The Effect of Copper Sulfate on the Cell Growth of Wild Type *CC-1690 Chlamydomonas reinhardtii*

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Abstract

Chlamydomonas reinhardtii is a unicellular green alga found around the world and intensely studied in biology. Due to the increasing demand in clean alternative energy, studies have been done on *Chlamydomonas reinhardtii* to optimize its ability to produce hydrogen gas and biofuels (Morowvat *et al*. 2009). Over the years, industrialization has led to increasing copper contamination in the environment (Jamers *et al*. 2006). In order to understand the optimal conditions for the growth of *Chlamydomonas reinhardtii* in a lab setting or in the environment, we sought to find out the effect of copper on the abundance of the wild-type form (CC-1690). Three treatments were used in our experiment: 0.0 mg/L [control], 0.6 mg/L and 1.2 mg/L of copper sulfate. For each treatment four replicates were setup and cell density measurements were taken through a 168 hour (7 day) time span. The results of our data collected showed no significant differences, but trends were noticed. Due to the lack of significant differences in our data, we failed to reject our null hypothesis which stated that an increase in the concentration of $CuSO_4$ increases or has no effect on the growth rate of *Chlamydomonas reinhardtii*. We believe this is due to *Chlamydomonas reinhardtii* being able to tolerate and benefit from the concentrations of $CuSO_4$ used in this experiment (Boswell *et al.* 2002).

Introduction

Organisms require trace amounts of essential micronutrients for optimal functioning of certain enzymes (Jamers *et al*. 2006). Copper (Cu) is an essential micronutrient for photosynthetic eukaryotes (Yamasaki *et al*. 2008). It contributes to cellular homeostasis by acting as a catalyst for redox reactions within a cell (Hill *et al*. 1996). Because of its multiple ionic states (Cu^{+2}, Cu^{+3}), copper can form stable complexes with proteins. Once formed these complexes can be altered by electron transfer, and in a reduced state they can be oxidized by oxygen (Hill *et al*. 1996). Although Cu is considered a nutrient in trace amounts it can also be

harmful to biota at concentrations only marginally greater than cellular requirements (Stoiber *et al*. 2009).

At moderately high concentrations in natural environments, Cu is considered to be an environmental stressor (Stoiber *et al*. 2009). Copper has the potential to bioaccumulate and can pose a threat to indigenous organisms (Jamers *et al*. 2006). Since the industrial revolution, Cu released into the environment has drastically increased, such that copper contamination and toxicity are becoming more frequent in surface waters and groundwater systems (Jamers *et al*. 2006). Even though these events are harmful to many organisms such as algae, invertebrates and fish, the major concern about the effect of copper is its ability to potentially harm humans (Jamers *et al*. 2006).

In order to better understand the effect of copper on organisms we chose to study growth rate patterns in response to copper. We used *Chlamydomonas reinhardtii*, a unicellular green alga, as our model organism (Stoiber *et al*. 2009). *C. reinhardtii* is a biflagellate eukaryote that contains a single chloroplast that takes up $^{2}/_{3}$ of the cellular volume (Stoiber *et al*. 2009). Currently, studies on *Chlamydomonas reinhardtii* have been focused on the production of alternative energy, due to its ability to produce hydrogen gas and to create biofuels (Morowvat *et al*. *2009).* Furthermore, *Chlamydomonas* is involved in photosensory and chemosensory processes (Stoiber *et al*. 2009), and is best studied with respect to the molecular mechanism of copper homeostasis (Yamasaki *et al*. 2008). For these reasons we chose to study the effect of copper concentration on the growth rate of *Chlamydomonas reinhardtii*.

Our null and alternative hypotheses are:

H_O: An increase in the concentration of $CuSO_4$ increases or has no effect on the growth rate of *Chlamydomonas reinhardtii.*

H_A: An increase in the concentration of $CuSO_4$ decreases cell growth rate in *Chlamydomonas reinhardtii.*

Literature supporting H_A:

Copper is considered a nutrient in trace amounts but can be harmful to biota at concentrations only marginally greater than cellular requirements (Stoiber *et al*. 2009).

