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Soil depth and crop determinants of bacterial communities under ten biofuel cropping systems

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ABSTRACT

Biofuel-cropping systems, projected for large land areas, can potentially change their soil microbiome and the ecosystem services they catalyze. We determined the bacterial community composition and relevant soil properties for samples collected after 6 crop years at 0–10 cm, 10–25 cm, 25–50 cm, and 50–100 cm under corn, switchgrass, *Miscanthus*, and restored prairie, as well as 0–10 cm under six additional candidate biofuel crops in replicate side-by side plots. Deep sequencing of the 16S rRNA-V4 region established that soil bacterial communities were significantly differentiated by depth as determined by proportional OTU abundance and composition, UniFrac distance, and taxonomic and indicator analyses. The cropping system significantly impacted bacterial community composition within the top three layers, with corn and switchgrass communities the most different within the 0–25 cm and 25–50 cm depths, respectively. The effects of crop type and depth co-mingled, likely attributed to differences in rooting depth and biomass among crops. Individual phyla demonstrated varying patterns with depth, with significant proportional decreases of *Proteobacteria*, *Actinobacteria*, *Planctomycetes*, and *Bacteroidetes* but proportional increases of *Firmicutes* from shallow to deep soils. The *Acidobacteria*, *Verrucomicrobia*, and *Chloroflexi* peaked in abundance in the middle layers, whereas *Thaumarchaeota* decreased in abundance. Importantly, some classes within the *Acidobacteria*, *Verrucomicrobia*, and *Firmicutes* followed contrasting patterns with depth suggesting that they have different ecological specializations. Poplar, followed by soils with perennial crops contained the most C in the surface soils, with data indicating that these differences will become more pronounced with time.

1. Introduction

Due to spatially and temporally heterogeneous chemical and structural properties, soil is the most complex and diverse microbial environment on earth, with microorganisms as its most abundant biological component (Daniel, 2005; Gans et al., 2005; Rodriguez and Konstantinidis, 2014). Soil bacteria play a critical role in biogeochemical cycling and are keystones to overall ecosystem function. It is well documented that soil bacterial community composition is influenced by soil properties, geographic distance, plant species, land use/management, and environment type (Daniel, 2005; Acosta-Martinez et al., 2008; Ushio et al., 2010; Caporaso et al., 2011; Rodrigues et al., 2013). However, most soil microbial ecology studies have focused solely on the surface horizons and were previously limited by low-resolution genetic profiling methodologies (Muyzer et al., 1993; Fierer and Jackson, 2006; Hartmann and Widmer, 2006; Wakelin et al., 2008). As

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such, there is currently little knowledge concerning soil bacterial community composition and diversity in deeper soil layers, where a significant proportion of microbial biomass resides (Fierer et al., 2003).

Biofuels are a sustainable alternative to fossil fuels and have the potential to address emerging energy demands while providing other ecosystem services, such as carbon sequestration, enhanced biodiversity, and reduced greenhouse gas emissions (Lemus and Lal, 2005; Hill et al., 2006; Robertson et al., 2008; Gelfand et al., 2013; Werling et al., 2014; Oates et al., 2015). Biofuel crops are projected to cover large areas of lesser-studied landscapes unsuited for food crop production (Gelfand et al., 2013), with ensuing changes in the soil microbiome. Meanwhile, soil microbes can promote biofuel feedstock yields directly or indirectly by fixing atmospheric nitrogen, increasing phosphorus acquisition, recycling nutrients, improving soil aggregation, and suppressing plant disease (Swift et al., 2004; Tiedje and Donohue, 2008). Near-surface and root zone soil microbial community composition is influenced by plant type due to the annual/perennial nature of the plants, root depth, differing root exudates, plant residue decomposability, and active recruitment of specific microbial taxa. In contrast, sub-surface soil microbial communities are likely more related to long-term C sequestration because subsoil organic matter is characterized by high mean residence times and enriched in microbially-derived carbon compounds (Rumpel and Kogel-Knabner, 2011; Liang et al., 2012a). Thus characterizing the impacts of biofuel cropping systems on both surface and sub-surface microbial communities is important in understanding long-term system sustainability and environmental impacts.

