Title: Characterization of Cyanobacterial Communities in Lakes Requires Consideration of Diurnal and Spatial Variation

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Abstract

Continental-scale increases in aquatic system eutrophication are linked with increased cyanobacteria threats to recreational water use and drinking water resources globally. While some guidance regarding monitoring is available, it is largely reactive and insufficient for proactive risk mitigation and management, which necessarily requires an understanding of the composition and dynamics of cyanobacterial communities in the aquatic system. Their distribution is impacted by several factors, including water column mixing and buoyancy regulation responses to light availability that create oscillatory diurnal migration patterns within the water column, creating challenges in the ability to accurately describe and quantify cyanobacterial densities. These dynamic fluctuations are not typically reflected in monitoring protocols, which frequently focus on surface depths and either ignore sampling time or recommend large midday timeframes (e.g., 10AM-3PM), thereby precluding accurate characterization of cyanobacterial communities. While diurnal vertical migration of cyanobacteria has been reported in marine and eutrophic freshwater systems, reports in oligotrophic freshwater lakes are scant and characterization have focused on individual 24-hour periods neglecting to consider day-to-day variability. These dynamics must be better understood and reflected in water quality monitoring guidance to advance drinking water risk management and source water protection approaches. To evaluate the impact of diurnal migrations and water column stratification on cyanobacterial abundance, communities were characterized using a multi-time point sampling series across a 48-hour period in a shallow well-mixed lake interconnected to a thermally stratified lake in the Turkey Lakes Watershed (Ontario, Canada). Amplicon sequencing of the V4 region in the 16S rRNA gene was performed to

characterize microbial community composition. Cyanobacteria were significantly represented in the microbial community in the midday and afternoon sampling times in the thermally stratified lake, but not in the well-mixed lake. Although the lakes in this study are interconnected, the cyanobacterial communities within them exhibited unique composition and distribution trends, thereby underscoring the importance of developing detailed sampling guidance to maximize the utility of cyanobacteria monitoring and better characterize and mitigate risk.

Keywords

Amplicon sequencing, diel cycles, vertical migration, buoyancy, algae, eutrophication

Highlights

- Water column stability impacts diurnal migrations of cyanobacteria
- Gas vacuolate taxa are more abundant at surface, but also present at depth
- Rain events can impact cyanobacteria distribution and impact detection
- Cyanobacteria distribution can vary significantly between interconnected lakes
- Cyanobacteria monitoring for risk management should incorporate time and depth

1. Introduction

Cyanobacteria are recognized as a threat to surface water quality because they can form dense blooms and produce secondary metabolites including taste and odor compounds (e.g., geosmin, 2-methyl isoborneol) and potent cyanotoxins (Harke et al., 2016; Huisman et al., 2018; Paerl, 2014; Vu et al., 2020). As a result of anthropogenic activities, cyanobacterial bloom frequency and intensity have been increasing (Huisman et al., 2018); climate change-exacerbated landscape disturbances (e.g., wildfires) further promote their proliferation (Emelko et al., 2016; Silins et al., 2014). Accordingly, as incidences of toxic blooms continue to increase (Huisman et al., 2018), water quality monitoring programs are needed to accurately characterize cyanobacterial communities in critical water supplies such as those relied upon for the provision of drinking water. The success of these monitoring programs relies on the ability to accurately characterize cyanobacterial communities and their distributions through incorporation of a comprehensive understanding on the adaptation of these organisms to environmental stress including the diurnal migrations in response to light and nutrient gradients (Huisman et al., 2018; Paerl, 2014).

The diurnal migration of cyanobacterial populations is driven by cellular characteristics (Naselli-Flores et al., 2021) and water column stability (Walsby et al., 1997), as summarized in Table 1. Diurnal migration rates (e.g., flotation and/or sinking rates) and vertical distribution of populations may be distinctive to individual taxa arising from characteristic differences in cell sizes or the presence of specialized cellular structures impacting cellular density (Naselli-Flores et al., 2021; Reynolds et al., 1987). However, these characteristic distributions are further impacted by water column stability

72 (Walsby et al., 1997) with the potential for oscillatory diurnal variation in the distribution
73 of cyanobacterial populations within systems of differing water column stability (Hunter
74 et al., 2008). Rapid changes caused by oscillatory diurnal variation in the distribution of
75 cyanobacterial populations can challenge detection if community structure and system
76 dynamics are not well characterized.

77 **Table 1**: A summary of characteristics of cyanobacteria and environmental conditions impacting buoyancy and spatial distribution in
78 the water column.

Characteristics of Cyanobacteria		
Characteristic	Example Taxa	Impact on Buoyancy
Cell Ballast Content	N/A	High photosynthetic rates result in accumulation of carbohydrates increasing cell density resulting in net downward migration (Chien et al., 2013; Hunter et al., 2008; Li et al., 2016; Westwood and Ganf, 2004)
Gas Vacuolate	*Microcystis* *Aphanizomenon* *Nostoc* *Anabeana* *Oscillatoria* *Coelosphaerium* (Staley, 1980; Walsby, 1981)	Provides positive buoyancy allowing for maintenance of position within the photic zone (Walsby et al., 1997). Decreased gas vacuole content after exposure to high light irradiance results in loss of buoyancy (Westwood and Ganf, 2004).
Small & Unicellular	*Synechococcus* *Cyanobium* *Synechocystis* *Cyanobacterium* (Sliwinska-Wilczeska et al., 2018)	Small cell size allows for maintenance of water column position (Reynolds et al., 1987; Śliwińska-Wilczewska et al., 2018).
Small & Colonial	*Aphanocapsa* *Aphanothece* *Chroococcus* *Coelosphaerium* *Cyanobium* *Cyanodictyon* *Merismopedia* *Romeira* *Snowella* *Tetracerus* (Sliwinska-Wilczeska et al., 2018)	Smaller colonies exhibit more random spatial movement with no clear diurnal pattern (Chien et al., 2013).

Large Colonial & Filamentous Forms	*Dolichospermum circinale* (Westwood and Ganf, 2004)	Larger colonies move more rapidly allowing for migration of greater depths (Reynolds et al., 1987; Westwood and Ganf, 2004) Sinking rates are also faster (Ganf, 1974)
Environmental Conditions		
Condition	Examples	Impact on Distribution
Water Column Stability	Thermal Stratification	Creates zonation in the water column frequently with nutrient depleted, light rich surface waters and light-limited, nutrient rich deep waters (Chien et al., 2013). Vertical migrations allow for access to optimal environmental conditions (Chien et al., 2013).
	Non-Stratified Water Columns	Wind induced mixing of the water column may result in homogeneous distributions (Frempong, 1981; Hunter et al., 2008; Wallsby et al., 1997).
	External Mixing Events – Storms	Storm events may result in downward mixing of communities (Walsby et al., 1997).
Light Availability	Daytime	Exposure to high light in the daytime results in loss in buoyancy with high photosynthetic rate and accumulation of carbohydrates resulting in downward migration (Ibelings et al., 1991).
	Nighttime	With light limitation and decreased photosynthetic rates, cellular carbohydrates are utilized resulting in decreased density and upward migration for light access in daytime (Ibelings et al., 1991).

