Cortney Borer

Honors Capstone Project

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Interactive Effects of Temperature and Nutrients on *Microcystis auerginosa* and *Anabaena flos-aquae* Cultures

Abstract

Harmful cyanobacterial blooms (HCBs) are occurring more often across the world, likely due to the effects of climate change. These blooms present a problem for environmental quality, human and animal health, and the state of freshwater ecosystems, as many of the species that make up blooms are toxic. Driving factors have been widely investigated, but little specific data exists for HCBs and the drivers of HCBs in Wyoming. This experiment focused on performing an experiment with Wyoming-relevant conditions and how they affect two common bloom species found in Wyoming: *Microcystis auerginosa* and *Anabaena flos-aquae.* Temperature and nutrient amounts, specifically the amounts of nitrogen and phosphorous, are two significant factors that are thought to be driving the increase in blooms. We tested the effect of three different levels of nitrogen and phosphorous, a high condition and a low condition along with a control, at three different temperatures in order to better understand the role these factors play in bloom growth. We used data from the Boysen Reservoir near Thermopolis and Shoshone, Wyoming to determine the temperatures we would use. Overall, there were few significant results. We found that temperature and nutrients interacted significantly to affect the *Anabaena flos aquae* cultures while a high nutrient level caused a decrease in the growth of *M. auerginosa* cultures.

Introduction

There has been a rise in harmful cyanobacterial blooms (HCBs) in recent years across the world, including in Wyoming reservoirs. This is a large concern in regards to environmental quality, human health, animal health, and the health of delicate freshwater ecosystems. Many of the most common species making up these HCBs, such as *Microcystis auerginosa*, produce toxins that are detrimental to the health of humans and animals, thus they hinder recreational use of the water bodies that they grow in, as well as causing other issues.

Increases in the frequency of HCBs across the world has been linked to climate change (Chapra et. al 2017). Higher temperatures have been associated with increased growth in some strains of cyanobacteria while being detrimental to others (O'Neil et. al 2012, Chapra et. al 2012, Richardson et. al 2019, Jöhnk et. al 2008). In addition, one study found that temperature can be used as a predictor for water quality, which is affected by HCBs (Collins et. al 2019). In this study, higher temperatures in particular were shown to have an effect on the presence of algae. Overall, warmer temperatures tend to increase the ability of cyanobacteria to dominate in shallow lakes (Kosten et. al 2012), and climbing temperatures due to climate change are predicted to cause the number of HCBs to continue to climb (Wells et. al 2020).

The level of nitrogen and phosphorous has also been shown to influence HCB growth, as well as other cyanobacteria functions (Herrero et. al 2001, Chen et. al 2020, Glibert et. al 2017). These two nutrients are vital to the growth and survival of cyanobacteria, and increased levels of both have been thought to have a significant effect on the growth and frequency of many species of cyanobacteria (Herrero et. al 2001, Chen et. al 2020). Nitrogen has been shown to have a significant effect on HCBs, not just in terms of growth but in terms of the toxicity of the blooms (Glibert et. al 2017). High levels of nutrients can also be detrimental to water ecosystems and water quality. For example, too much phosphorous can damage these ecosystems and degrade water quality (Hanson et. al 2020), as well as providing nutrients for HCBs. Eutrophication is also highly detrimental to water ecosystems and water quality and can increase the frequency of blooms (O'Neil et. al 2012). Finally, nutrients are not only

important to bolster blooms, but to control them as well, especially nitrogen and phosphorous (Paerl 2017). Understanding the effects of these factors can help inform management practices.

To better understand the effects of nitrogen, phosphorous, and temperature on HCBs in Wyoming, we examined the effect of different levels of nitrogen and phosphorous on the growth of two different strains of cyanobacteria: *Microcystis auerginosa* and *Anabaena flos aquae*. In addition, we also tested the effect of different temperature levels on each of our nutrient level conditions. *M. auerginosa* is one of the most commonly occurring cyanobacteria strains in blooms and is highly toxic to human and animals. This species of *Anabaena* is another frequently occurring species; unlike *M. auerginosa* it is filamentous and does not produce toxins (Note: other species of *Anabaena* do produce toxins, but *flos aquae* does not). These species are also two of the most commonly found species in blooms that have occurred in Wyoming. We tested the effect of temperatures and nutrient levels that were relevant to Wyoming on these two species.

Overall, this experiment examined the effect of rising temperatures and differing nutrient levels on the two different species in order to start forming a basis for how these factors might influence HCBs of *M. auerginosa* and *Anabaena flos-aquae* in Wyoming reservoirs. For this experiment, we tested three different nutrient levels against three different temperatures for a total of nine different conditions. We expected that as temperatures and nutrient levels rise, we would see more growth in our chosen species. In order to make this experiment more relevant to Wyoming, we chose temperature conditions and nutrient levels based on the average conditions present in Wyoming. These experiments will provide some knowledge about HCBs that is more applicable to Wyoming and opens the door to future research examining samples and other species present in Wyoming reservoirs.

Materials and Methods

Culturing

Experiments were conducted with cultures of both target species, which we obtained a 15mL aliquot of from the Culture Collection of Algae at The University of Texas at Austin (UTEX). Species were cultured in 2L flasks in Bold 3N medium and under constant light. The Bold 3N recipe we used for both the medium in the cultures and the medium in the experiment was also taken from UTEX. Cultures had a mixture of pre-made culture bought directly from the store and culture made in the lab by replicating the recipe. Cultures were continuously stirred during the growth period and additional medium was added about every week.

Experimental Design

For this experiment, we had three different temperature conditions, and we had three different nutrient conditions. We had a setup for each possible nutrient and temperature combination, which was nine different conditions with five replicates per condition. Our setup was kept on a twelve-hour light cycle. Twelve hours were spent in light, and twelve in darkness.

For our temperature conditions, we used static water baths set at three different temperatures: 17°C, 20°C, and 23°C. These temperatures were determined by taking the average annual temperatures of the sampling site for a concurrent experiment using reservoir samples, this sample site being the Boysen Reservoir by Thermopolis, Wyoming. The static baths were kept under constant light. Temperatures were kept constant by a setup of temperature monitors hooked up to solenoids, which would then be set to the desired temperature. This allowed an inflow of either hot or cold water, depending on what was needed to hold the temperature stable. Temperatures were monitored by deployed HOBO loggers. Temperatures were also taken with a DigiSense thermometer twice a week.

To achieve our different nutrient conditions, the Bold 3N medium recipe from UTEX was used, and the amounts of $NaNO_3$, K_2HPO_4, and KH_2PO_4 added to the recipe were altered 30% by volume, one where these components were reduced by 30% (our low condition) and one where they were increased by 30% (our high condition). The control replicates used regular medium made by following the recipe as one normally would.

In our first round of experiments, the soilwater medium ingredient was excluded from all three Bold 3N mediums used. It was included in the second round of experiments.

For our first experiment, experimental cultures were kept in glass pint bottles that were filled with 150mL of medium and dosed with 1mL of culture. These bottles were then submerged in our static water baths. 15mL of medium was poured off each bottle twice weekly and replaced with 15mL of fresh medium. At each temperature, we had a control for each species (n = 5) filled with the unaltered Bold 3N medium. The low condition (n = 5) and the high condition (n = 5) were filled with their corresponding altered medium (See Appendix I for photographs of our experimental setup).

