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3 **Title**

4 Spatiotemporal Dynamics of the Bacterial Microbiota and Methanotrophic Bacteria on Lotic

5 *Cladophora glomerata* (Chlorophyta)

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9 Anna Grant Birge Memorial Award – UW-Madison Center for Limnology

10 **Abstract**

11 The periphytic green alga *Cladophora glomerata* is found growing abundantly in dense
 12 mats in lakes and rivers worldwide, often co-occurring in eutrophic lakes with near-shore waters
 13 saturated in methane. This alga hosts a diverse microbial community, but the spatiotemporal
 14 dynamics of the alga's bacterial microbiota over a growth season have not been characterized. In
 15 this study, replicate samples of *Cladophora* were collected in 2014 from multiple locales in Lake
 16 Mendota at multiple times during the summer growth season to test the hypothesis that the
 17 bacterial community changes over time and is geographically heterogeneous. Genetic sequencing
 18 of epibiontic bacteria using the 16S rDNA biomarker showed significant differences in
 19 community structure and composition over time and space, suggesting a dynamic microbial
 20 community that is strongly influenced by sampling time and weakly by sampling site. Of
 21 particular importance are high diversity and relative abundance of likely methane-oxidizing
 22 (methanotrophic) bacteria, especially *Crenothrix*, *Methylobionas*, and *Methylocaldum*, which
 23 showed distinctive site preferences. Different patterns were observed in many aerobic

heterotrophic bacteria, such as *Meiothermus*, *Leadbetterella*, and *Flectobacillus* and non-oxygenic phototrophic bacteria such as *Rhodobacter*. Comparison to results of a similar 2011 study from the same site revealed a core bacterial assemblage that persists between years and over a growth season, but also opportunistic bacterial genera and ecological guilds whose populations increase, decrease, or peak over different timeframes. Evidence for a highly dynamic microbial community growing on *Cladophora glomerata* warrants further study to determine the most influential factors and how these factors influence freshwater macroalgae or related submerged photosynthetic organisms in environmental, industrial, or biotechnological systems.

Figures



Figure 1. Madison, WI, isthmus separating Lake Mendota and Lake Monona. Inset: Picnic Point with labeled collection sites North, Point, and South, (N, P, and S, respectively).

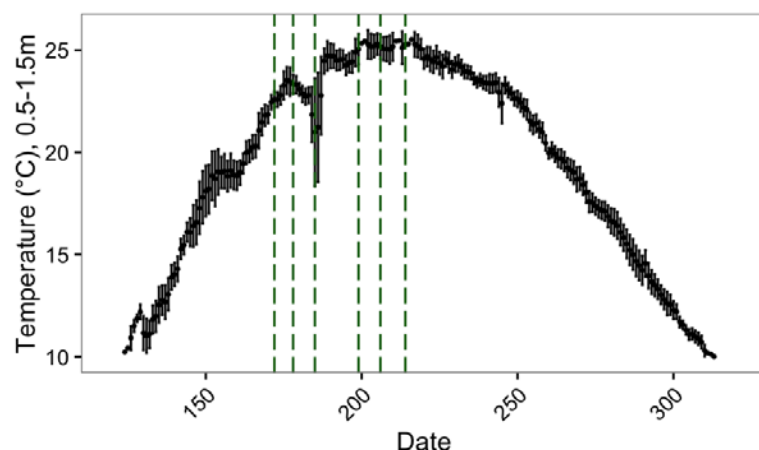


Figure 2. Estimated mean water temperatures in Lake Mendota from 2006 to 2016 at depths between 0.5m and 1.5m recorded by the Long Term Ecological Research buoy. Water temperature data for 2014 have been omitted due to instrumental malfunction that year. Dashed lines indicate dates of sample collection in 2014. Error bars \pm SE (n = 3-10).

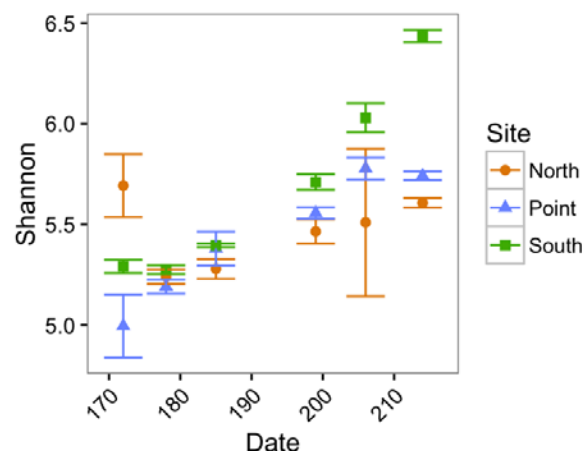


Figure 3. Mean alpha diversity as measured by the Shannon index of the bacterial microbiota of *Cladophora* from Picnic Point, Lake Mendota, WI, over during the summer of 2014. Error bars \pm SE (n=3).