The need to better describe cyanobacteria growth and behavior in freshwater systems is increasingly urgent because of climate variability and landscape change-associated impacts on freshwater systems. Global increases in eutrophication such as those described in recent continental-scale evidence from thousands of water bodies in the conterminous U.S. that led to the conclusion that dramatic reductions in the number of naturally oligotrophic streams and lakes have occurred since the turn of the century are likely linked to climate change driven extremes in precipitation and runoff that have exacerbated nutrient delivery to and primary productivity within these sensitive receiving waters (Stoddard et al., 2016). Effective risk management in response to the shifts requires accurate risk characterization. While the factors that lead to cyanotoxin production remain poorly understood, improved community characterization that reflects contemporary understanding of the diversity of cyanobacterial populations and their adaptations is essential to advancing the management of risks attributable to the presence of potentially toxic cyanobacteria in water supplies and better informing pre-emptive mitigation of potential health impacts and drinking water treatment challenges.

Critically, while diurnal vertical migration of cyanobacteria has been reported in marine (Olli, 1999) and eutrophic freshwater (Hunter et al., 2008; von Orgies-Rutenberg et al., 2018) systems, reports of diurnal vertical migration of cyanobacteria in oligotrophic freshwater lakes are scant. Previous diurnal characterization of cyanobacterial communities has utilized spectrophotometric analysis of chlorophyll-*a* and microscopic cell enumeration on samples to collected over a multi-time point sampling series, frequently limited to a 24-hour period (Frempong, 1981; Ganf, 1974; Gilbert et al., 2010; Ibelings et al., 1991; Olli, 1999; Shahraki et al., 2020). Studies using next-

generation sequencing technology to characterize diurnal variation in aquatic microbial communities are further limited in number and scope (Gilbert et al., 2010; Shahraki et al., 2020). While the use of multi-time point sampling series provides insights into the variability observed in community composition over a matter of hours, restriction to a single 24-hour period will not encompass the natural dynamic variability that aquatic systems may experience day-to-day. To fully characterize diurnal trends, sampling periods must extend outside of 24-hour periods and include consecutive days. Here, the impact of sampling time and depth on cyanobacterial community composition were evaluated using amplicon sequencing of the V4 region in the 16S rRNA gene. Taxonomic composition and community diversity analyses provided insights into diurnal trends in distribution and subsequently the impact of sampling time on detection of these organisms. Specifically, the fluctuations in cyanobacterial community composition were evaluated (i) over a multi-time point sampling period over a 48-hour window, and (ii) spatially within the water column of a stratified lake. The characterization of spatial and temporal trends present in cyanobacterial communities demonstrates the potential impact of sampling time and system specific conditions on detection and will provide critical insight into the development of cyanobacterial monitoring programs for oligotrophic freshwater systems.

2. Methods

2.1 Study Site: Turkey Lakes Watershed

The Turkey Lakes Watershed (TLW) Study was established in 1980 to investigate ecosystem effects of acidic atmospheric deposition; Jeffries et al. (1988) provide a comprehensive description of the physical characteristics of the watershed. In brief, it is

126 approximately 50 km north of Sault Ste Marie, Ontario on the Canadian Shield in an
127 uneven-aged tolerant hardwood and mixed conifer forest landscape (Jeffries et al., 1988).
128 It consists of four interconnected lakes fed by both first order streams and groundwater:
129 Batchwana Lake, Wishart Lake, Little Turkey Lake and Big Turkey Lake (Jeffries et al.,
130 1988; Figure S1). Except for Wishart, these lakes thermally stratify during summer and
131 winter annually. The lakes are classified as oligotrophic to mesotrophic, and
132 cyanobacteria are the dominant members of phytoplankton communities(Jeffries et al.,
133 1988). The shallow depth of Wishart Lake often results in complete wind-induced mixing
134 that precludes water column stratification; Little Turkey lake is deeper and regularly
135 undergoes thermal stratification (Figure S2).

136 *2.2 Sample Collection*

137 Water samples were collected from the deepest point in Little Turkey
138 (47°02’37.2”N 84°24’24.4”W) and Wishart (47°03’00.0”N 84°23’58.3”W) Lakes over a
139 two-day period in August 2018 (August 22nd and 23rd) across three time points on both
140 days: morning (8-9 a.m.), midday (12-1 p.m.) and afternoon (4-5 p.m.). Samples were
141 collected from Secchi depth, the water depth at which light penetration is approximately
142 1% of surface illumination is considered the maximum depth at which there is sufficient
143 light for photosynthesis (Bukata et al., 1988). In Wishart Lake, these samples were
144 collected from near the bottom (4 m) due to high water clarity. Secchi depths ranged from
145 4 to 5.25 m in Little Turkey Lake. Water samples were also collected at the surface (0 m)
146 in Little Turkey Lake on the first sampling day to evaluate potential temporal correlations
147 in cyanobacterial communities between depths.

Water samples collected using a Masterflex E/S portable sampler peristaltic pump were filtered initially through a 47 mm GF/C filter (Whatman plc, Buckinghamshire, United Kingdom). After vacuum filtration, 250 mL of filtered water were filtered a second time through a 0.22 µm Sterivex™ filter to collect additional microbes. The filters were stored at -20°C prior to DNA extraction. Sampling details are provided in Table S1.

2.3 DNA Extraction, 16S rRNA Gene Amplicon Sequencing

DNA was extracted using the DNeasy PowerSoil Kit (QIAGEN Inc., Venlo, Netherlands) following the manufacturer's protocol. In brief, elution buffer was added to spin columns for 15 minutes prior to elution of the DNA extract. DNA was quantified using a NanoDrop spectrophotometer (Table S1); absolute values were only accurate at DNA concentrations of more than 10ng/µl. The DNA extracts were submitted for amplicon sequencing using the Illumina MiSeq platform (Illumina Inc., San Diego, United States) at a commercial laboratory (Metagenom Bio Inc.,Waterloo, ON). Primers designed to target the 16S rRNA gene V4 region [515FB (GTGYCAGCMGCCGCGGTAA) and 806RB (GGACTACNVGGGTWTCTAAT)] (Walters et al., 2015) were used for PCR amplification. Amplicon sequencing of the DNA extracts was conducted using the Illumina MiSeq platform (Illumina Inc., San Diego, United States).

2.4 Sequence Processing & Library Size Normalization

The program QIIME2 (v. 2019.10; (Bolyen et al., 2019) was used for bioinformatic processing. Demultiplexed paired-end sequences were trimmed and denoised, including the removal of chimeric sequences and singleton sequence variants, using DADA2 (Callahan et al., 2016) to construct the amplicon sequence variant (ASV)

table. Taxonomic classification was performed using a Naïve-Bayes taxonomic classifier trained using the SILVA138 database (Quast et al., 2013; Yilmaz et al., 2014). Taxonomic assignments for amplicon sequence variants (ASV) classified as Cyanobacteria at the phylum level were manually curated to reflect taxonomic levels above the genus level according to AlgaeBase (Guiry and Guiry, 2022). Files from QIIME2 were imported into R (v. 4.0.1; R Core Team, 2020) for downstream analyses using *qiime2R* (v. 0.99.23; Bisanz, 2018). Initial sequence libraries were filtered to exclude ASVs that were taxonomically classified as mitochondria or chloroplast sequences using *phyloseq* (v. 1.32.0; (McMurdie and Holmes, 2012). For cyanobacterial community analysis, ASVs classified as Cyanobacteria at the phylum level were filtered to create libraries consisting of only cyanobacterial sequences. They were normalized to a library size of 370 reads.

