

The effect of increasing zinc concentration on the growth rate of *Chlamydomonas reinhardtii*

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ABSTRACT

Zinc is an essential nutrient required by organisms to survive, however, it can be toxic at high concentrations. Since zinc is also a common water pollutant, the objective of this study was to determine how increasing zinc concentration affects the growth rate of a common unicellular green alga species, *Chlamydomonas reinhardtii*. The experiment was conducted by exposing equal concentrations of *C. reinhardtii* cells to four different zinc concentrations (4.0 mg/L, 6.4 mg/L, 7.0 mg/L, and 10.0 mg/L) to see how this would influence their growth over time. Over a two week period we periodically counted the number of cells from our samples to see if there was a change in growth rate. Using a one-way ANOVA analysis we found the p-value to be less than 0.05, which supports our alternate hypothesis that increasing zinc concentration would increase the growth rate of *C. reinhardtii*. However, at our highest zinc concentration of 10.0 mg/L, a decline in growth was observed, therefore we were unable to support our alternate hypothesis. We proposed between 7.0 mg/L to 10.0 mg/L a toxic threshold concentration was reached, resulting in the decline in growth rate.

INTRODUCTION

Zinc is an essential nutrient required by organisms, ranging from bacteria to humans, due to its role in catalysis and in protein stabilization. However, excess concentrations of zinc are toxic since zinc can compete for metal binding sites in other proteins and indirectly produce damaging reactive oxygen species. Consequently, intracellular concentrations must be regulated to ensure zinc-containing proteins can function while zinc toxicity is avoided (King 2011).

Zinc is a common water pollutant that enters waterways through industrial plants, domestic wastewater, and run-off of zinc-containing soil. While the water toxicity of zinc is low for humans and animals, microscopic organisms are typically much more sensitive to heavy metal concentrations making zinc one of the most toxic metals to microscopic marine organisms

(Brauner *et al.* 2011). On the other hand, zinc also serves as an essential element that influences phytoplankton biota, and in turn, stimulates the growth of upper trophic levels that depend on phytoplankton as a food source (Malasarn *et al.* 2013). Understanding phytoplankton zinc tolerance is important to ensure zinc concentrations are maintained within levels that do not disrupt freshwater ecosystems. *Chlamydomonas reinhardtii* is a unicellular species of green algae that is widespread in freshwater habitats and can be used to study the relationship between zinc concentration and phytoplankton growth, as it has a quick generation time and simple life cycle that can be easily modeled under laboratory conditions (Humm and Wicks 1990).

Like other organisms, *C. reinhardtii* need metal cations, including zinc, as important cofactors for enzymes involved in both mitochondrial and chloroplast function. To maintain micronutrient metal homeostasis and prevent excess heavy metal concentrations, *C. reinhardtii* have developed a complex network of metal uptake, chelation, transport, storage and detoxification processes (Hanikenne *et al.* 2005). These processes involve metal transport proteins, which are essential for zinc metabolism and can be classified into two categories: cation diffusion facilitators (CDF) which increase the tolerance of zinc by removing zinc ions out of cells, and Irt-like protein (ZIP proteins) which move zinc into the cell (Malasarn *et al.* 2013).

In this study we exposed *C. reinhardtii* to varying concentrations of zinc in the form of zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), as a model to determine how zinc concentrations influence phytoplankton growth. We hypothesized:

H_A: An increase in the concentration of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ will increase the growth rate of *C. reinhardtii*.

H_O: An increase in the concentration of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ will decrease or have no effect on the growth rate of *C. reinhardtii*.

Prior research exposing *C. reinhardtii* to various zinc concentrations showed the organism to be relatively tolerant to zinc compared to other metals such as copper. (Danilov and Ekelund 2001). Consequently, in support of our alternate hypothesis, we believed that increasing zinc concentration would increase phytoplankton growth by improving enzymatic processes requiring zinc cofactors. We also believed that the *C. reinhardtii* would utilize a combination of CFD proteins and ZIP proteins to ensure that intracellular concentrations of zinc do not reach toxic levels by controlling the transport of zinc in and out of cells. However, we did not believe zinc concentrations would increase indefinitely without eventually hindering *C. reinhardtii*'s growth. We postulated that after some threshold level CFD proteins would reach their maximum capacity and be unable to counter the influx of zinc entering the cells. However, we did not expect to observe this threshold concentration during our experiment, since prior research determined that zinc toxification occurred at 40.3 mg/L of zinc sulfate which is well above the concentrations we used (Malasarn *et al.* 2013). At this threshold, intracellular concentrations of zinc would accumulate eventually leading to toxicity, and therefore, a reduction in growth.

