The Effect of $CuSO_4$ and Exposure Time on *Chlamydomonas reinhardtii* Population

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ABSTRACT

Chlamydomonas reinhardtii, a green algae, is one of many critical primary producers, making up the base of most aquatic food webs (Norambuena et al. 2-6). Any disruption to its populations, can in turn, affect other inhabitants of that ecosystem, including salmon (Müller-Navarra and Dörthe 489-505.). Copper acts as an essential compound when present in small quantities for many species, but becomes highly toxic past one's biological requirements (Gaetke et al. 147-163). This study aims to understand the impacts of different concentrations of copper sulfate and exposure time on a population of *C. reinhardtii.* It is predicted that the population of *C. reinhardtii* will be affected by exposure time and $CuSO_4$, respectively, and that there will be an interaction between time and copper concentration on the population. This was achieved by subjecting a population of *C. reinhardtii* to three separate treatments of $CuSO_4$ over time: a control of 0 μM, 100 μM, and 200 μM $CuSO_4$. By assessing the results obtained through similar studies in the literature, the following concentrations were chosen to best describe both a lower and a higher limit for the copper tolerance of *C. reinhardtii.* The study was conducted over an 11 day period, with a total of 8 sampling days. Through the use of a haemocytometer, cell counts for each treatment group were recorded approximately every 24 hours, and the samples were kept in an incubator between data collections. The data was analyzed using a two-way ANOVA test, which resulted in p-values of 3.68E-13, 1.63E-30, and 1.34E-19. Since the obtained p-values were smaller than α of 0.05, the three null hypotheses were rejected. This suggests that the individual factors of copper sulfate concentration and exposure time, as well as their interaction, affect *C. reinhardtii* population. While the impact of the 100 μM $CuSO_4$ treatment group was very similar to that of the 200 μM group, there was a significant decline in population between the control and these two treatment groups. As such, the further study of exact copper tolerance ranges is crucial in understanding how its anthropogenic input into aquatic systems, whether through mining activities, pesticide run-off or others, would affect the ecosystem (Willis and Bishop 37).

INTRODUCTION

This study aims to understand both the impacts of different copper sulfate concentrations and exposure time, as well as whether an interaction exists between these two factors, on a population of *Chlamydomonas reinhardtii*. This study also seeks to provide information that can be extrapolated to gain a better understanding of the factors influencing salmon abundance.

Salmon are integral to the ecosystem, culture, and economy of British Columbia (Lyons et al. 112-119). As a keystone species, they largely influence the survival and reproduction of many coastal species and are woven into many food webs (Wilson and Halupka 490). Furthermore, salmon are critical for B.C's Indigenous communities, providing both economic and spiritual support (Gerwing and McDaniels 261). However, many Indigenous territories have been experiencing significant reductions in salmon abundance in comparison to their history, mainly through habitat loss, migration barriers, and over-fishing (BC Wild Salmon Advisory Council). In fact, overall, salmon are currently in a seriously vulnerable state (BC Wild Salmon Advisory Council). Due to the changing climate and various additional factors resulting in different ocean conditions, salmon abundance is likely to continue to decrease (BC Wild Salmon Advisory Council). A reduction is seen across all salmon species and regions to various extents, reaching back to the 1950s, with reductions ranging from 14-45% (BC Wild Salmon Advisory Council).

To address the worrying reality of B.C.'s salmon population, it is critical to consider its food web. Phytoplankton are primary producers which make up the lowest trophic level for salmon in addition to various other aquatic invertebrates, and are important in the maintenance of dissolved oxygen levels in aquatic environments (Norambuena et al. 3). As a food source for

higher trophic levels, primary producers have a critical role in species' abundance and survival (Norambuena et al. 3). As such, the chosen organism for this study was *Chlamydomonas reinhardtii*; a single-celled green algae that is commonly used as a model species in biological studies (Boswell et al. 546). Its "model species" status is based on its short generation time, its ability to act as both a facultative autotroph and a heterotroph, as well as due to the wealth of genetic information already available on the species (Boswell et al. 546). *C. reinhardtii* has an observed doubling time of 5-8 hours (Harris 36). Being a highly adaptable species, it is found in various environments; primarily distributed in freshwater and topsoil worldwide (Merchant et al. 245-250). This species, along with other phytoplankton, play a crucial role in ecosystems through the transfer of nutrients up the food web (Norambuena et al. 4).