For the second round of experiments, we opted to increase the dose of culture used from 1mL to 5mL. In addition, as mentioned above, the medium for the second round of experiments included the soilwater medium that was absent in the first round.

Enumeration and Analysis

2mL sub-samples were taken twice weekly and examined using a spectrophotometer. Absorbance of the samples was measured at 665nm for both species (Fuad Hussain et. al 2020, Kosten et. al 2012, Richardson et. al 2019).

Feeding and sampling were done on the same days. 15mL was poured off each culture, then samples were taken, then 15mL of fresh medium was added back into each culture after all samples were taken (See Appendix II for a step-by-step outline of the enumeration and feeding protocol).

To analyze samples, absorbance levels were plotted over time for each condition to see if there were any significant trends. A one-way ANOVA statistical analysis was done on the absorbances from the final sample date to determine if there were any statistically significant differences in growth.

Standard Curve

A standard curve for *M. auerginosa* was created by manually enumerating the number of cells in different concentrations of culture in a 1mL Sedgewick Rafter slide under a microscope and taking the absorbance of the sample, then plotting the two on a graph (Fig. 1). Absorbance and cell count are

directly correlated because the color of the samples will darken (which will produce higher absorbance levels as more light is absorbed) as the concentration of cells increases, therefore absorbance can reliably be converted to estimated cell counts using this standard curve (See Appendix III for data table). We did not create a standard curve for *Anabaena*, so all results are given as absorbances.

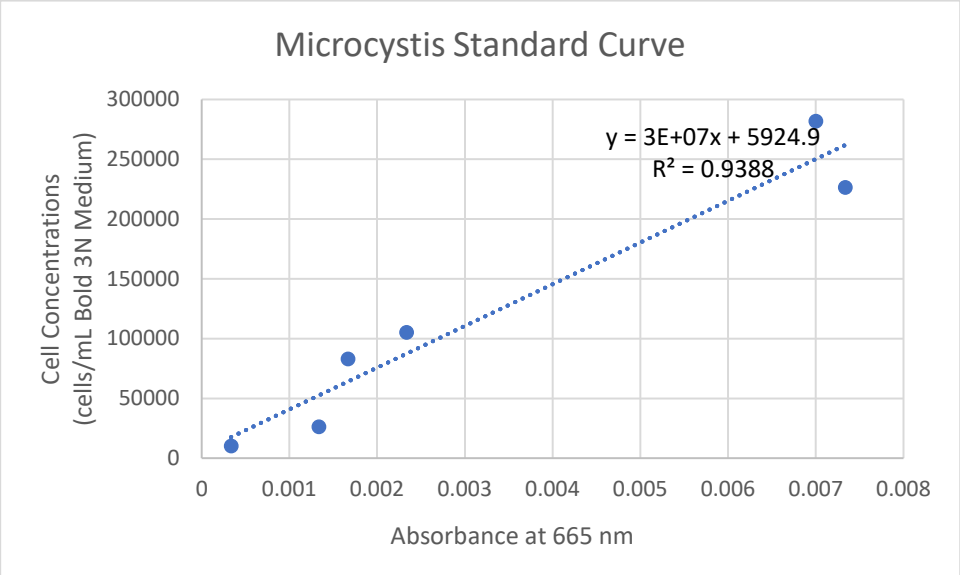


Fig. 1. A standard curve for *M. auerginosa* relating absorbance levels at 665nm to approximate cell concentration in Bold 3N medium.

Results

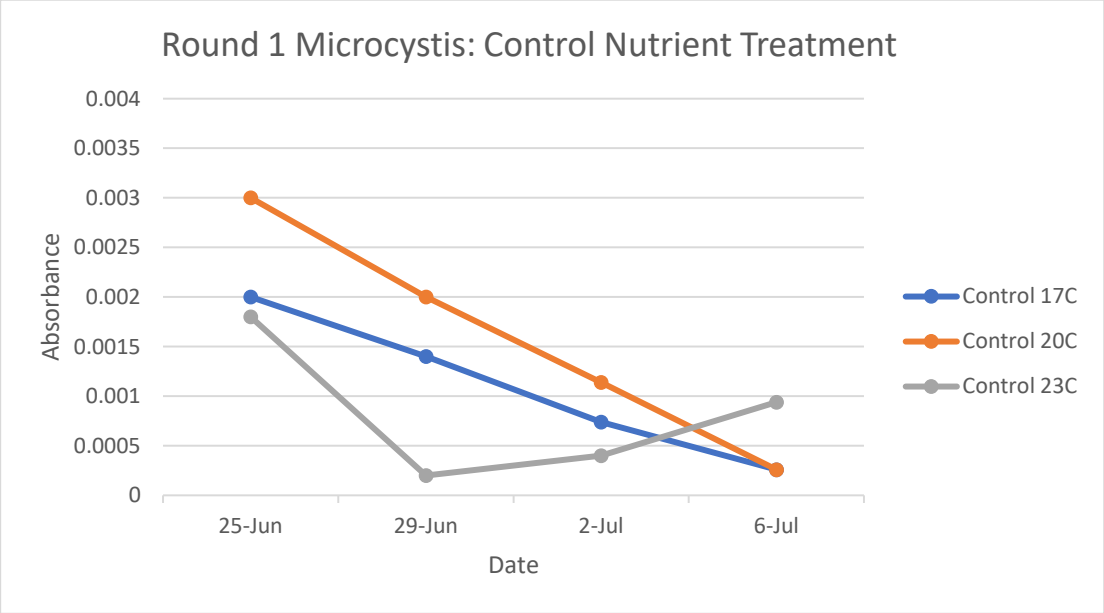
Microcystis auerginosa

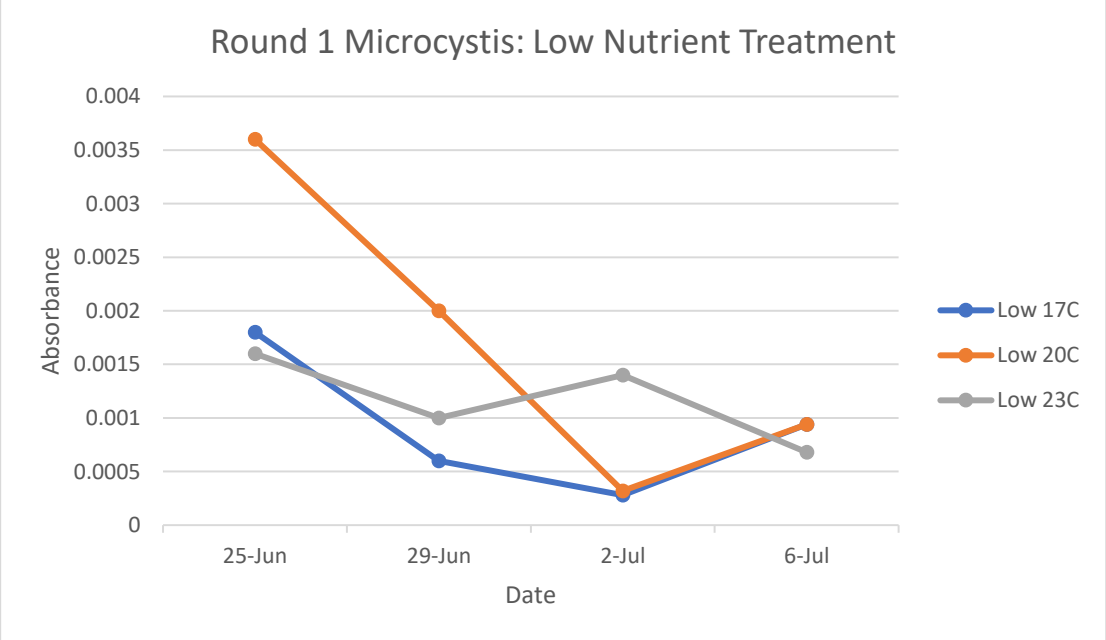
Growth trends through time for Round 1 showed limited growth, with absorbance levels decreasing slightly through time for both the control and the low nutrient conditions (Fig. 2). The high nutrient condition remained on average within the same range through time.

