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Survey of microbial urea degrader diversity in two freshwater ecosystems: Lake Shenandoah and the Shenandoah River

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Survey of microbial urea degrader diversity in two freshwater ecosystems: Lake Shenandoah and
the Shenandoah River

An Honors College Project Presented to
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College of Science and Mathematics
James Madison University

by Naomi Elizabeth Gilbert

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Accepted by the faculty of the Department of Biology, James Madison University, in partial fulfillment of the requirements for the Honors College.

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Abstract

One of the primary drivers of cyanobacterial harmful algal blooms (cHABs) in freshwater systems is nutrient loading, particularly of nitrogen and phosphorus. There has been an increased focus on assessing the role of nitrogen (N) in freshwater lakes and rivers that suffer cHABs. Urea, a widely-used, N-rich fertilizer, is a source of interest due to its abundance in freshwater ecosystems, primarily caused by anthropogenic nutrient loading. While recent work has shown that cHAB population succession may favor the toxic cyanobacterium *Microcystis* in urea-rich waters, the diversity of the associated bacterial community capable of degrading urea has yet to be determined. Therefore, we generated targeted sequence libraries of the gene encoding for the alpha subunit of the urease enzyme, *ureC*, from samples collected during summer (2015) from two model freshwater systems, Lake Shenandoah (LS) and the Shenandoah River (SR) (Virginia), to reveal potential urea-degrading members in threatened freshwater ecosystems. The total microbial community with urea-degrading capabilities was dominated by Proteobacteria in all samples, while Cyanobacteria was present in low abundance. This may be a result of the physical environment of LS and SR, or the low abundance of Cyanobacteria may be due to limitations in the custom database constructed for *ureC* amplicon analysis. LS and SR communities were similar overall, with the exception of a higher relative abundance of Acintobacteria *ureC* sequences in SR. Further analyses will aim to characterize more members with the *ureC* gene and expand upon the foundation built for community analyses based on *ureC*. These results provide better insight into the diversity of an important gene involved in bacterial urea degradation needed to resolve the microbial freshwater urea cycle.

Introduction

Introduction to Cyanobacterial Harmful Algal Blooms

Cyanobacterial harmful algal blooms (cHABs) are communities of algae containing toxic cyanobacteria that plague fresh, marine, and brackish water. Unlike beneficial algae which are crucial to the health of many aquatic ecosystems, cHAB-forming organisms include *Microcystis aeruginosa*, *Planktothrix spp.*, *Anabaena spp.*, *Nostoc spp.*, etc. that release cyanotoxins, such as microcystin and anatoxin. Accumulation of these toxins in the water column degrades aquatic ecosystems, harming biota in and around the body of water affected. For example, cyanotoxins in the Chesapeake Bay estuary (Virginia) were first detected in 2000s, and since then exceed World Health Organization guidelines for microcystin concentration (Tango and Butler, 2008)

Not only do cyanotoxins released by cHABs impact animals and other aquatic organisms, human health can be compromised when toxins exceed 0.3 µg/L-1.6 µg/L in drinking water according to Environmental Protection Agency (EPA) standards (EPA, 2015a). Microcystins have been found to induce liver problems in humans when ingested (Nishiwaki *et al.*, 1992; Falconer *et al.*, 1981) and are known to impact human health via irritation (mild skin rash), gastroenteritis, and liver disease (Bell and Cod, 1994). The EPA has previously filed health advisory reports after the detection of microcystins in drinking water (EPA, 2015b) and the City of Toledo was left without drinking water for three days due to microcystin in Lake Erie (Steffen *et al.*, 2017, *In review*). Additionally, cHABs negatively affect tourism and recreation due to their appearance, smell, and toxicity and therefore decrease both the economic and ecological value of areas surrounding the bloom. These consequences highlight the need to manage and reduce the occurrence of cHABs in order to preserve and restore freshwater and marine habitats worldwide.

Nutrients and cHAB Microbial Communities

Accelerated eutrophication of lake waters is linked to climatic and agricultural trends due to nutrient runoff, triggering massive algal blooms in recent years (Michalak *et al.*, 2013, Qin *et al.*, 2010). Increasing temperature due to climate change combined with elevated nutrient concentrations in the water column support the growth of harmful algal bloom species (Davis *et al.*, 2009). Specifically, anthropogenic sources of microbial growth-limiting nutrients have been shown to produce a favorable environment for cHABs (Schindler and Vallentyne, 2008), especially in freshwater environments (Paerl, 2001). If trends in nutrient loading persist, blooms are expected to recur with increasing intensity. Thus, managing anthropogenic nutrient pollution of lakes is an important step in controlling the occurrence of algal blooms for at-risk areas (Brookes and Carey, 2011; Paerl *et al.*, 2016). However, it is still poorly understood how specific forms of nutrients influence bloom severity and microbial community composition.

Both nitrogen (N) and phosphorus (P) are associated with the success of freshwater cyanobacterial blooms, and understanding the influence of both is key to managing algal blooms (Schindler, 1997, Conley *et al.*, 2009, Gobler *et al.*, 2010, Finlay *et al.*, 2010,). Bloom community structure can vary in response to the composition and availability of nutrients present in eutrophic waters (Anderson *et al.*, 2002, Heisler *et al.*, 2008). However, there is uncertainty and need for data about how N and P contribute to dominance of harmful microbial species (Schindler *et al.*, 2008, Scott and McCarthy, 2010). Historically, P has been considered the primary limiting nutrient in freshwater ecosystems (Schindler, 1977). However, in recent years it has been strongly suggested that a dual-nutrient management strategy focusing on both P and N is needed to prevent future bloom occurrence (Paerl *et al.*, 2016).

Non-atmospheric N may play an important role in the abundance of toxic cyanobacteria such as *Microcystis* spp. (Orr and Jones, 1998, Watanabe and Oishi, 1985), particularly at high temperatures (Codd and Poon, 1988). Furthermore, bloom growth has been stimulated in freshwater reservoirs and lakes during late summer and early fall months after enrichment with N sources, promoting toxin-producing species (Gobler *et al.*, 2007, Moisander *et al.*, 2009, Paerl *et al.*, 2015). With strong evidence of N influencing cHAB success, it is important to understand nutrient dynamics and its influence on cHAB communities by a thorough examination of how specific forms of N impact bloom development.