From Roman Danilov and Nils Ekelund's (2001) experiment on the effects of Cu^{2+}, $Ni^{2+,}$ $Pb^{2+,}$ Zn^{2+} and pentachlorophenol on photosynthesis and motility in *Chlamydomonas reinhardtii* , it was found that increasing copper concentration decreased the efficiency for photosynthesis in *Chlamydomonas*. Furthermore, they concluded that copper and pentachlorophenol are especially toxic to the photosynthetic apparatus of *C*. *reinhardtii* (Danilov and Ekelund 2001).

Methods

We performed our experiment over a one week period. We started the experiment with 500 mL of a pre-grown culture of *Chlamydonomas reinhardtii* with an initial cell concentration of 6.6×10^4 cells per liter. Throughout the experiment, we used sterile technique to prevent contamination.

The first step was to calculate the initial cell concentration. We gently swirled the *Chlamydomonas reinhardtii* culture flask for 1 minute; this was to thoroughly mix the culture so the cells would be evenly suspended in the media. We transferred 0.2 mL of the culture sample into three separate 1 mL micro-centrifuge tubes and added 20 µL of IKI buffer to fix each

sample. The IKI buffer helps to preserve the sample and prevent the cells from decaying. Then, we used the haemocytometer to determine the cell concentration of each of the three samples and used the average to determine the initial cell concentration of our starting culture (Figure 1).

Figure 1: Cell counts were done through a Zeiss Axio microscope with the sample on a haemocytometer.

The second step was to prepare the different treatment group replicates from the starting culture. Our total sample size for the experiment was 12. We used three treatment groups with different copper sulfate concentration: 0 mg/L (which served as the control group), 0.6 mg/L and 1.2 mg/L. For each treatment groups, we prepared four individual replicates. To prepare replicates, we added 35 mL of well-mixed culture sample into 12 separate 50 mL Erlenmeyer flasks. Then we added appropriate amount of copper sulfate solution to each flask. We added distilled water to some flasks to ensure that the volume in all flasks remained the same. After this step, the four flasks in the control group had a final copper sulfate

concentration of 0mg/L; the four flasks in the 0.6mg/L treatment group had a final copper sulfate concentration of 0.6mg/L; and the four flasks in the 1.2 mg/L treatment group had a final copper sulfate concentration of 1.2mg/L. We then covered the flasks with aluminum foil and placed in the incubator with temperature of 17˚C, light intensity of 1412 LUX, relative humidity of 83%, and light/dark interval of 14 hours light and 10 hours dark (Figure 2). The flasks were stored in the incubator for the entire one week period, during which time we only took the samples out of the incubators for 20 minutes when we collected samples for cell counting each time.

Figure 2: Represents the 12 samples consisting of 3 treatments and 4 replicates in the incubator that is at temperature of 17˚C, light intensity of 1412 LUX, relative humidity of 83%, and light/dark interval of 14 hours light and 10 hours dark.

The third step was to determine the cell concentration at different times throughout the week. We performed cell count at 24, 48, 72, and 168 hours after the initial placement of the culture flasks in the incubator. During each cell count, we first gently swirled the flasks for 1 minute to thoroughly mix the cultures. Then, from each flask, we transferred 0.2 mL of the sample into a 1 mL micro-centrifuge tube, added 20 µL of IKI buffer to fix the sample, and inverted the micro-centrifuge tube 20 times to thoroughly mix the sample and buffer. We then used the haemocytometer to count the number of organisms and calculated the cell concentration for each replicate sample.

The last step was to analyze the data. We calculated the interval growth rate, which was the change in cell concentration over the time between each cell count measurement. We calculated the cumulative growth rate over 168 hours as well. Then, we calculated the mean value and 95% confidence interval for the interval growth rates and cumulative growth rates for each treatment group. We plotted the values using bar graphs and scatter plots.

Results

The growth rate of *Chlamydomonas reinhardtii* was recorded for 3 treatments that had 4 replicates each, over a span of 168 hours. The cell count for each treatment was then averaged and converted to cell per milliliters as shown in Figure 3.

