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Prevalence and Persistence of Cyanotoxins in Lakes of the Puget Sound Basin

Cyanotoxin production in Spanaway Lake (Pierce County, Washington) during the summer of 2021

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Prevalence and Persistence of Cyanotoxins in Lakes of the Puget Sound Basin

Cyanotoxin production in Spanaway Lake (Pierce County, Washington) during the summer of 2021

by

William Hobbs

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Abstract

Cyanobacteria (or blue-green algae) are common in many inland waters worldwide. Several planktonic cyanobacteria species can produce toxins that harm humans, pets, and wildlife. Spanaway Lake in Pierce County (Washington) has a history of cyanobacterial blooms. The current project in Spanaway Lake (1) documented the succession of cyanobacteria from May to October, (2) measured toxins and toxin-producing genes in the water column, and (3) explored associations among nutrients, cyanobacteria communities, and cyanotoxin production.

Weekly profiling of the water column demonstrated that by early June, the water column was strongly stratified, and the bottom waters contained very little oxygen; there was early summer growth of cyanobacteria in the lake's bottom waters. In late July and early August, phycocyanin (PC, a cyanobacteria pigment) concentrations peaked at a depth of 5m, and subsequently, PC was measured throughout the water column until October. In the anoxic bottom waters and at the sediment surface, there was an increase of ammonia, iron, and dissolved inorganic phosphorus from the sediments beginning in early August; cyanobacteria growth had moved further up in the water column.

Microcystis aeruginosa was a small component of the cyanobacteria biomass but likely produced the microcystin measured in Spanaway Lake. The expression of the Microcystin genes (Mcy) was high between July and September. Genes covering all major microcystin-producing genera (McyE) and genes attributable to Microcystis (McyA) dominated. The microcystin concentration in the water positively correlated to McyA gene expression with a 2 – 3 week lag. There was a strong relationship between cyanobacteria growth (and microcystin production) and available nutrients. Cyanobacteria growth occurred mainly during periods of limited available nitrogen relative to phosphorus.

Introduction

Cyanobacteria (blue-green algae) are common in many inland waters worldwide. This diverse group of photosynthetic bacteria has a variety of life histories and habitat niches. The most widely recognized are the planktonic (open water) species. Some species can produce toxins (collectively called cyanotoxins) that harm humans and wildlife.

The success of cyanobacteria is linked to several environmental and physiological factors, including warm water temperatures, nutrient (nitrogen and phosphorus) enrichment of waters, the ability of cyanobacteria to store nutrients, thermal stratification of lake waters, and the ability of cyanobacteria to control their buoyancy (Hyenstrand et al. 1998; Paerl and Otten 2013). Recent genetic work has begun to define the specific characteristics of some regional cyanobacteria taxa, leading to a greater understanding of ecological niches and toxin production (Brown et al. 2016; Österholm et al. 2020; Dreher et al. 2021a). Quantitative polymerase chain reaction (qPCR) to amplify specific DNA sequences using complementary synthetic DNA molecules (primers) is now more common in environmental monitoring.

Spanaway Lake in Pierce County (Washington) has a history of cyanobacterial blooms. Previous monitoring of the blooms since 2007 has documented harmful concentrations of the cyanotoxin microcystin in shoreline accumulations (Figure 1). Microcystins (MCs) are hepatotoxins (affecting the liver) with over 200 variants or congeners. Seven congeners commonly comprise the bulk of the total MC in lake water (Catherine et al. 2017).

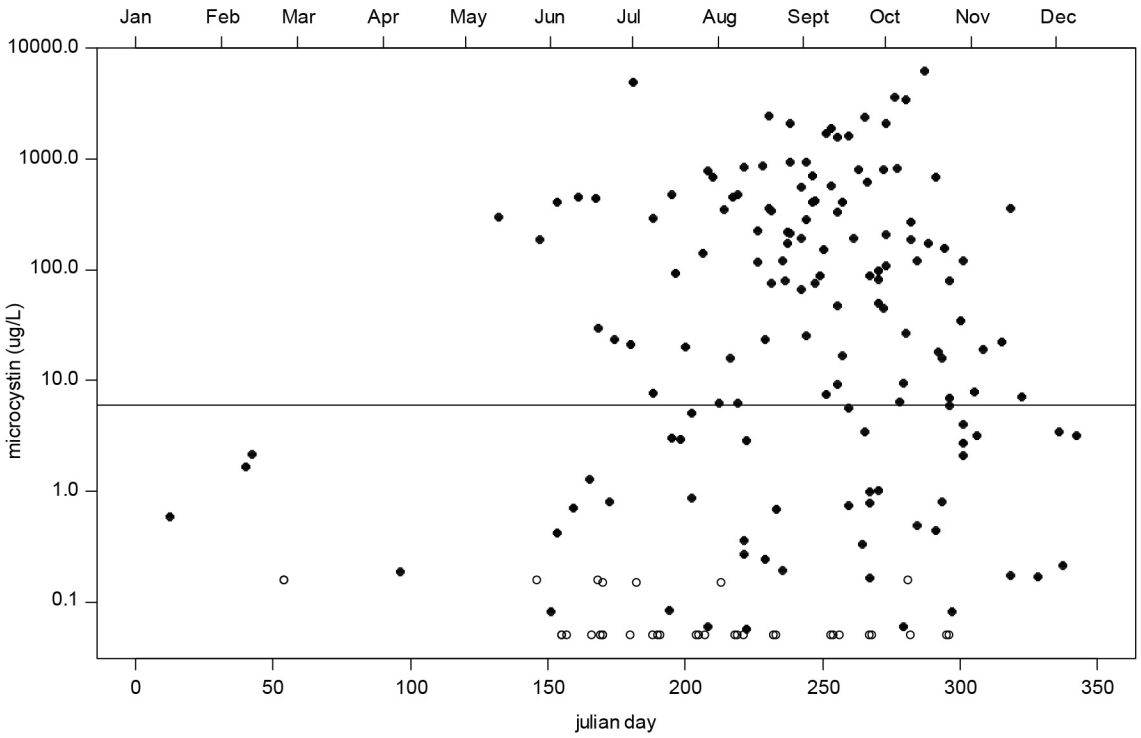

Figure 1. Concentrations of microcystin (µg/L) in Spanaway Lake from 2007 to 2022. Samples were collected through Ecology's Freshwater Algae Program (https://www.nwtoxicalgae.org/). The horizontal line is the 8 µg/L Department of Health state guideline for recreational use. Open circles represent results that are below the method detection limit.

The Washington State Department of Ecology (Ecology) has previously completed several studies on Spanaway Lake to investigate the monitoring and prevalence of MCs in different media throughout the lake. In 2018, Ecology and Washington State Department of Health collaborated on a project to assess the persistence and possible risk of cyanotoxins in public beach sediments (Preece et al. 2021). Samples from Spanaway Lake showed that MCs persisted in the beach sediments and porewaters well beyond a bloom in the surface water. However, MC concentrations in sediments did not represent a health risk to children or adults. Wildlife or pets were not assessed.

In 2019, Wong and Hobbs (2020) used a fluorometric probe to show a possible predictive relationship between MC concentration and the amount of phycocyanin pigment in the water. Phycocyanin is the main pigment (blue color) in cyanobacteria. In 2020, a sediment core was collected from Spanaway Lake, which showed that cyanobacteria have been present in the lake for at least the last ~150 years (Hobbs et al. 2022). Furthermore, the presence of MCs and MC-producing genes in the sediments from ~1850 show that toxins have also historically been present in the lake.

The goals of the 2021 Spanaway Lake study were to (1) document the temporal changes within the cyanobacteria from May – October, (2) measure toxins and toxin-producing genes in the water column, and (3) explore associations among nutrients, cyanobacteria communities, and cyanotoxin production.

Methods

Study Site

Spanaway Lake is an urban lake in Pierce County (Washington) (Figure 2). The lake has a long history of shoreline development and recreation. In the mid-1800s, a Hudson Bay Company subsidiary raised cattle, grain, and sheep on the shores of the lake. In the late 1800s, rail access to the lake was built, and the lake became a recreational destination. Today, much of the immediate lake shoreline is residential, with a park and golf course to the northeast of the lake. The surrounding watershed is mostly residential, with about 30% undeveloped land on the Joint Base Lewis-McChord (JBLM) southwest of the lake. It was not until the late 1990s that the golf course clubhouse and other park facilities were connected to a sanitary sewer system; the same is true of the Coffee Creek Condominiums adjacent to the main surface water inlet for the lake. All residences on the lake shore and many within the watershed rely on on-site septic systems (OSS). The watershed is estimated to have over 4000 OSS (Pierce County 2016).

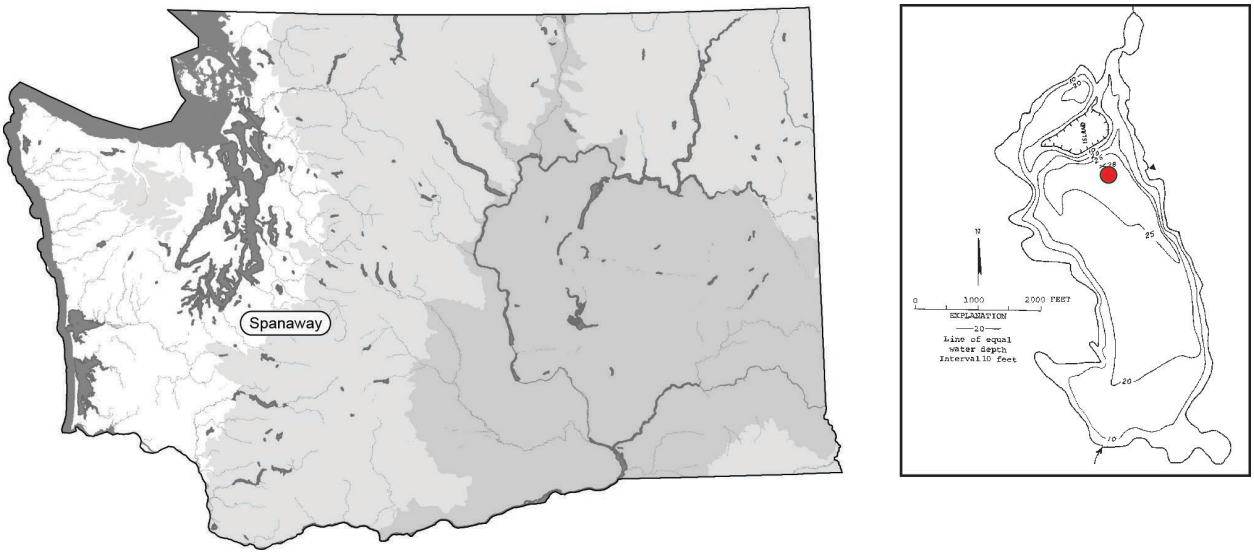

Figure 2. Site location map.
Washington State map showing the major biogeoclimatic zones and the location of Spanaway Lake in the Puget Sound Lowlands (left); a bathymetric map of the lake showing the sample location (red dot) at the deepest spot on the lake (right)

The lake is 110 ha (272 acres) in area and has a maximum and mean depth of 8.5 m (28 ft) and 4.9 m (16 ft), respectively. Spanaway Lake is a kettle lake, and the hydrology is heavily influenced by groundwater inputs, possibly up to around 65% of the inflow (Pierce County 2016). Surface water enters the lake from Coffee Creek at the southern end. The creek originates in a wetland complex on JBLM land. The primary surface outlet, Spanaway Creek, is at the north end of the lake, although it is also possible that a portion of the lake water leaves via groundwater in the north end of the lake (Pierce County 2016).

The lake's submerged plant community was assessed in 1996, 2002, 2008, 2009, and 2017 by the Washington State Department of Ecology. Pondweeds (*Potamogeton spp*.), muskwort, and water lilies have dominated the communities since 1996. The invasive plants, yellow flag Iris and reed canary grass, have been present since 1996; the invasive curly-leaf pondweed seems to have increased in density over time. There have been numerous applications of both glyphosate and diquat dibromide to reduce the density of pondweeds and water lilies.

Spanaway Lake supports largemouth and smallmouth bass, yellow perch, and rock bass populations. The Washington Department of Fish and Wildlife stocks rainbow trout. Other fish in the lake include common carp, pumpkinseed, sculpin, brown bullhead, and cutthroat trout (Caromile and Jackson 2002).

Field Methods

Weekly site visits occurred from mid-May through November 2021. The sample location was the deepest spot on the lake (Figure 2). A YSI EXO3 muti-parameter sonde was used to profile the water column every meter down to a depth of 8m, measuring pH, temperature, specific conductance, dissolved oxygen, chlorophyll (in reflectance units), and phycocyanin (in reflectance units). Probes were calibrated before each field measurement per the Ecology Standard Operating Procedure for multi-parameter sondes (Anderson 2016). The temperature profile was used to define the metalimnion (>1°C change in 1m depth) and the lower hypolimnetic waters.

A 2m integrated surface water sample was collected, homogenized, and split to analyze for the parameters shown in Figure 3. Sampling methods were consistent with the EPA's National Lakes Assessment (EPA 2022). Briefly, a 2m PVC pipe with a valve at the bottom was used to capture the upper epilimnetic lake waters. The water is discharged into a 2L amber HDPE bottle and shaken to homogenize. Sample containers for the parameters of interest were then filled on the shore. To collect the lower hypolimnetic waters, a Kemmerer bottle was lowered to a depth of 7m, triggered, and brought to the surface. The hypolimnetic waters were also transferred to a 2L amber HDPE bottle for subsampling on the shore.

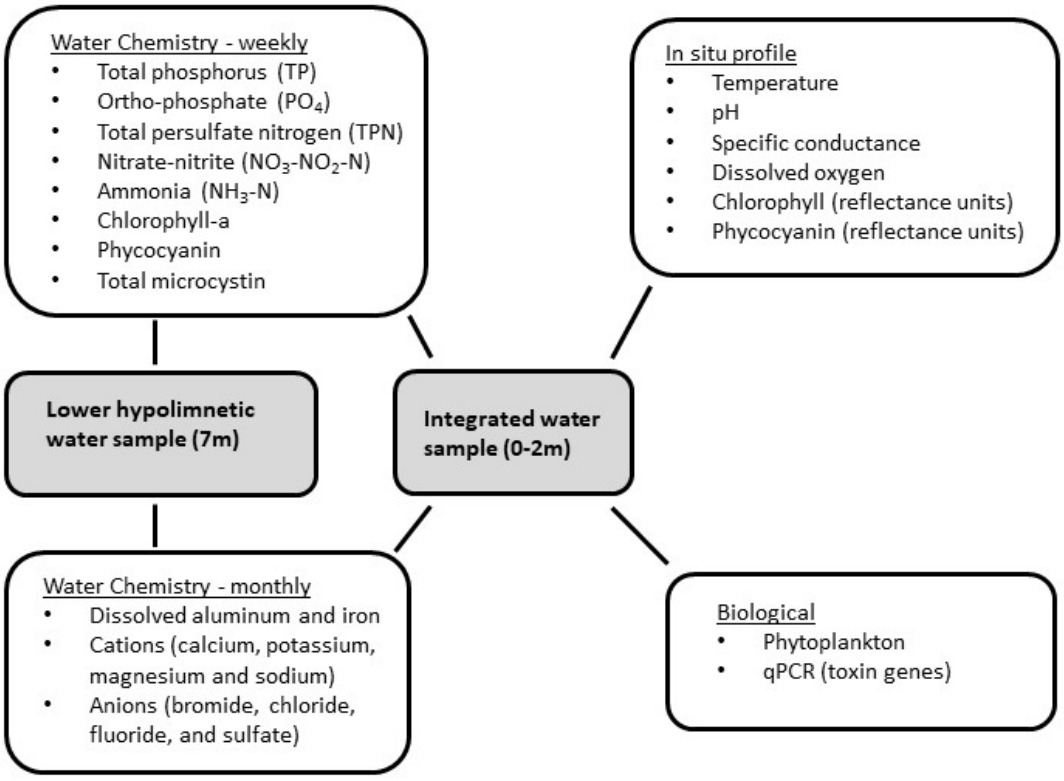

Figure 3. Schematic diagram showing the sampling design and frequency for the study.