Importance of Reactive Nitrogen in Freshwater systems

N is a key limiting nutrient for eutrophication in aquatic systems (Paerl *et al.*, 2014). Massive spikes in fixed N introduced into the system are mainly due to fertilizer from agricultural systems and industrial usage (Galloway *et al.*, 2008). When available, non-atmospheric sources of N (reactive N), such as nitrate (NO₃) and ammonium (NH₄), are the preferred N source for cyanobacteria due to the energetic costs to fix dinitrogen (N₂) (Herrero *et al.*, 2001). Therefore, freshwater systems with low N inputs tend to have communities dominated with diazotrophic cyanobacteria such as *Lynbya* spp. and *Anabaena* spp.. However, diazotrophic and non-diazotrophic cyanobacteria will effectively compete for reactive N when available (Moisander *et al.*, 2012). An influx of reactive N can shift the community structure where, in some cases, toxic algal bloom species dominate (Glibert *et al.*, 2006, Solomon *et al.*, 2012). Additionally, certain sources of reactive N may promote the dominance of the toxin cyanobacterium *Microcystis aeruginosa*, a common toxic species in cHABs (Davis *et al.*, 2010, Steffen *et al.*, 2014a).

Role of Urea in shaping bloom communities

Urea ($\text{CO}(\text{NH}_2)_2$), is a stable form of reactive N that purportedly contributes up to 50% or more of total N for microbial communities of aquatic systems (Solomon *et al.*, 2010). It is naturally found in ambient concentrations in the water, partly due to the regeneration via heterotrophic bacteria, but can also be introduced via external sources and impact N-cycling (Solomon *et al.*, 2010). Bacterioplankton can uptake urea utilizing using an ATP-binding cassette-type urea transporter and degrade it internally (Sachs *et al.*, 2006). Urea has been observed to largely satisfy the N needs and stimulate growth of plankton communities of several different species relevant in HABs (Anita *et al.*, 1991, Glibert and Terlizzi, 1999, Glibert *et al.*, 2006, Solomon *et al.*, 2010). The role that bacteria, specifically heterotrophic bacteria, play in urea cycling is still largely unknown.

Urea is suspected to have contributed to the development of harmful algal blooms via nutrient loading in both freshwater and marine ecosystems for over a decade (Glibert *et al.*, 2006). Anthropogenic loading of urea is prevalent and contributes to the total N of bodies of water it reaches, notably in the Chesapeake Bay (Lomas *et al.*, 2002). Urea is commonly found as a fertilizer and has been utilized over nitrate-based fertilizers over the past several decades (Figure 1). Due to its high efficiency and convenience, urea usage is expected to total 41,000,000 metric tons for 2013-2018 with East Asia, Africa and North America consuming the greatest percentages (Glibert *et al.*, 2006, Food and Agriculture, 2015). Investigating whether or not urea may be influencing cHAB community structure is important in understanding bloom development.

There is experimental evidence that the addition of urea to hypereutrophic freshwater systems can degrade water quality via increased algal biomass, toxin concentration, and presence

of toxin cyanobacteria (Finlay *et al.*, 2010, Davis *et al.*, 2010). When monocultures of several algal species were incubated with varying N sources, including urea, *M. aeruginosa* and the ubiquitous bacterium *Synechococcus spp.* cultures grew best when incubated with urea (Berman and Chava, 1999). In pure cultures, urea stimulated the growth of non-N₂ fixing bacteria (*M. aeruginosa*, *Planktothrix*) and prevented N₂-fixers (*Anabaena*) from growing (Finlay *et al.*, 2010), a result of “nitrogen control” in cyanobacteria (Herrero *et al.*, 2001). Not only was biomass increased in an experimental study by Davis *et al.*, 2010, microcystin toxin production increased 13-fold in summer months. Under incubation with different chemical forms of N (nitrate, urea, ammonium), the global transcriptional response of *M. aeruginosa* was unique in the urea treatment, where there was significant upregulation of gene transcription relative to the other treatments (Steffen *et al.*, 2014b).

In addition to the toxin-producing cyanobacteria community, heterotrophic bacteria, may also be influencing cHAB formation via nutrient transformation (Berg *et al.*, 2009, Morris *et al.*, 2008, Steffen *et al.*, 2012). Therefore, it is important to consider the entire microbial participants in cHABs when studying nutrient dynamics. Overall, it was worth noting that urea as a N source should be investigated as a driving factor for toxic bloom development.

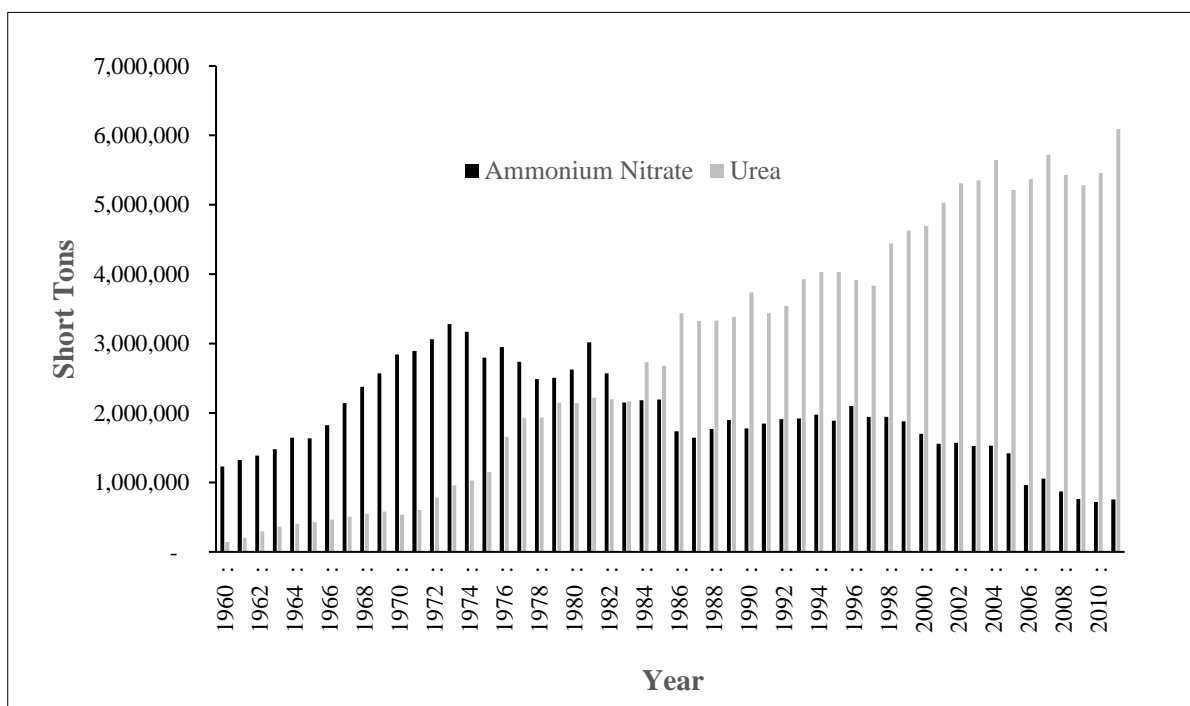


Figure 1. United States consumption of nitrogen materials from ammonium nitrate and urea from 1960 to 2011. A significant shift from consumption of N from ammonium nitrate to N from urea is apparent starting in 1984 and onwards. Data Source: <http://www.ers.usda.gov/data-products/fertilizer-use-and-price.aspx#26720>