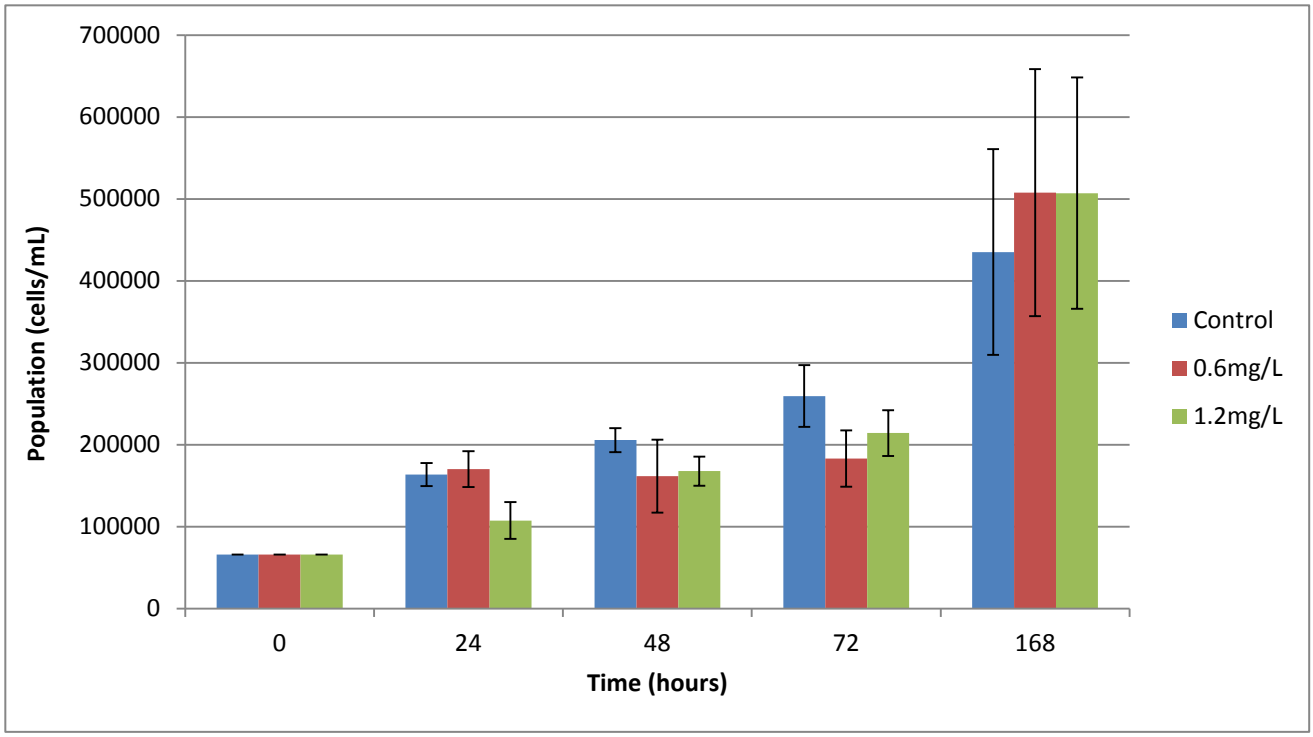

Figure 3: Mean cell count of *Chlamydomonas reinhardtii* over the course of 168 hours at 0.0, 0.6, 1.2 mg/L of $CuSO_4$. The mean was calculated from four replicates and 95% confidence intervals are represented by the error bars. (n=4 trials per treatment group)

Furthermore, Figure 3 shows that the control [0.0mg/L] and [0.6mg/L] of $CuSO_4$ treatments had a comparable cell division at a consistent rate between the time interval of 0 to 24 hours (66, 000 cells/ mL to 165 000 cells/mL respectively). During the same time interval it is evident that the 1.2 mg/L treatment had a slow cell growth (66, 000 cells/mL to 108 000 cells/mL) shown in Figure 3, but as time went on this treatment had a consistent cell growth (Figure 4). This analysis is further supported by Figure 4, which shows the interval growth rate between the control and the 0.6mg/L treatment of CuSO4 are in close proximity, for the interval between 0 to 24 hours.

