MIGHT RECYCLED WATER INHIBIT TOXIN-PRODUCING CYANOBACTERIA?

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A Thesis Submitted to the Faculty of the

DEPARTMENT OF SOIL, WATER AND ENVIRONMENTAL SCIENCE

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

2018

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ACKNOWLEDGEMENTS

The following project was completed with the support of family, friends, colleagues, and mentors during my studies in graduate college. I want to thank my wife, Sarah Lynch, for her unwavering support and confidence during my graduate studies. To Victoria Obergh, thank you for your patience and perseverance both in the laboratory and in the field, for without your aid, I would not have been as successful. I would like to convey my gratitude to Jean E. McLain, Ph.D., Kevin Fitzsimmons, Ph.D., Thomas Meixner, Ph.D., and Leif Abrell, Ph.D. for their mentorship and efforts as part of my graduate committee. To Dr. Jean E. McLain, thank you for the opportunity to earn a graduate degree at The University of Arizona and develop my skills in molecular biology, scientific investigation, and professionalism as a scientist.

DEDICATION

To my loving wife: Sarah Lynch

TABLE OF CONTENTS

LIST OF TABLES…….. 7

LIST OF FIGURES…… 8

ABSTRACT…….. 10

CHAPTER 1: LITERATURE REVIEW

1.1 Harmful Algal Blooms…… 12

1.1.1 Events

1.1.1.1 Lake Erie……... 13

1.1.1.2 Lake Okeechobee…….. 14

1.1.2 *Microcystis aeruginosa*…… 15

1.1.2.1 Microcystins…….. 17

1.1.2.2 MC-LR…….. 18

1.1.2.2.1 Methods for Detection

1.1.2.2.1.1 Quantitative Polymerase Chain Reaction (qPCR)..... 19

1.1.2.2.1.2 High Performance Liquid Chromatography Mass Spectrometry (HPLC-MS)…….……..….... 20

1.2 Water Quality in Relation to Algae

1.2.1 Physical and Chemical Parameters…….. 21

1.2.2 Inorganic Parameters (Metals)……. 23

1.3 Recycled Water Retention Ponds……..... 24

CHAPTER 2: RESEARCH METHODS

2.1 Sampling Locations…… 26

2.2 Sample Collection……... 28

2.3 Physical, Chemical, Inorganic, and Organic Analyses……..... 28

2.4 Sample Preparation and DNA Extraction……... 30

2.5 Molecular Analysis

2.5.1 Polymerase Chain Reaction…… 30

2.5.2 Real-Time Quantitative PCR……... 34

2.6 Data Analysis…….. 36

CHAPTER 3: MIGHT RECYCLED WATER INHIBIT TOXIN-PRODUCING CYANOBACTERIA?

3.1 Introduction……. 37

3.2 Materials and Methods

3.2.1 Locations and Collections…… 40

3.2.2 Sample Analyses…….. 42

3.2.3 Inorganic and Organic Methods……... 42

3.2.4 DNA Extraction and Purification……. 44

3.2.5 Molecular Methods…….. 44

3.3 Results

3.3.1 Molecular (*CYAN* and *mcyD*)……..… 46

3.3.2 MC-LR……. 47

3.3.3 Environmental Parameters……... 48

3.4 Discussion……... 52

3.5 Conclusions……. 55

APPENDIX A……...… 56

REFERENCES……. 64

LIST OF TABLES

Table 1: Metal isotopes analyzed by ALEC using ICP-MS……..... 29

Table 2: Primer and probe sequences…….……..… 34

Table 3: Metal isotopes analyzed by ALEC using ICP-MS (repeated)..…….. 43

Table 4: Primer and probe sequences used for qPCR...…… 45

Table 5: Concentrations for MC-LR detected in MAC samples…….. 48

Table 6: Metal isotopes measured in SW and MAC……...…….……..... 49

Table 7: Physical and chemical water parameters of SW over course of sampling…..……... 50

Table 8: Physical and chemical water parameters of MAC over course of sampling..……...…. 50

LIST OF FIGURES

Figure 1: Illustration of a general HPLC system (© 2018 Waters)…….. 20

Figure 2: Google Earth satellite image of SW retention pond and four sampling sites; SW1, SW2, SW3, and SW4, Tucson, AZ……... 27

Figure 3: Google Earth satellite image of MAC retention pond and four sampling sites; MAC1, MAC2, MAC3, and MAC4, Maricopa, AZ.……. 27

Figure 4: Illustration depicting reagents (template DNA, primer, DNA polymerase, and dNTPs) necessary for PCR (ThermoFisher Scientific)…….. 31

Figure 5: Illustration depicting exponential amplification of a single copy of template DNA (ThermoFisher Scientific)……. 32

Figure 6: Illustration depicting temperature-dependent steps (denaturation, annealing, and extension) during PCR (ThermoFisher Scientific)……... 32

Figure 7: Structure of microcystin with variable amino acid positions labeled X and Z (Tillett et al., 2000)……... 39

Figure 8: Google Earth satellite image of Sweetwater Wetlands retention pond and four sampling sites; SW1, SW2, SW3, and SW4 (repeat)……..... 41

Figure 9: Google Earth satellite image of MAC and four sampling sites, MAC1, MAC2, MAC3, and MAC4 (repeat)……... 41

Figure 10: Average *CYAN* molecular markers per 100 mL measured in pond samples over 6 months. Error bars represent standard deviation of quadruplet measurements…… 46

Figure 11: Average *mcyD* molecular markers per 100 mL measured in pond samples over 6 months. Error bars represent standard deviation of quadruplet measurements……... 47

Figure 12: Average temperature measured in each pond over sampling period. Error bars represent standard deviation of quadruplet measurements……... 51

Figure 13: Average pH measured in each pond over sampling period. Error bars represent standard deviation of quadruplet measurements……... 51

ABSTRACT

Cyanobacteria, or blue-green algae, is a phytoplankton phylum found in surface water bodies worldwide. For decades, blue-green algae has caused severe aesthetic water quality problems and induced water deoxygenation, leading to fish kills and other detrimental outcomes. Furthermore, some cyanobacterial genera, most notably several *Microcystis* species, are known to produce hepatotoxic peptides known as microcystins. Such toxin production is of critical and increasing public health concern, as hepatotoxic cyanobacterial blooms in freshwater lakes and streams have been implicated in human and animal sickness, and even death. Studies have correlated increased toxin production to enhanced temperature, nutrient concentrations, and light intensity, but research results examining microcystin toxin production in response to environmental stimuli have rarely been conclusive outside of the laboratory or over multiple seasons. Our research implemented advanced molecular techniques (real-time quantitative PCR) to detect and quantify cyanobacterial genes (*CYAN*) and toxin synthetase genes (*mcyD*) in water samples collected from a recycled water retention pond and a groundwater-filled pond. Water samples also underwent chemical and physical analyses to identify factors correlating to decreased toxin synthesis. Our results show similar concentrations of *CYAN* in both ponds but lower concentrations (sometimes below limits of detection) of *mcyD* levels in the recycled water retention pond. LC-MSMS showed average toxin concentrations of 6.702 ± 0.067 µg L^{-1} in the groundwater-filled pond while toxins were undetectable (detection limit ≥ 3 ppt) in the recycled water retention pond. Levels of *mcyD* in the groundwater pond were negatively correlated ($p \leq 0.05$) with Cr_{52}, a find that has been paralleled in previous research. Concentrations of Fe_{56} were significantly lower in the groundwater pond ($p \leq 0.05$), suggesting iron-limitation may have been

a contributing factor to microcystin-biosynthesis. Knowledge of the regulation of microcystin toxin biosynthesis may facilitate implementation of water management strategies to avoid environmental conditions that induce dangerous water quality conditions.

CHAPTER 1:
LITERATURE REVIEW

1.1 Harmful Algal Blooms

Harmful algal blooms (HABs) occur when aquatic photosynthetic plants and/or microorganisms overgrow and release toxins into the environment (NOAA, 2016). HABs develop at and near the surface of fresh, marine, and mixed-water bodies worldwide, affecting the ecology and economy of the localized region. Cyanobacteria, commonly referred to as blue-green algae, is a diverse phytoplankton phylum frequently responsible for toxic and non-toxic algal blooms. Environmental drivers of cyanobacterial blooms have been well-characterized; nutrient over-enrichment (eutrophication) of nitrate (NO_3^-) and phosphate (PO_4^{3-}) coupled with water temperatures of 25 °C and above are most often culpable for the competitiveness and proliferation of cyanobacteria over other phytoplankton (Paerl and Huisman, 2008). Environmental triggers that drive toxin-production from HABs however, are poorly understood. Scientists contend these events are complex and influenced by multiple water quality parameters including, but not limited to inorganic and/or organic compounds, water temperature, and light intensity (Heisler et al, 2008; O'Neil et al, 2012). Toxic secondary metabolites (toxins) produced in HABs impact "hepatopancreatic, digestive, endocrine, dermal, and nervous systems" of animals, including humans, who come into contact with contaminated water (Paerl and Otten, 2013). Climate change and global warming may further stimulate and enhance HABs (Paerl and Otten, 2013). The recurring frequency and threat of these damaging events in water bodies utilized for potable, irrigation, and recreational water are generating challenges for water resources management.

1.1.1 Events

1.1.1.1 Lake Erie

Despite initiatives to improve and restore water quality of the Great Lakes via the Great Lakes Water Quality Agreement (GLWQA) in 1972, and subsequent amendments in 1983, 1987, and 2012, Lake Erie has been experiencing recurring toxic and non-toxic algal blooms every summer since the 2000s. The GLWQA constitutes a commitment between Canada and the United States to enhance water quality programs to ensure the "physical, chemical, and biological integrity" of the Great Lakes through strategies such as nutrient reduction (EPA, 2018). Lake Erie is bordered by four American states (Michigan, New York, Ohio, and Pennsylvania) and one Canadian province (Ontario), and is regarded as the warmest and most biologically productive of the five Great Lakes (ODNR, 2016). It is revered as one of Ohio's most valuable natural resources because of its drinking water provision to approximately three million Ohio residents as well as its economic support via water-borne shipping and transportation, commercial and sport fishing, and other outdoor recreation (ODNR, 2016). Recognized as the twelfth largest freshwater lake in the world by surface area, Lake Erie provides drinking water to over 11 million people total (ODNR, 2016). Consequently, the negative impacts of algal blooms in this affected region have been far-reaching.

Negative impacts of widespread algal blooms are far more than aesthetic. Decomposing bloom material (biomass) induces water deoxygenation leading to fish kills, prompting beach closures with loss of fishing and other tourist activities (Patel & Parshina-Kotas, 2017). The enormity of algal-biomass generated during bloom events can also prevent recreational activities such as boating, thus hindering local economies (Patel & Parshina-Kotas, 2017).

When an algal bloom becomes toxic, additional challenges surface. Potable treatment technologies are not efficient at removal of the toxins. During the summer of 2014, the potable treatment plant for Toledo, Ohio issued a do-not-drink advisory to its 500,000 customer-base for approximately one week over concerns of elevated toxin concentrations post-treatment (Frankel, 2014). A culprit frequently identified during HABs in Lake Erie has been *Microcystis*, a cyanobacteria genera known to produce hepatotoxins referred to as microcystins (MC). *Microcystis* algae are commonly associated with HABs, not only in freshwater systems such as Lake Erie, but in mixed-water bodies as well (NOAA, 2017).

1.1.1.2 Lake Okeechobee

Lake Okeechobee is the largest freshwater lake in the state of Florida, comprising 730 square miles of surface area (FWC, 2018). It is a shallow and warm water body located approximately 100 miles north of the Florida Everglades. Sources of lake water are the tributaries Fisheating Creek, Taylor Creek, and the Kissimmee River, in addition to rainfall and runoff (FWC, 2018). Lake Okeechobee supports commercial and sport fishing, flood control, and provision of potable and irrigation water for most of south Florida (FWC, 2018). It is historically associated with massive inflow to the Florida Everglades, however, water levels are now managed by the U.S. Army Corps of Engineers in order to prevent flooding to downstream communities (Parker, 2016).

The Herbert Hoover Dike is a flood control system engineered in the 1930s by the U.S. Army Corps of Engineers that surrounds Lake Okeechobee. In cooperation with the South Florida Water Management District (SFWMD), the U.S. Army Corps of Engineers is responsible

for flood control, water supply, navigation, and the ecological health of the lake (SWFMD, 2018). When lake elevation is high, placing pressure on the aging dike system, water is diverted westward toward the Atlantic Ocean via the St. Lucie Canal, and eastward toward the Gulf of Mexico via the Caloosahatchee River.

Eutrophic conditions in Lake Okeechobee derived from runoff of the Kissimmee River watershed promotes a breeding ground for recurring *Microcystis* blooms. When bloom-infested water from Lake Okeechobee is discharged to the coastline of Florida and mixed with estuaries, the negative impacts of HABs are compounded. In 2016, public beaches in multiple counties in south Florida closed in response to a declaration of a state of emergency due to the overwhelming stench of an inch-thick layer of decomposing algal biomass (Neuhaus, 2016). These events have forced tough decisions in order to provide relief to coastal communities, leading the U.S. Army Corps of Engineers to reduce drainage from Lake Okeechobee (Reid, 2016).

