Identification of novel toxin-producing cyanobacteria in Iowan lakes

Micah Rain Fatka

Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/etd

Recommended Citation
https://lib.dr.iastate.edu/etd/18491

This Thesis is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Identification of novel toxin-producing cyanobacteria in Iowan lakes

by

Micah Rain Fatka

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Geology and Environmental Science

Program of Study Committee:
Elizabeth Swanner, Major Professor
Chris Harding
Kaoru Ikuma

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2021

Copyright © Micah Rain Fatka, 2021. All rights reserved.
# TABLE OF CONTENTS

| ACKNOWLEDGMENTS | iv |
| ABSTRACT | v |
| **CHAPTER 1. INTRODUCTION** | 1 |
| Ecology and Impact of Cyanobacteria | 1 |
| Cyanotoxins: Health Risks and Exposure Routes | 3 |
| Cyanotoxin and Cyanobacteria Relationship | 7 |
| Research Motivation, Goal, Design, and Contributions | 9 |
| **CHAPTER 2. MATERIALS AND METHODS** | 12 |
| Site Location and Sample Collection | 12 |
| Enzyme-Linked Immunosorbent Assay (ELISA) Toxin Measurements | 12 |
| Cultivation of Cyanobacterial Cultures | 14 |
| Screening Cyanobacterial Cultures for Toxigenic Strains | 15 |
| Fluorescence-assisted Sample Screening | 15 |
| Extraction and Purification of culture DNA | 16 |
| Toxin Gene Screening via Polymerase Chain Reaction | 17 |
| Further Investigation of a Putatively Toxic Cyanobacterial Culture | 18 |
| Confirmation of sxtI Amplicon Sequence via Cloning | 18 |
| Establishing Identity of putatively toxic isolate (Cloning of 16S rRNA gene) | 19 |
| Illumina Sequencing and Bioinformatics Analysis | 19 |
| Searching Microbial Community Dataset for Putatively Toxic Isolate | 20 |
| Microscopy on Putatively Toxic Enrichment | 20 |
| Fluorescent Assisted Cell Sorting | 21 |
| Cyanobacteria Community Analysis | 21 |
| **CHAPTER 3. RESULTS** | 22 |
| Toxin Measurements | 22 |
| Screening for Toxic Isolates | 24 |
| Cultivation and Most Probable Number | 24 |
| Flow Cytometry | 25 |
| Toxin Gene Screening | 26 |
| Further Investigation of Putatively Toxic Cyanobacterial Culture | 27 |
| Confirming sxtI Clone Insert Sequence | 27 |
| 16S rRNA paired-end reads from cloning | 28 |
| Microscopy | 30 |
| Flow Cytometry on Culture Containing sxtI Amplification Product | 30 |
| Community Dataset Analyses | 31 |
| Searching for Putatively Toxic Isolate in Larger Microbial Dataset | 31 |
| Cyanobacterial Community Structure | 32 |
| **CHAPTER 4. DISCUSSION** | 34 |
Isolation and Identification of a Putatively Toxic Cyanobacterial Strain .................................. 37
Denison Lake (Week 12) Cyanobacterial Community ................................................................. 47

CHAPTER 5. CONCLUSIONS ....................................................................................................... 51
REFERENCES .......................................................................................................................... 54
APPENDIX A. ELISA %CV VALUES ....................................................................................... 66
APPENDIX B. GROWTH UNIT RESULTS ............................................................................ 68
I would like to thank my advisor, Betsy Swanner, for providing me the opportunity to take part in this research project and for securing financial support from the EPA, which allowed me to explore the topics relating to this research. I would also like to thank my committee members, Kaoru Ikuma and Chris Harding, for their guidance and support throughout the course of this research. As this project was part of a larger research effort funded by the EPA, I also thank the collaborating members that took part and provided helpful suggestions for my work: Jaejin Lee, Adina Howe, Kaoru Ikuma, Xuewei Liang, Betsy Swanner, Tania Leung, along with Dan Kendall, Amy Buckendahl and field technicians, from the Iowa Department of Natural Resources.

Additional support came from Chiron Anderson, who assisted tremendously in helping me navigate the complexities involved in the bioinformatics analysis required for this work. A special thanks is extended to my lab members with whom I have worked, for providing feedback and friendship; especially to Zackry Stevenson and Tania Leung. Zak helped me think through and tackle troublesome issues that came up in lab, and Tania, also part of the EPA research group, provided both mentorship and friendship through these past years, for which I am truly grateful. I would like to thank my fellow Geology graduate students and other colleagues, for many memorable occasions, hilarious moments, and support. And last, but not least, I thank my family and friends for their love and support through this, and in every other endeavor, I have faced.
ABSTRACT

Cyanobacteria is a diverse phylum of photosynthetic bacteria with many species harboring the ability to produce toxins. Toxin-producing cyanobacteria are a growing ecological concern in freshwaters that has garnered much attention from prominent health agencies due to their global distribution and potentially life-threatening health impacts. The cyanotoxin microcystin is routinely detected in Iowa lakes as part of the Iowa Department of Natural Resources (IDNR) Beach Monitoring program. This study focused analytical efforts on lesser studied toxins (anatoxin-a, cylindrospermopsin, and saxitoxin) and aimed to isolate and identify cyanobacteria that produce these cyanotoxins in Iowan lakes.

While direct measurements of lake waters give insight into the presence and concentration of various toxins, determining which strains produce these toxins is complicated by several factors. Freshwater cyanobacteria grow in mixed communities, where many species are present. Different cyanobacterial strains are able to produce the same toxin types, and some strains produce multiple toxins. Many species have highly similar morphology, so microscopic identification cannot delineate toxic from non-toxic strains.

Cyanobacteria were enriched through serial dilutions on a selective growth medium. Individual strains were further isolated using flow cytometry. Cyanobacterial cultures were screened for toxin functional genes (anaC, cyrJ, sxtI) via PCR, which revealed three cultures containing the sxtI gene involved in saxitoxin production. Cloning of the sxtI insert from one sample and a subsequent search of its amino acid residues using NCBI’s BLASTx showed the closest sequence match was a carbamoyltransferase enzyme belonging to an uncultured Nostoc species. Amplification, cloning, and Sanger sequencing of the 16S rRNA gene, followed by a nucleotide search in NCBI’s BLASTn indicated that the most abundant organisms in the culture
were closely related to uncultured *Nostoc* strains. Microscopy revealed heterocysts and a complex life-cycle morphology, further corroborating the sequencing results that the culture contained organisms belonging to the genus *Nostoc*. The 16S rRNA and *sxtI* gene inserts showed the closest matches to uncultured *Nostoc* strains, indicating this organism is not yet taxonomically classified, and should be considered novel. Next steps include further purification and whole genome sequencing of the putatively toxic *Nostoc* enrichment culture to determine its species level classification. Verification of this strain’s ability to produce saxitoxin will be confirmed through searching its genome for the full *sxt* gene cluster, and through LC/MS/MS based analysis for detection of saxitoxin in the sample.
CHAPTER 1. INTRODUCTION

Ecology and Impact of Cyanobacteria

Cyanobacteria are a diverse phylum of photosynthetic bacteria that have successfully colonized both land and water in nearly all regions of the world, including polar, temperate, and tropical climates (Kleintech et al. 2014, Beaver et al 2018, León & Peñuela, 2019). Across these disparate habitats, cyanobacteria demonstrate the ability to produce toxins as secondary metabolites. These toxins, collectively termed ‘cyanotoxins’, are biohazardous compounds that pose a serious threat to the natural functioning of ecosystems (Rastogi et al., 2015), regional economies that are based in aquaculture (Merel et al., 2013), and cause adverse health effects in both aquatic organisms (Landsberg, 2002) and mammals (i.e. exposed wildlife, livestock, domestic pets, and humans; Dietrich et al. 2008). Due to the toxic effects these microorganisms are able to engender, cyanobacteria were included as emerging pathogens by the Organization for Economic Co-Operation and Development in 2005 (OECD, 2005).

Cyanobacteria are comprised of three different growth morphologies: unicellular, colonial, or filamentous. Unicellular cyanobacteria are single-celled organisms that are surrounded by a mucilaginous sheath, which acts to protect the cell under unfavorable environmental conditions. Colonial cyanobacteria are formed when single cells attach to themselves, and filamentous cyanobacteria consisting of vegetative cells (which perform photosynthesis) are linked together in chains. In certain species of filamentous cyanobacteria, cells can undergo differentiation to form akinetes (cold and drought resistant cells; Kaplan-Levy et al., 2010) and heterocysts (which allow the cyanobacteria to fix atmospheric nitrogen, N₂, under extremely low oxygen conditions) (Gallon 1992). Cyanobacteria also tolerate dramatic shifts in salinity (Joset et al., 1996) and are able to survive under low light intensity (Gan and Bryant, 2015). These adaptions allow
cyanobacteria to persist over long periods of time in environments where nutrients are scarce and temperature or light conditions are not favorable for growth. These adaptive mechanisms supply cyanobacteria with a large competitive advantage over other microorganisms (Dokulil and Teubner, 2000; Kaplan-Levy et al., 2010). Alternatively, favorable conditions such as high temperature, intense light, high pH and increased nutrient levels (particularly nitrogen and phosphorus), encourage these bacteria to rapidly multiply, forming cyanobacterial blooms (Paerl and Otten, 2013).

To date, there is no official consensus among the scientific community that exactly defines a bloom. For example, the United States Environmental Protection Agency (US EPA) has not established specific levels of cell concentrations that would constitute a bloom, but describes it as “visible coloration of a water body due to the presence of suspended cells, filaments and/or colonies and, in some cases, subsequent surface scums” (Fig. 1.1). On the other hand, the World Health Organization (WHO) has guidelines that consider both cell concentration and toxin levels (WHO 2003).

Despite the lack of an exacting definition, it is during these periods of excessive biomass development that some blooms are termed ‘toxic’. For a bloom to be considered toxic, the cyanobacteria from which they are composed must be toxigenic (harbor the ability to produce toxins) and environmental conditions are such that toxigenesis occurs (Sarazin et al., 2002). In temperate regions, modifications of natural habitats to crop fields and the application of fertilizers (i.e. nitrogen and phosphorus) both contribute to the presence of freshwater blooms dominated by cyanobacteria (Doubek et al., 2015; Dokulil and Teubner, 2000). As such, these regions are at an increased risk for their lakes to be affected by toxin levels that are dangerous to any exposed organisms.
Cyanotoxins: Health Risks and Exposure Routes

There are over 100 classified cyanotoxins that exhibit a wide range of chemical structure and health impacts. A PubMed search revealed the most commonly studied toxin types to date are anatoxin, cylindrospermopsin, microcystin, and saxitoxin (Figure 1.2), which was also demonstrated by Merel et al. (2013). Each toxin type has multiple structural forms, known as variants, and each of these variants demonstrate differing toxicity levels (Tonk et al., 2005, Casero et al., 2014, Wood et al., 2012, Sivonen et al., 2009). The various cyanotoxin types cause numerous effects in the human body as their chemical structure determines what organ is predominantly influenced, and thus, what physiological response occurs (Table 1.1). Cyanotoxins are typically contained inside of the bacterial cell (Carmichael, 1992), as they are produced intracellularly as secondary metabolites. These biohazards compounds are not used in primary metabolism, (which include processes like cell division or metabolism), but are utilized for other intracellular processes (Merel at al., 2013). Nonetheless, toxins produced by cyanobacteria are released into aquatic environments through a variety of mechanisms. For instance, there is evidence that cyanotoxins may be purposefully excreted to out-compete
Figure 1.2  Search results from PubMed illustrating the number of journal articles published pertaining to the most commonly studied cyanotoxins as of 2021 (total = 11032); anatoxin (ATX), aplysiasstin (APTX), beta-N-methylamino-L-alanine (BMAA), cylindrospermopsin (CYN), 2,4-diaminobutyric acid (DAB), lyngbyatoxin (LTX), microcystin (MC), nodularin (NOD), saxitoxin (STX).

Table 1.1  Summary of commonly studied toxins (grouped by class) with associated chemical structure, target organ, and resulting health consequences experienced by humans (compiled from: Sivonen et al., 2009, Codd et al., 2017).

<table>
<thead>
<tr>
<th>Toxin Class</th>
<th>Toxin Name</th>
<th>Chemical Structure</th>
<th>Target Organ</th>
<th>Health Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytotoxin</td>
<td>Cylindrospermopsin</td>
<td>Tricyclic guanidine alkaloid</td>
<td>kidney, liver</td>
<td>Gastroenteritis, liver inflammation, liver hemorrhage, kidney failure, pneumonia</td>
</tr>
<tr>
<td>Dermatoxins</td>
<td>Aplysiasstin</td>
<td>Indole alkaloid</td>
<td>skin</td>
<td>tumor promotion, skin irritation, eye irritation, headache, respiratory issues, fever</td>
</tr>
<tr>
<td></td>
<td>Lyngbyatoxin</td>
<td>Indole alkaloid</td>
<td>skin</td>
<td></td>
</tr>
<tr>
<td>Hepatotoxins</td>
<td>Microcystin</td>
<td>Cyclic Heptapeptide</td>
<td>liver</td>
<td>Diarrhea, vomiting, tumor promotion, liver hemorrhage, liver failure</td>
</tr>
<tr>
<td></td>
<td>Nodularin</td>
<td>Cyclic Pentapeptide</td>
<td>liver</td>
<td></td>
</tr>
<tr>
<td>Neurotoxins</td>
<td>Anatoxin-a</td>
<td>Bicyclic alkaloid</td>
<td>nervous system</td>
<td>Muscle twitching, muscle paralysis, burning, numbness, vertigo, respiratory paralysis leading to death</td>
</tr>
<tr>
<td></td>
<td>Anatoxin-a(S)</td>
<td>Phosphorylated cyclic N-hydroxyguanine</td>
<td>nervous system</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BMAA</td>
<td>Diamino Acid</td>
<td>nervous system</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saxitoxin</td>
<td>Alkaloid</td>
<td>nervous system</td>
<td></td>
</tr>
</tbody>
</table>
other photosynthetic organisms, causing effects like cell lysis, blistering, or growth inhibition (Legrand et al., 2003) or as a defensive mechanism to ward off herbivorous zooplankton known as grazers (Jang et al., 2013). Results from Pereira and Giani (2014) suggest these compounds may contribute to quorum sensing (an intercellular communication mechanism), as they were produced at significantly higher intracellular concentrations under high cellular densities, when compared to low density conditions. Moreover, toxins are introduced into aquatic systems upon cell death. When bacterial cells die, they undergo lysis—the physical breakdown of cellular components. As the cellular membrane breaks down, toxins are consequently released into the surrounding environment (Ross et al., 2006).

Cyanobacterial cell lysis could be triggered by natural death due to cell age (senescence), severe environmental stressors, predation by zooplankton, or the death of an entire bloom via human intervention (i.e. copper sulfate algaecide application; Fan et al., 2014). Since the concentration of toxins dissolved in the water column varies with respect to the number of cells dying at a given time, mode of cell death (natural versus human induced), should be considered as a contributing factor to the total concentration of toxins released into an aquatic system (Ross et al., 2006).

