# SOUTHERN APPALACHIAN PEATLANDS SUSTAIN UNIQUE ASSEMBLAGES OF ARCHAEA

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by
ASHLEY NICOLE HAWKINS

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APPROVED BY:

Dr. Suzanna L. Bräuer
Chairperson, Thesis Committee

Dr. Ece Karatan
Member, Thesis Committee

Dr. Gary Walker
Member, Thesis Committee

Dr. Sue L. Edwards
Chairperson, Department of Biology

Dr. Edelma D. Huntley
Dean, Cratis Williams Graduate School

**Abstract**

SOUTHERN APPALACHIAN PEATLANDS SUSTAIN UNIQUE ASSEMBLAGES OF ARCHAEA

Ashley Nicole Hawkins
B.S., North Carolina State University
M.S., Appalachian State University

Chairperson: Suzanna L. Bräuer

Numerous aspects of three peatlands (Pineola Bog, NC, Sugar Mountain Bog, NC, and Tater Hill Bog, NC) located along the Southern Appalachian highlands of western North Carolina were analyzed to elucidate the impact the Archaeal community present in these sites might have on future climatic warming events via the release of the greenhouse gas methane. It was initially hypothesized that members of the Archaeal community among these Southern Appalachian peatlands would be phylogenetically closely related to those identified in more northern or boreal peatlands, with a few minor differences in abundance. It was also hypothesized that, being in a temperate climate, these sites would experience seasonal shifts in the amount of methane produced and potentially in the composition of the Archaeal community. Quantitative analyses demonstrated a methanogenic community comprising roughly 50% of the Archaeal population and consisting of members of the orders Methanomicrobiales, Methanocellales, Methanosarcinales, and Methanobacteriales, as well as members of the uncultured Rice Cluster II. Both hydrogenotrophic and aceticlastic affiliated sequences were found, suggesting a diverse array of substrate utilization. A hydrogenotrophic *Methanobacterium* sp. was enriched from Pineola Bog using culture-based techniques, corroborating molecular data and indicating the presence of Methanobacteriales species (0.4%) in this peatland. In addition to the methanogenic population, many non-methanogenic species were also found among the peatlands both from DNA and cDNA analyses. Members of the deep-branching Euryarchaeota, distantly related to Thermoplasmatales, represented 5-18% of the sequences retrieved from each of the three sites. Sequences in this group included members of the recently proposed seventh order of

methanogens and included members of the Lake Dagow Sediment Cluster, Marine Benthic Group D, SM1K20, and Rice Clusters III and V. Crenarchaeota numbers were found to significantly contribute to the overall Archaeal community across sites but were most prominent in Pineola Bog, constituting roughly 50% of the total Archaeal population. Methane emission studies revealed the peak methane production to be just below (0-25 cm) the surface of the water table in each site, as was expected. Seasonal analysis of methane production demonstrated an increase in the amount of methane produced during the fall and winter months. Nutrient analysis of the study sites indicated a strong signature from the underlying bedrock of the area as concentrations of Fe, Al, and Na were at the high end of normal compared to other peatlands globally.

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## Dedication

This thesis is dedicated to my parents, Monique and Jerry Hawkins, and my sister, Caitlin Hawkins. Their continued support and encouragement have been a defining factor in my life.

## Table of Contents

## List of Tables

## List of Figures

## Foreword

The references, tables, and figures within this manuscript were prepared in accordance to the author submission requirements of Frontiers in Microbiology, the peer-reviewed journal co-published by Elsevier Publishers. Chapter Two is a manuscript intended for separate submission to the journal. As such, this chapter has been formatted independently.

# CHAPTER 1: INTRODUCTION

Wetland ecosystems vary widely in their characteristics, and as such harbor unique communities of microorganisms performing mutually dependent processes to the benefit of the system. A broad range of environments are considered peatlands, defined by a multitude of parameters such as nutrient status (nutrient-poor to nutrient-rich), vegetation (moss-dominated to grass- or tree-dominated sites), and water level (near surface water table to ~40 cm below the surface) (Gorham, 1992; Rydin, 2006; Yavitt, 2013). Because of this variety, and their unique properties, wetland systems are often studied independently of other ecological sciences such as estuarine and terrestrial ecology and have been found to provide several beneficial services to surrounding ecosystems as well as mankind (Mitsch, 2007). Nutrient concentration along with such variables as pH, chemical composition of the deposited plant detritus, and hydrological status are all major controllers on wetland dynamics and particularly microbial activity (Svensson, 1992; Yavitt, 2013). Although distributed throughout the world, one-third of all wetlands are found in North and South America, mostly in Canada and the United States ($2.37 \times 10^6$ $km^2$) with ~114.6 million hectares of wetland ecosystems in the United States alone (Mitsch, 2013).

Wetlands are the largest natural source of the greenhouse gas methane (Loulergue, 2008), emitted through complex physiological processes involving both plants and microorganisms and typically regulated by factors arising from both climatic and environmental factors (Cao, 1998; Chang, 2003; Zhang, 2013). Overall, natural and cultivated wetlands combined are said to represent approximately 40% of methane sources (Cao, 1998). With a global warming potential (GWP) of 23X

that of $CO_2$, methane has been shown to be responsible for approximately 20% of the direct radiative forcing when compared to all long-lived greenhouse gases (Zhang, 2013) and has shown an annual increase of 1% (Pearmen, 1986; Dlugokencky, 1994; Cao, 1998; Mosier, 1998). The waterlogged environment found in wetlands creates anoxic conditions that are conducive to the activity of methanogenic Archaea, the organisms responsible for producing the methane that is released to the atmosphere (Yavitt, 2013). Further, the uniquely adapted aquatic plant life present in waterlogged wetland systems plays a large part in the eventual release of methane to the atmosphere through effects on production, consumption, and transport via arenchymous chambers (Terazawa, 2007; Rice, 2010; Zhang, 2012). In addition to the transport of methane, wetland plants also produce exudates that impact redox states of the surrounding environment, thus affecting substrate availability and energy transformation of the soil microbial consortia and, indirectly, the abundance, diversity, and community structure of that same consortia (Song, 2008; Wang, 2013). Whereas northern peatlands represent approximately 3.5% of the world's total land area, they contain close to one-third of the soil carbon globally and play a large role in global carbon cycling (Gore, 1983; Roulet, 2000; Bridgham, 2006; Denman, 2007; Lai, 2009; Kolb, 2012). This could be attributed to the exceptionally low decomposition rate in northern peatlands due to lower temperatures resulting in a net carbon accumulation rate of 76 Tg C/yr (Lai, 2009). A critical question is whether or not these wetlands will continue to be carbon sinks or whether they will become net sources for carbon to the atmosphere in the face of global climate change.

Wetland ecosystems in the Northern Hemisphere have traditionally been considered some of the most important global sources of methane (Aselmann, 1989; Fung, 1991; Tyler, 1991; Westermann, 1993). However, lately there has been a growing interest in southern latitude wetland ecosystems as a model for climate-induced changes to wetlands worldwide such as increasing temperature and precipitation. In particular, mid-latitude and Arctic regions of peat are predicted to

contribute to a substantial rise in methane emissions due to climatic warming (Bloom, 2010; Bombonato and Gerdol, 2012; van Winden et al., 2012). These wetland ecosystems are at risk. For example, in the state of North Carolina alone, approximately 13% of the natural wetlands were drained for agricultural uses between 1970 and 1980 (Dahl, 1990; Heimlich, 1998; Morse, 2012), thus leaving sparsely dispersed wetland areas for study. More study of such temperate climates could reveal climatic changes affecting rainfall patterns and, as a result, affecting runoff and groundwater inflows to terrestrial wetlands (Mitsch, 2013). The potential for decreased precipitation and subsequent increased evapotranspiration due to climatic warming will most likely result in less-frequent flooding of existing wetlands, causing increased areas of oxygenation and methane release (Mitsch, 2013). Nutrients are also at risk of perturbation due to precipitation fluxes, revealing the potential for a shift in soil microbial community structure as well as activity and, as a consequence, the release of greater amounts of methane (Lu, 1999; Song, 2012). In this thesis, the abundance, composition, and activity of archaeal communities are described for three peatlands representative of these important and fragile Southern Appalachian peatland ecosystems.

# CHAPTER 2: SOUTHERN APPALACHIAN PEATLANDS HARBOR UNIQUE ASSEMBLAGES OF ARCHAEA


## Summary

Mid-latitude peatlands with a temperate climate are sparsely studied, and as such represent a gap in the present day knowledge base regarding Archaeal populations present in these environments and their roles in the environment. Phylogenetic analysis of the Archaeal populations among the three sites studied here reveal not only methanogenic species, but also significant populations of Thaumarchaeal/Crenarchaeal-related organisms of the uncultured Miscellaneous Crenarchaeotal Group (MCG) and the terrestrial group 1.1c, as well as deep-branching Euryarchaeota primarily related to sequences in the Lake Dagow Sediment and Rice Cluster V lineages. The Thaum/Crenarchaea and deep-branching Euryarchaea represented approximately 24-83% and 2-18%, respectively, of the total clones retrieved in each library, and methanogens represented approximately 14-72% of the clones retrieved. All three major groups (methanogens, Thaumarchaea/Crenarchaea, and deep-branching Euryarchaea) were detected in the RNA library, suggesting at least a minimum level of maintenance activity.


## Introduction

Wetland areas are major contributors to emission of the greenhouse gas methane (Cicerone, 1988; Chmura, 2002; Lai, 2009; Godin, 2012) and as such are partially responsible for the resulting environmental changes associated with greenhouse gas accumulation (Gorham, 1991; Kettunen et al.,

1999a; Christensen et al., 2003; Boucher et al., 2009). Because methane released from these wetland areas is of microbial origin (Kotsyurbenko, 2004; Whalen, 2005; Erkel, 2006; Jerman, 2009; Kato, 2010), it is important to increase our understanding of the associated microbial communities. The southern region of the United States is of special interest as mid-latitude wetlands are predicted to dramatically increase methane gas release as climate warming occurs (Bloom, 2010; Bombonato and Gerdol, 2012; van Winden et al., 2012).

Southern Appalachian peatlands are similar to their northern counterparts in biogeochemical cycling and vegetation (Wieder, 1981; 1985a; 1994), yet there are also distinct differences in succession, climate, biogeographic history, and some flora (Weakley and Schafale, 1994). Most notably, there are significant differences in rate of carbon mineralization (Yavitt, 1987), decomposition (Wieder, 1985a), net primary productivity (Wieder, 1983; 1988), and rates of greenhouse gas production (Yavitt, 1987).

Southern peatlands are also characterized by significant spatial heterogeneity in water input, pH, and vegetation (Weakley, 1995), as well as physical and chemical properties (Wieder, 1985a). Like Big Run Bog in West Virginia, most bogs further south in North Carolina receive some water seepage and thus hydrologically resemble fens (Wieder, 1985a). Yet, they differ from fens in that the water input is often acidic and oligotrophic (nutrient poor) (Weakley, 1995). In general, they are most similar to ombrotrophic (rain-fed) bogs, especially in overall vegetation, pH, and nutrient concentration, and thus are considered bogs (Wieder, 1985a; Weakley, 1995). Another major difference between northern and southern bogs in the U.S. is the relative age of the peat. Most northern bogs have been accumulating peat for the last 9,000 years, since the last glaciers retreated. However, bogs in Southern Appalachia have been found to contain much older peat that has been accumulating since the peak of the last glacial advance some 13,000-18,000 years ago, due to the fact that the southern mountains were not glaciated 18,000 years ago (Delcourt, 1985; Pittillo, 1995). Yet due to the relatively high rates of decomposition, the peat in southern bogs is generally much

shallower. For example, Pineola Bog is thought to contain the deepest peat layer in North Carolina with approximately two meters of accumulated peat (Weakley, 1995), whereas McLean Bog in New York has approximately six to eight meters of peat (Osvald, 1970).

To our knowledge, there have been no studies evaluating the microbial diversity in Southern Appalachian bogs. Further, northern bogs of Russia (Dedysh et al., 1998; Dedysh et al., 2001; Kotsyurbenko et al., 2007), Germany (Horn et al., 2003; Hamberger et al., 2008; Wüst et al., 2009), Scandinavia (Galand, 2005; Hoj et al., 2005), Canada (Dunfield et al., 1993; Roulet et al., 1993; Moore et al., 2008), and the United States (Basiliko et al., 2003; Cadillo-Quiroz et al., 2006) have received the most attention, followed by southern bogs of West Virginia and Maryland, which have received some biogeochemical study (Wieder et al., 1981; Wieder, 1985b; Yavitt et al., 1988; Wieder et al., 1989; Wieder et al., 1990). Yet bogs of North Carolina have rarely been studied (Richardson and Gibbons, 1993; Stewart Jr. and Nilsen, 1993; Weakley and Schafale, 1994). Southern Appalachian peatlands harbor a diversity of important and rare flora and fauna. Thus, efforts are warranted to conserve, understand, and research these richly diverse ecosystems.

**Experimental procedures**

*Site description*

Potato "Tater" Hill lower bog is a roughly 4.05 ha bog at ca. 1,300 meters elevation, located approximately 11 km northwest of Boone, NC (near N 36° 16' 33.96"; W 81° 41' 27.96"). Tater Hill experiences seep intrusions around the perimeter of the site; thus, the bog exhibits a nice biogeochemical gradient from higher to lower pH and from minerotrophic (spring- or stream-fed) to oligotrophic. Some adjacent seep areas are impacted by beaver dams. Tater Hill flora is marked by the presence of some bog species found throughout bogs in the United States, such as *Sphagnum* spp., *Drosera rotundifolia* (common sundew, found during this study), and *Vaccinium macrocarpon* (cranberry), as well as some species found only in the NC, TN, and VA areas, such as *Lilium grayi* (Gray's Lily) (Oakley, 2000). Other species found on site during the study period included the

following: *Carex* ssp., *Juncus* ssp. (*J. acuminatus, J. tenuis,* and *J. marginatus,* among others), *Luzula acuminata* var. *carolinae, L. echinata, Oxypolis rigidior, Mimulus ringens, Oenothera* ssp., *Eriophyllum virginicum, Osmundastrum cinnamomea (Osmunda cinnamomea), Salix sericea, Rhododendron* ssp. (*Rhododendron vaseyi, R. calendulaceum, R. maximum* among others), *Vaccinium* ssp., *Aronia prunifolia, Spiraea alba, Ilex verticillata,* and *Viburnum cassinoides* (Andrew Jenkins & Tim Metcalf, personal communication). Unlike the other study sites (described below), Tater Hill Bog is underlain by amphibolite metamorphic rock originating from ancient lava flows (Martin, 2007), and the soil is significantly richer and more minerotrophic, at least in some areas of the site. Consequently, Tater Hill Bog displayed both the highest pH on average (ca. 5.7), as well as the greatest variability (Table 1). Tater Hill is classified as a Southern Appalachian bog (typic subtype) by the most recent southern classification scheme (Schafale, 2012), although it would most likely be considered a moderate fen by northern classification methods (Mitsch, 2007). Due to its more minerotrophic nature, Tater Hill Bog was selected to contrast the typical Southern Appalachian bogs, such as Sugar Mountain Bog, that are found throughout the region.

Pineola Bog is located adjacent to the Linville River in the Pisgah National Forest (near N 36° 01’ 30.72”; W 81° 53’ 47.04”), at ca. 1,080 meters elevation. The site consists of approximately 47.35 ha, and, like Tater Hill Bog, it is reported to contain some species common to northern bogs such as *Vaccinium macrocarpon*, as well as other plants that are rare to the area: *Thelypteris simulata, Vaccinium macrocarpon, Carex trisperma, Arisaema triphyllum* ssp. *Stewardsonii,* and *Sphagnum fallax* (Smith, 2006). Pineola Bog contains the deepest (ca. two meters) reported peat layer in the region (Weakley and Schafale, 1994). Classified as a swamp-bog complex (typic subtype) according to the southern classification system (Schafale, 2012), Pineola was found to harbor a canopy of *Acer rubrum, Tsuga canadensis, Quercus rubra, Betula alleghaniensis, Liriodendron tulipifera, Betula lenta,* and *Aesculus flava,* as well as an understory of *Rhododendron maximum, Kalmia latifolia, Viburnum cassinoides, V. nudum, Sambucus canadensis, Clethera virginiana, Alnus serrulata,*

*Lobelia cardinalis,* several *Carex* ssp., *Osmundastrum cinnamomea (Osmunda cinnamomea), Juncus acuminatus, Xanthorhiza simplicissim,* and *Sphagnum* spp. (Andrew Jenkins & Tim Metcalf, personal communication).