Strategies which reduce eutrophication have always been a primary objective for preventing algal blooms, but the recurrence and severity of these events persist. Several *Microcystis* species have been implicated in HABs throughout Florida state, Lake Erie, and many other water bodies worldwide with one of the most notorious being *Microcystis aeruginosa* (*M. aeruginosa*).

1.1.2 *Microcystis aeruginosa*

Most commonly referred to as *Microcystis* in the media, *M. aeruginosa* has garnered mostly negative attention for cyanobacteria, but the benefits of cyanobacteria are well-

established in the scientific literature. Cyanobacteria are the oldest known oxygenic photoautotrophs according to Earth's fossil record, and are credited for engineering Earth's modern atmosphere beginning approximately 3.5 billion years ago (Paerl & Otten, 2013). The benefits of cyanobacteria also extend to soil ecosystems around the globe. In arid and semiarid climates where water, vegetation, and nutrients can become limited, cyanobacteria serve a pivotal role in engineering biological soil crusts (BSCs) (Chamizo et al., 2012). BSCs are a community that result from the association of soil particles, "cyanobacteria, algae, microfungi, lichens, and bryophytes" (Rosentreter et al., 2007). They occur at the soil surface and cycle nutrients such as N and P, increase soil fertility, inhibit wind and water erosion through improved soil structure, and provide microhabitats for other microorganisms.

M. aeruginosa is a planktonic species of freshwater cyanobacteria that has formed both toxic and non-toxic algal blooms on every continent except Antarctica (Harke et al., 2016). This unicellular prokaryote possesses a coccoid morphology which ranges 1 to 9 μm in diameter and aggregates during bloom formation (Harke et al., 2016). *Microcystis* genera may bloom when water temperatures exceed 15 °C, however, most cyanobacteria grow better and outcompete other phytoplankton at 25 °C and above (Harke et al., 2016; Paerl & Huisman, 2008). Eutrophic conditions caused by elevated nitrogen (N) and phosphorous (P) have favored algal blooms (Paerl & Otten, 2013). Traits such as intracellular gas vesicle formation allow *M. aeruginosa* to outcompete other phytoplankton by facilitating translocation through the water column to reach favorable light, nutrient, and temperature conditions (Brooks, 2001; Paerl & Huisman, 2008).

1.1.2.1 Microcystins

Microcystins are a diverse class of hepatotoxins produced intracellularly by the *Microcystis* genera which negatively impact the health of fish, birds, waterfowl, livestock, and humans who ingest contaminated water. Two variable amino acid positions on the cyclic heptapeptide structure give rise to over 100 different congeners of toxic and non-toxic microcystins (Harke et al., 2016). Upon cell death, lysis releases microcystins into the environment. Understanding microcystin-production is challenging because not all *M. aeruginosa* blooms result in their biosynthesis, nor are all microcystins toxic. Environmental parameters such as enhanced temperatures, solar radiation, N, P, and various metals have all been correlated with microcystin production, however, these findings have been based predominantly on culture-based studies that have not been reproduced or observed in the field (Harke et al., 2016).

Despite the complexity in drivers of microcystin production, there remains a strong desire to determine triggers that drive toxicity of harmful algal blooms to better safeguard public health. When humans come into contact with recreational or drinking water contaminated with toxic microcystins, they may experience illnesses that include gastroenteritis and/or hepatocyte necrosis (liver cell death), in addition to the risk of death for people suffering from liver disease (McElhiney & Lawton, 2005). Exposure can also occur when animals, including humans, consume aquatic plants and/or freshwater mussels in microcystin-affected water bodies since microcystins have shown to bio-accumulate (McElhiney & Lawton, 2005).

1.1.2.2 MC-LR

Of the multitude of variants of microcystins, the most notable is microcystin-LR (MC-LR), which contains leucine (L) and arginine (R) in the variable amino acid positions. MC-LR is one of the most common toxic variants of microcystins, inflicting illness and sometimes death on both wildlife and humans. Frequently detected in HABs worldwide, MC-LR disrupts metabolic activity in the liver by inhibiting protein phosphatases 1 and 2A thus contributing to the process of hepatotoxicity (Yoshizawa et al., 1990). A rare but extreme example of lethal human poisoning occurred in Caruaru, Brazil in 1996, in which 52 hemodialysis patients experienced acute liver failure and perished after consuming municipally treated water drawn from a bloom-infested lake (Yuan et al., 2006).

Although MC-LR in drinking water is not federally regulated in the U.S., some states such as Ohio have adopted their own sampling and monitoring protocols for microcystin concentrations. Potable treatment plants may subscribe to the World Health Organization (WHO) recommendation that MC-LR in drinking water should not exceed 1 µg/L (1 ppb) (WHO, 2003). However, some research has shown that MC-LR concentrations post-treatment can exceed the WHO recommended limit if water entering treatment contains elevated concentrations of toxins (Zamyadi et al., 2012). Understanding conditions that favor MC-LR production remains a key component for preventive mitigation.

1.1.2.2.1 Methods for Detection

1.1.2.2.1.1 Quantitative Polymerase Chain Reaction (qPCR)

Microcystin biosynthesis has been correlated with a 55 kb DNA cluster (*mcy*) encoding 10 open reading frames (ORFs), *mcyA-J* (Tillett et al., 2000). Target DNA sequence (gene) disruption showed direct involvement of ORF *mcyD* with MC-LR biosynthesis (Tillett at al., 2000). MC-LR synthetase gene markers (*mcyD*) in water samples can be detected and quantified through DNA amplification using quantitative polymerase chain reaction (qPCR), a sensitive molecular technique capable of detecting one molecular marker per sample.

Quantitative PCR (qPCR) is a useful technique in molecular biology for detecting target nucleic acid sequences specific to a microorganism. The technique involves multiple cycles of three temperature dependent steps; denaturation, annealing, and extension (MilliporeSigma Co., LLC, 2018). Denaturation breaks hydrogen bonds between complimentary bases of DNA to produce single strands. During the annealing step, primers specific to a portion of the target DNA sequence bind to their complimentary sequence. The extension step promotes the DNA polymerase to extend the primers and synthesize complimentary strands from the ones produced from denaturation. During qPCR, a fluorescing reporter molecule emits a signal "directly proportional to the quantity of exponentially accumulating PCR product molecules" to which the instrument can calculate the target DNA markers in the unknown sample (MilliporeSigma Co., LLC, 2018).

1.1.2.2.1.2 High Performance Liquid Chromatography Mass Spectrometry (HPLC-MS)

MC-LR is an organic, cyclic heptapeptide (seven amino acids) that can be detected and quantified using high performance liquid chromatography coupled with mass spectrometry (HPLC-MS). HPLC works by injecting an aqueous sample into a continuously flowing solvent that is carried through a column packed with chromatographic material (Figure 1). A high-pressure pump generates the desired flow rate in order to move the solvent through the column. Organic substances within the sample partition and separate in the column at different times depending on their affinity for either the solvent or chromatographic material. The column eluate passes through a detector wired to a computer station to produce a chromatogram of the sample's constituents. When coupled with mass spectrometry, the peaks (compounds) generated on the chromatogram can be analyzed and quantitated.

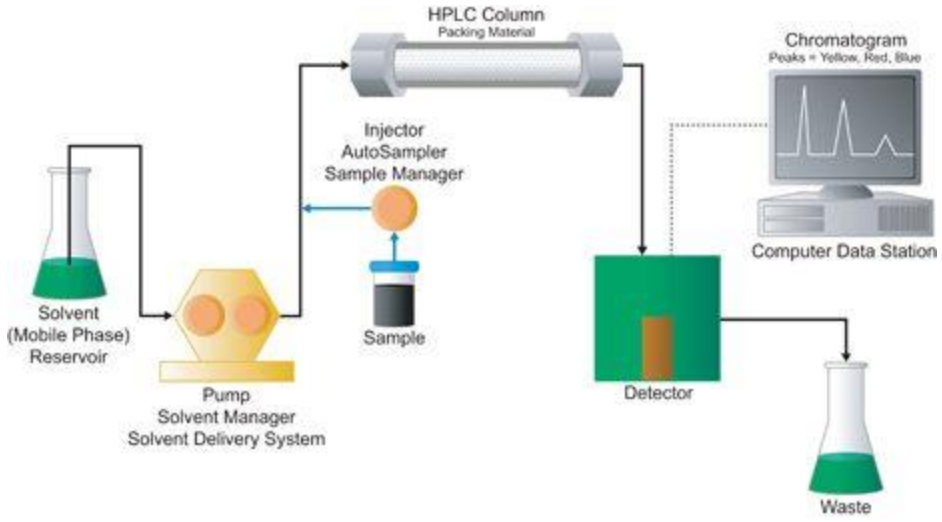

Figure 1: Illustration of a general HPLC system (© 2018 Waters).

1.2 Water Quality in Relation to Algae

1.2.1 Physical and Chemical Parameters

Toxic and non-toxic algal blooms occur when water quality parameters produce conditions complimentary to the competitive nature of the bloom-culprit. Despite the pre-existence of *M. aeruginosa* in water bodies throughout the year, favorable conditions brought on by seasonal changes promote faster growth rates and increased abundance that result in bloom formation. Physical water quality parameters such as light intensity, water temperature, turbidity, and electrical conductivity are variables that are either influenced or are impacted by bloom events. Chemical water quality parameters associated with bloom events may include but are not limited to pH (Hem, 1985).

Enhanced *M. aeruginosa* growth rates during HABs have been positively correlated to mean daily photosynthetically active radiation (PAR) (Tomioka & Komatsu, 2011). PAR constitutes visible light ranging 400-700nm and is fundamental to photosynthetic organisms like *M. aeruginosa*. Light intensity is a measurement of solar radiation received by the Earth's surface of which 99% is between 300-3000nm (LI-COR, 2015). Light intensity data can be gathered from cooperatives such as AZMET, The Arizona Meteorological Network, whose meteorological stations are equipped with instruments used for measuring solar radiation known as pyranometers (AZMET, 2018).

Water temperature has been shown to shift phytoplankton assemblages from eukaryotic green algae to *M. aeruginosa* and other harmful cyanobacteria at temperatures of 25 °C and above (Davis et al., 2009). As eukaryotic algae growth rates decline and/or stabilize at these temperatures, growth rates for *M. aeruginosa* optimize (Paerl & Otten, 2013). Increased

temperatures near the water surface intensify vertical stratification, allowing *M. aeruginosa* to exploit these conditions by remaining close to the surface via buoyancy control mechanisms. Non-buoyant phytoplankton remain below and are shielded from necessary solar radiation, thus removing competition for resources (Paerl & Otten, 2013). As a consequence of global warming, lakes and other water bodies may stratify earlier in the spring and linger later into autumn, thus compounding the exploitive behavior of *M. aeruginosa* and other harmful cyanobacteria (Paerl & Otten, 2013).

Turbidity is a physical water quality parameter positively influenced by increased growth of eukaryotic algae and aquatic cyanobacteria. Additionally influenced by inorganic particulates, colloids, and dissolved organic compounds, turbidity is a measure of the relative clarity of a solution (Swanson & Baldwin, 1965). It is characterized as the amount of light scattered by material in solution when light is passed through a sample (Swanson & Baldwin, 1965). Reported in nephelometric turbidity units (NTU), samples with high turbidity scatter high levels of light and thus possess higher NTU. Elevated *M. aeruginosa* and/or other algae concentrations increase turbidity and absorb more light, thus increasing localized water temperatures. This fosters a positive feedback mechanism by which harmful cyanobacteria like *M. aeruginosa* can exploit (Paerl & Huisman, 2008).

Electrical conductivity (EC) is a physical water quality parameter that describes the ability of a solution to conduct an electrical current (Hem, 1985). EC is a measure of conductance across a body of unit length and cross-section at a specified temperature (Hem, 1985). Reported in units of micro Siemens per cm (µm/cm) at a standard temperature of 25 °C, EC is determined by the presence of charged ionic species such as, but not limited to NO_3^- and

PO_4^{3-}. Ions conduct an electrical current proportional to their concentration, and thus conductance increases with increased ion concentrations (Hem, 1985).

M. aeruginosa blooms rely on and consume dissolved carbon dioxide (CO_2) for photosynthesis in the water column and/or the air-water interface, thus impacting pH. The pH of natural waters determines solubility and speciation of chemical constituents such as metals and nutrients in addition to their biological availability (Hem, 1985). This common chemical water quality parameter is quantified using the negative logarithm of the activity of free hydrogen ions (H^+) in solution and represents a measure of acidity (Hem, 1985). The chemical reaction between dissolved CO_2 and water impacts the pH of the solution, where enhanced CO_2 levels increase acidity ($pH \leq 7.0$), and lower CO_2 concentrations increase basicity ($pH \geq 7.0$) (Hem, 1985).

1.2.2 Inorganic Parameters (Metals)

Metals in the environment are inorganic elements released from rocks during chemical weathering processes (Garrett, R.G., 2000). Environmental parameters such as pH, redox potential, complexing anions, and organic ligands determine different forms (speciation) that metals assume, driving biological availability for microbes, plants, and animals (Garrett, 2000). Copper (Cu), iron (Fe), nickel (Ni), and zinc (Zn) are some of the metals that have been shown as essential for cyanobacteria, however, some metals such as cadmium (Cd) have been shown to be highly toxic (Baptista & Vasconcelos, 2006). Depending on metal speciation and respective concentrations, their effect on aquatic environments may shift phytoplankton communities and promote toxigenicity during algal blooms (Lukač & Aegerter, 1993).