Our objectives were to: i) determine shifts in the microbial community structure and diversity due to crop and depth, ii) determine taxonomic patterns, especially with depth since that presumably reflects the physiologies of the favored taxa, iii) determine indicator species for crop and depth and the drivers that correlate with them, and iv) where possible, infer potential function of the selected taxa. To do this, we determined soil microbial community composition after 6 crop years by Illumina MiSeq sequencing of 16S-V4 rRNA genes at four soil depths that covered the rooting zone and beyond of four primary candidate biofuel crops: corn, switchgrass, *Miscanthus*, and restored prairie as well as the surface soils from three other corn-based cropping systems and three other perennial biofuel crops.

2. Methods

2.1. Sample collection

Samples were collected post-harvest in November 2013 from ten biofuel cropping systems (G1 - G10) of the Great Lakes Bioenergy Research Center (GLBRC) Biofuels Cropping System Experiment (BCSE) at Kellogg Biological Station in southwest MI, USA. First established in 2008, the BCSE experimental design utilizes a randomized complete block design with 5 replicate blocks (30 × 40 m). The site had the same or very similar cropping history prior to establishing the current plot design in 2008. The soil is predominantly Kalamazoo loam (Fine-Loamy, Mixed, Semiactive, Mesic Type Hapludalf), a sandy loam with 47–56% sand. These cropping systems are continuous corn (G1), continuous corn + cover crop (G2), soybean in a soybean-corn rotation + cover crop (G3), corn in a corn-soybean rotation + cover crop (G4), switchgrass (*Panicum virgatum*, G5), *Miscanthus* (*Miscanthus* x *giganteus*, G6), native grass mix (G7), hybrid poplar (*Populus nigra* x *Populus maximowiczii*, G8), early successional (G9), and restored prairie (G10). Previously, G2, G3, and G4 were in a corn-soybean-canola rotation from 2008 to 2011. Details on cropping histories and species are given in Table S1. These cropping systems were selected to have a range of plant diversities and of external/management inputs.

Three 2.5-cm diameter intact soil cores were taken from each plot with a Geoprobe 540MT (Geoprobe Systems, Salinas, KS) and transported to a 4 °C cooler within 3 h of collection and stored there until processing, which usually occurred within one week of collection. Upon processing, soil cores were sectioned into 0–10 cm, 10–25 cm, 25–50 cm, and 50–100 cm slices from G1, G5, G6 and G10. Soils of the other systems were collected only at 0–10 cm. Each core section was sieved (4 mm) to remove roots and stones and then the three samples from the same plot and depth were combined into a single composite sample. Each composite sample was divided, with portions frozen for later DNA extraction, air-dried for nutrient analysis, and oven-dried at 60 °C for total carbon (C) and nitrogen (N) analysis. In total, 110 composite samples ((4 treatments × 4 depths + 6 treatments × 1 depth) × 5 plots) were collected.

2.2. Soil properties

To determine the total C and N content, sub-samples from each depth of each crop that had been oven-dried were pulverized and combusted in a Costech Elemental Combustion System 4010 (Costech Analytical Technologies, Valencia CA). To determine soil pH, potassium (K), phosphorus (P), calcium (Ca), and magnesium (Mg) concentrations, sub-samples that had been air-dried were analyzed at Michigan State University Soil and Plant Nutrient Laboratory using standard methods (http://extension.missouri.edu/explorepdf/specialb/sb1001.pdf).

2.3. DNA extraction, PCR amplification, and MiSeq sequencing

Soil samples were thawed and total DNA was extracted from 0.3 g portions of each soil sample using the MoBio PowerSoil Kit (MoBio, Carlsbad, CA), according to the manufacturer's protocol. The resulting DNA yield and quality were checked with an ND1000 device (NanoDrop, Wilmington, DE), followed by re-extraction and pooling of some samples with lower yields, which occurred for some 50–100 cm samples.