Samples were split into respective bottles and preserved, if necessary, immediately following collection. A dedicated filter tower was used to process a sample aliquot for qPCR, and a syringe filter was used to filter for particulate analysis. The filter tower was rinsed with deionized water prior to use and cleaned with alcohol following the sampling event. Filters for qPCR were placed immediately into 600 µL of RNAlater (Invitrogen) for preservation and frozen within an hour of sampling.

Laboratory Methods

Many of the laboratory methods used in this study are detailed in Table 1.

Table 1. Laboratory methods used in the study, including method detection and reporting limits.

Laboratory	Analyte	Method detection limit	Reporting limit	Sample prep method	Analytical (instrumental) method
KCEL[a]	Microcystin variants	0.04 µg/L	0.2 µg/L	KCEL SOP 469 [b]	KCEL SOP 473 [b]
KCEL[a]	Total microcystin: ELISA	0.15 µg/L	0.15 µg/L	KCEL SOP #465	ELISA-Abraxis ADDA (KCEL SOP #465)
IWS-WWU	Chlorophyll a	NA	0.004 mg/L	SM10200-H1	SM10200-H3
IWS-WWU[a]	phycocyanin	5 µg/L	5 µg/L	USEPA (2017b)	EPA (2017); Kasinak et al. (2015)
MEL	TP	0.005 mg/L	0.01 mg/L	SM4500-P B5	SM4500-P H
MEL	orthophosphate	0.005 mg/L	0.003 mg/L	SM4500 PG	SM4500-P G
MEL	NO_2-NO_3	0.005 mg/L	0.01 mg/L	SM4500NO3I	SM4500-NO3 I
MEL	NH_3-N	0.006 mg/L	0.01 mg/L	SM4500NH3	SM4500-NH3 H
MEL	Total persulfate N	0.013 mg/L	0.025 mg/L	SM4500-N B	SM4500-N B
MEL	Major cations and metals	0.025 µg/L	0.025 µg/L	EPA 200.7	EPA 200.7
MEL	Major anions	0.025–0.3 µg/L	0.025–0.3 µg/L	NA	EPA 300.0
EPA[a]	DNA and mRNA	0.5 ng/mL	97% identity match base pairs library	(sediment) Qiagen – Rneasy Powersoil total RNA kit	Agilent 2100 Bioanalyzer

KCEL = King County Environmental Lab;
IWS-WWU = Institute for Watershed Studies – Western Washington University;
MEL = Manchester Environmental Lab;
UC = University of California;
NH_3-N = ammonia as N;
NO_2-NO_3 = nitrite-nitrate as N;
[a] Analyses carried out using methods not accredited by Ecology and received a waiver under this project and study Quality Assurance Project Plan (Hobbs 2021).
[b] Mekebri et al. 2009

Microcystin compounds (MCs) were analyzed by King County Environmental Laboratory (KCEL). Sample preparation methods for ADDA ELISA consisted of freezing/thawing, sonication, and filtration. Microcystins were quantified using the Gold Standards Diagnostics Microcystin and Nodularin (ADDA) ELISA kit (Gold Standards Diagnostics PN 520011) following the manufacturer's directions. This ELISA kit quantifies all microcystin variants (but is calibrated using microcystin LR).

For two of the samples, the same prepared sample was further filtered and analyzed for seven specific microcystin variants (MC-RR, MC-YR, MC-LR, MC-LA, MC-LY, MC-LW, MC-LF, and nodularin) using an Agilent 1290 Infinity II LC system with a Poroshell EC-C18 3x100mm, 2.7µm analytical column (Agilent PN 695975-302) coupled to an Agilent 6470 Triple Quadrupole Mass Spectrometer. For each variant, a precursor ion and two of its products were monitored in positive MRM mode (Oehrle et al. 2010). External calibration with standards for each variant (Enzo Life Sciences) was used for quantification.

Analysis of filters for toxin-producing genes using qPCR was carried out by US EPA's Office of Research and Development, Cincinnati, OH. Samples were shipped on dry ice, frozen in 600 µL of RNAlater (Invitrogen). After thawing samples, the filters were aseptically moved to a lysing matrix A bead beating tube (MP biomedicals). The remaining cells in the RNAlater were centrifuged at 14,000g for 5 minutes to pellet the cells. Excess RNAlater was removed, and the cells pipetted into the bead tube with the filter. 600 µL of buffer RLT Lysis buffer from the Qiagen All Prep kit was added to the bead tubes, and the filters were bead beat using a mini-bead beater 16 (Biospec Products, Inc) for 1 minute. Samples were centrifuged at 12,000g for 1 min, and the supernatant was transferred to a DNeasy column. From this point, the manufacturer's protocol for the Qiagen AllPrep RNA/DNA mini kit was followed. Both the RNA and DNA were eluted with 100 µL of RNase-free water.

Reverse transcription for RT-qPCR was done using Applied Biosystem's High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, MA). Each reaction contained 5 µL of RNA, 2 µL of 10 × RT buffer, 2 µL of 10X primers, 0.8 µL of NTPs, 1 µL of Reverse Transcriptase, 1 µL of RNase Inhibitor, and 8.2 µL of molecular grade water.

We conducted qPCRs using an Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, California, USA). Each reaction mixture (final volume 20 µL) contained 10 µL of 2× qPCR SYBR® Green Master Mix (Life Technologies, Carlsbad, CA), 0.25 µM primers (each; Integrated DNA Technologies, Inc., Coralville, IA), and 2 µL of template DNA. The thermal cycling included 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C extension for 30 s, or the annealing temperatures (Table A-1).

Each qPCR was run with a 7-point standard curve ranging from 5 to $5x10^6$ copies/µL. The template for the standard curve was a linearized plasmid with the PCR target cloned into an Invitrogen™ pCR™4-TOPO™ Vector (Thermo Fisher Scientific Inc., Waltham, MA). The qPCR primers and targets can be found in Table A-1. The original DNA template and a 10-fold dilution of the DNA were run in the assays to detect inhibition in the samples. Inhibition was

defined by having less than 3.0 Ct values between the undiluted and diluted DNA aliquots used as templates. If inhibition was detected, the quantity for the diluted DNA template was used to estimate the gene copies in the sample; if no inhibition was detected the value for the undiluted DNA was reported.

We quantified the 16S rRNA of all cyanobacteria (16SCyano) and the *Microcystis* genus (16SMic). To target all cyanobacterial MC producers, we quantified mcyE (mcyEcyano) abundance and expression. We also targeted mcyE (mcyEMic) and mcyA (mcyAMic) genes specific to *Microcystis*. Additional toxin production was targeted by looking at the expression of anaC to target cyanobacterial anatoxin-a producers, sxtA to target cyanobacterial saxitoxin producers, and cyrA to target cyanobacterial cylindrospermopsin producers. To investigate N_2-fixation, we quantified the abundances and expression of the N_2-fixation gene nif in the genera *Dolichospermum* (née Anabaena) (Li et al. 2016) and Nostoc. We also quantified the abundances and expression of the inorganic phosphate scavenging gene pstS in *Dolichospermum*.

Phytoplankton samples were collected in 1L HDPE bottles and preserved immediately with Lugol's solution. Jim Sweet, Aquatic Analysts, analyzed the phytoplankton biovolume, density, and community structure. Phytoplankton were mounted on a microscope slide and enumerated with a Zeiss standard microscope (1000X, phase contrast). Only algae believed to be alive at the time of collection (intact chloroplast) were counted. A minimum of 100 algal units were counted. (Standard Methods 2020).

Numerical Methods

A hierarchical constrained cluster analysis, which is a cluster analysis constrained to time and based on the Bray-Curtis dissimilarity between samples, was used to delineate changes in the phytoplankton communities over time (Juggins 2017). This constrained analysis makes sure the temporal integrity of the time series is maintained.

The coherence between the time series for the microcystin concentrations in the surface waters and the abundance of Mcy genes was explored using two techniques: (1) a cross-correlation analysis between MC and time-lagged intervals for the Mcy genes and (2) a time alignment measurement distance, which quantifies the degree of temporal distortion between two time series (Mori et al. 2016; Folgado et al. 2018). The goal of this analysis was to determine if the data collected showed that the Mcy gene abundance lagged behind the concentrations of MC in the water column, as demonstrated in previous studies (Lu et al. 2020).

Results

Quality Assurance

The project QAPP outlined all measurement quality objectives (MQOs) (Hobbs 2021). Laboratory MQOs were met for this project, which included laboratory duplicates, matrix spikes, matrix spike duplicates where applicable, standard reference materials, and associated calibration and verification. One of the water samples analyzed for NH_3-N had an associated matrix spike with low recovery due to an insufficient spike amount; all other QC and instrument performance for the affected samples was acceptable, and the sample results were accepted.

Field duplicates were collected every month for most of the parameters. Laboratory and field quality control results, including relative percent differences (RPDs), are shown in Table A-2 through A-8. There were a few instances where the study MQOs were not met:

1. A duplicate of one of the hypolimnetic samples showed precision above the RPD threshold for iron and nitrate-nitrite. All laboratory QC was within acceptable limits for these samples, and no other field duplicates for these parameters were outside the study MQOs. Therefore, the results were accepted, and the final result was an average of the sample and duplicate.
2. A duplicate of one of the microcystin epilimnetic samples had an RPD of 87%. Variability in the measurement of total microcystin is not unusual because of differences in intracellular MC concentrations and spatial heterogeneity of cyanobacteria blooms (Wood et al. 2021). An average of the two concentrations was reported.
3. A field blank sample for phycocyanin had measurable pigment above the reporting limit. Investigation of this result led to a rejection of the result due to lab instrument error.

Fluorometric Probe Measurements

Previous work by Wong and Hobbs (2020) validated the use of continuous fluorometric measurements in Spanaway Lake to measure the phycocyanin (PC) and chlorophyll *a* (Chl *a*) content in surface waters. In the current study, the probe measurements were again validated using laboratory measurements of PC and Chl *a* on split water samples (Figure A-1). There was a strong linear relationship between field measured PC (in reflectance units, RFU) and laboratory concentrations of PC (Pearson $r = 0.86$, $p < 0.001$). Measurements of Chl *a* and probe measurements were also correlated ($r = 0.64$, $p < 0.001$). The lower correlation coefficient for the Chl *a* measurements is consistent with the previous Wong and Hobbs (2020) study. Additional investigation may be warranted to verify whether there is some interference from pheophytin or chlorophyll degradants when using Standard Methods for laboratory Chl *a*.

Probe measurements of PC in the water were also strongly positively correlated with the density of total cyanobacteria measured under the microscope in cells/ml ($r = 0.90$; $p < 0.001$). Lab

measurements of PC were also strongly correlated to total cyanobacteria density ($r = 0.8$; $p < 0.001$).

Water Column Profiles

All calibration and QC checks of the multi-parameter sonde met the study MQOs. Sampling began on May 10, 2021, and Spanaway Lake was already weakly stratified at the sample location (Figure 4). There was a clear thermal stratification with layers of upper waters (epilimnion), transitional waters (metalimnion), and lower waters (hypolimnion) evident. The thermocline in lakes is generally defined by the presence of layers in the water column with temperatures changing >1°C in 1m depth (Wetzel 2001). As the summer progressed the thermocline became larger and occupied more of the water column. The fall turnover, when the lake water column mixes completely due to temperature changes, occurred in mid-September. However, the thermal stratification appeared to be weakening in early September.

The water's dissolved oxygen (DO) content changed with biological activity and stratification. Early in the summer, there was ample DO throughout most of the water column, except near the sediment surface. As the water column stratified more strongly over the summer, the bottom waters (hypolimnion) became more isolated. As bacterial activity consumed the DO, concentrations dropped below 1 mg/L or near zero. In mid-July and early August, very high concentrations of DO were measured at a depth of ~5 – 6m, likely reflecting the presence of photosynthetic activity from algal growth. Throughout the summer, waters above 6m depth contained sufficient DO for fish.

The fluorometric probe that measures phycocyanin (PC, the main pigment in cyanobacteria) documented the growth of cyanobacteria in the lower waters of the lake (~7m) beginning in late June (Figure 4). By mid-July, the PC concentrations were roughly the same but higher in the water column (~ 5m). In late July and early August, PC concentrations peaked at a depth of 5m and subsequently, PC was measured throughout the water column until October.

The chlorophyll measured by the fluorometric probe captured the late spring (early June) phytoplankton bloom, the beginning of a deep chlorophyll maximum (DCM) in late June, and a peak in early to mid-July (Figure 4). Typically, the DCM will develop in stratified waters when a photosynthetically relevant amount of light penetrates the bottom of the thermocline, where nutrients are generally higher in the bottom waters (Fee et al. 1976; Leach et al. 2018). The DCM was spatially variable in the water column and appeared to dissipate in early August. However, an additional chlorophyll maximum was noticeable in mid-August at a depth of 5m; subsequently, fairly uniform chlorophyll was present throughout the water column.

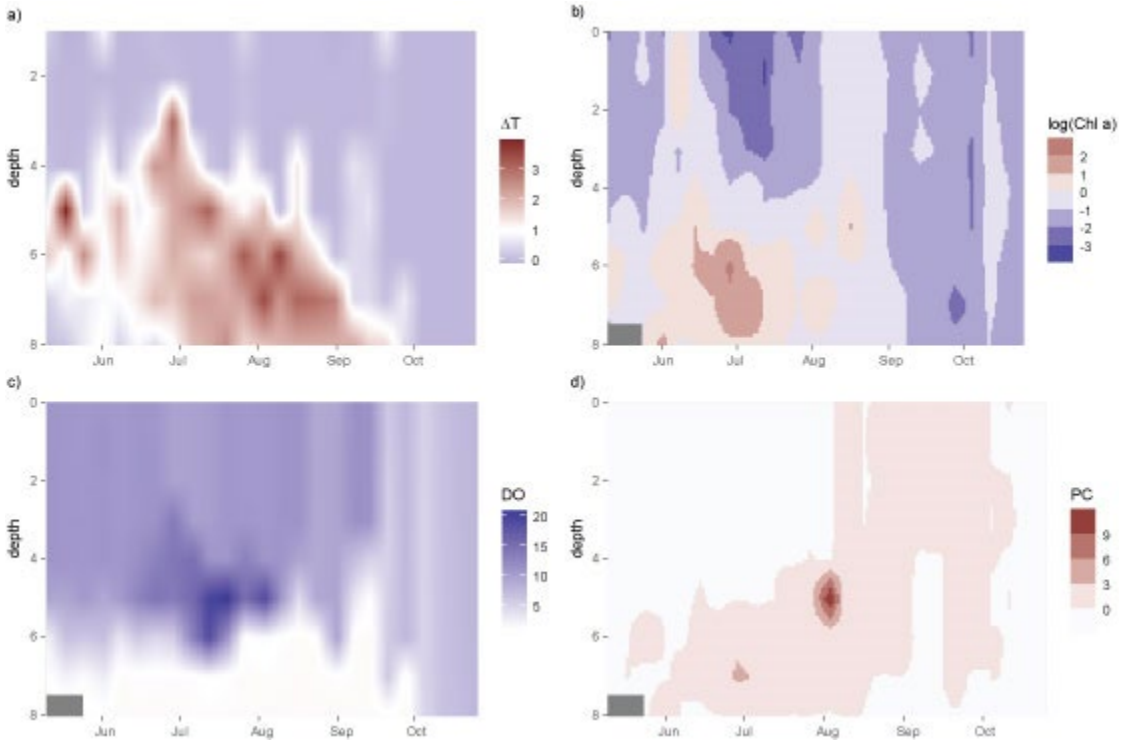

Figure 4: Contoured profiles of the a) thermocline (change in temperature in °C over 1m), b) chlorophyll a (RFU), c) dissolved oxygen (mg/L), and d) phycocyanin (PC in RFU).

