COVER SHEET

TITLE: Bacterial Community Spatial and Temporal Variation in a North Temperate Bog Lake

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ABSTRACT

Bacterial Community Spatial and Temporal Variation in a North Temperate Bog Lake

Bog lakes such as Mary Lake in northern Wisconsin are ideal systems to investigate microbial communities due to easily identified spatial boundaries and well characterized seasonal ecosystem dynamics. Multiple factors are thought to influence the bacterial communities within the lake, and our goal in this study was to analyze the spatial and temporal factors causing the variations between these populations. Molecular techniques and statistical analyses were performed to gain insight into which differences caused the greatest variability. The bacterial communities were analyzed based on conserved 16S ribosomal RNA sequences as well as on short base pair sequence reads obtained from a time series in 2009 in order to determine whether the bacterial community was more variable in space or in time. We found that while the upper and lower thermal layers of the bog were composed of similar microbial communities, these communities had different temporal patterns throughout the time series.

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Spatial and temporal variation of bacterial communities in a north temperate bog lake

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Humic bog lakes are ideal systems to investigate microbial communities due to easily identified spatial boundaries and well characterized seasonal ecosystem dynamics. Permanently stratified lakes such as Mary Lake in northern Wisconsin provide well-defined thermal layers with distinctive physical and chemical conditions, each thought to harbor distinct microbial communities. Multiple biotic and abiotic factors are thought to influence these communities, and the goal of this study was to identify the spatial and temporal factors that may be structuring communities in the bog, and to correlate variation among populations. Molecular techniques and multivariate statistical analyses were performed to gain a systems-level understanding of the variation and its drivers. The bacterial communities in the bog were analyzed with nucleotide fragments from the 16S-23S intergenic spacer region of the ribosomal RNA operon (ARISA) as well as short base pair sequence reads covering the fourth hyper variable region in the 16S ribosomal RNA gene obtained from a sample time series collected in 2009. Variation with lake depth, between thermal layers, and through time were calculated and assessed in order to determine whether the bacterial community was more dynamic in space or in time. We found that while the upper and lower thermal layers of the bog were composed of distinct microbial communities containing reoccurring populations, these communities had different occurence patterns across the time series, and were more variable in space than in time.

INTRODUCTION

According to traditional ecological theory, bacterial communities are shaped by variability in environmental conditions across space and time, representing habitat heterogeneity (Shade et al. 2008). In freshwater lake ecosystems, it has been demonstrated that both biotic and abiotic factors such as dissolved oxygen, pH (Lindstrom *et al.*,2005), trophic status (Yannarell *et al.*, 2003), landscape position, lake geography, (Yannarell and Triplett, 2005) and biotic interactions play a significant role in shaping bacterial communities. Small freshwater lakes are an ideal system to investigate microbial community dynamics because the seasonal ecosystem dynamics have been well characterized and the spatial boundaries are easily identified (Milferstedt et al. 2010). Temperate bog lakes in Northern Wisconsin are excellent examples of these small freshwater lake ecosystems, and can be classified according to annual mixing frequency (polymictic, dimictic, meromictic). Polymictic lakes mix vertically multiple times during the

year and stratify irregularly (Shade et al. 2008). Lake mixing in dimictic lakes occurs in the fall and spring due to differential warming and cooling of the upper and lower levels of the lake. These mixing events disrupt the chemical gradients that had been established in the winter and summer stratification periods, and combine the otherwise isolated water layers (Milferstedt et al. 2010). In permanently stratified lakes (meromictic), there are stark differences between the thermal layers (epilimnion and hypolimnion) in chemical and physical conditions. As such, each layer is known to harbor different bacterial communities (Shade et al. 2008).

The lower hypolimnion is generally cooler, nutrient-replete, thermally stable, and potentially anoxic, while the upper epilimnion is warmer, has a scarce availability of nutrients, is relatively oxygen-rich, and potentially affected by weather events and exposure to high solar radiation (Shade et al. 2008). Primary production and phytoplankton populations, which are large drivers of bacterial populations (Kent et al. 2006), are largely influenced by the availability of light; conversely the hypolimnion is largely unaffected by extrinsic drivers (Milferstedt et al. 2010). The deposition of bacterial immigrants from rainfall has also been suggested as a source of habitat heterogeneity, primarily affecting the epilimnion and increasing community variability (Jones and McMahon 2009). While there are numerous possible sources of community variability in temperate lakes, lake mixing has been shown to be the ultimate driver of community change because it destroys the vertical habitat heterogeneity of environmental parameters such as dissolved oxygen and temperature (Shade et al. 2008). For lakes that do not undergo a seasonal mixing event (meromictic lakes), permanent stratification leads to an epilimnion and hypolimnion with different physical and chemical conditions, which in turn leads to different bacterial communities (Shade et al. 2008). Mary Lake, a humic bog lake in Northern Wisconsin, USA, is one such meromictic lake (Table 1). Mary Lake is permanently stratified, thus presenting an ideal model for these differing bacterial communities due to the lack of seasonal disturbances (Milferstedt et al. 2010).

In this study, the bacterial communities in Mary Lake were analyzed through space and time based on conserved 16S ribosomal RNA sequences (ARISA) as well as on short base pair sequence reads obtained from a 2009 time series. Variations between lake depth, thermal layer, and through time were calculated and assessed in order to determine whether the bacterial community was more variable in space or in time. Methanotroph community dynamics were also analyzed to obtain a picture of one specific bacterial population and its seasonal variation.

Methanotrophic bacteria consume methane as their sole source of carbon and energy, and thus modulate its release into the atmosphere. Major sources of atmospheric methane are natural wetlands and bogs, and are important contributors to methane accumulation and global warming (Shade et al. 2008). Spatial and temporal diversity of methanotroph populations in the context of whole bacterial communities within the same freshwater system will give us a brief snapshot of their dynamics as well as contributions.

By looking at the bacterial community as a whole as well as distinct species-like groups (known as tribes) we were able to see how these populations change through time and space within a single freshwater system, and have a better idea of what causes the variations between the epilimnion and hypolimnion communities.

Table 1. Mary Lake characteristics.

