Journal of the North Carolina Academy of Science, 129(2), 2013, pp. 58–65

CULTIVABLE MICROBIAL DIVERSITY IN AN ACIDIC TEMPERATE LAKE, LAKE PHELPS, NC

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Abstract: Despite the immense benefit of freshwater habitats to the day to day activities of humans, their biological diversity remains understudied. Microbial diversity and community structure is critical to the study of ecological processes in these habitats. The naturally acidic oligotrophic lake, Lake Phelps, NC, was studied within the context of understanding sediment dwelling meiofauna using the cultivable microbial communities at three sites in the water column and sediment. Twenty-six bacterial isolates were cultivated, biochemically tested and sequenced. DNA sequence analyses of the 16S ribosomal subunit revealed 26 taxa representing three phyla (12 Proteobacteria, 12 Firmicutes, and 2 Bacteroidetes), four classes (12 Bacilli, 6 β-proteobacteria, 4 γ-proteobacteria, and 2 Flavobacteria), and six bacterial orders (12 Bacillales, 6 Neisseriales, 2 Burkholderiales, 4 Enterobacteriales, and 2 Flavobacteriales). Bacterial communities at the three sampling sites were distinct and this may be because of site-specific features. The lake is believed to have been formed from the fusion of two Carolina bays with differing geochemical factors contributing to unique microenvironments. Current studies are in progress to examine associated meiofauna within each microbial community.

Key Words: Freshwater bacteria; microbial biodiversity; cultivable bacteria; acidic lake.

INTRODUCTION

Non-extreme aquatic environments are dominated by bacteria (Hahn 2006). Molecular studies in limnology have gained momentum because of recent advances in molecular technology and improvements in methodology (Percent et al. 2008). Microbial communities differ not only in terrestrial and aquatic habitats but also between marine and freshwater systems. Bacteria play an important role in freshwater ecosystems by performing the tasks of decomposing organic matter, assisting in the cycling of nutrients, and forming the base of the microbial food web (Percent et al. 2008). They are ubiquitously distributed in all types of lakes, with varying intra and inter-lake diversity (Yarnell and Tripett 2004). Bacteria found in these environments are still understudied despite their roles in lake ecosystems (Hahn 2006; Tamakia et al. 2005).

Conditions in lakes have direct temporal and spatial effects on microbial communities. Factors that influence microbial communities in lakes include: water chemistry, water temperature, zooplankton predation, protistan predation, phytoplankton composition, organic matter supply, intensity of ultraviolet radiation, habitat size, and water retention time, among others (Linstrom et al. 2005). Studies of extreme conditions, such as extreme pH in acidic or alkaline lakes, could bring to light new extremophiles that potentially could be applicable to the development of new bioprocesses and novel microbial products (Johnson 1998). Anthropogenic effects have prompted intense study of artificially acidic lakes but the lesser studied naturally acidic lakes (Percent et al. 2008) could harbor bacteria that evolved within those natural systems.

Most studies focusing on acidic lakes emphasize recovery from anthropogenic acidification. A U.S. Environmental Protection Agency study on the anthropogenic effects of acidification concentrated on the Adirondack lakes in the state of New York. These lakes had pH values ranging from 4.86 to 7.76. Overall bacterial community structure could not be correlated with pH but certain bacterial classes were correlated with acidity and revealed that anthropogenic activities have minor influences on lake acidification when compared to naturally occurring humic material found in surrounding areas (Percent et al. 2008). Community richness and diversity were both correlated with ion concentrations which affected pH. α-proteobacteria was negatively correlated with increasing pH, contrary to Bacteroidetes and Flavobacteria, which were positively correlated, suggesting that bacterial diversity can be