There are several routes through which biota may be exposed to cyanotoxins. Common sources of human exposure include consumption of contaminated drinking water, food prepared with contaminated water, or direct ingestion of aquatic life (for instance, saxitoxin may be present in mussels, clams, oysters, and scallops, as it bioaccumulates in muscle tissues; Christensen and Khan, 2020). Accidental ingestion of toxins during recreational activities, dermal contact, and inhalation are additional exposure routes that readily occur through activities like swimming, boating, or fishing, in affected water systems, but may also occur through the use
of water containing cyanotoxins in domestic settings (such as showering using an untreated water supply; Christensen and Khan, 2020). Other exposure pathways include consuming cyanobacterial based dietary supplements, like *Spirulina* or *Aphanizomenon flos-aquae*; although the cyanobacteria harvested for supplements are usually not toxic themselves, the environment from which they are selected may contain toxins or toxic species that are not adequately removed during processing (Roy-Lachapelle et al, 2017). These supplements are not recognized as pharmaceutical products, and as such, do not undergo as stringent quality control measures (Petroczi et al., 2011). And, although not a frequent occurrence, one incident in Brazil involved the use of untreated surface-water in a hemodialysis procedure, causing the death of 65 renal patients by exposing them to microcystin (Carmichael et al., 2001). Following this incident, monitoring of cyanobacteria and their toxins in drinking water supplies became mandatory in Brazil (BRASIL, 2011). This event triggered the introduction of ordinance 2.914/11, that states cyanobacterial cell concentration exceeding 20,000 cells per milliliter requires an analysis of microcystin and saxitoxin in tap water and further encourages an analysis for anatoxin-a(S) and cylindrospermopsin. Due to the severity of health consequences presented by cyanotoxins and the multiple routes for human exposure, safety guidelines have been established by the WHO and US EPA.

In 2015, the US EPA established Health Advisories as part of the Drinking Water Protection Act for some of the known cyanotoxins (microcystin and cylindrospermopsin). These advisories are not enforceable by law, but are established as technical guidelines for federal and state officials to make decisions for the use of public drinking and recreational water when contamination arises. Table 1.2 shows the most recent Health Advisories as determined by the EPA for microcystin and cylindrospermopsin. The listed values are toxin concentration
thresholds that should not be exceeded for drinking water and exposure through recreational activity (via consumption or primary contact, respectively) due to the health consequences that these toxins have on humans. To further public safety, increased emphasis has been placed on monitoring water bodies susceptible to blooms through government agencies and issuing public warnings when toxin levels reach values above specified thresholds.

Table 1.2 Health Advisories established by the US EPA for drinking water and recreational waters. The EPA recommends that authorized officials use a 10-day assessment period.

<table>
<thead>
<tr>
<th>Cyanotoxin</th>
<th>Drinking Water Advisory</th>
<th>Recreational Advisory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>bottle-fed infants and pre-school children</td>
<td>children and adults</td>
</tr>
<tr>
<td>Cylindrospermopsin</td>
<td>0.7 µg/L</td>
<td>3.0 µg/L</td>
</tr>
<tr>
<td>Microcystins</td>
<td>0.3 µg/L</td>
<td>1.6 µg/L</td>
</tr>
</tbody>
</table>

Cyanotoxin and Cyanobacteria Relationship

Due to the negative impacts that cyanotoxins have on economies, ecosystem health, and biota, researchers have prioritized documenting the taxonomy of toxigenic cyanobacteria. Identification of strains common in toxic blooms may aid in predicting the timing of high toxin concentration incidents by pinpointing the biotic and environmental stressors that trigger toxigenesis; individual strains show variability in toxin producing capabilities (Wood et al., 2012), and there is evidence suggesting environmental triggers encouraging toxigenesis can vary between species and strains (Marmen et al., 2016; Wood et al., 2017).

Attempting to identify a toxic cyanobacterial species is a complex task, partly due to the relationship between currently described (taxonomically classified) species and the toxins they are able to produce. For instance, taxa from the genera *Dolichospermum*, *Microcystis*, *Nostoc*, *Oscillatoria*, *Planktothrix*, have all been shown to produce microcystin variants (Davis et al.,...
2009; Kurmayer et al., 2004; Rapala et al., 1997; Sivonen et al., 1992). Meanwhile, one species can produce different toxins; *Aphanizomenon flos-aquae* has been reported to produce cylindrospermopsin and multiple saxitoxin variants (Preußel et al. 2006; Ferreira et al., 2001). Further yet, one strain of a cyanobacterial species may contain the genes required to produce multiple types and variants of toxins, (Rapala et al., 1997, Wood et al., 2012, Sivonen et al., 1992). While far from exhaustive, Table 1.3 below shows additional examples of the complex relationship between freshwater cyanobacteria and toxins they have been established to produce at the species or strain level. Lastly, two strains of the same species may look morphologically identical under the microscope, while only one contains the genes required for toxin production (Wood et al., 2012). This makes the identification of toxin producing taxa through microscopy alone essentially impossible.

**Table 1.3** List of freshwater cyanobacterial species and/or strains that are capable of producing anatoxin (ATX), cylindrospermopsin (CYN), microcystin (MC), and saxitoxin (STX); toxins types shown include variants. Dots indicate confirmed production of toxin.

<table>
<thead>
<tr>
<th>Cyanobacterial Taxa</th>
<th>ATX</th>
<th>CYN</th>
<th>MC</th>
<th>STX</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aphanizomenon flos-aquae</em></td>
<td>*</td>
<td></td>
<td></td>
<td>*</td>
<td>Preußel et al. 2006</td>
</tr>
<tr>
<td><em>Aphanizomenon gracile</em></td>
<td></td>
<td></td>
<td>*</td>
<td></td>
<td>Casero et al., 2014</td>
</tr>
<tr>
<td><em>Aphanizomenon ovalisporum</em></td>
<td></td>
<td>*</td>
<td></td>
<td></td>
<td>Cires et al., 2013</td>
</tr>
<tr>
<td><em>Cuspidothrix issatschenkoi</em></td>
<td></td>
<td>*</td>
<td></td>
<td></td>
<td>Wood et al., 2007</td>
</tr>
<tr>
<td><em>Cylindrospermopsis raciborskii</em></td>
<td></td>
<td>*</td>
<td></td>
<td></td>
<td>Hawkins et al., 2001; Wood et al, 2003; Pomati et al., 2004</td>
</tr>
<tr>
<td><em>Dolichospermum cirrinalis</em></td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td>Mihali et al., 2009</td>
</tr>
<tr>
<td><em>Microcystis aeruginosa</em></td>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td>Long et al., 2001</td>
</tr>
<tr>
<td><em>Nostoc sp. Treb K1/5</em></td>
<td></td>
<td>*</td>
<td></td>
<td></td>
<td>Kust et al., 2018</td>
</tr>
<tr>
<td><em>Oscillatoria sp. PCC 6506</em></td>
<td>*</td>
<td>*</td>
<td></td>
<td></td>
<td>Mejean et al., 2010; Bormans et al., 2014</td>
</tr>
<tr>
<td><em>Phormidium sp. LP904c</em></td>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td>Shishido et al., 2019</td>
</tr>
<tr>
<td><em>Planktothrix agardhii</em></td>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td>Tonk et al., 2005</td>
</tr>
</tbody>
</table>

1 - previously classified as *Aphanizomenon*
2 – previously classified as *Anabaena*
Research Motivation, Goal, Design, and Contributions

The aim of this research is to isolate and identify novel (previously undescribed) toxin-producing cyanobacteria that inhabit lakes in Iowa, determine what toxin (or toxins) they are capable of producing, and establishing in what lakes the toxic strains are present. Iowan lakes, along with many states in the Midwest, have previously experienced bloom and toxin occurrences (including microcystin, anatoxin-a, cylindrospermopsin, and saxitoxin; Graham et al., 2010). This makes Iowan lakes primary candidates for the successful isolation and identification of toxin-producing cyanobacteria.

The project focus on lakes in Iowa is supported the collaborative effort of the IDNR’s Beach Monitoring Program. In this program, composite water samples are collected weekly from 39 beaches at lakes within Iowa’s State Parks, from Memorial Day through Labor Day. By combining laboratory and analysis efforts with the water sampling and data collection done by the IDNR, our hope is to obtain a more refined picture of the toxic cyanobacterial taxa present in the sites being evaluated.

Considering the details mentioned above, viewing lake water samples via microscopy will not provide enough evidence to identify a toxigenic cyanobacterial strain. As multiple strains have been shown to produce the same toxin types, toxin testing of water samples containing multiple cyanobacterial species will not give a clear indication of who is responsible for toxin production, nor will identification of cyanobacteria through molecular techniques. However, combining these methods to evaluate the relationship between cyanobacteria presence and toxin production could provide the level of necessary detail to better understand which species are producing which toxin (or toxins) in Iowan lakes.

The general procedure outlined for identifying novel toxic cyanobacterial isolates included obtaining lake water samples, testing raw lake water for toxins, cultivating
cyanobacteria in lab, establishing that cultivated cyanobacteria were in fact pure cultures, screening the isolates for toxin genes, establishing the identify of toxic strains, and using bioinformatics analysis to establish community composition of the lakes sampled. As neurotoxins of freshwater cyanobacteria are understudied compared to microcystin (Christensen and Khan, 2020), screening genes responsible for biosynthesis of anatoxin-a and saxitoxin, along with the cytotoxin, cylindrospermopsin, were prioritized to increase the odds of finding a novel toxin producing cyanobacterial strain.

Culturing and identifying novel toxic cyanobacterial strains also fill research gaps in respect to the lack of currently classified cyanobacteria. With the advance of High-Throughput Sequencing (HTS) techniques for DNA, the necessity of culturing bacterial strains for identification has been reduced; a pure culture is no longer required for DNA sequencing (Venter et al., 2004). This innovation provides a solution, as many bacteria (including cyanobacteria) are fastidious, and difficult to grow in a lab setting, particularly in isolation (Abed and Köster, 2005; Bruno et al., 2006). However, the need for purely cultured strains is not entirely replaced by HTS technologies (De Figueiredo et al., 2010; Rego et al., 2019). Without pure cultures of cyanobacterial strains, the ability to unambiguously determine their activity and significance in the environment, not just their genetic capacity, is lost, as metabolic analyses that measure growth rate and response to environmental stimuli require pure cultures that are maintainable in lab settings.

Isolating and taxonomically classifying novel cyanobacterial species will also fill knowledge gaps currently seen in the field of microbial ecology. Microbial community analyses utilize reference databases and sequencing information from environmental samples to classify sequences into Operational Taxonomic Units (OTUs). However, there is currently a large deficit in classified and cultured cyanobacterial sequences contained in the reference databases being
used. For instance, the sequence dataset used for this project contained ~65% uncultured or unclassified bacterial OTUs at the species level, highlighting a massive shortfall in the current landscape of microbial ecology. Indeed, it is estimated that there are $10^{11} - 10^{12}$ microbial species exist on Earth (Locey and Lennon, 2016), but documented sequencing information reveals only $10^5$ species are accounted for, and from which, only ~12,700 have been cultured (Marx, 2017). Knowing these vast numbers of microbial species of have not yet been classified, it is no surprise that OTU datasets may contain a large number of unclassified and uncultured cyanobacterial OTUs. The taxonomical classification of previously undescribed cyanobacterial taxa helps bridge the gap currently presented in this field.

The research for this thesis has also contributed to part of a larger collaborative project funded by the EPA. In learning about culturing conditions for cyanobacteria, I was able to receive and care for cyanobacterial isolates from culture collections, contributing to a published paper evaluating the effectiveness of different primer sets for detecting the \textit{mcyA} gene between various cyanobacterial genera (Improved detection of \textit{mcyA} genes and their phylogenetic origins in harmful algal blooms; Lee et al., 2020). Additional work done during my time as a Master’s student included liquid chromatography—tandem mass spectrometry (LC-MS/MS) evaluation of pigments in anoxygenic phototrophs (Detection, activity, and signatures of iron-based anoxygenic phototrophy in ferruginous Brownie Lake, MN; Lambrecht et al., in preparation), and creating a bioinformatics pipeline specific to the 18S rRNA region of eukaryotic organisms to be used for community composition analysis of phytoplankton (Phytoplankton dynamics of a seasonally anoxic and ferruginous lake: the Grosses Heiliges Meer in Germany; Swanner et al., currently in submission).
CHAPTER 2. MATERIALS AND METHODS

Site Location and Sample Collection

Lake water samples evaluated for this study were collected through the IDNR Beach monitoring program in 2019. This program collects raw lake water samples weekly from 39 beaches within Iowa State Parks, Memorial Day through Labor Day (15 weeks total). Composite freshwater samples collected from surface levels of lakes were stored in 1L plastic bottles and placed in coolers containing ice during transport. All samples collected by IDNR officials were divided between the IDNR and labs at Iowa State University, so that each respective organization could perform further analyses as needed.

Samples chosen for analysis in this study comprised a subset of lakes (n=9) exhibiting elevated microcystin levels in previous sampling years (Fig. 1). To further increase the odds of isolating and identifying a novel toxin-producing cyanobacterial strain, samples collected from July 16, 2019 to August 28, 2019 (weeks 9-15, respectively) were chosen for evaluation, as historical data showed dominance of Cyanobacteria during the later summer. This resulted in a total of 63 samples for the 2019 season. Upon receipt from sample collection, raw lake water samples were stored in plastic bottles at 4 °C and processed within 3 days of initial sample collection.

Enzyme-Linked Immunosorbent Assay (ELISA) Toxin Measurements

Samples from the subset of 9 lakes, weeks 9-15, collected from the 2019 season were analyzed for anatoxin-a and saxitoxin. Tests for anatoxin-a and saxitoxin were performed using their respective testing kit (Anatoxin-a microtiter plate and Saxitoxin (PSP) microtiter plate; ABRAXIS, PA, USA) according to manufacturer’s instructions. Raw lake water samples
Figure 2.1 Map of southwestern Iowa showing lake samples used for this study (indicated by red dots). The subset of nine lakes was chosen based off of previous data showing elevated microcystin levels. Blue dots indicate larger set of lakes sampled by the IDNR’s Beach Monitoring Program.

(10 mL) were collected on site and preserved with diluent according to manufacturer’s instructions, stored in glass vials wrapped in aluminum foil for protection from light, and placed in ice filled coolers while transported to the lab. Samples then underwent three freeze-thaw cycles for cellular digestion and subsequent release of intracellular toxins. The Limit of Detection (LOD) for anatoxin-a and saxitoxin in these assays is 0.1 µg/L and 0.015 µg/L, respectively. Samples and calibration standards were tested in duplicate in accordance with EPA
requirements (EPA method 546). Absorption of the color reaction was recorded at 450 nm using a Biotech Epoch2 plate reader.