Latitude	Longitude	Surface area [ha]	Max. depth [m]	Mixing regime	Average pH"
46°15'2	89°54'1	1.2	21.5	meromictic	5.6

"pH measured from 1 May 2008 to 19 August 2008

METHODS

Sample collection and processing

A total of 26 integrated and 31 depth discrete water samples from Mary Lake were collected weekly in 2009 during the ice-free season. These samples were collected by the North Temperate Lakes Microbial Observatory (NTL-MO) research team as a part of their efforts to understand the ecological dynamics of lakes in northern Wisconsin, bolstered by the North Temperate Lakes Long Term Ecological Research (NTL-LTER) site. Integrated water samples from both the epilimnion and hypolimnion of the lakes were collected, allowing opportunity for analysis on the spatial distributions of microbial communities at each time point. Depth-discrete samples from 24 June 2009 were also taken at 0.5-meter intervals throughout the water column in order to get a more depth-specific sample set.

Total DNA was extracted from the sample filters using the Bio 101 Fast DNA kit (QBiogene, Carlsbad, CA), following the manufacturers' instructions. The intergenic spacer region between the 16S and 23S rRNA genes was amplified from the total DNA using 6-FAM labeled universal 1406F primer (5'-TGYACACACCGCCCGT-3') and bacterial specific primer 23Sr (5'-GGGTTBCCCCATTCRG-3') (Fisher and Triplett, 1999; Yannarell et al., 2003). 5 ng of extracted DNA was used as template, and polymerase chain reaction conditions were as follows: denature at 94°C for 2 minutes, followed by 30 cycles of amplification at 94°C for 35 seconds, 55°C for 45 seconds, and 72°C for 2 minutes, with a final extension of 72°C for 2 minutes. PCR was conducted on a Mastercycler gradient thermocycler (Eppendorf, New York, New York).

Automated Ribosomal Intergenic Spacer Analysis (ARISA) was used to examine community bacterial fingerprints, allowing for the rapid estimation of microbial diversity and community composition within the lake (Fisher and Triplett, 1999), using McMahon Lab standard protocols (Shade et al. 2011). ARISA-PCR fragments ranging in size from 400 to 1,200 bp were discriminated and measured using capillary electropheresis conducted on a 3730xL ABI analyzer at the UW-Madison Biotechnology Center. Profiles were analyzed and aligned using GeneMarker software (SoftGenetics), and fragments

were grouped into operational taxonomic units (OTUs) based on these profile alignments (Shade et al. 2008).

Statistical Analysis

Individual peaks were relativized to total profile fluorescence to create a relative abundance matrix comprised of samples by operational taxonomic units (OTUs). The communities were then analyzed using the PRIMER v 6 software and standard community analyses, including analysis of similarity (ANOSIM) and non-metric multidimensional scaling (Clarke PRIMER software) (Luke et. al 2009), to search for patterns of occurrence and dynamics within the lake and through time. The 'heatmap.2' function in the R software for statistical computing (Iacus and Urbanek, 2005) was used to visualize the OTU persistence through time as a gradient grid (Shade et al. 2008).

Sequencing and sequence quality control

Initial quality filtering was performed using QIIME (Quantitative Insights Into Microbial Ecology, www.qiime.org). Sequences that were filtered included those with read lengths less than 75 base pairs, those containing ambiguous bases (N), and those with homopolymer stretches longer than six bases. After these quality-control criteria were implemented, 649,409 sequences were retained for further analysis.

OTU assignments and community analysis

The unique.seqs. command implemented in MOTHUR version 1.23.0 (www.mothur.org) was used to obtain a non-redundant set of sequences from high-quality reads. The resulting 132,429 unique sequences were aligned against Greengenes database including 4938 sequence entries. Aligned sequences were then filtered to screened to remove sequences less than 140 base pairs in length or sequences containing homopolymers longer than 6 base pairs. In addition, columns containing no information, those that corresponded to '.' or '-', were eliminated from all sequences. To reduce sequencing artifacts, a pre-clustering step was performed in MOTHUR using the pre.cluster command. This command bins sequences that are less abundant and differ from the dominant sequence type by less than 2%. These aligned sequences were again filtered to remove columns that corresponded to '.' or '-' in all sequences. Chimera checking was performed using U-CHIME within MOTHUR. Chimera sequences were detected with the self reference and removed. Reads were classified against a Greengenes database and any chloroplast reads were removed. After these quality filter steps, 459,449 sequences remained, 23,203 of which were unique. All samples were rarified to 5,000 total reads, and any sample dates that did not contain this many sequences after quality filtering were removed from analysis. There were 3 samples

that did not contain 5000 reads, and these samples were dropped from analysis. The samples that were excluded were all from the hypolimnion and sampled on May 30th, June 24th, and June 30th. The total number of sequences in the dataset was 130,000, 9,150 of which were unique. An uncorrected pairwise distance matrix was generated for the 9,150 sequences using the dist.seqs command in MOTHUR. Single linkage clustering was applied with a 97% sequence similarity cut off as described previously (Huse et al., 2010) to assign sequences into OTUs. A matrix of the OTU abundances for each sample was generated by classifying all OTUs defined at the 0.03 cut-off, combining the classify.seqs and classify.otus commands in MOTHUR.

Taxonomic placement

OTUs were attributed to taxonomic analyses by using a naive Bayesian approach implemented in the classify.seqs command in MOTHUR in combination with two template sequence databases and their corresponding taxonomic hierarchy frameworks (McDonald et al. 2011). The freshwater database uses a hierarchical naming structure (phylum/lineage/clade/tribe), the most refine taxon group being the tribe. This naming structure is similar to the Linnean taxonomy, and is based on phylogenetics. Tribes consist of groups of full-length sequences clustering as a monophyletic branch of a phylogenetic tree, with each sequence having over 97% sequence identity to another sequence of that branch (Newton et al. 2011). This classification system was created to maintain the phylogenetic context by which freshwater bacterial gene sequences have historically been identified, clustered and named, allowing the comparison the comparison of ecological observations among previously published freshwater surveys and our study (Newton et al., 2011). Thus, OTUs were first classified against this custom curated database. When an OTU was not assigned with a 70 percent confidence to the family level, it was reclassified using the Greengenes gold database with a classification confidence cutoff of 60 (Newton et al. 2011).