Sugar Mountain Bog is a very small (roughly 2.83 ha) oligotrophic bog within a larger 86.60 ha natural area located near Pineola Bog (near N 36° 04' 58.08"; W 81° 53' 47.04"), at nearly 1,100 meters elevation. This study site was chosen as a classic example of a Southern Appalachian bog of the typic subtype (Smith, 2006; Schafale, 2012). Flora present during the study period (2010-2012) included *Sphagnum* spp., herbaceous plants such as *Galax urceolata, Carex* ssp., *Lilium grayi, Scirpus* sp., and *Osmundastrum cinnamomea*, in addition to shrubs such as *Spiraea alba, Salix sericea, S. humilis, Viburnum cassinoides, Rhododendron maximum, Kalmia latifolia, Clethra accuminata, Hypericum densiflorum, Alnus serrulata, Rosa palustris,* and *Vaccinium corymbosum* (Andrew Jenkins & Tim Metcalf, personal communication). Rare and/or threatened plant species such as *Vaccinium macrocarpon, Carex trisperma, Epilobium ciliatum, Lycopodiella inundata, Lilium grayi,* and *Platanthera grandiflora* have also been reported at the site (Smith, 2006). This site exhibited the lowest pH at ca. 4.7 (Table 1). All three study sites were generally low in exchangeable ion concentrations. For example, sodium concentration was around 1-3 mg/L (ca. 90 μM) and potassium was around 20 μM, or 0.4-0.7 mg/L (described below), significantly higher than some northern peatlands, such as McLean Bog (Cortland, NY) with reported values of 1-4 μM. In contrast to other peatlands throughout the world (Mullen et al., 2000), aluminum, silicon, and iron were generally high (0.1-2.5 mg/L, 14-33 mg/L, and 0.4-11 mg/L, respectively), likely due to the nature of the bedrock in the area (Helmer, 1990). The potential methane production as determined in a previous study was within the range for northern bogs (Table 1). All three sites had areas of low pH (down to pH 4 or 5), and all three had water tables relatively close to the peat surface (0-15 cm; Table 1).

*Sampling procedures*

Each site was sampled during all four seasons between the summer of 2011 to the summer of 2012, and additional samples were also taken in the summer of 2010. During each sampling event, temperature, water table depth below the peat surface, and pH of the peat layer were measured. Peat samples were collected in airtight jars from 10-25 cm below the standing water table, corresponding to the depth of maximum methane production as determined at these sites previously (data not shown). Three plots were sampled within each site with the exception of Sugar Mountain, which was used for comparative purposes only. Samples were stored and transported at ambient temperature from study sites to the laboratory where they were subsampled for DNA analysis, dry weight calculations, soil slurries, and analyses of potential methane production. Additionally, aliquots of each peat sample were stored at -80 °C for nucleic acid extraction and molecular analyses. Pore water was collected from each site during the summer of 2012 and sent for ICP analysis at Cornell University (Cornell University, Ithaca, NY, USA).

*Nucleic acid extraction and cDNA synthesis*

Total genomic DNA was extracted from peat samples using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA 92010) per the manufacturer's instructions and stored at -20 °C or -80 °C for molecular analysis or long-term storage, respectively. Total RNA was isolated as according to the manufacturer's protocol using the TRIzol reagent (Invitrogen, Life Technologies, Grand Island, NY 14072) with slight modifications. Briefly, 0.3-0.5-g (fresh weight) aliquots of peat were added to 1.5-mL Eppendorf© tubes along with sterile ceramic beads and 750 µl of detergent/lysing solution and homogenized for 1 min at max speed using a MiniBeadbeater™ (Biospec Products, Oklahoma, USA). Homogenized samples were centrifuged for 5 min at 12,000 g and the supernatant transferred to a sterile 1.5 mL Eppendorf © tube. A second lysing step was then performed by the addition of 1 mL of TRIzol reagent to supernatant with subsequent repetitive pipetting. Phase separation was achieved by the incubation of samples for 5 min at 20 °C, after which

0.2 mL of chloroform was added. Tubes were vigorously shaken and incubated at 20 °C for an additional 3 min. Samples were centrifuged at 12,000 g for 15 min resulting in a separation of the mixture into a lower red, phenol-chloroform phase containing proteins, an interphase containing DNA, and a colorless upper aqueous phase containing the desired RNA. RNA was then precipitated by carefully transferring the upper aqueous phase to a new, sterile 1.5-mL Eppendorf © tube along with 0.5 mL of isopropyl alcohol and incubating samples at -20 °C for 10 min. Centrifugation was performed at 12,000 g for 10 min, after which the supernatant was discarded. The resulting RNA pellet was washed once with 1 mL of 75% ethanol and centrifuged at 7,500 g for 5 min and the supernatant was discarded. The RNA pellet was dried at room temperature for 10 min and dissolved in RNase-free water by passing the solution a few times through a pipette tip and incubating for 10 min at 60 °C. RNA was stored at -20 °C for no more than a week prior to analysis. First strand cDNA synthesis was performed using the OneStep RT-PCR Kit (Qiagen, Germany) according to the manufacturer's protocol.

*SSU rRNA and SSU rDNA gene amplification, cloning, and sequencing*

Archaeal SSU rRNA genes were amplified from extracted DNA or from cDNA using the Archaea-specific primer set Ar109f (5'-ACKGCTCAGTAACACGT-3') and Ar922r (5'-YCCGGCGTTGANTCCAATT-3') (Großkopf et al., 1998) and ARMAN-specific primer sets ARM979F (5'-TATTACCAGAAGCGACGGC-3') and ARM1356R (5'-AGGGACGTATTCACCGCTCG-3') (Baker, 2010) and ARMANF (5'-AGGCAGATGGCGGGGTAAC-3') and ARMANR (5'-CGCATTGACGTTGAGCGCC-3') (this study). The general Archaeal 25-µl PCR mixture contained: PCR Master Mix (0.05 U/µL *Taq* DNA polymerase, reaction buffer, 4 mM MgCl2), 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), 5 µM of each primer 109F and 922R, 3 ng of DNA, and $dH_2O$. The thermal profile for general Archaeal species was as follows: initial denaturation at 98 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 30 sec, primer annealing at 55 °C for 30 sec, and extension at 72 °C for 1.5

min. A final extension at 72 °C for 7 min ended the protocol. The ARMAN-specific thermal profile was as follows: A 45-sec denaturation step at 98 °C followed by 30 cycles of denaturation at 95 °C for 1 min, primer annealing at 57 °C for 1 min, and extension at 72 °C for 1.5 min. The protocol ended with an additional extension step at 72 °C for 7 min. Amplified products were visualized by gel electrophoresis in 1% agarose and stained with GelRed (Phenix Research Products, Candler, NC, USA). PCR reactions were conducted in triplicate and combined before cloning to reduce PCR bias. Amplicons were cloned using a TA cloning kit (TOPO 2.1; Invitrogen, Life Technologies, Grand Island, NY, USA) following the manufacturer's protocol. A total of four libraries were generated from DNA extracted from each of the following samples (the total number of sequences for each library is listed in parentheses along with the sampling date): Tater Hill DNA (90; 2011), Pineola DNA (164; 2011/2012), and Sugar Mountain DNA (88; 2011). Additionally, a cDNA library of 89 sequences was created from samples collected at Pineola in the summer of 2012, resulting in a total of five libraries. PCR amplification of successful transformants was performed in a 25-μL reaction containing: PCR Master Mix (0.05 U/μL *Taq* DNA polymerase, reaction buffer, 4 mM $MgCl_2$), 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), 15 μM of each primer (M13F-M13R), cell material, and $dH_2O$. The PCR protocol began with an initial denaturation at 95 °C for 10 min followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1.5 min, and extension at 72 °C for 3 min. A final extension at 72 °C for 7 min ended the protocol. PCR products were visualized on a 1% agarose gel via gel electrophoresis to ensure the expected product sizes. Sequencing was conducted using a Sanger platform at Beckman-Coulter Genomics (Danvers, MA).

*Sequence analysis and phylogenetics*

All forward and reverse sequences containing ≥400 bp were imported into Sequencher (Gene Codes Corporation, Ann Arbor, MI), and contiguous sequences were assembled from respective forward and reverse sequences from each clone. Resulting contigs were screened for chimeras using DECIPHER (Wright, 2012) and were compared to existing sequences using the BLAST tool

(http://blast.ncbi.nlm.nih.gov/) from the NCBI database. Sequence files along with a selection of top hits from the BLAST comparison were aligned using the SILVA aligner of the ARB program (http://www.arb-silva.de/aligner/). Sequence editing was performed using the BioEdit tool (Ibis Biosciences, Carlsbad, CA 92008). Phylogenetic trees were constructed by neighbor joining (NJ) and by the maximum-likelihood method. A distance matrix was generated using the Phylip package (http://evolution.gs.washington.edu/phylip.html) and used for subsequent operational taxonomic unit (OTU) assignment and NJ tree construction. The DOTUR computer program was used to generate OTUs and rarefaction curves for each data set. A 97% 16S rRNA sequence similarity threshold was used to define each OTU. NJ tree construction and subsequent maximum likelihood tree construction was performed using the Phylip package.

*Real-time quantitative PCR*

Due to the high percentage of clones belonging to the Crenarchaeota, abundance of Crenarchaeota and total Archaea were assessed for the Pineola and Tater Hill sites. Quantitative PCR (qPCR) was conducted with general Archaeal primers 967F and 1060R (Cadillo-Quiroz et al., 2006), Crenarchaeal-specific primers 771F and 957R (Ochsenreiter, 2003), and E1/E2 methanogen-specific primers A-gE372 and A-gE540aR (Cadillo-Quiroz et al., 2006). qPCR was performed on duplicate field samples in duplicate 20 µl reaction volumes containing the following: 9 µL SYBR Green Master Mix, 9.2 µL $dH_2O$, and 0.2 µM of each primer, on an Applied Biosystems$^{TM}$ 7300 Real-Time PCR System (Carlsbad, CA). Reactions were performed using two different dilution factors to account for PCR inhibitors. Reaction conditions were as follows: Initial denaturation at 94 ˚C for 5 min followed by 40 cycles of denaturation at 94 ˚C for 1 min, annealing at 54 ˚C for 1 min and elongation at 72 ˚C for 2 min. A final dissociation step followed that consisted of: 95 ˚C for 15 sec, 60 ˚C for 30 sec, and 95 ˚C for 15 sec. Efficiencies were between 80-110%. Calibration curves for quantification were generated using one of the following standards: 1) plasmid DNA containing the SSU rRNA gene from OREC-R1096 (Crenarchaea, range of $10^2$-$10^6$ target copies/µL) or 2) genomic DNA extracted

using the Qiagen Dneasy Blood and Tissue Kit (Valencia, CA) from *Methanoregula boonei* 6A8 (Archaea, range of $10^2$-$10^6$ target copies/μL). Circular plasmid standards have been reported to cause overestimation of sample cell number using quantitative PCR (Hou et al., 2010). To circumvent this potential issue, plasmid DNA standards were linearized by restriction digest using BssHII (New England BioLabs, Ipswich, MA).

**Results**

*Phylogenetic analysis of methanogenic archaea*

A large portion of the Euryarchaeota sequences recovered from the clone libraries at all three sites were related to the Methanosarcinales and the Methanomicrobiales. The globally abundant Methanoregulaceae-associated cluster of Methanomicrobiales dominated the methanogenic communities of the DNA libraries from both Sugar Mountain Bog (2011) and Pineola Bog in 2012 (Figure 1 and Table 2). The DNA libraries from Tater Hill Bog and Pineola Bog in 2011 were found to be dominated by the Methanosarcinales order of methanogens, which represented approximately 30% and 26% of the overall Archaeal community from each site, respectively (Table 2). The cDNA library from Pineola 2012 was also found to harbor an abundance of Methanosarcinales with roughly 40% of the Archaeal population being of this order (Table 2). Methanobacteriales clones ranged in abundance from 0-14% and were most dominant in the Sugar Mountain Bog samples. Members of the Rice Cluster II were also detected in Pineola Bog, but not in the other two sites. Compared to other Euryarchaeota or Crenarchaeaota, methanogenic species were found to dominate the overall Archaeal community in both Tater Hill (52% of the total sequences) and Sugar Mountain Bog (50%), as well as the cDNA library (71%) from Pineola Bog (Table 2).

*Phylogenetic analysis of deep-branching euryarchaea*

Many deep-branching Euryarchaeotal lineages were also identified in all three study sites from both DNA and RNA libraries. Among the species identified, Rice Cluster V (RC-V), Marine Benthic Group D, and the Lake Dagow Cluster were the most commonly represented groups across

sites (Figure 2 and Table 2). The RC-V group was found to dominate most libraries, constituting approximately 8%, 13%, 1%, and 3% of the overall archaeal community from Tater Hill Bog, Sugar Mountain Bog, and Pineola Bog 2012 DNA and RNA, respectively. However, the Marine Benthic Group D was also abundant and clones from this clade were highly represented (5%) in the DNA library from Pineola Bog in 2011. Overall, the Rice Cluster V group of deep branching Euryarchaea seem to represent the majority of the deep-branching, putatively non-methanogenic Euryarchaeal species.

*Phylogenetic analysis of Crenarchaea*

Crenarchaeaota represented at least 24% of the sequences in each library. However, the greatest number of taxa were found in the Pineola Bog site (Figure 3 and Table 2). The majority of Crenarchaeal sequences were phylogenetically related to Group 1.3 and represented from 22-31% of the sequences found in all DNA libraries from all three sites, whereas this group represented only 10% of the clones in the RNA library from Pineola Bog in 2012 (Table 2). Group 1.1c was found to dominate in this library and represented up to 39% of the overall archaeal community from the 2012 Pineola Bog DNA (Table 2). Overall, Thaum and Crenarchaeal-related sequences were found to dominate the Archaeal community among both DNA libraries of Pineola Bog and represented roughly 70-83% of the sequences in these libraries (Table 2). Sequences related to the nearly classified *Candidatus Microarchaeum acidiphilum* (ARMAN-2) were also identified only within Pineola Bog and are shown to group phylogenetically as a deep-branching Crenarchaeal relative (Figure 2).

*Quantification of Archaeal seasonal trends*

The estimated quantity of Archaeal cells showed high variation between different samplings, but did not appear to show any seasonal trend (Figure 4). For Pineola Bog samples, the median total number of Archaea was estimated at $1 \times 10^8$ cells per gram of soil (range was from $6 \times 10^7 - 1 \times 10^9$) and the mean number of Crenarchaea was estimated at $3 \times 10^8$ cells per gram of soil (range was from

$3 \times 10^6 - 1 \times 10^9$). Tater Hill estimates were consistently on half to two orders of magnitude lower and the median estimated number of total Archaea was $4 \times 10^7$ cells per gram of soil (range was from $1 \times 10^6 - 3 \times 10^8$), whereas the median Crenarchaeal numbers were estimated at $1 \times 10^6$ cells per gram of soil (range was from $6 \times 10^4 - 3 \times 10^7$) (Figure 4). Overall, the estimated number of Crenarchaeal target sequences rivaled that of total Archaeal sequences (Figure 4).

*Sequence coverage and species richness*

Estimates of sample completeness for each library resulted in a fairly consistent Good's coverage ranging from 70-85% of the total possible species present being identified (Table 3). Sampling efficiency was demonstrated by the creation of rarefaction curves from each library as well as all libraries combined and the curves approach a horizontal asymptote (Figure 5). The results reveal the possibility for greater sampling depth given that the limit of sampling was not reached. ACE and Chao1 estimates were calculated to determine the portion of the Archaeal community left unsampled as well as give an estimate of the true species diversity of the site, respectively. ACE and Chao1 estimates both reveal that Tater Hill Bog harbors the most, as yet, undiscovered species, as well as the most diverse population of Archaea, whereas Pineola Bog and Sugar Mountain Bog are more similar to each other (Table 3).

**Discussion**

*Community composition of the methanogenic Archaea*

Among the methanogen-related clones identified, members of the Methanomicrobiales dominated in the oligotrophic bogs, Pineola and Sugar Mountain Bog. The majority (63%) of the Methanomicrobiales clones retrieved clustered with *Methanoregula boonei* strain 6A8, the only formerly described cultured representative of acidophilic methanogens in the E2 group (Bräuer, 2006b; Bräuer et al., 2011). Originally called the R-10 group (Hales, 1996; Edwards et al., 1998), this group is ubiquitous and predominates in ombrotrophic bogs (Galand et al., 2005; Juottonen et al., 2005; Cadillo-Quiroz, 2010). Peat-associated organisms in the E2 group appear to be adapted to

acidic, nutrient-poor environments with low concentrations of exchangeable cations, and clones from this cluster were most abundant in the most acidic site, Pineola Bog (pH 4.7).

