The Effects of Copper Concentration on the Growth Rate of *Tetrahymena thermophila*

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Abstract

The growth of *Tetrahymena thermophila* can be affected by the concentration of heavy metals, like copper, in their environment. The focus of this study was to examine how *T. thermophila* responds to increasing concentrations of copper in the growing media. Treatments of *T. Thermophila* suspended in SSP medium and copper concentrations of 0 ppm, 1 ppm, 3 ppm, and 5 ppm were incubated at 35^{o} C while maintaining all other factors constant. Cell counts for T_0 were done before incubating. Cell counts for T_1 to T_4 were done after incubation in increments of 2.5 hours. T_5 was performed 24 hours after initial incubation and the last observation at T_6 was done at 26.5 hours after incubation. It was observed that the growth rate was slow from T_1 to T_3 and then the growth rate increased until the end of the experiment. The highest average growth rate was found to be in the concentration of 1 ppm with a growth rate of 7293 cells/hour and the lowest average growth rate was found in the treatment at 5 ppm with a growth rate of 6528 cells/hour. After analysis by a one-way ANOVA, the null hypothesis for the effects of copper concentration and growth rate was rejected. Further statistical post-hoc analysis using a Tukey test found there was a significant difference between mean growth rates in the 3 ppm and 5 ppm copper treatments. Further experiments must be performed over a longer period of time, more treatment groups, and more concentrations between 3 ppm and 5 ppm in order to determine the exact copper tolerance level for *T. thermophila.*

Introduction

British Columbia is home to North America's largest salmon run in the Adam's River running through Tsútswecw Provincial Park just north of Shuswap, B.C. (Androkovich 2015). However, salmon returning to the river to spawn have been in a steady decline for the past decades due primarily to human activities (Thorstad et al. 2008). One of the ways that human activity has affected salmon ecosystems is by affecting the growth of major food sources for zooplankton, which are a major food source for salmon (Engel 1976).

T. thermophila is a microorganism that forms the base of the aquatic ecosystem as it is a major food source for zooplankton (Hanley 2013, Hiltunen, Barreiro & Hairston 2012). A study on *T. thermophila* exposed to various copper concentrations concluded that copper was a toxic heavy metal that negatively impacted the growth of *T. thermophila* (Mortimer, Kasemets, & Kahru 2010). One source of contamination in British Columbia comes from Britannia Mine, one of the worst sources of heavy metal pollution in North America (Wilson, Lang, & Pyatt 2005). Heavy metals are of great concern for aquatic ecosystems because they are not biodegradable (Martín-González et al. 2005). In a study conducted by Hoffman and Cleffman, different factors, including heavy metals, were determined to affect the growth rate of *T. thermophila* (1981). Low concentrations of copper do not have an effect on the growth rate of *T. thermophila*, suggesting that the microorganism has adaptive mechanisms to extreme conditions (Frankel 2000). However, at higher concentrations of copper, the growth rate of *T. thermophila* is negatively impacted (Ruthven and Cairns 1973). Furthermore, at concentrations of 10 ppm of copper complete cell death is observed, suggesting that the organisms' capabilities to adapt to extreme conditions are limited (Schlenk and Moore 1994).

Thus the purpose of this study is to determine if the concentration of copper influences the growth rate of *T. thermophila*. Furthermore, investigating the effects of copper on *T. thermophila* will allow us to determine and better understand the effects that heavy metal pollution has on the aquatic ecosystem.

In this study, the concentrations of copper that were examined were 0 ppm, 1 ppm, 3 ppm, and 5 ppm. It was expected that the growth rate of the organism would

decline as the concentration of copper increased as studies suggest heavy metals have a detrimental effect on the growth of the microorganism (Schlenk and Moore 1994). The growth rate of *T. Thermophila* exposed to four different copper treatments was studied to investigate whether the concentration of copper in media does or does not have a significant effect on the growth rate of *Tetrahymena thermophila.*

Methods

Our starting stock solution containing *T.thermophila* had a concentration of $1.52x10^5$ cells/mL. The starting cell count was determined by counting cells using a hemocytometer and a compound microscope. They were at the stationary phase and not growing at this phase. To dilute the concentration of *T. thermophila* to $4.0x10^4$ cells/mL, 23.62 mL of new cell stock solution was combined with 73.68 mL of SSP media to make 100 mL of diluted stock cell solution.