In lakes, the pH profiles of the water column are often driven by the photosynthetic activity in the water and the cycling of aqueous inorganic carbon (i.e., the availability of dissolved CO_2). During periods of high algal productivity, dissolved CO_2 can result in proportionally more bicarbonate ions in the water, which shifts the pH higher (Wetzel 2001). This process is evident in the upper waters of Spanaway Lake during the summer as they become more productive (Figure 5). The upper waters had a mean (sd) pH of 8.1 (0.6), with a maximum of 9.1 in mid-August. The bottom waters of Spanaway Lake remained at a neutral pH (~7) throughout the period of stratification.

Specific conductance of the water is a measure of the total dissolved solids content or the ionic strength of the water. It is generally a conservative parameter that reflects the inputs of ions into the lake. During periods of stratification, higher ionic waters can accumulate at the bottom of lakes as solutes are diffusing from the sediments, or there is an input of higher ionic strength waters from groundwater. The Spanaway Lake profiles showed increased conductivity in the bottom waters, corresponding with the strength of water column stratification and anoxic conditions (Figure 5). Conductivity peaks at 371.8 µS/cm near the sediment surface in late August. Conductivity in the upper waters is fairly stable throughout the summer at a mean (sd) of 133.1 (8.2) µS/cm.

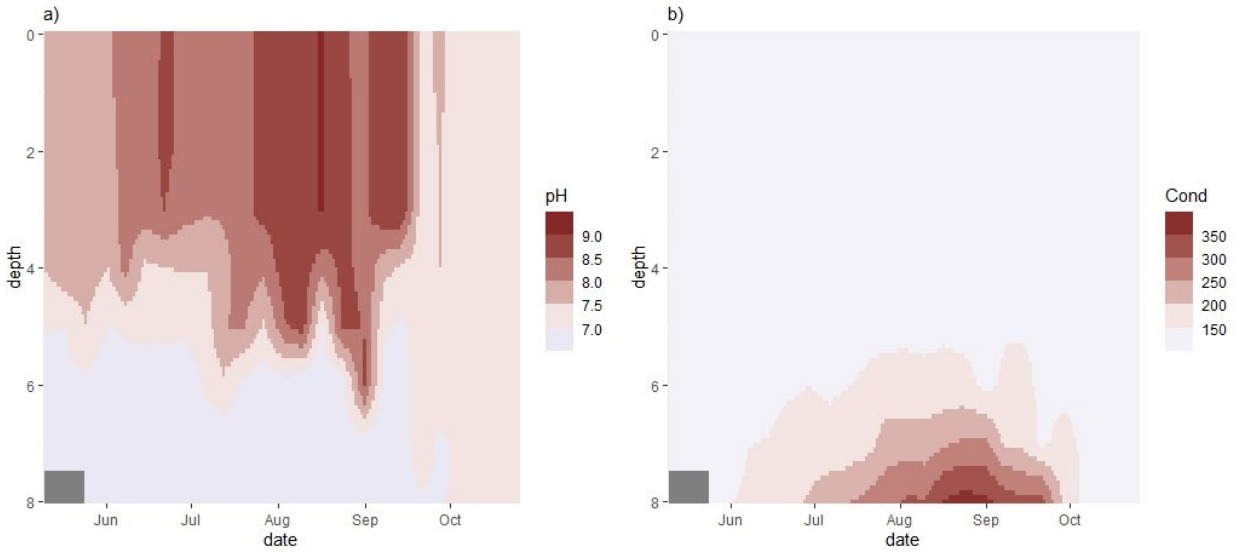

Figure 5. Contoured profiles of a) pH and b) specific conductance (µS/cm).

Water Chemistry

The concentrations of conservative ions and metals were measured monthly in the surface (0 – 2m) and bottom (7m) waters. Generally, these parameters describe water chemistry influenced by the lake's natural setting. In other words, the natural biogeochemistry is due to land cover, surficial geology, and hydrology. The dominant cations of the surface waters at the sample location were $Ca^{2+} > Na^{+} > Mg^{2+} > K^{+}$, while the dominant anions were $Cl^{-} > SO_4^{2-} > NO_3\text{-}NO_2$. Carbonate and bicarbonate were not analyzed (Figure 6). Conservative ions such as magnesium, chloride, sodium, and potassium showed little difference between the upper and lower waters. Ions and metals like calcium, iron, sulfate, and nitrate-nitrite, which are influenced by sediment-water processes or biological processes in the water column, showed greater variability and differences between the lower and upper waters. Dissolved aluminum, fluoride, and bromide were not detected in the water samples.

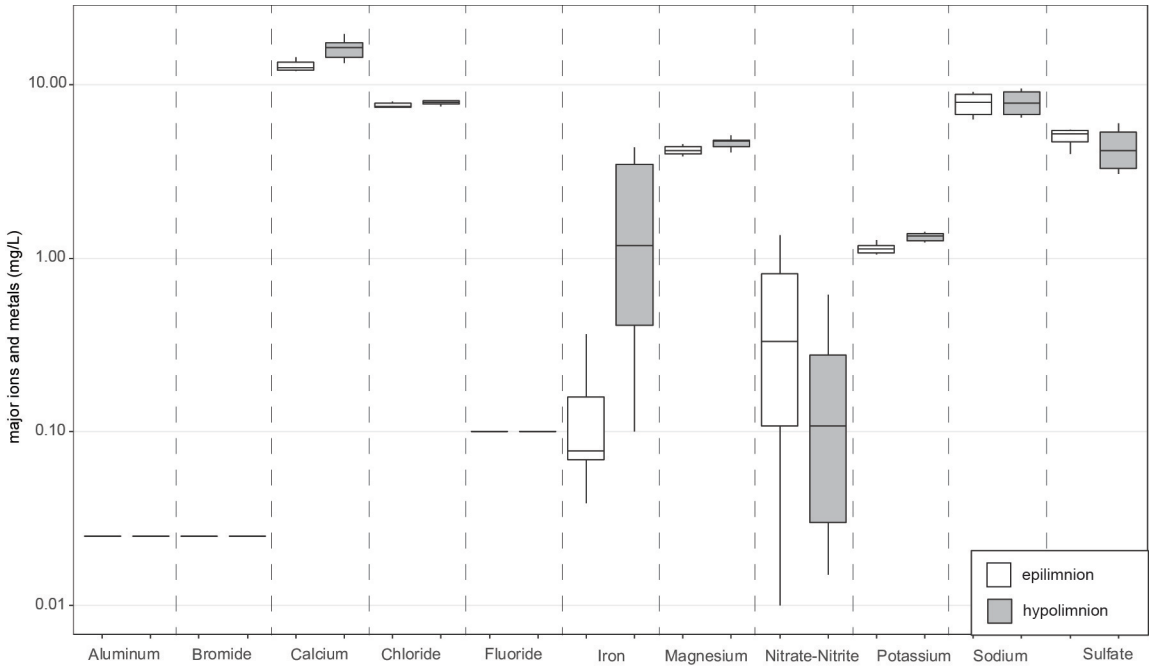

Figure 6. Box and whiskers plot of the major ions, aluminum, and iron in monthly water samples of the upper (white) and bottom (grey) waters.

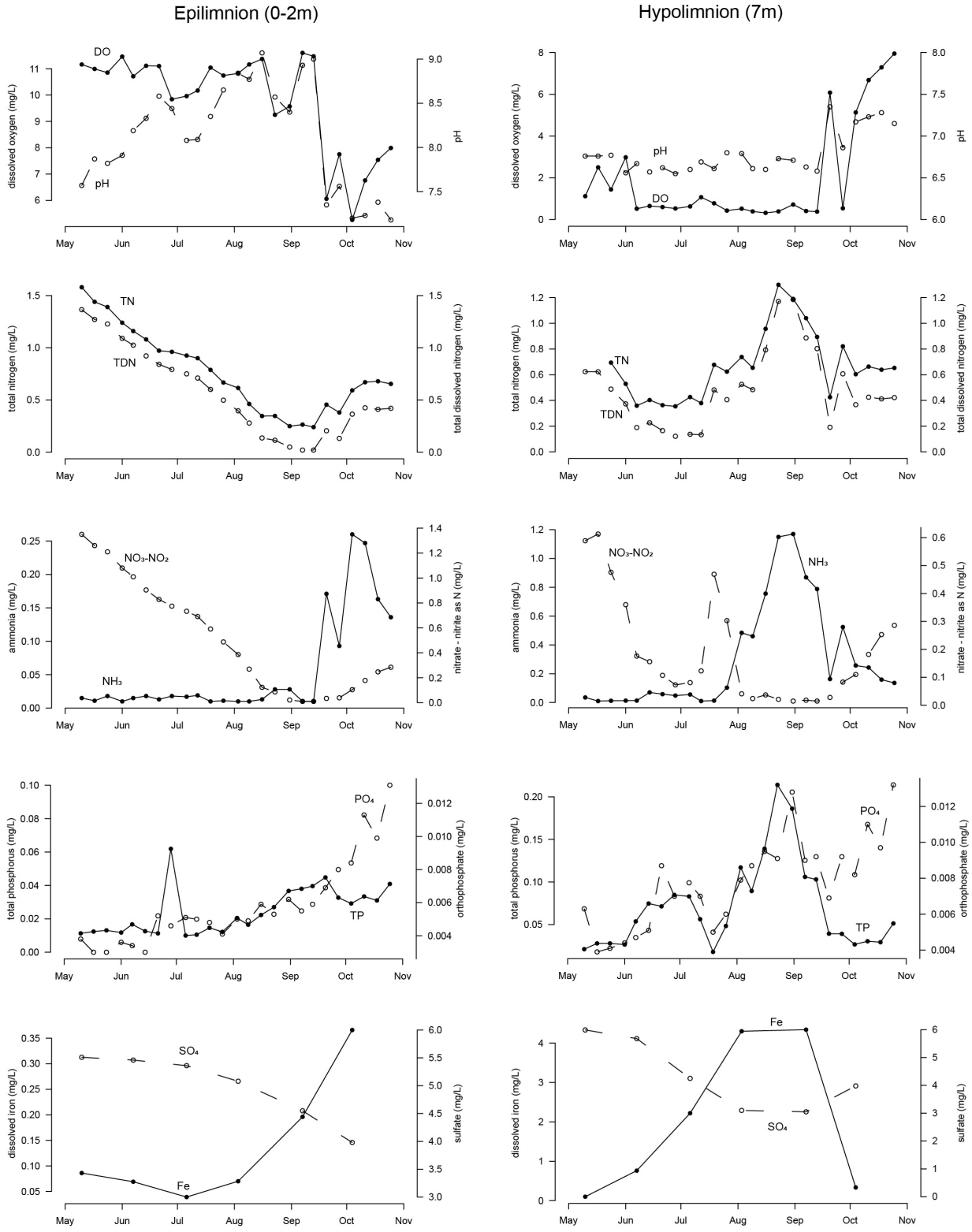

Figure 7. Trends of pH, dissolved oxygen, nutrients, dissolved iron, and sulfate in the upper waters (left side) and lower waters (right side).

Concentrations of the major nutrients nitrogen and phosphorus were measured as total nitrogen (TN), nitrate-nitrite as N (NO_3-NO_2-N), ammonia (NH_3), orthophosphate (PO_4), and total phosphorus (TP) (Figure 7). The inorganic forms of N (NO_3-NO_2-N and NH_3) made up most of the TN in the upper and lower waters. Ammonia was low and near analytical detection limits in the upper water for most of the sampling period, while NO_3-NO_2-N decreased steadily over the summer to levels below the detection limits. Ammonia in the bottom waters was below or near analytical detection limits from the beginning of the sampling period through mid to late July, when concentrations steadily increased and peaked in late August and early September. Concentrations of inorganic N in the upper and lower waters were similar after mid-September when the water column was mixed.

Total phosphorus concentrations in the upper waters ranged from <0.01 mg/L to 0.062 mg/L, while inorganic P (PO_4; orthophosphate) ranged from <0.003 mg/L to 0.013 mg/L. Generally, P concentrations steadily increased throughout the summer in the upper waters. In the bottom waters, TP ranged from 0.018 mg/L to 0.214 mg/L, and inorganic P ranged from 0.004 mg/L to 0.013 mg/L. TP and PO_4 concentrations in the bottom waters followed similar trends through the sampling period (Figure 7). In mid-July, P concentrations decreased to spring levels but then increased during August to peak concentrations in late August and early September. Phosphorus concentrations in the bottom waters decreased in early September as thermal stratification weakened until mixing occurred in mid-September.

The seasonal water column stratification and changes in the oxygen and redox potential of the lower waters and upper sediment surface influence many water chemistry variables. By early June, the water column is strongly stratified, and the bottom waters contain very little oxygen (median DO at 7m depth for June-August is 0.52 mg/L). In response to the anoxic conditions in the bottom waters, there is a decrease in nitrate and an increase in sulfate reduction (SO_4 concentrations decrease). During sampling, a strong sulfide (H_2S) odor (the by-product of sulfate reduction) was noted in the bottom waters. This reducing environment in the bottom waters and at the sediment surface yields an increase of ammonia from the sediments (Figure 7). Furthermore, the redox-sensitive metal iron is released from the sediments. During sampling, iron precipitate was noted in the bottom waters. Lastly, there is an increase in TP in the bottom waters as PO_4 is released from the sediments (Figure 7); it is possible that the soluble PO_4 was binding to the iron precipitates (FeO_3) in the bottom waters. Unlike nitrogen, the dissolved inorganic fraction of P (orthophosphate) comprises a small portion (roughly 10%) of the TP, suggesting that P was bound in mineral form (loosely with FeO_3 or strongly in sulfur or iron mineral lattices) or we were measuring a particulate organic P (e.g., phytoplankton).

Phytoplankton Communities

The density of phytoplankton over the sampling period showed a late spring bloom (May), a decrease in production in the early summer (June – July), and then sustained high production from early August through the end of September (Figure 8). The peak density was on August 9, with a second high density on September 13.

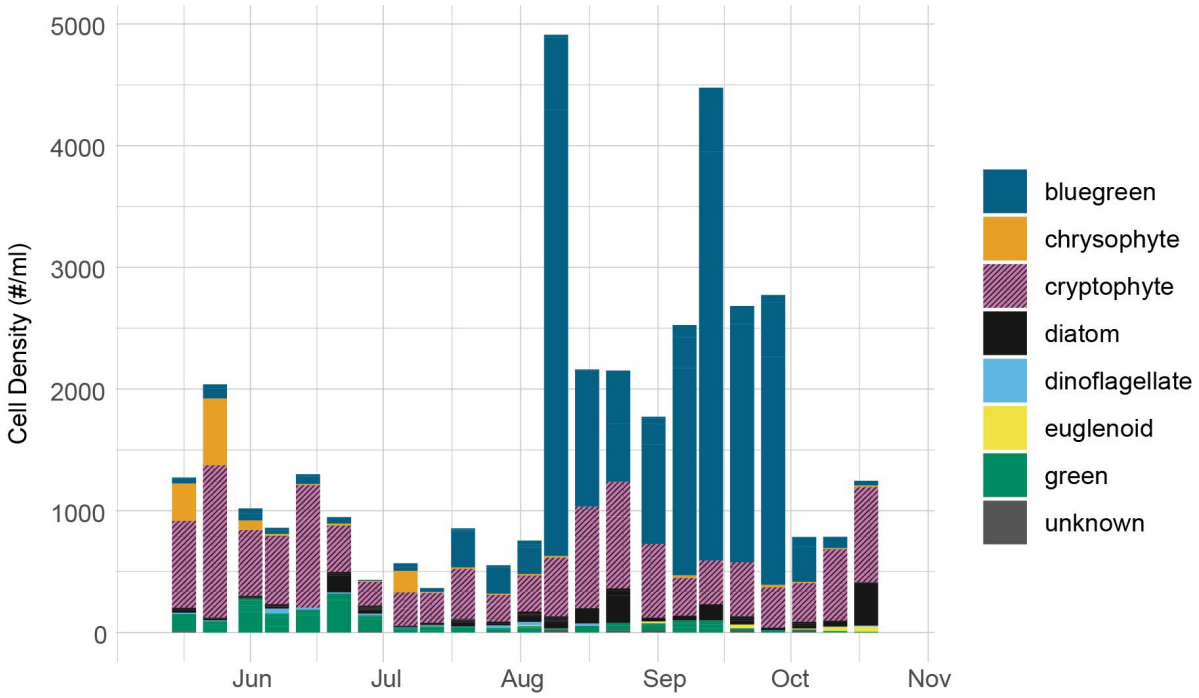

Figure 8. Stacked barplot showing the densities of phytoplankton communities over the sampling period.