Statistical analyses of tribe dynamics

To summarize seasonal patterns in the dynamic representation of amplicons from freshwater tribes, heatmpas were created for abundant OTU clusters. To determine which OTUs were abundant for each layer independently, a normal distribution was assumed, and a z-score was calculated for each OTU. OTUs that had a z-score larger than 2 were kept for comparison within the heatmaps. z-score is useful in comparing dynamics about populations as both the mean and standard deviation are used for the standardization (Legendre and Legendre, 1998; Yannarell and Triplett, 2005). Heatmaps and occurrence pattern plots were created in R (http://www.r-project.org/) using the ggplot2 package (Wickham 2009). Occurrence pattern plots were made by normalizing abundances using the maximum for each OTU, and

taking the variance to represent variability, and normalizing total abundance for each OTU using the largest overall OTU to represent abundance.

RESULTS

Depth Discrete Spatial Analysis

Samples were collected within Mary Lake on 24 June 2009 at half-meter intervals from 0 to 18 meters (with some replicates) and subsequently analyzed using ARISA. A Bray-Curtis similarity was created using PRIMER v 6 software, and an NMDS (Nonmetric Multidimensional Scaling) was performed and analyzed (Figure 1).

Mary Lake, because it is permanently stratified, has different physical and chemical properties associated with its upper (epilimnion) and lower (hypolimnion) layers. On this particular sample date, the average pH of the epilimnion was 4.99, while the average pH of the hypolimnion was 5.50. Average secchi depth was 1 meter. Figure 2 shows the temperature and dissolved oxygen curves for this sample date, with the thermocline at approximately 2 meters and a peak in dissolved oxygen at 1 meter.

Because the epilimnion and hypolimnion have such different physical conditions, it is expected that they will harbor distinct bacterial communities that are able to exist within each thermal layer. From the NDMS (Figure 1), it is clear that this hypothesis is correct, with three distinct clusters representing the epilimnion (0 to 1.5 meters), and two separate hypolimnion clusters. It is not clear which specific factors are causing these distinctions, but from this analysis we can conclude that on this sample date the epilimnion and hypolimnion harbored distinct bacterial communities.

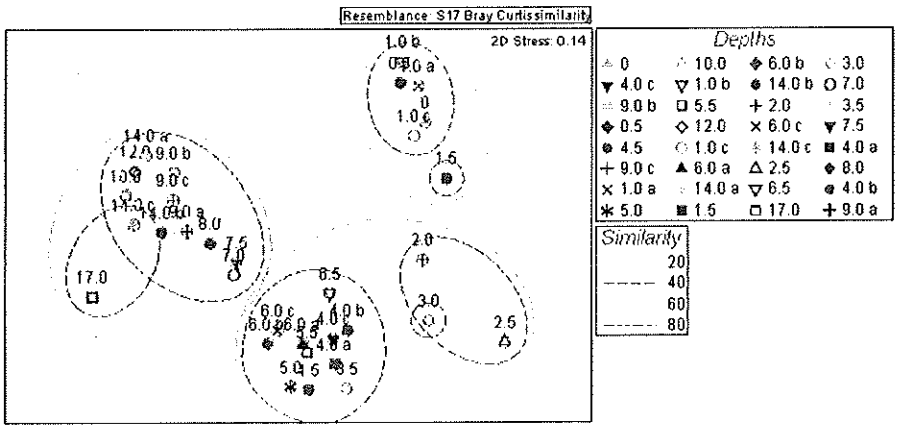


Figure 1. NDMS of bacterial community in Mary Lake on 24 June 2009. Samples were collected at half-meter intervals from 0 to 18 meters (some replicates), and analyzed using ARISA. Clustering is observed within the epilimnion (0 to 1.5 meters), near the thermocline (2 to 3 meters), and within the hypolimnion (3.5 to 18 meters). The hypolimnion (below 3 meters) is anoxic and lower in temperature, representing a different physical environment thus harboring a different bacterial community.

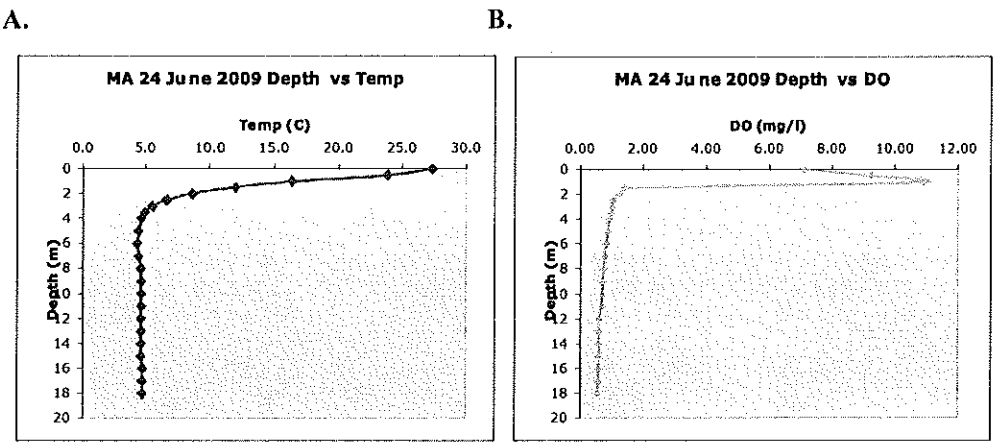


Figure 2. (A) Depth vs. Temperature curve for Mary Lake on 24 June 2009; temperature drops rapidly from 0 to 2 meters, then stays constant at around 4.6°C, thus the thermoclime on this sample date is around 2 meters, with the epilimnion between 0 and 2 meters and the hypolimnion at 2.5 to 18 meters. (B) Depth vs. Dissolved Oxygen (DO) curve for Mary Lake on 24 June 2009; DO spikes at 10.9 mg/l around the thermocline (1 meter), and then stays constant with depth in the hypolimnion at around 0.60 mg/l (anoxic).

Statistical Analysis of 2009 Time-Series

To look at Mary Lake from a temporal perspective, we used ARISA to analyze samples from a 2009 integrated time series, with samples

from both the epilimnion and hypolimnion. NMDS figures were generated for the whole lake (all samples included) (Figure 3), as well as epilimnion (Figure 4A) and hypolimnion (Figure 4B) specific figures.

