Novel Methods of Mitigation and the Evaluation of Wyoming Waterbodies for Cyanobacterial Bloom formation

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Project Summary

As the earth continues to experience climate change, greater amounts of Cyanobacteria are being found in run-off waterways throughout the state of Wyoming. Cyanobacteria is able to release toxins called Cyanotoxins into the surrounding water. The toxins released by the Cyanobacteria pose a threat to the safety of consuming water from water bodies in Wyoming and other places across the globe for both humans and animals.

Current eradication methods are costly and can end up being more detrimental to the environment, since introducing foreign metals can become toxic for most plants and animals at high concentrations. These metals can also create toxic sludge-like by products that will kill most living organisms in the environment, as well as become a competitor for many others. Methods are currently being studied to provide a more sustainable eradication approach that is cost effective and non-toxic to the environment. Prior research suggests that environmental factors play a role in causing Cyanobacteria to become toxic (Neilan). Several environmental factors that have been found to impact the expression of cyanotoxins include sunlight, CO2 concentrations, nitrate, phosphate and iron levels (Neilan). Out of these environmental factors, iron is unique because it can be controlled relatively easily in water bodies unlike sunlight and CO2 levels, and in the absence of iron, toxic blooms are more likely to form. It has been seen that phosphate and nitrate levels are inversely correlated with iron concentrations during toxic cyanobacterial blooms. Because Cyanobacteria release toxins at lower concentrations of Iron in the environment, this implies that there must be a threshold of Iron concentration that needs to be reached before blooms begin releasing cyanotoxins. We propose that Cyanotoxins must be released from Cyanobacteria when the iron concentrations in their environment drop below a threshold concentration.

As another method for controlling Cyanobacteria blooms, we propose that Cyanobacteria can be outcompeted for nutrients by other bacteria that utilize the same nutrients to grow. We have chosen to utilize Agrobacterium tumefaciens as the natural competitor of the blooms, due to its ability to compete with other bacteria for resources via secretion T6ss complex. The complex will be utilized because of its ability to interrupt interaction between microbes in larger microbial communities. Through culturing of Cyanobacteria in the lab with the presence of a growth competitor as well as altering iron concentrations in the environment, the goal of this research is to determine if *Agrobacterium tumefaciens* or iron are available methods to control harmful Cyanobacteria blooms (HCB) in Wyoming waterways.

Intellectual Merit: This research will further our understanding of how iron, nitrates and phosphates impact Cyanobacteria's fate when forming cyanotoxins. This research will further our understanding of natural competitors (Agrobacterium) for Cyanobacteria and how those competitors affect Cyanobacteria growth and bloom size. With this research, we could potentially determine the specific factors that are needed to induce the formation of cyanotoxins in Cyanobacteria, as well as methods for controlling growth in Wyoming waterways.

Broader Impacts: If a threshold concentration that needs to be maintained to prevent the formation of Cyanobacterial toxins is determined, this knowledge could be applied to natural water bodies to predict or even prevent the release of these toxins in water bodies across Wyoming. If competition is observed, then the research can be utilized as a possible method for cyanobacteria mitigation. This project will be conducted by 5 undergraduate students from the University of Wyoming who will see this research through from conception to completion while engaging the Wyoming population to increase knowledge and prevent incidents of exposure. Incorporating an outreach program to notify and educate the general public about cyanobacterial blooms will also help in the reporting and testing of Wyoming water bodies. As a diverse group of undergraduate researchers, this project will allow for the effective absorption of critical thinking and hands-on problem-solving techniques. As well as give back to science in the form of novel testing methods, mitigation techniques and standardization of cyanobacterial bloom conditions.