In our second round of experiments, the low and control nutrient conditions exhibited spikes and dips in their growth, first a spike, then a drop, then ending on a spike (Fig. 3). The high condition showed a steadier downward trend in absorbance readings. A significant effect of nutrients was found in this round of experiments ($F_{[2,39]} = 4.502$, $p = 0.017$), but no significant effect of temperature was

found (F [1,39] = 0.0019, p = 0.965), nor a significant interaction of nutrients and temperature (F [2,39] = 0.689, p = 0.508).

There was no significant effect of temperature (F [1, 39] = 1.51, p = 0. 23), nutrients (F [2, 39] = 1.33, p = 0. 28), nor was there a significant interaction between the two (F [2, 39] = 1.03, p = 0. 37) (Fig. 4). The high nutrient condition had a significantly lower mean growth in all three temperature conditions when compared to the other two conditions (Fig. 5).





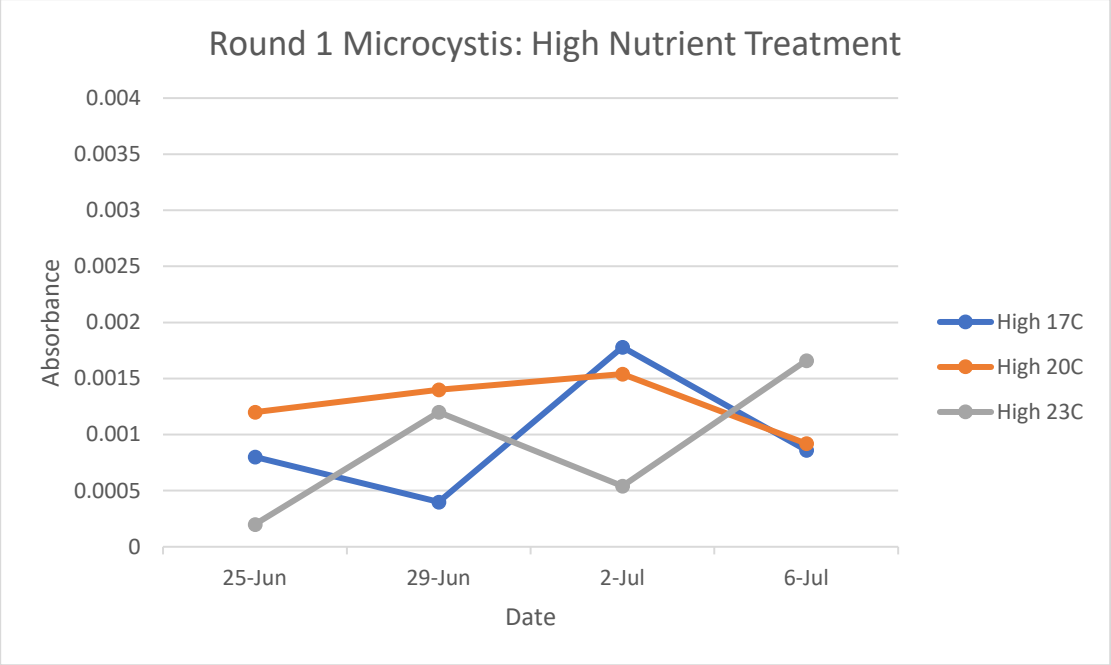
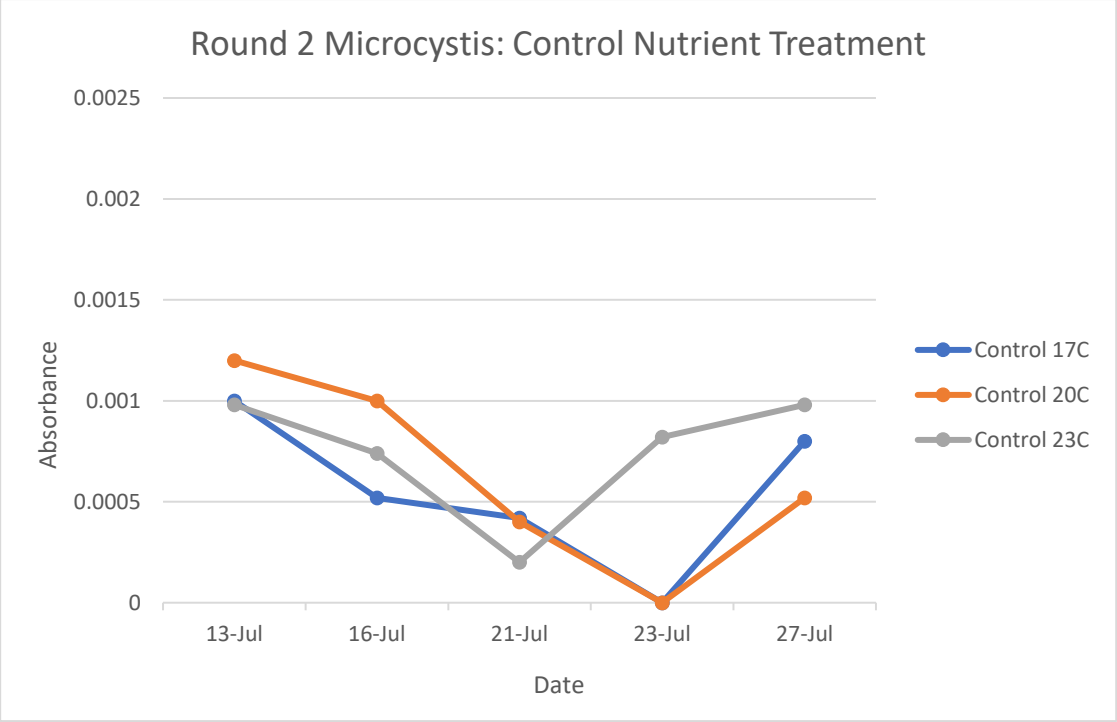
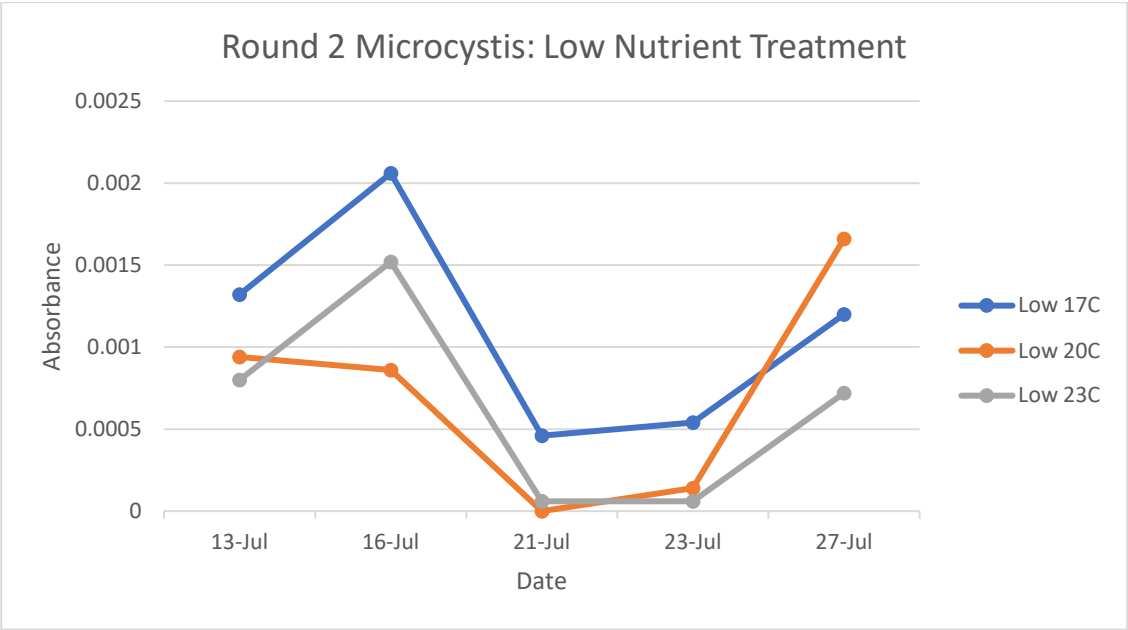