Three treatment groups and one control group were prepared with four replicates in each group. The treatment groups were diluted from 10 ppm Cu^{2+} solution to 1 ppm, 3 ppm, and 5 ppm. In Figure 1, the control, 0ppm solution was prepared by adding 5 mL of diluted *T. thermophila* stock and 5 mL of SSP media. The treatment groups were all added 5 mL of diluted *T. thermophila* stock. To prepare the 1 ppm treatment, 4 mL of SSP media and 1 mL of 10 ppm Cu^{2+} solution was added. To prepare the 3 ppm treatment, 2 mL of media and 3 mL of Cu^{2+} solution was added. To prepare the 5 ppm treatment, no SSP media was added and 5 mL of Cu^{2+} solution was added. Sterile technique was implemented while making the control and treatment groups.

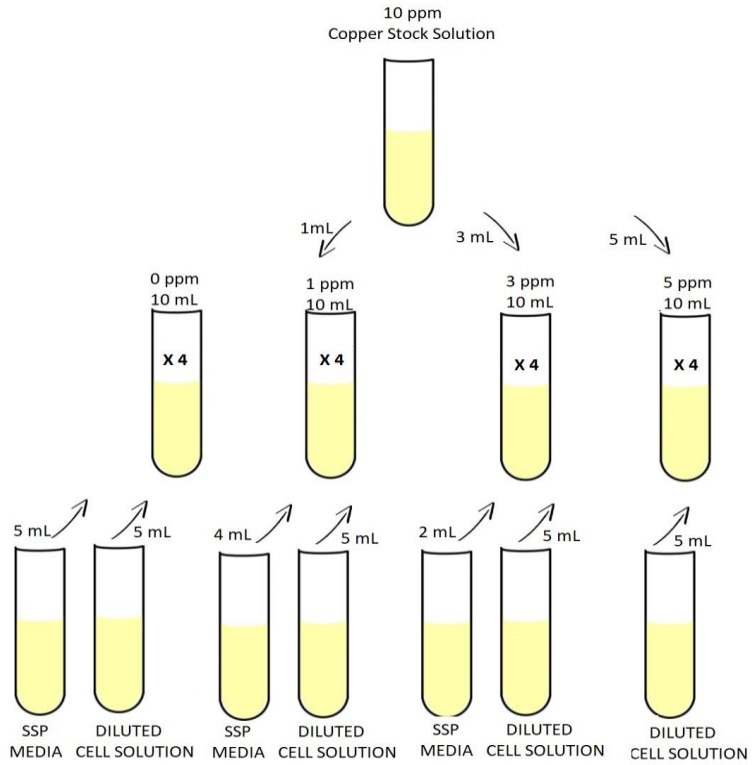

Figure 1. Schematic of Preparation of Control and Treatments.
Four replicates for 0 ppm, 1 ppm, 3 ppm, and 5 ppm.

Preparing the cells to be counted was done by micropipetting 10 µL of glutaraldehyde fixant and mixing 100 µL of the samples after vortexing (Figure 2). A hemocytometer, specifically for use with *T. thermophila*, was used. 20 µL of the fixated cells were added to the edge of the coverslip that was placed on top of a Fuchs-Rosenthal Haemocytometer. Each sample was counted three times. For the first two counts, the sample was vortexed and 20 µL of the sample was added to one side of the coverslip, then, the sample was vortexed again and another 20 µL of the sample was added to the other side of the coverslip. For the third count, we added the sample to one side of the coverslip and counted under the compound microscope.

