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Lineage-Specific Responses of Microbial Communities to Environmental Change

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A great challenge facing microbial ecology is how to define ecologically relevant taxonomic units. To address this challenge, we investigated how changing the definition of operational taxonomic units (OTUs) influences the perception of ecological patterns in microbial communities as they respond to a dramatic environmental change. We used pyrosequenced tags of the bacterial V2 16S rRNA region, as well as clone libraries constructed from the cytochrome oxidase C gene *ccoN*, to provide additional taxonomic resolution for the common freshwater genus *Polynucleobacter*. At the most highly resolved taxonomic scale, we show that distinct genotypes associated with the abundant *Polynucleobacter* lineages exhibit divergent spatial patterns and dramatic changes over time, while the also abundant *Actinobacteria* OTUs are highly coherent. This clearly demonstrates that different bacterial lineages demand different taxonomic definitions to capture ecological patterns. Based on the temporal distribution of highly resolved taxa in the hypolimnion, we demonstrate that change in the population structure of a single genotype can provide additional insight into the mechanisms of community-level responses. These results highlight the importance and feasibility of examining ecological change in microbial communities across taxonomic scales while also providing valuable insight into the ecological characteristics of ecologically coherent groups in this system.

Essential measures of community change assess the relative abundances and distributions of species through space and time (1). This poses difficulty for microbial ecologists, since quantification of community change has been hindered by both methodological limitations of sampling the vast richness of microbial communities and the lack of a species definition that identifies distinctive and unique ecological units (2–4). The sampling challenge may soon be overcome by next-generation sequencing of the conserved 16S rRNA gene (5–8). However, microbiologists are still faced with defining distinct units of diversity that are ecologically comparable to macrobial species.

To define units of diversity, microbial ecologists rely on clustering of 16S rRNA sequences into operational taxonomic units (OTUs). Ecological studies of microbial communities from a diversity of environmental systems ranging from the human microbiome (7) to deep-sea hydrothermal vents (8) typically classify OTUs using a single definition, most commonly where they share >97% sequence identity (9). However, it has become increasingly clear that using different taxonomic designations of OTUs can alter the perception of spatial or temporal community change and that important ecological dynamics are occurring among taxa distinguished at a higher resolution. For instance, changing 16S rRNA OTU designations from a wide variety of habitats uncovered different biogeographical patterns at different taxonomic scales (10). A single sequence similarity cutoff that is too low could falsely lump individuals that may respond differently to environmental stimuli, while a very high sequence similarity cutoff for producing OTUs could split taxa with false boundaries, resulting in the appearance of ecologically redundant taxa. The variation in ecology and evolutionary history of different lineages will produce ecological distinction at different taxonomic resolutions, which means that choosing a single OTU definition for any study will influence results in ways that cannot be anticipated *a priori*.

Examining the ways in which microbial communities respond to environmental change at different taxonomic scales will provide insight into the level of divergence at which microbial taxa are ecologically distinct or redundant (11). In particular, a dramatic environmental change, such as ecological disturbance, provides a useful tool to examine microbial response to environmental change (12). Similar responses to disturbance indicate ecological coherence and possible redundancy, while divergent responses indicate distinctiveness among taxa. This approach may provide insight for yet-uncultivable microorganisms, especially those for which we have limited understanding of their ecological roles or metabolic capabilities.

Freshwater lakes have become model systems for studying the effects of disturbance on microbial communities (13–15). It has been demonstrated that overturn, or mixing, of the stratified water column due to seasonal changes in temperature (14–17) or sporadic disturbances due to storm activity (13) overturns the stratified water column and creates a dramatic disturbance for the microbial community. Water column mixing destroys the physical, chemical, and biological gradients present when the water column is stratified into two thermal layers (18).

The microbial communities in freshwater lakes are complex and diverse but tend to be dominated by ubiquitous and relatively uncharacterized groups of *Actinobacteria* and the *Betaproteobacteria* genus *Polynucleobacter*. These two groups appear to occupy

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different niches in lakes (19) and exhibit sharply contrasting seasonal dynamics (20, 21). Both groups are known to circumscribe distinct clades with unique distribution patterns (22, 23). The differences in levels of microdiversity within these groups or in the ways that these groups respond to ecological disturbance have not been previously examined.

Here, we focus on an experimental whole-ecosystem disturbance in which a stratified water column of a dystrophic temperate lake was mixed mechanically (24). In a previous study on this system, the 16S rRNA gene was used to profile bacterial community composition with OTU designations at 97% 16S identity and phylotyping (i.e., using a sequence data set with a predefined taxonomy for classifying sequences) (25). These community-level analyses revealed changes in richness, taxonomic composition, and relative abundance of taxa before, during, and after the mixing event. The communities were ultimately resilient, recovering within 20 days. Here, we asked how altering the taxonomic resolution applied to the sequence data set uncovers new ecological patterns of microbial response to this dramatic whole-ecosystem disturbance focusing on comparisons between the two most prevalent lineages of *Actinobacteria* and *Polynucleobacter*.

MATERIALS AND METHODS

Artificial lake mixing. From 3 July to 10 July 2008, the highly stratified North Sparling Bog was mechanically mixed through buoyant forces (24). The changes in chemical conditions and aggregate measures of the bacterial community over the course of artificial lake mixing are described in detail elsewhere (25). In brief, the water column was destratified over the course of 8 days, changing the hypolimnion temperature from 6°C to 20°C. This large, rapid increase in temperature is assumed to be unprecedented in the entire history of the lake, and the temperature remained elevated until the water column became isothermal in early October 2008. The epilimnion cooled from 24.2°C to 20.3°C and returned to normal lake conditions 5 days after the artificial mixing event on 10 July. The dissolved oxygen (DO) concentration in the hypolimnion changed from below detection to near 3 mg/liter, and the epilimnion decreased from 7.0 to 3.2 mg/liter. DO concentrations within the hypolimnion returned to below detection 3 days after mixing stopped, while the DO in the epilimnion did not rebound until 15 July. Destratification altered concentrations of iron, sulfur, nitrogen, carbon dioxide, and methane within the water column, all of which rebounded within 20 days of mixing (25).

