The Effect of Copper Sulphate on the Swimming Speed of Wild Type CC-1690 *Chlamydomonas reinhardtii*

Emily Chorpita, Kimty Chowdhury, Safia Maher, Christina Melo

Abstract

To test the toxicity of copper sulphate ($CuSO_4$) on the swimming speed of *Chlamydomonas reinhardtii (C. reinhardtii)*, four different concentrations of $CuSO_4$ solution were added to the organism's growth medium. $CuSO_4$ concentrations of 1 ppm, 3 ppm, and 5 ppm were used, in addition to a control of 0 ppm, consisting solely of cell medium. Cell speed was determined by capturing the organism's movement using a DinoXcope camera inserted into a compound light microscope eyepiece and analyzing the videos using ImageJ with Fiji plugins software. A one-way ANOVA test returned an F statistic of $F_{(3,32)} = 4.5456$ and a p-value of 0.0092. The null hypothesis was rejected as there was no significant difference in swimming speed between different $CuSO_4$ treatments. The Tukey's HSD returned a p-value of 0.0222 between the 0 ppm and 1 ppm groups and a p-value of 0.0132 between the 0 ppm and 3 ppm groups, indicating a significant difference in the swimming speed between the control group and the 1 ppm and 3 ppm groups. Furthermore, a linear downwards trend was observed between the 0 ppm, 1 ppm, and 3 ppm groups. Understanding the effect of various $CuSO_4$ concentrations on the swimming speed of *C. reinhardtii* is important due to its impact on salmon, a keystone species that feeds on *C. reinhardtii* and plays a critical role in the ecosystem.

Introduction

Chlamydomonas reinhardtii (*C. reinhardtii*) are single-celled plant-like photosynthetic microorganism that swim with the aid of their two flagella (Richey & Qin, 2013). They are eukaryotic cells that contain a large chloroplast and silica exoskeleton (Harris, Stern & Witman, 2009). *C. reinhardtii* are found around the world, most commonly in freshwater systems (Harris et al., 2009). In previous studies, it has been shown that in the presence of heavy metals such as copper, *C. reinhardtii* experience detrimental effects (Danilov & Ekelund, 2001). Copper is a metal ion that has been used in a number of applications over the years such as alloys, tools, coins, and jewels (Solomon, 2009). Humans and many other organisms, such as fish and

shellfish, require tiny quantities (5-20 μg/g) of copper for carbohydrate metabolism and the functioning of over 30 enzymes (Solomon, 2009). It is also an integral part of the formation of haemoglobin and haemocyanin, which is the oxygen-transporting pigments in the blood of vertebrates and shellfish (Solomon, 2009). However, copper is extremely toxic at concentrations greater than 20 μg/g, which is equivalent to 20 ppm (Solomon, 2009). Specifically for *C. reinhardtii*, concentrations above 20 μg/g have been shown to cause the cells to burst and die (Solomon, 2009).

Based on recent popular news articles, there has been renewed interest to open a copper mine in the Bristol Bay area in Alaska (Bronstein, Devine, Griffin & Hackett, 2019). However, the waters in which the waste would drain into are the site of many salmon breeding grounds. There have been a number of studies, such as one by Woody and O'Neal (2012), that demonstrate the detrimental effects of a mine at such a site on the salmon population. Bristol Bay in particular has a concentration varying between 0.04-5.06 ppb (Woody & O'Neal, 2012). In Canadian freshwater, the concentration is anywhere from 1-8 ppb, and seawater concentrations are generally less than 1 ppb (Woody & O'Neal, 2012). Salmon, which spend parts of their lives in both freshwater and seawater, would be exposed to significantly higher copper concentrations if a copper mine were to be established.

In previous studies, it has been shown that there is a greater copper concentration in areas closer to copper mines (Davis Jr. et al., 2000). When these concentrations are between 10-20 ppb, it is observed to be lethal to fresh water fish in soft water (Woody & O'Neal, 2012). Soft water is defined as low concentrations of ions, particularly calcium and magnesium, in water (Weingärtner et al., 2000). In the past, there have been many instances of copper contamination

in relation to mine and storm water runoff such as Mineral Creek in Colorado with 410 ppb, with the highest being 4600 ppb in Vermont's Copperas Brook (Runkel, Bencala, Kimball, Walton-Day & Verplanck, 2009; Balistrieri, Seal, Piatak & Paul, 2007). All of these concentrations have been found to be lethal to fish and aquatic life in general (Eisler, 2000).