Clones affiliated with the family Methanosaetaceae in the Methanosarcinales order were particularly abundant (30%) in Tater Hill Bog and in the cDNA library from Pineola Bog (~40%), but were also fairly abundant (~3%) in Sugar Mountain Bog and Pineola Bog (3-8%) DNA libraries. Organisms of the Methanosaetaceae family are known to be aceticlastic (using acetate as their primary metabolic substrate), with a high affinity for acetate but low utilization rate (Min, 1989; Fukuzaki, 1990; Jetten, 1992; Conrad, 2002; Deppenmeier Uwe, 2007; Buan, 2010). Even though acetate was not specifically measured in this study, it can be assumed that acetate concentration is most likely fairly low in Pineola Bog and Tater Hill Bog given that the dominant methanogenic group present in the peat were those clustering in the Methanosaetacaea with *Methanosaeta concilli*. In contrast, only a few clones (4 total) clustered with *Methanosarcina acetivorans* (Figure 1), which is known to have a lower affinity for acetate and to require concentrations above 1 mM (Min, 1989; Jetten, 1992). Although acetate concentrations have been shown to increase transiently in some sites (Shannon and White, 1994; Duddleston et al., 2002), these studies rarely demonstrated acetate concentrations above 1 mM. The presence of Methanosarcina-related sequences would suggest that acetate concentrations may occasionally reach metabolically available concentrations (ca. 1 mM) either spatially or temporally as previously shown (Shannon, 1996).

Closely following the Methanosaetaceae in dominance were the Methanobacteriales which are a hydrogenotrophic group of methanogens. These organisms are commonly found in boreal (Kotsyurbenko, 2004) and temperate peatlands (McDonald, 1999; Upton, 2000; Basiliko et al., 2003; Metje, 2005; Cadillo-Quiroz et al., 2006; Lin et al., 2012; Yavitt, 2012); however, they are generally less abundant than the Methanomicrobiales. Early studies of Methanobacteriales enrichment cultures from peat suggested these members were less tolerant of acidity (Williams and Crawford, 1985; Horn et al., 2003). However, subsequent laboratory incubations of low pH and low temperature

demonstrated a substantial increase in members of the Methanobacteriacaea, suggesting that these organisms may be important in low pH, low temperature environments (Kotsyurbenko et al., 2007). Sugar Mountain Bog had the largest number of clones in the Methanobacteriales (14%), perhaps because it has the lowest pH of the sites. Finally, clones affiliated with hydrogenotrophic organisms of the RC-I/Methanocellales were detected in Sugar Mountain Bog (~8%) and Tater Hill Bog (1%), and those affiliated with Rice Cluster II were found in two of the Pineola libraries (5-7%). Overall, at least four orders of methanogens were detected in agreement with other researchers who generally find a dominance of the E1/E2 group of Methanomicrobiales (Hales, 1996; Galand, 2005; Cadillo-Quiroz, 2010; Kotsyurbenko, 2010; Kanokratana, 2011) and Methanosaeta/Methanosarcina species (Edwards, 1998; Basiliko, 2003; Kotsyurbenko, 2004; Galand, 2005; Lin et al., 2012), and occasionally detect other groups such as Rice Cluster I (RC-I) (Juottonen et al., 2005; Cadillo-Quiroz, 2010) and Rice Cluster II (Tyson et al., 2013, submitted).

*Deep-branching Euryarchaeota*

In addition to the methanogenic component of the Archaeal communities of each study site, roughly 12% of the clones retrieved fell within the deep-branching Euryarchaeota. These species are typically related to the Thermoplasmata and contain candidate methanogenic groups such as MBG-D (Paul, 2012) as well as verified methanogens affiliated with Rice Cluster III such as *Methanomassiliococcus huminyensis*. The majority of the sequences were found to belong to the Rice Cluster V group (RC-V), which represented approximately 3%, 8%, and 13% of the total Archaeal sequences recovered from Pineola Bog, Tater Hill Bog, and Sugar Mountain Bog, respectively (Table 2). The RC-V group of methanogens is an environmental cluster found ubiquitously throughout freshwater, inland habitats (Barberan, 2011), and they are probably not methanogenic (Chin, 2004). Roughly 1-2% of the total clones retrieved here clustered in the Lake Dagow Sediment Cluster (ca. 2%), the Marine Benthic Group D (up to 1.5%), Rice Cluster III (ca. 1%), and SM1K20. Previous studies have retrieved clones affiliated with Rice Cluster III (Paul,

2012), Rice Cluster V (Barberan, 2011), Marine Benthic Group D (Cadillo-Quiroz et al., 2006), and the Lake Dagow Sediment Cluster (Juottonen, 2008a) from peat. To the best of our knowledge, clones affiliated with SM1K20 have not previously been detected in peat, and, in fact, the top hits were affiliated with wetland, riverine, and estuarine rather than peatland environments. Interestingly, it has been inferred from molecular and phylogenetic analyses that organisms in Marine Benthic Group D (MBG-D) and Rice Cluster III contain mcrA and are likely to be involved in methanogenesis (Paul, 2012). However, more recent metagenomic analyses indicate a role for MBG-D organisms in peptide degradation (Lloyd, 2013). Unfortunately, the physiological roles of other clusters, such as RC-V and SM1K20, remain unknown, although SM1K20 have been associated with marine environments characterized as organic-lean (Durbin, 2012).

*Crenarchaeotal and Thaumarchaeotal lineages*

Crenarchaeal and Thaumarchaeal sequences were also recovered from each study site. Pineola Bog was revealed to contain the most abundant Crenarchaeal/Thaumarchaeal population with ~70-83% of all DNA-derived sequences obtained belonging to these phyla (Table 2), including two clones found to be related to the recently described *Candidatus* Microarchaeum acidiphilum ARMAN-2 (Baker, 2010). Among unsaturated terrestrial environments, Crenarchaea/Thaumarchaea have typically been shown to represent only a small portion of the overall Archaeal population (~0.5 to 3%) (Ochsenreiter, 2003). However, deep marine sediments with colder temperatures and slow decomposition rates have been demonstrated to harbor a dominant Crenarchaeal/Thaumarchaeal community (Teske, 2011; Kubo, 2012), and several clusters within these phyla have been associated with slowly decomposing, anoxic, low-energy environments (Durbin, 2012). Further, Thaum and Crenarchaeal-related sequences can represent a relatively large percentage of the total archaea in some peatlands, including roughly 70% of archaeal DGGE bands identified from eighteen distinct peatlands throughout Alaska and Massachusetts (Rooney-Varga, 2007), roughly half of archaeal SSU rRNA clones from an acidic peatland in Scandinavia (Metje, 2005) and 90% of the archaea in a

peatland in Brazil (Etto et al., 2012). Similarly, Thaumarchaea and Crenarchaeal sequences were found to dominate a forested acidic peatland in Slovenia (Stopnisek, 2010) and a forested peat swamp in Malaysia (Jackson et al., 2009).

Diversity of the Thaumarchaeal and Crenarchaeotal taxa was relatively high, with retrieved sequences clustering in terrestrial groups 1.1b and 1.1c as well as marine group 1.1a, SAGMCG-1, and groups 1.3, C3, pSL12, AK59, and the deep peat lineage. Clones in the MCG cluster 1.3 dominated among all sites and represented ca. 22-31% of the DNA libraries and 10% of the RNA library (Figure 3 and Table 2). Terrestrial group 1.1c, also partially known as Rice Cluster IV, was fairly abundant in Pineola Bog DNA libraries as well (13-39%) and was also detected (ca. 1%) in both the Tater Hill Bog DNA and Pineola Bog RNA libraries. Clones affiliated with the deep peat lineage were most abundant (2-12%) in the Pineola samples and were also detected (1%) in Tater Hill Bog, though not in Sugar Mountain Bog. Sequences clustering in terrestrial group 1.1c, Miscellaneous Crenarchaeotal Group (MCG) 1.3, and the deep peat lineage have been found in acidic peat soil in Finland (Juottonen, 2008a; Putkinen, 2009), in Slovenia (Stopnisek, 2010) and members of both the terrestrial group and MCG were also detected in Brazil (Etto et al., 2012). It has been shown that group 1.1c can be selected in low pH conditions and can predominate in acidic (ca. pH 4) soil types (Jurgens, 1997; Oline, 2006; Bomberg, 2007; Kemnitz, 2007; Lehtovirta, 2009). Thus, it is not too surprising to find this group in the acidic (pH 4.7–5.7) peat studied here.

Additionally, sequences were retrieved from several lineages less commonly reported in bogs, including marine group 1.1a, SAGMCG-1, and groups 1.3, C3, pSL12, and AK59. Most notably, the proportion of sequences clustering in the marine group 1.1a rivaled that of the deep peat lineage, with greater than 5% of the sequences from all three sites falling in this cluster. Interestingly, a sequence closely related to the newly described microarchaea *Candidatus Microarchaeum acidiphilum* (ARMAN-2) (Baker, 2010) was retrieved from the Pineola 2011 clone library. Using sequence data available for ARMAN-2 and related sequences retrieved from a Finnish peatland

(Juottonen, 2008a), we designed ARMAN-2-specific primers and retrieved sequences for a second OTU (Figure 3). Given that ARMAN-2 was originally identified from acid mine drainage (Baker, 2006), it is interesting to speculate as to the role this organism is playing in acidic peatland environments.

*Functional role of the Crenarchaeal-related organisms*

Despite current understanding that mesophilic Thaumarchaeota and Crenarchaeota are ubiquitous in cold, circumneutral, oxic oceanic waters (DeLong, 1992; Fuhrman, 1992) as well as in terrestrial (Buckley et al., 1998) and freshwater environments (Schleper, 1997), little is known about their metabolic functions (Koch et al., 2006; Juottonen, 2008a; Lehtovirta, 2009; Etto et al., 2012). Culturing studies indicate that some mesophilic Crenarchaea in marine and estuarine environments may be involved in ammonia oxidation (Könneke et al., 2005) and indeed Crenarchaeal amoA genes have been discovered in a wide range of environments (Lam, 2007; Beman, 2008; Tourna, 2008). Of the groups represented in our sequences, only two are known to carry amoA, 1.1a and 1.1b (Nicol, 2006). Group 1.1a is less common in peatlands and the relatively large proportion of clones in this group (>5%) suggests that nitrogen cycling may be somewhat important in these sites, despite being considered a minor process in most nutrient-poor, carbon-rich peat environments (Dedysh, 2011). Overall, the majority of the clones retrieved clustered in group 1.3 (MCG; >20%) and terrestrial group 1.1c (ca. 6%). Although speculative, it is likely that the dominant Thaumarchaea and Crenarchaea in peatlands may be involved in anaerobic fermentation. Indeed, genomic analyses of *Nitrosopumilus maritiumus* and *Cenarchaeum symbiosum* demonstrated the presence of predicted transporters for amino acids, peptides, and glycerol (Pester, 2011), and recent metagenomic analyses indicate a role for MCG organisms in peptide degradation (Lloyd, 2013), at least in deep-sea environments. Overall, Crenarchaeal populations present in peatlands represent potentially new functional groups of archaea, possibly playing important roles in the biogeochemical cycling of nutrients in these systems and as such warrant further study.

*Potentially active Archaeal groups*

In order to investigate the potential activity among archaeal groups, a cDNA clone library from extracted rRNA was constructed in the summer of 2012 and compared to a DNA control library. It was revealed that Methanosarcinales-related sequences represented ~40% of all sequences obtained (Table 2). Methanomicrobiales and RC-II were also represented in the library (23 and 7% respectively) as well as Thaum and Crenarchaeal-related groups, suggesting at least a minimum level of maintenance activity sometime near the time of sampling, and verifying that Crenarchaeotal-related organisms are not only present, but most likely active, functional members of the community. The over-representation of methanogen-related sequences in the RNA library compared to the two DNA libraries may suggest that the methanogens were more active than the Thaum and Crenarchaeal-related organisms. Alternatively, the abundance of methanogen-related rRNA may reflect a combination of larger cell volume and/or number of ribosomes per cell, since members of the MCG are reported to be quite small (0.4-0.5 µm coccoids) (Kubo, 2012). In contrast, Methanosaeta spp. are 2-6 µm long by 0.8-1.3 µm wide (Patel et al., 1990) and Methanosarcina cells are 1.5-2 µm coccoids (Bryant, 1987). By volume, Methanosaeta and Methanosarcina cells would be anywhere from 15-200 times larger than the typical Crenarchaeotal cell and could contain orders of magnitude greater numbers of ribosomes, suggesting that in our study, Crenarchaeota probably do represent a significant proportion of the microbial community by cell number, despite the fact that only 24% of the sequences retrieved from the rRNA library fell within this group. Overall, our findings support a growing body of evidence suggesting that Thaumarchaea and Crenarchaea may be numerically abundant in many different peatlands worldwide.

*Archaeal abundance*

The numerical abundance of Crenarchaeal-related sequences in both Tater Hill and Pineola was confirmed with qPCR analyses. Results estimated a median of roughly $10^8$ total Archaeal or Crenarchaeal cells per gram of soil in Pineola Bog, in line with previous studies in two northern

peatlands, which found up to $10^8$ total Archaeal cells per gram (Cadillo-Quiroz et al., 2006). The Archaeal communities of the wetlands studied here appeared to vary significantly in estimated cell numbers between samples, perhaps reflecting the site heterogeneity that is known to be a hallmark of southern peatlands. Overall, the bog site (Pineola, NC) demonstrated consistently greater numbers of estimated archaeal cells (median of $10^8$) when compared to the more circum-neutral site (Tater Hill, NC), which had a median number of $4 \times 10^7$ estimated total Archaea per gram of soil. Because it has been demonstrated that water table level has a significant impact on the Archaeal community of wetlands (Bridgham, 1992b; Bubier, 1995; Kettunen et al., 1999b; Weijers et al., 2004; Kotiaho, 2010; Webster, 2010), this could be the result of the slightly more stable water table level for Pineola. Given its forested topography, the site is protected from sudden flooding due to heavy rainfall and also from drying during the warmer months due to evaporation. The largest discrepancy between the sites was observed during the fall sampling season, when Tater Hill Bog demonstrated a dip in Archaeal numbers perhaps due to the open nature of the site that results in a decrease in the amount of leaf litter deposited during this time of increased bacterial decomposition (Clymo, 1965; Brock, 1989; Bardgett et al., 2003; Julies, 2010; Andersson et al., 2012).

*Sequence coverage and species richness*

Good's non-parametric coverage estimate revealed a sampling completeness of 70-85% in relation to the possible number of species that could be identified among the sample sites, suggesting that the majority of the major microbial taxa were most likely represented (Table 3). Rarefaction analyses indicated that the sequencing depth capacity of clone libraries was nearly exhausted by sequencing 400-500 clones across five libraries; fewer than 150 Archaeal taxa were identified. Results suggest that orders of magnitude deeper sequencing efforts would be required to discover a significantly higher diversity and identify rare community members, as is often revealed through next generation sequencing (Sogin, 2006). In contrast to some studies where acidic peat is sometimes predominated by the R10/E1/E2/Methanoregulacaea methanogens, our study revealed many distinct

methanogenic taxa as well as diverse taxa in the deep-branching Euryarchaea and Crenarchaeal-related sequences. The relatively high Archaeal sequence diversity and large number of distinct Archaeal taxa obtained here likely result from two main features. First, the degree of spatial heterogeneity in Southern Appalachian peatlands (Weakley and Schafale, 1994) is likely a contributing factor, since environmental heterogeneity is known to contribute to microbial diversity (Green, 2006). Additionally, low pH is known to limit diversity in soils (Fierer, 2006), including peatland soils (Kotsyurbenko, 2010). The overall pH averages measured for Pineola and Sugar Mountain (5.0 and 4.7, respectively) are at the higher end of that found in other oligotrophic bogs, which typically have pH values below 5, or sometimes as low as 2.6 (Mitsch, 2007). It is worth mentioning, however, that certain species of Archaea are often affiliated with low pH environments, such as group 1.1c Thaumarchaea (Lehtovirta, 2009) and the E1/E2 (or R10) group, or Methanoregulaceae (Hales, 1996; Bräuer, 2006b; Cadillo-Quiroz et al., 2006; Kanokratana, 2011); however, the overall diversity is generally low in acidic environments (Galand, 2005). Indeed, among the sites measured here, the location with the highest pH, Tater Hill (average pH of 5.7), had the largest species diversity estimates of 153 using ACE and 107 using Chao1, compared to 46-98 using ACE and 45-88 using Chao1 for libraries generated using samples from the other two sites with average pH values of 4.7 and 5.0. In line with our studies, microbial diversity was shown to increase along a gradient from pH 4.2 in an ombrotrophic bog to 5.1 in a mesotrophic fen in Finland (Juottonen et al., 2005). Similarly, a fen in Minnesota was found to have higher diversity than that of a nearby bog (Lin, 2012).