Bacterial 16S rRNA PCR amplification using a dual-index sequencing strategy (Kozich et al., 2013) was conducted to generate the amplicon library for MiSeq sequencing. Briefly, amplicons were generated in the reaction system consisting of 17 μl of AccuPrime *Pfx* SuperMix (Invitrogen, CA, USA), 1 μl of DNA (around 20 ng/μl), and 1 μl of each barcoded primer (10 μM). The fusion primer set with Illumina adapter and barcodes targeted the hypervariable V4 region with specific forward primer 5′-GTGCCAGCMGCCGCGGTAA and reverse primer 5′-GGACTACHVGGGTWTCTAAT (Caporaso et al., 2011). The cycling conditions were 95 °C for 2 min, 30 cycles of 95 °C for 20 s, 55 °C for 15 s, 72 °C for 1 min, and 72 °C for 10 min. PCR products were purified and normalized using the SequalPrep Normalization Plate Kit (Invitrogen, CA, USA), followed by pooling of 5 μl of each sample to produce a sample library. Sequencing was performed by the Research Technology Support Facility, MSU, for MiSeq paired-ends sequencing with the 250 bp kit (Standard v2 flow cell with 500 cycles). Raw sequences were deposited in the NCBI Sequence Read Archive under accession number PRJNA296796.

2.4. Bioinformatic analyses

Raw paired-end reads were assembled using the RDP Assembler (Cole et al., 2014) with a read Q score cutoff of 28. Primers were trimmed and the resulting reads (averaging 253 bp) were chimera

checked using UCHIME (Edgar et al., 2011) in *de novo* mode. OTU clustering was performed by UPARSE Following the pipeline, all chimera-free reads were demultiplexed into one file, clustered at 97% similarity, representative sequences generated, singletons removed, and a final OTU table was created. Representative sequences of OTUs were submitted for taxonomic classification by RDP Classifier at a confidence of 50% (Wang et al., 2007). OTUs classified as chloroplasts or unclassified at the domain level as *Bacteria* or *Archaea* were removed (For simplicity, "bacterial" is used throughout since *Archaea* accounted for less than 3% of the sequences). This did leave OTUs unclassified to a bacterial or archaeal phylum. All samples were re-sampled to 25,000 sequences per sample for downstream analyses. All bioinformatic analyses were performed using the High Performance Computational Center facilities at Michigan State University. All data files were organized using R package phyloseq (McMurdie and Holmes, 2013).

2.5. Statistical analyses

Differences in community structure were visualized using db-RDA based on generalized UniFrac distances ($\alpha = 0.5$), which were generated from representative sequences of OTUs by FastTree and the R package GUniFrac (Chen et al., 2012a). The significance was tested by PERMANOVA with 999 permutations (using the function adonis in R package vegan), and the correlations of soil properties to the db-RDA patterns were calculated using the envfit function in the R package vegan (Oksanen et al., 2015). Alpha diversity indexes, including observed richness (S_{obs}), Shannon diversity (H′), and Pielou's evenness (J′), were computed based on the OTU table using the vegan package as well. Shared OTUs were calculated and plotted using the R package VennDiagram (Chen, 2014). The indicator analysis based on genera (singleton and doubleton genera removed first) was conducted using R package indicspecies (De Caceres and Legendre, 2009) with 999 permutations and allowing for combinations of depths and crops, and the P-values were corrected for multiple comparison using R package qvalue (Dabney and Storey, 2015) with a false discovery rate of 10% ($q \leq 0.1$). Other measurements, including soil properties, were analyzed and plotted using the R package ggplot2 (Wickham, 2009), while NMDS of soil properties was performed using the vegan package. All one-way ANOVA tests were conducted by one-way.test and pairwise.t.test with P-value adjustment method of "BH" for multiple comparisons. Data were square-root- or log-transformed to meet normality and homogeneity of variance. Otherwise, kruskal in R package argicolae (Mendiburu, 2015) was used. Pearson's correlations and their significances of α-diversity indexes, abundances of individual phyla and classes to soil properties were calculated by functions of cor and cor. test in R package stats (R Core Team, 2014). These analyses were performed using R 3.1.2 (R Core Team, 2014).