As salmon are a keystone species, and thus an integral part of both terrestrial and marine ecosystems, any effects on their habitat or food source would pose a risk to the rest of the ecosystem. Many animals such as bears, eagles, and whales rely on salmon as a food source, furthering the essential role salmon play in the food chain (Farnell, 2019). In addition, salmon are a fundamental resource of British Columbia, resulting in economic prosperity. For these reasons, it is important to study the effects of copper sulphate on lakes, rivers, and the ocean on the primary producer of the food chain, *C. reinhardtii* (Sasso, Stibor, Mittag & Grossman, 2018).

As well, there has been an increase in copper entering the ecosystem not only through mine runoff but also secondary to the use of copper sulphate for control of algal blooms (Wurtsbaugh & Horne, 1982). The effectiveness of copper sulphate, dependent on water chemistry and copper activity in water, can be significantly reduced after its initial application (Jacob, Culver, Lanno & Voigt, 2016). Over the years, the repeated application of copper sulphate in lakes has led to high accumulation of copper in sediments, as copper is non-degradable (Jacob et al., 2016). The resuspension of copper in the sediments into the overlying water column during lake filling is extremely harmful to copper-sensitive fish species, particularly salmon (Jacob et al., 2016).

The purpose of this study was to examine how copper sulphate concentration affects the swimming speed of *C. reinhardtii*. According to a study done by Danilov and Ekelund (2001),

they found that copper was especially harmful to *C. reinhardtii* in relation to their photosynthetic efficiency. They tested this using motility as a marker for toxicity; meaning if *C. reinhardtii* are no longer able to move or their movement was compromised, then there was increasing toxicity. If *C. reinhardtii* are unable to move to areas with less copper, but can reach less concentrated areas through ocean currents, then they have a higher likelihood to be eaten by primary consumers. Since it is known that the copper toxicity levels for salmon are much lower than that of *C. reinhardtii*, it can be concluded that if the concentration is too high for *C. reinhardtii* to have normal motility functions, then salmon will certainly experience toxic effects as a result of consuming them through the trophic food chain (Woody & O'Neal, 2012).

To explore the effects of copper on swimming speed, *C. reinhardtii* was exposed to different concentrations of copper sulphate (1 ppm, 3 ppm, and 5 ppm) and their movement was compared to a control treatment (no copper sulphate, 0 ppm). It was predicted that *C. reinhardtii* will have a greater swimming speed in low or no copper sulphate concentrations (0 ppm and 1 ppm) than in higher copper sulphate concentrations (3 ppm and 5 ppm), as high copper sulphate concentrations are toxic and can impair their ability to move or result in apoptosis (de Carpentier, Lemaire & Danon, 2019). Based on this prediction, the null hypothesis was that a change in copper concentration does not affect the swimming speed of *C. reinhardtii*. Thus, the alternative hypothesis was that a change in copper concentration does affect the swimming speed of *C. reinhardtii*. Since the industrial revolution, copper concentrations have increased significantly (Hong, Candelone, Soutif, & Bouton, 1996). As a result, this study is relevant because *C. reinhardtii* are primary producers; their population influences the abundance and health of salmon and thus, all of the other organisms in the ecosystem (Sasso et al., 2018).

Methods

For this experimental study, *C. reinhardtii*, of wild type CC-1690, was cultured for approximately two weeks at the University of British Columbia, by Biology 342 Lab Technicians Mindy Chow and Chanelle Chow. A brief of the experimental methods is shown in Figure 1.