Twelve samples were collected from two water layers, the epilimnion (0 m) and hypolimnion (4 m), over six time points through the course of the disturbance experiment: before the mixing treatment began (−9), immediately after destratification was achieved (mix), and then at four postmixing time points at 3, 7, 11, and 20 days (25). Water samples were collected on 0.2-μm Supor-200 nylon membrane filters (Pall Life Sciences, Ann Arbor, MI) using a peristaltic pump that was rinsed between samples.

Amplification and sequencing. DNA extraction from the filters was performed with the FastPrep biogene kit (MP Biomedicals, Solon, OH). Pyrosequencing was performed using previously described methods (7, 25). Briefly, the V2 region of the 16S rRNA gene was amplified by PCR using the primers 27F and 338R with attached multiplex, error-correcting barcodes (26). Pyrosequencing was conducted using primer A on a 454 Life Science Genome Sequencer FLX instrument (Roche).

In order to resolve the genetic diversity of the genus *Polynucleobacter* beyond the 16S rRNA level, we used the protein-encoding gene cytochrome *c* oxidase, cbb3 type, subunit I (*ccoN*). DNA was extracted from filtered integrated water column samples using the Mo Bio UltraClean fecal DNA kit (Mo Bio Laboratories Inc., Carlsbad, CA). The primers Pnuc0453F (5′-CAGYCAATTTGCCATCGTTAC-3′) and Pnuc0453R (5′-GTCATGATGCCGTTGATC-3′) were designed using the genome sequence of *Polynucleobacter necessarius* subsp. *necessarius* STIR1 (NC_010531) and *Polynucleobacter necessarius* subsp. *asymbioticus* QLW-P1DMWA-1 (NC_009379). The gene fragment was amplified using a PCR with a final volume of 30 μl containing the final concentrations of 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate (dNTPs) (each), 0.4 μM primers (each), and 0.05 U of Phusion high-fidelity DNA polymerase F-530 (Finnzymes, MA). Thermocycler conditions consisted of an initial denaturation for 3 min at 98°C, followed by 30 cycles of 30 s at 98°C, 25 s at 58.1°C, and 25 s at 72°C, with a final extension of 10 min at 72°C. PCR products were cloned using the Zero Blunt TOPO PCR for Sequencing kit following the standard kit protocol (Invitrogen, Carlsbad, CA). All clones were submitted to the WM Keck Center for Comparative and Functional Genomics at the University of Illinois for Sanger sequencing of both ends of the vector insert.

Sequence analysis. Pyrosequencing reads were quality controlled based on quality scores, sequence length, primer mismatches, and length of homopolymers using QIIME version 1.1.0 (27). The filtered sequence data set was aligned with a Needleman-Wunsch pairwise alignment against a reconstruction of the SILVA SEED database as implemented in the software Mothur version 1.15.0 (28). A random subsample of 1,217 total reads for each sample was selected for further analysis in order to equalize the number of quality reads in each sample. No chimeras were found using the chimera.slayer command in Mothur version 1.19.0 with the SILVA Gold reference data set (29). We identified <0.5% of the 16S rRNA data set to be potential chimeras with Perseus (as implemented in Mothur), a database-independent method to detect chimeras (30). Both the *ccoN* and 16S rRNA data sets were hierarchically clustered in Mothur using the average-neighbor clustering algorithm. Sequence identity cutoffs for defining OTUs were chosen by comparing the diversity among all samples using the Bray-Curtis index (i.e., temporal and spatial beta diversity) and then comparing how these Bray-Curtis values changed based on the cutoff used, with the largest changes between cutoffs chosen as the taxonomic levels for our analyses (see Methods, Fig. S1, and Table S1 in the supplemental material). Only OTUs with ≥50 reads in the data set were used for all analyses (except number of OTUs in Fig. 4) in order to reduce the number of zeros (i.e., absences) in the data set, which can cause false correlations. This cutoff was chosen as a compromise between the number of absences in the data set and the number of OTUs (quantified at the 99.5% cutoff) removed from the data set (see Fig. S2 in the supplemental material). The number of OTUs assigned to each taxonomic level is listed in Table S2 in the supplemental material.

16S rRNA reads were classified with the classify.seqs command in Mothur, which utilized a naïve Bayesian classifier based on the reconstructed SILVA SEED database with SILVA classifications as a training data set for classifying sequences from the phylum to genus level. OTUs were assigned a taxonomic classification where 95% of an OTU's reads could be assigned to one classification. Otherwise, OTUs were labeled with an asterisk if they contained a mixture of classifications or were not labeled if the majority of reads could not be classified with confidence (bootstrap of >50). A detailed comparison of overlap between each taxonomic classification and OTU shows that the groups most often corresponded (see Fig. S3 in the supplemental material), although some were inconsistent (usually classifications were split into multiple hierarchical clusters). In addition, OTUs were classified using the new freshwater taxonomy (10). Figures based on this classification scheme are shown in Fig. S4 and S5 in the supplemental material.

ccoN sequences were assembled into contiguous fragments and manually checked for sequencing errors using Sequencher version 4.7 (Gene Codes Corporation, Ann Arbor, MI). The resulting 351 sequences had an average length of 788 nucleotides and were manually aligned with MacClade version 4.08 (31).

