12-2021

An Analysis of Environmental Conditions Impacting Cyanobacterial Algal Blooms in Drinking Water Sources in Upstate South Carolina

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AN ANALYSIS OF ENVIRONMENTAL CONDITIONS IMPACTING CYANOBACTERIAL ALGAL BLOOMS IN DRINKING SOURCES IN UPSTATE SOUTH CAROLINA

A Thesis
Presented To
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Environmental Toxicology

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by
Gregory Langlois
December 2021

Accepted by:
Peter van den Hurk, Committee Chair
Cindy Lee
James Strickland

Abstract

Maintaining water quality in reservoirs used for drinking water has been an issue in recent years due to the presence of algal blooms. Algal blooms are a perennially recurring problem that can have negative impacts on tourism, recreation, and overall water quality. Additionally, algal blooms will often produce an assortment of chemicals, some of which are hazardous to the health of humans, and some of which that, while relatively innocuous, result in unpleasant tastes and odors in water. Geosmin and 2-methylisoborneol are two taste and odor compounds that are notoriously difficult to treat out of drinking water sources by traditional methods like flocculation and screening. It is important to establish the time frame that odor causing algal blooms occur, and to determine the environmental conditions that drive excessive algal growth. Four freshwater lakes in Upstate South Carolina, Lake Whelchel, Lake Bowen, Lake Greenwood, and Lake Rabon, have had problems with cyanobacterial taste and odor compound causing blooms in recent years. Subsequently the goal of this research was to 1) establish any seasonal peaks in algal growth 2) establish any relationships between total algal growth and the presence of cyanobacteria 3) determine what, if any, environmental conditions influenced the growth of said blooms in each lake. The fourth and final project objective was to develop an ultraperformance liquid chromatography method for determining total geosmin and

2-methylisoborneol in environmental samples. While phosphorus is typically identified as the limiting nutrient in algal bloom growth, it was determined that Nitrogen, specifically ammonium and nitrates, were the primary drivers of algal blooms in sites with substantial algal growth, providing insight into how potentially harmful blooms can be managed in these drinking water sources in the future.

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1.0 Introduction

Maintaining water quality in drinking water reservoirs has been an issue in recent years due to the presence of algal blooms. Algal blooms are a perennially recurring problem in many freshwater lakes and reservoirs, that can have negative impacts on tourism, recreation, and overall water quality. Additionally, algal blooms will often produce an assortment of chemicals, some of which are hazardous to the health of humans, and some of which that, while relatively innocuous, result in unpleasant tastes and odors in water. These taste and odor compounds are also notoriously difficult for water treatment facilities to remove from drinking water sources with traditional treatment methods, and treatment oftentimes proves to be a costly endeavor. Two compounds of growing concern in many drinking water sources are the compounds geosmin, and 2-methylisoborneol, commonly referred to as MIB. While not dangerous, these two compounds produce an earthy, musty odor that is unappealing in both drinking water and for recreational water sport activities. In addition to taste and odor compounds, algal blooms can also produce a variety of harmful toxins. Of particular interest and concern is the compound microcystin produced by the genus of algae known as *Microcystis.* The two taste and odor compounds MIB and Geosmin and the toxins known as microcystins are metabolites of various species of cyanobacteria, commonly referred to as blue-green algae.

The following research was based on measurements of parameters selected to characterize the overall algal community of each site as well as characterizing specifically the cyanobacterial community of each site. Quantification of cyanobacteria and green algae was performed by analysis for total chlorophyll a, which would provide insight into the relative quantity of overall algae present in the four sampling sites. Quantification specifically for the

presence of total cyanobacteria was performed by measuring the total quantity of cyanobacteria DNA during each sampling period. Additionally, flow cytometry was used to quantify specific groups of algae in each water sample. These factors were compared against the overall quantity of nutrients present in each sample. Additionally, factors like rainfall and streamflow were examined for each site to determine any potential difference or similarity between the lakes. Because algal blooms can occur at varying times, the main objective of the following research was to compare these parameters against one another in order to achieve a better understanding of the specific time period of the year that freshwater drinking sources will be most susceptible to algal blooms, specifically cyanobacterial blooms, to determine when water quality management strategies should be employed to mitigate the production of potentially harmful compounds like microcystin and undesirable taste and odor compounds like geosmin and 2-methylisobornol. Once the time periods of greatest risk have been determined, a method of measuring the taste and odor compounds in the water column must be established to determine if the peak concentrations in these compounds coincide with the peak of algal growth, resulting in the need to develop an ultraperformance liquid chromatography method to measure these compounds.

1.1 Cyanobacteria Morphological Overview

Cyanobacteria, commonly referred to as blue-green algae, are a diverse and robust group prokaryotes that are named for the variety of pigments they contain. Distinct from other bacteria, Cyanobacteria contain chlorophyll-a as their primary pigment, but are referred to as blue-green algae owing to the presence of the blue phycocyanin pigments, an accessory light harvesting protein complex that functions in conjunction with chlorophyll. Some Cyanobacteria

can also contain Phycoerythrin, a red pigment. Because Cyanobacteria are prokaryotes, these pigments are contained in simple thylakoids (I.C. and Welker, 2021). Cyanobacteria come in a broad range of shapes and sizes, including spherical, ellipsoid, barrel shaped, cylindrical, conical, or disc-shaped (Chorus and Welker, 2021). Despite the numerous shapes that Cyanobacteria can take however, there are no known species of Cyanobacteria that have flagella, meaning that they lack mobility, and subsequently rely on the ability to change buoyancy using gas vesicles to adjust position in the water column (Walsby et al 1995).

Single cell Cyanobacteria have been identified, but most commonly cyanobacteria aggregate into multicellular forms. These aggregates can be entirely irregular clumps, or they can form neatly into sheets. One of the most common forms for these multicellular groups to take is long chains of cells that are referred to as filaments, which are then covered by a mucous sheath secreted by the cells. Aggregates of cells can appear as small clumps of just a few individual cells, or they can exist in groups of hundreds or thousands of cells that can be seen without using microscopy. With the constantly changing nature of microbiology, many identified species of cyanobacteria have been renamed, and to confuse matters further, cyanobacteria can exhibit morphological differences under different conditions (Watson et al., 2016).

1.2 The Compounds of Interest: Geosmin and 2-Methylisoborneol

Cyanobacterial blooms are notorious sources of volatile organic compounds in both freshwater and saltwater ecosystems. The two compounds of interest for this project are geosmin and 2-Methylisoborneol. These taste and odor compounds are difficult to remove from freshwater drinking sources. Normal purification processes like screening, flocculation, sedimentation, and chlorination have proven to be ineffective methods to completely remove these two compounds (Bruchet, 2004, Oh et al, 2017). While there have not been any observed and there are not believed to be any toxicological effects on humans of these compounds, suffice to say that their persistence and off-putting taste and odor is an issue for commercial, recreational, and public drinking water purposes.

Geosmin and MIB belong to the family of compounds called Terpenoids, a broad range of chemicals that can be found in almost all forms of organisms. Before cyanobacteria were identified as producers of these two compounds, the primary producer of geosmin and MIB was believed to be Actinomycetes, a phylum of bacteria typically found in soil (Gerber and Lechevalier, 1965). They can serve a variety of functions including as attractants or repellants between organisms, as protection from heat and light, and are most noted for fragrance and taste effects.

Geosmin and 2-methylisoborneol are secondary metabolites of isoprenoid pathways. There are two pathways for producing isoprenoids, the first being the mevalonate pathway. Geosmin was first proposed as a biproduct of the Mevalonate pathway by Bently and Meganathan (1981), and 2-Methylisoborneol was proposed as a byproduct of the Mevalonate

pathway by Dickschat et al (2007). The second pathway, simply referred to as the Non-Mevalonate pathway, was not thought to have the potential for the production of isoprenoids until Flesch and Rohmner proposed the Non-Mevalonate pathway as a potential route (1988). The proposed parent molecule for each compound is the same in each pathway however, each route is simply a different path to the same biproduct. Geosmin is derived from the cyclization of Farnesyl diphosphate, or FPP (Jiang et al 2007), and 2-Methylisoborneol is enzymatically synthesized from Geranyl Pyrophosphate (Giglio et al 2011).

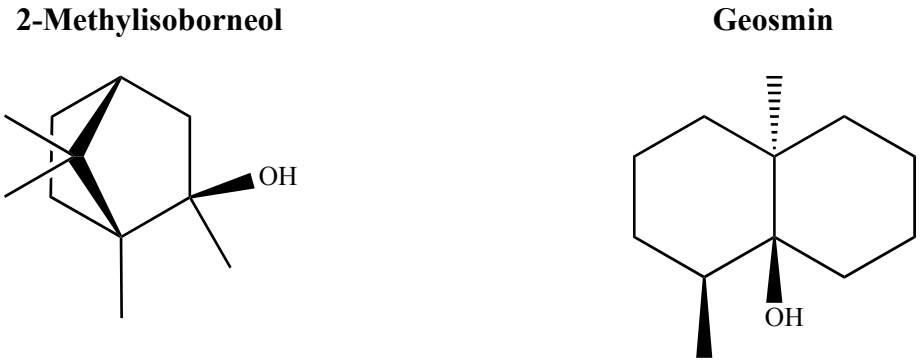


Figure 1. 2-Methylisoborneol and Geosmin, common taste and odor compounds produced by a wide variety of cyanobacteria species

1.3 Environmental Factors Influencing the Growth of Cyanobacteria

There are numerous processes that drive Eutrophication in freshwater sources. Eutrophication is the process via which excess nutrients drives the extreme growth of plants and phytoplankton which results in the decrease of oxygen levels, potentially causing the death

of animal life. Among the most important drivers of algal growth in freshwater systems is the overall nutrient composition of the water. Algal growth in freshwater lakes and reservoirs is under most circumstances limited by the relative amount of either nitrogen or phosphorus available for use. Since nitrogen is typically available in greater quantities, typically existing in a 16:1 proportion to phosphorus (Redfield, 1934), phosphorus is typically identified as the limiting nutrient in the process of eutrophication and subsequently is the target for nutrient management. Complexation with iron-based molecules like EDTA or ferric humate causes phosphorus to be less readily absorbed into phytoplankton, providing an avenue for nutrient management of algal blooms (Qui et al. 2014). These complexes then tend to settle into lake and stream sediment where they are inaccessible to free floating algae. There is however evidence that some species of cyanobacteria can accumulate, store, and recycle phosphorous, absorbing and storing excess amounts of phosphorus when quantities in water are high for use during any point in time when phosphorus levels may drop (Li and Dittrich, 2019). Nitrogen and phosphorus commonly enter water bodies via runoff from fertilizers and sewage. There are also species of Cyanobacteria capable of fixing atmospheric nitrogen, essentially providing an unlimited source of nitrogen for biological processes. Phosphorous is not readily available to cyanobacteria in the same manner that nitrogen is, which is why phosphorus is the most common target for management strategies seeking to mitigate algal blooms.

There is also growing evidence that the concentration of carbon available to cyanobacteria is a strong driver of algal bloom growth. Carbon speciation is dependent on the pH of the water that it is in, and recent studies have shown that HCO_3^- is the dominant form of available carbon in water with a pH range of 6 to 9, but also the most common form of carbon

taken up by cyanobacteria, and when available to cyanobacteria in large amounts, significantly increasing algal growth under these conditions (Liu et al 2016). This poses a large problem for the future of water quality management, since atmospheric carbon levels are increasing and subsequently more carbon is available in freshwater ecosystems.

1.4 Genetics

The gene clusters responsible for the production of geosmin, 2-methylisoborneol, as well as cyanotoxins such as microcystin and anatoxin, have been identified since the early 2000's (Cane and Watt, Gust et al), and PCR based techniques that target these specific gene sequences are now commonplace in water quality testing. The chemical precursors to the two compounds, farnesyl diphosphate for geosmin and geranyl pyrophosphate for MIB, are both acted on by monoterpene cyclase. The cluster of genes needed for the cyclization of farnesyl diphosphate was first identified by Cane and Watt in 2003. Amplification of this gene sequence and insertion into host E. Coli cells, and when incubated in a medium with farnesyl diphosphate, resulted in the cyclization of the farnesyl diphosphate into geosmin. Later studies revealed by Jiang et al in 2007 revealed that this is the result of a bifunctional enzyme that first transforms farnesyl diphosphate into germacradienol, which is then transformed into geosmin by the same enzyme, which was named geosmin synthase.

2-Methylisoborneol was speculated to the product of a two gene operon of sco7700 and sco7701 in cyanobacteria by Giglio et al in 2008. PCR was used to amplify the two presumed genetic sequences, which were then inserted into host E. Coli, and incubated with geranyl

pyrophosphate. The result was the formation of 2-methylisoborneol, confirming the research team's assumption of the two gene sequences involved in the production of the compound.

1.5 Project Objectives, Sampling Season, Site Descriptions

The overall purpose of this study was a fully characterization of algal blooms in drinking water sources in upstate South Carolina. The first goal of this project was to establish any potential seasonal peaks in algae growth for four sites. The second objective was to establish any relationship between total algal growth and the presence of cyanobacteria in the sites The third objective was to establish what, if any, environmental factors influenced the development of these algal blooms. The fourth objective of this project is to develop an ultraperformance liquid chromatography method for analyzing total geosmin and 2-methylisoborneol in environmental samples. (See appendix 1.)

The initial sampling season ran from April 25th of 2020 until September 26th of the same year. Samples were collected during the span of a single day approximately once every two weeks, for a total of twelve samples per lake over that five-month period. The sites selected were Lake Whelchel, Lake Bowen, Lake Greenwood and Lake Rabon, four drinking water sources in the Piedmont region of South Carolina. Specific sampling locations are denoted with a red dot. The site map below shows the location of each lake relative to one another. Lake Whelchel is represented by the red map marker, Lake Bowen is represented by the blue map marker, Lake Greenwood is represented by the green map marker and Lake Rabon is represented by the red map marker.

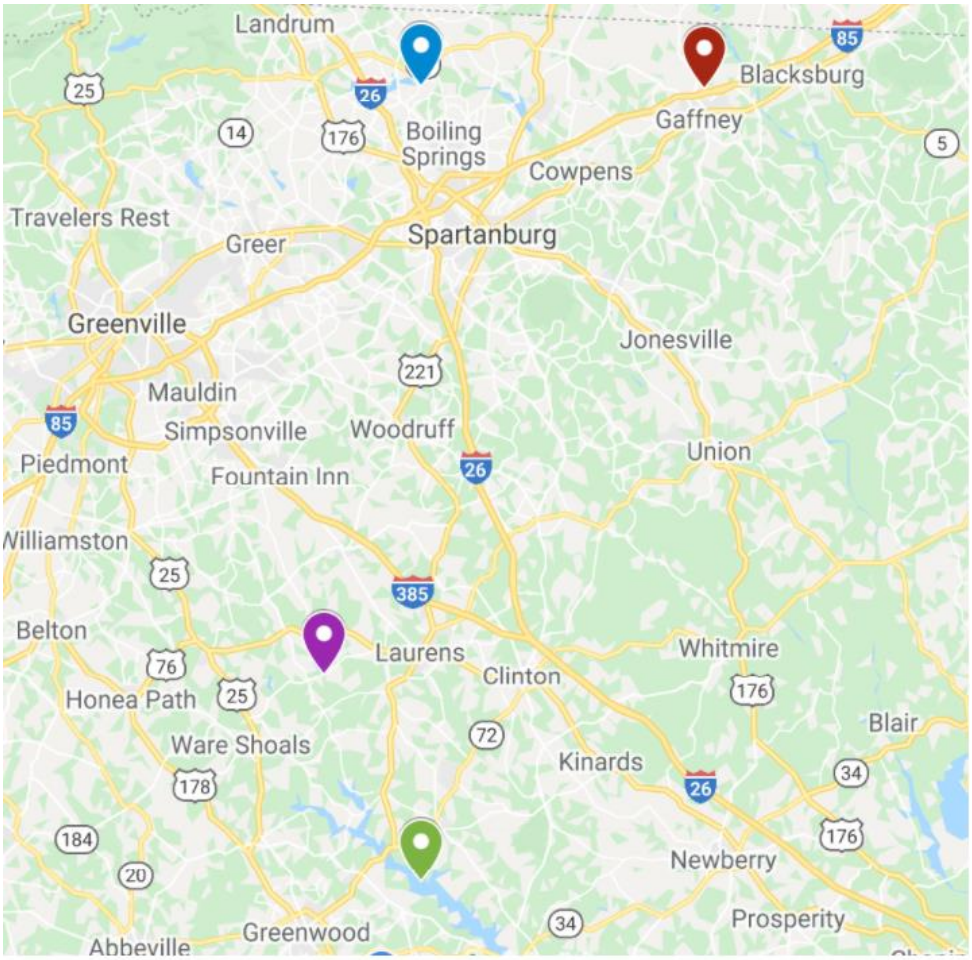


Figure 2. Map of all sites that were sampled in the Piedmont Region of South Carolina

Lake Whelchel is a 150-acre water body located near Gaffney, South Carolina. The water supply is occasionally supplemented by pumping in water from the Broad River. In addition to usage as a drinking water source for Gaffney, this lake is a popular fishing spot, and has a previous history of closure due to the presence of cyanobacteria.

Figure 3. Satellite map of Lake Whelchel, located in Gaffney, South Carolina

Lake Bowen is a 1534-acre water body located near Inman, South Carolina. This large lake is used for a variety of recreational purposes like boating and fishing, and is managed by the Spartanburg water system, providing drinking water to the residents of Spartanburg County. There are two South Carolina Department of Health and Environmental Control monitoring stations on the lake, and samples were taken from as close to one of these stations as possible. Lake Bowen has undergone sampling by Spartanburg County in coordination with the U.S. Geological Survey for the two taste and odor compounds geosmin and 2-methylisoborneol, with trace amounts of each found, most notably geosmin (Journey et Abrahamsen, 2008).

Figure 4. Satellite map of Lake Bowen, located in Inman, South Carolina

Lake Greenwood is a 11400-acre water body formed by the construction of a hydroelectric dam built across the Saluda River. A popular sportfishing and boating spot, most of the shoreline is taken up by residential homes and businesses. Water samples for this lake were taken from the immediate area surrounding the Lake Greenwood Drinking Water Treatment Plant.

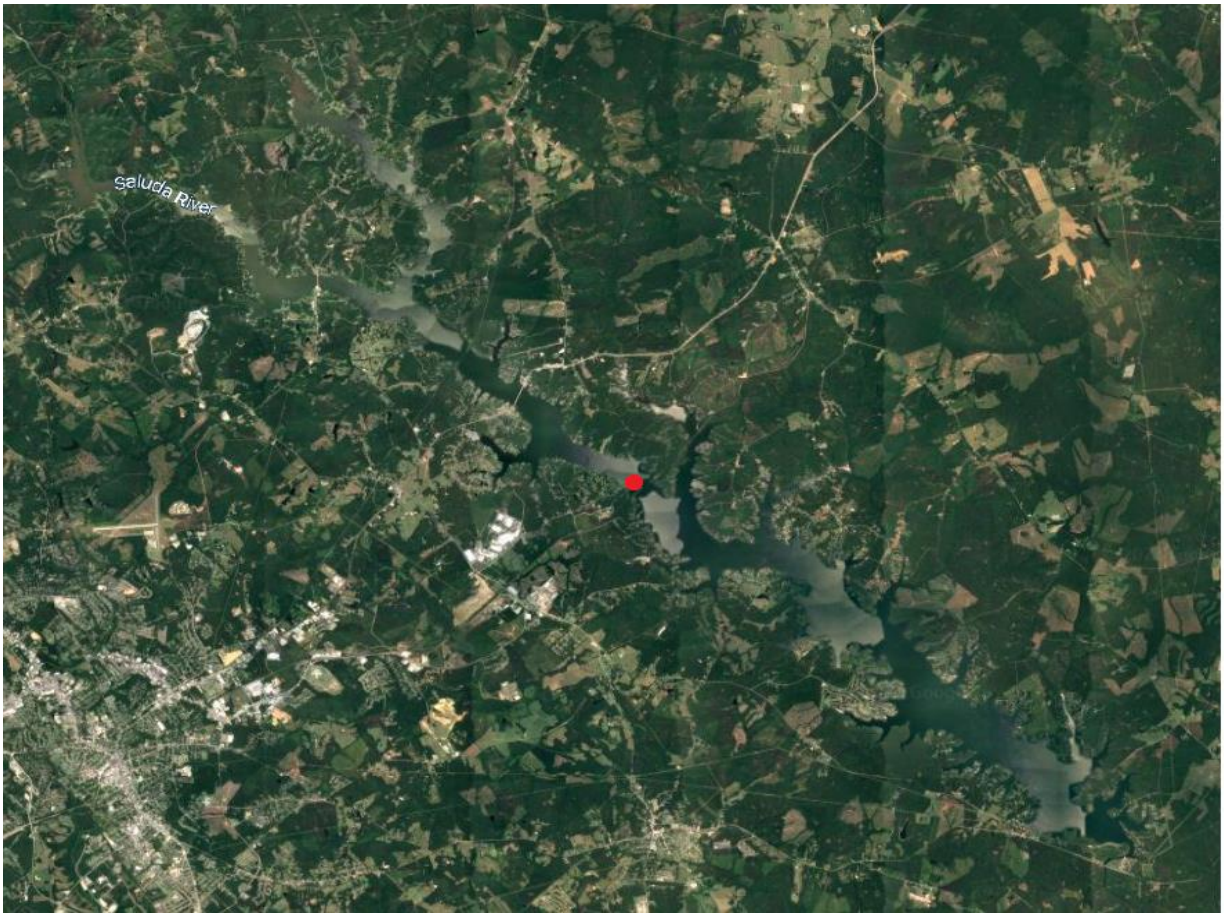


Figure 5. Satellite map of Lake Greenwood, located in Greenwood County, South Carolina

Lake Rabon is a 546-acre lake surrounded almost entirely by private homes. There is one park on the property that is accessible to the public for a fee, and a small section of shoreline commonly used for fishing. In August 2021, the South Carolina Department of Health and Environmental Control has posted warnings to the public of potential algal blooms in the lake, advising against swimming and fishing in the area. Subsequently, the popular fishing spots have been empty and traffic on the lake has slowed to a stop.

