LIMNOLOGY and OCEANOGRAPHY

ASLO

Limnol. Oceanogr. 9999, 2024, 1–17

doi: 10.1002/lno.12509

Mixing-driven changes in distributions and abundances of planktonic microorganisms in a large, oligotrophic lake

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Abstract

Temperate lakes experience variation in mixing and stratification that affects the distributions, activities, abundances, and diversity of plankton communities. We examined temporal and vertical changes in the composition of planktonic microorganisms (including Bacteria and Archaea) in oligotrophic Flathead Lake, Montana. Using a combination of approaches that included 16S rRNA gene sequencing and flow cytometric determination of cell abundances, we found that the microbial community was responsive to variations in stratification and mixing at time scales ranging from episodic (scale of days) to seasonal. However, the impact of such physical dynamics varied among taxa, likely reflecting taxa-specific responses to environmental changes that coincide with stratification and mixing (e.g., light availability and nutrient supply). During the early spring, periods of relatively short-term (< 7 d) intermittency in stratification and mixing influenced the vertical distributions of specific microbial taxa, notably including the cyanobacteria. These events highlight time scales of biological responses to high-frequency variations associated with lake stratification and mixing, particularly during the transition to the growing season in the early spring.

Planktonic microorganisms, defined here as Bacteria and Archaea, are central components of limnetic food webs and dominant contributors to the capture of energy and the cycling of elements in lakes (Cole et al. 1988; Stockner and Porter 1988; Cotner and Biddanda 2002). These microorganisms are phylogenetically diverse, relying on an array of metabolic strategies that include chemotrophy, phototrophy, autotrophy, heterotrophy, and mixotrophy (e.g., Zwart et al. 2002; Newton et al. 2011; Caliz and Casamayor 2014). Lake microorganisms are also responsive to environmental perturbations, making them sentinels of both progressive and abrupt changes to lake ecosystems (e.g., De Wever et al. 2005; Cabello-Yeves et al. 2018; Rohwer et al. 2023).

In high latitude and temperate aquatic ecosystems, microbial biomass, growth, and community composition vary seasonally, consistent with climatic variations in habitat structure (Crump et al. 2003; Cruaud et al. 2020). For example, seasonal variations in light and temperature have been shown to be important controls on lake plankton phenology (Adrian et al. 2006; Sommer and Lengfellner 2008). Seasonal modification of thermal structure, together with wind shear, influences lake stratification and mixing, with concomitant impacts on resource availability and competition among plankton communities (Sommer et al. 1986; Gaedke et al. 1998). Through seasonal development of the thermocline, warmer and sunlit epilimnetic waters are isolated from mixing with cooler and dark hypolimnetic waters. Partly in response, planktonic microorganisms in relatively deep lakes exhibit clear vertical structures (Urbach et al. 2001; Okazaki and Nakano 2016; Tran et al. 2021). With cooling through the fall and winter, vertical mixing homogenizes lake waters, impacting the distribution of organisms (Okazaki and Nakano 2016). Superimposed on such seasonal changes, episodic events (e.g., storms) can alter water column structure with associated impacts on plankton productivity and biomass distributions (Stockwell et al. 2020; Hampton et al. 2022). To date, it remains mostly unknown how such

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Additional Supporting Information may be found in the online version of this article.

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Author Contribution Statement: K.A.E.: Writing—original draft (lead); data curation (lead); writing—review and editing (equal); formal analysis (lead). J.R.R.: Investigation (lead); resources (lead); writing—review and editing (supporting). L.M.P.: Formal analysis (supporting); writing—review and editing (supporting). E.K.W.: Data curation (supporting); formal analysis (supporting); writing—review and editing (supporting). M.J.C.: Conceptualization (lead); funding acquisition (lead); formal analysis (supporting); writing—original draft (supporting); writing—review and editing (equal).

dynamics might alter the distribution of microorganisms, thereby impacting specific ecosystem functions.

For planktonic microorganisms in temperate lakes, much of the attention has focused on productivity, biomass, and community composition during the high light, stratified season (e.g., Hiorns et al. 1997; Oh et al. 2011; Skopina et al. 2015), with comparatively fewer studies investigating how distributions and abundances of microorganisms change over the year (but *see*, e.g., Garcia et al. 2013; Okazaki and Nakano 2016; Park et al. 2023). It remains unclear how changes in lake thermal structure might impact the phenology of planktonic microorganisms. In part, the phylogenetic and metabolic diversity exhibited by planktonic microorganisms complicates making predictions of how changes in lake physical structure will impact these organisms. However, given the central role of planktonic microorganisms in lake food webs and biogeochemical cycles (Cotner and Biddanda 2002), quantifying temporal variation in these communities is essential to improved understanding of the impact of lake vertical structure on the functioning of these ecosystems.

Flathead Lake is a large (482 km^2) weakly dimictic and oligotrophic lake in northwest Montana that has been the subject of sustained, time-series monitoring since 1977 (Stanford and Ellis 2002; Ellis et al. 2011; Elser et al. 2022). The oligotrophic nature of the lake is maintained by the relatively pristine and heavily forested surrounding watershed, local geology, and the relatively short residence time of water in the lake ($\sim$ 2.2 years; Ellis et al. 2011). Globally, oligotrophic lakes are increasingly under threat of eutrophication, with associated changes in lake chemistry and food web structure and function (Schindler and Vallentyne 2008; Schindler 2012). Hence, studying how planktonic microorganisms in oligotrophic systems respond to perturbation has increased urgency and importance.

We examined the impact of stratification-mixing dynamics on planktonic microorganisms. Flathead Lake is both relatively deep and presents robust seasonal patterns of stratification and mixing, providing an excellent system to examine the phenology of oligotrophic planktonic microorganisms and the responsiveness of this community to variations in the physical structure of the lake. Here, we address the following questions: What types of microorganisms inhabit Flathead Lake, and how do they vary with depth and in time? How variable are planktonic microorganisms in the epilimnion and the hypolimnion? Finally, which microbial taxa are particularly responsive to variations in lake physical structure, specifically mixing and stratification, and over what time scales?

Methods

Study site and sampling

Variations in relative and absolute abundances of planktonic microorganisms in Flathead Lake were assessed using 16S rRNA gene amplicon analyses together with flow cytometric enumeration of cells. Sampling was conducted at monthly or bi-monthly intervals over 2 yr (September 2016–November 2018) at a monitoring site termed Midlake Deep (47°52′N, 114°4′W, bottom depth 113 m) that has been the focus of long-term sampling in Flathead Lake since 1977 (Ellis et al. 2011). Midlake Deep is located in a deep trench that runs along the eastern side of the lake.

Immediately prior to the collection of water samples, sonde-based (OTT Hydrolab) vertical profiles of temperature, pH, conductivity, fluorescence, dissolved O_2, and turbidity were measured at 1 m intervals through the upper 30 m of the lake and 5 m intervals at depths between 30 and 90 m. The depth of the epilimnion (mixed layer) was calculated as the depth where water temperature differed by 0.5°C from the average temperature in the near-surface (between 0.5 and 3.5 m).

For insights into the frequency of episodic variation in stratification and mixing in Flathead Lake, we also analyzed a historical dataset of vertically resolved temperature profiles obtained using an Ice-Tethered Profiler (Toole et al. 2011) deployed from a buoy at Midlake Deep between December 2011 and January 2014. The profiler was equipped with a CTD sensor (Sea Bird Electronics 41CP), which measured temperature at approximately 0.3 m resolution between 11 and 90 m every 3 h for the duration of the deployment period. In addition, an internally logging temperature sensor (HOBO Water Temp Pro v2) was attached at 5 m depth to the buoy line, providing an hourly record of temperature throughout the period of deployment.

The downward flux of photosynthetically active radiation (PAR) through the upper 30 m of the lake was quantified using an underwater sensor (LI-COR LI-193). The resulting depth-dependent decrease in PAR was used to compute the attenuation coefficient (K_{PAR}; m^{-1}) by fitting a log-linear function to each vertical profile. The daily flux of PAR (mol photons m^{-2} d^{-1}) incident to the surface of the lake was derived by time-integrating 15-min interval measurements collected at a nearby shore-based weather station (47°52′23″N, 114°2′6″W; https://flbs.umt.edu/publicdata/). Together with K_{PAR}, the daily incident PAR flux was used to compute daily integrated downwelling PAR for each of the discrete depths where water samples were collected.