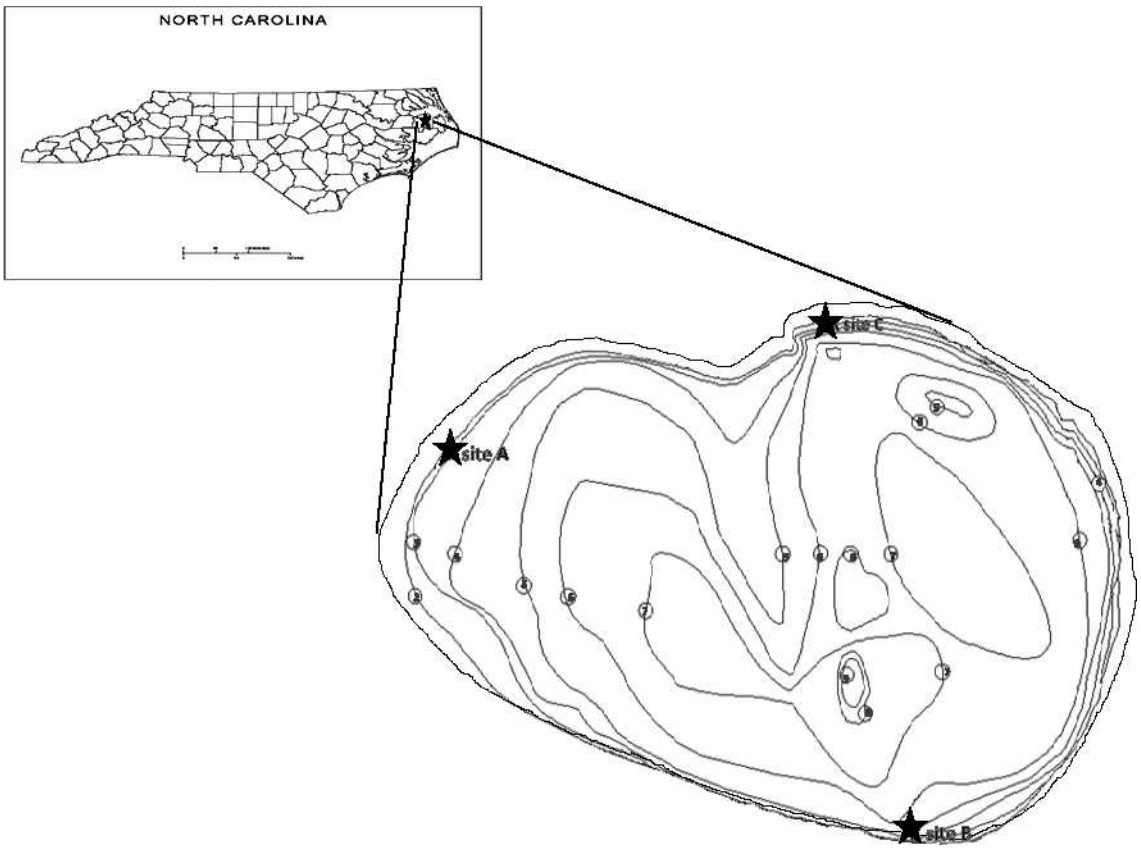

FIG. 1. Map of North Carolina with sampling site indicated in black star, and bathymetric map of Lake Phelps with locations of three sampling sites (black stars).

influenced by pH. While others, such as β-proteobacteria remained unaffected (Percent et al. 2008). This suggests that lakes have diverse bacterial communities, but are dominated by a small group of taxa (Hahn 2006).

This research explored the composition and diversity of microbial communities in the water column and sediment of a small acidic lake in North Carolina - Lake Phelps - in the context of the broader goal of exploring the relationship of bacterial communities with numerically dominant meiobenthic communities.

Study Area

Lake Phelps (35°77.24′N, 76°45.44′W) is a naturally acidic oligotrophic lake in the Pettigrew State Park system, North Carolina (Fig. 1). It is the second largest natural lake in the state with an area of 6,718 hectares. The lake has an average depth of 1.5 m and a maximum depth of 3 m. The lake water is clear with a pH of 4.1–4.6. The cause of acidity in the lake is unknown but is hypothesized to be from humic material and acid rain. It occupies one of the highest elevations in the area at 4 m. The lake is replenished by precipitation and maintained artificially at 3.0–3.5 m by water gates that flow into a canal from the lake. The lake is divided by a sandbar that rises 1.2 m above the bottom and extends southward to the middle of the lake, suggesting the lake was created through fusion of two adjacent bodies of water, possibly Carolina bays. To the west of this division is a regular and smooth bottom and to the east several depressions of 0.3–0.6 m. Sediment of the lake varies from brown and black mud to very fine sand. The mud was confirmed not to be peat but a fine organic detritus matter. A map from 1979 assumes that some movement of top layer of sediment has shifted slightly in the last 33 yrs. The three sites sampled consisted of organic matter stained sand (Site A), gray sand (Site B), and a mixture of organic stained sand and thick organic material similar to clay (Site C; Fig. 2). Elevated levels of mercury are reported from Lake Phelps, most likely from atmospheric deposition, leading to fish consumption advisories (Pasquotank river basin assessment 2011). Some houses are on the lake but most are used as a recreational facility (www.ncparks.gov) (Allen et al. 1979).