Urease and the *ureC* gene

Microbial usage of urea is enabled by the activity of the urease enzyme complex (Solomon *et al.*, 2010). Urease hydrolyzes urea to ammonia and carbon dioxide (Figure 2). Bacterial urease consists of three subunits: α - (~65-73 kDa), β - (~8-12 kDa), and γ -subunit (~8-10 kDa) and requires a nickel (Ni^{2+}) cofactor for activity (Mobley and Hausinger, 1989). The genes encoding these subunits are *ureC*, *ureB*, and *ureA*, respectively. Additionally, *ureD-G* genes are associated with urease activity and encode for accessory proteins that may facilitate the binding of Ni^{2+} (Mulrooney and Hausinger, 1990).

The gene targeted in this study, *ureC*, codes for the large α -subunit, which contains the active site of urease. Although the *ureC* gene has not yet been directly targeted in cHABs, there is evidence of urea-degrading activity by bacteria associated with toxic freshwater blooms (Belise *et al.*, 2016, Steffen *et al.*, 2012). Urea distribution and urease activity was studied experimentally in Lake Erie via 48-hour nutrient amendments to *in-situ* mesocosms in order to examine the role different forms of dissolved N played in stimulating phytoplankton biomass (Belise *et al.*, 2016). Results indicated a strong correlation between urease activity and microcystin toxin concentration as well as an increase in the cyanobacterial population (Belise *et al.*, 2016). Additionally, metatranscriptome data generated from a 2014 bloom in Lake Erie indicated active transcription of urea transporter (*urtA*) and metabolism (*ureC*) genes by *M. aeruginosa*. Evidence of both *ureC* gene transcription and urease enzyme activity by important species in cHABs elicits the need to generate a comprehensive study examining all of the potential urea-degrading bacteria in cHAB communities.

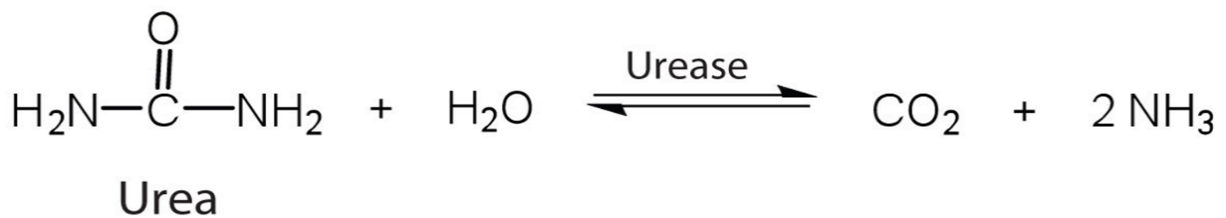


Figure 2. Hydrolysis of urea ($\text{CO}(\text{NH}_2)_2$) via the urease enzyme. One molecules of urea is degraded into carbon dioxide (CO_2) and two molecules of ammonia (NH_3).

Lake Shenandoah and the Shenandoah River

Lake Shenandoah (LS; Figure 3a) is located in Rockingham County near the City of Harrisonburg, Virginia, and serves as a valued fishing location for locals and tourists. Surrounded by residential homes and a golf course, the lake has suffered algal blooms where the cause points to anthropogenic nutrient loading (Virginia, 2015). Overall water quality and health of the lake has been an issue (Figure 3b). The Virginia Department of Game and Inland Fisheries has highlighted the risk of cyanobacterial algal blooms and looks forward to finding solutions to improve the water habitat (Virginia, 2015). Learning more about how external nutrient loading may be influencing bloom community members in Lake Shenandoah provides an important first step to restoring the health of the lake and will add to our understanding of cHABs in the Shenandoah Valley and the larger Chesapeake Bay Watershed. This lake serves as a model ecosystem in which high-throughput community analyses have yet to be done.

The Shenandoah River (SR) is the largest tributary of the Potomac River and consists of two forks. It runs through Virginia and a small portion of West Virginia, draining the Shenandoah Valley and Page Valley. Unfortunately, there have been numerous issues regarding the health of the river's ecosystem such as fish kills and annual algal blooms. The Virginia Department of Environmental Quality (VA-DEQ) deems the water impaired and polluted to the point where fishing, swimming, and drinking the water is unsafe (VA-DEQ, 2014). Cyanobacteria and species associated with toxin production are suspected to inhabit the algal blooms present throughout the river. Although this information has yet to be published, it has been recognized by a nonprofit organization dedicated to exposing the issues affecting the water quality of the Chesapeake Bay (<http://fosr.org/>). The Virginia Department of Game and Inland Fisheries (VDGIF) have also blamed toxic algal blooms as a potential source of abnormally high

fish kills (VDGIF, 2016). There have been efforts by the Shenandoah Riverkeeper, an advocacy group for healthy rivers, to mitigate the occurrence of algal blooms, yet little empirical research has been done to determine the most effective way to do so. With so much agricultural activity in the watershed, there is reason to suspect that excessive nutrient loading into the river may be leading to the nuisance blooms, or even to toxic blooms, however, this has never been assessed. Therefore, the Shenandoah River is a location of interest in investigating the influence of anthropogenic nutrient loading on toxic cyanobacterial algal blooms.