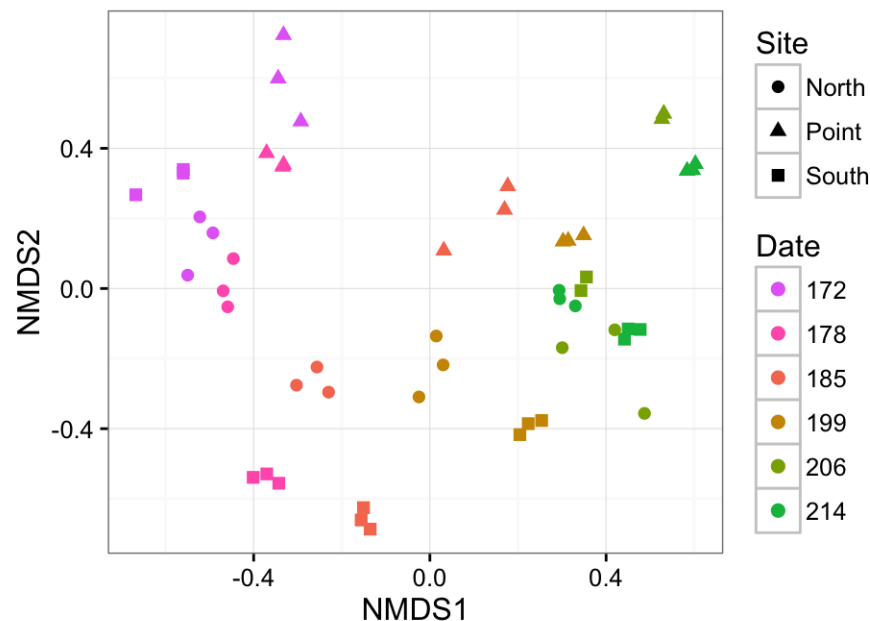


Figure 4. Non-metric multi-dimensional scaling (NMDS) ordination plot on UniFrac distances of all 16S rDNA sequenced from samples of *Cladophora* collected from Picnic Point, Lake Mendota, WI, during the summer of 2014. Color indicates collection date (Julian days 172 to 214), and shape indicates collection site (circle = North, triangle = Point, square = South). $k = 3$, stress = 0.0787.

