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## Interannual dynamics and phenology of bacterial communities in a eutrophic lake

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### *Abstract*

We investigated patterns of intra- and interannual change in pelagic bacterial community composition (BCC, assessed using automated ribosomal intergenic spacer analysis) over six years in eutrophic Lake Mendota, Wisconsin. A regular phenology was repeated across years, implying that freshwater bacterial communities are more predictable in their dynamics than previously thought. Seasonal events, such as water column mixing and trends in water temperature, were most strongly related to BCC variation. Communities became progressively less similar across years between the months of May and September, when the lake was thermally stratified. Dissolved oxygen and nitrate + nitrite concentrations were highly correlated to BCC change within and across seasons. The relationship between BCC and seasonal drivers suggests that trajectories of community change observed over long time series will reflect large-scale climate variation.

Bacteria underpin ecosystem function in freshwater lakes by mediating most biogeochemical cycling (Cotner and Biddanda 2002). However, surprisingly little is known of

what drives bacterial community composition (BCC) and diversity in lakes. Consequently, we lack information that may improve our ability to predict rates of biogeochemical transformation in lakes and the responses of freshwater ecosystems to environmental changes that alter BCC.

Cultivation-independent molecular techniques have enabled recent investigation of freshwater bacterial species distribution across space and time. Such studies have been conducted at several different spatial distributions: vertical (Urbach et al. 2001) and horizontal (Yannarell and Triplett 2004) within a lake, among lakes within a region (Yannarell and Triplett 2005), among lakes of the same trophic status (Lindstrom 2001) and of different trophic status (Lindstrom 2000; Yannarell et al. 2003), and across continents (Lindstrom et al. 2005). Many short-term temporal studies have documented seasonal BCC variation within a lake or between lakes (Lindstrom 2001; Crump et al. 2003; Kent et

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al. 2004). Hullar and coworkers (Hullar et al. 2006) described interannual seasonal patterns in benthic BCC over four years in a stream, and they related these patterns to temperature and dissolved organic carbon. Crump and Hobbie (2005) observed synchronous community dynamics over 2.5 years in two temperate rivers. Broadly defined phylogenetic groups (e.g., Betaproteobacteria) have been shown to reoccur across seasons and years (Pernthaler et al. 1998), and it was recently shown by Wu and Hahn (2006) that a *Polynucleobacter*-associated population reoccurs interannually across seasons.

Despite recent major advances in our ability to investigate the forces influencing freshwater BCC and diversity, few attempts have been made to conduct studies over multiple years at high temporal resolution (e.g., biweekly). As is the case for macroscale organisms and their ecosystems, patterns in microbial diversity and dynamics are the result of an interplay of forces acting at diverse scales of space, time, and biological complexity (Levin 2000). Long-term monitoring and extensive sampling efforts at appropriate scales are often required to reveal such patterns (Magnuson et al. 2005). The purpose of our study was to examine bacterial community dynamics over annual cycles and to relate these dynamics to chemical or physical forces that may influence freshwater microbial communities. This study also generated a long-term BCC data set that can in the future be used to build predictive models of bacterial community assembly and change.

## Materials and methods

**Lake characteristics**—Lake Mendota, Madison, Wisconsin (43°06'N, 89°24'W), is one of the most well-studied lakes in the world, and it is a Long Term Ecological Research Site affiliated with the Center for Limnology at the University of Wisconsin (Carpenter et al. 2006). It is dimictic and eutrophic with an average depth of 12.8 m, maximum depth of 25.3 m, and total surface area of 39.38 km<sup>2</sup>. It typically mixes in March–April, and again in October–November, but remains thermally stratified throughout the summer and winter months. Ice-on averages 119 d in winter, though this number appears to be gradually decreasing as a result of climate change (Magnuson et al. 2005).