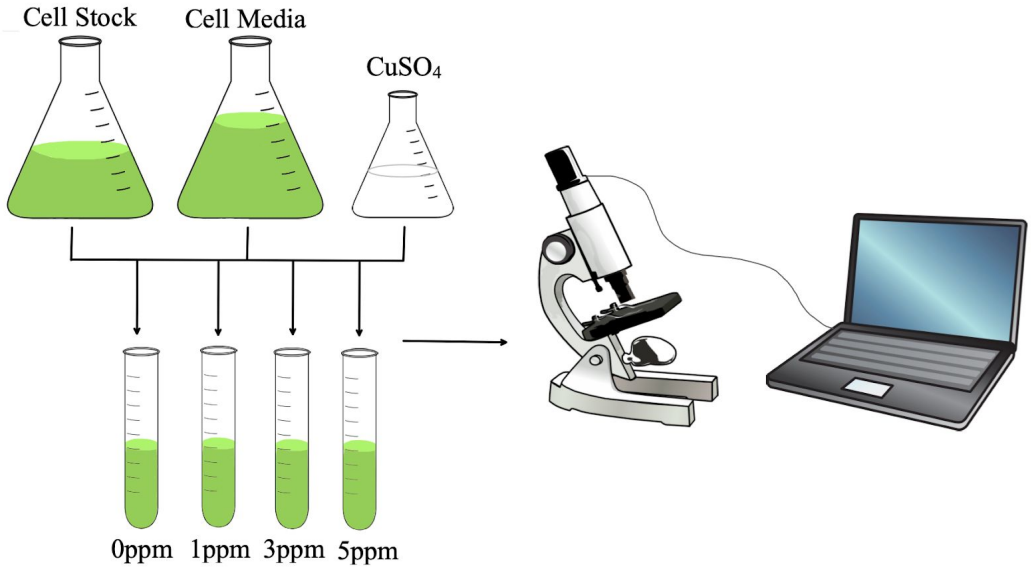


Figure 1. A schematic diagram of the experimental methods including the composition of the treatments: 0 ppm, 1 ppm, 3 ppm and 5 ppm $CuSO_4$ from the combination of *C. reinhardtii* cell stock, cell media and $CuSO_4$, respectively. Treatments were incubated at 20°C for one week, cell movement was measured by video collection using a DinoXcope camera in a compound light microscope and analyzed through the ManualTrack feature on ImageJ with Fiji plugins software.

Firstly, using Thermo Scientific micropipettes, 100 µL of *C. reinhardtii* culture and 10 µL of Prefer fixative were combined into a sterile Eppendorf tube and then resuspended to thoroughly mix. A Fisherbrand® microscope coverglass was then placed over the Hausser Scientific haemocytometer, with 20 µL of the culture and fixative mixture dispensed into the

chamber between the hemocytometer and the cover glass. The haemocytometer was inserted onto the stage of a Zeiss Axiostar Plus compound microscope set up through Köhler illumination and Phase contrast. Under the 10x objective lens, Fisherbrand® clicker-counters were used to count all the cells in the larger squares, including those on the edges, until 100 or more cells were counted. One group member completed all the counting, so as to eliminate potential bias. Dilution factor of square size was 1 x 10^3 for all runs due to only counting on the largest squares. The fixative dilution factor was calculated to be 1.1. An initial cell concentration of 6.3x 10^5 cells/mL was found, averaged from 3 runs using equation 1.

$$\textbf{Cell density } (\textbf{cells/mL}) = \frac{number\ of\ cells}{number\ of\ squares} \times dilution\ factor \times fixative\ dilution\ factor \quad (\mathbf{1})$$

Three replicates were then prepared for each copper sulphate ($CuSO_4$) treatment of 1 ppm, 3 ppm, and 5 ppm and the negative control of 0 ppm. These were prepared by performing dilution calculations using equation 2, with each final volume (V_2) being 8 mL. For the 1 ppm sample, 800 μL of $CuSO_4$ was measured using a Fisherbrand® 10 mL non-pyrogenic serological pipette, combined with 2 mL of cells and 5.2 mL of cell media into a test tube. For the 3 ppm sample, 2.4 mL of $CuSO_4$, 2 mL of cells and 3.6 mL of cell media were used. For the 5 ppm sample, 4 mL of $CuSO_4$, 2 mL of cells and 2mL of cell media were used. For the 0 ppm sample, 2 mL of cells and 6 mL of cell media were used.

$$C_1V_1 = C_2V_2 \quad (\mathbf{2})$$

All samples were labelled respectively on the test tube rack to ensure no mix ups, as shown in Figure 2. The samples were then incubated for one week at 20°C, starting on October

31, 2019.

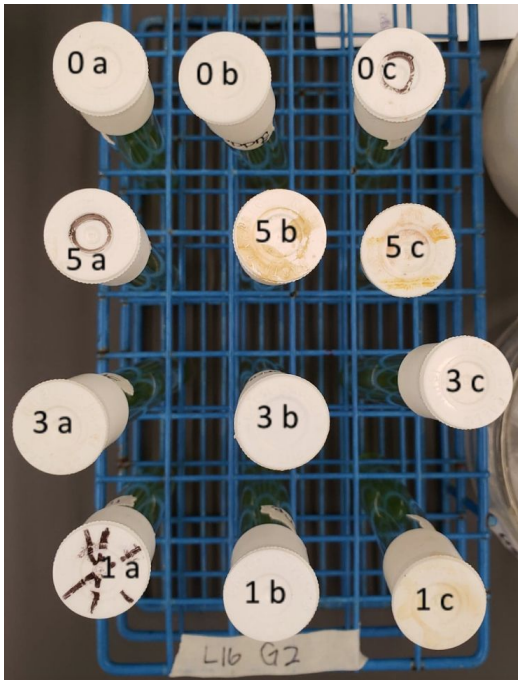
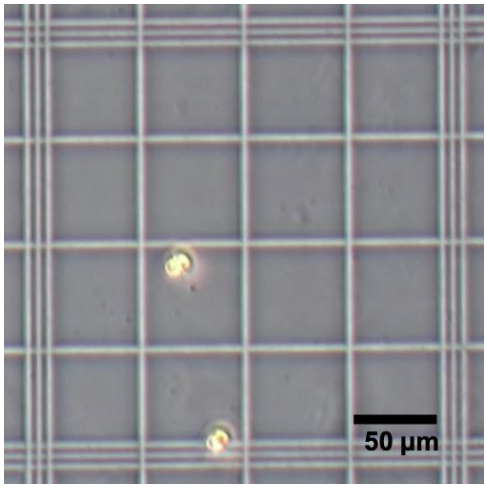


Figure 2. Labelling of test tubes for each replicate, 0 representing 0 ppm, 1: 1 ppm 3: 3 ppm, 5: 5 ppm (of $CuSO_4$). A, B and C represent replicates.

