Investigating the Use of qPCR and Water Chemistry to Analyze Microcystin Production by Cyanobacteria in Wyoming Lakes

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Project Summary

The occurrence of cyanobacterial blooms has been increasing around the world for the past few decades (Paerl & Paul 2012). The cyanotoxins that are produced by these blooms can be fatal to both humans and animals. In the U.S., the most commonly found cyanotoxins in lakes with cyanobacterial blooms are microcystins (Svircev et al. 2019). Current microcystin testing methods are expensive and time-consuming, and they have no ability to predict a rise in toxins. The microcystin synthetase E (*mcyE*) gene is required for the synthesis of every type of microcystin and can be measured as a proxy for the number of microcystin-producing cells in a water sample (Jungblut & Neilan 2006). We hypothesize that in both laboratory cultures and in lakes there will be an increase in the number of *mcyE* genes detected by qPCR before the concentration of microcystins in the water increases. Temperature and water chemistry are also linked to bloom formation and toxin production (Paerl 2013, Magonono et al. 2018). We hypothesize that in lakes, *mcyE* count and microcystin concentration correlate with pH, electrical conductivity (EC), temperature, dissolved oxygen, total organic carbon (TOC), dissolved organic carbon (DOC), and dissolved organic nitrogen (DON).

The first goal of this study is to identify any relationships between *mcyE* gene count and microcystin over time. To accomplish this, we will take daily samples from West Granite Springs Reservoir, which has an active cyanobacterial bloom, as well as from cultures grown from the lake's microbiota. The microcystin concentration of each sample will be measured using an enzyme-linked immunoassay (ELISA) and the number of microcystin-producing organisms will be counted by qPCR of the *mcyE* gene. The results of these tests will be compared to find any relationships between *mcyE* count and microcystin concentration over time. Our second goal is to determine if there is a relationship between microcystin concentration, *mcyE* gene count, and the chemical properties of the water. Three lakes with blooms will be sampled weekly and data collected by qPCR, ELISA, and chemical analysis will be compared to find any measurements of water chemistry that correlate with microcystin production.

Intellectual merit: This research will expand scientific understanding of the relationship between changes in cyanotoxin gene count and future toxin production, as well as advance knowledge of the relationship between toxin-producing blooms and water chemistry. Furthermore, this information has the potential to be used to predict dangerous levels of toxins before they occur, allowing for more informed decisions regarding water safety by citizens and the Wyoming DEQ.

Broader impacts: This research will promote broad social benefits through the improvement of cyanobacterial bloom monitoring and toxin warning systems. Our findings have the potential to lead to the development of fast, cheap, predictive tests for cyanotoxins. This will allow citizens to interact with water bodies in Wyoming more safely as measures are taken to provide better information about potentially toxic water. Additionally, this research will be led by undergraduate students and will facilitate learning and practice of designing, conducting, and presenting research.

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Statement of Problem and Significance

Cyanobacterial blooms have been a growing concern for the past few decades, especially as they have become more prevalent due to increased nutrients in water bodies and to other shifting environmental factors due to climate change and human activity (Paerl & Paul 2012). The main concern is that many of these blooms produce cyanotoxins, which are toxic to humans, pets, wildlife, plants, and livestock and cause disruptions in both water and soil ecosystems (Corbel et al. 2014). Cyanotoxins can have a variety of effects on humans and can include gastrointestinal symptoms, liver inflammation, pneumonia, dermatitis, and even death (Cheung, Liang, and Lee 2013). Because blooms occur in water sources that are used for recreation, drinking water, and agriculture, these toxins pose a danger to a wide range of people.

A major issue in monitoring water bodies and protecting the public from cyanotoxins is the lack of ability to determine the risk of a toxic bloom. Cyanobacterial blooms are dynamic and unpredictable, and it is common for an originally harmless bloom to begin producing toxins. If warnings of toxic water are issued unnecessarily, they cause undue disruptions to recreation and other uses of the water and potentially lead to economic impacts, but if a warning is not issued, there are potential risks for pets, livestock, or even humans ingesting toxins. In Wyoming, satellite imagery and on-site sampling are being used to monitor cyanobacterial blooms. However, blooms can grow and produce toxins before they are visible on satellite imagery, and commonly used methods of toxin detection, such as ELISA, are expensive and time-consuming (Roberts et al. 2020, U.S. EPA 2021). Detecting changes in water chemistry or *mcyE* concentration in a cyanobacterial bloom may provide an easier way to determine if the bloom is producing toxins (Magonono et al. 2018). A method of monitoring blooms with less frequent testing, simpler methods, and more accurate results would allow for better warnings and more efficient use of resources.

This research aims to help the Wyoming Department of Environmental Quality (DEQ) improve the monitoring of cyanobacteria in lakes by analyzing the relationship between the number of toxin-producing cells and future concentrations of microcystins and by examining changes in water chemistry during toxic and non-toxic blooms. We will measure and compare changes in water pH, EC, TDS, temperature, dissolved oxygen, TOC, DOC, DON, *mcyE* concentration, and microcystin concentration. We will use qPCR to quantify levels of microcystin-producing cyanobacteria by counting copies of the *mcyE* gene in water samples. By measuring *mcyE* gene count and microcystin concentration over time, we will examine the ability of *mcyE* count to predict increases in microcystin concentration. By measuring changes in water chemistry, a significant change in the water may be an indicator of a rise in the relative amount of toxins.

Our research into the relationships between multiple factors in lakes with harmful cyanobacteria blooms may provide alternative methods to determining toxicity, thus making it easier and more cost-effective to detect toxins. This improved methodology may also allow for earlier and more informed warnings to be issued to the public about hazardous water. Our research will further the understanding of the relationship between toxin gene counts and the

potential for toxin production and the relationships between different aspects of water chemistry and current toxin production.