METHODS

Cell Media and Replicate Preparation

Since we were interested in determining how increasing zinc concentration influence *C. reinhardtii* growth, we increased the concentration of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ by different amounts in three different culture media above a standard culture medium, which had no additional $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ added. We had to ensure that enough zinc was in the growth media for the phytoplankton to survive, but not in excess that would cause the cells to die too quickly. While Malasarn *et al.* (2013) determined that zinc toxification occurred at concentrations of 40.3 mg/L, other research

estimates that toxicity can be seen at concentrations as low as 0.04 mg/L depending on environmental conditions such as pH and algal population size (Mikulic and Beardall 2014). Based on previous study of green algae under similar conditions to our experiment we approximated that we could expose *C. reinhardtii* to up to 10.0 mg/L of ZnSO₄·7H₂O without causing harm (Rai *et al.* 1982). The different treatment groups are summarized in Table 1 and our control is the standard culture medium with a zinc concentration of 4.0 mg/L.

Table 1. Treatment conditions and corresponding replicates for each treatment group.

Treatment Group	Concentration of ZnSO ₄ ·7H ₂ O
Replicates 1 – 4 (Control)	4.0 mg/L
Replicates 5 - 8	6.4 mg/L
Replicates 9 – 12	7.0 mg/L
Replicates 13 - 16	10.0 mg/L

The initial cell count of *C. reinhardtii* in the stock medium was determined to be 3.96 x10⁵ cells/mL. We performed serial dilution on the stock media in order to obtain an estimated cell count of 1.50 x 10⁵ cells/mL. To prepare the media for each treatment, we used 300 mL (4 treatments x 4 replicates x 15 mL per Erlenmeyer flask) of the stock medium that *C. reinhardtii* are typically grown in, which is summarized in Figure 1. We then added different concentrations of zinc and culture media, as depicted in Figure 2, in the form of ZnSO₄·7H₂O to reach our final treatment concentrations listed in Table 1. After this process, the starting cell count for each treatment was approximately 7.5 x10⁴ cells/mL. This was repeated so that we obtained four replicates per treatment, each 30 mL in volume, yielding 16 replicates in total. We labeled each replicate, as detailed in Table 1, to keep track of which replicates corresponded with which treatment group. The samples were stored in an incubator at 17°C and under a constant light

intensity of 4,750 lux. We also counted our samples in the same laboratory to ensure other abiotic factors, such as temperature, were kept constant.

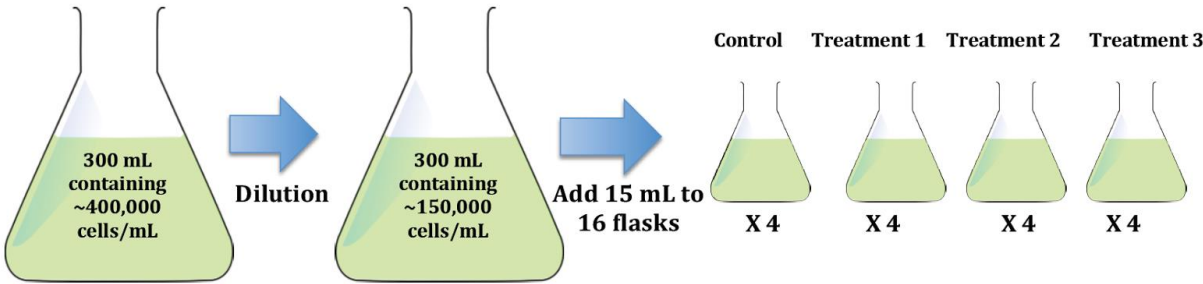


Figure 1. The dilution of the stock medium and initial preparation of our control and treatment groups.