Figure 6. Satellite map of Lake Rabon, located in Laurens South Carolina

2.0 Data Collection, Sample Analysis, Results

Cyanobacteria are a ubiquitous environmental presence in freshwater lakes and reservoirs. Environmental conditions such as the presence of various nutrients as well as rainfall events have the potential to influence the growth of multiple cyanobacterial species. Additionally, peaks in the growth of cyanobacterial algal blooms are known to occur during the spring and summer. Using samples collected during the Spring and Summer of 2020, a visualization of the temporal dynamics of cyanobacterial growth as well as nutrient analysis was established for each of the four selected sites to establish a relationship between the presence of Nitrates, Ammonium, and Phosphorus in the water column and cyanobacteria bloom growth in the sites. Additionally, a relationship between the overall quantity of cyanobacteria and other algal species has been established.

2.1 Methods and Materials

Samples were taken from each site once every two weeks, the dates of which are reflected in the following graphical data, for a period of roughly five months, from mid-March to the end of August, in order coincides with the typical blooming timeframe for cyanobacteria in freshwater lakes. Samples were taken from the shoreline of each lake as close to the intake of local municipal drinking water treatment facilities as was possible, in one liter glass bottles at a depth of roughly one foot, which were then stored in an ice filled cooler and returned to campus. Specific sampling locations were taken as close to drinking water treatment facility intake points as possible. The typical sampling trip takes approximately six hours to complete, after which samples were returned to campus and processed within 24 hours of collection.

2.2 Analysis for Total Chlorophyll a

Sample water was suction filtered in 250mL aliquots onto 47mm diameter microfiber glass filters (Whatman). After filtration, each filter was individually wrapped in aluminum foil and frozen at -20°C. After storage, the filters were cut into small pieces of less than 3mm and soaked for 24 hours in 10 mL of a solution of 90% acetone (Sigma-Aldrich) and 10% deionized water in 12 mL borosilicate glass test tubes to extract the chlorophyll from the filters. Some of the 10mL of this solution was used to rinse the scissor blades used to cut the filters, as well as the aluminum foil that the filters were stored in to ensure that all chlorophyll was included in each sample. During this soaking period each test tube was stored in the refrigerator at 4 degrees Celsius. After this 24-hour period, each test tube was centrifuged for five minutes at 1000g, and the supernatant collected and transferred to a new test tube. Each sample was analyzed using a spectrophotometer, collecting the absorbances of each sample at 750, 664, 647, and 630 nanometers (Thermo Scientific Genesys 20). Between each measurement the spectrophotometer was blanked using a solution of 90% acetone and 10% deionized water. Because chlorophyll is subject to degradation under light and heat, during analysis all samples were kept on ice and covered from light.

In addition to analysis via spectrophotometer, total chlorophyll content was analyzed using fluorescence. The extraction method for this measurement was the same as for analysis via spectrophotometer, thus only a single filter was required for each measurement. From each sample, 300 µL of supernatant from each sample were placed in all black polypropylene 96-well plates in replicates of three. These plates were then read using a BioTek Synergy H1 Hybrid

plate reader with an excitation wavelength of 436 nanometers and an emission wavelength of 680 nanometers.

A standard curve was constructed for this fluorescence measurement using 1 milligram of chlorophyl a standard dissolved into 50 milliliters of 90% acetone and 10% deionized water in a volumetric flask. This stock solution was serial diluted into 25%, 5% 1% and 0.2% solutions, with a 0% solution of only 90% acetone included as a blank.

2.3 Nutrient Analysis

After filtering samples onto glass fiber filters, the excess water was collected and transferred into conical centrifuge tubes and frozen and -20 degrees Celsius. Samples were analyzed by The Paerl Lab at the University of North Carolina Chapel Hill. Samples were analyzed following standard operating procedures set by Lachat instruments using a QuikChem 8500 to perform flow injection analysis. QuikChem method 1-115-01-3-A was followed for determination of total phosphorus. QuikChem method 10-107-10-1-C was followed for determination of Nitrate/Nitrite. QuikChem method 10-107-06-1-J was followed for determination of Ammonia.

2.4 Algal Fixation and Flow Cytometer Analysis

Flow cytometry was performed by Dr. Ryan Paerl at North Carolina State University Raleigh. Surface water samples for FCM analysis were fixed with glutaraldehyde (0.25% final conc.) and placed for at least 15 minutes in the dark before storage at -80°C. Samples were kept frozen and then shipped to North Carolina State University campus for analysis. Thawed FCM samples were analyzed using a Guava EasyCyte HT (Millipore) flow cytometer equipped with blue (488 nm) and red (600 nm) excitation lasers. GuavaSoft (Millipore) was used for data analysis and

specifically counting events based on red fluorescence due to excitation by the blue laser. Distinct phytoplankton populations were identified based on autofluorescence from blue and red laser excitation as well as forward scatter (FSC; proxy for cell size) (Liu et al., 2013; Paerl et al., 2020) (Supplemental Figures X, Y). Populations were primarily identified based on red fluorescence from blue and red excitation lasers, which respectively distinguishes Chlorophyll *a* and phycocyanin content of populations. Yellow fluorescence from the blue excitation laser was also used to distinguish populations, specifically putative *Microcystis* cells (Haeyoung et al., 2021; Otsuka et al., 1998) (Supplemental Figure Z). For enumeration of phycocyanin-rich *Synechococcus*-like cells (PC-SYN) and small eukaryotic phytoplankton (Large1) secondary gating based on FSC was used to exclude events that are likely abiotic particles. The limit of quantification for FCM analysis was 8.13 cells mL^{-1} based on detection of a single cell from sample volume processed (considering the average volume processed for 10 random samples).

2.5 Quantitative PCR for the Determination of Total Cyanobacterial DNA

Quantitative PCR was performed on samples from each site for each sampling date. 25 ml aliquots of sample water was suction filtered over membrane filters with a 0.8 um pore size (Whatman), and were then promptly placed inside individual bead lysis tubes, which were then frozen at -20 degrees Celsius. Samples were thawed for analysis and homogenized using a bead mill beater for 5 minutes. Samples were analyzed using the CyanoDTec kit for the detection of total cyanobacteria DNA from Phytoxigene, which includes individual tubes of dehydrated master mix, as well as standards for the generation of a standard curve. All water referred to in

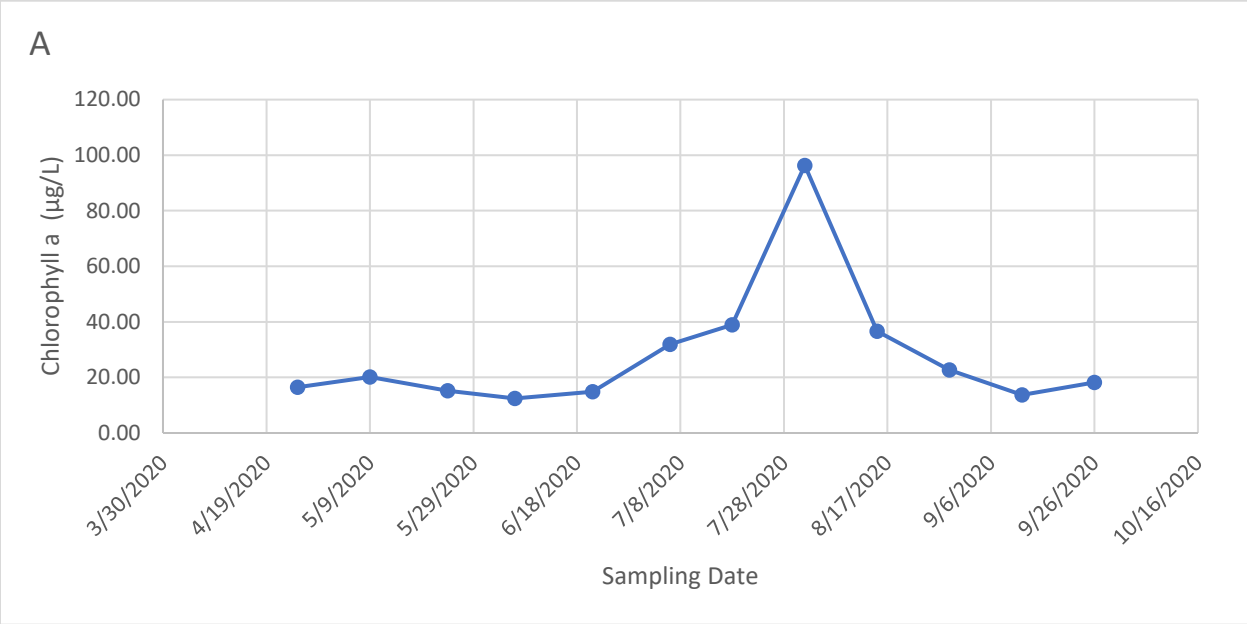
this section is PCR grade. Dehydrated master mix tubes are reconstituted using 80 ul of water per tube and vortexed. Standards are made by pipetting 5 microliters of each standard, beginning with the most concentrated, into separate wells and adding 20 microliters of master mix into each well. A negative control containing only master mix was added for additional sensitivity. For sample analysis, 20 microliters of master mix was added to each well, after which 5 microliters of sample was added to each well, and pipetted up and down to ensure mixing. The following parameters were used for PCR thermocycling: 2-minute initial denaturation at 95°C, 15 second denaturation at 95°C, 30 second annealing/extension phase at 60°C. Set number of cycles to 40.

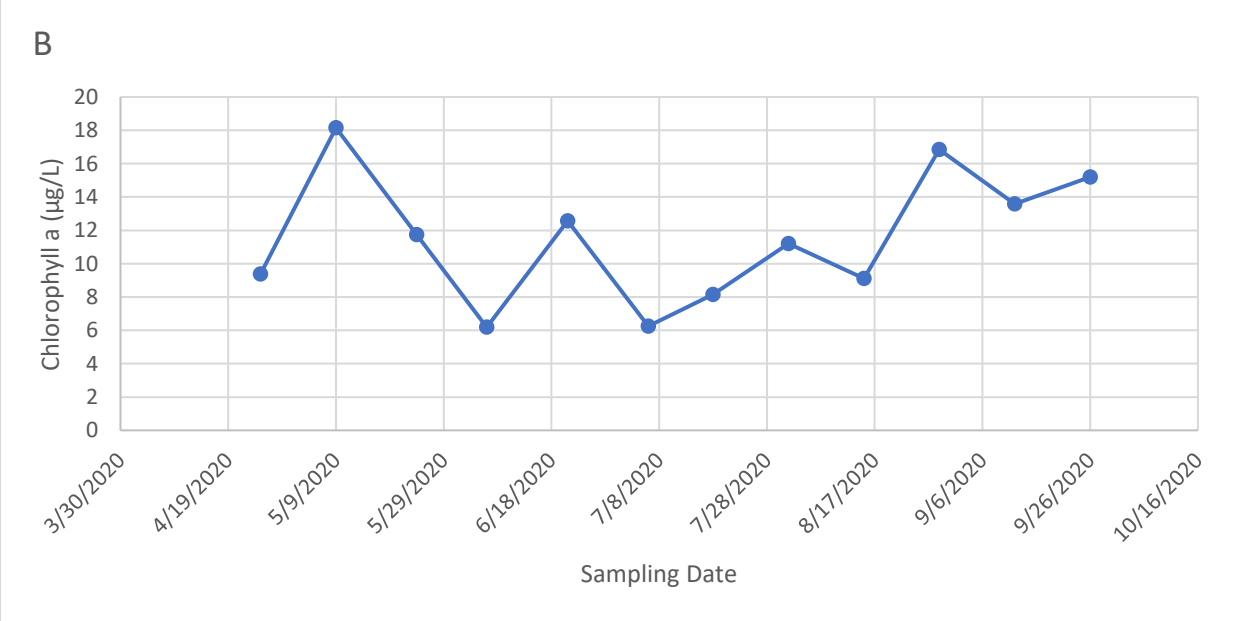
3.0 Results

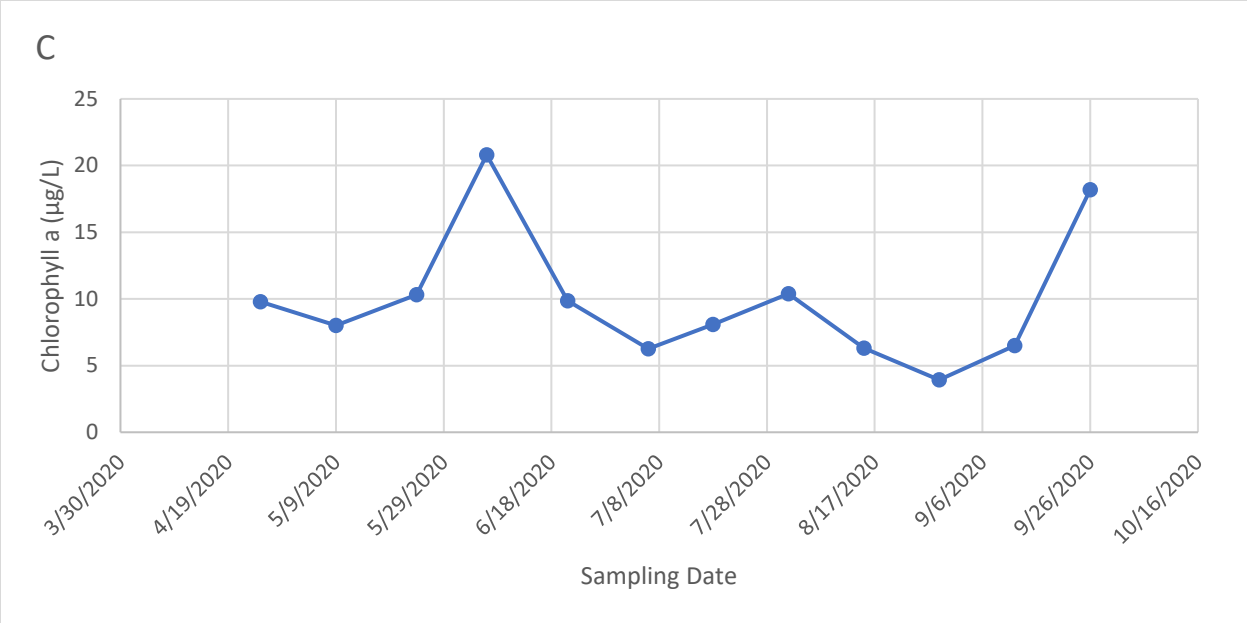
3.1 Total Chlorophyll a Results

Peaks in chlorophyll a concentration were observed for all four sites (Figure 1). Total Chlorophyll a concentration was obtained using fluorescence as well as ultraviolet visible spectroscopy to ensure accuracy. For simplicity the following results are fluorescence measurements, and the correlation with the UV/Vis measurements is provided (Figure 2). Lake Whelchel maintained the strongest coefficient of determination with an R^2 value of 0.9636. The second strongest correlation was observed in Lake Rabon with an R^2 value of 0.9021. Lake Bowen had an R^2 value of 0.8094. Lake Greenwood had the weakest correlation strength with an R^2 value of 0.7458. Lake Whelchel maintained a consistent concentration of approximately 20 g/L through spring until July, when the chlorophyll a concentration began to increase, peaking on August 1st with a concentration of 96.34 µg/L. In September, the level of chlorophyll a returned to roughly 20 µg/L. Lake Bowen had two small seasonal peaks, in spring on May 9th and in summer on August 29th. Dissimilarly to the other sites, chlorophyll a concentration fluctuated over the course of the sampling season. The largest observed concentration for the season was 18.15 µg/L, and the lowest observed was 6.19, which proved to be the smallest difference between the maximum and minimum observed concentrations for any of the four sites. Late spring and summer peaks were also observed in Lake Greenwood. The largest observed concentration was 20.8 µg/L on June 6th, but there was a second peak of 18.2 µg/L observed on September 29th. The lowest observed concentration was 3.93 µg/L. Like Lake Bowen, Lake Greenwood had a relatively small range between the maximum and minimum observed values for the season when compared to Lake Whelchel. Lake Rabon had a small

spring peak of 18.1 µg/L, and a larger late summer peak. At the end of the sampling season, the chlorophyll a concentration in Lake Rabon appeared to be increasing, with the largest observed concentration for the season observed on September 29th of 36.6 µg/L.







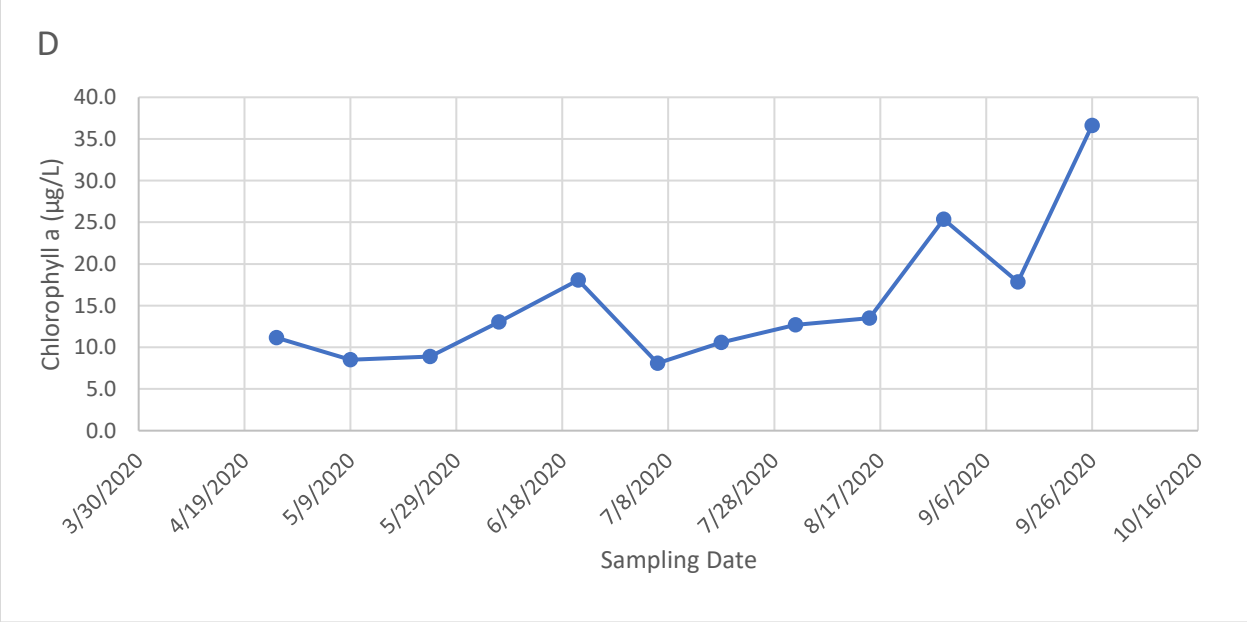


Figure 7. Chlorophyll a concentration fluctuations over the duration of the 2020 sampling season. Lake Whelchel (A) had the largest observable chlorophyll peak and the highest average concentration overall. Lake Bowen (B) and Lake Greenwood (C) had minor fluctuations, but the quantity stayed relatively constant during the season. Lake Rabon (D) maintained stable chlorophyll a content during the summer, with an increase in the Fall