2.5 Cyanobacterial Communities - Taxonomic Composition & Diversity Analyses

Community composition was assessed at the taxonomic order level and relative abundances were visualized using a heatmap produced with *mirlyn* (Cameron and Tremblay, 2020). The implementation of relative abundances of taxonomic classifications of ASVs does not require additional library size normalization. Relative abundances were randomized across phyla within samples to identify significantly dominant groups within the microbial community in relation to sampling time with a Bonferroni correction.

To confirm the taxonomic classification performed by the Naïve-Bayes classifier, a phylogenetic tree was constructed in MEGA X (Kumar et al., 2018) using cyanobacterial reference sequences and sequences from samples (Figure S3). Sequences classified to the genera highlighted in guidelines and resources for sampling protocol

deigns (Graham et al., 2008; Vidal et al., 2021) including *Microcystis*, *Dolichospermum* (as *Anabaena* in Graham et al., 2008), *Aphanizomenon*, *Pseudanabaena*, and *Synechococcus* were selected for further evaluation. Notably, other taxa contributed to the compositional structure at the order level but were excluded from this analysis due to the frequent water quality management focus on bloom forming and toxic taxa. In addition to the aforementioned genera, sequences classified to the following were also included *Radiocystis* because of its 16S rRNA genes that are identical to *Microcystis* (Vidal et al., 2021) and toxicity (Vieira et al., 2003), and *Cyanobium*, which is a potentially toxic picocyanobacterial genera (Śliwińska-Wilczewska et al., 2018) that was detected in high relative abundances. Cyanobacterial orders were grouped by morphology as follows: (i) Unicellular Taxa – Synechococcales (*Cyanobium*, *Synechococcus*), (ii) Colonial – Nostocales (*Microcystis*, *Radiocystis*), and (iii) Filamentous – Nostocales (*Dolichospermum*, *Anabaena*, *Aphanizomenon*) and *Pseudanabaena* (Order – Synechococcales) to characterize the potential variation in diurnal movements dependent on cell size and shape. Notably, the genus *Pseudanabaena* initially was classified as Oscillatoriales based on the filamentous morphology, but recent genomic sequencing and examination of ultrastructural characteristics has resulted in reclassification into the order Synechococcales (Vidal et al., 2021). ASVs classified to the genus *Pseudanabaena* were included with other filamentous taxa. Relative abundances of the cyanobacterial sequences were visualized using a heatmap and evaluated based on unicellular, filamentous, or colonial morphologies to characterize taxa-specific diurnal trends

As amplicon sequencing only indirectly and partially represents source diversity and clustering, amplification, and the lack of certainty about how many zeros should be

included in the data compromise statistical inference about source diversity (Schmidt et al., 2021), community diversity analyses were performed using *mirlyn* on libraries that were repeatedly rarefied(Cameron et al., 2021) . The Shannon Index (Shannon, 1948), an alpha diversity metric, was analyzed for sample comparison to identify trends in sample diversity as a function of time. Rarefied libraries were also used for beta-diversity analyses and rarefied libraries were transformed using a Hellinger transformation (Legendre and Gallagher, 2001). Hellinger transformed data were used to calculate Bray-Curtis distances (Bray and Curtis, 1957) used in principal component analysis (PCA).

2.6 Data Availability

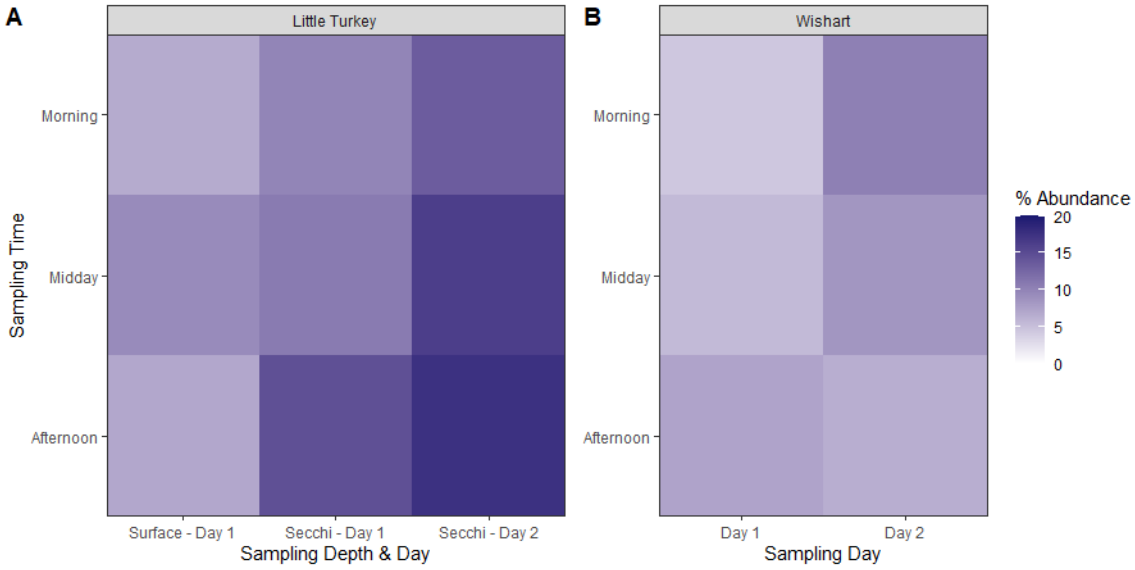
Sequence data analyzed in this study is available in the European Nucleotide Archive (ENA) under study accession ERP134980.

3. Results & Discussion

3.1 Diurnal Variation in Cyanobacterial Communities

Cyanobacterial relative abundances were higher in Little Turkey Lake than Wishart Lake (Figure 1A; Table 2). In Little Turkey Lake, the increase in relative abundance of cyanobacteria exhibited diurnal trends with significant representation in the microbial community on both sampling days during the afternoon sampling time point ($p = 0.011$, $p = 0.001$, respectively; Table S2) and midday on the second sampling day ($p = 0.0006$), but not the first day ($p = 0.27$). Further variation between the representation of cyanobacteria in the microbial community of Little Turkey Lake was observed in morning sampling, between the first ($p = 1$) and second sampling days ($p = 0.28$) indicating differences in microbial community composition between consecutive days (Figure S4). Unlike Little Turkey Lake, Wishart Lake did not exhibit a consistent and

240 recurring increase in the relative abundances from morning to afternoon; cyanobacteria
241 were not significantly represented in the microbial community at any point and their
242 relative abundance remained consistent over the period of evaluation ($p = 1$) (Figure 1B;
243 Table 2).