The succession of phytoplankton throughout sampling was well described by weekly collections of phytoplankton from the upper waters and a few samples of phytoplankton from the lower waters. All samples were preserved at the time of collection and received in good condition at the lab. Generally, the specimens were identified down to species level with the following exceptions: green algae in the *Chlamydomonas* genus, chrysophytes in the *Chromulina*, *Mallomonas* and *Dinobryon* genus, and dinoflagellates in the *Glenodinium* genus. Cyanobacteria were present in the water column throughout the sampling period, ranging from 4% to 99% of the algal biovolume (μm^3/ml) and 4% to 87% of the algal density (cells/ml).

Cluster analysis of the phytoplankton communities defined five significant periods of succession during the summer into the fall (Figure A-2). The general succession of phytoplankton (expressed as biovolume) at the sampling site during 2021 was as follows (Figure 9):

- Late spring to early summer (May 10 – June 14), the phytoplankton community was dominated by cyanobacteria (*Aphanizomenon flos-aquae* and *Dolichospermum flos-aquae*), cryptophytes (*Cryptomonas erosa* and *Rhodomonas minuta*) and chrysophytes (*Dinobryon sertularia*).
- Early to mid-summer (June 21 – July 12), the diatoms *Fragilaria crotonensis* and *Asterionella formosa* became more prominent in the community.
- Mid- to late summer (July 19 – August 23) was strongly dominated by the cyanobacteria species and the diatom *F. crotenensis*. In the late summer, the cyanobacteria *Dolichospermum planctonica* and *Microcystis aeruginosa* were also part of the community.

- Late summer to early fall (August 31 – October 4) was strongly dominated by the cyanobacteria previously listed, particularly *A. flos-aquae* and *D. planctonica*.
- Fall (October 11 – 28) was when the water column had mixed, and the phytoplankton community was more diverse with species of diatoms, cryptophytes, cyanobacteria, and dinoflagellates.

Figure 9. Phytoplankton communities over the sampling period in the upper waters. Proportions are based on biovolume (µm^3/mL).

Four hypolimnetic (bottom) water samples were also analyzed for phytoplankton communities (Figure 10). At the beginning of June (6/1), the cyanobacteria *Aphanizomenon flos-aquae* dominated the hypolimnetic community along with the cryptophyte *Rhodomonas minuta* and *Cryptomonas erosa*. The following week (6/7), through the end of June, the cryptophytes dominate the community with the cyanobacteria *Oscillatoria limosa,* a minor part of the community with *A. flos-aquae*. In mid-July (7/12), the cyanobacteria species *A. flos-aquae* and *Microcystis aeruginosa* dominated the community with the dinoflagellate *Ceratium hirundinella* and the crysophytes *Dinobryon sertularia* and *Mallomonas sp*.

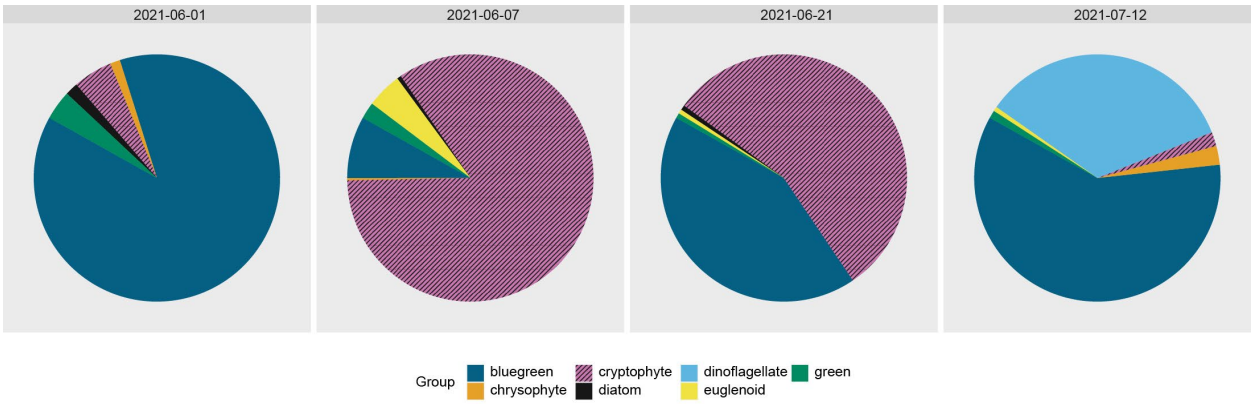

Figure 10:Proportions of phytoplankton groups in four samples collected from the bottom waters in June and July 2021.

The cyanobacteria growth in the surface waters was generally low in the spring through early summer and dominated by *Aphanizomenon flos-aquae* and *Dolichospermum flos-aquae* (Figure 11). On July 12, *Microcystis aeruginosa* was identified in larger numbers for the first time, after which *Dolichospermum planctonica* became more prominent than *D. flos-aquae*. Peak cyanobacteria production on August 9 was dominated by *A. flos-aquae*. Over the summer growing period, the cyanobacteria community of Spanaway Lake was dominated by species that belong to the Nostocales, which is an order of cyanobacteria genera that can fix and use nitrogen gas (N_2, from the atmosphere or dissolved in the water column). The *Anabaena-Dolichospermum-Aphanizomenon* clade is known as the ADA clade and are filamentous, planktonic cyanobacteria. Recent genetic work has begun to define the specific characteristics of the ADA clade (Österholm et al. 2020; Dreher et al. 2021a). The ADA clade generally contains genes responsible for producing gas vesicles that allow buoyancy in plankton, genes responsible for nitrogen fixation, genes responsible for producing the pigment phycocyanin, and a lack of genes for producing microcystin. Some strains identified from Oregon by Dreher et al. (2021b) produce microcystin. However, the ADA clade is not usually associated with microcystin production. Some strains from Washington State can produce anatoxin-a (Hobbs et al. 2021; Brown et al. 2016), but anatoxin-a was not present in Spanaway Lake.

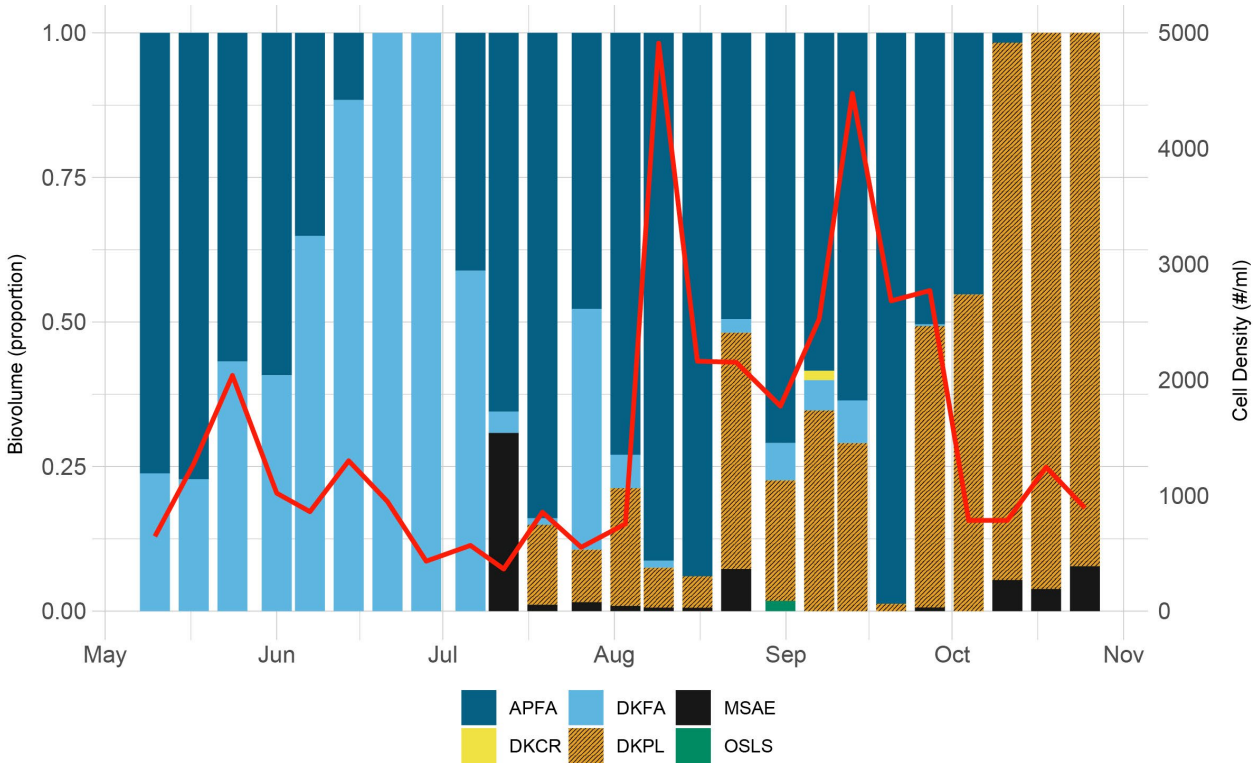

Figure 11. Stacked bar plot showing the proportions of the dominant cyanobacteria species in the community over the summer of 2021.
The red line details the cell density over the sample period. APFA = *Aphanizomenon flos-aquae*; DKFA = *Dolichospermum flos-aquae*; MSAE = Microcystis aeruginosa; DKCR = *Dolichospermum circinalis*; DKPL = *Dolichospermum planctonica*; OSLS = *Oscillatoria limosa.*

Microcystins and qPCR

Water samples analyzed for cyanotoxins, anatoxin-a and microcystin, were collected by Ecology and the Tacoma-Pierce County Health Department (TPCHD) during the summer of 2021 (Figure 12). Typically, TPCHD collects samples at public shorelines on Spanaway Lake (e.g., recreational beaches and the boat launch). Microcystin concentrations in shoreline accumulations were much higher than in integrated open-water samples. Health departments generally target shoreline accumulations as a possible worst-case scenario for public exposure. Only one sample collected during this study from the open water (pelagic) area of the lake had MC concentrations over the state recreational guidance level (8 µg/L); the sample was collected from a 7m depth on September 7 and had a concentration of 14.6 µg/L. Concentrations in the open water samples were highest during August through mid-September (Figure 12). MC concentrations in shoreline accumulations reached a maximum of 875 µg/L.

The laboratory also analyzed some of the samples by LCMS to understand the congener composition of microcystins (Figure 12). All samples were dominated by the compound MC-LA, consistent with other microcystin studies in Spanaway Lake (Hobbs et al. 2022; Preece et al. 2022). MC-LA is one of the more toxic congeners, with a half-life of 1.5 to 8.5 days in the

environment (Zastepa et al. 2014). The half-life and degradation of MC-LA depend on the bloom composition and the form of the MC compound (particulate or dissolved). An understanding of the different MC compounds and gene markers is not well established.

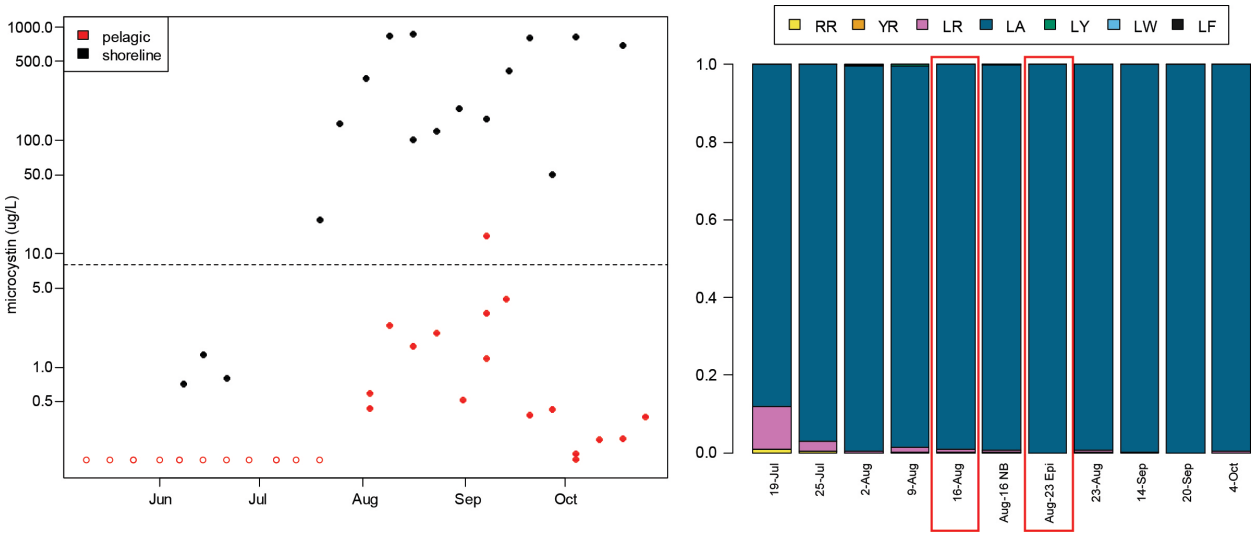

Figure 12. Microcystin concentrations and composition in Spanaway Lake during the summer of 2021.

(Left) Scatterplot of microcystin concentrations in Spanaway Lake during the summer of 2021. Red dots are open water (pelagic) samples, and black dots are shoreline accumulations collected by Tacoma-Pierce County Health Department. The horizontal dashed line is 8 µg/L — the state recreational guideline for microcystin (Right) Congener composition of the microcystin in select shoreline samples; the two samples highlighted in the red boxes are from the pelagic location.

The abundance of gene DNA and the reverse transcription (RT) of the genes were quantified using quantitative polymerase chain reaction (qPCR). The RT-qPCR is a measure of gene expression and has been used successfully in studies of cyanobacteria and toxin production (Lu et al. 2020; Barón-Sola et al. 2016; Ngwa et al. 2014). Microcystin genes (Mcy) and nitrogen (N_2) fixation genes (Nif) all showed a steady increase through July, peaking in August (Figure 13). The expression of the Mcy genes between July and September was dominated by the Mcy genes that cover all major microcystin-producing genera (McyE) and genes that are attributable to *Microcystis* and *Planktothrix* (McyA); however, *Planktothrix* does not appear to be found in Spanaway Lake. The abundance of McyE and McyA were strongly correlated (r = 0.909; Figure A-3).

The Mcy DNA increased in mid to late July, peaking in August to September (Figure 13). The expression of the Mcy genes occurred throughout the summer and into the fall. There was a possible periodicity to the Mcy gene expression. Gene abundance tended to rise and then fall sharply over a few weeks or a month, dropping to levels near detection in the spring and fall. However, an auto-correlation analysis of the time series of the McyA gene showed no significant periodicity.