Introduction and Background

Relevant Literature

The prevalence of toxic cyanobacterial blooms has been increasing for many years, and scientific research into the problem has expanded as well, especially since the early 2000s (Svircev et al. 2019). The rise of toxin-producing cyanobacteria has been influenced by many factors, including climate change and other human influences on environmental conditions in water bodies (Boopathi and Ki 2014, Paerl and Paul 2012). Studies have found that eutrophication, rising temperatures, and increasing atmospheric carbon dioxide all favor cyanobacterial growth and bloom formation (Paerl and Paul 2012, Panksep et al. 2020). Additionally, the amount of toxin produced per cell is higher in warmer water, suggesting that global warming may lead to more toxic blooms (Paksep et al. 2020).

Between 2017 and 2019, there were at least 321 emergency department visits in the United States that can be attributed to harmful algal and cyanobacterial blooms (Lavery et al. 2021). The data in this report came from a database that contains information from only 70% of all emergency room visits in the U.S., so the total number of cases is likely even higher (Lavery et al. 2021). Cyanotoxins are also a major hazard for pets and wildlife. A study of reports from 2016 to 2018 found that 89% of animals that had contact with a harmful cyanobacterial bloom died (Roberts et al. 2020). Because toxic cyanobacteria blooms are increasing in frequency and prevalence around the world, monitoring bodies of water used for irrigation, drinking, or recreation has become even more important to preventing cyanotoxin poisonings.

In North America, the most common types of cyanotoxins are microcystins, anatoxin-a, saxitoxins, and cylindrospermopsin (Boopathi and Ki 2014). In most cases, scientists and government agencies must rely on testing for the presence of these toxins by ELISA or LC-MS/MS to evaluate the danger of a cyanobacterial bloom (Panksep et al. 2020). To be effective, these methods require the ability to sample the body of water frequently, which is a huge use of resources and may not be possible when monitoring a large area. Additionally, these tests are expensive, time-consuming, and may not detect all variants of the toxin (Pacheco et al. 2016). There are some proxy measurements that are currently used for cyanotoxin risk, including spectrophotometry of chlorophyll-a, biomass measurements, and microscopic counts of cyanobacteria, but these methods cannot differentiate toxin-producing and non-toxin-producing bacteria (Panksep et al. 2020, Pacheco et al. 2016, Ngwa et al. 2014). Recently, qPCR has been used in studying cyanobacteria because it can be used to detect cyanotoxin genes, allowing only toxin-producing cells to be counted. Studies have found that qPCR provides a more accurate cell count than other methods, and can identify toxin-producing bacteria before toxin concentration in the water is high enough to be detected (Pacheco et al. 2016, Ngwa et al. 2014).

Most studies that have used qPCR to analyze harmful cyanobacterial blooms have focused on whether it is a reliable indicator of current cyanotoxin concentration or have used it

for other purposes entirely, such as analyzing species diversity (Ngwa et al. 2014, Panksep et al. 2020, Moraes et al. 2021). One notable exception is a 2020 study, which used qPCR along with RT-qPCR to develop an early warning system for microcystin concentration (Lu et al. 2020). This study was able to predict microcystin concentrations exceeding the EPA health advisory limit one week before it occurred and to detect microcystin gene expression in early-stage blooms about three weeks before toxin was detectable in the water (Lu et al. 2020). This research intends to further investigate the use of qPCR as a predictive tool for monitoring cyanobacterial blooms.

A potential proxy for measuring toxin concentration is monitoring changes in water chemistry. Bloom formation is both precipitated by and precipitates changes in the water chemistry and the environment in which it resides. For example, high nitrogen levels have been implicated in contributing to blooms (Magonono et al. 2018). Some data has also shown that when the temperature is high, the growth of cyanobacteria is promoted (Paerl 2013, Magonono et al. 2018). Additionally, there is some evidence that a high pH, above 8.4, allows cyanobacteria in the genus *Microcystis* to begin producing microcystins (Magonono et al. 2018). We aim to combine chemical water testing methods and toxin-producing gene detection methods with microcystin concentration measurement to determine a relationship. Using this information to monitor changes in the water chemistry may allow for detection of dangerous levels of microcystin without the need for the traditional laboratory tests.