MATERIAL AND METHODS

Sampling.—Water column and sediment samples were taken in triplicate from three separate sites (Fig. 1) about 10 m from where the water meets the shoreline (Allen 1979). A telescoping pole was used to obtain a 20 cm core

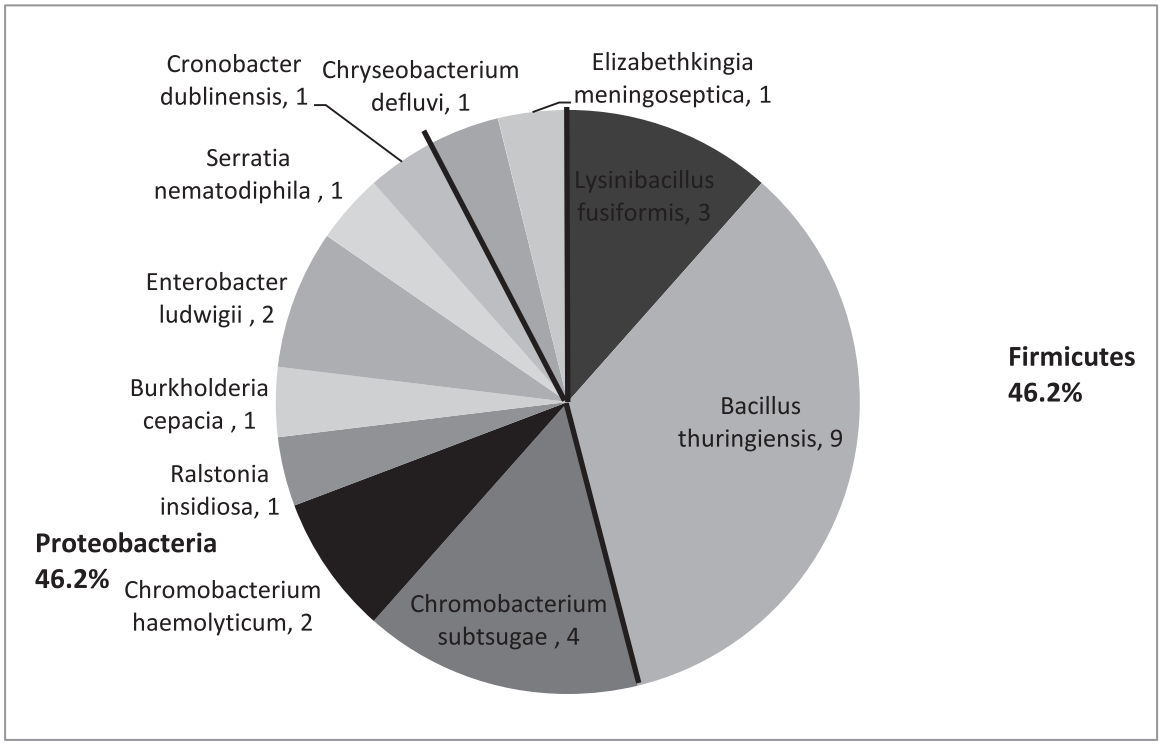

FIG. 2. Cultivable bacterial community composition of Lake Phelps.

of sediment samples and collected in polyethylene bottles. Five hundred milliliter of water samples were collected 10 cm deep after 4 rinses. Distances from the shore ensured that samples were taken from sediment permanently covered by water. Samples for microbial study were placed in a cooler with ice to ensure no degradation occurred during the approximately 1.5 hr drive back to Elizabeth City State University (ECSU). Water samples were immediately fixed using 10% HCl (v/v).