The control and the 0.6mg/L treatment of CuSO4, between the intervals of 24 to 48 hours had a reduction in the cell population, followed by a consistent cell division from the

interval between 48 to 168 hours shown in Figure 4. These consistent growth rates in all treatments correlate to our observations made during the experiment on the medium of our culture going from a clear fluid at time 0 to a green opaque fluid by the end of the experiment, by then green granules seen by the naked eye had precipitated to the bottom of the flask which held our cultures throughout the experiment.

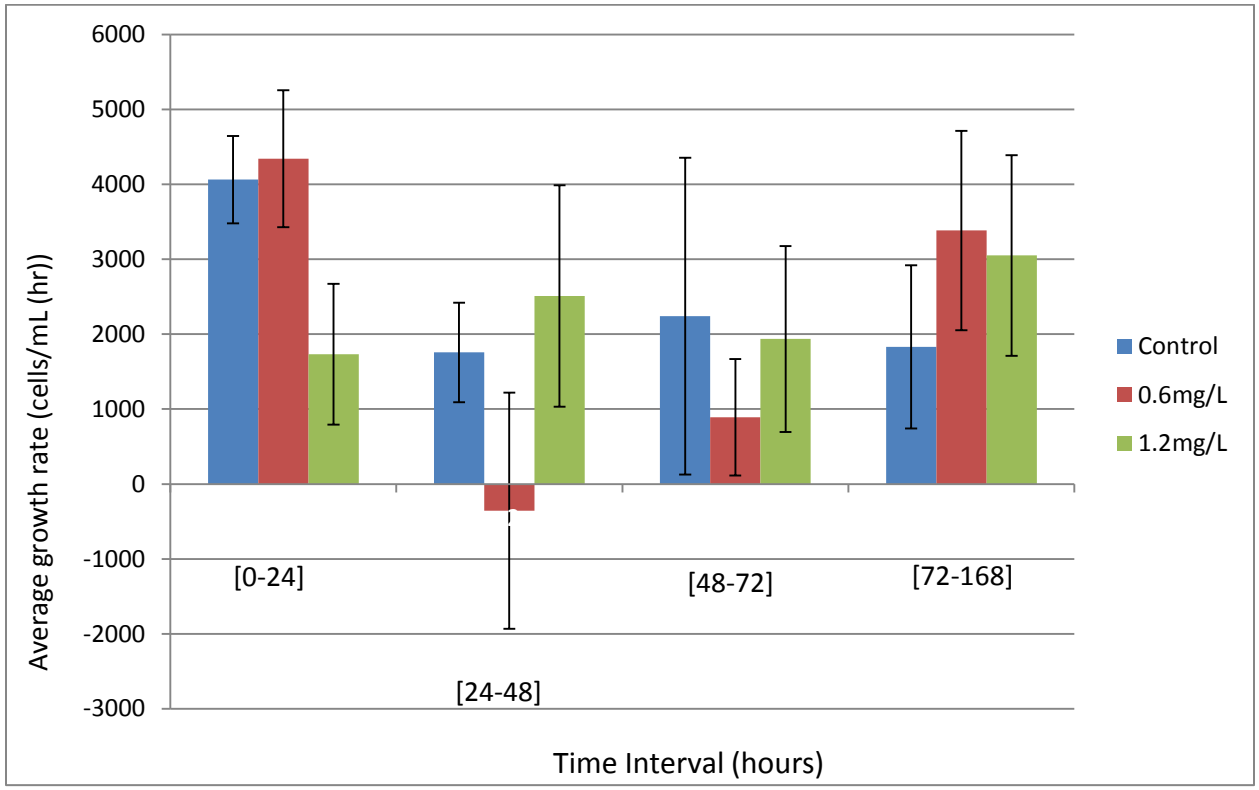

Figure 4: Mean cell count of *Chlamydomonas reinhardtii* over the course of intervals [0-24 hrs], [24-48 hrs], [48-72 hrs], [72-168 hrs] at 0.0, 0.6, 1.2 mg/L of $CuSO_4$. The mean was calculated from four replicates and 95% confidence intervals are represented by the error bars. (n=4 trials per treatment group)

Lastly, as shown in Figure 5, there was no significant difference in the overall cumulative cell growth in the three different treatments, but there seems to be a trend that shows a higher

cumulative cell growth in the 1.2mg/L treatment. All three treatments averaged at 2500 cells per milliliter with their 95% confidence intervals overlapping seen in Figure 5.