**Sample collection**—Lake water was collected over the deepest part of the pelagic zone (~24 m) every two weeks during the open-water phase, as described originally (Yannarell et al. 2003) but with minor modifications (Yannarell and Triplett 2005). Bacteria were recovered by filtration on 0.2- $\mu$ m polyethersulfone filters (Pall-Supor-200, Gelman), without prefiltration. Filters were frozen at -80°C and stored prior to deoxyribonucleic acid (DNA) extraction. Physical and environmental parameters of Lake Mendota were measured by the North Temperate Lakes Long Term Ecological Research (NTL-LTER) project at the University of Wisconsin (UW)—Madison Center for Limnology every two weeks. These parameters are available through the NTL-LTER website at <http://www.limnology.wisc.edu>.

**Sample processing**—Total community DNA was extracted from filters using the QBiogene Bio101 FastDNA kit, using manufacturer's instruction with modification as described previously (Yannarell et al. 2003; Yannarell and Triplett 2005). Intergenic spacer regions between the 16S and 23S ribosomal ribonucleic acid (rRNA) genes were then amplified from the community DNA using primers 1406F (5'-TGYACACACCGCCCGT-3', universal 16S rRNA gene, 6-FAM labeled), and 23Sr (5'-GGGTTBCCCCATTCRG-3', bacterial-specific 23S rRNA gene) (Yannarell et al. 2003). Polymerase chain reaction (PCR) was conducted on an Eppendorf Mastercycler gradient thermocycler (Eppendorf Scientific), and conditions were as follows: 2 min initial denaturation at 94°C, 30 cycles of 35 s denaturation at 94°C, 45 s annealing at 55°C, 2 min elongation at 72°C, and a final extension for 2 min at 72°C. One microliter of extracted DNA was used as a template for PCR.

**Bacterial community fingerprinting**—We examined BCC using automated ribosomal intergenic spacer analysis (ARISA), with minor modifications (Yannarell et al. 2003; Yannarell and Triplett 2005). Briefly, ARISA exploits the length variation in the intergenic spacer region to create a bacterial community profile in which different populations are represented by amplified DNA fragments of different lengths. Amplified DNA was diluted 1:1 with sterile double-distilled H<sub>2</sub>O, and 1  $\mu$ L of this dilution was added to 10  $\mu$ L formamide and 0.4  $\mu$ L of fluorescently labeled custom internal size standard (range: 100 base pair [bp] to 2,000 bp at 50 bp intervals; Bioventures). Denaturing capillary electrophoresis was carried out on the amplified community DNA using an ABI 3700 Genetic Analyzer at the University of Wisconsin—Madison Biotechnology Center. Profiles were analyzed using GeneScan 3.1.2 and aligned with Genotyper 2.5 (PE Applied Biosystems). To account for minor run-to-run variations in fragment length associated with the limits of instrument resolution, peaks were grouped together to form operational taxonomic units (OTUs) based on the profile alignments. The signal strength (i.e., peak area) of each individual peak was normalized by dividing that peak area by the entire summed profile fluorescence (area) and expressing each peak as a relative proportion of the observed community (Yannarell and Triplett 2005). Although PCR-based techniques are not generally considered to be quantitative due to differential amplification of taxa (Wintzingerode et al. 1997), we used normalized peak area as a proxy for relative abundance because presence-absence data transformations are known to significantly distort such community data sets (Yannarell and Triplett 2005). Furthermore, normalized ARISA peak area has been found to relate well to quantitative relative abundance determined using flow cytometry in other aquatic environments (Brown et al. 2005). When replicates were available (~50% of all samples), normalized fluorescence values were averaged across samples prior to further analysis. The fluorescence threshold for inclusion in the data set was set at 50 fluorescent units (peak height) above a baseline determined by signal-to-noise criteria.

*Multivariate analysis to detect patterns in BCC*—Correspondence analysis (CA) was conducted to search for patterns in BCC change over time, using sample-OTU matrices and the Canoco for Windows software package version 4.5.1 (Ter Braak and Smilauer 2002). Intersample distances were calculated using biplot scaling, and rare OTUs were down-weighted during analysis by the Canoco program. Briefly, this transformation reduces the abundances of species in proportion to their frequencies, where the most common species has a frequency of A, and species less common than A/5 are down-weighted (Hill 1979).