**Conclusions**

The study described here is one of the first molecular studies to focus mainly on temperate Southern U.S. peatlands and as such presents a unique perspective into variables specific to warmer climates. Results obtained from the experimental investigation reported here add to the knowledge base surrounding wetland habitats and their roles in numerous environmental aspects as well as reveal

new information relevant to 1) using more southerly, temperate peatland sites as a proxy for studying the effects of potential climate warming events and 2) the variation in microbial community structure/activity amongst varying climates. Archaeal numbers remain unchanged throughout the seasonal shifts and appear to have adapted to the environment and the variables unique to the region, as indicated by quantitative data taken throughout the duration of the sampling. Community members present among the study areas demonstrate the abundance of methanogenic Archaea present and active among sites, indicating the potential for increased methane emissions from such sites. The presence of an abundance of Crenarchaeotic sequences adds to a growing body of literature suggesting that these organisms may be more ubiquitous in peatlands than has previously been understood. However, the function of these organisms has yet to be elucidated.

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**Figures and Tables**

**Table 1.** General characteristics of the peatland study sites. The values are reported either as an average or as a range encompassing the entire sampling period between the summer of 2010 and the winter of 2012. Potential methane production rates were determined in a previous study.

| | Pineola | Tater Hill | Sugar Mountain |
|---|---|---|---|
| Wetland Description | Oligotrophic forested swamp-bog complex | Minerotrophic Southern Appalachian Bog | Oligotrophic Southern Appalachian Bog |
| Temperature Range (°C) | 5.0-15.5 | 4.5-16.5 | 4.5-16.5 |
| pH average (range) | 5.0 (4.0-5.6) | 5.7 (4.9-6.8) | 4.7 (3.9-5.3) |
| Vegetation | *Sphagnum* spp., *Pinus palustris, Acer saccharum, Rhododendron maximum, Vaccinium macrocarpon,* | *Sphagnum* spp., *Rhododendron* spp., *Vaccinium macrocarpon, Carex* spp., *Juncus* spp., *Lilium grayi* | *Sphagnum* spp., *Rhododendron maximum, Vaccinium corymbosum, Rosa palustris, Carex* spp. |
| Water Table Range (cm below surface) | 2.5-15 | 0.0-7.5 | 0.0-7.5 |
| Potential Methane Production (mmol/L $CH_4$ $d^{-1}$) | 0.006 | 0.029 | 0.014 |

**Table 2.** Phylogenetic representation of the Archaeal community present in all three sample sites as estimated from clone sequences retrieved.

| | Tater Hill Bog, NC | Sugar Mtn. Bog, NC | Pineola Bog, NC | | |
|---|---|---|---|---|---|
| | | | DNA 2011 | DNA 2012 | RNA 2012 |
| **Methanogens** | | | | | |
| *Methanomicrobiales* | 17.86% | 25.64% | 6.58% | 10.89% | 23.33% |
| *Methanocellales* | 1.19% | 7.69% | 0.00% | 0.00% | 0.00% |
| Rice Cluster II | 0.00% | 0.00% | 5.26% | 0.00% | 6.67% |
| *Methanosarcinales* | 29.76% | 2.56% | 7.90% | 3.96% | 40.00% |
| *Methanobacteriales* | 3.57% | 14.10% | 0.00% | 0.00% | 1.11% |
| | **52.38%** | **50.00%** | **19.74%** | **14.85%** | **71.11%** |
| **Deep Euryarchaea** | | | | | |
| Rice Cluster V | 8.33% | 12.82% | 2.63% | 0.99% | 3.33% |
| Lake Dagow Sediment Cluster | 3.57% | 2.56% | 1.32% | 0.99% | 1.11% |
| Marine Benthic Group D | 0.00% | 0.00% | 5.26% | 0.00% | 0.00% |
| Rice Cluster III | 0.00% | 2.56% | 0.00% | 0.00% | 0.00% |
| SM1K20 | 1.19% | 0.00% | 1.32% | 0.00% | 0.00% |
| | **13.09%** | **17.95%** | **10.53%** | **1.98%** | **4.44%** |
| **Crenarchaea** | | | | | |
| Deep Peat Group | 1.19% | 0.00% | 11.84% | 3.97% | 2.22% |
| Thaumarchaea Grp C3 | 1.19% | 1.28% | 0.00% | 0.00% | 0.00% |
| Unidentified Miscellaneous Crenarcha (MCG) | 0.00% | 0.00% | 6.58% | 0.00% | 1.11% |
| MCG Grp 1.3 | 26.19% | 21.80% | 30.26% | 30.69% | 10.00% |
| Thaumarchaea pSL12 | 0.00% | 0.00% | 1.32% | 0.00% | 0.00% |
| Thaumarchaea AK59 | 0.00% | 0.00% | 2.63% | 0.00% | 0.00% |
| Grp. 1.1a | 4.76% | 7.69% | 2.63% | 7.92% | 7.78% |
| Grp. 1.1b | 0.00% | 0.00% | 0.00% | 0.00% | 1.11% |
| SAGMCG-1 | 0.00% | 1.28% | 0.00% | 0.99% | 1.11% |
| Grp. 1.1c | 1.19% | 0.00% | 13.16% | 38.61% | 1.11% |
| ARMAN | 0.00% | 0.00% | 1.32% | 0.99% | 0.00% |
| | **34.52%** | **32.05%** | **69.74%** | **83.17%** | **24.44%** |

**Table 3.** Similarity-based OTUs and species richness estimates.

| | | | Cluster Distance | | | |
|---|---|---|---|---|---|---|
| | | | 0.03 | | | |
| **Year of Sample** | **Sample Site (library type)** | **Number of Sequences** | **OTU** | **ACE** | **Chao 1** | **Good's Coverage** |
| 2011 | Pineola (DNA) | 78 | 31 | 68 | 88 | 76% |
| 2011 | Tater Hill (DNA) | 90 | 37 | 153 | 107 | 70% |
| 2011 | Sugar Mtn. (DNA) | 88 | 34 | 98 | 73 | 75% |
| 2012 | Pineola (DNA) | 93 | 25 | 46 | 45 | 85% |
| 2012 | Pineola (RNA) | 91 | 34 | 90 | 76 | 77% |

**Figure 1.**

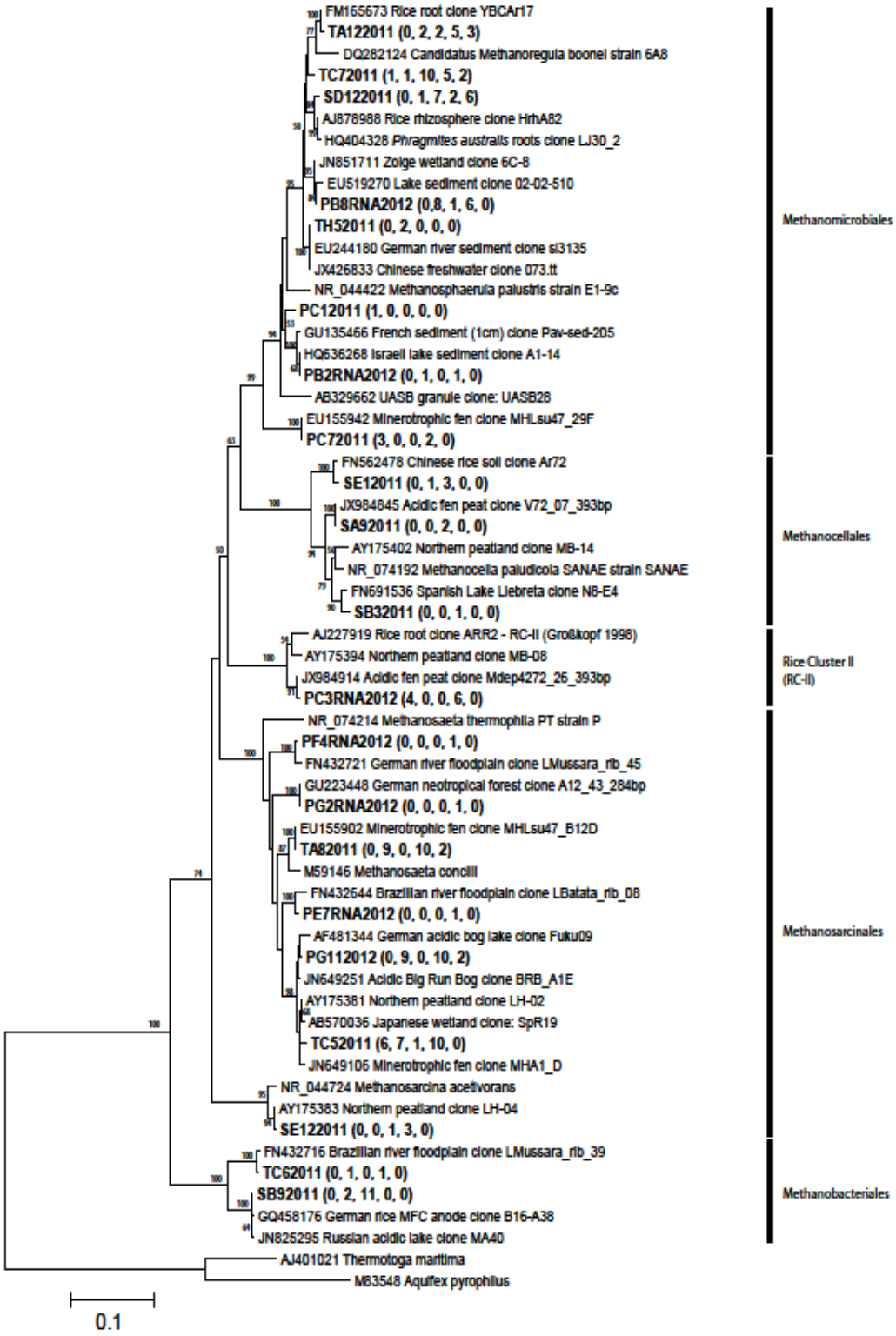
FM165673 Rice root clone YBCAr17
TA122011 (0, 2, 2, 5, 3)
DQ282124 Candidatus Methanoregula boonei strain 6A8
TC72011 (1, 1, 10, 5, 2)
SD122011 (0, 1, 7, 2, 6)
AJ878988 Rice rhizosphere clone HrhA82
HQ404328 Phragmites australis roots clone LJ30_2
JN851711 Zoige wetland clone 6C-8
EU519270 Lake sediment clone 02-02-510
PB8RNA2012 (0,8, 1, 6, 0)
TH52011 (0, 2, 0, 0, 0)
EU244180 German river sediment clone sl3135
JX426833 Chinese freshwater clone 073.tt
NR_044422 Methanosphaerula palustris strain E1-9c
PC12011 (1, 0, 0, 0, 0)
GU135466 French sediment (1cm) clone Pav-sed-205
HQ636268 Israeli lake sediment clone A1-14
PB2RNA2012 (0, 1, 0, 1, 0)
AB329662 UASB granule clone: UASB28
EU155942 Minerotrophic fen clone MHLsu47_29F
PC72011 (3, 0, 0, 2, 0)
FN562478 Chinese rice soil clone Ar72
SE12011 (0, 1, 3, 0, 0)
JX984845 Acidic fen peat clone V72_07_393bp
SA92011 (0, 0, 2, 0, 0)
AY175402 Northern peatland clone MB-14
NR_074192 Methanocella paludicola SANAE strain SANAE
FN691536 Spanish Lake Liebreta clone N8-E4
SB32011 (0, 0, 1, 0, 0)
AJ227919 Rice root clone ARR2 - RC-II (Großkopf 1998)
AY175394 Northern peatland clone MB-08
JX984914 Acidic fen peat clone Mdep4272_26_393bp
PC3RNA2012 (4, 0, 0, 6, 0)
NR_074214 Methanosaeta thermophila PT strain P
PF4RNA2012 (0, 0, 0, 1, 0)
FN432721 German river floodplain clone LMussara_rib_45
GU223448 German neotropical forest clone A12_43_284bp
PG2RNA2012 (0, 0, 0, 1, 0)
EU155902 Minerotrophic fen clone MHLsu47_B12D
TA82011 (0, 9, 0, 10, 2)
M59146 Methanosaeta concilii
FN432644 Brazilian river floodplain clone LBatata_rib_08
PE7RNA2012 (0, 0, 0, 1, 0)
AF481344 German acidic bog lake clone Fuku09
PG112012 (0, 9, 0, 10, 2)
JN649251 Acidic Big Run Bog clone BRB_A1E
AY175381 Northern peatland clone LH-02
AB570036 Japanese wetland clone: SpR19
TC52011 (6, 7, 1, 10, 0)
JN649106 Minerotrophic fen clone MHA1_D
NR_044724 Methanosarcina acetivorans
AY175383 Northern peatland clone LH-04
SE122011 (0, 0, 1, 3, 0)
FN432716 Brazilian river floodplain clone LMussara_rib_39
TC62011 (0, 1, 0, 1, 0)
SB92011 (0, 2, 11, 0, 0)
GQ458176 German rice MFC anode clone B16-A38
JN825295 Russian acidic lake clone MA40
AJ401021 Thermotoga maritima
M83548 Aquifex pyrophilus
0.1
Methanomicrobiales
Methanocellales
Rice Cluster II
(RC-II)
Methanosarcinales
Methanobacteriales

**Figure 2** .


SB52011 (0, 0, 2, 0, 0)
AJ867612 Swiss lake water clone M01
AM495334 German rice root clone LL1Root_09
TB62011 (0, 1, 1, 0, 0)
GU257278 German freshwater sediment clone OTRo_D1_78
AB686512 Japanese rice field soil clone: R8
AM712540 Permafrost peat clone Sv-66 - RC-V (Bhattaral 2012)
FR820765 Spanish Lake Liebreta clone ANNA-C8
PA102012 (0, 0, 0, 0, 1)
AY531695 German lake sediment clone Dg2002_T_16 - RC-V (Chan 2005)
PE4RNA2012 (0, 0, 0, 1, 0)
GU257225 German freshwater sediment clone OTRo_D1_9
JF262363 Yellowstone lake clone YLA079
PD112011 (1, 0, 0, 0, 0)
DQ310451 Canadian Mackenzie River clone mrR2.67 - RC-V (Galand 2006)
JN820176 Cave ferromanganese deposit clone G06
HE613643 Spanish ferromanganese biofilm clone 09T2A-G5
SG12011 (0, 0, 1, 0, 0)
AJ867624 Swiss lake water clone M33
TF52011 (0, 1, 0, 0, 0)
EU244238 Canadian Lake Mackenzie clone CaS1b.b08
SF112011 (0, 0, 1, 0, 0)
SC102011 (0, 0, 1, 0, 0)
AM503250 German rice root clone LL_Koral_38
JN853711 Swiss lake sediment clone KS8_3a
AM503238 German rice root clone LL_Koral_23 - RC-V (Bhattaral 2012)
AY133914 German Lake Dagow sediment clone LDS26
TC12011 (0, 1, 0, 0, 0)
PE12011 (1, 0, 0, 0, 0)
JQ315353 Russian oil field clone FwtA_otu6_3
EU244296 Canadian Mackenzie River clone CaR3b.f12
PA6RNA2012 (0, 0, 0, 1, 0)
JQ792392 Canadian oligotrophic lake sediment clone ALAKE10_44
SG32011 (0, 0, 1, 0, 0)
GU257074 German freshwater sediment clone OTRo_A1_20
SE52011 (0, 0, 1, 0, 0)
TG72011 (0, 1, 1, 0, 0)
FR865353 Chinese rice field soil clone t30d34La7
SH52011 (0, 0, 1, 0, 0)
AJ227951 German rice soil clone ABS3 - RC-V (Großkopf 1998)
DQ310411 Canadian Mackenzie River clone mrR1.22
TE122011 (0, 1, 0, 0, 0)
GU257184 Freshwater sediment clone OTRo_O1_23
JF835243 Brazilian river water clone IG2F06
PD3RNA2012 (0, 0, 0, 1, 0)
GU047745 Wetland clone AL.59
DQ310383 Canadian Mackenzie River clone mrR1.51 - RC-V (Galand 2006)
TA42011 (0, 1, 0, 0, 0)
AB107814 Japanese paddy soil clone:TN-1
AB380105 Japanese soil clone: IU-FSC-17_Ar-A026
TE42011 (0, 1, 0, 0, 0)
AJ548932 Oligotrophic fen clone FenF-16S
JN825241 Russian acidic lake clone DA65
PG62012 (0, 0, 0, 1, 1)
JN825231 Russian acidic lake clone DA36
AY133915 German lake sediment clone LDS27 - LDS (Bhattaral 2012)
JF431710 Chinese lake sediment clone 30-10-T6
TD22011 (0, 1, 1, 0, 0)
AM503240 German rice root clone LL_Koral_26
TG12011 (1, 2, 1, 0, 0)
DQ310396 Canadian Mackenzie River clone mrR1.39
EF014581 Canadian Lake Mackenzie clone CaS1s.50
EU155986 Minerotrophic fen clone MH1100_E4B
PA92011 (4, 0, 0, 0, 0)
AM851081 Alpine lake sediment clone Cad915-20
DQ301914 Acidic peatland clone CBd-305F
JF789814 Freshwater wetland clone EC081.16
AJ576232 Landfill leachate clone GZK56 - VADIN (Paul 2012)
EU910624 Sediment clone G16 - TMEG-1 (Paul 2012)
AJ576219 Landfill leachate clone GZK24 -TMEG-2 (Paul 2012)
FM957983 Chinese rice paddy soil clone NA5AR42 - RC-III (Paul 2012)
SG102011 (0, 0, 2, 0, 0)
JN605146 Estuarine sediment clone 87B915r4 - SM1K20 (Breuker 2013)
TE102011 (0, 1, 0, 0, 0)
FJ479771 Zolge wetland clone 7C-6
PC52011 (1, 0, 0, 0, 0)
EF014531 Canadian Mackenzie River clone CaR3s.23
AJ401021 Thermotoga maritima
M83548 Aquifex pyrophilus
0.1
Rice Cluster V (RC-V)
Lake Dagow Sediment Group (LDS)
Marine Benthic Group D (MBG-D)
VADIN
TMEG
Rice Cluster III (RC-III)
SM1K20