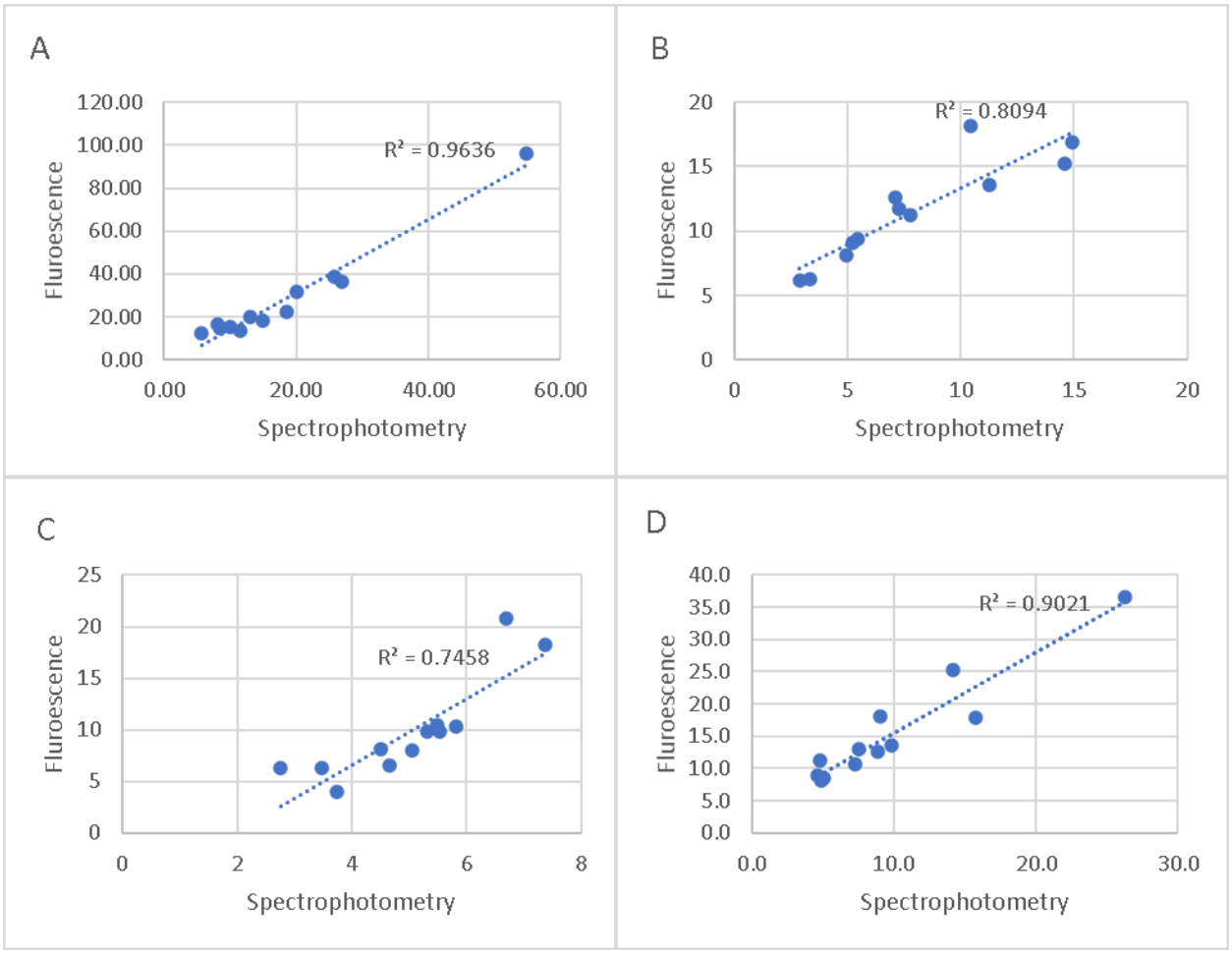
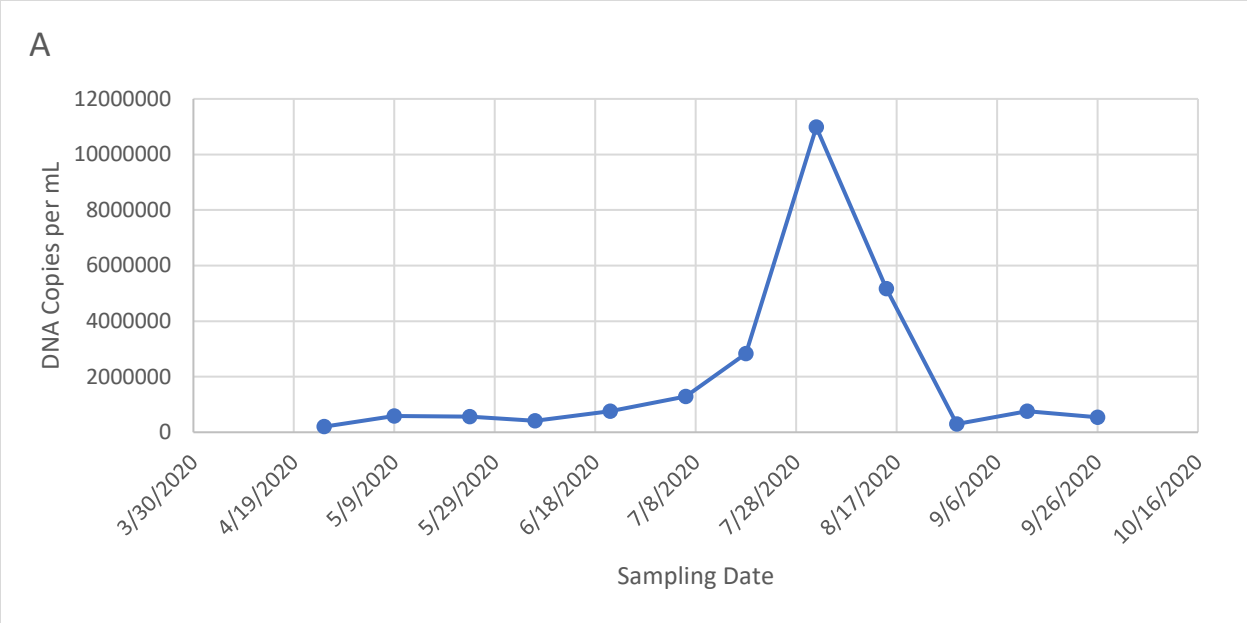


Figure 8. Correlation of fluorescence and UV/Vis measurements for chlorophyll a content. Both measurements are strongly correlated with one another in all four lakes. All values are in µg/L The strongest correlation is observed for Lake Whelchel (A). Moderate correlations were observed for Lake Bowen (B) and Lake Greenwood (C). A strong correlation was also observed in Lake Rabon (D)

3.2 qPCR analysis for total cyanobacteria DNA

Fluctuations in total cyanobacteria DNA content for each site was similar to those for chlorophyll a. Lake Whelchel presented little DNA for cyanobacteria during the spring through summer until July, when the quantity of DNA in samples reached a sharp peak of just over 10.8 million copies of DNA per millilitre of sample August 1st. This peak was quickly reduced back to levels similar to the spring time by the end of September. Lake Bowen consistetly displayed the presence of cyanobacteria DNA over the course of the sampling season with a peak of approximately 704,000 copies per millilitre on June 21st. Lake Bowen had the smallest observed differenec between its largest and smallest values for total DNA, with an approximately 320000 copy difference between the maximum and minimum values. Lake Greenwood had a peak of 1,120,000 copies occuring on June 6th, which then decreased over the next month before returning to a similar peak of approximately 1,024,000 copies milliliter before decreasing for the rest of the season. Lake Rabon had a consistent increase of DNA over the coures of the season, culminating in a large peak occuring only July 17th of roughly 1,440,000 copies per milliliter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DNA Copies per mL
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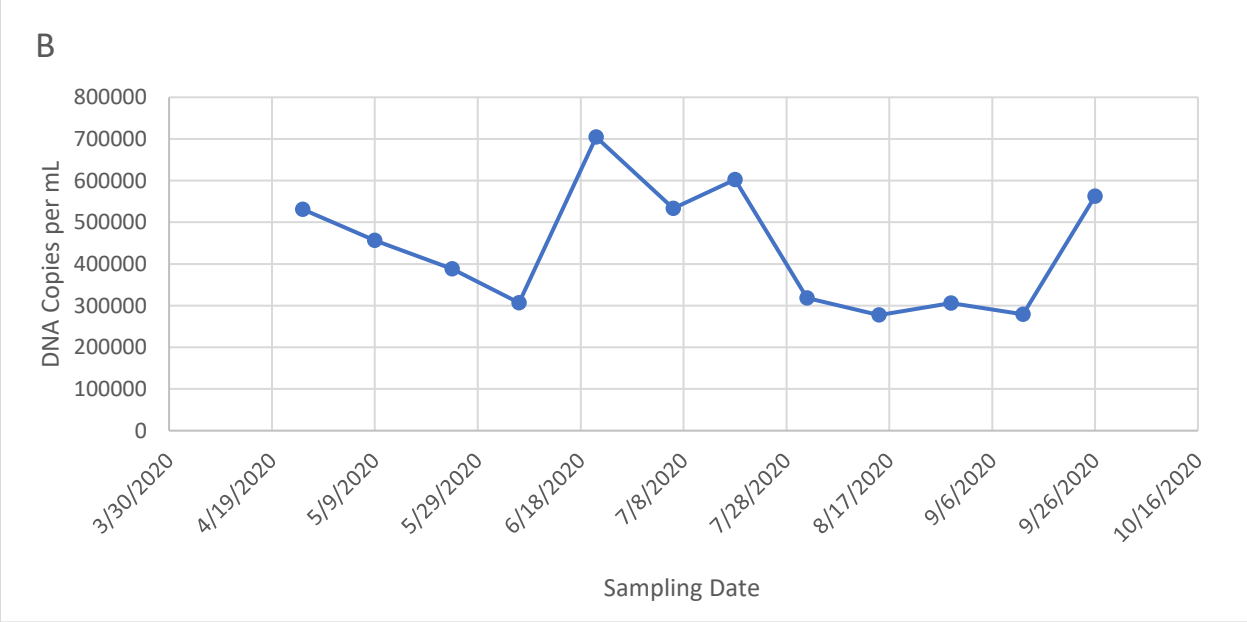
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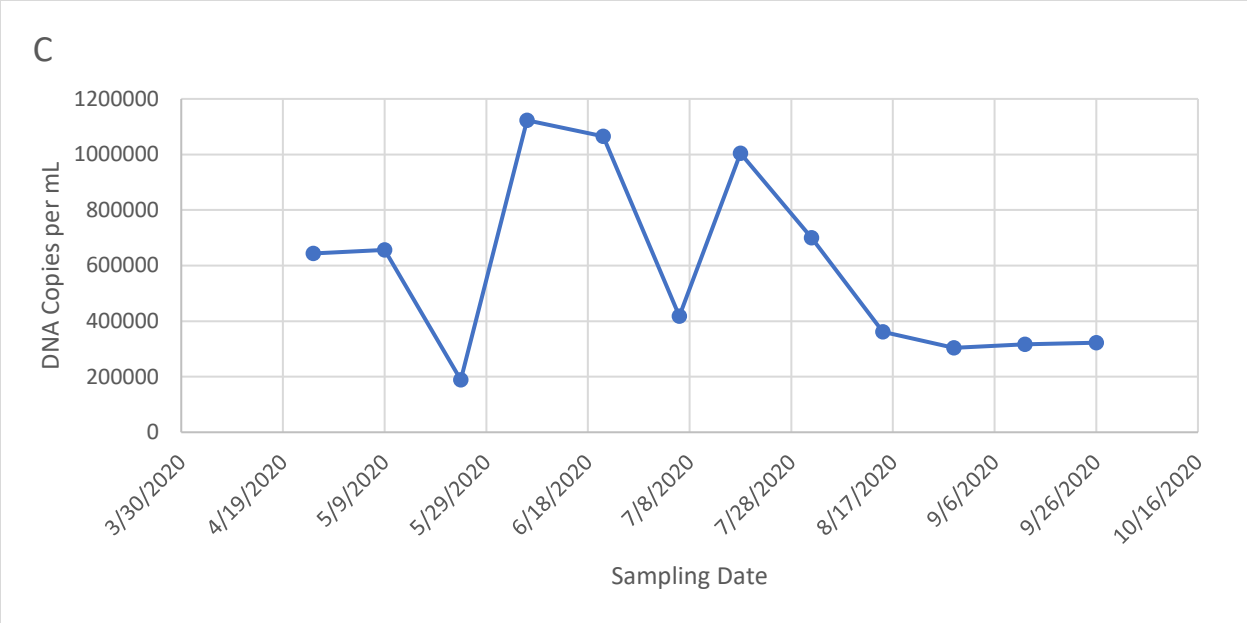
10/16/2020
Sampling Date



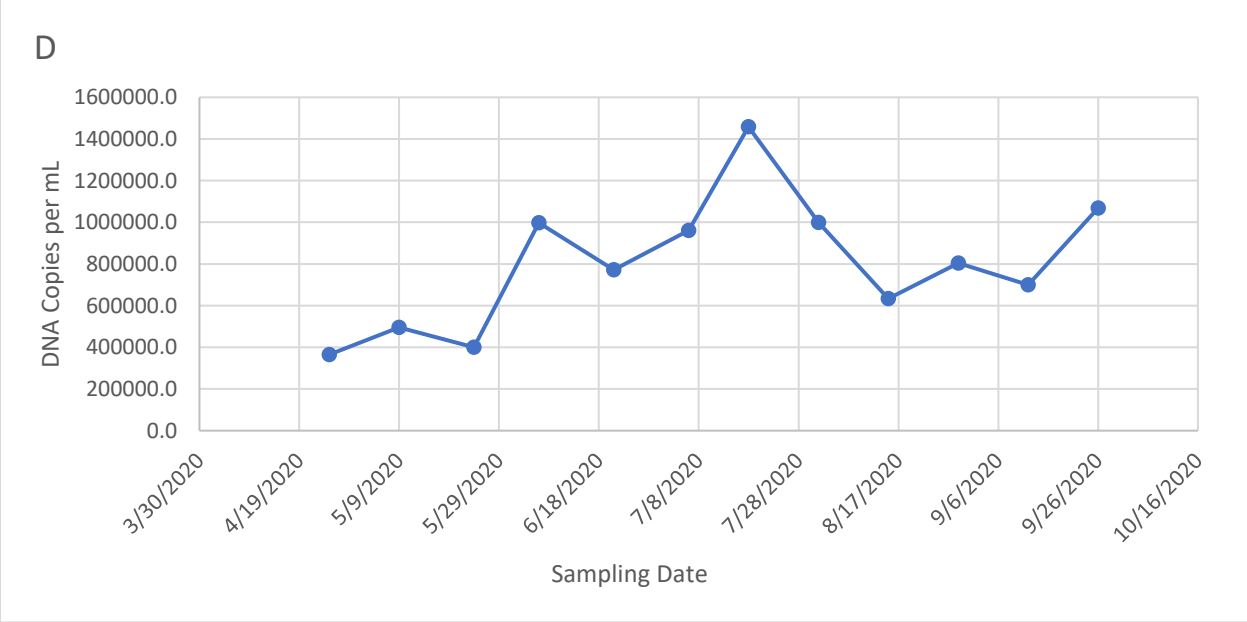


Figure 9. DNA copies per mL of site water. Lake Whelchel (A) has the largest peak of cyanobacteria. Lake Bowen (B) and Lake Greenwood (C) expressed similar temporal peaks, with slightly more cyanobacteria in Greenwood. Lake Rabon (D) had a large mid-July peak similar to Bowen and Greenwood.

3.3 Flow Cytometry

Flow cytometry analysis of site water demonstrated unique algal population changes for each lake, and distinct algal populations in Whelchel and Rabon that were not present in Bowen and Greenwood. Flow cytometry data was visualized in the form of scatter plots, where individual cells are represented by dots based on their fluorescence under various wavelengths and colors of light. Cells fluoresce differently based on their pigmentation and can therefore be sorted into groups referred to as gates, which are represented by the different color dots seen on the scatter plots. The cells that are of greatest interest are represented by the pink regions of dots on the scatterplots, which represent phycocyanin rich cyanobacteria. The other region of interest is the neon green regions seen in Lake Whelchel and Lake Rabon, representing *Microcystis* cells. The regions labeled R4 represent small phycoerythrin rich cells, likely *Synechococcus* cells, which are a type of cyanobacteria. The regions labeled R6 likely represent eukaryotic phytoplankton, or possibly chlorophytes. The R3 region could possibly be cyanobacteria but are likely large phytoplankton. Finally, Lake Bowen contains an R7 region that could be aggregated unicellular cyanobacteria but could also be nanoeukaryotes.

Phycocyanin rich large cyanobacteria, referred to here as R2 cells, and phycoerythrin rich *synechococcus* like cells, referred to as R4 cells, were present in all four sites. Early in spring, Lake Whelchel had low levels of cyanobacteria, which then began to increase at the beginning of summer. The concentration of large cyanobacteria fluctuated over the course of the summer and into fall, with a peak of 187111 cells per mL on June 21st, and *synechococcus* cells peaked in mid-August with a concentration of approximately 168000 cells per mL. Lake Bowen also had low observable concentrations of large cyanobacteria cells early in the spring, which rose

sharply on June 21st to almost 160000 cells per mL, which coincided with the peak in *synechococcus* cells which was 185000 cells per mL. This peak was temporally consistent with the peak for total cyanobacteria DNA, as well as a small increase in chlorophyll a content. Cyanobacteria cells concentration remained elevated until the beginning of August, at which point the concentration gradually decreased into fall. Lake Greenwood exhibited a similar pattern, with low quantities of large cyanobacteria cells early in the spring, which then rose consistently into late summer with minor fluctuations, resulting in an August 1st peak of approximately 180000 cells per mL. Lake Greenwood had a temporally different peak for synechoccous cells compared to large cyanobacteria, with a September 29th peak of 168000 cells per mL. Lake Rabon also had small quantities of large cyanobacteria early in the spring, rising consistently through the summer. Like chlorophyll a, the peak for cyanobacteria cells was observed on the last sampling date for the season, approximately 280000 cells per mL, and a June 21st peak of 158000 *synechococcus* cells per mL, which coincided with a small spring peak in chlorophyll a and total cyanobacteria DNA

Lake Whelchel and Lake Rabon contained observable *Microcystis* cells late in the sampling season. Lake Whelchel had very low observable levels of *Microcystis* during the spring, with cells present but in very small quantities. Beginning July 6th, the level of *Microcystis* cells began to increase with a peak of 107634 cells per mL on August 1st, which coincides with both the chlorophyl a peak and total cyanobacteria DNA peak for Lake Whelchel. Additionally, during this time, the South Carolina Department of Health and Environmental Control issued a public notice that the levels of the toxic compound microcystin in Lake Whelchel had exceeded the

water quality standard threshold and an advisory against water recreation was put in place. Lake Rabon contained low observable quantities of *Microcystis* over the entirety of the sampling season, which also peaked on September 29th at a concentration of 8000 cells per mL. Additional figures below show examples of dot plots produced from samples taken from Lake Whelchel (Figure 5) and Lake Rabon (Figure 6), both of which have clearly defined regions of cells consistent with *Microcystis* cells which are represented by the neon green regions of dots, while the dot plot from Lake Bowen (Figure 7) does not.

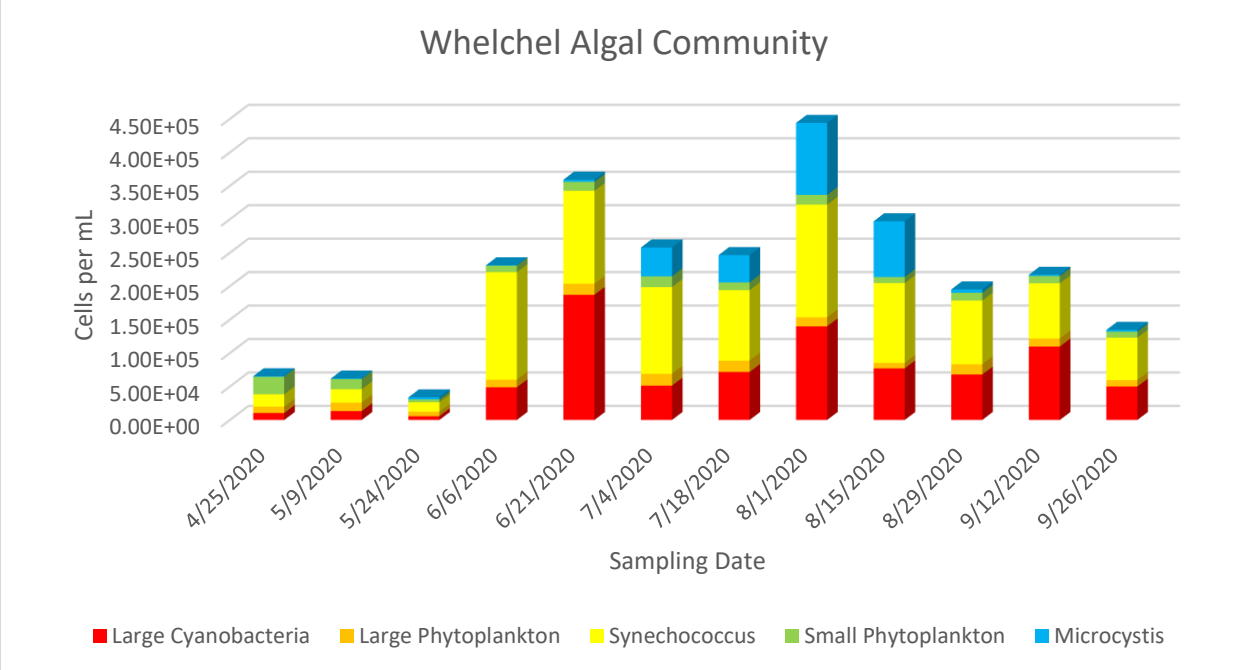


Figure 10. Putative characterization of the algal population in Lake Whelchel via flow cytometry. Lake Whelchel had a large observable Microcystis population that was not observed in other sites