Cultivation of Cyanobacterial Cultures

The growth medium used for this study was BG-11 (both solid and liquid), as it has been optimized for the growth of oxygenic photoautotrophs (Vaara 1979), along with a BG-11 based buffer (containing the standard BG-11 recipe, minus the addition of vitamins, trace metals, ferric ammonium citrate, sodium carbonate, and dipotassium phosphate). Additionally, there was a modified form of BG-11 used for the initial cultivation of samples, in which the nutrient concentration was reduced by 10-fold, as an attempt to better match lake water nutrient conditions. To exclude the growth of phototrophic eukaryotic organisms, the antibiotic cycloheximide (0.1 g/L) was added to all BG-11 growth medium (Vaara et al. 1979).

The cultivation method used was a Most Probable Number (MPN) technique, which served to both serially dilute cells to cultivate abundant organisms and to quantify the abundance of cells cultivated from environmental samples (Prasana et al., 2006). To estimate the abundance of cells in the original samples, 96-well plates were scored according to their growth response upon cultivation. To cultivate cyanobacteria, 10 mL of lake water sample from each collection site was aliquoted into a sterilized glass tube. Then, a 10-fold serial dilution of each sample was performed aseptically. This required 1 mL of undiluted freshwater sample being added to 9 mL of buffer and vortexed. This process was repeated to end with a final concentration of 10^{-11} mL of sample. 96-well plates containing 500 µL BG-11 (nutrient reduced) solid agar were then inoculated with 50 µL aliquot (raw sample and dilution series) per well, such that the sample aliquot was 10% of the total volume in each well. Samples were transferred using a multichannel
pipettor; agar was penetrated with pipette tips and inoculum was injected steadily as pipettor was removed, such that inoculum was distributed into the solid agar. Each sample dilution series was replicated in seven rows, while the last row in the 96-well plate was uninoculated only with buffer mixture, to serve as a control for the presence contamination.

Plates were incubated at 25°C with 6.5 W and 10 W fluorescent bulbs (2700K and 5000K, respectively; Rippka 1988). This light treatment provided wavelengths across the spectral PAR band to promote growth of oxygenic photoautotrophs. Transparent plastic covers were placed over plates to prevent agar desiccation. MPN plates were scored after ~3 months of incubation, and MPN estimates were calculated using the EPA MPN calculator (https://mostprobablenumbercalculator.epa.gov/index.html) using a 95% Cornish and Fisher Confidence Limit, with 30 maximum iterations. Results were reported as Growth Units. Following MPN assessments, individual wells containing visible signs of growth were transferred into 10 mL sterilized class tubes containing standard BG-11 liquid medium, placed on a shaker set at 100 rpm, and allowed to incubate under the conditions mentioned above. Successful cultures were transferred several generations.

**Screening Cyanobacterial Cultures for Toxigenic Strains**

**Fluorescence-assisted Sample Screening**

Twenty cultures were screened for purity using flow cytometry (FC) based on fluorescent properties as well as size. As cyanobacteria are photosynthetic, they contain pigments used to capture and convert sunlight into energy. While flow cytometry typically utilizes dyes for its analyses, this innate feature of Cyanobacteria allows for the characterization of cells by laser excitation and subsequent emission (fluorescence). The presence of cyanobacteria was verified by fluorescence response of allophycocyanin (APC), a particular pigment exhibited by all
cyanobacteria. As pigments will only fluoresce at a specific emission under a defined wavelength, pigments types and relative amounts can be differentiated based on their fluorescence intensity. This method was utilized to distinguish the presence of multiple cyanobacterial morphotypes, but as screening was carried out only with autofluorescence, other unpigmented bacterial organisms were likely not identified. APC fluorescence was measured at 670/30 nm with excitation wavelength 673 nm. Multiple cyanobacterial taxa contained in the sample were identified using excitation wavelengths (488 and 633 nm) and fluorescence values measured at 675/26 nm and 780/60 nm (Table 2.1). Samples were submitted to Iowa State University Office of Biotechnology Flow Cytometry Facility and run on BD FACSCanto (San Jose, CA) factory direct instrumentation with no secondary modifications.

Table 2.1 Fluorescence channels with corresponding excitation wavelength and fluorescence values; APC pigment used as an indicator for cyanobacterial cells (FL3). Fluorescence channels (FL1, FL2, and FL4) distinguished cyanobacterial cells containing different pigment proportions.

<table>
<thead>
<tr>
<th>Fluorescence Channel</th>
<th>Excitation Wavelength (nm)</th>
<th>Fluorescence (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL1</td>
<td>488</td>
<td>675/26</td>
</tr>
<tr>
<td>FL2</td>
<td>488</td>
<td>780/60</td>
</tr>
<tr>
<td>FL3</td>
<td>633</td>
<td>670/30</td>
</tr>
<tr>
<td>FL4</td>
<td>633</td>
<td>780/60</td>
</tr>
</tbody>
</table>

Extraction and Purification of culture DNA

DNA was extracted from culture samples that were considered pure according to flow cytometry results (14 of 20 samples), using the DNeasy PowerBiofilm Kit (Qiagen) (Gaget et al., 2016). Extraction was done primarily to according to the manufacturer’s instructions, however, the incubation step (at 65 °C) was extended from to 5 minutes to 12 minutes. Extracted DNA was purified using E.Z.N.A Cycle Pure Kit (Omega Bio-tek, Inc.). Concentration and quality measurements of purified DNA samples were recorded at 260/280 nm using an Epoch2 spectrometer (BioTek). Purified DNA products were stored at -20 °C.
Toxin Gene Screening via Polymerase Chain Reaction

To determine which enrichments possibly contained toxin-producing cyanobacteria, the presence of toxin functional genes for anatoxin-a, cylindrospermopsin, and saxitoxin were evaluated using polymerase chain reaction (PCR) (Table 2.2) on purified DNA of the 14 cyanobacterial enrichments. Cycling began with a denaturing step at 94 °C for 5 min., followed by 30 cycles of denaturing at 94 °C for 30 s, primer annealing at 53.5, 50.3, 59, 55 °C, (for primer sets anaC-gen, anaXgen, cyrJ, and sxt1, respectively) for 30 s, and final extension at 72 °C for 10 minutes. Positive control template sequences were obtained from NCBI GenBank (anaC, CP011456.1; cyrJ, FJ418586.4; sxt1, EU439559.1). Synthetic oligonucleotides of these sequences were synthesized by Integrated DNA Technologies (IDT). Gel electrophoresis (1 % agarose gel with 100 µL/L SyberSafe in 1X Tris-acetate-EDTA [TAE] buffer) results were viewed using a UV Transilluminator (BioDoc-It System UVP).

Table 2.2 Table listing toxins synthesized by targeted functional genes and their respective primer pairs used for amplification, along with expected amplicon length. Orientation of primers listed as forward (F) and reverse (R).

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Functional Gene</th>
<th>Primer Pair/ Orientation</th>
<th>Primer Sequence (5' - 3')</th>
<th>Amplicon Length (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anatoxin-a</td>
<td>anaC</td>
<td>anxgen F</td>
<td>ATGGTCAGAGGTTTTACAAG</td>
<td>861</td>
<td>Rantala-Yinlen et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anxgen R</td>
<td>CGACTCTTAATCATGCATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anatoxin-a</td>
<td>anaC</td>
<td>anaC-gen F</td>
<td>TCTGGTATTCAGTCCCCCTCTAT</td>
<td>366</td>
<td>Rantala-Yinlen et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>anaC-gen R</td>
<td>CCCAATAGGCTGCATCAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cylindrospermopsin</td>
<td>cyrJ</td>
<td>cyrJ F</td>
<td>TTCTCTCTCTCCCTCTCTTTATATC</td>
<td>561</td>
<td>Mazmouz et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cyrJ R</td>
<td>GCTACGCTGTACCAAGGGGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saxitoxin</td>
<td>sxt1</td>
<td>sxt1 F</td>
<td>GCTTACTACCACGATAGTGCTGCG</td>
<td>1669</td>
<td>Kellman et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sxt1 R</td>
<td>GGTTCGCCGCGACATTAAA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Further Investigation of a Putatively Toxic Cyanobacterial Culture

Confirmation of sxtI Amplicon Sequence via Cloning

To confirm the result obtained from PCR amplification of the sxtI region, cloning was performed on the sxtI amplicon, using the sxtI primers under the same conditions as mentioned above. The resulting PCR amplicons were purified using PureLink Quick Gel Extraction & PCR purification kit (Invitrogen), and purified PCR products were cloned into a plasmid vector containing an ampicillin-resistant marker gene using the TOPO TA cloning kit (ThermoFisher). Vectors containing the sxtI gene insert were then transformed into One Shot Top10 chemically competent E. coli cells using the TOPO TA cloning kit (ThermoFisher) and plated onto LB agar containing ampicillin (100 µg/ml). Selected colonies (n=8) were tested for the correct insert size using PCR with primers M13F (5’-CAGGAAACAGCTATGAC-3’) and M13R (5’-GTAAACCGACGGCCAG-3’) and gel electrophoresis (1 % agarose gel with 100 µL/L SybrSafe in 1X Tris-acetate-EDTA [TAE] buffer). M13 PCR products of the appropriate amplicon size were excised from gel and cleaned using the gel extraction protocol form PureLink Quick Gel Extraction & PCR purification kit (Invitrogen). Concentrations of purified samples were measured using a Qubit 3.0 fluorometer. Samples were submitted to the Iowa State University DNA facility for Sanger sequencing with T3 and T7 primer promoters provided in the pCR 4-TOPO kit (ThermoFisher Scientific). Forward and reverse sequences provided from Sanger sequencing were evaluated with FastQC and trimmed. High-quality, single-end reads were unable to be made into paired-end reads due to the length of the target gene (Table 2.2). As such, each single-end read (forward and reverse) were searched for in NCBI using BLASTx (Altschul et al., 1997) in the non-redundant protein sequence database.
Establishing Identity of putatively toxic isolate (Cloning of 16S rRNA gene)

To identify which organisms were present in sample 20, a sample that showed amplification of the sxtI gene, near full-length 16S rRNA gene copies were obtained from PCR amplification using the universal bacterial primers 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-TACGGYTACCTGTTACGACTT-3’), and the cloning process was completed as described above (section 2.5.3). Forward and reverse sequences provided from Sanger sequencing were evaluated with FastQC and trimmed; single-end reads were combined into paired-end reads (n=3) using Unipro Ugene software. Paired-end reads were compared against NCBI BLASTn (Zhang et al., 2000) standard nr/nt database. Paired-end reads (3 of 3) were aligned with TCoffee (Notredame et al., 2000) through EMBL-EBI (Madeira et al., 2019).

Illumina Sequencing and Bioinformatics Analysis

To determine the microbial community composition, DNA was extracted from lake water samples for amplicon sequencing of the 16S rRNA gene. In this process, 200 to 300 mL composite samples were filtered on 0.22 µm pore size hydrophilic polyethersulfone (PES) membranes and placed into 5mL Bead tubes. Filters were frozen at -80°C. DNA was extracted using the MagAttract PowerWater DNA/RNA kit (QIAGEN, Germantown MD., USA) following manufacturer instructions. Illumina MiSeq was performed by the Sequencing Facility at Argonne National Lab using the EMP 16s Illumina amplicon protocol (Caporaso et al., 2018). Sequencing the V4 region of the 16S rRNA gene was completed via primer pair 515F (5’-GTGCCAGCMGCCGCGGTAA-3’) and 805R (5’-GACTACVGGGTATCTAA-3’). Processing was performed on de-multiplexed sequences using the standard protocol in Mothur version 1.44.1 (Schloss et al. 2009); amplicon pairs were checked for quality (quality score >25, error < 1%, ambiguities removed), assembled, and aligned to the SILVA v138 database (Quast et
al., 2013). Any sequences less than 245 and more than 265 base pairs were removed. Chimeras were checked with VSEARCH version 2.13.3 (Rognes et al., 2016), and sequences preclustered up to 2 nucleotide differences between sequences. Operational taxonomic units (OTUs) were clustered at 97% similarity and 80% confidence threshold. The taxonomy of each OTU was assigned using the SILVA NR v138 database. All subsequent analyses were performed in R/Rstudio version 4.0.2.

**Searching Microbial Community Dataset for Putatively Toxic Isolate**

Command Line Blast (version 2.9 with default parameters) was used to search for the 16S rRNA sequence of a putatively toxic isolate in the larger microbial community dataset. An additional dataset was generated in mothur containing individual sequences and OTU representative sequences, using the ‘bin.seqs’ and ‘getotu.rep’ commands. The highest percent matches were reviewed to establish if homology could be inferred between the highest matches found in the larger community dataset and the 16S rRNA sequence from the putatively toxic isolate.

**Microscopy on Putatively Toxic Enrichment**

To characterize the purity of the enrichment, a Leica DM6B Leica Microsystems (Mannheim, Germany) was to view the sample using bright field illumination (BF), polarized light (POL), and fluorescence of unstained (autofluorescent) cells. A composite image created using filter cubes A4, L5, RHO and I3, (corresponding to excitation wavelengths 360/40, 480/40, 546/10, and 450-490 nm, respectively). Cell dimensions were measured using ImageJ version 1.53. Varying cell sizes, morphologies, and fluorescent properties were used as an indicator that more than one organism was present in the culture.
Fluorescent Assisted Cell Sorting

Target cells were sorted by adjusting the FC parameters to select according to wide Forward Scatter Width signal (FCS-W). Before undergoing the FC selection process, cells were disaggregated according to the optimal method shown by Avezedo et al. (2012); a 4 minute sonication period of cells contained in suspension, followed by the addition of isolation solution FCB5 (Triton-X100, .5% in distilled water) to culture suspension at a 2:1 ratio (isolation solution: culture suspension). Final cell suspensions were filtered through a 40 µm nylon filter before sorting. Upon sorting, cells were collected into BG-11 liquid medium, aseptically transferred into glass flasks, and incubated under the previously mentioned conditions.

Cyanobacteria Community Analysis

Using the package ‘phyloseq’, as implemented in RStudio, all OTUs not belonging to the phylum ‘Cyanobacteria’, were filtered for further analysis. Only samples that had corresponding toxin concentration data (the subset of 9 lakes sampled between weeks 9 through 15) were used in community evaluation and statistical analyses, and low abundance OTUs (<1x10^-5) were removed. After read counts were evaluated, correction for differential sequencing depth was done by normalizing the number of reads in each sample using median sequencing depth.
CHAPTER 3. RESULTS

Toxin Measurements

To determine if the selected toxins were present in the lakes being evaluated for toxin-producing cyanobacteria, ELISA tests were completed for each sampling site from Week 9 to Week 15 of the 2019 sampling season. The percent of detected values, mean detected values, and median detected values (both by week and by site) were calculated using detected values only. The coefficient of variability (%CV) between sample replicates is listed in Appendix A.