As shown in Figure 3, the hypolimnion samples cluster very close together, meaning the samples are more similar in composition and not largely variable with time. The epilimnion samples cluster farther apart, and are in fact separated into two distinct clusters (early summer and late summer months), suggesting the upper layer bacterial composition is more variable with time.

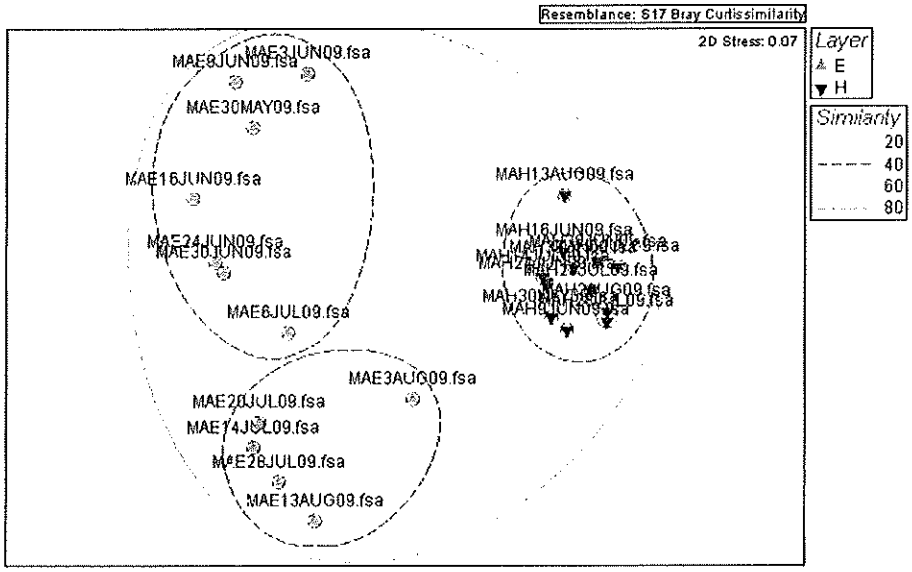


Figure 3. NMDS of both epilimnion and hypolimnion samples from May, June, July and August of 2009. Epilimnion samples are depicted as green triangles, and hypolimnion samples as blue triangles. From this figure it is clear that the epilimnion and hypolimnion contain distinct bacterial communities across the summer months, with the early months in the epilimnion clustering separately from the later months (more variable), and all hypolimnion samples clustering very close together (more stable).

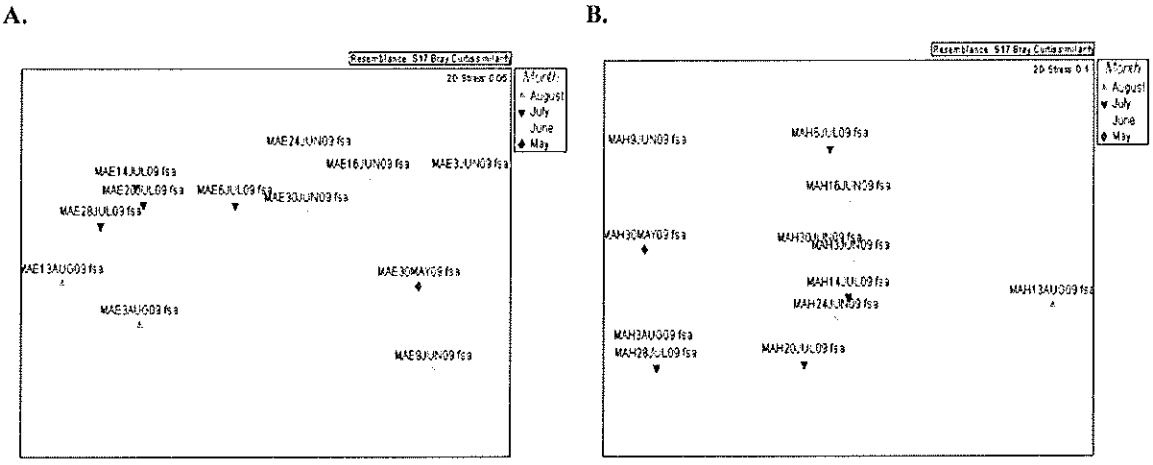


Figure 4. (A) NDMS of epilimnion samples from Mary Lake throughout the summer months of 2009. There is a clear temporal trend as the summer progresses, which is consistent with the fact that the epilimnion is more variable (less similar) and communities would be subject to change temporally. (B) NDMS of hypolimnion samples from Mary Lake throughout the summer months of 2009. There is no clear temporal trend throughout the summer, which is consistent with the fact that the hypolimnion remains relatively stable throughout the year and we would thus not expect the communities to change in a temporal trend.

By separating the samples by later and performing NMDS analyses, it is evident that the epilimnion follows a temporal pattern throughout the summer months, whereas the hypolimnion follows no such pattern, and no distinct clustering of months (Figure 4). This is reflected by the previous NDMS (Figure 3) which shows the epilimnion samples being much more variable (less similar) than the hypolimnion samples, which were much more similar and thus less variable. Therefore, it is expected that the hypolimnion would not follow a temporal pattern since the communities remain relatively stable throughout the year. This can be correlated to the temperature and dissolved oxygen curves (Figures 2A and 2B), which show a very stable hypolimnion environment throughout its depth. Because Mary Lake is meromictic, there is no mixing event to disrupt this stable environment, and thus we would expect to see stable thermal and dissolved oxygen conditions through time at these depths.

Average Pairwise Similarity Analysis

Average pairwise community similarities were calculated using Bray-Curtis similarity values from ARISA analyses in PRIMER.6. Values were calculated for the epilimnion and hypolimnion separately throughout the entire 2009 time series, and then by month (June, July, August). May was not included in the month-only calculations due to the fact that there was only one sample date in May, thus an average pairwise similarity could not be performed. It was not grouped with June to be consistent with previous protocols. We then calculated the similarities by month within lake layer. These data represent a temporal aspect of the bacterial community. We also used the depth-discrete data from 24 June 2009 to calculate the average pairwise similarities for the Mary Lake-specific epilimnion (0 to 2 meters) and hypolimnion (2.5 to 18 meters) on that date, giving us a look at spatial variability of the bacterial community. Figure 5 is a graphical representation of these data.