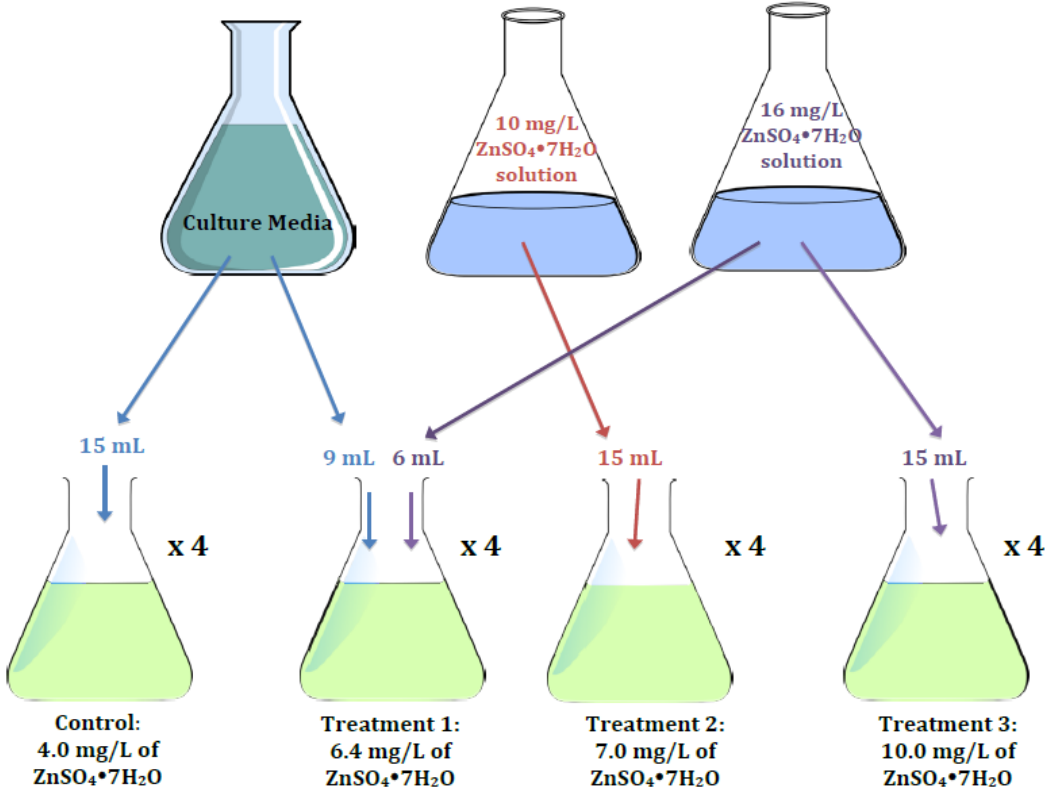


Figure 2. The preparation of our control and treatment groups to obtain the final concentrations of ZnSO₄·7H₂O.

Cell Counts

We performed cell counts of each replicate in six sessions over the course of fourteen days (Humm and Wicks 1990). To reduce measurement error, we randomized the cell counts so

that each researcher would count a different set of replicates at random rather than the same replicates each time. To prepare the cell counts we added 100 μL of each replicate and 10 μL of Lugol's solution (IKI) for fixation to a microcentrifuge (mcf) tube. After, we pipetted 10 μL of this mixture onto a haemocytometer and placed a coverslip to observe cells under a compound microscope. We used clickers to aid the counting process. When counting the cells, we first counted cells on the entire nine-grid area on the haemocytometer. As the cells proliferated, it became more efficient to count cells in fewer grids. We also ensured that when performing our cell counts, we did not count very small or irregularly shaped objects, as these were likely to be cell fragments or contamination.

Qualitative Measurements

Since *C. reinhardtii* are very small (roughly 10 μm in diameter) organisms it was hard to observe any noticeable qualitative changes throughout our experiment other than colour changes as seen through the Erlenmeyer flasks and the overall cell abundance observed under the microscope. We also took note of observations under the microscope such as movement, shape and other behaviours of the cells.

Quantitative Measurements

Using our cell count data we made four separate graphs for each treatment group: for each of these graphs we plotted the cell count of each of the replicates as a function of time. We then determined the linear slope to determine the growth rate for each replicate. Using this information, the mean growth rate, standard deviation, and 95% confidence interval for each treatment group was calculated. We then applied a one-way ANOVA test to determine if the mean growth rates between treatment groups were significantly different.

RESULTS

From the data that were collected, there was a large degree of variation in cell abundance for each treatment at each measurement day. Therefore, it was more accurate to analyze the data using growth rate rather than mean cell abundance.

For the first five days, the average cell abundance was steady and gradually increased. From day five to day ten, a huge increase in average cell abundance was observed for all treatments. From day 10 to day 12, there was a drop in average cell abundance for all treatment except for the 6.4 mg/L zinc treatment.

As seen in Figure 3 and Table 2, the 7.0 mg/L zinc treatment had the highest average growth rate and the 6.4 mg/L zinc treatment had the lowest average growth rate. The control and 10.0 mg/L treatment had higher variance than the 6.4mg/L and 7.0mg/L treatment. Therefore, their growth rates are more scattered from the mean than the 6.4 and 7.0 mg/L zinc treatments.

From a one-way ANOVA analysis, the p-value of 0.0158 for growth rate at different zinc concentrations is less than the critical value of 0.05. The confidence interval of the 4.0 mg/L zinc (control) and the 7.0 mg/L zinc treatments do not overlap which is also the case between the 6.4 mg/L and 7.0 mg/L zinc treatment groups (Figure 3).