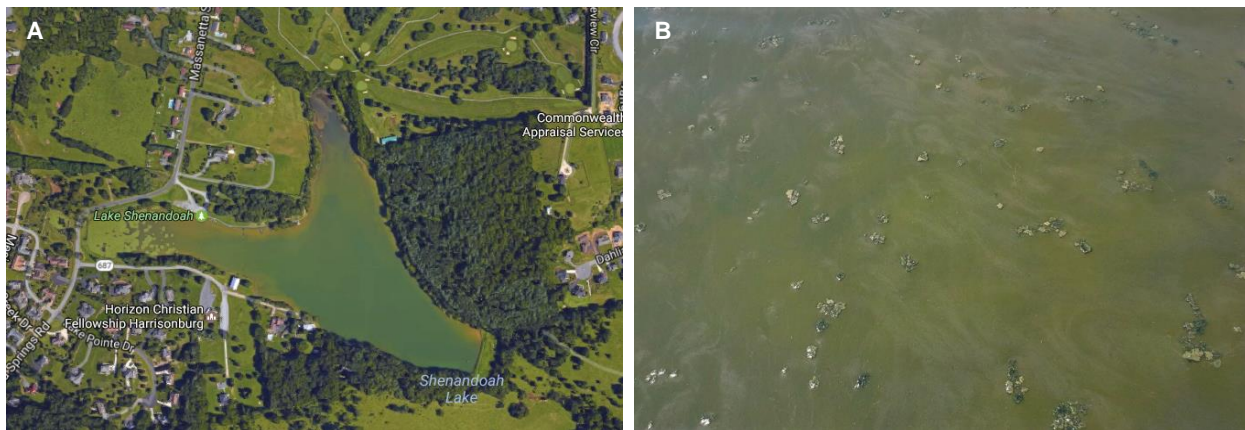


Figure 3. Lake Shenandoah study site. (A) Google maps image of Lake Shenandoah, Rockingham VA (2017). (B) Photo image of the water in Lake Shenandoah.

Project Goals and Aims

The community of urea degrading bacteria in LS and SR was characterized to develop a foundation for understanding the role of urea in freshwater ecosystems. Due to increased calls from both the scientific community and the public to manage and mitigate cHABs in lake environments, a better understanding of how the community is impacted by specific forms of limiting nutrients is needed. It is unclear “who” in these communities has the ability to use urea,

or if urea may be promoting the persistence of harmful members in these systems. This is largely because high throughput analyses that assess the community based upon functional genes involved in urea degradation is lacking. Therefore, we surveyed solely the urea degrading community by generating and sequencing amplicon libraries of the *ureC* gene in LS and SR samples over a temporal scale. This approach is as an essential first step needed to reveal key players in urea cycling, and ultimately contribute to future projects examining the functions of these members in relation to urea.

Materials and Methods

Sample Collection and DNA Extraction

During the summer of 2015, water samples were taken biweekly from LS in Rockingham, VA and the SR from June to September using a sterile 0.2 µm filter. DNA was extracted from each filter using the MoBio PowerWater® DNA Isolation Kit and stored for analysis. A subset of the samples was selected based upon quality to assess the community of urea degraders. Nutrient and environmental data were collected during sampling trips using the EXO2 Multiparameter SONDE (YSI, Ohio) water quality monitoring platform.

Targeted Metagenomics and Sequencing

To amplify the *ureC* gene, a universal primer set (HEDWIG (forward) 5'-GCTATCGGTCTCAAACCTTCAYGARGAY- TGGGG-3' and cTINP (reverse) 5'-GCAATACCATGCGCAATC- GCNGCNGGRTT DATNGT-3) was employed (Baker *et al.*, 2009). This primer set was previously demonstrated to target over 400 bacterial urease sequences from aquatic systems with the two sites being nearly universally conserved. Due to low DNA concentrations following PCR, a final volume of 100 µL was needed for each sample, done in duplicate, consisting of 0.5 µM of 2X EconoTaq® DNA Polymerase (Lucigen), Template DNA, 1 µM HEDWIG primer, 1µM cTINP primer, and sterile Milli-Q water. DNA extracted from the reference strain *Microcystis aeruginosa* NIES 843 was used as a positive control where water (no DNA) was used as a negative control. PCR was carried out in a cycle of 1 min at 80°C, followed by 24 cycles of: denaturing for 30 s at 95°C, annealing for 1 min at 55°C initially, decreasing 0.3°C per cycle (end at 47.5°C at the last cycle), elongation of 2 min at 72°C. After the 24 cycles, and a 10 min final elongation at 72°C. PCR clean-up using the UltraClean® PCR Clean-Up Kit

(MOBIO Laboratories, Inc.) was done for each sample. Positive amplification of the product was confirmed with gel electrophoresis. The concentration of DNA and sample quality was obtained using the Qubit dsDNA HS Assay Kit.

Due to the presence of non-specific binding (primer dimers) caused by the primers, primer dimer removal was performed at Lucigen Corporation (NGS Services). An additional step to increase DNA concentration in each sample was also performed at Lucigen (Nextara XT DNA Library Preparation Kit). Amplicons were sequenced using the Illumina Mi-Seq platform.

Microbial Community Analysis

ureC amplicon sequences were processed and analyzed using the QIIME 1.9.1 software pipeline (Caporaso *et al.*, 2010) with necessary adjustments made to accommodate functional gene (*ureC*) diversity rather than the commonly assessed 16S rRNA gene diversity. Before performing the workflow, a custom reference database was constructed to replace the use of more common databases (greengenes, SILVA) typically used in QIIME. The database consists of two parts:

1. A fastA formatted file containing sequences from a NCBI BLAST search using the targeted portion of the *ureC* sequence from the reference strain *M. aeruginosa* NIES 843. ~4000 hits from ~70-100% similarity with the reference were aligned and exported into a FASTA format using the program *MEGA* version 4 (Tamura *et al.*, 2007).
2. An ID-to-taxonomy file containing the taxonomic string (phylum;class;order;family;genus;species) of each entry in the database. This file was generated with the Python script *entrez-qiime.py* (Baker, 2016) using the

database file and data from the NCBI taxonomy database
(<https://www.ncbi.nlm.nih.gov/taxonomy>).

Raw Illumina reads were prepared by quality filtering and joining demultiplexed samples. Operational Taxonomic Units (OTUs) were picked (open-reference) and grouped (determined at $\geq 97\%$ similarity) using the QIIME default, USEARCH (Edgar, 2010). Taxonomic assignment of representative OTUs was determined by clustering against the custom database ($\geq 50\%$ similarity) using the QIIME default, RDP Classifier (Wang *et al.*, 2007). Raw taxonomic abundance data was converted for visualization into BIOM formatted bar charts (McDonald *et al.*, 2012) and adjusted using Microsoft Excel.

Results

Environmental Conditions

Water conditions of LS and SR were monitored over the 2015 summer sampling months for each sample assessed. Environmental data were collected using the EXO2 Multiparameter SONDE (YSI, Ohio) and included water temperature ($^{\circ}\text{C}$), water conductivity ($\mu\text{s}/\text{cm}$), salinity (psu), chlorophyll concentration ($\mu\text{g}/\text{L}$) and fluorescence (RFU), blue-green algae phycocyanin (BGA-PC), and total dissolved solids (mg/L). Data from July 13, 2015 to August 18, 2015 were collected for LS samples, and data on September 11, 2015 were collected for the SR sample (Table 1). SR BGA-PC and chlorophyll fluorescence was not reported for SR (Figure 4). The general trends of selected environmental conditions (Temperature, Chlorophyll concentration, and BGA-PC) over time for LS and SR were derived from the SONDE data taken daily over the entire summer (Figure 4).