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Project Description

Statement of Problem Significance

Cyanobacteria, which is commonly referred to as blue green algae, has recently become an issue of global prominence, this is most likely due to driving factors such as climate change (Neilan et. al, 2013). Though cyanobacteria and their toxins have been known and associated with overabundance of nutrients for quite a while, it was not until recently that we started testing for incursions into headwater states, such as Wyoming. Cyanobacteria can exhibit harmful cyanobacterial blooms or HCBs, that emit a foul smell when they decay and produce cyanotoxins that have been known to kill household animals and livestock (Sukenik et.al, 2021). Certain strains of Cyanobacteria are able to produce microcystins, one of the many toxins cyanobacteria can produce, which are capable of inhibiting covalent binding of protein phosphatases, cell death, and structural disruption of cytoskeleton elements (Tian et. al, 2021). There is also preliminary data displaying further chronic complications relating to exposure to the toxins that impact the cardiac, nervous, and reproductive systems. These toxins can be transmitted through drinking/ingesting contaminated water or consuming material exposed to the algae. Outdoor recreation, tourism, and ranching are all impacted by contaminated water not only by having restricted access to water if an area becomes infected, but if the toxins are ingested the repercussions could be as serious as death. Fortunately not all Cyanobacteria blooms are toxic, but a non-toxic bloom can become toxic in the right conditions. The main resources that influence the growth and potentially the toxicity of Cyanobacteria includes nitrogen, phosphorus, iron, CO2, and light (Neilan). Unfortunately, there is limited knowledge in the science world on the specifics of how much of each resource is needed to cause a Cyanobacteria bloom to become toxic. Because of the lack of data on testing it is not well known the range of Wyoming water bodies containing blue-green algae and to what extent they are affected. In understanding the specifics of what resources induce Cyanobacteria blooms to become toxic, more proactive measures can be taken in Wyoming's water bodies to prevent Cyanobacterial toxins from being expressed and released into the water through monitoring the resources that are in these water bodies and controlling the levels of resources that are present in each water body.

Current measures to eradicate blooms utilize harsh chemicals or the introduction of metals into water bodies is harmful to the natural flora. New research is emerging with different options of eradication being the introduction of horseradish root oil or different strains of macroalgae. Determining an eradication/control method that lessens the overall impact to the existing environment as well as being cost effective would help to combat the toxin in waterways. One method that has proven effective in causing competition and elimination of blooms has been the use of *Agrobacterium tumefaciens* (*A.tumefaciens*). *A.tumefaciens* is most known for altering genes in many plants to cause them to grow tumors where the bacteria can replicate. A lesser known ability of agrobacteria is the delivery of T6ss effector proteins which can “antagonize” other bacteria as well as inhibit interactions between the bacteria in larger communities (Munoz et. al, 2021).

Relevant Literature

The first organism to utilize oxygen as a growth factor was cyanobacteria almost 3 billion years ago and can be attributed as the creator of life as we know today (Gomelsky, 2021). Without cyanobacteria blooms, Earth would be devoid of an ozone layer and organisms would be greatly impacted by harmful UV rays from the sun. As the earth begins to heat up and the climate becomes riddled with more metals and pollutants, cyanobacteria blooms have become increasingly prevalent in fresh water runoff. The increased prevalence of cyanobacteria blooms is creating major problems with water supplies due to the blooms producing toxins that are harmful to the natural fauna, agriculture, and any recreational activities in contaminated areas (Tian 2021). Cyanobacteria is able to release toxins called Cyanotoxins into the surrounding water. There are several toxins that can be released from Cyanobacteria. These toxins include: hepatotoxins microcystin and nodularin, the cytotoxin cylindrospermopsin, the neurotoxins anatoxin and saxitoxin, and the dermatotoxin lyngbyatoxin. The most common of these toxins is Microcystin-leucine-arginine (MCLR) which is also the most toxic of the toxins. The gene that codes for the microcystin toxin is well known and is a common indicator of whether the Cyanobacteria has become toxic or not (Neilan, 2012). High expression of microcystin is correlated to high concentrations of nitrogen and phosphorus (Vezie), and high intensity of light (Neilan,2012). It has also been determined that the concentration of iron is inversely proportional with the release of microcystin (Fujii, 2010). Also, the microcystin gene has a central regulatory region that contains sequence motifs for a ferric uptake regulator which points to iron playing a role in controlling the microcystin gene epigenetically (Neilan, 2012). As seen in a previous study of *Microcystis aeruginosa*, when grown in depleted iron levels (10 nM Fe) microcystin levels increased notably (Alexova, 2011).