Fe, for example, is one of Earth's most abundant metals and can thus have a marked effect on growth rate and toxin production of *M. aeruginosa* (Lukač & Aegerter, 1993). Cyanobacteria require Fe for major physiological functions such as photosynthesis, N-fixation, respiration, and chlorophyll synthesis (Boyer et al., 1987). Fe-deficiency can stress microorganisms like *M. aeruginosa*, and has been shown to reduce growth rate and induce microcystin synthesis (Lukač & Aegerter, 1993; Sevilla et al., 2008). Other metals have also been reported to induce microcystin production including Ni, molybdenum (Mo), lead (Pb), and chromium (Cr) (McLain & Rock, 2012).

Occurrence of algal blooms have frequently been associated with eutrophic conditions, water temperature, and light intensity, however, field-based studies correlating microcystin production with inorganics such as metals are incipient (Baptista & Vasconcelos, 2006). The ability to further identify trends in metals' influence on algal bloom growth and toxigenicity may support improved water management practices.

1.3 Recycled Water Retention Ponds

Occurrence of microcystins in recycled water retention ponds may pose a risk to public and wildlife health, as water scarcity in the American southwest has fueled interest in the use of recycled water to replenish groundwater supplies via managed aquifer recharge ponds. Recycled water retention ponds are used to naturally filter municipally-treated wastewater before transport to groundwater recharge basins. When needed, replenished groundwater can be pumped back up through extraction wells and distributed accordingly.

Retention ponds can be engineered as constructed wetlands because they provide wildlife habitat and outdoor recreational activities in addition to their functions that contribute to the biological, chemical, and physical health of the environment. Wetlands are both naturally occurring and man-made areas defined by the presence of saturated soils for at least part of the year, specialized plants (hydrophytes), and water (EPA, 2018). Wetlands serve both the environment and wastewater treatment process through flood abatement, sediment trapping, and N and P removal (Zedler & Kercher, 2005).

Despite the ability for wastewater treatment plants (WWTP) to effectively treat wastewater according to standards set by the State, municipal wastewater can still load important levels of trace metals into the environment (Santos & Judd, 2010). Metals display a range of physico-chemical behaviors that impact their bioavailability, mobility, toxicity, and fate in aqueous systems, and thus metal speciation is an important factor determining its removal during wastewater treatment (Bubb & Lester, 1991). Some metals have been shown to be effectively removed during wastewater treatment (Cd, Cr, and Pb), whereas other metals have shown to persist for consequent release into the environment (Cu and Ni) (Buzier et al., 2006).

The objective of this research was to correlate microcystin production in samples obtained from a recycled water retention pond and a groundwater-filled retention pond to water quality parameters and determine any significant contributory differences between the two sites. Results of this work could facilitate the development of predictive models to forecast toxic algal blooms, facilitating public health advisories throughout the U.S.

CHAPTER 2:

RESEARCH METHODS

2.1 Sampling Locations

Water samples were collected from a recycled wastewater recharge basin at Sweetwater Wetlands (SW, Tucson, AZ; 32º12'41"N, 111º01'24"W) (Figure 2) and a groundwater-filled irrigation retention pond at the Maricopa Agricultural Center (MAC, Maricopa, AZ; 33º03'49"N, 111º58'43"W) (Figure 3). At Sweetwater Wetlands, one sample (SW1) was collected on each sampling date at an exposed portion of the pond's edge adjacent to the dirt pathway along the northern edge of the pond. Dense vegetation limited all other pond access to the pier where the other three samples (SW2-SW4) were collected in a clockwise fashion. Beginning from the north-end of the MAC pond, four samples (MAC1-MAC4; Figure 3) were collected from the western shore along a southward transect approximately 100 paces apart.

Figure 2: Google Earth satellite image of SW retention pond and four sampling sites; SW1, SW2, SW3, and SW4, Tucson, AZ.

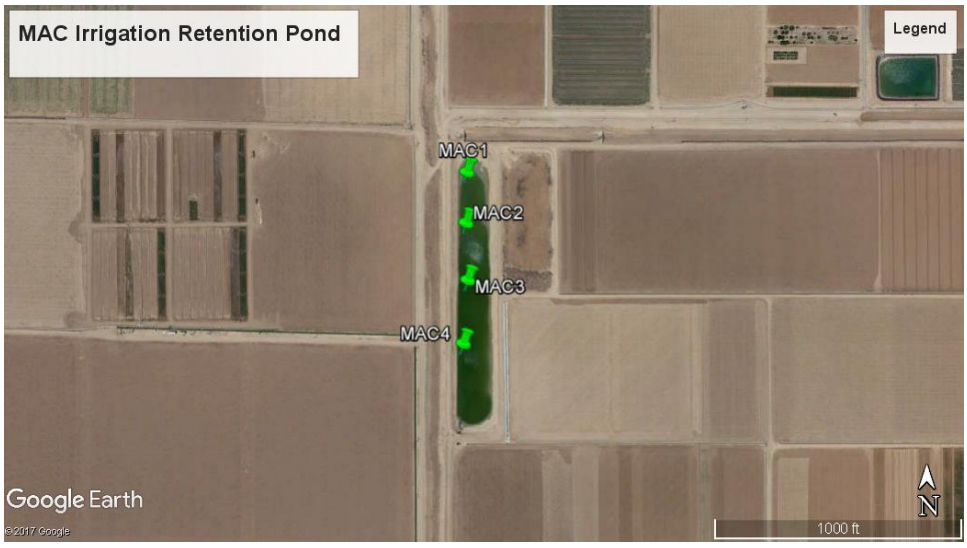

Figure 3: Google Earth satellite image of MAC retention pond and four sampling sites; MAC1, MAC2, MAC3, and MAC4, Maricopa, AZ.

2.2 Sample Collection

Between May and September, 2017, monthly water samples were collected between 8:00am and 12:00pm. Over the length of the project, a total of 24 water samples were collected from each pond, totaling 48 pond samples. On sampling dates, water was collected at the surface using a grab sampling technique with a 24 foot reach pole (Nasco Inc., Fort Atkinson, WI) and 1 L sterile Nalgene® plastic bottles. Samples were immediately stored on ice for transport to the University of Arizona laboratory in Tucson, AZ (University of Arizona, Tucson, AZ).

2.3 Physical, Chemical, Inorganic, and Organic Analyses

At time of field collection, temperature, turbidity, and conductivity measurements were obtained using a field thermometer (Fisher Scientific, Pittsburgh, PA), Milwaukee/Martini-Instruments Turbidity Meter (MI 415) (Milwaukee Instruments, Inc., Rocky Mount, NC), and a Traceable® Conductivity Meter (VWR International, LLC, Radnor, PA) respectively. At the University of Arizona laboratory, pH values were obtained with a Corning pH Meter 430 (Corning Inc., Corning, NY). All equipment was calibrated according to manufacturer protocol prior to sample collection.

For metal analysis, a single pond sample was produced from each location on sampling dates by compositing 25 mL of each sub-sample and filtering 15 mL into a Metal Free Centrifuge Tube (VWR® International, LLC, Radnor, PA) via 0.45 µm Nylon Syringe Filters (VWR® International, LLC, Radnor, PA). The analyses for metals listed in Table 1 were performed by the Arizona Laboratory for Emerging Contaminants (ALEC) at the University of

Arizona, Tucson, AZ using inductively-coupled plasma mass spectrometry (ICP-MS; Elan DRC II, Perkin Elmer).

Table 1: Metal isotopes analyzed by ALEC using ICP-MS.

Metal	Abbreviation	Atomic Mass
Beryllium	Be	9
Aluminum	Al	27
Vanadium	V	51
Chromium	Cr	52
Manganese	Mn	55
Iron	Fe	56
Cobalt	Co	59
Nickel	Ni	60
Copper	Cu	63
Zinc	Zn	66
Arsenic	As	75
Selenium	Se	78
Molybdenum	Mo	95
Silver	Ag	107
Cadmium	Cd	111
Tin	Sn	118
Antimony	Sb	121
Barium	Ba	137
Lead	Pb	208

For organic analysis (MC-LR), 50 mL aliquots of each water sample were subjected to three freeze/thaw cycles of -80 °C and 37 °C to lyse cells and release total MC-LR into solution. Water samples were then filtered into sterile 50 mL conical tubes using 1.5 µm Nylon Syringe Filters (Thermo-Scientific™, Rockwood, TN). The analysis for MC-LR was performed at ALEC using liquid chromatography-tandem mass spectrometry (LC-MSMS; Dionex Ultimate

3000, Dionex-Thermo). A stock solution of MC-LR (dilution series spanning 374 ppb to 3 ppt) was used as a positive control (Sigma-Aldrich Co., LLC, St. Louis, MO).

2.4 Sample Preparation and DNA Extraction

Bacterial biomass was isolated by filtering each pond sample onto Whatman® 0.45 µm pore size membrane filters using a Millipore vacuum filtration system. In between samples the Millipore apparatus was sterilized with ethanol and flame. Due to varying turbidity between individual samples, which could range from 0.5 Nephelometric Turbidity Units (NTU) to 890 NTU any given day, between 75 mL and 500 mL of each water sample was filtered for downstream DNA extraction. To improve DNA recovery during extraction, each filter was cut into four pieces and stored together in a 15 mL conical vial at 4 ºC. DNA extraction was performed using a MOBio Laboratories, Inc. PowerBiofilm® DNA Isolation Kit (MOBio Laboratories, Inc., Carlsbad, CA) according to the included instruction manual. DNA extracts were stored (-80 ºC) until further processing.

2.5 Molecular Analysis

2.5.1 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a molecular method in which a gene marker is detected by amplifying a target DNA sequence. The process which requires a DNA template, oligonucleotide primers specific to the DNA target, DNA polymerase, and free deoxynucleoside triphosphates (dNTPs), produces exponentially greater copies of the specified DNA sequence for qualitative and/or quantitative analysis (Figure 4 and 5). Both analyses rely on temperature-dependent steps in which DNA denatures, anneals, and extends (Mullis et al., 1986). The three-

step process is repeated for multiple cycles until the target sequence can be visualized (PCR) or quantified (qPCR).

During the first step (denaturing), hydrogen bonds that occur between complementary nucleotide bases which create the double helix shape, are destroyed by a high temperature of 95 ºC (Figure 6). This step may last for 1-3 minutes and is critical for separating DNA into single-stranded nucleotide templates for upcoming annealing and extension. Next, the temperature is lowered to a point at which oligonucleotide primers can hybridize the template through attachment at homologous sequences (Mullis et al., 1986). Afterwards, a slight increase in temperature initiates the third and final step by triggering the DNA polymerase to extend the primers and finish assembling a copy of the source DNA.

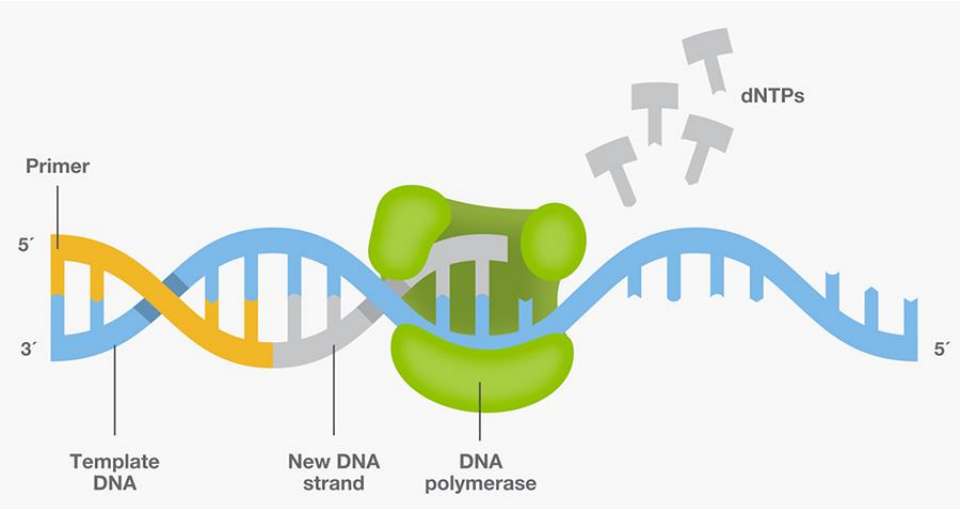

Figure 4: Illustration depicting reagents (template DNA, primer, DNA polymerase, and dNTPs) necessary for PCR (ThermoFisher Scientific).