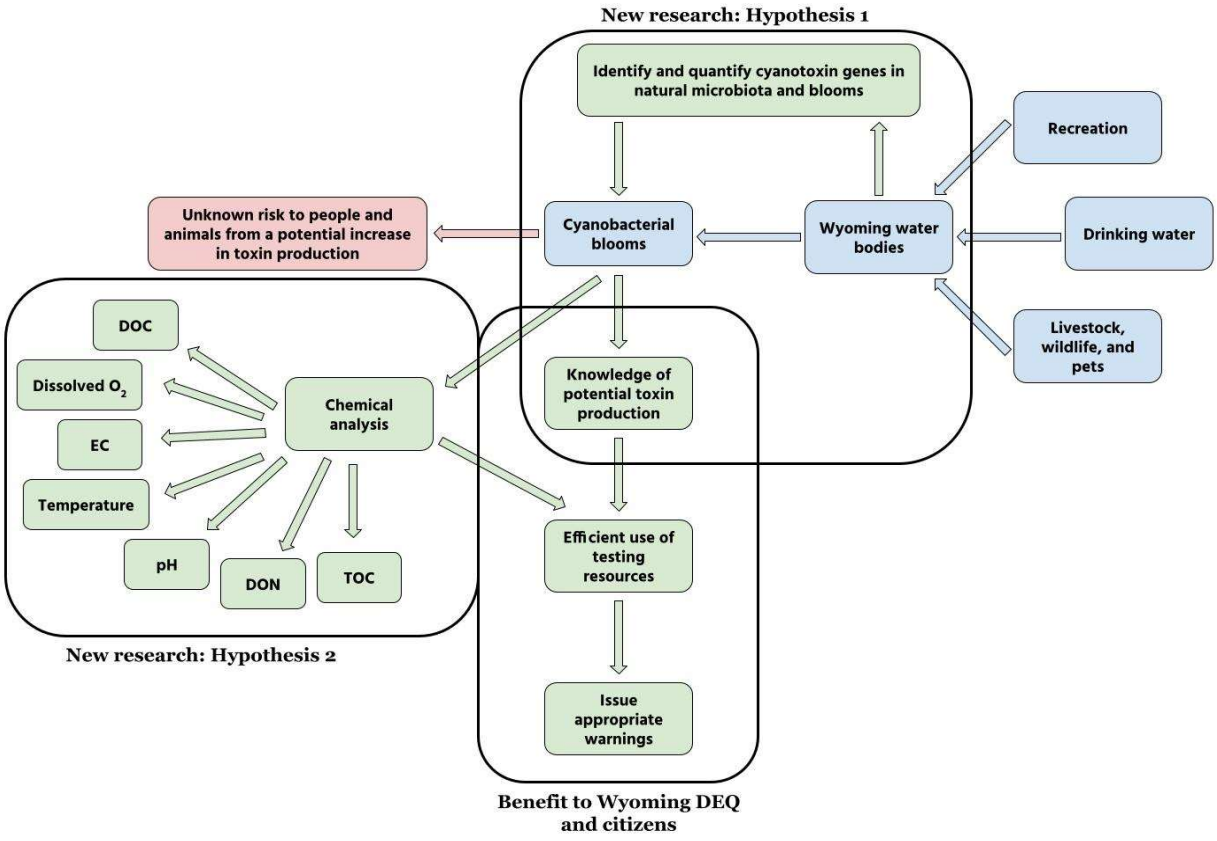
Preliminary Data

Our community partner for this research, the Wyoming DEQ, monitors Wyoming water bodies for cyanobacterial blooms and cyanotoxins using satellite imagery and water sampling. For each identified bloom, they issue a toxin advisory or bloom advisory. Sampling water from each of these categories will be key to understanding the changes in the water body that may be present. For this research, we will be collecting samples from Leazenby Lake, which currently has a toxin advisory with a microcystin concentration of 0.634ug/L, West Granite Springs Reservoir, which is currently under a bloom advisory but does not have detectable microcystins, and Twin Buttes Lake, which has a very recently confirmed bloom with low levels of microcystin.

The DEQ has been testing the active bloom in West Granite Springs Reservoir since July 21, 2021. Their data indicates that the level of microcystins in the water was below the amount they could detect with their testing methods (<0.15 μg/L) as of August 24, 2021. Their testing also revealed that the dominant cyanobacteria species present in the bloom is *Aphanizomenon flos-aquae.* This species is not known to produce microcystins, but other microcystin-producing species are often found in the same areas (Lyon-Colbert et al. 2018). According to the Wyoming DEQ, another lake in Wyoming, Woodruff Narrows Reservoir, had an active bloom composed primarily of *Aphanizomenon flos-aquae* and was issued a toxin warning on September 1, 2021 with a reported microcystin concentration of 93.05 μg/L.

Our observations of West Granite Springs Reservoir have confirmed that the west causeway where the DEQ did their sampling appears to still have a high concentration of cyanobacteria, as of September 19, 2021. The floating clumps of *Aphanizomenon flos-aquae* pictured on the DEQ's website were not visible from the shore on this date, but the water was turbid and green, especially in areas sheltered by rocks.

Conceptual Model



Justification of Approach and Novel Methods

The collection of water samples by submerging sterile collection bottles a few inches under the lake's surface has been established in many studies of cyanobacteria in lakes (Lu et al. 2020, Ngwa et al. 2014). We will use bottles that have been sterilized with bleach to avoid contamination of our samples by DNA. The glass bottles and mason jars we will use follow sampling procedures provided by the Wyoming DEQ for measuring toxin concentration.

Due to a lack of literature on the subject, we have developed a novel method for growing cyanobacteria cultures from lake water. To include the full diversity of the bloom in our cultures, a small sample of lake water will be collected, then the microbes will be collected by

centrifugation and transferred into liquid media. In addition to this, other cultures composed of half media and half lake water will be made. We will collect our samples from whichever cultures appear to have the best growth after five days of growth. The media and growth conditions have been proven to grow a variety of cyanobacteria genera (James 2012). For long-term preservation, samples will be frozen using DMSO as a cryoprotectant, which has been found to provide the highest rate of recovery (Rastoll et al. 2013).

To process the lake and culture samples, the microorganisms to be used for qPCR will be separated from the water to be used for microcystin measurements using methods established by previous studies (Moraes et al. 2021, Cordeiro et al. 2021). These samples will be stored until they are tested using methods described in the literature that can successfully preserve cyanobacteria and microcystins for several weeks (Moraes et al. 2021, Almuhtaram et al. 2018).

qPCR of the *mcyE* gene has been well established as a method for counting microcystin-producing cells (Lu et al. 2020, Ngwa et al. 2014, Pacheco et al. 2016, Panksep et al. 2020). We have selected primers for amplification of *mcyE* that have been tested for specificity across multiple cyanobacteria genera and successfully used to monitor microcystin producers in a lake (Jungblut and Neilan 2006, Lu et al. 2020). For microcystin concentration measurements, we have chosen to use a commercial ELISA kit from Attogene. ELISA is a commonly used method and is effective at detecting microcystins at levels below what is considered hazardous by the Environmental Protection Agency (U.S. EPA 2021).

For water chemistry analysis, pH, EC, TDS, temperature, and dissolved oxygen will be measured at the time of sampling using a YSI pH/conductivity meter. TON, DOC, and DOC will be measured by the Ecology Biogeochemistry Core Lab (EcoBGC) at the University of Wyoming.