Water samples for flow cytometry and plankton deoxyribonucleic acid (DNA) were collected using either 2 or 3 L opaque Van Dorn water sampling bottles (Aquatic Research Instruments) closed at discrete depths using a messenger. Samples were collected from five depths (5, 10, 50, 90 m, and at a variable depth where fluorescence was maximal, referred to here as the Chl max). For sampling the upper lake (< 30 m), the Van Dorn sampler was affixed to a handline marked at 0.5 m intervals; for deeper depths (50 and 90 m), the Van Dorn sampler was affixed to wire that was spooled onto an electric winch and fed through a calibrated meter wheel. Water was subsampled from the Van Dorn sampler into acid-washed, 2 L

polyethylene bottles. Water for subsequent laboratory measurements of chlorophyll *a* (Chl *a*) concentrations was collected as 0–30 m depth-integrated samples using a 30 m long, 12.7 mm diameter hose. Water was homogenized from the hose into 10 L polyethylene carboys, then subsampled into duplicate 1 L acid-washed amber bottles. All samples were stored in a cooler in the dark until transport to the lab (< 5 h).

Measurements of nutrients, Chl *a*, and cell concentrations

In the shore-based laboratory, samples for measurements of dissolved inorganic nutrient concentrations were filtered through a 47 mm diameter, 0.45 μm pore size nitrocellulose filter (Millipore Durapore) and stored frozen. Filters were pre-rinsed with 100 mL of MilliQ water and again with sample water prior to filtering water. Concentrations of nitrate + nitrite (N + N) and soluble reactive phosphorus (SRP) were measured colorimetrically using a segmented flow analyzer (Astoria-Pacific Astoria2; Strickland and Parsons 1972). For Chl *a* concentrations, 2 L of lake water was filtered onto 47 mm diameter, glass fiber filters (Whatman GF/F; nominal 0.7 μm pore size). Chl *a* on filters was extracted with a 90% acetone solution, which included a filter grinding step to facilitate extraction; extracts were quantified by absorption at 664 and 750 nm on a spectrophotometer, including correction for phaeopigments (Lorenzen 1967; Strickland and Parsons 1972).

Abundances of cyanobacteria and non-pigmented Bacteria and Archaea (restricted to size ranges between approximately 0.2 to < 10 μm) were determined using an Attune Acoustic Focusing flow cytometer. Lake water samples (2 mL) were fixed with paraformaldehyde (0.8% final concentration) for 10 min at room temperature and then frozen at −80°C until analysis. Each sample was analyzed twice: once to quantify cyanobacteria and again following the addition of SYBR Green I DNA stain to quantify total microorganism abundances. Cyanobacteria were detected via Chl *a* autofluorescence using a blue (488 nm) excitation laser (20 mW) and a 640 nm longpass emission filter, and further characterized based on the autofluorescence of phycoerythrin using the same blue laser and the R-phycoerythrin emission filter (574 and 26 nm bandwidth; Olson et al. 1988). SYBR-stained cells were identified using the blue excitation laser and a 530 nm emission filter. Non-pigmented cell abundances were calculated as the difference between the total cells (SYBR stained) and Chl *a* containing cells.

DNA extraction, amplification, and sequencing of 16S rRNA genes

Water samples (400–500 mL) for subsequent extraction of plankton DNA were filtered sequentially through in-line 25 mm, 3 μm pore size polycarbonate filters and then onto 25 mm, 0.2 μm pore size polyethersulfone filters (Supor) using a peristaltic pump. Filters were placed in 2 mL microcentrifuge tubes containing 600 μL of cell lysis buffer (MasterPure DNA Purification Kit; Biosearch Technologies Inc. LGC) and 100 μL each of 0.1 mm and 0.5 mm zirconium beads, and frozen at −80°C until DNA was extracted.

The 0.2 μm filters were thawed, placed in a mechanical bead beater, and agitated for 2 min, followed by DNA extraction and purification following the MasterPure DNA Purification kit (Lucigen Corporation) manufacturer's recommendations. The concentration of DNA in each extract was fluorometrically quantified using the Invitrogen Qubit High Sensitivity dsDNA Kit (Thermo Fisher Scientific). The V4-V5 hypervariable region of the 16S rRNA genes was PCR amplified using primers recommended by Parada et al. (2016): 515F-Y (5′-GTGYCAGCMGCCGCGGTAA) and 926R (5′-CCGYCAATTYMTTTRAGTTT). PCR reactions were run in triplicate and examined for correct size using an agarose gel (1% wt vol^{-1}). Amplicons were purified using the ENZA Cycle Pure Kit (Omega Bio-tek), and samples were pooled to approximately equimolar proportions in 2 libraries and then sequenced at the University of Montana Genomics Core on an Illumina MiSeq using the MiSeq 500/PE250 v2 kit (Illumina).

Amplicon informatics and statistical analyses

Mothur v.1.42.3 (Schloss et al. 2009; Schloss 2019) was used to process the resulting 16S rRNA amplicon sequences. Sequences were clustered to 99% sequence similarity using an abundance-based greedy clustering method (Schloss et al. 2009; Schloss 2019). 16S rRNA genes were then analyzed using the TaxAss pipeline (Rohwer et al. 2018), which enabled comparison of sequences using both SILVA v.132 (Quast et al. 2012; Yilmaz et al. 2014) and FreshTrain 15 June 2020 (Newton and McMahon 2011; Rohwer et al. 2018) rRNA gene databases. The TaxAss pipeline uses blastn to identify groups of sequences with high percent identities (≥ 98%) and low percent identities (< 98%) compared to FreshTrain 15. As described in Rohwer et al. (2018), sequences with percent identities ≥ 98% were subsequently classified using the FreshTrain 15 June 2020 database, while those with percent identities < 98% were classified using SILVA v.132.

After classification, samples were subsampled to 10,000 sequences. One sample, collected from the Chl max on 07 August 2017, had fewer than 10,000 sequences and was removed from subsequent analyses. We used the R package "vegan" (v.2.5.7) to subsample and calculate operational taxonomic unit (OTU) diversity metrics, including Shannon's diversity (H'). We also computed Jaccard distance (one minus the ratio of shared OTUs relative to the total number of OTUs between two samples, where higher values indicate less similar samples) for each sampling date, comparing dissimilarities in community composition of OTUs in samples collected from 10, 50, 90 m, and the Chl max to samples collected from 5 m. Seasonality in OTUs was determined using the Lomb–Scargle periodogram (Lomb 1976; Scargle 1982; Lambert et al. 2019) using the R package "lomb" (v.2). We define a class of microorganisms as "seasonal" if the resulting p value was ≤ 0.05 with a

period between 350 and 400 d (i.e., an annual cycle). The environmental factors most strongly correlated with the microbial community composition of Flathead Lake were computed using the "ADONIS" function from the R package "vegan."

We utilized DESeq2 (Love et al. 2014) to compute log2 fold-changes in the relative abundances of OTUs from the seven major phyla in Flathead Lake across both depth and mixed layer depth (deeper or shallower than the photic zone, 30 m). Relative abundances of OTUs from samples collected in the upper 30 m of the lake (including 5, 10 m, and the Chl max) were compared to those at deeper depths (50 and 90 m). In addition, log2 fold changes in OTU relative abundances were computed to compare periods when the lake was stratified against periods where the lake was either fully mixed or inversely stratified.

Finally, we computed Jaccard distances as a function of days between samples to identify time scales in community dissimilarity. For this analysis, every possible pair of samples for each of the discrete sampling depths was used to compute Jaccard distances as a function of the days between samples. We then fit a sinusoidal function with a period of 365 d (representing an annual cycle), including a saturation function to allow for interannual variability, to the Jaccard distances. The amplitude of the resulting sine function represents the strength of seasonality. This approach is similar to the pseudo-autocorrelation function described in Fuhrman et al. (2015).

Results

Seasonality in mixing and cell abundances

Over the 2-yr study period, Flathead Lake underwent clear seasonal mixing-stratification dynamics typical of a temperate lake (Fig. 1). Surface water temperatures varied from 1°C in the winter to 21.5°C in the summer, while deep waters (90 m) were more stable, varying between 2.4°C and 5.5°C (Fig. 1). During the early summer, the depth of the epilimnion shoaled to < 5 m, while in the winter the lake was fully mixed (Fig. 1; Supporting Information Table S1). Occasionally, during the winter, the lake appeared inversely stratified where temperatures in the near-surface waters cooled to nearly 1°C with warmer (~ 3°C) waters at depth (Fig. 1). The open waters of Flathead Lake rarely freeze, and no ice cover was observed at our sampling site during the study. The depth of the Chl max varied between 10 and 32 m, progressively deepening from