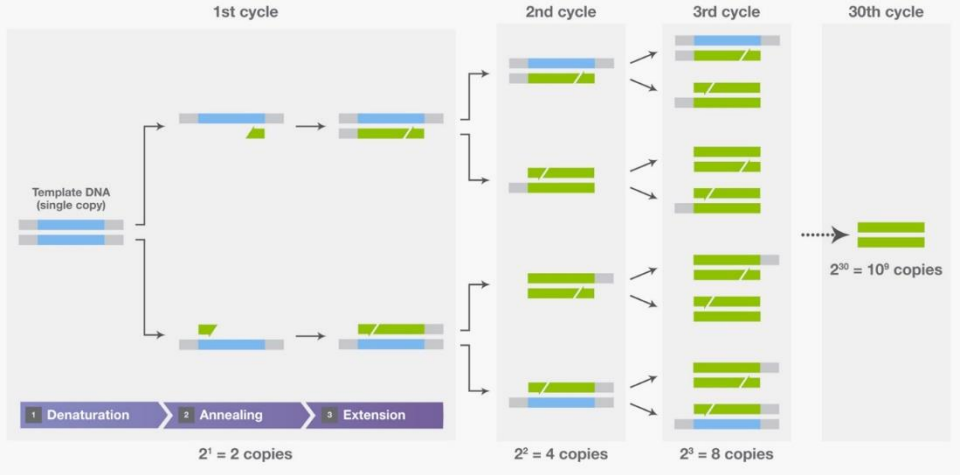

Figure 5: Illustration depicting exponential amplification of a single copy of template DNA (ThermoFisher Scientific).

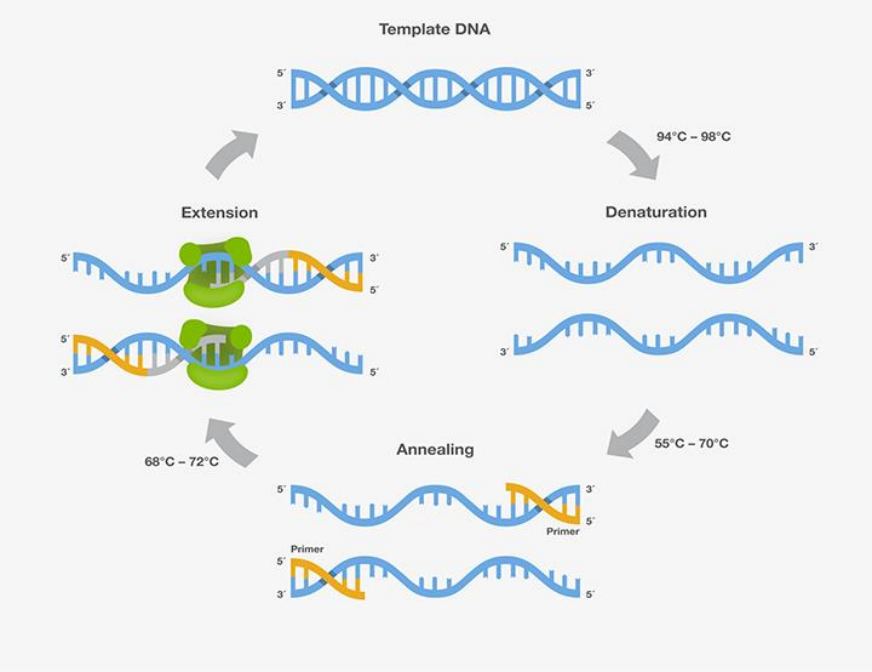

Figure 6: Illustration depicting temperature-dependent steps (denaturation, annealing, and extension) during PCR (ThermoFisher Scientific).

To determine presence or absence of cyanobacteria and toxic *Microcystis spp*., all DNA extracts were analyzed for cyanobacterial (CYAN) and toxin synthetase (*mcyD*) gene markers via PCR. To maintain a sterility, all PCR work was performed within a Fisher Scientific PCR Workstation.

For each sample, two PCR reactions were carried out to detect *CYAN* markers using primers *CYAN* 108F and *CYAN* 377R; and *mcyD* markers using primers *mcyD*F and *mcyD*R (Table 2).

All PCR reactions were carried out in 25 µL volume comprising of 9.5 µL Nuclease Free Water, 12.5 µL GoTaq® Green Master Mix (Promega Corporation, Madison, WI), 400 nM Forward Primer (Table 2), 400 nM Reverse Primer (Table 2), and 1 µL of extracted DNA. The temperature protocol for each reaction conducted in a Mastercycler® Pro (Eppendorf, Hamburg, Germany) thermal cycler consisted of an initial denaturation step for 5 minutes at 95 ºC followed by 50 cycles of 94 ºC for 30 seconds (denaturation), 56 ºC for 60 seconds (annealing), 72 ºC for 30 seconds (extension), and a final extension step of 72 ºC for 15 minutes.

PCR products were subjected to gel electrophoresis using 1.5% agarose gels. The target base pair for CYAN and *mcyD* gene markers were 267 bp and 297 bp respectively (Kaebernick et al., 2000) and was verified using a Mini DNA Ladder (25bp – 650bp) under UV illumination.

Table 2: Primer and probe sequences.

Primer	Sequence (5'- 3')	Reference
CYAN 108F	ACGGGTGAGTAACRCGTRA	Urbach et al. (1992)
CYAN 377R	CCATGGGCGGAAAATTCCCC	Nűbel et al. (1997)
mcyDF	GGTTCGCCTGGTCAAAGTAA	Kaebernick et al. (2000)
mcyDR	CCTCGCTAAAGAAGGGTTGA	Kaebernick et al. (2000)
CYAN – Probe	(6FAM)CTCAGTCCCAGTGTGGCTGNTC(TAM)	Rinta-Kanto et al. (2005)
mcyD - Probe	(6FAM)ATGCTCTAATGCAGCAACGGCAAA(TAM)	Rinta-Kanto et al. (2005)

2.5.2 Real-Time Quantitative PCR

To quantify *CYAN* and *mcyD* gene markers, all samples were analyzed via quantitative polymerase chain reaction (qPCR). Unlike standard PCR which allows for detection of DNA upon completion of polymerization, qPCR detects and quantifies gene copies in real-time using a fluorogenic probe.

During the extension phase of qPCR, the oligonucleotide probe emits a signal when cleaved from the DNA target by the DNA polymerase. Corresponding emissions from a series of log dilutions of known standard DNA (300,000 markers to 3 markers per µL of standard) (see section 2.5.4) allow the number of gene copies present in the environmental samples to be calculated.

For each sample, two qPCR reactions were carried out to detect CYAN and *mcyD* gene markers using the same primers previously mentioned, with the addition of a 0.1 mM probe for

each target (Table 1). All qPCR reactions were facilitated in an AB Applied Biosystems Step One™ Thermocycler (Life Technologies, Grand Island, NY) using MicroAmp™ Fast Optical 48-Well Reaction Plates (0.1 mL). Each 25 µL reaction comprised of 8.5 µL Nuclease Free Water, 12.5 µL BIO-RAD SsoAdvanced™ Universal Probes Supermix, 400 nM Forward Primer (Sigma-Aldrich Co., LLC, St. Louis, MO) (Table 2), 400 nM Reverse Primer (Sigma-Aldrich Co., LLC, St. Louis, MO) (Table 2), 0.1 mM probe (Table 2), and 1 µL of extracted DNA.

The temperature protocol for the *CYAN* gene marker during qPCR was the same as previously discussed, however the temperature protocol for the *mcyD* gene marker during qPCR was as follows: initial denaturation of 95 ºC for two minutes followed by 45 cycles of 95 ºC for 30 seconds and 55 ºC for one minute.

In lieu of pure cultures, quantitative PCR standards were created from PCR products of the target size. ExoSAP-IT® PCR Product Cleanup Reagent (Affymetrix®, USB® Products, Cleveland, OH) was used according to manufacturer protocol to hydrolyze excess primers and dNTPs in PCR products for downstream cloning.

PCR products for each target DNA sequence were used as templates for blunt-end cloning using a CloneJet™ PCR Cloning Kit (Thermo Fisher Scientific, Waltham, MA). Cloning kit product containing the target DNA was inserted into the host plasmid and transformed into *E.cloni*® 10G SOLOs Chemically Competent Cells (Lucigen Corporation, Middleton, WI) through growth overnight at 37 ± 0.5 ºC on LB agar with ampicillin. Ampicillin is an antibiotic used during the cloning process to promote growth of a pure culture of ampicillin-resistant *E. coli* for higher plasmid extraction efficiency. Bacterial cells containing the plasmid insert were isolated from the agar and inoculated in LB broth with ampicillin for growth overnight again at 37 ± 0.5 ºC. The following day, bacterial cells grown in the LB broth

were pelleted and their plasmids were extracted using a GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich Co., LLC, St. Louis, MO). Plasmid extract concentration for each target gene marker were measured with a ThermoFisher Scientific NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) for calculation of serial log dilutions of 300,000 to 3 gene markers per 1 µl. Target base pair lengths for *CYAN* (267 bp) and *mcyD* (297 bp) were added separately to the 2974 bp length of the *E. coli* plasmid (containing ampicillin insert) to achieve quantitative standards.

2.6 Data Analysis

Correlations and 2-Sample T-tests of statistical significance ($p \leq 0.05$) for molecular markers, MC-LR concentrations, physical and chemical water quality parameters, and metal concentrations were calculated by Minitab Release 14.20 Statistical Software (Minitab® Statistical Software, State College, PA).

Chapter 3:

MIGHT RECYCLED WATER INHIBIT TOXIN-PRODUCING CYANOBACTERIA?

3.1 Introduction

Water scarcity in the American southwest has fueled interest in the use of recycled water for potable and/or irrigation water demands (EPA, 2017). Recycled water is treated wastewater that is commonly used as irrigation for public parks and golf courses, coolant for industrial processes, and groundwater recharge for increased water sustainability. Groundwater recharge projects typically possess a series of retention ponds that naturally filter municipally-treated wastewater before transport to recharge basins. Groundwater recharge projects can generate wetlands which provide wildlife habitat, outdoor recreational activities, and biological, chemical, and physical functions that contribute to improved water quality (EPA, 2017). Wetland biota such as specialized plants (hydrophytes) and microbial communities, including planktonic cyanobacteria, contribute to enhanced water quality through sediment trapping and removal of nitrogen (N), phosphorous (P), and other contaminants that may be present in recycled water (Zedler & Kercher, 2005).

Wastewater treatment facilities can effectively treat wastewater, however, municipal wastewater can still load important levels of trace metals into the environment (Santos & Judd, 2010). Metals are inorganic elements released from rocks during chemical weathering processes (Garrett, R.G., 2000). Some have been shown as essential to cyanobacteria, such as copper (Cu), iron (Fe), nickel (Ni), and zinc (Zn). Others have shown to be highly toxic, such as cadmium (Cd). Metals that have been shown to be effectively removed from wastewater include

chromium (Cr), lead (Pb), and Cd, whereas other metals may have shown to persist treatment for consequent release into the environment (Cu and Ni) (Buzier et al., 2006).

Microcystis aeruginosa is a planktonic species of cyanobacteria known to form harmful algal blooms (HABs) in water-bodies worldwide (Harke et al., 2016). Although *M. aeruginosa* has garnered negative attention, the benefits of cyanobacteria are well-established in the literature. According to the Earth's fossil record, cyanobacteria are the oldest known oxygenic photoautotrophs (Paerl & Otten, 2013). Cyanobacteria are credited for the engineering of our modern atmosphere beginning approximately 3.5 billion years ago, and continue to contribute to primary production in aquatic and terrestrial environments today (Paerl & Otten, 2013).

During favorable conditions, *Microcystis spp.* of cyanobacteria are known to outcompete other phytoplankton, overgrow, and form algal blooms. Eutrophication and water temperatures of 25 °C and above are most often correlated with increased *Microcystis* proliferation over other phytoplankton (Paerl & Huisman, 2008). Under certain conditions, *Microcystis* genera produce secondary metabolites known as microcystins, which negatively impact hepatopancreatic systems of animals, including humans, who ingest contaminated water (Paerl and Otten, 2013). Environmental drivers of cyanobacterial blooms (including *Microcystis*) have been well-characterized, however, environmental triggers that drive toxin-production from HABs are poorly understood.

Microcystins belong to a diverse group of monocyclic heptapeptides synthesized intracellularly by *Microcystis spp.* of cyanobacteria. The microcystin structure contains two variable amino acid positions which affect toxicity (Figure 7). Over 100 different microcystins of varying toxicity have been identified (Harke et al., 2016). Microcystin-producing strains of

cyanobacteria contain a 55 kilobase pair (kb) synthetase gene cluster (*mcy*) responsible for biosynthesis of the toxin (Tillett et al, 2000).

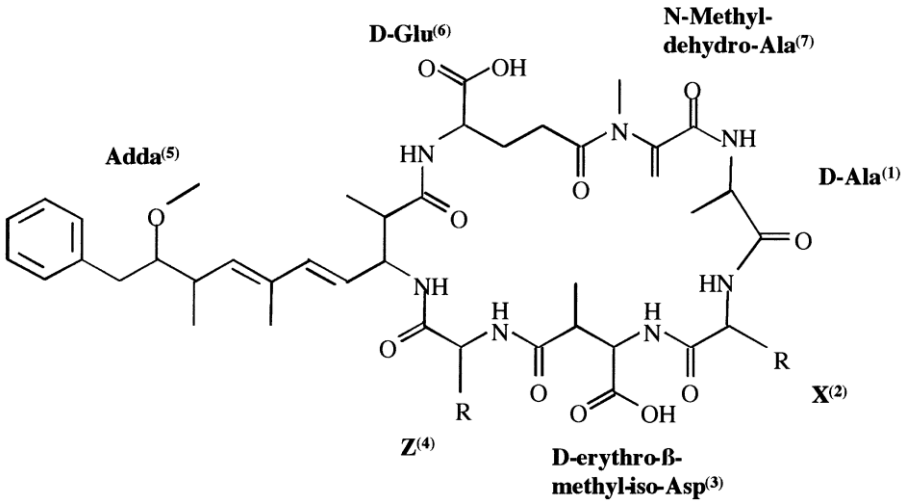

Figure 7: Structure of microcystin with variable amino acid positions labeled X and Z (Tillett et al., 2000).