Samples plotted in each ordination were classified by various parameters of interest, including mixing status, season, year, and month. When applicable, Bonferroni corrections were made when testing for significant differences among multiple comparisons. Lake mixing status was determined using temperature and oxygen depth profiles from the NTL-LTER database (<http://www.limnology.wisc.edu>). The bottom of the epilimnion was defined to be the point at which water temperature decreased by more than approximately 3°C over 2 m or less. Under such conditions, the lake was considered to be stratified. When the water-column temperature profile was homogeneous with depth (less than 3°C difference across the water column), the lake was considered fully mixed. Season at the time of sample collection was also defined by mixing status: when the lake mixed after winter stratification, these sampling times were designated “spring,” and when the lake mixed after summer stratification, these sampling times were designated “fall.”

Similarity matrices were created from pair-wise comparisons of area-normalized ARISA profiles, using the Bray-Curtis coefficient ( $S_{17} = 1 - D_{14}$  of Legendre and Legendre (1998). Analysis of similarity (ANOSIM) was then used to test the hypothesis that communities within annual or seasonal groups were more similar to each other than to communities classified as a different group (Clarke and Gorley 2001). The same classifications that were used for CA (mixing status, season, month, year) were used to define groups of samples for ANOSIM. ANOSIM generates a test statistic ( $R$ ) to indicate the degree of separation between groups, where a score of 1 indicates complete separation and 0 indicates no separation.

## Results

*Annual bacterial community richness and compositional variability*—BCC was assessed in 83 samples using ARISA. Over the six-year data set, a total of 218 OTUs was observed, with an average of 63 taxa per sample (Fig. 1A). No trends were apparent in annual richness across years, although mean richness per sample in 2002, 2003, and 2004 was significantly higher than in 2000, 2001, and 2005. The extent of compositional variation within each year was calculated using mean centroid distance derived from a correspondence analysis (Legendre and Legendre 1998). A lower mean centroid distance represents less dispersion within the cluster. The interannual mean centroid distance for Lake Mendota BCC was 0.54, while the intra-annual

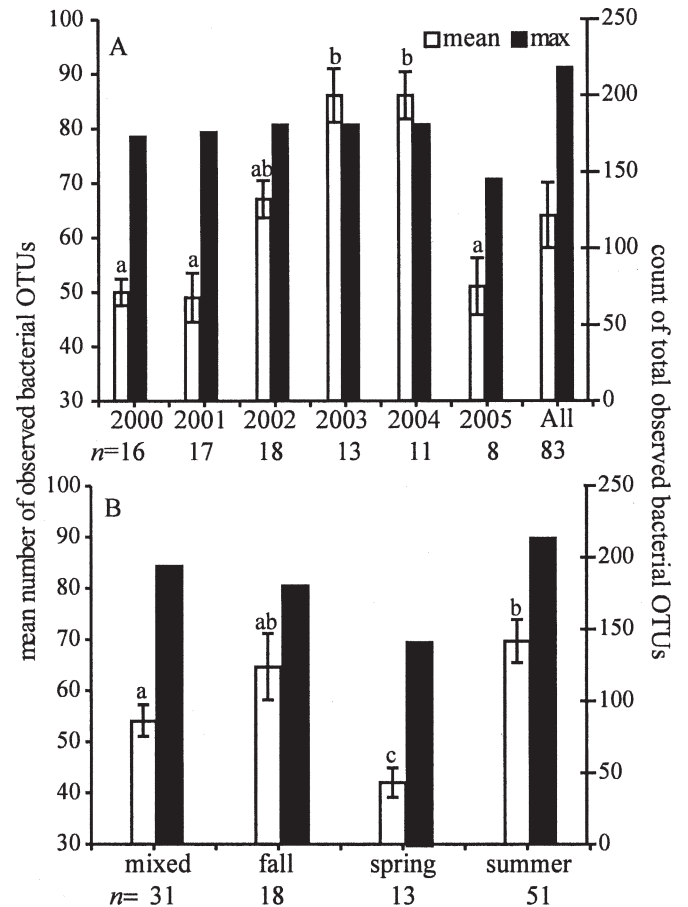


Fig. 1. Summary of bacterial OTU richness in Lake Mendota over the six-year study. This histogram depicts mean (left scale) and maximum (right scale) observed bacterial richness, as detected by ARISA. Samples were collected approximately every two weeks during the open-water phase from 2000 to 2005 and then classified according to mixing status and season. Error bars are standard error calculations. Letters above the histogram bars represent which groups are distinguishable at Bonferroni-corrected alpha levels. (A) There are no noticeable trends between mean richness and year, though 2003 and 2004 communities were generally more rich than other years ( $\alpha = 0.025$ ). (B) Spring communities were significantly less rich than fall or summer communities ( $\alpha = 0.05$ ).