Humans come into contact with the toxin through drinking contaminated water, ingesting fish (including shellfish) or vegetables grown around toxic blooms, or using toxic algae as dietary supplements (Tian 2021). The toxins are known for causing GI tract problems, neural toxicity, liver toxicity, and spontaneous cellular apoptosis. If they don't result in death many of the toxins have been linked to cancers of the liver, kidney, brain, and a few more. Ingestion of the toxins in most cases is fatal and there is no current treatment for combating the toxin. The concentration of Cyanobacteria and toxins are poorly regulated, due to the surveying of water bodies along with testing water toxicity is a strenuous, time-consuming process which can only determine blooms after they have become toxic or will display images of blooms with no indication of toxicity. Some testing methods that are currently being researched include studying sediment near bodies of water that are known to have had blooms in years past due to "Many studies...suggest that sediment serves as the major inoculum for subsequent summer blooms." (Bácsi et. al, 2021). Other methods involve testing the waters for the presence of nitrogen and Phosphorus which are two key growth factors for cyanobacteria. The state of Wyoming utilizes aerial pictures of water bodies to monitor the spread/prevalence of blooms in the water. There is a call to action for creating better testing methods that will help to discover toxic blooms before

they start producing toxins as well as discovering methods that can safely contain and kill blooms.

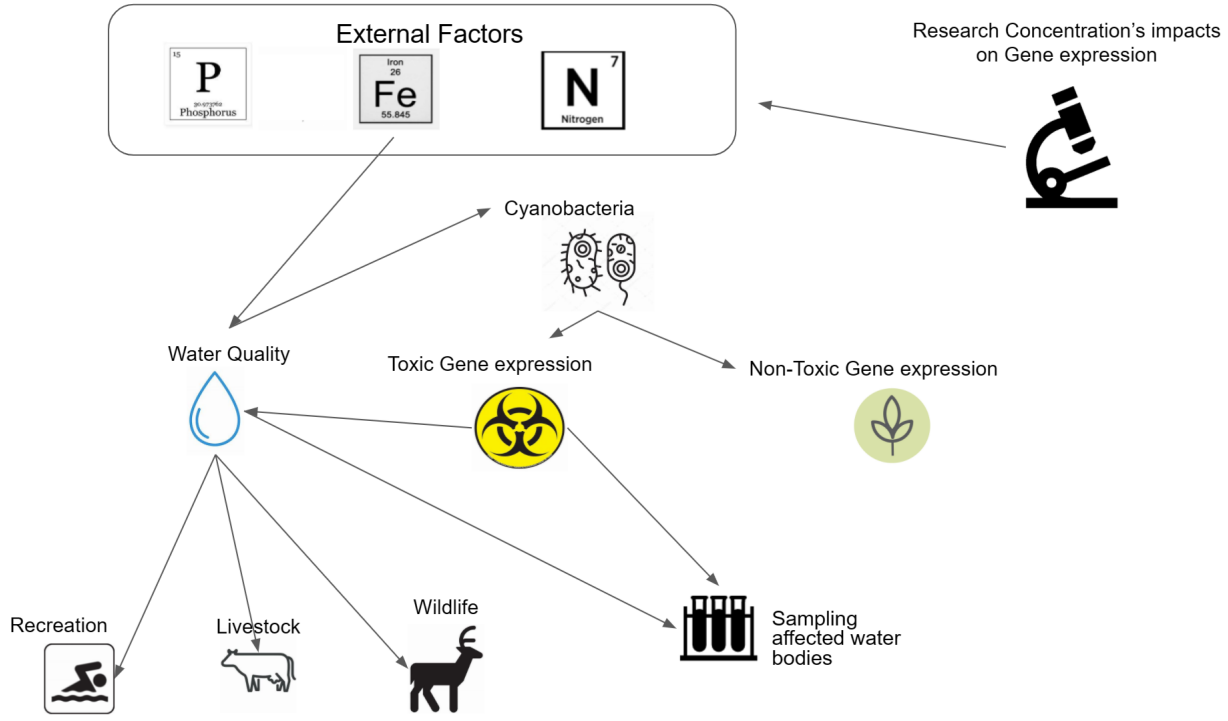
Current measures for containing or eliminating the blooms utilizes many harsh chemicals which impact the environment by killing the blooms and some surrounding flora. One harsh method utilizes metal (Aluminum, iron, or Copper) compounds to remove phosphorus which is a necessary growth factor for the bacteria. A second method utilizes chemicals to create toxic oxygen species that become reactive under light which are used to aggravate and eliminate the blooms. A final method utilizing toxic metals is traditional herbicides. These methods have harmful effects including: the production of toxic sludge, irritation or toxicity to other organisms, and the formation of toxic by-products (Bácsi et al, 2021). Alternative methods are being currently researched on how to create effective measures to eradicate blooms utilizing natural ingredients that don't produce any harmful effects on the environment. One method being researched utilizes different strains of naturally occurring macroalgae to inhibit growth of cyanobacteria via secretion of allelopathic compounds. Allelopathic compounds are secondary metabolites which have been used to eliminate competitors in a shared environment with the macroalgae. The macroalgae has been shown to reduce Cyanobacteria growth as well as restrict the growth of other species in the combined environment. This method shows less direct harm to the natural flora but still presents undesirable effects which are being studied further before the method becomes effective (Budzałek et al, 2021). Another method currently being researched utilizes horseradish root to contain blooms and reduce bloom size, which once again won't disrupt the natural flora while effectively eliminating toxic blooms. The research focuses on the roots production of the secondary metabolite- Glucosinolates which produces Isothiocyanates that have antibacterial properties. These enzymes can affect oxidative phosphorylation (main energy production mechanism for Cyanobacteria) and damage to DNA (Bácsi et al, 2021). The study showed a measurable decrease in bloom size as well as the ability to be used as a growth inhibitor on a small scale model. Further research is being done on how to bring this method to a larger scale for effective use in major waterways.