To measure final cell concentration, and to ensure the correct amount of growth for the cells to swim freely, the test tubes were removed from incubation on November 7, 2019 and examined in the lab. 100 µL of *C. reinhardtii* was sampled from each test tube by Thermo Scientific micropipettes and mixed thoroughly with 10 µL of Prefer fixative in Eppendorf tubes to prevent any movement and continual cell division. The same procedure to count the initial cell densities was performed for each of the 12 samples. It was concluded that cell growth was sufficient to allow for free movement of the cells, which was then measured.

To measure swimming speed, a DinoXcope camera was inserted into the eyepiece of a Zeiss AxioStar Plus compound light microscope set up through Köhler illumination and Phase contrast, and its USB inputted into a MacBook Pro (Figure 1). 20 µL of each sample was dispensed onto a Hausser Scientific haemocytometer and a Fisherbrand® microscope coverglass

was placed on top, allowing 1 minute for cell acclimation, as shown in Figure 3a. Light and temperature were measured at the beginning and end of measurements. To randomize selection of the cells, the slide was moved around for 5 seconds using the stage control without observing through the lens. The video was recorded for 30 seconds, and then the process was repeated two additional times on the same slide for a total of 3 runs per replicate, for example: 3C1, 3C2, 3C3. Three cells' velocities were analyzed in each frame, for a sample size of 3 per each treatment, which were then averaged for a total of 3 measurements per replicate. These cells were chosen randomly by rolling a dice, which numbers, 1 through 6, pertained to a box of 1 through 6 on the x and y axis as shown in Figure 3b. This was repeated for a total number of 36 videos which were then analyzed in the ManualTrack feature from ImageJ software with Fiji plugins.

a) 

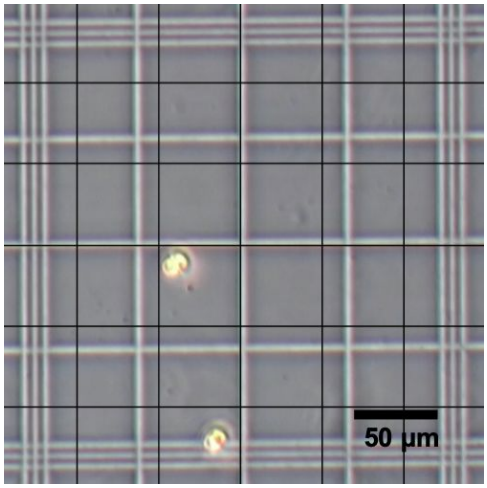
b) 

Figure 3. (a) A screenshot of a segment of the 3 ppm replicate C, sample 1 (3C1) DinoXcope video recording, taken at 10X magnification. The lines represent grid lines from the haemocytometer and the circular, yellow-green objects represent *C. reinhardtii* cells. **(b)** The 6 by 6 grid, in black, used for randomized cell selection.

This program allowed for the manual tracking measurement of cell velocity between frames, as shown in Figure 4. Within the 30 second video clip, 10 or so frames were analyzed and then averaged to provide a velocity of the designated cell, calculated on Microsoft Excel. A

one-way ANOVA and Tukey Kramer test were then run on the data to determine significance.