Research Plan

Objectives

- To improve methods for cyanobacterial bloom monitoring to allow for more efficient, cheaper, and more informative testing
- To further understand the relationship between *mcyE* gene count and future microcystin concentration in lakes and in culture
- To discover if qPCR can be used to accurately predict an increase in microcystin production
- To further understand the relationships between microcystin concentration, *mcyE* concentration, and water chemistry
- To determine if the measurement of pH, EC, TDS, temperature, dissolved oxygen TOC, DOC, or DON is an accurate proxy for microcystin concentration
- To share our findings with the Wyoming DEQ and aid them in sharing more useful data about cyanobacterial bloom safety with the public

Specific Aims

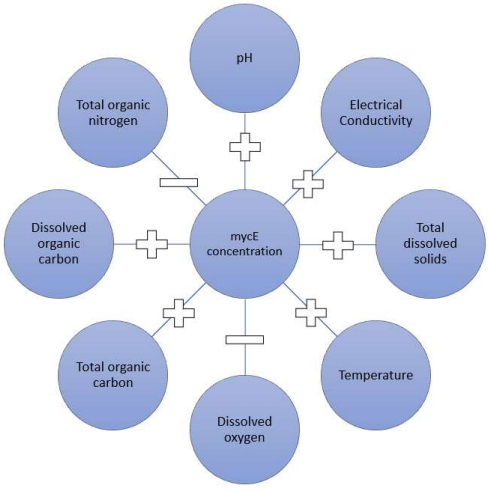
- We will measure microcystin concentration using ELISA and *mcyE* gene count using qPCR over time and compare the results to establish the relationship between them
- We will develop a qPCR based water testing method that will provide advance warning of dangerous microcystin levels in lakes using the relationship between *mcyE* gene count and microcystin concentrations
- We will compare the relationship between *mcyE* gene count and microcystin concentration found in a lake and in cultures
- We will measure *mcyE* count and microcystin concentration, using qPCR and ELISA respectively, in weekly samples from three lakes
- We will measure pH, EC, TDS, temperature, and dissolved oxygen using a field meter at the time of each weekly sample collection and get TOC, DOC, and DON measurements from EcoBGC
- Water chemistry, microcystin concentration, and *mcyE* count from the same time and location will be compared to determine if there is a relationship between them

Hypotheses

1a: The number of *mcyE* genes detected by qPCR will increase four to seven days prior to an increase of microcystins

1b: The correlation between *mcyE* count and microcystin concentration will be the same in culture and in a lake

2a:



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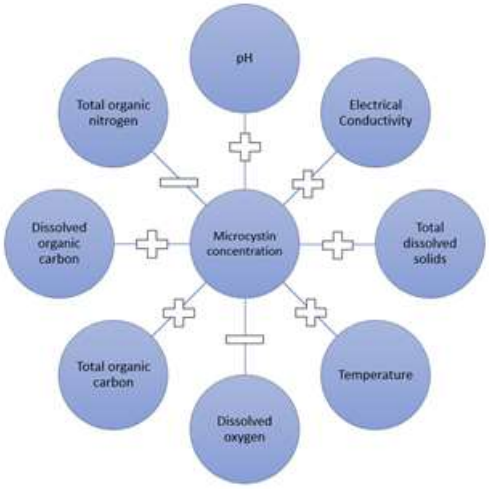
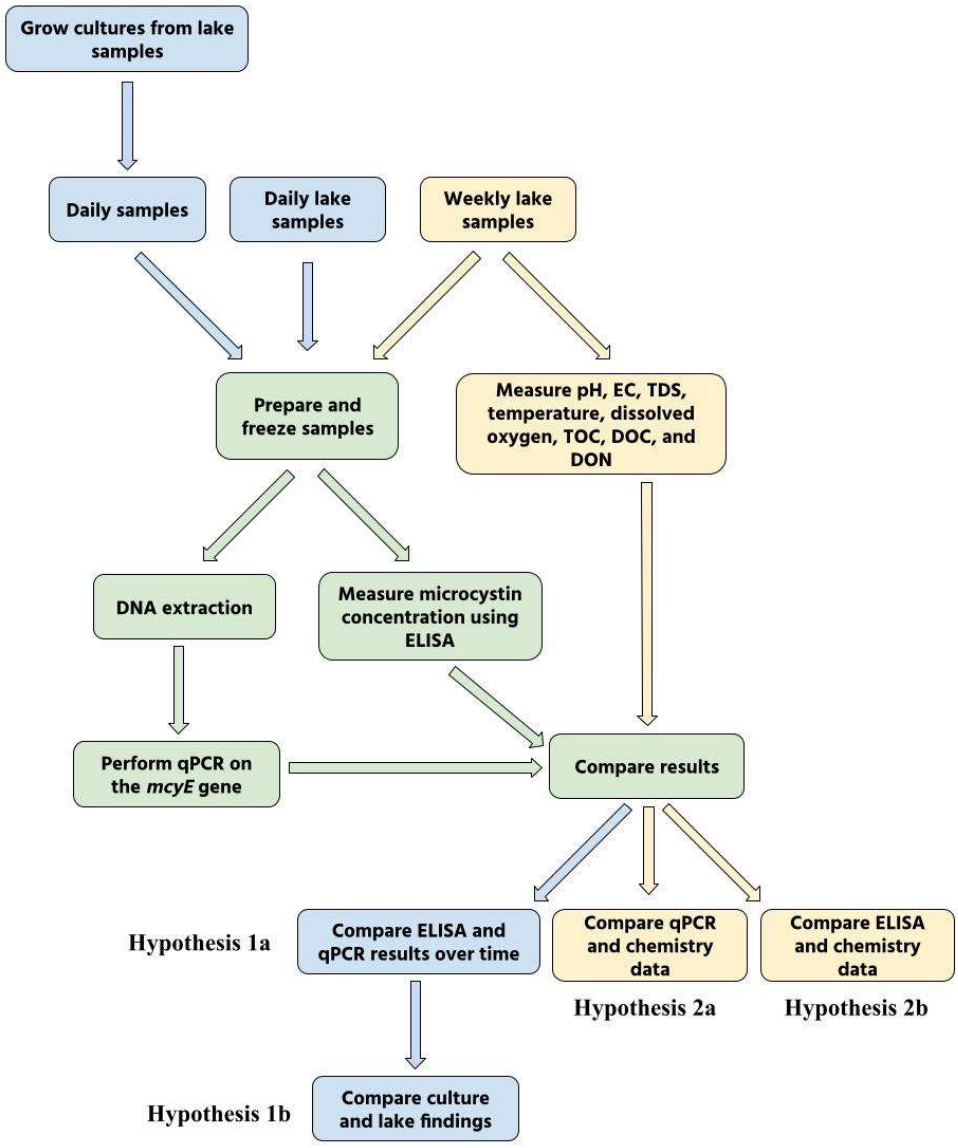


Research Design Schematic



Materials and Methods

Sample collection and storage

For hypothesis 1, samples will be collected from West Granite Springs Reservoir. This lake was selected using the map of current harmful cyanobacterial bloom advisories maintained by the Wyoming DEQ and the Department of Health. The reservoir has an active but not yet toxic cyanobacterial bloom, allowing us to monitor conditions similar to what the qPCR toxin prediction method we hope to develop could be used for in the future.