Agrobacterium Tumefaciens (*A.tumefaciens*) is a bacteria that is most commonly known for altering genes of plants to create large tumors that facilitate replication of the bacteria. *A.tumefaciens* also has the ability to secrete proteins that are able to antagonistically engage with other bacteria and facilitate competitive behaviors. These proteins are part of a system called T6ss which is utilized for competition and other functions. T6SS protein is utilized when the bacteria comes in direct contact with another organism, once covalently bound core components and core effectors can initiate protein secretion. One major function that affects bacteria is the ability to break down peptidoglycan walls or cellular membranes. A second key function is the ability to degrade bacterial nucleic acid. The final key function of T6SS that makes A.tumefaciens a good competitor is the ability to uptake DNA from other lysed cells to integrate into its own genes or be utilized as a carbon source. With this machinery and the ability to produce biofilms, *A.tumefaciens* could be a method for eradicating other bacteria in an environment (Wu et. al, 2018).

Preliminary Data

Wyoming Department of Environmental Quality (DEQ) has primarily used satellite imagery to determine if a bloom is present then collected some preliminary data over the last few months, and have classified different bodies of water under either current toxin advisory, current bloom advisory, or under investigation. In Wyoming, Leazenby Lake is now being classified as a current toxin advisory, and a current bloom advisory. West granite springs is currently under a bloom advisory, and Twin Buttes Lake is classified as under investigation. As of each water body's last sampling time as done by Wyoming DEQ, West Granite Springs Reservoir has a microcystin concentration of <0.15ug/L, while Leazenby Lake has a microcystin concentration of 0.634ug/L and Twin Buttes Lake has shown to have low levels of microcystin but enough to be under investigation. This information highlights the current problems Wyoming is facing with cyanotoxins in it's water bodies. Regarding this specific research, a potential starting concentration of Iron that may induce the formation of cyanotoxins has been expressed. A prior research team was able to find that ≤ 2.5 μM iron caused *Microcystis aeruginosa* Cyanobacteria to release 20-40% more cyanotoxins (Lukac).

Conceptual Model



Justification of Approach

To identify a threshold concentration of iron that must be maintained to prevent the formation of Cyanobacterial blooms, Cyanobacteria has to be grown in the different concentrations of iron and tested for toxicity. Microcystins are great cytotoxins to test for because they comprise the largest and most structurally diverse group of cyanobacterial toxins and they can be easily tested for by using ELISA (Neilan). To be able to identify the role other environmental factors play into

the expression of cyanotoxins, one of the factors needs to be isolated and studied in how it influences the formation of cyanotoxins to be used as a control group. In this experiment, iron is the most logical factor to study as the control. Once the control is studied and the role of what it plays in inducing the formation of cyanotoxins, other variables can be studied to see how they and the control impact the expression of toxicity. In this experiment, phosphorus, light, nitrogen, and CO2 all work as variables that are studied to see how they impact the control iron threshold that prevents the release of cyanotoxins.

For this experiment we will utilize Bristol's broth as our medium to cultivate Cyanobacteria blooms as well as utilize imagej for visualization of the colonies. Our method for culturing using Bristol's broth is supported by Ward's Science guide to handling Cyanobacteria and other Algae in a lab. This broth also supports the addition of other nutrients which is necessary for portions of this experiment. Bristol's broth contains chemicals that are readily available in our lab which allows for the ability to synthesize new broth at any time, allowing for multiple tests to be run and new broth to be made up in case of contaminated cultures.