Sample Recovery, Quality Filtering, and OTU picking

A total of nine samples from LS and SR were recovered after sample processing and Illumina sequencing (Table 2). Only LS sample #3 (collected July 13) and LS sample #6 (collected Aug-10th) were obtained in duplicate. There were 95,000 to 348,000 amplicons recovered from LS and SR samples, with an average sequence length of 238 nucleotides (Table 2). All samples had sequences removed after the quality filtering step in QIIME due to short read length (Table 2). There were a total of 68,433 different OTUs observed throughout the samples and all samples had a subset of their sequences assigned an OTU (Table 2).

Table 1. Environmental data collected using the EXO2 SONDE during Lake Shenandoah and Shenandoah River sampling dates. Conductivity, salinity, chlorophyll (Chlor) relative florescence units (RFU), and Total Dissolved Solid (TDS) concentration of the water are reported.

Date	Conductivity ($\mu\text{s}/\text{cm}$)	Salinity (psu)	Chlor (RFU)	TDS (mg/L)
30-Jun	N/A	N/A	N/A	N/A
13-Jul	562.29	0.26	0.94	357.67
21-Jul	589.77	0.26	0.60	356.90
4-Aug	555.92	0.25	0.93	345.59
10-Aug	518.75	0.25	1.50	334.08
18-Aug	562.33	0.25	0.64	342.80
11-Sept (SR)	439.35	0.21	N/A	291.59

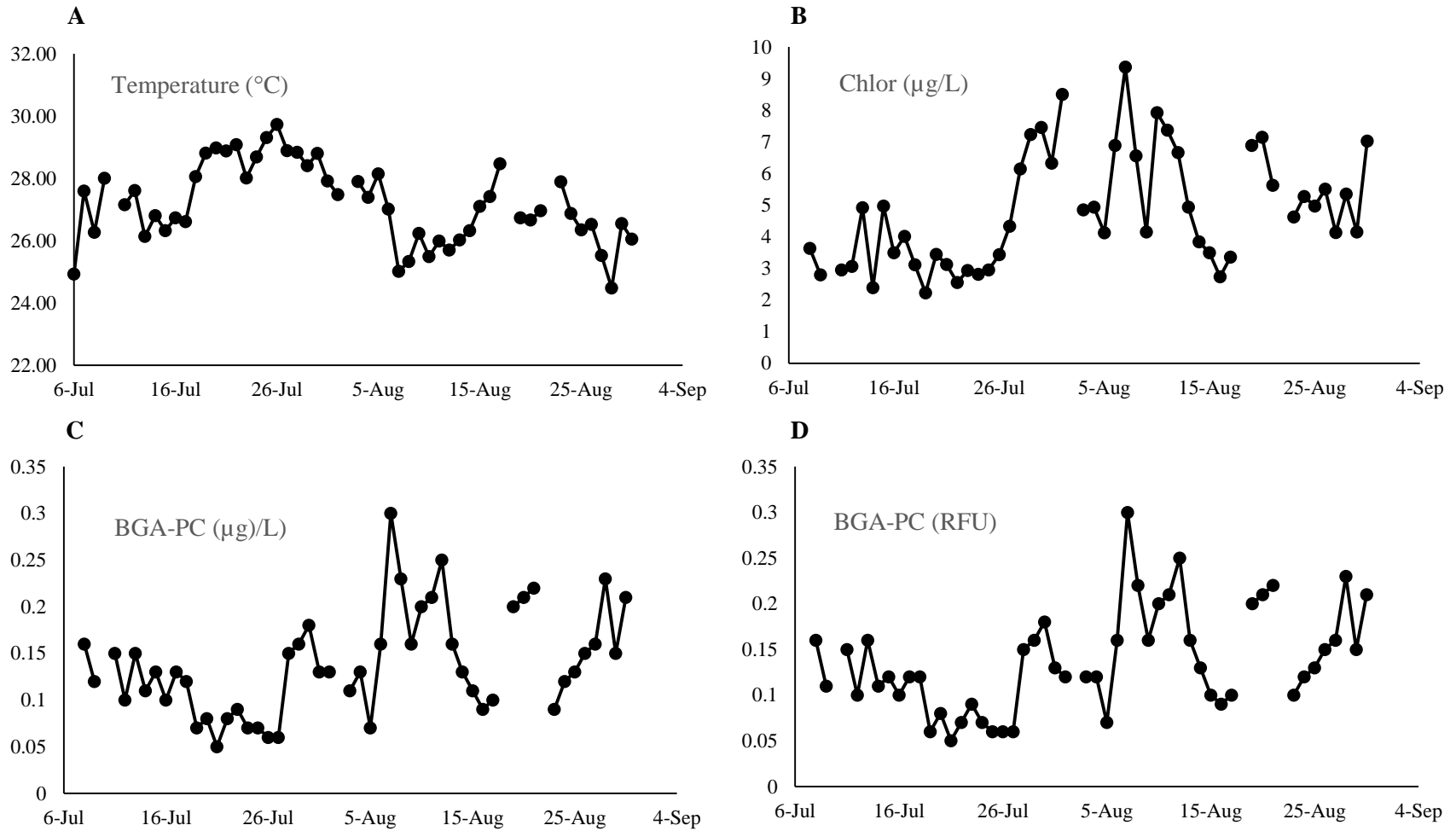


Figure 4. Environmental trends in the water quality of Lake Shenandoah (June-Sept). A) Temperature (°C) trends. B) Trends in Chlorophyll (Chlor) concentration (µg/L) . C) Trends in blue-green algae phycocyanin (BGA-PC) concentration (µg/L) D) BGA-PC relative fluorescence units (RFU).

Table 2. Sample identification and sequence information for Lake Shenandoah (LS) and Shenandoah River (SR) amplicon libraries. Mean sequence length shown after quality filtering in QIIME. Total number of sequences written shown after removal of short read length in QIIME. OTUs clustered at $\geq 97\%$ similarity using the QIIME default USEARCH.