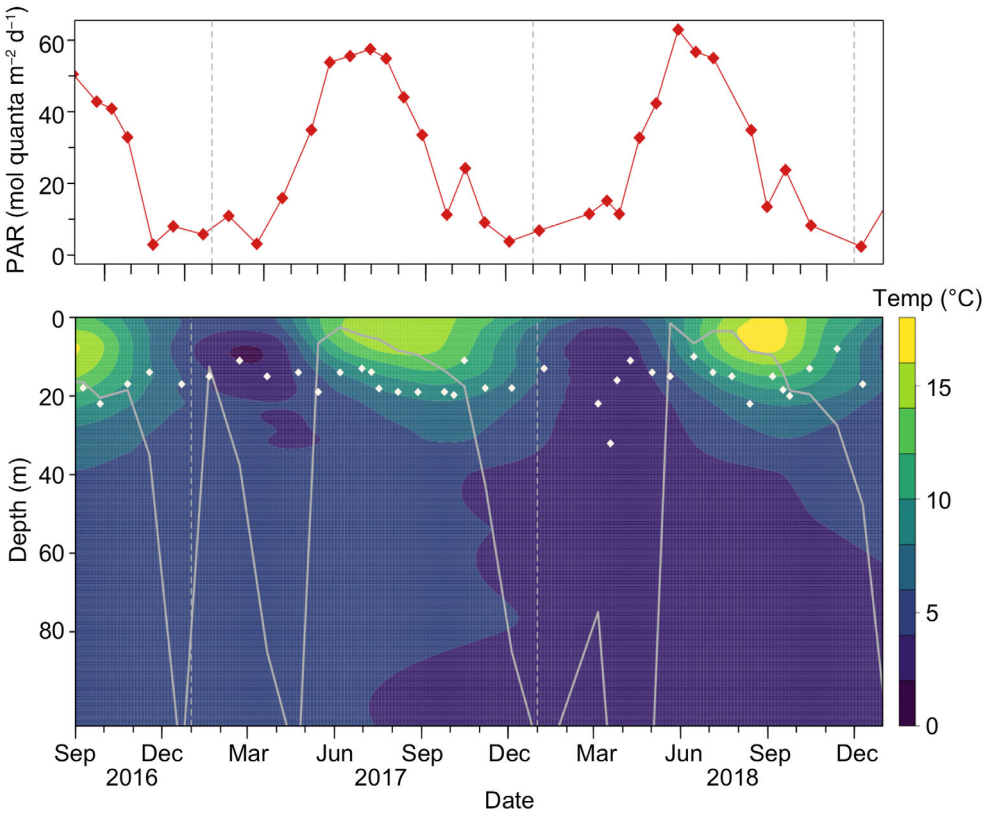

Fig. 1. Temporal variation in light and thermal structure in Flathead Lake. Daily incident PAR flux (top panel) and vertical profiles of temperature (bottom panel) during the study period. Vertical dashed lines denote 01 January of each year. Filled white symbols represent the depth of the Chl max and the continuous gray line represents the mixed layer depth.

summer into fall (Supporting Information Table S1). The lake was persistently well oxygenated; although dissolved O_2 decreased in the hypolimnion through the summer, concentrations in the lake remained consistently above 82% of the air-saturation value (Supporting Information Table S1).

Chl *a* and N + N concentrations varied seasonally, while SRP concentrations were persistently below detection (< 25 nmol L^{-1}) throughout the water column. Chl *a* concentrations for the upper 30 m of the lake varied more than 25-fold, ranging between 0.1 and 2.6 μg Chl L^{-1}, with peak concentrations in July (Supporting Information Table S1). N + N concentrations at 5 and 90 m converged when the lake was fully mixed (averaging $\sim$ 3.5 μmol N L^{-1}); however, when the lake stratified, N + N concentrations at 5 m were below detection (< 100 nmol L^{-1}) by late summer, increasing to $\sim$ 6 μmol N L^{-1} at 90 m.

Daily incident PAR was highly seasonal, peaking ($\sim$ 55–60 mol quanta m^{-2} d^{-1}) in June and July and decreasing up to 20-fold in the winter (Fig. 1; Supporting Information Table S1). Daily fluxes of PAR at the Chl max were less seasonal (in part due to variability in the depth of this feature), varying $\sim$ 10-fold through the year (Supporting Information Table S1).

Abundances of non-pigmented planktonic microorganisms were relatively invariant with depth and time. In the epilimnion and Chl max, abundances increased $\sim$ 1.5-fold during the summer and fall, reaching abundances of 1.1×10^6 cells mL^{-1} (Fig. 2). In the hypolimnion, cell abundances remained relatively constant (averaging 5.4×10^5 cells mL^{-1}) throughout the year. During periods when the lake was well mixed, abundances were similar at all depths sampled, consistent with mixing-driven homogenization of plankton (Fig. 2). Cyanobacteria abundances were more variable with depth and time than non-pigmented cells. In the epilimnion, cyanobacterial cell abundances appeared bi-modal, increasing threefold in the late spring, then decreasing through the summer, and increasing again in the early fall (Fig. 2). In contrast, at the Chl max cyanobacteria peaked in the summer, reaching concentrations upwards of 1.7×10^5 cells mL^{-1} (Fig. 2). In the hypolimnion, cyanobacteria were lower in abundance ($\sim 1.0 \times 10^4$ cells mL^{-1}), with the notable exception of abrupt increases during the early spring

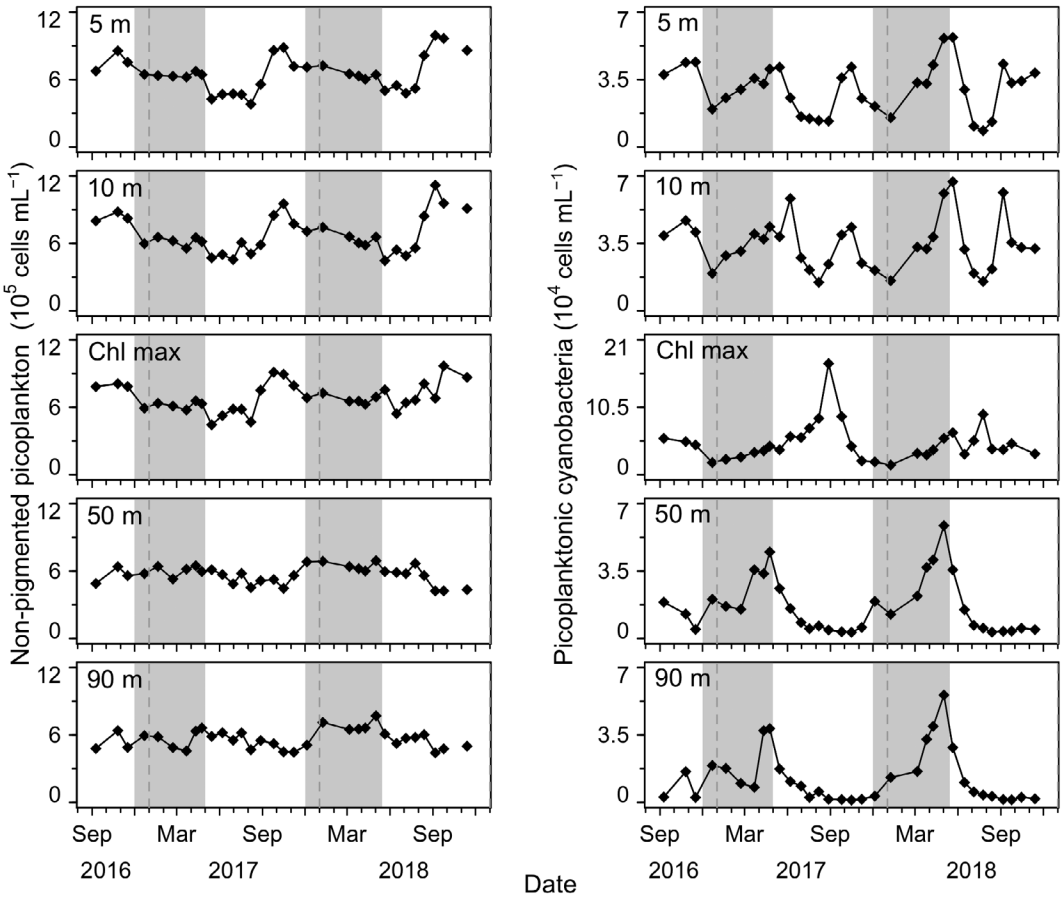

Fig. 2. Variations in planktonic microorganism abundances at different sampled depths. Non-pigmented microorganisms (left panels) varied less across depths and time than cyanobacteria (right panels). Different depths are depicted in different rows. Note differing scales for cyanobacteria sampled at the depth of the Chl max. Gray shading represents periods when the lake was fully mixed or inversely stratified, and vertical dashed gray lines represent 01 January.

(April and May), when abundances increased approximately six-fold. During these early spring periods, both water temperatures and cell abundances were vertically invariant, consistent with a well-mixed lake.