3. Results

3.1. Soil properties

Soil chemical properties differed with depth and among cropping systems (Fig. 1 and Table 1). NMDS ordination (Fig. 1) showed that samples separated primarily by depth (PERMANOVA, $P < 0.001$). Crop was a minor factor (PERMANOVA, $P = 0.037$), and there was no significant interaction between crop and depth (PERMANOVA, $P = 0.750$). From the surface soil (0–10 cm) to the bottom (50–100 cm), soil total C and total N contents decreased (ANOVA, $p < 0.001$) by 12 and 7 times, respectively. The pH was also significantly lower in the bottom layer (ANOVA, $p < 0.01$). Soil K and P concentrations were significantly higher in the surface soil (ANOVA, $p < 0.001$), while Ca and Mg first increased with depth and then decreased (highest at 25–50 cm, ANOVA, $p < 0.001$). Moreover, continuous corn (G1) generally had significantly lower C, N, pH, Ca, and Mg than the other biofuel crop systems (especially the poplar, G8) at 0–10 cm depth (Table 1), while switchgrass (G5), *Miscanthus* (G6), native grass mix (G7), and restored prairie (G10) contained significantly lower K than corn-related soils (especially the continuous corn, G1). There were no significant differences in soil characteristics among the continuous corn, switchgrass, *Miscanthus* and the restored prairie in the deeper layers.

3.2. Diversity measures

A total of 5,006,088 high quality reads were obtained after assembly which produced 20,179 unique OTUs at a 97% sequence similarity cutoff, 18,354 of which occurred in samples from G1, G5, G6, and G10 (Fig. S1). Shared "universal" OTUs (found in samples from all four of these crops at all four depths) accounted for 31% (5655) of the total (Fig. S1). Surface soils contained the highest proportion (12%) of exclusive OTUs, followed by the bottom layer (8%), while the intermediate layers accounted for the least (5 and 6% each).

For the four crops sampled over depth, bacterial diversity (H′), observed number of species (S_{obs}), and evenness (J′) were highest in the top 10 cm of soil and decreased significantly in each deeper layer (Fig. 2, left panels, ANOVA, $\alpha < 0.05$). Comparing crops within each soil layer, bacterial diversity indexes were significantly lower in corn soils than switchgrass in the 10–25 cm layer (Fig. 2, right panels), but otherwise no marked differences were found among crops (Fig. 2 and Fig. S2). For all data combined, bacterial diversity indexes were strongly associated with soil total C and N content (Fig. S3).

3.3. Community structures

There were significant differences in microbial community structure among the ten crops in the 0–10 cm layer ($p < 0.001$, Table 2). On ordination, annual corn treatments were separated from the perennial crop treatments by the first db-RDA axis (Fig. 3A). Continuous corn (G1) was separated from corn treatments with cover crop by axis 2 (Fig. 3A), and continuous corn with cover crop (G2) was separated from corn with cover crop in rotation (G3 and G4) by axis 3 (Fig. 3B). Similarly, two clusters were observed within the six perennial systems: monocultures G5, G6, and G8 (switchgrass, *Miscanthus*, poplar) and polycultures G7, G9, and G10 (native grass mix, early successional, and restored prairie) (Fig. 3A). Furthermore, G8 was separated from the other monocultures by axis 3 (Fig. 3B). Thus, six groups in total are distinguishable. Correlations between site scores and environmental variables were relatively weak but statistically significant except for K; values for all but P were lower for the corn sites (Fig. 3 and Table S2).

Ordination of all samples for the four crops sampled with depth (G1, G5, G6, and G10) indicated that their microbial communities were shaped primarily by factors associated with depth (Fig. 4A). Site scores were significantly correlated with all measured environmental variables, especially C ($r^2 = 0.91$) and N ($r^2 = 0.88$) (Table S2). While such depth related factors played a dominant role in shaping communities, PERMANOVA revealed a significant interaction between depth and crops (Table 2), leading us to make further tests for each separate soil layer. These results indicated that crops affected soil communities for all but the deepest (50–100 cm) layer (Table 2).