Sample ID	Date Collected	Mean sequence length (nt)	# Sequences before quality filtering	Total # Sequences written	# Sequences assigned an OTU
LS1	Jun 30 2015	231	278,146	252,457	224,253
LS3A	Jul 13 2015	251	112,630	72,235	63,798
LS3B	Jul 13 2015	238	257,951	213,377	185,006
LS4	Jul 21 2015	232	347,688	328,041	285,814
LS5	Aug 04 2015	238	95,318	67,062	61,738
LS6A	Aug 10 2015	234	182,915	163,284	142,697
LS6B	Aug 10 2015	230	208,877	190,283	166,996
LS7	Aug 18 2015	250	274,366	173,596	146,348
SR1	Sep 11 2015	235	252,900	223,133	196,334

Urea-degrading Microbial Community

Taxonomy assignment and abundance of OTUs in each sample were determined in QIIME using the custom *ureC* reference database. A threshold of $\geq 50\%$ sequence similarity of the OTU sequence and reference sequence in the database was set due to the limitations of the database. Thus, “unassigned” OTUs made up 21.2% of the total community in the samples (Table 3)

The most abundant OTUs were of the phylum Proteobacteria, comprising 75.5% of the total urea-degrading community throughout all samples (Table 3, Figure 5a). Betaproteobacteria, Gammaproteobacteria, Alphaproteobacteria, and “Other” comprised 27.2%, 16.6%, 16.5%, and 14.4%, respectively of the Proteobacteria community (Table 3, Figure 5a). Burkholderiales made up 24% of the Betaproteobacteria community (Figure 5b), with *Burkholderia* being the most abundant genus (12.9%) (Table 4). Pseudomonadales made up 12.9% of the Gammaproteobacteria community, with *Pseudomonas* as the most abundant genus (12.9%) (Table 4). In the Alphaproteobacteria phylum, class Rhizobiales was the most abundant (10.3%) (Figure 5b), with *Rhizobium* being the most abundant genus (2.1%) (Table 4).

The rest of the urea-degrading community was mostly comprised of Firmicutes (1.20%), Cyanobacteria (0.90%), Actinobacteria (0.80%), and Deinococcus-Thermus (0.20%) (Table 3). Bacillales was the most abundant class in the Firmicutes phylum (Figure 5b), with *Bacillus* being the most abundant genus (0.90%) (Table 4). *Synechococcus* (0.4%) and *Chroococcidiopsis* (0.2%) were the most abundant genera of Cyanobacteria (Table 4). Several genera in small abundances made up the Actinobacteria phylum community, with *Streptomyces* the most abundant (0.3%) (Table 4).

The community composition shown is relatively consistent in June through July, but shifts in the beginning of August towards a community with more unassigned taxa and Firmicutes, and less Betaproteobacteria (Figure 5). However, another shift in the community was observed in the second sampling date of August where Betaproteobacteria and Alphaproteobacteria dominate (Figure 5). The microbial community of the Shenandoah River (SR1) reflects that of LS, with the exception of a higher relative abundance of Actinobacteria in SR (Figure 5).

Table 3. Phylum and class taxonomy assigned to abundant OTUs in total. Phylum is shaded in grey and corresponding class shown below in white. Total abundance shown is the average of all the samples.

Taxon (phylum; class)	Total Abundance (%)
Proteobacteria	75.5
Betaproteobacteria	27.2
Gammaproteobacteria	16.6
Alphaproteobacteria	16.5
Other	14.4
Deltaproteobacteria	0.50
Epsilonproteobacteria	0.30
Unassigned	21.2
Firmicutes	1.20
Bacilli	1.20
Cyanobacteria	0.90
Cyanophyceae	0.90
Actinobacteria	0.80
Actinobacteria	0.80
Deinococcus-Thermus	0.20
Deinococci	0.20

Table 4. Phylum and genus ranking assigned to abundant OTUs in total. Phylum is shaded in grey and genera within each phylum is shown below in white. Total abundance shown is the average of all the samples.

Taxon (phylum; genus)	Total Abundance (%)
Betaproteobacteria	27.2
<i>Burkholderia</i>	12.9
<i>Other (Burkenholderiales)</i>	5.70
<i>Other</i>	1.50
<i>Nitrosomonas</i>	1.00
<i>Bordetella</i>	0.80
<i>Pandoraea</i>	0.60
Gammaproteobacteria	16.6
<i>Pseudomonas</i>	12.9
<i>Other</i>	1.70
<i>Enterobacter</i>	0.50
<i>Klebsiella</i>	0.30
<i>Azobacter</i>	0.30
Alphaproteobacteria	16.5
<i>Rhizobium</i>	2.10
<i>Other</i>	2.60
<i>Bradyrhizobium</i>	1.70
<i>Mesorhizobium</i>	0.90
<i>Asospirillum</i>	0.90
Other	14.4
Unassigned	21.2
Firmicutes	1.20
<i>Bacillus</i>	0.90
Cyanobacteria	0.90
<i>Synechococcus</i>	0.40
<i>Chroococidiopsis</i>	0.20
Actinobacteria	0.80
<i>Streptomyces</i>	0.30
<i>Other</i>	0.10
<i>Micrococcus</i>	0.10
Deinococcus-Thermus	0.20
<i>Deinococcus</i>	0.20