Microorganism community composition and seasonality

The planktonic microorganism community was dominated by seven bacterial phyla (Actinobacteria, Proteobacteria, Bacteroidetes, Planctomycetes, Cyanobacteria, Verrucomicrobia, and Chloroflexi), each representing > 1% of the total community. Representatives of the Armatimonadetes, Nitrospirae, and Acidobacteria were found in lower relative abundances (typically < 1%). Archaea comprised a relatively small fraction (averaging < 0.1%) of the total sequences and included members of the phyla Thaumarchaeota, Nanoarchaeaeota, and Crenarchaeota.

Members of the phylum Actinobacteria were consistently a large fraction of the community (relative abundances ranged from 22% to 73%) irrespective of depth or time of sampling (Fig. 3). Actinobacteria OTUs belonging to the acI B1, acI A7, and acIV A tribes were among the most abundant members of the planktonic microbial community, while other OTUs

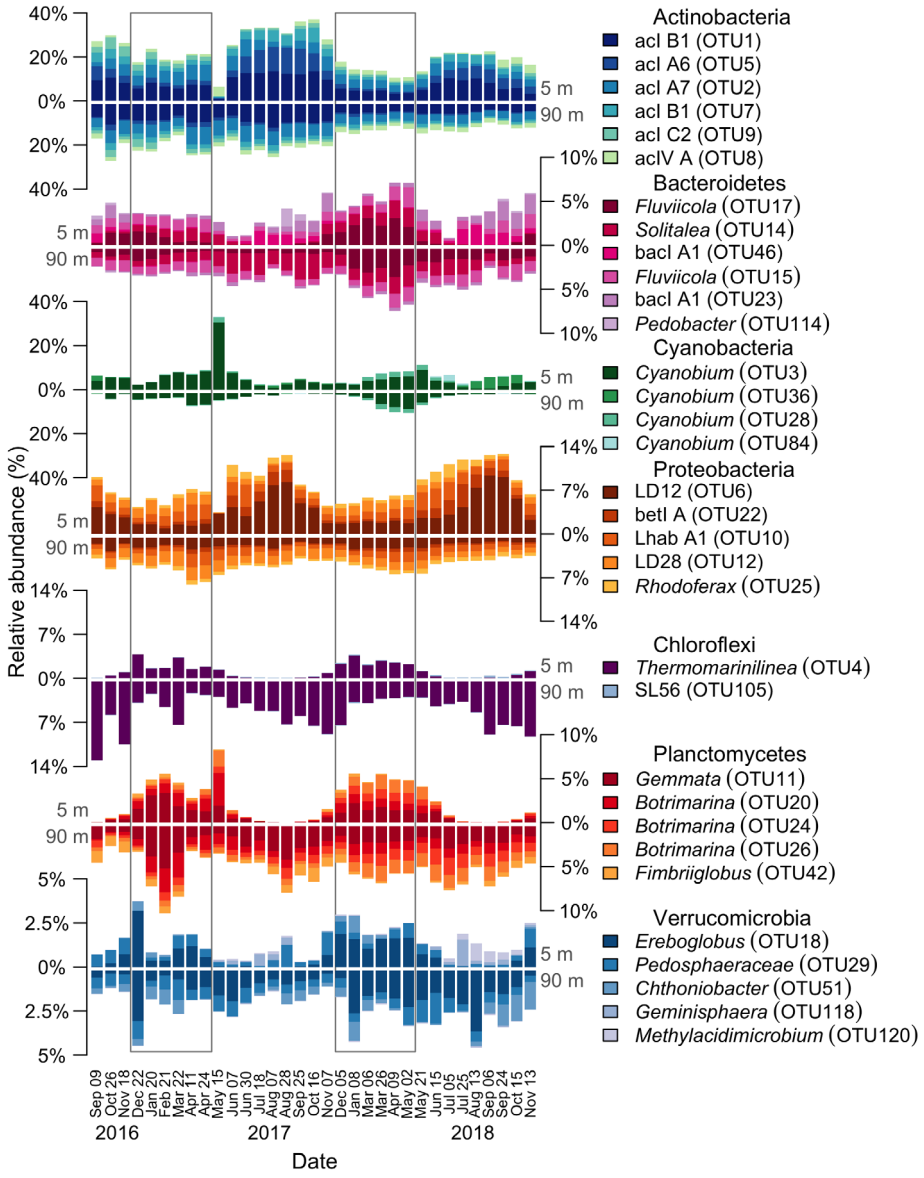

Fig. 3. Vertical structure and temporal variability in % relative abundance among the seven most abundant bacterial phyla in Flathead Lake. Upward bars in each plot represent relative abundances of OTUs from samples collected at 5 m, while downward bars represent relative abundances at 90 m. The lowest resolved taxonomic classification (clade or tribe) for each OTU is listed. Gray boxes represent periods when the lake was fully mixed or inversely stratified.

belonging to the acI B1, acI A7, acI A6, and acI C2 tribes were present but in lower relative abundances (Fig. 3). While most Actinobacteria OTUs demonstrated little depth- or season-differentiation, some (e.g., OTUs belonging to the acI A6 and acIV A tribes; Fig. 3; Supporting Information Fig. S1; Supporting Information Table S2) varied with depth and lake mixing (e.g., log2 fold-changes of 1.77 for the acI A6 member OTU and 1.18 for the acIV A member OTU in the epilimnion relative to the hypolimnion, $p < 0.01$; Supporting Information Table S2). OTUs belonging to the Bacteroidetes phylum also demonstrated high relative abundances (ranging from 4% to 20% of the total community; Fig. 3; Supporting Information Table S3). In some cases, Bacteriodetes OTUs were significantly greater in the hypolimnetic waters (e.g., OTU17 and OTU14, members of the clades *Fluviicola* and *Solitalea*; log2 fold-changes of 1.84 and 2.40, $p < 0.01$, respectively; Supporting Information Table S2), while other members of Bacteroidetes (e.g., OTU46 and OTU23, both members of the bacI A1) had significantly greater relative abundances in the epilimnion compared to the hypolimnion (log2 folds of 4.06 and 1.59, respectively, $p < 0.01$), and higher abundances when the lake was stratified compared to periods of deep mixing (log2 fold-changes of 1.35 and 1.36, respectively, $p < 0.01$; Fig. 3; Supporting Information Table S2).

In contrast, several taxa demonstrated pronounced depth and seasonal variation. Not surprisingly, members of the Cyanobacteria were largely restricted to the upper waters of the lake and were highly variable on seasonal time scales (Fig. 3; Supporting Information Fig. S2). Cyanobacteria averaged 7% of the total sequences, but ranged between 0.3% and 40%, depending on the time of year and depth sampled. Among the Cyanobacteria, 9 of the top 10 most abundant OTUs belonged to the genus *Cyanobium*, and many of these OTUs varied seasonally (Fig. 4; Table 1). For example, a single *Cyanobium* OTU (OTU3) contributed ~6–10% of the total sequences throughout the water column during the early spring, reaching peak relative abundances (> 30%) in the near-surface waters in May 2017 (Figs. 3, 4). This OTU was