For the 0–10 cm layer, ordination separated samples for the four crops in the same way as when all 10 crops were included. G5 and G6 clustered together, G1 and G10 separately (Fig. 4B). Only four of

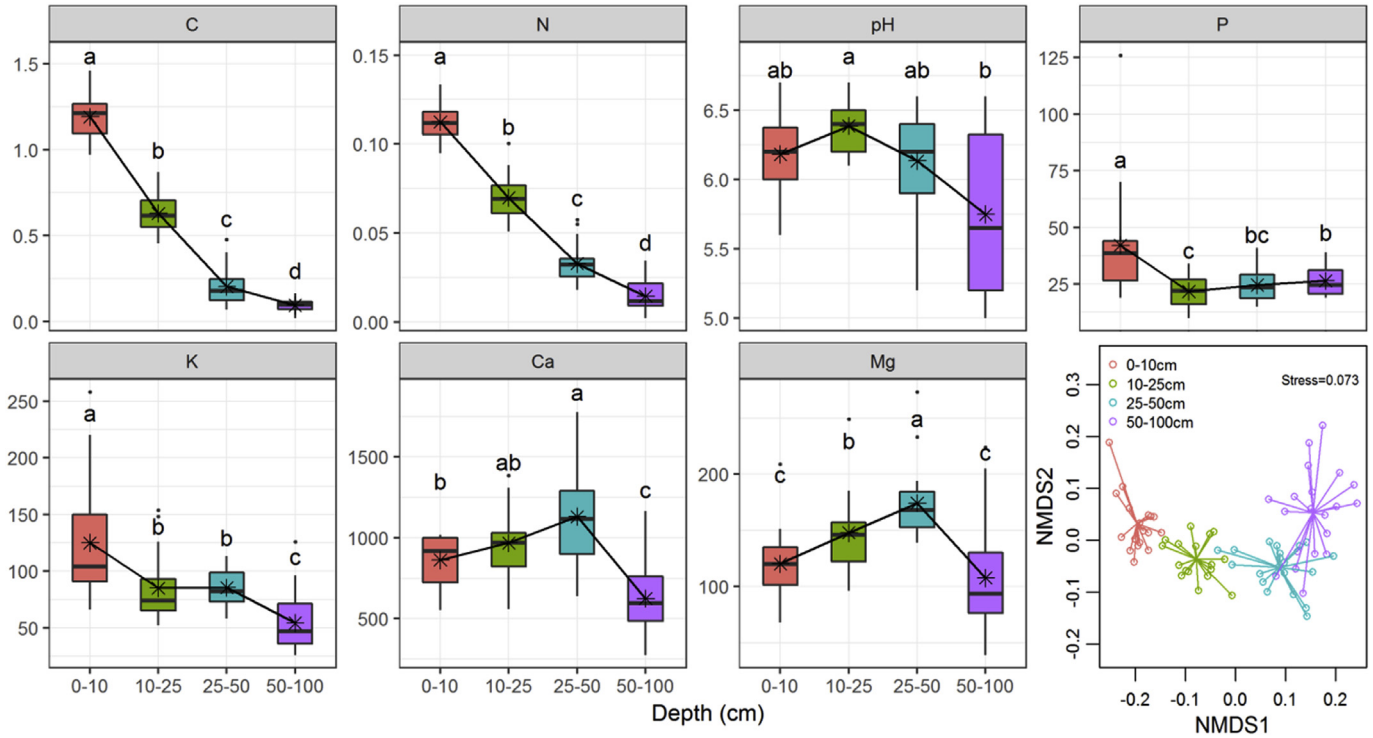

Fig. 1. Comparisons of soil chemical properties among depths under four crops (G1, G5, G6, and G10), and NMDS ordination based on these properties. The bottom and top of a box are the 25th and 75th quartiles, the horizontal line within a box is the median, and the ends of the whiskers are the limits of the distribution as inferred from the upper and lower quartiles. Dots are outliers. Black stars are means. Letters indicate the ANOVA grouping among depths. Both soil C and N are in units of g/100g (weight %), while P, K, Ca, and Mg are all in units of μg/g (ppm).