Organic matter determination.—The Loss on Ignition (LOI) method was used. Samples were dried in a Vulcan A-1750 oven (Ney Tech) at 100°C for 24 hr and weighed. Samples were then burned in a furnace at 500°C for 5 hr and weighed. Organic matter was calculated by subtracting ash weight from dry weight after furnace treatment (Campos 2010).

Bacterial isolation.—We used standard serial dilution methods to isolate bacteria from water and sediment samples. Either, 1g of sediment or 1ml of water sample was mixed in 10ml of sterile H_2O and serially diluted until a 10^{-10} dilution was obtained. One-hundred micro-liters of each dilution was spread on duplicate nutrient agar plates (Difco Laboratories) and incubated at room temperature (20 ± 2°C) which resembled Lake Phelps environmental temperature, for 5 days then bacterial colonies were counted, and morphologically distinct colonies distinguished by color, shape, margin, elevation, and texture were isolated in pure culture. Frozen stock was prepared from the isolated colonies from 18–24 hr. growth on LB broth (U.S. Biological) mixed in a 50/50 v/v ratio with 50% sterile glycerol, and stored at −80°C.

Bacterial count.—DAPI staining was used to enumerate bacteria of L. Phelps water, in addition to the bacterial counts obtained from the spread plate method as already described under bacterial isolation. Two hundred milliliters of sample were centrifuged to concentrate bacteria, removing 199 ml of water of supernatant. Settled bacteria were vortexed and resuspended. Nine microliter of bacterial suspension was stained with 1 µl DAPI for microscopic counting. Sixteen fields at 40× magnification, after resuspension of bacteria, were used. Counting of bacteria was done using Olympus IX51 Fluorescent microscope.

Biochemical characterization.—Bacterial characterization was conducted using traditional tests and rapid identification kits. Gram staining, growth in differential/selective media (i.e., MacConkey, Phenylethyl Alcohol Agar), oxidase, and catalase tests were performed according to established protocols (Leboeff et al. 2005). Gram negative bacteria were further evaluated using biochemical tests from the rapid identification Enterotube II system (BD Diagnostic Systems).

Identification of bacteria using 16S rDNA sequence.—DNA was extracted from fresh pure cultures of the bacterial isolates grown on LB broth for 24 hr, utilizing the MoBio UltraClean DNA isolation kit according to manufacturer's instructions. Successful extraction of DNA was verified by visualization after electrophoresis

Table 1. Cultivated Lake Phelps bacteria from water and sediment by site.

		Sites					
		Water			Sediment		
Bacterial Strains	GenBank Accession	A	B	C	A	B	C
Lysinibacillus fusiformis strain EAPL01	JX500173						x
Bacillus thuringiensis strain EAPL02	JX500174				x		
Chromobacterium subtsugae strain EAPL03	JX500175		x				x
Bacillus thuringiensis strain EAPL04	JX500176			x		x	x
Bacillus thuringiensis strain EAPL05	JX500177			x		x	x
Chryseobacterium defluvii strain EAPL06	JX500178			x			
Chromobacterium subtsugae strain EAPL07	JX500179		x				
Bacillus thuringiensis strain EAPL08	JX500180			x			
Lysinibacillus fusiformis strain EAPL09	JX500181			x			
Enterobacter ludwigii strain EAPL10	JX500182		x				X
Bacillus thuringiensis strain EAPL11	JX500183						X
Cronobacter dublinensis lausanensis strain EAPL12	JX500184						X
Chromobacterium haemolyticum strain EAPL14	JX500185			x			
Burkholderia cepacia strain EAPL15	JX500186		x				
Chromobacterium subtsugae strain EAPL16	JX500187		x				
Bacillus thuringiensis strain EAPL17	JX500188					x	
Enterobacter ludwigii strain EAPL18	JX500189		x				X
Lysinibacillus fusiformis strain EAPL19	JX500190						X
Ralstonia insidiosa strain EAPL20	JX500191		x				
Elizabethkingia meningoseptica strainEAPL21	JX500192		x				
Serratia nematodiphila strain EAPL22	JX500193		x				
Chromobacterium subtsugae strain EAPL23	JX500194		x				X
Bacillus thuringiensis strain EAPL24	JX500195					x	
Chromobacterium haemolyticum strain EAPL25	JX500196		x				
Bacillus weihenstephanensis strain EAPL26	JX500197				x		
Bacillus aerophilus strain EAPL27	JX500198						X