Anatoxin-a was present at detectable levels at all sites in six of the seven weeks (Table 3.1) and saxitoxin was present at detectable levels in at least one lake per week (Table 3.2). Anatoxin-a showed detectable amounts in all lakes for Week 14 (mean of detected values = 0.273 µg/L) and Week 15 (mean of detected values = 0.238 µg/L). Brushy Creek showed the greatest number of detectable values of anatoxin-a through the sampling period (71%), while the highest mean value of detectable anatoxin-a was from Green Valley Lake (1.098 µg/L). Saxitoxin showed detectable amounts in all lakes during Week 11 (mean of detected values = 0.034 µg/L). Denison lake showed the greatest number of saxitoxin detections through the sampling period (86%), while the highest mean value of detectable saxitoxin was from Lake Anita (0.031 µg/L).
Table 3.1. Summary of anatoxin-a concentrations (µg/L). Values above the detection level of ELISA kit (0.1 µg/L) are listed in bold.

<table>
<thead>
<tr>
<th>Lake</th>
<th>Week 9</th>
<th>Week 10</th>
<th>Week 11</th>
<th>Week 12</th>
<th>Week 13</th>
<th>Week 14</th>
<th>Week 15</th>
<th>% detections by site</th>
<th>mean of detections</th>
<th>median of detections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake of Three Fires</td>
<td>0.024</td>
<td>0</td>
<td>0.017</td>
<td>0.076</td>
<td>0.072</td>
<td>0.612</td>
<td>0.128</td>
<td>29%</td>
<td>0.434</td>
<td>0.37</td>
</tr>
<tr>
<td>Green Valley</td>
<td>0.027</td>
<td>0</td>
<td>0.006</td>
<td>0.204</td>
<td>0.304</td>
<td>0.207</td>
<td>0.536</td>
<td>57%</td>
<td>1.098</td>
<td>0.255</td>
</tr>
<tr>
<td>Big Creek</td>
<td>0.126</td>
<td>0</td>
<td>0.022</td>
<td>0.021</td>
<td>0.234</td>
<td>0.215</td>
<td>NA</td>
<td>50%</td>
<td>0.491</td>
<td>0.215</td>
</tr>
<tr>
<td>Denison</td>
<td>0.01</td>
<td>0.023</td>
<td>0.053</td>
<td>0.035</td>
<td>0.234</td>
<td>0.19</td>
<td>0.189</td>
<td>43%</td>
<td>0.457</td>
<td>0.19</td>
</tr>
<tr>
<td>Black Hawk</td>
<td>0.017</td>
<td>0</td>
<td>0.076</td>
<td>NA</td>
<td>0.229</td>
<td>0.25</td>
<td>0.123</td>
<td>50%</td>
<td>0.449</td>
<td>0.229</td>
</tr>
<tr>
<td>North Twin East</td>
<td>0.004</td>
<td>0.01</td>
<td>0.004</td>
<td>0.07</td>
<td>0.08</td>
<td>0.231</td>
<td>0.266</td>
<td>29%</td>
<td>0.382</td>
<td>0.248</td>
</tr>
<tr>
<td>North Twin West</td>
<td>0.006</td>
<td>0.005</td>
<td>0.003</td>
<td>0.014</td>
<td>0.038</td>
<td>0.162</td>
<td>0.204</td>
<td>29%</td>
<td>0.285</td>
<td>0.183</td>
</tr>
<tr>
<td>Brushy Creek</td>
<td>0.205</td>
<td>0.021</td>
<td>0.143</td>
<td>0.014</td>
<td>0.141</td>
<td>0.258</td>
<td>0.189</td>
<td>71%</td>
<td>0.772</td>
<td>0.189</td>
</tr>
<tr>
<td>Lake Anita</td>
<td>0.016</td>
<td>0</td>
<td>0.354</td>
<td>0.013</td>
<td>0.271</td>
<td>0.334</td>
<td>0.266</td>
<td>57%</td>
<td>0.960</td>
<td>0.302</td>
</tr>
</tbody>
</table>

% detections | 22%     | 0%       | 22%     | 11%     | 67%     | 100%    | 100%    |                      |                    |
Detection mean      | 0.166   | -        | 0.249   | 0.204   | 0.236   | 0.273   | 0.238   |                      |                    |
Detection median    | 0.166   | -        | 0.249   | 0.204   | 0.234   | 0.231   | 0.197   |                      |                    |

Table 3.2. Summary of saxitoxin concentrations (µg/L). Values above the detection level of ELISA kit (0.015 µg/L) are listed in bold.

<table>
<thead>
<tr>
<th>Lake</th>
<th>Week 9</th>
<th>Week 10</th>
<th>Week 11</th>
<th>Week 12</th>
<th>Week 13</th>
<th>Week 14</th>
<th>Week 15</th>
<th>% detections by site</th>
<th>mean of detections</th>
<th>median of detections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake of Three Fires</td>
<td>0.031</td>
<td>0</td>
<td>0.022</td>
<td>0.022</td>
<td>0.033</td>
<td>0</td>
<td>0</td>
<td>57%</td>
<td>0.027</td>
<td>0.0265</td>
</tr>
<tr>
<td>Green Valley</td>
<td>0.03</td>
<td>0</td>
<td>0.019</td>
<td>0.021</td>
<td>0.042</td>
<td>0</td>
<td>0.009</td>
<td>57%</td>
<td>0.028</td>
<td>0.0255</td>
</tr>
<tr>
<td>Big Creek</td>
<td>0</td>
<td>0</td>
<td>0.034</td>
<td>0.015</td>
<td>0.036</td>
<td>0</td>
<td>NA</td>
<td>50%</td>
<td>0.028</td>
<td>0.034</td>
</tr>
<tr>
<td>Denison</td>
<td>0.017</td>
<td>0.025</td>
<td>0.059</td>
<td>0.02</td>
<td>0.022</td>
<td>0.008</td>
<td>0.015</td>
<td>86%</td>
<td>0.026</td>
<td>0.021</td>
</tr>
<tr>
<td>Black Hawk</td>
<td>0.02</td>
<td>0.005</td>
<td>0.022</td>
<td>0.033</td>
<td>0.018</td>
<td>0.013</td>
<td>0</td>
<td>57%</td>
<td>0.023</td>
<td>0.021</td>
</tr>
<tr>
<td>North Twin East</td>
<td>0.006</td>
<td>0.018</td>
<td>0.043</td>
<td>0</td>
<td>0.013</td>
<td>0.014</td>
<td>0</td>
<td>43%</td>
<td>0.031</td>
<td>0.0305</td>
</tr>
<tr>
<td>North Twin West</td>
<td>0</td>
<td>0.022</td>
<td>0.042</td>
<td>0.001</td>
<td>0.13</td>
<td>0.023</td>
<td>0.014</td>
<td>43%</td>
<td>0.029</td>
<td>0.023</td>
</tr>
<tr>
<td>Brushy Creek</td>
<td>0</td>
<td>0.02</td>
<td>0.035</td>
<td>0.02</td>
<td>0</td>
<td>0</td>
<td>0.011</td>
<td>43%</td>
<td>0.025</td>
<td>0.02</td>
</tr>
<tr>
<td>Lake Anita</td>
<td>0.017</td>
<td>0</td>
<td>0.03</td>
<td>0.013</td>
<td>0.047</td>
<td>0.028</td>
<td>0.014</td>
<td>57%</td>
<td>0.031</td>
<td>0.029</td>
</tr>
</tbody>
</table>

% detections | 22%     | 44%      | 100%    | 56%     | 67%     | 22%     | 13%     |                      |                    |
Detection mean      | 0.023   | 0.021   | 0.034   | 0.022   | 0.033   | 0.026   | 0.015   |                      |                    |
Detection median    | 0.020   | 0.020   | 0.034   | 0.021   | 0.035   | 0.026   | 0.015   |                      |                    |
Screening for Toxic Isolates

Cultivation and Most Probable Number

To estimate the concentration of culturable cyanobacteria in original lake water samples, an MPN approach was used. MPN plates were scored for visible growth of cyanobacteria ~3 months after initial plating and incubation. As many cultures were composed of filamentous cyanobacteria growing in clusters (not as individual cells), the calculated values are given as Growth Units/mL of culturable cyanobacteria in the original (undiluted) lake water sample (Appendix B). After MPN plates were scored, individual wells were aseptically transferred into glass tubes containing standard BG-11 medium for further cultivation.

From the 2019 sampling season, 20 cyanobacterial cultures were produced. Many of the MPN plates showed no signs of visible growth (Appendix B), and not all transferred cultures grew successfully. As such, multiple cultures collected from high concentration MPN wells representing the same Site and Week were used for downstream analyses (Table 3.3).

Table 3.3. Sample key of successfully grown cyanobacterial cultures transferred from MPN plate wells. All 20 cultures were used for subsequent analyses.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Enrichment Site/Week Number</th>
<th>Sample Number</th>
<th>Enrichment Site/Week Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Anita Week 13</td>
<td>11</td>
<td>Denison Week 12</td>
</tr>
<tr>
<td>2</td>
<td>Anita Week 13</td>
<td>12</td>
<td>Denison Week 12</td>
</tr>
<tr>
<td>3</td>
<td>Anita Week 14</td>
<td>13</td>
<td>Denison Week 13</td>
</tr>
<tr>
<td>4</td>
<td>Big Creek Week 14</td>
<td>14</td>
<td>Denison Week 15</td>
</tr>
<tr>
<td>5</td>
<td>Big Creek Week 15</td>
<td>15</td>
<td>Green Valley Week 10</td>
</tr>
<tr>
<td>6</td>
<td>Black Hawk Week 9</td>
<td>16</td>
<td>Green Valley Week 11</td>
</tr>
<tr>
<td>7</td>
<td>Black Hawk Week 10</td>
<td>17</td>
<td>North Twin East Week 13</td>
</tr>
<tr>
<td>8</td>
<td>Black Hawk Week 11</td>
<td>18</td>
<td>North Twin East Week 14</td>
</tr>
<tr>
<td>9</td>
<td>Black Hawk Week 15</td>
<td>19</td>
<td>North Twin West 14</td>
</tr>
<tr>
<td>10</td>
<td>Brushy Creek Week 14</td>
<td>20</td>
<td>Three Fires 10</td>
</tr>
</tbody>
</table>
Flow Cytometry

Before further analyses were completed, cultures collected from the 2019 sampling season (Table 3.4) were subject to flow-cytometry. The confirmation of cyanobacteria in cultures was attributed to an APC-H peak (near $10^4$) on the x-axis, where the number of cells containing APC is visible on the y-axis (Fig. 3.1). Peaks near $10^1$ were indicative of non-APC events (i.e. non-cyanobacterial cells, dead cells, or any debris derived from culture). As APC fluorescence was compared to additional pigments represented by three fluorescence channels (FL1, FL2, and FL4), fluorescence signals displaying a linear pattern were depicted as axenic cyanobacterial cultures (Fig. 3.2). Cultures with multiple cyanobacterial taxa (populations) were indicated by clustering of different pigment types separated by their fluorescence intensity; P1, P2, and P3 represent three different cyanobacterial taxa in culture (Fig. 3.3).

![Figure 3.1](image.png)

**Figure 3.1.** Single parameter histogram of flow cytometry results; APC-H count. Peak near $10^4$ confirms the presence of cyanobacteria in cultures; peak displayed near $10^1$ are non-APC events (including non-cyanobacterial cells or debris).
Figure 3.2 Flow cytometry results comparing pigment fluorescence signals. Dot plots compare APC fluorescence signal (y-axis) to additional pigments represented by fluorescence channels FL1, FL2, and FL4 (x-axes). Linear patterns indicate an axenic cyanobacterial culture.

Figure 3.3 Flow cytometry results comparing pigment fluorescence signals. Dot plots compare APC fluorescence signal (y-axis) to additional pigments represented by fluorescence channels FL1, FL2, and FL4 (x-axes). Clustering patterns indicate culture containing multiple cyanobacterial taxa (denoted as P1, P2, and P3).

Toxin Gene Screening

PCR screening for toxin functional genes on the 14 isolates determined to be pure by flow cytometry yielded no results from anaCgen, anaX-gen, or cyrJ primer sets, as indicated by the absence of amplified material upon viewing gel electrophoresis results. However, results from PCR did indicate material amplified from the sxtI primer set corresponding to the
appropriate control bp size (1669 bp) in three samples; Denison Week 12 (n=2) and North Twin East Week 14 (n=1), (Fig. 3.4). One of the duplicate samples from Denison Week 12 (Sample 12) was chosen for further analysis based on its higher saxitoxin concentration (0.02 µg/L) compared to North Twin East Week 9 (0.006 µg/L).

![Figure 3.4. Gel electrophoresis image from PCR targeting gene partially responsible for saxitoxin (sxtI) production from 14 cyanobacterial DNA samples; + and – indicate positive and negative controls respectively; L indicates DNA ladder.](image)

**Further Investigation of Putatively Toxic Cyanobacterial Culture**

**Confirming sxtI Clone Insert Sequence**

As PCR results indicated the presence of a cyanobacterial strain containing the saxitoxin functional gene in Sample 12 (Denison Week 12), further investigation was warranted to determine its purity, taxonomy, and to confirm the sxtI PCR amplification product. As a first step, cloning was performed on the amplification product provided from the sxtI primers.

The forward and reverse reads returned from Sanger sequencing clone inserts containing the sxtI gene were not able to be joined into paired-end reads due to the length of the gene (1669 bp) exceeding 2X the average sequencing length from each primer (700-800 bp). However, searching the single-end reads (forward and reverse) in NCBI using BLASTx (Altschul et al., 1997) returned the top match for each single-end read, showing the highest similarity to a carbamoyltransferase protein belonging to Nostoc sp. MG11 (Table 3.5).
Table 3.4. Results from running single-end reads \textit{sxTI} clone inserts from Sample 12 (Denison Week 12) against NCBI nr protein sequence database using BLASTx. Amino acid with highest percent similarity is shown; clone number 4 was not successfully sequenced.

