Effect of phosphorus concentration on growth rate of C*hlamydomonas reinhardtii*

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Abstract

Phosphorus is an element that exists naturally in living organisms and the environment. The levels of phosphorus in ecosystems is impacted by human activities such as run-off from agricultural fertilizers, industrial waste, and mining, to name a few. This in turn affects the organisms living in that habitat. Unicellular algae, *Chlamydomonas reinhardtii*, are a key part of the salmon food web and are heavily influenced by freshwater nutrient levels. In this study, the relationship between the growth of *C. reinhardtii* and phosphorus concentration in the growth media was evaluated. Average growth rate was calculated using the measured cell densities in different treatment groups of varying phosphorus concentration over a two week period. Using a one-way ANOVA on growth rates of different treatment groups, it was determined that there is a statistically significant increase in growth rate of *C. reinhardtii* at higher phosphorus concentrations, with the 45mg of phosphorus/L treatment having the largest growth rate.

Introduction

Humans shape the environment in many ways, sometimes intentional, other times unintentional. We must be conscious of the impact of our actions on the environment, as the changes incurred can heavily affect ecosystems and their inhabitants. In several ways, the pollutants that are created from humans enter ecosystems uncontrolled and cause damage. A typical example is of the plastics that humans release en-masse into beaches and the ocean via dumping. Further are instances of oil spills in the ocean.

Often, plastics and other wastes are the result of industries and people purposely dumping into the environment, while oil spills and chemicals usually come about unintentionally. A key distinction between the various environmental wastes is whether said waste comes from a point source or nonpoint source. Point sources are when the pollutants come from one source, such as a single pipe, spill, conduit. Nonpoint source pollutants are those that do not come from one distinct source, such as run-off (Environmental Protection Agency). Run-off is a typical example where chemicals enter ecosystems externally, such as from industrial areas, construction zones, and farms, and has significant consequences for neighboring habitats.

Run-off occurs when the amount of water going into an area is greater than the amount absorbable (Sharpley 920). Excess water drains through the area into other habitats, and as it goes, picks up and carries nutrients, minerals, and pollutants with it. One common source of harmful run-off is from farm fertilizer and soil. Farm fertilizers contain phosphorus, nitrogen, and sulfur which are beneficial for plant growth, but run-off from farms contributes significantly to pollution. A report by the European Environmental Agency (EEA) states that in Europe, agriculture is responsible for about 50-80% nitrogen pollution and 25-75% of phosphorus pollution (32). These nutrients are naturally occurring in nature, but only in low concentrations. They are considered limiting nutrients in aquatic life and run-off increases their concentrations above natural levels, a process known as eutrophication. This can lead to various changes such as rapid growth of algae, referred to as algal blooms. These blooms can cut off light from reaching deep into waters, affecting both plants and light-sensitive animals. Also, the high levels of photosynthesis during day can deplete inorganic carbon, raising water pH to dangerous levels for aquatic life (Chislock et al.). When these algal blooms die, their decomposition consumes a lot of dissolved oxygen and can render the water hypoxic or anoxic. These areas of low oxygen are

essentially dead zones where very little life can survive. Finally, some algae also produce toxins, which harm other inhabitants as well as humans (Chislock et al.).

Understandably, the effects human activity has on the environment can be quite destructive. Such issues affect many communities, even here in the Lower Mainland of BC, Canada. Much of our wildlife depends on the availability of key nutrients from the land and presence of keystone species. One species particularly affected by the disruption of aquatic life is the salmon, which is important to local cultures, economy, and environment. Salmon have long been used as a source of food and an indicator of the health of the ecosystem by First Nations of BC (Columbia River Inter-Tribal Fish Commision), but also hold cultural and ritualistic significance to the people as well. Further, the BC seafood industry, which had a wholesale value of $1.749 billion in 2017, depends on the sale of salmon, which contributed $199 million in 2017 from just wild commercial salmon (BC Seafood Industry, 6). Finally, as a keystone species, salmon provide nutrition for much of the ecosystem by being preyed upon, scavenged, or decomposing and releasing nutrients into the environment (Wipfli et al. 1507).

In most cases, disturbing one species will lead to most other species of the same habitat being affected. For example, algae feeds into many lower food trophic levels, which in turn feed into higher ones and so one. Salmon too are a part of this food web at higher levels, and so they also rely on algae, despite ironically being endangered by algal blooms (Figure by NOAA, GLERL., shown in Food Chains and Food Webs, Lumen Learning).

