The effect of copper sulfate toxicity on cell growth rate of *Tetrahymena thermophila*

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Abstract: An investigation on the toxicity of copper sulfate ($CuSO_4$) on the growth rate of the ciliated protozoan, *Tetrahymena thermophila* was conducted. Previous studies reveal that that the organism has developed a resistance to heavy metal toxicity, with copper sulfate being one of the most common metals *T. thermophila* encounters. The growth rate after 24 hours has been measured in these studies, but there is little information on the initial response of the cells with heavy metal exposure. This study observed the cell growth within a 12-hour period at 3 hour increments. It involved three treatments of copper sulfate – 0 ppm (control), 1 ppm and 3 ppm, with 4 replicates each treatment. The growth rate of *T. thermophila* was determined by the cell counts during the growth phase, and the statistical significance of the results was analyzed using the ANOVA test. It was found that at 0 ppm, 1 ppm and 3 ppm, the growth rate was around 350000 cells/mL/hour, 320000 cells/mL/hour, and 140000 cells/mL/hour respectively. The ANOVA test produced a p-value of 0.00074, which indicated a significant difference amongst the three treatments. Further analysis using the 95% confidence intervals between individual treatments showed that there is no significant difference between 0 ppm and 1 ppm copper sulfate treatment; however, the 3 ppm treatment showed a significant difference compared to the 0 and 1 ppm treatments. Based on the statistical results, the null hypothesis was rejected, indicating a decrease in *T. thermophila* growth rate with an increase in copper sulfate concentrations. Thus, the alternative hypothesis was supported.

Introduction

Tetrahymena thermophila is a unicellular eukaryotic cell that commonly inhabits freshwater environments (Collins and Gorovsky 2005). *T. thermophila* belong to the Ciliated Protozoa group, a group found to be sensitive to the effects of heavy metals (Ruthven and Cairns 1973). In the laboratory, these types of cells are often compared to human cells for their large size, as they are about 50 µm wide, and similar cellular processes (Wloga and Frankel 2012).

Copper is a heavy metal found to have toxic effects on humans and in aquatic environments if found in excess concentrations (de Romaña 2011; Rico *et a*l. 2008; Ryu *et al*. 2011). In humans, chronic copper exposure can result in damage to cell membranes,

proteins and DNA (de Romaña 2011). Exposure to copper can come from water contamination due to heavy metals from various sources, like sewage waste (Ryu *et a*l. 2011). In aquatic habitats, heavy metals are found to induce oxidative stress in ciliated protozoans, decreasing its population growth (Rico *et a*l. 2008). With *T. thermophila* living in aquatic habitats, this species is often used as a eukaryotic model to study the effects of pollution on aquatic environments and to determine the water quality. By monitoring the effect of copper on *T. thermophila* cells, we are able to determine the effect of heavy metals on microorganisms and we may use our findings to compare its consequences on human cells.

The objective of this experiment was to determine the effect of copper sulfate concentrations on the population growth of *T. thermophila*. Our null hypothesis stated that an increase in copper sulfate concentrations would result in an increase or would have no effect on *T. thermophila* growth rate. Our alternate hypothesis stated that an increase in copper sulfate concentrations would result in a decrease in *T. thermophila* growth rate. In a study conducted by Schlenk and Moore (1994), *T. thermophila* cells experienced the greatest toxicity to copper at higher copper sulfate concentrations. Schlenk and Moore (1994) only observed the effect of copper sulfate concentrations after 24 hours, but did not measure the effect of copper for a shorter time period.

Method

T. thermophila was extracted from stock solution containing $2.5x10^5$ cells/mL that was originally cultivated in a growth medium in the lab at room temperature (22°C). Three $CuSO_4$ treatments, each containing four replicates, were set up. The first treatment

was the control, with no $CuSO_4$ in the medium, so the $CuSO_4$ concentration was 0 parts per million (ppm). To prepare this, 3 mL of stock *T. thermophila* was mixed with 1.5 mL water and 1.5 mL double stock medium in four 6 mL test tubes.

