ABSTRACT

Title of Thesis: **BACK TO EARTH: MOLECULAR APPROACHES TO MICROBIAL ECOLOGY MUST CONSIDER SOIL MORPHOLOGY AND PHYSICOCHEMICAL PROPERTIES**

Glade Arthur Dlott, Master of Science, 2015

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This project studied the influence of different long-term agricultural management regimes on soil microbial communities, and compared survival strategies of individual prokaryotic OTUs in diverse soils subjected to long-term incubation. Together these would show whether alterations to microbial communities affect rates of soil carbon cycling. Agricultural soils were sampled at arbitrary depths above and below the plow layer, and relative abundances of microbes were measured using high-throughput sequencing. 'Activity' (rRNA:rDNA) ratios were calculated for individual OTUs identified by high-throughput sequencing of tropical rainforest and temperate cornfield soils after incubation for one year with differing water and carbon availabilities. It was found that depth controls microbial communities to a greater degree than agricultural management, and that the characterization of microbial trophic strategies might be complicated by the often-ignored DNA preservation potential of soil. The study highlights the need for holistic approaches to testing hypotheses in modern microbial ecology.

BACK TO EARTH: MOLECULAR APPROACHES TO MICROBIAL ECOLOGY MUST CONSIDER SOIL MORPHOLOGY AND PHYSICOCHEMICAL PROPERTIES

by

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Table of Contents

List of Tables

Chapter II

List of Figures

Chapter II

Chapter III

Appendices

Appendix A

Appendix B

Chapter I – Introduction and Literature Review

Soil microbial ecologists have historically been limited by the tools needed to study community structure, and to link changes in microbial β-diversity to ecosystem function (van Elsas and Boersma, 2011). The 'great plate count anomaly', in which it was recognized that the majority of environmental organisms were not culturable with techniques common to microbiology of the time, was first noted almost a century ago (Staley and Konopka, 1985), and studying the diversity and function of 'unculturable' organisms remains a major challenge in microbial ecology today (Pham and Kim, 2012). However, with the advent of high-throughput sequencing and bioinformatics (e.g. 454 Pyrosequencing, Illumina, QIIME), researchers have been able to study the detailed composition of microbial communities quickly and relatively cheaply for the first time (Hamady et al., 2008; Caporaso et al., 2011). The last five years has seen an explosion in research on soil microbial communities, including: how they are affected by external factors, and how they affect larger ecosystem processes (Fierer et al., 2007; Lauber et al., 2009; Koljalg et al., 2013).

Major research efforts have focused on the impacts of environmental changes on microbial communities, and possible influences of these community alterations on ecosystem functioning. A particularly vibrant area of this research has focused on ways in which soil microbes process and store carbon (C) (Trivedi et al., 2013) and the ways anthropogenic influences, especially climate change (Bardgett et al., 2008; Allison et al., 2010) could affect these rates. As soil is a major sink of global C, accounting for approximately 2.2 trillion metric tons worldwide (Batjes, 1996), changing rates of

biochemical C processing may have far-reaching relevance for atmospheric C levels, and therefore is a matter of global concern.

In agroecosystems, continued human activity directly impacts both soil microbial communities and soil C storage, and changes in agricultural management style could alter these impacts on a large geographic scale. Despite their central role in ecosystem functioning, the community structure of soil microbes has long been understudied as a functionally relevant factor in agronomy (Schimel and Schaeffer, 2012). While next-generation sequencing has spurred increased exploration of the links between agricultural management, C storage, and microbial community composition and function, studies of agroecosystems in recent years have often neglected key factors affecting these relationships. Many studies in microbial ecology homogenize across soil depth (Shange et al., 2012) and/or sample only the surface 5-10 cm of soil (Roesch et al., 2007; Lauber et al., 2009), despite widespread evidence of the importance of depth effects on C form and storage in agricultural soils (Syswerda et al., 2011) and on microbial communities in natural soils (Eilers et al., 2012). At the same time, studies in agronomy and soil science which acknowledge and account for depth and soil morphology often do not feature microbial community analyses, nor do they handle and store samples in a way that allows for these analyses to be performed (Follett et al., 2013). The historic separation of the fields of microbiology and agronomy has thus created a gap in our understanding of these systems.

The wide variety of possible agricultural management styles and cropping rotations (Hartmann et al., 2014), methods of microbial analyses (van Elsas and Boersma, 2011), and geographic variation add additional layers of complexity to this field. Both

no-till and USDA organic management systems have been promoted to increase soil conservation and sustainability of agricultural systems (Lal, 1997; Spargo et al., 2011). USDA organic management forbids the use of synthetic chemicals, including most mineral fertilizers. Therefore, plant nitrogen (N) needs must be met with naturally occurring sources of N. Manures, composts, and legume cover crops are generally used – all biochemically complex, C-linked forms of N. This is in contrast to the biochemically homogeneous, readily available mineral fertilizers used in 'conventional' agriculture, including no-till systems. It has been shown that microbial communities in systems using macromolecular C-linked N are significantly different (Li et al., 2012) and more diverse (Hartmann et al., 2014) than those that use chemical fertilizer. However, another study did not detect a significant difference in the diversity of functional genes between organic and conventional soils (Xue et al., 2013).

While USDA organic systems use C-linked fertilizer, they also generally involve intensive tillage regimes (Gomiero et al., 2011). Historic observations indicate that increased tillage leads to increased rates of microbial activity and C mineralization; however, recent studies suggest otherwise (Acosta-Martínez et al., 2010; Spargo et al., 2011; Wickings et al., 2011). Especially under organic management, it seems that C inputs are high enough to offset losses by mineralization (Gomiero et al., 2011). The results of these studies may be confounded by the lack of consideration for soil depth. When accounting for depth, the greater surface C storage found under no-till (Lal, 1997) is balanced by greater C storage in the subsurface in tilled soil (Angers et al., 2007; Baker et al., 2007). Depth is now considered an important factor in soil C dynamics (Fontaine et al., 2007; Xiang et al., 2008; Rumpel and Kögel-Knabner, 2011). Approximately 30-60%

of terrestrial soil organic matter is stored below 30 cm (Batjes, 1996), and both crop type and tillage can affect soil C at depth (Wright et al., 2007). However, it has not, to the author's knowledge, been studied as a controlling factor on soil microbe populations in agricultural soil. Studies on paired microbial communities, activities, and C and N forms at depth are needed to evaluate the potential influence of microbial community in C cycling across the entire soil profile.

Another focus in studying the relationship between soil microbial ecology and soil C processing is aimed at describing the survival strategies of the newly uncovered 'unculturable' bacterial and archaeal majority in terrestrial soils (Fierer et al., 2007; Goldfarb et al., 2011). Survival strategy – the methods by which microbes obtain energy and reproduce, while adapting to or avoiding dangers – determines the functional diversity of microbial communities (Schimel and Schaeffer, 2012). Currently, the most widely used dichotomy for describing different survival strategies of soil microbes is the copiotroph – oligotroph model (Lauro et al., 2009). Copiotrophs are defined by their ability to make use of large pulses of nutrients by reproducing rapidly when nutrients are plentiful, only to die off or enter resting states when the nutrient source is depleted. Conversely, oligotrophs maintain small but stable populations, making use of small concentrations of nutrients and allocating most of their energy to cellular growth rather than reproduction.

Fierer et al., (2007) proposed that these strategies are conserved at the phylum level for some bacteria, and provided observational and limited experimental support for this hypothesis. Since then, many studies have followed this example, and common associations have been found between oligotrophic soils and the prevalence of certain

bacterial phyla, particularly *Acidobacteria* (Lee et al., 2008; Fierer et al., 2011). Additionally, some phyla (*Betaproteobacteria, Gammaproteobacteria, Bacteroidetes, Actinobacteria*) are consistently found in copiotrophic soils, and proliferate rapidly when labile carbon sources are added (Cleveland et al., 2007; Eilers et al., 2010; Goldfarb et al., 2011).

Dominant survival strategy in a soil community – either oligotrophic or copiotrophic – might shift with changes in agricultural management regimes (Li et al., 2012). Most studies of soil microbial communities have found that soil management changes cause significant and abiding shifts in community structure (Allison and Martiny, 2008). If the dominant survival strategy changes, the rates of C transformations and the eventual fate of the C in the soil may change (Schimel and Schaeffer, 2012). This could make the dominant survival strategy in a community relevant to soil C sequestration.

However, the experimental evidence for highly conserved 'oligotrophy' in soils is tenuous. Genes controlling C decomposition are highly dispersed amongst soil microbes (Martiny et al., 2012), undercutting assumptions that changing archaeal and bacterial community would change rates of C processing in soil. Furthermore, the majority of 'oligotrophic' bacteria are classified based on observational data (Fierer et al., 2007; Pascault et al., 2013). There is little experimental data to suggest a repeatable definition or archetypal example of oligotrophy. The few studies that attempt to clearly place bacterial populations on an oligotrophic-copiotrophic gradient find not two, but five or six discrete survival strategies (Lauro et al., 2009).

This research had two components: the first investigated the links between agricultural management practices, C and N dynamics, and archaeal, bacterial, and fungal community structure, sampling by arbitrary depth fractions both above and below the plow layer to demonstrate the influence of both soil depth and morphology on edaphic factors and microbial α- and β-diversity. The second was a manipulative study that directly investigated the basis for oligotrophy in bacterial taxa. Together these studies explore soil microbes in agro-ecosystems at an unprecedented resolution: in the first experiment, in terms of physical and sequencing depth; in the second, in terms of the survival strategies of individual archaeal and bacterial OTUs.

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Chapter II

Title: Dispersal patterns of bacteria and archaea differ with depth in an agricultural field containing a plow layer (Ap horizon)

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Abstract

In spite of known differences in the composition of microbial communities across soil depth, most studies fail to characterize microorganisms from soils deeper than 5-20 centimeters. The few that address soil depth tend to use arbitrary depth increments, and do not divide samples by soil horizon boundaries. Previously tilled agricultural soils generally feature a well-defined plow layer (Ap horizon) that provides an opportunity to examine the role of vertical soil heterogeneity on changes in microbial β-diversity, and the distribution of different microbial groups within the profile. Illumina sequencing was used to characterize bacteria and archaea by targeting a region of the 16S rRNA gene, and to characterize fungi by targeting a region of the ITS. Microbial communities were compared to each other across depths and agricultural management regimes, and correlated to a variety of physical and chemical soil characteristics. The relative abundance of fungal OTUs generally decreased consistently with depth, though several abundant OTUs were most prevalent immediately above the plow layer. In contrast,

bacterial and archaeal abundance showed one of four clearly defined distribution patterns with increasing depth: constant decrease, constant increase, enrichment above the Ap horizon, or enrichment below the Ap horizon. The significant effects of proximity to the Ap horizon on the distribution and changes in β-diversity highlight the need to examine microbes in deeper soil communities, and to explicitly account for soil horizonation when sampling these communities.

Introduction

There is a growing body of literature suggesting that microbial community composition is shaped by both microscale edaphic features such as pH (Lauber et al., 2009; Nemergut et al., 2013), total carbon (Nemergut et al., 2008; Shange et al., 2012), and soil texture (Lauber et al., 2008), and larger-scale soil morphological features such as vegetation and parent material (Michel and Williams, 2011; Yarwood et al., 2015). Although evidence is mixed, several studies have also linked microbial community β-diversity to ecosystem function (Allison and Martiny, 2008). Unfortunately, the majority of microbial ecology studies focus on β-diversity across horizontal space, and generally ignore soil depth (Fierer et al., 2003; Bru et al., 2011). Most studies using high-throughput sequencing have sampled only the top 5-20 cm of soil, and have homogenized across this depth. In the relatively few cases in which depth has been considered using these methods, β-diversity between surface and sub-surface soils was as high for both fungi (Bahram et al., 2015) and bacteria (Eilers et al., 2012) as that of surface soils between continents. To the authors' knowledge, there has not yet been a similar depth-structured study of fungal, archaeal, and bacterial communities characterized using Illumina sequencing.

Even when depth is studied, it is usually fractionated arbitrarily, with little consideration for soil horizonation (Michel and Williams, 2011). The vertical boundaries of soil horizons, which may feature sharp gradients between widely differing chemical and physical conditions, may fluctuate in depth even across a small horizontal area (Grüneberg et al., 2010; Soil Survey Staff, 2010). By homogenizing across depth, the inherent variability of the soil structure is masked and the accuracy of correlations between edaphic factors and microbial communities is lowered. An understanding of microbial diversity with explicit consideration for soil morphology is particularly important when attempting to explore the ecological niches of uncultured microbes. Experimental field data with an explicit depth component may more accurately suggest the ecological functions and niches of different microbial groups (Nunan et al., 2007; Vos et al., 2013).

Agricultural soils present an opportunity to better understand the magnitude of depth effects, as tilled soils at a minimum should have depth effects due to the plow layer (Ap Horizon). Studying agricultural soils is complex, however, due to the wide variety of management practices. Recent papers examining surface soils have shown that populations of both bacteria and fungi are greater under organic management compared to no-till or conventional management (Wu et al., 2015), and microbial diversity can be significantly affected by management regime (Hartmann et al., 2014). The study reported here tested the effects of agricultural management on physical, chemical, and microbial characteristics. High-throughput sequencing was used to characterize soil fungal, bacterial, and archaeal communities. The study system is a long-term agricultural experiment where grain is grown under a variety of management systems: no-till,

conventional chisel till, and USDA organic management. We sampled at arbitrary depth increments both above and below the plow layer (Ap horizon). Edaphic properties known to influence microbial community assembly were measured and enzyme assays were used to assess function. We had three hypotheses: 1) In all three management regimes, the diversity of microbial communities will significantly differ by depth and treatment: communities from soils above the plow layer will be more diverse than those below, and communities in no-till soils will be less diverse than organic soils above the plow layer. 2) Microbial β-diversity will significantly differ across every depth fraction, and will be significantly affected by treatment in all depths above the plow layer. 3) Depth will have a greater effect on bacterial and archaeal community composition than on fungal communities.

Results

Soil cores (0-60 cm) were sampled from experimental agricultural plots of the Farming Systems Project in Beltsville, MD (Cavigelli et al., 2008). Individual cores were split into 0-5 cm, 5-10 cm, 10 cm- plow layer (Ap horizon), Ap horizon-30 cm, and 30-60 cm increments, and homogenized. The depth of the Ap horizon was determined by color gradient, and ranged in depth from 12 to 26 cm, depending on the individual core (**Figure 1**).

A variety of physical and chemical factors were measured (**Table 1**; **Appendix A**, **Tables S1-3**). A linear mixed effect model (Randomized Complete Block Design within each treatment) showed that all physical and chemical variables (except NH_4^+-N, **Table S2**) significantly differed ($p < 0.05$) by depth. However, a repeated measures model

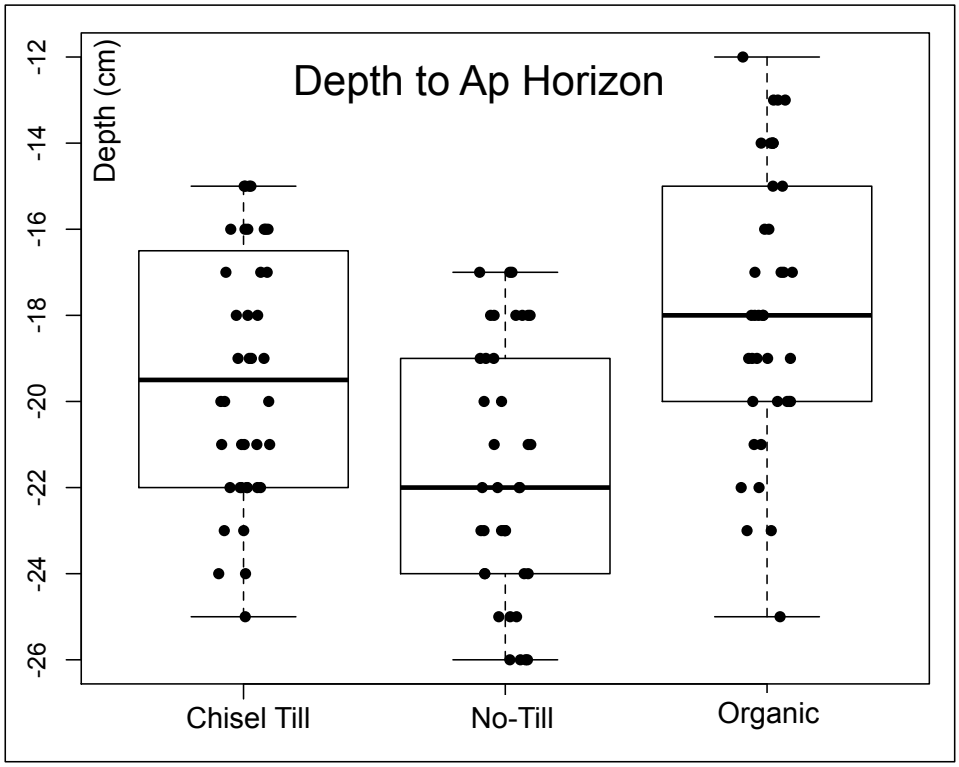


Figure 1. Box plots show the depth of the Ap horizon as determined by abrupt color change within individual cores taken from soils under chisel-till, no-till, or USDA organic management. For each management regime, four replicate field plots were sampled. Within each plot, 10 cores (100 cm deep, 2.5 cm diameter) were removed.

Table 1. Texture, pH, and total carbon and nitrogen of soils under each agricultural management regime at each of five depth increments (N=4, $\alpha = 0.05$). Letters show significance groupings within each treatment for each factor.

Treatment	Depth	Sand (%)	Silt (%)	Clay (%)	pH	Total C (%)	Total N (%)	C:N Ratio
Chisel Till	0-5 cm	24.5 ± 2.4 a	58.4 ± 1.8 a	17.1 ± 1.7 b	6.37 ± 0.11 bc	1.36 ± 0.07 a	0.13 ± 0.009 a	10.6 ± 0.2 a
	5-10 cm	23.2 ± 2.3 ab	59.0 ± 1.3 a	17.8 ± 1.8 b	6.47 ± 0.04 ab	1.42 ± 0.16 a	0.13 ± 0.013 a	11.1 ± 0.2 a
	10 cm-Ap	21.3 ± 2.3 b	59.7 ± 2.0 a	19.0 ± 1.3 b	6.81 ± 0.11 ab	0.88 ± 0.09 b	0.08 ± 0.010 b	10.7 ± 0.2 a
	Ap-30 cm	16.0 ± 2.6 c	54.2 ± 1.4 b	29.8 ± 2.2 a	7.00 ± 0.14 a	0.30 ± 0.03 c	0.04 ± 0.004 c	8.2 ± 0.3 b
	30-60 cm	15.9 ± 4.1 c	50.6 ± 0.5 c	33.5 ± 4.2 a	5.79 ± 0.33 c	0.18 ± 0.03 c	0.03 ± 0.004 c	6.5 ± 0.1 c
No Till	0-5 cm	23.4 ± 1.7 a	60.2 ± 1.1 a	16.4 ± 1.1 b	6.28 ± 0.12 c	1.70 ± 0.06 a	0.16 ± 0.007 a	10.8 ± 0.1 ab
	5-10 cm	23.2 ± 2.2 a	59.3 ± 1.5 a	17.5 ± 1.4 b	6.39 ± 0.16 bc	1.23 ± 0.10 b	0.12 ± 0.010 b	10.4 ± 0.2 ab
	10 cm-Ap	21.8 ± 1.7 a	59.3 ± 1.3 a	18.9 ± 1.3 b	6.81 ± 0.09 ab	0.95 ± 0.14 c	0.08 ± 0.006 c	11.2 ± 0.8 a
	Ap-30 cm	14.2 ± 2.2 b	54.6 ± 1.5 b	31.2 ± 2.6 a	6.86 ± 0.07 a	0.34 ± 0.04 d	0.07 ± 0.005 d	9.4 ± 0.7 b
	30-60 cm	12.6 ± 3.4 b	50.6 ± 2.2 b	36.8 ± 5.0 a	5.77 ± 0.18 d	0.22 ± 0.01 d	0.03 ± 0.003 d	7.3 ± 0.5 c
Organic	0-5 cm	22.1 ± 1.4 a	59.1 ± 1.0 a	18.8 ± 1.5 b	6.55 ± 0.14 b	1.66 ± 0.04 a	0.15 ± 0.007 a	11.2 ± 0.7 a
	5-10 cm	22.2 ± 1.2 a	59.1 ± 1.1 a	18.7 ± 1.5 b	6.94 ± 0.10 ab	1.53 ± 0.07 a	0.15 ± 0.008 a	10.4 ± 0.3 ab
	10 cm-Ap	21.7 ± 1.3 a	58.1 ± 1.5 a	20.2 ± 1.6 b	7.04 ± 0.13 a	1.17 ± 0.07 b	0.11 ± 0.007 b	10.6 ± 0.2 a
	Ap-30 cm	17.9 ± 2.0 b	51.5 ± 0.7 b	30.6 ± 1.6 a	7.06 ± 0.18 a	0.29 ± 0.02 c	0.03 ± 0.002 c	9.0 ± 0.9 b
	30-60 cm	14.5 ± 2.7 b	50.4 ± 2.2 b	35.1 ± 4.2 a	5.75 ± 0.18 c	0.17 ± 0.02 c	0.03 ± 0.002 c	6.4 ± 0.3 c

showed that there were no significant effects of treatment on any measured edaphic factor (except bulk density, $p = 0.047$) across all depths.