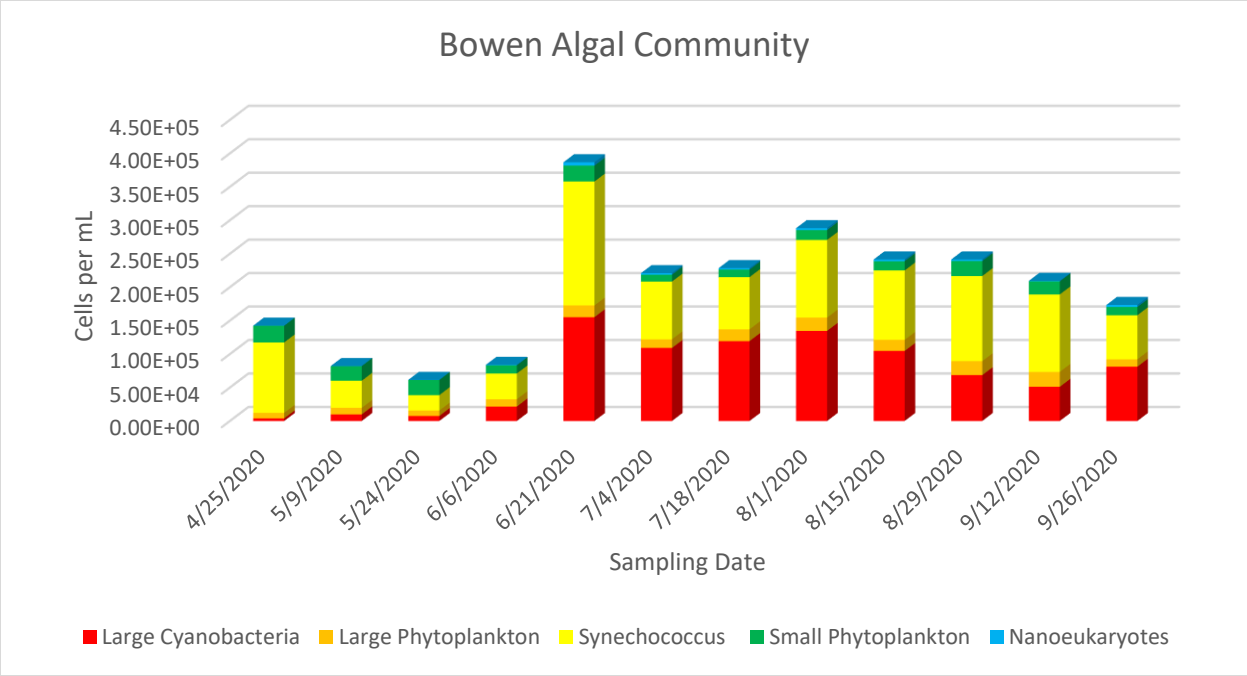


Figure 11. Putative characterization of the algal population in Lake Bowen via flow cytometry. Lake Bowen had relatively small cyanobacteria populations in comparison to other sites

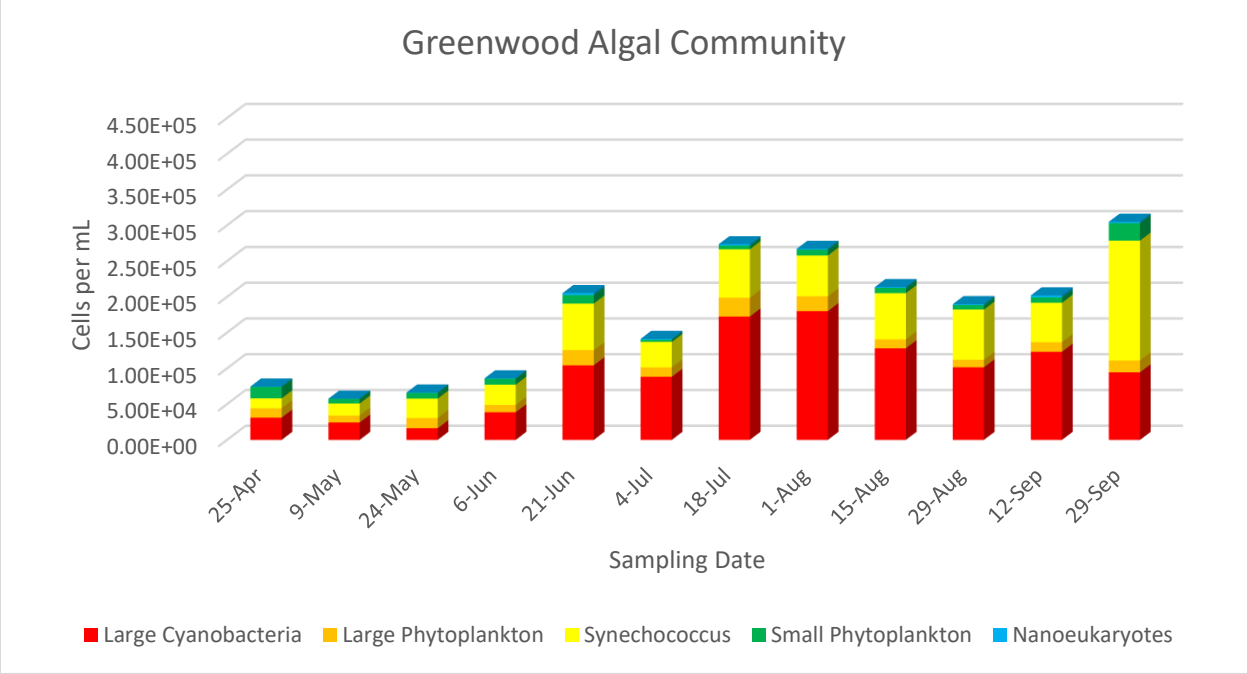


Figure 12. Putative characterization of the algal population in Lake Greenwood. Like Bowen, Greenwood had lower quantities of cyanobacteria in comparison to the two other sites

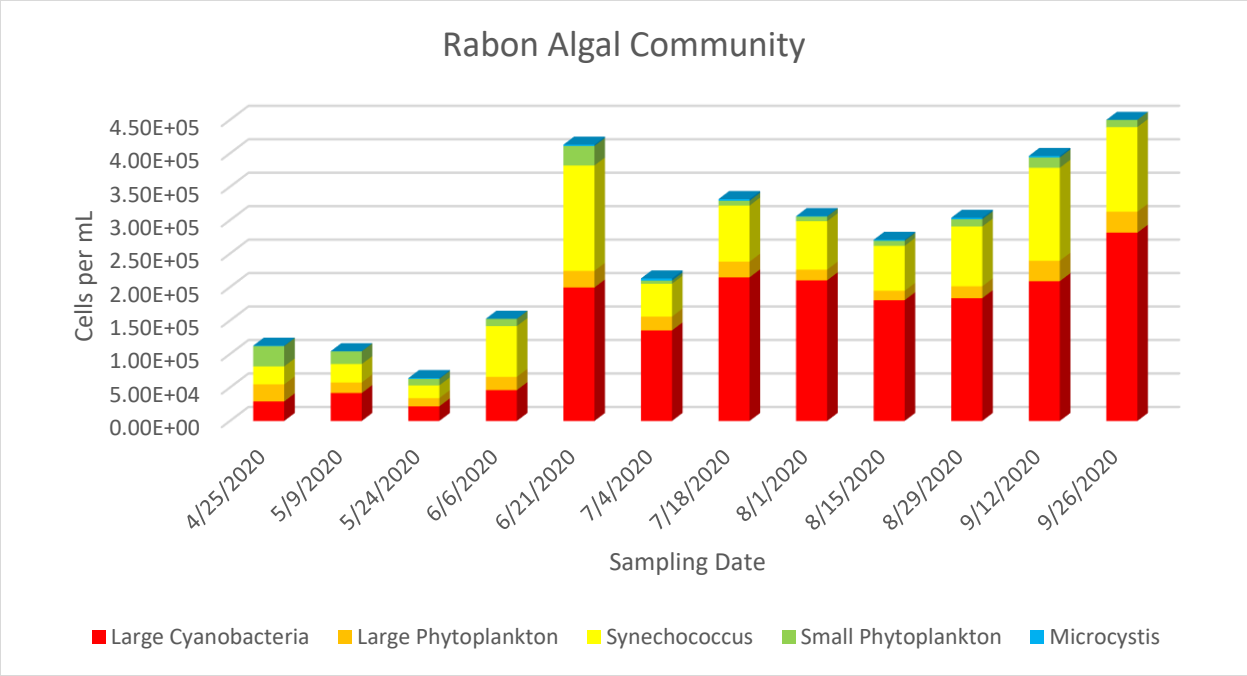


Figure 13. Putative characterization of the algal population in Lake Rabon. Despite low quantities of chlorophyll a, Rabon maintained higher quantities of cyanobacteria, particularly in comparison to Lake Bowen and Lake Greenwood

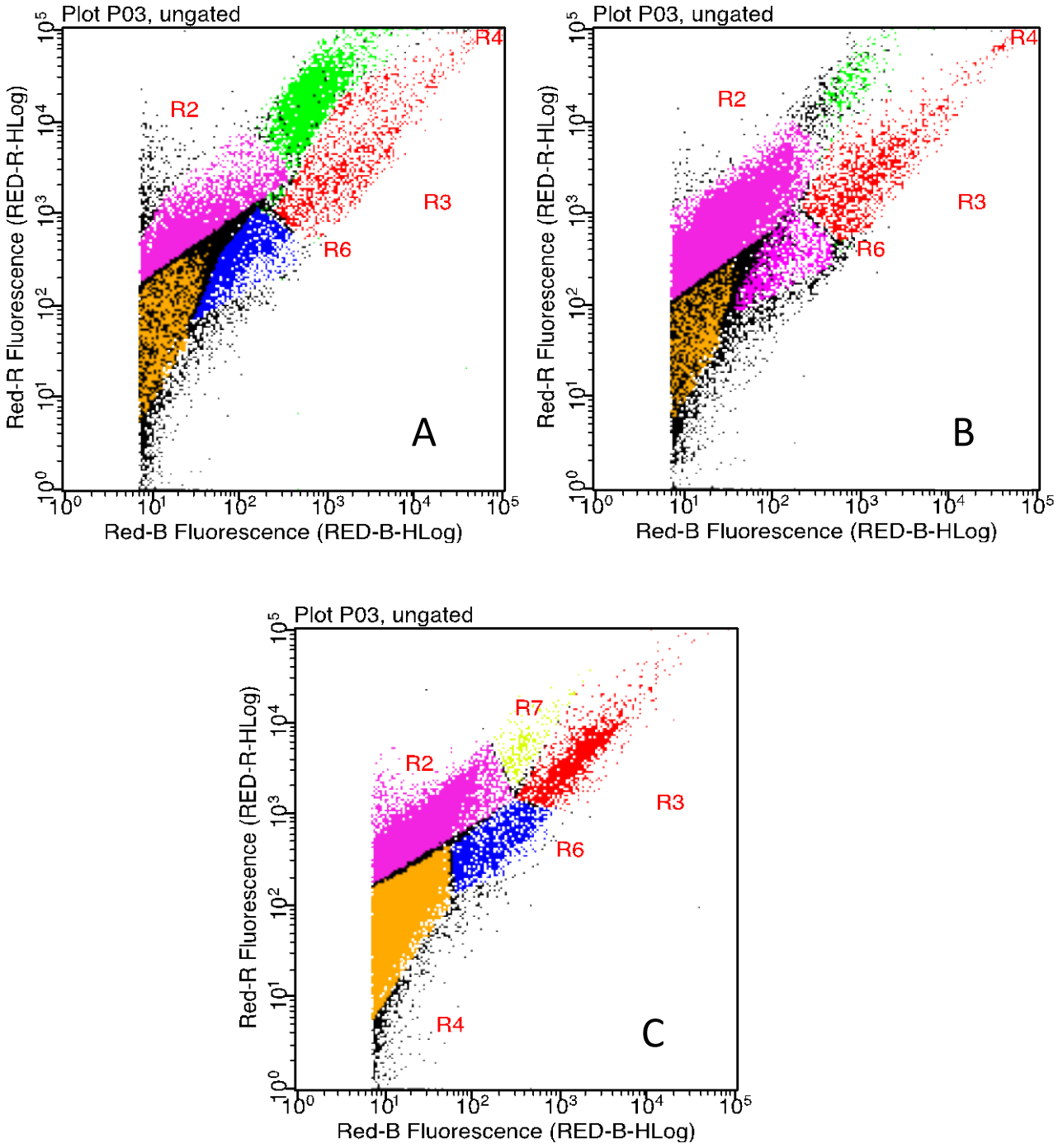
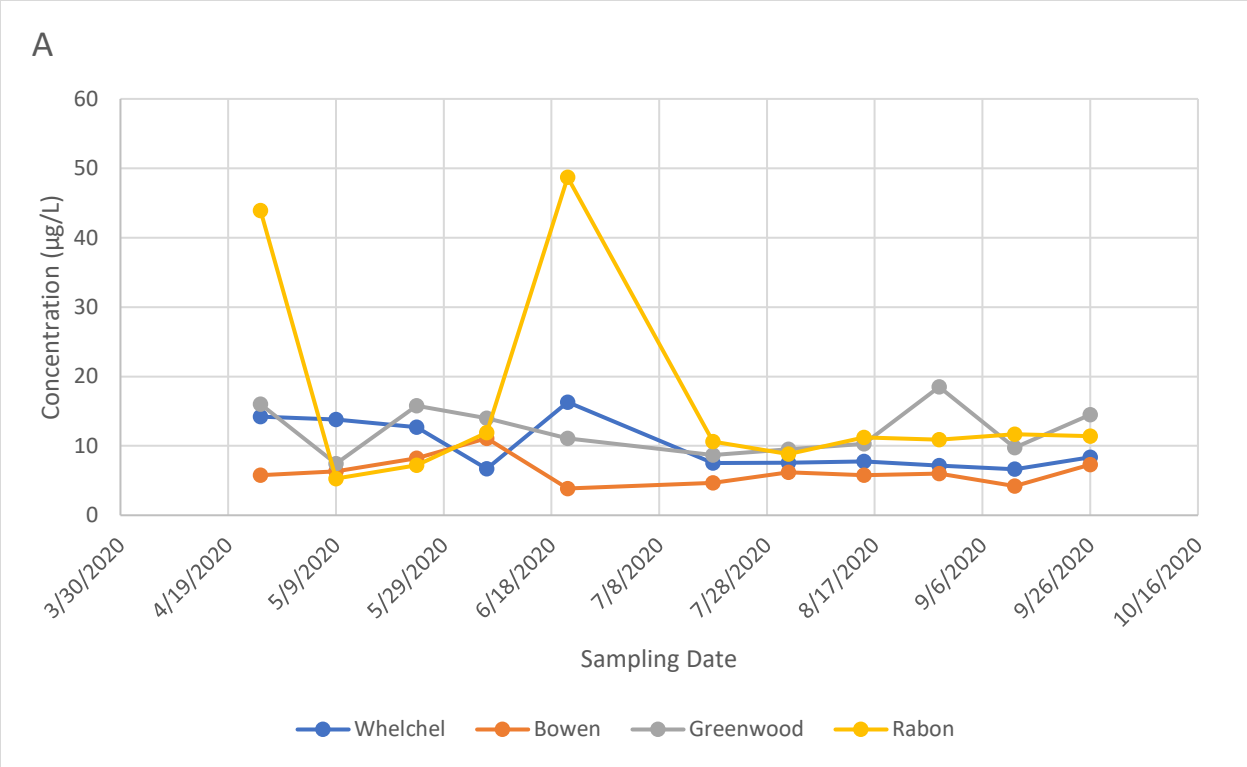
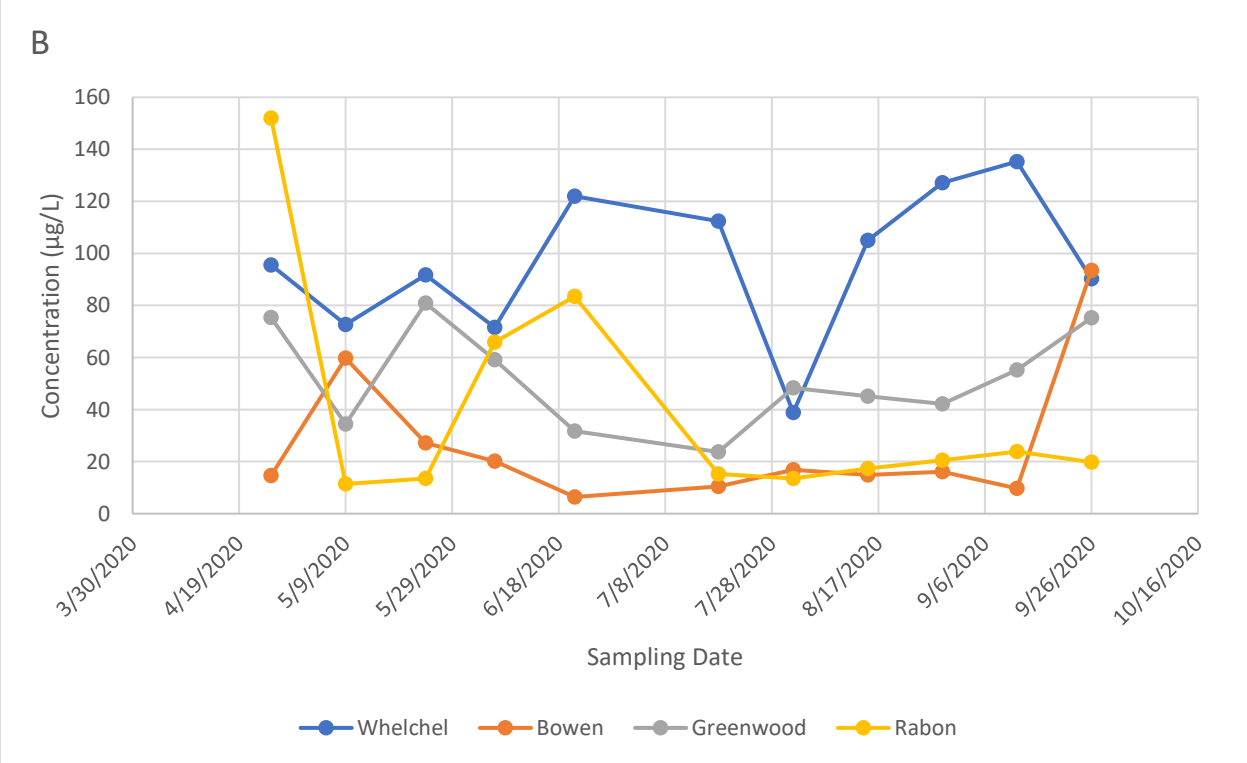


Figure 14. Examples of dot plots created via flow cytometry detection of cell fluorescence. The first plot is representative of Lake Whelchel (A), and has a large cluster of neon green dots, representing the large Microcystis population. This population was mirrored in Lake Rabon (B), but in a smaller quantity. Lake Greenwood (C) did not have any observable Microcystis population and subsequently is lacking this region of the dot plot

3.4 Nutrient Analysis

The observed patterns for nutrient fluctuations for each site were generally the same throughout the sampling season with some exceptions. Lake Whelchel began the season with high concentrations of nitrates, which steadily decreased over the course of the season reaching a low on July 18th, before a slight increase into the Fall. Levels of ammonium were relatively constant over the entirety of the season, with a slight decrease on August 1st. Levels of phosphorus were consistently low the entire season. Lake Bowen began the season with elevated levels of nitrogen oxides which decreased to almost nonexistent levels over the course of the season before a very slight increase in the Fall. Ammonium and phosphorus levels also began low and maintained consistently low concentrations during the season before increasing very slightly in the Fall. Lake Greenwood maintained a pattern consistent with the previous two lakes for all three nutrients. High levels of nitrogen oxides were observed early in the season, which decreased to low levels with a slight increase in the Fall. Ammonium and phosphorus levels stayed low with minimal fluctuations during the season with very slight increases in the Fall. The patterns observed in Lake Rabon differed from the other three sites. Lake Rabon began the season with elevated levels of nitrogen oxides, ammonium and phosphorous compared to the other three lakes, and after decreases in all three nutrients, a spike in each was observed in late Spring/ early Summer that gradually decreased into low concentrations, which was consistent with the pattern observed in the other sites.


A
Concentration (µg/L)
60
50
40
30
20
10
0
3/30/2020
4/19/2020
5/9/2020
5/29/2020
6/18/2020
7/8/2020
7/28/2020
8/17/2020
9/6/2020
9/26/2020
10/16/2020
Sampling Date
Whelchel
Bowen
Greenwood
Rabon


B
Concentration (µg/L)
160
140
120
100
80
60
40
20
0
3/30/2020
4/19/2020
5/9/2020
5/29/2020
6/18/2020
7/8/2020
7/28/2020
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9/6/2020
9/26/2020
10/16/2020
Sampling Date
Whelchel
Bowen
Greenwood
Rabon

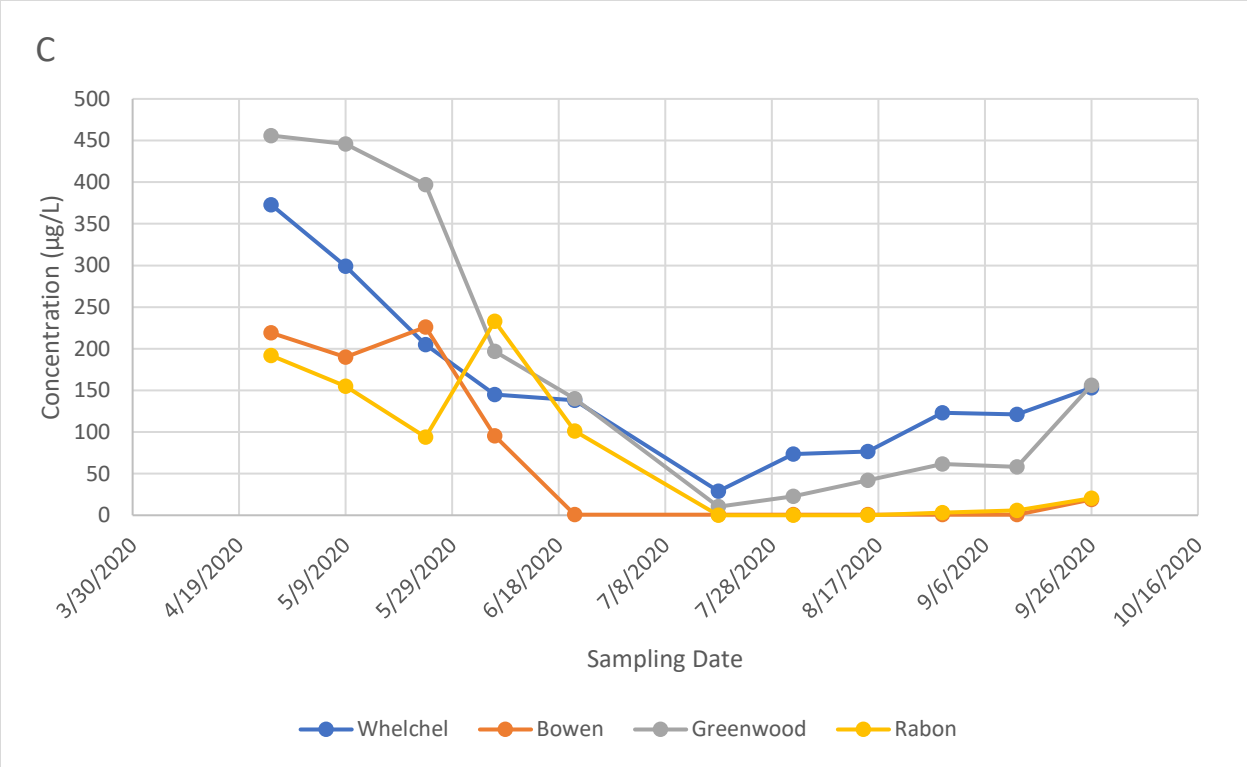

Figure 15. Nutrient fluctuations for each site. Phosphorus (A) was the most consistent across all four sites, maintaining similar quantities in each site throughout the season, with the exception of two periods in the spring for Lake Rabon. Ammonium (B) had substantial fluctuations for each site. Nitrates (C) followed a consistent pattern for each site, with high initial values that uniformly decreased into the summer months

3.5 Total DNA vs Chlorophyll a Content

A strong positive correlation between total cyanobacteria DNA and Chlorophyll content was observed for Lake Whelchel, with an R^2 value of 0.9256. Very weak positive correlations were observed for Lake Greenwood and Lake Rabon with R^2 values of 0.1409 and 0.0574 respectively. Interestingly, Lake Bowen showed a weak negative correlation between cyanobacteria DNA and Chlorophyll a content, with an R^2 value of 0.0025.