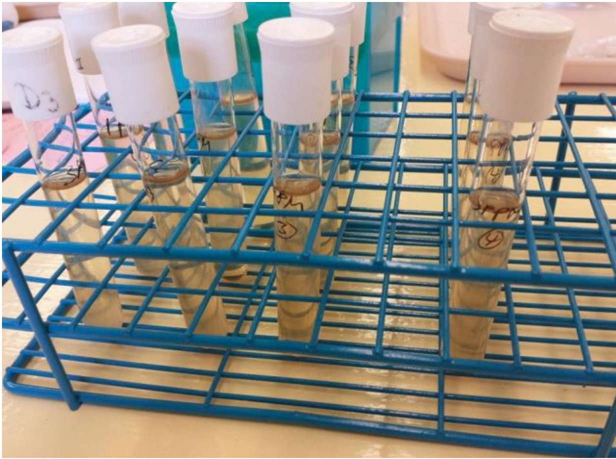

Figure 1. *T. thermophila* in varying treatments of copper sulphate.

Then, the 1 ppm solution was prepared with 3 mL stock *T. thermophila* mixed with 1.5 mL 0.004176 M $CuSO_4$ solution and 1.5 mL double stock medium in four 6-mL test tubes. Similarly, the 3 ppm solution was prepared with 3 mL stock *T. thermophila* mixed with 1.5 mL 0.012528 M $CuSO_4$ solution and 1.5 mL double stock medium in four 6-mL test tubes. Figure 1 shows the replicates in their media. Furthermore, all test tubes were sterilized by flame prior and during transfer of sample to avoid contamination.

The growth rate was quantified using the AXIOSTAR compound microscope by determining the cell count via haemocytometer. The cell count was measured every 3 hours, for 12 hours starting at the initial time of addition of $CuSO_4$. The interval of 3 hours was chosen because it allowed enough time for the cells to grow in the medium, as well as provide enough data points to conduct a test for statistical significance.

Firstly, each test tube was mixed by finger vortex to ensure the solution was well mixed. In addition, each pipetting was done at the center of the test tube to ensure consistency. Each cell count was carried out by adding a 20 μL sample of each treatment

group to 2 µL of glutaraldehyde. The glutaraldehyde fixed the cells in the medium, to allow counting. 20 µL of this mixture was then placed on a haemocytometer with a cover slip. The cells were counted at three random large squares, each containing 16 smaller squares, in the haemocytometer. Thus, there would be 12 cell counts for each treatment at each time increment. An average of these 12 cell counts was multiplied by the dilution factor, and then used to determine the final cell concentration in the medium. All the test tubes were placed in the same rack, and were kept in the same environment throughout the 12 hours to reduce any environmental fluctuations.

Referring to Figure 3, the time span from 0 to 6 hours demonstrated the growth phase; therefore it was used for determining the growth rate of *T. thermophila.* This was done by plotting the cell concentration versus time graph to apply the logarithmic regression line to determine the slope that illustrated the growth rate. Using the growth rate, the one-way analysis of variance (ANOVA) was conducted to determine if there was a significant difference between the treatments. Moreover, if ANOVA tested positive, a 95% confidence interval (CI) would be carried out to determine which specific treatment is different from the others.

Results

During the course of the experiment, all the solutions stayed light brown in colour, which was the colour of the medium at the start. As time passed, more and more white flakes started settling to the bottom of the vials. These represented dead cells, and this amount increased with the $CuSO_4$ concentration, as well as towards the later hours (9 hours and onward).