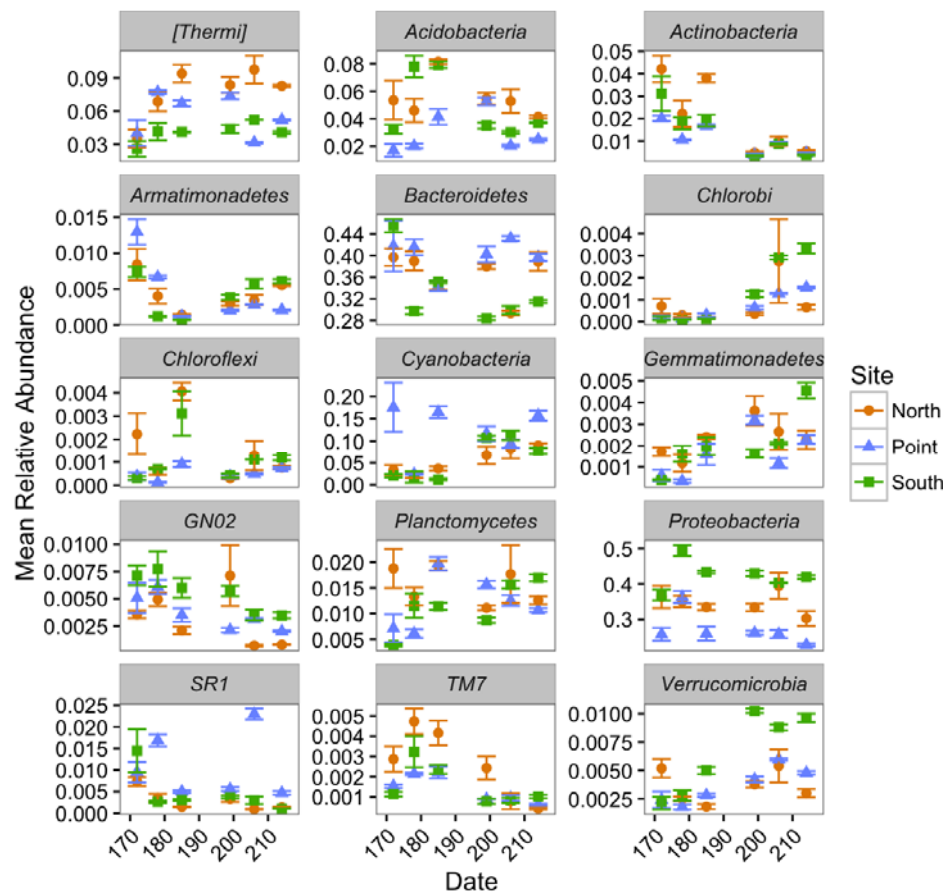


Figure 5. Mean relative abundances of dominant phyla of the microbiota of *Cladophora* from Picnic Point, Lake Mendota, WI, during the summer of 2014. Error bars \pm SE (n=3).