To protect the environment and key species like the salmon, we must better understand, and then better control, human activity so as to not harmfully disrupt natural processes. Many studies have examined the effects of run-off pollutants and minerals on algae growth. Typically, phosphorus, nitrogen, and sulfur are used to observe the growth and motility of various algae,

one such example being *Chlamydomonas reinhardtii*. The aforementioned elements are easily manipulated as oxyanions, such as phosphate, and are non-toxic to the algae, which makes them good variables in tests. *C. reinhardtii* is an ideal lab organism to test due to its ease of culturing and relatively quick reproduction rate (Harris 363). Further, *C. reinhardtii*, studying *C. reinhardtii* also provides insight into salmon conditions, owing to the algae being a part of salmon food web.

Several studies have shown relationships between phosphorus, sulfur, and nitrogen levels and various aspects of *C. reinhardtii*. For instance, Wang et. al found that within specific ranges of nitrogen and phosphorus levels, *C. reinhardtii* reached optimal growth with maximum protein content and larger chloroplast sizes (Wang 5762-5770). Several other studies found on The Expedition, and open journal system, have been done in a similar fashion; observing the growth of the algae under various nutrient levels such as sulfur and nitrogen. Yet, we could not find any study testing just phosphorus content on *C. reinhardtii* growth. This is a large part of our motivation to study the relationship between *C. reinhardtii* growth and phosphorus content of the growth media. By studying this relationship, we can gain an understanding of the nutrient ranges in which *C. reinhardtii* achieves optimal growth, as well as how significant the difference in growth is between various nutrient levels.

C. reinhardtii will be cultured in 4 treatments, with 4 replicates each, of varying phosphorus concentrations. The lowest concentration will serve as a control, which is unaltered culture media of nutrient concentration ~1.5 mg P / L. The other treatments will be of increased concentrations of 5, 15, and 45 mg P / L. Samples will be obtained five times from each replicate over a period of two weeks and cell counts will be performed on each sample to gauge cell density over time for each treatment. Analysis will provide an average growth rate for each

replicate (final cell density - initial density), for which the significance of the rates will be assessed using One-Way Anova. One-way Anova will be used despite the experiment lacking several required key assumptions since this test is still the most appropriate for our type of data comparison. As such, our analysis will compare, between each treatment, the mean of the average growth rates of each replicate. Our null and alternative hypotheses are as follows:

H_0: There is no significant difference in the mean of the average growth rates of *C. reinhardtii* between treatments.

H_A: There is a significant difference in the mean of the average growth rate of *C. reinhardtii* between treatments

Owing to the various articles and background on our and similar topics, we predict that there will be a difference in the mean of the average growth rate of *C. reinhardtii* between treatments.

Methods

Overview

To determine the relationship between growth rate of *C. reinhardtii* and phosphorus concentration in the growth media, four different treatment groups were made at varying phosphorus levels as follows: control = 1.6mg P/L, treatment 1 = 5.0mg P/L, treatment 2 = 15mg P/L, and treatment 3 = 45 mg P/L. Each treatment group had four replicates, and an initial cell concentration of about $5.0x10^4$ cells/mL. The experiment was run for two weeks and cell density was calculated six times over the course of the experiment. Following is an explanation of how the experiment was setup, how cell density was measured, and how the data was analyzed.

Preparation

The initial concentration of *C. reinhardtii* in growth media was unknown and therefore needed to be determined. To find the number of cells, 10 µL of IKI fixative was pipetted into an Eppendorf tube, to which 100 µL of the initial *C. reinhardtii* in growth media solution was added. After using the pipet to mix the solution in the Eppendorf tube, 10 µL of the mixture was pipetted onto a hemocytometer and viewed under a compound microscope. The initial cell concentration was determined to be $2.2x10^4$ cells/mL, which was lower than the desired concentration of $5.0x10^4$ cells/mL for the treatments. The *C. reinhardtii* in the initial growth media need to removed and resuspended in the treatment growth media at a concentration of $5.0x10^4$ cells/mL.