Accounting for possible PCR and sequencing errors. We took care to ensure that sequence processing did not bias our results at the finest level of taxonomic resolution (99.5%). We found very few potential chimeras in the 16S rRNA data set with Chimera Slayer and Perseus (implemented

in Mothur) (28, 29). Furthermore, we did not perform any analyses that relied on rare divergent subtypes. We found 87 of 95 OTUs to be present in multiple samples, which suggested little to no signal of chimeras in the data set if they occurred. A second concern is errors introduced into the sequence by DNA polymerase that may be amplified depending upon when they occurred. The 99.5% cutoff allowed for ~1 single nucleotide polymorphism (SNP) error since the median fragment length of the 16S rRNA gene data set was 202 nucleotides (nt) (minimum, 166 nt; maximum, 227 nt), and the average-neighbor clustering algorithm employed has been shown to more accurately produce clusters that are reflective of genetic distance than other clustering algorithms. This algorithm reduced the number of singleton or doubleton taxa that varied from other OTUs by 1 SNP to <1% of OTUs (32). Thus, any erroneous OTUs should be aggregated with a true OTU. Also, PCR errors should occur randomly among samples, but most OTUs were present in multiple samples, which suggests little to no influence of PCR error in their distribution. Finally, the majority of our analyses focused on change in abundance among taxa and relied on an abundance cutoff of ≥50 total reads in the data set, which greatly reduced the prevalence of less abundant and potentially erroneous reads.

Two additional types of errors result from the sequencing process. The first occurs from errors, again by the DNA polymerase, but this time during the bead amplification. This is unlikely to influence our results, again due to the clustering algorithm and abundance cutoffs used throughout our analysis. The second results from changes in abundance due to pseudoamplification of sequences during the bead amplification process (33). Sanger sequencing of a different phylogenetic marker (*ccoN*) confirmed that changes in dynamics of a single lineage did not result from pseudoamplification.

Statistics. Patterns of change in abundance among taxa were compared using Spearman partial rank-order correlations while conditioning for depth (0 m or 4 m) or time (rank-order of time points). Significance values for correlations were adjusted using the false discovery rate (Q value) to account for multiple hypothesis testing (34). We defined strong, significant correlations as a $|\rho|$ value of >0.6 and a Q value of <0.05 for comparing temporal and spatial changes of taxon abundance. Partial correlations were calculated using the Stats packages in R. Correlations in abundance among taxa were visualized with Cytoscape (35). Collector's curves were produced based on the observed richness with the collect.single command in Mothur. The scripts used to compare the hierarchical clustering and phylotyping methods at each taxonomic scale (see Fig. S3 and S5 in the supplemental material) are available at http://www.life.illinois.edu/whitaker/.

Heat maps, bar plots, and line plots were created in R with the Heatplus, APE, and ggplot2 packages (36–38).

Nucleotide sequence accession numbers. All 77 unique *ccoN* gene sequence types are available at GenBank under accession numbers JX944304 to JX944380.

RESULTS

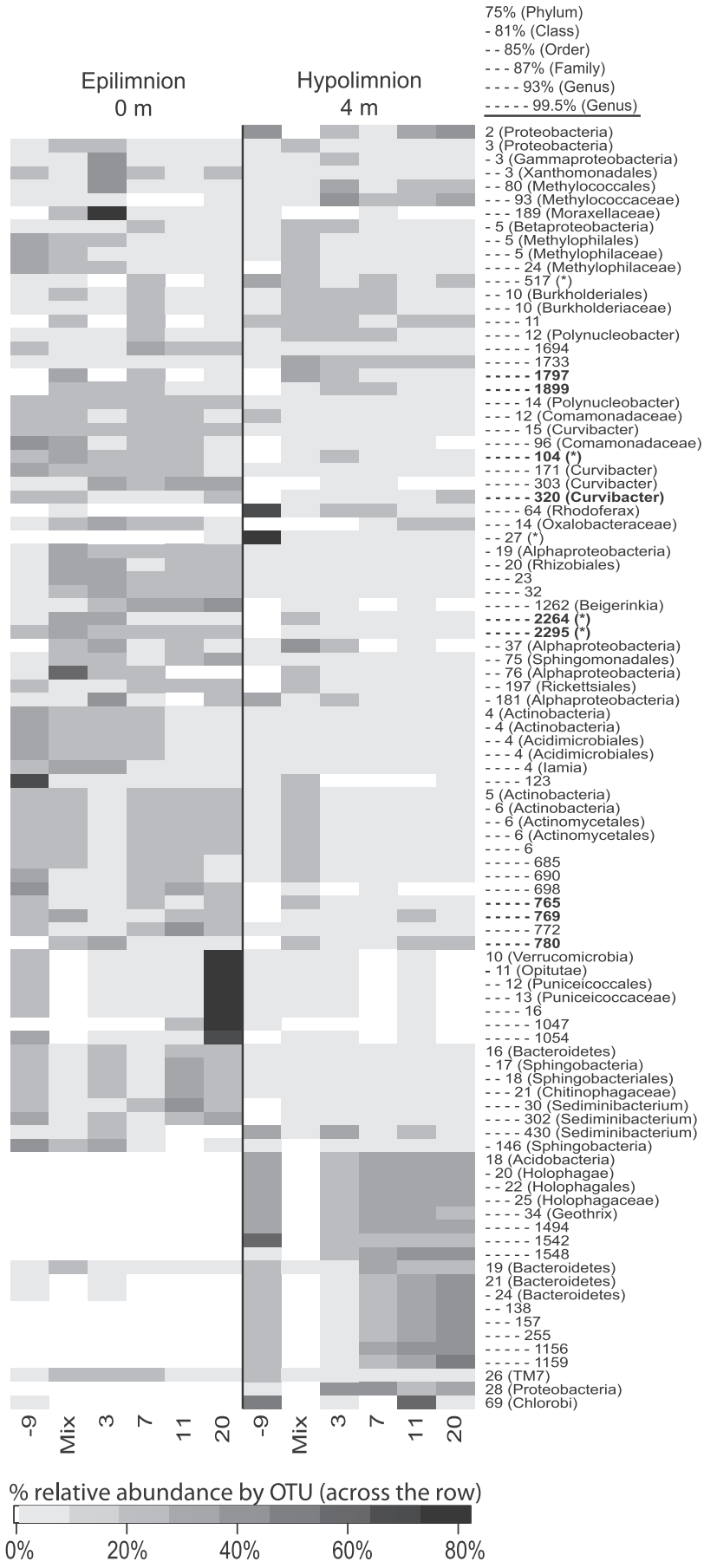
Ecological patterns vary across taxonomic scales. The relative abundance of taxa defined at increasingly stringent OTU definitions (from 75% to 99.5%) were calculated for 12 samples that differ in space (epilimnion and hypolimnion) and time (six different times) throughout the whole lake mixing experiment. Collector's curves produced from OTUs defined at each cutoff (either all samples or each time point separately) showed complete or nearly complete sampling of the standing diversity except at the 99.5% cutoff (data not shown). Figure 1 shows a qualitative assessment of the change in relative abundance of taxa when they are binned at different taxonomic scales. Throughout the text, we will refer to OTUs using numerical codes derived from the hierarchies on the right side of Fig. 1. For example, OTU 3.3.3.80.189 was assigned to *Moraxellaceae* within the *Gammaproteobacteria*, while OTU 3.3 represents all reads classified as *Gammaproteobacteria*. As parental OTUs were split into daughter OTUs, new patterns emerged (Fig. 1) in some lineages. For example, the sister taxa OTU 3.3.3.80.189 and 3.3.3.80.93 in the *Gammaproteobacteria*, defined by 87% sequence divergence, showed different spatial distributions, being found predominantly in the epilimnion and the hypolimnion, respectively. As shown in Fig. 1, while most sister taxa show similar responses to mixing disturbance, differences occurred in both space and time and at all taxonomic levels. For example, OTU 3.3 (*Gammaproteobacteria*) differed from OTU 3.5 (*Betaproteobacteria*) in their temporal change in abundance across the time course of the mixing experiment.