Illumina sequencing returned a total of 9.9 million 16S rRNA sequences from the V4-V5 region and 5.9 million ITS sequences from an area targeting ITS-1. After quality filtering, 7.3 and 5.0 million sequences (respectively) remained for use in further analyses. In four samples from the 30-60 cm depth fraction, no high quality ITS sequences remained after filtering, and these samples were excluded from further analysis. Missing samples included two samples under organic treatment, one under no-till, and one under chisel-till, ensuring an *n* of at least 2 for statistical analysis. There were an average of 121,000 sequences per sample for bacteria and archaea, and 88,500 for fungi. There tended to be approximately 5-9 times as many unique OTUs for bacteria than fungi in each sample, and 12-17 times as many bacterial OTUs as archaeal OTUs (**Table 2**). Species richness significantly differed with depth ($p < 0.001$ for archaea, $p = 0.0012$ for bacteria, $p < 0.001$ for fungi), but not between treatments. In pairwise comparisons, only the 5 cm-Ap horizon and 30-60 cm depths significantly differed in archaeal OTUs, and the 0-10 cm and 30-60 cm depths significantly differed for bacteria ($p < 0.05$). In contrast, fungal species count decreased significantly with increasing depth, from a maximum 996 OTUs in the 0-5 cm depth fraction to 474 OTUs from 30-60 cm. Pairwise comparisons by depth also showed that there were significantly ($p = 0.0026$) more unique fungal OTUs under both Chisel-Till (930 ± 20) and Organic (910 ± 24) management than No-Till (794 ± 13) between 5-10 cm.

Table 2. The average number of unique OTUs per sample at each depth fraction (N = 12) ± SE. Due to missing values, N = 8 at 30-60 cm. Bacterial and archaeal sequences were considered OTUs at 97% similarity, and fungi were considered unique at variable similarity (97-99%) according to the curated UNITE dynamic database (Koljalg et al., 2013).

		Archaea	**Bacteria**	**Fungi**
Depth	0-5 cm	290 ± 18 ab	4921 ± 206 a	996 ± 31 a
	5-10 cm	360 ± 32 a	5414 ± 287 a	878 ± 21 b
	10 cm-Ap	348 ± 36 a	4296 ± 296 ab	584 ± 17 c
	Ap-30 cm	260 ± 39 ab	4426 ± 526 ab	477 ± 24 d
	30-60 cm	182 ± 28 b	3145 ± 523 b	475 ± 49 cd

Combined archaeal and bacterial communities of every depth increment were significantly different from each other ($p < 0.001$, A-statistic = 0.526, **Figure 2A**). Communities in the 0-5 cm and 5-10 cm depth fractions were the most similar to each other ($p < 0.001$, $A = 0.116$). Moving down the soil profile, communities became increasingly distinct, with the two most divergent communities being those immediately above and below the Ap horizon ($p < 0.001$, $A = 0.420$). There were no significant differences in communities associated with agricultural management regime across all depths ($p = 0.17$, $A = 0.012$). However, when analyzed individually, there were significant differences between treatments in the 0-5 cm ($p = 0.0037$, $A = 0.244$), 5-10 cm ($p < 0.001$, $A = 0.439$), and 10 cm-Ap Horizon ($p = 0.037$, $A = 0.134$) depth fractions. According to PerMANOVA analysis, there was a significant effect of treatment nested within depth on 16S-based communities ($p< 0.001$, both treatment and depth factors). In the 10 cm-Ap horizon, there was also a significant difference between communities in different experimental blocks ($p = 0.038$, $A = 0.169$), a trend that also held for the Ap horizon-30 cm ($p < 0.001$, $A = 0.404$) and 30-60 cm ($p = 0.001$, $A = 0.239$) depths. Pairwise comparisons show that these differences were mostly due to divergence between communities in organic and no-till systems. Organic and no-till communities diverged at the 0-5 cm ($p = 0.008$, $A = 0.292$), 5-10 cm ($p = 0.008$, $A = 0.444$), and 10 cm-Ap horizon ($p = 0.017$, $A = 0.190$) depths.

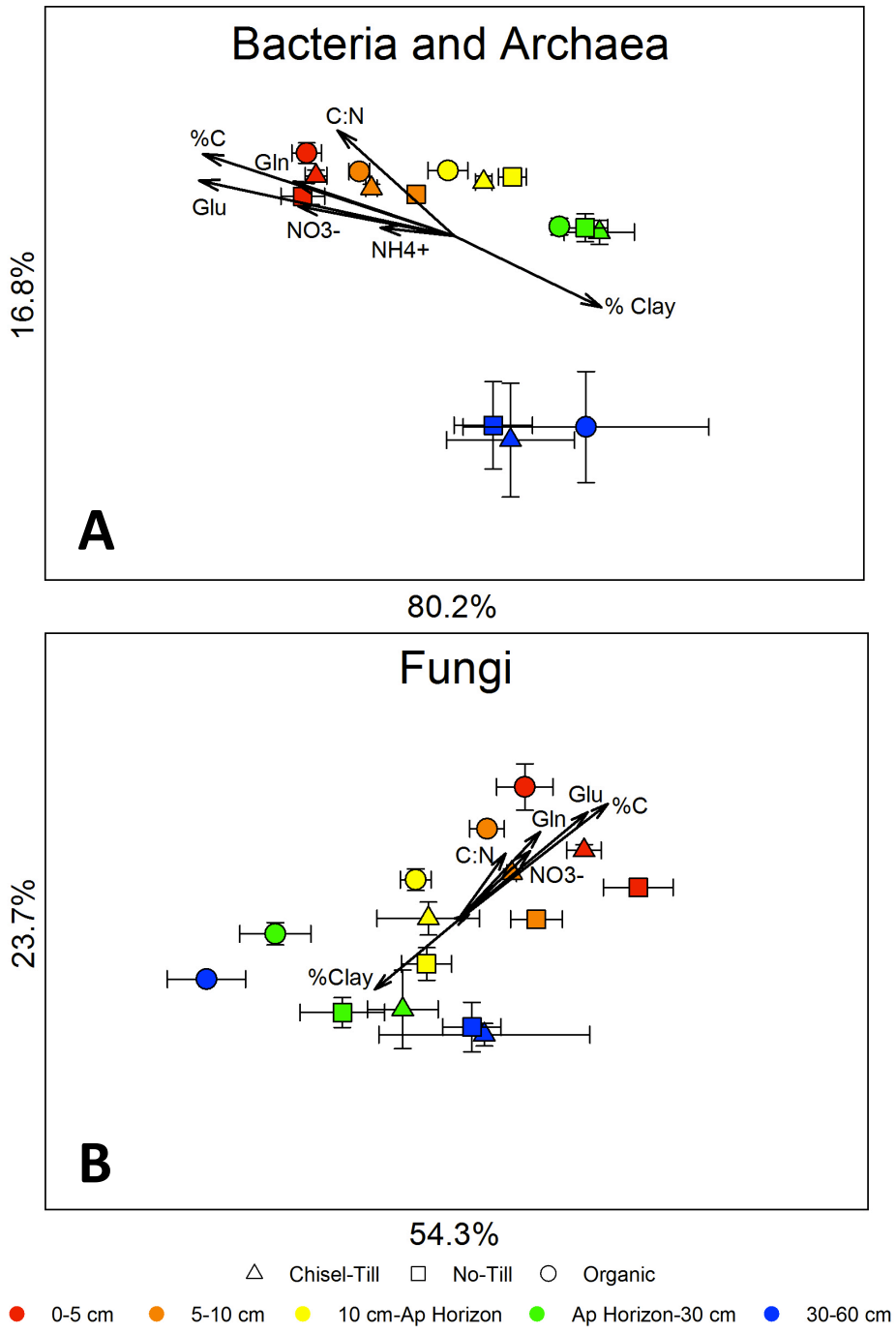


Figure 2. Non-metric multidimensional (NMS) ordinations representing **A**) archaeal and bacterial communities and **B**) fungal communities from three different agricultural management regimes at each of five depth increments. Each point represents one treatment group ($2 > N > 4$), error bars represent standard errors of the means. Vectors show factors correlated to soil properties (Glu – glucosidase activity, Gln – glucosaminidase activity); all are significant ($p < 0.001$).

The β-diversity in 16S-based communities was correlated with factors related to soil depth (**Table 3**). All factors were significant ($p < 0.05$) predictors of community composition except for pH. The strongest predictors of surface communities were total C ($r = 0.770$) and glucosidase activity ($r = 0.776$), while subsurface communities were correlated with increasing clay content ($r = -0.588$).

In contrast to bacteria and archaea, fungal communities differed both by depth and treatment among all samples (**Figure 2B**). Communities of every depth class and treatment were significantly different from each other ($p < 0.05$, A statistics $0.081 < A < 0.412$), with the exception of the 0-5 cm and 5-10 cm depths ($p = 0.066$). While treatment effects on fungal communities were stronger in fungal communities, depth effects were weaker, especially between communities immediately above and below the Ap horizon ($p < 0.001$, $A = 0.12$, **Figure 2B**). A-statistics, which describe the magnitude of the distinction between tested groups, were 0.12, 0.33, 0.42, and 0.34 for communities of neighboring, successive descending depths in 16S-based communities. In ITS-based communities, A-statistics for these same comparisons were 0.04, 0.10, 0.12, and 0.08.

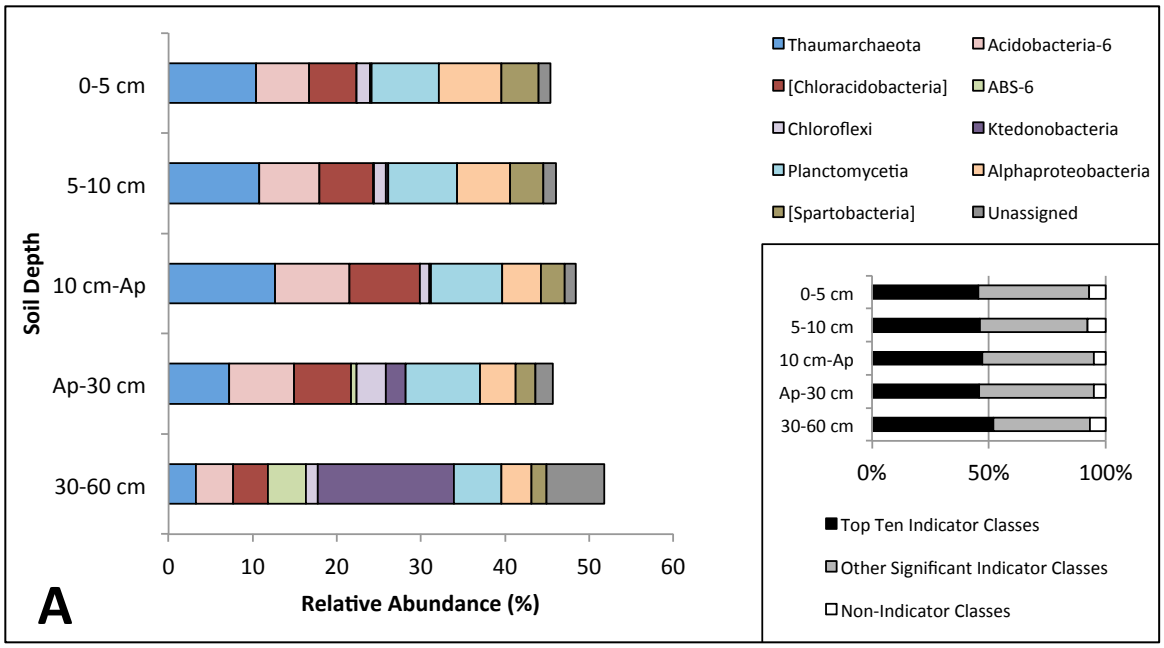
ITS-based communities showed generally similar relationships to edaphic factors as those for the 16S-based communities (**Table 3**). All correlations were significant except for pH ($p = 0.12$) and NH_4^+ concentration ($p = 0.14$). Again, factors related to depth were the primary predictors of community structure, with total C ($r = -0.812$) correlated to surface communities, and % clay ($r = 0.617$) correlated with subsurface communities.

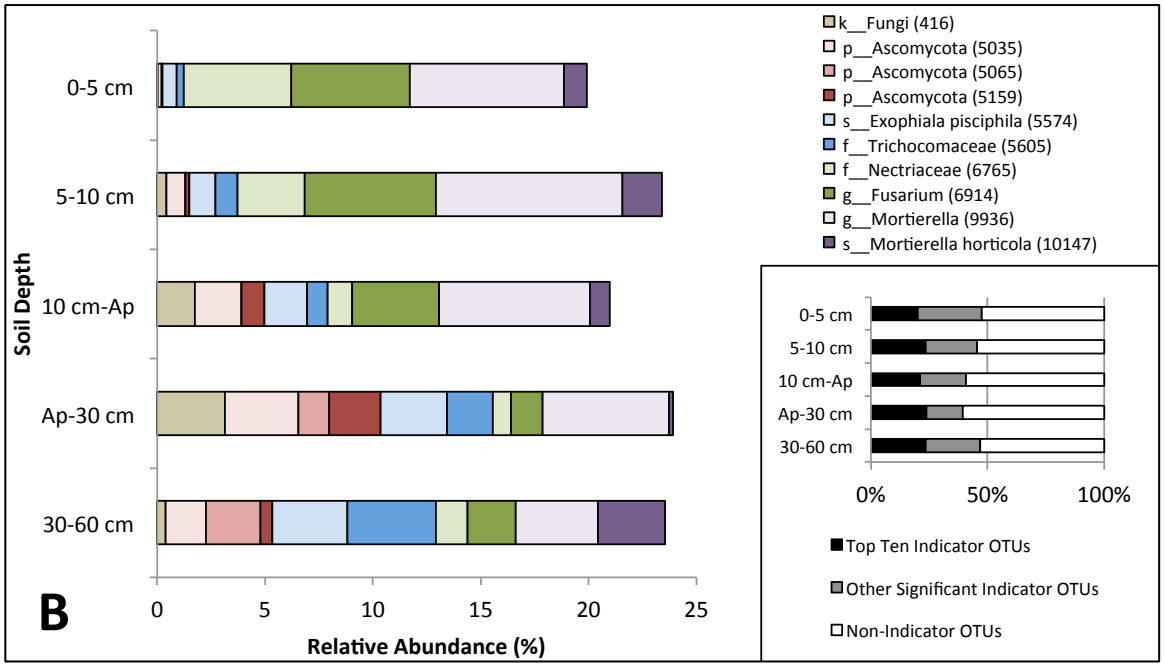
Table 3. Correlations between edaphic factors and 16S rRNA bacterial and archaeal communities and ITS fungal communities.

	Factor	r	p
16S	% Clay	-0.588	< 0.001
	pH	-0.075	0.572
	NO_3^-	0.607	< 0.001
	NH_4^+	0.417	0.001
	Total %C	0.770	< 0.001
	C:N Ratio	0.525	< 0.001
	Glucosidase	0.776	< 0.001
	Glucosaminidase	0.616	< 0.001
ITS	% Clay	0.617	< 0.001
	pH	-0.212	0.117
	NO_3^-	-0.628	< 0.001
	NH_4^+	-0.198	0.143
	Total %C	-0.812	< 0.001
	C:N Ratio	-0.617	< 0.001
	Glucosidase	-0.782	< 0.001
	Glucosaminidase	-0.708	< 0.001

Of the 238 classes of bacteria and archaea identified in our samples, 134 were significant indicators of depth fractions (**Figure 3A**), while 21 were significant indicators of treatments across all depths (*data not shown*). Together these significant depth indicator classes accounted for between 73-97% of the total community, while significant treatment indicator classes only ranged between 0 – 4.2% of the total community in all samples, and these were therefore analyzed within individual depth fractions. This revealed 101 significant ($\alpha = 0.05$) indicator classes, 75 for treatments from depths above the Ap horizon, and 26 for experimental blocks from depths between 10-60 cm. Average abundances of indicator classes between treatments were subtle, while those in blocks were more dramatic (**Appendix A, Figure S1 A-F**). Especially notable were the differing abundances of members of phylum GAL-15 and acidobacterial classes *Acidobacteria*-6 and *Acidobacteria* iii1-8 between blocks in the Ap-30 cm depth, *Chloracidobacteria* in the 30-60 cm depth fraction, and the extreme variability of *Ktedonobacteria* in all depths below the Ap horizon.

