Bacterial community structure and response to nitrogen amendments in Lake Shenandoah (VA, USA)


ABSTRACT

Microbial processes are critical to the function of freshwater ecosystems, yet we still do not fully understand the factors that shape freshwater microbial communities. Furthermore, freshwater ecosystems are particularly susceptible to effects of environmental change, including influx of exogenous nutrients such as nitrogen and phosphorus. To evaluate the impact of nitrogen loading on the microbial community structure of shallow freshwater lakes, water samples collected from Lake Shenandoah (Virginia, USA) were incubated with two concentrations of either ammonium, nitrate, or urea as a nitrogen source. The potential impact of these nitrogen compounds on the bacterial community structure was assessed via 16S rRNA amplicon sequencing. At the phylum level, the dominant taxa in Lake Shenandoah were comprised of *Actinobacteria* and *Proteobacteria*, which were not affected by exposure to the various nitrogen treatments. Overall, there was not a significant shift in the diversity of the bacterial community of Lake Shenandoah with the addition of nitrogen sources, indicating this shallow system may be constrained by other environmental factors.

**Key words** | bacterial community, lake, nitrogen

INTRODUCTION

Microbial activity drives nutrient cycling in freshwater ecosystems, which is crucial to maintaining proper ecosystem function. Unfortunately, our understanding of the forces that shape bacterial community structure, particularly in...
shallow lake ecosystems, remains limited. A wide range of abiotic factors have been found to constrain bacterial community ecology in these systems (Sinistro et al. 2015; Li et al. 2017), although the same phyla (Actinobacteria, Bacteroidetes, Cyanobacteria, Proteobacteria) appear to dominate almost universally in shallow freshwater lakes, regardless of trophic status (Lew et al. 2015; Dai et al. 2016; Tang et al. 2017). Seasonality in particular has been shown to be a critical factor in driving bacterioplankton community structure in freshwater systems (Newton & McMahon 2011; Peura et al. 2012; Tang et al. 2017). However, diverse metabolic requirements in bacterioplankton communities suggest structure is likely also shaped by nutrient availability, particularly in eutrophic systems (Wilhelm et al. 2011; Davis et al. 2015). In smaller, shallower lakes, nutrients also appear to drive bacterial community structure (Li et al. 2017).

Due to their sensitivity to abiotic perturbation, freshwater ecosystems are considered sentinels for environmental change. Furthermore, the structure and function of microbial communities may be the first to change during a disruptive event such as an influx of exogenous nutrients. The introduction of nitrogen (N), largely from anthropogenic sources via runoff, is considered a prominent threat to freshwater ecosystem health (Paerl et al. 2011). Recently, the potential impact of organic nitrogen sources such as urea within aquatic communities has been recognized due to the increase in their application as nitrogen fertilizers around the world (Staley et al. 2018). In some systems, urea contributes >50% of total dissolved nitrogen, suggesting that its impact on ecosystem function needs to be studied universally across aquatic ecosystems (Harrison et al. 1985). Currently we have little understanding of how aquatic microbial communities respond to inorganic (e.g. nitrate and ammonium) versus organic nitrogen (e.g. urea) sources, while exposure to these different N-forms may result in unique impacts on community structure and function, particularly in those systems dominated by phytoplankton (Steffen et al. 2014; Harke et al. 2016). Previously, addition of ammonium has stimulated enrichment of Betaproteobacteria (Barlett & Leff 2010), while nitrate addition has resulted in decreased abundance of this taxon (Ren et al. 2016). However, the number of studies directly linking the impact of nitrogen sources on bacterial community composition in freshwater ecosystems is limited, particularly those solely examining nitrogen.

In this study, we exposed water samples collected from a shallow impoundment in the Chesapeake Bay watershed to a variety of nitrogen sources at different concentrations to determine the impact of nitrogen influx on the bacterial community structure. The Chesapeake Bay watershed encompasses >165,000 km² in the mid-Atlantic region of the United States and is heavily impacted by anthropogenic nutrient loads. Changes to bacterial community structure of the chosen system, Lake Shenandoah (Virginia, USA), were assessed using high-throughput Illumina sequencing of 16S rRNA amplicon libraries of bottle-incubated water samples amended with varying concentrations of ammonium, nitrate, or urea. The results of this study represent the first assessment of the microbial community structure of this lake or any system in the Shenandoah River region of the Chesapeake Bay watershed and expand our understanding of the drivers of bacterial community composition in shallow lake ecosystems.