The extent of difference in patterns between sister taxa was quantified using change in rank order of taxon abundances (number of reads) between samples (Fig. 2; see also Fig. S6 in the supplemental material). Overall, a total of 34.9% of sister taxa displayed strong positive correlations ($\rho \geq 0.6$, $Q < 0.05$), while 13.7% showed strong negative correlations ($\rho \leq -0.6$, $Q < 0.05$) when ranked for abundance in the 12 samples from six time points in two water layers. As shown in Fig. 2, strong positive correlations and negative correlations occurred at most taxonomic scales (75%, 85%, 93%, and 99.5%). The 93% cutoff showed the greatest number of strong negative correlations among sister OTUs, particularly in the *Betaproteobacteria* (Fig. 2, Table 1).

The highest resolution of 99.5% cutoff showed the largest number of strong positive correlations among taxa when controlling for depth or time, which suggested similar ecological preferences among groups resolved at this scale (Fig. 2). The majority of these strong positive correlations originated from the OTU 5 lineage, which was composed of only one abundant OTU at each cutoff until OTU 5.6.6.6.6 split at the 99.5% cutoff into 7 abundant and often temporally or spatially correlated OTUs (see Fig. S6 in the supplemental material). In contrast, some taxa exhibited ecological distinction at the finest taxonomic scale. For example, the two most abundant OTUs in the data set, OTUs 3.5.10.10.12.1733 and 3.5.10.10.12.1694, which are assigned to the PnecC subcluster within the *Polynucleobacter* genus (19, 39), displayed a negative correlation when controlling for time, suggesting a preference for specific water layers (Fig. 2, Table 1). These two *Polynucleobacter* OTUs are within the OTU 3 lineage, in which many of the negative correlations among sister taxa occurred, suggesting that ecological distinction is prevalent throughout the lineage's hierarchy (Table 1).

Further resolving the distinction between *Polynucleobacter* genotypes. We further investigated the differences between closely related taxa observed in the dominant *Polynucleobacter*-affiliated OTU 3.5.10.10.12. To do this, we used a heat map to view the change in relative abundance of reads within this OTU over the course of the mixing experiment and found a population dominated by two relatively highly abundant OTUs, with each segregated in different water layers (Fig. 3A). Both OTUs were highly abundant in both layers during the artificial mixing of the water column but quickly rebounded to the relative abundances in each layer seen prior to mixing. These prevalent OTUs differed by one nucleotide out of 202 (~99.5% identical), providing an example of ecological differentiation occurring at a particularly fine scale.

To test whether we could further resolve these two prevalent *Polynucleobacter* OTUs beyond the single nucleotide change in the 16S rRNA marker, we compared the rRNA patterns to those of