One of the most concerning toxic variants detected in HABs worldwide is microcystin-LR (MC-LR); which contains the amino acids leucine (L) and arginine (R) in the variable positions. Biosynthesis of MC-LR has been correlated with a segment (*mcyD*) of the microcystin gene cluster (Tillett et al., 2000). MC-LR is a known inhibitor of protein phosphatases within liver cells of animals, including humans; disrupting metabolic processes and inflicting illness and sometimes death (Yoshizawa et al., 1990). In 1996, 52 hemodialysis patients experienced acute liver failure and died after consuming potable water containing MC-LR that was drawn from a bloom-infested lake in Caruaru, Brazil (Yuan et al., 2006). Occurrence of MC-LR in recycled water retention ponds can pose a risk to wildlife, as well as humans, that come into contact with the water.

The overall objective of this project was to determine if differences in the water quality parameters (including metals, temperature, pH, and others) between a recycled water pond and a groundwater-filled pond are correlated with microcystin-production. Molecular markers specific to cyanobacteria and a microcystin-synthetase gene (*mcyD*) in water samples collected from a recycled water retention pond and a groundwater-filled pond were quantified over six discrete sampling events. Analysis of the MC-LR toxin was performed to determine if presence of *mcyD* genes correlated with MC-LR biosynthesis. Collection of environmental data and water quality parameters could facilitate the development of predictive models to forecast toxic algal blooms, facilitating public health advisories throughout the U.S.

3.2 Materials and Methods

3.2.1 Locations and Collections

This study analyzed water quality in a recycled wastewater recharge basin at Sweetwater Wetlands (SW, Tucson, AZ; 32º12'41"N, 111º01'24"W) (Figure 8) and a groundwater-filled irrigation retention pond at the Maricopa Agricultural Center (MAC, Maricopa, AZ; 33º03'49"N, 111º58'43"W) (Figure 9). At Sweetwater Wetlands, one sample (SW1) was collected on each sampling date at an exposed portion of the pond's edge adjacent to a dirt trail along the northern edge of the pond, whereas the other three samples (SW2-SW4) were collected from a pier that extended out into the south end of the pond. At MAC, four samples (MAC1-MAC4) were collected on each sampling date from the western shore along a southward transect.

Figure 8: Google Earth satellite image of Sweetwater Wetlands retention pond and four sampling sites; SW1, SW2, SW3, and SW4.

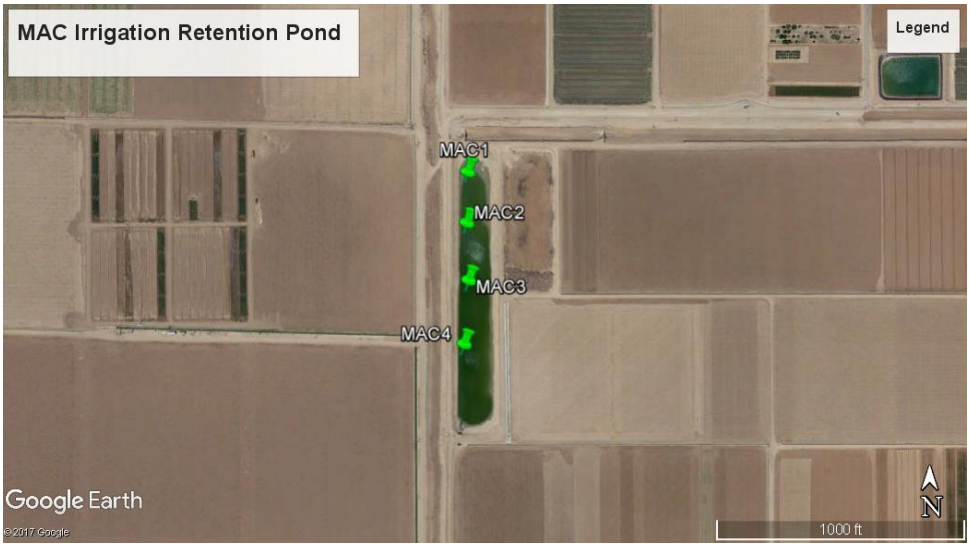

Figure 9: Google Earth satellite image of MAC and four sampling sites, MAC1, MAC2, MAC3, and MAC4.

Water sample collections occurred monthly between May and September, 2017. Over the length of the project, a total of 24 water samples were collected from each pond, totaling 48 pond samples. Water was collected at the surface using a grab sampling technique with a 24 foot reach pole (Nasco Inc., Fort Atkinson, WI) and 1 L sterile Nalgene® plastic bottles. Samples were stored on ice during transport to the University of Arizona laboratory in Tucson, AZ.

3.2.2 Sample Analyses

Chemical and physical parameters measured in each water sample included temperature, turbidity, pH, and electrical conductivity. At time of field collection, temperature, turbidity, and conductivity measurements were obtained using a field thermometer (Fisher Scientific, Pittsburgh, PA), Milwaukee/Martini-Instruments Turbidity Meter (MI 415) (Milwaukee Instruments, Inc., Rocky Mount, NC), and a Traceable® Conductivity Meter (VWR International, LLC, Radnor, PA) respectively. At the University of Arizona laboratory, pH values were obtained with a Corning pH Meter 430 (Corning Inc., Corning, NY).

3.2.3 Inorganic and Organic Methods

For inorganic (metals) analysis, a single pond sample was produced from each location on sampling dates by compositing the respective sub-samples and filtering 15 mL into a Metal Free Centrifuge Tube (VWR® International, LLC, Radnor, PA) via 0.45 µm Nylon Syringe Filters (VWR® International, LLC, Radnor, PA). The analyses for metals listed in Table 3 were performed by the Arizona Laboratory for Emerging Contaminants (ALEC) at the University of

Arizona, Tucson, AZ using inductively-coupled plasma mass spectrometry (ICP-MS; Elan DRC II, Perkin Elmer).

Table 3: Metal isotopes analyzed by ALEC using ICP-MS.

Metal	Abbreviation	Atomic Mass
Beryllium	Be	9
Aluminum	Al	27
Vanadium	V	51
Chromium	Cr	52
Manganese	Mn	55
Iron	Fe	56
Cobalt	Co	59
Nickel	Ni	60
Copper	Cu	63
Zinc	Zn	66
Arsenic	As	75
Selenium	Se	78
Molybdenum	Mo	95
Silver	Ag	107
Cadmium	Cd	111
Tin	Sn	118
Antimony	Sb	121
Barium	Ba	137
Lead	Pb	208

For organic analysis (MC-LR), 50 mL aliquots of each water sample were subjected to three freeze/thaw cycles of -80 °C and 37 °C to lyse cells and release total MC-LR into solution. Water samples were then filtered into sterile 50 mL conical tubes using 1.5 µm Nylon Syringe Filters (Thermo-Scientific™, Rockwood, TN). The analysis for MC-LR was performed at ALEC using liquid chromatography-tandem mass spectrometry (LC-MSMS; Dionex Ultimate

3000, Dionex-Thermo). A stock solution of MC-LR (dilution series spanning 374 ppb to 3 ppt) was used as a positive control (Sigma-Aldrich Co., LLC, St. Louis, MO).

3.2.4 DNA Extraction and Purification

Bacteria were isolated by filtering each pond sample onto Whatman® 0.45 µm pore size membrane filters via a Millipore vacuum filtration system. Due to varying turbidity between individual samples, which could range from 0.5 Nephelometric Turbidity Units (NTU) to 890 NTU on any given day, between 75 mL and 500 mL of each water sample was filtered for downstream DNA extraction. Filters were stored in 15 mL conical vials at 4 ºC until extraction. Total DNA extraction from the filters was performed using a MOBio Laboratories, Inc. PowerBiofilm® DNA Isolation Kit (MOBio Laboratories, Inc., Carlsbad, CA) according to manufacturer instructions. DNA extracts were stored (-80 ºC) until further processing.

3.2.5 Molecular Methods

To quantify cyanobacteria and toxic *Microcystis spp*., all DNA extracts were analyzed for cyanobacterial (*CYAN*) and toxin synthetase (*mcyD*) gene markers using quantitative polymerase chain reaction (qPCR). Quantitative standards (ranging from 300,000 to 3 markers per µL) for qPCR were created from PCR products of the target size (*CYAN* and *mcyD* gene markers were 267 bp and 297 bp respectively) (Kaebernick et al., 2000).

For each water sample, two qPCR reactions were carried out to detect *CYAN* and *mcyD* gene markers using primers and probes listed in Table 4. All qPCR reactions were performed using an AB Applied Biosystems Step One™ Thermocycler (Life Technologies, Grand Island,

NY) using MicroAmp™ Fast Optical 48-Well Reaction Plates (0.1 mL). Each 25 µL qPCR reaction was comprised of 8.5 µL Nuclease Free Water, 12.5 µL BIO-RAD SsoAdvanced™ Universal Probes Supermix, 400 nM Forward Primer (Sigma-Aldrich Co., LLC, St. Louis, MO) (Table 4), 400 nM Reverse Primer (Sigma-Aldrich Co., LLC, St. Louis, MO) (Table 4), 0.1 mM probe (Bio-Rad Laboratories, Inc., Hercules, CA) (Table 4), and 1 µL of extracted DNA.

Corresponding emission of fluorescence from a standard curve of serial log dilutions (300,000 to 3 markers per µL) allowed for quantification of unknown samples.

Table 4: Primer and probe sequences used for qPCR.

Primer	Sequence (5'- 3')	Reference
CYAN 108F	ACGGGTGAGTAACRCGTRA	Urbach et al. (1992)
CYAN 377R	CCATGGGCGGAAAATTCCCC	Nűbel et al. (1997)
mcyDF	GGTTCGCCTGGTCAAAGTAA	Kaebernick et al. (2000)
mcyDR	CCTCGCTAAAGAAGGGTTGA	Kaebernick et al. (2000)
CYAN – Probe	(6FAM)CTCAGTCCCAGTGTGGCTGNTC(TAM)	Rinta-Kanto et al. (2005)
mcyD - Probe	(6FAM)ATGCTCTAATGCAGCAACGGCAAA(TAM)	Rinta-Kanto et al. (2005)

3.2.6 Data Analysis

Correlations and 2-Sample T-tests of statistical significance ($p \leq 0.05$) for molecular markers, MC-LR concentrations, physical and chemical water quality parameters, and metal concentrations were calculated by Minitab Release 14.20 Statistical Software (Minitab® Statistical Software, State College, PA).

3.3 Results

3.3.1 Molecular (*CYAN* and *mcyD*)

Molecular markers (*CYAN* and *mcyD*) were quantified over six months (Figures 10 & 11 respectively). Over the course of sampling, *CYAN* markers were slightly higher in the groundwater pond (MAC) than the recycled water pond (SW), averaging 1.06 x 10^{12} and 5.59 x 10^{11} markers 100 mL^{-1} respectively, but this difference was not significant (p = 0.158). *CYAN* markers at SW displayed an increasing trend between April and July compared to MAC, with a noted decrease occurring in September for both ponds (Figure 10).

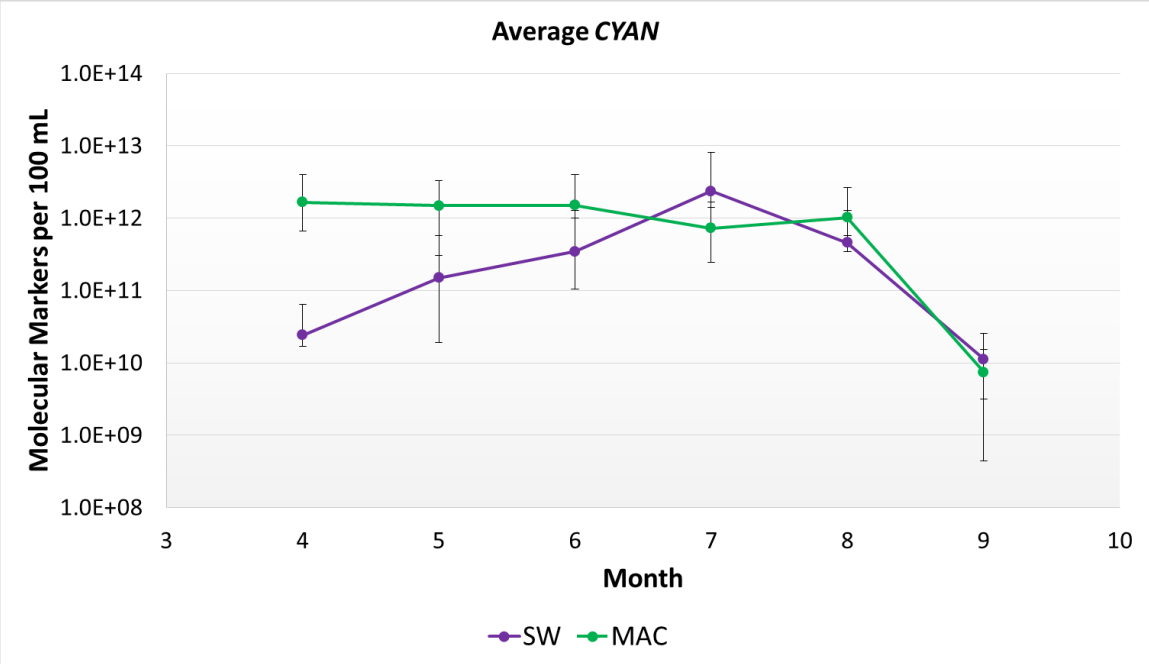

Figure 10: Average *CYAN* molecular markers per 100 mL measured in pond samples over 6 months. Error bars represent standard deviation of quadruplet measurements.