Research Plan

Objectives

- To determine if there is a threshold concentration of Iron ions in water bodies of Cyanobacteria that induce the Cyanobacteria to release toxins.
- To find the specific threshold of Iron ions that must be present to prevent the formation and release of Cyanobacterial toxins.
- To explore how Iron testing in water and Iron supplements could potentially suppress the release of cyanotoxins from Cyanobacteria.
- To utilize the knowledge of Iron concentration thresholds to suggest an easy test to predict the likelihood of toxic Cyanobacteria bloom formation.
- To determine if *agrobacterium tumefaciens* can inhibit Cyanobacteria in a controlled environment.
- To set up outreach within the local community to help inform and educate residents about dangers to personal health and that of their animals.

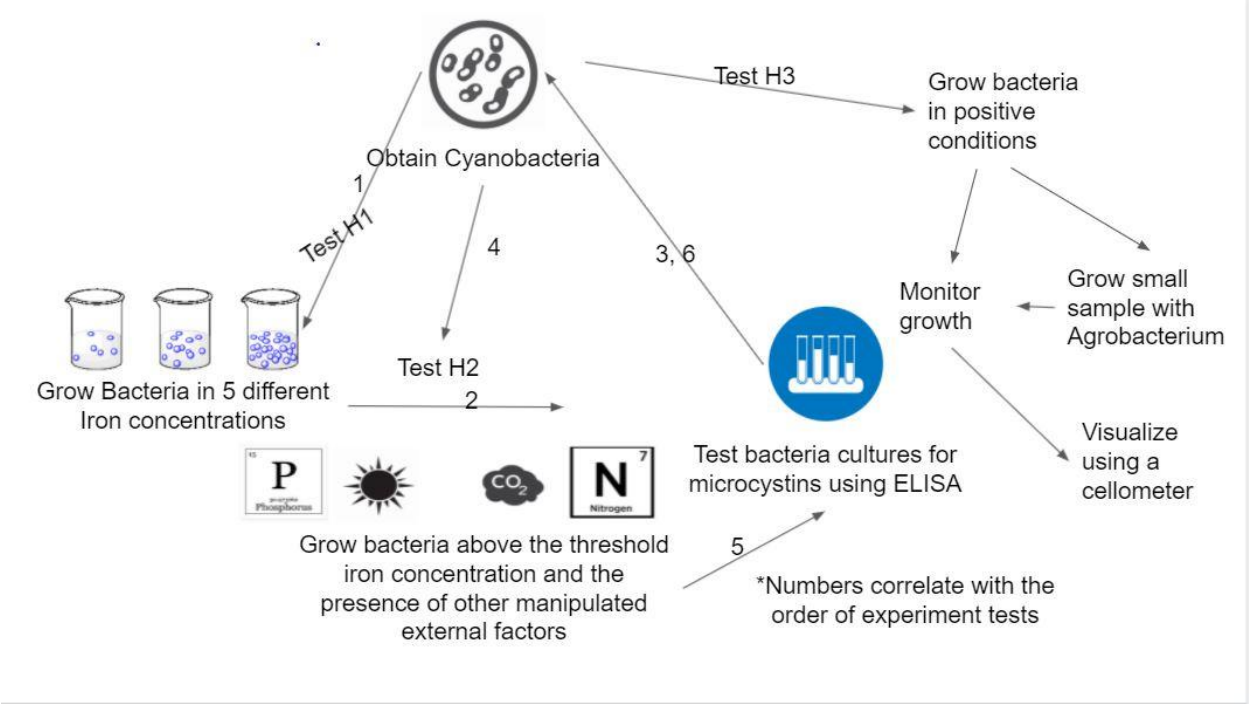
Hypotheses

H1: Mycrocystins will be released from Cyanobacteria at a detectable level when the iron concentrations in solution drop below a measurable concentration termed a threshold.
H2: The threshold concentration of Iron is able to induce the formation of Cyanobacterial toxins independent of sunlight, dissolved CO2, Nitrogen or Phosphorus levels.
H3: If *Agrobacterium tumefaciens* is added to a media containing Cyanobacteria, the Cyanobacteria cell numbers will decrease ~~due to competition f… nutrient~~s.