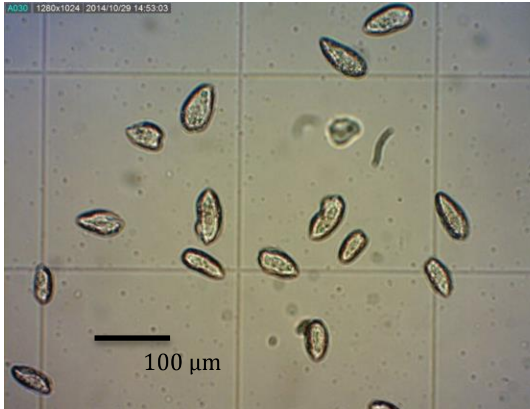

Figure 2. *T. thermophila* cells in 0 ppm $CuSO_4$ on a haemocytometer, using a microscope at 100x magnification

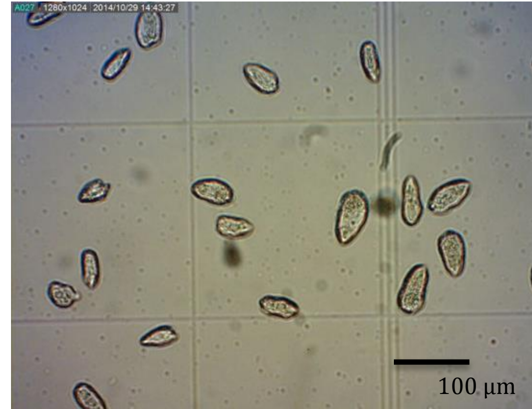

Figure 3. *T. thermophila* cells in 3 ppm $CuSO_4$ on a haemocytometer, using a microscope at 100x magnification

Figure 2 and Figure 3 show the cells in treatments of 0 ppm and 3 ppm $CuSO_4$ respectively, and it can be noted that the cells at 0 ppm are more oblong and oval-shaped, whereas the cells at 3 ppm are more round.