Epilimnion
0 m
Hypolimnion
4 m
75% (Phylum)
- 81% (Class)
- - 85% (Order)
- - - 87% (Family)
- - - - 93% (Genus)
- - - - - 99.5% (Genus)
2 (Proteobacteria)
3 (Proteobacteria)
- 3 (Gammaproteobacteria)
- - 3 (Xanthomonadales)
- - 80 (Methylococcales)
- - - 93 (Methylococcaceae)
- - - 189 (Moraxellaceae)
- 5 (Betaproteobacteria)
- - 5 (Methylophilales)
- - - 5 (Methylophilaceae)
- - - - 24 (Methylophilaceae)
- - - - 517 (*)
- - 10 (Burkholderiales)
- - - 10 (Burkholderiaceae)
- - - - 11
- - - - 12 (Polynucleobacter)
- - - - - 1694
- - - - - 1733
- - - - - 1797
- - - - - 1899
- - - - 14 (Polynucleobacter)
- - - 12 (Comamonadaceae)
- - - - 15 (Curvibacter)
- - - - - 96 (Comamonadaceae)
- - - - - 104 (*)
- - - - - 171 (Curvibacter)
- - - - - 303 (Curvibacter)
- - - - - 320 (Curvibacter)
- - - - 64 (Rhodoferax)
- - - 14 (Oxalobacteraceae)
- - 27 (*)
- 19 (Alphaproteobacteria)
- - 20 (Rhizobiales)
- - - 23
- - - - 32
- - - - - 1262 (Beigerinkia)
- - - - - 2264 (*)
- - - - - 2295 (*)
- - 37 (Alphaproteobacteria)
- - 75 (Sphingomonadales)
- - 76 (Alphaproteobacteria)
- - 197 (Rickettsiales)
- 181 (Alphaproteobacteria)
4 (Actinobacteria)
- 4 (Actinobacteria)
- - 4 (Acidimicrobiales)
- - - 4 (Acidimicrobiales)
- - - - 4 (Iamia)
- - - - 123
5 (Actinobacteria)
- 6 (Actinobacteria)
- - 6 (Actinomycetales)
- - - 6 (Actinomycetales)
- - - - 6
- - - - - 685
- - - - - 690
- - - - - 698
- - - - - 765
- - - - - 769
- - - - - 772
- - - - - 780
10 (Verrucomicrobia)
- 11 (Opitutae)
- - 12 (Puniceicoccales)
- - - 13 (Puniceicoccaceae)
- - - - 16
- - - - - 1047
- - - - - 1054
16 (Bacteroidetes)
- 17 (Sphingobacteria)
- - 18 (Sphingobacteriales)
- - - 21 (Chitinophagaceae)
- - - - 30 (Sediminibacterium)
- - - - 302 (Sediminibacterium)
- - - - 430 (Sediminibacterium)
- 146 (Sphingobacteria)
18 (Acidobacteria)
- 20 (Holophagae)
- - 22 (Holophagales)
- - - 25 (Holophagaceae)
- - - - 34 (Geothrix)
- - - - - 1494
- - - - - 1542
- - - - - 1548
19 (Bacteroidetes)
21 (Bacteroidetes)
- 24 (Bacteroidetes)
- - 138
- - - 157
- - - - 255
- - - - - 1156
- - - - - 1159
26 (TM7)
28 (Proteobacteria)
69 (Chlorobi)
-9
Mix
3
7
11
20
-9
Mix
3
7
11
20
% relative abundance by OTU (across the row)
0%
20%
40%
60%
80%

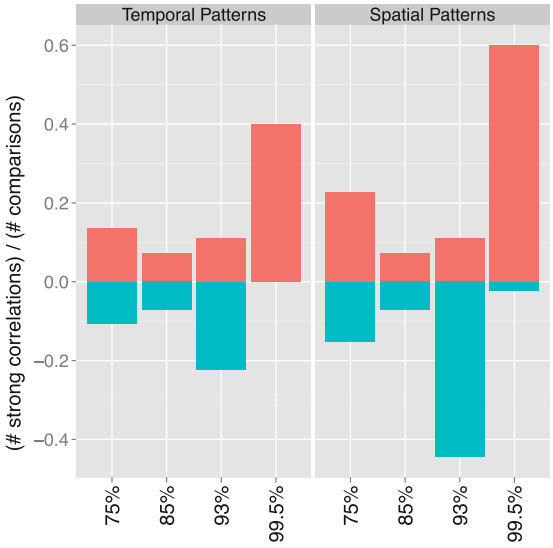


FIG 2 Summarizing correlations of ecological preference among sister taxa. Correlations of change in abundance of sister OTUs when controlling for depth (temporal patterns) or time (spatial patterns) were summed at each cutoff and represented in the bar graph as the number of strong correlations among sister OTUs normalized by the total number of comparisons made among sister OTUs. Negative and positive correlations are shown in red and blue, respectively. The 81% and 87% cutoffs are not shown because no strong correlations were found. See Fig. S6 in the supplemental material for a network diagram displaying correlations among OTUs.

protein-encoding marker *ccoN*, which encodes a fragment of cytochrome *c* oxidase, cbb3 type, subunit I (*ccoN*), specifically found within the genus *Polynucleobacter*. We obtained a total of 351 partial gene sequences from the surface epilimnion population. The relative abundances of each unique sequence observed in the epilimnion of each sample were visualized with a heat map and compared to the 16S rRNA heat map (Fig. 3B). The alternative phylogenetic marker *ccoN* resolved a pattern of succession similar to that observed in Fig. 3A, demonstrating robust patterns across these two different markers. There was a single dominant genotype in the epilimnion before mixing. As seen using the 16S rRNA marker, a new abundant genotype was introduced at mixing that we infer is linked to the prevalent 16S rRNA genotype from the hypolimnion. Following the mixing event, this second genotype decreased in frequency over time as the relative abundances of both types return to their predisturbance distribution. The *ccoN* gene was chosen as a marker for differentiating populations, and so it is unlikely that sequence variation between the genotypes contributed to their ecological differentiation. In agreement with this assumption, we found the *ccoN* fragment for the two dominant genotypes to differ by 7 amino acids (aa) (262 aa total), which were all confined to the middle of the gene, where no functional domains are present according to the NCBI Conserved Domain Database (40).