**Figure 3 .**

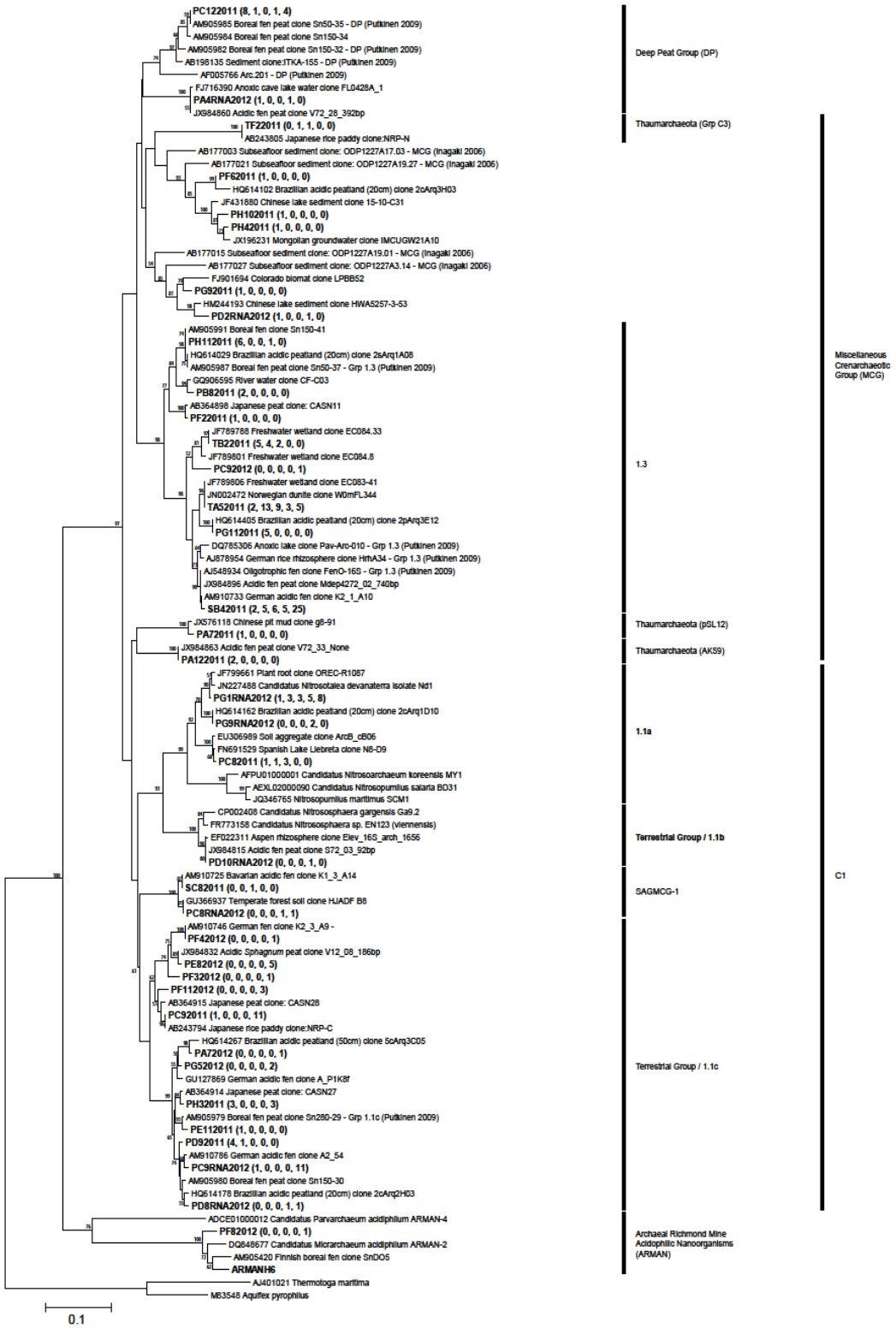
PC122011 (8, 1, 0, 1, 4)
AM905985 Boreal fen peat clone Sn50-35 - DP (Putkinen 2009)
AM905984 Boreal fen peat clone Sn150-34
AM905982 Boreal fen peat clone Sn150-32 - DP (Putkinen 2009)
AB198135 Sediment clone:ITKA-155 - DP (Putkinen 2009)
AF005766 Arc.201 - DP (Putkinen 2009)
FJ716390 Anoxic cave lake water clone FL0428A_1
PA4RNA2012 (1, 0, 0, 1, 0)
JX984860 Acidic fen peat clone V72_28_392bp
TF22011 (0, 1, 1, 0, 0)
AB243805 Japanese rice paddy clone:NRP-N
AB177003 Subseafloor sediment clone: ODP1227A17.03 - MCG (Inagaki 2006)
AB177021 Subseafloor sediment clone: ODP1227A19.27 - MCG (Inagaki 2006)
PF62011 (1, 0, 0, 0, 0)
HQ614102 Brazilian acidic peatland (20cm) clone 2cArq3H03
JF431880 Chinese lake sediment clone 15-10-C31
PH102011 (1, 0, 0, 0, 0)
PH42011 (1, 0, 0, 0, 0)
JX196231 Mongolian groundwater clone IMCUGW21A10
AB177015 Subseafloor sediment clone: ODP1227A19.01 - MCG (Inagaki 2006)
AB177027 Subseafloor sediment clone: ODP1227A3.14 - MCG (Inagaki 2006)
FJ901694 Colorado biomat clone LPBB52
PG92011 (1, 0, 0, 0, 0)
HM244193 Chinese lake sediment clone HWA5257-3-53
PD2RNA2012 (1, 0, 0, 1, 0)
AM905991 Boreal fen clone Sn150-41
PH112011 (6, 0, 0, 1, 0)
HQ614029 Brazilian acidic peatland (20cm) clone 2sArq1A08
AM905987 Boreal fen peat clone Sn50-37 - Grp 1.3 (Putkinen 2009)
GQ906595 River water clone CF-C03
PB82011 (2, 0, 0, 0, 0)
AB364898 Japanese peat clone: CASN11
PF22011 (1, 0, 0, 0, 0)
JF789788 Freshwater wetland clone EC084.33
TB22011 (5, 4, 2, 0, 0)
JF789801 Freshwater wetland clone EC084.8
PC92012 (0, 0, 0, 0, 1)
JF789806 Freshwater wetland clone EC083-41
JN002472 Norwegian dunite clone W0mFL344
TA52011 (2, 13, 9, 3, 5)
HQ614405 Brazilian acidic peatland (20cm) clone 2pArq3E12
PG112011 (5, 0, 0, 0, 0)
DQ785306 Anoxic lake clone Pav-Arc-010 - Grp 1.3 (Putkinen 2009)
AJ878954 German rice rhizosphere clone HrhA34 - Grp 1.3 (Putkinen 2009)
AJ548934 Oligotrophic fen clone FenO-16S - Grp 1.3 (Putkinen 2009)
JX984896 Acidic fen peat clone Mdep4272_02_740bp
AM910733 German acidic fen clone K2_1_A10
SB42011 (2, 5, 6, 5, 25)
JX576118 Chinese pit mud clone g8-91
PA72011 (1, 0, 0, 0, 0)
JX984863 Acidic fen peat clone V72_33_None
PA122011 (2, 0, 0, 0, 0)
JF799661 Plant root clone OREC-R1087
JN227488 Candidatus Nitrosotalea devanaterra isolate Nd1
PG1RNA2012 (1, 3, 3, 5, 8)
HQ614162 Brazilian acidic peatland (20cm) clone 2cArq1D10
PG9RNA2012 (0, 0, 0, 2, 0)
EU306989 Soil aggregate clone ArcB_cB06
FN691529 Spanish Lake Liebreta clone N8-D9
PC82011 (1, 1, 3, 0, 0)
AFPU01000001 Candidatus Nitrosoarchaeum koreensis MY1
AEXL02000090 Candidatus Nitrosopumilus salaria BD31
JQ346765 Nitrosopumilus maritimus SCM1
CP002408 Candidatus Nitrososphaera gargensis Ga9.2
FR773158 Candidatus Nitrososphaera sp. EN123 (viennensis)
EF022311 Aspen rhizosphere clone Elev_16S_arch_1656
JX984815 Acidic fen peat clone S72_03_92bp
PD10RNA2012 (0, 0, 0, 1, 0)
AM910725 Bavarian acidic fen clone K1_3_A14
SC82011 (0, 0, 1, 0, 0)
GU366937 Temperate forest soil clone HJADF B8
PC8RNA2012 (0, 0, 0, 1, 1)
AM910746 German fen clone K2_3_A9 -
PF42012 (0, 0, 0, 0, 1)
JX984832 Acidic Sphagnum peat clone V12_08_186bp
PE82012 (0, 0, 0, 0, 5)
PF32012 (0, 0, 0, 0, 1)
PF112012 (0, 0, 0, 0, 3)
AB364915 Japanese peat clone: CASN28
PC92011 (1, 0, 0, 0, 11)
AB243794 Japanese rice paddy clone:NRP-C
HQ614267 Brazilian acidic peatland (50cm) clone 5cArq3C05
PA72012 (0, 0, 0, 0, 1)
PG52012 (0, 0, 0, 0, 2)
GU127869 German acidic fen clone A_P1K8f
AB364914 Japanese peat clone: CASN27
PH32011 (3, 0, 0, 0, 3)
AM905979 Boreal fen peat clone Sn280-29 - Grp 1.1c (Putkinen 2009)
PE112011 (1, 0, 0, 0, 0)
PD92011 (4, 1, 0, 0, 0)
AM910786 German acidic fen clone A2_54
PC9RNA2012 (1, 0, 0, 0, 11)
AM905980 Boreal fen peat clone Sn150-30
HQ614178 Brazilian acidic peatland (20cm) clone 2cArq2H03
PD8RNA2012 (0, 0, 0, 1, 1)
ADCE01000012 Candidatus Parvarchaeum acidiphilum ARMAN-4
PF82012 (0, 0, 0, 0, 1)
DQ848677 Candidatus Micrarchaeum acidiphilum ARMAN-2
AM905420 Finnish boreal fen clone SnDO5
ARMANH6
AJ401021 Thermotoga maritima
M83548 Aquifex pyrophilus
0.1
Deep Peat Group (DP)
Thaumarchaeota (Grp C3)
1.3
Thaumarchaeota (pSL12)
Thaumarchaeota (AK59)
1.1a
Terrestrial Group / 1.1b
SAGMCG-1
Terrestrial Group / 1.1c
Archaeal Richmond Mine Acidophilic Nanoorganisms (ARMAN)
Miscellaneous Crenarchaeotic Group (MCG)
C1

**Figure 4.**

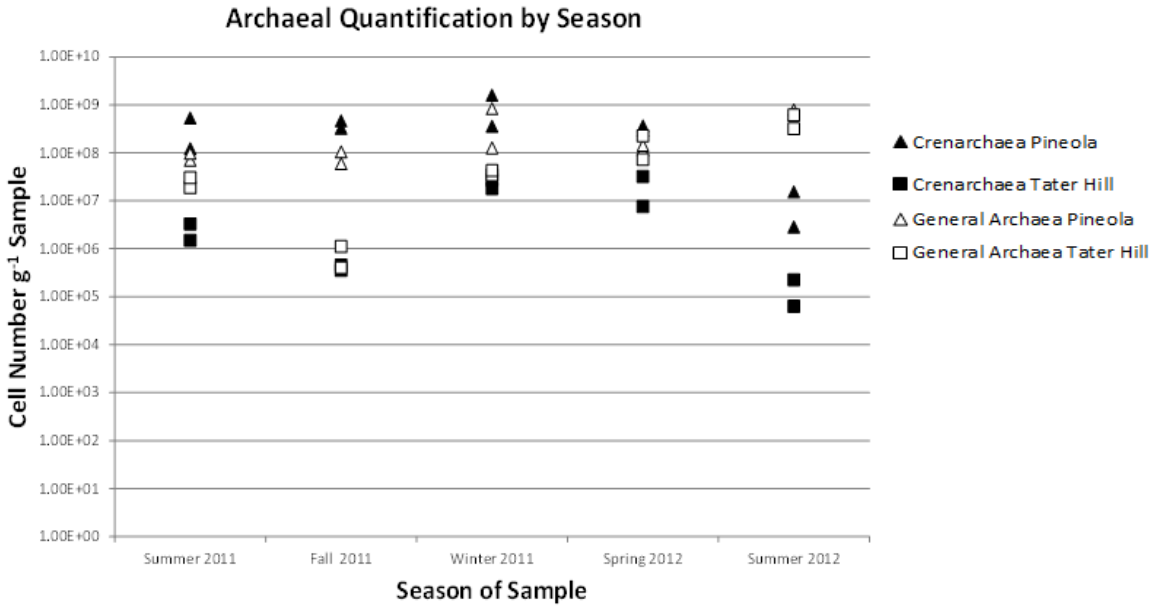


**Figure 5.**

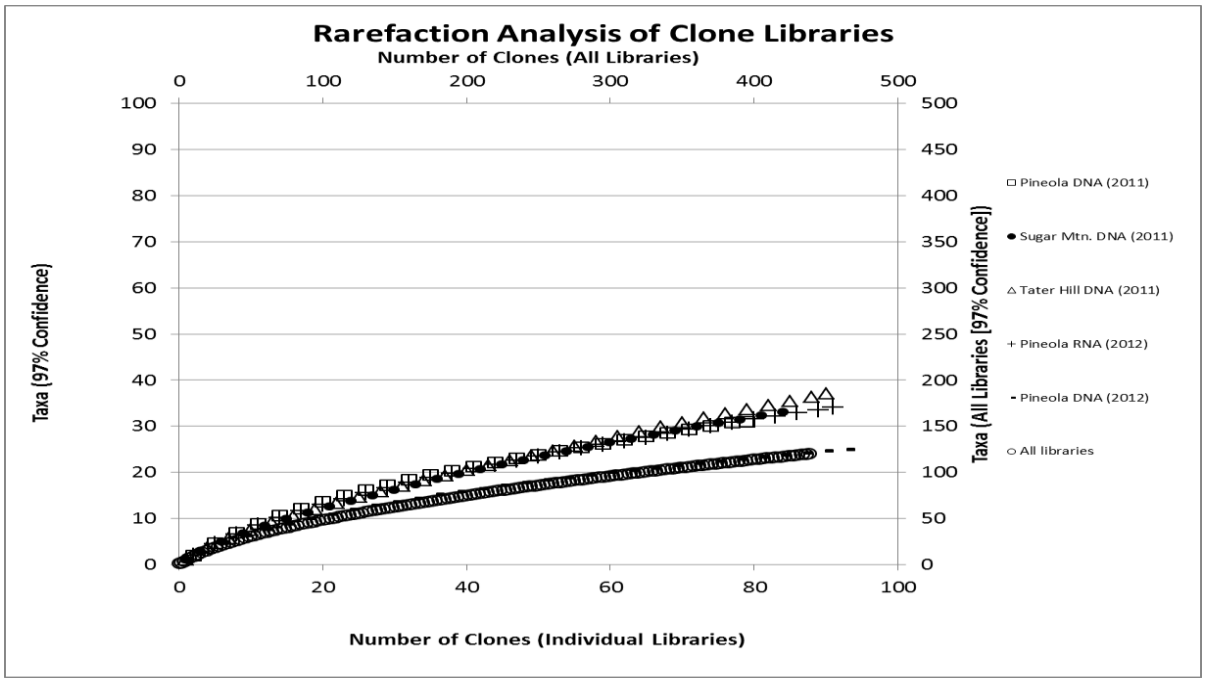

**Figure 1.** Neighbor-joining tree demonstrating the phylogenetic relationship of Methanogenic Euryarchaeotal SSU rRNA gene sequences retrieved from Pineola Bog, Avery County, NC, and Tater Hill Bog, Watauga County, NC, and Sugar Mountain Bog, Avery County, NC. Sequences represent both DNA and cDNA sequencing libraries, and only representative sequences of each operational taxonomic unit (OTU) are included in this tree. The number of sequences represented by each OTU is given in parentheses in the following arrangement: 1) DNA sequences from Pineola Bog (2011), 2) DNA sequences from Tater Hill Bog (2011), 3) DNA sequences from Sugar Mountain Bog (2011), 4) RNA sequences from Pineola Bog (2012) 5) DNA sequences from Pineola Bog (2012). Alignments were created using the ARB-based online SILVA aligner. Dendrogram was created using the PHYLIP package. *Thermotoga maritima* and *Aquifex pyrophilus* were used as outgroups. Bootstrap values represent 100 replicates with values >50 and supported by maximum likelihood analysis.