Treatment Setup

The flask containing 200 mL of *C. reinhardtii* in initial growth media was evenly distributed to eight, 50 mL Falcon centrifuge tubes. These Falcon centrifuge tubes, each filled with about 25 mL of solution, were then centrifuged on the highest setting for five minutes. ~~While this was happening,~~ dilutions of WC media were made for each treatment group. Stock WC media had 1.6 mg of phosphorus per mL of solution, and was used for the control. The phosphorus enriched WC media had 45 mg of phosphorus per mL of solution, and was used for the third treatment group. To make the WC media for the first treatment group, 46.03 mL of stock WC media was mixed with 3.97 mL of phosphorus enriched WC media to obtain 50 mL of WC media with 5.0 mg of phosphorus per mL. The WC media for the second treatment group was made by mixing 34.52 mL of stock WC media with 15.48 mL of phosphorus enriched WC media to obtain 50 mL of WC media with 15 mg of phosphorus per mL.

The contents of the Falcon centrifuge tubes were then decanted to isolate the pellet of *C. reinhardtii*. Next, 10 mL of stock WC media (1.6mg P/L) was pipetted into two of the Falcon tubes containing the *C. reinhardtii* pellet, and vortexed for 30 seconds to resuspend the cells. These two Falcon tube solutions of *C. reinhardtii* were combined into one Falcon tube and vortexed for another 30 seconds. This was repeated with the other three WC media solutions to obtain a total of four Falcon tubes with cells suspended in growth media of four different phosphorus concentrations. The concentration of cells in each of the four Falcon tubes was then determined using a hemocytometer. Corresponding WC media was added to each Falcon tube to reach a final concentration of $5.0x10^4$ cells/mL. The cell concentration was counted one more time for each Falcon tube, and the data was recorded as the initial cell concentration for each treatment group.

Next, 4.5 mL of solution from the Falcon tube containing the control treatment was pipetted into four glass 15 mL test tubes, and labelled accordingly. This was repeated for the Falcon tubes containing treatment 1, treatment 2, and treatment 3, resulting in a total of 16 test tubes. The four treatment groups, each with four replicates, were put in an incubator at 20°C. The incubator cycled 12 hours with the UV lights on, 12 hours with the UV lights off each day.

Data Collection

On six days over the course of the two week experiment, cells were collected for counting. On each collection day, the test tubes were vortexed for about 10 seconds, and 100 µL of solution was pipetted into an Eppendorf tube with 10 µL of IKI fixative. This was repeated for each of the 16 test tubes, and each Eppendorf was labelled with the corresponding treatment group, replicate number, and collection day. The Eppendorf tubes with the collected samples were stored in the fridge at 4°C until counting day, which occurred at the end of each week. On

counting day, each of the Eppendorf tubes were vortexed for about 10 seconds, and 10 µL was pipetted onto a hemocytometer, which was viewed under a compound microscope to count the cells. A handheld tally counter was used when counting the cells, and the data was recorded.

Data Analysis

Using Microsoft Excel, the cell density was calculated for each recorded cell count. The cell density for each treatment and day was found by determining the average of all the replicates. For example, the cell density for treatment 1 on the first sample day was found by averaging the cell density of treatment 1 replicate A, B, C and D of that respective sampling day. Excel was also used for a one-way ANOVA test to statistically analyze the mean cell growth for each treatment group, and to graph the cell growth data which can be seen in the results section. Lastly, a post-hoc Tukey's honestly significant difference (HSD) test was performed using astatsa.com online tool to determine which treatment groups differed from the results of the one-way ANOVA test.

Results

The average cell growth for each treatment over the two week experiment are as follows: T_0: $1.43x10^5$ cells/mL, T_1: $2.16x10^5$ cells/mL, T_2: $2.83x10^5$ cells/mL, T_3: $3.07x10^5$ cells/mL. One-way ANOVA statistical analysis of mean growth between the four treatment groups resulted in a p-value of 0.002491, and a corresponding F value of 8.659649 and F critical of 3.490295. A subsequent Tukey's HSD post-hoc test was performed at the alpha = 0.05 significance level. The Tukey's HSD only showed a statistically significant difference between the means of treatment 0 and treatment 2, with a corresponding p-value = 0.0089637, and the means of treatment 0 and

treatment 3, with a p-value = 0.0028149. The mean growth between the other treatment groups resulted in a p-value greater than alpha = 0.05, as determined by Tukey's HSD post-hoc analysis.