Genotype specificity of response to lake mixing. One of the most striking changes observed previously in the microbial community in response to mixing was a reduction in phylogenetic diversity and number of OTUs observed in the hypolimnion on the day of mixing (day mix) (Fig. 4 and reference 25). Our analysis of highly resolved taxa (see above) shows that this drop in richness co-occurs with a dramatic increase in a single *Polynucleobacter*-affiliated OTU (3.5.10.10.12.1733) that we identified previously as specific to the hypolimnion. This OTU increased by more than 2-fold (163 to 381 16S rRNA reads) from day −9 to day mix, which corresponded to the *Proteobacteria* OTU 3 increasing from 49% of the total sample reads at day −9 to 67% at day mix. The increase in number of sequences affiliated with this one resolved taxon dramatically decreased the observed phylogenetic diversity and richness of this community.

Highly resolved taxa show differences in response to disturbance in the hypolimnion. Previous work had reported that the bacterial communities in this experiment were not resistant to disturbance but were resilient over the course of 20 days (25). We quantified how splitting OTUs into highly resolved taxa altered assessments of community resistance (from the predisturbance time point, day −9) and resilience (return to predisturbance) in terms of their presence and absence profiles in both water layers through time (Tables 2 and 3). This is a slightly different definition of resistance and resilience than used in the community-level analyses of Shade et al. (25), which assessed changes in relative abundances in addition to presence and absence at a single taxonomic scale. We tested for a bias toward an increased number of absences for OTUs with lower total abundances, which may influence our measurements of resistance and resilience, especially when subdividing OTUs at increasingly fine taxonomic resolutions. We found a weak correlation between OTU abundance and number of absences (Spearman, $\rho = -0.42$, $P = 0.02$), indicating that our measurements of resistance and resilience were not substantially influenced by such a bias. In addition, the number of absences decreased minimally when increasing our OTU abundance cutoff beyond ≥50 reads, suggesting that we have mitigated most spurious absences caused by low OTU abundance (see Fig. S2 in the supplemental material).

Using these definitions of resistance and resilience, the majority of taxa were resistant to mixing disturbance when binned at the 75%, 81%, 85%, and 87% levels (pattern A), although the community profile of "not resistant but resilient" (pattern B) was seen for a minority of individual taxa. One striking difference between the patterns of community response to mixing was identified in the hypolimnion, where we observed that 42% of the 99.5%-resolved taxa displayed a pattern of "introduced at mix" (pattern E). These OTUs were not categorized as resistant because they were not observed before mixing and also were not resilient because they did not return to undetectable levels after 20 days (Fig. 1, bold labels). All but three (out of 9) of these OTUs were detected in the epilimnion prior to mixing.

FIG 1 Divergent ecological patterns between parent and daughter taxa. The heat map shows relative abundances (number of 16S rRNA reads) of each OTU defined at a 75%, 81%, 85%, 87%, 93%, or 99.5% sequence identity cutoff. Relative abundances are normalized by OTU to show relative change in abundance of each OTU over time and depth. OTUs were assigned a taxonomic classification if 95% of the reads in an OTU were classified identically or labeled with an asterisk if mixed. OTUs were left unlabeled if the reads they contained could not be classified with confidence to the specified taxonomic resolution. Bold labels highlight OTUs defined at the 99.5% cutoff that show the "opportunistic starting at mix" pattern as shown in Table 1. A similar figure using the freshwater classifications from Newton et al. (19) is shown in Fig. S4 in the supplemental material.

TABLE 1 OTUs within each lineage that showed a strong negative correlation[a]

Cutoff (%)	Pattern	OTU identifier	Classification
85	Temporal	3.5.27 and 3.5.5	*Betaproteobacteria*
93	Temporal	3.5.10.10.11 and 3.5.10.10.14	*Polynucleobacter*
93	Temporal	16.17.18.21.30 and 16.17.18.21.430	*Sediminibacterium*
85	Spatial	3.5.27 and 3.5.5	*Betaproteobacteria*
93	Spatial	3.5.10.10.11 and 3.5.10.10.14	*Polynucleobacter*
93	Spatial	3.5.10.12.15 and 3.5.10.12.64	*Comamonadaceae*
93	Spatial	3.5.5.5.24 and 3.5.5.5.517	*Methylophilaceae*
93	Spatial	16.17.18.21.30 and 16.17.18.21.430	*Sediminibacterium*
99.5	Spatial	3.5.10.10.12.1694 and 3.5.10.10.12.1733	*Polynucleobacter*

[a] The table lists the OTUs in each lineage (i.e., 75% cutoff OTUs) that show strong negative correlations (as summarized in Fig. 2, blue bars). See Fig. 1 for the OTU classification methodology. See Fig. S6 in the supplemental material for a network diagram displaying correlations among OTUs.

DISCUSSION

We have shown that assessing microbial community sequence diversity through time and space across different taxonomic scales uncovers novel patterns of response to environmental change. In addition, we have shown that there are differences in the ecological coherence (i.e., similar patterns in space and time) at different taxonomic scales between abundant *Actinobacteria* and *Polynucleobacter* OTUs in this freshwater community (Fig. 2 and Table 1; see also Figure S6 in the supplemental material) in ways that support the ecological distinction between these two abundant and ubiquitous but uncharacterized groups of microorganisms. These results demonstrate the importance of varying taxonomic resolution in culture-independent studies of microbial communities in order to identify ecologically distinct units that correspond to environmental change.

When split at the 99.5% sequence identity cutoff, most OTUs demonstrated a coherent response to environmental change. In particular, OTUs such as 5.6.6.6.6 (*Actinobacteria*) resolved a large number of daughter OTUs (seven) with significant positive correlations in their stable abundance patterns through space and time (all except for OTU 5.6.6.6.6.780) (see Fig. S6 in the supplemental material). This suggested that at least in response to this disturbance, these OTUs were highly redundant. Previous work has shown that the *Actinobacteria* have stable populations through resistance to desiccation, predation, and dramatic variations in organic carbon load; therefore, this disturbance may not present ecological parameters that differentiate these closely related lineages if they exist (20, 41, 42). Such high levels of coherence between finely resolved taxa have been seen in plant and animal data sets and were suggested to result from low competition relative to environmental variables (43). This apparent redundancy in ecological responses at the finest taxonomic resolution may indicate neutral divergences within most OTUs at this taxonomic level (44). Alternatively, closely related, nonredundant taxa may covary because they are interdependent or because each covaries with independent environmental stimuli that change in the same way in response to disturbance. It is important to note that ecological redundancy among taxa observed in this study may not hold for other changes in environmental stimuli, and further work with different disturbances in a diversity of systems will help to understand the robustness of this pattern.