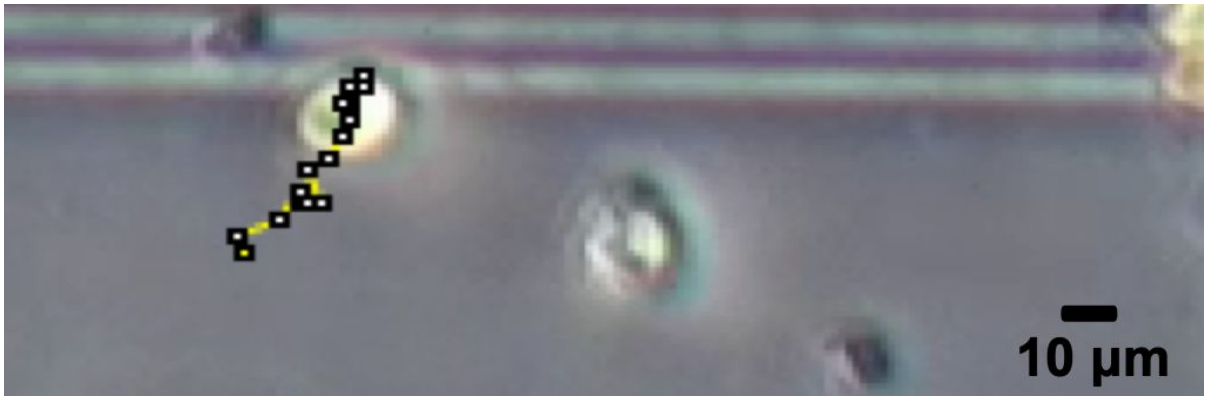


Figure 4. Treatment 1 ppm, replicate C, sample 3, cell 3 (1C3-3); an example of the ManualTrack feature found on ImageJ software with Fiji plugins.

Results

For each $CuSO_4$ treatment, there were 3 replicates, with 3 cells selected for tracking of swimming speed (n = 3). A one-way ANOVA test returned an F statistic of $F_{(3,32)} = 4.5456$ and a p-value of 0.0092. Based on these results, the null hypothesis was rejected that there was no significant difference in swimming speed between different $CuSO_4$ treatments.

The Tukey's HSD returned a p-value of 0.0222 between the 0 ppm (control) and 1 ppm group, and a p-value of 0.0132 between the 0 ppm and 3 ppm groups. This indicates that there was a significant difference in the swimming speed between the control group and the 1 ppm and 3 ppm groups. The resulting p-value for the difference between the control group and the 5 ppm group was not significant at 0.0726. The differences between the 1 ppm and 3 ppm groups, 1 ppm and 5 ppm groups, and 3 ppm and 5 ppm groups were also not significant at p=0.9967, p=0.9547, and p=0.8866, respectively.

Figure 5 illustrates the average swimming speed and 95% confidence intervals for 0 ppm, 1 ppm, 3 ppm, and 5 ppm $CuSO_4$ treatments to be M = 0.8073 μm/sec, 95% CI [0.2381, 1.3765],

M= 0.115 μm/sec, 95% CI [-0.0118, 0.2418], M= 0.0682 μm/sec, 95% CI [0.0132, 0.1232], and M= 0.2322 μm/sec, 95% CI [0.1481, 0.3163], respectively. Figure 5 depicts a noticeable trend in the averages of the different treatment groups: the 0 ppm group (control) appears to have the highest average swimming speed, and a linear downwards trend is obvious between the 0 ppm, 1 ppm, and 3 ppm groups.

The observed *C. reinhardtii* showed similarity in the movement types observed on the DinoXcope. The light intensity was consistent at approximately 415 lux across all replicates and a constant temperature of 20°C was measured. Most cells did not move at all, and a number moved slightly in place (slow vibrations) or slightly shifted their position. However, several cells were observed to move in rapid, zig-zag movements or in circles before darting off screen. The highest amount of movement and the greatest variation of movement patterns was observed in the 0 ppm group, followed by the 5 ppm group. Most cells in the 1 ppm and 3 ppm groups did not move at all, or moved slightly or very slowly. The 3 ppm group showed the least amount of movement. All treatments contained relatively the same number of cells, and all cells appeared similar in size, shape and colour (small, circular, and bright yellow-green).