Fig. 2. Growth trends through time of the averaged replicates of the A) control, B) low nutrient, and C) high nutrient *Microcystis* cultures in Round 1. No significant trends were found in the absorbance levels over time.





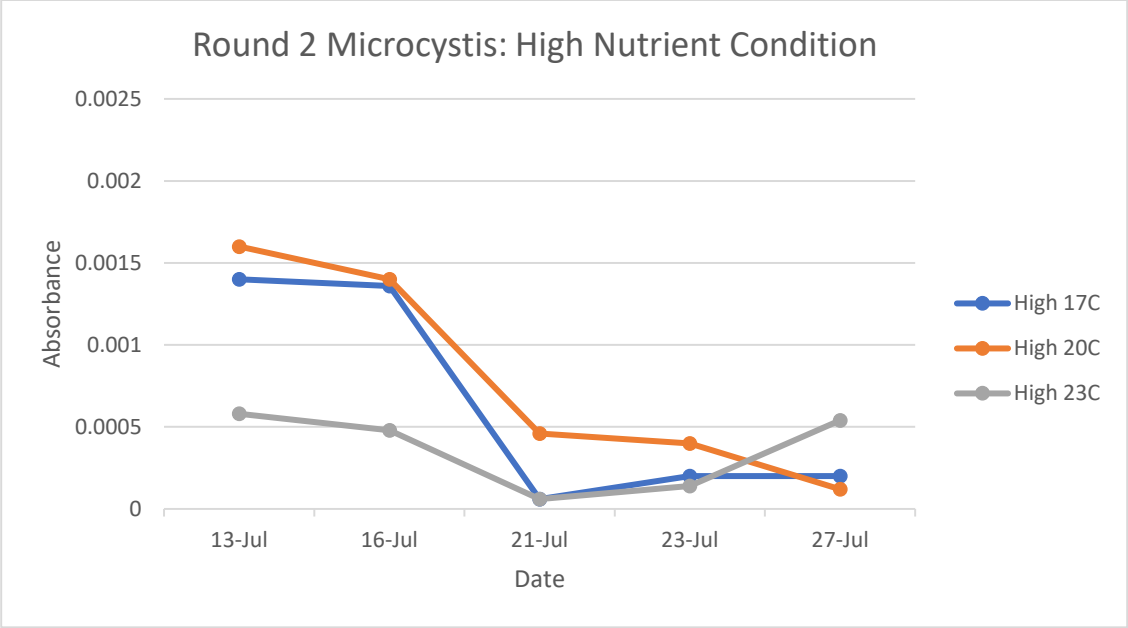


Fig. 3. Growth through time of the averaged replicates of the A) control, B) low nutrient, and C) high nutrient *M. auerginosa* cultures from Round 2. No significant trends were found.

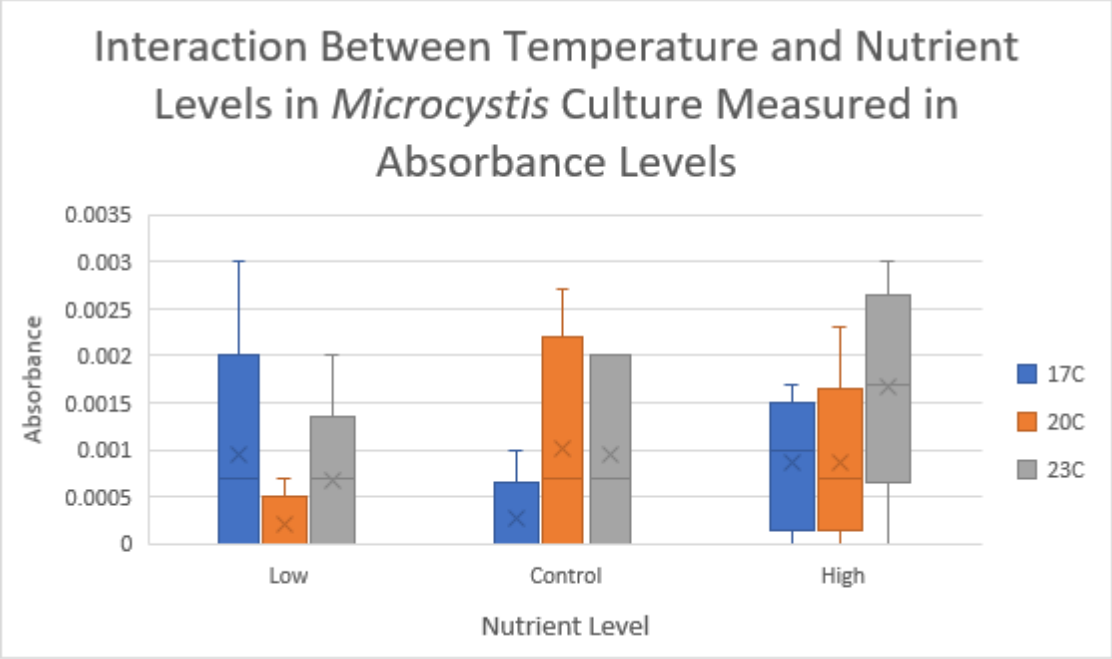


Fig. 4. A box and whisker plot of the final absorbance levels of the *M. auerginosa* cultures of Round 1. No statistically significant difference was found between the means of the different nutrient levels, the different temperature treatments, or in nutrient-temperature interactions.