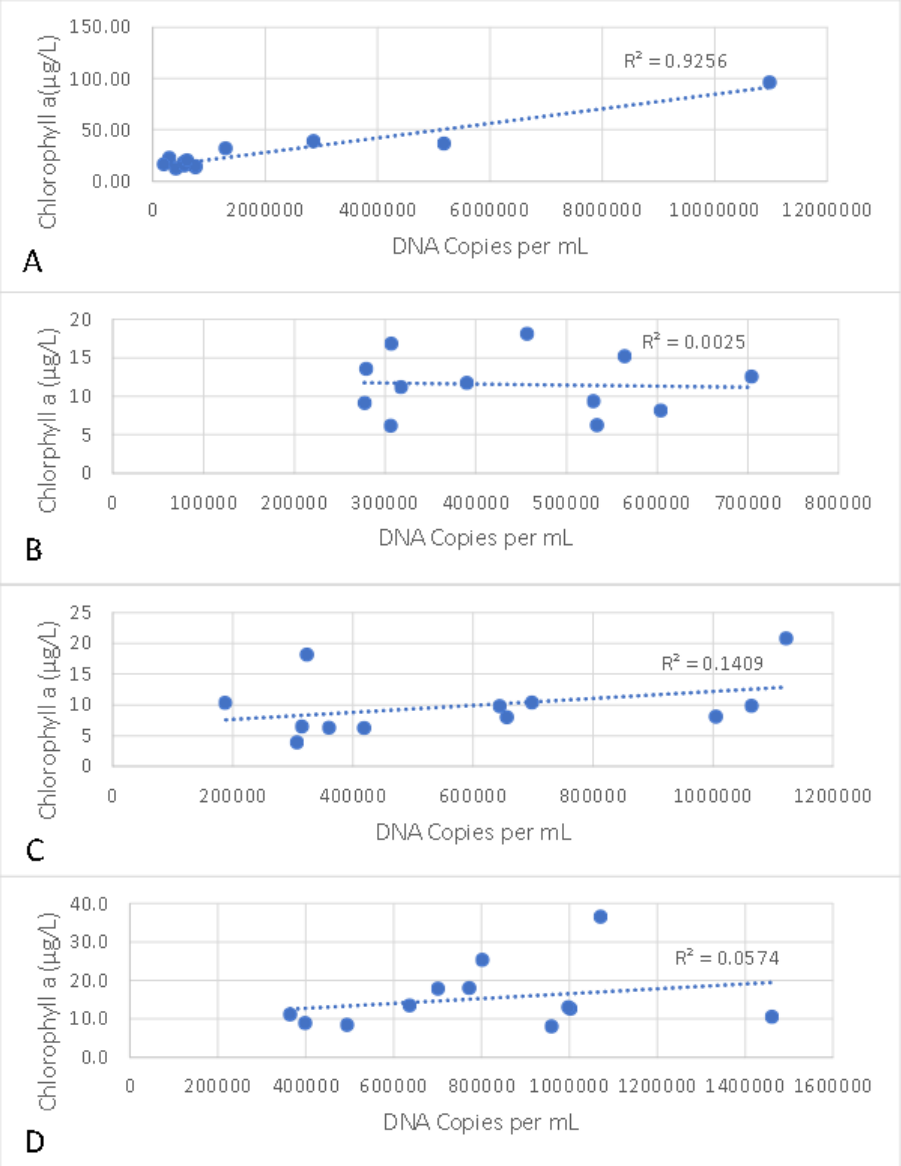

Figure 16. Correlations between total chlorophyll a content and total cyanobacteria DNA. Lake Whelchel (A) had the strongest correlation. Lake Bowen (B) Lake Greenwood (C) and Lake Rabon (D) all had extremely weak correlations, suggesting a lack of relationship between cyanobacterial presence and the overall quantity of chlorophyll a in those three sites

3.6 Chlorophyll a Content and Total Cyanobacteria cell Count

Cyanobacteria cells were grouped together using the data obtained from flow cytometry results. Total cyanobacteria cells consist of Synechococcus cells, larger cyanobacteria, and in the case of Whelchel and Rabon, *Microcystis* cells. A moderate correlation between the total number of cyanobacteria cells per mL of site water and chlorophyll a content was observed in Lake Whelchel (Figure A), with a correlation strength of 0.4271. The correlation between the total number of cyanobacteria cells and chlorophyll a content was almost nonexistent in Bowen (Figure B) and Greenwood (Figure C) with negative correlations of 0.1157 and 0.0022 respectively. Lake Rabon had the strongest relationship between total cyanobacteria cells and chlorophyll a content, with a moderately strong positive correlation strength of 0.4817.

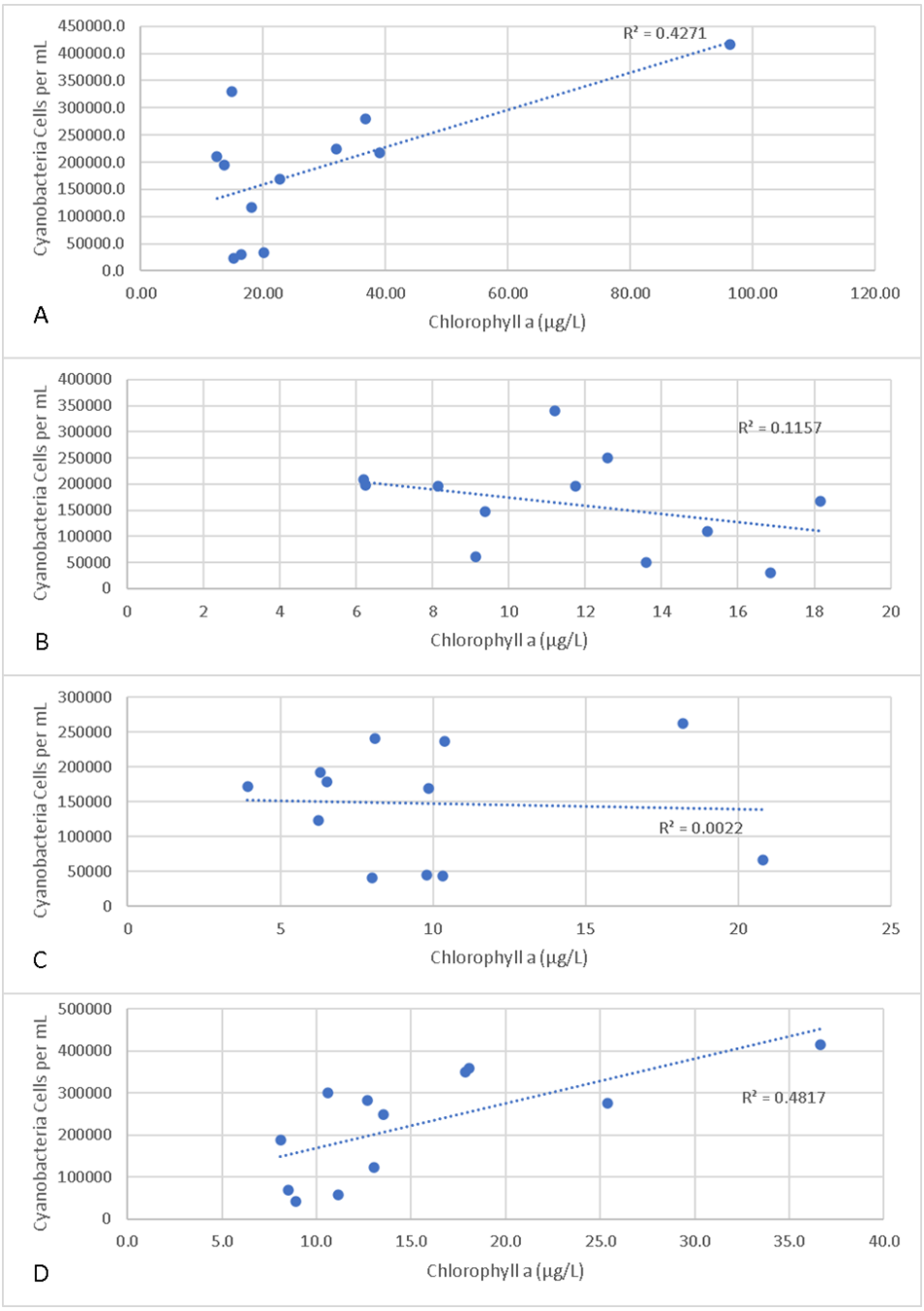

Figure 17. Correlations between the number of cyanobacteria cells present in each site and the total chlorophyll a content of each lake. Lake Whelchel (A) had a moderate correlation between the two parameters. Lake Bowen (B) and Lake Greenwood (C) both had extremely weak negative correlations between the two values. Lake Rabon (D) had a moderately strong correlation similar to that observed in Lake Whelchel

3.7 Rainfall

Rainfall data was provided by the United States Geological Survey. The monitoring station selected was USGS Rain Gauge number 345109082171900 at Gary Pittman Park in Wade Hampton, SC. This station is central to all four lakes and is a good representation of the rainfall events for the general region. The sampling season was characterized by consistent rainfall throughout the early spring, with heavy rainfall events throughout the month of May. Rainfall events became sparser in the month of June, followed by a long period of no rainfall in July. At the end of July there were two large rainfall events followed by a return to dry conditions in the region that continued into September.

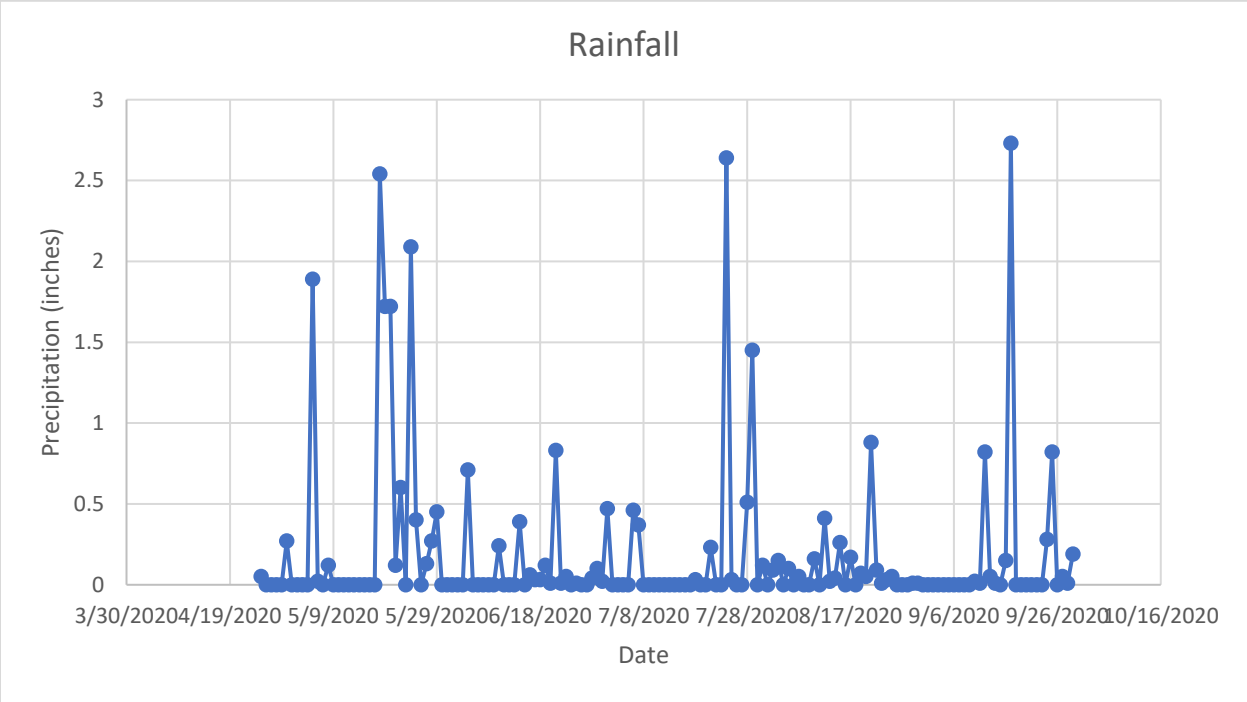

Figure 18. Rainfall data selected from a location central to all four sites. Gary Pittman Park in the Greenville area of South Carolina was selected due to its generally central location and USGS monitoring station

3.8 Streamflow

Stream and River discharge data was available for each site except for Lake Whelchel through the United States Geological Survey. Stream discharge was similar for all available monitoring sites, which is of particular interest considering discrepancy in size among the lakes. The stream flow data for each location have a sizable peak in late May into early June, which then decreases to consistently lower discharge during July. The discharge spikes in late July and early August before decreasing, with one final spike in the month of September. This pattern is consistent for all three sites that data was available for, including both monitoring stations for Lake Rabon, and is consistent with the rainfall pattern observed in the previous section. There was not a monitoring station or data available for Lake Whelchel.

Lake Bowen

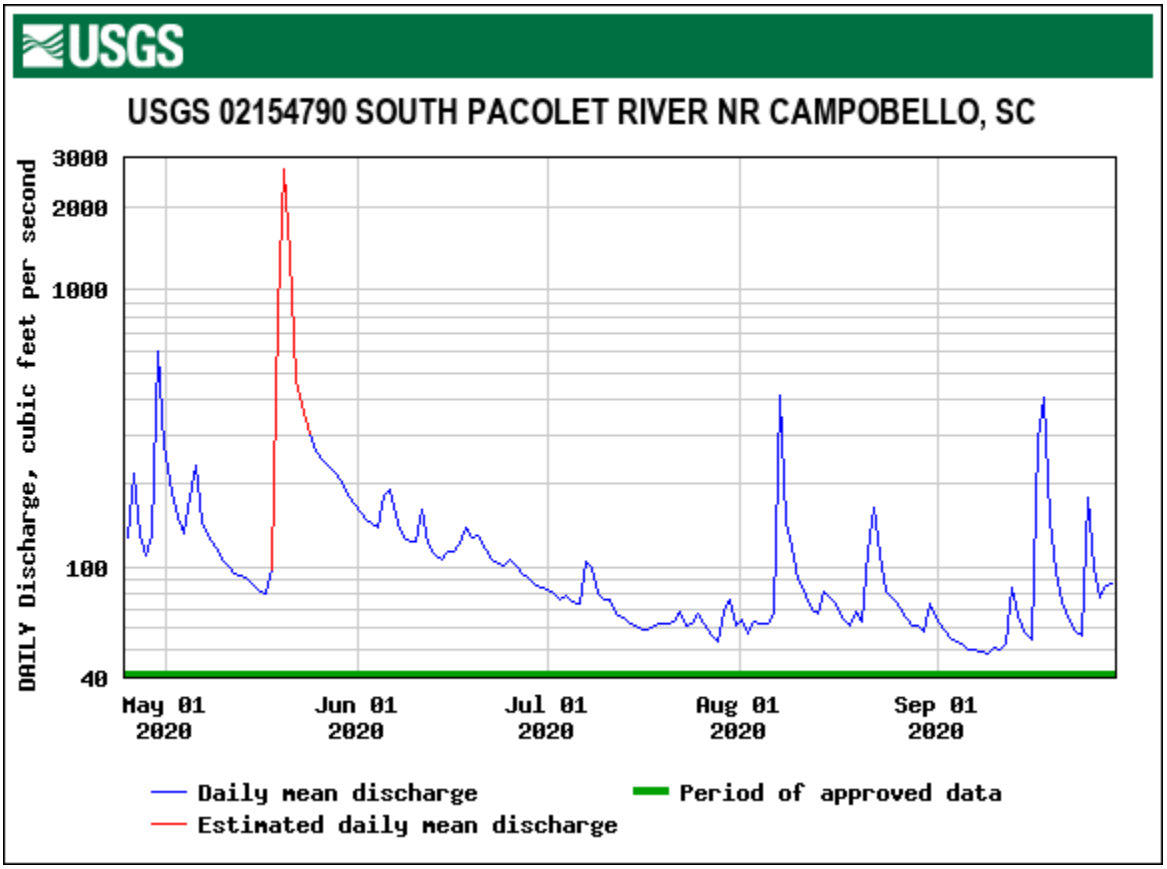

Figure 19. Streamflow data for May 2020 to the end of September 2020 for Lake Bowen. Increases in streamflow data were consistent with the pattern of rainfall in the region

Lake Greenwood

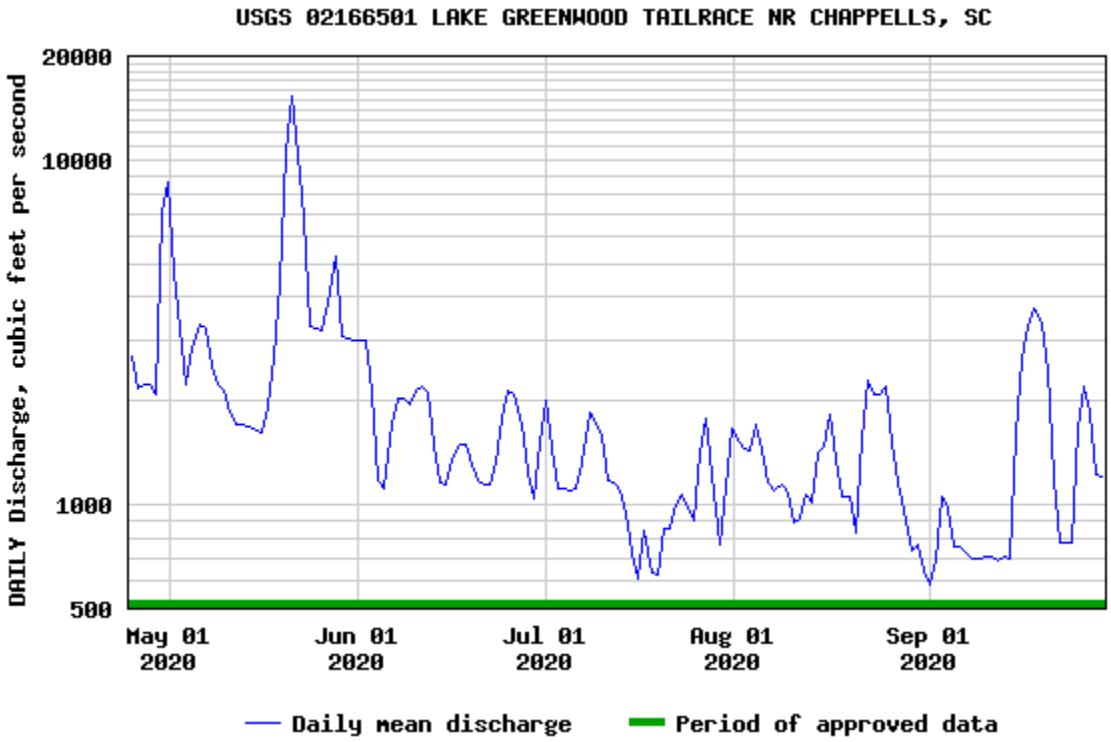

Figure 20. Streamflow data for May 2020 to the end of September 2020 for Lake Greenwood. Increases in streamflow data were consistent with the pattern of rainfall in the region