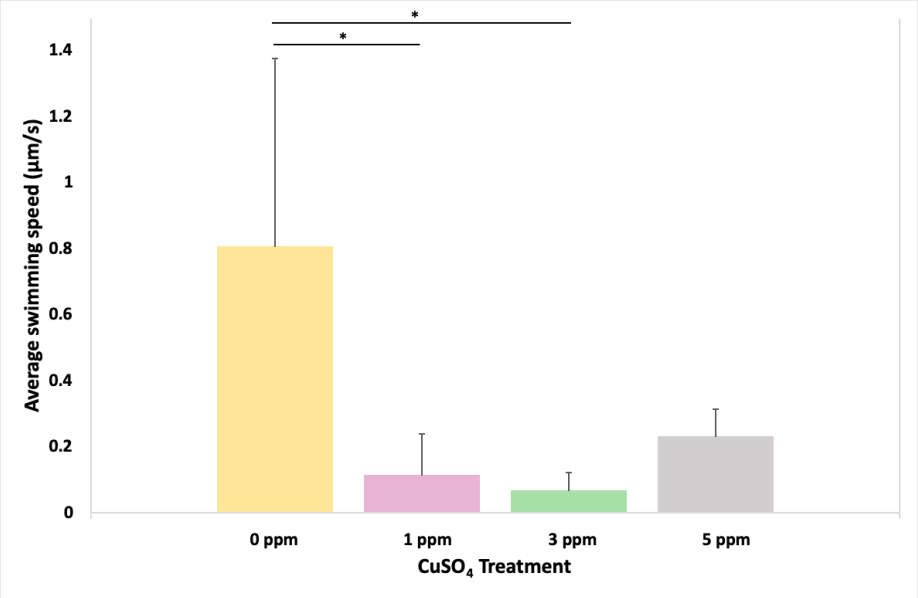


Figure 5. The average swimming speed of *C. reinhardtii* grown in four different $CuSO_4$ treatments (n = 3) with error bars representing 95% confidence interval. A one-way ANOVA test returned an F statistic of $F_{(3,32)} = 4.5456$ and a p-value of 0.0092. Based on these results, the null hypothesis was rejected that there was no significant difference in swimming speed between different $CuSO_4$ treatments. The Tukey's HSD returned a p-value of 0.0222 between the 0 ppm (control) and 1 ppm group, and a p-value of 0.0132 between the 0 ppm and 3 ppm groups. This indicates that there was a significant difference in the swimming speed between the control group and the 1 ppm and 3 ppm groups. * = p-value < 0.05.

Discussion

The main purpose of this study was to determine the effect of different $CuSO_4$ concentrations on the swimming speed of *C. reinhardtii*, in light of recent proposals to build a controversial copper mine in Bristol Bay, Alaska (Bronstein et al., 2019). The mean swimming speed in the negative control (0 ppm) was visibly higher than that of all of the other $CuSO_4$ treatments, as seen in Figure 5. This aligns with the prediction that little to no $CuSO_4$ is optimum for *C. reinhardtii* health.

A visible downward trend was observed between the 0 ppm, 1 ppm and 3 ppm groups; however, the 5 ppm group had a higher mean swimming speed than the 1 ppm and 3 ppm

groups. Every attempt possible was made to minimize bias through randomization; however, it is possible that in the random selection process, faster moving cells were more frequently chosen while collecting data from the 5 ppm group. However, it is more likely that the limited sample size of 3 has resulted in statistical results that are prone to error and thus may not be reliable. This observation could also be due to mechanisms within *C. reinhardtii* that respond to heavy metals. One such study done by Wei, Zheng, Liu, and Yang (2011) looked at how the tolerance of *C. reinhardtii* could change due to heme oxygenase-1 (HO-1). HO-1 is an enzyme the is coded in *C. reinhardtii* that responds to stress from heavy metals. To understand the role HO-1 plays in the regulation of heavy metals, the researchers used expression vectors that contained HO-1 to increase the amount of HO-1 that was expressed (Wei et al., 2011). This overexpression of HO-1 exhibited antioxidative effects, protecting the cells from the harm that heavy metals can inflict through protection against oxidation (Wei et al., 2011). This demonstrates that there is a response mechanism in place for heavy metal toxicity, which may have been part of the reason that higher speeds were observed at the 5 ppm concentration.

Other response mechanisms to heavy metal toxicity that may have influenced the results includes the glutathione cycle as seen in the study by Stoiber, Shafer, and Armstrong (2010). Stoiber et al. (2010) analyzed differing amounts of glutathione, an antioxidant, in response to different concentrations of copper. It was shown that *C. reinhardtii* that were in solutions with higher concentrations of copper also had larger concentrations of glutathione to respond to this stress (Stoiber et al., 2010). This increase in glutathione increases the antioxidant capacity of the cell thus protecting it from the detrimental effects of heavy metals such as copper (Stoiber et al., 2010). This further supports that the small sample size may not be the only reason that higher

average speed was observed in the 5 ppm treatment, as it is possible that an antioxidative response was occuring.

The finding of decreasing swimming speed with increasing $CuSO_4$ concentration is consistent with a study conducted by Cao et al. (2015) in which the researchers demonstrated that increased copper concentrations resulted in decreased photosynthesis rates in green algae. Photosynthesis is important to green algae as it is their source of energy, and without it they are not able to perform daily functions such as moving around their environment. Cao et al. (2015) were able to demonstrate through increasing the concentration of copper in which their green algae was grown, an increase in malondialdehyde (MDA) which is a marker for oxidative stress, further demonstrating the detrimental effects that copper has on the motility of *C. reinhardtii*. Based on previous studies, it would be expected that swimming speed would decrease as cells are exposed to increased environmental stressors which negatively impacts their ability to survive.