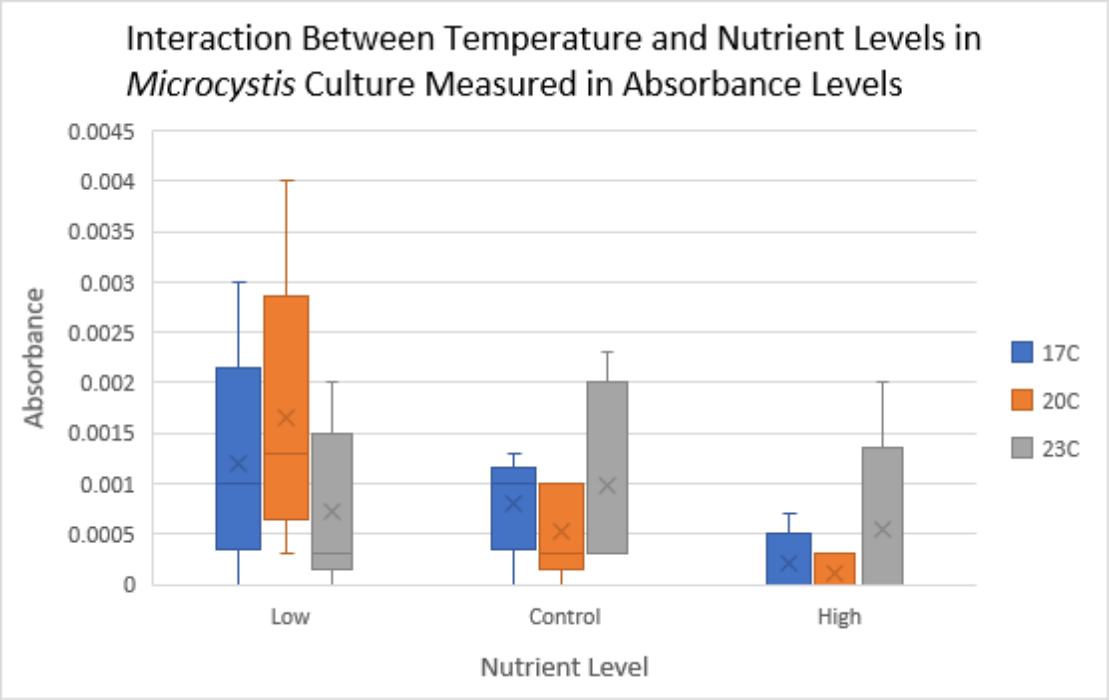
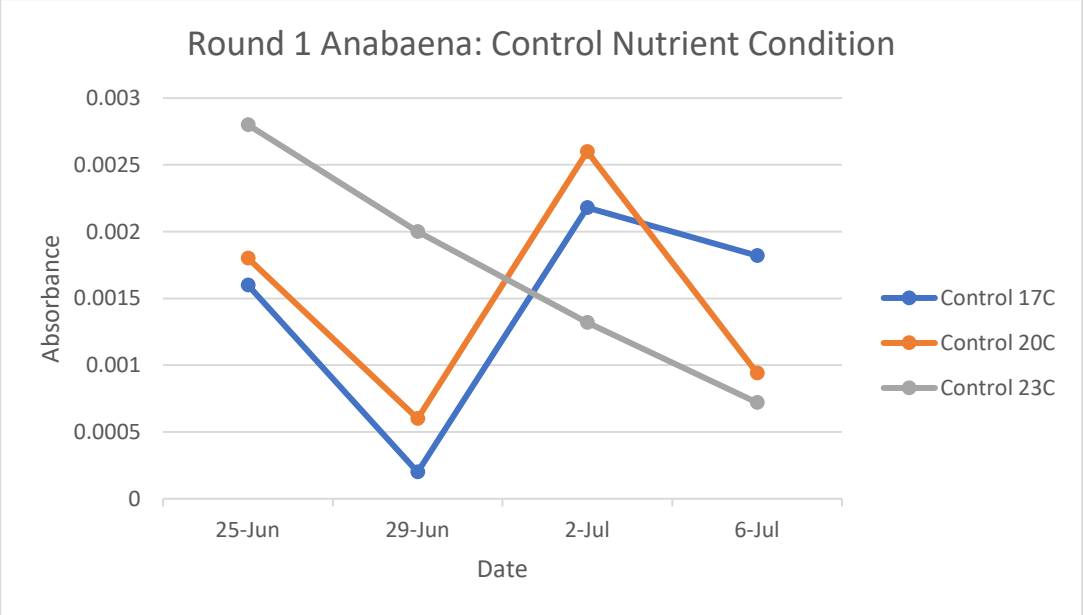


Fig. 5. A box and whisker plot of the final absorbance levels of the *M. auerginosa* cultures of Round 2. A significant difference was found in the means of the different nutrient levels (p = 0.017). The high nutrient condition has significantly lower means than the other two conditions.

Anabaena flos-aquae

The first round of this experiment yielded no significant differences in trends through time (Fig. 6). In the second round of experiments, no significant differences in trend through time were seen (Fig. 7).

There was no significant effect of temperature (F [1,39] = 0.669, p = 0.418), no significant effect of nutrients (F [2,39] = 1.634, p = 0.208), nor was there a significant interaction between temperature and nutrients in our first round of experiments (F [2,39] = 0.0207, p = 0.980) (Fig. 8). There was a significant interaction between temperature and nutrients in our second round of experiments. (F [2,39] = 3.787, p = 0.0314) (Fig. 9). There was no significant effect of temperature in round two (F [1,39] = 3.150, p = 0.0837), nor was there a significant effect of nutrients (F [2,39] = 0.203, p = 0.817).