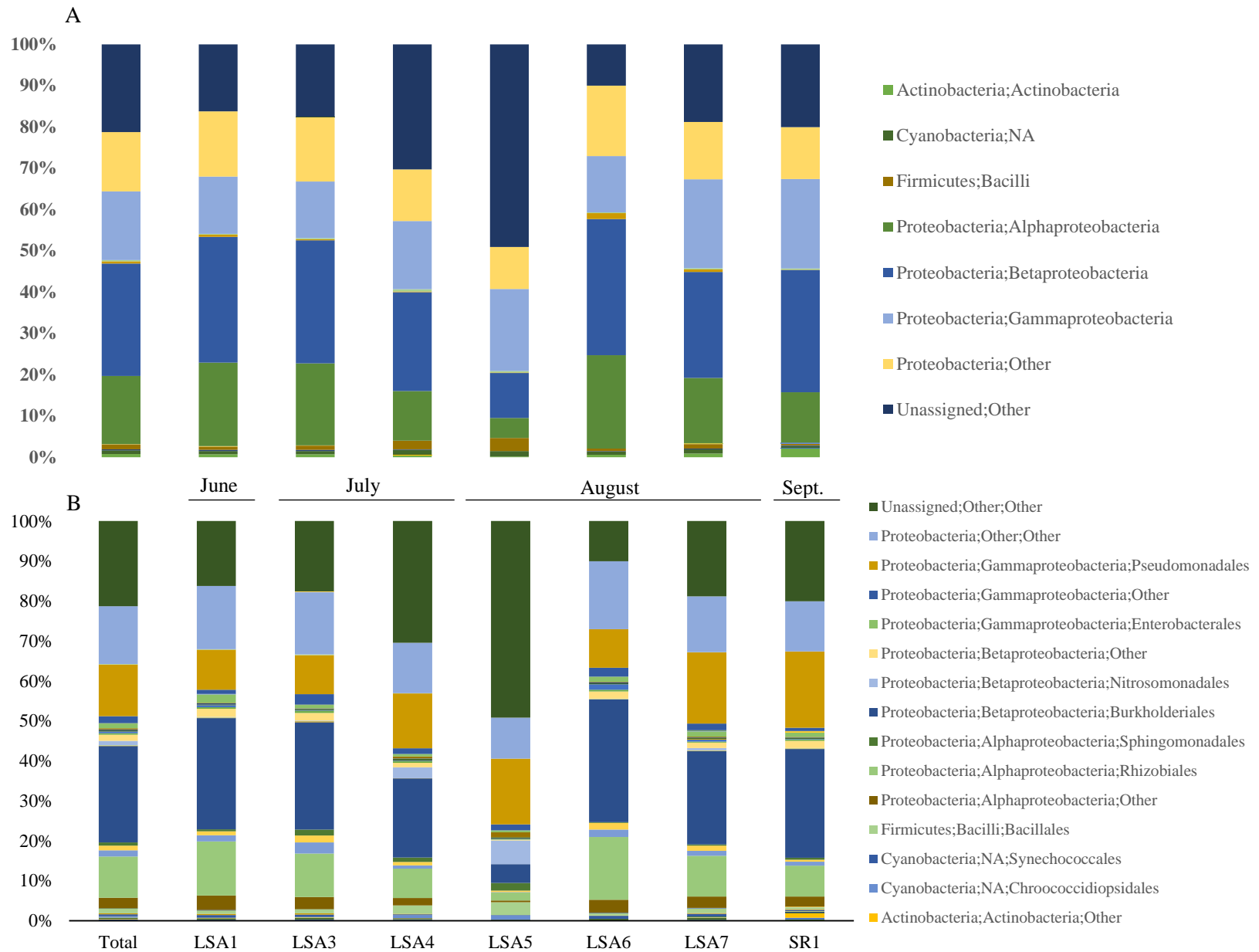


Figure 5. Relative abundance of LS and SR urea-degrading taxa grouped by sampling month (June-August). Legends display most abundant taxa found in total. Sequence information can be found in Table 2. A) Relative abundance of taxa on the *class* level. B) Relative abundance of taxa on the *order* level.

Discussion

Purpose of Study

Experimentally, urea has been shown to increase the growth of species related to cHABs, resulting in a population shift leading to dominance of non-N₂ fixers such as *M. aeruginosa* (Finlay *et al.*, 2012, Davis *et al.*, 2010). Therefore, the role of anthropogenic urea in the eutrophication of freshwater ecosystems needs to be further assessed (Glibert *et al.*, 2006). However, no previous studies have utilized high-throughput community analyses to examine the diversity and abundance of taxa with a gene important to urea degradation in bacteria (*ureC*). This study is the first to characterize the community of urea degrading bacteria in a eutrophic lake and river through targeted metagenomics (16S) analysis of *ureC*.

Lake Shenandoah (LS) and the Shenandoah River (SR) were sampled during summer months (2015) to study urea-degrader bacterial diversity. These systems are surrounded by, and therefore influenced by, both agricultural and urban areas. Currently, these freshwater systems are being monitored by state agencies due to the quality of the water and reported fish and bird kills, which may suggest the presence of toxic algae (VA-DGIF, 2015, 2016), but no empirical analyses of the microbial communities have been completed. Therefore, these study sites were chosen as model ecosystems to examine the urea-degrading microbial community of threatened freshwater environments.

Environmental Conditions of Lake Shenandoah/Shenandoah River

Environmental data collected at each sampling date showed higher water temperature, and a peak of blue-green algae (BGA) phycocyanin (PC) in August (Figure 4b and 4d). cHABs are comprised of BGA that include toxic cyanobacteria such as *M. aeruginosa* and *Anabaena*

spp. The presence of blue-green algae in LS and SR are expected with higher temperature, increased nutrient concentration, and other factors. Nutrient concentrations, such as ammonium and nitrate and others, could not be determined due to technical issues with the EXO SONDE. However, a nutrient analysis to determine the concentrations of importance nitrogenous compounds, including urea, in water samples should be done in order to gain a better understanding of the environmental conditions driving microbial community structure.

Sample Preparation and Custom Database Limitations

The *ureC* gene was amplified in a total of nine different samples taken from June to September 2015 to examine the community composition over a temporal scale. 95,000 to 348,000 amplicons were generated from each sample, resulting in a mean sequence length of 238 nucleotides after paired-end read joining (Table 2). The universal primers that were used in this study were designed by Baker *et al.* 2010, and targeted a 617-632 base pair region of *ureC*. PCR amplification using these primers resulted in one ~600 bp fragment for each samples, but only a fraction of the targeted portion of *ureC* was sequenced. This is due to the library preparation methodology for targeted gene sequencing with the Illumina Mi-Seq platform that is meant to sequence smaller fragment lengths.

The QIIME (Quantitative Insights into Microbial Ecology) bioinformatics pipeline was used in this study to examine the microbial communities of LS and SR. Quality filtering of the LS and SR raw reads, and open reference OTU-picking resulted in a smaller subset of dataset that was used in the taxonomic assignment step (Table 2). A minimum threshold of $\geq 50\%$ sequence similarity of each representative OTU to the reference database was set instead of the QIIME default of $\geq 90\%$ to confirm a hit to the database. Additionally, a large percentage (21.2%) of the OTU's in the total community were unassigned (Table 2). While unassigned taxa

are not rare in community studies, their high abundance in this study may be due to the limitations of the custom *ureC* reference database. Unlike studies of the well-characterized nitrogen fixation gene, *nifH* (Heller *et al.*, 2014), there is currently no collection of aligned *ureC* sequences in an established database. Furthermore, there is a limited availability of *ureC* genes derived from whole genome sequences in the NCBI database (used to create a QIIME-compatible reference) that complement a wide variety of species, narrowing the scope of species in the database. Thus, a more comprehensive database representing *ureC* of a wider variety of taxa should be constructed by examining *ureC* clone library studies to gain better coverage.