distances were as follows: 0.58 (2001) < 0.61 (2000) < 0.73 (2005) < 0.74 (2004) < 0.76 (2002) < 0.80 (2003).

Differences in community richness were further investigated in mixed versus stratified samples. Bacterial communities sampled during spring mixing were generally less rich than those sampled during fall mixing or stratification (Fig. 1B). To determine whether specific OTUs were unique to a season, we cross-compared the community composition of each season. More than half of the OTUs were observed during all three seasons, although around 10% were unique to the summer stratified condition (Fig. 2). Fall and summer shared more common OTUs with each other than either shared with the spring.

*Seasonal patterns in BCC*—Patterns in Lake Mendota bacterial community dynamics were identified using

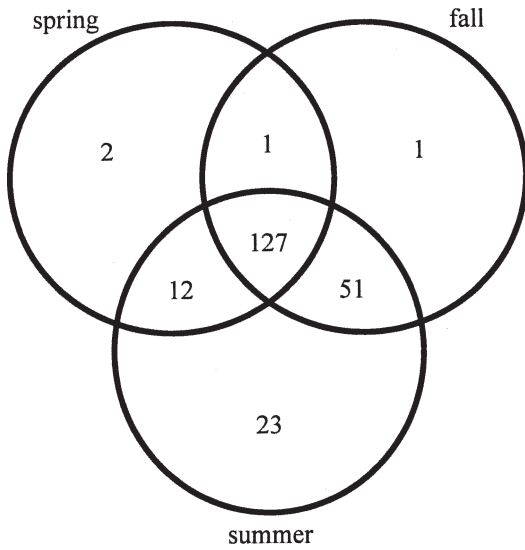


Fig. 2. Total observed OTUs common to bacterial communities classified by season and mixing status. There were 218 total taxa observed from all samples over six years.

multivariate statistics. CA was performed to ordinate samples based on BCC so that seasonal patterns over time could be described and to identify potential environmental drivers of change (Fig. 3). We found the same patterns in CA ordinations whether presence-absence or relative

fluorescence values were used in the analysis (data not shown). Relative abundance data were used in all presented analyses unless otherwise noted. The potential advantages of using relative fluorescence instead of presence-absence data for these kinds of analyses are discussed further elsewhere (Yannarell and Triplett 2005).

Samples classified by year revealed only a weak distinction between years (Fig. 3A; ANOSIM,  $R = 0.222$ ,  $p < 0.001$ ). However, bacterial communities sampled within a particular month were similar among all years (Fig. 3B; ANOSIM,  $R = 0.464$ ,  $p < 0.001$ ). The CA plot illustrates a predictable interannual seasonal pattern in BCC. Environmental variables that most strongly correlated with the first CA axis included oxygen, temperature, and nitrate + nitrite (0.82, -0.76, and 0.78, respectively).

Since monthly groups are not ecologically relevant beyond their use to represent a seasonal timescale, we instead grouped samples by mixing status, which could be a stronger predictor of bacterial community dynamics. However, the ANOSIM  $R$  statistic ( $R = 0.228$ ,  $p < 0.001$ ) indicated minimal separation between mixed and stratified samples.