For 10 consecutive days, sampling from the lake will be done at two locations along the shore in areas with a visible concentration of cyanobacteria. According to the guidelines supplied by Wyoming DEQ, all water samples will be collected using glass sample bottles and frozen

within 8 hours of collection. Samples will be taken by submerging the sampling container below the surface. For DNA extraction, a 350 mL sample will be collected in a 1-pint mason jar that has been sterilized with 10% bleach and autoclaved. The water will be filtered through a 0.2-micron filter and the collected microbes stored following the methods used by Moraes et al. (2021). For microcystin analysis, small samples will be collected in 60 mL amber glass bottles and frozen until used for ELISA according to the method described by Almuhtaram et al. for dissolved cyanotoxin measurements (2018).

For hypothesis 2, water samples will be collected from three different water bodies at different advisory levels as specified by the Wyoming DEQ. Leazenby Lake is under a toxin advisory, West Granite Springs Reservoir is under a bloom advisory, and Twin Buttes Lake has very recently been issued a bloom advisory. Water samples from two locations in each lake will be collected once a week for three weeks. Samples for DNA extraction and microcystin analysis will be collected and processed using the same methods as for hypothesis 1. For measurement of TOC, DOC, and DON by the EcoBGC, a 50 mL falcon tube will be filled with water from each sample site. These will be stored at -20°C within 8 hours after collection and kept frozen until they are sent to the EcoBGC for analysis. A YSI pH/conductivity meter will be used to measure pH, dissolved oxygen, EC, and temperature of the water at the time of each collection.

Culturing

To prepare the cultures, two 45 mL samples from West Granite Springs Reservoir will be taken from an area of dense bloom. The samples will be centrifuged at 2000 rpm for 10 minutes, the water poured off, and the pellets resuspended in two 1 L Erlenmeyer flasks containing 500 mL of autoclaved Bristol's medium. To increase the likelihood of successfully culturing the lake's microbiota, two cultures will also be made by combining 250 mL of lake water with 250 mL of Bristol's medium. All of the cultures will be kept at room temperature (20-25 °C) and under low-intensity natural sunlight. To allow for gas exchange, the bottle openings will be loosely covered with sterile aluminum foil. After one week, samples from the cultures will be frozen with 3% DMSO for preservation as described by Rastoll et al. (2013).

Two cultures with good growth will be selected for sampling. Samples will be collected each day for ten days beginning seven days after the cultures are started, or when growth becomes visible. For DNA extraction and ELISA, a 1 mL sample will be collected from each culture using a sterile pipet. The sample will be centrifuged at 5000 rpm for 10 minutes. The supernatant will be collected and transferred to a new sterile microcentrifuge tube to be used for ELISA (Cordeiro et al. 2021). The pellet will be stored for DNA extraction. Each of these samples will be stored under the same conditions as the samples from West Granite Springs Reservoir.

DNA extraction and qPCR

DNA extraction from the filters will be done using the Omega bio-tek kit following the manufacturer's instructions. DNA extracts will then be stored at -20 C until it is utilized for

qPCR. qPCR will then be performed using the primers for cyanobacteria *mcyE* designed and tested by Jungblut and Neilan (2006) and SYBR Green qPCR master mix. We will use the real-time PCR system in the Genome Technologies lab (GTL) at the University of Wyoming with the assistance of the staff there. In qPCR, the number of amplification cycles before fluorescence is detectable, known as the cycle threshold (Ct), is used to quantify the number of copies of the target gene in the original sample. Each of our samples will be run in triplicate and the average Ct measure will be used for analysis.

Toxin measurements

In order to lyse cells and release toxins, the samples collected for microcystin measurement will be frozen and thawed three times, following the procedure outlined by the EPA (U.S. EPA 2021). The total microcystin concentration in each sample will be measured using a commercial microcystin ELISA kit following the manufacturer's instructions. Each sample will be tested in duplicate. We will use Microsoft Excel to interpret the data and will take the average of the two ELISA tests to use for analysis, assuming the standard deviation and coefficient of variation indicate that there was no error in the ELISA setup (CV less than 20%).

Analysis and Expected Results

Files containing the data from qPCR, chemical analysis, and spectrophotometry on the ELISA plates will be saved to GoogleDrive. For the qPCR data, a lower Ct measure will indicate a higher number of *mcyE* genes present in the samples. Absorbance data from the ELISA tests will be obtained using a plate reader according to the manufacturer's instructions for the kit and analyzed to determine concentration as described by the manufacturer.

We will analyze the data for hypothesis 1 using the methods of Lu et al. as a guide (2020). We expect to find a significant positive correlation between *mcyE* gene count and future microcystin concentration. We expect that any significant increase in the number of *mcyE* genes detected by qPCR is followed by a higher microcystin concentration in samples from the same source collected four to seven days later. We also expect that if this correlation does exist, the time between an increase of *mcyE* and a rise in microcystin concentration will be the same in the lake and in cultures. If there is no increase of *mcyE* count over the sampling period, we expect that there will not be an increase in microcystin concentration either. If unexpected results are found, they would indicate that there is not a consistent relationship between *mcyE* gene count and future microcystin concentration. This would suggest that qPCR is not a reliable method for predicting increases in microcystin concentration. This could be due to the influence of environmental factors on cyanotoxin production, which may have a greater role in the amount of microcystin released by a bloom than that of the number of toxin-producing cells.