In contrast to the majority of taxa, OTU 3.5.10.10.12 (assigned to the *Polynucleobacter* genus within the *Proteobacteria* phylum) had an overall abundance very similar to OTU 5.6.6.6.6 but contained only four daughter OTUs at the 99.5% cutoff. Two of these OTUs were prevalent and were found in opposite thermal layers, indicating that in our experimental system, this OTU was comprised of two nonredundant taxa. This niche diversification may have resulted from strong competitive interactions that drive ecological differentiation within this group. Recently, a study found

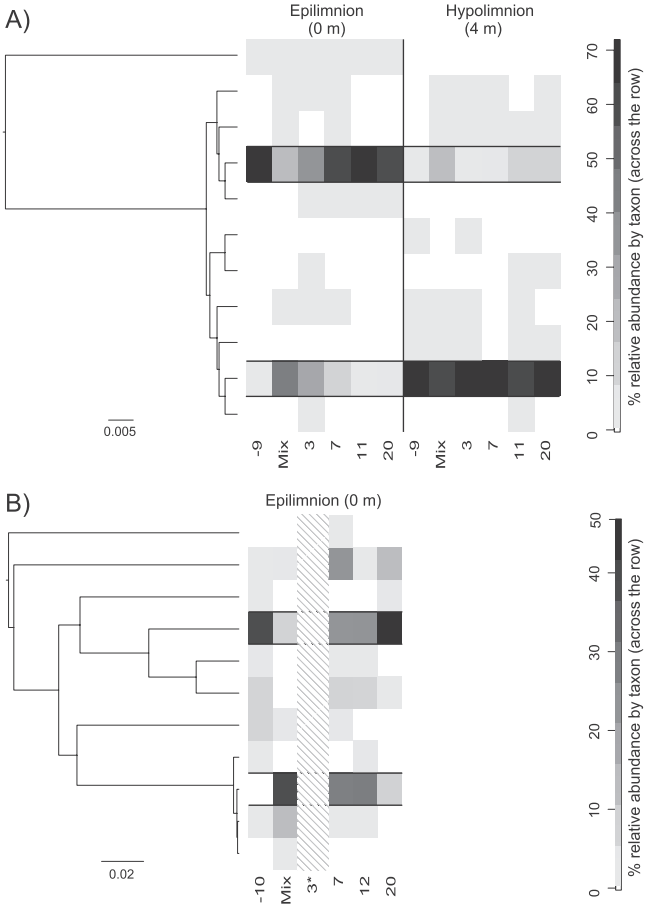


FIG 3 Assessing ecological differences among highly related genotypes of *Polynucleobacter* using two phylogenetic markers. Each heat map and dendrogram depicts the relative abundance of each dereplicated sequence (genotype) over time and the genetic similarity among genotypes using the V2 16S rRNA pyrosequencing data (A) and the protein-encoding phylogenetic marker (*ccoN*) (B). Abundances are relative to the total number of 16S rRNA sequences in each sample. Genotypes represent unique 16S rRNA sequences that were classified as belonging to the *Polynucleobacter* genus (A) and unique *ccoN* sequences amplified with primers specific for the *Polynucleobacter* genus (B). The two dominant genotypes in the 0-m and 4-m depths are outlined in each heat map. Asterisks and striped boxes signify that a *ccoN* clone library was not constructed for day 3 at 0 m.

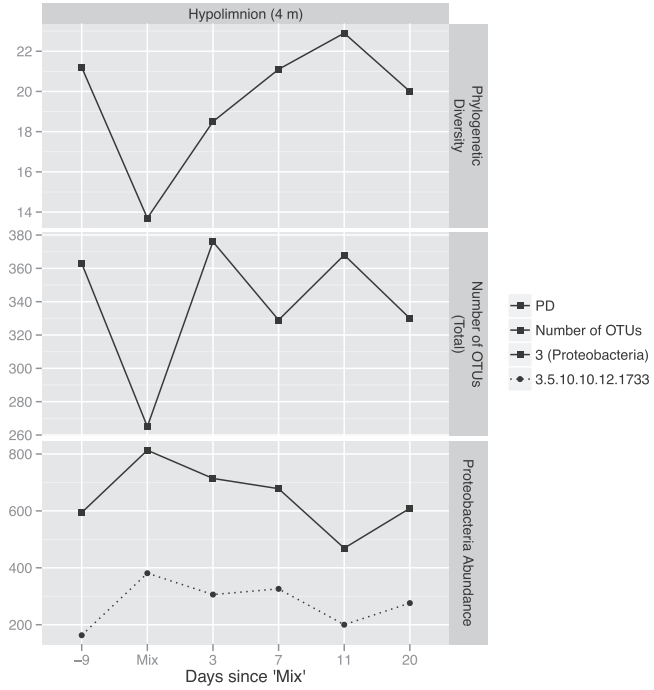


FIG 4 A bloom of one highly resolved OTU corresponds to a drop in community diversity. The top and middle plots show change in phylogenetic diversity and richness (number of OTUs) in the hypolimnion, with OTUs defined at the 99.5% sequence identity cutoff used for both analyses (comparable to Shade et al. [25]; Fig. 3). The lower plot shows relative abundance (number of reads) of OTU 3.5.10.10.12.1733, the most abundant OTU defined at the 99.5% cutoff, and OTU 3, the most abundant OTU in the data set.

niche differentiation among *Polynucleobacter* PnecC OTUs that were >99% identical by 16S rRNA. This specialization occurred along gradients of pH, DOC, and other important parameters spanning a large number of freshwater habitats (45). In the same study, there were strains that preferred either oxic or anoxic layers of a lake water column, suggesting that *Polynucleobacter* taxa are commonly ecologically distinct in other lake ecosystems as well as in our humic lake. Again, it is important to note that the divergence among the *Polynucleobacter* OTUs was in response to this particular disturbance. Alternative scenarios of environmental change may cause divergence between other taxa, while potentially not producing the divergence between the *Polynucleobacter* strains observed in this study.