<table>
<thead>
<tr>
<th>sxT/ Read</th>
<th>Description</th>
<th>e-value</th>
<th>Percent Similarity</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1_forward</td>
<td>carbamoyltransferase [Nostoc sp. MG11]</td>
<td>2.00E-130</td>
<td>92.08%</td>
<td>WP_193197365.1</td>
</tr>
<tr>
<td>1_reverse</td>
<td>carbamoyltransferase [Nostoc sp. MG11]</td>
<td>2.00E-97</td>
<td>98.95%</td>
<td>WP_193197365.1</td>
</tr>
<tr>
<td>2_forward</td>
<td>carbamoyltransferase [Nostoc sp. MG11]</td>
<td>3.00E-126</td>
<td>96.76%</td>
<td>WP_193197365.1</td>
</tr>
<tr>
<td>2_reverse</td>
<td>carbamoyltransferase [Nostoc sp. MG11]</td>
<td>7.00E-96</td>
<td>98.85%</td>
<td>WP_193197365.1</td>
</tr>
<tr>
<td>3_forward</td>
<td>carbamoyltransferase [Nostoc sp. MG11]</td>
<td>4.00E-155</td>
<td>95.45%</td>
<td>WP_193197365.1</td>
</tr>
<tr>
<td>3_reverse</td>
<td>carbamoyltransferase [Nostoc sp. MG11]</td>
<td>3.00E-128</td>
<td>98.70%</td>
<td>WP_193197365.1</td>
</tr>
<tr>
<td>4_forward</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4_reverse</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5_forward</td>
<td>carbamoyltransferase [Nostoc sp. MG11]</td>
<td>2.00E-153</td>
<td>96.62%</td>
<td>WP_193197365.1</td>
</tr>
<tr>
<td>5_reverse</td>
<td>carbamoyltransferase [Nostoc sp. MG11]</td>
<td>2.00E-98</td>
<td>97.46%</td>
<td>WP_193197365.1</td>
</tr>
<tr>
<td>6_forward</td>
<td>carbamoyltransferase [Nostoc sp. MG11]</td>
<td>1.00E-144</td>
<td>96.15%</td>
<td>WP_193197365.1</td>
</tr>
<tr>
<td>6_reverse</td>
<td>carbamoyltransferase [Nostoc sp. MG11]</td>
<td>2.00E-113</td>
<td>99.52%</td>
<td>WP_193197365.1</td>
</tr>
<tr>
<td>7_forward</td>
<td>carbamoyltransferase [Nostoc sp. MG11]</td>
<td>2.00E-103</td>
<td>98.51%</td>
<td>WP_193197365.1</td>
</tr>
<tr>
<td>7_reverse</td>
<td>carbamoyltransferase [Nostoc sp. MG11]</td>
<td>6.00E-142</td>
<td>95.67%</td>
<td>WP_193197365.1</td>
</tr>
<tr>
<td>8_forward</td>
<td>carbamoyltransferase [Nostoc sp. MG11]</td>
<td>3.00E-133</td>
<td>96.41%</td>
<td>WP_193197365.1</td>
</tr>
<tr>
<td>8_reverse</td>
<td>carbamoyltransferase [Nostoc sp. MG11]</td>
<td>7.00E-115</td>
<td>97.22%</td>
<td>WP_193197365.1</td>
</tr>
</tbody>
</table>

16S rRNA paired-end reads from cloning

From the seven clone inserts that underwent Sanger sequencing, three were successfully joined into paired-end reads, while the remaining single-end reads were unsuccessfully joined.

The three paired-end reads (12a, 12b, and 12c) containing the nearly complete 16S rRNA gene (~1465 bp) and single-end reads were searched for using BLASTn (Zhang et al., 2000) in the NCBI nr database. Paired-end read similarity was \( \geq 99.23\% \) to \textit{Nostoc} sp. 5n, \( \geq 99.02\% \) to \textit{Nostoc} sp. 2-07, and \( \geq 99.02\% \) to \textit{Nostoc} sp. VP2-08 (Table 3.6). Single-end reads showed similar matches, with the exception of a 98.84\% similar identity to an uncultured bacterium clone 16S rRNA gene (read 3R). Multiple Sequence Alignment of paired-end reads was done using TCoffee (Notredame et al., 2000) through EMBL-EBI (Madeira et al., 2019). The percent similar identity between the three paired-end reads was \( \geq 98.88\% \) (Table 3.6).
Table 3.5 Results from running 16S rRNA gene paired-end reads obtained from clone inserts and single-end reads against NCBI nr sequence database using BLASTn. Top three matches are presented for each paired-end read (12a, 12b, 12c) while the highest match for each single-end read is listed for each forward (F) and reverse (R) read.

<table>
<thead>
<tr>
<th>Description</th>
<th>Percent Similarity</th>
<th>E-value</th>
<th>Ascension Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>12a</td>
<td>Nostoc sp. 5N-02c partial 16s rRNA gene</td>
<td>99.23%</td>
<td>0.0</td>
</tr>
<tr>
<td>12a</td>
<td>Nostoc sp. 2-07 partial 16s rRNA gene</td>
<td>99.02%</td>
<td>0.0</td>
</tr>
<tr>
<td>12a</td>
<td>Nostoc sp. VP2-08 partial 16s rRNA gene</td>
<td>99.02%</td>
<td>0.0</td>
</tr>
<tr>
<td>12b</td>
<td>Nostoc sp. 5N-02c partial 16s rRNA gene</td>
<td>99.86%</td>
<td>0.0</td>
</tr>
<tr>
<td>12b</td>
<td>Nostoc sp. 2-07 partial 16s rRNA gene</td>
<td>99.64%</td>
<td>0.0</td>
</tr>
<tr>
<td>12b</td>
<td>Nostoc sp. VP2-08 partial 16s rRNA gene</td>
<td>99.64%</td>
<td>0.0</td>
</tr>
<tr>
<td>12c</td>
<td>Nostoc sp. 5N-02c partial 16s rRNA gene</td>
<td>99.78%</td>
<td>0.0</td>
</tr>
<tr>
<td>12c</td>
<td>Nostoc sp. 2-07 partial 16s rRNA gene</td>
<td>99.56%</td>
<td>0.0</td>
</tr>
<tr>
<td>12c</td>
<td>Nostoc sp. VP2-08 partial 16s rRNA gene</td>
<td>99.56%</td>
<td>0.0</td>
</tr>
<tr>
<td>2F</td>
<td>Nostoc sp. VP2-08 partial 16s rRNA gene</td>
<td>98.95%</td>
<td>0.0</td>
</tr>
<tr>
<td>2R</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3F</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3R</td>
<td>Uncultured bacterium clone rRNA075 16S rRNA gene</td>
<td>98.84%</td>
<td>0.0</td>
</tr>
<tr>
<td>4F</td>
<td>Nostoc sp. 2-07 partial 16s rRNA gene</td>
<td>98.66%</td>
<td>0.0</td>
</tr>
<tr>
<td>4R</td>
<td>Nostoc sp. 2-07 partial 16s rRNA gene</td>
<td>98.97%</td>
<td>0.0</td>
</tr>
<tr>
<td>5F</td>
<td>Nostoc sp. 2-07 partial 16s rRNA gene</td>
<td>99.22%</td>
<td>0.0</td>
</tr>
<tr>
<td>5R</td>
<td>Nostoc sp. 5N-02c partial 16s rRNA gene</td>
<td>98.51%</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Table 3.6 Multiple Sequence Alignment similarity matrix of paired-end reads obtained from 16S rRNA clone inserts(n=3); aligned using TCoffee through EMBL-EBI.

<table>
<thead>
<tr>
<th></th>
<th>12a</th>
<th>12b</th>
<th>12c</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>12a</strong></td>
<td>100</td>
<td>98.89</td>
<td>98.88</td>
</tr>
<tr>
<td><strong>12b</strong></td>
<td>98.89</td>
<td>100</td>
<td>99.51</td>
</tr>
<tr>
<td><strong>12c</strong></td>
<td>98.88</td>
<td>99.51</td>
<td>100</td>
</tr>
</tbody>
</table>
Microscopy

Images obtained under Bright Field and Polarized Light revealed the presence of a filamentous cyanobacterium containing heterocysts (Fig 3.5a and 3.5b), while the composite image (Fig 3.5c) indicated the presence of filamentous cells displaying red coloring and single cells with green-blue color. When viewed solely under the I3 wavelength filter cube, single cells exhibited yellow fluorescence, distinct from the red fluorescence shown by filamentous cells.

Figure 3.5. Microscopy images (40x) taken of Sample 12; a) Bright field image of culture displaying filaments; b) Polarized image revealing heterocysts; c) composite image indicating presence of non-filamentous cells (blue) and filamentous cyanobacteria (red).

Flow Cytometry on Culture Containing sxtI Amplification Product

Flow cytometry results from Sample 12 yielded two distinct populations based on their FSC-W signal (Fig. 3.6). Population 1 (P1) contained smaller (<5 µm) cells, while cells from population 2 (P2) displayed filamentous morphology with wider FSC-W. Filamentous cells from P2 were sorted based on their FCS-W signal, and collected into BG-11 liquid growth medium for further cultivation.
Figure 3.6. Flow cytometry results from sample 12 displaying smaller FSC-W (P1) and filamentous cells (P2). P2 cells were sorted according to their wider FSC-W signal and collected in BG-11 liquid growth medium for further cultivation.

Community Dataset Analyses

Searching for Putatively Toxic Isolate in Larger Microbial Dataset

Executed through command line, BLAST (version 2.9) was used to find the highest percent match between the putatively toxic culture containing 16S rRNA gene sequence and sequences in the pertaining to the V4 region of the 16S rRNA gene contained in the larger microbial dataset. The top match revealed a 96.838% similarity across 253 bases. When searched in NCBI BLASTn, the OTU containing this sequence showed 100% similarity to the cyanobacterium *Gloeotrichia longicauda* SAG 32.84, belonging to order Nostocales.
Cyanobacterial Community Structure

The cyanobacterial community structure was obtained from Illumina Miseq amplification of the V4 region of the 16S rRNA gene, followed by sequence quality control and taxonomic classification using the bioinformatics software, mothur. Further analyses completed through R/Rstudio revealed Cyanobacteria to compose 43.5% (31 OTUs) of the total bacterial community composition in Lake Denison (Week 12). The top three dominant Cyanobacterial taxa assigned to OTUs were *Cyanobium* PCC 6307 (32.89%), *Nodosilinea* PCC 7104 (28.76%), and *Planktothrix* NIVA-CYA15 (25.5%) (Table 3.8). Of the 31 OTUs present in the Lake Denison Week 12 sample, 15 were unclassified at the genus level or higher. Three of the 16 classified strains were identified as toxigenic: *Planktothrix* NIVA-CYA15 (microcystin), *Synechocystis* SAG 90.79 (exopolysaccharides), and *Nodularia* PCC 9350 (nodularin) (Table 3.8). As cyanobacterial taxa have been subject to extensive taxonomical reclassification, classified strains were searched in NCBI’s taxonomy browser for previous names (i.e. classifications) to ensure all listed strains were identified for their toxin producing potential.
Table 3.7 Cyanobacterial community composition results from Lake Denison (Week 12). Strains are presented by their percent relative abundance of the total cyanobacterial community. Potential for strain toxicity is listed as non-toxic (is not able to produce toxins), NO (no observed toxins during toxicity evaluation), NE (Not Established; limited studies pertaining to strain and/or toxicity), NA (for unclassified strains). Toxin types are listed for axenic strains that have been positively determined to produce toxins in laboratory studies; Exopolysaccharides, (EPS), Microcystin (MC), Nodularin (ND). Taxa belonging to order Nostocales are listed in bold.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Strain</th>
<th>Relative Abundance</th>
<th>Potential Toxicity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanobium</td>
<td>gracile</td>
<td>PCC 6307</td>
<td>32.89%</td>
<td>non-toxic</td>
<td>Glowacka et al., 2011</td>
</tr>
<tr>
<td>Nodosilinea</td>
<td>nodulosa</td>
<td>PCC 7104</td>
<td>28.76%</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>Planktothrix</td>
<td>agardhii</td>
<td>NIVA CYA15</td>
<td>25.20%</td>
<td>MC</td>
<td>Tooming-Klunderud et al., 2013</td>
</tr>
<tr>
<td>Sphaerospermopsis</td>
<td>aphanizomenoides</td>
<td>BCCUSP55</td>
<td>3.49%</td>
<td>NO</td>
<td>Mota 2017</td>
</tr>
<tr>
<td>Unclassified</td>
<td>Unclassified</td>
<td>Unclassified</td>
<td>1.66%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Unclassified</td>
<td>Unclassified</td>
<td>Unclassified</td>
<td>1.62%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Microcystis</td>
<td>aeuruginosa</td>
<td>PCC 7914</td>
<td>1.51%</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>Snowella</td>
<td>Litoralis</td>
<td>0TU37S04</td>
<td>1.19%</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>Cylindrospermopsis</td>
<td>raciborskii</td>
<td>CRJ1</td>
<td>0.46%</td>
<td>non-toxic</td>
<td>Chonudonkul et al., 2004</td>
</tr>
<tr>
<td>Unclassified</td>
<td>Unclassified</td>
<td>Unclassified</td>
<td>0.45%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Pseudanabaena</td>
<td>Biceps</td>
<td>PCC 7429</td>
<td>0.42%</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>Unclassified</td>
<td>Unclassified</td>
<td>Unclassified</td>
<td>0.39%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Synechocystis</td>
<td>aquatilis</td>
<td>SAG 90.79</td>
<td>0.25%</td>
<td>EPS</td>
<td>Flamm and Blaschek, 2014</td>
</tr>
<tr>
<td>Unclassified</td>
<td>Unclassified</td>
<td>Unclassified</td>
<td>0.24%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Dolichospermum</td>
<td>circinale</td>
<td>NIES41</td>
<td>0.22%</td>
<td>NO</td>
<td>Lyra et al., 2001; Beltran and Neilan, 2000</td>
</tr>
<tr>
<td>Aphanizomenon</td>
<td>flos aqua</td>
<td>MDT14a</td>
<td>0.21%</td>
<td>NO</td>
<td>Driscoll et al., 2017</td>
</tr>
<tr>
<td>Limnolyngbya</td>
<td>circumcreta</td>
<td>CHAB4449</td>
<td>0.18%</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>Cuspidothrix</td>
<td>issatschenkoi</td>
<td>LMECYA 163</td>
<td>0.17%</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>Unclassified</td>
<td>Unclassified</td>
<td>Unclassified</td>
<td>0.11%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Unclassified</td>
<td>Unclassified</td>
<td>Unclassified</td>
<td>0.11%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Unclassified</td>
<td>Unclassified</td>
<td>Unclassified</td>
<td>0.10%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Synechocystis</td>
<td>sp.</td>
<td>PCC-6803</td>
<td>0.08%</td>
<td>non-toxic</td>
<td>Morris et al., 2014</td>
</tr>
<tr>
<td>Limnothrix</td>
<td>Unclassified</td>
<td>Unclassified</td>
<td>0.06%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Unclassified</td>
<td>Unclassified</td>
<td>Unclassified</td>
<td>0.06%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Sympothoece</td>
<td>euryhalinus</td>
<td>PCC 7002</td>
<td>0.05%</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>Unclassified</td>
<td>Unclassified</td>
<td>Unclassified</td>
<td>0.03%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Unclassified</td>
<td>Unclassified</td>
<td>Unclassified</td>
<td>0.02%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Synechocystis</td>
<td>Unclassified</td>
<td>Unclassified</td>
<td>0.02%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Nodularia</td>
<td>spumigena</td>
<td>PCC 9350</td>
<td>0.01%</td>
<td>NOD</td>
<td>Lyra et al., 2005</td>
</tr>
<tr>
<td>Unclassified</td>
<td>Unclassified</td>
<td>Unclassified</td>
<td>0.01%</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Unclassified</td>
<td>Unclassified</td>
<td>Unclassified</td>
<td>0.01%</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 4. DISCUSSION

While microcystin is routinely measured by the IDNR Beach Monitoring Program, other toxins including cylindrospermopsin, saxitoxin, and anatoxin have been detected in some Iowa lakes (Graham et al., 2010). These toxins are of emerging concern due to their adverse health effects in biota, including humans, but we lack an understanding of which organisms produce them. As toxic blooms dominated by cyanobacteria are a major concern in freshwaters, the goal of this research was to isolate novel toxin-producing cyanobacteria present in Iowa’s lakes. By measuring these additional toxins in a set of nine Iowan lakes that routinely experience toxic blooms, lakes with understudied toxins were targeted for the enrichment, isolation, and identification of novel toxin-producing cyanobacteria.