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245 **Figure 1**: Heatmap depicting the composition of cyanobacterial communities at the order
246 level across a multi-time point sampling series in a stratified (Little Turkey) and non-
247 stratified (Wishart) lake. Amplicon sequence variants of the V4 region of the 16S rRNA
248 gene classified to the phylum Cyanobacteria were selected to examine the contribution of
249 cyanobacterial communities to the bacterial community at (A) surface waters and Secchi
250 depth in Little Turkey Lake, and (B) Secchi depth in Wishart Lake. At Secchi depth,
251 cyanobacteria exhibited increased abundances later in the day in the stratified lake but no
252 consistent diurnal trend in the non-stratified lake.

253 **Table 2**: Relative abundances of cyanobacteria within the microbial community and subsequent composition of cyanobacteria
254 communities. Values were rounded to two decimal points and excluded groups that were present at less than 1% abundance.

Taxonomic Group	Day 1			Day2		Sampling Time
	Little Turkey		Wishart	Little Turkey	Wishart	
	Secchi	Surface				
Cyanobacteria	9.91	6.67	4.39	13.42	10.34	Morning
	10.74	9.30	5.47	16.28	8.47	Midday
	14.62	6.97	7.31	17.53	6.46	Afternoon
Chroococcales	33.55	26.98	2.43	15.66	2.69	Morning
	10.18	26.13	3.19	12.54	4.03	Midday
	16.27	23.41	1.87	16.30	3.59	Afternoon
Chroococcaceae	12.00	7.13	-	2.85	1.41	Morning
	2.20	9.35	1.09	3.93	1.04	Midday
	4.99	8.58	-	5.52	1.79	Afternoon
Microcystaceae	15.33	16.25	1.08	7.62	-	Morning
	4.41	13.26	-	4.01	1.55	Midday
	5.52	10.66	1.09	5.18	1.79	Afternoon
Nostocales	1.33	1.81	-	-	-	Morning
	0.72	1.08	-	-	0.22	Midday
	0.70	2.20	-	0.17	-	Afternoon
Oscillatoriales	-	-	-	-	-	Morning
	-	-	-	-	-	Midday
	-	-	-	-	-	Afternoon
Synechococcales	62.03	66.09	91.89	82.00	90.28	Morning
	87.77	69.93	93.10	85.70	89.67	Midday
	81.19	71.24	89.86	81.39	90.54	Afternoon

Coelosphaeriaceae	2.93	1.24	1.35	3.20	1.29	Morning
	2.36	1.92	-	2.32	-	Midday
	4.29	3.34	-	2.82	-	Afternoon
Merismopediaceae	1.07	1.41	-	-	-	Morning
	-	1.09	-	-	-	Midday
	-	-	-	1.11	-	Afternoon
Pseudanabaenaceae	-	-	4.32	-	2.58	Morning
	-	-	14.37	-	1.85	Midday
	-	-	2.34	-	8.46	Afternoon
Synechococcaceae	61.87	66.42	87.57	82.35	87.70	Morning
	88.05	70.47	78.74	85.86	87.82	Midday
	81.44	71.37	87.52	81.60	82.11	Afternoon

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In Little Turkey Lake, the significant representation of cyanobacterial sequences in the afternoon suggests the occurrence of daily vertical migrations of cyanobacteria in the water column. This observation is consistent with previous reports of diurnal vertical migration arising from buoyancy regulation in response to environmental changes (Howard, 2001). Water column stratification in Little Turkey Lake likely creates light limited environments at deeper depths. The increase in relative abundances of cyanobacterial sequences that was observed at Secchi depth at midday and in the afternoon is consistent with the downward migration of surface populations to avoid high light irradiance (Olli, 1999) and increased cellular density through accumulation of photosynthetic products (Chu et al., 2007; Xiao et al., 2012).

Unlike Little Turkey Lake, Wishart Lake exhibited similar relative abundances of cyanobacterial sequences throughout the multi-time point sampling series due to the shallow non-stratified water column. Vertical distribution of cyanobacteria in shallow lakes that are not stratified have been shown to be less dependent on the light cycle (Ibelings et al., 1991) supporting the non-temporal response observed in Wishart Lake. Although Wishart and Little Turkey Lake are interconnected and are located within the same watershed, both lakes exhibited differing relative abundances and diurnal trends indicating the dynamic nature of cyanobacterial communities in individual systems. While the scope of this study is still limited by the number of sampling days and sites, the variation observed between sampling times on these days exhibits the potential dynamic nature and distribution of aquatic microbial communities that can occur between consecutive days. This warrants the development of sampling protocols and supports the

278 notion that universal sampling strategies are near impossible to design (Pobel et al.,
279 2011).

280 In addition to examining the daily trends of cyanobacterial relative abundances at
281 Secchi depth, samples collected at the surface of Little Turkey Lake were assessed over
282 the three time points in a single day to identify diurnal trends arising between different
283 depths within a stratified water column. At the surface, relative abundances varied over
284 time (6.67 – 9.30%; Figure 1A; Table 2) but were consistently lower than those at Secchi
285 depth. Unlike the Secchi depth community, the relative abundances of cyanobacterial
286 sequences at the surface were not significantly represented in the bacterial community
287 over the sampling period ($p = 1$) indicating no changes over time (Figure S5). The
288 markedly different cyanobacterial relative abundances detected between surface and
289 Secchi depths underscore the importance of including across a range of depths in
290 monitoring protocols focused on risk management so that cyanobacterial communities are
291 not underestimated; notably, in the present investigation, surface sampling alone—which
292 is a common water monitoring sampling approach (Graham et al., 2008)—would have
293 severely underestimated cyanobacterial abundance. Accordingly, these data demonstrate
294 that diurnal migrations of cyanobacteria necessitate the need for discrete depth sampling
295 across the water column so that cyanobacterial communities can be accurately
296 characterized, and risk can be effectively managed. While discrete depth sampling
297 provides important insights regarding cyanobacterial community membership and its
298 variability across the depth of the water column (and thus represents significant
299 advancement over sole reliance on surface sampling), it is impossible to characterize true
300 population size, diversity, and distribution in the absence of full depth profiles.

Cyanobacteria sequence libraries were repeatedly rarefied to a normalized library size of 370 to explore diurnal trends in community diversity (Figure 2). The diversity of the cyanobacterial community in Little Turkey Lake at Secchi depth indicated small fluctuations that were not linked to specific diurnal responses. For example, the Shannon Index decreased from morning to afternoon on the first sampling day but increased on the second day. Alternatively, diversity peaked at midday in Wishart Lake (Figure 2A). In contrast, in Little Turkey Lake, surface communities were less diverse than those Secchi depth in the morning (Figure 2B). Later in the day, however, the Shannon Index was almost equivalent at these two depths. While the diversity of communities at the surface remained relatively consistent over the course of a day, the diversity at Secchi depth slightly decreased by afternoon of the first day, suggesting potential for downward migration in the stratified layer during the afternoon sampling period. These data underscore that cyanobacteria sampling protocol designs should include community characterization across the depth of the water column at various times during the day to ensure that the complete genetic and functional diversity of cyanobacterial communities is reflected to inform risk management.