in a 1% agarose gel (MoBio UltraClean Agarose) stained with 1% (v/v) ethidium bromide and observed under a Foto/phoresis UV transilluminator (Fotodyne) and concentration determined using a Thermo Scientific Biomate 5 spectrophotometer. The DNA obtained was PCR amplified using the Taq PCR Master Mix Kit (Qiagen) and universal primers (27F 5′-3-AGA GTT-TGATCCTGGCTCAG and 1492R 5′-3′GGTTACC-TTGTTACGACTT) in a Techne TC-3000 thermocycler (Lane et al. 1985). PCR conditions were 94°C for 2 min and 40 cycles of denaturation at 95°C for 30 s, annealing at 63°C for 30 s, and extension at 72°C for 1 min. DNA amplicons were visualized via electrophoresis as explained above and molecular weight of the products was verified using an exact Gene 1kb DNA ladder (Fisher Scientific). The PCR products were cleaned with the MoBio UltraClean PCR clean-up kit (MoBio) following manufacturer's protocol, and sequencing analysis conducted by Retrogen Inc. (San Diego, CA) using a next generation capillary ABI 3730 sequencer (Applied Biosystems). DNA baser (Heracle BioSoft SRL) sequence assembler software was used to obtain a consensus for each sequence. Consensus sequences were entered into BLAST. Nearest matches to sequences were obtained with a minimum level of 97% (Stackebrandt and Goebel 1994).

Phylogenetic analysis.—Phylogenetic analysis was done using the Molecular Evolutionary Genetics Analysis 5 (MEGA 5) software (Tamura et al. 2011). Nucleotide sequences were aligned using the multiple sequence alignment method (MUSCLE) (Edgar 2004; Tamura et al. 2011). A maximum-likelihood tree was constructed with 1000 bootstrap value repetitions using the Tamura-Nei model (Tamura and Nei 1993).

Chemical analysis.—Chemical analysis was completed at RTI International (Durham, NC). Water samples were filtered to remove suspended sediment and each sample was mildly digested in the presence of high-purity nitric acid and then analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-OES). Sediment samples were dried and ground, and aliquots of each sample were subjected to an aggressive microwave digestion procedure in the presence of high-purity nitric and hydrofluoric acids and then analyzed by ICP-OES.

RESULTS

Cultivable community structure.—Twenty-six strains were isolated from our samples and of those 26, all represented distinct taxa (Table 1). They represented three phyla: 12 Proteobacteria, 12 Firmicutes, and 2 Bacteroidetes; four classes: 12 Bacilli, 8 β-proteobacteria, 4

γ-proteobacteria, and 2 Flavobacteria; and six bacterial orders: 12 Bacillales, 6 Neisseriales, 2 Burkholderiales, 4 Enterobacteriales, and 12 Flavobacteriales. Relative proportion of isolated bacteria is Proteobacteria 46.2%, Firmicutes 46.2%, and Bacteroidetes 7.6% (Fig. 3). Bacilli represent 80.0% of the cultivable microbial diversity in sediment samples, with γ-proteobacteria representing the remaining 20.0%. Microbial diversity in water samples comprised 53% β-proteobacteria, 23.5% Bacilli, 17.5% γ-proteobacteria, and 6% Flavobacteria.

Distribution of bacteria by site.—Eleven bacteria were isolated from sediment only and nine from the water only column and six were cultivated from both. We cultivated one isolate from Site A, and 15 isolates from each of the sites B and C (Table 1).

Organic matter and bacterial count.—Total organic matter in sediment was less than 1% for each of the three sites (Site A = 0.6%; Site B = 0.8% and Site C = 0.5%). Bacterial counts were significantly higher using the DAPI staining method than by traditional plate count methods. An average of 1.54×10^5 cells/mL in water and 1.64×10^5 cells/mL in sediment were found by DAPI count and 3.78×10^2 cells/mL in water and 50 cells/mL in sediment by the plate count method. (Table 2)

Chemical analysis.—Site B sediment had lower Ca: 9.9 μg/mL, K: 56.49 μg/mL, Mg: 0.96 μg/mL, and Na: 36.1 μg/mL than sites A(Site A: Ca: 25.69 μg/mL, K: 129.5 μg/mL, Mg: 4.95 μg/mL, and Na: 152.43 μg/mL) and C (Ca: 37.7 μg/mL, K: 129.22 μg/mL, Mg: 9.02 μg/mL, and Na: 159.82 μg/mL) (Table 3). Solute concentration in water was lower in site B than sites A and C with the exception of K at site C: 2.91 μg/mL. Chloride concentration in water was close to ten times higher in site B than either of the two other sites, A or C (Table 3).