The values of anatoxin-a and saxitoxin measured through the current study were similar to concentrations found by Graham et al. (2010), who evaluated twenty-three surface water samples from Midwestern lakes (MN, IA, MO, KS), collected over a 1-week period in August 2006. Graham et al. found saxitoxin at detectable levels in two of Iowa’s lakes (Beeds Lake = 0.19 µg/L, Rock Creek = 0.04 µg/L), and reported the median saxitoxin detected value for all Midwestern lakes as 0.03 µg/L. These values are comparable to the weekly median saxitoxin detections in the present study, ranging from 0.020 to 0.035 µg/L (Table 3.2). In the current study, weekly anatoxin-a median detection levels by ELISA ranged from 0.166 to 0.249 µg/L. Anatoxin-a was analyzed by Graham et al. (2010) through liquid chromatography-tandem mass spectrometry (LC-MS/MS); three of the ten Iowan lakes contained anatoxin-a (Beeds Lake = 0.29 µg/L, Clear Lake = 9.5 µg/L, and East Okoboji = 0.02 µg/L), and the median detected level of all lakes was 0.16 µg/L.
Similar to Graham et al. (2010), the values reported in this study reflect both extracellular and intracellular toxins. Lake water samples underwent 3 freeze-thaw cycles before ELISA measurements were taken to lyse cells and release intracellular toxins. Thus, toxin concentration results reported in this study are not strictly reflective of extracellular toxins in raw lake water samples. Nonetheless, the risk for human exposure through ingestion of cells should be considered; water in Iowa used for consumption is chemically treated and filtered, making accidental ingestion through recreational activities the likeliest route for human exposure (Codd et al., 2017; Kubickova et al., 2019).

In 2015, the EPA developed Health Advisories relating to cylindrospermopsin and microcystins. However, EPA regulatory limits for human freshwater exposure to anatoxin-a and saxitoxin are not currently established. In absence of federal guidelines, the Oregon Health Authority (OHA) developed exposure threshold values—their evaluation determined human Tolerable Daily Intake (TDI) should not exceed 0.1 µg/kg-day and 0.05 µg/kg-day, for anatoxin-a and saxitoxin, respectively (Farrer et al., 2015). Data relating to human dosage and resulting toxicity effects of anatoxin-a and saxitoxin are limited (Christensen and Khan, 2020), however studies relating to exposure in mice are available. In mouse toxicological studies, the lethal dose (LD50) of anatoxin-a is 200-250 µg/kg body weight via intraperitoneal (i.p.) injection (Carmichael et al., 1990), and saxitoxin LD50 is 5.5-10 µg/kg body weight (Chorus and Bartram, 1999). However, the oral dose for both toxins is greater than the i.p. dose (e.g. anatoxin-a oral LD50 for mice is >5000 µg/kg body weight; Chorus and Bartram, 1999). In humans, the oral dose for saxitoxin ranges from 7 to 15 µg/kg body weight (Geraci et al., 1989).

These studies highlight both the potency of saxitoxin, and that the concentrations of the toxins found in the current study are relatively low compared to the amount required for lethality.
However, accidental oral consumption during recreational activities or drinking of untreated waters may lead to non-lethal symptoms causing pain and discomfort. Considering the risks posed by anatoxin-a and saxitoxin, and the lack of data regarding oral exposure to these toxins in larger mammals, further oral exposure studies and regulatory guidelines for human exposure of these toxins should be developed.

Though the risk for oral exposure to anatoxin-a and saxitoxin may be low in the lakes evaluated, saxitoxins are known to accumulate in the tissues of aquatic life often consumed by humans. Bioaccumulation has been well established for marine environments (Carmichael et al., 1985; Cusick and Sayler, 2013), and saxitoxins were also found to accumulate in the tissues of freshwater fish and shellfish by Giovannardi et al. (1999) and Calado et al. (2019). As anatoxin-a and saxitoxin have been found by Graham et al. in 2006, and from the current study in 2019, additional studies relating to the bioaccumulation of these neurotoxins in Iowan wildlife would be highly encouraged for future research.

Many studies report the expected increase of marine and freshwater toxic blooms (both magnitude and frequency) in response to climate change and other human activities (i.e. increased eutrophication and natural habitat destruction) (Griffith and Gobler, 2020; Gobler 2020; Glibert et al., 2014; Huisman et al., 2018). Of the lakes sampled in the current study, all showed anatoxin-a and saxitoxin levels at or above detectable limits at least once through the sampling period. With the predictions of bloom response to climate change in mind, these lakes could accumulate higher toxin levels in years to come. Future monitoring efforts for the toxins evaluated in this study are recommended for Iowan lakes used for recreation or drinking water.
Isolation and Identification of a Putatively Toxic Cyanobacterial Strain

To accurately identify toxin-producing cyanobacteria from the collected lake water samples, methodology was used to first enrich only cyanobacteria and then to isolate individual strains. The addition of cycloheximide to all growth media eliminated eukaryotic organisms successfully, as none were observed through microscopy. An MPN method was implemented to serve as a tool for quantification and isolation of culturable cyanobacterial taxa (Prasana et al., 2006). However, one assumption of this method is that culturable organisms must grow as separate cells and not in clusters. As the majority of cyanobacteria cultured from this study grew as filamentous and/or colonial morphotypes, the resulting estimations are reported as Growth Units (Table 3.3). While Prasana et al. (2006) determined the MPN method accurate for isolation of cyanobacterial organisms, it should be noted that axenic cyanobacterial cultures are particularly difficult to obtain, especially from filamentous strains (Abed and Köster et al., 2005; Bruno et al., 2006; De Figueiredo et al., 2010), and as such, the cultured cyanobacteria in this study required further isolation steps through fluorescence-based flow cytometric analysis and cell sorting.

Establishing culture conditions for the successful growth of cyanobacteria in the lab setting emerged as a challenging aspect of this project. The agar containing samples collected from the 2018 season desiccated in the culture plates, leaving only a few samples from which to choose. To amend this issue for the 2019 sampling season, transparent plastic covers were placed over plates to prevent agar desiccation. However, in the 2019 season, the nutrient concentration of the cyanobacteria’s growth medium was lowered by 10-fold as an attempt to simulate the nutrient conditions found in Iowa’s lakes. This value was obtained by taking the average of nitrogen and phosphorus across lakes through the previous sampling season, and in retrospect, was not
reflective of individual lake habitats or of optimal growth conditions. While this action likely contributed to the low yield of cultivatable cyanobacterial strains (Table 3.4) the culturing process was not completely unsuccessful; by lowering the Nitrogen (N) concentration in the culture plates, we selected for diazotrophic (N-fixing) cyanobacteria.

To date, research has been overwhelmingly focused on planktonic species (Fig 4.1) (Wood et al., 2020; Husk and Nieuwenhuis, 2019), and diazotrophic cyanobacteria found in freshwater are typically filamentous (Bergman et al., 1997). Benthic cyanobacteria are found to inhabit temperate lakes (Wood et al., 2010; Husk and Nieuwenhuis, 2019), and the biofilm they produce is a complex matrix that can cause interference with successful DNA extraction (Gaget et al., 2017). The PowerBiofilm Kit was proven by Gaget et al. to be effective for DNA extraction from benthic organisms; in the current study, this kit allowed for successful DNA extraction of benthic cyanobacteria, possibly eliminating biases towards planktonic species.

As benthic filamentous toxic strains are understudied in temperate lake environments (Husk and Nieuwenhuis, 2019), the combination of N limitation in culture media to select for diazotrophs and the PowerBiofilm extraction kit allowing for DNA obtained from benthic organisms, allowed for the selection and molecular characterization of an underrepresented cyanobacterial strain.

The molecular techniques applied in this study, PCR and direct Sanger sequencing, required cultures to be axenic. If more than one species or strain is present in a sample, the DNA extracted will constitute that of multiple organisms. As such, it becomes difficult to resolve which strain harbors toxin producing genes and taxonomical identification through gene sequencing yields low quality and/or inaccurate results. For this reason, cultures were screened for the presence of
multiple cyanobacteria through flow cytometric analysis. The presence of non-APC events observed from this analysis (Fig. 3.1) possibly included non-cyanobacterial cells,

indicating that cultures contained bacterial organisms other than cyanobacteria. Low-quality results from initial sequencing attempts could attributed to this factor. However, without the use of dyes, it was not possible to discriminate between living non-cyanobacterial cells or dead cyanobacterial cell debris as the causative agents responsible for producing this peak.

Figure 4.1. Number of publications on planktonic and benthic cyanobacteria each year since 1990. These data were obtained by searching the following databases: Web of Science Core Collection, Biological Abstracts, BIOSIS Citation Index, Derwent Innovation Index, KCI- Korean Journal Database, MEDINE, Russian Science Citation Index, and SciELO Citation Index. Keywords used are given in Table S1. The search field to display was set to topic and time span was set to all years. Create marked lists (function on web of science interface) was used to exclude duplicate results. (Figure and caption from Wood et al., 2020)
While flow cytometry has successfully been used to assess cyanobacterial community structure at the species level (Patel et al., 2019) the parameters were based on axenic reference strains with known pigment fluorescence signals. Patel et al. were able to determine the presence of reference species in lake water samples, yet, non-reference species were labeled as “closely related” and were not clearly demarcated by their fluorescence intensity. As the cyanobacterial cells in the current study were not identified with DNA sequencing prior to flow cytometry, it is possible that different taxa (or perhaps even strains) containing the same pigment profiles were not truly delineated using this technique. From this analysis, 6 of the 20 cultures showed multiple clusters in their fluorescence profiles, indicating multiple distinct cyanobacterial populations. Thus, the remaining 14 cultures regarded as axenic were screened for the presence of toxin functional genes via PCR. However, flow cytometry, as applied in this study, likely could not be used to distinguish between closely-related strains or morphotypes.

Not all samples with toxins detected using the ELISA testing kit (Table 3.1 and 3.2) appeared to contain the toxin functional genes screened for in this study. For instance, saxitoxin was detected in the sample belonging to Lake Anita (Week 13; 0.047 µg/L), however, sxtI was not amplified from DNA extracted from the enrichment produced from this sample. There are several possible explanations for this result: 1) the culture enriched from this lake water sample did not contain the toxigenic strain responsible for saxitoxin production, 2) the primers used for this study were too specific at the genus level to capture the full diversity of all toxin gene sequences existing in the lake water samples from which cultures were derived, and/or 3) ELISA tests are nonspecific and detect multiple variants, degradants, and precursor molecules that may not be related to the sxtI gene. Regarding primer bias, the sxtI primer pair developed by Kellman et al., (2008), was designed from sequences of closely-related saxitoxin producers belonging to
the order Nostocales (Anabaena circinalis AWQC131C, Aphanizomenon flos-aquae NH-5, Cylindrospermopsis raciborskii T3) and one strain belonging to the order Oscillatoriales, Lyngbya wollei (Farlow). As many of the known saxitoxin producers belong to orders Nostocales and Oscillatoriales (i.e. Aphanizomenon, Cuspidothrix, Cylinrospermopsis, Cylindrospermum, Dolichospermum, Phormidium, Raphidiopsis, Scytonema, etc.) (Ballot et al., 2010; Ballot et al., 2016), it is possible that sxtI orthologs belonging to phylogenetically distant genera would not be amplified by the primer set used in this study. Finally, as the selected primers screened for genes involved in anatoxin-a, cylindrospermopsin, and saxitoxin synthesis, it is possible that gene sequences coding for variants would not be amplified.

To identify the putatively toxigenic strain containing sxtI, the 16S rRNA gene was amplified from DNA extracted from Sample 12 and directly sequenced with the Sanger method. However, the returned sequence reads were of low quality, indicating the presence of multiple 16S rRNA amplicons due to various bacterial taxa (cyanobacteria or otherwise) contained in the culture. As a result, the 16S rRNA gene products were cloned to determine if multiple organisms were present in the sample, and which organism(s) were most abundant.

Results from searching cloned inserts in NCBI revealed one single-end read as an uncultured bacterium clone 16S rRNA (Table 3.6). This clone showed 98.84% similarity to the single-end read, and lower percent matches from this read were listed as alpha-proteobacteria. As such, the low-quality reads from Sanger sequencing of the un-cloned 16S rRNA amplicons were likely to have been affected by contamination with a non-cyanobacterial organism. The remaining clones had close matches within the genus Nostoc, indicating that these organisms were likely the dominant members of the enrichment. Multiple sequence alignment between paired-end reads 12a and 12c were 98.88% identical (Table 3.6), leaving room for the possibility of multiple
(closely-related) *Nostoc* strains contained in Sample 12 (Konstantinidis & Tiedje, 2005). If the presence of multiple *Nostoc* strains is confirmed, the physiological similarity (i.e. pigment profiles) of these strains could be why they were not detected during the initial flow cytometry screening process.

Analysis of 16S rRNA paired-end reads obtained from clone inserts showed the highest similarity to partial 16S rRNA genes belong to several unclassified *Nostoc* strains (Table 3.5) isolated from a study done by Cuzman et al. (2010). Although these strains were grouped into a highly supported phylogenetic cluster, their species level taxonomy remains unknown (Cuzman et al., 2010). These strains, originating from monumental fountains in Spain with water sourced from the Darro River, are diazotrophic as indicated by the presence of heterocysts identified through microscopy (Cuzman et al., 2010).

Although the band amplified by the *sxtI* primer was of the correct size, sequencing was required to confirm that the amplicon sequence was truly the *sxtI* gene, and not a result of non-specific amplification from the primer pair used. Similar to the initial 16S rRNA amplicon sequences, low-quality results from initial Sanger sequencing of the PCR product from Sample 12, initiated cloning of the gene fragment specific to the *sxtI* primer pair to separate the amplicons. After Sanger sequencing of clone inserts, the resulting sequences were converted to amino acid residues and searched for in NCBI using BLASTx. Each forward and reverse read obtained from cloning showed the highest percent identity to a carbamoyltransferase enzyme (ranging from 92.08% to 99.52% similarity) belonging to the unclassified *Nostoc* sp. MG11 (Table 3.5).