Similarly, as aforementioned, the study conducted by Danilov and Ekelund (2001) lends support that copper is particularly toxic to *C. reinhardtii* in relation to their photosynthetic efficiency. Using motility as a marker of toxic stress, they found that increased levels of copper were detrimental to their photosynthetic abilities. *C. reinhardtii* would thus be unable to acquire the necessary energy to move. This implies that if *C. reinhardtii* lose the ability to move, continued exposure to dangerous levels of toxicity would result. Higher copper levels were also shown to decrease the amount of oxygen present, further indicating a decrease in photosynthesis and thus, cell movement.

Not only have previous studies shown that copper affects this organism's ability to

photosynthesize, but also numerous other biologically important functions including their ability to take up nutrients. In a study conducted by Wurtsbaugh and Horne (1982), it was demonstrated that high levels of copper affected both nitrogen and carbon fixation mechanisms for algae. Another study conducted by Mosulen et al. (2003) further inquired into the effects of copper on sulphate and nitrate assimilation, which would alter nutrient uptake. In this study, it was demonstrated that an increase in copper showed a decrease in both sulphate and nitrate uptake (Mosulen et al., 2003). This is due to sulfur-starvation, leading to the reduction of nitrate uptake as the mechanisms that consume nitrate are unable to be activated without sulfur (Mosulen et al., 2003). These results can be related back to this study by means of providing nutrition which would be converted to energy for movement. By limiting carbon fixation, *C. reinhardtii* photosynthesis would be compromised. This further supports previous studies in which their ability to photosynthesis and produce energy plays a major role in their ability to move.

This study is relevant as *C. reinhardtii* serve as a food source to salmon in the aquatic food chain and consequently impact their survival. The abundance of salmon is in decline globally, with a growing concern in British Columbia due to their integral role in the aquatic ecosystem as a keystone species. There are many drivers of the decline in salmon population; however, the association between salmon and *C. reinhardtii* is significant (Blossom, 2001). *C. reinhardtii* are primary producers, forming the base of the aquatic food chain. They integrate into the ecosystem with their photosynthetical abilities, making food for all other aquatic organisms from inorganic matter (Blossom, 2001). Copper concentration is observed to increase in the ocean due to natural and anthropogenic processes. This increase reduces *C. reinhardtii*'s ability to swim (Saison et al., 2010). As the swimming speed of *C. reinhardtii* decreases, its ability to

elude prey also decreases, leading to a larger likelihood of capture and death. A reduction in *C. reinhardtii* population causes an immediate depletion in food supply for salmon, eventually leading to changes in the dynamics of the trophic structure (Blossom, 2001). It is also crucial that copper concentrations are kept low to avoid further disturbances to the salmon population caused by copper bioaccumulation in salmon through trophic levels.

There were three main sources of uncertainty and variation during the experiment. The first source of uncertainty was the limited sample size. Only 3 replicates were measured per $CuSO_4$ treatment, giving a sample size of 3 which is not adequate for reliable statistical analysis nor robust enough to minimize error in data collection. The second source of uncertainty and variation is the method used to track cell movement. Unfortunately, CellTrack is no longer available for use, and so ManualTrack on ImageJ with Fiji plugins was used to allow for manual cell tracking. This meant that movement needed to be tracked in 300 frames per 30 second video clip. Instead, movement was tracked every 20 slides and an average was calculated per clip. This may have introduced error because the tracked movement may have been precise, but not accurate. In addition, two group members completed this analysis, which may introduce personal bias in the measurement of cell location. Personal bias may have been better minimized by having only one member of the group conduct all analyses. The final source of uncertainty and variation concerns the exclusion of movement-tracking for extremely fast moving cells because they were difficult to capture, even when analyzing frame-by-frame. As such, the data is largely representative of the slow and average moving cells, with only a few faster moving cells captured by chance in the frames.

For further research, more data could be obtained by increasing the number of replicates

which would in turn increase the sample size and reduce error. A more sensitive and perhaps automatic cell tracking tool should be used in future studies, such that data is as accurate as possible and better represents the population as a whole. There was deviation when measuring the swimming speed of *C. reinhardtii* in 5 ppm of copper sulphate. Investigation as to whether higher copper sulphate concentration indeed benefits *C. reinhardtii* should be researched to disentangle this deviation. Lastly, further studies should investigate how smaller concentrations of copper sulphate affect the growing capacity of *C. reinhardtii* and the respective impact on fish, particularly salmon, in the ecosystem.

Conclusion

In conclusion, with an F statistic of $F_{(3,32)} = 4.5456$ and a p-value of 0.0092, the null hypothesis was rejected that there is no significant difference in swimming speed between different $CuSO_4$ treatments. The Tukey's HSD further supported a significant difference in swimming speed between the control group and the 1 ppm and 3 ppm groups. The average swimming speed of the 5 ppm group was observed to be lower than the control group, although higher than the 1 ppm and 3 ppm groups and not significant. The prediction that *C. reinhardtii* swimming speed decreases with increased $CuSO_4$ concentrations was supported, except for the 5 ppm group which might be due to antioxidative response mechanisms. Furthermore, the overall effect of the tested $CuSO_4$ concentrations on the swimming speed of *C. reinhardtii* indicates that the tested concentrations of 1 ppm and 3 ppm would be detrimental to salmon and thus the ecosystem as a whole.