DISCUSSION

We recognize the limitations of the bacterial community profile; it was restricted to only cultivatable forms, had a low number of isolates, provided a small fraction of the total diversity (Amann et al. 1995), and represented only one sampling. Nevertheless, this cultivable community profile provides a baseline and comparative platform for similar extreme habitats. Albeit at a rudimentary level it also adds to the existing knowledge of lentic ecosystems in general and lays a foundation for further investigation of microbial communities in Lake Phelps.

The cultivated bacteria from Lake Phelps represent typical freshwater phyla found in many lakes (Hiorns 1997; Newton 2011; Percent et al. 2008; Zwart 2002): in that they include bacteria from the phyla Proteobacteria, Firmicutes, and Bacteriodetes. These larger groups are similar in most freshwater lakes but vary in the quantity of species per subgroups. Our study found bacterial compositions similar to the Adirondack, Wisconsin, and Northern European studies (Hiorns 1997; Newton et al. 2011; Percent et al. 2008).

Lake Phelps has a higher number of Proteobacteria. Proteobacteria typically dominate most lakes (Newton et al. 2011). β-proteobacteria, the most abundant Proteobacteria in Lake Phelps, are known to be found in alkaline and acidic environments (Newton et al. 2011; Burkert et al. 2003; Methe et al. 1998). β-proteobacteria are found year round in most lake types suggesting a rapid adaptation to environmental changes. (Burkert et al. 2003). They are most studied of all freshwater bacteria and have only been isolated in water samples and none in sediment of lakes (Newton et al. 2011; Yarnell et al. 2009). Similar to Lake Phelps, Lake Fuchskuhle in Germany, a naturally acidic humic lake, cultivated clade II β-proteaobacteria abundant in its surface waters (Burkert et al. 2003). *Chromobacterium sp.* belong to this clade and represent several of cultivated β-proteobacteria studied. This could possibly indicate that clade II β-proteobacteria prefer acidic humic waters like those of Lake Phelps and Lake Fuchskeuhle. γ-proteobacteria were found in mainly marine aquatic systems but have been isolated in freshwater (Burkert et al. 2003). γ-proteobacteria were found as the dominant group for the first time in the Adirondack study in Cascade Pond and Icehouse Lake, but little is known on what controls dominance of this group (Percent et al. 2008). They are believed to be copiotrophs and are thought of as externally introduced to lake environments (Newton et al. 2011). It is not known if these bacteria were native or introduced. Current literature does not suggest a role for γ-proteobacteria in freshwater.

Bacteriodetes found in Lake Phelps are usually found during high production times in lake systems. Sampling time could have affected the quantity of Bacteriodetes. Their abundance may be related to the appearance of phototrophic protists which were not sampled in our study (Newton et al. 2011). Bacteriodetes are typically particle associated bacteria and degrade biopolymers such as carbohydrates and proteins (Thomas et al. 2011). The Adirondack study also found low number of Bacteriodetes in number similar to our study (Percent et al. 2008).

Firmicutes are a less dominant type of freshwater bacteria that represent another large Lake Phelps phyla. Bacilli were the only Firmicutes cultivated from Lake Phelps, but were abundant enough to dominate Lake Phelps. This suggests seasonal influences contribute to their success. Firmicutes were not present in the Adirondack study, but have been found in Lake Kawah Ijen, a volcanic lake in Indonesia (Lohr et al. 2006). Minimal research has been done on this phyla in lake ecosystems. *Bacillus* often feed on organic matter and