Because mixing status was not a strong predictor of BCC, we investigated whether both mixing and seasonality were related to community structure. To determine if bacterial communities present during spring-mixed conditions could be distinguished from those present during fall-mixed conditions, a CA biplot was constructed without the samples from stratified conditions (Fig. 4). The strong

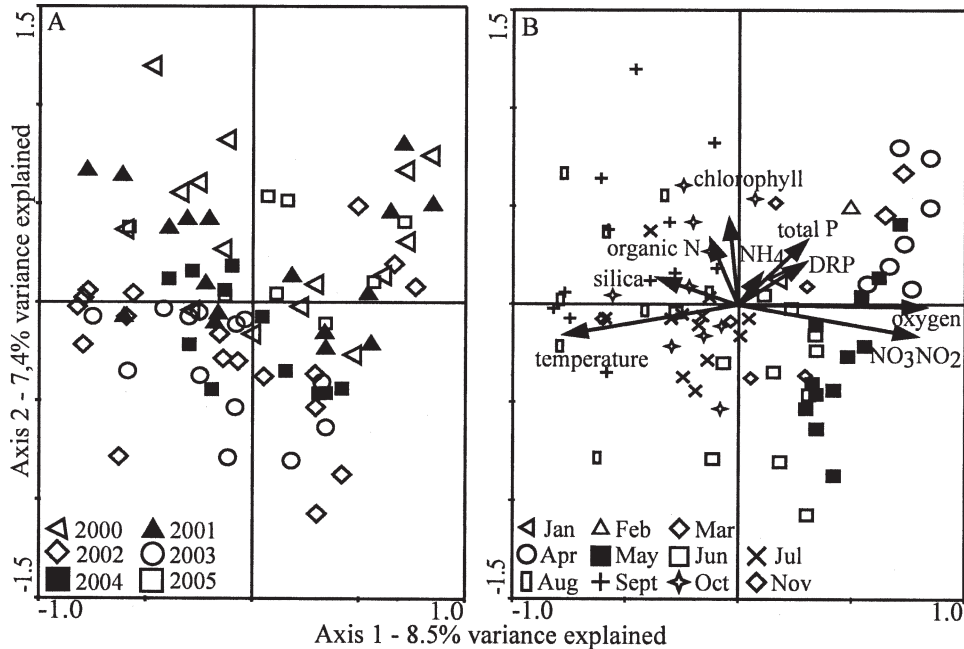


Fig. 3. Correspondence analysis (CA) plots illustrating bacterial community phenology in Lake Mendota over six years. Each symbol represents a bacterial community from a different sample date, and the distance between any two symbols represents the similarity between two communities represented. Samples were grouped based on when they were collected: (A) by year or (B) by month. No obvious pattern is apparent in (A). In contrast, (B) shows a distinct progressive pattern of community change (or phenology) that is conserved across all six years. A suite of environmental parameters is included in (B) to observe their potential relationships to BCC dynamics. The length and direction of each arrow indicate the degree of correlation with the ordination axes.

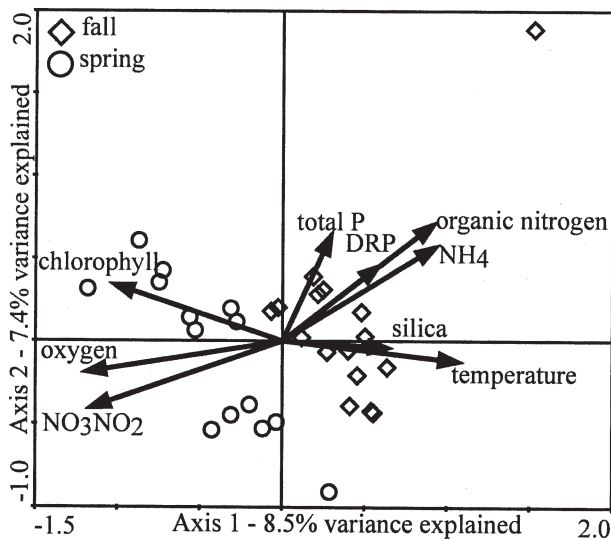


Fig. 4. Seasonal patterns in Lake Mendota BCC over six years. This ordination was constructed using CA and includes only communities sampled while the lake was not stratified. The communities were classified by season.

separation of spring and fall mixed samples was supported by a high ANOSIM  $R$  value of 0.528 ( $p < 0.001$ ). High oxygen, nitrate + nitrite, and chlorophyll values were correlated with the axes that distinguished spring mixed communities, while total Kjeldahl nitrogen and temperature were correlated with the axes that distinguished fall mixed communities.