The dynamics of highly resolved taxa can provide additional insight into the possible mechanisms of community change. Previous work found that there was a decrease in hypolimnion richness during the mixing (25) and attributed this decrease to a reduction in the abundances of strict anaerobic taxa when oxygen was introduced (25). Here, we additionally found that an increase in a single *Polynucleobacter* lineage, OTU 3.5.10.19.12.1733, appeared to contribute to the richness decrease. Although we cannot quantify the extent to which the two mechanisms contributed to the measured decrease, the rapid change in abundance of this *Polynucleobacter* OTU during the mixing is consistent with the opportunistic lifestyle of *Polynucleobacter* lineages, which have been shown to be highly susceptible to specific changes in grazing, nutrient source and concentrations, and oxygen requirements

TABLE 2 Pattern key for Table 3[a]

Pattern (letter in Table 3)	Presence/absence pattern key					
	−9	Mix	3	7	11	20
Resistant (A)	X	X	X	X	X	X
Not resistant but resilient (B)	X		X	X	X	X
Not resistant, not resilient (C)	X					
Transient (D)		X				
Opportunistic starting at mix (E)		X	X	X	X	X
Opportunistic after mix (F)			X	X	X	X
Sporadic (G)	X		X		X	

[a] Response patterns to the lake mixing disturbance were defined by presence (X) or absence (no X) before mixing, immediately after mixing, or the following postmix time points. The "sporadic" pattern is defined as taxa that were present only in some of the later postmix time points (days 3, 7, 11, and 20). The "not resistant, not resilient" pattern is defined as taxa that were present at day −9 but not present in any postmix time points. See Table 3 for pattern quantification at each taxonomic level.

(19). There is also evidence that *Polynucleobacter* lineages can be very dynamic over relatively short temporal windows. For example, in a similarly dystrophic lake, the PnecC subcluster of *Polynucleobacter* changed from as low as ~5% to as high as ~60% of the total bacteria abundance in the span of days or weeks (46).

We found some OTU-level exceptions to the community-level overall pattern of resilience when defining OTUs at the 99.5% sequence identity cutoff. Only at this taxonomic resolution was there a large number (42%) of OTUs to be absent before mixing began but were found continuously afterward (Table 2). Most of these OTUs were detected in the epilimnion prior to mixing, which suggests that these OTUs were introduced during mixing and then persisted in the lower water layer. Persistence of these OTUs may have been due to increased temperature or other environmental variables that were different pre- and postmix in the hypolimnion but was not correlated with a change in oxygen concentration, which quickly returned to premixing levels (25).

Although several new patterns may be resolved at finer taxonomic resolutions, many studies do not assess dynamics of taxa at a fine scale because of perceived problems associated with se-

TABLE 3 Patterns of resistance and resilience at different taxonomic resolutions

Water layer	Level/cutoff (%)	% of OTUs in the community[a]						
		A	B	C	D	E	F	G
Epilimnion (0 m)	75	67	11	11	0	0	0	11
	81	58	8	8	0	0	0	25
	85	61	6	6	0	6	0	22
	87	52	5	5	0	14	0	24
	93	48	4	8	4	8	0	28
	99.5	69	3	0	3	7	0	17
Hypolimnion (4 m)	75	50	33	0	0	0	0	17
	81	53	33	0	0	0	0	13
	85	55	18	0	0	9	5	14
	87	52	16	0	0	8	4	20
	93	45	17	0	0	7	3	17
	99.5	22	17	0	0	42	0	19

[a] A, resistant; B, not resistant but resilient; C, not resistant, not resilient; D, transient; E, opportunistic starting at mix; F, opportunistic after mix; E, sporadic. See Table 2 for pattern descriptions.

quencing error (e.g., reference 47). We demonstrated coherence of patterns detected using 454-tag pyrosequencing of the V2 region of the 16S rRNA gene and a significantly more divergent marker (*ccoN*), suggesting that for *Polynucleobacter*, the 16S rRNA gene is sufficient for understanding dynamics at a more resolved taxonomic level. The same may be true for other genera and other protein-encoding markers and should be explored for taxa of interest in other environments.

16S rRNA pyrosequencing provides the ability to deeply sample diverse microbial communities. Through analysis of change in relative abundance among taxa resolved at various taxonomic scales, these data sets can be used to identify ecologically relevant units of diversity in various environmental and experimental systems. Identifying such units will improve our understanding of how particular lineages and the entire community respond to environmental change. Our study clearly demonstrates that defining ecologically relevant taxonomic units can provide novel perspective to microbial dynamics that are otherwise unobserved in a community-level analysis. While closely related sequences are commonly assumed to be redundant evolutionary variants, we show that ecological changes occur at this scale.

In conclusion, this work has provided a nonarbitrary method for defining ecologically relevant taxonomic units informed by disturbance responses, has clearly demonstrated that different bacterial lineages may demand different taxonomic unit definitions, and has shown that redefining ecologically relevant taxonomic units brings novel perspective to microbial dynamics that are otherwise unobserved in a community-level analysis.

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