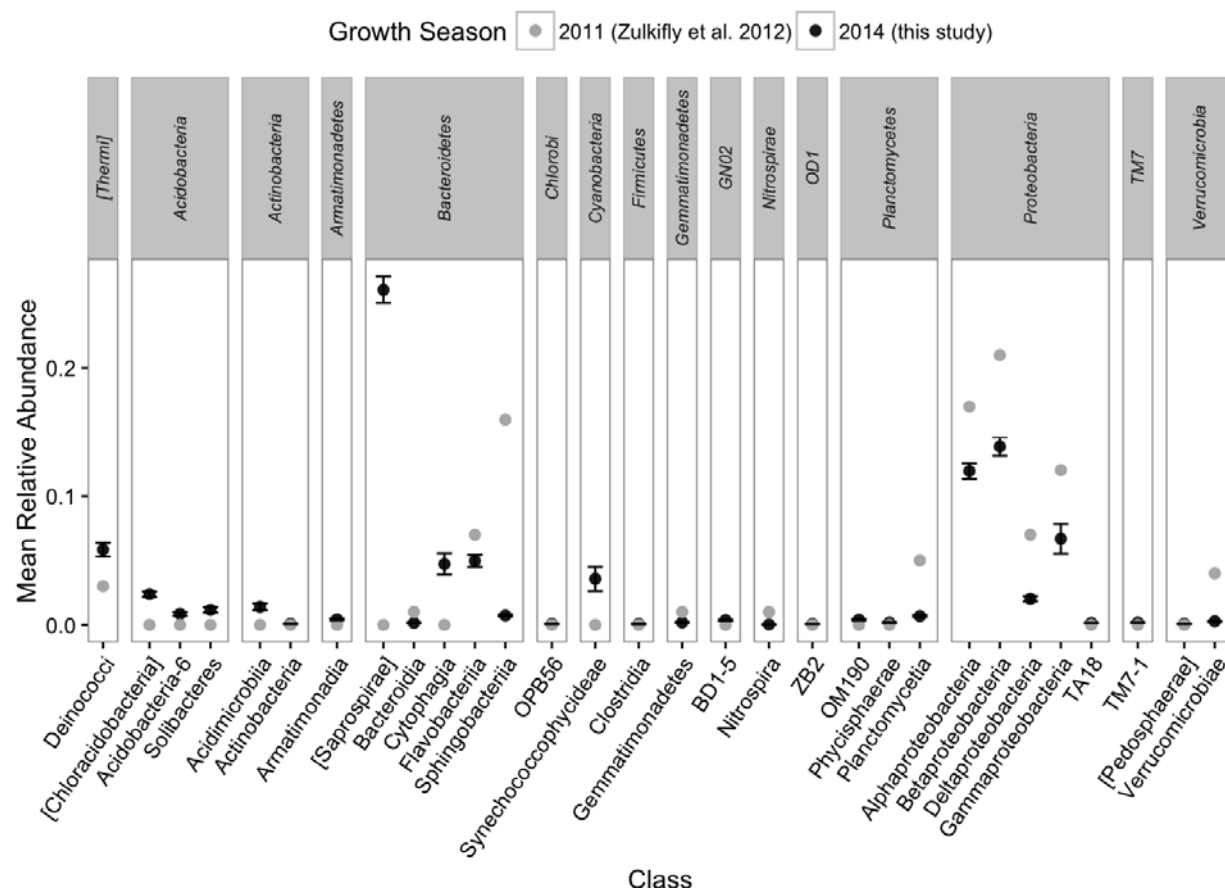


Figure 6. Comparison of relative abundances of major classes detected by Zulkifly, *et al.* (2012) from 16S rDNA amplicon assessment of *Cladophora* microbiota from Picnic Point, Lake Mendota, WI, in the summer of 2011 (grey points) and this 16S amplicon analysis of the microbiota of the same alga from the same site in the summer of 2014 (black points). For data from the replicated current study, error bars \pm SE (n=18).

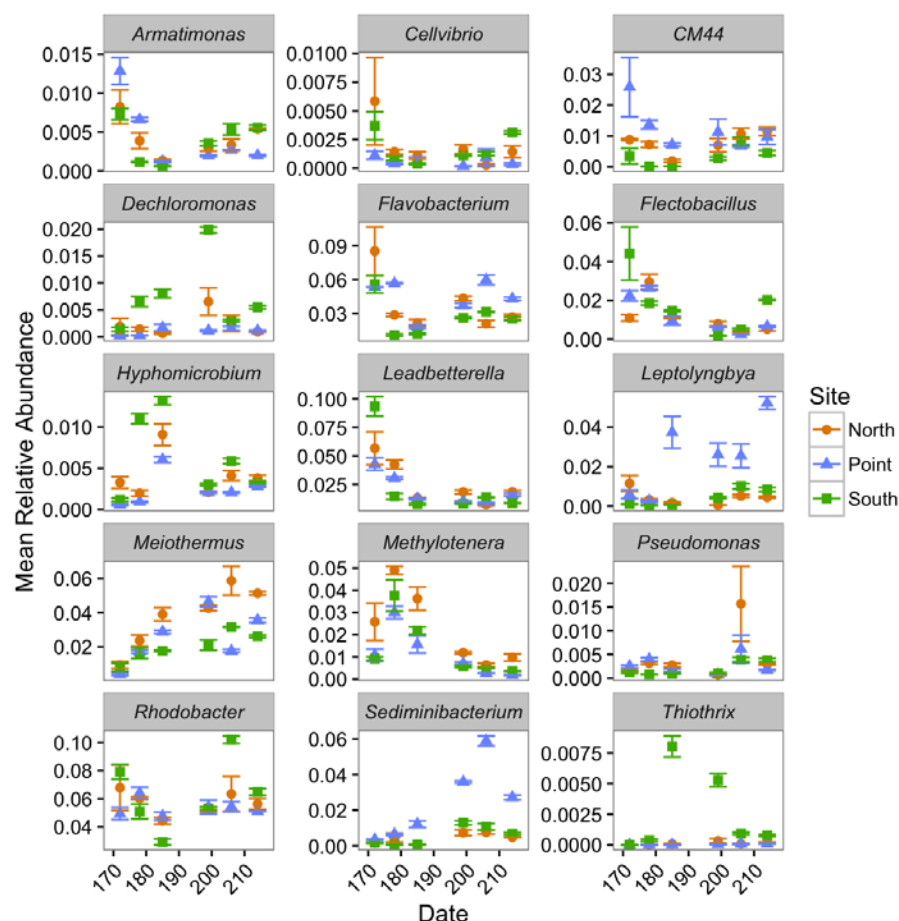


Figure 7. Mean relative abundance of dominant, non-methanotrophic genera of the bacterial microbiota of *Cladophora* from Picnic Point, Lake Mendota, WI, during the summer of 2014, inferred from 16S rDNA. Error bars \pm SE (n=3).