To further explore bacterial community phenology during the summer months (Fig. 3B), we searched for patterns in BCC among summer stratified samples alone. A CA biplot of summer stratified samples showed a clear trajectory of BCC change between May and September (Fig. 5), with increasing interannual variability later in the summer and fall. May and June BCC were less dispersed and more consistent across years than those communities that occurred during the late summer and fall months. With the exception of July, each subsequent month had a less predictable (more dispersed) bacterial community than the previous. However, despite the higher variability around the mean centroid, the late communities continued to follow the directional trajectory. This indicates a general increase in interannual differences with increasing time from spring mixis to fall mixis.

## Discussion

It should be noted that our analysis of BCC was based on a proxy for relative abundance of individual populations that was derived from PCR-amplified community fingerprints. This type of approach suffers from a number of methodological limitations that preclude accurate quantitative interpretation of BCC (Wintzingerode et al. 1997). However, ARISA profiles based on proportional fluorescence have recently been used to successfully describe BCC over time and space in a variety of aquatic habitats (Hewson and Fuhrman 2004; Yannarell and Triplett 2005;

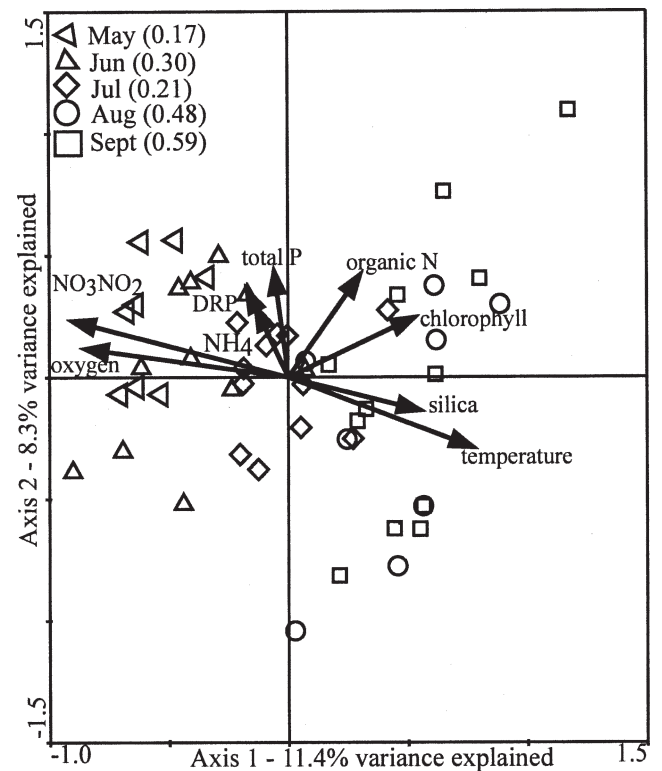


Fig. 5. Summer-stratified seasonal pattern in Lake Mendota BCC over six years. This ordination was constructed using CA and includes only communities sampled during summer stratification. The communities were then classified by month in order to interpret temporal trends. The numbers in parentheses represent the mean centroid distance, which quantifies dispersion within a classification. A mean centroid distance closer to one indicates a highly dispersed cluster, while a number closer to zero indicates a tighter cluster.

Newton et al. 2006). The high-throughput nature of the technique makes it a valuable tool despite its established limitations (Danovaro et al. 2006). Furthermore, it is encouraging that a recent comparison of ARISA peaks and quantitative data based on direct cell counts showed that quantification of certain abundant taxa by ARISA was more accurate than may be expected (Brown et al. 2005).

*Lake seasonal dynamics and bacterial community phenology*—Predictable, interannual patterns of BCC change were observed in Lake Mendota. These data showed that bacterial communities occurring at similar seasonal time points but across years were more similar to each other than to bacterial communities observed at different time points within a year (Figs. 3, 5). Early summer-stratified communities were consistent across years, but became less so in late summer. These phenological trends were repeated across our six-year study.