Specific Aims

- To grow Cyanobacteria in specific iron concentrations to identify the concentration that induces microcystin release.
- Create an artificial environment in the lab that closely resembles the conditions of naturally growing Cyanobacteria blooms.
- Determine the specific threshold of Iron that needs to be obtained to inhibit the release of cyanotoxin microcystins from Cyanobacteria blooms.
- Find what types of toxins are released under certain concentrations of Iron.
- Test if this threshold Iron concentration that prevents the formation of Cyanobacterial toxins remains constant under other changes in environmental factors like Sunlight, dissolved CO2 concentrations, Nitrogen, and Phosphorus levels.
- *Agrobacterium tumefaciens* will be added to a culture, from a freeze-down, to determine their impact on cell numbers in media compared to cell count in a non-competitive culture.

Research Design Schematic



Methods and Materials for H1 and H2

Growing Cyanobacteria cultures: The Cyanobacteria *Microcystis* was obtained from UTEX. The Cyanobacteria cultures will be grown in erlenmeyer flasks with Bristol's broth media recipe (Ward's). This media will be assembled in the lab using the recipe on the PDF in the references below. Bacteria will be grown under light at room temperature and occasional swirling in the flask. Cyanobacteria will then be introduced into 5 environments with differing concentrations of iron ions. The concentrations of iron tested will be 10nM, 25nM, 50nM, 75nM, and 100nM. The

concentrations will be assembled in 250mL Erlenmeyer flasks. To make the different Iron concentrations, a stock solution of Iron will be made in a liter solution by adding 2.702 grams FeCl3*6H20 to a 1000ml volumetric flask. Distilled water will be added to the rest of the flask until 1000mL solution is made creating a .01M Fe3+ solution. 1 mL of the .01M Fe3+ solution will be added to 999mL distilled H20 to create a 1x10-5M Fe3+ solution. From there, I will add distilled water to the respective volumes of 1x10-5M Fe3+ until 150mL solution is made of the correct Iron concentrations. For 10nM=150uL 1x10-5Fe3+ solution, 25nM=375uL solution, 50nM=750uL solution, 75nM=1,125uL solution, 100nM=1,500uL solution to make 150mL test broth solutions. These test solutions will be autoclaved to ensure that nothing else is growing in the varying Iron concentration solutions. The Cyanobacteria will grow in their respective Iron concentration environments over a 2 week span and will then be tested for the presence of microcystins using an ELISA test. Once the cultures are tested, we will have determined the lowest concentration of iron needed to prevent the release of microcystins in the culture. After the threshold concentration is determined, another experiment will be conducted using the highest concentration of Iron needed to suppress the release of microcystins. There is a potential correlation between microcystins being released in the presence of higher nitrogen, and phosphorus levels. We will test the threshold by puting the lowest amount of iron concentration needed to prevent toxicity while introducing higher nitrogen and phosphorus levels in each test sample. Each test sample will have the same concentration of iron and one of the other variables changed.

Methods and Materials for H3

To test our hypotheses, we obtained two strains of Cyanobacteria (Microcystis and Anabaena) from the Wyoming Ashleigh Pilkerton in University of Wyoming's Krist Lab. Two erlenmeyer flasks are filled with 500ml of Bristol's broth. We will be using the Bristol's broth recipe presented by Ward's Science in their instruction manual Working with Algae and Cyanobacteria. For Bristol's broth we will utilize $NaNO_3$, $CaCl_2$, $MgSO_4$, K_2HPO_4, KH_2PO_4, and NaCl. Blooms will be aerated (via a stir bar and stir plate) and placed near natural sunlight to help promote growth. Once a bloom has formed, 25ml of the culture will be removed and placed into separate erlenmeyer flasks (2 flasks). To one sample we will add *A.tumefaciens,* the agrobacterium was cultured to stationary phase. Part of the *A.tumefaciens* culture will be reserved to perform a standard plate count to determine specific titer. The *A.tumefaciens* was received from an outside source and frozen to preserve the strain. Once the culture has been able to persist for a week, cell count will be taken utilizing a cellometer. We will utilize three tests per culture to determine an average cell count per culture. The usage of the cellometer will be overseen by Dr. Zhaoje Zhang at the University of Wyoming Microscope facility.