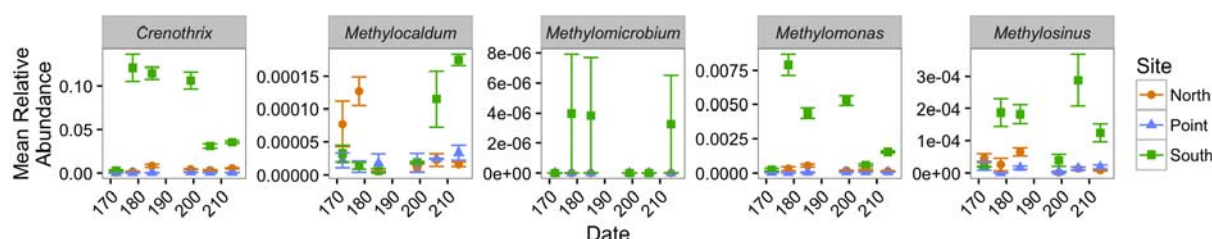


Figure 8. Mean relative abundance of methanotrophic genera of the bacterial microbiota of *Cladophora* from Picnic Point, Lake Mendota, WI, during the summer of 2014, inferred from 16S rDNA. Error bars \pm SE (n=3).

Introduction

Cladophora glomerata is a periphytic green macroalga that is abundant in freshwaters worldwide, particularly in environments affected by eutrophication. This alga commonly occurs in near-shore lake waters and co-occurs with the energy-rich substrate methane (Bastviken *et al.*, 2004; Raymond *et al.*, 2013), has a changing surface area influenced by seasonal climatic factors and wave action (Bergey *et al.*, 1995), and supports dense microbial biofilms (Niederdorfer *et al.*, 2016; Zulkifly *et al.*, 2012). Metagenomic analysis has shown that microbiota occupying surfaces of *Cladophora* display surprising taxonomic and functional diversity, including many lineages of eukaryotic microorganisms and methanotrophic, nitrogen-fixing, and cellulose-degrading bacteria (Graham *et al.*, 2015). Because this macroalga is common and abundant worldwide, particularly in environments impacted by eutrophication, and interacts strongly with biogeochemical cycles and other organisms, these holobionts have been described as ecological engineers (Zulkifly *et al.*, 2013). It follows that spatiotemporal variation of the associated bacterial community might prove to be ecologically significant or relevant to potential biotechnological applications (Hoover *et al.*, 2011).

The epibiontic microbial communities of living submerged freshwater photosynthetic hosts, such as the microbiota of algae (Knack *et al.*, 2015; Fisher *et al.*, 1998) and aquatic macrophytes (He *et al.*, 2012) are dominated by aerobic heterotrophs and photoautotrophic microbes, suggesting that these assemblages are commensal or contribute to the health of the host. Metabolic exchanges among taxa within the *Cladophora* microbiome have been hypothesized to include algal use of nitrogen compounds and vitamin B₁₂ produced by bacterial associates (Gerloff & Fitzgerald, 1976), and bacterial, protist, and microfauna use of oxygen and organic carbon produced by the photosynthetic host (Zulkifly *et al.*, 2012; Graham *et al.*, 2015).

Methanotrophic bacteria, for example, require oxygen and produce carbon dioxide, thereby helping associated photosynthesizers to cope with dissolved inorganic carbon limitations in the water column.

Some past studies have focused on microbiota of potential pathogenicity to humans that are associated with nearshore lake sediments or on-shore decomposing drift *Cladophora* contaminated by shorebird wastes (Davies *et al.*, 1995; Alm *et al.*, 2003; Badgley *et al.*, 2011; Byappanahalli *et al.*, 2003; Chun *et al.*, 2017). By contrast, our study focused on the spatiotemporal dynamics of this highly-branched and abundant host, particularly dynamics of bacterial associated with the globally significant process methane oxidation. To achieve this goal, we examined attached, living *Cladophora* from multiple microsites and multiple time points through the growth season to explore the potential ecological importance of associated bacterial communities, particularly methanotrophs. We tested the hypotheses that the taxonomic composition of epibiontic bacterial communities of *Cladophora* (1) significantly changes over the growth season and (2) is spatially heterogeneous within short distances.

Materials and Methods

Site Description and Sampling Strategy

Picnic Point is a shoreline peninsular feature of Lake Mendota, Dane Co., WI (43°05'21.7"N 89°24'56.5"W, Figure 1). Attached biomass dominated by the species *C. glomerata* (Graham *et al.*, 2015) was sampled from Julian day 172-214, from a northern aspect (N), southern aspect (S), and point (P), 100m apart. The Point site was the most exposed and least shaded sampling site among the three sampling sites. Although *Cladophora* occurs from the surface to variable depths within the water column, for this study, sampling was restricted to depths of 1.0-1.5m. Water temperature at the depth of algal collection increased over the