Acting as an essential compound when present in small quantities for many species, but turning highly toxic past biological requirements, makes copper sulfate a biologically intriguing compound to study (Gaetke et al. 147-163). Generally speaking, $CuSO_4$ is commonly used as an herbicide and fungicide; specifically targeting invasive aquatic plants and eradicating roots (Richardson 93-97). It is highly soluble in water, making it easily distributed within the environment and bioavailable to many species (Ahsanullah and Florence 41-45). In terms of algae, sensitivity to copper can range based on the species and has been used by humans in the past to control some algal blooms, most effectively that of blue-green algae, which tend to be the most sensitive (Gibson 513-518). According to the findings of Boswell et al., $CuSO_4$ was not toxic to *C. reinhardtii* up to a level of 100 μM, whereas cell growth was inhibited at the 150 μM $CuSO_4$ mark, with only a small number of cells surviving through tolerance development. These findings were kept in consideration when determining the parameters for this study.

This study directly relates to B.C.'s salmon population through analysis of how the input of copper into the environment affects phytoplankton, as well as how copper exposure can directly impact salmon health. The study of copper's effects is very relevant, especially here in B.C., where it is a metal of interest through our past and present mining activities. Copper can be released into the environment through direct copper mining, as well as indirectly when rocks with high metal concentrations are exposed to rain and air (Foy 3). The latter can produce acid mine drainage, since metals are often found in rocks containing sulphide minerals (Foy 3). Following excavation, the sulphides become exposed and get oxidized by water and oxygen (Foy 3). This produces acidic conditions which dissolve the metals, making them easily distributed through runoff into streams or rivers, if not controlled (Foy 3). This copper discharge is a cause for concern in aquatic systems, being that it is highly toxic to fish and other organisms (Foy 3). Specifically, with salmon, an increase of just two to eight parts per billion above natural stream copper levels can impair their sense of smell, which is important in their search for mates, their ability to avoid predators, and their successful return to spawning grounds (Foy 3). The biggest spill in Canada's history happened in 2014 at the Mount Polley Copper-Gold Mine, here in B.C (Foy 3). This spill was responsible for spilling 25 million cubic metres of solid and liquid waste into major salmon rearing areas (Foy 3).

The introduction of excess copper to aquatic environments can also affect the phytoplankton population, which in turn, affects those that feed on them, including salmon (Brand et al. 225-250). High copper concentrations above biological requirements, can reduce the phytoplankton populations upon which salmon feed and depend on for the maintenance of stream oxygen levels (Norambuena et al. 5-8). Oxygen is critical for salmon's biological

processes, including swimming performance and migration, and hypoxic conditions have been correlated to major salmon die-offs (BC Wild Salmon Advisory Council). The understanding of the factors that influence salmon wellbeing are therefore crucial to ensuring their survival.

For analysis purposes, this study consists of three null hypotheses. Firstly that *C. reinhardtii* cell population over different $CuSO_4$ concentrations will be the same. Secondly that *C. reinhardtii* cell population over different exposure times will remain the same. Lastly that there is no interaction between exposure time and different $CuSO_4$ concentrations of *C. reinhardtii* cell population. It is predicted that the population of *C. reinhardtii* will be affected by exposure time and $CuSO_4$, respectively, and that there will be an interaction between time and copper concentration on the population.

MATERIALS AND METHOD

Cultures of *Chlamydomonas reinhardtii* were obtained from the biology laboratory technicians at the University of British Columbia. Our starting materials included an Erlenmeyer flask containing 10 mL of 400 μM $CuSO_4$. Additionally, we received 60 mL of the standard media for *C. reinhardtii*, the recipe for which is provided in Appendix I on page 13. The initial concentration of the culture was calculated using a haemocytometer and a cell count of 6.78E5 cells/mL was estimated. The culture was then diluted to achieve a total desired concentration of 5.00E4 cells/mL (Harris 36), which was achieved by mixing 3.69 mL of culture with 46.31 mL of media.

All work stations were sterilized prior to the conduction of the experimental procedures. This included the use of 70% ethanol, gloves, and an open ethanol flame. Samples were only

opened when necessary and kept under the flame at all times. The mouth of the treatment tubes were flamed whenever they were exposed. These conditions were necessary to prevent any contamination within the samples.

The experiment consisted of three treatments, including a control, and were each replicated four times to ensure accuracy and consistency. Following the dilution, each of the 12 test tubes were filled with 3.0 mL of the diluted culture using a micropipette. The cultures were then treated with copper sulfate concentrations of 0 µM, 100 µM and 200 µM.