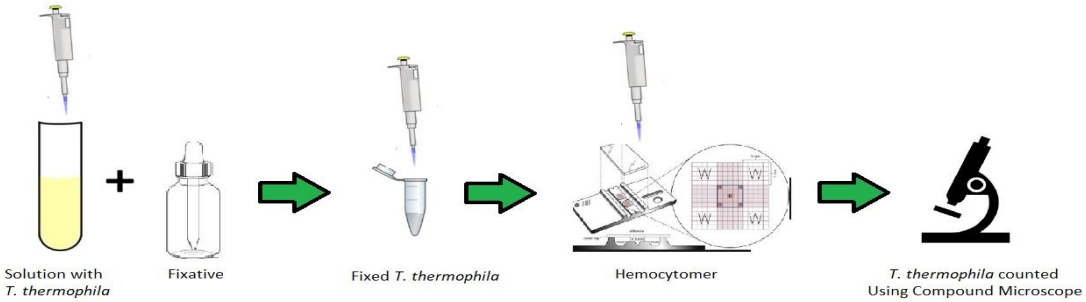

Figure 2.. Schematic of Preparation of *T. thermophila* for Counting Using Hemocytometer

Immediately after preparation and before incubating the first count was done, T_0. Then, the samples were incubated at 35^o C for 2.5 hours and then counting of all replicates three times was repeated to determine T_1. The process was repeated every 2.5 hours until T_4. The samples were incubated overnight at 35^o C. T_5 was performed 24 hours after initial incubation. The samples were once again incubated at 35^o C for 2.5 hours and the final count, T_6, was determined at a final incubation time of 26.5 hours.

The hemocytometer consisted of 16 big squares composed of 16 smaller squares each. The cells in each big square were counted, not counting the cells that were on the top and the right edges (Figure 3). The cells were counted until the counted cells were at least 50. The exact number was recorded and the number of squares it took to reach the count to determine the number of cells per square. After each count, we removed the coverslip and washed the hemocytometer with alcohol.

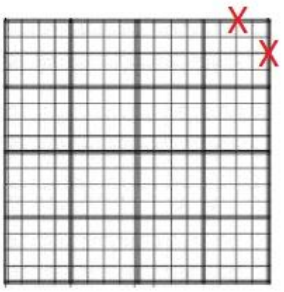

Figure 3. Hemocytometer for counting cells. No cells on the top or right edge were counted

After collecting all the required data it was analyzed using a one-way ANOVA test for significance and further statistical analysis of the data was done using a post-hoc Tukey test to find significance between the treatment groups

Results

The log of mean cell density measured in cells per millilitre was plotted against time to show the growth of *T. Thermophila* in the four different Cu^{2+} treatments (Figure 4). In the first ten hours, T_0-T_4, of the experiment, the cell count of *T. Thermophila* in all four treatments showed an increasing trend. Initially, it was observed that the four treatments were visually identical. Since the 10 ppm Cu2+ solution that was diluted for the treatments and the *T. Thermophila* medium were both yellow and transparent, it was not possible to differentiate the treatments with the naked eye. After 24 hours, T_5, it is evident that the cells have entered the stationary phase as shown in Figure 4.