Taxonomic Structure of the Urea-Degrading Microbial Community

LS and SR urea-degrading community structures were assessed at multiple taxonomic levels. Generally, the taxonomic structure was consistent throughout the summer collection, with the exception of the sample collected on August 4, 2015 (Figure 5). There were more unassigned OTUs than there were OTUs assigned to Proteobacteria relative to the other communities (Figure 5). This may be due to issues with the quality of the amplicon library in this sample. This sample contained the fewest number of raw Illumina reads, and a large fraction of the sequences was removed following QIIME quality filtering (Table 2). Although a dissimilar relative abundance of taxa in the community cannot be fully attributed to a reduced number of sequences in the sample, it is worth noting that this sample did not match the quality of the others.

The communities in both LS and SR were dominated by the phylum, Proteobacteria (Table 3). Proteobacteria are present in many different environments and have diverse metabolic functions. *Burkholderia*, a genus that occupies a wide range of niches and environments (Coeyne and Vandamme, 2003), was the most abundant genus of the class, Betaproteobacteria (Table 4). *Pseudomonas* was the abundant genus in the Gammaproteobacteria phylum, and *Rhizobium*, a

symbiotic nitrogen fixer commonly found in soil, was most abundant in the Alphaproteobacteria phylum (Table 4). The presence of a high number of *Rhizobium* suggests a large contribution of soil bacteria from runoff surrounding LS and SR. However, the high abundances of Proteobacteria in all of the samples is not surprising given the ubiquitous nature of the phylum.

In contrast, the phylum Cyanobacteria was found in small abundance (0.9%) throughout all of the samples (Table 3), with the genus *Synechococcus* comprising the cyanobacterial majority (Table 4). It was surmised that a greater number of OTU's would hit to cyanobacterial species in the database due to the abundance of blue-green algae phycocyanins detected in LS (Figure 4). This lack of cyanobacterial urea-degraders in the community may be an artifact of the limited coverage in the custom reference database where cyanobacterial *ureC* sequences are underrepresented, thus leading to a large abundance of unassigned taxa. For example, clone libraries targeting the 16S rRNA and *ureC* gene constructed on these 2015 LS and SR samples indicated the presence of *Cyanobium* and *Synechocystis* (Fried *et al.*, 2017 *unpublished*), two cyanobacterial genera possibly underrepresented in the custom *ureC* database. The inclusion of more *ureC* genes from these organisms detected in LS and SR, as well as a wider range of cyanobacterial *ureC* sequences is necessary to further resolve this issue.

The lack of cyanobacterial urea-degraders in the community may alternatively be due to the nature of the environment in LS and SR, where Proteobacteria are likely to dominate. As reported in Steffen *et al.* (2012), nitrogen fixation and urea metabolism genes in cHABs of a hypereutrophic, shallow lake, Lake Tai (China), were mostly contributed by Proteobacteria, rather than Cyanobacteria, which comprised the majority of these genes in Lake Erie. The microbial communities of LS may be similar to that of Lake Tai were the aquatic community reflects the sedimentary community due to a shallow water depth allowing sediment

resuspension (Zhu *et al.*, 2007), resulting in the dominance of heterotrophic bacteria. This study highlighted the importance of heterotrophic bacteria as well as cyanobacteria in nitrogen cycling and urea metabolism, and how varying microbial communities may infer similar function in geographically isolated environments. Thus, the need to further study the involvement of heterotrophic bacteria as well as of cyanobacteria in nitrogen uptake and cycling in these systems is necessary.

Community Variation over a Temporal Scale

Temporal shifts in the structure of the potential urea degrading bacterial community in LS and SR were assessed from June to August/September (Figure 5). The community composition was similar between June and the first sampling date in July, but shifted during late July and early August. Here, an increased abundance of unassigned taxa and reduced abundance of Proteobacteria taxa occurred (Figure 5a). Notably, a decrease in the abundance of orders Burkholderiales and Rhizobiales, and an increase in Nitrosomonadales in early August (Aug 4) occurred (Figure 5b). It is known that Rhizobiales contains symbiotic N-fixing bacteria while Nitrosomonadales commonly consist of nitrifying bacteria (Garrity *et al.*, 2006). Although there is no conclusive evidence to support the shift in nitrogen fixing to nitrifying (transformation of ammonium to nitrate) bacteria due to nutrient changes, nitrogen flux in the water column due to external input (nutrient loading) could have played a role in altering LS microbial communities in this way.

Another noteworthy shift in the community occurred during mid-August, where the abundance of assigned OTUs decreased dramatically, and Proteobacteria dominance reemerged, along with an increased abundance of Rhizobiales (Figure 5). Again, due to the lack of nutrient data in this study, it is not definitive if nutrient availability played a role in this shift.

Cyanobacterial OTUs remained at low abundance in total, however were highest towards the end of August (Aug 10 and 18) where BGA-PC's were relatively high (Figure 4c and d). Lastly, the SR microbial community (Sept 11) generally reflected that of LS communities, with the exception of the high abundance (relative to LS), of Actinobacteria in this sample (Figure 5). The low abundance of this phylum in all LS samples is surprising due to the typical presence of Actinobacteria in lakes as well as rivers (Glöckner et al., 2000; Zwart et al., 2002). In lakes, Actinobacteria may be outcompeted by Cyanobacteria under high levels of inorganic nutrient availability, high organic matter, and high temperature (Ghai *et al.*, 2014). The possibility of these conditions in LS cannot be ruled out, thus potentially suppressing the presence of Actinobacteria.

Future directions

The goal of this study was to characterize the community of bacteria capable of degrading urea in two model freshwater ecosystems. Given evidence that indicates a shift in freshwater microbial communities that are dominated with cyanobacteria after the addition of urea (Finlay *et al.*, 2010; Belise *et al.*, 2016), we sought to examine if the taxa in this targeted community were predominantly cyanobacteria, or predominantly heterotrophic bacteria. This study should be applied to environments in which cHABs regularly occur to assess the members with the capability to degrade urea that may be exacerbating bloom severity. For example, Lake Erie (Canada-U.S border) and Lake Tai are commonly studied lakes due to the frequent occurrence of cHABs and nutrient loading due to surrounding agricultural activity (Paerl and Otten, 2013), and there has been evidence of the influence of urea on bloom growth and composition (Steffen *et al.*, 2014a, 2017 *in review*).

Resolving the phylogenetic composition of urea degrading bacteria is an important first step in assessing the role that urea plays to shape the microbial community structure in freshwater ecosystems threatened with nutrient loading and cHABs. In addition to screening lakes such as Lake Erie and Lake Tai, other future projects may include examining *ureC* gene expression in LS and SR identified taxa. Moreover, investigating the transport of urea from the environment into bacterial cells will add better context to urea uptake and cycling in lakes and rivers. Ultimately, this study adds to a greater project that seeks to determine the role urea may play in promoting harmful algal blooms, leading to better management strategies and bloom modelling.

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