summer, starting around 21°C on Julian day 160 and peaking around 26°C on Julian day 220 (Figure 2). Near-shore regions of Picnic Point and other areas of Lake Mendota were supersaturated with methane in 2014, varying from 190ppm at mid-summer, peaking at 350ppm in late August, then dropping to 20 ppm in October (Luke Loken, University of Wisconsin Center for Limnology, personal communication). A total of 52 samples of *Cladophora* were collected, three from each site once per week for two three-week periods, one near the beginning and the other toward the end of the 2014 growth season (Julian days 172, 178, 185, 199, 206, and 214). Using ethanol-sterilized tools and nitrile gloves, whole algal filaments were removed from rocks and underwater debris and placed into 4-oz. sterile Whirl-Paks (Nasco, Fort Atkinson, WI) containing local lake water and a headspace of air and transported to the lab in an insulated pack, where they were immediately processed.

Sample Processing and DNA Extraction

To remove planktonic microbial cells and unattached microfauna, as in previous studies of *Cladophora* microbiota (Zulkifly *et al.*, 2012; Graham *et al.*, 2015), algal filaments were washed three times in fresh autoclave-sterilized SD11, a defined medium closely resembling the chemical composition and pH of Lake Mendota water (Graham *et al.*, 1996). All filaments of *Cladophora* were trimmed to the oldest 2 cm of basal cells near holdfasts, in order to consistently sample the colonized microbiota from equivalent locations, and avoiding newer, less highly colonized host cells at growing tips (as in Zulkifly *et al.*, 2012 and Graham *et al.*, 2015). The resulting algal materials were placed in 1.5 mL Eppendorf Safe-Lock tubes, and total genomic DNA was extracted on the same day using the DNEasy Plant Mini Kit (Qiagen) amended with 100μL of 100mg/ml lysozyme (Sigma-Aldrich) solution to increase access to DNA of bacterial cells with recalcitrant cell walls (Yuan *et al.*, 2012). All DNA was frozen from

the date of collection and extraction until September 16, 2014, when samples were transported to the University of Wisconsin Biotechnology Center for 16S rDNA amplification and amplicon sequencing. 16S rDNA is both highly conserved across the domain Bacteria and contains variable regions, making amplified reads of this gene ideal for conducting a broad taxonomic census of bacterial microbiota (Ward *et al.*, 1990).

Sequencing and Annotation of 16S Amplicons

Variable regions V3 and V4 of 16S rDNA were targeted as part of the 16S Metagenomic Sequencing Library Preparation Protocol, Par #15044223 Rev. B (Illumina Inc., San Diego, California, USA), using forward primer S-D-Bact-0341-b-S-17 and reverse primer S-D-Bact-0785-a-A-21 (Klindworth *et al.*, 2013). Paired end, 250 bp sequencing was performed using the Illumina MiSeq Sequencer and a MiSeq 500 bp (v2) sequencing cartridge. Images were analyzed at the University of Wisconsin-Madison Biotechnology Center using the standard Illumina Pipeline, version 1.8.2. QIIME (Caporoso *et al.*, 2010; v.1.9.1) were used with UCHIME (Edgar *et al.*, 2011, v.4.2) and Mothur (Schloss *et al.*, 2009, v.1.36.1) to trim, cluster, and annotate all reads after merging forward and reverse paired ends. A total of 11,541,927 reads were obtained. 441,017 reads were removed because they were too short for identification after quality trimming and 12,861 chimeras were found and also removed. Taxonomy was assigned to the remaining 11,100,910 reads (a mean of 213,479 reads per sample) using the SILVA database (Quast *et al.*, 2012; v.123).

Statistical Analyses

The Shannon index of alpha diversity (Wilhm, 1970) was calculated to measure site- and time-specific bacterial species diversity. The Shannon index accounts for relative abundance and evenness across OTUs in a community (Sager & Hasler, 1969). The test for non-parametric

multivariate analysis of variance (NPMANOVA; Anderson, 2001) was used to detect significant differences among microbial communities. Non-metric multi-dimensional scaling (NMDS) was used to cluster microbial communities by a calculated UniFrac dissimilarity distance matrix (Lozupone *et al.*, 2005). In an effort to reduce PCR bias that could inflate downstream OTU abundance metrics and indices, raw read abundances were normalized to relative abundances, which are ratios of the reads of an OTU to all reads in each sample. All calculations and tests were completed using the R packages Phyloseq (McMurdie & Holmes, 2013) and vegan (Dixon, 2003).