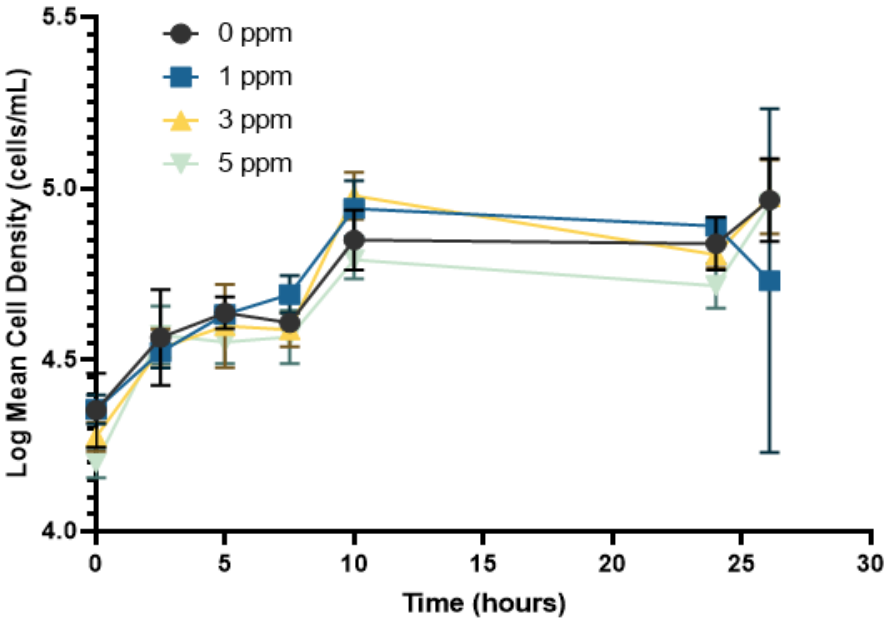

Figure 4. Time series plot of log of mean cell density in cell/mL of *T. Thermophila* for four different concentrations of Cu^2 treatments (0 ppm, 1 ppm, 3 ppm, 5 ppm) over 26.5 hours. Each line represents each treatment (n=4). Error bars represent standard deviation.

Additionally, at T_6, after incubating for 26.5 hours, cells were observed as white precipitates at the bottom of the test tubes before they were vortexed (Figure 5a). The number of cells present and visible with the naked eye in the test tubes was inversely proportional to the concentration of Cu2+ in the treatments, the higher the concentration of Cu2+ the fewer precipitates present. After vortexing, the solutions became cloudy with the most cloudiness at 0 ppm and the least at 5 ppm (Figure 5b).

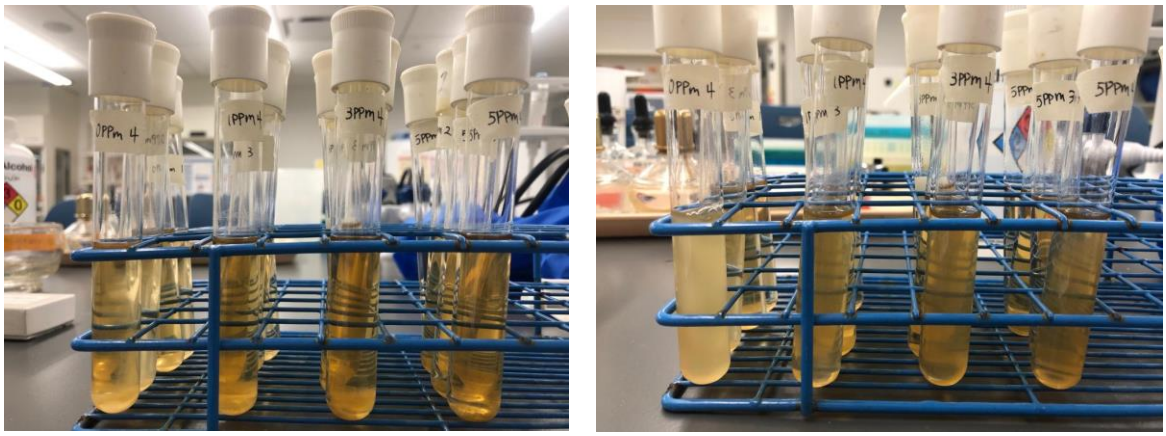

Figure 5. Test tues representing four different concentrations of Cu^{2+} treatments in the same order for both pictures from left to right: 0 ppm, 1 ppm, 3 ppm, and 5 ppm. Image on the left **(5a)** shows precipitates of cells at the bottom of the test tubes prior to vortexing. The most amount of cells were observed at 0 ppm Cu2+ treatment and precipitates decreased as the concentration of Cu2+ increased. Image on the right **(5b)** shows the test tubes after vortexing, the solutions became cloudy.