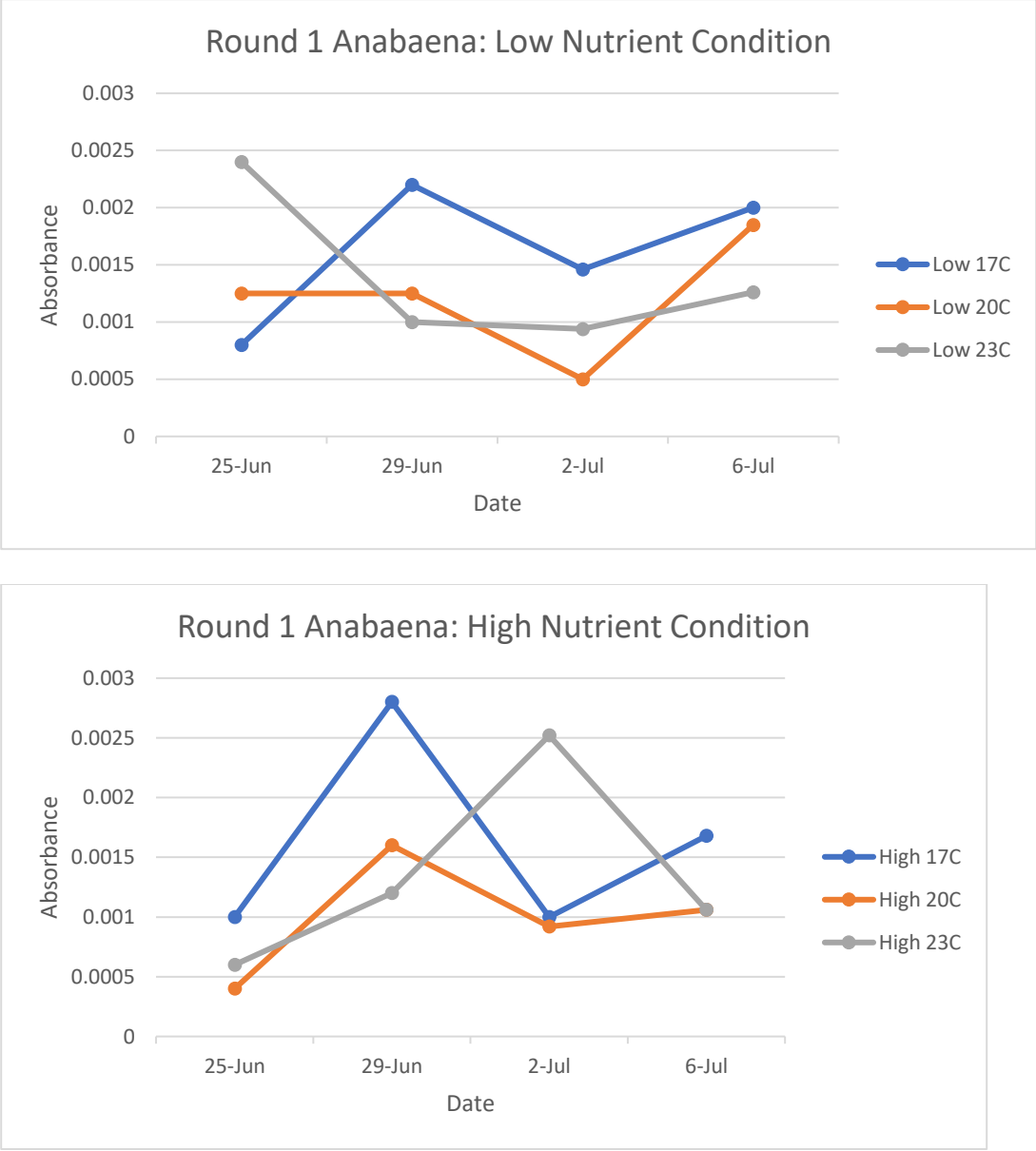
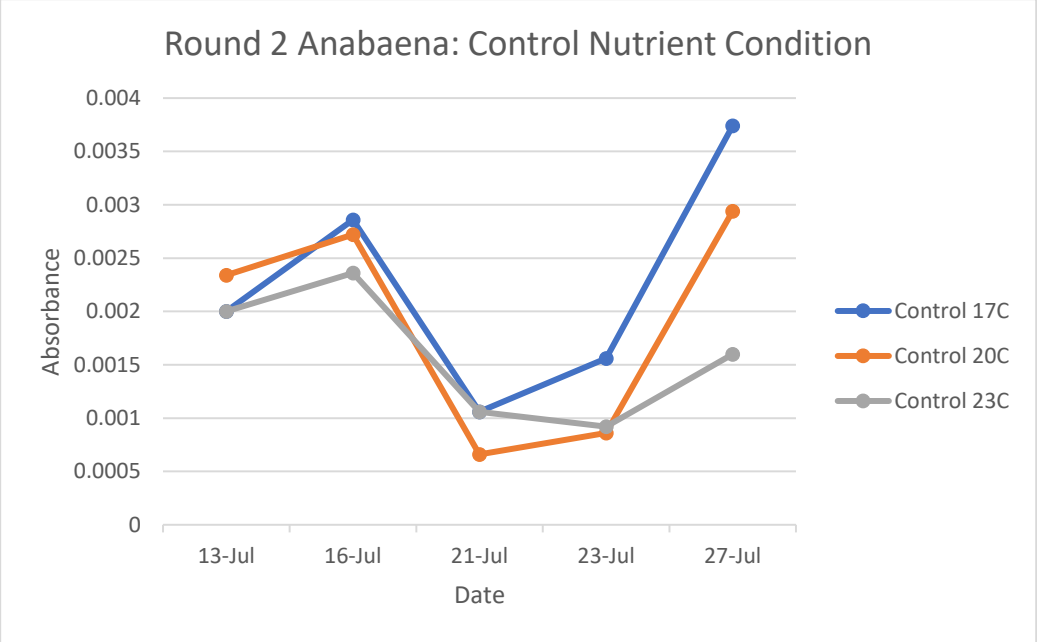
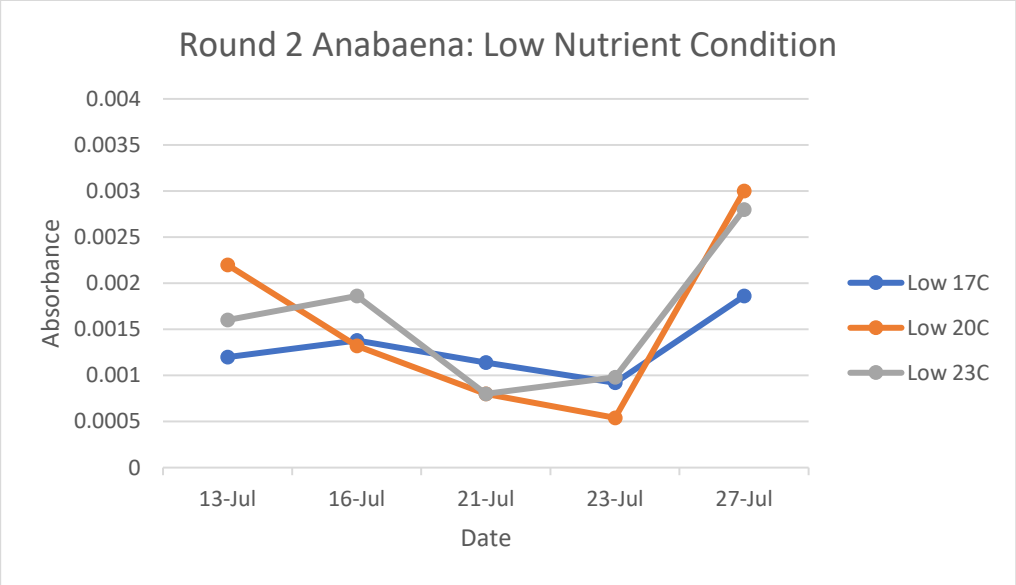


Fig. 6. Growth trends through time of the averaged replicates of the A) control, B) low nutrient, and C) high nutrient *Anabaena* cultures in Round 1. No significant trends were found in the absorbance levels over time.