The Nif genes specific to the *Dolichospermum* genus were strongly expressed throughout the sampling period but were particularly active in the surface waters in early June and mid-September (Figure 13). The Nif genes specific to Nostoc were also strongly expressed near the end of the summer, peaking in mid-September. There were no detections of the genes associated with the production of anatoxin-a (anaC), cylindrospermopsin (cyrA), saxitoxin (sxtA), or paralytic shellfish toxins (PstS). The lack of anatoxin-a was consistent with toxin monitoring since 2007, where this toxin has not been measured in shoreline accumulations of cyanobacteria.

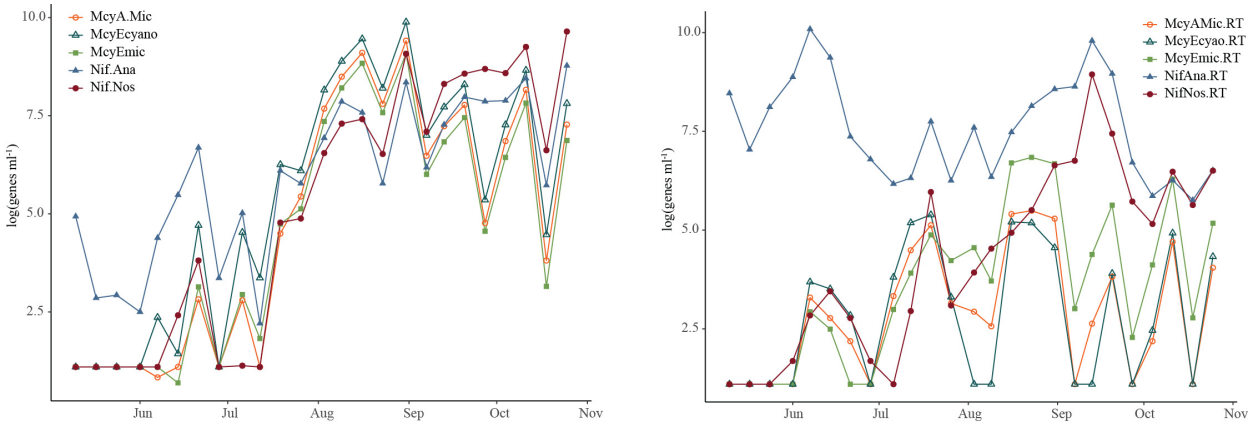

Figure 13. Time series line plots of the qPCR (left) and qPCR-RT (right) results for the microcystin and nitrogen fixation genes.
Samples were collected weekly.

Comparing the time trends of the McyA-RT gene and the microcystin concentration in the waters using a cross-correlation analysis showed an approximate two-to-three-week lag of the measured MC following gene expression (Figure 14). An additional time alignment analysis (Folgado et al. 2018) found a lag of 2.5 time periods (weeks), confirming the cross-correlation analysis. Previous work comparing the time trends of microcystins to the Mcy and Mcy-RT genes has found that the abundance of genes precedes the toxin by approximately a week (Lu et al. 2020; Duan et al. 2022). The potential utility of monitoring for the Mcy-RT genes is to provide an early warning of the presence of MCs in the water.

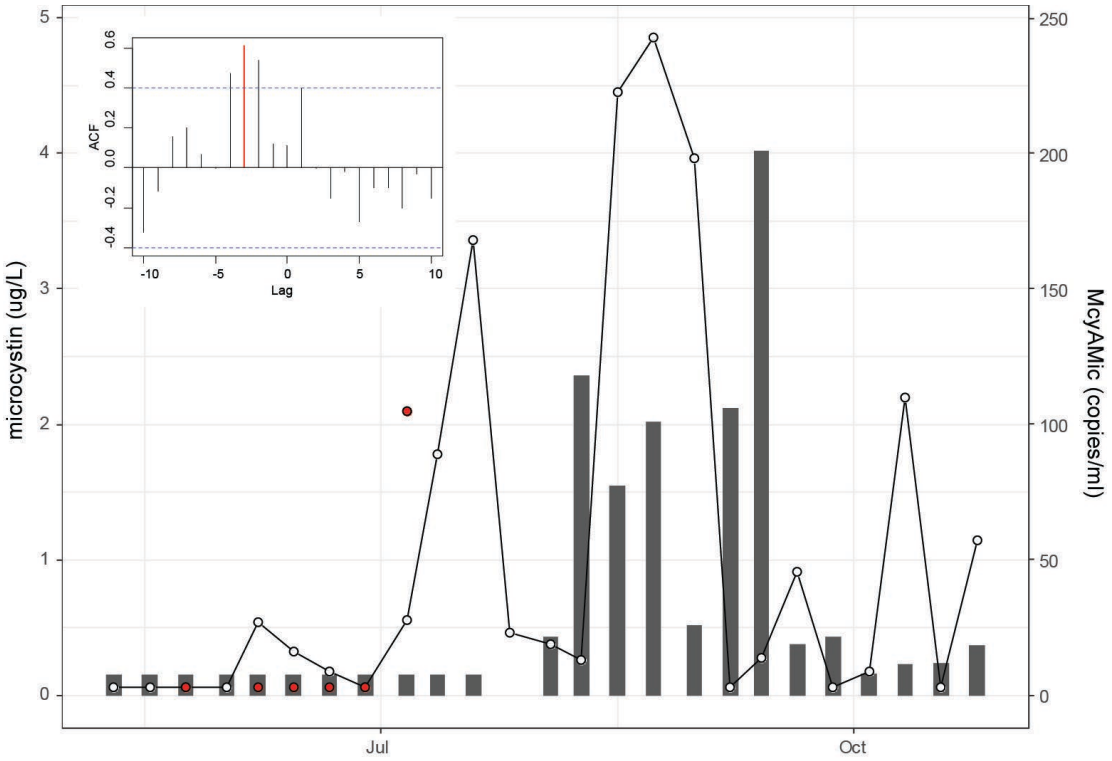

Figure 14. Time series of microcystin concentration and McyA gene copies.
A bar graph of the microcystin concentration time series with a line graph of the McyAMic gene abundance measured in the epilimnion (0 – 2m) over the summer. The McyA Mic abundance in the hypolimnion in the early summer is shown as red dots. Inset: cross correlation analysis of MC and the McyAMic gene; the highest score is for a lag of -3 (red line).

Discussion

Cyanobacteria Succession

Cyanobacteria are a major part of the phytoplankton community in Spanaway Lake for most of the growing season. Multiple species dominate the community in the peak months of August through October. This finding is similar to previous studies conducted in 2012 (Jacoby et al. 2015) and 2014 (Pierce County 2016). In the Pierce County study (2016), cyanobacteria were found to be a dominant part of the phytoplankton community throughout the year.

The cyanobacteria communities in Spanaway Lake are dominated by the ADA (*Anabaena-Dolichospermum-Aphanizomenon*) clade. The dominance of the ADA clade in Spanaway Lake in 2021 is consistent with previous studies (Jacoby et al. 2015; Wong and Hobbs 2019). Generally, the cyanobacteria within this clade are filamentous and contain gas vesicles for vertical migration in the water column. Some can fix dissolved nitrogen gas, and very few strains contain the genes necessary to produce microcystin. It's not uncommon for *Microcystis aeruginosa* to be present in the cyanobacteria community with the ADA clade (Caldwell Eldridge et al. 2013; Zhang et al. 2020). *Microcystis aeruginosa* has a very plastic genome and a wide range of environmental tolerances; it is a widespread microcystin-producing species (Frangeul et al. 2008; Humbert et al. 2013). In previous investigations of the cyanobacteria in Spanaway Lake, a *Woronichinia* sp. was also noted in more near-shore accumulations (Jacoby et al. 2015; Wong and Hobbs 2019).

M. aeruginosa and many species from the ADA clade overwinter at the bottom of lakes on the sediment surface, rising in the water column during summer and autumn when temperatures, light, and nutrients are suitable (Reynolds and Rogers 1976; Li et al. 2016). The profile data of phycocyanin in Spanaway Lake shows the seasonal rise of cyanobacteria beginning in late June and then occupying the water column from early August through September. *M. aeruginosa* is also known to migrate daily within the water column when light or nutrients may become limiting at certain depths (Brookes and Ganf 2001). The deep chlorophyll maximum (DCM) at a depth of 5 – 6m in the early part of the summer (mainly July) also documents the higher algal production (and higher dissolved oxygen) in the deeper stratified waters. The DCM forms when ample light penetrates the deeper waters at the thermocline, where nutrients tend to accumulate.

Microcystin Production

The recent work of Österholm et al. (2020) and Dreher et al. (2021a, 2021b) has demonstrated the genetic composition for toxin production by regionally relevant cyanobacteria taxa. Microcystin production in Spanaway is likely from *Microcystis aeruginosa*. *M. aeruginosa* is known to contain the Mcy genes analyzed in our sampling of Spanaway Lake (Jungblut and Neilan 2006; Dittman et al. 2013). There is a strong positive correlation between the density of *M. aeruginosa* and microcystin concentrations ($r = 0.81$; $p = 0.002$). The percent density of *M.*

aeruginosa across all the samples ranges from 0.5% to 43.6%, and biovolume ranges from 0.1% to 8.6%. Therefore, the proportion of *M. aeruginosa* in the phytoplankton community is small, based on biovolume and density.

Within the ADA clade, only a few *Dolichospermum* strains seem to possess the ability to produce microcystin; these strains were identified from a few Oregon lakes. While we cannot be certain that the dominant ADA species in Spanaway Lake (*Aphanizomenon flos-aquae*, *Dolichospermum flos-aquae,* and *Dolichospermum planctonica*) are not producing microcystin, it seems unlikely. To verify this, we would need to investigate the metagenome of the individual species present in the lake.

The coherence between the time series of the gene McyA (and McyE) and microcystin concentration, with a 2 – 3 week lag, adds to existing research documenting the use of Mcy abundance as a tool for early detection of microcystin. Lu et al. (2020) documented a one-week lag between McyA and McyE genes and MC concentrations in an Ohio reservoir during the summer of 2015. The cell abundance and presence of MC-producing genes precede the intracellular production of MC and accumulation in the waters.

Temperature and Nutrient limitations

Temperature and nutrients limit and drive the growth of cyanobacteria. Changes in these drivers affect the success of different cyanobacteria genera. The growth of *M. aeruginosa* increases as temperatures warm, and so does the vertical buoyancy and rate of vertical movement in the water column (You et al. 2018). When sampling in 2021 began, the lake was already stratified, with bottom waters much cooler than the upper waters (Figure 15). By late June, the thermocline (greatest amount of temperature change with depth) began to deepen, and the surface waters reached approximately 26 °C. You et al. (2018) modeled the optimal (greatest) growth and buoyancy for *M. aeruginosa* to occur at 27.5 °C. In early July, we observed the largest proportion of *M. aeruginosa* in the surface waters and documented a large rise in algal production and phycocyanin in the deeper waters. As the upper waters continued to warm throughout the summer and the lower waters remained thermally isolated and cool, *M. aeruginosa* continued to bloom with *Aphanizomenon flos-aquae* in the upper waters and at depth (~5m) near the deep chlorophyll maximum.

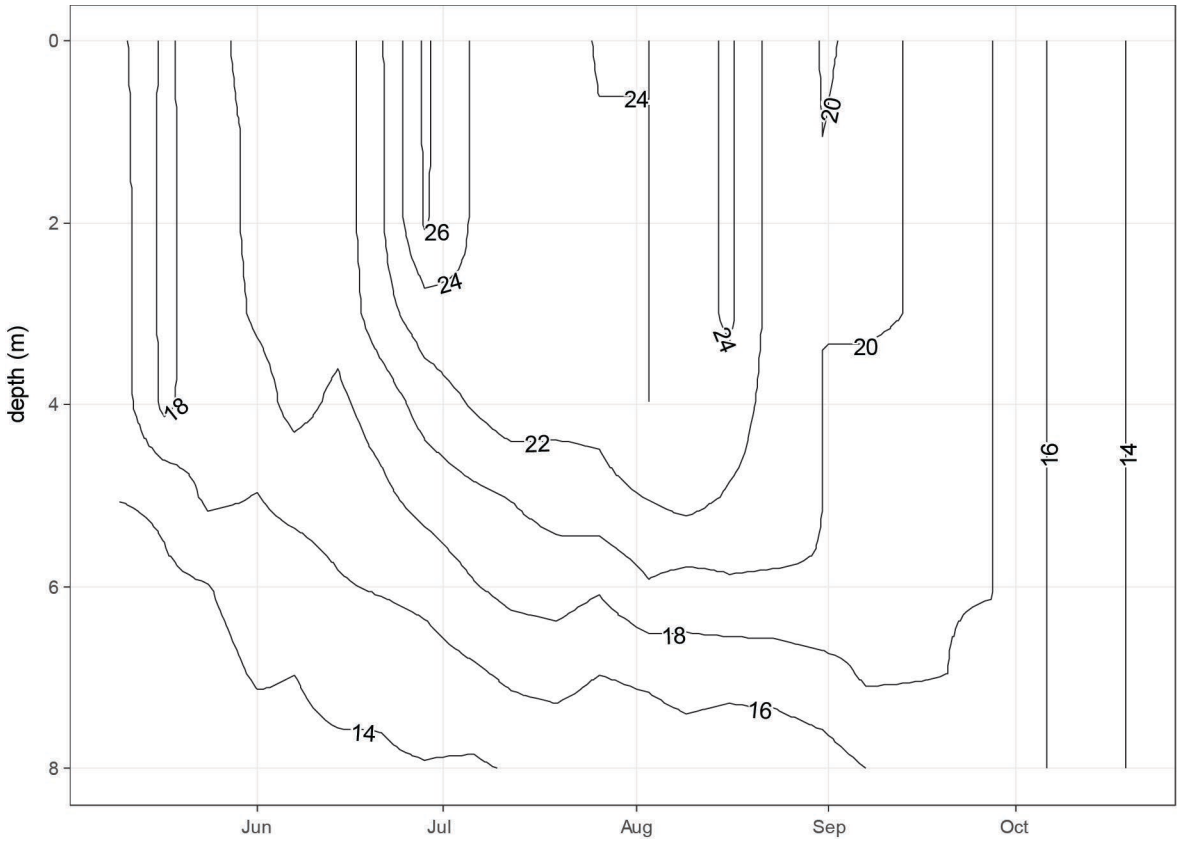

Figure 15. Thermal profile of the water column at the sample location throughout the study.

Temperatures are in °C.

Nutrient requirements of cyanobacteria are complex and vary throughout the growing season. There have also been some studies on how nutrient limitations might influence the production of toxins (Jacoby et al. 2015; Scott et al. 2013; Ginn et al. 2010). In a regional study by Jacoby et al. (2015), which included Spanaway Lake, a mass ratio of total N to total P (TN:TP) below 25 was associated with the presence of MC. In a study of Canadian lakes, elevated concentrations of MCs were more likely at a TN:TP between 15 and 20 (Orihel et al. 2012; Scott et al. 2013). A classification regression tree analysis for the 2021 Spanaway dataset, with MC concentrations as the response variable and TN:TP and temperature as the explanatory variables, showed higher MC concentrations at a TN:TP below 15.5 (Figure 16).