Fungal indicator species were assessed at the OTU level, due to the greater ability to connect fungal OTUs to ecological functions (**Figure 3B and C**). These accounted for less of the total community than in bacteria and archaea, with greater variability between samples. Significant depth indicators made up 24.1 - 66.1% of community abundance, and significant treatment indicators accounted for 7.0 - 57.1%. There were a total of 600 significant ($\alpha = 0.05$) indicator species for depth, and 464 for treatments, with 87 shared OTUs significant in both categories. Proportions of phylum membership amongst significant indicator OTUs were similar in each category: depth indicators were 38.8% *Ascomycota*, 8.5% *Basidiomycota*, 1.0% *Chytridiomycota* and 4.8% *Zygomycota*, with


Soil Depth
0-5 cm
5-10 cm
10 cm-Ap
Ap-30 cm
30-60 cm
0
10
20
30
40
50
60
Relative Abundance (%)
A
Thaumarchaeota
Acidobacteria-6
[Chloracidobacteria]
ABS-6
Chloroflexi
Ktedonobacteria
Planctomycetia
Alphaproteobacteria
[Spartobacteria]
Unassigned
0%
50%
100%
Top Ten Indicator Classes
Other Significant Indicator Classes
Non-Indicator Classes


Soil Depth
0-5 cm
5-10 cm
10 cm-Ap
Ap-30 cm
30-60 cm
0
5
10
15
20
25
Relative Abundance (%)
B
k__Fungi (416)
p__Ascomycota (5035)
p__Ascomycota (5065)
p__Ascomycota (5159)
s__Exophiala pisciphila (5574)
f__Trichocomaceae (5605)
f__Nectriaceae (6765)
g__Fusarium (6914)
g__Mortierella (9936)
s__Mortierella horticola (10147)
0%
50%
100%
Top Ten Indicator OTUs
Other Significant Indicator OTUs
Non-Indicator OTUs

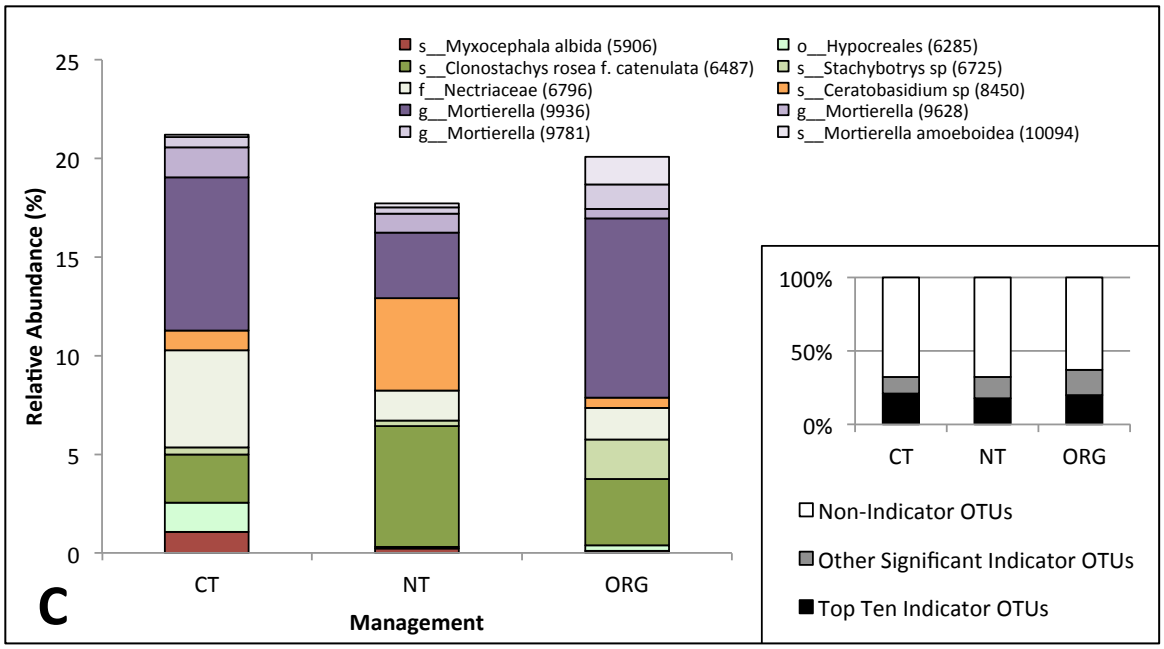


Figure 3. Stacked bar graphs show average abundances of **A**) bacterial and archaeal classes and **B**) fungal OTUs with the top 10 greatest absolute ranges in average abundance between depth fractions (N = 12). **C** shows fungal OTUs with the top 10 greatest absolute ranges in average abundance between agriculture management regimes across all depth fractions (N = 20). Only significant indicator species ($\alpha = 0.05$, PC-ORD, (McCune and Mefford, 2011) for either depth (**A** and **B**) or treatment (**C**) were considered. Inset stacked bar graphs show relative proportions of the top ten indicator classes (main graph), proportions of all other significant indicator species, and non-indicator species. Fungal OTUs are identified at the finest taxonomic resolution assigned by the RDP classifier (k – kingdom, p – phylum, c – class, o - order, f – family, g – genus, s – species) with a unique internal identification number. Only one fungal OTU (g_Mortiella 9936) is common to both **B** and **C**.

46.8% unclassifiable beyond the kingdom level. Treatment indicators were 41.2% *Ascomycota*, 13.6% *Basidiomycota*, 1.1% *Chytridiomycota* and 4.7% *Zygomycota*, with 39.4% unclassified remaining.

Changing patterns of indicator species relative abundance with increasing depth were characterized into four distribution patterns: 1) constant decrease with increasing depth, 2) constant increase with depth, 3) greatest abundance immediately above the Ap and 4) greatest abundance immediately below the Ap horizon (Figure 4). In bacteria and archaea, half of the identified classes were most abundant at the surface and constantly decreased (51 of 104). The remaining classes were split evenly between the other three distribution patterns (17, 19, and 17 in the 10 cm-Ap, Ap-30 cm, and 30-60 cm depth fractions, respectively). Archaeal class *Thaumarchaeaota* and bacterial classes *Acidobacteria*-6 and *Chloracidobacteria* were highest above the Ap horizon, while *Chloroflexi* and *Planctomycetia* were highest below the Ap horizon. Classes AB-6 and *Ktedonobacteria*, as well as sequences that could not be assigned to any known taxonomy, were highest at depth. Distribution patterns of individual classes tended to be diverse within each of their respective phyla (**Figure 4**). Within the archaea, only *Thaumarchaeota* were abundant above the Ap horizon. In contrast, crenarchaeotal classes MBGA and MGC, as well as euryarchaeal class *Methanomicrobia* were most abundant at depth. Phylum *Ktedonobacteria* was the most dramatic indicator of depth, but the majority of classes in phylum *Chloroflexi* were also most abundant below the Ap horizon. The only phyla with more than two component classes in which all classes were indicators for the same depth were *Bacteroidetes* (8 classes, surface) and *Cyanobacteria* (8 classes, surface).

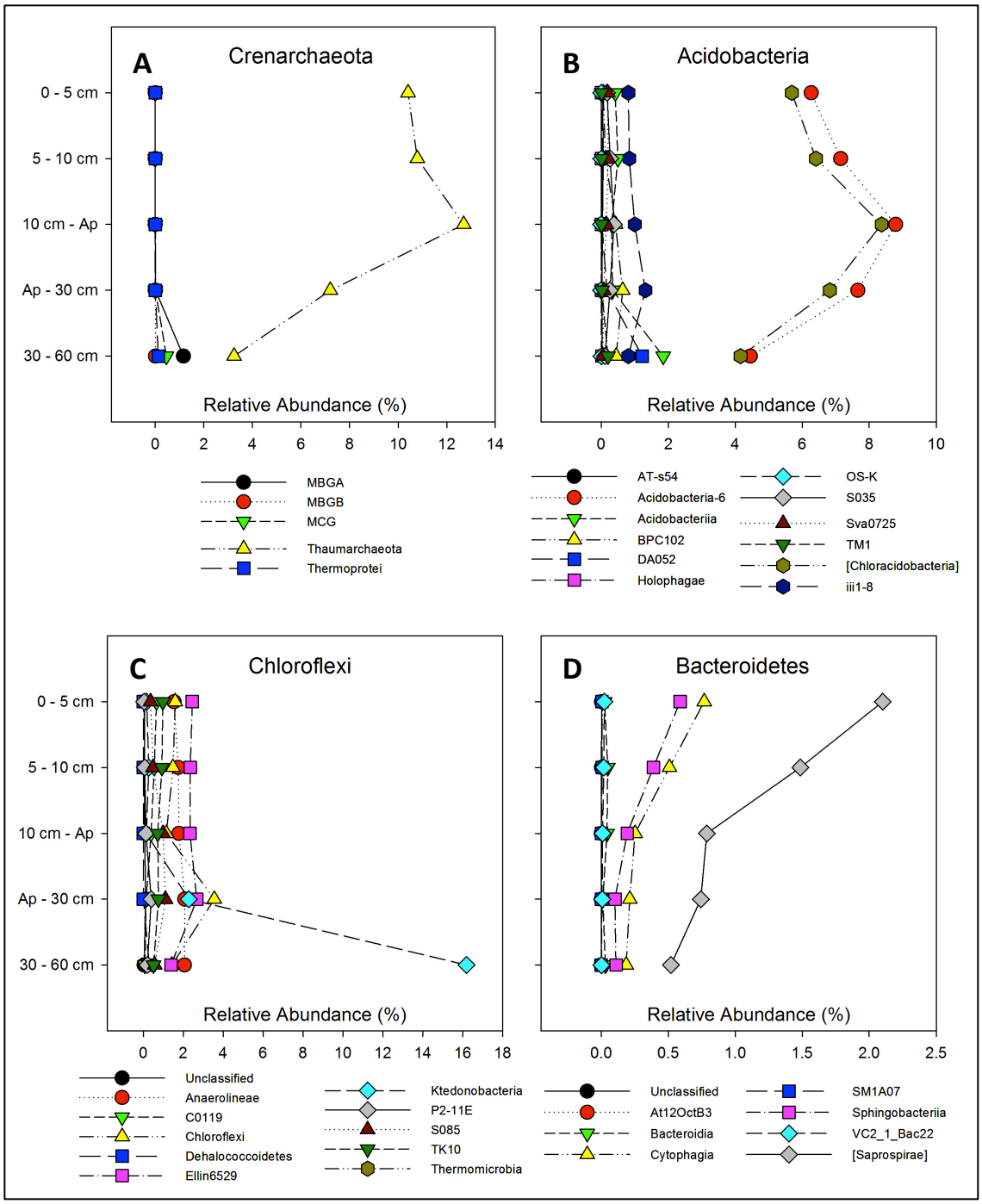


Figure 4. Line graphs show average abundances of all classes (including putative classes) within phyla *Crenarchaeota* (**A**), *Acidobacteria* (**B**), *Chloroflexi* (**C**), and *Bacteroidetes* (**D**) in each of five depth increments. Only significant indicator species ($\alpha = 0.05$, PC-ORD, (McCune and Mefford, 2011) for depth were considered.

Distribution patterns of fungal indicator species were more consistent compared to bacteria and archaea. Most fungal indicator species had the highest relative abundance at the soil surface and decreased with increasing depth (264 of 336). An additional 43 indicator species were most abundant immediately above the Ap horizon. Unlike in bacteria and archaea, proportions of depth patterns did not differ much between phyla, with proportions of members of *Ascomycota*, *Basidiomycota*, *Zygomycota*, and unidentifiable fungi all having approximately the same distributions as above.

Though most fungal OTUs were most abundant at the surface, abundance patterns of the top 10 greatest absolute ranges between depth increments were diverse (**Figure 3B**). Two members of *Ascomycota* (one family *Nectriaceae*, one genus *Fusarium*) and two members of *Zygomycota* (one genus *Mortierella*, one species *Mortierella horticola*) were relatively most abundant at the surface and decreased with depth. Indicator species for treatments did not overlap with those for depth, except in the case of the most abundant *Mortierella* species (**Figure 3C**). This OTU, along with two other *Mortiella* sp. and an unidentified *Stachybotrys* sp. were most abundant in organic plots. One final *Mortiella* species, as well as two members of order *Hypocreales* and one *Myxocephala albida* were most abundant under chisel-till management. *Clonostachys rosea* and an unidentified *Ceratobasidium* species were most abundant under no-till management.

Discussion

We observed ~4500 bacterial OTUs in each sample of agricultural soil (**Table 2**). This is an order of magnitude higher than other reports based on clone libraries (Upchurch et al., 2008) or pyrosequencing (Hartmann et al., 2014). Higher species richness in the current study is likely due to a number of generated sequences several

orders of magnitude higher than those commonly received from other sequencing methods. Species richness was greater at the soil surface compared to depth for archaea, bacteria, and fungi, partially supporting our first hypothesis (**Table 2**). Greater microbial diversity in surface communities is consistent with previous studies with both lower (Will et al., 2010; Michel and Williams, 2011) and higher (Eilers et al., 2012) spatial resolutions. This greater surface diversity likely results from more possible niches at the surface compared to the subsurface (Vos et al., 2013). Our results reinforce previous findings that fungal diversity decreases with increasing depth (Jumpponen et al., 2010; Bahram et al., 2015), but the only significant difference in archaeal and bacterial diversity was between the surface soils (0-10 cm or 5 cm-Ap horizon) and the deepest sampled layer (30-60 cm). This alone contradicts previous studies that have shown significant differences in diversity across smaller vertical distances (Eilers et al., 2012).

Hartmann et al. (2014) reported an increase in bacterial and archaeal species richness when agricultural management included manure addition. In contrast, 16S-based OTU richness did not differ between the farming treatments at any depth within our study. We did find significantly greater fungal diversity under both chisel-till and organic management than no-till, although this only held true at the 5-10 cm depth. Our results suggest that agricultural management affects microbial community species richness above the plow layer, but has no effect below this anthropogenic disturbance.

Similarly, β-diversity of both 16S and ITS-based communities significantly differed between agricultural management regimes at every depth above the Ap horizon (**Figure 2A and B**), supporting our second hypothesis. The dramatic difference observed between soils just above and below the plow layer highlights the need to sample and

study soils using horizon boundaries, rather than arbitrary depths (Grüneberg et al., 2010). The importance of sampling agricultural soil by Ap horizon boundary has been previously reported (Syswerda et al., 2011), though its location has been found not to vary in depth across experimental fields. In contrast, we found that Ap horizon depth in individual cores was variable (**Figure 1**). Although it did not significantly differ by management regime, pairwise comparisons showed that it significantly varied from plot to plot, with average depths ranging from 15.7 to 23.9 cm. Dividing individual cores above and below this horizon was important in capturing the changing edaphic factors across the horizon boundary. These included a precipitous drop in total C and N, and a dramatic increase in average percent clay (**Appendix A, Table S1**).

Soil morphology is important in shaping soil microbial communities because it represents a history of mineral deposition, chemical illuviation, and disturbance (Soil Survey Staff, 2010; Michel and Williams, 2011). Factors known to correlate with changes in microbial community structure include: pH (Lauber et al., 2009), moisture (Barnard et al., 2013), C availability (Eilers et al., 2010), and the mineralogy of parent material (Yarwood et al., 2015). All these factors are in turn related to the weathering processes of soil and can differ between soil horizons.

Sampling from above and below the Ap horizon revealed the vertical distribution of common agricultural soil microbes, and allowed us to detect putatively identified microbes that have traditionally not been reported as abundant in agricultural soils, such as *Ktedonobacteria* (**Figure 3A and B**). Depth sampling showed that *Thaumarchaeota* (Fuhrman, 1992), the most abundant class of archaea in these soils, was common in surface soils and most abundant immediately above the Ap horizon. This contrasts with

previous findings (Hansel et al., 2008; Eilers et al., 2012) showing Thaumarchaeota to be more abundant at depth in forest soils. The majority of studies of *Thaumarchaeota* have focused on their proposed role as widespread ammonia-oxidizers due to the prevalence of ammonia monooxygenase (amo) genes in their genome (Pester et al., 2011). While these organisms were first discovered and most researched in marine settings, they are abundant in agricultural soils, and have been shown to have nitrifying roles in terrestrial ecosystems (Zhang et al., 2010; Pratscher et al., 2011). Though it is generally assumed that *Thaumarchaeota* exclusively use ammonia, there are few physiological studies of *Thaumarchaeota,* and the diversity of metabolic strategies in the class has not been experimentally tested. Therefore, it is possible that these microbes could use other substrates, or that some members could be facultative ammonia-oxidizers (Pester et al., 2011).

The two most abundant acidobacterial classes (*Acidobacteria*-6 and *Chloracidobacteria*) were also most abundant above the Ap horizon. *Acidobacteria*-6 is widely found in terrestrial soils (Jones et al., 2009; Foesel et al., 2014), but *Chloracidobacteria* is not. The closest related known organism to the unidentified OTUs in class *Chloracidobacteria* is the phototrophic *Chloracidobacterium thermophilum*, isolated from a microbial mat in Yellowstone (Bryant et al., 2007). This depth-structured distribution pattern of *Acidobacteria*-6 has been previously noted in a weathered shale saprolite (Hansel et al., 2008), but is contradicted by findings of greater abundance in the A horizon of grassland soils (Will et al., 2010) and no distinguishable pattern in forest soils (Eilers et al., 2012), showing that the vertical distribution of this group is highly dependent on ecotone.

Observing abundance patterns of archaeal and bacterial classes may provide insight into the ecological diversity of their respective phyla. It has been stated that, for certain bacteria, general trophic strategies are conserved at the phylum level (Philippot et al., 2010), a claim that has been especially explored for *Acidobacteria* (Fierer et al., 2007). Here we show this to be the case only in two common groups: *Bacteroidetes* and *Cyanobacteria,* in which all component classes are most abundant at the soil surface and decrease with increasing depth (**Figure 4**). This can be explained in that all members of cyanobacteria are photosynthetic, one of a few highly conserved functions (Martiny et al., 2013), and require direct light to function. This also provides further evidence for *Bacteroidetes'* status as generally copiotrophic, adapted to taking advantage of large amounts of easily available C to reproduce quickly (Fierer et al., 2007; Eilers et al., 2012). However, most other bacterial and archaeal phyla were composed of classes with diverse patterns of abundance across depth, especially *Acidobacteria*. The high niche diversity of members of Acidobacteria is increasingly reported, but the ecological functions of most of its component classes are mostly unknown (Eichorst et al., 2011). Patterns of abundance across depth, in addition to space (Jones et al., 2009) may provide greater context for future experiments of the specific functions of individual members of *Acidobacteria*, rather than the generalized activity of the entire phylum.

Compared to archaea and bacteria, fungal communities between depth increments were less distinct, supporting our third hypothesis. This may be due to the filamentous morphology of many fungi, which gives them access to resources from a larger area than bacteria (Vos et al., 2013). Though fungal communities of successive depths were less distinct from each other than archaeal and bacterial communities, fungal communities

above and below the Ap horizon were still the most distinct (**Figure 2B**). This shows that soil morphology and horizonation may have significant effects even on communities of organisms with the ability to span horizon boundaries (Taylor et al., 2014).

Though most fungal OTUs were most abundant at the soil surface, several of the most abundant OTUs increased in abundance with increasing depth, and peaked below the Ap horizon or from 30-60 cm (**Figure 3B**). Of those that were identifiable past phylum level, both belonged to family *Eurotiales*: one unidentified *Trichocomaceae*, and one member of *Exophiala pisciphila*. Both are molds commonly found in soils, and are likely saprophytic (Webster and Weber, 2007). In contrast, the two most abundant OTUs decreasing with depth belonged to family *Nectriaceae*, one in genus *Fusarium*, both commonly saprophytic or parasitic to plants (Webster and Weber, 2007). Together, these suggest that increasing depth in agricultural soils may be associated with a decrease in plant parasite abundance, and an increase in saprophytes.

Though many recent studies have drawn links between edaphic features and microbial communities in an attempt to suggest possible niches for organisms, we show that homogenizing across depth may obscure these relationships. Both depth and changes in soil morphology are correlated with significant changes in microbial community dynamics, and taking into account soil horizon boundaries can improve interpretations of effects of anthropogenic changes on both edaphic properties and soil microbial communities. The Ap horizon is an important heterogeneity that appears to support communities that differ in β-diversity. Even after 11 years of no-till management, the Ap horizon remains distinct and still affects microbial community structure. Future studies

should take into account this important soil feature and investigate the legacy of tillage over longer time frames.

Experimental Procedures

Soils were sampled in August 2013 from the Farming Systems Project (FSP) in Beltsville, MD (39.03° N, 76.90° W). The climate is humid subtropical bordering humid continental, with an average annual precipitation of approximately 1110 mm, and average temperature of 12.8 °C. Soil types at the study site are Christiana (fine, kaolinitic, mesic Typic Paleudults), Keyport (fine, mixed, semiactive, mesic Aquic Hapludults), Matapeake (fine-silty, mixed, semiactive, mesic Typic Hapludults), and Mattapex (fine-silty, mixed, active, mesic Aquic Hapludults) silt loams (Spargo et al., 2011). All soils are designated as Ultisols with variable amounts of clay in the subsoil, and variable average water table depths (Soil Survey Staff, 2014).