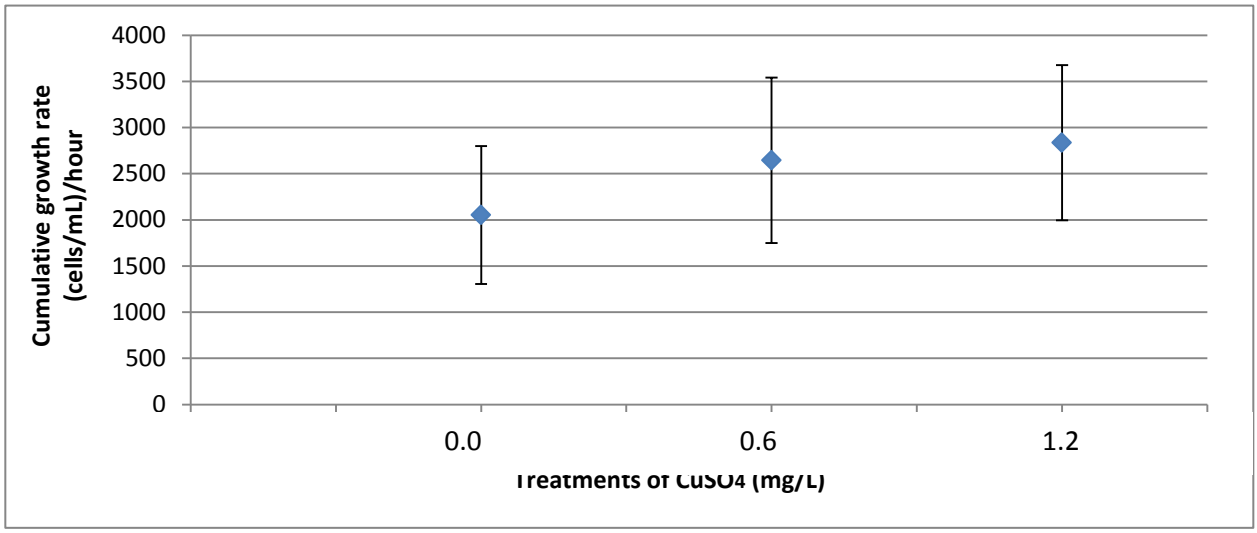

Figure 5: Overall cell growth rate of *Chlamydomonas reinhardtii* over the course of 168 hours at 0.0, 0.6, 1.2 mg/L of $CuSO_4$. The overall cell growth is the final cell count subtracted by the initial cell count divided by the overall time. The mean was calculated from four replicates and 95% confidence intervals are represented by the error bars. (n=4 trials per treatment group)

Sample calculation for Control Replicate #1:

$$\textbf{Average Proportion} = \frac{\sum(Proportions)}{Number\ of\ replicates} = \frac{(1650000+158889+182111+147889)}{4} = \mathbf{163472}$$

Standard deviation =

$$\sqrt{\frac{\sum(Proportions - average)^2}{Number\ of\ replicates - 1}}$$

$$=$$

$$\sqrt{\frac{((165000 - 163472)^2 + (158889 - 163472)^2 + (182111 - 163472)^2 + (147889 - 163472)^2))}{3}}$$

$$= \mathbf{14301}$$

$$\textbf{95\% Confidence Intervals} = 1.96\left(\frac{Standard\ Deviantion}{\sqrt{\#\ of\ replicates}}\right) = 1.96\left(\frac{14301}{\sqrt{4}}\right) = \textbf{14015}$$

Discussion

Based on the results, we failed to reject our null hypothesis that an increase in copper sulfate concentration will decrease the growth rate of *Chlamydomonas reinhardtii*. However, we did observe three general trends in the change of growth rate of *Chlamydomonas reinhardtii* in respect to change in copper sulfate concentration and time.