Hypothesis 1a

Expected Results
- A significant rise in *mcyE* is followed by a rise in microcystins
- No rise in microcystins without an increase in *mcyE* first

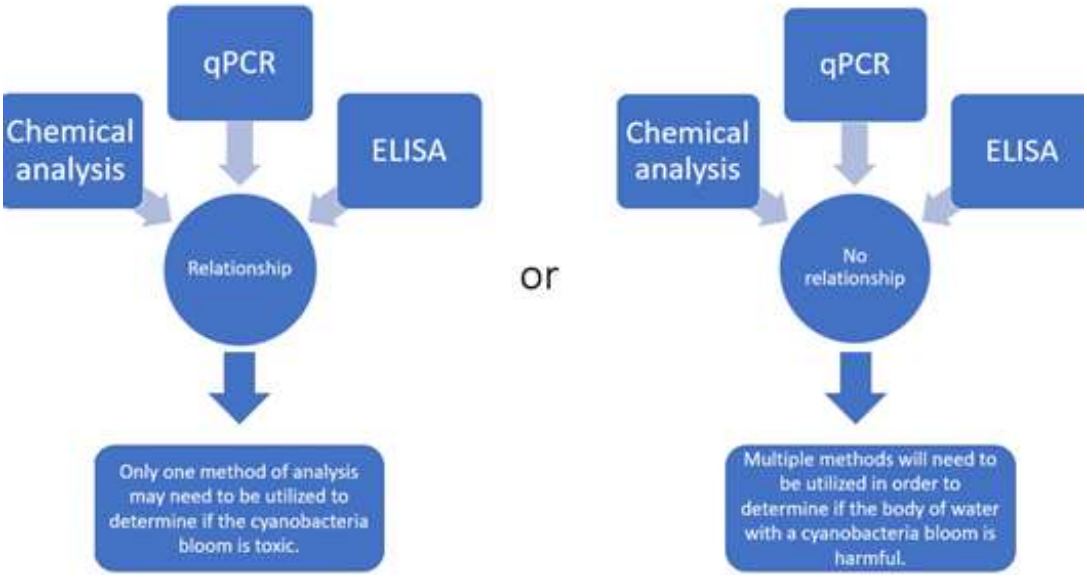
Unexpected Results
- A significant increase of *mcyE* with no following increase in microcystins
- An increase of microcystins without a prior increase in *mcyE*

Hypothesis 1b

Expected Results
- The time between *mcyE* increase and microcystin increase is the same in the lake and in culture
- The relationship between *mcyE* and microcystins either exists or does not in the lake and in culture

Unexpected Results
- The time between *mcyE* increase and microcystin increase is different in the lake and in culture
- The relationship between *mcyE* and microcystins is found only in the lake or only in culture

For hypothesis 2, we expect that there will be a significant relationship between the amount of *mcyE* genes present, microcystin concentration, and some chemical property (pH, EC, TDS, temperature, dissolved oxygen, TOC, DOC, DON). An unexpected result would be finding no significant correlation between the amount of *mcyE* genes present, microcystin concentration, and any of the chemical properties. If the analysis of results shows that there is a relationship, there is potential to develop a testing method in which only one of these measurements is needed to accurately test for the presence of microcystins in a water body. This would significantly reduce the amount of time and money that is required to test water bodies for dangerous levels of microcystins. If no significant correlation is found, it would indicate that measurement of pH, EC, TDS, temperature, dissolved oxygen, TDS, TOC, DOC, or DON is not a reliable indicator of microcystin concentration in water.



A scatter plot or similar graphic, like the one below, will be generated to show the results of each hypothesis. These graphs will be constructed for each sample location. The results from all the analyses will be compared for each sample location to determine what relationships are present between mcyE concentration, microcystin concentration, and chemical properties (pH, EC, TDS, temperature, dissolved oxygen, TDS, TOC, DOC, DON) of the water.

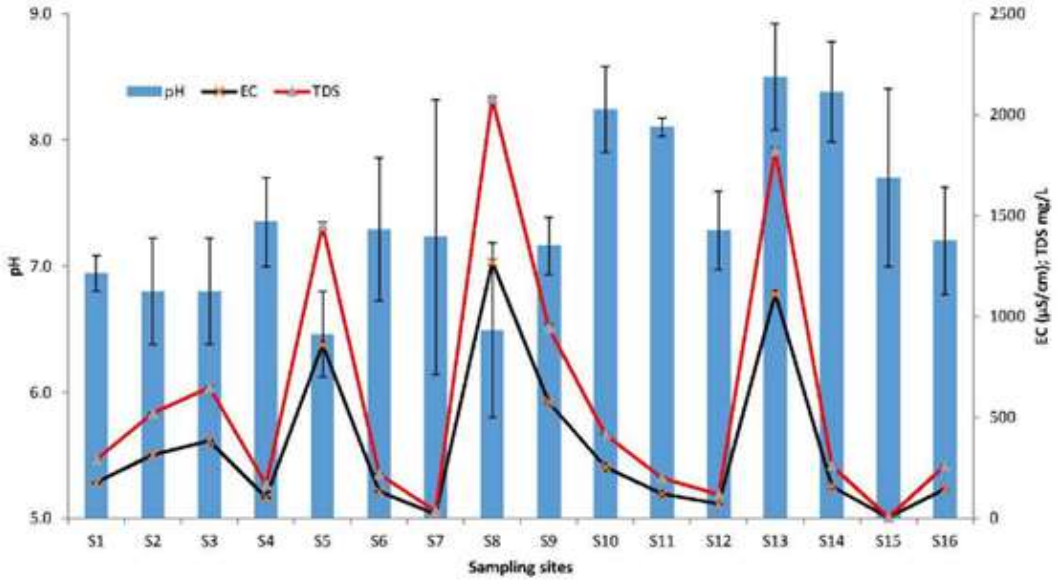


Figure 1. Average values of the physical characteristics of the river sediments of the 18 sampling sites. Whiskers reflect standard error. EC: electrical conductivity; TDS: total dissolved solids.