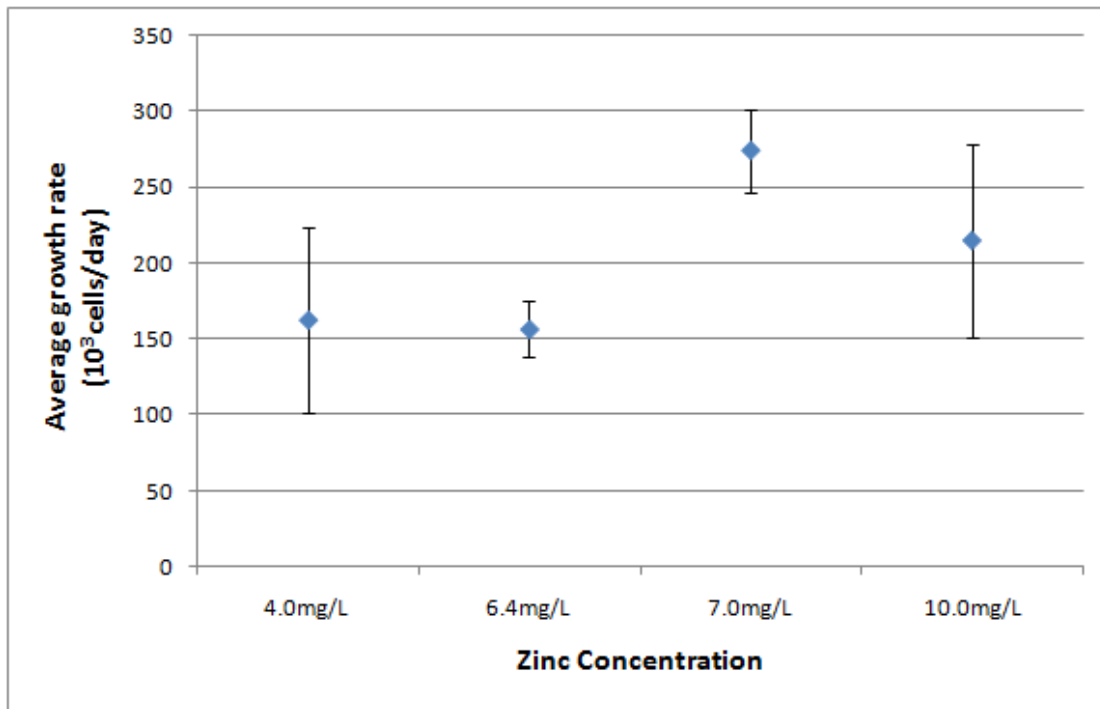


Figure 3. The effect of increasing zinc concentration on average growth rate (cells/day) of *Chlamydomonas reinhardtii* over a 12 days growth period at different concentrations (n =4 per treatment). Error bars represent 95% confidence intervals.

Table 2. Growth rate, variance, standard deviation, and 95% confidence intervals for all treatments

Treatment Groups	Growth Rate (cells/day)	Variance	95% Confidence Interval (cells/day)
4.0 mg/L (Control)	1.62×10^5	3.92×10^9	$1.62 \times 10^5 \pm 6.14 \times 10^4$
6.4 mg/L	1.56×10^5	3.58×10^8	$1.56 \times 10^5 \pm 1.85 \times 10^4$
7.0 mg/L	2.74×10^5	7.56×10^8	$2.74 \times 10^5 \pm 2.69 \times 10^4$
10.0 mg/L	2.15×10^5	4.24×10^9	$2.15 \times 10^5 \pm 6.38 \times 10^4$

DISCUSSION

Our ANOVA analysis determined a p-value less than 0.05 implying that our growth rates are statistically different and that we should reject our null hypothesis in support of our alternate

hypothesis, which stated that increasing zinc concentration increases the growth rate of *C. reinhardtii*. However, from Figure 3, we observed that the growth rate at our highest zinc concentration of 10.0 mg/L declined, which contradicts this statement. Consequently, we were not able to support our alternate hypothesis based on our ANOVA results. From our findings, a more accurate conclusion is that increasing zinc concentration increased the growth rate of *C. reinhardtii* up until a threshold concentration for zinc toxification, which we predicted was between 7.0 mg/L and 10.0 mg/L where a downward slope was observed. While we initially did not expect to observe toxification in our experiment due to the high zinc tolerance observed in a study on *C. reinhardtii* by Malasarn *et al.* (2013), other studies predict that toxification can occur at concentrations as low as 0.04 mg/L (Mikulic and Beardall 2014). The reason for the large variability in zinc tolerance in prior studies is likely due to the fact that toxification depends on many environmental variables, such as pH and algal population density (Rai *et al.* 1982).

