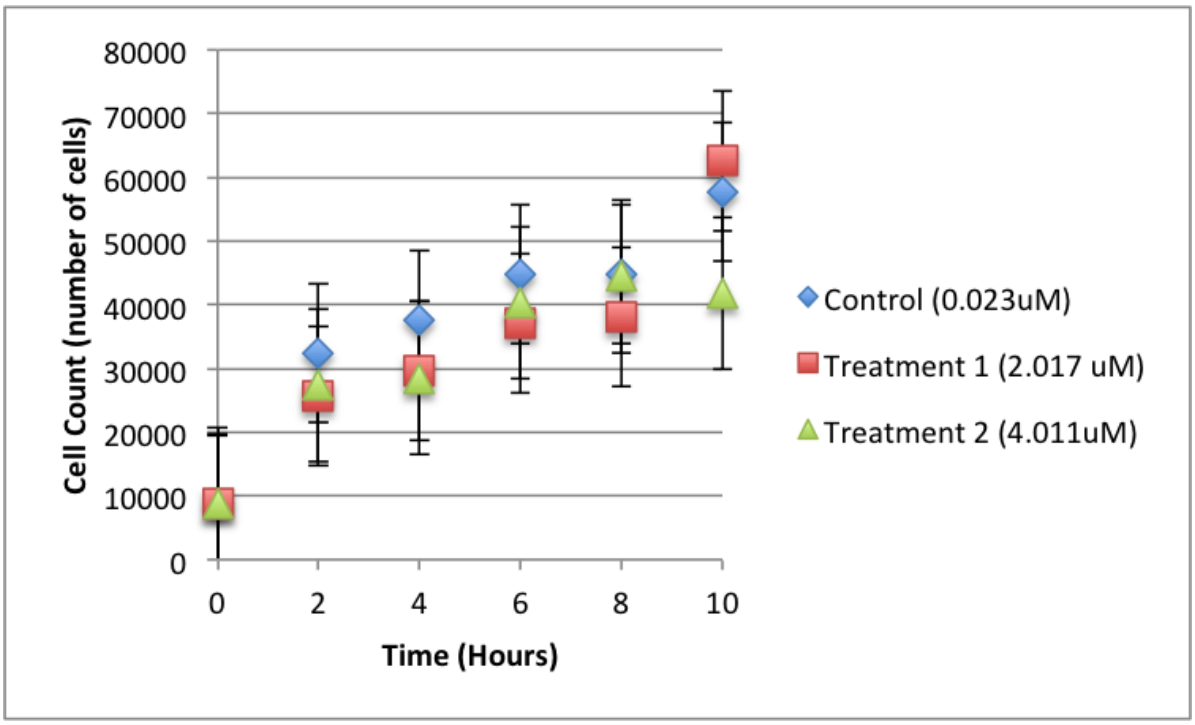

Figure 2: Population size of all three treatments over 12 hours of treatment. The blue, red and green points represent data for the control, 2.0117M treatment and the 4.011M treatment, respectively. Confidence intervals are included for each point. Data points represent the mean counts taken from all three replicates for each treatment at the given time.

Sample Calculations:

To Find Actual Cell Amount (e.g. Control at 2 hours):

Actual Cell Count = (average of control A, B, and C)*312.5*1000*(1.1mL/1000mL)

=94.33*312.5*1000*(1.1mL/1000mL) = 32427.08 Cells

To find confidence intervals:

Standard Deviation =

$\sqrt{(E((x-X)^2)/N)}$= 33319.18861 cells

CI=1.96*(standard deviation/$\sqrt{(n)}$)=1.96*33319.18861/6= 10884.26828 cells

Figure 2 suggests a trend of increasing population over time for all of the treatments. However, a closer analysis reveals slight variations in each treatment. From hour one to three, there seemed to be a slow increase or slight decrease in cell number for all three treatments, after a rapid increase in the first hour. From three to seven hours, the growth recovered from the previous lag and the figure shows a steady increase in population in all three treatments. The control and treatment two showed a rapid decrease in population from seven to nine hours, whereas treatment one recovered from this rapid decline by about eight hours. After the latter decrease in population, all three treatments begin a fast recovery and increased population. Confidence intervals overlapped in Figure 2 for all treatments in each time period and variance between treatments was at its maximum during hours nine and ten in contrast to a relatively constant variance between treatments from hours one to eight. The final population in number of cells for the control, treatment one and treatment two at 25 hours were: $1.43*10^6$, $1.41*10^6$, and $1.51*10^6$, respectively.

During our experiment, we were able to see large amount of iron precipitates aggregated on the bottom of the test tubes (see Figure 3). Also some kinds of precipitates were visible under the compound microscope that were suspected as being iron precipitates (see Figure 4).