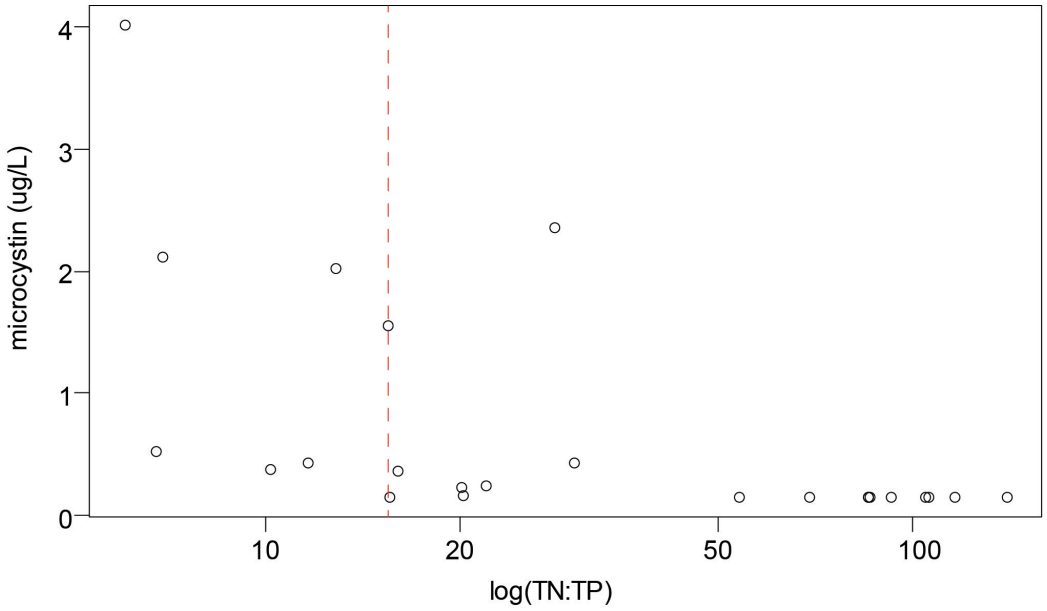

Figure 16. Relationship between microcystin and TN:TP.
TN = total nitrogen and TP = total phosphorus. The vertical dashed red line is at TN:TP = 15.5, as defined by a regression tree analysis using TN:TP and temperature.

Phosphorus is generally assumed to be the main limiting nutrient for algal growth in lakes (Schindler 1977). This appeared to be the case in the surface waters from the beginning of the sample period until early August. In contrast, the bottom waters were possibly P-limited in late May, but P did not appear to be limiting throughout the summer. Bioavailable P, as orthophosphate (PO_4), was generally at low concentrations in the upper and lower waters. The trend of PO_4 over the summer was similar to the TP trend, suggesting that similar processes were influencing both parameters. Given the nutrient ratios and ample cyanobacteria growth in the bottom waters, there was enough bioavailable P supply from the sediments or iron-bound P in bottom water particulates to support algal growth. However, cyanobacteria growth seemed to be strongly influenced by N availability.

The availability of dissolved inorganic nitrogen (DIN) declined steadily over the growing season in the upper waters of Spanaway Lake. By the end of July and into August, the algal growth in the upper waters was likely limited by nitrogen and phosphorus and then N-limited in September (Figure 17). Indeed, the growth of cyanobacteria (indicated by phycocyanin concentrations) had a strong negative relationship with the ratio of DIN:TP ($r = -0.91$; $p<0.001$) (Figure 17), where a lower DIN:TP indicates a N-limitation. Interestingly, algal growth in the bottom waters of Spanaway seemed to be N-limited for most of the sampling period, which may have been due to a strong demand for inorganic N as cyanobacteria were growing in the lower waters of the lake along with an adequate supply of P. Perhaps one of the main reasons for the strong association of N-limitation and cyanobacteria was the production of nitrogen-rich microcystin compounds, requiring available inorganic N to synthesize. It is also noteworthy that the Mcy gene is regulated by nitrogen supply, and the expression of the genes is highest under N-limitation or stress (Ginn

et al. 2010). As N-limitation became more prominent and cyanobacteria growth (phycocyanin) increased, so did the concentration of MC in the water (Figure 17) ($r = 0.82$; $p < 0.001$).

In Spanaway Lake, there was a dominance of species that can fix N_2 gas (from the atmosphere or from in-lake N-cycling), but this N input was insufficient to overcome the N-limitation (also described by Paerl and Scott, 2010). The qPCR results from Spanaway showed an increased abundance of nitrogen fixation (Nif) genes and toxin-producing genes beginning in August and into September when N-limitation was the greatest.

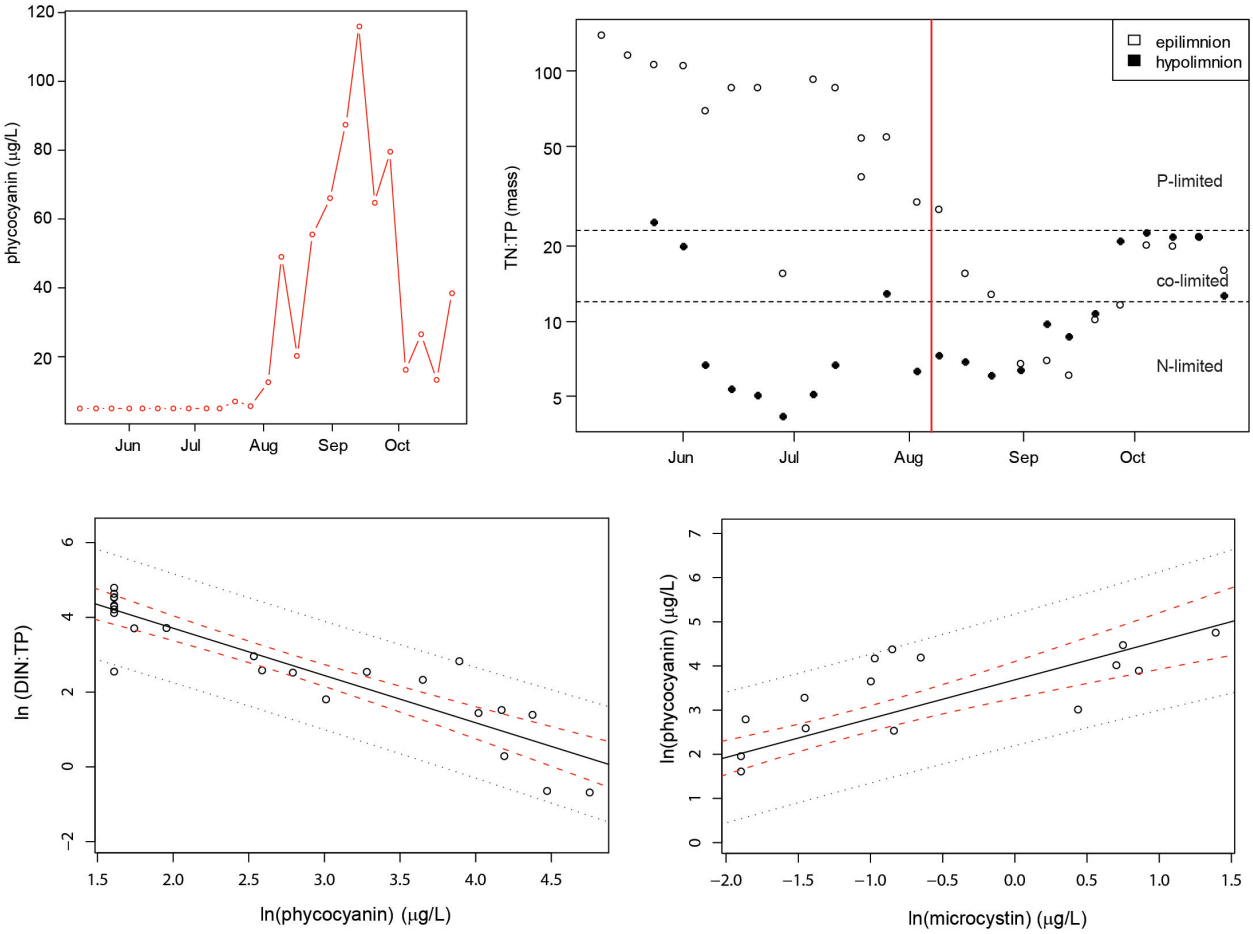

Figure 17. Relationships among cyanobacteria growth, nutrient limitations, and microcystin production.

(upper left) the phycocyanin concentrations in the water over the sampling period (µg/L); (upper right) the TN:TP in the upper (epilimnion) and bottom (hypolimnion) waters over the sample period, the vertical red line delineates the rapid increase in phycocyanin; (lower left) log-log relationship of DIN:TP and phycocyanin, 95% confidence intervals are dashed red lines and predictive confidence intervals are dashed blue lines; (lower right) log-log relationship of phycocyanin and microcystin (µg/L).

Conclusions

The succession of cyanobacteria was monitored in Spanaway Lake from May through October 2021 at the deepest spot on the lake. Numerous water quality parameters were measured weekly throughout the water column, along with an assessment of phytoplankton communities, cyanotoxins, and toxin-producing genes. The following observations can be made:

- Profiles of the main cyanobacteria pigment phycocyanin (PC), using a lab-validated fluorometric probe, documented the early summer growth of cyanobacteria in the bottom waters of the lake. In late July and early August, PC concentrations peak at a depth of 5m, and subsequently, PC is measured throughout the water column until October.
- By early June, the water column was strongly stratified, and the bottom waters contained very little oxygen. A decrease in nitrate and an increase in sulfate reduction occurred. This reducing environment in the bottom waters and at the sediment surface yielded an increase of ammonia, iron, and dissolved inorganic phosphorus from the sediments beginning in early August. Cyanobacteria growth had moved further up in the water column at that time.
- There were five main periods of phytoplankton succession over the sampling period. Cyanobacteria were always a part of the community but overwhelmingly dominant from August through October.
- Cyanobacteria communities were dominated by *Aphanizomenon flos-aquae*, *Dolichospermum flos-aquae,* and *Dolichospermum planctonica*; all species that can fix nitrogen gas (N_2), control their buoyancy, and do not produce microcystin. *Microcystis aeruginosa* was a small component of the cyanobacteria biomass but produced the microcystin in Spanaway Lake.
- Microcystin genes (Mcy) and nitrogen (N_2) fixation genes (Nif) all showed a steady increase through July, peaking in August. The expression of the Mcy genes between July and September was dominated by the Mcy genes covering all major microcystin-producing genera (McyE) and genes attributable to *Microcystis* (McyA).
- The microcystin concentration in the water was positively correlated to McyA gene expression with a 2 – 3 week lag.
- There was a strong relationship between cyanobacteria growth (and microcystin production) and available nutrients. Cyanobacteria growth occurred mainly during periods of limited available N, which may reflect the use of inorganic N by cyanobacteria. Furthermore, the Mcy genes are regulated by nitrogen supply, and the expression of the genes was highest under N-limitation or stress.

Recommendations

Following this study, the following recommendations can be made:

- Nitrogen plays an important role in cyanobacteria growth and microcystin production. Lake management actions should consider both N and P budgets and management, which may require refining the understanding of internal loading.
- This monitoring study took place in one location on the lake. While it provides a detailed look at the cyanobacteria and microcystin production over the summer, conditions may vary in the shallower locations of the lake. Further understanding of the cyanobacteria dynamics and nutrient limitations across the lake would be beneficial.
- Follow-up work on Mcy gene expression, nutrient limitations, and microcystin production is a possible next step in monitoring cyanotoxins in Washington lakes.

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Glossary, Acronyms, and Abbreviations

Glossary

Chlorophyll *a*: One of the primary pigments in algae responsible for the acquisition of energy to allow photosynthesis to occur. It is a form of chlorophyll.

Conductivity: A measure of water's ability to conduct an electrical current. Conductivity is related to the concentration and charge of dissolved ions in water.

Cyanobacteria: Group of ancient and diverse microorganisms that have prokaryotic (bacterial) cell structure. They are photosynthetic and act like algae, but are classified as a Eubacteria. They are found in marine and freshwater. They are also called cyanophyta, blue-green algae and toxic algae.

Cyanotoxin: A term used to describe the toxins produced by cyanobacteria. It is a general term that refers to many different compounds. The toxins are a secondary metabolite produced by cyanobacteria.

Diel: Of, or pertaining to, a 24-hour period.

Dissolved oxygen (DO): A measure of the amount of oxygen dissolved in water.

Epilimnion: The warm upper layer of a body of water with thermal stratification, which extends down from the surface to the Thermocline, which forms the boundary between the warmer upper layers of the epilimnion and the colder waters of the lower depths, or Hypolimnion. The epilimnion is less dense than the lower waters and is wind-circulated and essentially homothermous.

Hypolimnion: The lowermost, non-circulating layer of cold water in a thermally stratified lake or reservoir that lies below the Thermocline, remains perpetually cold and is usually deficient of oxygen.

Metalimnion: The middle layer of a thermally stratified lake or reservoir. In this layer there is a rapid decrease in temperature with depth. Also referred to as Thermocline.

Microcystin: class of toxins produced by certain freshwater cyanobacteria. So far, approximately 150 different isomers of microcystin have been observed. They are hepatotoxins, which affect the liver. They are produced mainly by genus *Microcystis*.

Parameter: Water quality constituent being measured (analyte). A physical, chemical, or biological property whose values determine environmental characteristics or behavior.

pH: A measure of the acidity or alkalinity of water. A low pH value (0 to 7) indicates that an acidic condition is present, while a high pH (7 to 14) indicates a basic or alkaline condition. A pH of 7 is considered neutral. Since the pH scale is logarithmic, a water sample with a pH of 8 is ten times more basic than one with a pH of 7.

Phycocyanin: a pigment-protein complex from the light-harvesting phycobiliprotein family, along with allophycocyanin and phycoerythrin. It is an accessory pigment to chlorophyll. Phycocyanins are found in cyanobacteria.

qPCR: quantitative polymerase chain reaction is a molecular laboratory technique which monitors and quantifies a targeted DNA molecule.

Thermocline: The intermediate summer or transition zone in lakes between the overlying Epilimnion and the underlying Hypolimnion, defined as that middle region of a thermally stratified lake or reservoir in which there is a rapid decrease in temperature with water depth. Typically, the temperature decrease reaches 1°C or more for each meter of descent (or equivalent to 0.55°F per foot).

Watershed: A drainage area or basin in which all land and water areas drain or flow toward a central collector, such as a stream, river, or lake at a lower elevation.

303(d) list: Section 303(d) of the federal Clean Water Act requires Washington State to periodically prepare a list of all surface waters in the state for which beneficial uses of the water – such as for drinking, recreation, aquatic habitat, and industrial use – are impaired by pollutants. These are water quality limited estuaries, lakes, and streams that fall short of state surface water quality standards and are not expected to improve within the next two years.

Acronyms and Abbreviations

Ecology	Washington State Department of Ecology
EIM	Environmental Information Management database
EPA	U.S. Environmental Protection Agency
MEL	Manchester Environmental Laboratory
qPCR	quantitative polymerase chain reaction
RPD	relative percent difference
SOP	standard operating procedures
WRIA	Water Resource Inventory Area

Units of Measurement

°C	degrees centigrade
genes/mL	number of gene copies per milliliter
mg/L	milligrams per liter (parts per million)
RFU	relative fluorescence units (in situ probe)
µg/L	micrograms per liter (parts per billion)
µS/cm	microsiemens per centimeter, a unit of conductivity

Appendices

Appendix A. Supplemental Materials

Table A-1. qPCR primer sequences, targets, and annealing temperatures.