Similarity between communities across sampling times, lake sites, and sampling depths was analyzed using Bray-Curtis dissimilarity and visualized using a PCA ordination on the rarefied data (Figure 2C). Cyanobacterial communities within lakes were found to be more similar within lakes than between lakes, with distinct clusters formed for Little Turkey and Wishart Lake. Additional similarity was observed within lakes between sampling time points across sampling days, except in the morning on the first day, which is discussed below in *Section 3.3*. The innate dissimilarity between

sampling depths and sampling sites in interconnected lakes within the same watershed further emphasizes the need for sampling approaches that can sufficiently capture ecological community structures that may ultimately contribute to signaling the potential for bloom formation, or toxin or taste and odor production.

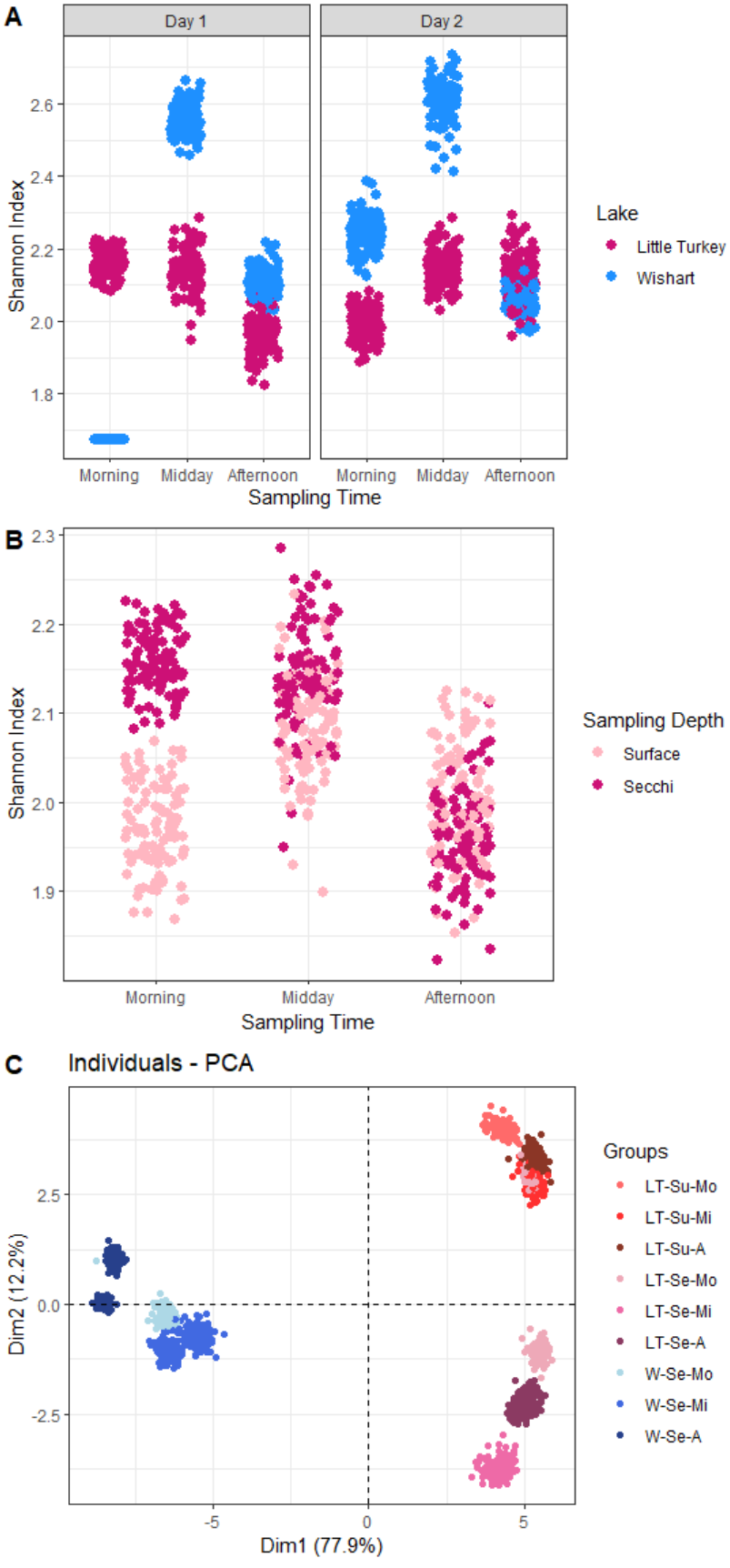

Figure 2: Alpha and beta-diversity analyses of cyanobacterial communities collected across a multi-time point sampling series in a stratified (Little Turkey) and non-stratified lake (Wishart). Amplicon sequence variants classified to the phylum Cyanobacteria were filtered to characterize diversity within cyanobacterial communities. The Shannon Index was calculated on rarefied libraries to evaluate the effects of (A) lake site and sampling time on community diversity and (B) sampling depth and sampling time on community diversity. (C) The Bray-Curtis dissimilarity metric was used to explore similarities in communities between sampling times (Morning = Mo, Midday = Mi, Afternoon = A), lake site (Little Turkey = LT, Wishart = W) and sampling depth (Su = Surface, Se = Secchi) demonstrating unique communities between sampling depth and lake site.

3.2 Diurnal Trends of Bloom Forming & Toxic Cyanobacterial Taxa

Taxa specific diurnal responses were evaluated by assessing the taxonomic and ASV composition of the cyanobacterial communities. A total of 41 ASVs classified to the phylum Cyanobacteria were identified across the samples collected; these included taxa belonging to the cyanobacterial orders Chroococcales, Nostocales, and Synechococcales (Table 3) and are contextualized below based on their respective morphologies.

Table 3: Taxonomic classification of potentially bloom-forming and toxic Cyanobacteria classified amplicon sequence variants in the diurnal sampling series.

ASV	Taxonomic Classification	Lake Site
ASV819	Nostocales	Little Turkey
ASV822	Nostocales	Little Turkey
ASV824	Nostocales	Little Turkey
ASV838	Synechococcaceae	Wishart
ASV839	Synechococcaceae	Little Turkey & Wishart

ASV842	Synechococcaceae	Wishart
ASV843	Synechococcaceae	Wishart
ASV846	Synechococcaceae	Little Turkey & Wishart
ASV848	Synechococcaceae	Little Turkey & Wishart
ASV849	Synechococcaceae	Wishart
ASV850	Synechococcaceae	Little Turkey & Wishart
ASV853	Synechococcaceae	Wishart
ASV855	Synechococcaceae	Little Turkey & Wishart
ASV857	Synechococcaceae	Wishart
ASV858	Synechococcaceae	Wishart
ASV859	Synechococcaceae	Wishart
ASV865	Synechococcaceae	Little Turkey & Wishart
ASV866	Synechococcaceae	Little Turkey & Wishart
ASV869	Synechococcaceae	Wishart
ASV921	Synechococcaceae	Wishart
ASV919	*Radiocystis*	Little Turkey & Wishart
ASV913	*Microcystis*	Little Turkey
ASV 914	*Microcystis*	Little Turkey & Wishart
ASV806	*Pseudanabaena*	Wishart