**Figure 2.** Neighbor-joining tree allowing reference of the phylogenetic relationship of deep-branching Euryarchaeotal SSU rRNA gene sequences retrieved from Pineola Bog in Avery County, NC, Tater Hill Bog in Watauga County, NC, and Sugar Mountain Bog in Avery County, NC. Sequences represent both DNA and cDNA sequencing libraries and only representative sequences of each operational taxonomic unit (OTU) are included in this tree. The number of sequences represented by each OTU is given in parentheses in the following arrangement: 1) DNA sequences from Pineola Bog (2011), 2) DNA sequences from Tater Hill Bog (2011), 3) DNA sequences from Sugar Mountain Bog (2011), 4) RNA sequences from Pineola Bog (2012), 5) DNA sequences from Pineola Bog (2012). Alignments were created using the ARB-based online SILVA aligner. Dendrogram was created using the PHYLIP package. *Thermotoga maritima* and *Aquifex pyrophilus* were used as outgroups. Bootstrap values represent 100 replicates and values are shown for node values both >50 and supported by maximum likelihood analysis.

**Figure 3.** Neighbor-joining tree demonstrating the phylogenetic relationship of Crenarchaeotal SSU rRNA gene sequences retrieved from Pineola Bog, Avery County, NC, and Tater Hill Bog, Watauga County, NC, and Sugar Mountain Bog, Avery County, NC. Sequences represent both DNA and cDNA sequencing libraries and only representative sequences of each operational taxonomic unit (OTU) are included in this tree. The number of sequences represented by each OTU is given in parentheses in the following arrangement: 1) DNA sequences from Pineola Bog (2011), 2) DNA sequences from Tater Hill Bog (2011), 3) DNA sequences from Sugar Mountain Bog (2011), 4) RNA sequences from Pineola Bog (2012), 5) DNA sequences from Pineola Bog (2012). Alignments were created using the ARB-based online SILVA aligner. Dendrogram was created using the PHYLIP package. *Thermotoga maritima* and *Aquifex pyrophilus* were used as outgroups. Bootstrap values represent 100 replicates with values >50 and supported by maximum likelihood analysis.

**Figure 4.** Quantification of Archaeal populations by real time quantitative PCR shown in relation to season. Square symbols = Tater Hill, NC; Triangle symbols = Pineola, NC; Solid symbols = Crenarchaeota; Open symbols = General Archaea.

**Figure 5.** Rarefaction analysis of all clone libraries obtained from all three study sites. Every third data point is shown to allow improved visualization.

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# CHAPTER 3: SUMMARY, CONCLUSIONS, AND BROADER IMPACTS

## Introduction

Through the course of my research, I sought to address questions regarding the sparsely studied Southern Appalachian wetland ecosystems of North Carolina and their importance in global climate change. In particular, my objectives were to answer questions relating to the microbial communities found in these environments and the role of these organisms in methane cycling within the system (Chapter Two). Throughout the course of study, several variables were identified as having influence on the underlying microbial consortia. Here, the summary of these results is reported along with conclusions that take into account the full scope of the work and suggestions for potential future avenues of research. I will also present potential opportunities for a broader impact of this work on the field of wetland microbial ecology and climate studies.

## Summary

These thesis reports findings related to the characterization of a unique microbial consortia present in the wetland ecosystems of the Southern Appalachian region of North Carolina, along with potential methane emission fluctuation data dependent on seasonal and depth changes. Findings from this investigation revealed communities of microorganisms distinctly associated with non-northern peatland ecosystems. Each site included in this study harbored microbial communities distinct from those of more northern U.S. sites such as Michigan Hollow fen and McLean Bog as shown by Smemo and Yavitt (Smemo, 2006) and Bräuer, Yavitt and Zinder (Bräuer, 2004), respectively. As demonstrated with microcosm experiments, potential methane production from study sites revealed a seasonal dependence. Previous studies have shown that methanogenesis is dependent on hydrology

as well as temperature (Verville, 1998; Grunfeld, 1999), both of which could be playing important roles in the fluctuation of methane revealed in this study. Despite the consistent evidence for ongoing methanogenesis, a surprising abundance of Crenarchaeota was also noticed among sites. One site, Pineola Bog in Avery County, NC, was found to contain the highest abundance of Crenarchaeota (as determined by molecular sequencing and subsequent quantitative PCR) among the sites studied here when compared to numbers typically reported (Lehtovirta, 2009) and other study sites. In general, results from multiple experimental procedures indicate both that methanogenic communities in more southerly regions are actively producing methane throughout the year at multiple depths and that Crenarchaeota could potentially play an essential role in wetland ecosystems.

Given the scarcity of studies focusing on non-northern (or mid-latitude) wetlands, many points still need to be addressed regarding the variables affecting microbial community structure as well as Archaeal methanogenesis. As such, some suggestions for future research areas are included here with a focus on determining environmental variables specific to southern U.S. regions affecting methanogenesis and the community structure of the resident microorganisms. First, although wetlands are commonly known to harbor a vast array of methanogenic Euryarchaea (Groβkopf, 1998; Abreu et al., 2001; Basiliko, 2003; Cadillo-Quiroz, 2008; Emerson, 2012), little is known about communities of Crenarchaea potentially present in abundance among peatlands. Crenarchaeota are known to have nitrogen-reducing capabilities (Pester, 2011); however, not much is known about their role in wetland ecosystems. Thus, Crenarchaeal populations present in wetlands represent a potential new functional group of Archaea with possibly important roles in the overall metabolism of the systems and as such warrants further study.

Second, bacterial involvement in the mediation of methanogenic pathways is recognized among researchers (Beman and Francis, 2006; Almeida, 2009; Amaral-Zettler, 2011; Bardhan et al., 2012). However, few studies address the study of Archaeal and bacterial contributions as an intertwined system. Bacteria are known to have a wide variety of optimal conditions as well as

functions (Goodwin, 1987; Daane et al., 2001; Buesing and Gessner, 2006; Caffrey et al., 2007; Hamberger et al., 2008; Jia and Conrad, 2009; Boden, 2011) and as such are likely to contribute to the overall carbon flow of wetland ecosystems in diverse and regionally dependent ways. Given the concern for environmental warming and the effects such warming might have on peatlands in more temperate zones, bacterial consortia should be taken into account when addressing the variables affecting methanogenesis in future lines of study.

Finally, the continued efforts to isolate methanogenic species as well as novel wetland-associated species (such as ARMAN) are needed to fully elucidate the role of each member of peatland microbial communities. Such efforts should focus on the improvement and development of existing and new lab-based techniques adapted to the extreme conditions in which these organisms are found to exist. Such studies should shed light on the role of little-known contributors to the carbon cycling of peatland environments and the variables that could potentially perturb the delicate balance between the members of wetland microbial consortia.

**Conclusions**

Much of the research available to date regarding peatland microbial community structure as well as the methane dynamics of the system has been conducted in northern and boreal regions (Gorham, 1991; Bubier, 1995; Basiliko, 2003; Fisk et al., 2003; Yavitt, 2005; Cadillo-Quiroz, 2006; Cadillo-Quiroz, 2008; Godin, 2012). Very few studies exist that focus on more temperate zones (Bridgham, 1992a; Abreu et al., 2001; Bardhan et al., 2012), where climatic warming is predicted to have the greatest impact (Bloom, 2010; Bombonato and Gerdol, 2012; van Winden et al., 2012).

Additionally, the majority of the aforementioned studies do not extend past the Cranberry Glades region of West Virginia, thus excluding the potential contributions of more southerly sites. The work contained within this thesis provides the first investigation of Archaeal populations in Southern Appalachian peatlands in the United States. It has long been recognized that colder, boreal peatlands and those located in more temperate zones have distinctive communities of microbes that

are influenced by environmentally linked variables. Even among the close regional peatlands in this study, differences were seen when the communities of Pineola Bog of Avery County, NC, are compared with that of Tater Hill Bog of Watauga County, NC (Chapter Two). Despite this discrepancy, similar functional roles are still noted between the microbial communities of the two sites in regards to the methanogenic pathway.

Because these warmer climate peatlands could contribute significantly to the overall methane cycling, more studies are needed that address the dynamics of the unique and fragile peatlands found in more temperate regions.

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# APPENDIX A: CULTURING OF METHANOGENIC COMMUNITIES PRESENT AT THE WATER TABLE


## Summary

Culture-based techniques are often a key step in identifying novel or uncharacterized microbial species. Media designed previously for the isolation of methanogenic species was utilized in an attempt to isolate key methanogenic species present in Southern Appalachian wetland regions. One common methanogen was enriched for during this study and was shown to group closely with the previously identified SWAN-1 methanogen when subjected to phylogenetic analyses. Evidence of activity was detected via the production and analysis of methane via gas chromatography. Further culturing techniques will be needed to fully isolate a member of the Archaeal population present in Southern Appalachian wetlands.


## Introduction

Climate warming has been a growing concern for many years (Boucher et al., 2009; Bloom, 2010) and has sparked the need for comprehensive studies into the causes, effectors, and controls over the production and emission of greenhouse gases. Methane is an important component of the consortia of greenhouse gases thought to have the most impact on the environmental equilibrium (Brook, 2005). Wetlands have become a focal point when addressing the potential methane emissions that might occur with increased warming as several studies have demonstrated wetlands to be the largest source of naturally occurring methane and one of the largest sources overall (Cicerone, 1988; Le Mer, 2001). Methane cycling in wetlands has thus become a topic of much concern over the recent years and research into the causes of methane fluctuations is become increasingly common (van Winden et al., 2012; Zhang, 2013).

Methanogenic Archaea are key players in the global methane cycle and as such present a unique opportunity to study the process of methane formation and emission from terrestrial habitats. To further elucidate their metabolic and biochemical capabilities, the ability to isolate individual species in pure culture is a vital component of ongoing research. Recent increases in the concern over potential climate warming events have stimulated the need for more in-depth research focused on methane-producing organisms and the variables affecting their growth and metabolic activities. Conditions closely resembling the natural habitat of methanogens are likely to provide the most realistic interpretations of the function and capabilities of these organisms in regards to how they might react and adapt to a future climate warming event.

**Experimental procedures**

*Field description*

Pineola Bog, located in Avery County, NC, is classified as a swamp-bog complex (typic subtype) according to the southern classification system (Schafale, 2012). Groundcover is dominated by *Sphagnum* sp., *Carex* sp., and *Juncus* sp. The water table exhibits little fluctuation and is typically 2.54-5.08 cm below the surface of the peat. The pH of the peat layer is acidic and ranges from 4.0-5.0 throughout the year.

*Sampling procedures*

Samples of peat were collected from just below the water table level at the zone of anoxia from three separate sub sites within the Pineola Bog study site in the summer of 2011 (between the months of May to August). Care was taken to maintain anaerobiosis throughout the sampling procedure and in the laboratory.

*Cultivation of methanogenic Archaea*

Samples to be used for enrichment studies were placed in an anaerobic glove box (Coy Laboratory Products, Ann Arbor, MI, USA) no more than 48 hrs after collection to maintain little to no oxygen exposure. Approximately 10 g of peat was then homogenized using a handheld immersion

blender (Cuisinart, East Windsor, NJ, USA) and approximately 20 mL of anaerobic deionized water. Once homogenized, 1 mL of peat slurry was added to 5 mL of either Peat Medium 1 or Peat Medium 2 in anaerobic balch tubes, two types of media previously formulated for the growth and propagation of methanogenic organisms present in peat (Bräuer, 2006a and references therein), that had been previously flushed with inert nitrogen gas ($N_2$) to ensure anaerobic conditions. Peat Medium 1 contained the following additional minerals (in mg $L^{-1}$): 1.5 KCl, 13.6 $KH_2PO_4$, 26.8 $NH_4Cl$, 0.024 $CoCl_2x5H_2O$, 0.075 $ZnCl_2$, 0.019 $H_3BO_3$, 0.024 $NiCl_2x6H_2O$, 0.024 $Na_2Mo_4x2H_2O$, 1.344 $FeCl_2x4H_2O$, 0.026 $MnSO_4x4H_2O$, 1.556 $MgSO_4$, 2.336 $CaCl_2x2H_2O$, 0.009 $CuSO_4x5H_2O$, 3.446 $AlK(SO_4)_2x12H_2O$. Sterile anaerobic additions were made resulting in the following final concentrations: 1.0 mM titanium (III) nitrilotriacetate, 5 mM Homopipes ($pK_a$ = 4.7 at 28 °C, filter-sterilized stock solution adjusted to pH 5.5), 0.5 mM coenzyme-M (2-mercaptoethanesulfonic acid), 0.2 mM sodium acetate, 0.2 g $L^{-1}$ yeast extract, and a vitamin solution (Balch, 1979; Bräuer, 2006a; b; Bräuer et al., 2006) and Peat Medium 2 (described previously) (Cadillo-Quiroz et al., 2008). Inoculated cultures were sealed with butyl rubber stoppers and crimped closed to prevent introduction of oxygen to the culture. Samples were then flushed with an 80/20% mixture of $N_2/CO_2$ gases (Airgas, Radnor, PA, USA) for approximately 1 min to eliminate any oxygen introduced during the transfer process and also to provide carbon dioxide as a potential substrate for methanogenesis. A headspace of 99% pure $H_2$ gas was added to each enrichment culture for approximately 30 sec as a potential substrate for methanogenesis and to increase the partial pressure of the culture. Enrichment cultures were incubated at 28 °C for 4 weeks before transfer of 1 mL to another 5 mL of either Peat Medium 1 or 2 as described above. After three transfer events, enrichment cultures were tested for the presence of methane via gas chromatography.

*Gas chromatography*

Headspace samples from each tube were taken with a gas-tight syringe (Hamilton Co., Nevada, USA) approximately every 48 hours for two weeks. Each headspace measurement consisted

of 200 mL of headspace gas analyzed with a Shimadzu Model GC-2014 using a FID (150 °C), and a Porapak N column (100 °C) using an external set of methane standards. Before analysis, the tubes and standards were shaken vigorously for approximately 1 min to ensure headspace and sediment $CH_4$ concentration equilibrium. Methane production was determined for each sample and recorded as mmol/L.