Lake Rabon

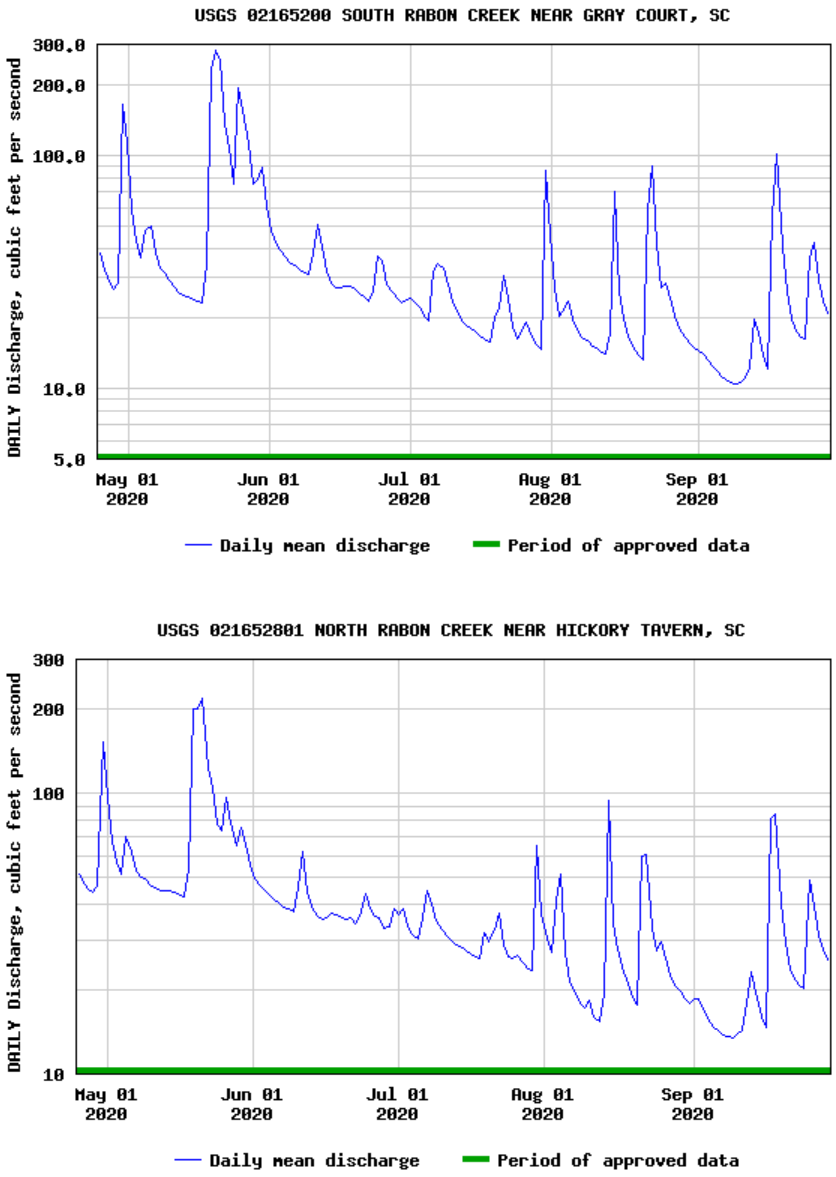

Figure 21. Streamflow data for May 2020 to the end of September 2020 for Lake Rabon. Increases in streamflow data were consistent with the pattern of rainfall in the region for both monitoring stations

3.9 Nutrient to Chlorophyll a and Cyanobacteria DNA Correlations

Correlations were determined between individual nutrients and chlorophyll a and total cyanobacteria DNA for each site. The strongest correlations for Lake Whelchel were for Chlorophyll a and ammonium, followed by the relationship between total cyanobacteria DNA and ammonium. This relationship was observed once again in negative correlations between chlorophyll a and cyanobacteria DNA with nitrates, suggesting an inverse relationship with algal growth and the general presence of nitrogen. These two correlations proved to be the strongest of any nutrient to either chlorophyll a or total cyanobacteria DNA in any site. In general, ammonium or nitrates represented stronger correlations than phosphorus for all four sites, in terms of the relationship between both chlorophyll a and total cyanobacteria DNA. This suggests that for all four sampling locations that the presence of nitrogen generally impacted the fluctuations of algae to a greater degree than the presence of phosphorus, although it should be noted that the correlations between these variables are moderate at best, as a single nutrient is unlikely to be responsible for the propagation of an entire algal community.

Lake Whelchel Nutrient and Chlorophyll a Correlations

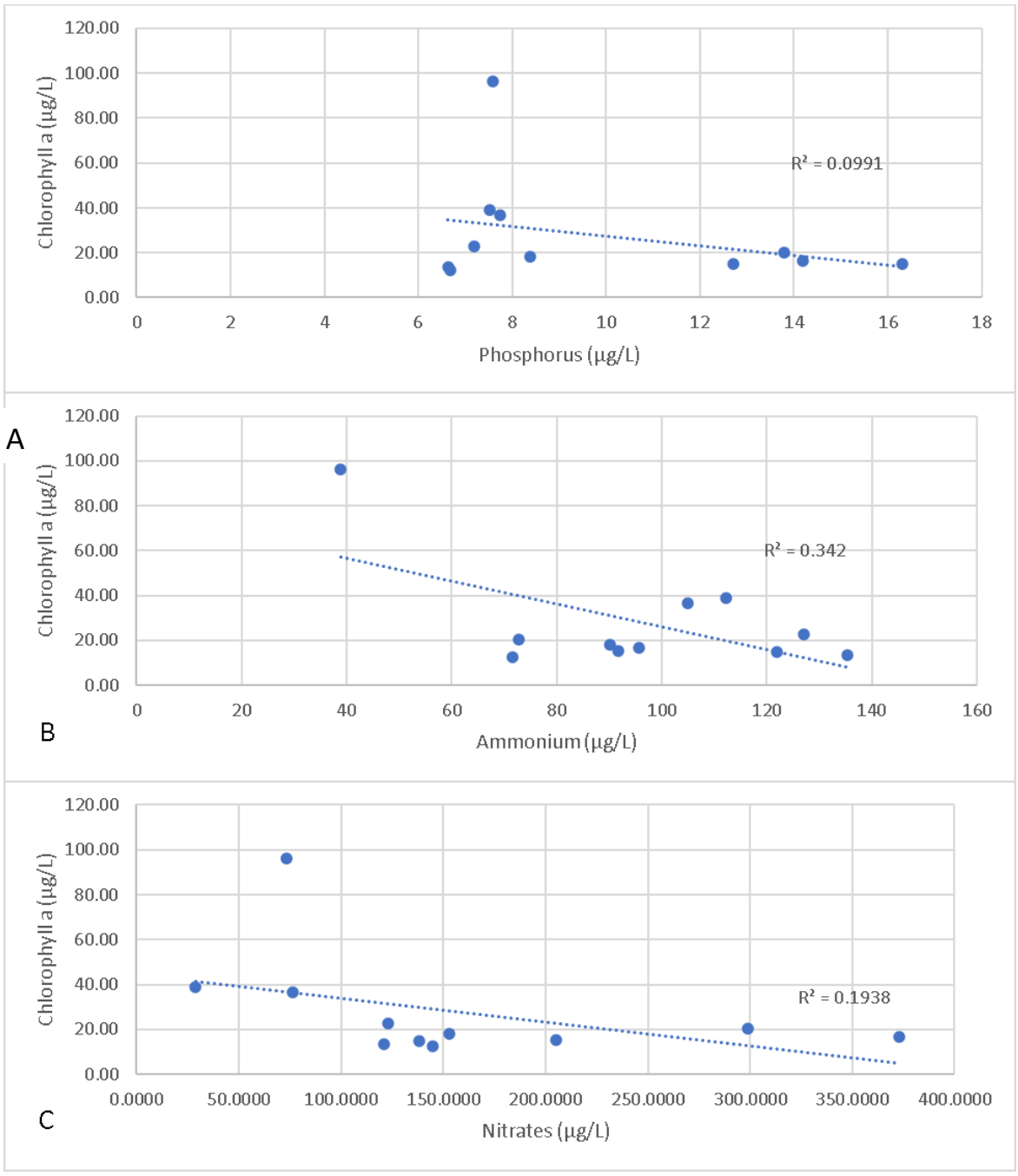

Figure 22. Correlations between total chlorophyll a and nutrients in Lake Whelchel. Correlation strength was weak with phosphorus (A), moderate with ammonium (B) and weaker with nitrates (C)

Lake Whelchel Nutrient and Total Cyanobacteria Correlations

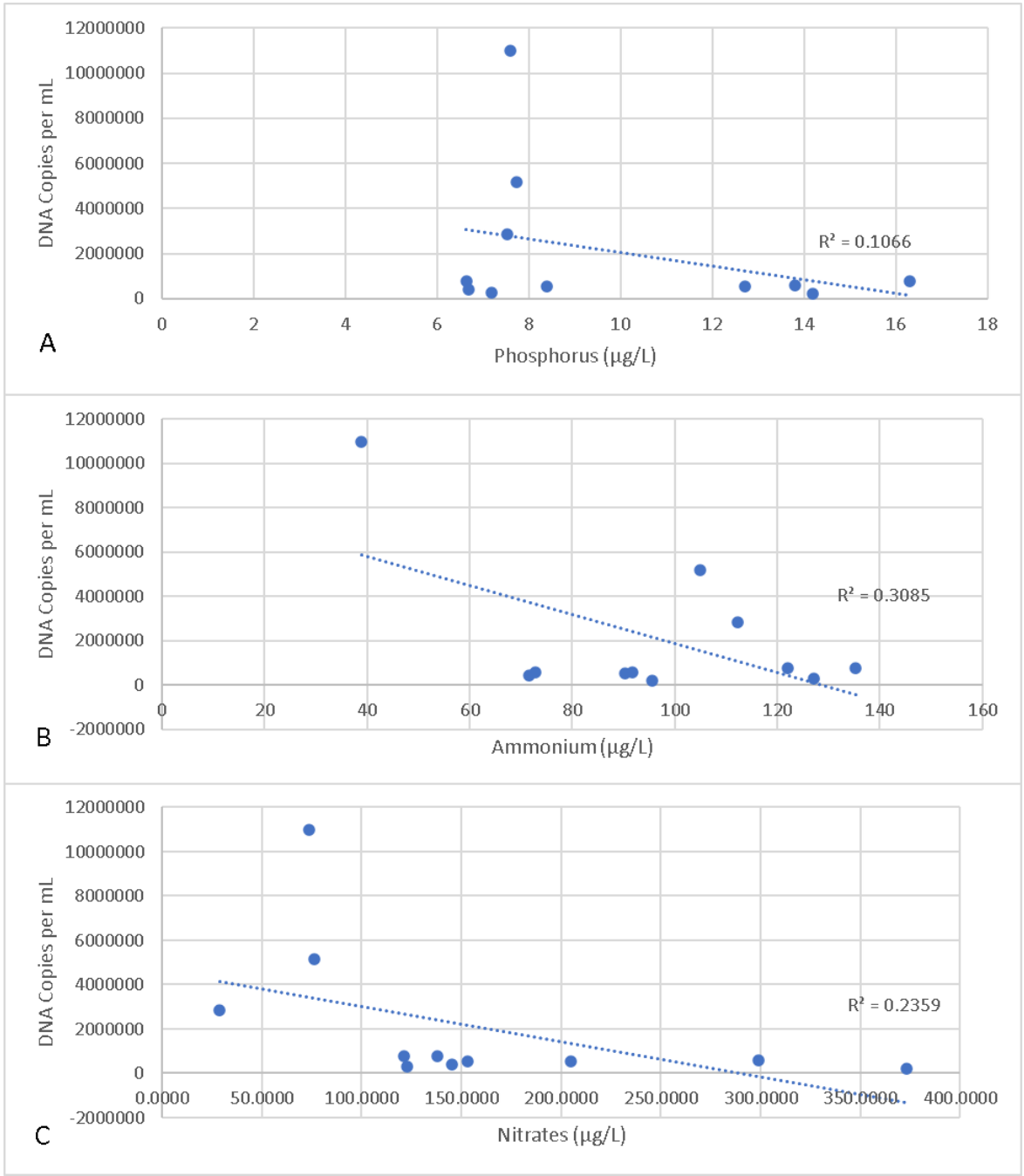

Figure 23. Correlations between total cyanobacteria DNA and nutrients in Lake Whelchel. Correlation strength was weak with phosphorus (A), moderate with ammonium (B) and weaker with nitrates (C)

Lake Bowen Nutrient and Chlorophyll a Correlations

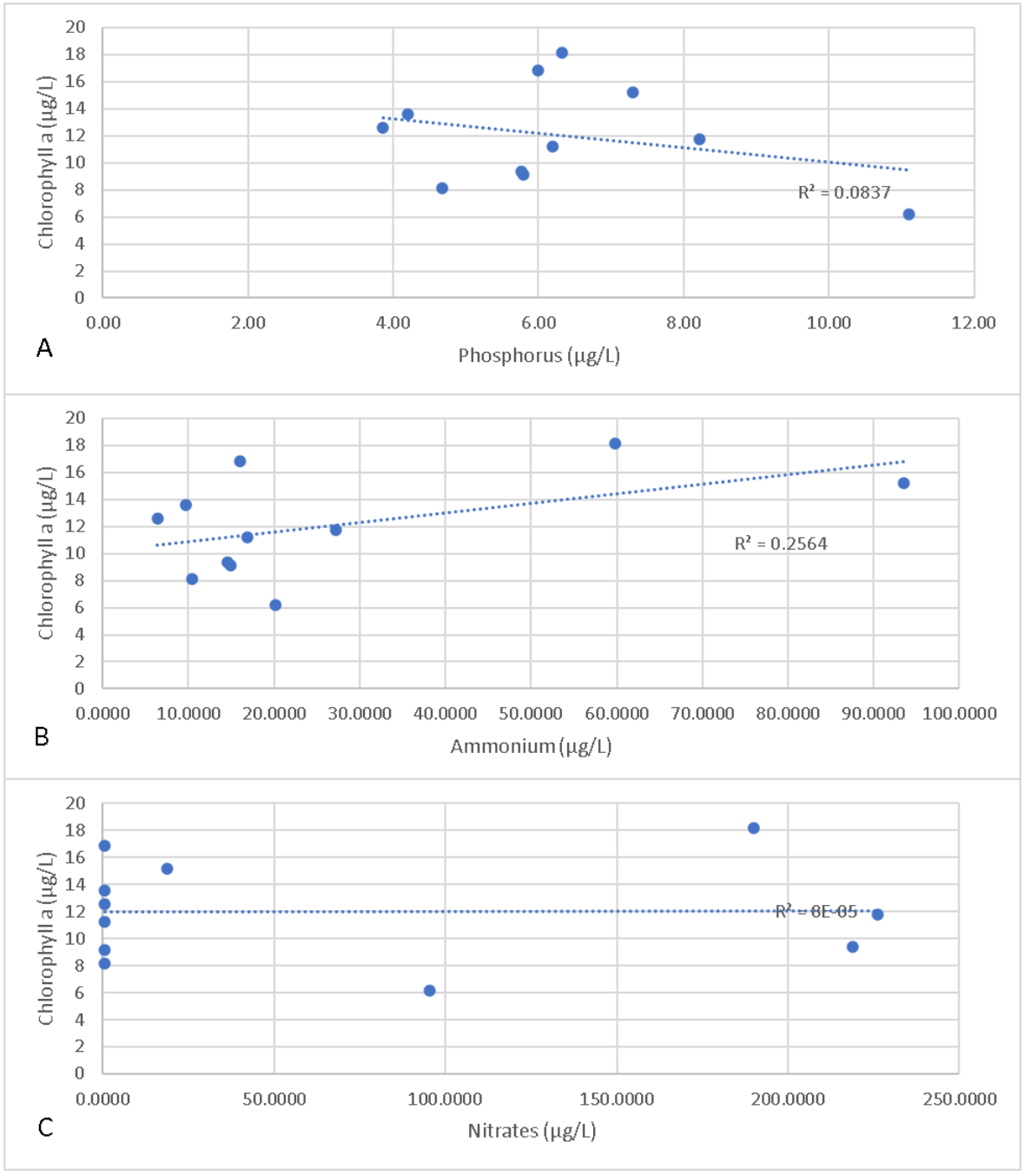

Figure 24. Correlations between total chlorophyll a and nutrients in Lake Bowen. Correlation strength was weak with phosphorus (A), approaching moderate with ammonium (B) and extremely weak with nitrates (C)

Lake Bowen Nutrient and Total Cyanobacteria Correlations

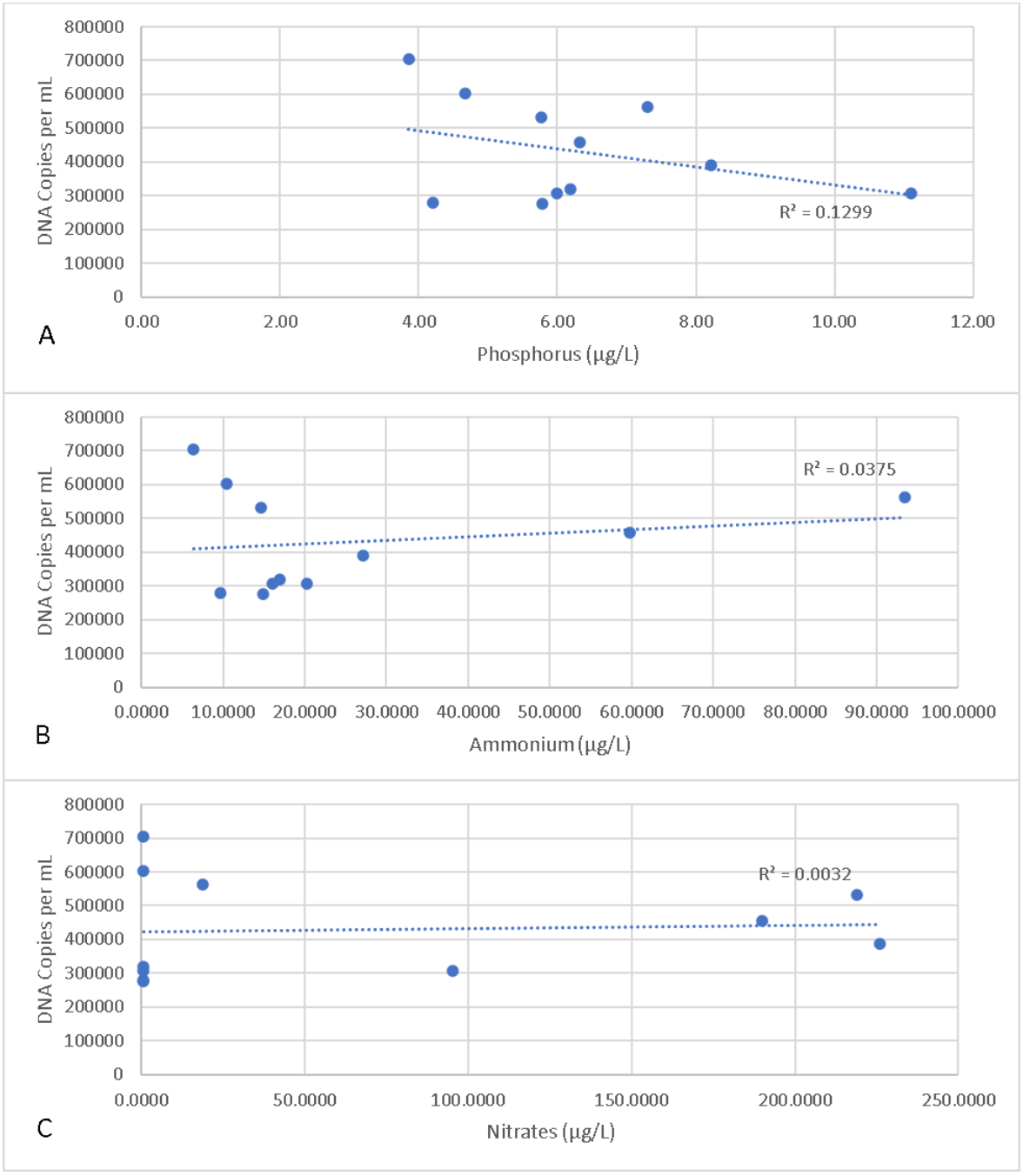

Figure 25. Correlations between total cyanobacteria DNA and nutrients in Lake Bowen. Correlation strengths were extremely weak for phosphorus (A) ammonium (B) and nitrates (C)