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349 *3.2.1 Unicellular Taxa*

350 Irrespective of time of day, both lakes were dominated by sequences classified to
351 the order Synechococcales (Figure 3), and family Synechococcaceae (Table 2), which
352 includes unicellular picocyanobacterial genera such as *Synechococcus* and *Cyanobium*.
353 The dominance of Synechococcaceae classified ASVs over the sampling period indicates
354 a lack of distinct diurnal migrations. This result is not surprising because smaller sized
355 taxa are able to maintain water column position better than larger colonial and

356 filamentous taxa (Śliwińska-Wilczewska et al., 2018; Yamamoto and Nakahara, 2006). In
357 Little Turkey Lake, Synechococcaceae ASVs were found in higher relative abundances at
358 Secchi depth, possibly due to the higher abundance of gas vacuolate taxa at the surface
359 (discussed below in *Section 3.3.2*). Notably, the high relative abundances of
360 Synechococcaceae ASVs at Secchi depth include the presence of potentially toxic
361 picocyanobacterial taxa that would be overlooked by sampling programs reliant only on
362 surface sampling.

363 While the cyanobacteria communities in both lakes were dominated by the ASVs
364 classified to the family Synechococcaceae, substantial differences in composition were
365 observed between Little Turkey (Figure 4A) and Wishart Lakes (Figure 4B). Specifically,
366 Little Turkey Lake included 7 ASVs and Wishart Lake included 17 ASVs, thereby
367 demonstrating considerable differences in community composition between systems
368 within the same watershed. ASV848 was observed consistently in high relative
369 abundances in both lakes and ASV846 was noted in high relative abundances in Little
370 Turkey Lake. Despite belonging to the same taxonomic family, these ASVs exhibited
371 distinct diurnal patterns in abundance. For example, ASV848 exhibited a decrease in
372 abundance at midday, while ASV846 peaked. Although Wishart Lake exhibited general
373 homogeneity in diurnal variation of cyanobacteria relative abundances, individual ASVs
374 exhibited unique diurnal responses. For example, ASV855, ASV859, ASV850, and
375 ASV849 were absent in morning periods on both sampling days but were present in the
376 midday or afternoon. The consistent and ephemeral occurrence of the different
377 Synechococcaceae ASVs would likely be missed by traditional monitoring approaches.

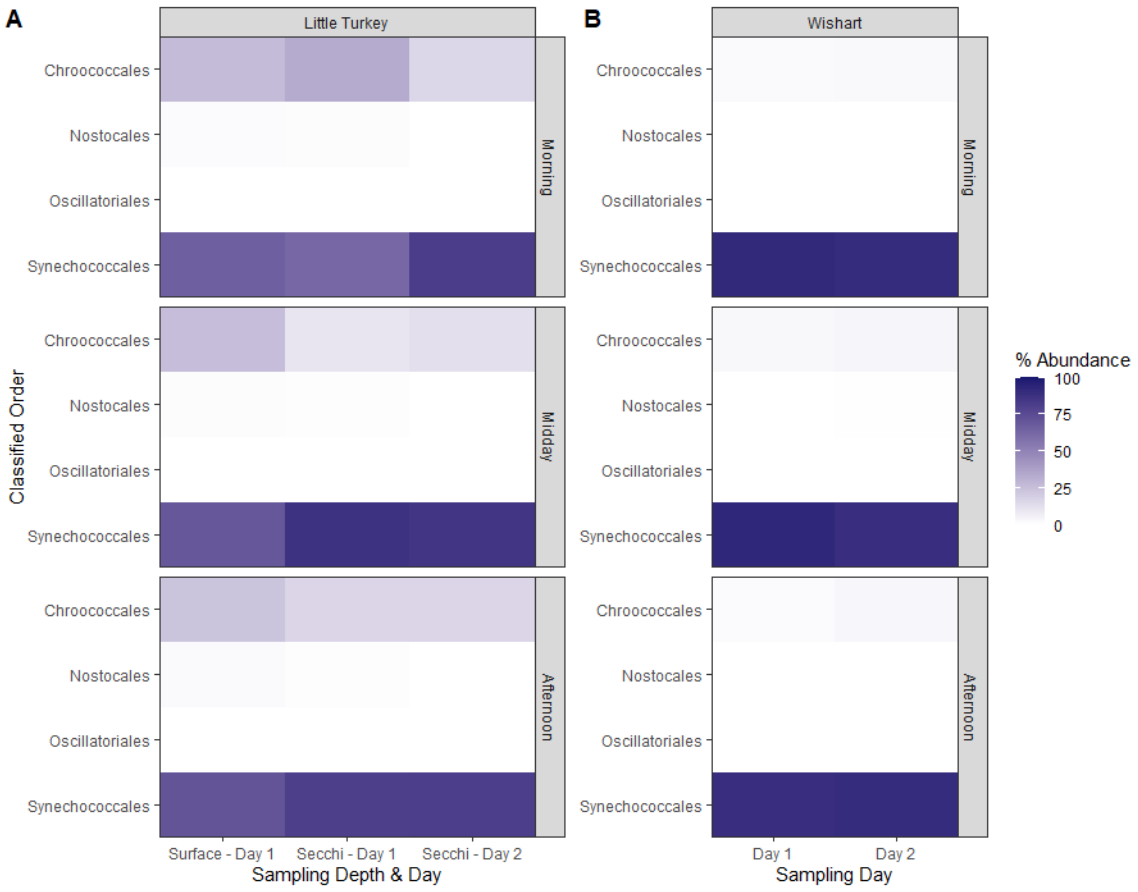


Figure 3: Heatmap depicting the composition of cyanobacterial communities at the order level across a multi-time point sampling series in a stratified (Little Turkey) and non-stratified (Wishart) lake. Amplicon sequence variants of the V4 region of the 16S rRNA gene were classified to the phylum Cyanobacteria were selected to examine the taxonomic composition of cyanobacterial communities at (A) surface waters and Secchi depth in Little Turkey Lake, and (B) Secchi depth in Wishart Lake. Cyanobacterial communities in both lakes were consistently dominated by the order, Synechococcales which contains potentially toxic picocyanobacterial genera.