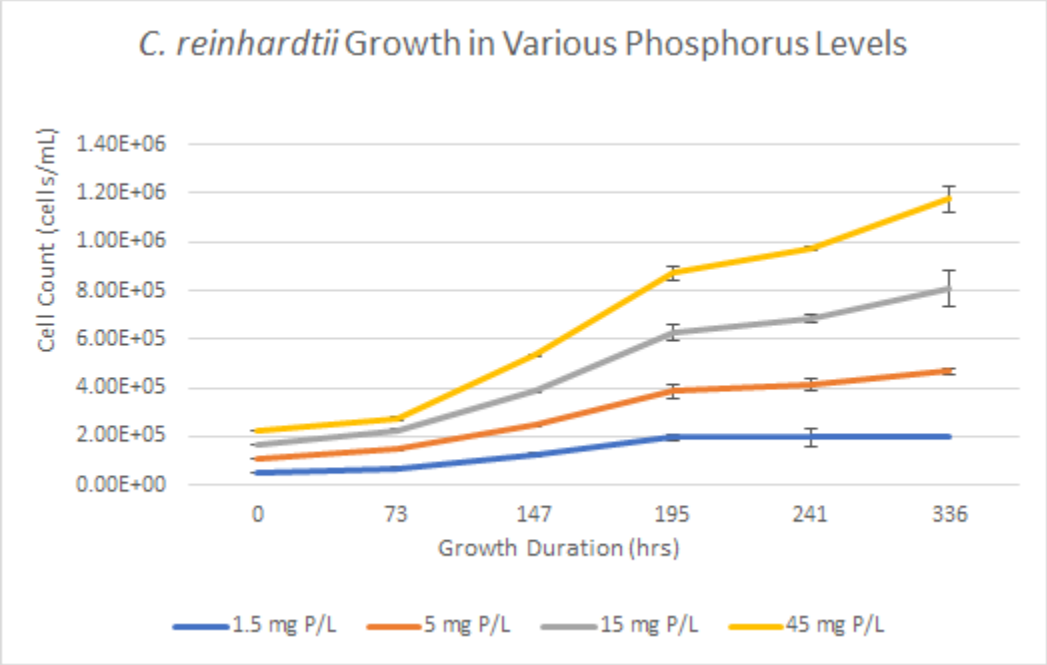

Figure 1: Comparison of the growth of *C. reinhardtii* in the four different treatment groups. Each data point represents the average cell density of the replicates for each sample. The error bars represent the 95% confidence interval of each data point.

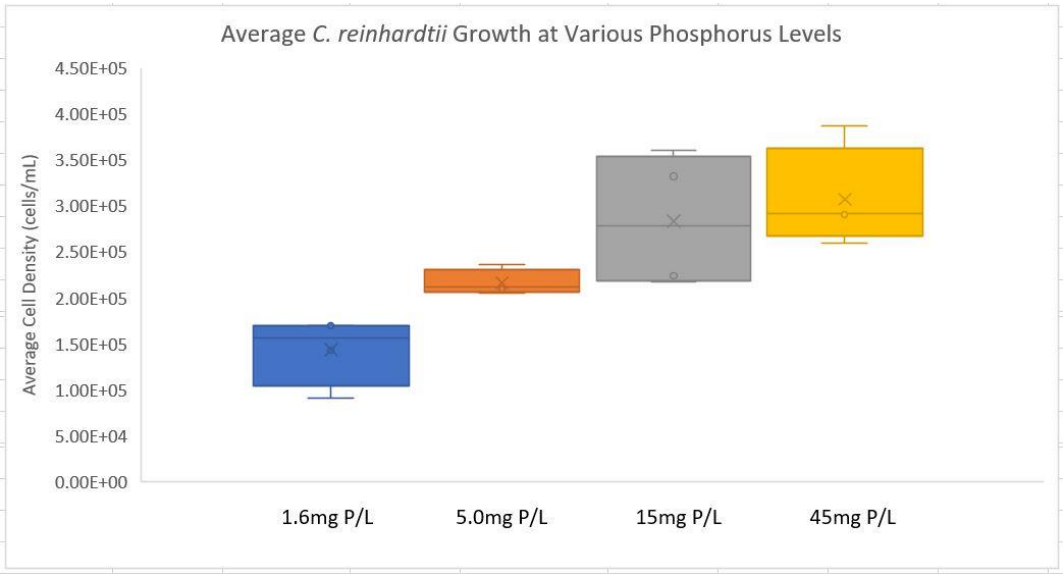

Figure 2: Box and whisker plot for comparison of average growth of *C. reinhardtii* for each of the four different treatment groups.