Figure 5 shows that the hypolimnion is more similar (and thus less variable) than the epilimnion throughout the summer 2009 time series, which is consistent with our previous results. Because Mary Lake is permanently stratified, we would expect that the lower hypolimnion would not be affected by weather events and other extrinsic disruptions as much as the upper epilimnion would. By month, the bacterial communities increase in similarity from June to July and then decrease from July to August, thus increasing in variability.

When we analyzed the data spatially using the depth-discrete data, we found the opposite result. The epilimnion depths (0 to 2 meters) were more similar (less variable) than the hypolimnion depths (2.5 to 18 meters). While at first glance these results seem to conflict with the temporal epilimnion and hypolimnion data, they are consistent with the fact that the bacterial community at 3 meters is likely much different from the community at 17 meters, thus giving a lower average pairwise similarity and showing greater variation than the epilimnion depths, which are only from 0 to 2 meters. Furthermore, we know

that the hypolimnion water chemistry varies significantly with depth while the epilimnion is more homogeneous. The temporal epilimnion and hypolimnion values were calculated using integrated samples, thus the 2.5 to 18 meter bacterial communities were grouped together as one whole sample, not individual depths.

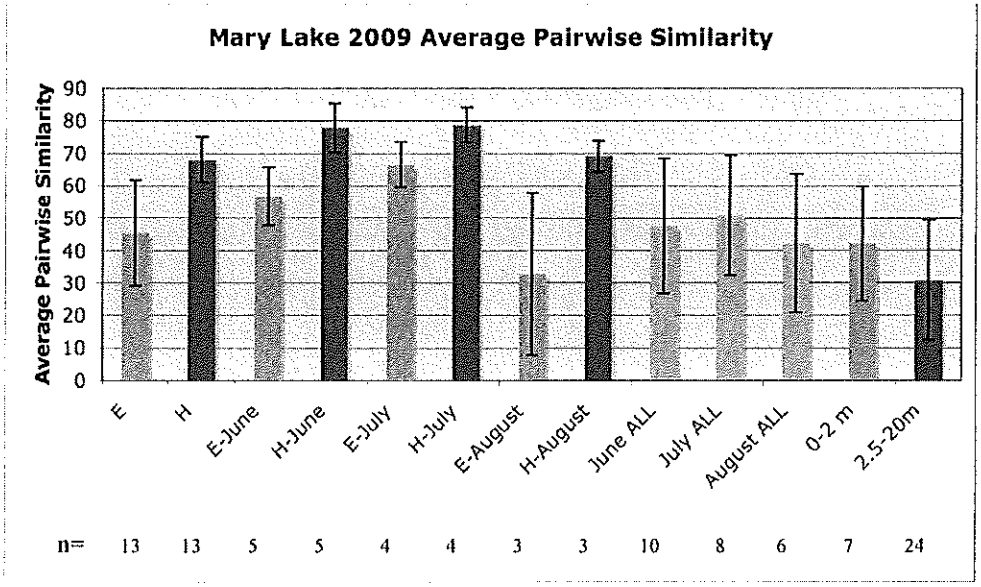


Figure 5. Average pairwise similarity analysis of the Mary Lake 2009 time series. E is epilimnion-specific data (blue) while H is hypolimnion-specific data (red). Green bars represent data with both epilimnion and hypolimnion data separated by month. The 0-2 meter and 2.5 to 20 meter bars used the depth-discrete samples from 24 June 2009 to get a spatial representation of the bacterial community, rather than a temporal representation. The y-axis represents the average pairwise similarity (0 to 100), with 0 being completely dissimilar and 100 being completely similar. The sample size (n) of each category is listed below the corresponding bar.

Sequence-based Analysis

16S rRNA gene tag sequence data was quality filtered in QIIME and MOTHUR, and taxonomies were assigned to OTUs using a custom curated freshwater database (Newton et al. 2011) and the Greengenes database (McDonald et al. 2011). The output files were further analyzed using the R statistical software package (http://www.r-project.org/), and relative abundance heatmaps for the epilimnion and hypolimnion sequencing data were generated (Figure 6). The relative abundance of reads associated with each OTU was normalized by log transformation. Doing so allowed us to visualize the change in relative abundance of each OTU relative to the rest of the chosen OTUs, instead of plotting raw relative abundance. The inclusion of an OTU in the heatmaps was determined by performing a z-score cut-off. An OTU was only included if its abundance was more than 2 standard deviations above the mean OTU abundance.