Lake Greenwood Nutrient and Chlorophyll a Correlations

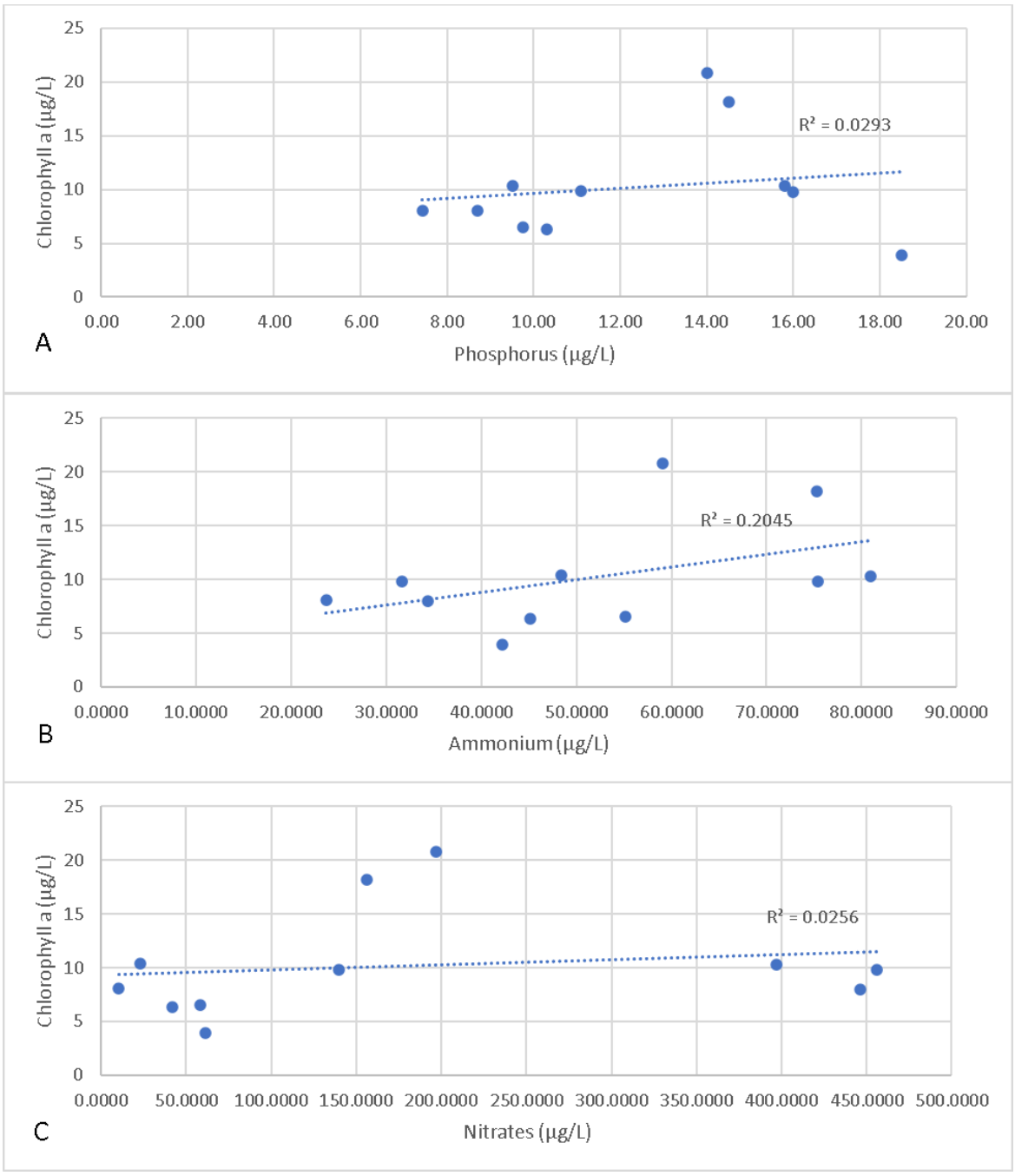

Figure 26. Correlations between total chlorophyll a content and nutrients in Lake Greenwood. Correlation strength was weak for phosphorus (A), approached moderate but still weak for ammonium (B) and was weak for nitrates (C)

Lake Greenwood Nutrient and Total Cyanobacteria Correlations

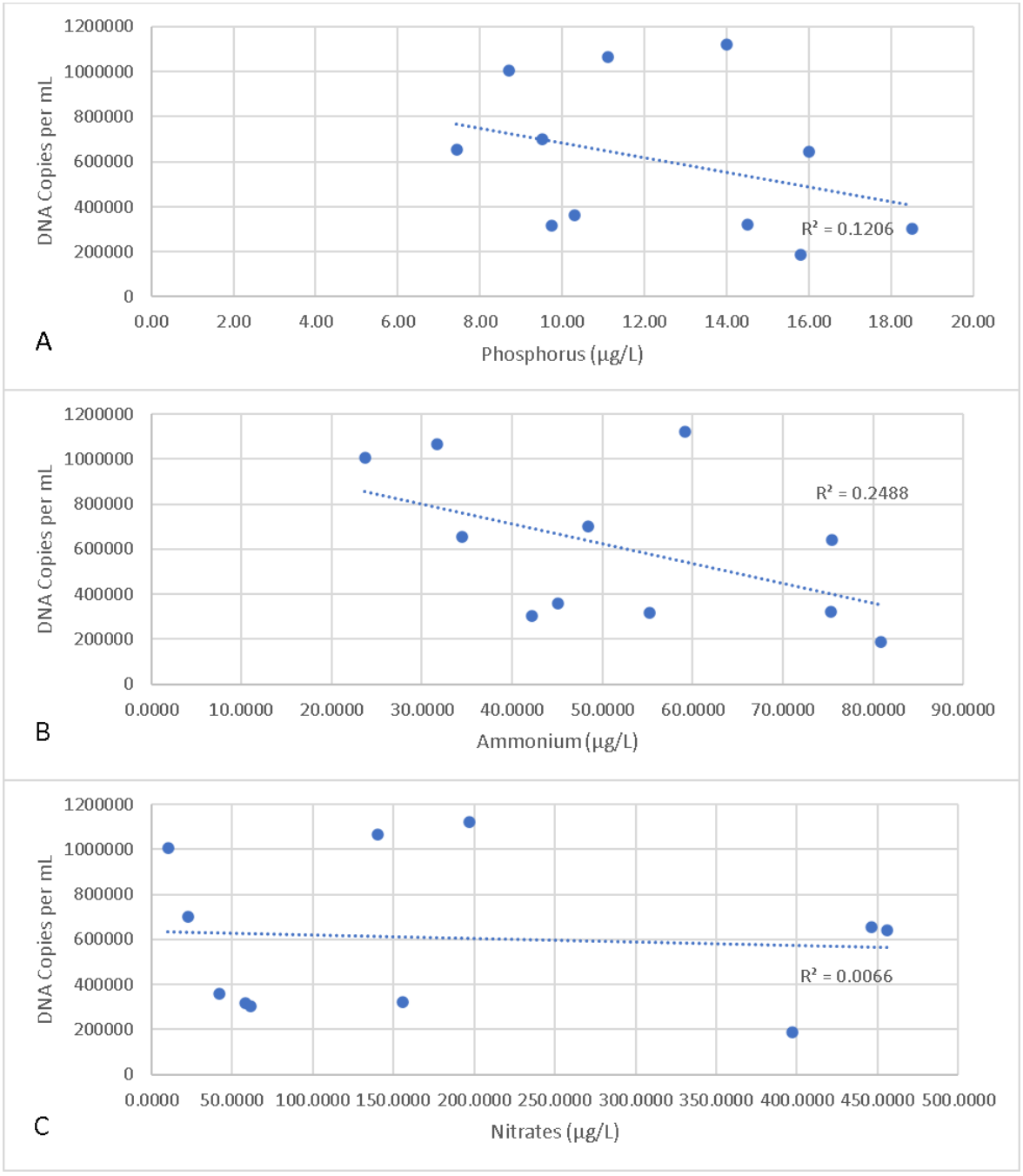

Figure 27. Correlations between total cyanobacteria DNA and nutrients in Lake Greenwood. Correlation strength was weak for phosphorus (A), approaching moderate for ammonium (B) and weak for nitrates (C)

Lake Rabon Nutrient and Chlorophyll a Correlations

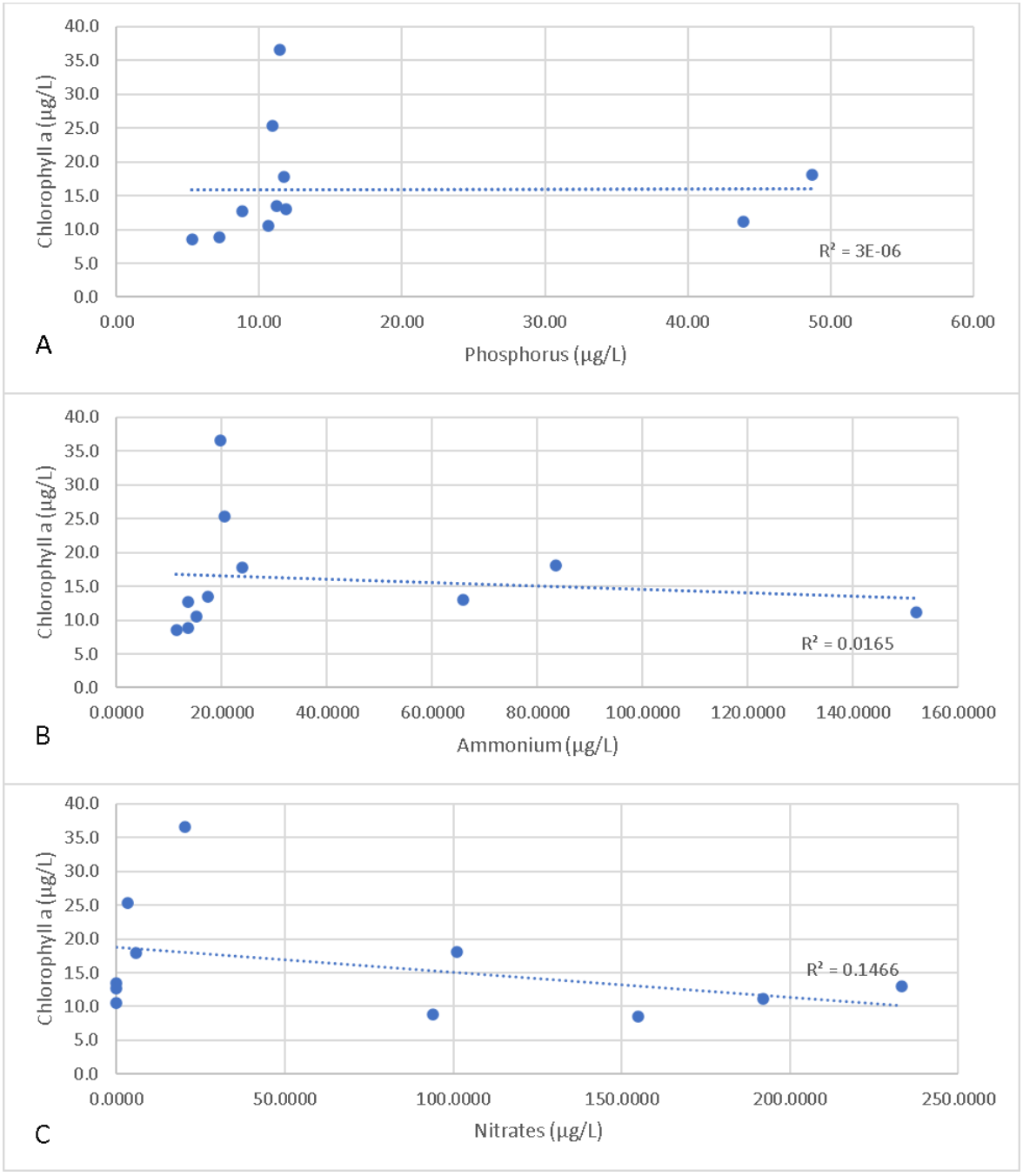

Figure 28. Correlations between total chlorophyll a content and nutrients in Lake Rabon. Correlation strengths were weak for phosphorus (A), ammonium (B) and nitrates (C)

Lake Rabon Nutrient and Total Cyanobacteria Correlations

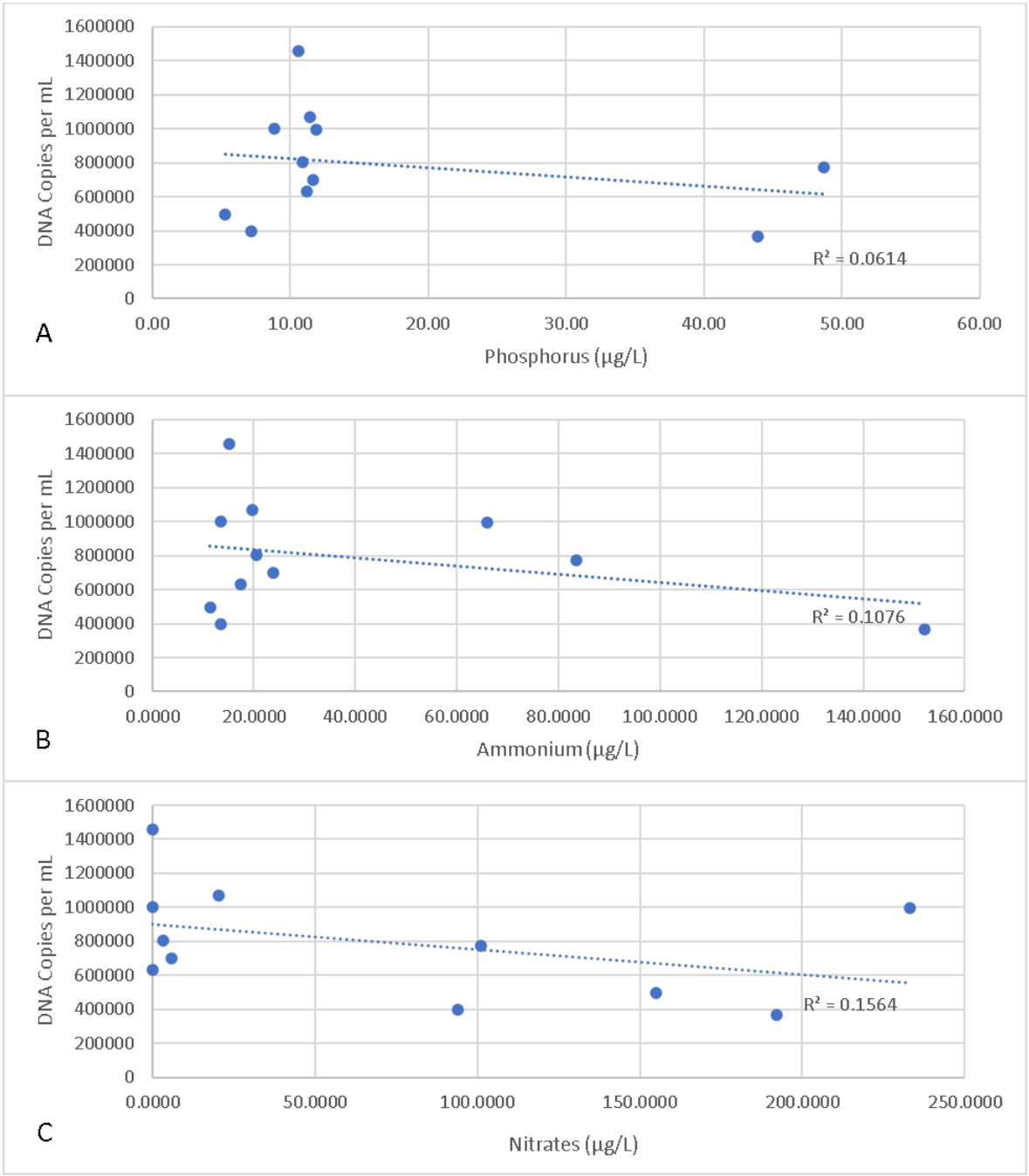

Figure 29. Correlations between total cyanobacteria DNA and nutrients in Lake Rabon. Correlation strengths were weak for phosphorus (A), ammonium (B) and nitrates (C)

4.0 Discussion

4.1 Environmental Conditions and Algae Growth

This study confirmed typical seasonal dynamics for nutrients in each of the four sites. Denitrification is a common seasonal event in freshwater lakes according to USGS freshwater lake surveys, typically beginning in the spring and decreasing to lowest levels in the summer months. Ammonium decreases are typically associated with increasing water temperatures and the incorporation of ammonium into algal biomass, which was again consistent with the results from this samplings season. Finally, relatively consistent levels of phosphorus is a common seasonal event during the summer months. The results from each site reflected these well-established dynamics except for the occasional spike in certain nutrients which may be attributed to a variety of anthropogenic sources, such as fertilizer runoff considering the agricultural nature of the region. The quantity of each nutrient is for the most part higher in the two smaller sites, Lake Whelchel and Lake Rabon, which are substantially smaller compared to Lake Greenwood and Lake Bowen. It is interesting to note that Lake Whelchel is substantially smaller than any other site and yet the concentration of the nitrates and ammonium was high compared to the other sites, and the concentration of phosphorus was similar for all the sites. The raised levels of nutrients in Lake Whelchel relative to the size of the lake is likely a contributing factor to the higher concentrations of algae growth observed compared to the other sites. The trend of algae growth and nutrient fluxes in Lake Whelchel is also more consistent with observed patterns in other studies, where the peak of algal growth coincides with lows in concentrations of nitrates and nitrogen (Dalu and Wasserman). Lake Bowen and Lake Greenwood both maintained consistent albeit low concentrations of phosphorus over the

duration of the season and had lower concentrations of ammonium than the smaller sites. Lake Greenwood did have a high nitrate concentration at the beginning of the season but was quickly reduced as seasonal denitrification occurred. Not only did Lake Greenwood contain very little cyanobacteria, as reflected by the qPCR results, the site also contained very little chlorophyll a in general, suggesting that algal growth in the lake is limited or more widely distributed than in the smaller sites. This is reflected in the results for Lake Bowen, which had observably lower concentrations of nutrients when compared to the smaller lakes, particularly lake Whelchel, and emphasizes that larger bodies of water are likely to have reduced or more widely spaced algal growth.

The correlations between total cyanobacteria DNA/Chlorophyll a content and nutrients trended negative and weak at best, but it is difficult to specify a single nutrient that is responsible for driving cyanobacterial blooms. Typically, phosphorus is recognized as the limiting nutrient in driving algal growth, but it is also recognized that cyanobacteria are efficient at both storing and recycling phosphorus to continue growing. In other lakes studies have shown that thresholds exist for the minimum amount of phosphorus needed to drive substantial algal growth. A concentration of 30 µg/L was established as the threshold for driving substantial algal growth in Lake Okeechobee. At the 30 µg/L the risk of significant algal blooms increased and maximum risk began when phosphorus concentrations exceeded 60 µg/L (Walker and Havens, 1995). There were only two samples taken over the entirety of the sampling season with a phosphorus concentration of over 20 µg/L, and these peaks, both observed in Lake Rabon, were short lived and immediately returned to typical levels in a two-week span. Other studies have shown phosphorus concentrations nearly fivefold of observed conditions for the four sites,

averaging over 100 µg/L of phosphorus for lakes with particularly intensive algal blooms (Xu et al 2015). Phosphorus quantities remained relatively constant, and subsequently it is difficult to say with any certainty that the limiting nutrient in any of the four sites was phosphorus. Recent studies recognize that the constantly shifting dynamics of lakes due to rainwater inputs and subsequent overland runoff means that it is possible for phosphorus and nitrogen to be colimiting (Piranti et al, 2018). Despite this, it would be difficult to say that phosphorus is the main driver of algae blooms in any of these systems. It would seem most likely that the consumption of nitrates is driving the increase in chlorophyll a concentration and consequential increase in biomass in Lake Whelchel, considering that the pattern of denitrification is near identical temporally to the algal bloom's maximum point in that lake.

A contributing factor to the lower nutrient content in Greenwood and Bowen is the streamflow dynamics of the source rivers for each lake. Lake Greenwood is fed by the Saluda and Reedy Rivers and Lake Bowen is fed by the South Pacolet River, and have substantially more streamflow than Whelchel and Rabon, which dilutes the relative quantity of nutrients in the water column through nutrient dilution. Additionally, rainfall events further dilute large water bodies to a greater degree than smaller water bodies, providing a further explanation for lower nutrient concentration for Greenwood and Bowen.

Additional confidence in the accuracy of our nutrient data is provided by historical data as the result of a USGS study performed in Lake Bowen from August to September in the year 2005 (Journey and Abrahamsen, 2008). Total phosphorus concentrations for those two months ranged consistently between 10 and 20 µg/L for water samples taken near the surface of the lake, which was very consistent with our reported values. Chlorophyll a content for surface

samples during those two months was typically around 6 µg/L, which was slightly less than our reported values, which were typically between 10 and 20 µg/L. Our results are similarly reflected in other USGS studies performed on drinking water reservoirs elsewhere in South Carolina. Bushy Park Reservoir near Charleston, South Carolina was surveyed from September 2013 to April of 2015 (Conrads et al, 2018). This reservoir is approximately 800 acres, which is roughly half the size of Lake Bowen, but still substantially sized when compared to Whelchel and Rabon. Chlorophyll a concentration ranged between 6.4 and 11.2 µg/L. Total nitrogen content ranged between 330 µg/L to 470 µg/L. Phosphorus concentrations were between 20 µg/L and only reached a maximum of 65 µg/L, during which time the chlorophyll content was 11.2 µg/L.

4.2 Differing Cyanobacterial Speciation

Flow cytometry and detection of total cyanobacteria DNA provided insight into the composition of the overall algal community in each lake. The strong correlation between chlorophyll a content and cyanobacteria in Lake Whelchel shows clearly that the dominant species of algae in that lake is cyanobacteria, a large proportion of which consists of *microcystis* cells. One study surveying the relative abundance of cyanobacteria in relation to other algal species in water treatment facilities in freshwater reservoirs in Sudan showed that in the summer months the ratio of cyanobacteria to all other algal species was over 2:1 (Kheiralla et al, 2014).In the other three sites the algal population is more equally proportioned. The considerably lower concentration of Cyanobacterial DNA in Lake Bowen and Lake Greenwood paired with the very

weak correlations between the total chlorophyll content and cyanobacterial DNA reaffirms that the populations of algae in Bowen and Greenwood consist of a larger relative proportion of other algae species such as chlorophytes and large species of phytoplankton, or freshwater diatoms. Flow cytometry results also showed a more equal distribution of algae types in Bowen and Greenwood but it is important to be aware that flow cytometry distinguishes cells from one another on the basis of fluorescence and cell size, so the divisions between cell types is not totally accurate. Some cells may be abnormally large or have variations in pigmentation and may subsequently be included in an incorrect group.