The FSP is a long-term experiment testing the effects of agricultural management style and crop rotation length on soil properties and farm profitability. Soils were sampled from chisel-till, no-till, and USDA organic plots in each of four experimental blocks on the site. Only plots under three-year rotations were sampled (**Table 4**). Further details of the agricultural management of the FSP can be found in Spargo et al., 2011.

Ten cores (2.5 cm diameter, 100 cm depth) within plastic sleeves were taken from each of twelve plots by backing a hydraulic probe 10 feet into the edge of each plot and taking three cores at 0°, 90°, and 180° orientations, backing a further 10 feet into the plot and repeating, then backing another 10 feet into the plot and taking two cores at 0° and 180°. An additional 10 cores were taken at random points within the sampling area with a

Table 4. Agricultural management practices of the Farming Systems Project (adapted from (Spargo et al., 2011).

Management Practice	**Cropping System**		
	No Till (NT)	Chisel Till (CT)	Organic (ORG)
Crop rotation †	C-r-S-W/S	C-r-S-W/S	C-r-S-W-v
Primary tillage ‡	None	Ch	D, MB, or Ch
Weed control §	Herbicides	Herbicides	RH, RC
Fertility ¶	N, P, K	N, P, K	GM, AM, K

† - C corn, S soybean, W wheat, W/S wheat followed by double-cropped soybean; r rye cover crop, v hairy vetch. No till and chisel till followed a 2-year C-W/S rotation from 1996 to 2000.
‡ - D disk, MB moldboard plow, Ch chisel plow
§ - RH rotary hoe, RC row cultivator
¶ - N urea ammonium nitrate, P triple super phosphate, K potassium sulfate, GM green manures, AM animal manures

10 cm push probe to ensure enough soil in the 0-5 cm and 5-10 cm depth fractions for all analyses. Meter-deep soil cores were covered and transported to a lab in Beltsville for subsampling.

Soils were immediately extruded from plastic cores and divided into 0-5 cm, 5-10 cm, 10 cm-Ap Horizon, Ap Horizon-30 cm, and 30-60 cm depth fractions in sterile conditions. The depth of Ap horizon was judged core-by-core by the abrupt color change. All depth fragments for all 10 cores from each plot were collected into sterile containers, and the extra 0-5 cm and 5-10 cm cores taken from the field were combined with those from the cores. Combined core fractions were homogenized with a 4 mm sieve, then subsampled and stored for further analysis: samples for DNA extraction were frozen at -20 °C, samples for NO_3^- and NH_4^+ extraction and enzyme assays were stored at 4 °C, samples for moisture correction and particle size analysis were oven-dried at 105 °C for 24 hours, and those for all other analyses were allowed to air-dry at room temperature for three days.

Bulk density was calculated by dividing the oven-dry corrected mass of aggregated soil depth fractions from each plot by the calculated volume of the soil cores. Soil texture was determined using the pipette method of particle size analysis (Gee and Bauder, 1986). Suspensions (2:1) of 5.0 grams of soil in 10.0 mL of deionized H_2O were allowed to equilibrate for 30 minutes, after which pH was measured using an Accumet AB15 soil pH meter with a glass electrode (Fisher Scientific, Waltham, MA). Total C and N from air-dry soil samples were measured using dry combustion with a LECO CHN 2000 analyzer (LECO Corporation, Lakeville, MI). NO_3^- and NH_4^+ were extracted simultaneously from 5.0 grams of field-moist soil (stored at 4 °C for no more than 2

weeks) using 25 mL of 2 M KCl. Soil suspensions were shaken for 1 hour on a reciprocating shaker, and filtered through Whatman #2 filters into scintillation vials. Extractable NO_3^- and NH_4^+ were quantified colorimetrically using an autoanalyzer (Lachat, Loveland, CO).

Activities of β-d-glucosidase (EC 3.2.1.20) and N-acetyl-β-d-glucosaminidase (EC 3.2.1.52) were determined using a modified version of the microplate method detailed in Popova and Deng, 2010. Modifications were as follows: 1.00 g oven-dry equivalent mass of field moist soil was mixed with 100 mL of deionized H_2O for 30 minutes using a magnetic stir bar in a glass dish measuring 10 cm in height and 15 cm in diameter. H_20 was used in place of 60 mM 2-(N-morpholino)ethanesulfonic acid (MES), and 50 μL 0.3 M MES buffer was added to individual microplate wells to bring the total concentration of MES in the wells to 60 mM. Additionally, 6.0 mM solutions of p-nitrophenyl-N-acetyl-β-d-glucosaminide and 10.0 mM solutions of p-nitrophenyl-β-d-glucoside substrates were used in place of 60 mM concentrations, as these were found to be the upper limits of solubility for these compounds in H_2O. Enzyme assays were performed within 3 months of sampling, but all samples were performed within a week and a half of each other, due to time taken to optimize assay protocols.

DNA was extracted from soils using MoBio "Powerlyzer" Powersoil DNA isolation kits (MoBio Laboratories, Carlsbad, CA) according to manufacturer's instructions. Briefly, 0.25 g of frozen soils were added to bead-beating tubes and shaken using a FastPrep-24 Instrument (MP Biomedical, Solon, OH) set at 5.5 m/s for 45 seconds. In four samples of soil from 30-60 cm depths, DNA yields using this kit were too low for amplification, and MoBio "Powermax" DNA isolation kits were used instead

(MoBio Laboratories, Carlsbad, CA). Kits were used according to manufacturer's instructions with the following alteration: bead-beating was performed at room temperature by shaking on a reciprocating shaker at maximum speed for 30 minutes. DNA was quantified using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA). Extracts were diluted to 2.0 ng/μL for amplification and sequencing. The V4-V5 region of the 16S rRNA gene was amplified using 505F – 806R universal primers (Caporaso et al., 2012), and a region of the fungal ITS gene was amplified with modified ITS1f - ITS2 fungal primers (Smith and Peay, 2014), both amplified according to published amplification parameters. Both sets of primers had Illumina adaptor overhang sequences: 5' TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG – Forward Primer – 3', and 5' GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G – Reverse Primer – 3'. Amplified products were cleaned and prepared for Illumina sequencing using the 16S Metagenomic Sequencing Library Preparation protocol (Part # 15044223 Rev. B, support.illumina.com). Both 16S and ITS PCR products were cleaned of oligonucleotides using AMPure XP beads (Beckman Coulter, Pasadena, CA), 8-nucleotide indexes and Illumina sequencing indices were attached using a Nextera XT Index Kit (Illumina, San Diego, CA), and final products were cleaned a final time using AMPure XP beads (Beckman Coulter, Pasadena, CA). Two μL of each cleaned final amplified product was combined into one pooled sample for each target (16S or ITS), which was quantified in triplicate using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA) before being diluted into two aliquots of 10 nM and 1.0 ng/μL. The 1.0 ng/μL dilution of combined 16S and ITS samples was analyzed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) to test for product purity and appropriate

amplicon length (variable for ITS sequences) prior to sequencing. Sequencing of the 10 nM dilutions was performed using a MiSeq Desktop Sequencer (Illumina, San Diego, CA) at the Center for Genome Research and Biocomputing at Oregon State University. Spikes of 5% and 10% PhiX were included on 16S and ITS plates respectively, to prevent sequencing errors. Paired-end sequences (250 bp) were generated and demultiplexed at Oregon State, and downloaded at the University of Maryland, College Park for further processing.

Paired-end sequences were joined using the fastq-join function within the ea-tools bioinformatics software package (Aronesty, 2011), implemented within the QIIME pipeline (Caporaso et al., 2010). All default settings were used, but minimum base pair overlap was increased to 50 from 6. As demultiplexed samples had been stripped of indexes, primers were removed, quality controls were implemented, and sequences were labeled with experimental data by processing them individually with the split_libraries.py function. All default settings were used, except the –z truncate_remove option was enabled to remove all sequences without a completely intact reverse primer. Individual sequences were then concatenated, and OTUs were picked using UCLUST through the pick_otus.py function (97% similarity) (Edgar, 2010). The most recent release of the greengenes database (May 2014 release, greengenes.org, McDonald et al., 2012) was referenced in 16S sequence clustering, and the UNITE dynamic database (October 9, 2014 release, unite.ut.ee, Koljalg et al., 2013) was used to cluster ITS sequences. Likewise, taxonomy was assigned 16S sequences using default settings (UCLUST, greengenes reference), though fungal taxonomy was assigned using the RDP classifier, referencing the UNITE database (Cole et al., 2009). OTU tables for 16S and ITS were

generated with the make_otu_table.py function, and taxonomy summaries were generated using summarize_taxa.py.

OTU tables and taxonomy summaries were used to calculate Sorenson distance matrices and perform multidimensional statistics using PC-ORD ver. 6.0 (McCune and Mefford, 2011). Distinctions in archaeal, bacterial, and fungal community structures by depth and treatments were evaluated using Nonmetric Multidimensional Scaling (NMS). NMS ordinations were plotted on two axes, with 250 iterations to the final ordination. Significant differences between groups were determined by Multi-Response Permutation Procedures (MRPP). Both significance of indicator species, and overall significance of treatment and depth effects (evaluated using PerMANOVA) were evaluated within PC-ORD. Significance of depth and treatment effects on soil physical, chemical, and biochemical properties were evaluated using the lme() function within the nlme package in R (Pinheiro et al., 2007). Post-hoc pairwise comparisons of individual groups were performed with the glht() function within the R 'multcomp' package (Hothorn et al., 2014). All results were considered significant at $\alpha = 0.05$.

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Chapter III

Title: Microbial rRNA:rRNA gene ratios may be unexpectedly low due to extracellular DNA preservation in soils

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Abstract

We tested a method of estimating the activity of detectable individual bacterial and archaeal OTUs within a community by calculating ratios of absolute 16S rRNA to rDNA copy numbers. We investigated phylogenetically coherent patterns of activity among soil prokaryotes in non-growing soil communities. 'Activity ratios' were calculated for bacteria and archaea in soil sampled from a tropical rainforest and temperate agricultural field and incubated for one year at two levels of moisture availability and with and without carbon additions. Prior to calculating activity ratios, we corrected the relative abundances of OTUs to account for multiple copies of the 16S gene per genome. Although necessary to ensure accurate activity ratios, this correction did not change our interpretation of differences in microbial community composition across treatments. Activity ratios in this study were lower than those previously published (0.0003 – 210, logarithmic mean = 0.24), suggesting significant extracellular DNA preservation. After controlling for the influence of individual incubation jars, significant differences in activity ratios between all members of each phylum were observed.

Planctomycetes and *Firmicutes* had the highest activity ratios and *Crenarchaeota* had the lowest activity overall. Our results suggest that greater caution should be taken in interpreting soil microbial community data derived from extracted DNA. Indirect extraction methods may be useful in ensuring that microbes identified from extracellular DNA are not erroneously interpreted as components of an active microbial community.

Highlights

- We calculated 'activity ratios' for all OTUs detected in incubated soil samples.
- We found significantly different overall activity ratios for some phyla.
- Low activity ratios suggested significant extracellular DNA preservation.
- Indirect DNA extraction may help distinguish extracellular DNA from active microbes.

Keywords

Activity, eDNA, iDNA, Oligotrophy, Preservation

1. Introduction

High-throughput sequencing of microbial DNA has become increasingly common in environmental microbial ecology over the last decade (Caporaso et al., 2011). These techniques are increasingly being used to explore the uncultured majority of microbes in soils and sediments, and apply macro-ecological theories to the microbiome (Fierer et al., 2012). For example, some studies have proposed that ecological niches and environmental life-strategies of environmental microorganisms may be coherent at high taxonomic levels (i.e. phyla) (Fierer et al., 2007; Philippot et al., 2010), and broadly divisible into "copiotrophs" and "oligotrophs". Theorized copiotrophs (i.e. *β-Proteobacteria, Firmicutes, and Bacteroidetes*) make up a greater proportion of microbial

communities in soils with higher carbon (C) availability (Cleveland et al., 2007), and their relative abundance increases rapidly when exposed to readily available C sources (Goldfarb et al., 2011). Conversely, theorized oligotrophs (i.e. *Acidobacteria* and *Verrucomicrobia*) are more abundant in low C systems (Fierer et al., 2007), and do not increase in population after the addition of available C (Goldfarb et al., 2011). This copiotroph-oligotroph dichotomy has become an increasingly accepted conceptual framework in microbial ecology, supported by the identification of defining genomic traits of a model copiotroph and oligotroph (Lauro et al., 2009), and the high abundance of genes related to high carbon affinity and desiccation resistance in selected *Acidobacteria* genomes (Ward et al., 2009).

Studies investigating phylogenetic conservation of ecological functions in environmental microbes tend to have two limitations, however. The first applies chiefly to studies investigating trophic strategy. Many experiments showing a lack of increased growth in response to the addition of labile C feature incubation times from days (Goldfarb et al., 2011) up to a month (Fierer et al., 2007). Some dormant bacteria can stochastically awaken and form a rapidly growing population after months or even years of inactivity (Buerger et al., 2012). A lack of response to one specific stimulant (labile C) over a period of less than a year may not be useful as experimental evidence of an oligotrophic survival strategy. The second limitation applies more generally to studies that rely on sequencing and quantifying 16S rRNA genes (rDNA) rather than 16S rRNA itself. Characterizing rDNA does not necessarily reflect the active community, only the potential for activity (Lennon and Jones, 2011; DeAngelis and Firestone, 2012), because it includes living, dormant, and non-viable organisms.

Studies have reported that microbial β-diversity can differ significantly between rDNA- and rRNA-based soil communities (Angel et al., 2013; Baldrian et al., 2012; DeAngelis and Firestone, 2012). This may in part be due to high-quality extracellular DNA (eDNA) preserved in soil and sediment, either sorbed to clay minerals (Ogram et al., 1988) or as a component of biofilms (Bockelmann et al., 2006; Alawi et al., 2014). Additionally the number of 16S gene copies is variable between different organisms' genomes, ranging from one to fifteen copies in bacteria and archaea (Kembel et al., 2012). Not considering multiple rDNA copies could cause overestimation of relative abundance of some OTUs, thus affecting β-diversity results. Although 16S copy number correction has been shown to be useful in studies of nematodes (Darby et al., 2013) the tools needed to correct 16S rDNA for bacteria and archaea are relatively new (Kembel et al., 2012; Langille et al., 2013) and the use of these tools is not widely published in the study of soil microbial communities.

The study reported here uses a culture-free specific activity method (Kemp et al., 1993) to identify active members of microbial communities in incubated soils. We included sequencing and quantification of both rDNA and rRNA to estimate 'activity ratios' for soil bacteria and archaea. Bacterial pure culture studies have shown that, in many (but not all) cases, active cells contain more ribosomes than those that are less active, resulting in a higher ratio of rRNA to rDNA (Blazewicz et al., 2013). Calculating this ratio in a mixed community requires quantification of both 16S rDNA and rRNA, followed by multiplication of absolute quantities by the relative abundance of a group as determined by sequencing. The accuracy of this ratio depends upon correcting the 16S rDNA for number of copies per genome, and on sufficient sequencing depth to reflect

true α- and β-diversities of sequenced communities (Caporaso et al., 2011). In theory, the resulting 'activity ratio' represents the average number of ribosomes present in all cells for a given OTU. This can then be used as a measurement of the general protein production capacity of individual taxa, including those that are difficult to culture or whose specific functions are currently unknown (Blazewicz et al., 2013). Similar methods have been in use for decades, but have been applied either in observational studies (Brettar et al., 2011; Campbell and Kirchman, 2012) or pure cultures (Kemp et al., 1993; Muttray et al., 2001). To the authors' knowledge, no study has yet applied these techniques to entire soil communities in experimental microcosms.

Soil rRNA and rDNA was extracted from soils taken from two geographically distinct areas and incubated at two levels of moisture availability, and with or without added C for one year. We tested three hypotheses: 1) Microbial community composition as determined by rRNA sequencing will be more similar across treatments compared to composition determined from rDNA sequencing. 2) Correcting for 16S gene copy number will make a statistically significant difference in a DNA-based measure of microbial community β-diversity. 3) Putative copiotrophic and oligotrophic groups will be distinguished by the magnitude and variability of their activity ratios in each of six treatment groups: activity ratios of copiotrophic groups will be lower and highly variable between treatments; those of oligotrophic groups will be higher and more constant. As these incubations will result in universally oligotrophic microenvironments after one year, oligotrophic organisms should have higher activity ratios than copiotrophs. Furthermore, copiotrotrophs should respond to the addition of labile carbon, while oligotrophs should not.

2. Materials and Methods

2.1. Soil Descriptions

Soils were sampled from two geographically distinct sites. A forested slope in the El Yunque National Forest in Rio Grande, Puerto Rico (18° 19' 54.57" N, 65° 46' 28.12" W), hereafter referred to as the 'Rio Grande' soil, was sampled in February 2011. Soil was taken from a moderately steep, north-facing convex contour, and could be classified as either the Yunque or Los Guineos series, both of which are very-fine, kaolinitic, isothermic Humic Hapludox (Soil Survey Staff, 2014). The second soil was sampled in late July 2011, from a cornfield near the USDA-ARS Integrated Cropping Research Laboratory in Brookings, South Dakota, USA (44° 20' 27.94" N, 96° 47' 17.31" W), hereafter the 'Brookings' soil. Soil in the area is mapped as a Kranzburg-Brookings complex. The Kranzburg series is classified as fine-silty, mixed, superactive, frigid Calcic Hapludolls, while the Brookings series is classified as fine-silty, mixed, superactive, frigid Pachic Hapludolls (Soil Survey Staff, 2014). The Rio Grande and Brookings soils were sampled to depths of 30 cm and 15 cm, respectively. A thin layer of organic material (O horizon) was scraped away from the surface of the Rio Grande soil prior to sampling. Approximately 48 hours passed between sampling at both sites and storage at the USDA-ARS Beltsville Agricultural research Center in Beltsville, Maryland, USA. Soils were stored at 4 °C until laboratory analysis and incubation.

2.2. *Incubation Conditions*

Soils from each site were bulked and passed through a 4 mm sieve before assignment to one of three incubation treatments for each soil, with three replicates for each treatment. Soils were incubated for one year in glass jars at 25 °C with the following

adjustments: (1) -0.25 MPa water potential with the addition of 0.005 g of dried, powdered *Zea mays* shoot (C/N = 40.1) per gram of dry soil, (2) -0.25 MPa water potential, and (3) -2.5 MPa water potential. These levels were chosen to represent surface soil conditions expected in summer months in 1) a temperate agricultural soil with one season's corn crop plowed under to a depth of 15 cm, 2) a temperate agricultural soil, and 3) a desert soil or an agricultural soil during a drought year. Moisture corrections were made gravimetrically based on soil water potential vs. volumetric water curves. These were constructed using a WP4 Dewpoint Potentiometer (Decagon Devices, Pullman, WA). A small hole was drilled through the top of each lid and covered with filter paper to allow continuous gas exchange with the atmosphere.

Following incubation, 1-g subsamples of each replicate soil treatment were removed from jars and placed into sterile 10-mL plastic tubes containing 1-mL of LifeGuard solution (MoBio Laboratories, Carlsbad, CA). Preserved soils were stored at -20 °C until extraction.

2.3. Soil Chemical and Physical Analyses

Physical and chemical tests were performed on representative samples from each soil. The pH of each soil was measured in a 2:1 suspension of 0.01 M $CaCl_2$ using an ion-selective probe. Textures were measured using the hydrometer method of particle size analysis (Orr and Gee, 2002) (Appendix B, Table S4).

Total C and N were measured on representative samples of pre- and post-incubation soil treatments using dry combustion with a LECO CHN 2000 analyzer (LECO Corporation, Lakeville, MI) (Appendix S2, Supplementary material). As the

Brookings soil appeared to contain carbonates, samples of this soil were subjected to acid fumigation (Harris et al., 2001) prior to measurement.