Kellman et al. (2008) showed the *sxtI* primer pair (the same used in the current study) to amplify a sequence coding for an *O*-carbamoyltransferase enzyme. This enzyme adds a
carbamoyl group to the hydroxymethyl side chain of the saxitoxin precursor (Fig. 4.2) (Kellman et al., 2008; Kellman and Neilan 2007), and is related to enzymes that produce nodulation factors (Jabbouri et al., 1988) and antibiotics (Coque et al., 1995). A study done by Murray et al. (2011), listed the sxtI gene to code for an enzyme belonging to the carbamoyltransferase gene family when evaluating the evolutionary history of the sxt gene cluster (Table 4.1). According to the National Library of Medicine’s controlled vocabulary thesaurus, Medical Subject Headings, carbamoyl transferases are a group of enzymes that function to catalyze the transfer of carbamoyl groups in biosynthetic pathways.

The protein search result in this study provides evidence that a cyanobacterium from Sample 12 contains the sxtI sequence that encodes for a carbamoyltransferase enzyme. As such, this enzyme could fulfill the role of transferring a carbamoyl group to the saxitoxin precursor molecule. Additionally, the amplicon product was of the expected size relative to the synthetic sxtI control. Results from Sanger sequencing the 16S rRNA amplicon clone inserts revealed the identity of the dominant inserts recovered through cloning to be Nostoc at the genus level, and the sequence coding for the carbamoyltransferase enzyme was a close match to an uncultured Nostoc. The Nostoc genus is part of the order of Nostocales, which as mentioned above, include the cyanobacterial taxa known to produce saxitoxins. As of yet, no Nostoc sp. has been identified in culture as a saxitoxin-producing cyanobacterium. However, as of 2020, there are an estimated 15 identified cyanobacterial saxitoxin producing species; compared to the current number of known anatoxin-a producers (41) (Khan and Christensen 2020), it seems reasonable that not all saxitoxin-producing cyanobacteria have currently been identified.

Nostoc sp. morphology has been shown to display a complex life cycle (Fig. 4.1), as axenic strains are documented to contain various growth stages including filaments of different
lengths and sizes, colonial cells, and single cells, along with filamentous cells with heterocysts and akinetes (Johansen and Casamatta, 2005; Sant’ Anna et al., 2007). These same features were exhibited by the enrichment culture from Denison Week 12; light microscopy revealed the presence of heterocysts (Fig. 3.5b) and a generally complex morphology including filaments of different shapes and sizes, clusters of round cells, and single cells (Fig. 3.5a). From the composite fluorescence image (Fig. 3.5b), two distinct cell types emerged based on their coloration. Cells growing in clusters appeared as blue-green, while filamentous cells appeared red. The indication of multiple taxa from the composite image required further steps to obtain an axenic culture containing the putative saxitoxin producer.

As the currently identified saxitoxin producers belong to the order Nostocales, the cells displaying filamentous morphology were inferred to be the putative saxitoxin producer as morphological features of the cells in culture are consistent with *Nostoc* spp. Additionally, as 16S rRNA clones were identified as *Nostoc* spp. and the sxtI clone sequences matched to *Nostoc* spp., it was inferred that the filamentous cells were the appropriate cells to be targeted for purification via flow cytometry. The culture was sorted for isolation of the *Nostoc* sp. based on wider FSC-W signal (larger size) displayed through flow cytometric screening (Fig. 3.6). Selected filamentous cells were collected into vials containing BG-11 medium upon the sorting process for further cultivation.
Figure 4.2. Enzymatic pathway responsible for saxitoxin production in cyanobacteria (grey spheres represent enzymes). The *sxtI* gene, found to code for an O-carbamoyltransferase enzyme, transfers a carbamoyl group to the saxitoxin precursor molecule. (Figure: Murray et al., 2011).
Table 4.1. Gene and gene families in the sxt Cluster and their putative role in the biosynthetic pathway of saxitoxin production. The amplicon produced from sxtI primer pair used in the current study showed the highest similarity to a carbamoyl transferase enzyme. (From: Murray et al., 2011)

<table>
<thead>
<tr>
<th>Gene or gene family</th>
<th>Putative Role</th>
<th>Gene or gene family</th>
<th>Putative Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>sxtA</td>
<td>Polyketide synthase</td>
<td>sxtN</td>
<td>Sulfotransferase</td>
</tr>
<tr>
<td>sxtB</td>
<td>Cytidine deaminase</td>
<td>sxtO</td>
<td>ASK</td>
</tr>
<tr>
<td>sxtC</td>
<td>Unknown</td>
<td>sxtP</td>
<td>Integrins</td>
</tr>
<tr>
<td>sxtD</td>
<td>Sterole desaturase</td>
<td>sxtQ</td>
<td>Unknown</td>
</tr>
<tr>
<td>sxtE</td>
<td>Unknown</td>
<td>sxtR</td>
<td>Transferase</td>
</tr>
<tr>
<td>sxtF/M</td>
<td>MATE</td>
<td>sxtPER</td>
<td>Permease</td>
</tr>
<tr>
<td>sxtG</td>
<td>Amidinotransferase</td>
<td>sxtS</td>
<td>Phytanoyl diox</td>
</tr>
<tr>
<td>sxtH/T/diox</td>
<td>Phenylprop dioxygenase</td>
<td>sxtU</td>
<td>Alcohol dehydrogenase</td>
</tr>
<tr>
<td>sxtI</td>
<td>Carbamoyltransferase</td>
<td>sxtV</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>sxtJ</td>
<td>Unknown</td>
<td>sxtW</td>
<td>Ferredoxin</td>
</tr>
<tr>
<td>sxtK</td>
<td>Unknown</td>
<td>sxtX</td>
<td>Cephalosporin hydroxylase</td>
</tr>
<tr>
<td>sxtL</td>
<td>GDSL lipase</td>
<td>Orf</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Figure 4.3. Complex morphology displayed by two axenic *Nostoc* strains throughout their growth stages. a) Light microscope morphology of *Nostoc* CM1-VF14, scale bar = 10 µm (figure and captions from Johansen and Casamatta, 2005); A – Hormogonia; B – Germling colonies; C – Microscopic spherical colonies; D – Spherical colony showing compartmentalized mucilage. b) Light microscope morphology of *Nostoc viride*, scale bar = 10 µm, except when indicated (Figure and captions from Sant’ Anna et al., 2007); A – Few typical trichomes; B – Macroscopic type colonies (mucilaginous mass); D – Parts of trichomes with intercalary heterocytes; E – Two trichomes with developing akinetes; G – Terminal part of fertile trichome; H – Chain of liberated akinetes; I – Detail of ripe akinete.
The 16S rRNA gene sequence of the putatively toxic *Nostoc* species was searched via BLAST in the larger community dataset containing partial 16S rRNA amplicons from all study lakes for all weeks. Finding this sequence would allow for finding the potentially toxigenic strain in lakes other than Lake Denison Week 12 and determining its relative abundance. However, the 16S rRNA amplicon of this strain was not contained in the larger dataset generated through Mothur. The closest match was ~96% similar, approximate to the order level, and from which homology cannot be confirmed.

Possible reasons why the potentially toxic strain was not found in the larger data set include primer specific design bias; the primers used for high-throughput sequencing determination of the microbial community composition (515F and 805R) are universal and amplify a wide variety of bacterial and archaeal taxa. However, Lee et al. (2017) demonstrated that primers modified for water quality studies, also targeting 16S rRNA hypervariable regions, will select for a larger number of Cyanobacterial taxa with increased sensitivity and identification; using modified primers Cyanobacteria and Proteobacteria phyla accounted for ~95% of all sequences obtained from NGS runs, compared to ~50% using standard universal primers. The use of universal primers to fully evaluate the whole microbial community is an acceptable choice, but could contribute to the absence of the Cyanobacterial 16S rRNA amplicons in the larger community dataset.

Additionally, DNA extraction bias is a significant factor in microbial community and diversity research. Many studies have been completed to compare DNA extraction methods of 16S rRNA amplicon sequencing in bacteria across microbiome types, finding that the chosen method typically results in statistically significant differences in the community profiles.
generated (Han et al., 2019, Xue et al 2018; Teng et al., 2018). Specifically, one study relating to marine periphyton biofilm diversity (which includes Cyanobacteria) found that the number of OTUs, families, and genera were statistically significant between four DNA extraction methods; the relative abundance of Cyanobacterial genera (Nostocophyicdea, Nostocales, Nostocaceaa) was also statistically significant between extraction methods (Corcoll et al., 2017).

The lake water sample containing the putatively toxic Nostoc sp. was also evaluated for the presence of additional saxitoxin producers using bioinformatics analyses as implemented through Mothur. The community composition revealed the three dominant cyanobacterial taxa in this lake account 86.85% of the cyanobacterial community, and are not known to produce saxitoxin (Table 3.7). Interestingly, the third most dominant taxon was *P. agardhii* CYA15, a strain that is able to produce microcystin, but no microcystin was detected in this sample from ELISA testing.

Because homology could not be inferred from the BLAST search, it is difficult to ascertain if the potentially toxigenic strain identified in this study is a large component of the whole cyanobacterial community. As previously mentioned, the known saxitoxin producers belong to the order Nostocales. However, not all taxa classified under Nostocales were classified at the strain, species, or even genus level. Without lower-level classification, it becomes impossible to establish whether or not these taxa could be contributing to the saxitoxin concentration found from ELISA testing. Following the trends that are shown from this result, an assumption could be made that the Nostoc sp. found in this study would compose less than 4% of the relative abundance, as all other Nostocales fall below this threshold.

From the MPN analysis, the estimated amount of Growth Units from culturable bacteria in the original sample was 7.843 GU/ml. This value is relatively low compared to other samples
collected from Lake Denison, and toxin concentrations observed for anatoxin-a and saxitoxin were relatively low in the 2019 season, which could be attributed to low levels of cyanobacterial toxin-producers. However, the low Growth Unit values of these organisms in lake water samples may suggest factors other than species dominance play a larger role in the concentration of the observed toxins.

Indeed, as increased emphasis has been placed on the timing and prediction of toxic blooms, several important factors have emerged that do not include species dominance as the only contributor to toxin concentrations. First, the gene copy number of a single cell could increase due to environmental factors that trigger upregulation of its transcription (Davis et al., 2009, Kaebernick et al., 2002). This implies that toxin levels could increase regardless of the number of cells harboring the ability to produce toxins. Second, multiple strains are able to produce the same toxin type (Rapala et al., 1997, Wood et al., 2012, Sivonen et al., 1992)—this creates the potential for additive effects of toxin accumulation, and toxigenesis induced from environmental factors (such as light, pH, and temperature) is varied at the strain level. Thus, if toxins are produced at differing levels between multiple strains, causative relationships between OTUs and toxins could be statistically insignificant.

A solution to this issue may lie in the copy number of selected toxin functional genes. Davis et al. (2009) found that molecular quantification of mcyD-possesing Microcystis was statistically correlated with microcystin in every lake studied and was found to be a better predictor of environmental microcystin levels than total cyanobacteria, total Microcystis, chlorophyll-a or other factors. Additionally, Lu et al. (2019) was able to track cyanobacterial species succession and verify toxic bloom formation using genes associated with Nitrogen
fixation \((nifDKH)\), phosphorus scavenging \((pstSCB)\), and the cyanotoxin genes \(mcyG\) and \(ana-sxtA\), for toxic *Microcystis* and *Anabaena*, respectively.

Toxin gene sequences can vary between taxa resulting in primer sets displaying differential selectivity at the genera level (Lee et al., 2020). Experimental verification of toxin gene sequences across broad taxonomic groups is highly necessary for successful studies geared toward metagenomic analyses of cyanobacterial blooms. Confirmation of the sequences found in the \(sxt\) gene cluster provided through whole genome sequencing of the *Nostoc* sp. found in this study could provide this result.
CHAPTER 5. CONCLUSIONS

In this study, 14 cyanobacterial cultures were generated from an MPN cultivation approach targeting cyanobacteria from lakes in Iowa. These cultures were screened for their ability to produce anatoxin-a, cylindrospermopsin, and saxitoxin using PCR primers for key genes involved in the biosynthesis of these compounds. Cloned 16S rRNA sequences from the enrichment generated from Lake Denison water sampled during Week 12 of the 2019 sampling season showed the highest 16S rRNA gene similarity with uncultured *Nostoc* spp. This enrichment also produced amplicons for the *sxtI* gene involved in saxitoxin biosynthesis. The amino acid residues predicted from cloned DNA sequences recovered from this culture match to an uncultured *Nostoc* carbamoyltransferase enzyme, suggesting that the enriched *Nostoc* sp. may also contain saxitoxin biosynthesis genes. Cells in culture exhibited filamentous morphologies and heterocysts and additional complex life-cycle morphology associated with known *Nostoc* spp. Taken together, these data strongly suggest that the Denison Week 12 enrichment contains a cyanobacterium strain of genus *Nostoc* with saxitoxin producing potential. The 16S rRNA and *sxtI* gene sequence searches in NCBI did not reveal species level classification, indicating this particular species has not yet been taxonomically classified and should be considered novel.

The 16S rRNA amplicon from Sample 12 was not found in the larger microbial community dataset; the highest percent match was ~96% similar, which does not allow for inferring homology at the species level. Estimating the relative abundance of the cultured *Nostoc* sp. within the cyanobacterial community becomes speculative; the OTUs assigned to the order Nostocales fall below 4% of the total community, an assumption that this *Nostoc* sp. follows the same trend could be made. The finding that the putatively toxic *Nostoc* isolate was not present in
the environmental sample could represent bias in the DNA extraction and 16S rRNA amplification procedures for filamentous cyanobacteria, in particular.

Microscopy revealed the presence of distinct cell types that were not clearly delineated from initial flow cytometric screening. Cells exhibiting filamentous morphology were thought to be the putative saxitoxin-producer, as the majority of cloned inserts matched closest to *Nostoc* spp., the *sxtI* amino acid residues showed highest similarity to a carbamoyltransferase enzyme belonging to *Nostoc*., and the currently identified saxitoxin producers belong to the order Nostocales. As such, filamentous cells were collected through additional flow cytometric analysis based on FSC-W signal for further analyses to be completed.

Multiple sequence alignment between clone inserts 12a and 12c were 98.88% similar indicating that closely related *Nostoc* strains may be contained in culture (Konstantinidis & Tiedje, 2005). If these clone inserts represent two *Nostoc* strains, their morphology could be highly similar (Marquardt and Palinska, 2007). As filamentous cells were collected from additional flow cytometric analysis, the possibility that multiple *Nostoc* strains existing in this culture could be explored through additional cloning experiments based on the 16S rRNA region. Furthermore, comparison of the less conserved adjacent intergenic transcribed spacer (ITS) region, could allow for a greater level of discrimination between strains that share highly-similar 16S rRNA gene regions (Marquardt and Palinska, 2007).