Primer	Sequence	Target	Annealing Temp
Cyan108F	acgggtgagtaacrcgtra	16S all cyano	56
Cyan 377R	ccatggcggaaaatttccc	16S all cyano	56
Micr 209F	atgtgccgcgaggtgaaacctaat	Microcystis 16S genus	64
Micr 409R	ttacaatccaaagaccttcctccc	Microcystis 16S genus	64
HepF	tttggggttaacttttttgggcatagtc	All Microcystin producers	54
HepR	aattcttgaggctgtaaatcgggttt	All Microcystin producers	54
mcyA-MSF	atccagcagttgagcaagc	Microcystin/Microcystis specific	60
mcyA-MS2R	gccgatgtttggctgtaaat	Microcystin/Microcystis specific	60
AnaCF1	ggtcctgggttgatgacagg	general anatoxin	60
AnaCR1	cggtagccccgactcttaatc	general anatoxin	60
cyrAf7	AACACGGCTTTGAGGTCTATC	general cylindro	64
cyrAr7	GAAGTAATCCTCACAGGTTCCC	general cylindro	64
sxtA F1	GCG GGA CTT TAT GCT CTA CTA C	general saxotoxin	60
sxtA R1	TAC TCC GTC ATC GGC ATT TG	general saxotoxin	60
McyE Mic F3	gtt atg ttt gcc ggc tcc ta	Toxic Microcystis	60
McyE Mic R3	gtg cct aga ctt aag ggt tga g	Toxic Microcystis	60
Nif Ana F6	ATGCCTATCCGTGAAGGTAAAG	nif in Anabaena	60
Nif Ana R6	CCACCGGAGTGAGCATATTT	nif in Anabaena	60
Nif Nos F3	ATCGTTCAACACGCAGAATTG	nif in Nostoc	60
Nif Nos R3	TCATCCATTTCGATAGGTGTGG	nif in Nostoc	60
PstS F3	TGGAATGTTACCAGCAGGAATAA	pstS in Anabaena (mainly A. flos-aquae)	60
PstS R3	AGTGCTGCTTGACGTAAACT	pstS in Anabaena (mainly A. flos-aquae)	60

Table A-2. Nutrient (mg/L) and phycocyanin (µg/L) laboratory quality control samples.

Analysis Date	Type	Nitrate-Nitrite as N	Ammonia	Total Persulfate Nitrogen	Ortho-Phosphate	Total Phosphorus	Phycocyanin
5/20/2021	MS	NA	NA	NA	93	102	NA
5/20/2021	Lab Dup	0.049	0.01	0.094	0.0043	0.0166	NA
5/26/2021	DUP RPD	4	NC	0.5	0.4	2	NA
5/26/2021	MS	96	97	94	109	103	NA
5/26/2021	Lab Dup	0.262	0.023	0.361	0.0064	0.0161	NA
5/26/2021	DUP RPD	0.5	5	5	9	2	NA
5/25/2021	MS	96	97	94	103	101	NA
5/25/2021	Lab Dup	0.262	0.023	0.361	0.0056	0.0134	NA
5/25/2021	DUP RPD	0.5	5	5	1	2	NA
6/16/2021	MS	94	96	92	105	104	NA
6/16/2021	Lab Dup	0.251	0.01	0.382	0.019	0.0203	NA
6/16/2021	DUP RPD	0.7	NC	3	2	19	NA
6/22/2021	MS	98	94	98	96	100	NA
6/22/2021	Lab Dup	0.102	0.01	0.194	0.0034	0.029	NA
6/22/2021	DUP RPD	0.7	10	0.4	5	0.2	NA
7/1/2021	MS	96	94	93	102	100	NA
7/1/2021	Lab Dup	0.076	0.018	0.398	0.0051	0.0169	NA
7/1/2021	DUP RPD	1	7	5	1	3	NA
6/24/2021	MS	95	90	97	104	100	NA
6/24/2021	Lab Dup	0.031	0.01	0.074	0.003	0.0147	3.78
6/24/2021	DUP RPD	1	NC	10	NC	0.3	NC
7/1/2021	MS	96	94	93	102	100	NA
7/1/2021	Lab Dup	0.076	0.018	0.398	0.0051	0.0169	NA
7/1/2021	DUP RPD	1	7	5	1	3	NA
7/19/2021	MS	98	101	98	95	100	NA
7/19/2021	Lab Dup	0.969	0.076	1.03	0.0043	0.0597	NA
7/19/2021	DUP RPD	0.2	0.7	4	8	4	NA
7/12/2021	MS	96	99	100	96	99	NA
7/12/2021	Lab Dup	0.179	0.01	0.275	0.0049	0.01	NA
7/12/2021	DUP RPD	0.3	NC	0.07	5	NC	NA
7/19/2021	MS	98	93	97	100	105	NA
7/19/2021	Lab Dup	0.131	0.01	0.25	0.003	0.0105	NA
7/19/2021	DUP RPD	2	NC	5	NC	0.8	NA
7/30/2021	MS	96	93	97	105	100	NA
7/30/2021	Lab Dup	0.187	0.01	0.252	0.0083	0.0133	7.25
7/30/2021	DUP RPD	0.3	NC	7	6	9	1.8
8/11/2021	MS	99	94	98	95	103	NA
8/11/2021	Lab Dup	0.066	0.014	0.289	0.0036	0.012	NA
8/11/2021	DUP RPD	0.05	8	8	12	2	NA
8/20/2021	MS	101	101	103	108	102	NA

Analysis Date	Type	Nitrate-Nitrite as N	Ammonia	Total Persulfate Nitrogen	Ortho-Phosphate	Total Phosphorus	Phycocyanin
8/20/2021	Lab Dup	0.039	0.481	0.757	0.007	0.0143	NA
8/20/2021	DUP RPD	4	0.6	3	6	35	NA
9/2/2021	MS	99	103	97	104	97	NA
9/2/2021	Lab Dup	0.011	0.01	0.045	0.003	0.0172	NA
9/2/2021	DUP RPD	NC	NC	44	NC	4	NA
9/2/2021	MS	101	98	107	109	94	NA
9/2/2021	Lab Dup	0.042	0.025	0.14	0.01	0.023	17.9
9/2/2021	DUP RPD	20	4	3	15	4	8.9
9/2/2021	MS	79	44	82	104	93	NA
9/2/2021	Lab Dup	0.082	0.029	0.342	0.0091	0.0302	NA
9/2/2021	DUP RPD	4	3	2	0.4	11	NA
9/16/2021	MS	95	99	88	96	95	NA
9/16/2021	Lab Dup	0.767	0.01	0.881	0.0058	0.035	NA
9/16/2021	DUP RPD	1	NC	7	7	5	NA
9/16/2021	MS	97	91	95	100	92	NA
9/16/2021	Lab Dup	0.103	0.01	0.025	0.0058	0.038	NA
9/16/2021	DUP RPD	0.6	NC	NC	10	0	NA
9/16/2021	MS	86	85	78	102	94	NA
9/16/2021	Lab Dup	0.01	0.01	0.042	0.003	0.0379	NA
9/16/2021	DUP RPD	NC	NC	NC	NC	4	NA
10/13/2021	MS	93	93	96	105	92	NA
10/13/2021	Lab Dup	0.66	0.021	0.642	0.0088	0.0451	NA
10/13/2021	DUP RPD	0.1	3	3	0.4	0.7	NA
10/6/2021	MS	93	90	98	102	91	NA
10/6/2021	Lab Dup	0.072	0.172	0.01	0.0302	0.0328	NA
10/6/2021	DUP RPD	0.5	3	NC	0.9	0.4	NA
10/20/2021	MS	99	90	109	105	94	NA
10/20/2021	Lab Dup	0.158	0.01	0.258	0.151	0.0327	16.7
10/20/2021	DUP RPD	0.6	NC	3	0.07	11	1.7
10/28/2021	MS	100	94	109	99	92	NA
10/28/2021	Lab Dup	0.724	0.01	0.82	0.007	0.033	NA
10/28/2021	DUP RPD	0.04	NC	3	1	1	NA
11/3/2021	MS	93	104	98	102	92	NA
11/3/2021	Lab Dup	0.313	0.016	0.423	0.0191	0.033	NA
11/3/2021	DUP RPD	1	1	2	0.2	1	NA
11/17/2021	MS	94	96	97	102	76	NA
11/17/2021	Lab Dup	0.061	0.01	0.082	0.013	0.0384	NA
11/17/2021	DUP RPD	2	NC	3	0.7	6	NA

NC = not calculated; NA = not analyzed; MS = matrix spike recovery (%); Lab Dup = laboratory duplicate; DUP RPD = relative percent difference for lab duplicate; red shaded box is outside study MQOs due to an insufficient spike.

Table A-3. Field quality control samples for nutrients (mg/L), chlorophyll *a* (µg/L), phycocyanin (µg/L), and microcystins (µg/L).

Sample Location	Depth (m)	Sample Date	Lab ID	TPN	NH_3-N	NO_3-NO_2 as N	PO_4	TP	Chl a	PC	MCs
MRL	—	—	—	0.025	0.01	0.01	0.003	0.01		5	0.15
MQO (RPD %)	—	—	—	20	20	20	20	20	30	30	40
SPANLK-HYPO	7	6/1/2021	2106043-02	0.529	0.013	0.36	0.0044	0.0265	3.33	BDL	NA
SPANLK-HYPO DUP	7	6/1/2021	2106043-03	0.541	0.012	0.362	0.0044	0.0268	3.32	BDL	NA
DUP RPD	—	—	—	2.2%	NA	0.6%	NC	NC	0.4%	NC	NC
SPANLK-HYPO	7	7/6/2021	2107024-02	0.387	0.044	0.066	0.0076	0.082	NA	NA	NA
SPANLK-HYPO DUP	7	7/6/2021	2107024-03	0.465	0.067	0.096	0.0079	0.0841	NA	NA	NA
DUP RPD	—	—	—	18.3%	NC	**37.0%**	NC	2.5%	—	NC	NC
SPANLK-EPI	0–2	7/12/2021	2107025-01	NA	NA	NA	NA	NA	1.73	BDL	NA
SPANLK-EPI DUP	0–2	7/12/2021	2107025-03	NA	NA	NA	NA	NA	1.68	BDL	NA
DUP RPD	—	—	—	NC	NC	NC	NC	NC	3.2%	NC	NC
SPANLK-HYPO	7	8/3/2021	2108024-02	0.738	0.484	0.041	0.0079	0.117	NA	NA	NA
SPANLK-HYPO DUP	7	8/3/2021	2108024-03	0.742	0.481	0.042	0.0081	0.121	NA	NA	NA
DUP RPD	—	—	—	0.5%	0.6%	NC	NC	3.4%	NC	NC	NC
SPANLK-EPI	0–2	8/9/2021	2107025-01	NA	NA	NA	NA	NA	20.47	55.5	NA
SPANLK-EPI DUP	0–2	8/9/2021	2107025-03	NA	NA	NA	NA	NA	22.51	42.5	NA
DUP RPD	—	—	—	NC	NC	NC	NC	NC	9.5%	26.5%	NC
SPANLK-EPI	0–2	9/7/2021	2109024-01	0.264	0.01	0.01	0.0055	0.038	27.74	87.5	3.04
SPANLK-EPI DUP	0–2	9/7/2021	2109024-04	0.254	0.01	0.01	0.0059	0.0503	28.74	88.5	1.19
DUP RPD	—	—	—	3.9%	NC	NC	NC	NC	3.5%	1.1%	**87.5%**
SPANLK-EPI	0–2	10/4/2021	2110012-01	0.592	0.26	0.104	0.0084	0.0292	7.42	16.3	0.155
SPANLK-EPI DUP	0–2	10/4/2021	2110012-04	0.645	0.26	0.103	0.0083	0.0276	6.96	15.7	0.174
DUP RPD	—	—	—	8.6%	0.0%	1.0%	NC	NC	6.4%	NC	NC
SPANLK-EPI	0–2	10/25/2021	2110015-01	0.653	0.136	0.286	0.0132	0.0513	11.91	38.5	NA
SPANLK-EPI DUP	0–2	10/25/2021	2110015-03	0.659	0.136	0.288	0.0131	0.0434	9.51	23.5	NA
DUP RPD	—	—	—	0.9%	0.0%	0.7%	NC	NC	22.4%	NC	NC

MRL = method reporting limit; DUP RPD = relative precent difference for field duplicate; NA = not analyzed; NC = not calculated; BDL = below detection limit; red shaded box is outside study MQOs.

Table A-4. Laboratory quality control samples for metals and anions.

Analysis Date	Type	Magnesium	Sodium	Aluminum	Calcium	Iron	Potassium	Bromide	Chloride	Fluoride	Sulfate
5/20/2021	MS	100	110	103	99	103	104	103	115	100	110
5/20/2021	Lab Dup	NA	NA	NA	NA	NA	NA	0.025	7.41	0.1	5.52
5/26/2021	DUP RPD	NC	NC	NC	NC	NC	NC	NC	0.2	NC	0.1
6/16/2021	MS	98	112	102	96	102	103	102	102	99	103
6/16/2021	Lab Dup	NA	NA	NA	NA	NA	NA	0.025	7.38	0.1	5.45
6/16/2021	DUP RPD	NC	NC	NC	NC	NC	NC	NC	0.1	NC	0.2
7/12/2021	MS	102	109	104	101	104	106	102	101	99	103
7/12/2021	Lab Dup	NA	NA	NA	NA	NA	NA	0.025	7.35	0.1	5.33
7/12/2021	DUP RPD	NC	NC	NC	NC	NC	NC	NC	0.5	NC	0.6
8/20/2021	MS	95	104	104	80	99	104	101	101	98	102
8/20/2021	Lab Dup	NA	NA	NA	NA	NA	NA	0.025	7.55	0.1	5.09
8/20/2021	DUP RPD	NC	NC	NC	NC	NC	NC	NC	0.03	NC	0.1
9/16/2021	MS	100	107	105	99	105	105	104	97	100	106
9/16/2021	MSD	97	99	103	91	103	102	103	96	99	102
9/16/2021	Lab Dup	NA	NA	NA	NA	NA	NA	0.025	7.96	0.1	4.55
9/16/2021	DUP RPD	NC	NC	NC	NC	NC	NC	NC	0.08	NC	0.09
10/20/2021	MS	97	99	100	93	99	98	103	87	98	104
10/20/2021	Lab Dup	NA	NA	NA	NA	NA	NA	0.025	7.91	0.1	3.98
10/20/2021	DUP RPD	NC	NC	NC	NC	NC	NC	NC	0.03	NC	0.004

NA = not analyzed; NC = not calculated; MS = matrix spike recovery (%);MSD = matrix spike duplicate (%); Lab Dup = laboratory duplicate; DUP RPD = relative precent difference for lab duplicate.

Table A-5. Field quality control samples for metals and anions (mg/L).

Sample Location	Depth	Sample Date	Lab ID	Aluminum	Calcium	Iron	Potassium	Magnesium	Sodium	Bromide	Chloride	Fluoride	Sulfate
MRL	—	—	—	0.025	0.025	0.025	0.25	0.025	0.025	0.025	0.1	0.1	0.3
MQO (RPD)	—	—	—	30	30	30	30	30	30	30	30	30	30
SPANLK-HYPO	7	6/7/2021	2106042-02	0.025	15.9	0.762	1.33	4.68	9.18	0.025	7.66	0.1	5.68
SPANLK-HYPO DUP	7	6/7/2021	2106042-03	0.025	16.1	0.892	1.33	4.72	9.19	0.025	7.69	0.1	5.72
DUP RPD	—	—	—	NA	1.3%	15.7%	0.0%	0.9%	0.1%	NA	0.4%	NA	0.7%
SPANLK-HYPO	7	7/6/2021	2107024-02	0.025	17.6	1.82	1.41	4.79	9.44	0.025	7.91	0.1	4.34
SPANLK-HYPO DUP	7	7/6/2021	2107024-03	0.025	17.5	2.62	1.38	4.76	9.34	0.025	7.87	0.1	4.16
DUP RPD	—	—	—	NA	0.6%	36.0%	2.2%	0.6%	1.1%	NA	0.5%	NA	4.2%
SPANLK-HYPO	7	8/3/2021	2108024-02	0.025	19.6	4.3	1.42	5.07	6.55	0.025	8.08	0.1	3.1
SPANLK-HYPO DUP	7	8/3/2021	2108024-03	0.025	19.1	4.17	1.42	4.92	6.41	0.025	8.09	0.1	3.08
DUP RPD	—	—	—	NA	2.6%	3.1%	0.0%	3.0%	2.2%	NA	0.1%	NA	0.6%
SPANLK-EPI	0–2	9/7/2021	2109024-01	0.025	13.7	0.196	1.19	4.55	6.52	0.025	7.96	0.1	4.55
SPANLK-EPI DUP	0–2	9/7/2021	2109024-04	0.025	13.6	0.196	1.16	4.52	6.47	0.025	7.97	0.1	4.54
DUP RPD	—	—	—	NA	0.7%	0.0%	NA	0.7%	0.8%	NA	0.1%	NA	0.2%
SPANLK-EPI	0–2	10/4/2021	2110012-01	0.025	14.3	0.366	1.27	4.42	7.29	0.025	7.91	0.1	3.98
SPANLK-EPI DUP	0–2	10/4/2021	2110012-04	0.025	14	0.36	1.24	4.32	7.18	0.025	7.92	0.1	3.99
DUP RPD	—	—	—	NA	2.1%	1.7%	NA	2.3%	1.5%	NA	0.1%	NA	0.3%

DUP RPD = relative precent difference for field duplicate; MRL = method reporting limit; NA = not analyzed ; red shaded box is outside study MQOs

Table A-6. Equipment blank samples for nutrients, metals, and anions.