2.4. Processing ribosomal RNA and DNA

2.4.1. Simultaneous extraction of rRNA and rDNA

At the beginning of the experiment DNA was extracted from each replicate incubation jar using a MoBio Powersoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA). DNA was stored at -20 °C for one year. Following the one-year incubation DNA and rRNA were extracted together from samples preserved in LifeGuard using a MoBio RNA Powersoil Total RNA Isolation Kit with the additional DNA elution step, according to manufacturer's protocol (MoBio Laboratories, Carlsbad, CA). DNA and rRNA extracts were stored at -20 °C and -80 °C respectively until further use. Extracted rRNA was transcribed to cDNA using an Invitrogen SuperScript® III First-Strand Synthesis Kit according to manufacturer's protocol (Life Technologies, Grand Island, NY).

2.4.2. Quantification of rRNA and rDNA

Bacterial and archaeal 16S rDNA and 16S rRNA (via cDNA) were measured with qPCR of the V4 region of the 16S ribosomal subunit using primers F515 (5' – GTG CCA GCM GCC GCG GTA A – 3') and R806 (5' – GGA CTA CVS GGG TAT CTA AT – 3') (Caporaso et al., 2011). These were the same primers used in sequencing. KiCqStart SYBR green qPCR ReadyMix with ROX (Sigma-Aldritch, St. Louis, MO) was used with a StepOnePlus Real-Time PCR System (Life Technologies, Grand Island, NY). Amplification was carried out with a 5 minute denaturation step at 95 °C followed by 40

cycles of 5-second denaturation at 95 °C, 15-second annealing at 54 °C, and 10-second extension at 72 °C.

2.4.3. Sequencing and assigning taxonomy to PCR-amplified rRNA and rDNA

Sequencing was conducted using 454 pyrosequencing on post-incubation bacterial and archaeal communities. DNA and cDNA samples were amplified with barcoded 16S 515F and 806R primers, using 10-bp barcodes for identification (Caporaso et al., 2010). Amplified, barcoded samples were pooled and sent to Duke University for 454 Titanium pyrosequencing (454 Life Sciences, Branford, CT). Sequences of amplified genes were processed and classified using the QIIME bioinformatics package (Caporaso et al., 2010). In total, 744,565 sequences were generated in pyrosequencing, of which 371,392 were removed in quality filtering. The remaining 373,173 sequences were split amongst 36 individual samples – 18 each of RNA and DNA. The minimum sequence count was 3,711, and maximum was 19,487, with a mean of 10,366 quality-filtered sequences. Barcodes and reverse primers were then removed, and OTUs were picked at 97% similarity using the pick_closed_reference_otus.py command, with default settings (UCLUST). OTU taxonomy was assigned by comparing sequences to the greengenes database (greengenes.lbl.gov).

2.5. 16S rRNA gene copy number correction

We corrected for variable 16S rRNA gene copy number amongst incubated bacteria and archaea using the normalize_by_copy_number.py function within the bioinformatics program PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, ver. 1.0.0 (Langille et al., 2013). This divides the abundance of every OTU in a user-supplied OTU table by the known (or PICRUSt-

extrapolated) copy number abundance for all OTUs in the latest greengenes database (May 2013 release).

2.6. Activity ratio calculation

Following copy number correction, activity ratios were calculated for every OTU identified in both rRNA and rDNA-based communities in each sample. First, we multiplied the relativized abundance of each OTU within the overall community by the rRNA or rDNA abundance for each sample. This gave an absolute number of 16S rRNA (ribosomes) and corrected 16S rDNA (total cells) for every OTU in every sample. The ratio of these two numbers therefore gives an estimate of the average number of ribosomes per cell for each OTU detected by sequencing.

2.7. Statistics

Differences in rDNA and rRNA-based bacterial and archaeal community structures within different soils and incubation treatments were evaluated by Nonmetric Multidimensional Scaling (NMS) using PC-ORD ver. 6.0 (McCune and Mefford, 2011), NMS ordinations were plotted on two axes, with 250 iterations to the final ordination. Significant differences in community structures between treatment groups were determined using Multi-Response Permutation Procedure (MRPP), also within PC-ORD. Statistical significance of incubation effects on rDNA and rRNA copy numbers was evaluated using linear mixed effect models with the "nlme" package in R (Pinheiro et al., 2007). Significance of the influence of phylum membership on 16S gene copy number correction factors of individual OTUs, and on activity ratios of individual OTUs, were tested with the aov() function in R (R Core Team, 2014). Post-hoc pairwise comparisons

of correction factors and activity ratios were performed with the HSD.test() function within the R 'agricolae' package (de Mendiburu and de Mendiburu, 2014).

3. Results

3.1. Incubation effects on rRNA and rDNA-based communities

The dominant factor determining composition in both rDNA- and rRNA-based communities was soil type (Figure 5). We calculated separate ordinations for each of the two soils, as their communities were too divergent to observe effects of incubation treatments when plotted together. Low moisture and C additions resulted in community composition differences in the Rio Grande soil, but only low moisture potential significantly affected composition in the Brookings soil ($\alpha = 0.05$). Ribosomal RNA-based communities were distinct from the rDNA-based communities in all cases, and individual replicates were always more variable in rRNA-based communities. Communities based on rRNA in each of the two soils did not converge during the one-year incubation.

3.2. 16S gene copy number correction effects

Correcting rDNA-based community composition for 16S gene copy number had an observable but insignificant effect ($\alpha = 0.05$) on overall community composition, as measured by NMS and MRPP (Figure 5A and 5B). The average correction factor for members of phyla present in all samples ranged from 2.4 (*Planctomycetes*) to 3.6 (*Tenericutes*), with no statistically significant differences between phyla as estimated by Tukey's HSD pairwise comparisons from a one-way ANOVA ($\alpha = 0.05$).

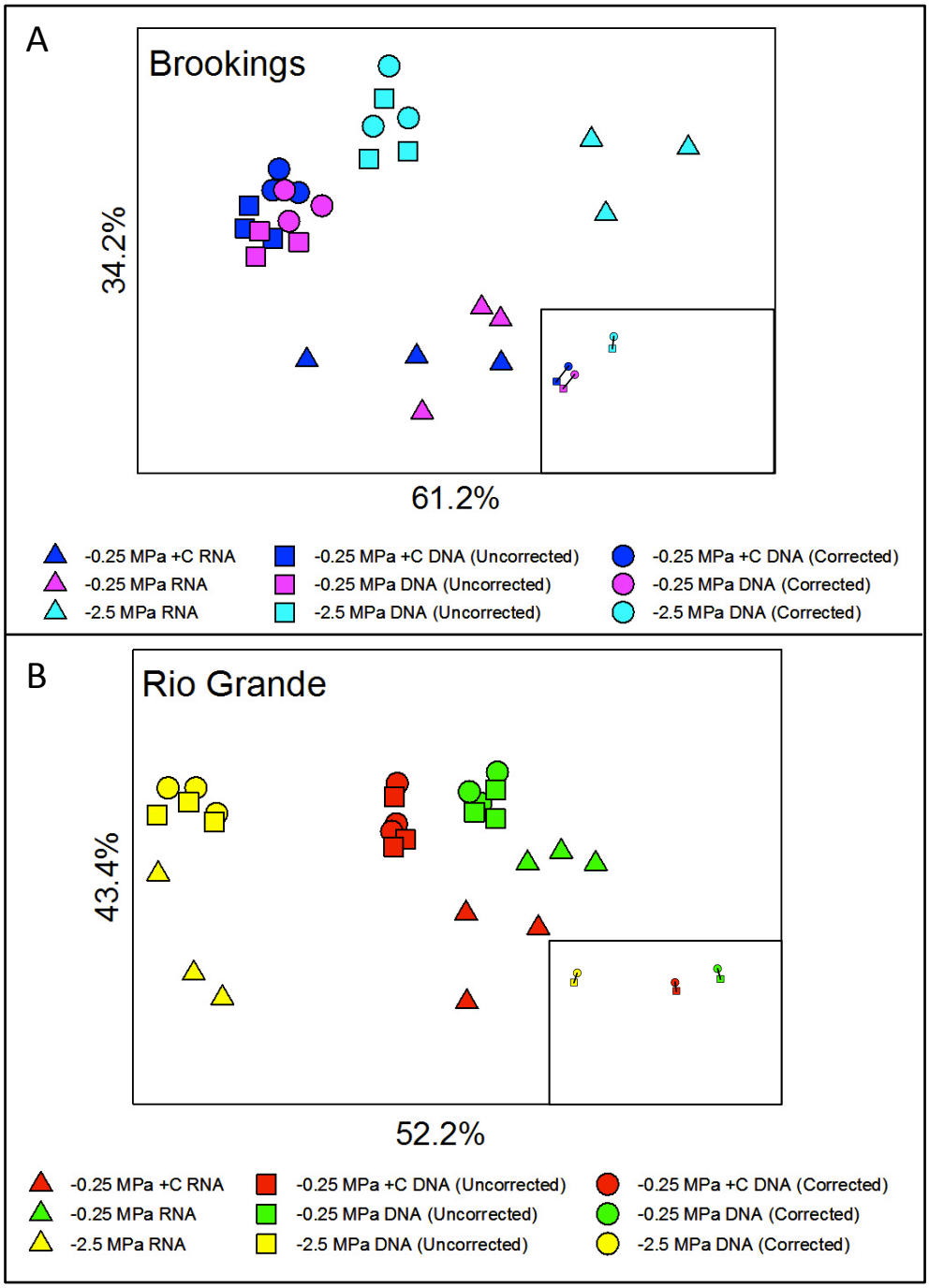

Figure 5. Nonmetric Multidimensional Scaling (NMS) ordinations show 16S rRNA gene (rDNA)-based bacterial and archaeal communities in A) Brookings and B) Rio Grande soil samples. Relative abundances of OTUs in 'Corrected' rDNA communities have been corrected for 16S rDNA copy numbers per genome using PICRUSt (Langille et al., 2013), while 'Uncorrected' communities are unaltered. Inset panels show the effect of copy number correction on rDNA-based communities, with black lines connecting the centroids of each set of replicates for each treatment (N=3).

3.3. Composition of rRNA and 16S gene copy number corrected rDNA communities

Community differences between soils were driven predominantly by the relative abundances of members of archaeal phyla *Euryarchaeota* and *Crenarchaeota,* and bacterial groups *Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes*, and *γ*- and *α*-*Proteobacteria* (Figure 6A and 6B). *Bacteroidetes* was universally more abundant in the Brookings soil (14.0% vs. 3.5%), while *α-Proteobacteria* was more prevalent in the Rio Grande soil (14.0% vs. 5.2%) (Figure 6A and 6B). Differences between rRNA and rDNA-based communities in each soil were subtle, with average relative abundances of rRNA:rDNA ranging from 0.5:1 (*γ-Proteobacteria* in Rio Grande, *Euryarchaeota* in both soils) to 1.7:1 (*Acidobacteria* in Rio Grande) (Figure 6A and 6B).

3.4. Quantification of 16S rRNA and rDNA

Efficiency of qPCR reactions ranged from 96-99%, as measured against a dilution series of a plasmid standard. All results were normalized to copy number per gram of soil, and log-transformed before analysis to satisfy the assumption of normality. There were significantly ($p = 0.0001$) more 16S rDNA copies in incubated soils (mean 2.40*10^11 copies/g soil) than pre-incubated soils (mean 9.52*10^10 copies/g soil), and significantly ($p < 0.0001$) less rRNA (mean 8.59*10^10 copies/g soil) than rDNA copies in incubated soils (Figure 7). We did not observe significant differences between treatment groups ($\alpha = 0.05$, data not shown), however (Figure 7).

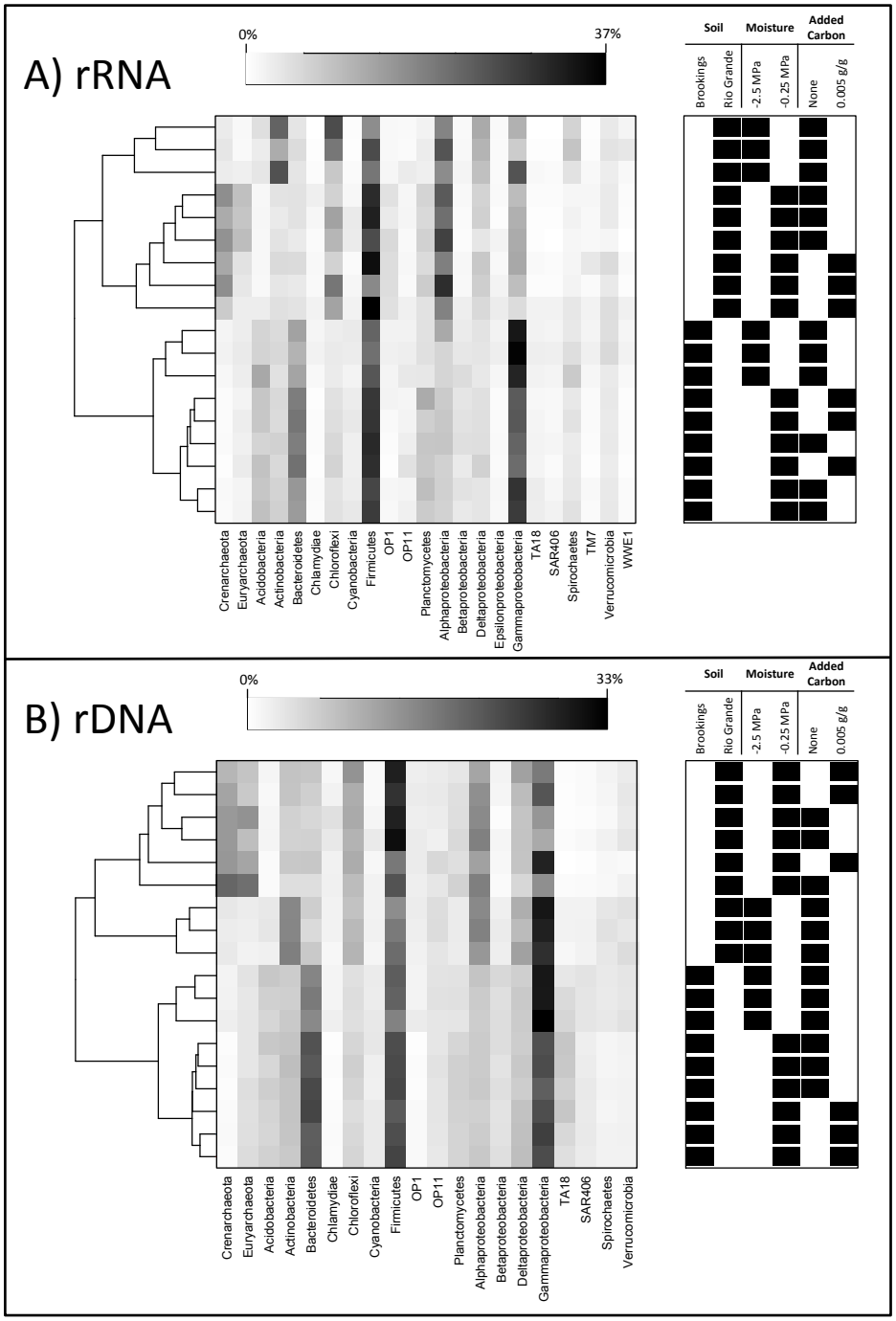

Figure 6. Heatmap shows the relative abundances of phyla (and *Proteobacteria* classes) for A) 16S rRNA and B) rRNA gene (rDNA)-based communities in all incubated soil samples. The color of bars represents 0% (white) to A) 37% or B) 33% (black) abundance. Binary annotation blocks to the right of heatmaps show the properties of each sample when read horizontally – black boxes indicate the soil type, moisture treatment, and carbon treatment for each sample. Dendrograms left of heatmaps show groupings of samples based on Bray-Curtis distance matrices across all phyla. Only phyla that comprised at least 1% abundance in at least one sample are shown.

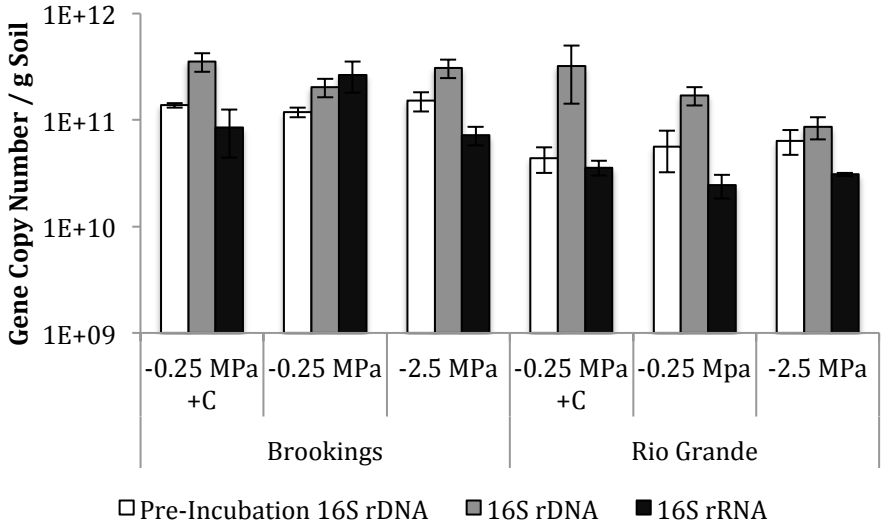

Figure 7. Copy numbers of 16S rRNA gene (rDNA) and rRNA in soil samples, as quantified by qPCR. Error bars show standard error of the mean for three replicates per treatment.

3.5. Analysis of activity ratios between treatments and phyla

The total number of copies of 16S rDNA and rRNA in each sample was multiplied by the relative community abundances for each OTU in that sample to calculate activity ratios. Activity ratios were lower than 1:1 for the majority of OTUs, though were highly variable both within and among treatments and phyla. There were no significant treatment effects on activity ratios, nor significant patterns of activity among phyla at the treatment level, due to the extreme variability of activity ratios in individual incubation replicates (Appendix B, Figure S2).

When the influence of individual treatment jars was removed and ratios were calculated for individual OTUs (97% similarity), however, bacterial and archaeal phyla significantly ($\alpha = 0.05$) differed in mean activity ratios (Figure 8). Pairwise comparisons were calculated with Tukey's HSD test from a one-way ANOVA testing the effect of phylum membership on log-transformed activity ratios, with incubation jars as a nested factor. Members of *Planctomycetes* had the highest average activity ratio (mean = 0.320), and members of *Crenarchaeota* had the lowest (mean = 0.096). *Acidobacteria* and all component classes of *Proteobacteria* fell between these two extremes. Despite these differences, activity ratios of individual OTUs within each phylum were variable over up to five orders of magnitude, and outliers on either side of the distribution were common.