Firstly, we found that the overall cumulative growth rate, which is the change in cell number per hour over 168 hours period, increases with increasing copper sulfate concentration. Although the difference is not statistically significant at 0.05 alpha level, the general trend is fairly clear. This result was contradictory to our alternative hypothesis which predicted that increasing copper sulfate concentration in the culture medium would decrease the growth rate of *Chlamydomonas reinhardtii*. There are several possible explanations for this result. The most probable reason would be that the copper concentrations in the treatment groups were within the tolerable trace amount range that is actually beneficial to *Chlamydomonas reinhardtii*. Since copper is an essential ion required for photosynthesis in algae and plants, the presence of trace amounts of copper in the growing medium increases the growth rate of *Chlamydomonas reinhardtii* (Yamasaki *et al*. 2008). Boswell *et al*. (2002) found in their study that copper sulfate concentration below 100 µM led to observations of normal growth patterns of *Chlamydomonas reinhardtii* cultures. Since our copper concentration in the treatment groups were only 3.76 µM (0.6 mg/L) and 7.52 µM (1.2 mg/L), which were below the

100 µM toxicity limit, it should have not had a negative impact on the growth of *Chlamydomonas reinhardtii*.

The third trend we observed is that for the 1.2 mg/L treatment group, the interval growth rate from 0 to 24 hours was significantly lower than the interval growth rate of the other two groups; the growth rate increased after 168 hours (Figure 5). The significantly lower initial growth rate of the 1.2mg/L treatment group may be a result of higher toxicity of higher copper sulfate concentration. The subsequent steady increase in growth rate may indicate the presence of an on-going natural selection process: cells who survived in the toxic environment (they had higher copper tolerance level) are selected to produce a population of cells with tolerance to the 1.2mg/L copper concentration level, and these cells then followed a normal population growth pattern (Boswell *et al.* 2002).

There are a number of possible sources of errors in our experimental procedures; this may be another cause for the inconsistencies between our result and results from other relevant studies. For instance, we used a 25 mL graduated cylinder to transfer the 35mL of sample into small flasks; therefore we had to do 2 transfers to get the 35mL amount. Along with the fact that it is harder to get exact volumes using the graduated cylinder, it is very likely that the volume of sample in each replicate flask differ slightly. Inaccurate amount of copper sulfate added to samples, inadequate mixing before taking samples and errors in counting the cells using the haemocytometer are other examples of possible sources of error. Such errors can result in differences in the measurement of cell density and as a result can affect the overall

observed trend of cell population growth. They also led to larger confidence intervals which make the result statistically not significant. Small sample size was another major limitation of our study, and it also led to larger confidences intervals. An experiment with higher number of replicates may be able to show statistically significant relationships.

Conclusion

The result of our experiment failed to reject our null hypothesis. Although the results were not statistically significant, we found a general trend that is contradictory to our alternative hypothesis: the trend suggests that an increase in copper concentration in the medium leads to increase in the cumulative population growth rate of *Chlamydomonas reinhardtii*. This finding is consistent with some other relevant studies on this topic and contradictory to others. The major differences among the findings lie on the differences in the observed copper tolerance level of the *Chlamydomonas reinhardtii* cultures studied. Although copper is an essential nutrient required in trace amount by the photosynthesis processes in eukaryotic cells, concentration slightly above the tolerance level will lead to severe adverse impact on *Chlamydomonas reinhardtii* population (Yamasaki *et al*. 2008) (Stoiber *et al*. 2009). Since the industrial revolution, heavy metal pollution of water has been becoming an increasingly common environmental concern (Jamers *et al*. 2006). Therefore, experiments that use *Chlamydomonas reinhardtii* as model organism to study the effect of high copper concentration on freshwater organisms can provide important knowledge and insights on the problem (Yamasaki *et al*. 2008).

Acknowledgements

We would like to take this opportunity to express our gratitude and deep regards to our course instructor Dr. Carol Pollock for her guidance, knowledge and patience through the process of designing this experiment. We also like to express a deep sense of gratitude to our teaching assistant Diana Rennison for helping countless times throughout this experiment answering questions and providing feedback, and to our lab technician Mindy Chow for providing valuable insight and the preparation of materials that were required for this experiment. Lastly, we would like to thank University of British Columbia for offering Biology 342, and the facilities that allowed this experiment to happen.

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