Figure 1 *(Magonono et al. 2018)*

Timeline

	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Order materials	XX							
Communicate with DEQ partners	XX		XX		XX		XX	XX
Reachout to EcoBCG to plan analysis	XX							
Organize use of qPCR system with the GTL	XX	XX						
Weekly lake sampling		XX	XX	XX				
Weekly field measurements		XX	XX	XX				
Culturing		XX	XX	XX				
Daily lake sampling		XX	XX					
Daily culture sampling			XX	XX				
DNA Extraction			XX	XX				
qPCR troubleshooting				XX				
ELISA					XX	XX		
qPCR					XX	XX		
Data analysis							XX	XX

References

Almuhtaram, Husein , Yijing Cui, Arash Zamyadi, and Ron Hofmann. 2018. “Cyanotoxins and Cyanobacteria Cell Accumulations in Drinking Water Treatment Plants with a Low Risk of Bloom Formation at the Source.” *Toxins* 10[11]: 430. https://doi.org/10.3390/toxins10110430
This article provides a method for storing water samples that preserves microcystins for several weeks. Found through the Web of Science database.

Boopathi, Thangavelu and Jang-Seu Ki. 2014. “Impact of Environmental Factors on the Regulation of Cyanotoxin Production” *Toxins* 6: 1951-1978. https://doi.org/10.3390/toxins6071951
This review collects data on each group of cyanotoxins and explains the environmental factors that have been found to influence production of each of them. It also points out many inconsistencies and contradicting results found in different studies. Found on NCBI.

Cheung, M. Y., Liang, S., & Lee, J. (2013). Toxin-producing Cyanobacteria in Freshwater: A Review of the Problems, Impact on Drinking Water Safety, and Efforts for Protecting Public Health. *Journal of Microbiology*, *51*(1), 1–10. https://doi.org/10.1007/s12275-013-2549-3
This paper was found via Web of Science. This article is important because it discusses the broader impacts of cyanobacteria and the specific illnesses that cyanotoxins can cause. It goes in-depth about what illnesses are caused and how severe it can be. This is pertinent to the work to ensure that the audience understands the significance and the impact of harmful cyanobacterial blooms and why readers should care about this research.

Corbel, S., C. Mougin, and N. Bouaicha. 2014. “Cyanobacterial toxins: Modes of actions, fate in aquatic and soil ecosystems, phytotoxicity and bioaccumulation in agricultural crops.” *Chemosphere,* 96: 1–15. https://doi.org/10.1016/j.chemosphere.2013.07.056
This study provides a thorough review of the literature regarding the effects of cyanotoxins on plants and ecosystems. Found using the Web of Science database.

Cordeiro, Rita, Joana Azevedo, Rúben Luz, Vitor Vasconcelos, Vítor Gonçalves, and Amélia Fonseca. 2021. “Cyanotoxin Screening in BACA Culture Collection: Identification of New Cylindrospermopsin Producing Cyanobacteria.” *Toxins* 13[4]: 258. https://doi.org/10.3390/toxins13040258
This study provided a method for collecting cyanotoxins from small liquid samples by centrifugation. Found using the Web of Science database.

James, Daniel E.. 2012. “Culturing Algae.” Carolina Biological Supply Company. https://www.carolina.com/teacher-resources/Document/culturing-algae-instructions/tr29112.tr
This booklet provides an overview of growth conditions that are ideal for growing cyanobacteria. Found using Google.

Jungblut, Anne-Dorothee and Brett A. Neilan. 2006. “Molecular identification and evolution of the cyclic peptide hepatotoxins, microcystin and nodularin, synthetase genes in three orders of cyanobacteria.” *Archives of Microbiology* 185: 107-114. https://doi.org/10.1007/s00203-005-0073-5
These researchers designed and tested primers for amplifying the *mcyE* gene with high specificity across many genera of cyanobacteria, which will be used for this study. Found through the article by Lu et al. (2020).

Lavery, Amy M., Lorraine C. Backer, Virginia A. Roberts, Jourdan DeVies, and Johnni Daniel. 2021. “Evaluation of Syndromic Surveillance Data for Studying Harmful Algal Bloom-Associated Illnesses — United States, 2017–2019” *CDC Morbidity and Mortality Weekly Report* 70[35].
This report summarizes all of the confirmed and probable deaths and emergency department visits due to cyanotoxins in the US over a two year period. Found through NCBI email alerts.

Lu, Jingrang, Ian Struewing, Larry Wymer, Daniel R.Tettenhorst, Jody Shoemaker, and Joel Allen. 2020. “Use of qPCR and RT-qPCR for monitoring variations of microcystin producers and as an early warning system to predict toxin production in an Ohio inland lake.” *Water Research* 170. https://doi.org/10.1016/j.watres.2019.115262
This study developed an early warning system for MC concentration which used qPCR and RT-qPCR. The results indicated that MC transcription can be detected up to three weeks before toxin levels in the water rise. They also found that qPCR could be used to determine when toxic blooms are not likely to occur. Found using the Web of Science database.

Lyon-Colbert, Amber, Shelley Su, and Curtis Cude. 2018. “A Systematic Literature Review for Evidence of *Aphanizomenon flos-aquae* Toxigenicity in Recreational Waters and Toxicity of Dietary Supplements: 2000–2017” *Toxins* 10: 254. doi:10.3390/toxins10070254
This article provides an overview of toxins found in lakes with cyanobacterial blooms consisting primarily of *Aphanizomenon flos-aquae.* Found using GoogleScholar.

Magonono, Murendeni, Paul Johan Oberholster, Addmore Shonhai, Stanley Makumire, and Jabulani Ray Gumbo. 2018. “The Presence of Toxic and Non-Toxic Cyanobacteria in the

Sediments of the Limpopo River Basin: Implications for Human Health.” *Toxins*. https://doi.org/10.3390/toxins10070269.
Found via NCBI explains the differences in sediment samples collected and shows using PCR to determine if the cyanobacterial isolates (16S sequencing) are capable of producing toxins and if they are. Shows what the differences in the sediment (pH, EC, and TDS) relate to whether or not the cyanobacteria present produce toxins or not. Has a nice external chart that relates all of the cyanobacteria results (separate from the sediment data.