**METHODS**

Sample site and collection

Lake Shenandoah is a shallow (~1 m, with a maximum depth of 7.6 m), 36-acre impoundment located in Rockingham County, Virginia, USA (38.3804°N, 78.8358°W). Sampling occurred on 5 September 2016 at 11.30 a.m. Eastern Standard Time. Environmental parameters were measured with a YSI EXO2 Sonde (YSI, Yellow Springs, Ohio, USA). Water samples were collected in 2.5 or 5.0 L sterile carboys and returned to the James Madison University campus for incubation. Incubations were set up within 1 hour of sample collection.

Incubation conditions

Five hundred millilitres of whole lake water were immediately filtered on a 0.2 µM polycarbonate filter (Millipore) in duplicate and flash frozen to act as the Environmental Control sample. Five hundred millilitres of lake water were aseptically transferred to sterile, acid washed 2 L Erlenmeyer flasks. This was performed in duplicate for each treatment. An exogenous nitrogen (N) source was added to a final concentration of either 10 µM or 50 µM as urea, ammonium chloride, or potassium nitrate. A no-amendment treatment was done as a Bottle Control. Flasks were incubated at 26 °C in a 12:12 light/dark cycle for 48 hours (Davis et al. 2010, 2015).

DNA extraction, library prep, and sequencing

After 48 hours, samples were concentrated onto 0.2 µM polycarbonate filters and DNA was extracted using the DNeasy
PowerWater Kit (Qiagen). DNA concentration and quality were assessed using a NanoVue Plus (GE Healthcare). The 16S rRNA gene amplicons were generated using the 27f and 1492r primers with a reaction mixture of 1.5 µL of DNA, 2.0 µM of both the forward and reverse primer, 12.5 µL of 2x EconoTaq Master Mix and 8.5 µL of molecular grade water (DeLong 1992). The polymerase chain reaction (PCR) program consisted of an initial 5 minutes at 95 °C, followed by 35 cycles of 95 °C for 1 minute, 50 °C for 30 seconds, and 72 °C for 90 seconds. PCR products were purified using the QIAquick PCR Purification kit (Qiagen, Maryland, USA). Library construction (Nextara XT Library Preparation Kit, Illumina) and sequencing on the Illumina MiSeq platform were performed by Lucigen (Wisconsin, USA). Sequence libraries were generated as paired-end fastq files and are available on the NCBI sequence read archive under Project Number SRP160139.

**Sequence analysis**

Forward reads (Muturi et al. 2016; Bletz et al. 2017) of the paired-end demultiplexed fastq files were analyzed using QIIME 2 version 2018.6 (Caporaso et al. 2010; Kuczynski et al. 2012; Bolyen et al. 2018). The DADA2 pipeline available through QIIME 2 was used to detect and correct sequence data, as well as to trim sequences where appropriate, as determined by a drop in the quality score to below a predetermined threshold. Taxonomic units are referred to as amplicon sequence variants (ASVs) rather than operational taxonomic units due to the use of the DADA2 pipeline (Callahan et al. 2017). These areas of lower quality were removed by using the denoise-single command (Callahan et al. 2016). Taxonomic analysis of the sequences was then conducted by using the Greengenes version 13.5 data set to train the Naïve Bayes classifier for use in the feature-classifier plugin on the V4 region of the 16S amplicon (DeSantis et al. 2006). This taxonomic analysis was performed at a minimum percent identity of 70% (Swanson et al. 2010; Chaparro et al. 2015; Mason et al. 2014). Setting the percent identity threshold at 70% provided a classification for sequences that matched a minimum of 70% of the reference data set used. This percent identity threshold was applied to identification at all taxonomic levels. QIIME 2 was also used in the alpha- and beta-diversity analyses of sequence data via the QIIME diversity plugin. The core-metrics-phylogenetic command was used at a sampling depth of 52,381 in order to maximize the amount of sequences retained per sample for analysis. Overlap of ASV presence/absence between samples was visualized using UpSet (Lex et al. 2014). Individual ASVs had to be present in at least one of the duplicate samples to be included.

**RESULTS AND DISCUSSION**

Anthropogenic activity can disrupt nutrient cycling dynamics in aquatic ecosystems. Since the nineteenth century, human activity has dramatically reshaped the global nitrogen cycle, which has had cascading effects on the abundance and form of this nutrient within aquatic food webs. We examined the effect of an influx of various exogenous nitrogen sources (urea, nitrate, and ammonium) on the bacterial community of Lake Shenandoah, a small impoundment in the Shenandoah Valley of Virginia, located in the Chesapeake Bay watershed.

**Environmental conditions**

Samples were collected from a depth of 0.33 m in Lake Shenandoah on 5 September 2016. At the time of sample collection, the water temperature was 25.0 °C and conductivity was 366.56 µS/cm. Chlorophyll was 3.24 µg/L and phycocyanin was 0.19 µg/L. All measurements were made with a YSI EXO2 Sonde (YSI, Yellow Springs, Ohio, USA).