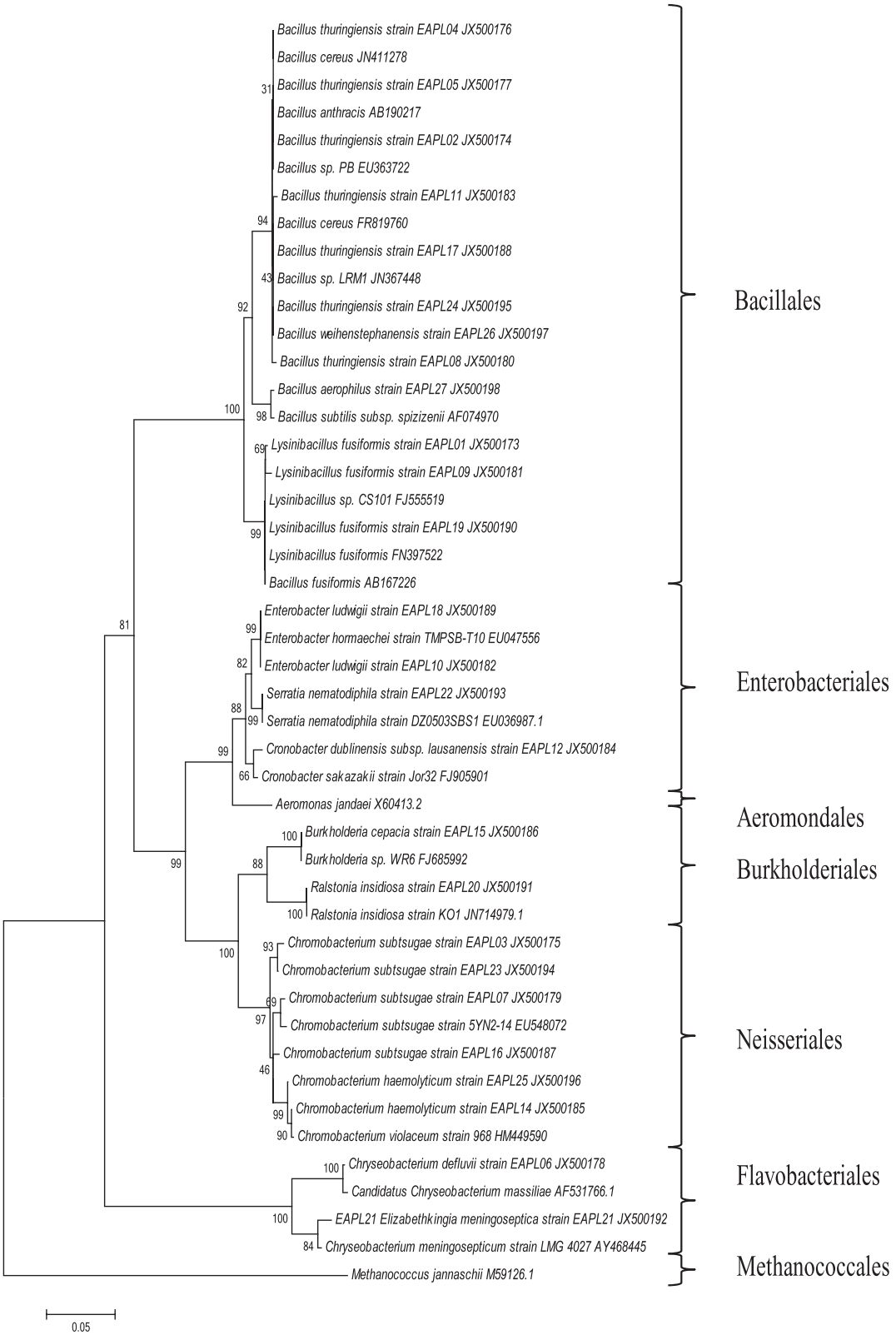

FIG. 3. Phylogenetic relationship of isolated bacteria of Lake Phelps based on 16S using maximum-likelihood tree constructed with 1,000 bootstrap value using the Tamura-Nei model.

Table 2. Comparative data of total bacterial count using plate count method and direct DAPI staining.

	Plate count (cfu/ml)			DAPI count (cells/ml)		
	Site A	Site B	Site C	Site A	Site B	Site C
Water	2.55×10	2.16×10^2	9.3×10	8.70×10^4/mL	2.40×10^5	1.36×10^5
Sediment	Not available	2.2×10^4	7.8×10^4	3.0×10^5/mL	2.74×10^5	9.20×10^4

contribute to insect microflora and obtain nutrients from their hosts (Dillon and Dillion 2003). *Burkholderia*, a Proteobacteria, and *Bacillus thurnigenisis* are associated with agriculture processes such as biocontrol and plant-growth (Lee et al. 2010). The presence of these bacteria could suggest that nearby agriculture is influencing the environment of Lake Phelps.