Discussion

In this experiment, the change in the average growth rate of *C. reinhardtii* in response to varying phosphorus concentrations was investigated. After performing statistical analysis on our data using a one-way ANOVA test, we are able to reject the null hypothesis (F > F-crit) and provide support for the alternate hypothesis. Our null hypothesis, that there is no significant difference in the means of the average growth rates of *C. reinhardtii* between treatments of varying phosphorus concentrations, is reported to have a p-value of 0.00249 which is smaller than the 0.05 significance level. This means that there is a significant difference in the means of the average growth rates of *C. reinhardtii* between treatments of varying phosphorus concentrations. A Tukey's HSD post-hoc analysis only revealed a statistically significant difference between the mean growth rates of T_0 and T_2, and between the mean growth rates of T_0 and T_3.

Looking at figure 1 above, C. *reinhardtii* responded to the different phosphorus treatments as expected for most of the experiment duration. We can see that all four treatments, from our control media to our enriched media, followed the common growth curve of unicellular organisms. The lag phase, where the *C. reinhardtii* were metabolically active but not reproducing, was present up to roughly the 73 hour growth point. The *C. reinhardtii* were preparing for replication by synthesizing the necessary proteins and increasing in size (Bailey). Starting at the 73 hour growth mark, we can see the exponential phase where replication took place. There was high metabolic activity as internal and external components of the cell were produced in order to divide (Bailey). At the 195 hour growth mark, it is uncertain whether or not the exponential phase was continuing or transitioning into the stationary phase, as we can see a small increase in slope beyond the 241 hour mark. If stationary phase was reached, this is where

nutrient conditions decreased due to heavy uptake and cell death rates increased to where they were near the growth rates (Bailey). However, it is possible that an upward trend and larger slope increase would have been observed if the experiment was carried on over a longer period of time.

From the Tukey post-hoc test, we state that cell growth is statistically different when comparing some treatments, but not others. In the situations where it did differ, we can reject the associated null hypothesis. From the results of this test, a statistically significant difference was only found between the mean growth rates of T_0 and T_2, and between the mean growth rates of T_0 and T_3, while all other comparisons were deemed insignificant. However, there are clear trends in the data that point towards larger growth rates when increasing phosphorus concentration. By looking at p-values obtained from the Tukey test for all treatment comparisons, larger phosphorus concentration differences between treatments produced p-values that were much closer to the significance level. This suggests that phosphorus concentration does have an effect on *C. reinhardtii* growth rate, however, only when comparing large enough differences in phosphorus conditions. It is likely that, if the treatments were prepared with larger variations in phosphorus concentrations, more statistically significant relationships would have been obtained.

Our results were consistent with our prediction that the growth rate of *C. reinhardtii* would increase with increasing phosphorus concentrations. Phosphorus is an essential nutrient for living organisms and is involved in numerous biological functions. Phosphorus plays a role in nucleic acid synthesis (DNA production), cellular energy transfer and storage in the form of ATP, the formation of reducing agents, as well as the critical formation of the phospholipid bilayer (Kamalanathan et al. 1509-1520). This is a good explanation for why limiting

phosphorus concentrations would result in lower growth rates of *C. reinhardtii*. It would interfere with the synthesis of many different cellular components, and result in a limited ability of cells to synthesize the sufficient materials and resources needed for cell replication.

C. reinhardtii are a part of the salmon food web and are found in marine waters where adult salmon reside, as well as freshwater locations where young salmon grow and develop. Since *C. reinhardtii* reside in the same ecosystems as salmon do, changes in their growth rates can greatly impact the salmon population. Our results, that *C. reinhardtii* growth rates increase with increasing phosphorus concentration, suggest that areas which receive phosphorus concentrated runoff water from agricultural lands will result in algae blooms and large increases in microalgae populations. Although a larger *C. reinhardtii* population would lead to a more available food source for lower trophic levels, which has an indirect positive effect on salmon, an overabundance of *C. reinhardtii* will likely have an overall negative effect on salmon. Eutrophication and an overabundance of *C. reinhardtii* will result in anoxic waters, as more organic matter in the water body will need to be decomposed by decomposing bacteria upon death of microalgae, and this process requires oxygen (Yang et al. 197-209). Oxygen levels may become insufficient to sustain the salmon population.