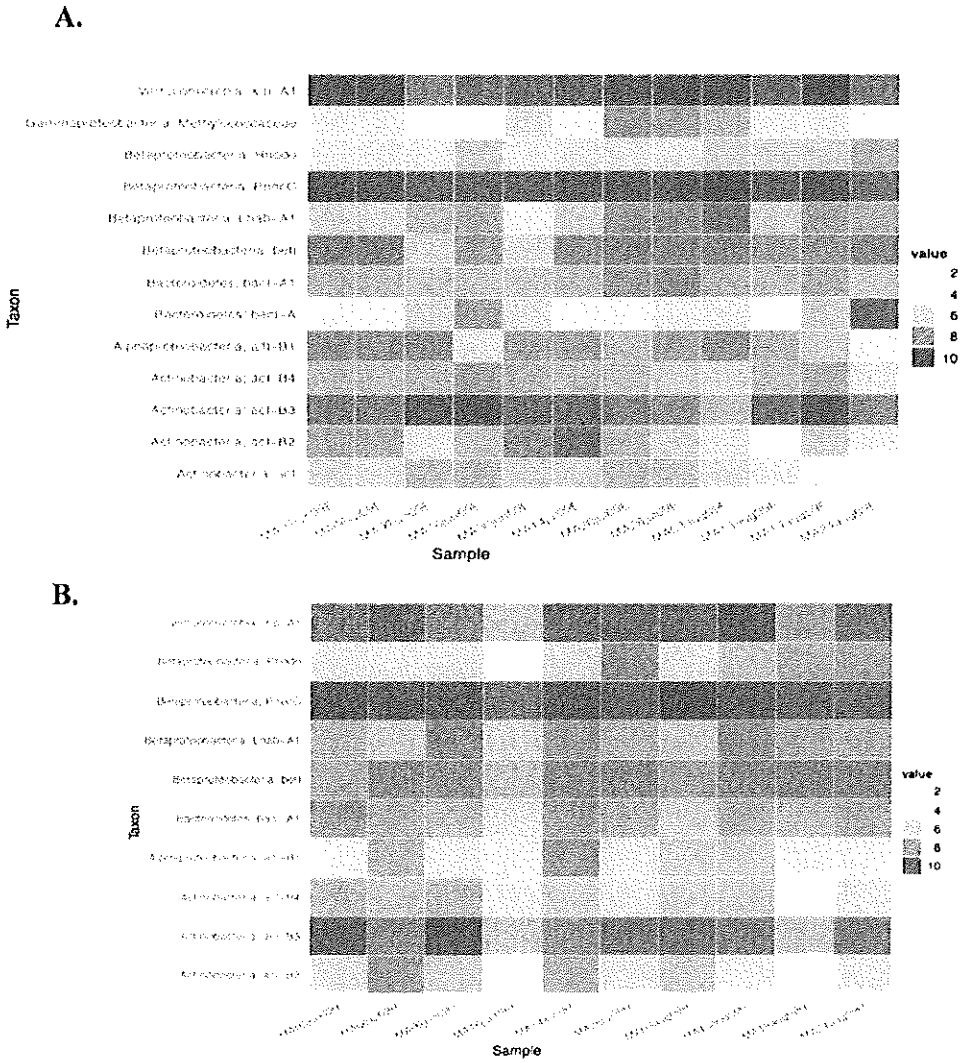


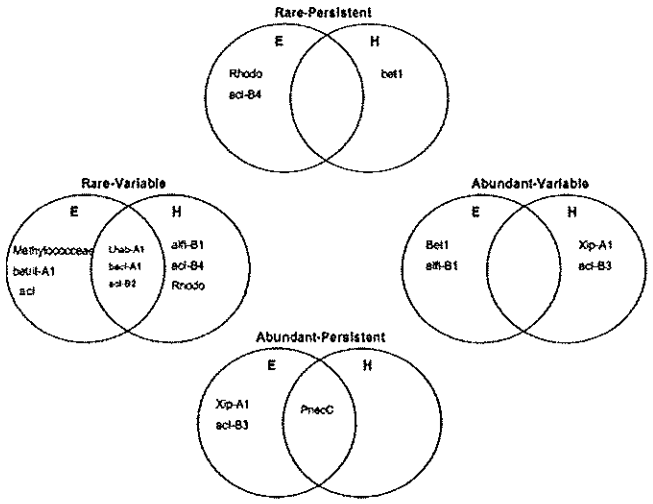
Figure 6. (A) Heatmap corresponding to Mary Lake epilimnion Illumina sequencing data. The most abundant (z score greater than 2) OTUs are listed next to their relative abundance data, which are represented by color on a scale of white (lowest abundance) to dark blue (highest abundance). The taxonomy of each OTU is shown to the left of the heatmap. The phylum is listed first, followed by its most finely resolved taxonomic assignment. **(B).** Heatmap corresponding to Mary Lake hypolimnion sequencing data. The order presented for each OTU is determined in the heatmap function of R, where OTUs are clustered based on similar patterns.

From these figures, we are able to visualize the relative abundances of each dominant OTU in both layers relative to each other, giving us a picture of how these populations change relative to one another throughout the time series. Between the epilimnion and the hypolimnion analyses, there do not appear to be any distinct seasonal patterns for all OTUs within each layer. However, each OTU can be categorized based on its occurrence pattern in each layer; rare versus abundant, and persistent versus variable. We were able to form four categories based off of these occurrence patterns; rare-persistent, rare-variable, abundant-persistent, and abundant-variable. Each OTU falls within one of these four categories, and some OTUs fall

in the same category between lake layers (Figure 7A). Persistent OTUs are those that show consistent abundance levels (high or low) across the time series, while variable OTUs showed fluctuations in these abundance levels.

To represent this quantitatively, we created a four-quadrant plot using tag sequencing data to display these occurrence patterns, with each quadrant representing one of the four patterns previously mentioned (Figure 7B). The x-axis represents variability, with 'persistent' as the left quadrants and 'variable' as the right quadrants. The y-axis represents abundance, with the top quadrants as 'abundant' and the bottom quadrants as 'rare'. Thus, we see the same four occurrence patterns discussed previously from a quantitative standpoint.

A.



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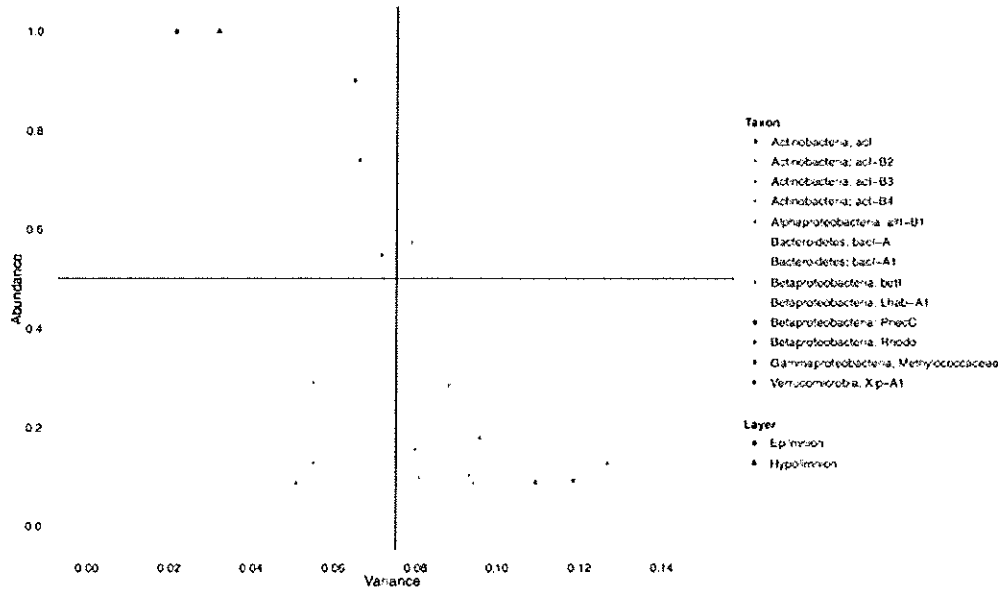