Analysis and Expected results for H1 and H2

At the end of the experiment I would expect one of the cultures between 10nM and 100nM iron would induce microcystin formation to narrow in on the threshold iron concentration that

induced to xicity found in . With a concentration of iron at 100nM iron without microcystin formation, I would expect higher levels of nitrogen and phosphorus to cause microcystins to be released into the media. If however microcystins are not released into the media, this would be a very interesting result which may have some important beneficial implications. If this threshold is not impacted by varying concentration levels of nitrogen and phosphorus, this would indicate that testing bodies of water for iron could be an important way to determine if a bloom has the potential to become toxic or not. This could also offer a quick fix to preventing toxic blooms from forming by adding iron to bodies of water above the threshold iron concentration.

Analysis and Expected Results for H3

Data analysis will be performed via a cellometer which will be analyzed using the counting feature in the program. The images as well as the calculated data will be saved as jpeg and Tiff on a secure flash drive as well as a secure Google Drive. All data and information will be readily available to collaborators and community partners.

Table 1: Expected and Unexpected results of Cyanobacteria growth in an environment with and without the presence of *A.tumefaciens*

	Environment with *A.tumefaciens*	**Environment without *A.tumefaciens***
Cyanobacteria Growth	**Expected:** Cyanobacteria growth will be inhibited due to competition by *A.tumefaciens* and less colonies will be visualized through imageJ. **Unexpected:** Cyanobacteria population will thrive and no inhibition has occurred.	**Expected:** Cyanobacteria colonies will thrive and imageJ will display a large number of colonies. **Unexpected:** Cyanobacteria colonies will barely grow and few colonies will be visualized using imageJ.

Table 2: Expected and Unexpected results of Cyanobacteria growth in an environment with A.tumefaciens at different nutrient concentrations

	Environment with *A.tumefaciens* and standard concentration	**Environment with *A.tumefaciens* and varying nutrient concentrations**
Cyanobacteria Growth	**Expected:** Cyanobacteria growth will be inhibited by *A.tumefaciens* and few colonies will be seen via imageJ.	**Expected:** The decrease in nutrient concentration will see a decrease in Cyanobacteria colonies due to being outcompeted by

	Unexpected: Cyanobacteria populations will thrive and no inhibition has occurred.	*A.tumefaciens.* **Unexpected:** Cyanobacteria will outcompete *A.tumefaciens* and more colonies will be visualized by imageJ.

Timeline for H1 and H2

Week	Tasks	Overall picture
9/29-10/3	● Create a controlled environment in the lab Cyanobacteria can grow ○ Make a stock environment ○ Make environment to test each sample	Start
Week 1 10/4-10/10	● Add increasing concentrations of iron to each sample (10, 25, 50, 75, 100 uM) of Cyanobacteria ● Let the Cyanobacteria grow	Grow Cyanobacteria in different Iron concentrations
Week 2 10/11-10/17	● Continue letting the Cyanobacteria grow	Growth
Week 3 10/18-10/24	● Test the samples for the presence of microcystins	Test Cyanobacteria samples for microcystins
Week 4 10/25-10/31	● Keep the concentration of iron just above the threshold and change the concentration levels of nitrates and phosphates	Study how other factors impact the determined threshold iron concentration that induces Cyanotoxin release
Week 5 11/1-11/7	● Continue letting the Cyanobacteria grow	Growth
Week 6 11/8-11/14	● Test for microcystin toxins in the samples to see if changing other external factors impacted the iron threshold that kept cyanotoxins from being formed	Test Cyanobacteria samples for microcystins
11/15-11/18	● Finish Testing for microcystins ● Clean up lab materials	Finish

Timeline for H3

Week	**Tasks**
Week 0 - Preliminary	- Make up test broth - Order *A.tumefaciens* - Add Cyanobacteria to broth - Freeze *A.tumefaciens* and Cyanobacteria
Week 1 - Testing	- Freeze *A.tumefaciens* - If unable to, this is time reserved to find a new method of promoting growth. - Add *A.tumefaciens* to media to cultivate
Week 2 - Testing	- Add *A.tumefaciens* to one Cyanobacteria culture - Allow Cyanobacteria to grow with *A.tumefaciens*
Week 3 and 4 - Testing and Data Collection	- Visualize via Cellometer - Plot data in graphs and collect visuals of cultures
Week 5 - Review	- Allows for longer periods if needed for growth of cyanobacteria in broth.