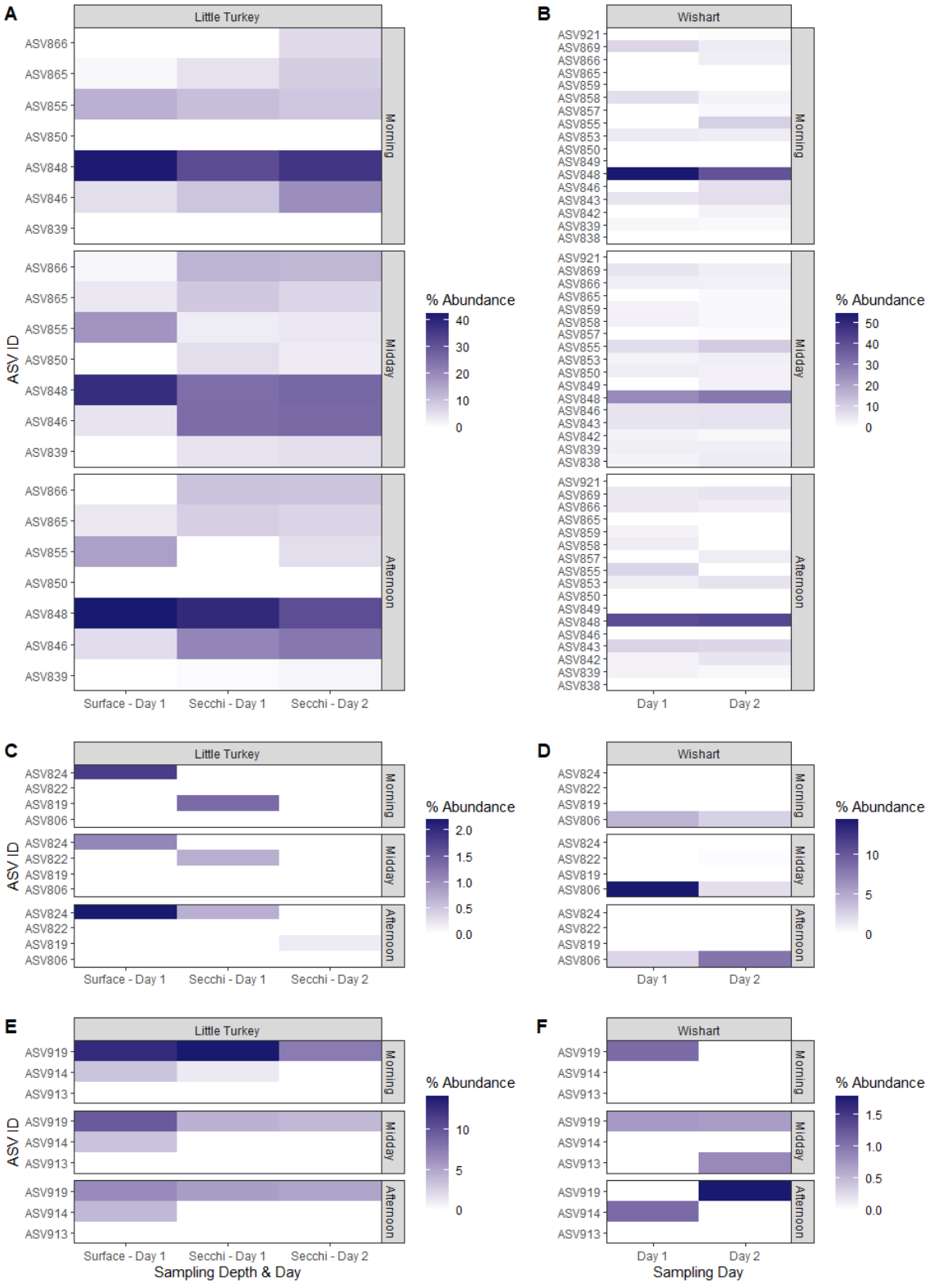
387

388 *3.2.2 Colonial & Filamentous Taxa*

389 Chroococcales and Nostocales were observed in lower relative abundances in
390 Little Turkey (Figure 3A) and Wishart Lake (Figure 3B) but nonetheless contributed to
391 the differences in compositional structure of the cyanobacterial communities in these
392 lakes. Little Turkey Lake had a larger Chroococcales population than Wishart Lake
393 (Figure 2; Table 2). Nostocales sequences were detected in Little Turkey Lake but were
394 largely undetected in Wishart Lake. Further uniqueness of cyanobacteria communities
395 between systems was also observed at the ASV level. Specifically, ASV806 (classified to
396 the genus *Pseudanabaena*) was only detected in Wishart Lake (Figure 4D) and
397 Nostocales ASVs (ASV824, 822 819) were only detected in Little Turkey Lake (Figure
398 4C). Further unique ASV composition was observed with the detection of ASV913
399 (classified to the genus *Microcystis*) in Wishart Lake at very low relative abundances
400 (Figure 4F), but larger relative abundances of ASV914 (classified to the genus
401 *Microcystis*) and ASV919 (classified to the genus *Radiocystis*) in Little Turkey Lake
402 (Figure 4E).

403 Within samples, sequences attributed to taxa that contain gas vesicles, including
404 *Microcystis, Dolichospermum,* and *Aphanizomenon,* were detected. Sequences classified
405 to these genera including ASV824 (classified to the genus *Dolichospermum*), and
406 ASV914 (classified to the genus *Microcystis*), were consistently detected at the surface in
407 Little Turkey Lake demonstrating positive buoyancy regulation due to the presence of gas
408 vesicles. Diurnal variation associated with downward migration during the daylight was
409 exhibited in the surface populations of ASV919 (classified to the genus *Radiocystis*) as a

consistent decrease in abundance was observed from morning to afternoon exhibiting the expected diurnal migration trend. While taxa with gas vesicles were detected regularly in surface samples of Little Turkey Lake, ASV819 (classified to the genus *Dolichospermum*) and ASV822 (classified to the order Nostocales) were only detected at Secchi depth indicating that gas vacuolate taxa may be found deeper in the water column and supporting the distribution of cyanobacteria through the photic zone and not only as surface accumulations (Graham et al., 2008). The diurnal presence of taxa with gas vesicles at depths other than the surface provides not only empirical, but mechanistic justification for expanding cyanobacteria monitoring programs designed to water supplies used for potable water production and recreation.



420

Figure 4: Heatmap depicting the relative abundances of individual amplicon sequence variants of interest across a multi-time point sampling series in a stratified (Little Turkey) and non-stratified (Wishart) lake. Amplicon sequence variants (ASV) of the V4 region of the 16S rRNA gene classified to the phylum Cyanobacteria to highlight diurnal responses of common toxic and bloom forming genera including Synechococcaceae (*Synechococcus, Cyanobium*), Nostocales (*Anabaena*, *Aphanizomenon*), *Pseudanabaena* and Microcystaceae (*Microcystis*, *Radiocystis*). Relative abundances of individual ASVs in cyanobacterial community composition for (A/B) unicellular taxa (Synechococcaceae), (C/D) filamentous taxa (Nostocales & *Pseudanabaena*), and (E/F) colonial taxa (Microcystaceae) to identify taxa specific diurnal responses.

3.3 Rainfall Impacts on Cyanobacteria Distribution

Cyanobacterial communities at Secchi depth in the morning of the first sampling day were highly similar to the surface communities in Little Turkey Lake, a phenomenon that was not observed subsequently (Figure 2C). Although similar order level composition in cyanobacterial communities across sampling times at surface and Secchi depth was observed (Figure 3A), the relative abundance of the order Chroococcales, was higher at Secchi depth in the morning of the first sampling day, and similar to that observed at the surface. Similarly, ASV919 (classified to the genus *Radiocystis*) and ASV914 (classified to the genus *Microcystis*) were detected at higher relative abundances in the morning period of the first sampling day (Figure 4E). Notably, a heavy rainfall event occurred during the overnight period prior to the first sampling day. Storm events have been previously associated with downward mixing of cyanobacterial communities

in lakes (Walsby et al., 1997); thus, it is possible that the overnight storm redistributed the surface communities across the water column in Little Turkey Lake, otherwise atypical observation of community similarity at surface and Secchi depths on the morning of the first sampling day.