Moraes, Munique, Raphaella Rodrigues, Louise Schlüter, Raju Podduturi, Niels Jørgensen, and Maria Calijuri. 2021. "Influence of Environmental Factors on Occurrence of Cyanobacteria and Abundance of Saxitoxin-Producing Cyanobacteria in a Subtropical Drinking Water Reservoir in Brazil" *Water* 13[12]: 1716. https://doi.org/10.3390/w13121716
This study tests a new approach for using qPCR on STX genes to monitor potentially toxic blooms. It confirms that toxin gene count by qPCR is a reliable method of counting potentially toxin bacteria and explains the benefits they found to using qPCR to monitor cyanobacterial blooms. Found on NCBI.

Ngwa, Felexce, Chandra Madramootoo, and Suha Jabaji. 2014. “Comparison of cyanobacterial microcystin synthetase (*mcy*) E gene transcript levels, *mcyE* gene copies, and biomass as indicators of microcystin risk under laboratory and field conditions.” *MicrobiologyOpen* 3[4]: 411– 425. https://doi.org/10.1002/mbo3.173
This study provides evidence that *mcyE* copies, counted using qPCR, are correlated with microcystin concentrations and are a better indicator of MC concentration in environmental samples than mycE transcripts, chlorophyll a levels, phytoplankton biomass, and microscopic counts. Found on NCBI.

Pacheco, Ana, Iame A. Guedes, and Sandra M. Azevedo. 2016. "Is qPCR a Reliable Indicator of Cyanotoxin Risk in Freshwater?" *Toxins* 8[6]:172. https://doi.org/10.3390/toxins8060172
This review article explains the shortcomings of current toxin measurement methods and explores current research into qPCR as a method of measuring toxin concentrations. It finds that there are many benefits to qPCR, mainly the ability to differentiate toxin and non-toxin producing bacteria. It also concludes that qPCR cannot yet be used as a sole measurement of current toxin levels because only two-thirds of the studies they analysed found a consistent correlation between the two measurements. Found using the Web of Science database.

Paerl, H.W. and V.J. Paul. 2012. “Climate change: Links to global expansion of harmful cyanobacteria.” *Water Research*, 46 [5]: 1349–1363. https://doi.org/10.1016/j.watres.2011.08.002

This review article explores how different factors altered by climate change are affecting cyanoHABs. It provides evidence that nutrient loading, rising temperatures, and increasing atmospheric carbon dioxide favor cyanobacterial growth. It also states some effects of this problem, including contaminating water used for irrigation, drinking, recreation, and fisheries. Found on NCBI.

Paerl, Hans W., and Timothy G. Otten. 2013. “Harmful Cyanobacterial Blooms: Causes, Consequences, and Controls.” *Microbial Ecology* 65 (4): 995–1010. https://doi.org/10.1007/s00248-012-0159-y.
This paper was found via NCBI and it provides details on the results of chemical changes to help guide the hypotheses. It also effectively shows many factors that relate to cyanoHABs and whether they may increase or decrease blooms. Also states that microcystin is the most prevalent cyanotoxin.

Panksep, Kristel, Marju Tamm, Evanthia Mantzouki, Anne Rantala-Ylinen, Reet Laugaste, Kaarina Sivonen, Olga Tammeorg, and Veljo Kisand. 2020. "Using Microcystin Gene Copies to Determine Potentially-Toxic Blooms, Example from a Shallow Eutrophic Lake Peipsi" *Toxins* 12[4]: 211. https://doi.org/10.3390/toxins12040211
This study found that *mcyE* copy number can be used to accurately counts potentially toxic cyanobacteria and is also significantly correlated with MC concentration in the water. It also mentions that qPCR can be used in future studies to measure the effects of environmental factors on toxin production potential. Found using the Web of Science database.

Rastoll, M.J., Y. Ouahid, F. Martín-Gordillo, V. Ramos, V. Vasconcelos, and F. F. del Campo. 2013. “The development of a cryopreservation method suitable for a large cyanobacteria collection.” *Journal of Applied Phycology,* 25: 1483-1493. https://doi.org/10.1007/s10811-013-0001-z
This article describes a method for cryofreezing cyanobacteria with a high recovery rate. Found using GoogleScholar.

Roberts, Virginia A, Marissa Vigar, Lorraine Backer, Gabriella E Veytsel, Elizabeth D Hilborn, Elizabeth I Hamelin, Kayla L Vanden, et al. 2020. “Surveillance for Harmful Algal Bloom Events and Associated Human and Animal Illnesses-One Health Harmful Algal Bloom System, United States, 2016-2018.” Vol. 69. https://www.cdc.gov/habs/pdf/.
This paper was found on Web of Science. It is very specific with the number of illnesses and types due to contact with cyanotoxins. Discusses what is being tested when someone/animal has symptoms of toxins. A lot of good statistics about how many cases of illness result and what types. Knowing the severity of these illnesses is important for the reader to fully understand what happens if harmful cyanobacteria blooms are not

caught in time, and somebody/animal drinks or is in the body of water that has toxins in it.

Svirčev, Z., Lalić, D., Bojadžija, S., Tokodi, N., Backovic, D., Chen, L., Meriluoto, J., Codd, G.. 2019. “Global geographical and historical overview of cyanotoxin distribution and cyanobacterial poisonings.” *Archives of Toxicology,* 93: 2429–2481.
This review article analysed the findings of 468 articles on cases of harmful cyanobacterial blooms and cytotoxin poisonings. It provides extensive data on where each type of cyanotoxin has been found, as well as breakdowns of every cyanotoxin poisoning reported in scientific literature the researchers could find. Found using GoogleScholar.

United States Environmental Protection Agency. 2021. “Cyanobacterial Harmful Algal Blooms (CyanoHABs) in Water Bodies.” https://www.epa.gov/cyanohabs
This website provides a general overview of cyanobacterial blooms. It also gives access to pages with the EPA’s guidelines for dangerous toxin levels for drinking water and recreation, as well as testing methods. I accessed this page directly.