In order to test whether the difference in the four treatments was statistically significant and to verify the prediction for the experiment, a one-way ANOVA was performed. The independent variable is Cu^{2+} concentrations. From the one-way ANOVA, the F-statistic was 3.809 when only considering Cu^{2+}concentration as our factor. This was then compared to $F_{3, 12}$ distribution. The resulting p-value was 0.0396. Tukey's multiple comparisons test was performed after the one-way ANOVA. Since there are four different concentrations of Cu^{2+} treatments, a total of six comparisons were made between the treatments: "0 ppm vs. 1 ppm", "0 ppm vs. 3 ppm", "0 ppm vs. 5 ppm", "1 ppm vs. 3 ppm", " 1 ppm vs. 5 ppm", and "3 ppm vs. 5 ppm". The only

comparison that had a p-value smaller than the alpha level of 0.05 was between 3 ppm and 5 ppm Cu^{2+} treatments with the adjusted p-value of 0.0343 with 12 degrees of freedom. The remaining five comparisons had p-values that were higher than the alpha value with the same degrees of freedom (Figure 6).

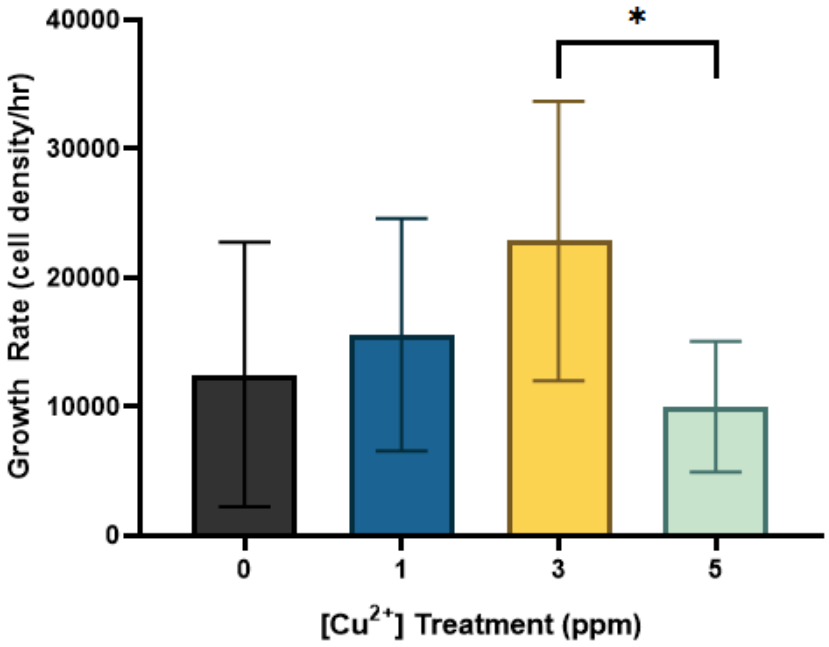

Figure 6. Bar graph of the growth rate of *T. Thermophila* at four different Cu2+ treatments. Each bar represents each treatment (n=4). Error bars show the 95% confidence intervals. One-way ANOVA was performed resulting F statistic of 3.184 and p-value of 0.0631. * indicates p-value significance at alpha = 0.05.

Discussion

From our statistical analysis using one-way ANOVA test, the p-value for Cu^{2+} was found to be 0.0396, which is smaller than our alpha level of 0.05. Therefore, we obtained sufficient evidence to reject the null hypothesis and provides support for the alternative hypothesis that the concentration of Cu^{2+} does have a significant effect on the growth rate of *T. thermophila.* From Tukey's multiple comparisons tests, it was found that the significance lies between the groups at 3 ppm and 5 ppm. Since

excessive levels of Cu^{2+} can result in cell death due to oxidative stress (Pulido and Parrish, 2003), it was predicted that the growth rate of *T. thermophila* would decrease as Cu^{2+} concentrations increased. As the difference between mean growth rates for Cu^{2+} concentrations between 3 ppm and 5 ppm was determined to be significant, this suggests that the Cu^{2+} tolerance level for *T. thermophila* lies between 3 to 5 ppm. Since *T. thermophila* was grown for a period of 26.5 hours in this experiment, it may not be long enough for *T. thermophila* to be affected by the relatively low Cu^{2+} concentration. A study by Schlenk and Moore (1994) found that survival rates were significantly lower for copper sulfate concentrations above 3 ppm at 48, 72, and 96 hours, which suggests that running our experiment for a longer period may have resulted in observed significance.