**Bacterial community composition**

A total of 5,497,748 reads were generated across 14 sequence libraries. After demultiplexing and quality control functions, 4,928,941 total ASVs were used for analyses. Across all libraries, 44.5% of reads were classified as unassigned, with the rest being classified as part of 31 unique bacterial phyla (Figure 1(a)). Only seven phyla comprised the majority of ASVs, with *Actinobacteria* being the most dominant phylum in six of the seven treatments (Figure 1(a)). Members of the *Actinobacteria* were almost exclusively comprised of the *Actinomycetales*, making up 34.6% of total ASVs (Figure 1(b)). *Proteobacteria* were the second most abundant phylum across all samples, making up 15.6% of all ASVs on average. At the order level, composition of the *Proteobacteria* community varied slightly between samples, with the ASVs classified as *Sphingomonadales* being most abundant in all treatments except the Environmental Control and the 50 µM ammonium treatment (Figure 1(c)). The *Bacteroidetes* were most dominant in the Bottle Control samples, comprising 15.2% of ASVs compared to an average of 6.8% of ASVs across all other treatments (Figure 1(a) and 1(c)). The 10 µM ammonium treatment had a uniquely high abundance...
of Verrucomicrobia (14.2%) compared to the rest of the treatments (average 2.4%).

At the phylum level, the bacterial community was largely unresponsive to nitrogen amendment, regardless of the type or concentration of nitrogen applied (Figure 1). Members of the Actinobacteria and Proteobacteria phyla remained dominant across all treatments, both of which have been found to be the dominant bacterial phyla in shallow freshwater systems previously (McLaughlin et al. 2018; Woodhouse et al. 2015; Tang et al. 2017). Members of the Actinomycetales, including the cluster ACK-M1, comprised almost all of the Actinobacteria ASVs in this study. This observation is similar to those made in freshwater lakes worldwide (Ghai et al. 2014; Tang et al. 2017; Chopyk et al. 2018). Freshwater Actinobacteria have streamlined genomes with phosphate uptake capabilities and have been strongly negatively correlated with the presence of Cyanobacteria in previous metagenomes, a finding also reflected in our own study (Ghai et al. 2014; Kang et al. 2017). While previous work has demonstrated that Actinobacteria in freshwater mesocosms significantly decrease when nitrate and phosphorus are added, this phylum did not decrease significantly from the control treatments when the different nitrogen forms were added. This may be due to the differences in experimental design: the mesocosm experiments were conducted on a long-term scale (8.5 years) and phosphorus was added in conjunction with nitrogen (Ren et al. 2016). Additionally, Lake Shenandoah is surrounded by potential...
non-point sources of N runoff, including a golf course, and therefore may have been replete with N already, resulting in little to no impact on the overall community structure.

*Verrucomicrobia* have been found to comprise up to 20% of total bacterial populations in lakes (Cabello-Yeves et al. 2017). Our data suggest that their abundance is more limited in Lake Shenandoah, although they were enriched for in the 10 µM ammonium treatment (Figure 1). They have also previously been shown to be negatively correlated with nitrate levels in freshwater sediments (Zhang et al. 2016).
2013), a pattern which is also reflected in our data (Figure 1). Previous studies on the metabolic capabilities of aquatic members of this phylum have largely been dedicated to understanding their ability to degrade a variety of complex carbohydrates (Cabello-Yeves et al. 2017; Tran et al. 2018). However, Tran et al. (2018) demonstrated expression of genes involved in ammonium uptake in under-ice freshwater Verrucomicrobia.

The 10 most abundant families across all samples are members of these four phyla. The actinobacterium ACK-M1 was by far the most abundant family in most samples, with the exception of the Bottle Control samples (Figure 2(a)). It was particularly dominant in the urea and nitrate treatments, comprising up to 27.7% of all ASVs in an individual sample. Members of the Comamonadaceae and the Flavobacteriaceae were more abundant in the control samples than the treatment samples (Figure 2(a)). The members of Sphingomonadales (Figures 1(c) and 2(a)) have the capability to degrade a broad array of hydrocarbons. These organisms are also known to inhabit oligotrophic environments, suggesting that carbon may be a key factor in shaping the overall bacterial community in Lake Shenandoah (Aylward et al. 2013; Kertesz et al. 2017). The total family composition was at least 80% similar across all replicates with the exception of three, which were at least 60% similar to all samples (Figure 2(b)).