Throughout our two-week collection period, there were many limitations and sources of uncertainty that we encountered. As mentioned above, we started our experiment by determining the number of cells of *C. reinhardtii* present in the solution that was given to us, and then we isolated the cells via centrifuge. It is likely that during the decanting process of removing the solution containing our pellet, small amounts of the liquid were left behind. We did not have time to fully dry out our pellet, so the undesired default media was carried over into our new treatments. Another source of uncertainty was that we did not regulate the phosphorus

concentrations in each of our treatments throughout the experiment. After preparing the solutions of desired phosphorus concentrations on day 1 of the experiment, we did not change the treatments in anyway, and we performed our experiment assuming that the concentrations stayed the same. It is likely that the phosphorus concentrations would have decreased over time, as it was being used up by *C. reinhardtii*. Different treatments showed larger growth rates of *C. reinhardtii* over time, so as the experiment proceeded, higher cell density treatments would have resulted in greater uptake of phosphorus. Furthermore, there were some errors in our data due to the counting of cells using a hemocytometer. The number of cells viewed on the hemocytometer varied, as some areas of the grid had cells evenly dispersed throughout where as other areas showed large amounts of cells grouped together, and sometimes cells would form very dense clusters. Also, before taking replicates from the treatment test tubes, we noticed that there was a dark green ring around the inside of the test tube at the top of the liquid media. This indicated that some *C. reinhardtii* were accumulating at the surface of the media, rather than dispersing throughout the media. To counter this, we vortexed each treatment tube for up to ten seconds, however, a faint green ring still remained in most cases. This would have affected *C. reinhardtii* cell densities and resulted in underestimation and overestimation of growth rates at different treatments. Lastly, since we took turns counting different samples, there would have been slight variation in counting techniques as well.

Conclusion

This experiment suggests that phosphorus concentration has a significant effect on the growth rate of *C. reinhardtii*. We observed the growth rate of *C. reinhardtii* to increase with increasing phosphorus concentrations, as although phosphorus was present in all treatments, *C.*

reinhardtii were slightly more phosphorus limited in treatments with lower phosphorus concentrations than those in treatments with higher phosphorus concentrations. Therefore, we conclude that agricultural areas with phosphorus concentrated runoff waters due to fertilizer use, would result in a tremendous growth increase of *C. reinhardtii*.

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Appendix

Components	**Concentration (g/L)**	**Use for 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 below	1.0
Na_3citrate $2H_2O$	100.0	1.0
NH_4NO_3	120.0	2.5
Trace Metals (10x)		
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 1: Initial growth media composition

Components	**Concentration (g/L)**	**Use for culture (mL/L)**
$CaCl_2\ 2H_2O$	36.80	1.0
$MgSO_4\ 7H_2O$	37.00	1.0
$NaHCO_3$	12.60	1.0
$K_2HPO_4\ 3H_2O$	11.40	1.0
$NaNO_3$	85.00	1.0

Table 2: Stock WC Media composition

Components	Concentration (g/L)	Use for culture (mL/L)
$CaCl_2$ $2H_2O$	36.80	1.0
$MgSO_4$ $7H_2O$	37.00	1.0
$NaHCO_3$	12.60	1.0
K_2HPO_4 $3H_2O$	331.61	1.0
$NaNO_3$	85.00	1.0

Table 3: Phosphorus enriched WC Media composition

Treatment Pairs	Tukey HSD p-value	Tukey HSD Inference
T0 vs T1	0.2214930	insignificant
T0 vs T2	0.0089637	$p < 0.05$
T0 vs T3	0.0028149	$p < 0.05$
T1 vs T2	0.2804313	insignificant
T1 vs T3	0.0981666	insignificant
T2 vs T3	0.8999947	insignificant

Table 4: One-way ANOVA post hoc Tukey's HSD test results from astatsa.com online calculator

Treatment	Mean cell density growth (cells/mL)	95% Confidence Interval
T0: P[1.6mg/L]	$1.43x10^5$	$3.23x10^4$
T1: P[5.0mg/L]	$2.16x10^5$	$1.19x10^4$
T2: P[15mg/L]	$2.83x10^5$	$6.39x10^4$
T3: P[45mg/L]	$3.07x10^5$	$4.76x10^4$

Table 5: Mean cell density for each treatment over two week experiment with corresponding 95% confidence interval