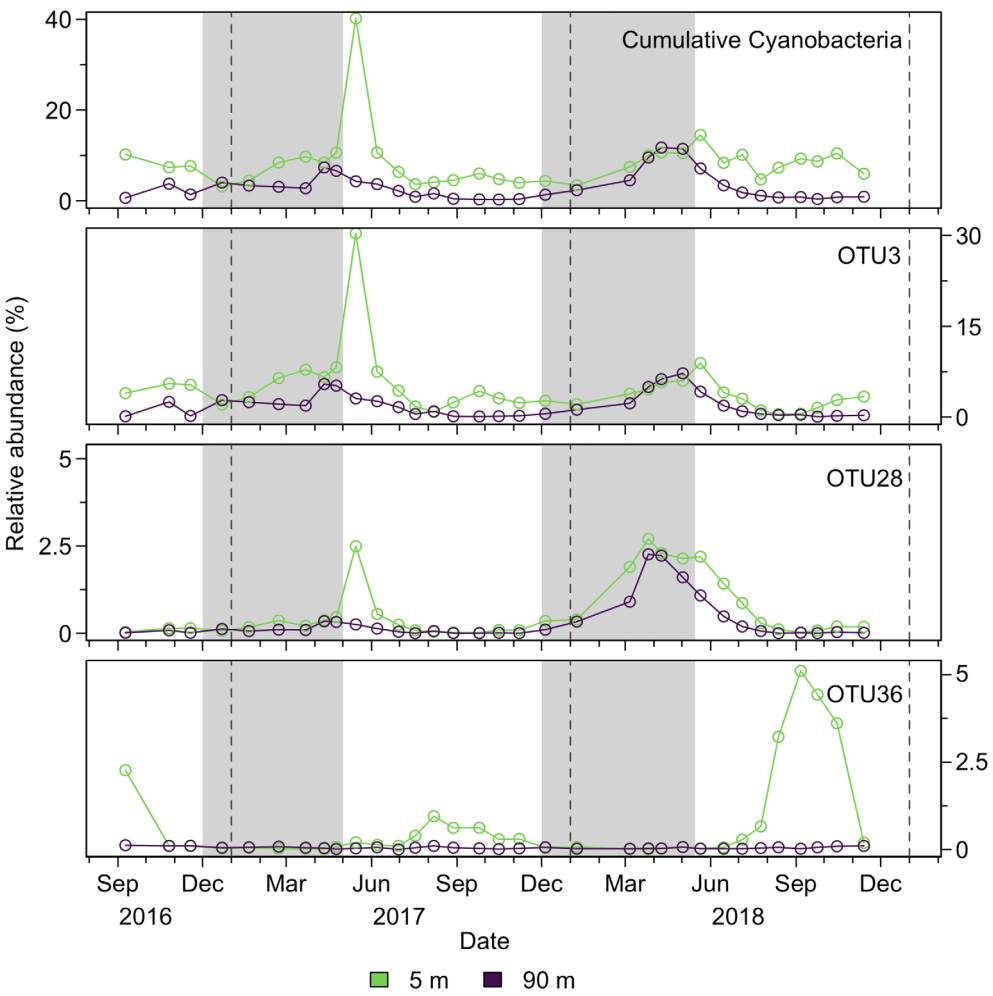

Fig. 4. Temporal variation at 5 and 90 m in the % relative abundance of Cyanobacteria. Depicted are cumulative relative abundances for all OTUs in the phylum and the three most abundant OTUs clustering among the genus *Cyanobium*: OTU3, OTU28, and OTU36.

Table 1. Relative abundances (%), seasonality, and phylogenetic affiliations of the top 20 most abundant OTUs. Also depicted are maximum, minimum, and standard deviation of mean relative abundances for all depths and sampling times. Seasonality at both 5 and 90 m was determined using a Lomb–Scargle periodogram; *p* values are shown for those OTUs which were determined to exhibit significant seasonality ($p < 0.05$, periodicity of between 350 and 400 d). Dashes indicate OTUs did not exhibit significant seasonality ($p > 0.05$). A version of this table, which includes the top 200 most abundant OTUs is available in the Supporting Information Table S3.

	Relative abundance (%)			Seasonality *p* value		Taxonomy						
OTU	Mean	Max	SD	5 m	90 m	Kingdom	Phylum	Class	Order	Lineage	Clade/genus	Tribe/ species
OTU1	6.75	15.11	3.05	-	-	Bacteria	Actinobacteria	*Actinobacteria*	*Frankiales*	acI	acI B	acI B1
OTU2	4.67	9.68	1.82	-	-	Bacteria	Actinobacteria	*Actinobacteria*	*Frankiales*	acI	acI A	acI A7
OTU3	4.05	30.34	3.59	-	< 0.01	Bacteria	Cyanobacteria	*Oxyphotobacteria*	*Synechococcales*	*Cyanobiaceae*	*Cyanobium*	*PCC 6307*
OTU4	2.7	12.51	2.44	< 0.01	< 0.01	Bacteria	Chloroflexi	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	*Thermomarinilinea*	*Lacunifontana*
OTU5	2.51	12.19	2.53	< 0.01	-	Bacteria	Actinobacteria	*Actinobacteria*	*Frankiales*	acI	acI A	acI A6
OTU6	2.24	9.37	1.7	< 0.01	0.03	Bacteria	Proteobacteria	*Alphaproteobacteria*	*SAR11 clade*	alfV	alfV-A	LD12
OTU7	2.19	6.72	1.3	< 0.01	-	Bacteria	Actinobacteria	*Actinobacteria*	*Frankiales*	acI	acI B	acI B1
OTU8	1.67	4.64	0.76	-	-	Bacteria	Actinobacteria	*Acidimicrobiia*	*Microtrichales*	acIV	acIV A	Unclassified
OTU9	1.43	5.87	1.06	0.02	-	Bacteria	Actinobacteria	*Actinobacteria*	*Frankiales*	acI	acI C	acI C2
OTU10	1.39	3.92	0.71	-	< 0.01	Bacteria	Proteobacteria	*Gammaproteobacteria*	*Betaproteobacteriales*	betI	*Limnohabitans* (betI A)	Lhab A1
OTU11	1.37	4.79	1	< 0.01	-	Bacteria	Planctomycetes	*Planctomycetacia*	*Gemmatales*	*Gemmataceae*	*Gemmata*	Unclassified
OTU12	1.22	5.04	0.59	< 0.01	-	Bacteria	Proteobacteria	*Gammaproteobacteria*	*Betaproteobacteriales*	betIV	betIV A	LD28
OTU13	1.07	2.45	0.53	< 0.01	-	Bacteria	Actinobacteria	*Actinobacteria*	*Frankiales*	acI	acI A	acI A7
OTU14	0.98	3.47	0.75	< 0.01	-	Bacteria	Bacteroidetes	*Bacteroidia*	*Sphingobacteriales*	*Sphingobacteriacea*	*Solitalea*	Unclassified
OTU15	0.95	2.28	0.42	-	-	Bacteria	Bacteroidetes	*Bacteroidia*	*Flavobacteriales*	bacV	Unclassified	Unclassified
OTU16	0.92	3.11	0.51	< 0.01	-	Bacteria	Actinobacteria	*Acidimicrobiia*	*Microtrichales*	acIV	acIV A	Iluma A2
OTU17	0.91	3.23	0.83	< 0.01	-	Bacteria	Bacteroidetes	*Bacteroidia*	*Flavobacteriales*	bacV	*Fluviicola*	Unclassified
OTU18	0.91	3.47	0.78	< 0.01	-	Bacteria	Verrucomicrobia	*Verrucomicrobiae*	*Opitutales*	*Opitutaceae*	*Ereboglobus*	Unclassified
OTU19	0.84	2.11	0.46	< 0.01	-	Bacteria	Actinobacteria	*Actinobacteria*	*Frankiales*	*Sporichthyaceae*	acI A	acI A7
OTU20	0.82	3.7	0.65	0.05	-	Bacteria	Planctomycetes	*Planctomycetacia*	*Pirellulales*	*Pirellulaceae*	*Botrimarina*	Unclassified

significantly more abundant in the epilimnion than hypolimnion (log2 fold-change of 1.07, $p < 0.01$; Supporting Information Table S2); however, it demonstrated high relative abundances during the spring and fall (log2 fold-change of 0.65, $p < 0.01$; Supporting Information Table S2). Other members of *Cyanobium* (e.g., OTU36) were prevalent (2–6%) in the early fall as the epilimnion cooled and deepened (Figs. 3, 4), demonstrating significant preference for the epilimnion (log2 fold-change of 2.77, $p < 0.01$; Supporting Information Table S2) and stratified periods (log2 fold-change of 3.37, $p < 0.01$; Supporting Information Table S2).

OTUs belonging to the class *Alphaproteobacteria* and order *Betaproteobacteria* also demonstrated depth and seasonal dependence. For example, members of the alphaproteobacterial LD12 tribe were greater in the epilimnetic waters (ranging 1.7–10.7% of the total sequences) than in the hypolimnion (relative abundances 0.6–2.7%; log2 fold-change of 0.82, $p < 0.01$; Supporting Information Table S2). Similarly, some OTUs belonging to the order *Betaproteobacteria*, including members affiliated with the LD28 clade of the *Methylophilaceae*, demonstrated elevated relative abundances in the well-lit upper waters of the lake during the stratified months (log2 fold-change between epilimnion and hypolimnion of 1.28, $p < 0.01$; Supporting Information Table S2), decreasing with in the winter (log2 fold-change of 0.72, $p < 0.01$; Fig. 3; Supporting Information Table S2).