**Variation in individual community members**

Despite no significant difference in community makeup at the phylum level, individual members of the bacterial community varied between nitrogen treatments. Excluding unassigned ASVs, there were a total of 9,988 unique ASVs identified across all samples. The vast majority of these ASVs (85.0%) were only identified in one single treatment, with 54.0% only identified in one of the two control treatments (Figure 3). The number of unique ASVs in the two control treatments (Environmental Control and Bottle Control) far exceeded those in the nitrogen amended treatments, at 76.8% and 75.2% of the treatments’ total ASVs respectively (Figure 3). The greatest number of ASVs shared between samples was 282, shared between the control treatments (Figure 3). Only 89 ASVs were shared between all treatments.
seven treatments, although this is the greatest number shared between any individual N treatment in the experiment (Figure 3). Zero ASVs were shared exclusively between all of the N treatments (Figure 3). Of the N treatments, the most diverse in terms of number of ASVs identified were the urea treatments, followed by the nitrate treatment, with the ammonium treatments resulting in the fewest number of ASVs identified (Figure 3). Of the 89 ASVs shared between all treatments, only three individual ASVs comprised >1% of the total community when averaged across all samples. These three individuals were an ACK-M1 ASV (6.6%), a Microbacteriaceae ASV (2.0%), and a Prosthecobacter ASV (1.3%).

**Bacterial community diversity**

Interestingly, the differences in ASV abundance were not reflected in the diversity measurements, as differences in $\alpha$-diversity metrics were largely insignificant across treatments (Figure 4). This is not unique to our study, as seasonality has been shown to be a stronger driver of diversity than single nutrient availability in freshwater lakes (Peura et al. 2012). Even when nutrients have been found to drive bacterial community composition in shallow lakes, total carbon has a stronger impact than either nitrogen or phosphorus on community structure (Li et al. 2011). Previous work using DNA-SIP in Arctic marine waters demonstrated that members of the Proteobacteria and Firmicutes are able to incorporate 15-N urea during winter months, suggesting including a functional metric in our analysis may have broadened our insight into the influence of nitrogen sources on community structure and physiology (Connelly et al. 2014). In soil systems, addition of urea only shifted $\alpha$-diversity at levels 10–50 times higher than those applied in our treatments; however, application of urea to soil communities resulted in a shrinkage of diversity of the bacterial community (Staley et al. 2018). Additionally, this large observed difference may also be due to deeper sequencing for one of the duplicates each for samples Bottle Control and Environmental Control, which had to be resequenced due to initial sample loss.

Community richness ($\alpha$-diversity) was relatively stable despite nitrogen amendments, as only the ammonium condition was significantly different from the control treatment (Figure 4). As bacterial community composition has been found to be tightly coupled with primary producers in freshwater systems, and the nitrogen amendments did not substantially shift the population of primary producers (at least at the prokaryotic level), this could be reflected in the static bacterial community richness. At the individual ASV level, there was quite a bit of heterogeneity both between samples and within the same treatments, which could have tempered changes in measured community richness.

Both metrics used to measure $\alpha$-diversity were higher for the control treatments than any of the N treatments. Among the N treatments, the urea treatments had a slightly higher diversity compared to the other two (ammonium or nitrate). In fact, for both measurements of $\alpha$-diversity, only the ammonium treatment was significantly lower than the control treatments using the Shannon metric ($p < 0.05$; Figure 4).
\(\beta\)-diversity was compared between treatments using both phylogenetic (UniFrac) and non-phylogenetic (Bray–Curtis distance) ordination. Larger separation between control and nitrogen treated samples occurred using the Bray–Curtis non-phylogenetic method than with the unweighted UniFrac method (Figure 5).

Previous work in eutrophic systems suggests that both seasonality and availability of multiple nutrients (carbon and phosphorus in addition to nitrogen) may drive larger shifts in bacterial community structure in lakes (Newton & McMahon 2011; Peura et al. 2012). As these samples do not reflect a seasonal gradient, but rather a single snapshot of the community, possible large shifts in bacterial community structure during spring or fall seasons may not have been captured (Newton & McMahon 2011; Ávila et al. 2016). Given that single nutrient additions have failed to stimulate large shifts in lake bacterial community structure, it is unsurprising that we demonstrated a similar community response to nitrogen additions in Lake Shenandoah (Newton & McMahon 2011).

CONCLUSIONS

The structure of the bacterial community within Lake Shenandoah, particularly the dominant phyla (Actinobacteria and Proteobacteria), were similar to other freshwater lake ecosystems worldwide. Addition of various nitrogen sources did not significantly shift the bacterial community of the surface waters of the system. This observation suggests that nitrogen may not be limiting in Lake Shenandoah, and other nutrients such as phosphorus or carbon or seasonality may constrain the system; however, more detailed biochemical studies of the system will be required to define the nutrient profile of the lake.

ACKNOWLEDGEMENTS

This work was supported by the Eppley Foundation for Research. We would like to thank Taylor Wright for assistance with sample collection and Steve Cresawn for assistance with computing access for data analysis.

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