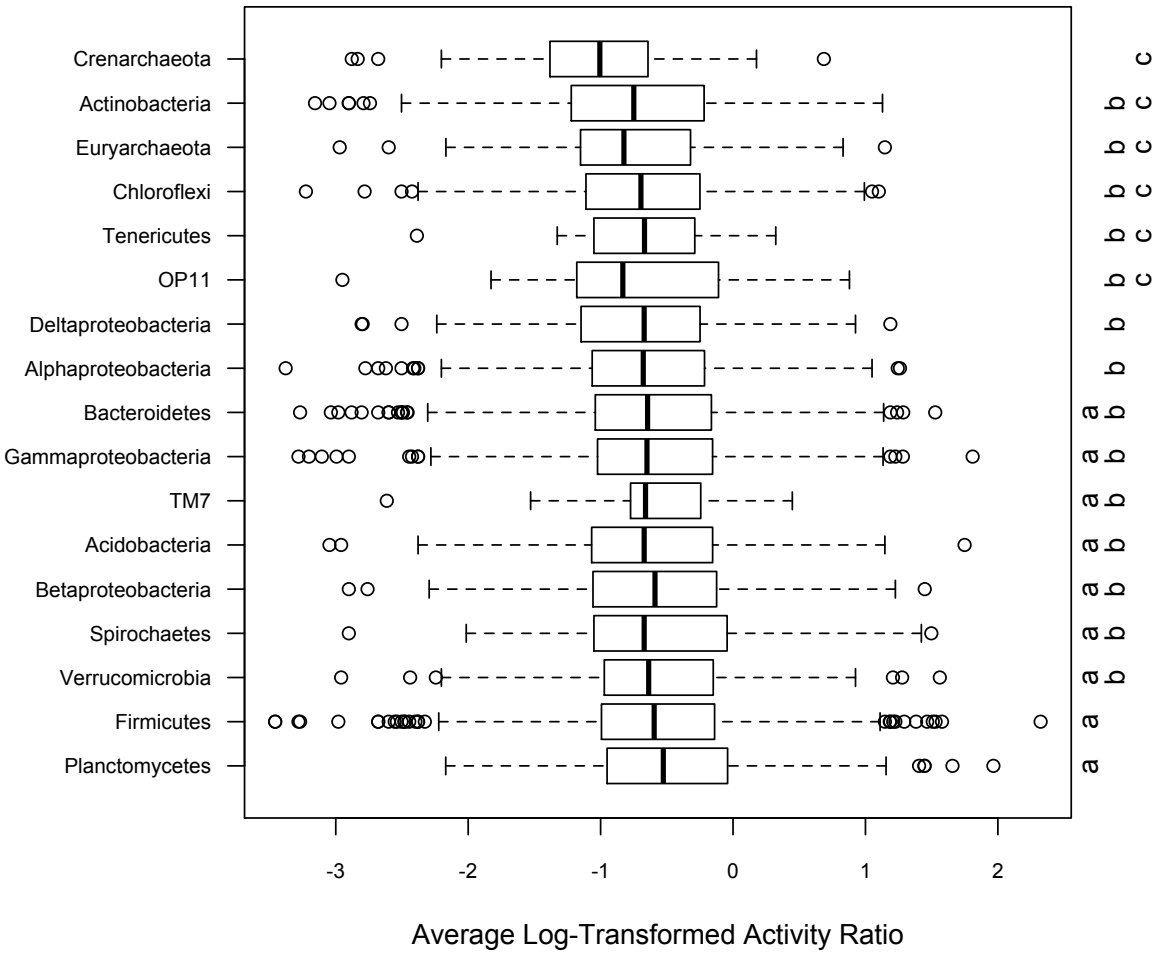

Figure 8. Boxplot shows average rRNA:rRNA gene ratios, corrected for absolute numbers of RNA and DNA quantified using qPCR ('activity ratios') of all putative bacterial and archaeal OTUs present in at least one replicate of each treatment group, divided by phylum membership. Letters show significance groupings of pairwise comparisons using Tukey's HSD within a one-way ANOVA (α=0.05). Log-transformed activity ratios of individual OTUs were nested within sample jars to control for the strong effects of individual treatment replicates. Phylum *Proteobacteria* was split into component classes *α-, β-, γ-*, and *δ-Proteobacteria*.

4. Discussion

Correction for 16S gene copy number is a built-in function of the PICRUSt functional gene prediction package (Langille et al., 2013), which has been used to investigate the functional diversity of bacterial communities in humans (David et al., 2013), salamanders (Loudon et al., 2013), and river water (Staley et al., 2014). Kembel et al. (2012) showed that 16S gene copy correction of individual OTUs can affect measures of abundance for several taxa (*Cyanobacteria Group II*, *Alteromonadales*) within marine bacterial communities, as well as the structure of hierarchical clustering of communities sampled from the human microbiome. However, we did not find that this correction made a significant difference in measures of overall community composition in incubated terrestrial soils (Figure 5). Although this correction has a large influence on selected individual groups (e.g. *Thermodesulfobacteriales* vs. *Sphingobacteriales*, two orders in our samples with the most extreme mean correction factors of 4.7 and 1.8, respectively), in general the influence of a few OTUs with many copies of the 16S gene was drowned out by the vast majority of closely related OTUs with 1-2 copies. It is feasible that 16S copy number correction might significantly impact measurements of community structure in communities with less diversity than those studied here (we putatively identified 4,069 OTUs among all samples). While correction had little effect on rDNA community composition, applying the correction was necessary to ensure accurate rRNA:rDNA ratios. Failure to make this correction would have led to activity ratios lower by a factor of 2.5 (averaged across all OTUs).

Calculated activity ratios were much lower than we expected. Published values for calculated specific activities of bacteria range from 3 (uncultured *Acidobacteria,*

Foesel et al., 2014) to 2,006 (cultured *E. coli,* Bremer and Dennis, 1996). In contrast, in this study activity ratios were lower than 1 for 82% of OTUs, and lower than 0.1 for 28% of OTUs, with a logarithmic average of 0.24. As activity ratios describe the average number of ribosomes per cell, this suggests that at least 76% of rDNA sequences assigned to OTUs represented extracellular DNA (eDNA) within biofilms or bound to organic or inorganic components of the soil matrix (Alawi et al., 2014). As living cells may contain many ribosomes, the actual proportion of eDNA in our samples may have been higher than 76%.

These data raise questions about the validity of DNA-based approaches to answer some questions in soil microbial ecology; however, our interpretation depends on several experimental assumptions. First, we must assume 100% recovery of both rRNA and rDNA from the soil, or at a minimum assume that the percent recovery is similar between the two molecules. Like many labs, we routinely use MoBio extraction kits that have been shown to be effective at DNA extraction (Whitehouse and Hottel, 2007), but do not typically spike samples to assess recovery. Similarly, prior to qPCR we assumed 100% reverse transcription of rRNA. A select group of rRNA extracts were subjected to a second reverse-transcription and quantification using the same kit, and produced comparable values (data not shown). Presumably any bias in extraction or reverse transcription is consistent between samples, allowing for relative comparisons between treatments.

Our data suggest a large amount of extracellular DNA (mean = 1.51*10^11 copies/g soil) was preserved for up to a year, with fidelity high enough to be sequenced and assigned taxonomy according to the greengenes database (greengenes.lbl.gov). Our

long, relatively dry incubation conditions may have allowed a degree of DNA preservation at the upper end of what could be expected in natural environments. However, long-term DNA preservation in soil has been extensively reviewed in terms of sorption and complexation (Nielsen et al., 2006; Pietramellara et al., 2009), the potential for microbial transformation (Levy-Booth et al., 2007; Nielsen et al., 2007), and exploitation in archeology (Pääbo et al., 2004). When added to non-sterile soil, pure DNA can be used by bacteria for transformation for a period of hours to days (Nielsen et al., 2007), after which point it becomes unavailable due to a combination of sorption to mineral surfaces, complexation with organic compounds, and enzymatic degradation. Soils with mildly acidic to neutral pH and high clay mineral content, particularly of montmorillonite, have no practical upper limit to DNA sorption potential (Ogram et al., 1988). Once sorbed to clay surfaces, DNA is at least 100-400 times less susceptible to enzymatic degradation than free DNA (Cai et al., 2006), due partly to the high sorption affinity clay particles also have for DNAses (Khanna and Stotzky, 1992). Amplifiable 20-500 bp fragments of DNA can persist in soils for several months to several years (Nielsen et al., 2006), up to eleven millennia (Epp et al., 2012). As extracellular microbial DNA is likely to be released in close proximity to clay particles (Miltner et al., 2012) or within macro- or microaggregates (Blaud et al., 2012), it may be even more protected than laboratory studies using additions of pure DNA or bacterial inoculum would otherwise suggest (Schimel and Schaeffer, 2012). Although an extensive literature exists to support the stabilization of DNA in soil and sediment (Alawi et al., 2014; Ceccherini et al., 2009; Corinaldesi et al., 2008), this has largely been ignored in DNA based microbial ecology studies.

Consistent with previous studies (Angel et al., 2013; DeAngelis et al., 2013; DeAngelis and Firestone, 2012), we observed significant differences between 16S rRNA- and rDNA-based measures of β-diversity in all treatment groups (Figure 5A and 5B). Counter to our hypothesis that the composition of 'active' rRNA-based communities in samples of different soil types incubated in the same environmental conditions for one year would converge, the rRNA communities remained distinct between soil types. We cannot explain the relative contributions of factors responsible for these differences in our study, but wide-ranging studies of bacterial and archaeal community have generally found that pH, C and N availability, soil texture, and management history correlate with changes in β-diversity at the phylum level (Fierer et al., 2012); it is likely that a combination of these factors are responsible for overall community differences here.

Due to the high variability of activity ratios in individual incubation replicates, we were not able to discern trophic strategies between OTUs based on activity ratios between treatments (Appendix B, Figure S2). We did however observe significant differences in activity ratios amongst phyla after accounting for variation due to individual treatment replicates (Figure 8). Although activity ratios were low and variable across all phyla, there were coherent patterns in activity ratios across broad taxonomic groups. This may support previous assertions that ecological function is broadly conserved at the phylum level (Philippot et al., 2010; Lennon et al., 2012). We were unable to test for this conservation at lower taxonomic levels (with the exception of the large *Proteobacteria* classes), as these were not represented in all samples, likely due to insufficient sampling depth. Rarefaction curves of OTU counts for each of our incubated

samples show that we did not approach total sequence coverage for any of the studied communities (Appendix B, Figure S3).

The phyla with the highest mean activity ratios were *Firmicutes* and *Planctomycetes* (Figure 8). Both have been previously found to have high relative abundance in soils subjected to long-term desiccation (Barnard et al., 2013). As many members of *Firmicutes* form endospores in response to harsh environmental conditions (i.e. *Clostridia*, which comprised 13% average community abundance and 70% of total *Firmicutes* abundance per sample) their ranking here may be a result of their superior ability to form resistant resting states (Setlow, 2007). Much less is known about *Planctomycetes* (Fuerst and Sagulenko, 2011). Cultured *Planctomycetes* have double-layered membranes, analogous to eukaryotic organelles, and (in some cases) non-peptidoglycanous cell walls (Lage et al., 2013). Their distinctive cell wall adaptations may give them an increased ability to adapt to prolonged, harsh conditions. On the other hand, *Crenarchaeota* had the lowest mean activity ratio, nearly half that of the next lowest phylum (*Actinobacteria*, mean activity ratio = 0.18). In our samples, most *Crenarchaeota* (93%) were members of the *Miscellaneous Crenarchaeotal Group* (MCG). MCG was only recently described through 16S gene sequencing, and is widespread in many oligotrophic marine sediments (Gagen et al., 2013; Kubo et al., 2012). The activity ratios calculated here demonstrate the need for greater distinction between the concepts of oligotrophy as 'resistant to change', vs. 'adapted to oligotrophic environments' (Barnard et al., 2013; Lennon et al., 2012). Though these are distinct survival strategies, they are often conflated, with 'model' oligotrophs possessing both (Lauro et al., 2009).

Calculating and interpreting activity ratios should be done with caution. Many studies use rRNA:rDNA ratios as a proxy for metabolic activity at the time of extraction, but this may not be valid in all circumstances (Blazewicz et al., 2013). While higher activity ratios indicate higher rates of cellular division in some organisms (Bremer and Dennis, 1996), they indicate dormancy in others (Sukenik et al., 2011). Here, we show that in addition to the critiques made in Blazewicz et al. (2013), quantification of the rDNA and rRNA copy numbers is vital to get an accurate picture of both the size and composition of rRNA and rDNA-based communities. More studies of cultured representatives are needed to understand the functional relevance of activity ratios in individual taxa.

4.1. Conclusions

The activity ratios of individual OTUs were significantly different between some phyla in our soil incubations, but our results suggest that extracted soil DNA may not only represent active and dormant members of the microbial community. Rather our data provide evidence for the presence of extracellular DNA in soil. In order to validate the low activity ratios reported here, further incubations tailored to test the degree to which microbial DNA can be preserved in terrestrial soils under natural environmental conditions should be conducted. Long incubations with multiple time points could be used to determine the rate at which environmental microbes become inviable, and of eDNA accumulation. To improve the accuracy of ecological interpretations of molecular data, indirect extraction techniques should also be considered. Separation of intact microbial cells from the soil matrix prior to RNA and DNA extraction would ensure that communities are represented only by active or dormant organisms. These methods have

been used successfully for more than a decade in studies of soil metagenomics (Gabor et al., 2003) and marine sediment communities (Alawi et al., 2014), but have been superseded by bead-beating extraction kits in the larger field of environmental microbial ecology. While these kits conveniently and consistently return larger yields (Whitehouse and Hottel, 2007), they also may extract DNA of unknown age and provenance.

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Chapter IV - Conclusions

Research reported here addressed two main objectives: 1) study the effects of agricultural management on soil microbial communities, with potential implications for rates of carbon (C) sequestration under different management systems, and 2) use molecular methods to investigate survival strategies (and potential strategies of C use) of individual, mostly uncultured archaeal and bacterial OTUs. Chapter 2 reports that management style significantly affects microbial communities, though community composition is much more influenced by depth than agricultural management. Chapter 3 shows that attempts to use molecular methods to describe microbial trophic strategies might be complicated by the often-ignored DNA preservation potential of soil. Together, these studies demonstrate that the sampling techniques and experimental tools of modern microbial ecology may lead to over- or misinterpretation of results, and that a multi-pronged, holistic approach is needed to make further advances in the field.

Chapter 2 demonstrated the importance of consideration for spatial scale and soil morphology in sampling. The results validate similar findings that bacterial and archaeal (Eilers et al., 2012) and fungal (Bahram et al., 2015) communities significantly differ in both α- and β-diversity by depth, a fact that has been widely recognized in a variety of ecosystems including forest (Goberna et al., 2005), grassland (Fierer et al., 2003), and tundra (Kim et al., 2014) soils. Unfortunately most studies still tend to ignore depth (Michel and Williams, 2011), even when their reported aim is to discover basic environmental factors influencing microbial community assembly (Lauber et al., 2009). We found that splitting depth fractions by soil horizon, rather than arbitrary depth increments, produced an abrupt distinction in edaphic factors and 16S-based community

composition across a depth gradient of approximately 5-10 cm. While there is no way to determine whether we would have arrived at identical conclusions by sampling 10-20 cm and 20-30 cm depth fractions instead of above and below the Ap horizon in individual cores, our sampling method certainly improved our accuracy in correlating microbial communities to the conditions of their immediate environments (Grüneberg et al., 2010; Michel and Williams, 2011) .

Despite the high sampling resolution of this study, the heterogeneity of potential microbial niches in terrestrial soil is greater than any field study could reasonably account for (Vos et al., 2013). This is best demonstrated by the anaerobic conditions at the center of soil aggregates as small as 4 mm (Sexstone et al., 1985), reflected in this study by the small but detectable proportion of methanogens at every depth sampled. Additionally, unless soil is saturated, capillary forces from water films immobilize unicellular microbes, pinning them to soil particle surfaces (Or et al., 2007; Dechesne et al., 2010). This can produce microscale niche homogeneity in which non-filamentous microbes may be unable to access resources beyond their immediate surroundings. An accurate measurement of microbial communities and the specific terrestrial soil environments they inhabit requires microscale techniques (Nunan et al., 2007; Vos et al., 2013) that are generally incompatible with field-level agricultural experiments. In any study involving the homogenization of many grams of soil across multiple centimeters, measured bacterial and archaeal communities may represent a diversity of niches and functions analogous to that of an entire watershed in terms of terrestrial macroecology.

Whereas the study in Chapter 2 was designed to quantify these broad-scale relationships, Chapter 3 was designed to draw conclusions of the trophic strategies of

individual OTUs using molecular techniques. Instead, this study highlighted the limitations of purely molecular approaches to studying basic ecological functions of uncultured microbes. The results of this study suggested the potential for undisturbed, relatively dry soils to preserve large amounts of amplifiable microbial DNA, showing that the assumption that 16S amplicons necessarily represent living microbes may not hold in some circumstances. Evidence for long-term preservation of DNA in soil is widespread (Nielsen et al., 2007; Pietramellara et al., 2009), though its relative importance in different environments is unknown. Despite the potential impact of this effect on interpretations of microbial q-PCR and sequencing data, it is rarely acknowledged or controlled for in large-scale studies of microbial ecology. The implications of DNA preservation may expand and complicate the power of molecular techniques to measure communities, depending on the circumstances. On the one hand, DNA preservation may allow researchers to study historic communities, potentially spanning many thousands of years in dry, cold climates (Pääbo et al., 2004; Mackelprang et al., 2011). Preserved DNA also may reduce our ability to make definite conclusions that a community represented by 16S gene sequences has any relationship to current, measurable environmental conditions (Peay, 2014).

Both of these experiments demonstrate limitations of the ways high-throughput sequencing is currently used, and of the conclusions that can be drawn from these data (Blazewicz et al., 2013; Vos et al., 2013; Peay, 2014). The ability to cheaply and quickly identify the relative proportions of millions of OTUs in hundreds of samples has invited widespread speculation on their ecological niches and potential environmental functions (Fierer et al., 2012). There has been a drive to sequence communities from increasingly

novel environments in stated attempts to better understand the factors controlling microbial distribution, or suggest their possible functions, while maintaining novelty (Roesch et al., 2007; Fierer et al., 2012; Pessi et al., 2014). Studies tend to be unable to make conclusions beyond correlation and speculation, as few members of several dominant microbial groups (e.g. *Acidobacteria, Chloroflexi, Planctomycetes*) have been sequenced (Pham and Kim, 2012). Lack of sequenced representatives may also force researchers to describe patterns of community abundance at arbitrary phylogenetic levels (i.e. phylum) that may be inappropriate to differentiate coherent functions (Martiny et al., 2012). While the exploration of ecological theory across spatial scales should be a priority of microbial ecologists, without significant knowledge of the spatiotemporal environment experienced by microbes and a database of cultured representatives (Peay, 2014), our ability to infer ecological functions from molecular data alone will remain tentative.

A different experimental focus is needed to advance the field of soil microbial ecology. A method for improving our ability to draw less ambiguous conclusions from experiments is a greater focus on soil depth, especially when used to connect microbial community composition to other soil features including horizon boundaries and mineralogy. Microbial community characterization should also include both rRNA and rRNA genes so as to differentiate potentially active components of microbial communities from extracellular DNA. Although it is outside the scope of this thesis, other improvements to the current standards of experimental design could include the use of stable isotopes or BrdU to distinguish active microorganisms from the large majority of dormant cells. Additionally, though it has become less widely published in

environmental microbiology, microscopy and X-ray tomography should be considered as viable methods of studying the spatial environments inhabited by soil microbes.

Microbial ecologists have summarized the state of their field following the widespread adoption of high-throughput sequencing technology as a burst of interest in the basic question, "who's there?" This was followed by phases of "why are they there?" and "what are they doing?", analogous to the second and third chapters of this thesis. However, many researchers skipped over the issue of what "there" is, and what "there" means in the context of individual organisms and communities. A better understanding of the physical and chemical habitats of soil microbes will aid in unraveling their diversity and interactions, with other microbes and their environment. While large-scale sequencing efforts will continue to provide huge amounts of ecological data, paired studies at a microbial scale are needed to interpret the results in meaningful ways.

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Appendix A.

Table S1. Soil physical characteristics – bulk density and texture of soils under each agricultural management regime at each of five depth increments (N=4, $\alpha = 0.05$). Letters show significance groupings within each treatment for each factor.