Figure 7. A. Venn Diagram depicting the four occurrence pattern categories of the most abundant OTUs in the epilimnion and hypolimnion; rare-persistent, rare-variable, abundant-variable, and abundant-persistent. OTUs that had the same occurrence pattern in both the epilimnion and hypolimnion were placed in the overlapping area between circles, demonstrating their consistent pattern between thermal layers. **B.** Graphical representation of occurrence pattern distributions. Abundance is displayed on the x-axis (rare on the left, abundant on the right), and variance is displayed on the y-axis (variable on the top, persistent on the bottom). Abundant-variable is the top-right quadrant, abundant-persistent the bottom-right, rare-persistent the bottom left, and rare-variable the top-left. The epilimnion OTUs are represented as circles, and the hypolimnion as triangles, with each OTU a distinct color.

Methanotroph community dynamics

In order to get an even deeper look into the community dynamics within Mary Lake during the summer of 2009, we targeted methanotroph-specific sequences from the 16S rRNA gene tag sequencing dataset and analyzed the changes in abundance. Interestingly, Type II methanotrophs (of the Alphaproteobacteria class) were not present at a detectable level, and thus we did not include them in this figure. Type I methanotrophs

(of the Gammaproteobacteria class) were abundant at different levels in the epilimnion and hypolimnion and at different sample dates throughout the time series (Figure 8). These Type I methanotrophs had seasonal dynamics in both layers, with a low relative abundance in the spring and fall, but a higher abundance in the summer. While the abundance patterns do not directly overlap between lake layer, the seasonal dynamics are the same for these methanotrophs.

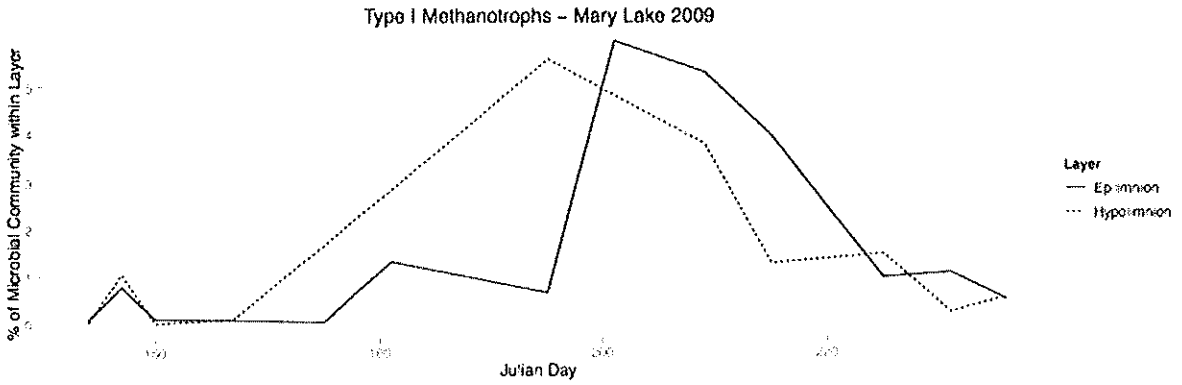

Figure 8. Type I methanotroph relative percent abundance throughout the 2009 time series in the epilimnion and hypolimnion of Mary Lake. Both the epilimnion and hypolimnion show similar patterns of methanotroph abundances, the epilimnion is more variable in abundance than the hypolimnion, and reaches a slightly higher abundance peak than the hypolimnion.

DISCUSSION

Our depth-discrete ARISA data analysis revealed that there is indeed a stark difference between bacterial communities in the epilimnion, metalimnion, and hypolimnion of Mary Lake. The hypolimnion in Mary Lake is relatively anoxic, and much cooler than the epilimnion, thus we would expect the two communities to differ based on the differing chemical and physical compositions of their respective layers. There are many other possible contributing factors, such as different P and N levels within each layer, presence of a deep chlorophyll maximum, alternative electron acceptor/donor pairs (e.g. iron, sulfide, sulfate), or methane levels. More research must be done to pinpoint specific causes for these apparent differences.

Analysis of integrated epilimnion and hypolimnion samples from the 2009 time series also revealed a stark difference in bacterial community composition. Figure 3 shows the samples clustered together by lake layer, with the hypolimnion cluster much less variable (thus clustering closer together) than the epilimnion samples, which cluster much farther apart and are thus less stable (more variable). This makes sense in the context of meromictic lakes such as Mary, in which the upper epilimnion is more affected by extrinsic factors such as weather events. Previous studies have also suggested that hypolimnion communities are more strongly driven by vertical gradients in electron acceptor availability, while the epilimnion communities are more strongly driven by biotic intra-layer competition (Shade et al. 2008). Because the epilimnion is exposed to more environmental variability, we would expect the bacterial communities within this layer to be more variable as well.

This conclusion was once again supported by a layer-specific statistical analysis. Figure 4A shows epilimnion samples as a function of sample month, with a clear temporal pattern as the time series progresses. Although meromictic lakes like Mary are permanently stratified, the epilimnion is only 2 meters deep and is thus exposed to weather events, making it plausible that the bacterial communities would be subject to change as the season progressed. However, from Figure 4B it is clear that there is no such seasonal pattern with the hypolimnion community samples, which do not seem to be clustered by month at all. This result is supported by the fact that the hypolimnion communities are statistically very similar through time in relative abundances (discussed below), and is thus not very variable compared to the epilimnion. This similarity in community structure is supported by the hypolimnion's isolation from external factors, and the fact that Mary Lake is permanently stratified.