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Appendix

Equipment List: *Chlamydomonas reinhardtii* (wild-type CC-1690, L16B2), Hausser Scientific Fuchs Rosenthal Ultra Plane hemocytometer, Fisherbrand® microscope coverglass, Thermo Scientific Finnipipette F1 pipettes with various volume limits (P10, P20, P100, P1000), Fisherbrand® micropipette tips, Fisherbrand® clicker-counters, KIMTECH Kimwipes, Eppendorf tubes, Erlenmyer flask, test tubes, test tube racks, 20 °C incubator, Fisherbrand® 10 mL non-pyrogenic serological pipette, Zeiss Axiostar Plus compound microscope (#1512), DinoXcope camera (#7), ImageJ software, MacBook Pro laptop, Fisher thermometer, Lux meter, dice and Fisher Scientific nitrile gloves.

Chemical List: Prefer fixative by Anatech Ltd., wild-type CC-1690 *Chlamydomonas reinhardtii* stock solution, Copper sulphate ($CuSO_4$), growth medium, distilled water (dH_20)

Hazards: Due to the toxicity of $CuSO_4$, extra precaution was followed during use. Gloves were worn during use, and hands were thoroughly washed post-use to prevent potential irritation. $CuSO_4$ was disposed of down the sink with plenty of water.

Table 1a. Recipe for wild-type CC-1690 *Chlamydomonas reinhardtii* culture, prepared by Mindy Chow and Chanelle Chow at the University of British Columbia. Each solution was added slowly, one at a time, into distilled water and mixed well, and finally autoclaved once at the *C. reinhardtii* cycle.

Component	**Stock Solution (g/L)**	**Used in Culture (mL/L)**
KH_2PO_4	20.0	5.0
K_2HPO_4	26.0	5.0
$FeCl_3$	12.5	1.0
$MgSO_4$ - $7H_2O$	60.0	5.0

$CaCl_2$	95.0	0.5
Trace Metals	See Table 1b.	1.0
Na_3citrate-$2H_2O$	100.0	1.0
NH_4NO_3	120.0	2.5

Table 1b. The trace metal components, at 10X, included in the stock solution, in g/L.

Trace Metal (10X)	**Stock Solutions (g/L)**
H_3BO_3	4.0
$ZnSO_4$-$7H_2O$	4.0
$MnSO_4$-$4H_2O$	1.6
$COCl_2$-$6H_2O$	0.8
$CuSO_4$	0.16
NH_4Moltbdate	0.8

Table 2. Amounts of designated solutions added to each of the four treatments.

Treatment	**Amount of Cell Stock Added (mL)**	**Amount of Cell Media Added (mL)**	**Amount of Copper sulphate Added (mL)**
0 ppm	2	6	0
1 ppm	2	5.2	0.8
3 ppm	2	3.6	2.4
5 ppm	2	2	4

Table 3. Output of the ANOVA using R software (version 3.5.1) for the four $CuSO_4$ treatments (n=3) of 0 ppm, 1 ppm, 3 ppm, and 5 ppm. Resulting values for degrees of freedom (df), sum of squares (Sum Sq), mean of squares (Mean Sq) the test statistic F, and the p-value are shown.

	df	**Sum Sq**	**Mean Sq**	**F-Stat**	***p***
Between Groups	3	3.1466	1.0489	4.5456	0.0092
Within Groups	32	7.3839	0.2307		

Total:	35	10.5305			

Table 4. Output of the Tukey-Kramer test using R software (version 3.5.1) for the four $CuSO_4$ treatments (n=3) of 0 ppm, 1 ppm, 3 ppm, and 5 ppm. Resulting values for the difference between means (diff), lower (lwr) and upper (upr) bounds of the 95% confidence intervals, and the p-value are shown.

Comparison	**diff**	**lwr**	**upr**	***p***
0 ppm x 1 ppm	0.6919	0.0784	1.3054	0.0222
0 ppm x 3 ppm	0.7391	0.1257	1.3527	0.0132
0 ppm x 5 ppm	0.5751	-0.0384	1.1887	0.0726
1 ppm x 3 ppm	0.0473	-0.5662	0.6608	0.9967
1 ppm x 5 ppm	0.1168	-0.4967	0.7303	0.9547
3 ppm x 5 ppm	0.1640	-0.4494	0.7776	0.8866