Treatment	Depth	Bulk Density (g/cm^3)	Sand %	Silt %	Clay %
Chisel Till	0-5 cm	1.30 ± 0.04 c	24.5 ± 2.4 a	58.4 ± 1.8 a	17.1 ± 1.7 b
	5-10 cm	1.37 ± 0.02 bc	23.2 ± 2.3 ab	59.0 ± 1.3 a	17.8 ± 1.8 b
	10 cm-Ap	1.48 ± 0.05 ab	21.3 ± 2.3 b	59.7 ± 2.0 a	19.0 ± 1.3 b
	Ap-30 cm	1.54 ± 0.04 a	16.0 ± 2.6 c	54.2 ± 1.4 b	29.8 ± 2.2 a
	30-60 cm	1.56 ± 0.03 a	15.9 ± 4.1 c	50.6 ± 0.5 c	33.5 ± 4.2 a
No Till	0-5 cm	1.25 ± 0.04 c	23.4 ± 1.7 a	60.2 ± 1.1 a	16.4 ± 1.1 b
	5-10 cm	1.35 ± 0.04 b	23.2 ± 2.2 a	59.3 ± 1.5 a	17.5 ± 1.4 b
	10 cm-Ap	1.42 ± 0.02 b	21.8 ± 1.7 a	59.3 ± 1.3 a	18.9 ± 1.3 b
	Ap-30 cm	1.55 ± 0.04 a	14.2 ± 2.2 b	54.6 ± 1.5 b	31.2 ± 2.6 a
	30-60 cm	1.56 ± 0.01 a	12.6 ± 3.4 b	50.6 ± 2.2 b	36.8 ± 5.0 a
Organic	0-5 cm	1.40 ± 0.06 bc	22.1 ± 1.4 a	59.1 ± 1.0 a	18.8 ± 1.5 b
	5-10 cm	1.35 ± 0.02 c	22.2 ± 1.2 a	59.1 ± 1.1 a	18.7 ± 1.5 b
	10 cm-Ap	1.50 ± 0.04 ab	21.7 ± 1.3 a	58.1 ± 1.5 a	20.2 ± 1.6 b
	Ap-30 cm	1.60 ± 0.01 a	17.9 ± 2.0 b	51.5 ± 0.7 b	30.6 ± 1.6 a
	30-60 cm	1.61 ± 0.01 a	14.5 ± 2.7 b	50.4 ± 2.2 b	35.1 ± 4.2 a

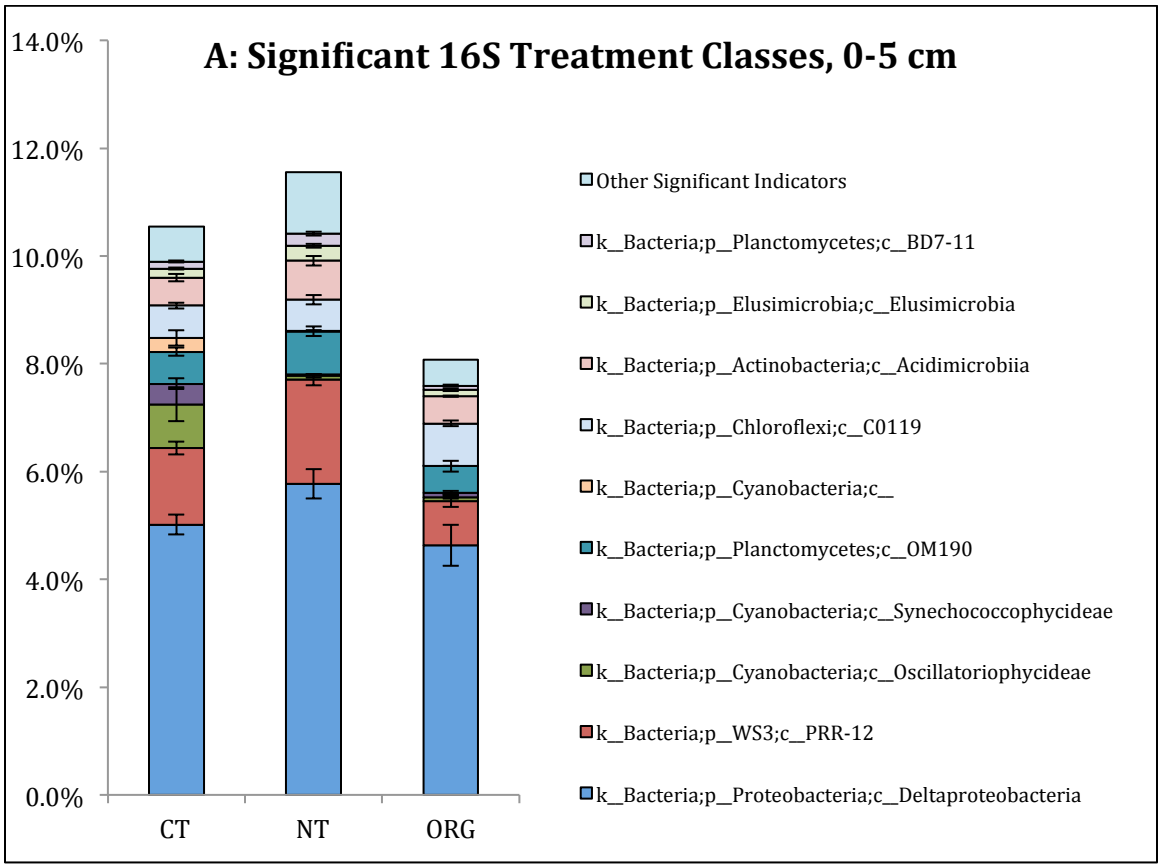
Table S2. Soil chemical characteristics – pH, C and N of soils under each agricultural management regime at each of five depth increments (N=4, $\alpha = 0.05$). Letters show significance groupings within each treatment for each factor.

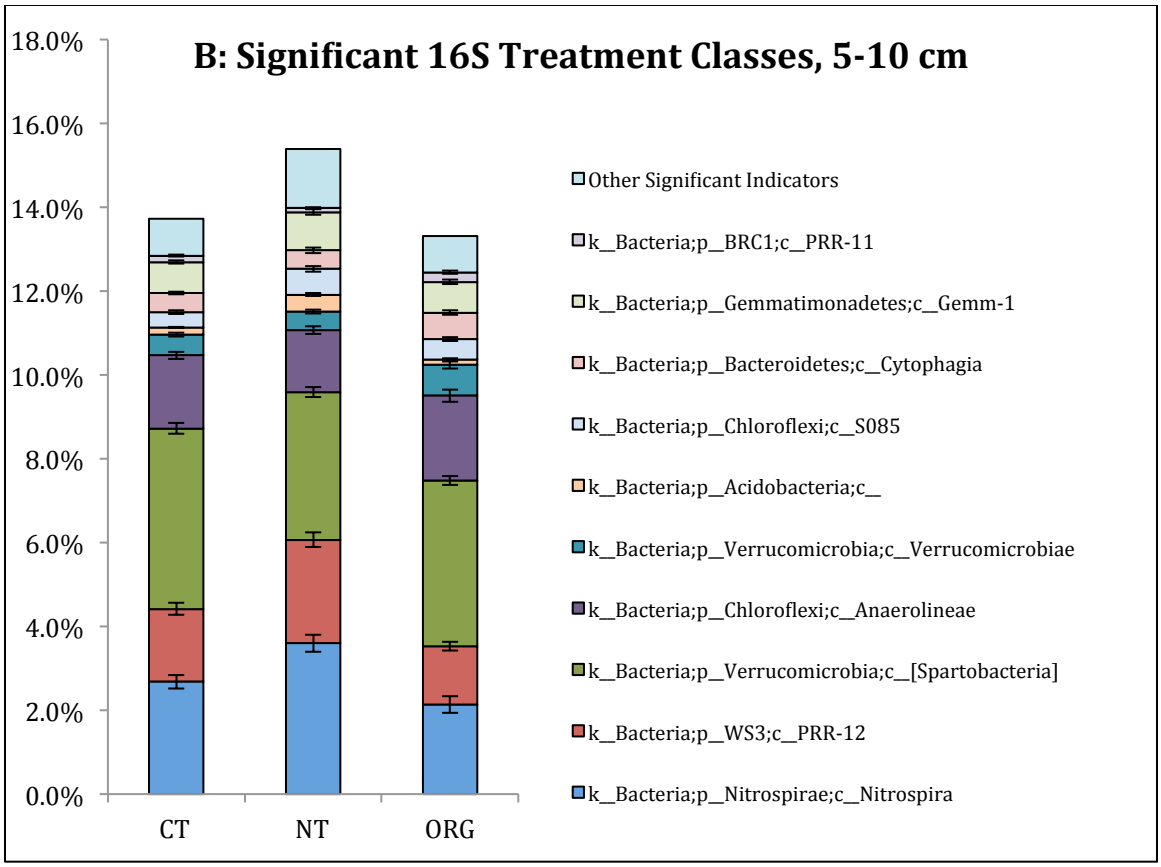
Treatment	Depth	pH	NO_3^--N (ug/g soil)	NH_4^+-N (ug/g soil)	Total N %	Total C %	C:N Ratio
Chisel Till	0-5 cm	6.37 ± 0.11 bc	7.57 ± 1.04 a	2.01 ± 0.64 a	0.13 ± 0.009 a	1.36 ± 0.07 a	10.6 ± 0.2 b
	5-10 cm	6.47 ± 0.04 ab	4.77 ± 0.47 b	1.64 ± 0.79 a	0.13 ± 0.013 a	1.42 ± 0.16 a	11.1 ± 0.2 a
	10 cm-Ap	6.81 ± 0.11 ab	3.37 ± 0.37 bc	1.07 ± 0.12 a	0.08 ± 0.010 b	0.88 ± 0.09 b	10.7 ± 0.2 a
	Ap-30 cm	7.00 ± 0.14 a	1.72 ± 0.24 c	1.04 ± 0.16 a	0.04 ± 0.004 c	0.30 ± 0.03 c	8.2 ± 0.3 b
	30-60 cm	5.79 ± 0.33 c	1.57 ± 0.32 c	1.19 ± 0.34 a	0.03 ± 0.004 c	0.18 ± 0.03 c	6.5 ± 0.1 c
No Till	0-5 cm	6.28 ± 0.12 c	4.15 ± 0.46 a	2.12 ± 0.46 a	0.16 ± 0.007 a	1.70 ± 0.06 a	10.8 ± 0.1 ab
	5-10 cm	6.39 ± 0.16 bc	3.20 ± 0.44 b	1.05 ± 0.21 b	0.12 ± 0.010 b	1.23 ± 0.10 b	10.4 ± 0.2 ab
	10 cm-Ap	6.81 ± 0.09 ab	3.03 ± 0.53 b	1.18 ± 0.09 b	0.08 ± 0.006 c	0.95 ± 0.14 c	11.2 ± 0.8 a
	Ap-30 cm	6.86 ± 0.07 a	2.33 ± 0.38 bc	1.16 ± 0.23 b	0.07 ± 0.005 d	0.34 ± 0.04 d	9.4 ± 0.7 b
	30-60 cm	5.77 ± 0.18 d	1.74 ± 0.36 c	1.32 ± 0.27 ab	0.03 ± 0.003 d	0.22 ± 0.01 d	7.3 ± 0.5 c
Organic	0-5 cm	6.55 ± 0.14 b	7.46 ± 1.76 a	1.65 ± 0.69 ab	0.15 ± 0.007 a	1.66 ± 0.04 a	11.2 ± 0.7 a
	5-10 cm	6.94 ± 0.10 ab	4.92 ± 1.29 b	1.35 ± 0.24 ab	0.15 ± 0.008 a	1.53 ± 0.07 a	10.4 ± 0.3 ab
	10 cm-Ap	7.04 ± 0.13 a	3.22 ± 1.10 bc	2.58 ± 0.73 a	0.11 ± 0.007 b	1.17 ± 0.07 b	10.6 ± 0.2 a
	Ap-30 cm	7.06 ± 0.18 a	1.80 ± 0.82 c	1.14 ± 0.14 ab	0.03 ± 0.002 c	0.29 ± 0.02 c	9.0 ± 0.9 b
	30-60 cm	5.75 ± 0.18 c	2.04 ± 0.46 c	0.80 ± 0.29 b	0.03 ± 0.002 c	0.17 ± 0.02 c	6.4 ± 0.3 c

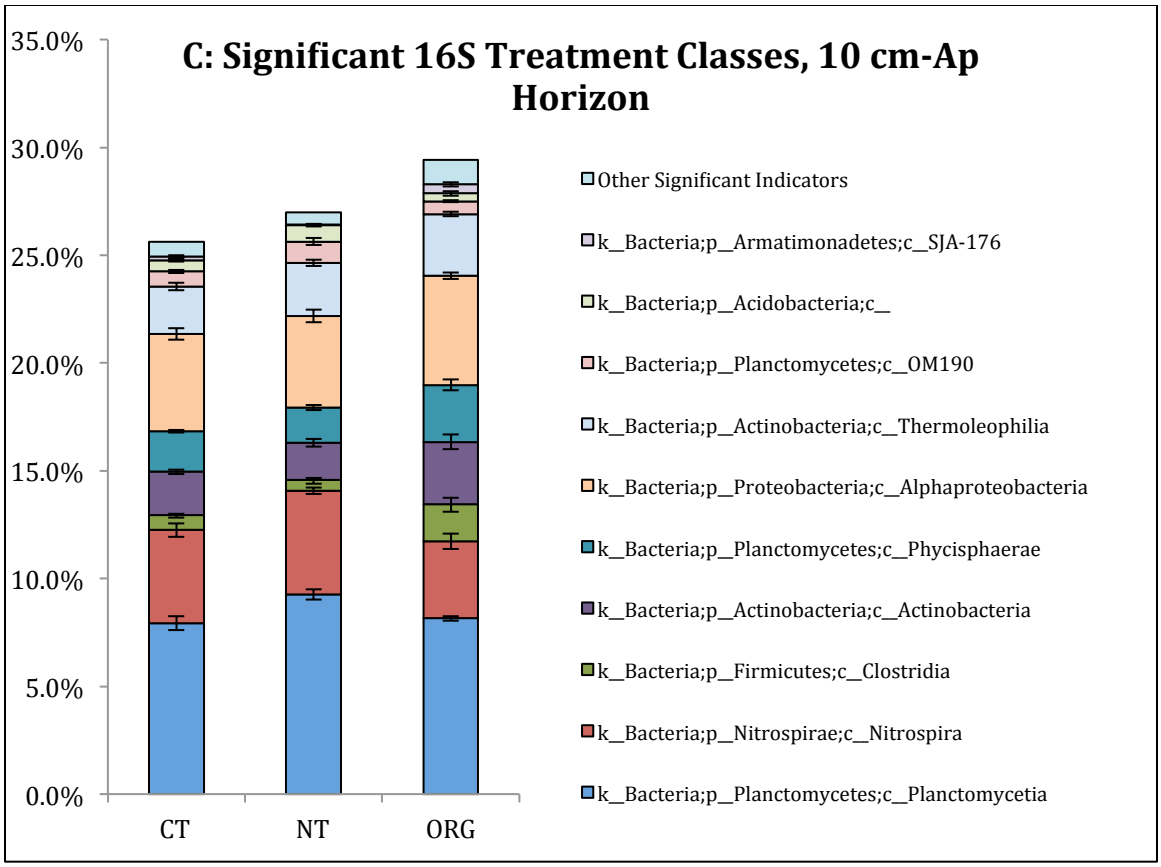
Table S3. Soil biochemical characteristics – enzyme activities of soils under each agricultural management regime at each of five depth increments (N=4, $\alpha = 0.05$). Letters show significance groupings within each treatment for each factor.

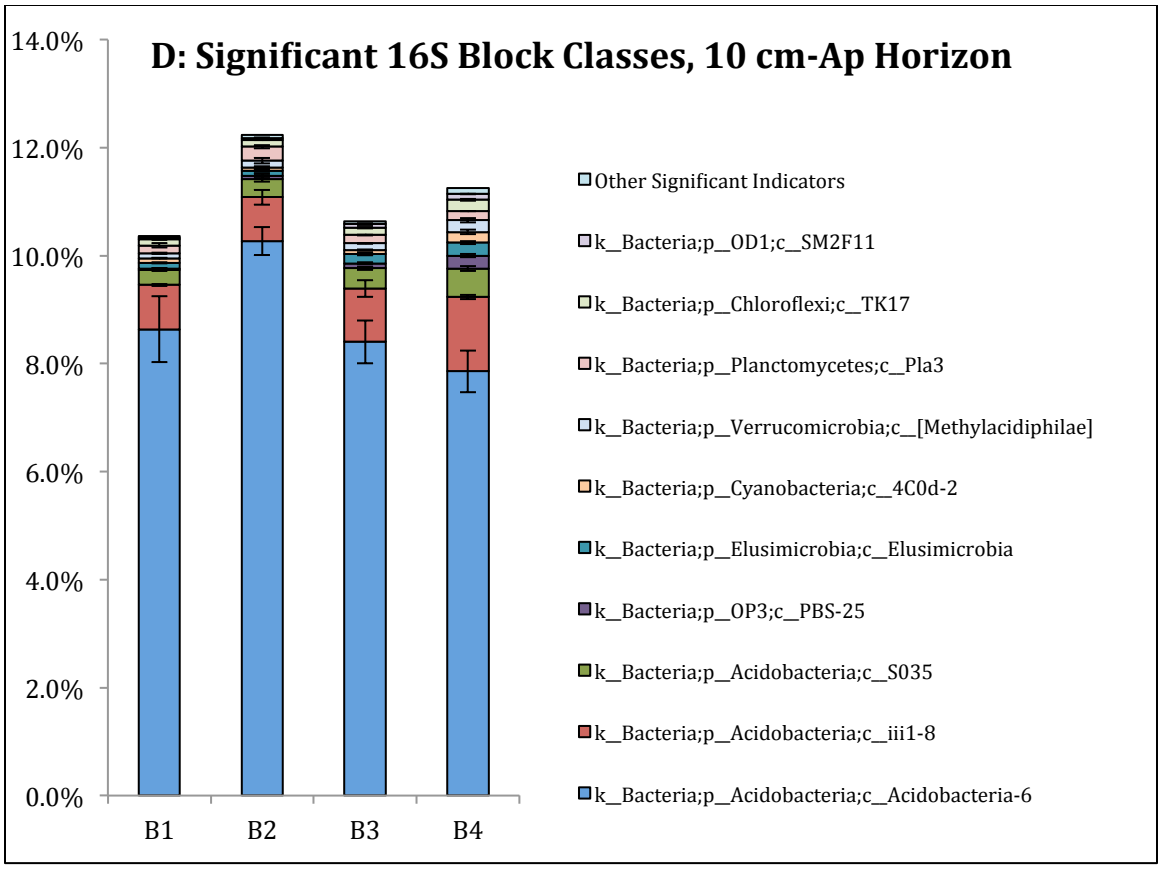
Treatment	Depth	Glucosidase (mg pnit/kg soil/hr)	Glucosaminidase (mg pnit/kg soil/hr)
Chisel Till	0-5 cm	272.7 ± 30.3 a	132.4 ± 5.8 a
	5-10 cm	278.8 ± 13.6 a	125.7 ± 5.8 a
	10 cm-Ap	121.8 ± 12.6 b	159.4 ± 35.0 a
	Ap-30 cm	42.5 ± 4.0 c	63.6 ± 4.8 b
	30-60 cm	38.1 ± 3.9 c	50.8 ± 11.2 b
No Till	0-5 cm	356.4 ± 37.0 b	158.7 ± 5.5 a
	5-10 cm	253.4 ± 35.0 c	132.4 ± 14.4 a
	10 cm-Ap	103.2 ± 11.9 d	88.3 ± 14.7 b
	Ap-30 cm	59.0 ± 6.3 ad	65.0 ± 2.2 b
	30-60 cm	56.2 ± 19.8 a	61.0 ± 5.9 b
Organic	0-5 cm	404.5 ± 43.5 a	185.7 ± 12.0 a
	5-10 cm	287.9 ± 53.3 b	213.9 ± 33.0 a
	10 cm-Ap	211.7 ± 34.0 b	100.1 ± 22.3 b
	Ap-30 cm	38.9 ± 4.2 c	66.3 ± 4.3 b
	30-60 cm	40.0 ± 4.5 c	71.7 ± 11.4 b