Unlike Little Turkey Lake, distributions of cyanobacteria in Wishart Lake did not exhibit obvious impacts of the rainfall event. High dissimilarity in community composition was not observed between the morning of the first sampling day and subsequent time points (Figure 2C) and taxonomic composition of communities at the order level were consistent across sampling times (Figure 3B). Previous diurnal studies on freshwater microbial communities did not show an impact of meteorological conditions on bacterial abundances (Filippini et al., 2008) indicating that the response to meteorological conditions may be system specific. The potential for system specific responses to external mixing events as demonstrated in Little Turkey and Wishart Lake further signifies the importance of utilizing unique protocols tailored to system dynamics.

3.4 Drinking Water Reservoir Monitoring Implications

The observation of diurnal vertical migration of cyanobacteria in oligotrophic lakes reported herein emphasizes the importance of incorporating sampling time into monitoring protocols that do not utilize depth integrated sampling. Here, cyanobacterial relative abundances continued to fluctuate between the hours of 9 a.m. and 4 p.m. demonstrating that large, generalized time frames are too broad to apply universally to varying aquatic systems. Consequently, limiting sample collection to a single time point or discrete depth in stratified lakes may result in vast underestimation of cyanobacterial relative abundances. While cyanobacteria sampling protocols and guidelines do

467 sometimes speak to the time and depth of sample collection, guidance is often vague and
468 refers to collection of "surface waters" at times "later in the day" or from "10 a.m. to
469 3 p.m." (Table 4).

470 **Table 4**: A summary of cyanobacteria monitoring sampling protocol features and
471 potential impact on detection.

Sampling Protocol Design Feature	References	Impact on Detection
Recommended Sampling Time		
No optimal sampling time advisory	Chorus et al., 2000; Chorus and Bartram, 1999; Graham et al., 2008; Lake and Management, 2015	Inconsistent and/or arbitrary sampling time will result in variation in the detection of cyanobacteria populations.
10 a.m. – 3p .m.	Klamath river blue green algae working group, 2009; University of New Hampshire - Center for Freshwater Biology, 2010	Cyanobacterial populations will experience fluctuations during the large timeframe. Samples collected at 10 a.m. will differ in composition from samples collected at 3 p.m. due to water column migration resulting in the potential for missed detection or underrepresentation of cyanobacteria populations.
"Later in the day"	Water Quality Research Australia and Global Water Research Coalition, 2009	Sample collection time is dependent on user interpretation of "later in the day" which creates bias from the interpretation of sampling protocol.
Morning (for surface sampling only)	Ministry for the Environment and Ministry of Health., 2009	Sampling of surface water in the morning period is appropriate due to known trends in water column migration of cyanobacteria populations.
Recommended Sampling Depth		
Integrated water column depth sample	Klamath river blue green algae working group, 2009; Colorado Lake and Reservoir Management, 2015; Ministry for the Environment and Ministry of Health., 2009; Ohio Environmental Protection Agency, 2013; Sarnelle et al., 2010; University of New	Integrated depth sampling accounts for the potential of daily variation in cyanobacteria populations.

	Hampshire - Center for Freshwater Biology, 2010	
Surface waters	Sarnelle et al., 2010	Surface water should only be sampled in morning periods in lakes with stratified water columns.
1 metre below surface	Graham et al., 2008	
0 – 50 cm below surface	Klamath river blue green algae working group, 2009; Ministry for the Environment and Ministry of Health., 2009; Sarnelle et al., 2010	

472

While depth integrated sampling is sometimes suggested and allows for characterization of cyanobacterial communities across the water column with a single sample (Ministry for the Environment and Ministry of Health., 2009; Newcombe, 2009; Ohio Environmental Protection Agency, 2013; Sarnelle et al., 2010; University of New Hampshire - Center for Freshwater Biology, 2010), trade-offs in sensitivity are recognized, but not been well described. Moreover, insights to community dynamics including factors that may contribute to bloom formation, toxin production, or risk mitigation may be obscured or confounded using these approaches. In contrast, discrete sampling allows for the characterization of associated heterogeneities in cyanobacterial populations (Vidal et al., 2014) and focus on specific points of interest such as water treatment plant intakes (Graham et al., 2008).

Critically, the increased availability and rapidly decreasing costs of NGS tool have eliminated most of the barriers to their wider use in resource management and the drinking water industry. The insights enabled by these approaches allow for proactive rather than reactive management of drinking water supplies (Chapman, 2010). It is important to note that although amplicon sequencing has revolutionized our ability to study microbial communities, these data do not quantify abundance of community members. Rather, they only indicate community composition and relative abundance (Gloor et al., 2017, 2016); this introduces challenges in the interpretation of changes in community structure. For example, an observed decrease in relative abundances of cyanobacteria, may only be an artifact of increased relative abundances of sequences classified to other taxonomic groups and not be representative of an actual absolute decrease in cyanobacterial populations. Traditional cell enumeration techniques (e.g.,

flow cytometry; Patel et al., 2019) or other molecular techniques (e.g., qPCR; Chiu et al., 2017) can be used to supplement amplicon sequencing with cyanobacterial community density data, however.

The design of sample collection schemes is closely linked to the utility of the information that is delivered using these approaches. The analysis provided herein has delivered clear evidence to demonstrate the need for integrating multi-timepoint, multi-depth discrete sampling guidance into lake and reservoir monitoring programs to proactively understand cyanobacteria community dynamics and hopefully signal change inform risk management associated with the potential for cyanotoxin production. This work further emphasized that cyanobacteria are present in oligotrophic lakes and their community structure varies (i) diurnally, (ii) across the depth of the water column, and (iii) between different lakes that are closely interconnected within the same watershed. Ignoring this variability and reducing sample numbers can lead to a false sense of security and missed opportunities to identify and mitigate changes in trophic status and associated risks such as toxin or taste and odor production, especially in sensitive, oligotrophic systems.

4. Conclusions

- The potential for diurnal migration should be reflected in cyanobacterial monitoring programs through the inclusion of multiple-sampling times or conducting sampling at an ecologically significant time of day.
- Sampling of lakes should not be restricted to the surface and should include discrete sampling and multiple depths to reflect spatial heterogeneity of cyanobacterial communities present in the water column.

- The positive buoyancy of cyanobacteria with gas vesicles may frequently be concentrated at the surface, but is not limited to this depth, with occurrence at deeper depths in the water column.
- Rainfall or wind induced mixing events such as those observed in this investigation may significantly impact cyanobacterial community composition and distribution.
- Cyanobacterial monitoring may be enhanced through the incorporation of system characteristics (e.g., thermal stratification) and characterization of communities for design of sampling protocols that are system specific.

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