Parameter	MRL	SPANLK-BLK	SPANLK-BLK
Sample Date	—	5/10/2021	6/7/2021
Lab ID	—	2105047-03	2106042-04
Aluminum	0.025	0.025	0.025
Calcium	0.025	0.05	0.057
Iron	0.025	0.025	0.025
Potassium	0.25	0.25	0.25
Magnesium	0.025	0.025	0.025
Sodium	0.025	0.291	0.154
Bromide	0.025	NA	0.025
Chloride	0.1	NA	0.1
Fluoride	0.1	NA	0.1
Sulfate	0.3	NA	0.3
Total Persulfate Nitrogen	0.025	0.025	0.025
Ammonia	0.01	0.01	0.01
Nitrate-Nitrite as N	0.01	0.01	0.01
Ortho-Phosphate	0.003	0.003	0.003
Total Phosphorus	0.01	0.01	0.01

MRL = method reporting limit; NA = not analyzed; red shaded box is outside study MQOs.

Table A-7. Transfer blank samples for chlorophyll a and phycocyanin.

Sample Location	Sample Date	Lab ID	Chl a	PC
MRL	—	—	NA	5.0
SPANLK-BLK	6/7/2021	2106042-04	0.017	0.44
SPANLK-BLK	7/12/2021	2107025G-1	1.489	BDL
SPANLK-BLK	8/9/2021	2108025-04	0.071	1.15
SPANLK-BLK	9/7/2021	2109024-5	0	0.32
SPANLK-BLK	10/4/2021	2110012-05	0	0.31
SPANLK-BLK	10/25/2021	2110015-04	0.024	19.6

Red shaded box is outside study MQOs; MRL = method reporting limit;
BDL = below detection limit; NA = not applicable

Table A-8. qPCR field quality control results — blank field filters.

Sample	2106042-blank 6/7/2021	2108024-blank 1 8/3/2021	2110012-blank 10/4/2021	2110015-blank 10/25/2021
16S-Mic	0	0	0	0
16SMic-RT	0	0	20.61	below detection
McyA-Mic	0	0	0	0
McyAMic-RT	0	0	0	0
Sxt	0	0	0	0
Sxt-RT	0	0	0	0
AnaC	0	0	0	0
AnaC-RT	0	0	0	0
16SCyano	0	0	0	0
16SCyano-RT	461.05	below detection	54.02	12.82
McyEmic	0	0	0	0
McyEmic-RT	0	0	0	0
McyEcyano	0	0	0	0
McyEcyao-RT	0	0	0	0
Nif-Ana	0	0	0	0
NifAna-RT	0	0	0	0
Nif-Nos	0	0	below detection	0
NifNos-RT	0	0	0	0
Psts	0	0	0	0
Psts-RT	0	0	0	0
Cylindro	0	0	0	0
Cylindro-RT	0	0	0	0

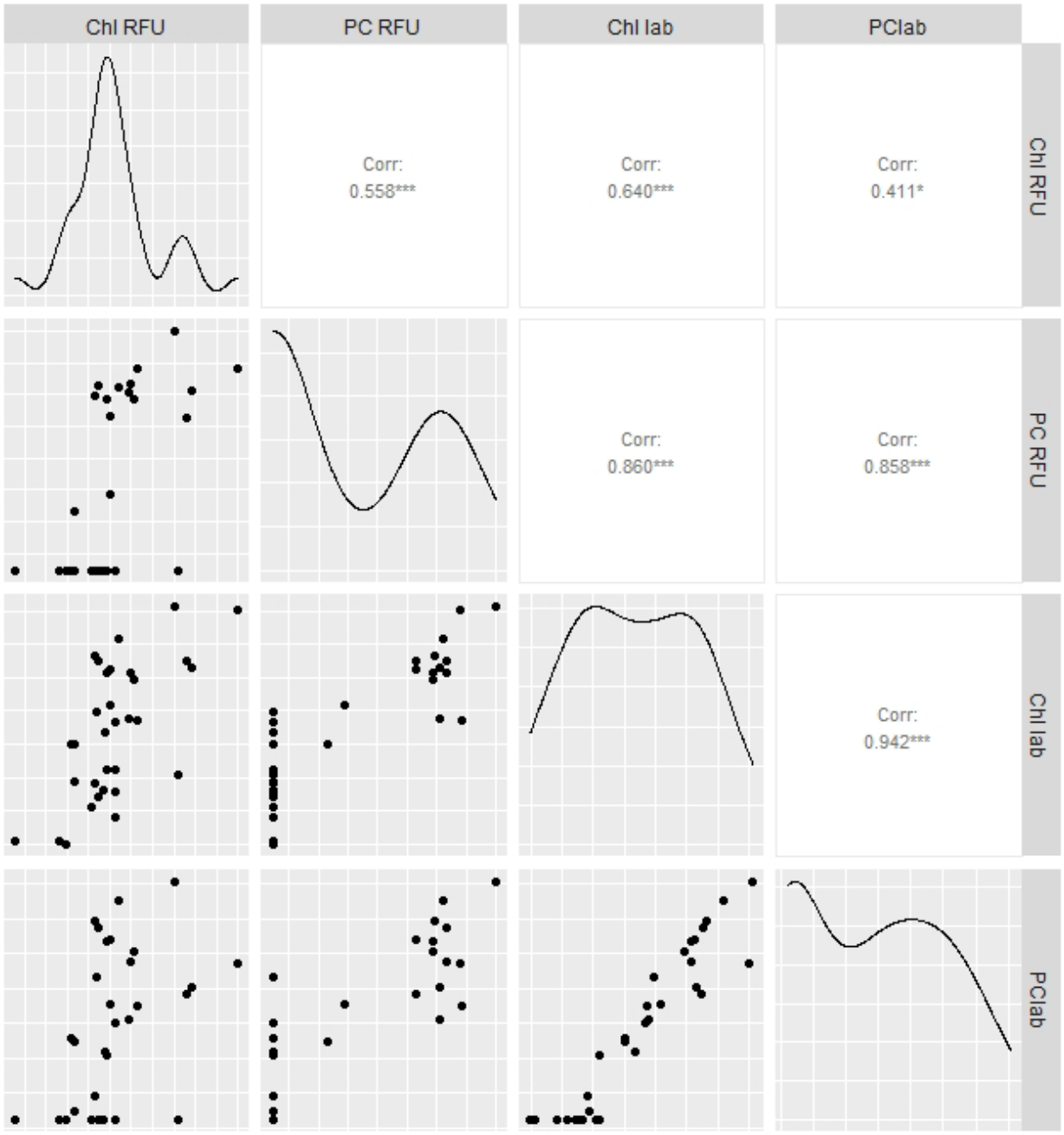

Figure A-1. Pearson correlation matrix for probe-measured and lab-measured pigments. All data are log-transformed.

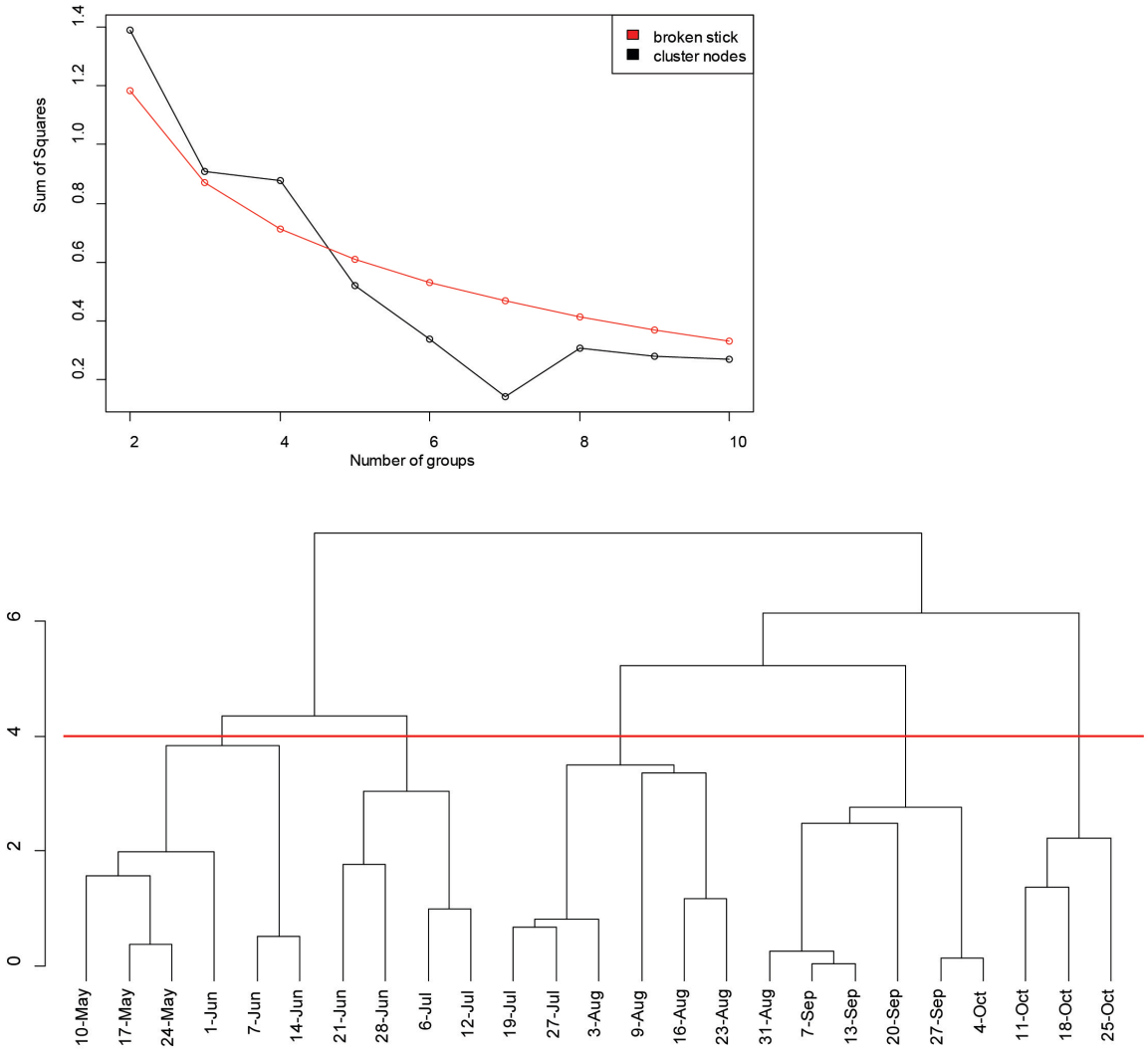

Figure A-2. Hierarchical cluster analysis constrained to the time of the phytoplankton communities in Spanaway Lake during the sampling period.

A random broken-stick model (upper plot) was conducted to determine the number of significant groups in the data, denoted by the red horizontal line on the cluster dendrogram.

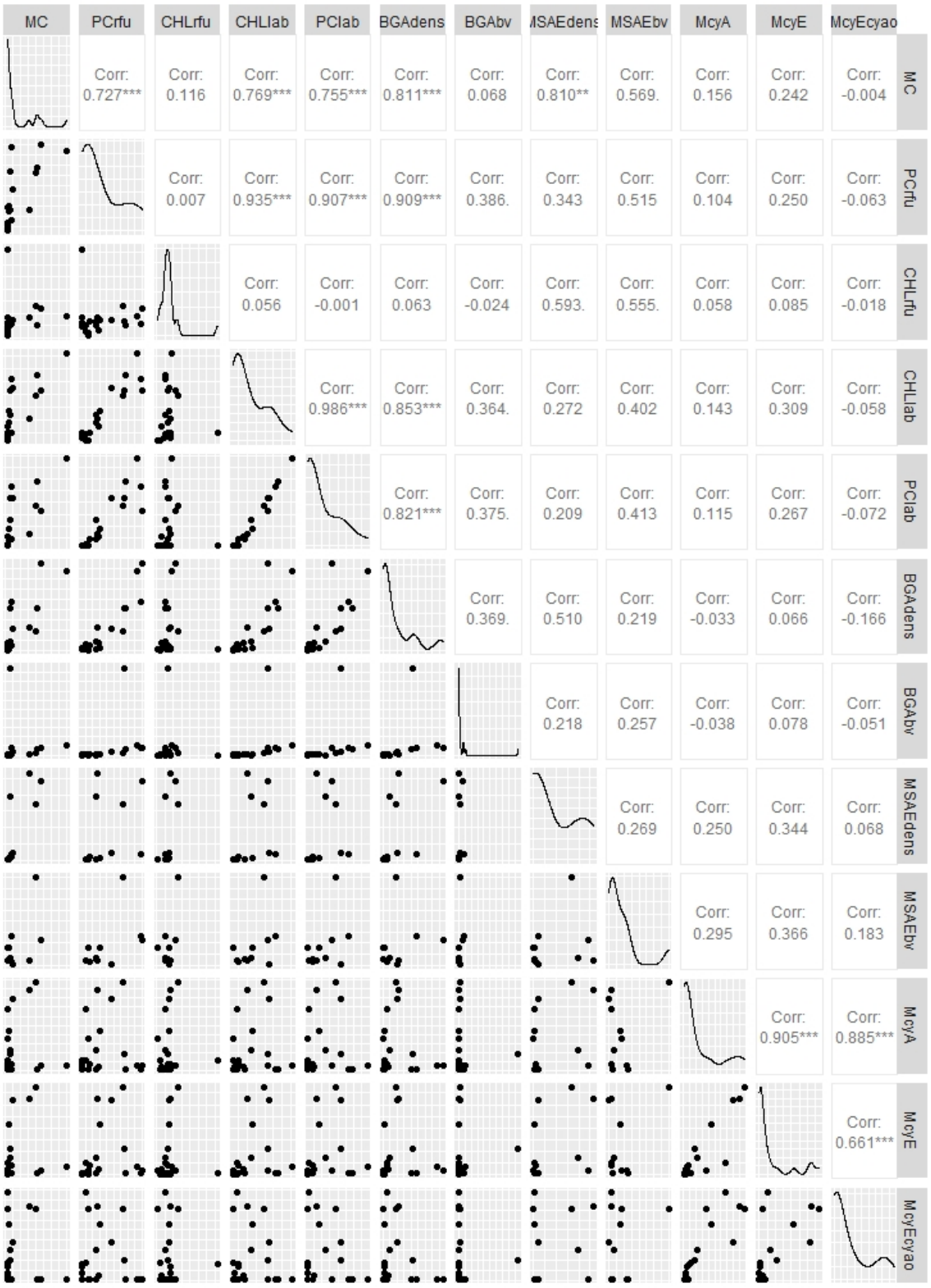

Figure A-3. Screening correlation matrix of untransformed data among pigments, cyanobacteria production, and Mcy genes.