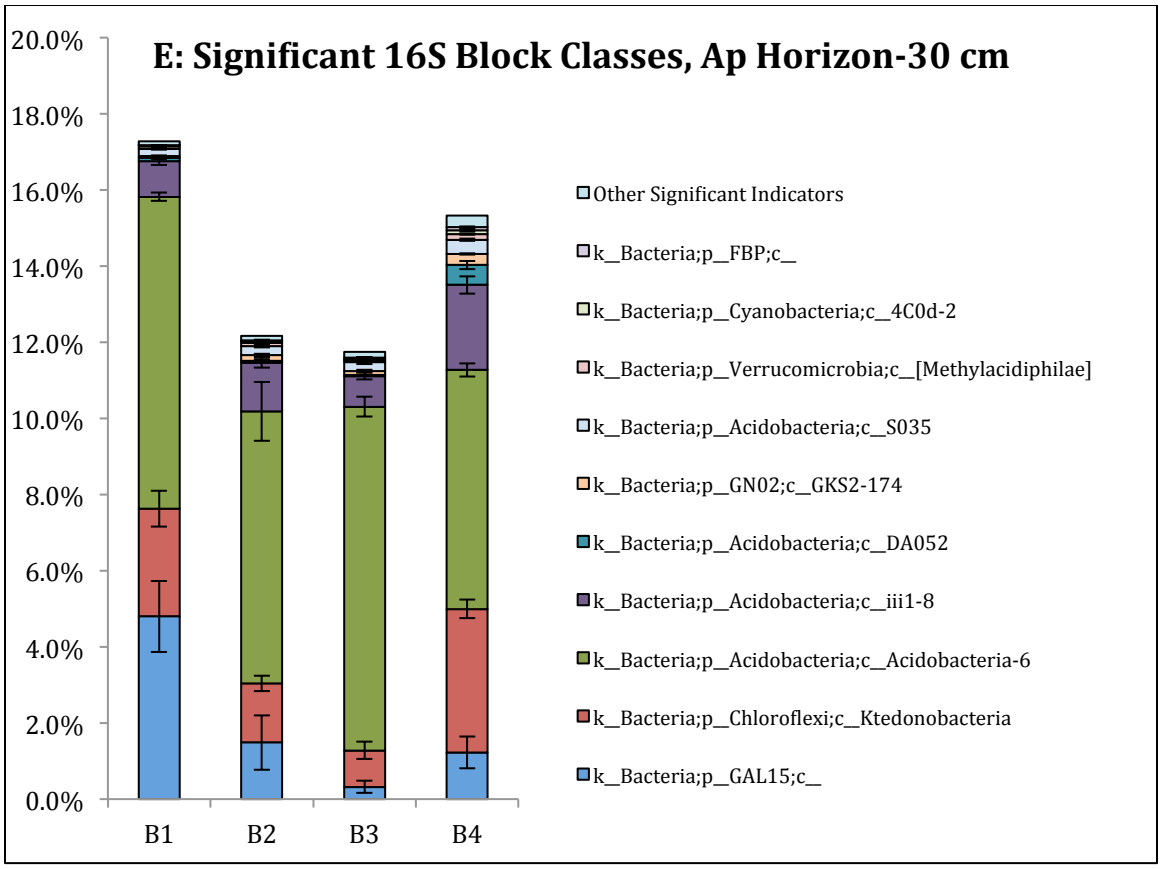
Figure S1. Stacked bar graphs show average abundances of bacterial and archaeal classes with the top 10 greatest absolute ranges in average abundance between agricultural management regimes (**A, B, C,** N = 3) or experimental blocks (**D, E, F,** N = 4) within each of five depth fractions. Only significant indicator species (α = 0.05, PC-ORD, (McCune and Mefford, 2011) for either treatment (**A, B, C**) or block (**D, E, F**) were considered. Error bars show standard error of the mean.

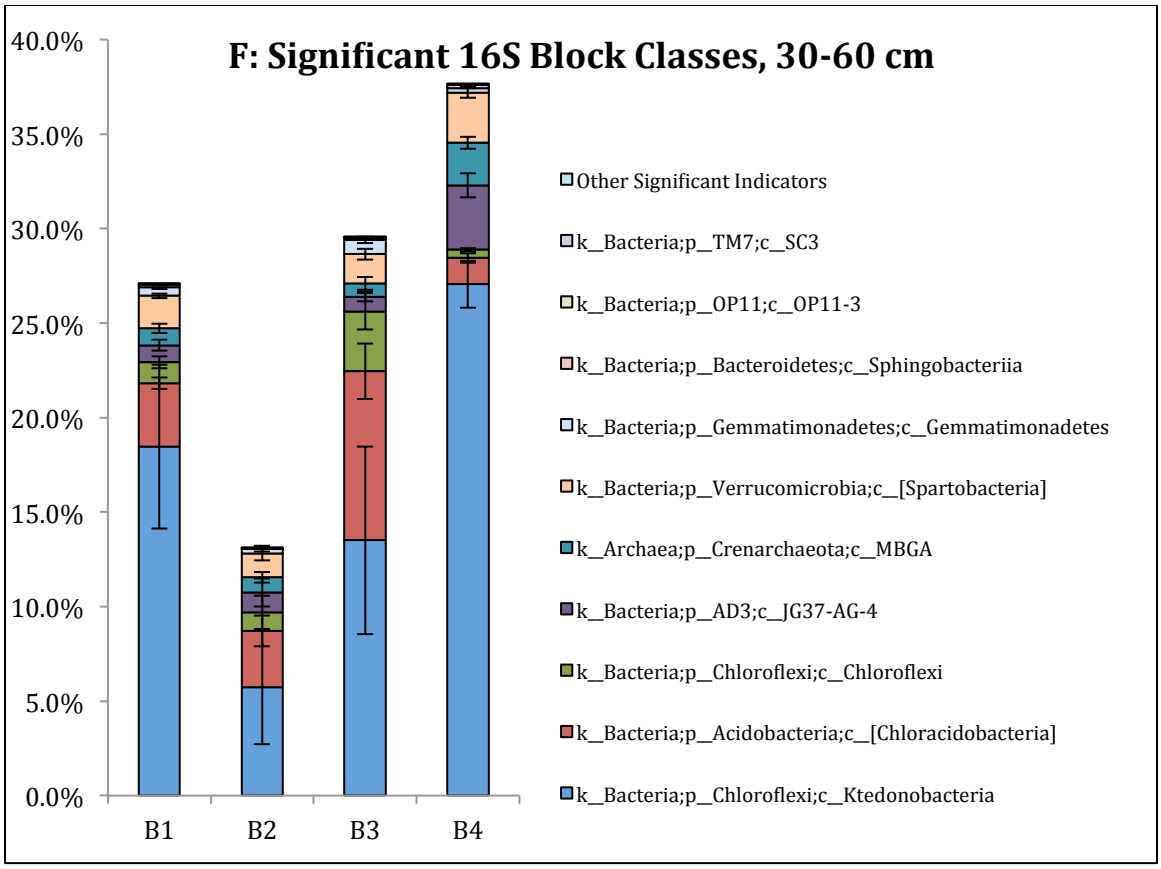



B: Significant 16S Treatment Classes, 5-10 cm
18.0%
16.0%
14.0%
12.0%
10.0%
8.0%
6.0%
4.0%
2.0%
0.0%
CT
NT
ORG
Other Significant Indicators
k__Bacteria;p__BRC1;c__PRR-11
k__Bacteria;p__Gemmatimonadetes;c__Gemm-1
k__Bacteria;p__Bacteroidetes;c__Cytophagia
k__Bacteria;p__Chloroflexi;c__S085
k__Bacteria;p__Acidobacteria;c__
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae
k__Bacteria;p__Chloroflexi;c__Anaerolineae
k__Bacteria;p__Verrucomicrobia;c__[Spartobacteria]
k__Bacteria;p__WS3;c__PRR-12
k__Bacteria;p__Nitrospirae;c__Nitrospira


C: Significant 16S Treatment Classes, 10 cm-Ap Horizon
35.0%
30.0%
25.0%
20.0%
15.0%
10.0%
5.0%
0.0%
CT
NT
ORG
Other Significant Indicators
k__Bacteria;p__Armatimonadetes;c__SJA-176
k__Bacteria;p__Acidobacteria;c__
k__Bacteria;p__Planctomycetes;c__OM190
k__Bacteria;p__Actinobacteria;c__Thermoleophilia
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria
k__Bacteria;p__Planctomycetes;c__Phycisphaerae
k__Bacteria;p__Actinobacteria;c__Actinobacteria
k__Bacteria;p__Firmicutes;c__Clostridia
k__Bacteria;p__Nitrospirae;c__Nitrospira
k__Bacteria;p__Planctomycetes;c__Planctomycetia


D: Significant 16S Block Classes, 10 cm-Ap Horizon
14.0%
12.0%
10.0%
8.0%
6.0%
4.0%
2.0%
0.0%
B1
B2
B3
B4
Other Significant Indicators
k__Bacteria;p__OD1;c__SM2F11
k__Bacteria;p__Chloroflexi;c__TK17
k__Bacteria;p__Planctomycetes;c__Pla3
k__Bacteria;p__Verrucomicrobia;c__[Methylacidiphilae]
k__Bacteria;p__Cyanobacteria;c__4C0d-2
k__Bacteria;p__Elusimicrobia;c__Elusimicrobia
k__Bacteria;p__OP3;c__PBS-25
k__Bacteria;p__Acidobacteria;c__S035
k__Bacteria;p__Acidobacteria;c__iii1-8
k__Bacteria;p__Acidobacteria;c__Acidobacteria-6


E: Significant 16S Block Classes, Ap Horizon-30 cm
20.0%
18.0%
16.0%
14.0%
12.0%
10.0%
8.0%
6.0%
4.0%
2.0%
0.0%
B1
B2
B3
B4
Other Significant Indicators
k__Bacteria;p__FBP;c__
k__Bacteria;p__Cyanobacteria;c__4C0d-2
k__Bacteria;p__Verrucomicrobia;c__[Methylacidiphilae]
k__Bacteria;p__Acidobacteria;c__S035
k__Bacteria;p__GN02;c__GKS2-174
k__Bacteria;p__Acidobacteria;c__DA052
k__Bacteria;p__Acidobacteria;c__iii1-8
k__Bacteria;p__Acidobacteria;c__Acidobacteria-6
k__Bacteria;p__Chloroflexi;c__Ktedonobacteria
k__Bacteria;p__GAL15;c__


F: Significant 16S Block Classes, 30-60 cm
40.0%
35.0%
30.0%
25.0%
20.0%
15.0%
10.0%
5.0%
0.0%
B1
B2
B3
B4
Other Significant Indicators
k__Bacteria;p__TM7;c__SC3
k__Bacteria;p__OP11;c__OP11-3
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia
k__Bacteria;p__Gemmatimonadetes;c__Gemmatimonadetes
k__Bacteria;p__Verrucomicrobia;c__[Spartobacteria]
k__Archaea;p__Crenarchaeota;c__MBGA
k__Bacteria;p__AD3;c__JG37-AG-4
k__Bacteria;p__Chloroflexi;c__Chloroflexi
k__Bacteria;p__Acidobacteria;c__[Chloracidobacteria]
k__Bacteria;p__Chloroflexi;c__Ktedonobacteria

Appendix B.

Table S4. Texture and pH of homogenized soils before assignment to incubation chambers. Texture measured using the pipette method; pH measured in a 2:1 suspension of 0.01 M $CaCl_2$.

Soil	**Texture**			**pH**
	Sand (%)	Silt (%)	Clay (%)	
Brookings	38	27	35	7.20
Rio Grande	46	32	22	3.99

Table S5. Total C and N content of pre- and post-incubated soils. Labels show the incubation conditions and soil type for each sample analyzed. Only the -2.5 MPa treatments were analyzed by individual replicate; others were subsampled prior to assignment to incubation chambers.

Sample	**Pre-Incubation**			**Post-Incubation**		
	Total %C	Total %N	C/N Ratio	Total %C	Total %N	C/N Ratio
Brookings						
-0.25 MPa +C	2.65	0.23	11.54	2.26	0.25	9.2
-0.25 MPa	2.74	0.25	10.81	2.43	0.25	9.74
-2.5 MPa 1	2.71	0.27	10.18	2.67	0.26	10.28
-2.5 MPa 2	2.74	0.25	11.17	2.36	0.25	9.44
-2.5 MPa 3	2.47	0.25	9.98	2.47	0.23	10.6
Rio Grande						
-0.25 MPa +C	5.75	0.47	12.16	5.49	0.48	11.41
-0.25 MPa	5.64	0.49	11.45	5.42	0.48	11.38
-2.5 MPa 1	5.30	0.46	11.45	5.46	0.47	11.53
-2.5 MPa 2	5.20	0.46	11.33	5.3	0.46	11.53
-2.5 MPa 3	5.38	0.46	11.78	5.6	0.49	11.44

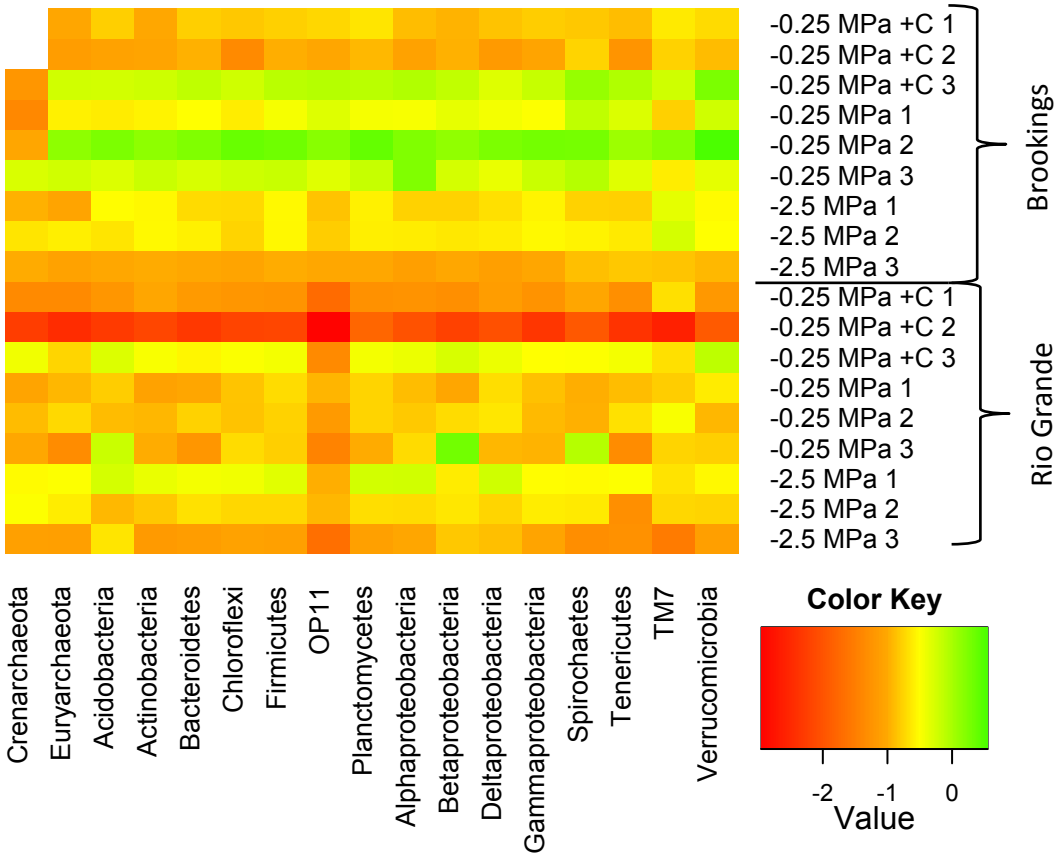


Figure S2. Heatmap shows log-transformed activity ratios of every phylum present in at least one replicate of each treatment group. Red shows untransformed values of less than 0.1, yellow shows values from 0.1 to 1.0, and green shows values greater than 1.0. Despite slight differences in patterns of activity between phyla, the primary factor affecting activity ratio is the specific jar in which the community was incubated, and groupings do not seem to conform to coherent patterns in either incubation conditions or phylogenetic relatedness. Members of phylum Crenarchaeota were not present in Brookings treatments -0.25 MPa +C replicates 1 or 2.

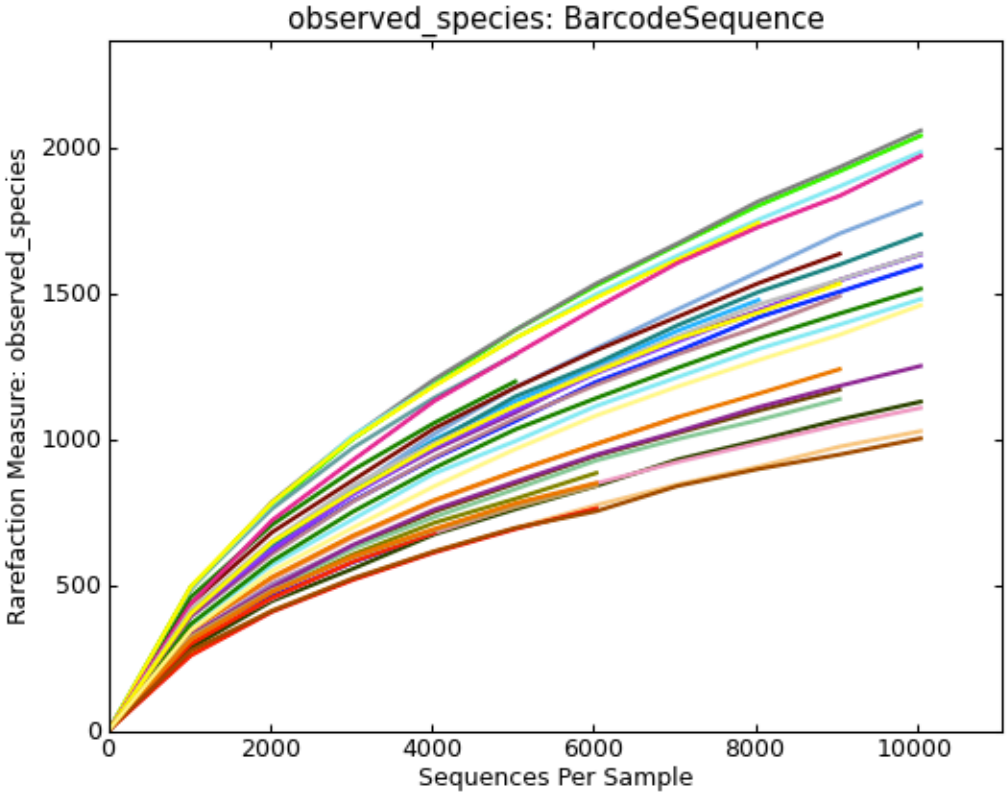


Figure S3. Alpha rarefaction plot of observed species obtained from each of 36 extractions (rRNA genes and reverse-transcribed cDNA from rRNA for each of 18 samples), using the alpha_rarefaction.py workflow script within the QIIME pipeline.

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