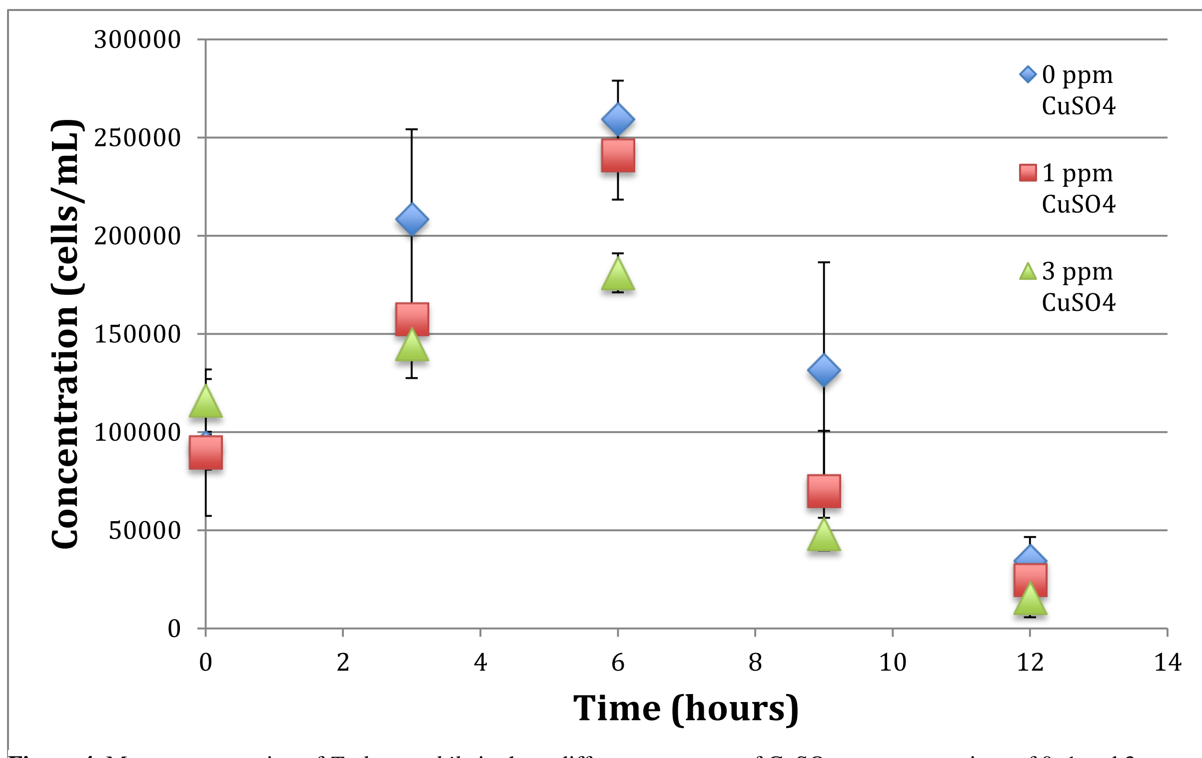

Figure 4. Mean concentration of *T. thermophila* in three different treatment of $CuSO_4$, at concentrations of 0, 1 and 3 ppm. The error bars show a 95% confidence interval for each of the means.

Based on Figure 4, the results show that the growth phase of *T. thermophila* primarily occurs during the first six hours, as there was a high increase in cell concentration in that time frame. This represented the log phase, and the concentration from 0 to 6 hours was used to calculate the growth rate of *T. thermophila*. It can also be noted that the 3 treatments all had the same initial concentration. At 0 ppm, 1 ppm and 3 ppm, the cell count seemed to peak at 6 hours, after which the cells decreased exponentially in number until 12 hours. The $CuSO_4$ concentration of 0 ppm showed the greatest increase in cell count, and the concentration of 3 ppm showed the least increase in cell count.