The hypolimnetic waters were inhabited by different planktonic microorganisms, including members of the phyla Chloroflexi (relative abundances ranging 1.9–13.0%), Planctomycetes (4.8–17.5%), and Verrucomicrobia (3.1–14.7%). One of the most abundant hypolimnetic OTUs clustered among the Chloroflexi genus *Thermomarinilinea*. This OTU was a prominent contributor to communities in the meta- and hypolimnion, reaching peak relative abundances (up to 13%) in these deeper waters during the summer and early fall (Fig. 3; Supporting Information Fig. S3). With the onset of winter mixing, relative abundances of this same OTU averaged ~ 2% throughout the water column (Fig. 3). Microorganisms belonging to the classes *Planctomycetacia* and *Phycisphaerae* of the Planctomycetes were also significantly more abundant in the hypolimnion than the epilimnion (log2 fold-changes of five most abundant OTUs ranged from 2.68 to 4.77, $p < 0.01$; Fig. 3; Table 1). This included organisms in the lineages *Gemmataceae* and *Pirellulaceae*, which demonstrated only weak seasonality in the hypolimnion (Table 1)

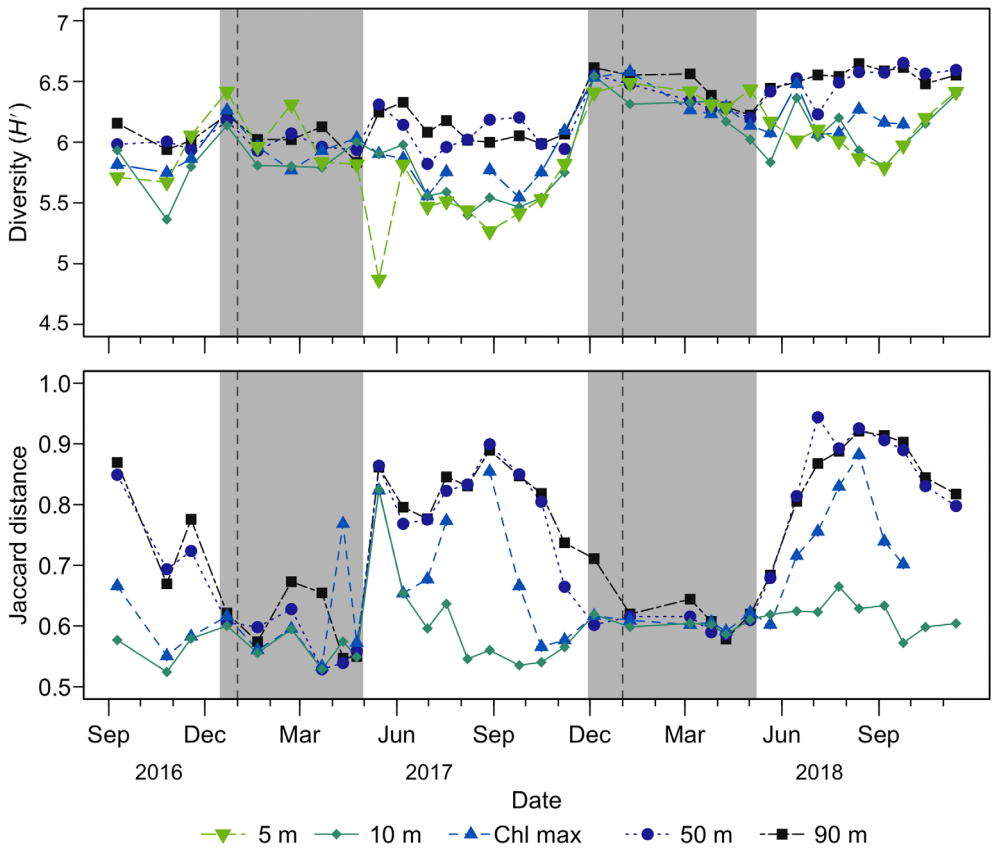

Fig. 5. Shannon index (H') and Jaccard distance converge among sampled depths during periods of mixing and inverse stratification. Shannon H' (top) and Jaccard distance (bottom) at 10 m, the Chl max, 50 m, and 90 m calculated relative to 5 m. Gray shaded boxes indicate periods where the lake was fully mixed or inversely stratified.

but varied in the upper lake consistent with mixing-driven entrainment from depth (Fig. 3). Consistent with the flow cytometric analyses, members of the phylum Cyanobacteria also increased in the hypolimnion in the early spring, during periods where the lake appeared fully mixed, where collectively Cyanobacteria comprised up to 11.7% of the hypolimnetic community (Fig. 3).

Seasonal variation in vertical structure and diversity

Microorganism diversity (Shannon's diversity; H') was significantly greater in the hypolimnion (50 and 90 m) than the epilimnion (5 , 10 m, and Chl max; *t*-test, $p < 0.01$), a pattern driven by decreasing community diversity in the epilimnion with the onset of stratification in the spring (Fig. 5). Values of H' at discrete depths varied seasonally, with values of H' greatest in the hypolimnion during the summer, converging as the mixed layer deepened in the fall (Fig. 5). The Jaccard distance was computed to compare how microorganism communities at each depth varied in time relative to the community at 5 m (Fig. 5), as well as every pairwise comparison between depths. These analyses revealed depth-dependent changes that coincided with shoaling and deepening of the epilimnion (Fig. 5). Relative to 5 m, dissimilarity between depths converged when the lake was well-mixed, consistent with mixing-driven vertical homogenization of communities. During the period when the lake was inversely stratified (between January and April of 2017), dissimilarity values increased (relative to 5 m) at deeper depths. ADONIS tests identified temperature and depth as the strongest potential drivers underlying variation in compositional changes (Supporting Information Fig. S4).

We also used a model of Jaccard dissimilarity at each of the discrete depths to identify time scales modifying planktonic

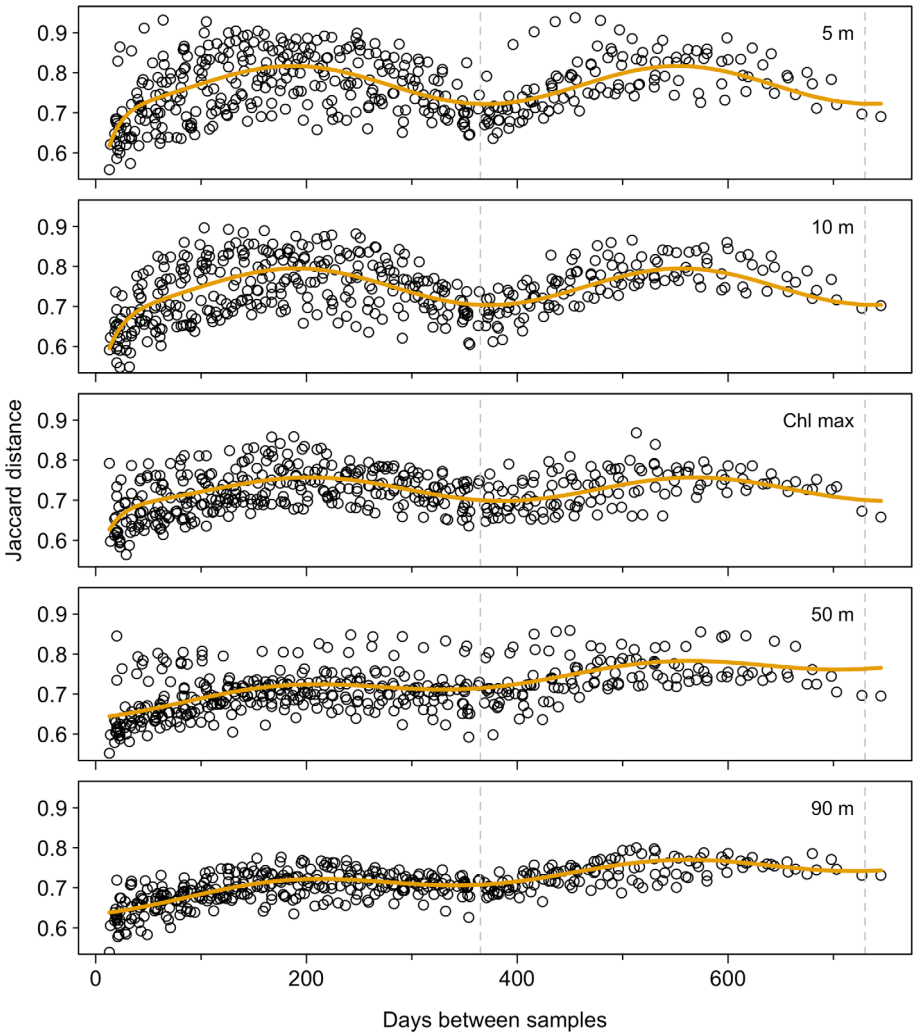

Fig. 6. Seasonal oscillations in microbial community structure dampen with depth. Pairwise Jaccard distance between every possible pair of samples from each depth, plotted by the days between samples. Each data point represents the mean dissimilarity between paired samples collected *n* days apart (where values of *n* are depicted on the *X*-axes). The sinusoidal pattern evident with a period of 365 d (dashed gray line) reflects seasonally recurring fluctuations in microorganism community composition. Orange lines depict additive sine and saturation function fits to the resulting dissimilatory time series.

microorganism communities. Microorganism communities in the deeper waters exhibited dampened seasonal variability relative to the near-surface waters. In the upper lake (e.g., 5 and 10 m) dissimilatory fluctuated with a period of approximately 365 d (Fig. 6). At deeper depths (e.g., 50 and 90 m) dissimilatory values demonstrated weaker seasonal fluctuations, with the magnitude of seasonality (amplitude of the sine) approximately half of that observed in the epilimnion (Fig. 6). Lomb–Scargle seasonal analyses revealed that approximately half (46% and 51% at 5 and 10 m, respectively) of the OTUs in the epilimnion varied seasonally, while only 20% of the OTUs varied seasonally at depth (Table 1; Supporting Information Table S3).