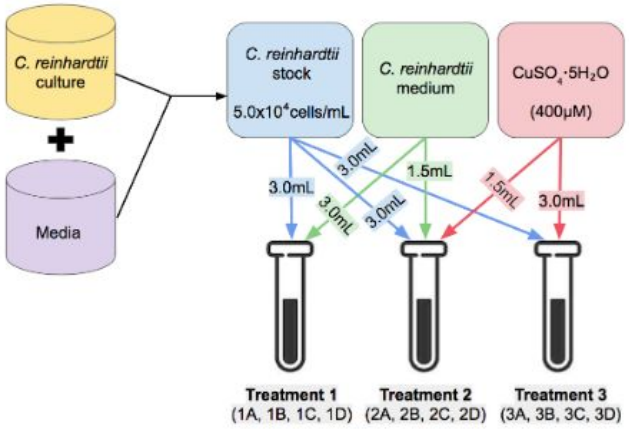

Figure 1. A diagram of the experimental procedure, indicating the exact volumes of each material added to the corresponding treatment groups.

As illustrated in figure 1, Treatment 1 had a one-to-one ratio of the culture and media (3mL of each), serving as the experimental control. Treatment 2 had 3.0 mL of culture, 1.5 mL of media, and 1.5 mL of $CuSO_4$ to achieve a total concentration of 100µM $CuSO_4$. Treatment 3 had a one-to-one ratio of culture and $CuSO_4$ to achieve a total concentration of 200µM $CuSO_4$. Each test tube had a final volume of 6 mL. Cultures were then grown in an incubator at 20°C,

with alternating exposure to light and darkness, each for periods of 12 hours. This mimicked natural environmental conditions in which *C. reinhardtii* typically grow (Boswell et al. 547).

During each data collection, 10µL of Lugol's Iodine (IKI) was added to each of the 12 fresh plastic vials, using a micropipette, and 100µL of each sample was mixed in. IKI was used as a fixative in order to allow for an accurate cell count (Yang et al. 2016). Next, the plastic vials were vortexed at maximum speed for 10 seconds, in order to ensure thorough mixing of IKI. Sufficient time was allocated for the samples to settle, in order to allow for the proper functioning of IKI.

Lastly, a haemocytometer slide was loaded with 10µL of each sample for the counting of cells. A Zeiss Axio compound microscope, with the 10X lens, was used for a total magnification of 100X. Three readings were conducted on the largest grid of the haemocytometer slide (3 mm x 3 mm) by different individuals. Any cell counts that were outside of 10% of each other were re-counted, and an average cell count was recorded per sample. Physical observations of the organisms were also noted. The test tubes were incubated immediately upon sample collection up until the next collection was to be conducted.

This process was repeated on days 2 through 11. Data collection was conducted approximately every 24 hours, but did not occur on weekend days, due to inaccessibility to the lab. All plastic sample vials containing any remaining solutions, were stored in a refrigerator at 4°C for the duration of our experiment, in the case that a recount was necessary. Wastes were disposed of appropriately, based on possible contamination by chemicals and biological materials.

Following the collection of cell counts for each treatment group, with their respective four replicates, an average cell count was established per treatment. A growth curve for *C. reinhardtii* was produced by plotting the log of the average cell concentration against time. The log was used since several of the counts differed by more than 10 times, and this produces a more visually significant graph. For the data analysis, a two-way ANOVA test was conducted in order to assess the effects of $CuSO_4$ concentration levels, as well as exposure time, on the population of *C. reinhardtii*, and their interaction.

RESULTS

For each of the three copper sulfate treatment groups, there were four replicates ($n = 4$). While the data was found to be normally distributed, an F-test found that variance was not equal, even following the application of several mathematical transformations. For the purpose of this study, a 2-way ANOVA test was nonetheless conducted. The two-way ANOVA test returned a p-value of 3.68E-13 for the first null hypothesis that different $CuSO_4$ concentrations would not affect the *Chlamydomonas reinhardtii* population. A p-value of 1.63E-30 was obtained for the second null hypothesis that exposure time does not affect the population. Finally, a p-value of 1.34E-19 was obtained for the third null hypothesis that there is no interaction between exposure times and $CuSO_4$ on the population of *C. reinhardtii*. Based on a 0.05 significance level, all three null hypotheses are rejected.