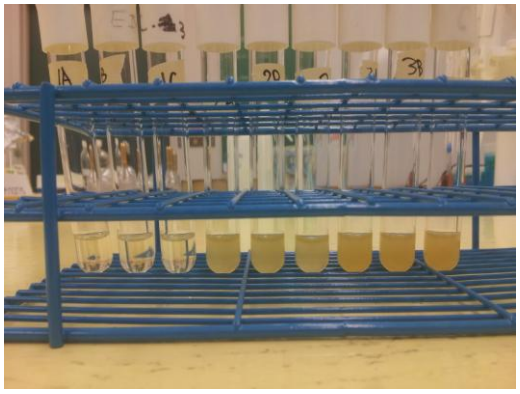

Figure 3: This is 2.2mL of growth medium in the test tubes. Precipitates of Fe(III) can be viewed in growth medium due to their distinctive color. Fe(III) concentration increases from left to right. Yellowish color is the Fe(III) precipitates.

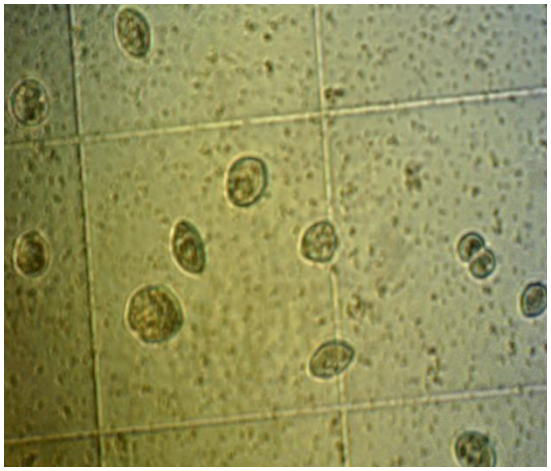

Figure 4: Sample of Treatment 2 viewed under compound microscope. The black dots are suspected Fe(III) precipitates suspended around *T. thermophila.*

Discussion

Iron is an essential trace element for many organisms and their availability in the environment will promote or limit the growth of iron-dependent organisms. However, iron can also be toxic for organism if excessive amount is present (Suhr-Jessen *et al.* 1982). Due to this dual impact of iron, our experiment focused on observing population growth of *Tetrahymena thermophila* in response to increasing Fe(III) concentration.). Based on the data (see Figure 2), there are general trends of increasing population for all three treatments, 0.024μM of Fe(III), 2.017μM of Fe(III), and (4.011μM of Fe(III.

However, purely basing on the cell numbers it seems like control group shows the most growth and Treatment 2 shows least growth. For control and Treatment 1 group seems to experience log-phase of growth at eight hours of incubations and onward but when these two different populations reached its carrying capacity is not clear (Figure 2). For Treatment 2 group it is not clear when the log-phase was initiated since there are no data from 11 hours to 24 hours (Figure 2). Looking at the slope of log-phase of control and Treatment 1 group by taking the slope at eight and ten hours of incubations, Treatment 1 group seems to have steeper slope than the control group. Therefore it is possible to state that Treatment 1 group had faster growth rate than the control group. However, the difference in growth rate of these three treatment groups is not statistically significant since their 95% confidence interval all overlapped with each other. Therefore, we failed to reject the null hypothesis and failed to support the alternate hypothesis.

As stated in alternate hypothesis, it was expected that Treatment 2 with 4.011μM of Fe(III) would show highest growth rate. But the data showed exactly the opposite (Figure 2). If we look at Figure 2, Treatment 2 had the lowest cell growth and also did not yet entered the log phase compare to control and Treatment 1 group. This unexpected outcome can be explained by looking at biological explanation. Suhr-Jessen *et al.* (1982) suggested that *T. thermophila* can multiply in 1.8 – 4μM of Fe(III) but Fe(III) concentration above 4μM can be toxic for the organism. In addition, according to Rasmussen *et al.* (1984), $FeCl_3$ is very toxic for *Tetrahymena* so that only one type of clone could survive in the concentration ranging from 1 and 7-10μM. In our study, *T. thermophila* in Treatment 3 had Fe(III) concentration above 4μM (4.011μM). This additional 0.011μM of Fe(III) in Treatment 2 might be toxic enough to suppress the

growth of *T. thermophila.* However, at 25 hours of incubation *T. thermophila* showed highest population compare to other two treatments. Treatment 2 had cell population of 1.51×10^5 cells meanwhile control group had the least growth with 1.43×10^5 cells. This rapid increase in cell population in Treatment 2 suggests that *T. thermophila* were able to respond to higher Fe(III) concentration and their log-phase growth happened later around at 15 hours of incubation. Since there are no data to show exactly when the *T. thermophila* in Treatment 2 experienced log-phase growth, it is possibly that log-phase growth could have happened before or after 15 hours of incubation.