Round 2 Anabaena: Control Nutrient Condition
0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
Absorbance
13-Jul
16-Jul
21-Jul
23-Jul
27-Jul
Date
Control 17C
Control 20C
Control 23C


Round 2 Anabaena: Low Nutrient Condition
0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
Absorbance
13-Jul
16-Jul
21-Jul
23-Jul
27-Jul
Date
Low 17C
Low 20C
Low 23C

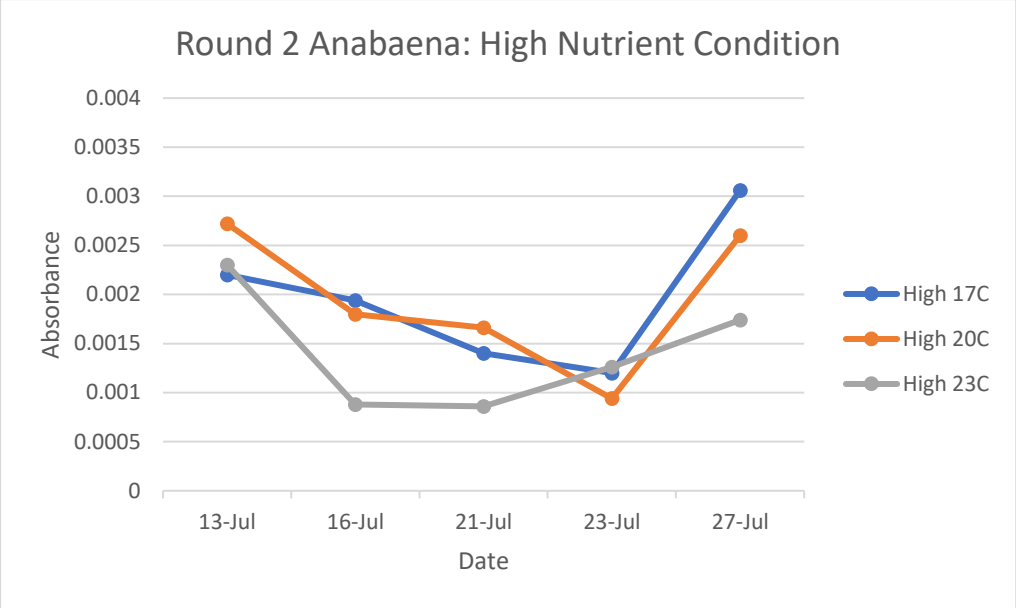


Fig. 7. Growth trends through time of the averaged replicates of the A) control, B) low nutrient, and C) high nutrient *Anabaena* cultures in Round 2. No significant trends were found in the absorbance levels over time.

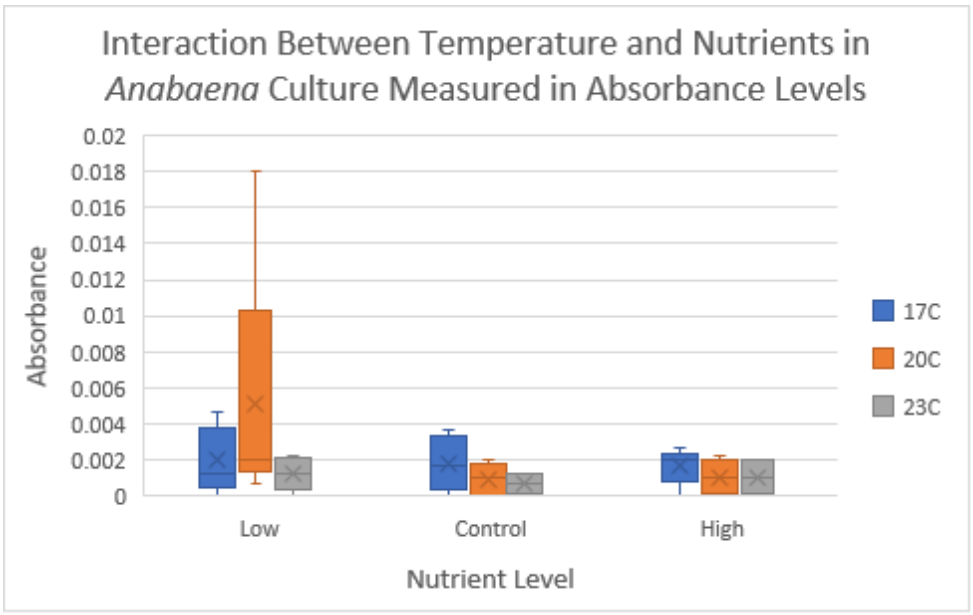


Fig. 8. A box and whisker plot of the final absorbance levels of the *Anabaena* cultures of Round 1. No statistically significant difference was found between the means of the different nutrient levels, the different temperature treatments, or in nutrient-temperature interactions.

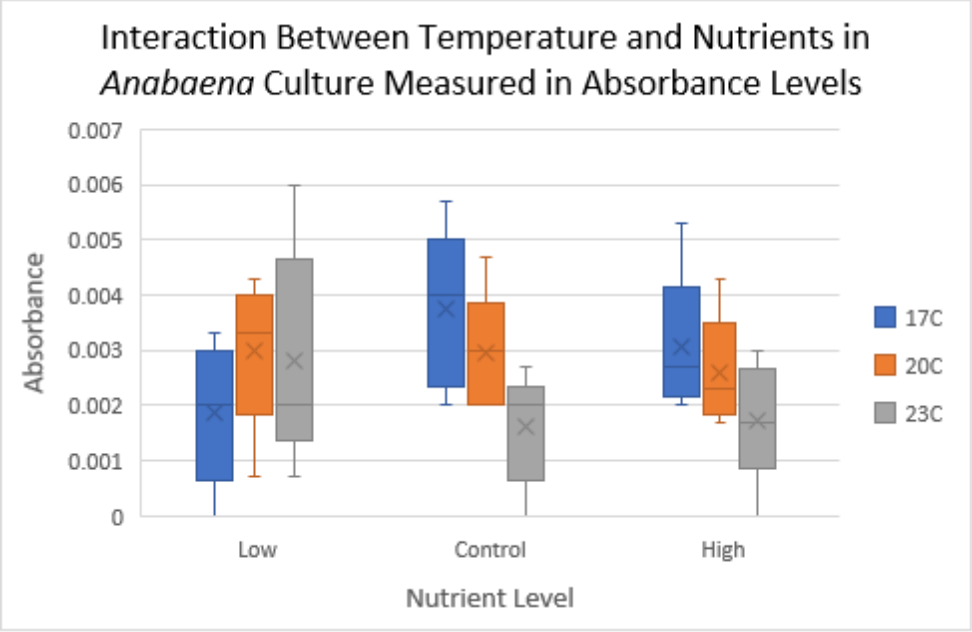


Fig. 9. A box and whisker plot of the final absorbance levels of the *Anabaena* cultures in Round 2. A significant interaction between temperature and nutrients was found ($p = 0.031$). At the low nutrient level, absorbance increases with temperature. At the control and high nutrient levels, absorbance decreases as the temperature rises.

Discussion

Overall, there were few significant results from this experiment. Our data in the second round seemed to show that nutrients do influence the species *M. auerginosa*, specifically, that higher levels of nutrients lead to lower levels of growth, which was the opposite of what we had hypothesized would happen. This also goes against much current research that indicates that higher levels of nutrients cause an increase of growth in this species (Herrero et. al 2001, Chen et. al 2020, Glibert et. al 2017, O'Neil et. al 2012). In addition, the *Anabaena* cultures were shown to be influenced by both temperature and nutrients, and that both factors interact to influence the growth of this species. At the lower nutrient levels, higher temperatures increase the absorbance of our samples, while at the control and high

nutrient levels we see higher temperatures causing a decrease in absorbance. This interaction needs to be explored further.