In order to determine the instances where differences and interactions are occuring, post-hoc analysis was conducted using the Bonferroni method at a 0.05 significance level. Upon analysis on the impact of copper sulfate, a significant difference was found when comparing both

treatment 2 (100 µM $CuSO_4$) and treatment 3 (200 µM $CuSO_4$) with the control (0 µM $CuSO_4$). That being said, there was no significant difference found in the population between treatment 2 and treatment 3. Furthermore, the analysis on exposure time showed that there was a significant difference in cell population on days 4 through 9. The growth curve of *C. reinhardtii* is illustrated in Figure 2, which shows the log of the average cell concentration on the y-axis and exposure time on the x-axis. There is an observed difference between the control and treatments 2 and 3, and the asterisks represent the significance found on days 4 through 9.

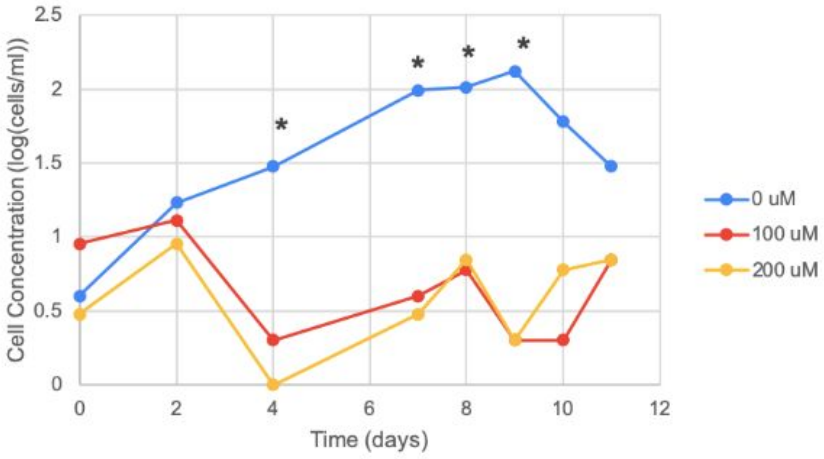

Figure 2. Log of the average cell concentration (cells/mL) of *C. reinhardtii* over a period of 12 days. Each number represents the average (± standard error of the mean) of four replicates. The asterisks indicate significance found for the corresponding exposure times ($\alpha = 0.05$) between the control and treatment groups 2 and 3.

DISCUSSION

Following analysis of the data, all three null hypotheses were rejected due to observed p-values of less than the significance level ($\alpha = 0.05$), aligning with the initial prediction for the study. This suggests that there is an effect on *Chlamydomonas reinhardtii* populations due to

increasing copper sulfate concentrations, as well as due to exposure time. Furthermore, these results suggest that an interaction does exist between $CuSO_4$ concentrations and exposure time, on the number of *C. reinhardtii* cells observed. Through statistical testing on exposure time, it can be concluded that it does play a role on the population, specifically with differences being observed on Day 4 through Day 9, as represented by asterisks in Figure 1. Additionally, the data shows that there is a significant decrease in population size between the control and both the 100 μM and 200 μM $CuSO_4$ treatment groups. That being said, there was no significant difference observed between the population sizes of the 100 μM and 200 μM $CuSO_4$ treatment groups. This differs slightly from the findings reported by Boswell et al., which stated that there was no significant impact on cell growth at the 100 μM $CuSO_4$ level, and that cell growth was only inhibited once the 150 μM mark was reached. The authors did address that their strain of *C. reinhardtii* may have developed an increased tolerance; therefore, explaining the delayed response to the presence of copper. In comparison, this current study suggests that *C. reinhardtii* population can be negatively affected at copper sulfate concentrations of as low as 100 μM. Upon obtaining these results, further literature searches were conducted and a study by Prasad et al. was found and analyzed. The study showed similar results in that there was a significant effect reported at the 100 μM $CuSO_4$ mark. Even more interestingly, this study also reported a significant effect observed at 50 μM $CuSO_4$. This clearly shows the need for further studies in this area, with the testing of additional copper concentrations, with smaller increments of possibly 25 μM. This would allow for a clearer suggestion of *C. reinhardtii*'s copper tolerance range, since no concrete evidence yet exists.