To determine statistically whether Mary Lake was more variable in space or in time, we used the Bray-Curtis community similarity metric to perform average pairwise similarity analyses with different sets of samples. Figure 5 summarizes these results in graphical form, showing once again that the hypolimnion is more similar (and less variable) than the epilimnion. We defined "through space" as the depth discrete sample analyses depicted as 0 to 2 meters (epilimnion) and 2.5 to 20 meters (hypolimnion). "Through time"

is the remaining integrated sample analyses separated by lake layer, and then by month within lake layer. From this analysis, we found that the spatial data were less similar (more variable) than the temporal data, and we conclude that Mary Lake is more variable in space than in time. Because Mary is permanently stratified and quite deep for a humic bog lake, this finding could be due to a number of characteristics; the fact that the lake does not mix as do seasonally-mixed lakes means that Mary is likely to be very similar from season to season, thus we would not expect much variability temporally. Also, because Mary is so deep (21.5 feet), there are multiple niches for communities to occupy, as well as vertical gradients in electron acceptor availability, giving rise to more bacterial population variability by depth (space).

Deep 16S rRNA gene tag sequencing analysis revealed distinct differences in occurrence patterns for each dominant OTU in both the epilimnion and hypolimnion. These occurrence patterns were categorized as rare-persistent, rare-variable, abundant-persistent, and abundant variable (Figures 7A and 7B). While most of these OTUs fell into different categories between the epilimnion and hypolimnion, some showed the same occurrence pattern in both thermal layers. For example, PnecC was abundant and persistent in both the epilimnion and hypolimnion throughout the 2009 time series. PnecC is commonly found in freshwater systems, thus this result is not surprising (Salcher et al. 2008). Also, Lhab-A1, bacI-A1, and acI-B2 are all rare and variable in both the epilimnion and hypolimnion, perhaps because they are affected by seasonal dynamics within the lake or by competition from other bacterial groups.

Figure 7B shows these patterns in a four-quadrant plot, each quadrant representing one of the occurrence patterns. Interestingly, each abundant taxon from the sequencing data that was characterized as abundant in this dataset was clustered close together on the plot in terms of lake layer (epilimnion and hypolimnion). Above the x-axis (variability), these OTUs (characterized as abundant) were clustered together as pairs. However, below the x-axis (characterized as rare) these pairs were no longer obviously discernable, and some OTUs were characterized differently between the epilimnion and hypolimnion. This is consistent with our Venn diagram analysis, as some OTUs showed the same occurrence patterns between the epilimnion and hypolimnion, and others displayed different patterns. This could once again be due to factors such as biological competition or nutrient availability between thermal layers, with ubiquitous (abundant) bacteria more competitive than their more rare and variable community members. While further studies are necessary to determine the causes of these fluctuations in abundance, these occurrence patterns allow us to catch a glimpse of the most abundant bacterial communities within each thermal layer of the lake and how these communities change over time.

To get a close look at one specific bacterial community within Mary Lake, we analyzed methanotrophic bacteria-specific sequences from our dataset and plotted their abundance throughout the time series based on lake layer. From this analysis, we found that while both communities undergo similar seasonal changes, the hypolimnion is less variable than the epilimnion in these changes. Figure 8 shows the

same overall trend in relative percent abundance of Type I methanotrophs, demonstrating seasonal dynamics consistent between the epilimnion and hypolimnion. Both layers display a low relative abundance in the fall and spring, with a higher abundance in the summer. This similar seasonal pattern may be seen due to the fact that methanotrophs are generally found around the anoxic/oxic border at the thermocline; thus, there is a possibility that the epilimnion and hypolimnion samples contained water from the opposing layer, thus these results could have been affected by sampling errors and a solid conclusion is not possible in this situation. A notable aspect of this figure, however, is that there is a relatively large peak in methanotrophs in the middle of the summer time series in both layers, which could reflect methane availability. More studies are needed to confirm this, but it is nevertheless an interesting point that deserves further exploration. It is also interesting to note that Type II methanotrophs were barely detected in this analysis, perhaps due to methane levels or other physical and chemical factors.

Overall, this study provided insight into the spatial and temporal dynamics of the bacterial communities within a single freshwater bog lake. The deep hypolimnion in this meromictic lake was less variable than the shallow epilimnion, most likely due to its proximity to the lake surface and thus being more affected by extrinsic factors such as weather events and seasonal climate variation. We also found that these bacterial communities were more variable in space (by depth) than through time, which is reflective of Mary Lakes' permanent stratification, and its inability to mix seasonally. While future studies are needed to specify which physical and chemical factors are the drivers of this variability (and lack thereof), this study is a good starting point, confirming that permanent stratification can lead to stark differences in bacterial communities.

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APPENDIX

Sample List: LakeID_SampleDate_LakeLayer/Depth

Depth Discrete:

MA_24June09_0m
MA_24June09_0.5m
MA_24June09_1.0m_a
MA_24June09_1.0m_b
MA_24June09_1.0m_c
MA_24June09_1.5m
MA_24June09_2.0m
MA_24June09_2.5m
MA_24June09_3.0m
MA_24June09_3.5m
MA_24June09_4.0m_a
MA_24June09_4.0m_b
MA_24June09_4.0m_c
MA_24June09_4.5m
MA_24June09_5.0m
MA_24June09_5.5m
MA_24June09_6.0m_a
MA_24June09_6.0m_b
MA_24June09_6.0m_c
MA_24June09_6.5m
MA_24June09_7.0m
MA_24June09_7.5m
MA_24June09_8.0m
MA_24June09_9.0m_a
MA_24June09_9.0m_b
MA_24June09_9.0m_c
MA_24June09_10.0m
MA_24June09_12.0m
MA_24June09_14.0m_a
MA_24June09_14.0m_b
MA_24June09_17.0m

Integrated:

MA_30May09_E
MA_03June09_E
MA_09June09_E
MA_16June09_E
MA_30June09_E
MA_06July09_E
MA_14July09_E
MA_20July09_E
MA_28July09_E
MA_03Aug09_E
MA_13Aug09_E
MA_19Aug09_E
MA_24Aug09_E
MA_30May09_H
MA_03June09_H
MA_09June09_H
MA_16June09_H
MA_30June09_H
MA_06July09_H
MA_14July09_H
MA_20July09_H
MA_28July09_H
MA_03Aug09_H
MA_13Aug09_H
MA_19Aug09_H
MA_24Aug09_H