However, there were many possible confounding factors that could have altered our results, and that weakens the integrity of our results. Leaving out the soilwater medium ingredient from the first round of experiments could have changed how the species grew, the amounts of culture we used could have been too small, and our experimental conditions may not have been extreme enough to produce a result in the purchased cultures. Another issue we may have encountered was an unintentional dilution effect from the way that we fed our species. Pouring off the old medium could have resulted in some bacteria being removed as well, which would have lowered our absorbance readings. Another potential issue is that the water baths could have made it difficult for the light to reach our samples, which would have hampered the growth of our samples. Any of these issues could have skewed our results and caused our cultures not to grow or caused them not to grow as well as they could have.

In addition, many of our readings were very small numbers, and they were within the error bounds of the spectrophotometer we were using (±0.003), which severely limited the accuracy and reliability of our results. This is, perhaps, the most likely suspect for much of the error we may have seen in this experiment, and in the future, it may be helpful to either find ways past the limitations of this machine, or to use a different tool to measure our results. One option would be manually enumerating all our samples, which was not done in this experiment for the sake of expediency, that would ensure much higher accuracy than we saw in this work. Another potential tool would be to use a fluorometer. There are companies that make fluorometers specifically for measuring HCBs, such as the Turner company, and it may be able to overcome the limitations of the spectrophotometer.

Given these potential limitations, limited growth across all treatments, and the differing results between rounds 1 and 2, there are limited broader conclusions from this work. The finding in one experiment that nutrients might lead to lower growth is especially surprising and contradicts much research that has been done with these species. Many studies have shown that nutrients, specifically

nitrogen and phosphorous, are very influential in the growth of HCBs (Herrero et. al 2001, Chen et. al 2020, Glibert et. al 2017). An increase in these nutrients tends to lead to an increase in the number of blooms (O'Neil et. al 2012). Nutrient control has also been suggested as a viable method for controlling HCBs (Paerl 2017), indicating its great significance.

There are many options going forward from this experiment. In the future, it may be beneficial to test the effects of temperature and nutrients separately. In addition, it may also be beneficial to run the experiment for longer in order to allow the cultures more time to grow. Another option would be to run this same experiment with other species found in Wyoming. Finally, further efforts could be made to more closely replicate the average climate conditions in Wyoming, such as using samples of cyanobacteria taken from affected lakes and reservoirs in Wyoming.

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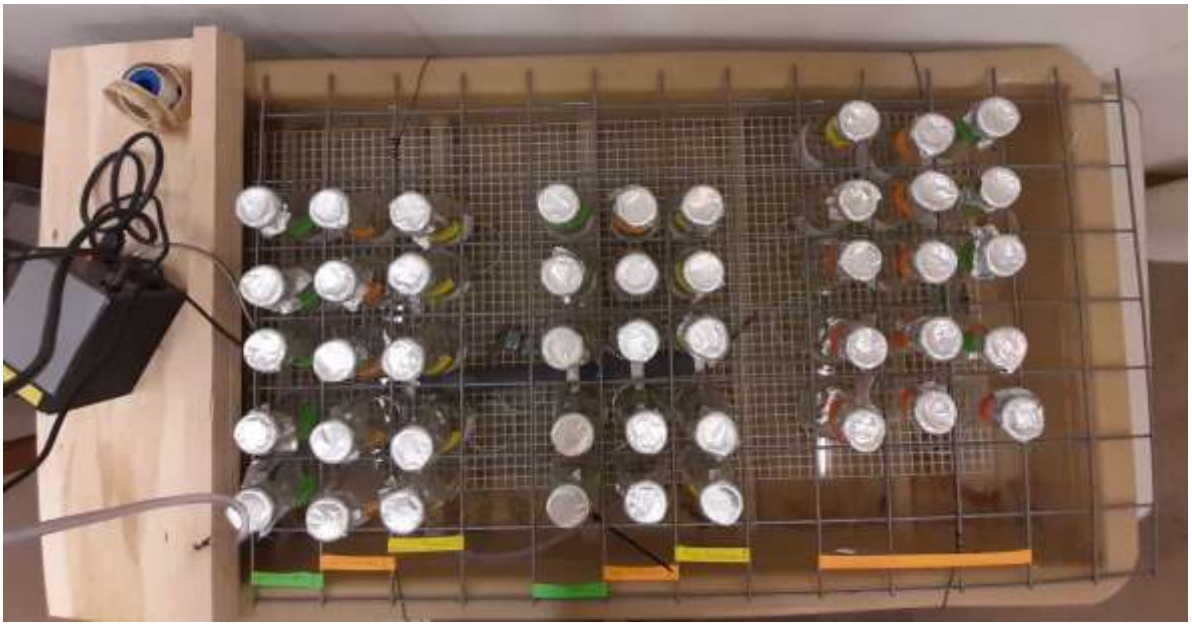
Appendix I: Experimental Design Photos

The leftmost tank was the 17℃ tank, the middle tank was 20℃, and the rightmost blue tank was 23℃.



Bottles labeled in green were the control nutrient condition, orange were low nutrient condition, and yellows were high nutrient condition. The first three rows were the *Microcystis* cultures, the second set of three rows were the *Anabaena* cultures, and the last three rows were the bottles for an experiment that was being run concurrently to mine.

Appendix II: Sample Collection and Feeding Protocol

1. 15mL of medium was poured out of the sample bottles. Bottles were not shaken or otherwise disturbed beforehand to reduce the risk of pouring off cyanobacteria cells.
2. Bottles were then swirled somewhat forcefully to stir up the bacteria.
3. 2mL subsamples were taken from each bottle after they were swirled and used to obtain absorbance readings.
4. Absorbance readings were taken at 665nm for both species.
5. Subsamples were then placed in a waste container.
6. 15mL of fresh medium were then added back into the bottles after all the samples were taken.
7. The waste accumulated that day was then killed with approximately 15mL of 200 proof ethyl alcohol.

Appendix III: Data Tables for Standard Curve

Absorbance Readings

	1.5mL Culture	1.25mL Culture	1mL Culture	0.75mL Culture	0.5mL Culture	0.2mL Culture	0.1mL Culture	0.05mL Culture
Rep_1	0.007	0.007	0.009	0.002	0.002	0.002	0.001	0
Rep_2	0.007	0.007	0.009	0.003	0.002	0.002	0	0*
Rep_3	0.007	0.008	0.009	0.002	0.001	0	0	0.002
Mean	0.007	0.007333333	0.009	0.002333333	0.001666667	0.001333	0.000333	0.000667

*****Read as -0.001 but was changed to 0 as per protocol, which stated that any negative readings were to be interpreted as zeros.

Average Cell counts per mL

1.5mL Culture	**1.25mL Culture**	**1mL Culture**	**0.75mL Culture**	**0.5mL Culture**	**0.2mL Culture**	**0.1mL Culture**	**0.05mL Culture**
281960	226460	122400	105180	82980	26420	10280	3560

Data for Plot Microcystis Standard Curve

	X	Y
	Absorbance at 665 nm	Cell concentration (cells/mL Bold 3N Medium)
1.5mL Culture	0.007	281960
1.25mL Culture*	0.007333333	226460
1mL Culture	0.009	122400
0.75mL Culture	0.002333333	105180
0.5mL Culture	0.001666667	82980
0.2mL Culture	0.001333333	26420
0.1mL Culture	0.000333333	10280
0.05mL Culture	0.000333333	3560

*Was excluded from the final graph