**Results and Discussion**

*Enrichment of methanogenic organisms from wetland environments*

Previously formulated media designed to promote methanogenic growth (Bräuer, 2006a) was used in the enrichment of methanogenic Archaea from Pineola Bog, Avery County, NC. Because these organisms are slow growing, multiple transfers of the enrichment were performed over the course of several months. After approximately four weeks of growth, the last transfer was subjected to gas chromatography (GC) analysis to measure any methane produced in the headspace. Enrichments were shown to produce methane and as such were determined to have allowed the dominant methanogen to propagate. Enrichment cultures were screened for identification and one sequence was obtained that, when subjected to a BLAST search, was closely related to a previously identified methanogenic organism, SWAN-1 (Figure A1).

Results from the initial attempt at methanogenic enrichment from Pineola Bog suggest the dominance of SWAN-1-like Archaea, a *Methanobacterium*. However, when compared to phylogenetic analysis of the Pineola study site (Chapter 2) the Methanobacteriales are shown to only represent 13% of all Euryarchaeal sequences from the area. This discrepancy is likely due to the fact that acidophilic members of the Methanobacteriales are more readily culturable (Patel et al., 1990; Horn et al., 2003; Kotsyurbenko et al., 2007) compared to the more fastidious members of other methanogenic groups such as the Methanoregulaceae (Bräuer, 2004; Yashiro, 2011) and Methanocellacea (Sakai, 2008).

Increasing knowledge of culturing and isolation techniques is an important aspect of microbiological research and is of particular importance to Archaeal research given the relatively small number of methanogenic organisms isolated in pure culture (Bräuer, 2006b; Nichols, 2008; Bräuer et al., 2011). By further understanding the biochemical functional roles played by methanogenic Archaea in wetland systems, scientists will be better equipped to address issues surrounding their production of methane and its eventual emission into the atmosphere as a greenhouse gas.

## Figures and Tables

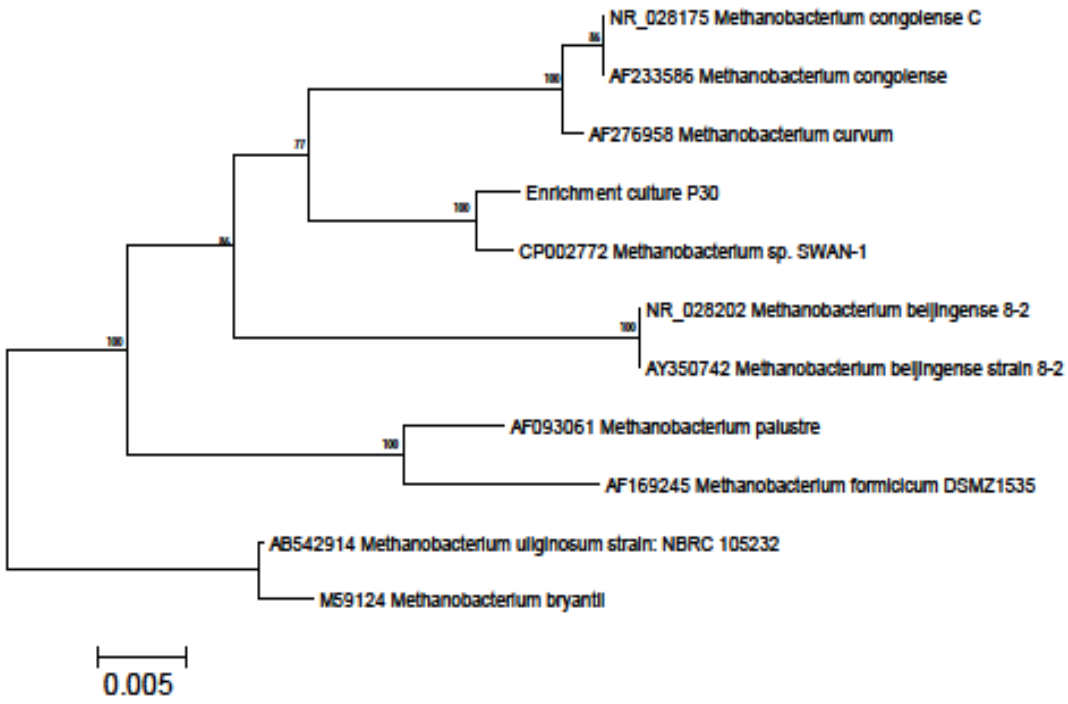


**Figure A1.** Neighbor-joining tree demonstrating the phylogenetic relationship of SSU rRNA gene sequences retrieved from Pineola Bog, Avery County, NC, and enriched for in-laboratory incubations. Alignments were created using the ARB-based online SILVA aligner. Dendrogram was created using the PHYLIP package. *Methanobacterium uliginosum* and *Methanobacterium bryantii* were used as outgroups. Branch lengths indicate the expected number of changes per sequence position (see scale bar). Bootstrap values represent 100 replicates with values >50 and supported by Maximum Likelihood analysis.

# APPENDIX B: VERTICAL ANALYSIS OF ARCHAEAL COMMUNITIES AND ESTIMATION OF POTENTIAL $CH_4$ EMISSIONS

## Summary

Vertical analysis of archaeal populations revealed no significant change in abundance of methanogenic members of the E1/E2 group of the Methanomicrobiales, or in that of Crenarchaea or total Archaea, despite the presence of a distinct optimum for potential methane production around 0-30 cm below the water table. Potential methane production using endogenous substrates were within the range determined for other peatlands globally and averaged 0.006, 0.014, and 0.029 mmol/L $CH_4$ $d^{-1}$ for Pineola, Sugar Mountain and Tater Hill, respectively. Seasonally, the highest rates of potential stimulated methane production were found in fall 2011. Nutrient analysis of the study sites indicated a strong signature from the underlying bedrock of the area as concentrations of Fe, Al, and Na were at the high end of normal compared to other peatlands globally.

## Introduction

Changes in methane emissions into the atmosphere have been documented for many years (Cao, 1998; Brook, 2005; Loulergue, 2008; Boucher et al., 2009; Bloom, 2010) and have the potential to accelerate climatic warming events. It has been proposed by researchers that an increase in overall global temperature could cause a rise in the amount of methane produced from natural sources such as wetlands (Bloom, 2010; Mitsch, 2013) and that this effect could be particularly obvious in more southerly, temperate areas (Bloom, 2010; Bombonato and Gerdol, 2012; van Winden et al., 2012). As a result of this increase in emissions, temperature increases would be enhanced by the influx of methane as a greenhouse gas into the atmosphere, thus causing a feedback loop to be set in motion. Because of growing concern as to the effect future methane emissions may have on the environment,

studies have begun to focus on the regional characteristics seemingly having an effect on methane production and emission (Bridgham, 1992a; Bubier, 1995). Numerous variables have been identified to date that cause shifts in the methane production and emission potential from wetland areas (Hobbie, 1992; Bubier, 1995; Christensen et al., 2003; Strom, 2005; Yavitt, 2005; Keller, 2006; Webster, 2010; Kim et al., 2012). It is unclear, however, whether these influencing variables are causing perturbations among the underlying microbial community structure or their ability to actively produce methane gas. This uncertainty highlights the need for more in-depth studies regarding specific variables and their effects on microbial community structure and activity.

Many studies have demonstrated the controlling effects of both hydrology and vegetation on methane production and emission (Strom, 2003; 2005; Webster, 2010). These variables are highly site dependent and can vary greatly even within the same geographical region. Because of this, it is difficult to determine a general trend among the effects and therefore each site or region should be addressed separately. Both water level and plant life not only influence methane production, but have subsequently been shown to enhance methane emission to the atmosphere as well (Bubier, 1995). Dissolved methane in the water column of waterlogged peatlands can erupt into the environment when subjected to pressure changes in a process termed *ebullition* (Brook, 2005). This typically occurs when the water column becomes supersaturated with dissolved methane. Plant life also plays a role in facilitating methane release to the environment. Some methane produced below the surface in the anoxic layers of peat can bypass the oxygenated layer of surface peat where methane-oxidizing bacteria reside and pass directly into the atmosphere via arenchymous structures within the stems of vascular plants. These variables and many more present the need for continued in-depth studies of methane production and emission in wetlands and the influencing factors controlling these processes.

## Experimental procedures

### *Field description*

Pineola Bog, located in Avery County, NC, is classified as a swamp-bog complex (typic subtype) according to the southern classification system (Schafale, 2012). Groundcover is dominated by *Sphagnum* sp. and *Carex* sp. The water table exhibits little fluctuation and is typically 2.54-5.08 cm below the surface of the peat. The pH of the peat layer averaged 4.9 (range 4.25-5.6) throughout the course of this investigation from summer 2010 through summer 2012. Sugar Mountain bog, located in Avery County, NC, is classified as a Southern Appalachian Bog (typic subtype) (Schafale, 2012) that is possibly in transition. Dominating plant species include *Rhododendron maximum, Rosa palustris, Sphagnum* sp., and *Carex* sp. The pH of this site is acidic and averaged around 4.9 (range 3.9-5.3) throughout seasonal and moisture changes during the course of this study. Tater Hill Bog, located in Watauga County, NC, is classified as a Southern Appalachian bog (typic subtype) by the most recent southern classification scheme (Schafale, 2012), although it would most likely be considered a moderate fen by northern classification methods (Mitsch, 2007). Plant species populating the sample area include *Rhododendron* spp., several *Vaccinium* spp., *Carex*, and *Juncus* spp. The pH of sample sites ranged from 4.9-6.0 (average 5.7) from summer 2010 to summer 2012.

### *Potential methane production*

Potential methane production was measured as described previously (Bräuer, 2004). Briefly, 1g subsamples of peat were transferred into each 150 x 18 mm Balch tube (Bellco Glass Co., Vineland, NJ, USA) in an anaerobic chamber. To each tube 9 mL of deionized, anaerobic water was added. Tubes were shaken to release residual methane and sealed with butyl rubber stoppers, crimped to maintain anaerobiosis. Tubes were then flushed on the bench top with an 80/20 gas mixture of $N_2/CO_2$ to provide the necessary carbon dioxide for methanogenesis to occur. The following amendments were made: 1) no addition, 2) 10 psi of $H_2$, or 3) 10 psi $H_2$ plus 10 mg/L of Rifampicin.

Tubes for soil slurry incubations were prepared in triplicate and were incubated (on a rotary shaker) at 28 °C in the dark. After one week of incubation, headspace samples from each tube were taken with a gas-tight syringe (Hamilton Co., Nevada, USA) approximately every 48 hours for two weeks. Each headspace measurement consisted of 200 mL of headspace gas analyzed with a Shimadzu Model GC-2014 using a FID (150 °C) and a Porapak N column (100 °C) using an external set of methane standards. Before analysis, the tubes and standards were shaken vigorously for approximately 1 min to ensure headspace and sediment $CH_4$ concentration equilibrium. Methane production was determined for each sample and recorded as mmol/L.

*Molecular analysis*

DNA extraction and quantification were carried out as described in Chapter 2.

*Elemental analysis of peat pore water*

Pore water was collected from each site during the summer of 2012 and sent for inductively coupled plasma mass spectroscopy (ICP or ICP-MS) analysis at Cornell University (Cornell University, Ithaca, NY, USA).

**Results and Discussion**

*Vertical analysis of potential methane production in peat*

Maximum potential methane production varied from just below the water table down to ca. 40 cm (data not shown), but was often found at ca. 25 cm below the standing water table (Figure B1). These data corroborate other studies that have found peak methane production rates just below (ca. 0-33 cm below) the water table, where litter decomposition provides a consistent nutrient supply, but the peat is also sufficiently anaerobic to support methanogenic growth (Edwards, 1998; Galand et al., 2002; Kotsyurbenko, 2004; Galand, 2005; Cadillo-Quiroz, 2006; Sun, 2012). These results can also be observed in the daily rate of methane production for each site (Table B1).

*Vertical analysis of Archaeal community numbers*

Real-time quantitative PCR was performed on peat samples from varying depths among sample sites to elucidate whether the Archaeal community structure changed depending on depth below the water table. Vertical analyses of the peat profile revealed no significant trends in abundance of E1/E2 group methanogens (Figure B2). This is in contrast to at least one other study that was able to demonstrate a peak for E1/E2 methanogens around 10-20 cm below the water table (Cadillo-Quiroz, 2006). Similarly, there was no significant change in crenarchaeal abundance with depth.

*Potential seasonal methane production*

Potential methane production using endogenous substrates (no addition) averaged 0.006, 0.014, and 0.029 mmol/L $CH_4$ $d^{-1}$ for Pineola, Sugar Mountain and Tater Hill, respectively (Table B2). These numbers are within the range reported for 23 peatlands throughout Alaska and Massachusetts, which varied from no production or 0.001 up to 0.096 mmol/L $CH_4$ $d^{-1}$ (Rooney-Varga, 2007), as well as those determined for peatlands in New York State, which varied from 0.020 up to 0.181 (Sun, 2012). Stimulated methane production rates in the presence of $H_2$ or $H_2$/Rifampicin increased the potential methane production rates up to ca. 30 fold (Tables B2 and B3), in line with studies by Sun et al. that demonstrated a stimulation up to 17.5 fold (Sun, 2012). Interestingly, the ratio of $H_2$ stimulated to endogenous rates of methane production were highest for Sugar Mountain Bog for samples taken in 2010 (Table B2), perhaps due to the dominance of hydrogenotrophic groups of Methanomicrobiales, Methanobacteriales, and Methanococcales found in this peatland (see Table 2 in Chapter 2).

Potential methane production was also measured throughout a seasonal cycle to determine any effects on methanogenesis influenced by seasonal changes. Seasonal analysis of methane potential revealed a spike in methane production during the fall sampling both in Pineola Bog and Tater Hill Bog (Figure B3). Since the autumnal months are characterized by increased litter

deposition, the increase in potential methane production rates in fall could be a result of increased nutrient influxes resulting from decomposition activity. This is in contrast to studies of peatlands in upstate New York, which demonstrated the highest rates in summer, consistently across several years of sampling (Bräuer, 2006a; Sun, 2012), most likely due to the contribution of root exudates, which have been shown to contribute directly to methanogenesis (Conrad et al., 2008). Another study of a boreal fen in Finland found the highest methane production rates in the cold winter months, most likely due to the build-up of substrates when methanogenic rates slowed in the colder months (Juottonen, 2008b). It was also observed that methane production in Pineola Bog seemed to drop off during the summer months, perhaps due to a lowering of the water table level as a result of evaporation (Figure B3). These observations are supported by the average measured rates of potential $H_2$-stimulated methane production from Pineola, Tater Hill, and Sugar Mountain peatlands as well (Table B3).

*Archaeal seasonal quantification of community members present in a second Southern Appalachian bog*

A second bog site, Sugar Mountain Bog, was investigated as a comparison for the more heavily forested Pineola Bog. Quantification of the Archaeal community present in this bog site was within the range determined for Pineola and Tater Hill and fell between $3 \times 10^6 – 3 \times 10^8$ and between $3 \times 10^6 – 2 \times 10^8$ for Crenarchaea and total Archaea, respectively (Table B4). Surprisingly, qPCR results revealed a maximum during the winter months that was significantly reduced during the spring months. This may be due to reduced water level fluctuations during the winter months because of freezing, which may have allowed methanogenic organisms to grow and duplicate without periods of oxygenation. In the spring months, thawing and rainfall could cause significant water level flux and introduce sudden oxygenation events to the methanogenic community, thus halting growth and metabolic activity during those periods.

*Pore water ICP analysis*

In the present study, pore water samples were taken from all study sites during the summer months for analysis of nutrient status via inductively coupled plasma mass spectroscopy (ICP-MS) analysis to elucidate any notable nutrient deficits or toxicity that might affect microbial activity, as it has been previously shown that microbial community structure and perhaps activity is linked to the biogeochemical gradients present in bogs and fens (Lin et al., 2012).

The ICP chemical analyses of the wetland pore water are reported in Table B5. Sodium concentrations were within the expected range according to a literature review by Bourbonniere as seen in Table B5 (Bourbonniere, 2009). Acidic wetlands have been shown to have low ionic strength when compared to more neutral ecosystems (Likens, 1967; Malli, 1998; McLaughlin, 2010). Therefore, it was not overly surprising to find a low amount of sodium in both Pineola Bog and Tater Hill Bog when compared to typical culture media concentrations (Stevenson, 2004). In contrast to some northern ombrotrophic bogs where sodium concentrations are extremely low (1-4 uM; Bräuer, 2004), the concentrations at Pineola Bog and Tater Hill were roughly 30-70 fold greater, and yet still below that required by traditional media recipes designed to cultivate methanogenic species. It has previously been shown that other (non-acidiphilic) methanogenic cultures require sodium for growth (Perski et al., 1982) due to a sodium-ion dependent enzyme in methanogenesis (Deppenmeier, 2002). Thus, these data lend credence to the hypothesis that methanogens in acidic peatlands are uniquely adapted to low ionic strength environments and can grow in the presence of minimal sodium concentrations (Bräuer, 2006b).