Intermittency in stratification and plankton distributions

The observation that absolute and relative abundances of cyanobacteria increased in the deep waters of the lake during the early spring, when the thermal structure of the lake indicated the lake was well mixed, prompted us to explore possible mechanisms underlying these patterns. Based on the rRNA gene sequence analyses, the increased cyanobacterial abundances appeared driven by increases in specific members of *Cyanobium* (OTU3 and OTU28; Fig. 4). We explored the possibility that these observations reflected periods of weak stratification that permitted growth of specific cyanobacteria in the well-lit waters of the lake, followed by wind-driven mixing prior to our sampling. To examine whether there was precedent for such intermittent stratification and mixing in the early spring, we examined a higher frequency record of vertical temperature profiles collected at Midlake Deep in 2012 and 2013. The resulting dataset revealed several occurrences of intermittent stratification-mixing events occurring in April 2013 (Fig. 7). Specifically, there were two to three periods where temperatures in the upper 10 m warmed (as much as 0.3°C) relative to deeper waters (indicative of weak stratification), followed by periods when wind speeds increased abruptly over 45 km h^{-1}, and the lake thermal structure was re-homogenized for 1 to 2 d. However, similar intermittent mixing-stratification dynamics, which drive shallow stratification above 50 m, were not observed during the onset of full vertical mixing in the late fall, despite similar wind conditions.

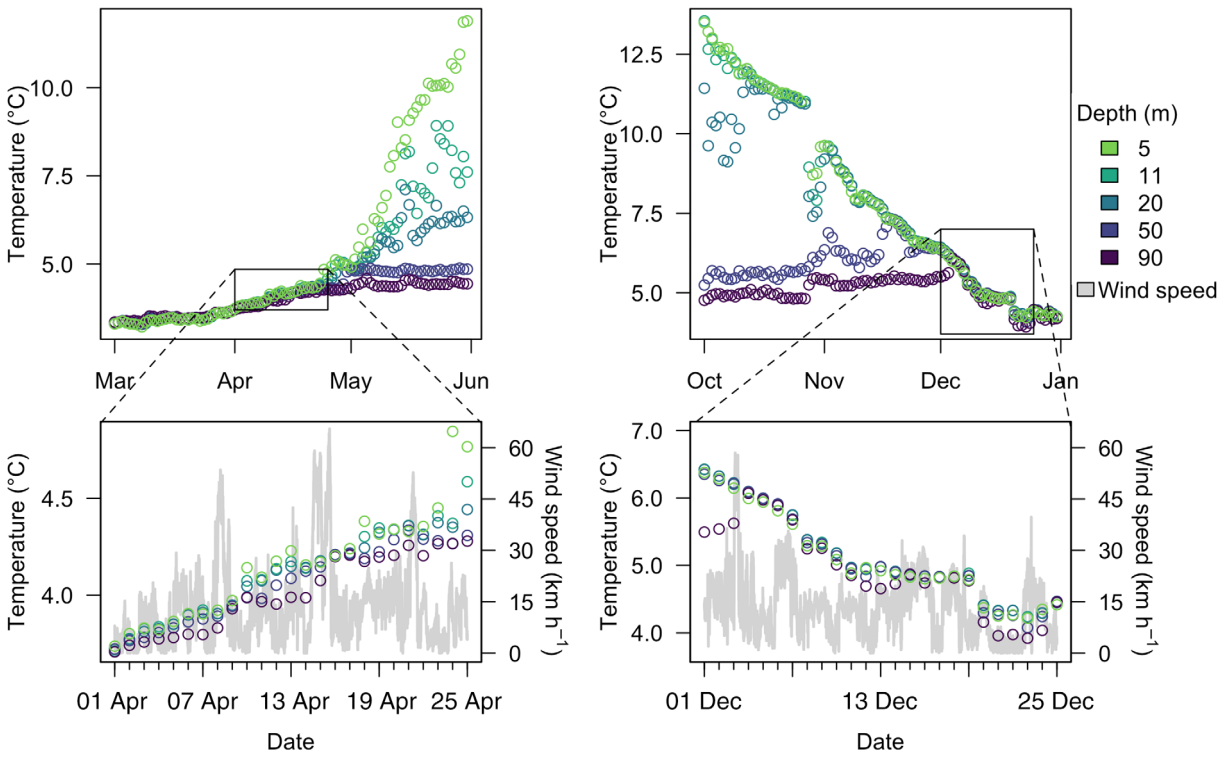

Fig. 7. Temperatures in Flathead Lake during the early spring and fall demonstrate periods of weak stratification followed by intermittent convective mixing. Upper panels depict the mean daily temperatures between March and May 2013 (left) and October through December 2013 (right). Lower panels depict mean daily temperatures from 01 April to 25 April (left) and 01 December to 25 December (right). Note difference in temperature scales among panels. The gray line in both lower plots represents wind speed (km h^{-1}). Days where temperatures at all depths converge on a single value are consistent with periods of deep mixing; periods of stratification appear as times when temperatures diverge across depths. Fluctuations between stratified and mixed periods coincide with variations in wind speed, with lower wind speeds permitting stratification and high winds (above 45 km h^{-1}) resulting in deep mixing.

Discussion

The community of microorganisms in Flathead Lake are similar to those described in other relatively deep and low nutrient aquatic systems, including predominance of putative oligotrophic taxa that exhibit seasonally dependent vertical structure (Park et al. 2023). Although absolute abundances of non-pigmented planktonic microorganisms were relatively constant with depth and time, both relative abundances of specific taxa and community diversity varied seasonally and vertically. Through time-resolved sampling, our study provides evidence that intermittent variations in stratification and mixing are important controls on vertical distributions of microorganisms in the early spring. Such findings highlight linkages between seasonally predictable variation in climate and its role in controlling variation in resource availability and point to the importance of regional and local weather as higher frequency controls on lake habitat structure.

Flathead Lake microbial community

Several patterns emerged from this study that are consistent with other relatively deep and oligotrophic temperate lakes (e.g., Baikal, Tanganyika, Crater, Biwa, Zurich; Urbach et al. 2001; De Wever et al. 2005; Cabello-Yeves et al. 2018). Absolute abundances of non-pigmented planktonic microorganisms demonstrated only modest temporal and vertical changes; however, relative abundances of numerous microbial lineages varied seasonally and with depth. The two strongest predictors of variation in community structure were temperature and depth (Supporting Information Table S3), despite seasonal variation in concentrations of N + N. The vertical structure of microorganism communities varied with progressive strengthening or weakening of the lake thermocline. This dynamic resulted in the emergence of taxa that were largely restricted to the warm and well-lit regions of the upper lake and other taxa that were more abundant in the cool and dark waters of the hypolimnion.

Cyanobacteria in Flathead Lake were dominated by members of the genus *Cyanobium*; these microbes were among the most abundant taxa in sunlit-waters. *Cyanobium* are often highly abundant in oligotrophic lakes, observed as small ($\sim 1\ \mu$m) phycoerythrin- and phycocyanin-containing cells (Padisák et al. 1997; Callieri and Stockner 2002; Nwosu et al. 2021). In Flathead Lake, abundances of cyanobacteria in the epilimnion appeared bi-modal, with peak abundances in the early spring and fall, while abundances in the deeper Chl max increased in the mid- to late-summer. These changes coincided with variation in the relative abundances of different members of the *Cyanobium*, likely pointing to differences in adaptive traits among different lineages. During the early spring and fall, proliferation of *Cyanobium* lineages may reflect competitive advantages under conditions when nutrient concentrations (i.e., N + N) are relatively elevated, light fluxes are intermittent and low, and temperatures are relatively cool. In contrast, *Cyanobium* OTUs prevalent through the mid-summer are likely better adapted to the warmer, low nutrient, and high-light conditions. Other studies have also found that individual *Cyanobium* species demonstrate unique temporal and spatial patterns, including spring, summer, and fall specialists (e.g., Nwosu et al. 2021). Together, our observations point to different ecotypes of *Cyanobium* segregating to use the seasonally and vertically varying niche space within the Flathead Lake photic zone.

As has been described in other lakes (e.g., Salcher et al. 2011), *Alphaproteobacteria* belonging to the LD12 tribe of SAR11 were prevalent in Flathead Lake and exhibited preference for the epilimnion. The LD12 tribe are often dominant members of freshwater microbial communities (Urbach et al. 2001; Newton et al. 2011; Salcher et al. 2011). Isolation and genome sequencing of members of the LD12 tribe point to restricted, aerobic, chemoheterotrophic metabolism (Salcher et al. 2011; Henson et al. 2018). Consistent with the observed vertical distribution in Flathead Lake, members of the LD12 have been shown to possess the capability of phototrophy via rhodopsins (Atamna-Ismaeel et al. 2008; Pinhassi et al. 2016).