The mechanisms responsible for the observed pattern of decreasing interannual similarity through the summer months could include interannual differences in climatic forcing, annual extinction and/or immigration, or human activities on and around the lake. Further long-term observation and ultimately experimentation are required

to determine which of these (or other yet-identified factors) cause such interannual variation.

*Lake mixing regime as an indicator of BCC*—Lake trophic status (Lindstrom 2000; Yannarell et al. 2003; Yannarell and Triplett 2004), pH (Lindstrom et al. 2005), and hydrology (Yannarell and Triplett 2005) are three important determinants of freshwater pelagic BCC. We propose that lake mixing regime is an additional important control on BCC, both over time and across lakes, as originally suggested by Yannarell et al. (2003). Within the constraints imposed by eutrophic conditions, dimictic lakes can be expected to select for bacterial communities capable of surviving transitions between high and low temperature, nutrient and oxygen availability, and light penetration, such as those transitions that occur during seasonal mixis. Bacterial communities from polymictic or meromictic lakes are not necessarily expected to have the same predictable composition and dynamics, regardless of trophic status. This question should be examined further by searching for phenological patterns in BCC across lakes of different and similar mixing regimes.

A general seasonal pattern of BCC dynamics was also observed in two dimictic lakes, but not in a polymictic lake, during a two-year study that included Lake Mendota (Yannarell et al. 2003). However, the patterns observed were less pronounced than in our study, likely because BCC data were analyzed using a presence-absence transformation instead of normalized fluorescence, and also because it included a much shorter sampling period (two years rather than six).

*Proximate drivers of BCC*—Our results consistently highlight the importance of three environmental variables (dissolved oxygen, nitrate + nitrite, and water temperature) in distinguishing Lake Mendota bacterial communities. High oxygen and nitrate + nitrite concentrations were closely correlated to spring-mixed and early summer-stratified communities. Prior to spring mixing in Lake Mendota, nitrate + nitrite levels are typically at maximum, while competition between bacteria and phototrophic phytoplankton are at a minimum (Brock 1985). As mixing occurs, ammonia is released from the sediments and transported into the upper water column, stimulating growth. Competition between phytoplankton and bacteria for nitrogen and electron acceptors (oxygen and nitrate + nitrite) likely plays a role in controlling bacterial community phenology during late spring, though further studies are required to determine if such biotic interactions affect BCC and/or overall bacterial abundance. A closer examination of bacterial populations and their specific interaction with the nitrogen cycle in lakes should be conducted to better understand this potential driver of BCC.

Temperature had the strongest relationship to BCC—it was most highly correlated with the first axis in all ordinations. Our findings agree with multiple studies that have demonstrated the influence of temperature or seasonal changes on bacterial respiration, production, and community composition (Kent et al. 2004; Crump and Hobbie

2005). These observations combined with the link between water temperature and water-column stability suggest that thermal structure is a primary control on BCC.

Only a few samples in our data set were collected during winter stratification. The winter freezing of Mendota, and subsequent snow coverage, prevents light from penetrating to the water column and limits primary production (Brock 1985). Results from this study suggest that freezing and overwintering may act as a bottleneck to bacterial community richness. The resulting “output” spring communities after ice-off were less rich and distinct in composition from the “input” of the fall communities. While studying Lake Baikal, Goldman and colleagues (Goldman et al. 1996) observed a correlation between timing of ice-off and subsequent lake stratification, and they related these hydrodynamic properties to biological productivity and nutrient flow. Similarly, in Lake Mendota, ice cover could play a role in resetting BCC, resulting in the spring bacterial communities observed each year. Though we have found no direct correlation between ice-off time and Lake Mendota BCC, we plan to explore this and the roles of other seasonal factors in future analyses.

Multiple studies have documented the correlation between phytoplankton and bacterial community dynamics, particularly at weekly or more frequent timescales (Worm et al. 2001; Kent et al. 2004; Newton et al. 2006). In our data set, no relationship was found between phytoplankton community composition and BCC at the biweekly timescale (data not shown). A more temporally resolved data set may be necessary to reveal links between phytoplankton species and BCC in Lake Mendota.