A possible explanation for the decline in the *C. reinhardtii* population is damage caused to the chloroplasts of the cells upon the absorption of free copper (Che et al. 923). As a result, photosynthesis is inhibited and the cells lack appropriate functionality. The study by Che et al. found that photosynthesis and respiration are the most important primary metabolic processes and are the main sources of production of reactive oxygen species (ROS). When the copper absorbs, it concentrates within the chloroplasts and disrupts the oxygen-evolving complex (OEC) in the photosynthetic electron transport chain (Che et al. 923). OEC is a water-oxidizing enzyme that is involved in the photooxidation of water during the light reactions of photosynthesis (Che et al. 923). This damage ultimately causes the inhibition of photosynthetic electron transport.

An area of uncertainty within the results exists as shown at Day 11 on Figure 2. The sudden increasing trend observed within treatment groups 2 and 3 are somewhat perplexing. Further studies would benefit from a longer study period and more consistent data collection times to address the fate of the population past Day 11. As water is a polar solvent, the addition of $CuSO_4$ will result in its dissociation into positively charged copper ions and negatively charged sulphate ions (Manahan 51). A proposed rationale for the sudden increase observed at Day 11 is the possible presence of chelating ligands, which would bind to the free copper ions; therefore, decreasing their bioavailability (Manahan 69).

The obtained results may have been influenced by a variety of sources of error. Some variance may exist within the reported cell counts, based on discrepancies in counting methods conducted by different individuals and varying interpretations of applicable cells for the count. While the intention of having different individuals conduct counts was to account for inaccuracies within one individual's counts, this may have resulted in the opposite effect and

have reduced precision. The cell counts were also conducted using the largest reference grid on the haemocytometer slide (3mm x 3mm) at 100X magnification. This method was kept consistent throughout all cell counts; however, this large reference grid may have reduced precision within the larger cell populations. Furthermore, despite ensuring sterile working conditions throughout the procedure, it is likely that some contamination occurred during transfer of materials. Moreover, due to a time limitation throughout this study, it was not possible to conduct data collections at the exact same time and with consistent hour intervals. Data was collected at approximately 24-hour time intervals, with the exception of weekend days which could not be accounted for due to inaccessibility to the lab. Seeing as exposure time was a main factor of the study, it would be beneficial to conduct cells counts on a more consistent basis.

CONCLUSION

In summary, with a p-value of 3.68E-13 for the first null hypothesis, one of 1.63E-30 for the second null hypothesis, and 1.34E-19 for the third, all null hypotheses are rejected at 0.05 significance. This concludes that both the individual factors of copper sulfate concentration and exposure time, affect the population of *Chlamydomonas reinhardtii*, and that an interaction exists between the two factors and population. This is aligned with the initial prediction that an effect on the population will result from changes in both $CuSO_4$ and exposure times. Changes to a *C. reinhardtii* population, will in turn, impact the distribution abundance of salmon. We can extrapolate these results to salmon, and hypothesize that a decline in *C. reinhardtii* populations could have a negative effect on salmon and other organisms that are part of the same food web or

ecosystem. This is plausible based on *C. reinhardtii*'s role as a primary producer and its contribution to providing oxygen for aquatic environments.

ACKNOWLEDGEMENTS

A special thank you to our course instructor, Jordan Hamden, for inspiring the motivation behind this study. We would also like to acknowledge the guidance and feedback provided by our teaching assistant, Carol Sato. Lastly, the study would not have been possible without the provision of resources acquired through the lab technicians Mindy Chow, and Chanelle Chow. We appreciate the commitment of our peers throughout the peer-review process, as well as during poster presentations. Lastly, we acknowledge the University of British Columbia for providing us with this research opportunity.

APPENDIX I:

Chlamydomonas Maintenance

	Stock Solutions	Use for culture
KH_2PO_4	20.0g/L	5.0ml/L
K_2HPO_4	26.0g/L	5.0ml/L
$FeCl_3$	12.5g/L	1.0ml/L
$MgSO_4$ - $7H_2O$	60.0g/L	5.0ml/L
$CaCl_2$	95.0g/L	0.5ml/L
Trace Metals	see below	1.0ml/L
Na_3citrate-$2H_2O$	100.0g/L	1.0ml/L
NH_4NO_3	120.0g/L	2.5ml/L

Trace metals (10X)

H_3BO_3	4g/L
$ZnSO_4$-$7H_2O$	4g/L
$MnSO_4$-$4H_2O$	1.6g/L
$COCl_2$-$6H_2O$	0.8g/L
$CuSO_4$	0.16g/L
NH_4Moltbdate	0.8g/L

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