Over the course of sampling, *mcyD* markers were always higher in MAC than in SW, averaging 2.44 x 10^7 and 1.66 x 10^3 100 mL^{-1} respectively over six months (t = -6.79; $p \leq 0.001$) (Figure 11). A spike in *mcyD* markers was observed during July and August at MAC, whereas *mcyD* markers were below detection limits in June and July at SW (Figure 11)

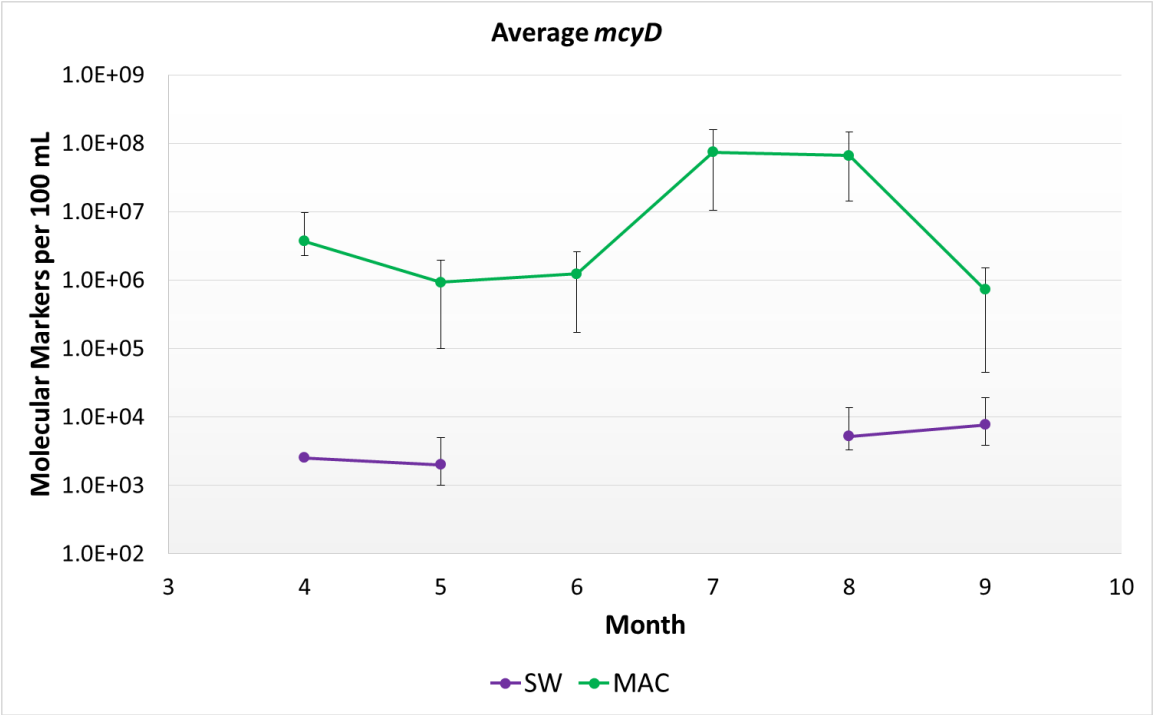

Figure 11: Average *mcyD* molecular markers per 100 mL measured in pond samples over 6 months. Error bars represent standard deviation of quadruplet measurements.

3.3.2 MC-LR

Though all 48 samples were analyzed for MC-LR, the toxin was detected only in waters collected from MAC during July and August, averaging 6.762 ± 0.021 and 6.643 ± 0.022 µg L^{-1} respectively (Table 5). Although the highest water temperatures in MAC occurred during July

and August, MC-LR concentrations were negatively correlated with temperature (r = -0.795; p = 0.018).

Table 5: Concentrations for MC-LR detected in MAC samples. Parentheses for average concentrations represent standard deviation. MC-LR levels in the remaining 40 water samples collected over six months were below limits of detection.

Sample	MC-LR [µg L^{-1}]
MAC 4-1	6.794
MAC 4-2	6.752
MAC 4-3	6.749
MAC 4-4	6.753
MAC 5-1	6.631
MAC 5-2	6.642
MAC 5-3	6.673
MAC 5-4	6.624
MAC 4 AVG	**6.762 (0.021)**
MAC 5 AVG	**6.643 (0.022)**

3.3.3 Environmental Parameters

Statistical analysis performed between each parameter listed in Table 6 with *CYAN* and *mcyD* molecular markers (Figures 9 and 10) revealed significant positive correlations between SW *CYAN* markers and metals: V_{51} (r = 0.901; p = 0.014), As_{75} (r = 0.882; p = 0.020), and Sb_{121} (r = 0.921; p = 0.009). *CYAN* markers in MAC revealed significant negative correlations with Zn_{66} (r = -0.868; p = 0.025), and Ba_{137} (r = -0.861; p = 0.028), while MAC *mcyD* markers revealed a significant negative correlation with Cr_{52} (r = -0.908; p = 0.012).

Statistical analysis performed on inorganic parameters (metals) listed in Table 6 between each pond revealed higher concentrations of: V_{51} in MAC (t = -5.52; p = 0.003), Mn_{55} in SW (t = 2.64; p = 0.046), Fe_{56} in SW (t = 6.89; p = 0.001), As_{75} in MAC (t = -6.81; p = 0.001), and Se_{78} in MAC (t = -4.07; p = 0.010) than SW.

Table 6: Metal isotopes measured in SW and MAC. Values represent average of six monthly samples (standard deviation in parentheses).

Metal	SW [µg L^{-1}]	MAC [µg L^{-1}]
Be_{9}	0.02 (0.04)	0.01 (1.79)
Al_{27}	1.35 (0.71)	3.17 (1.79)
V_{51}	3.09 (2.55)	10.04 (2.43)
Cr_{52}	0.81 (0.45)	1.40 (0.57)
Mn_{55}	31.84 (28.90)	0.45 (0.39)
Fe_{56}	33.42 (10.30)	2.32 (1.92)
Co_{59}	1.21 (2.00)	0.69 (1.00)
Ni_{60}	2.15 (1.11)	1.07 (0.86)
Cu_{63}	0.37 (0.23)	0.86 (0.57)
Zn_{66}	9.16 (7.50)	3.19 (4.39)
As_{75}	4.22 (2.85)	13.56 (1.74)
Se_{78}	1.20 (0.61)	1.95 (0.49)
Mo_{95}	5.17 (2.85)	3.83 (0.66)
Ag_{107}	0.01 (0.00)	0.01 (0.00)
Cd_{111}	0.02 (0.01)	0.02 (0.01)
Sn_{118}	0.04 (0.02)	0.15 (0.30)
Sb_{121}	0.34 (0.18)	0.48 (0.29)
Ba_{137}	54.40 (27.64)	32.94 (9.98)
Pb_{208}	0.03 (0.03)	0.06 (0.07)

Statistical analysis performed between each parameter listed in Tables 7 and 8 with *CYAN* and *mcyD* molecular markers in each pond revealed a negative correlation between *CYAN* markers in MAC and conductivity (C) (r = -0.847; p = 0.033). Average temperature and turbidity values were lower in SW compared to MAC, but their differences were not significant

($p = 0.073$ and $p = 0.675$ respectively). Increased pond temperatures were observed in SW between June and August, whereas water temperatures in MAC showed a slight increase during July and August. MAC showed significantly higher pH values compared to SW ($t = 7.26$; $p \leq 0.001$), but conductivity showed no significant difference (0.113).

Table 7: Physical and chemical water parameters of SW over course of sampling. Each value represents an average of four measurements (standard deviation in parentheses). Turbidity measurement of 229.5 (440.4) at SW was largely influenced by a single collection at sampling site SW1 (890.00 NTU). This most likely due to a noticeable drop in water elevation at only site SW1 on that sampling date.

SW	T (°C)	T (NTU)	pH	C (mS)
4	21.5 (1.0)	10.2 (17.7)	7.4 (0.0)	0.94 (0.05)
5	22.3 (1.4)	4.6 (3.4)	7.4 (0.0)	1.25 (0.04)
6	29.7 (2.5)	229.5 (440.4)	7.9 (0.4)	1.23 (0.05)
7	30.3 (2.1)	10.6 (5.7)	8.4 (0.8)	1.15 (0.04)
8	28.6 (1.6)	16.7 (4.0)	7.6 (0.4)	1.13 (0.05)
9	22.4 (2.1)	21.3 (14.5)	7.5 (0.1)	1.10 (0.12)

Table 8: Physical and chemical water parameters of MAC over course of sampling. Each value represents an average of four measurements (standard deviation in parentheses).

MAC	T (°C)	T (NTU)	pH	C (mS)
4	25.5 (0.6)	40.8 (3.5)	10.2 (0.1)	1.13 (0.02)
5	28.8 (0.4)	34.2 (3.6)	9.1 (0.7)	1.07 (0.03)
6	28.2 (0.9)	35.3 (4.4)	9.8 (0.0)	1.12 (0.02)
7	29.9 (0.3)	38.0 (6.0)	9.3 (0.0)	1.13 (0.02)
8	30.7 (0.2)	23.6 (2.2)	9.3 (0.0)	1.13 (0.03)
9	23.1 (0.6)	27.4 (4.1)	9.3 (0.1)	1.78 (0.03)

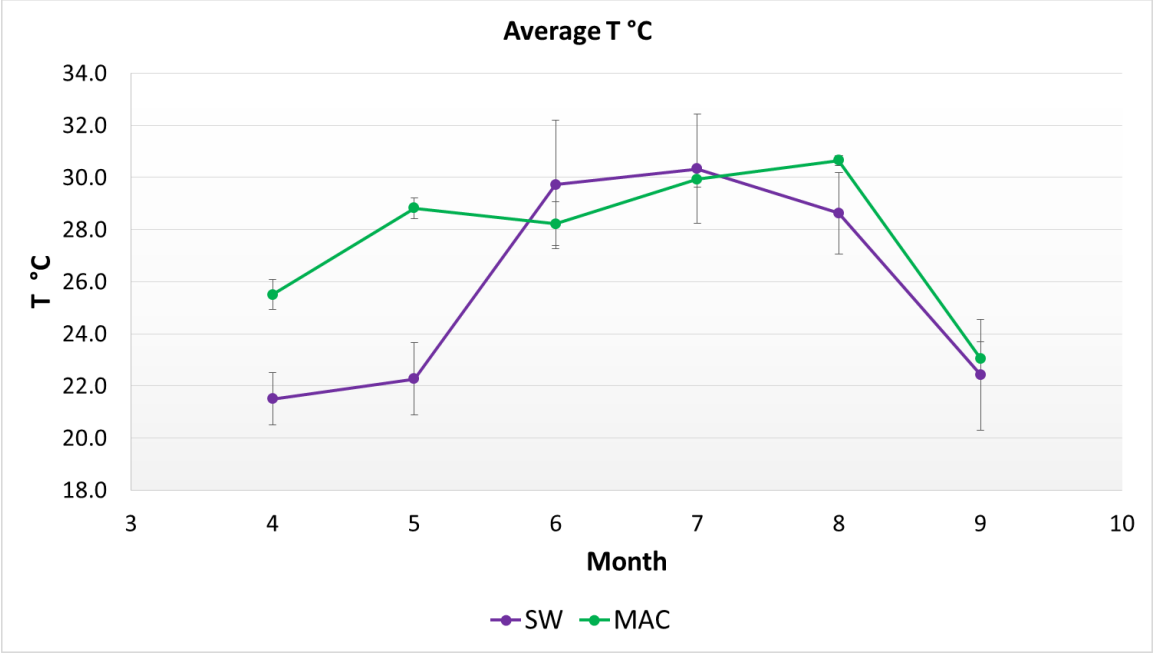

Figure 12: Average temperature measured in each pond over sampling period. Error bars represent standard deviation of quadruplet measurements.