References

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Accessed through the University of Wyoming Library database. This article clarified what has already been found regarding nitrogen and phosphorus both being found to have higher concentration in the presence of cyanotoxins.

Lukac, M., and Aegerter, R. (1993) Influence of trace metals on growth and toxin production of Microcystis aeruginosa. Toxicon 31: 293–305.

Accessed through the University of Wyoming Library database. This article gives a framework of past experiments done that have indicated where a threshold concentration of iron to induce a cyanotoxin bloom could be.

Ward’s Science (2021). www.wardsci.com/www.wardsci.com/images/Wards_Working_with_Algae_Cyanobacteria_Literature.pdf

Accesed through pdf. This contains the Bristol media protocol we will use grow our Cyanobacteria cultures.

Budzałek G, Śliwińska-Wilczewska S, Klin M, Wiśniewska K, Latała A, Wiktor JM. 2021. Changes in Growth, Photosynthesis Performance, Pigments, and Toxin Contents of Bloom-Forming Cyanobacteria after Exposure to Macroalgal Allelochemicals. Toxins (Basel). 13(8):589. doi: 10.3390/toxins13080589. PMID: 34437460; PMCID: PMC8402365.

This study was accessed through NCBI. This research was what sparked the notion of utilizing a competitive bacteria or other algae against the Cyanobacteria blooms. The methods used in this research were referenced when determining our overall methods.

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This study was accessed through NCBI. This research helped to form an overall background for cyanotoxins and their ability to affect other organisms. This was utilized to form the basis for our broader impact statements and the global impact Cyanobacteria presents.

Tian H, Jin J, Chen B, Lefebvre DD, Lougheed SC, Wang Y. 2021. Depth-Dependent Spatiotemporal Dynamics of Overwintering Pelagic *Microcystis* in a Temperate Water Body. Microorganisms. 9(8):1718. doi: 10.3390/microorganisms9081718. PMID: 34442797; PMCID: PMC8399979.

This study was accessed through NCBI. This research was utilized to form a background for how Cyanobacteria can persist through multiple seasons as well as highlighting where toxins can be harbored in the environment.

Bácsi I, Gonda S, Nemes-Kókai Z, B-Béres V, Vasas G. Horseradish Essential Oil as a Promising Anti-Algal Product for Prevention of Phytoplankton Proliferation and Biofouling. 2021. Plants (Basel). 10(8):1550. doi: 10.3390/plants10081550. PMID: 34451595; PMCID: PMC8400301.

This study was accessed through NCBI. The study was another resource used to identify possible avenues to test for natural elimination methods. The study also highlights the negative effects of the traditional eradication methods.

Wu CF, Smith DA, Lai EM, Chang JH. 2018. The Agrobacterium Type VI Secretion System: A Contractile Nanomachine for Interbacterial Competition. Curr Top Microbiol Immunol. 418:215-231. doi: 10.1007/82_2018_99. PMID: 29992360.

This study was accessed through NCBI. This study was utilized to determine our use of Agrobacterium as the competitor. The study highlights the bacteria's natural competitive mechanisms and how it had been utilized in other trials to outcompete other bacteria.

Munoz M, Cirés S, de Pedro ZM, Colina JÁ, Velásquez-Figueroa Y, Carmona-Jiménez J, Caro-Borrero A, Salazar A, Santa María Fuster MC, Contreras D, Perona E, Quesada A, Casas JA. 2021. Overview of toxic cyanobacteria and cyanotoxins in Ibero-American freshwaters: Challenges for risk management and opportunities for removal by advanced technologies. Sci Total Environ. 761:143197. doi: 10.1016/j.scitotenv.2020.143197. Epub 2020 Oct 27. PMID: 33160675.

This study was accessed through NCBI. A major component to our research was to figure out the problems that present eradication methods presented to the natural environment and how our method had to be less invasive/destructive.

Gomelsky M. 2021. Origins of Life. Microbial Phylogeny, Lecture.

This was accessed through attendance of a molecular physiology class given at the University of Wyoming. This information was utilized as a basic understanding of the importance of Cyanobacteria in evolution and how Cyanobacteria has persisted for millions of years.

Alexova R, Fujii M, Birch D, Cheng J, Waite TD, Ferrari BC, et al. Iron uptake and toxin synthesis in the bloom-forming microcystis aeruginosa under Iron Limitation. Environmental Microbiology. 2011;13(4):1064–77.

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