The phylum Actinobacteria is absent from Phelps Lake. Actinobacteria are known to flourish in late fall and winter and could possibly be replaced by Betaproteobacteria during times of high algal blooms (Yannarell and Kent 2009). The time of year or methodology could have played a role in the absence of Actinobacteria, though this group has been seen in lakes of all trophic states and the epilimnia of lakes globally (Newton et al. 2011).

Sites A and C represent the west side of the lakes sand bar and site B represent the east side of the lake sandbar in Lake Phelps. As the possible result of two Carolina bays merging, Lake Phelps might have two different environments with different evolutionary tracks. Site A sediment sample counts could not be included because 1) the control plates from this site were contaminated, 2) a few plates of water sample showed obvious contamination, and 3) the counts of the remaining were very low. Bacterial counts for water at site B were greater than counts for water at sites A and C. The higher counts were true for both DAPI and plate count methods. A higher frequency of isolated water bacteria we observed at Site B may suggest a possible abundance of β-proteobacteria at this site. Site B sediment, on the other hand, contained only three Firmicutes. This area of the lake is against the Pocosin National Park and has little contact with agricultural land, whereas the other sites are very close in proximity to agricultural industry.

Lake Phelps has a diverse bacterial community that is best explained by the combination of two cation environmental variables, K and Mg that explain the microbial community, as reflected in the higher correlation value of 0.599. Total ionic concentrations influence the species of organisms that can survive in the lake, in addition to affecting many important chemical reactions that occur in the water (Horne et al. 1994). This varying difference in chemistry within the lake supports the notion that lake bacterial communities are dependent on niche microenvironments, rather than the overall lake ecosystem. The difference in chemistry of the sites could be related to the division of Lake Phelps as two separate Carolina Bays and the continual division by the sandbar until ultimately merging into one lake. Carolina Bays are acidic unexplained phenomenon whose origins are still unknown. These elliptical shallow depressions remain understudied. Newman and Schalles (1990) surveyed Carolina Bays from Georgia to North Carolina to analyze water chemistry. Values for each bay in this study were different because of specific external conditions, but, Na was the most abundant cation, followed by Ca, Mg, and finally K at each site similar to results at Lake Phelps (Newman and Schalles 1990).

Our findings of microbial communities in L. Phelps dominated by Proteobacteria suggest a general pattern in microbial communities among naturally acidic, oligotrophic inland water bodies (Andersen et al. 2012). In this regard, our results will enrich knowledge about lake ecosystems in general and microbial communities in Lake Phelps in particular. As a result, a detailed analysis of the L. Phelps' ecosystem, including extensive chemical analysis from both sides of the sandbar and correlation of associated meiofauna with its microbial communities, is warranted. Furthermore, a comparison of nearby lakes to Lake Phelps would be beneficial to have a better understanding of processes at work in shaping microbial communities in inland water bodies of the region.

Table 3. Sediment and water chemical analysis of the three sampling sites in Lake Phelps, NC.

Site	[Ca] 396.8 (μg/mL)	[K] 769.8 (μg/mL)	[Mg] 285.2 (μg/mL)	[Na] 589.5 (μg/mL)	[Si] 251.6 (μg/mL)	[Chloride] (ng/mL)	[Nitrate] (ng/mL)	[Sulfate] (ng/mL)
Site A Water	8.16	3.22	2.57	11.5	1.13	360	1,156	29,160
Site A Sediment	25.69	129.5	4.95	152.43				
Site B Water	7.86	3.15	2.49	10.95	2.71	31,240	928	27,340
Site B Sediment	9.9	56.49	0.96	36.1				
Site C Water	8.37	2.91	2.84	11.8	2.88	347	1,081	29,380
Site C Sediment	37.7	129.22	9.02	159.82				

Acknowledgements: This work was supported in part by the U.S. Army Research Laboratory and U.S. Army Research Office under Contract number W911N7-08-1-0402 and the U.S. National Science Foundation Award Number 0808632.

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