*Aquatic bacterial phenologies and regional- or global-scale change*—The observed Lake Mendota bacterial community phenology could be used as a model for bacterial community dynamics in eutrophic lakes. Trophic status is known to be a strong predictor of pelagic BCC (Lindstrom 2000; Gasol et al. 2002; Yannarell et al. 2003). Lake Mendota is often referred to as a primary example of anthropogenic-induced eutrophication caused by regional land-use change, which has been an ongoing process documented since 1889 (Brock 1985). To develop predictive models that reflect the influence of trophic status on bacterial community dynamics, the phenology presented here should be compared with those of other lakes in differing stages of eutrophication.

Future models could also help predict the implications of BCC shifts over time for larger-scale lake processes, such as food web dynamics. Little is understood about the long-term interplay between macro-scale and micro-scale aquatic communities. For example, predictions could be made about the potential effect of BCC regime shifts on macrophyte or fish populations as more long-term studies of BCC and dynamics become available and are linked to other data sets.

Analyses of long-term data sets have revealed evidence for links between large-scale weather events such as El Niño, or long-term changes in these events, and altered patterns of aquatic community dynamics among zooplankton (Edwards and Richardson 2004; Winder and Schindler

2004), phytoplankton (Winder and Schindler 2004; Li et al. 2006), and bacterioplankton (Li et al. 2006). Climate change appears to alter average lake temperatures, as well as intra- and interannual variation in water temperature and duration of ice cover (Arhonditsis et al. 2004; Magnuson et al. 2005). In Lake Mendota and other southern Wisconsin lakes, ice-off dates are known to vary with El Niño events (Anderson et al. 1996), and they are occurring increasingly earlier, presumably because of global warming (Magnuson et al. 2005). Interestingly, two El Niño events occurred during our sampling period, one strong event over 2002–2003, and a weak event in 2004. Speculatively, these events may be linked to increases in BCC richness over those years. The observations of Magnuson et al. (2005), taken together with our six-year data set, suggest that microbial community dynamics are influenced by long-term climatic trends. Several more years of sampling Lake Mendota BCC may reveal patterns created by such drivers. Notably, others have proposed that microbial communities are good indicators of regional anthropogenic and global climate change in aquatic ecosystems (Paerl et al. 2003).

*The importance of hierarchical drivers on BCC*—This study contributes to an emerging conceptual framework that accounts for multiple interacting drivers that structure lake bacterial communities at different scales of space and time (Yannarell and Triplett 2005). These drivers include both biotic and abiotic factors, as well as intrinsic and extrinsic forces. Abiotic components include geography, lake landscape position, hydrology (drainage or seepage lake classification), seasonal and weather events, trophic status, mixing regime, and clarity (Yannarell and Triplett 2005). Biotic factors may include competition and trophic interactions (Kent et al. 2004; Newton et al. 2006), or the effect of virioplankton (Goddard et al. 2005). These forces vary in relative importance at different spatial and temporal scales. Some are secondary to others, but all may play a role in shaping the dynamics of the lake bacterial community. In Lake Mendota, within the previously established constraints of higher-level filters (for example, trophic status, landscape position, and pH), we have demonstrated that seasonal mixing and nitrogen availability are also important predictors of BCC.

A predictable bacterial community phenology in Lake Mendota was observed over six years. Seasonal factors such as water temperature and water-column stability appeared to be strong drivers of community change over monthly timescales, which may have masked or filtered other previously examined controls on BCC that act on daily to weekly timescales, such as food web interactions.

Besides temperature and mixing status, the environmental variables that correlated best with BCC dynamics included oxygen and nitrate + nitrite in the spring-mixed and early summer-stratified times. Despite the similar hydrodynamic state of the lake during spring and fall mixing, the spring and fall bacterial communities were distinct in composition. We observed less interannual similarity in BCC in the late summer than in early summer. BCC and dynamics seemed to “reset” after ice coverage.

Long-term observations of microbial community dynamics are useful for detecting intra-annual phenologies, and they may prove to be important for understanding and predicting ecosystem response to regional and global change.

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