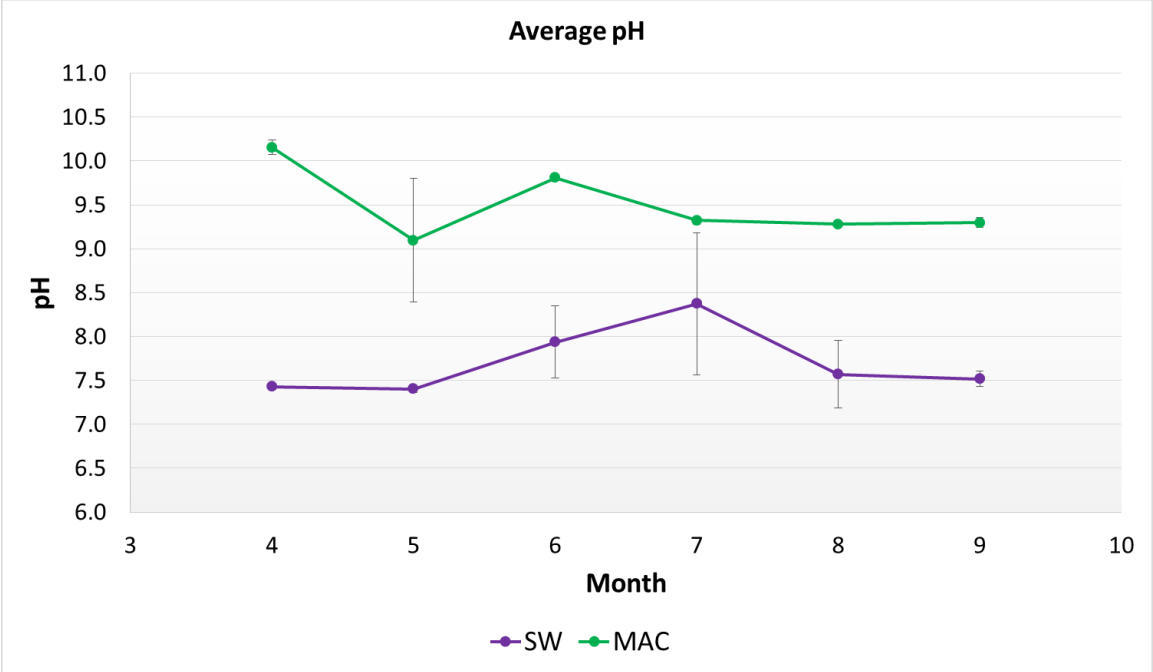

Figure 13: Average pH measured in each pond over sampling period. Error bars represent standard deviation of quadruplet measurements.

3.4 Discussion

This study examined the molecular markers specific to *CYAN* and *mcyD* genes over the course of six months in a recycled water retention pond and a groundwater-filled irrigation retention pond. Levels of *CYAN* in both ponds shared similar orders of magnitude with each other, suggesting that the difference in water source did not negatively impact cyanobacteria presence. Microcystin synthetase genes, *mcyD*, were always detected in MAC, with levels reaching three to four orders of magnitude greater than in SW. Strong seasonal trends of both molecular markers were not observed, however, levels of *CYAN* increased slightly from April to July in SW, followed by a decrease of *CYAN* levels during August in both ponds.

Pond samples were analyzed to determine if presence of *mcyD* markers correlated to MC-LR production. MC-LR production was detected only in samples collected from MAC during July and August, with concentrations six times greater than the potable water limit of 1.0 µg L^{-1} recommended by the World Health Organization (WHO). Water temperatures in MAC reached their highest average during July and August (29.9 & 30.7 °C respectively), correlating significantly with MC-LR concentrations (r = -0.795; p = 0.018). This finding supports previous studies that showed microcystin production increases between 26 and 32 °C (O'Neil et al., 2012). Although the difference in average water temperature between the two ponds was not significant, the absence of vegetation and shade at the MAC pond is the most likely reason for higher average water temperatures at MAC.

Although no significant correlation was observed between MC-LR concentrations and metals, *mcyD* levels in MAC showed a significant negative correlation with Cr_{52} that parallels a previous finding (r = -0.908; p = 0.012) (McLain & Rock, 2012). Cr_{52} is known to negatively impact cyanobacteria by inhibiting photosynthesis, however, Cr_{52} was not significantly

correlated with *CYAN* levels in either SW or MAC ($p = 0.843$, $p = 0.285$ respectively) (Wang et al., 2013). Cr_{52} was on average higher in MAC than in SW (1.40 ± 0.57 vs 0.81 ± 0.45 ppb), but this difference was not significant ($p = 0.079$). Interestingly, Cr_{52} was lowest in MAC during July and August, falling to 0.68 and 0.76 ppb. This noted decrease of Cr_{52} in MAC coincided with MC-LR synthesis, suggesting that increased concentrations (although only slightly) of Cr_{52} in MAC that occurred during all other sampling dates inhibited MC-LR synthesis.

Iron is one of Earth's most abundant metals and has been shown to affect microcystin-production and growth rate of *Microcystis aeruginosa* (Lukač & Aegerter, 1993). Fe_{56} was significantly lower ($p = 0.001$) in MAC when compared to SW, but it was not significantly correlated with *mcyD* levels in MAC ($p = 0.780$) or SW ($p = 0.646$). Despite the insignificant correlations, lower concentrations of Fe_{56} in MAC may have contributed to MC-LR synthesis.

CYAN markers in SW were positively correlated with V_{51}, As_{75}, and Sb_{121}, indicating increased levels of *CYAN* with higher concentrations of those metals. *CYAN* levels in MAC however, were negatively correlated with Zn_{66} and Ba_{137}, indicating an inverse relationship. Some metals may elicit a toxic effect, however, depending on their oxidation-state and concentration, cyanobacteria may accumulate, detoxify, or metabolize metals (Baptista & Vasconcelos, 2006).

Vanadium-containing nitrogenases are enzymes present in cyanobacteria necessary for nitrogen fixation, whereas arsenic and antimony have been associated with photosynthesis and growth rates (Rehder, D., 2015). Studies have shown high levels (10 mg L^{-1}) of As(III) to inhibit growth and photostem II (PSII) activity of *M. aeruginosa*, however, low concentrations (such as those detected in both ponds) of As(III) have no inhibitory effect (Wang & Zhang, 2012). Similar to As(III), Sb(V) has also been shown to have adverse effects on growth, pigment

content, and PSII, however, low concentrations (≤ 5 mg L^{-1}) of Sb(V) can stimulate *M. aeruginosa* growth (Wang & Pan, 2012). Zinc is known as essential to cyanobacteria which supports the correlation with *CYAN* levels in MAC (Baptista & Vasconcelos, 2006). SW contained slightly higher concentrations of Zn_{66}, however, the difference in Zn_{66} between ponds was not significant ($p = 0.131$).

Average water temperatures in SW were cooler than in MAC, but this difference was not significant ($p = 0.073$). Cooler water temperatures at SW could be attributed to wetlands' ability to moderate temperature extremes (Zedler & Kercher, 2005). Higher average turbidity values at MAC may have fostered a positive feedback mechanism in which higher turbidity absorbs more sunlight and increases water temperature. Turbidity, as well as pH, is positively associated with algal growth, and higher average turbidity and pH in MAC suggests that algal concentrations may be greater than in SW. However, *CYAN* levels were similar in each pond.

Quantification of chlorophyll α, a pigment necessary for photosynthesis that is found in algae and cyanobacteria could be employed in future methodologies to measure photosynthetic activity (Hambrook Berkman & Canova, 2007). Photosynthetic activity removes dissolved CO_2 and thus raises pH. Qualitatively assessing how green the pond water is can be subjective, and will not provide the same level of confidence as chlorophyll α measurements. Quantitating chlorophyll α provides unbiased measurements that can be used for statistical analysis that lend support to observations such as significantly higher pH values in MAC.

3.5 Conclusions

The results of this study revealed significant differences in temperature, pH, select metals (including Fe_{56}), *mcyD* markers, and MC-LR biosynthesis between SW and MAC. The negative correlation between *mcyD* markers in MAC with Cr_{52} has been paralleled in a previous study, suggesting Cr_{52} may play a role in microcystin-production. However, microcystin-production is complex and may not be controlled by a single environmental variable, but most likely the simultaneous occurrence of many. It has been shown in previous studies that iron limitation may be a strong contributing factor for *mcyD* presence and MC-LR biosynthesis. Despite the insignificant correlation ($p > 0.05$) between *mcyD* levels or MC-LR toxin concentrations with Fe_{56}, lower levels of Fe_{56} in MAC may have contributed to microcystin-production. The differences in water quality parameters between SW and MAC did not contribute to differences in *CYAN* levels, however, the differences may have inhibited microcystin-production in SW.

Forecasting toxigenicity during algal blooms has been challenging for water professionals, and thus measures to prevent HABs have focused on reducing nutrient loading (N & P) which favors algal growth. Past studies have concentrated on the roles of N and P in relation to microcystin-production, however, the results of this study suggest metals (including but not limited to Cr_{52} and Fe_{56}) may play critical contributory roles. Recycled water ponds provide a unique opportunity for increased understanding of toxin-producing cyanobacteria. Culture-based studies are rarely reproducible in the field, however, the differences in water quality parameters and environmental characteristics of a recycled water pond and a groundwater-sourced pond could facilitate the development of predictive models to forecast toxic algal blooms.

APPENDIX A

Table 1a: Sampling dates and environmental parameters measured over course of sampling at SW and MAC.

Sample	Date	Time Collected	Temperature (˚C)	Turbidity (NTU)	Conductivity (mS)	pH
SW 1-1	4/25/2017	8:19 AM	20	36.69	0.96	7.39
SW 1-2	4/25/2017	8:26 AM	22	0.74	0.966	7.46
SW 1-3	4/25/2017	8:30 AM	22	0.5	0.87	7.43
SW 1-4	4/25/2017	8:33 AM	22	2.83	0.971	7.43
MAC 1-1	4/25/2017	11:40 AM	25	37.77	1.113	10.25
MAC 1-2	4/25/2017	11:44 AM	26	40.93	1.126	10.12
MAC 1-3	4/25/2017	11:51 AM	25	45.55	1.133	10.06
MAC 1-4	4/25/2017	11:56 AM	26	38.63	1.151	10.19
SW 2-1	5/24/2017	7:50 AM	20.2	9.58	1.196	7.37
SW 2-2	5/24/2017	8:05 AM	23.1	3.23	1.271	7.42
SW 2-3	5/24/2017	8:15 AM	22.8	3.26	1.279	7.37
SW 2-4	5/24/2017	8:25 AM	23	2.2	1.268	7.46
MAC 2-1	5/24/2017	10:25 AM	28.5	34.6	1.038	8.04
MAC 2-2	5/24/2017	10:30 AM	29	32.3	1.109	9.52
MAC 2-3	5/24/2017	10:40 AM	29.3	30.93	1.047	9.41
MAC 2-4	5/24/2017	10:50 AM	28.5	39.1	1.071	9.41
SW 3-1	6/20/2017	10:32 AM	26.1	890	1.156	7.35
SW 3-2	6/20/2017	10:45 AM	30.3	13.06	1.244	7.96
SW 3-3	6/20/2017	10:52 AM	31	3.63	1.245	8.18
SW 3-4	6/20/2017	11:00 AM	31.5	11.13	1.258	8.26
MAC 3-1	6/20/2017	8:20 AM	27.6	40.05	1.135	9.85
MAC 3-2	6/20/2017	8:23 AM	27.4	34.44	1.123	9.82
MAC 3-3	6/20/2017	8:35 AM	29.1	37.07	1.088	9.77
MAC 3-4	6/20/2017	8:40 AM	28.8	29.73	1.12	9.79
SW 4-1	7/25/2017	10:30 AM	27.2	3.77	1.2	7.21
SW 4-2	7/25/2017	10:42 AM	31.3	17.42	1.141	8.63
SW 4-3	7/25/2017	10:48 AM	31.1	12.21	1.146	8.56
SW 4-4	7/25/2017	10:55 AM	31.7	9.02	1.101	9.09
MAC 4-1	7/25/2017	8:16 AM	29.9	46.46	1.111	9.38
MAC 4-2	7/25/2017	8:18 AM	29.6	35.47	1.14	9.32
MAC 4-3	7/25/2017	8:36 AM	30.3	32.74	1.128	9.31
MAC 4-4	7/25/2017	8:40 AM	29.9	37.19	1.147	9.28

SW 5-1	8/29/2017	11:20 AM	26.3	19.39	1.204	7.11
SW 5-2	8/29/2017	11:32 AM	29.2	11.5	1.111	7.47
SW 5-3	8/29/2017	11:40 AM	29.6	20.36	1.11	8.04
SW 5-4	8/29/2017	11:50 AM	29.4	15.71	1.097	7.66
MAC 5-1	8/29/2017	9:02 AM	30.5	25.92	1.114	9.29
MAC 5-2	8/29/2017	9:06 AM	30.5	22.18	1.157	9.28
MAC 5-3	8/29/2017	9:20 AM	30.9	25.1	1.11	9.26
MAC 5-4	8/29/2017	9:25 AM	30.7	21.29	1.156	9.29
SW 6-1	9/26/2017	11:30 AM	19.5	8.04	1.167	7.61
SW 6-2	9/26/2017	11:39 AM	22.8	11	1.174	7.51
SW 6-3	9/26/2017	11:50 AM	22.8	27.04	0.93	7.54
SW 6-4	9/26/2017	11:55 AM	24.6	38.99	1.136	7.4
MAC 6-1	9/26/2017	9:03 AM	22.6	30.41	1.766	9.26
MAC 6-2	9/26/2017	9:06 AM	22.4	31.07	1.775	9.34
MAC 6-3	9/26/2017	9:20 AM	23.5	26.03	1.758	9.35
MAC 6-4	9/26/2017	9:24 AM	23.7	22.2	1.828	9.24

Table 2a: Volume filtered for each pond sample, DNA extraction date, and DNA concentration measurements.