Results

Epibiontic bacterial communities sampled from Lake Mendota (WI, USA) *Cladophora glomerata* at six time points and three microsites during the growing season of 2014 included 51,928 total possible OTUs present in the 52 samples of 16S rDNA sequenced reads. There was a mean of 5,104 OTUs per sample and a maximum of 7,819 OTUs at the South site on Julian day 214. Taxonomy assignments yielded several thousand annotated OTUs, most of which were rare, but 14 genera achieved relative abundances $\geq 1.0\%$ (Figure 7). Within these abundant OTUs, there was substantial spatial and temporal variation. OTUs assigned to the genus *Hyphomicrobium* reached a mid-June peak before declining in July and August. Conversely, *Pseudomonas* and *Meiothermus* increased in relative abundance more slowly than other genera, peaking in late summer. The Point site was notable for higher relative abundances of *Leptolyngbya* and *Sediminibacterium*. *Leadbetterella* exhibited an early-summer peak followed by slow decline, a temporal pattern common among most genera detected in the Lake Mendota *Cladophora* microbiota. The most abundant likely methanotrophic genera, *Crenothrix* and *Methylomonas* peaked in mid-June before declining in July and August and were also more

prominent in the South site than other sampled locations (Figure 8). *Methylocaldum* was more relatively abundant at the North site at the beginning of the growth season but became more prominent at the South site by the end of the growth season. The lower-abundance methanotrophic bacterial genera *Methylomicrobium* and *Methylosinus* were also found in greater abundance in the south site.

The non-parametric multivariate analysis of variance test showed that the bacterial microbiota of *Cladophora* differed significantly over the growth season (pseudo $F = 17.4$, $R^2 = 0.414$, $p < 0.001$) and between sites (NPMANOVA, pseudo $F = 21.2$, $R^2 = 0.207$, $p < 0.001$). Shannon diversity indices showed a gradual net increase over time at the Point site (starting at 5.0 and ending at 5.7) and South site (starting at 5.3 and ending at 6.4), but the North site instead initially decreased from 5.7 to 5.2 and ended at 5.6 (Figure 3). Samples also clustered more strongly by collection date than site (Figure 4).

The five dominant bacterial phyla present in the *Cladophora glomerata* microflora over the sampling period, in decreasing rank by relative abundance, include *Bacterioidetes*, *Proteobacteria*, *Deinococcus-Thermus* ([*Thermi*]), *Cyanobacteria*, and *Actinobacteria* (Figure 5). *Bacterioidetes* decreased at the South site but remained stable at sites North and Point. Relative abundance of *Proteobacteria* remained constant throughout the growth season, but the South site displayed the greatest abundance and the Point site displayed the lowest relative abundance of this phylum. [*Thermi*] was initially below 5% relative abundance, and increased at all sites over the growth season, but diverged mid-summer by site, where relative abundance fell back to 5% at the South and Point sites but remained at 8% at the North site. *Actinobacteria* began with 2-5% relative abundance but decreased in all sites to near zero contribution to late-summer microbial communities on *Cladophora*.

The bacterial census performed by Zulkifly *et al.* (2012), which also employed 16S rDNA amplicons to assess *Cladophora*-associated bacteria at the same locale, indicated relative abundances of *Proteobacteria* classes that were nearly identical to those observed in this current replicated study (Figure 6). However, the 2014 sampling revealed a much larger relative abundance of candidate phylum [*Saprospirae*], and large differences occurred across additional classes, most notably among the classes of *Bacterioidetes*. In 2011 the relative abundance of *Cytophaga* was somewhat lower and relative abundance of *Sphingobacteria* much higher than we observed for 2014. Additionally, in 2014 *Planctomycetes* and *Verrucomicrobiae* were lower in relative abundance than in 2011.

Discussion

The goals of this study were to test the hypotheses that *Cladophora glomerata*-associated bacteria display temporal and spatial variance, using samples of the alga from three sites in Lake Mendota (WI, USA) obtained at six times during the growth season of 2014, taxonomically assessed with the 16S rDNA biomarker. Results of the genetic sequencing analysis indicated a highly dynamic bacterial community more strongly influenced by sampling time, which accounted for 41% of the variance. Sampling site accounted for only 20% of the variance observed, which was expected considering the close proximity of sample sites used in this study.