Since the tested Cu^{2+} concentrations of 1 and 3 ppm were found to be insignificant, this suggests that these levels are tolerable for sustaining a healthy population of *T. thermophila*. Since *T. thermophila* are food sources for plankton and subsequently form the bottom of the food chain, any potential population changes could ultimately affect keystone species, like salmon, that rely on plankton. (Carrick et al, 1991). Our study suggests that the tested concentrations of 1 and 3 ppm Cu^{2+} are tolerable for *T. thermophila*, and would likely have no effect on the salmon population, at least regarding specifically the salmon's ability to gather food. Though 5 ppm Cu^{2+} was found to have a significant effect on the growth rate of *T. thermophila*, more research would be needed to determine whether this population decrease would affect salmon populations.

Understanding how *T. thermophila* reacts under various concentrations of Cu^{2+} would allow better utilization of *T. thermophila* as a whole-cell biosensor. Whole-cell biosensors are single-cell organisms used to detect heavy metals in the environment that test for bioavailability and genotoxicity (Amaro 2011). Utilizing *T. thermophila* as a whole-cell biosensor could reduce potential human exposure (Amaro 2011). This study could provide data on the parameters and conditions that *T. thermophila* could be used as a whole-cell biosensor, as well as ways to better modify the organism.

The amount of dilution and micropipetting which occurred in this experiment means that error due to uncertainty can propagate. Though it is likely that this error would have no statistically significant effect on the results of our data analysis. Ensuring that the solutions containing *T. thermophila* were properly mixed was done using a vortex mixer, though this method could be improved by vortexing all solutions for the same period of time to reduce any inconsistencies in mixing.

Improvements to the experimental method could be done by using a microscope camera and using software such as ImageJ to count the number of *T. thermophila.* This would help reduce human error due to fatigue and miscounting, and would also provide a way to confirm the counts if necessary. Another improvement would be to reduce the time between each count. The time between T_4 and T_5 was 14 hours, which would have resulted in multiple doubling events, and may have resulted in inaccurate growth rates. Taking more measurements between T_4 and T_5 would result in more accurate growth rates, as well as being able to determine if any replicates were transitioning from the exponential phase to the stationary phase.

Since the results from our experiment show Cu^{2+} concentrations between 3 to 5 ppm to have a significant effect on the growth rate of *T. thermophila*, a future iteration of this experiment could be done to investigate a smaller range in concentration by testing Cu^{2+} concentrations between 3 and 5 ppm. Alternatively, future studies could test how abiotic factors which *T. thermophila* are exposed to, such as light or pH levels, in conjunction with differing Cu^{2+} concentrations could affect growth rate, as Cu^{2+} uptake by *T. thermophila* may be affected by these factors.

Conclusion

The One-way ANOVA was performed resulting in a significant difference among mean growth rates at four Cu^{2+} treatments. The F-statistic of 3.184 and the p-value of 0.0631 from this analysis provided sufficient evidence to reject the null hypothesis at the alpha level of 0.05. Our prediction that the increasing Cu^{2+} concentration in the media would decrease the growth rate of *T. Thermophila* was supported by the data obtained from this study. After the Tukey's multiple comparisons test, a p-value significance at an alpha level of 0.05 was found between the mean growth rate at 3 ppm and 5 ppm. Therefore, it is evident from this study that Cu^{2+} concentrations over 3 ppm negatively affect the growth rate of *T. thermophila.* Moreover, it is our suggestion that Cu^{2+} levels should be monitored in creeks and tributaries since *T. thermophila* belong to the base of the food web which could affect keystone species such as salmon.

Acknowledgments

We would like to thank the Biology department of the University of British Columbia for offering this lab and giving us the opportunity to participate in this project. A special thanks to our instructor Dr. Jordan Hamden for his guidance and input on our experimental design and for being available to continuously support us throughout the experimental procedure. Thank you to the teaching assistants as well. They were available to answer any questions and give us insight into better techniques, especially, Tessa Blanchard for her help during our set-up procedure. Thank you to Mindy Chow and Chanelle Chow for preparing the cell cultures and all of the equipment needed to perform the experiment and for their input on improving our list of materials and the experimental procedure.

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