Silicon concentrations in both sites as well as aluminum concentrations in Pineola Bog surpassed the typical ranges reported by Mullen et al. in 2000 (Table B5). Higher concentrations of silicon and aluminum were not unexpected since initial stages of soil formation (as generally occurs in the Southern Appalachian region) often result in high silicon and high aluminum. Specifically, the weathering of metamorphosed quartz and feldspar at Pineola Bog would be expected to generate the

high silicon and high aluminum concentrations that were detected here. Additionally, the low pH of both sites (Table 1 Chapter 2) may promote higher concentrations of extractable Si and Al (Helmer, 1990). Results also revealed relatively high concentrations of Fe in both sites, particularly Tater Hill Bog. The high iron in Tater Hill would be expected due to weathering of amphibolite, which contains hornblend. Further, the low pH of both Pineola and Tater Hill likely contributes to higher concentrations of dissolved iron in the pore water, as has been shown in other freshwater systems (Kato, 2009; Kato, 2012). Phosphorous concentrations were on the low end (Table B5). Low phosphorous may be the result of binding with aluminum and iron, which may prevent extraction (Zhang, 2002).

## Figures and Tables

**Table B1.** Potential methane production with depth for soil slurries amended with $H_2$ and Rifampicin using peat collected from either Pineola or Tater Hill in the summer of 2012. Ranges (±) indicate standard error.

| | Pineola (Avery Co., NC) mmol/L $CH_4$ $d^{-1}$ | Tater Hill (Watauga Co., NC) mmol/L $CH_4$ $d^{-1}$ |
|---|---|---|
| 0-5cm below wt* | 0.044 ± 0.007 | 0.249 ± 0.001 |
| 15-25cm below wt* | 0.058 ± 0.009 | 0.507 ± 0.301 |
| 30-45cm below wt* | 0.020 ± 0.019 | 0.101 ± 0.022 |
| 50-60cm below wt* | 0.032 ± 0.011 | 0.075 ± 0.016 |

*wt = standing water table at the time of sampling

**Table B2.** Potential methane production for soil slurries with no amendment, or amended with $H_2$, using peat collected from either Pineola, Tater Hill, or Sugar Mountain Bog in the summer of 2010. Values represent methane rates at the optimum sampling depth, which varied from just below the water table down to ca. 40 cm. Triplicate field samples were collected from all field sites, and the parentheses indicate standard error. The ratio for the rate of methane production with added substrate versus un-amended control was also calculated.

| | Endogenous rates (mmol/L $CH_4$ $d^{-1}$) | $H_2$ stimulated rates (mmol/L $CH_4$ $d^{-1}$) | Ratio of $H_2$ stimulated to endogenous |
|---|---|---|---|
| Pineola, NC | 0.006 (0.002) | 0.007 (0.004) | 1.2 |
| Sugar Mtn., NC | 0.014 (0.003) | 0.099 (0.024) | 7.07 |
| Tater Hill, NC | 0.029 (0.005) | 0.046 (0.010) | 1.6 |

**Table B3.** Potential methane production for soil slurries amended with $H_2$ and Rifampicin using peat collected from Pineola, Tater Hill, or Sugar Mountain Bog from summer 2011 through summer 2012. Triplicate field samples were collected from all field sites except for Sugar. Triplicate laboratory replicates were also made, and the numbers in parentheses indicate standard error.

| | Pineola (Avery Co., NC) mmol/L $CH_4$ $d^{-1}$ | Tater Hill (Watauga Co., NC) mmol/L $CH_4$ $d^{-1}$ |
|---|---|---|
| Summer ‘11 | 0.131 ± 0.103 | 0.111 ± 0.047 |
| Fall ‘11 | 1.00 ± 0.409 | 1.96 ± 1.60 |
| Winter ‘11 | 0.428 ± 0.152 | 0.369 ± 0.256 |
| Spring ‘12 | 0.211 ± 0.091 | 1.06 ± 0.257 |
| Summer ‘12 | 0.285 ± 0.389 | 0.471 ± 0.227 |

**Table B4.** Real-time quantitative PCR estimates of Archaeal cell numbers found in Sugar Mountain Bog, Avery County, NC. Ranges (±) indicate variation around the mean.

| | Sugar Mountain Crenarchaeota* | Sugar Mountain General Archaea* |
|---|---|---|
| Summer 2011 | 8.19E+06 ± 6.38E+06 | 6.40E+07 ± 1.36E+07 |
| Fall 2011 | 2.14E+07 ± 8.73E+05 | 1.98E+08 ± 2.33E+07 |
| Winter 2011 | 3.13E+08 ± 1.16E+08 | 2.30E+08 ± 4.58E+07 |
| Spring 2011 | 3.16E+06 ± 7.16E+05 | 2.72E+06 ± 1.08E+05 |

* Average of two analytical replicates

**Table B5.** Chemical analysis of pore water samples collected from Sugar Mountain Bog, compared to the two other sites reported in Chapter 2. Concentrations of components were determined by ICP analysis and are reported as an average. No replicates were conducted for Sugar Mountain Bog samples.

| | Pineola (Avery Co., NC) | Tater Hill (Watauga Co., NC) | Sugar Mountain (Avery Co., NC) | Average values in the literature |
|---|---|---|---|---|
| | mg/L* | mg/L* | mg/L | mg/L |
| Phosphorous (P) | 0.153 ± 0.083 | 0.210 ± 0.252 | 0.00 | 0.01-11[+] |
| Sodium (Na) | 2.96 ± 1.08 | 1.23 ± 0.293 | 1.54 | 0.05-76[+] |
| Aluminum (Al) | 2.49 ± 1.61 | 1.42 ± 1.82 | 0.12 | 0.003-2.3[§] |
| Potassium (K) | 0.727 ± 0.186 | 0.737 ± 0.370 | 0.42 | 0.05-21[+] |
| Calcium (Ca) | 0.907 ± 0.361 | 1.74 ± 0.757 | 0.34 | 0.8-179[+] |
| Iron (Fe) | 1.85 ± 0.920 | 11.0 ± 11.9 | 0.40 | 0.012-18[§] |
| Silicon (Si) | 28.3 ± 6.92 | 32.8 ± 27.1 | 14.14 | 0.01-16[§] |
| Sulfur (S) | 0.670 ± 0.288 | 1.12 ± 1.09 | 0.21 | 0.03-40[+] |

* Average of three replicates
[+] = Bourbonniere (2009); [§] = Mullen et al. (2000)

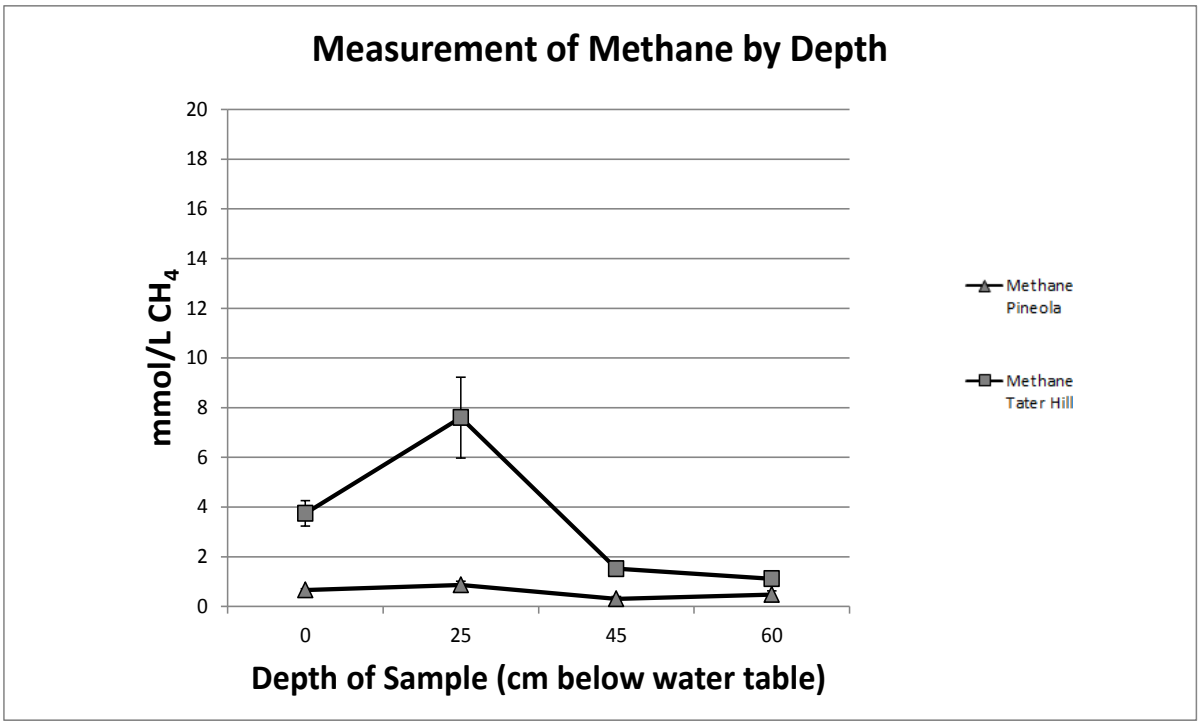


**Figure B1.** Potential methane production as measured by gas chromatography according to depth below the standing water table at the time. Square symbols = Tater Hill, NC; Triangle symbols = Pineola, NC.

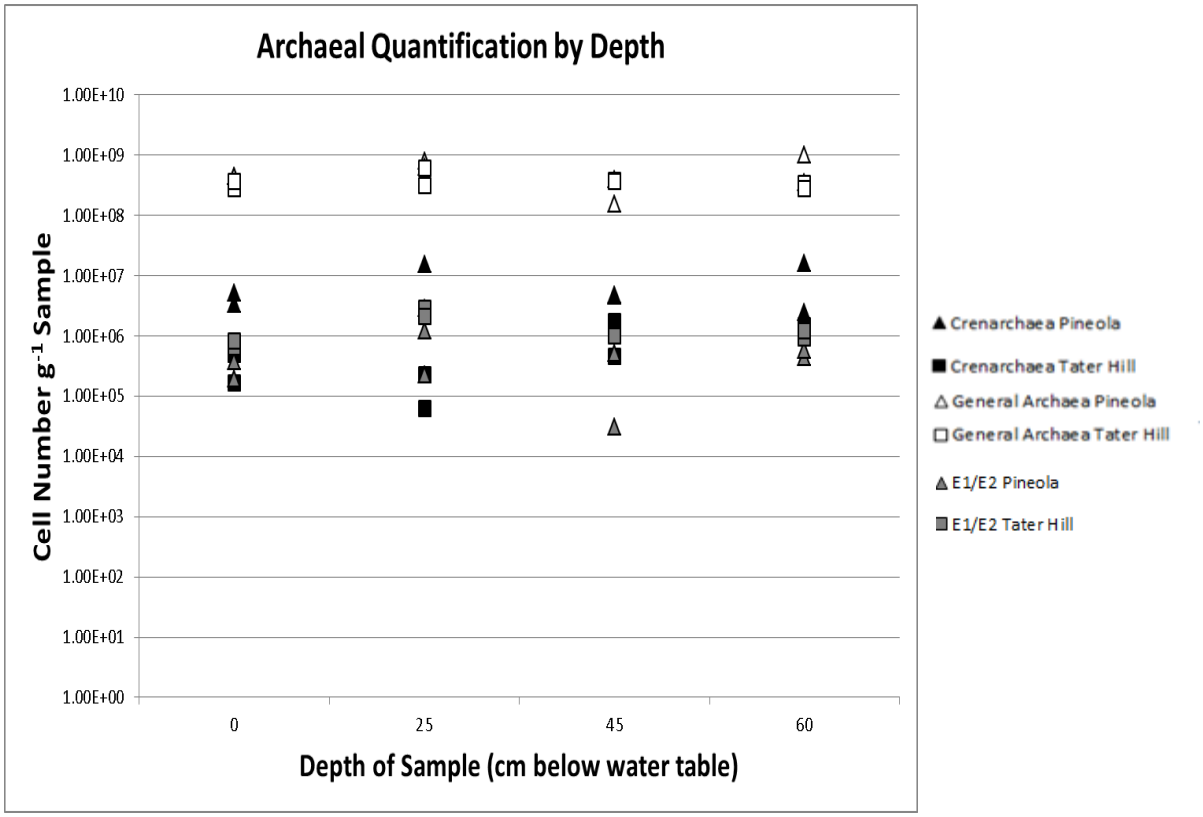


**Figure B2.** Quantification of Archaeal populations by quantitative PCR shown in relation to season. Square symbols = Tater Hill, NC; Triangle symbols = Pineola, NC; Solid symbols = Crenarchaeota; Open symbols = General Archaea; Shaded symbols = E1/E2 clade.

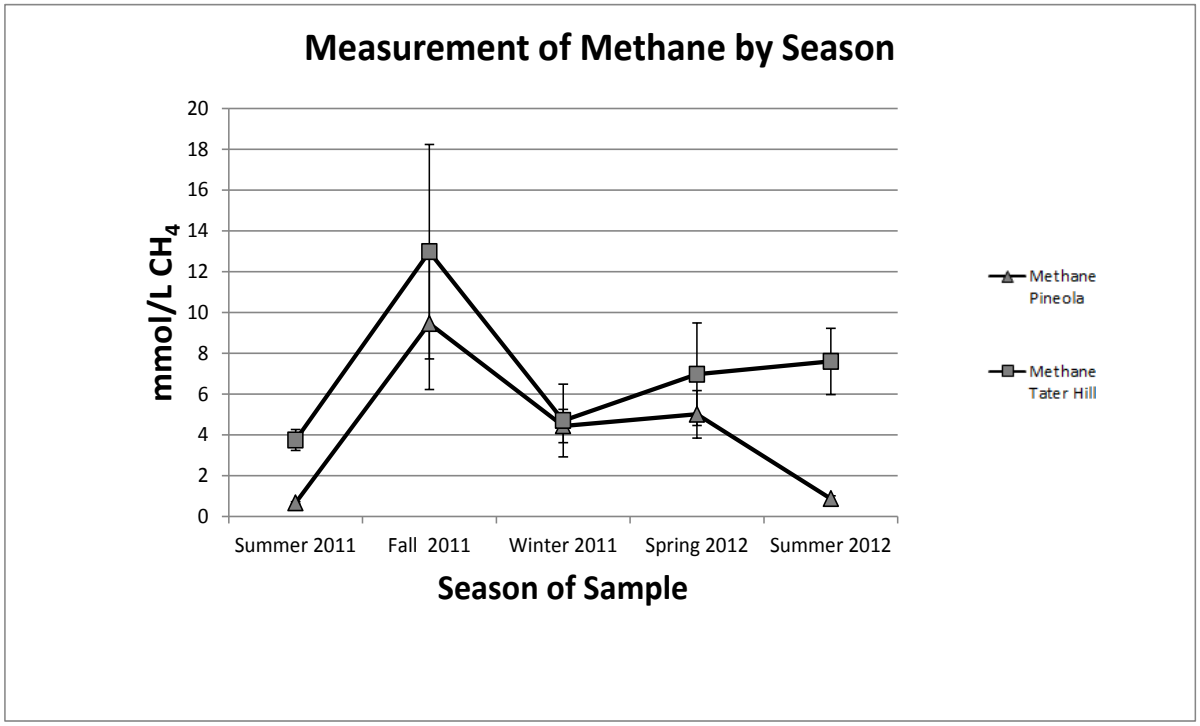


**Figure B3.** Potential methane production at ca. two weeks for soil slurries amended with $H_2$ and Rifampicin using peat collected from either Pineola or Tater Hill from summer 2011 through summer 2012 at 0-25 cm below the water table. Triplicate field samples were collected from both field sites. Square symbols = Tater Hill, NC; Triangle symbols = Pineola, NC.

## Vita

Ashley Hawkins was born in Winston-Salem, North Carolina on the 10th of May, 1987. Ashley graduated from North Carolina State University with a B.S. in Microbiology in 2010. Ashley came to Appalachian State University in August 2010 in pursuit of a M.S. in Cell and Molecular Biology with Dr. Suzanna L. Bräuer. Upon completion of her degree in August 2013, Ashley will pursue an MBA at Appalachian State University.