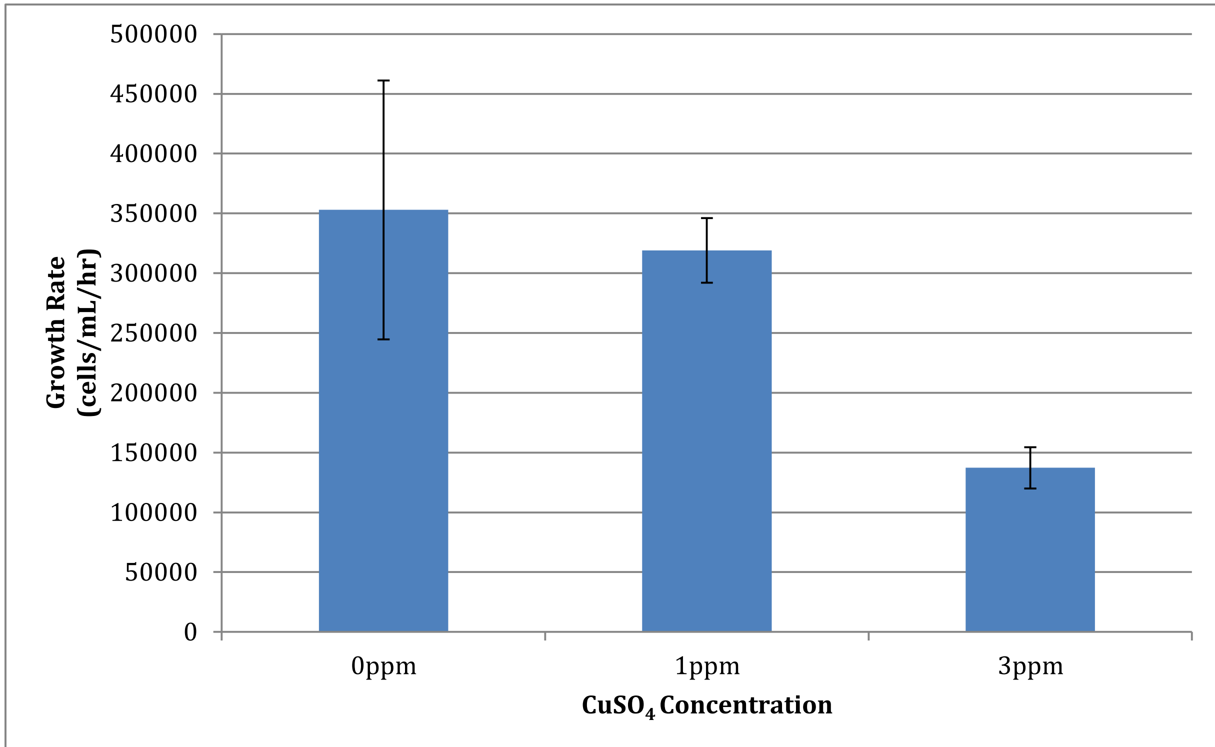

Figure 5 Mean *T. thermophila* growth rate at the 3 $CuSO_4$ treatments (0, 1, and 3 ppm). The error bars show a 95% confidence interval for each of the three different treatments.

Figure 5 shows the mean growth rate in cells/mL/hour of *T. thermophila* cells at the 3 different concentrations of $CuSO_4$. The highest growth rate of the cells was at 0 ppm concentration, at around 350,000 cells per mL per hour. At 1 ppm, we see a slight decrease in growth rate, at 320,000 cells per mL per hour. 3 ppm showed the lowest growth rate, at 140,000 cells per mL per hour.

The ANOVA test provided a p-value of 0.00074 and an F-calculated value to be 12.17. The F-critical value was 4.25, hence our results showed a significant difference. Respectively, $CuSO_4$ concentrations of the 0 ppm, 1 ppm, and 3 ppm had a confidence interval of $\pm$108210 cells/mL/hr., $\pm$26983 cells/mL/hr., and $\pm$17250 cells/mL/hr. Based on the 95% interval, there were overlaps in the 0 ppm and 1 ppm results, but there was a significant difference between the treatments of 0 ppm and 3 ppm.

Discussion

Our p-value from the ANOVA was 0.00074, and since this was less than the p-critical of 0.05, we rejected our null hypothesis and provided support for our alternative hypothesis, which stated that an increase in $CuSO_4$ concentration decreases the growth rate of *T. thermophila*. This was in accordance with previous studies that showed the negative effects of heavy metals, such as copper, on cell viability (Schlenk and Moore 1994). The ANOVA told us that there was a general significant difference in the treatments; however it did not differentiate within the 0, 1, and 3 ppm concentrations. Thus a 95% confidence interval of the mean growth rate of each individual treatment was used to determine significant difference within each treatment. Figure 5 shows a confidence interval overlap in the 0 ppm and 1 ppm treatments, as it was $\pm$108210

cells/mL/hr and $\pm$26983 cells/mL/hour for the respective treatments. But, there was no overlap in the 3 ppm treatment, which was $\pm$ 17250 cells/mL/hour, with 0 ppm or 1 ppm treatments. As a result, the treatments of 0 ppm and 1 ppm were significantly different from the 3 ppm treatment.

The lack of significant difference in the 0 ppm and 1 ppm treatments may be due to the resistant to copper toxicity by *T. thermophila* at low concentrations; this suggests that there is an intracellular mechanism responsible for this resistance (Schlenk and Moore 1994). Heavy metals, such as $CuSO_4$, bind and denature cellular membrane proteins, which may trigger a change in intracellular function (Gadd and Griffiths 1977). This change may be the binding of integral protein or receptor protein that hinders the cell from obtaining nutrients. Sexual reproduction of *Tetrahymena* requires conjugation where the two cell pairs and forms a temporary junction. This binding requires recognition proteins on cell surfaces that could have been hindered if heavy metals bind and change confirmation of this junction site.