In the case of Lake Whelchel, the concentration of chlorophyl a and subsequent increase in the total cyanobacteria population, *Microcystis* represents roughly one third of the entire cyanobacterial community, meaning that *Microcystis* represents a substantial portion of the overall algal community in this Lake. This is of particular interest when considering the environmental conditions surrounding Lake Whelchel at the peak of the bloom season, where nitrates were at their lowest available concentrations and phosphorus was still at relatively low concentrations. Studies have postulated that when environmental conditions are suboptimal for algal communities, characterized by conditions like limited nutrient availability, that microcystin producing strains of cyanobacteria are better able to propagate than non-microcystin producing cells, which suggests some benefit to survivability for the cells that are able to produce microcystin over the cells that cannot (Briand et al. 2008). Subsequently it seems likely that the *Microcystis* community in Lake Whelchel was able to out compete the other species of algae given suboptimal environmental conditions. Additionally, *Microcystis* has been documented displaying the ability to alter its position in the water column in order to

obtain sufficient quantities of sunlight exposure, and directly compete for position close to the surface with other algae species (Yang et al 2012). Further still, a potentially significant reason why *Microcystis* represented a large proportion of the algal community of Lake Whelchel and not the community of Lake Rabon, is that *Microcystis* have been observed to be stimulated to a greater degree by the presence of nitrogen rather than the presence of phosphorus (Kim et al 2020). *Microcystis* blooms have shown that the levels of microcystin in algal blooms that were caused by heightened levels of phosphorus was not different than control groups (Kim et al 2020). Cell densities of Microcystis in controls treated with an excess of nitrogen however were found to be substantially higher than in the control groups. One reason for this is that *Microcystis* cells are not heterocysts, meaning that they cannot fix atmospheric nitrogen like some other species of algae, and instead rely on the presence of nitrates and ammonium in the water column, and are able to offset nitrogen deficits by storing nitrogen (monchamp et al 2014). Once the nitrogen available in the water column has been consumed, the time it takes for total biomass to return to typical levels is quick as *Microcystis* communities can no longer sustain themselves and return to typical levels (Shen et al 2020). Regarding Lake Whelchel and Lake Rabon, the two sites with *Microcystis* cells present, the quantity of phosphorus present in each lake was near identical during the time of the sampling season when *Microcystis* cells were observed, with concentrations of around 10 µg/L, which as stated previously in this discussion, is likely not high enough to drive substantial algal bloom growth. The key difference between the two sites however is the substantially higher concentration of both nitrates and ammonium present in Lake Whelchel around the time of the year when conditions for algal blooms, specifically blooms involving *Microcystis*, occurred. The already relatively low nitrogen

concentration at the beginning of the spring, paired with the natural occurrence of denitrification in Lake Rabon and subsequent near absence of nitrates and ammonium, did not provide the conditions necessary for substantial *Microcystis* algal bloom growth, and the lack of substantial quantities of phosphorus in all sites did not provide enough nutrients for substantial growth of other species of algae who's growth is typically stimulated by the presence of phosphorus, such as diatoms and chlorophytes . The summation of the environmental factors observed over the course of this study reasonably explain why Lake Whelchel was the only site with a substantial albeit short lived algal bloom, whereas the lack of nutrients in the other sites explains the limited algal growth over the course of the spring and summer.

5.0 Conclusion, Recommendations, and Future Research

The first goal of this project was to establish any potential seasonal peaks in algae growth for the four sites. In Lake Greenwood and Lake Bowen, no substantial peak was observed, with fluctuations occurring regularly over the season. Lake Rabon saw a small but steady increase in algal growth. Lake Whelchel saw substantial growth during the end of July and August. The second objective was to establish any relationship between total algal growth and the presence of cyanobacteria in the sites. Correlations between the total presence of cyanobacteria and the total content of chlorophyll a varied depending on site. The third objective was to establish what, if any, environmental factors influenced the development of these algal blooms. It is likely that the discrepancy among the sites is due to the variation of available nutrients and the species of algae present in distinct lakes and water layers. Considering that all four lakes generally experienced the same air temperature, rainfall and similar streamflow patterns given their size in relation to one another, the deciding factor in the severity of algal growth in the four sites was likely determined largely by nutrient content and the algal communities already present in each lake, and potentially other factors that were not recorded during the first sampling season like temperature and pH. Nutrient conditions in Lake Whelchel were optimal for a large cyanobacterial algal bloom and were particularly appropriate for the growth of *Microcystis* cells. Lake Rabon had a small observed algal bloom but was likely unable to experience larger growth owing to limited nutrient availability. Despite having the greatest concentration of bioavailable nitrogen, Lake Greenwood does not have an observable *Microcystis* community at least in the area that was sampled and was therefore seemingly unable to support a *Microcystis* algal bloom during this sampling period. Additionally, low bioavailable phosphorus content in Lake Greenwood inhibits the growth of other species of

algae. Finally, Lake Bowen generally had the lowest measured nutrient content, the lowest chlorophyll a content and the lowest cyanobacterial presence. In summary, the nutrient load in Lake Whelchel, specifically the excess quantities of aqueous nitrogen species, was substantial enough to drive algal blooms that largely consisted of cyanobacteria. In Lake Bowen, Lake Greenwood, and Lake Rabon, nutrient load conditions were not substantial enough to drive excessive growth of algae, and therefore these lakes did not experience algal blooms during the sampling period.

Continuation of nutrient load management for Lake Bowen and Lake Greenwood will be sufficiently appropriate for controlling algal communities. Lake Whelchel and Lake may benefit from further limitations to introducing nitrogen-based fertilizers into surrounding farmlands. As stated previously, phosphorus is not likely to be a significant driver of algal growth in these two lakes, though if water quality managers in these areas were interested the use of chelating agents like EDTA or ferric humate could be employed to further sequester phosphorus from the aquatic environment. Additional future research could explore which specific algal species in these sites are responsible for producing taste and odor compounds to develop a management strategy specifically aimed at taste and odor compound producers. Finally, development of a management strategy for controlling *Microcystis* populations is a potential avenue of research that would help to reduce public exposure to potentially harmful chemical.

The project objective of establishing a method for the analysis of taste and odor compounds is still in progress. Establishing a separation for the individual compounds isolated in methanol has been achieved, but a technique for extracting the compounds from water samples is still in development for use on the instrument available. Solid phase microextraction, a technique

used in the analysis of these compounds using gas chromatography, is one avenue currently being explored. Future research regarding water samples will include additional parameters like water temperature, conductivity, and pH. A second sampling season began in March 2021 and ended at the beginning of October 2021. The addition of a Sonde YSI probe for the 2021 sampling season will provide these additional parameters. One potential area of difficulty regarding these additional parameters, and even parameters from this sampling season, is that pH is directly dependent on temperature. A single sampling trip takes most of a day, so it is not possible to take samples at the same time, which will provide a degree of variation that is out of our control. Additional support was provided by the Clemson University Department of Environmental Engineering and Earth Sciences, providing us geosmin and 2-methylisoborneol measurements for a portion of the season, including the month of August and September when blooms are expected to be the most severe. Microscopy of site samples could also provide additional insight into algal community speciation for each lake, which could help determine further management strategies, as responses to algaecides may vary depending on species. Finally, measurements of the compound microcystin measurements via quantitative PCR could be added to provide an overview of how toxins are released temporally in relation to algal blooms.

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Appendix A: Method Development

Ultraperformance Liquid Chromatography Determination of Geosmin and 2-Methylisoborneol

Introduction

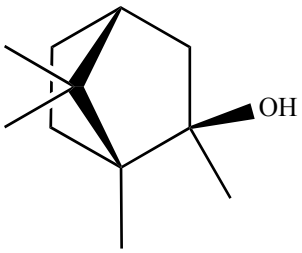
Geosmin and 2-methylisoborneol (MIB) are common secondary metabolites produced by cyanobacterial blooms in freshwater lakes, and the potentially the most ubiquitous and frequently reported taste and odor compounds (Jüttner and Watson, 2007). These compounds are nontoxic but are considered a nuisance for communities that rely on freshwater lakes as a source of drinking water, as they add a musty, earthy odor to water that proves difficult for drinking water treatment facilities to remove via traditional methods. Monitoring and measuring the presence of these compounds is of particular interest to identify when the compounds will be at their highest concentrations so that water treatment facilities can develop strategies for mitigating the issue at hand, either via limiting the presence of cyanobacteria or by developing water treatment strategies that may only need to be used intermittently when the compounds are at their peak. Determining if the concentrations of these compounds is associated with any specific environmental condition could potentially help develop an environmental control strategy for water quality managers.

Geosmin and MIB are both terpenes. These compounds are cyclical aromatic hydrocarbons commonly produced by plants and bacteria. Geosmin is bicyclic with two hydrocarbon rings (Jiang et al 2007), while MIB has an irregular structure for a terpene, with a single ring and a bridge that connects both sides of its single ring (Giglio et al 2011). Both structures are very stable and are

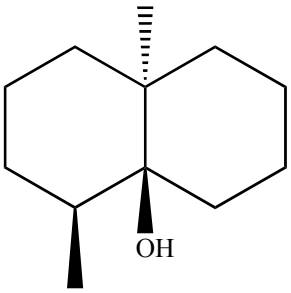
resistant to traditional methods of chemical decay like oxidation, screening, and flocculation (Bruchet 2004, Oh et al, 2017). These strategies may be effective for the removal of cyanobacterial blooms, but they typically fail to break down and remove the compounds from source water.

The source of these compounds is typically cyanobacterial blooms in freshwater lakes. Additionally, actinobacteria in soil are known producers of these compounds (Gerber and Lechevalier, 1965), although there is only speculation whether these bacteria are significant contributors of geosmin and MIB into the water column, which would have to be measured via groundwater flow into streams and lakes. Both geosmin and MIB are known secondary metabolites of the synthesis of isoprenoids in cyanobacteria, a class of compounds with a wide variety of biological functions. There are two established pathways to produce these two isoprenoid compounds, one referred to as the mevalone pathway and one simply referred to as the non mevalone pathway (Bentley et Meganathan, Dickschat et al, Flesch and Rohmner) diphosphate, which is then metabolized into MIB via 2-MIB synthase.

2-Methylisoborneol



Geosmin



One established method for measuring geosmin and MIB in water samples is via gas chromatography interfaced with mass spectrometry. This method involves the use of solid phase microextraction to remove the compounds from water samples, after which they are injected directly into the gas chromatography apparatus. Establishing a method via the use of ultraperformance liquid chromatography was a decision made based on the availability of instruments.

Materials and methods

For our method, an ultraperformance liquid chromatograph (**UPLC**; Acquity H-Class, Waters Inc.) coupled to a tandem mass spectrometer (**MS/MS**; XevoTQ-S, Waters Inc.) fitted with an atmospheric pressure chemical ionization (**APCI**, Waters Inc.) probe was used. Supelco. geosmin and MIB standards were purchased from Sigma Aldrich in 1 mL vials. Standards came premixed in 100 ug/mL volumes in methanol. UPLC grade methanol from Sigma Aldrich was purchased for use as mobile phase. UPLC grade water was purified in lab.

Using the parameters established by Bedner and Saito, initial peaks for each compound were established. A trial-and-error process of altering the time and percentage of each eluent was performed until each peak was sufficiently separated from one another. A standard curve was then established by creating a series of 5 serial dilutions in a 1:10 ratio and integrating the area under each peak.

Results and Discussion

Our method for monitoring the presence of geosmin and 2-methylisoborneol as it exists now was adapted from Bedner and Saito (2020). By adapting this method and adjusting it to fit the instrument available, separation of individual peaks was achieved. Separation is achieved over the course of a three-minute run for each sample, using geosmin and 2-methylisoborneol standards in methanol. It was determined that the best mobile phase for this separation is UPLC grade water and methanol. The first peak in the separation was determined to be 2-methylisoborneol. After the 2-methylisoborneol peak, three individual peaks can be fully separated from one another, which were determined to be three different stereoisomers of geosmin, which was determined based on the structure of the compound. The following conditions were selected for the UPLC and MS/MS method based on repeated trial and error experiments.

Table 1: UPLC MS/MS method parameters.

UPLC Column (Waters Inc.) Parameters				
Type	*Particle Size (mm)*	*Diameter x Length (mm)*	*Temperature (°C)*	*Part #*
Acquity BEH C18	1.7	2.1x 50	65	186002350

UPLC Eluent Program			
Time (min)	*Flow Rate (mL/min)*	*% Water*	*% Methanol*
0.00	0.75	25	75
0.15	0.75	25	75
1.50	0.75	15	85
2.00	0.75	15	85
2,01	0.75	25	75
3.0	0.75	25	75

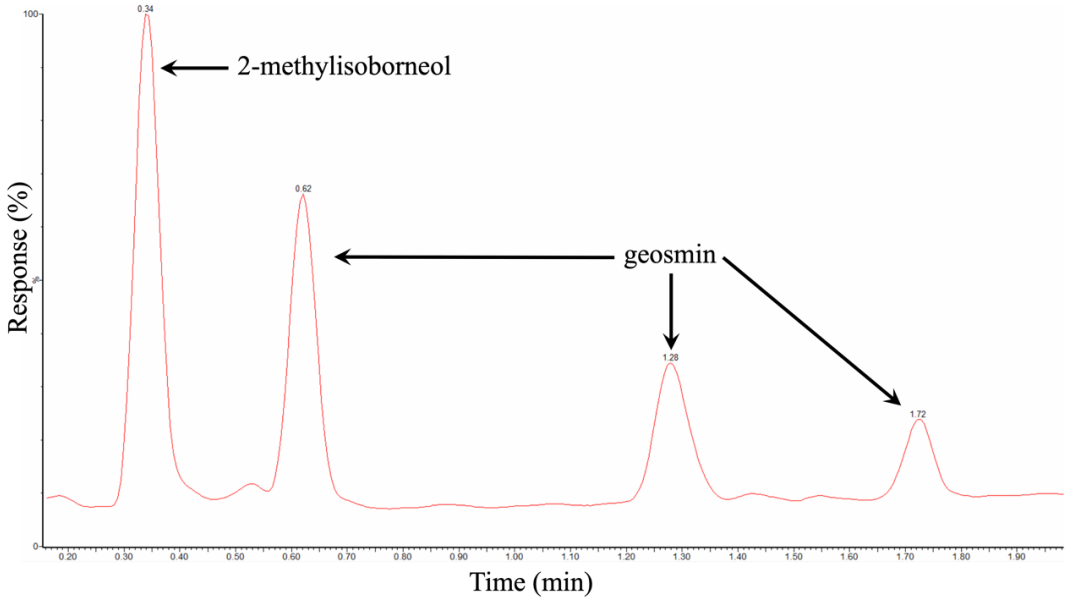
Ionization Source (APCI) Parameters	
Source Temperature (°C)	*Desolvation Gas Flow (L/min)*
600	1000

MS/MS Parameters							
Compound	*Molecular Weight, g/mol*	*Parent Ion, m/z*	*Quantifier Ion, m/z*	*Qualifier Ion, m/z*	*Cone voltage, V*	*Collision Energy, V (quantifier ion)*	*Collision Energy, V (qualifier ion)*
MIB	168.28	168.28	95.12	67.02	26	14	22
Geosmin	182.31	162.97	81.12	92.57	2	14	18

Using the parameters described in table 1, separation of MIB and geosmin was achieved from standard solutions (Figure 1). As reported in Bedner and Saito (2020), our method results in the geosmin standard separating into three distinct peaks. Bedner and Saito (2020) suggests that the geosmin is reacting to the stationary phase of the column leading to separation of different isomers. Geosmin (Figure 2) contains three chiral centers leaving open the possibility of enantiomers that may account for more than one chromatographic peak representing geosmin.

2-Methylisoborneol was represented by one peak as in Bedner and Saito (2020). However, MIB also has three chiral centers (Figure 2) offering the possibility of MIB enantiomers. In fact, during a recent UPLC MS/MS experiment (not reported herein) using the MIB parent ion, 168.28 *m/z* and an in-source degradation ion, 150.97 *m/z* (water molecule loss), a second MIB peak has been identified. Further work is needed to determine how these multiple peaks will need to be handled for quantification of geosmin and MIB in environmental water samples. Bedner and Saito (2020) handled the situation by modifying the procedure to obtain only one peak for geosmin. However, this approach ignores the unique chemistry that may exist for each isomer and thus, potential environmental impacts.

Figure 1. Chromatographic (Total Ion Count) separation and detection of geosmin (71.88 ng on column) and MIB (0.69 pg on column) per the parameters in Table 1.



The current method (Figures 3 and 4) is capable of measuring geosmin and MIB over a linear range of concentrations of 18 to 575 ng and 0.34 to 5.5 ng on column, respectfully. It is apparent from the standard curve results, that there is a difference (approximately 100-fold) in sensitivity of the method in measuring geosmin and MIB. Bedner and Saito (2020) reported a similar issue with their method. The apparent difference in sensitivity may be due to ionization and/or chromatographic separation efficiency differences for the two molecules.

As the method currently exists, limits of detection and quantitation are not yet established. Before these limits can be established further development of the assay for increased peak resolution and instrument sensitivity must be established and the assay must be validated. The standard curves (Figures 3 and 4) generated from the preliminary method indicate that geosmin can be quantified down to 18 ng and MIB can be quantified down to 0.34 ng on column. Without any method to concentrate samples, instrument sensitivity is not able to detect the compounds in environmental waters samples. Given the report of Bedner and Saito (2020) attaining detection limits in the low part per billion (ng/L) range, we are confident that adjustments to MS/MS parameters, chromatographic separation and sample preparation method will result in similar if not better sensitivity.

Figure 3. 2-Methylisoborneol standard concentrations curve.

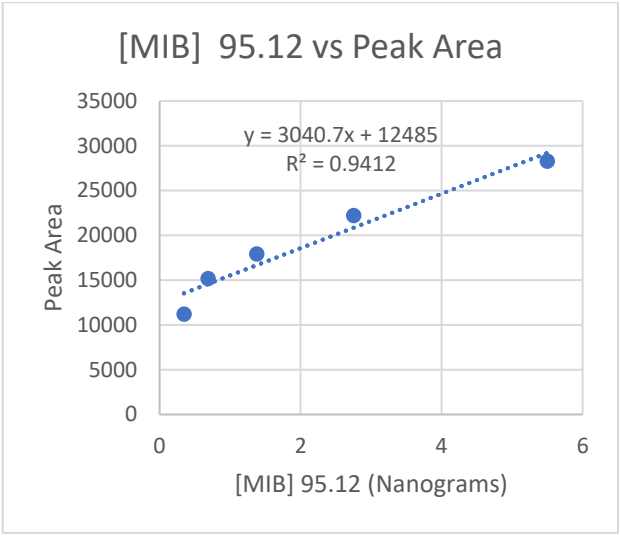
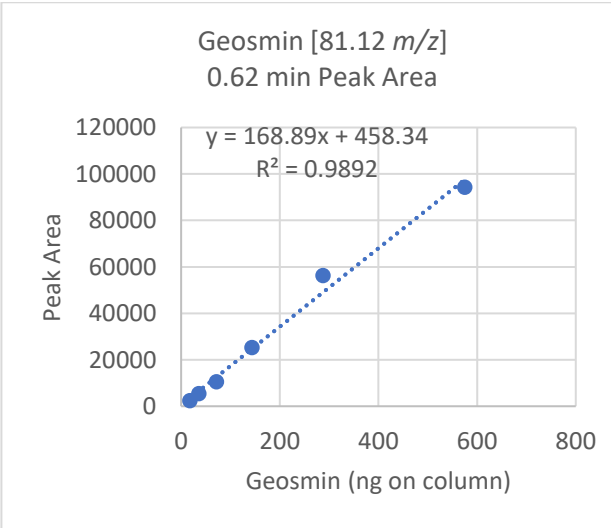
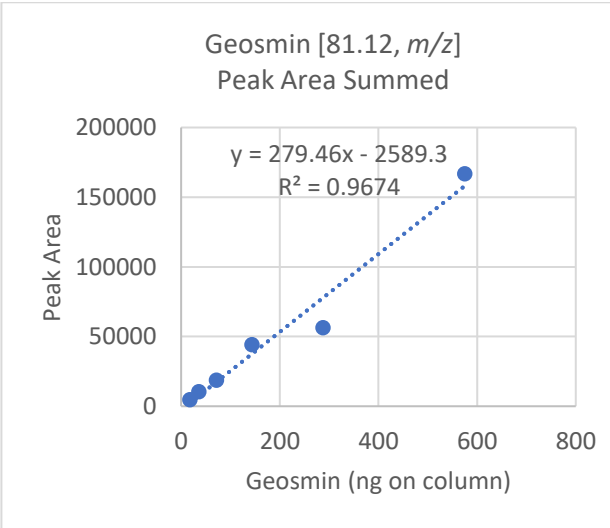


Figure 4. Geosmin standard concentration curves. Panel A – Geosmin concentration versus 0.62 min peak area under the curve. Panel B – Geosmin concentration versus summed areas under the curve for the three geosmin peaks.

Panel A



Panel B



There are several steps left to finish for establishing a standard analysis of water samples for geosmin and 2-methylisoborneol using this instrument. Improving the assay sensitivity and fully validating the method using standards is yet to be completed, as is selecting an internal standard for the instrument that is appropriate for our purposes. Additionally, several methods for concentrating the chemicals of interest out of environmental samples is still being explored. Solid phase microextraction is one method that is commonly used for this measurement using gas chromatography. Other more traditional solid phase extraction materials are also being explored as potential avenues of concentrating geosmin and 2-methylisoborneol.