Whether or not the *Nostoc* strain containing the *sxtI* amplification product is truly capable of producing saxitoxin could potentially be confirmed through LC/MS/MS (or similar) analysis. However, this would require successful induction of toxigenesis in the lab setting and the abiotic factors that promote saxitoxin production may vary between species. Studies have shown that N limitation (Casero et al., 2014), high Na⁺ and pH (10 mM and pH 9 respectively; Ongley et al.
2016), culture age (Dias et al., 2002), light availability (Yin et al., 1997), and temperature changes (Castro et al., 2004), all play roles in the production of saxitoxin and its analogues in cyanobacteria. As the putatively toxic *Nostoc* sp. was enriched on BG11 medium with lowered inorganic nitrogen, recreating these culture conditions could be explored to induce toxigenesis.

An important consideration from this study is the carbamoyltransferase enzyme found in the *Nostoc* strain could fulfill roles other than saxitoxin production (Jabbouri et al., 1988; Coque et al., 1995), and due to the potential deletion of partial gene clusters through evolutionary time, the presence of one toxin functional gene does not always indicate the entire gene cluster required for toxin production is present (Dittman et al., 2013). The presence of genes belonging to the functional *sxt* gene cluster, verified through whole genome sequencing (WGS), could provide additional support for the saxitoxin producing capabilities of the *Nostoc* strain.

Lastly, WGS based analyses could also establish the species and strain level taxonomy of this uncultured *Nostoc* strain. Obtaining the whole genome of the *Nostoc* sp. allows for classification based on phylogenetic reconstruction (based on 16S rRNA) and genomic signatures (Average Amino acid Identity, Average Nucleotide Identity, and Genome-to-Genome Distances; Walter et al., 2017). Comparing the genomic sequence information of this strain to complete cyanobacterial genomes already available in public databases would allow for the full identification of the novel *Nostoc* sp. found in this study.
REFERENCES


### APPENDIX A. ELISA %CV VALUES

<table>
<thead>
<tr>
<th>Anatoxin-a</th>
<th>%CV</th>
<th>Lake/Week number</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.333</td>
<td>12_Denison</td>
<td>0.557</td>
</tr>
<tr>
<td>9_ThreeFree</td>
<td>0.009</td>
<td>12_NTE</td>
<td>9.05</td>
</tr>
<tr>
<td>9_Green Valley</td>
<td>11.461</td>
<td>12_Three Fires</td>
<td>9.731</td>
</tr>
<tr>
<td>9_Big Creek</td>
<td>14.475</td>
<td>12_Green Valley</td>
<td>7.52</td>
</tr>
<tr>
<td>9_Denison</td>
<td>0.219</td>
<td>12_Anita Fdup</td>
<td>0.207</td>
</tr>
<tr>
<td>9_Black Hawk</td>
<td>0.59</td>
<td>Control</td>
<td>4.346</td>
</tr>
<tr>
<td>9_NTE</td>
<td>1.245</td>
<td>13_NTE</td>
<td>16.243</td>
</tr>
<tr>
<td>9_NTW</td>
<td>2.032</td>
<td>13_NTW</td>
<td>3.79</td>
</tr>
<tr>
<td>9_Brushy Creek</td>
<td>15.814</td>
<td>13_Black Hawk</td>
<td>8.274</td>
</tr>
<tr>
<td>9_Three Fires Fdup</td>
<td>0.384</td>
<td>13_Big Creek Fdup</td>
<td>4.996</td>
</tr>
<tr>
<td>9_Anita</td>
<td>0.247</td>
<td>13_Brushy Creek</td>
<td>9.528</td>
</tr>
<tr>
<td>10_Brushy Creek</td>
<td>0.508</td>
<td>13_Denison</td>
<td>0.277</td>
</tr>
<tr>
<td>10_NTW</td>
<td>0.665</td>
<td>13_Big Creek</td>
<td>7.604</td>
</tr>
<tr>
<td>10_NTE</td>
<td>0.282</td>
<td>13_Green Valley</td>
<td>6.143</td>
</tr>
<tr>
<td>10_NTE Fdup</td>
<td>2.988</td>
<td>13_Anita</td>
<td>20.178</td>
</tr>
<tr>
<td>10_Big Creek</td>
<td>0.633</td>
<td>13_Three Fires</td>
<td>1.019</td>
</tr>
<tr>
<td>10_Green Valley</td>
<td>0.599</td>
<td>14_Anita</td>
<td>6.042</td>
</tr>
<tr>
<td>10_Three Fires</td>
<td>1.409</td>
<td>14_NTE</td>
<td>0.983</td>
</tr>
<tr>
<td>10_Anita</td>
<td>0.363</td>
<td>14_NTW</td>
<td>16.134</td>
</tr>
<tr>
<td>10_Black Hawk</td>
<td>0.319</td>
<td>14_Three Fires</td>
<td>5.832</td>
</tr>
<tr>
<td>10_Denison</td>
<td>7.277</td>
<td>14_Green Valley</td>
<td>12.761</td>
</tr>
<tr>
<td>11_Brushy Creek</td>
<td>2.037</td>
<td>14_Brushy</td>
<td>7.313</td>
</tr>
<tr>
<td>11_Big Creek</td>
<td>3.385</td>
<td>14_Big Creek</td>
<td>12.922</td>
</tr>
<tr>
<td>11_Anita</td>
<td>1.218</td>
<td>14_Denison</td>
<td>1.673</td>
</tr>
<tr>
<td>11_Three Fires</td>
<td>0.321</td>
<td>14_Black Hawk</td>
<td>5.607</td>
</tr>
<tr>
<td>11_Black Hawk</td>
<td>0.447</td>
<td>14_NTW Fdup</td>
<td>6.005</td>
</tr>
<tr>
<td>11_Denison</td>
<td>2.778</td>
<td>15_Anita</td>
<td>19.446</td>
</tr>
<tr>
<td>11_NTE</td>
<td>1.675</td>
<td>15_Three Fires</td>
<td>1.916</td>
</tr>
<tr>
<td>11_NTW</td>
<td>0.373</td>
<td>15_NTW</td>
<td>7.451</td>
</tr>
<tr>
<td>11_Three Fires</td>
<td>1.963</td>
<td>15_Green Valley</td>
<td>16.465</td>
</tr>
<tr>
<td>11_Green Valley</td>
<td>0.232</td>
<td>15_Black Hawk</td>
<td>4.369</td>
</tr>
<tr>
<td>11_Back Hawk</td>
<td>0.978</td>
<td>15_Anita</td>
<td>1.888</td>
</tr>
<tr>
<td>12_Anita</td>
<td>1.925</td>
<td>15_NTE</td>
<td>5.133</td>
</tr>
<tr>
<td>12_Brushy Creek</td>
<td>0.186</td>
<td>15_Green Valley Fdup</td>
<td>1.043</td>
</tr>
<tr>
<td>12_NTW</td>
<td>0.326</td>
<td>15_Denison</td>
<td>5.582</td>
</tr>
<tr>
<td>12_Big Creek</td>
<td>1.403</td>
<td>15_Brushy Creek</td>
<td>0.859</td>
</tr>
<tr>
<td>Lake/Week number</td>
<td>Saxitoxin</td>
<td>Lake/Week number</td>
<td>Saxitoxin</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------</td>
<td>------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Control</td>
<td>0.702</td>
<td>12_Big Creek</td>
<td>2.19</td>
</tr>
<tr>
<td>9.Brushy Creek</td>
<td>0.305</td>
<td>12_Denison</td>
<td>4.886</td>
</tr>
<tr>
<td>9.NTW</td>
<td>2.185</td>
<td>12_NTE</td>
<td>2.712</td>
</tr>
<tr>
<td>9_Denison</td>
<td>3.171</td>
<td>12_Anita Fdup</td>
<td>3.431</td>
</tr>
<tr>
<td>9_Big Creek</td>
<td>9.864</td>
<td>13_NTW</td>
<td>11.148</td>
</tr>
<tr>
<td>9_Green Valley</td>
<td>13.645</td>
<td>13_Black Hawk</td>
<td>19.802</td>
</tr>
<tr>
<td>9_Three Fires</td>
<td>21.714</td>
<td>13_Big Creek Fdup</td>
<td>3.012</td>
</tr>
<tr>
<td>9_Three Fires Fdup</td>
<td>1.059</td>
<td>13_Brushy Creek</td>
<td>7.851</td>
</tr>
<tr>
<td>9_Anita</td>
<td>4.579</td>
<td>13_Denison</td>
<td>26.311</td>
</tr>
<tr>
<td>10_Brushy Creek</td>
<td>3.278</td>
<td>13_Big Creek</td>
<td>16.04</td>
</tr>
<tr>
<td>10_NTW</td>
<td>4.338</td>
<td>13_Green Valley</td>
<td>4.474</td>
</tr>
<tr>
<td>10_NTE</td>
<td>4.871</td>
<td>13_Anita</td>
<td>3.6</td>
</tr>
<tr>
<td>10_NTE Fdup</td>
<td>5.012</td>
<td>13_Three Fires</td>
<td>6.624</td>
</tr>
<tr>
<td>10_Big Creek</td>
<td>1.796</td>
<td>14_Anita</td>
<td>4.308</td>
</tr>
<tr>
<td>10_Green Valley</td>
<td>7.487</td>
<td>14_NTE</td>
<td>13.265</td>
</tr>
<tr>
<td>10_Three Fires</td>
<td>0.172</td>
<td>14_NTW</td>
<td>4.446</td>
</tr>
<tr>
<td>10_Anita</td>
<td>0.119</td>
<td>14_Three Fires</td>
<td>0.13</td>
</tr>
<tr>
<td>10_Black Hawk</td>
<td>3.293</td>
<td>14_Green Valley</td>
<td>1.418</td>
</tr>
<tr>
<td>10_Denison</td>
<td>20.416</td>
<td>14_Brushy</td>
<td>0.144</td>
</tr>
<tr>
<td>11_Black Hawk</td>
<td>16.687</td>
<td>14_Big Creek</td>
<td>6.699</td>
</tr>
<tr>
<td>11_Denison</td>
<td>0.709</td>
<td>14_Denison</td>
<td>6.567</td>
</tr>
<tr>
<td>11_NTE</td>
<td>6.144</td>
<td>14_Black Hawk</td>
<td>2.546</td>
</tr>
<tr>
<td>11_NTW</td>
<td>5.599</td>
<td>14_NTW Fdup</td>
<td>7.963</td>
</tr>
<tr>
<td>11_Three Fires</td>
<td>0.133</td>
<td>15_Anita</td>
<td>7.248</td>
</tr>
<tr>
<td>11_Green Valley</td>
<td>2.61</td>
<td>15_Three Fires</td>
<td>5.624</td>
</tr>
<tr>
<td>11_Three Fires Fdup</td>
<td>2.526</td>
<td>15_NTW</td>
<td>19.705</td>
</tr>
<tr>
<td>11_Anita</td>
<td>4.554</td>
<td>15_Green Valley</td>
<td>15.29</td>
</tr>
<tr>
<td>11_Big Creek</td>
<td>0.757</td>
<td>15_NTE</td>
<td>0.322</td>
</tr>
<tr>
<td>11_Brushy Creek</td>
<td>0.393</td>
<td>15_Black Hawk</td>
<td>1.773</td>
</tr>
<tr>
<td>12_Black Hawk</td>
<td>17.122</td>
<td>15_Brushy</td>
<td>15.654</td>
</tr>
<tr>
<td>12_Anita</td>
<td>22.223</td>
<td>15_Denison</td>
<td>9.137</td>
</tr>
<tr>
<td>12_Brushy Creek</td>
<td>3.108</td>
<td>15_Anita</td>
<td>17.906</td>
</tr>
<tr>
<td>12_NTW</td>
<td>4.91</td>
<td>15_Green Valley</td>
<td>2.225</td>
</tr>
</tbody>
</table>
APPENDIX B. GROWTH UNIT RESULTS

Most Probable Number results for each collection Site and Week from the 2019 sampling season; reported values represent estimations of Growth Units contained in 1 mL of undiluted lake water sample. Lower and Upper Confidence Levels were established using Cornish and Fisher Limits with 30 maximum iterations. Plates that did not exhibit visible growth are labeled as ‘no growth’.

<table>
<thead>
<tr>
<th>Lake</th>
<th>Week</th>
<th>Growth Units/mL</th>
<th>Lower 95% CL</th>
<th>Upper 95% CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anita</td>
<td>9</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.576</td>
<td>0.277</td>
<td>5.319</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>14.215</td>
<td>5.063</td>
<td>26.363</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>3.001</td>
<td>0.275</td>
<td>8.021</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.576</td>
<td>0.277</td>
<td>5.319</td>
</tr>
<tr>
<td>Big Creek</td>
<td>9</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.001</td>
<td>0.275</td>
<td>8.021</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>3.001</td>
<td>0.255</td>
<td>8.021</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>10.996</td>
<td>3.286</td>
<td>21.221</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>9.42</td>
<td>2.453</td>
<td>18.702</td>
</tr>
<tr>
<td>Black Hawk</td>
<td>9</td>
<td>10.996</td>
<td>3.286</td>
<td>21.221</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.42</td>
<td>2.453</td>
<td>18.702</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>10.996</td>
<td>3.286</td>
<td>21.221</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>7.846</td>
<td>1.659</td>
<td>16.17</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>10.544</td>
<td>3.044</td>
<td>20.499</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>10.996</td>
<td>3.286</td>
<td>21.221</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>9.42</td>
<td>2.453</td>
<td>18.702</td>
</tr>
<tr>
<td>Brushy Creek</td>
<td>9</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1.507</td>
<td>0.275</td>
<td>5.157</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>3.12</td>
<td>0.275</td>
<td>8.235</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>10.996</td>
<td>3.286</td>
<td>21.221</td>
</tr>
<tr>
<td>-------</td>
<td>---------</td>
<td>---------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>10</td>
<td>9.42</td>
<td>2.453</td>
<td>18.702</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>10.996</td>
<td>3.286</td>
<td>21.221</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>7.843</td>
<td>1.657</td>
<td>16.165</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>21.496</td>
<td>9.259</td>
<td>38.268</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>10.996</td>
<td>3.286</td>
<td>21.221</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>15.796</td>
<td>5.96</td>
<td>28.907</td>
<td></td>
</tr>
<tr>
<td>Green Valley</td>
<td>9</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>11.492</td>
<td>3.554</td>
<td>22.011</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>4.682</td>
<td>0.275</td>
<td>10.956</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>North Twin East</td>
<td>9</td>
<td>1.5</td>
<td>0.275</td>
<td>5.141</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>4.502</td>
<td>0.275</td>
<td>10.648</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>4.504</td>
<td>0.275</td>
<td>10.652</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>North Twin West</td>
<td>9</td>
<td>1.5</td>
<td>0.275</td>
<td>5.141</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.501</td>
<td>0.275</td>
<td>5.142</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1.5</td>
<td>0.275</td>
<td>5.141</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.5</td>
<td>0.275</td>
<td>5.141</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>3</td>
<td>2.891</td>
<td>8.019</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>3.001</td>
<td>0.275</td>
<td>8.021</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Three Fires</td>
<td>9</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.011</td>
<td>0.275</td>
<td>8.039</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1.576</td>
<td>0.277</td>
<td>5.319</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>no growth</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>