The intracellular zinc content of the cells must remain homeostatic to ensure that zinc related proteins still function while excess zinc is avoided. In *C. reinhardtii*, this is accomplished through the development of chelation, metal uptake, storage, detoxification, and transport processes. The first network of control is referred to as assimilation, where intracellular zinc levels control the expression of transporters at the plasma membrane. The second network of control is distribution and storage, where zinc is transported into the secretory pathway through vacuoles and the Golgi apparatus when zinc is in excess (Eide 2006). The transporters involved in these processes belong to two families, the Irt-like transport protein (“ZIP”) and cation diffusion facilitators (“CDF”). The ZIP transport protein carry zinc and/or other metal ion substrates from the extracellular space or organelle lumen into the cytoplasm. The CDF family, on the other hand, transports zinc and/or other metal ions from the cytoplasm into the lumen of

intracellular organelles or to the outside of the cell (Malasarn *et al.* 2013). The expression of these types of transporters are therefore critical for homeostasis, survival, and growth of *C. reinhardtii*, and are more active at higher concentrations of zinc due to overexpression. In our results, we determined maximal growth at 7.0 mg/L zinc treatment, which we assume is the optimal concentration that allows *C. reinhardtii*'s enzymatic processes to be carried out, while excess concentrations are stored or taken out of the cell by transport proteins, as described above. Above this concentration, between 7.0 mg/L to 10.0 mg/L, the decline in growth rate is likely due to transport proteins being at maximum capacity and therefore unable to control excess intracellular levels of zinc. Excess intracellular concentrations of zinc are attributed to a decline in cell abundance and growth due to excess zinc ions competing for metal binding sites in other proteins (Malasarn *et al.* 2013). Furthermore, excess zinc ions can generate reactive oxygen species which can damage photosystem II in photosynthetic organisms (Rai *et al.* 1982).

As previously mentioned, zinc tolerance depends on a number of environmental factors, most notably pH and algal population density (Rai *et al.* 1982). Zinc toxicity tends to decrease under acidic conditions and when algal populations are higher since more cells are available to absorb excess zinc ions. Malasarn *et al.* (2013) may have found a high zinc toxification level of 40.3 mg/L because their cell culture was more acidic and had a higher abundance than ours. In addition while $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ was used as our source of zinc, Malasarn *et al.* (2013) used ZnSO_4 mixed with ethylenediaminetetraacetic acid (EDTA) to act as a buffer for trace elements. Chemicals in the EDTA used by Malasarn *et al.* (2013) contain MnCl_2 , FeCl_3 , and CuCl_2 along with a medium filled with Tris acetate-phosphate, which may be the reason why our resulting toxic concentration levels are different. The difference in chemicals may produce different reactions and have different interactions with the *C. reinhardtii*, resulting in inconsistent data.

In the case of 10.0 mg/L ZnSO₄·7H₂O, even though it had the highest concentration of zinc, its confidence interval overlaps with all of the other treatments' confidence intervals leaving us unable to ascertain whether the growth rate was either greater, less than or equal to the other treatments (Figure 3). The growth rate seen with the 4.0 mg/L also has overlapping confidence interval resulting in a growth rate value that can be greater, less or equal to the 6.4 mg/L and 10.0mg/L ZnSO₄·7H₂O (Figure 3). The overlaps in our confidence intervals could be due to human error, such as improper sterile technique, insufficient mixing of cells during extractions, and miscounting of *C. reinhardtii* cells. Nonetheless, insufficient mixing could be a reason for why the growth rate of 10.0 mg/L is higher than 7.0 mg/L, and for the large variance seen on the 4.0 mg/L and 10.0 mg/L in Figure 3.

CONCLUSION

Our results showed a statistically significant increase in growth rate following treatment of *C. reinhardtii* with increasing zinc concentration. However, due to the growth rate of the highest (10.0 mg/L) zinc treatment being lower than that of 7.0 mg/L treatment, we cannot support our alternate hypothesis. There are few experiments that have determined the relationship between zinc levels and growth rate of *C. reinhardtii* and a definitive concentration that leads to zinc toxicity. Our results suggest that there is a zinc threshold level for toxicity around 10.0 mg/L for *C. reinhardtii* and may have implications for pollution management in freshwater systems. Further investigation is necessary to determine the specific threshold zinc concentration that leads to toxification under different environmental conditions.

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