Taxonomic assessment at multiple classification levels indicated that the microbiota of Lake Mendota *Cladophora* has a relatively static core bacterial community that includes representatives of the phyla *Bacterioidetes*, *Proteobacteria*, *Deinococcus-Thermus* ([*Thermi*]), *Cyanobacteria*, and *Actinobacteria*. The consistent increase in alpha diversity observed across all sites over time suggests an accumulation of colonists over time. The increase and decrease in relative abundance found at all taxonomic levels also suggests successions of microbial guilds,

although direct interactions are not detectable without experimental study. However, because we focused on the same region of algal biomass over the growing season (the 2 cm of basal filament above the holdfast), fresh colonization may have been a weaker a driver of community composition compared to successional dynamics. Potential causes of the observed differences in alpha diversity and differences in community composition between sites include shore aspect, sunlight protection, and differences in human activity among sites. NPMANOVA results showing larger differences among sampling times than among sampling sites are also evidence against a single dominant microbial community that remained unchanged over the growth season.

Although there are limitations in the methods of this study to infer the function of each reported genus (Langille *et al.*, 2013) and additional limitations to indirectly infer environmental gradients, some genera showed patterns of change over the growth season that might be ecologically significant. For example, the rapid increase followed by slow decrease in the relative abundances of the likely methanotrophs *Crenothrix* (Stoecker *et al.*, 2006), *Methylocaldum* (Islam *et al.*, 2015), and *Methylomonas* (Ogiso *et al.*, 2012) suggest that methane concentrations might have changed over time, possibly reflecting differences in emissions from sediments. *Methylomicrobium* (Brantner *et al.*, 2002) and *Methylosinus* (Fox *et al.*, 1989) were found in lower relative abundance, but these genera contributed to the unexpectedly high South site by the end of diversity of methanotrophic bacteria living on *Cladophora*. The studied eutrophic lake is very rich in other nutrients such as dissolved organic carbon, phosphorus, and nitrogen (Beverdors *et al.*, 2013; Carpenter *et al.*, 2014; Karatayev *et al.*, 2012), all of which likely contributed to supporting the putatively hardy copiotrophs *Meiothermus* (Tindall *et al.*, 2010), *Sediminibacterium* (Kang *et al.*, 2016), *Pseudomonas*

(Klausen *et al.*, 2003), and *Flectobacillus* (Hwang *et al.*, 2006), found growing in high relative abundance on *Cladophora*. The phototrophic cyanobacterium *Leptolyngbya* (Shimura *et al.*, 2015) occurred in greater relative abundance at the site having the least shade. By contrast, the non-oxygenic phototrophic bacterium *Rhodobacter* (Hiraishi *et al.*, 1996) did not show site preference.

Understanding that *Cladophora* hosts a dynamic bacterial microbiota that likely has important ecological impacts, such as methanotrophy, could have important biogeochemical and technological implications. For example, alga-bacterial co-cultures have been employed to transform methane-rich biogas into useful materials (van der Ha, *et al.*, 2012), and bacteria of highly various yet unknown functions could be co-cultured using algae as a host for industrial applications, such as hydrogen-producing photobioreactors (Berberoglu & Pilon, 2010). This knowledge could also influence the management of lakes, rivers, and wetlands where filamentous algae grow abundantly and are often viewed solely as a nuisance (Suplee *et al.*, 2009; Vodacek, 2012). By contrast, the present study is consistent with earlier work (Graham *et al.*, 2015) in revealing that this holobiont performs potentially important ecosystem services such as methane oxidation.

In summary, among sampling times over a single growth season or between years of algal growth, the core microbial community of *Cladophora* seems relatively stable, but specific bacterial taxa exhibited different patterns in relative abundance over time. Our hypothesis that the taxonomic composition of associated bacterial communities of *Cladophora* significantly changes over the growth season was not disproven, but, contrary to our hypothesis, communities from different locations were largely similar when sampled in relatively close proximity. Methanotrophic bacteria were found to be abundant enough to warrant further study of their

influence on the algal host's methane-rich aquatic environments and global biogeochemistry.

This study provides a foundation for future experimental studies designed to determine which

conditions of the environment drive structural and temporal shifts in the composition of the

microbial community, as well as assessments of the relative role of interspecific competition and

symbioses among and between microbial species associated with this algal host.

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Supplementary Materials

Sequences are deposited at the National Center for Biotechnology Information (NCBI)

Short Reads Archive (SRA) under accession ###.

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