The onset of stratification also influenced relative abundances of microorganisms in the hypolimnion, including members of Chloroflexi, Verrucomicrobia, and Planctomycetes. These deep-dwelling taxa were less seasonally variable than their shallower relatives, consistent with the relative stability of the cold, dark, hypolimnion habitat. The predominance of such taxa in the hypolimnion has been described in other relatively deep lakes (Salmaso 2010; Pollet et al. 2011; Cabello-Yeves et al. 2018). One of the most abundant OTUs in our study, a member of the *Thermomarinilinea* clade of Chloroflexi, was strongly overrepresented in the hypolimnion relative to the epilimnion. This is consistent with previous studies, which have shown these microbes to be prominent members of aphotic zone bacterial communities in relatively deep lakes (Urbach et al. 2001; Okazaki et al. 2013; Denef et al. 2016). Genome reconstructions indicate the potential for varied forms of metabolism that span chemoheterotrophy, including methylotrophy, and anoxygenic phototrophy among aquatic Chloroflexi (Denef et al. 2016; Mehrshad et al. 2018).

In contrast to such clear vertical and seasonal structuring, four of the six most abundant members of the Actinobacteria appeared not to demonstrate strong depth differentiation. For example, several OTUs in the acI family of Actinobacteria maintained high relative abundances irrespective of variations in mixing, light, and nutrient concentrations, a finding consistent with prior studies in temperate lakes (Glöckner et al. 2000; Newton et al. 2007). Actinobacteria are often small (cell volumes as small as $< 0.1\ \mu m^3$; Hahn et al. 2003; Neuenschwander et al. 2018), possess highly streamlined genomes (genome sizes range 0.8 to 1.6 Mb; Kang et al. 2017), and often grow slowly (Patin et al. 2016). Some of these organisms have been shown tolerant to UV light (Warnecke et al. 2004; Barka et al. 2016) and possess light-harvesting

rhodopsin proteins for energy generation (Sharma et al. 2009; Dwulit-Smith et al. 2018). These latter findings may underlie the slight epilimnetic preference demonstrated by two of the six abundant Actinobacteria OTUs. Taken together, such adaptations may drive the dominance of Actinobacteria in oligotrophic lakes across time and depth.

Temporal variability in the epilimnion vs. hypolimnion

Perhaps not surprisingly, bacterial communities at shallower depths exhibited greater seasonal variability than communities in the deeper waters, and consistent with previous studies (Kurilkina et al. 2016; Salmaso et al. 2018) epilimnion communities exhibited lower diversity (e.g., Shannon H'). During the winter when the lake was deeply mixed, the diversity and composition of microorganism communities most closely resembled the hypolimnetic community. It is possible that the combination of cold temperatures and reduced solar energy input during the winter (due to the combination of shorter daylength, lowering of the incident angle of sunlight, and deep mixing reduction in daily light flux) yields conditions like those found in the hypolimnion during the summer. Alternatively, mixing of the larger volume of hypolimnetic waters with the epilimnion during the winter may effectively dilute cell concentrations of shallow-dwelling taxa, resulting in a community more like the hypolimnetic community. Similar results have been reported for Lake Baikal (Kurilkina et al. 2016) and Lake Gara (Salmaso 2010), where increased diversity in the winter was linked to mixing introduction of hypolimnetic communities.

Short-duration mixing-stratification

Our time-series sampling also highlighted the importance of intermittent physical dynamics occurring on daily-to-weekly scales, superimposed on more robust seasonal variability, as controlling microbial communities. During the early spring (April and May), both absolute and relative abundances of cyanobacteria increased throughout the dark waters of the lake. The vertical distributions of cyanobacteria and uniform temperature structure during these periods were consistent with the lake being well mixed. Similar findings have been reported in other temperate lakes during the early spring (e.g., Padisák et al. 1997). Given the presumed photosynthetic metabolism of cyanobacteria and the low daily light flux these cells would experience during deep mixing, these observations were perplexing. If the lake was still deeply mixed, as the temperature structure implied, the obvious question is what conditions led to the apparent increase in cyanobacteria throughout the lake waters during the early spring?

By leveraging a vertically resolved, high frequency temperature dataset from Flathead Lake, we found that periods of intermittent stratification followed by wind-driven mixing were regular features of the early spring. Our findings suggest that short duration periods (on the scale of days) in the early spring where the near-surface waters of the lake are warming and winds are low enable the establishment of weak stratification, which would increase the average daily light flux experienced by planktonic microorganisms in the upper lake. Such conditions could overcome light limitation of photosynthesis during periods of deep mixing, promoting the expansion of photosynthetic biomass (Sverdrup 1953). Such short periods of stratification, on the scale of days (3–6 d), appear long enough to enable increases in the abundances of cyanobacteria in the upper lake; however, the weakly stratified conditions appear overcome by wind-driven mixing (e.g., wind speeds in excess of 45 km h^{-1}), redistributing planktonic microorganisms from the near-surface waters to deeper depths. These dynamics appear to occur at scales shorter than our once- to twice-monthly sampling frequency.

Notably, we do not see evidence for similar vertical redistribution of plankton in the late fall despite evidence that the lake experiences short periods of weak stratification followed by deep mixing. We suspect this reflects the lower daily flux of light incident to the lake during the fall compared to the early spring. Such findings point to the importance of considering time scales of microbial growth, together with scales of stratification and mixing, as key to understanding the contemporaneous vertical structure of microbial communities in temperate lakes. Our findings also point to the importance of considering the recent history of local weather (e.g., high wind events) as an additional control, superimposed on seasonal variability, on contemporaneous distributions of plankton in temperate lakes. A number of studies have found weather events can be key drivers to lake plankton communities, particularly phytoplankton, through vertical mixing or supply of nutrients (Langenberg et al. 2003; Stockwell et al. 2020; Hampton et al. 2022). Our results highlight the potential value that biologically focused sampling could provide for insights into the recent history of the physical structure (i.e., stratification-mixing) of the lake, emphasizing the value of time-resolved sampling for mechanistic insight into processes shaping the distributions of microorganisms in aquatic systems.

Conclusion

We examined how variation in stratification and mixing affected the composition and distributions of microorganisms in a large, oligotrophic temperate lake. Consistent with other deep lakes, the microorganism communities in the epilimnion and hypolimnion differed, and with the onset of convective mixing in the winter, the structure of communities in the upper lake converged on those observed at depth. While the types of microorganisms appear largely consistent with other relatively deep oligotrophic lakes, our time-series sampling yielded new insights into time scales underlying variability in these organisms, including highlighting the importance of daily-to-weekly scale events as modifiers of lake habitat structure.

Data availability statement

16S rRNA gene amplicon sequences are available via GenBank (Accession #PRJNA973100). Physical and biogeochemical data from Flathead Lake are available through the Flathead Lake Biological Station public data portal (https://flbs.umt.edu/newflbs/research/flbs-public-data/) under the Flathead Lake hydrolab data ("Temp," "CHLa," "D.O.0.1") and Flathead Lake weather data (YB Point "Wind Speed"), with additional chemistry data available under "Flathead chemistries—Lake". Vertical profiler data are available from the Woods Hole Oceanographic Institution ice-tethered profiler data page for mission LMP46-FHL (https://www2.whoi.edu/site/itp/data/completed-missions/lmp46-fhl/) and the stationary 5 m temperature data are available from Zenodo (https://doi.org/10.5281/zenodo.7972586). Flow cytometric absolute abundance data are available from Zenodo (https://doi.org/10.5281/zenodo.8358244). R scripts used to generate figures and process data are available in a GitHub repository (https://github.com/kevans27/FlatheadLake16S).

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Acknowledgments

This work benefitted from the Flathead Monitoring Program (FMP), specifically the efforts of several people at the Flathead Lake Biological Station, including Tyler Tappenbeck, Jim Craft, Adam Baumann, Sydni Racki, Evan Bilbrey, Shawn Devlin, and Tom Bansak. In addition, we are grateful to Drs. Bonnie Ellis and Jack Stanford for their years of leadership of FMP and for making the ice-tethered profiler data available. FMP receives support from the State of Montana and philanthropic donations. Feedback from Robert O. Hall Jr. (FLBS), James J. Elser (FLBS), Scott R. Miller (UM), and Royce C. Engstrom (UM) improved this manuscript. This research was supported in part by the US National Science Foundation (grant DEB-1951002 to M.J.C.) with additional support from the Simons Foundation (SCOPE grant # 721221 to M.J.C.) and a scholarship provided by Joan Schleicher to K.A.E.

Conflict of Interest

None declared.

Submitted 30 May 2023
Revised 06 October 2023
Accepted 01 January 2024

Associate editor: Michaela M Salcher