While carrying out the experiment, we noted a difference in the cell sizes of *T. thermophila* in the 3 treatments. In the 0 ppm $CuSO_4$ treatment, the cells were elongated and measured to be 0.05 mm wide; in the 1 ppm $CuSO_4$ treatment the cells were slightly round; and the 3 ppm $CuSO_4$ treatment, the cells were more rounded and slightly bigger at 0.1 mm wide. The observations suggest that for 0 ppm and 1 ppm $CuSO_4$ treatments, cells are growing fast and are not reaching the larger size like the cells for the 3 ppm $CuSO_4$ treatment. The 3 ppm cells growing in size may suggest that they have more time to accumulate food, taking longer for them to divide, leading to greater cell size. It could be that the cells in the 3 ppm media are dying at a faster rate than at lower CuSO4

concentrations due to the toxicity affecting the reproduction, so the cells that do survive have less competition, and thus have easier access to nutrient supplies (Laakso *et al.* 2003).

T. thermophila is exposed to copper in its freshwater habitat (Georgopoulos *et al*. 2001; Rico *et a*l. 2008). Copper is released into this environment via a myriad of sources, including industrial waste, domestic water waste, wood production, phosphate fertilizer production, forest fires and combustion processes (Georgopoulos *et al.* 2001). Harrison (1998) reported that copper concentrations in freshwater are low (0.1-15.6 ppb) but are higher in freshwater sediments (0.4-796 ppm). The concentration in sediments in freshwater sources could be harmful for aquatic organisms, especially once processes such as chelation or sorption affect the bioavailability of copper (Harrison 1985). When the threshold concentration is reached, usually due to anthropogenic activities, heavy metals, like copper, will have toxic effects on aquatic organisms.

Comparing our experiment to Schlenk and Moore (1994), our observations were done from the time interval from 0 to 12 hours as opposed to Schlenk and Moore (1994) who observed from 24 to 96 hours. Our experiment provides an insight on the sudden effects of changes of environment on *T. thermophila* over a short period of time prior to the population having adapted to the new environment.

There are several sources of human error that may have influenced our results. To begin with, the setting for the counting intervals was carried out in an open lab. Environmental and abiotic factors, such as room temperature, may affect the cell growth. Room temperature fluctuates throughout the day, as there are slightly higher temperatures

during midday and slightly lower temperatures during the morning and night. Since *T. thermophila* cells have an optimum temperature, the fluctuating temperatures could increase or decrease cell growth rates. (De Coninck *et al.* 2004). The act of finger vortexing to mix the cells may affect the cell growth due to the vibration. As the cell culture grows, it tends to accumulate at the bottom of the test tube, leading to a concentration gradient that increases over time. This would require longer finger vortexing to mix the cells in the medium and obtain accurate samples. Cells that feel too much vibration may collapse (Hellung-Larsen *et al.* 1992). We can aim to reduce these errors by using a water bath to maintain a constant temperature, and not conducting the finger vortex multiple times. Furthermore, biological variance also exists as a source of error, as all cells do not grow and reproduce in exactly the same way. Having 4 replicates for each treatment and measuring the cell count thrice for each replicate reduced this variance, but a small error still exists since these are biological beings.

It would be interesting to look at the same treatments conducted on the mutant-type of *T. thermophila*, and how similar the results of the wild type and mutant could be. The mutant has a defect in the oral apparatus development leading to a lower vacuole count, so copper toxicity could affect the mutant in a more defective way since it is already slower in nutrient uptake. We could also test the effect of different heavy metals, such as cadmium and iron. Industrial waste runoff has a combination of many heavy metals, so an investigation could be done on other heavy metals, or a mixture of them. Schlenk and Moore's (1994) study showed that cadmium had more severe effects on *T. thermophila* growth rate, leading to a 100% mortality rate after 24 hours of exposure to 2 ppm cadmium, whereas a 20% mortality at higher copper concentrations.

Conclusion

We conclude that copper sulfate toxicity does have an effect on growth rate of *T. thermophila*, as we rejected the null hypothesis. We support our alternate hypothesis, which states that an increase in $CuSO_4$ concentration decreases the growth rate of our model organism.

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