The ability of *T. thermophila* to multiply can be influenced by the change in the environment (Rasmussen *et al.* 1984). In this experiment, a factor that changes the environment is the Fe(III) concentration. As depicted in Figure 2, the control group showed the largest population growth in general compare to other treatments since they were already adapted to their Fe(III) level. This is because *T. thermophila* in the control solution was directly taken from the original medium so an acclimation period was not necessary. However, *T. thermophila* in the iron-enriched media were exposed to a new environment with higher Fe(III) concentration and therefore an acclimation period was required before cell division could occur. According to Rasmussen *et al.* (1984), *Tetrahymena* were able to multiply even at 1000μM of Fe(III) if they were given a sufficient acclimation period coupled with a gradual increase in Fe(III). Rasmussen *et al.* (1984) further stated that the ability of *Tetrahymena* to grow at higher Fe(III) concentration was because of their physiological adaptation (acclimation) rather than gene selection process (mutation). It is acclimation and not adaptation because when *Tetrahymena* grown in high-iron media were subcultured into iron-less media and then

back to the high-iron media, *T. thermophila* lost its tolerance at high Fe(III) concentration. Referring back to Figure 2, *T. thermophila* in Treatment 1 and 2 showed slow growth rate compare to control group since they required an acclimation period. For Treatment 1, *T. thermophila* seems to acclimate fast to their new environment since they were able to enter log-phase at the same time as control group did at eight hours of incubation. However, *T. thermophila* in Treatment 2 did not show any tendency to enter log-phase within the first 10 hours of incubation. This indicates that *T. thermophila* may still be acclimating. By 25 hours of incubation, *T. thermophila* in Treatment 2 had the highest population size compare to other treatments suggesting that they have acclimated to the environment and entered their log-phase growth between 10 - 25 hours of incubation.

Enzymes are very important for cell metabolism so they are directly related to cell growth and multiplication. Athavale *et al.* (2012) found that Fe(II) could replace Mg(II) for RNA folding and catalysis and therefore enhance the metabolism of *T. thermophila*. This is possible because, Fe(II) has a molecular structure that is very similar to Mg(II) so that enzymes catalyzing the RNA folding could accept Fe(II) instead of Mg(II) (Athavale *et al*. 2012). Based on this assumption, increasing Fe(III) concentration should also aid RNA folding and catalysis since Fe(III) could exist in equilibrium with Fe(II). However, as discussed earlier, the data from our experiment does not strongly indicate that high Fe(III) concentration enhances the growth of *T. thermophila* (see Figure 2). One of the possible reasons why Fe(III) may have not aided RNA folding as Fe(II) have since our solution was under an aerobic environment; where the growth medium was exposed to the air. Athavale *et al.* (2012) carried out their experiment under an anoxic environment

where Fe(III) could be reduced to Fe(II) under the low oxygen environment. Also Fe(II) is more soluble in the water and that makes it readily available for *T. thermophila* (Athavale *et al.* 2012). If our experiment were carried out under anoxic environment, the log phase of the growth curve in Treatment 1 and 2 might have occurred earlier and the growth curves might have had steeper slopes.

Unlike what Athavale *et al.* (2012) stated on iron promoting RNA folding, there are alternate arguments suggesting that Fe(III) could prohibit enzyme activity (Rasmussen *et al.* 1984). Ferric is insoluble in water unlike Fe(II) (Rasmussen *et al.* 1984). The aggregation of iron precipitates happens due to polymerization of ferric hydroxide complex that is formed Fe(III) with other oxygen containing molecules such as sulfates and phosphates (Rasmussen *et al.* 1984). According to Rasmussen *et al.* (1984), ferric hydroxide complex in the nutrient medium causes cross-linking reaction within the *T. thermophila*. Once cross-linking reaction happens within *T. thermophila;* it will prohibit enzyme activity by restricting bond movements in the enzyme (Rasmussen *et al.* 1984). This cross-linking reaction possibly happened to some *T. thermophila* in Treatment 1 and 2 and limited the metabolism for growth and slowed the efficacy of multiplication.

One of the reasons why *T. thermophila* failed to show increased growth rate under higher Fe(III) concentration is because sufficient time was not provided for *T. thermophila* to acclimate to the changes in Fe(III) concentration (Rasmussen *et al.* 1984). Secondly, the concentration of 4.011 μM of Fe(III) could be too concentrated and could have been toxic for *T. thermophila* (Suhr-Jessen *et al.* 1982). Thirdly, Fe(III) may have not been used for essential metabolism such as RNA folding and catalysis to aid faster growth of *T. thermophila* (Athavale *et al.* 2012). Lastly, Fe(III) may have formed ferric

hydroxide complexes that can cause cross-linking reactions within *T. thermophila.* This reaction limits the activity of the enzymes through restricting the motion of the bonds. By lowering the activity of enzyme the growth of *T. thermophila* in Treatment 1 and 2 would have been restricted and therefore showed slower rate of growth compare to the control group.

Conclusion

We failed to reject the null hypothesis and therefore failed to support the alternate hypothesis. This is because 95% confidence interval of three treatments: control with Fe(III) concentration of 0.024μM, Treatment 1 with 2.011μM of Fe(III), and Treatment 2 with 4.011μM of Fe(III) all overlapped. So the difference between the population growth of these three treatments with different Fe(III) concentration is not statistically significant.

Acknowledgements

We would like to thank Dr. Carol Pollock for her help throughout the various stages of our experiment, including the planning stages. We would also like to thank our T.As- Haley Kenyon, and especially Mindy Chow for her help with the multiple calculations required before we could begin our experiment. Lastly, thanks to UBC for providing us with the opportunity to take Bio 342, and facilitating our study.

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