Sample	**Volume of Sample Filtered (mL)**	**DNA Extraction Date**	**DNA Concentration Avg**	**DNA 260/280 Avg**
SW 1-1	350	4/27/2017	51.5	1.83
SW 1-2	500	4/27/2017	19.1	1.68
SW 1-3	500	4/27/2017	18.8	1.71
SW 1-4	500	4/27/2017	25.6	1.75
SW 2-1	350	4/27/2017	43.7	1.82
SW 2-2	500	4/27/2017	51.6	1.86
SW 2-3	500	4/27/2017	83.7	1.85
SW 2-4	500	4/27/2017	47.9	1.82
SW 3-1	75	5/25/2017	52.9	1.83
SW 3-2	450	5/25/2017	39.7	1.80
SW 3-3	450	5/25/2017	45.5	1.81
SW 3-4	450	5/25/2017	46.2	1.84

SW 4-1	400	5/25/2017	76.9	1.83
SW 4-2	350	5/25/2017	97.3	1.84
SW 4-3	250	5/25/2017	52.6	1.81
SW 4-4	350	5/25/2017	43.8	1.84
SW 5-1	500	6/21/2017	35.1	1.81
SW 5-2	450	6/21/2017	35.2	1.81
SW 5-3	350	6/21/2017	40.5	1.81
SW 5-4	500	6/21/2017	44.0	1.84
SW 6-1	500	6/21/2017	107.7	1.82
SW 6-2	400	6/21/2017	105.0	1.82
SW 6-3	300	6/21/2017	108.6	1.84
SW 6-4	250	6/21/2017	138.2	1.85
MAC 1-1	100	7/27/2017	79.6	1.84
MAC 1-2	100	7/27/2017	79.3	1.84
MAC 1-3	100	7/27/2017	80.4	1.87
MAC 1-4	100	7/27/2017	53.5	1.86
MAC 2-1	150	7/27/2017	99.8	1.84
MAC 2-2	150	7/27/2017	85.2	1.85
MAC 2-3	100	7/27/2017	74.2	1.83
MAC 2-4	100	7/27/2017	75.5	1.86
MAC 3-1	150	8/31/2017	53.5	1.79
MAC 3-2	150	8/31/2017	79.1	1.83
MAC 3-3	150	8/31/2017	91.9	1.85
MAC 3-4	150	8/31/2017	134.4	1.84
MAC 4-1	200	8/31/2017	45.1	1.82
MAC 4-2	200	8/31/2017	57.3	1.81
MAC 4-3	200	8/31/2017	78.8	1.83
MAC 4-4	200	8/31/2017	50.7	1.82
MAC 5-1	200	10/2/2017	65.7	1.79
MAC 5-2	200	10/2/2017	48.1	1.81
MAC 5-3	200	10/2/2017	58.6	1.79
MAC 5-4	200	10/2/2017	45.7	1.73
MAC 6-1	500	10/2/2017	182.1	1.85
MAC 6-2	450	10/2/2017	108.5	1.84
MAC 6-3	450	10/2/2017	166.1	1.84
MAC 6-4	450	10/2/2017	124.5	1.84

Table 3a: *CYAN* markers quantified over course of sampling at SW and MAC.

Sample	**Dilution Factor**	***CYAN* Quantity Avg**	***CYAN* Markers/100mL**
SW 1-1	1.0E+04	1.6862E+04	4.8176E+10
SW 1-2	1.0E+04	1.1566E+04	2.3131E+10
SW 1-3	1.0E+04	6.6259E+03	1.3252E+10
SW 1-4	1.0E+04	5.7993E+03	1.1599E+10
MAC 1-1	1.0E+04	1.0319E+05	1.0319E+12
MAC 1-2	1.0E+04	1.2746E+05	1.2746E+12
MAC 1-3	1.0E+04	2.5514E+05	2.5514E+12
MAC 1-4	1.0E+04	1.7579E+05	1.7579E+12
SW 2-1	1.0E+04	1.7903E+05	5.1152E+11
SW 2-2	1.0E+04	1.3948E+04	2.7896E+10
SW 2-3	1.0E+04	1.2743E+04	2.5486E+10
SW 2-4	1.0E+04	1.6397E+04	3.2794E+10
MAC 2-1	1.0E+04	2.6216E+05	1.7477E+12
MAC 2-2	1.0E+04	1.5587E+05	1.0391E+12
MAC 2-3	1.0E+04	1.5537E+05	1.5537E+12
MAC 2-4	1.0E+04	1.5739E+05	1.5739E+12
SW 3-1	1.0E+04	9.1534E+04	1.2205E+12
SW 3-2	1.0E+04	1.7220E+04	3.8268E+10
SW 3-3	1.0E+04	3.9852E+04	8.8561E+10
SW 3-4	1.0E+04	1.3545E+04	3.0099E+10
MAC 3-1	1.0E+04	3.1638E+05	2.1092E+12
MAC 3-2	1.0E+04	2.7442E+05	1.8294E+12
MAC 3-3	1.0E+04	3.0640E+05	2.0427E+12
MAC 3-4	1.0E+04	3.1456E+03	2.0970E+10
SW 4-1	1.0E+06	2.9254E+04	7.3136E+12
SW 4-2	1.0E+06	1.5772E+03	4.5062E+11
SW 4-3	1.0E+06	3.2574E+03	1.3030E+12
SW 4-4	1.0E+06	1.3877E+03	3.9648E+11
MAC 4-1	1.0E+06	1.6086E+03	8.0432E+11
MAC 4-2	1.0E+06	1.8193E+03	9.0967E+11
MAC 4-3	1.0E+06	7.3360E+02	3.6680E+11
MAC 4-4	1.0E+06	1.6147E+03	8.0737E+11
SW 5-1	1.0E+06	7.4415E+02	1.4883E+11
SW 5-2	1.0E+06	1.2256E+03	2.7235E+11

SW 5-3	1.0E+06	1.6833E+03	4.8093E+11
SW 5-4	1.0E+06	4.6803E+03	9.3605E+11
MAC 5-1	1.0E+06	1.6303E+03	8.1517E+11
MAC 5-2	1.0E+06	9.2701E+02	4.6351E+11
MAC 5-3	1.0E+06	1.9803E+03	9.9017E+11
MAC 5-4	1.0E+06	3.6460E+03	1.8230E+12
SW 6-1	1.0E+06	3.8962E+01	7.7923E+09
SW 6-2	1.0E+06	3.9256E+01	9.8140E+09
SW 6-3	1.0E+06	3.6768E+01	1.2256E+10
SW 6-4	1.0E+06	3.7784E+01	1.5113E+10
MAC 6-1	1.0E+06	3.3948E+01	6.7896E+09
MAC 6-2	1.0E+06	3.5025E+01	7.7833E+09
MAC 6-3	1.0E+06	3.3915E+01	7.5367E+09
MAC 6-4	1.0E+06	3.4387E+01	7.6415E+09

Table 4a: *mcyD* markers quantified over course of sampling at SW and MAC. Samples in which *mcyD* genes fell below detection limits (3 markers per µL) noted as undetected (und).

Sample	**Dilution Factor**	***mcyD* Quantity Avg**	***mcyD* Markers/100mL**
SW 1-1	1.0E+00	und	und
SW 1-2	1.0E+00	1.4223E+01	2.8445E+03
SW 1-3	1.0E+00	7.1433E+00	1.4287E+03
SW 1-4	1.0E+00	1.6303E+01	3.2607E+03
MAC 1-1	1.0E+00	2.8734E+03	2.8734E+06
MAC 1-2	1.0E+00	2.4714E+03	2.4714E+06
MAC 1-3	1.0E+00	7.0659E+03	7.0659E+06
MAC 1-4	1.0E+00	2.3976E+03	2.3976E+06
SW 2-1	1.0E+00	und	und
SW 2-2	1.0E+00	und	und
SW 2-3	1.0E+00	9.9920E+00	1.9984E+03
SW 2-4	1.0E+00	und	und
MAC 2-1	1.0E+00	1.6090E+03	1.0727E+06
MAC 2-2	1.0E+00	1.2716E+03	8.4776E+05

MAC 2-3	1.0E+00	8.8648E+02	8.8648E+05
MAC 2-4	1.0E+00	8.9083E+02	8.9083E+05
SW 3-1	1.0E+00	und	und
SW 3-2	1.0E+00	und	und
SW 3-3	1.0E+00	und	und
SW 3-4	1.0E+00	und	und
MAC 3-1	1.0E+00	2.0096E+03	1.3397E+06
MAC 3-2	1.0E+00	2.0124E+03	1.3416E+06
MAC 3-3	1.0E+00	1.4669E+03	9.7790E+05
MAC 3-4	1.0E+00	1.8441E+03	1.2294E+06
SW 4-1	1.0E+00	und	und
SW 4-2	1.0E+00	und	und
SW 4-3	1.0E+00	und	und
SW 4-4	1.0E+00	und	und
MAC 4-1	1.0E+00	1.7122E+05	8.5612E+07
MAC 4-2	1.0E+00	1.5461E+05	7.7305E+07
MAC 4-3	1.0E+00	1.2166E+05	6.0832E+07
MAC 4-4	1.0E+00	1.4257E+05	7.1286E+07
SW 5-1	1.0E+00	1.7870E+01	3.5739E+03
SW 5-2	1.0E+00	3.0451E+01	6.7669E+03
SW 5-3	1.0E+00	und	und
SW 5-4	1.0E+00	und	und
MAC 5-1	1.0E+04	1.1301E+01	5.6505E+07
MAC 5-2	1.0E+04	1.7486E+01	8.7428E+07
MAC 5-3	1.0E+04	1.2604E+01	6.3021E+07
MAC 5-4	1.0E+04	1.1620E+01	5.8102E+07
SW 6-1	1.0E+00	3.8414E+01	7.6828E+03
SW 6-2	1.0E+00	und	und
SW 6-3	1.0E+00	und	und
SW 6-4	1.0E+00	und	und
MAC 6-1	1.0E+02	3.3078E+01	6.6155E+05
MAC 6-2	1.0E+02	3.4350E+01	7.6333E+05
MAC 6-3	1.0E+02	3.3196E+01	7.3770E+05
MAC 6-4	1.0E+02	3.3497E+01	7.4438E+05

Table 5a: SW metal isotopes measured by ALEC.

Metal	Apr	May	Jun	Jul	Aug	Sep
Be_{9}	0.10	nd	nd	nd	nd	nd
Al_{27}	nd	1.48	2.13	1.49	1.42	1.60
V_{51}	0.79	2.47	2.44	8.05	1.77	3.00
Cr_{52}	0.003	0.79	1.26	0.78	1.18	0.88
Mn_{55}	0.14	0.85	38.31	34.96	77.76	39.00
Fe_{56}	49.7	21.23	35.07	29.26	39.53	25.73
Co_{59}	5.29	0.25	0.38	0.50	0.49	0.33
Ni_{60}	0.01	2.76	2.50	2.28	3.20	2.14
Cu_{63}	0.01	0.67	0.44	0.53	0.33	0.23
Zn_{66}	0.09	19.97	3.10	15.59	8.96	7.23
As_{75}	0.19	4.08	4.31	9.12	3.88	3.75
Se_{78}	0.86	1.02	2.38	1.00	0.69	1.26
Mo_{95}	nd	5.61	8.30	7.12	5.23	4.76
Ag_{107}	0.01	0.002	0.01	0.01	0.01	0.00
Cd_{111}	0.004	0.03	0.02	0.03	0.01	0.01
Sn_{118}	nd	0.05	0.04	0.06	0.03	0.04
Sb_{121}	0.31	0.34	0.32	0.68	0.24	0.16
Ba_{137}	0.09	52.21	75.22	68.19	66.26	64.44
Pb_{208}	nd	0.07	nd	0.04	nd	0.04

Table 6a: MAC metal isotopes measured by ALEC.

Metal	Apr	May	Jun	Jul	Aug	Sep
Be_{9}	0.05	nd	nd	nd	nd	nd
Al_{27}	1.56	4.23	2.85	3.70	5.77	0.88
V_{51}	5.49	11.60	12.51	9.94	10.24	10.45
Cr_{52}	2.13	1.73	1.69	0.68	0.76	1.42
Mn_{55}	1.11	0.48	0.39	0.02	0.10	0.61
Fe_{56}	1.05	0.77	4.36	3.76	0.01	4.00
Co_{59}	2.72	0.24	0.21	0.29	0.20	0.46
Ni_{60}	2.56	0.42	0.44	0.54	0.86	1.63
Cu_{63}	2.02	0.64	0.51	0.67	0.57	0.74

Zn_{66}	1.33	0.98	0.85	1.68	2.19	12.10
As_{75}	14.66	15.22	12.65	14.22	14.18	10.46
Se_{78}	2.39	1.97	2.64	1.48	1.41	1.79
Mo_{95}	4.36	3.21	3.76	2.90	4.24	4.51
Ag_{107}	0.01	0.002	0.01	0.01	nd	0.01
Cd_{111}	0.01	0.03	0.01	0.01	0.01	0.02
Sn_{118}	0.75	0.01	0.01	0.02	nd	0.07
Sb_{121}	1.06	0.30	0.28	0.40	0.42	0.41
Ba_{137}	23.45	25.58	30.68	45.61	27.03	45.30
Pb_{208}	0.08	0.10	nd	0.03	nd	0.18

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