Table 1
Comparisons of soil chemical properties among crops at 0–10 cm. Letters indicate the ANOVA grouping among crops.

Crops[#]	C[*]	N[**]	pH[**]	P	K[**]	Ca	Mg[***]
G1	1.148 ± 0.118^{b}	0.111 ± 0.009^{c}	5.9 ± 0.2^{c}	57 ± 43	173 ± 56^{a}	748 ± 148	85 ± 11^{c}
G2	1.265 ± 0.172^{ab}	0.120 ± 0.013^{bc}	6.4 ± 0.7^{ab}	48 ± 24	148 ± 35^{ab}	992 ± 248	131 ± 40^{abc}
G3	1.208 ± 0.115^{b}	0.117 ± 0.013^{bc}	6.2 ± 0.3^{abc}	45 ± 16	140 ± 42^{abc}	914 ± 159	126 ± 24^{ab}
G4	1.195 ± 0.172^{b}	0.116 ± 0.015^{bc}	6.2 ± 0.2^{abc}	50 ± 15	149 ± 26^{ab}	938 ± 163	125 ± 12^{b}
G5	1.141 ± 0.170^{b}	0.110 ± 0.013^{c}	6.3 ± 0.1^{abc}	33 ± 13	97 ± 17^{c}	863 ± 167	124 ± 12^{b}
G6	1.257 ± 0.152^{ab}	0.114 ± 0.013^{bc}	6.1 ± 0.1^{bc}	40 ± 9	111 ± 62^{c}	935 ± 83	126 ± 15^{b}
G7	1.292 ± 0.126^{ab}	0.124 ± 0.011^{bc}	6.4 ± 0.5^{abc}	33 ± 7	111 ± 22^{bc}	1056 ± 57	156 ± 16^{a}
G8	1.558 ± 0.295^{a}	0.148 ± 0.024^{a}	6.4 ± 0.1^{ab}	54 ± 10	182 ± 45^{a}	1000 ± 107	145 ± 3^{ab}
G9	1.365 ± 0.135^{ab}	0.136 ± 0.010^{ab}	6.5 ± 0.2^{ab}	47 ± 30	169 ± 51^{a}	996 ± 225	172 ± 17^{a}
G10	1.208 ± 0.109^{b}	0.116 ± 0.012^{bc}	6.5 ± 0.1^{a}	35 ± 12	110 ± 29^{bc}	915 ± 138	154 ± 37^{ab}

[#]G1 and G2 are annually continuous corn and continuous corn + cover crop, respectively; while G3 and G4 are soybean + cover crop and corn + cover crop, respectively, in a soybean-corn rotation. G5 - G10 are perennially switchgrass, *Miscanthus*, native grass mix, poplar, early successional, and restored prairie, respectively. Significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

the environmental variables were significantly ($\alpha \leq 0.05$) associated with the site scores. K was positively associated with the corn samples while pH, Mg, and Ca were positively associated with the other crops. Clustering of crops was different for the deeper depths. In the 10–25 cm layer, G5 and G10 clustered together while G1 and G6 formed separate clusters (Fig. 4C). In the 25–50 cm layer, G5 formed a separate cluster, and while the other crops show some separation in Fig. 4D, no differences among them were found by PERMANOVA (data not shown). There were no significant correlations between environmental variables and site scores for depths other than 0–10 cm, and no significant differences among crops for the 50–100 cm layer (Table 2).

Generalized UniFrac distances between pairs of soil layers increased with the distance between the layers being compared (Fig. S4A). Pairwise comparisons of distances between cropping systems also showed that bacterial communities were more distinct in the top three layers (0–50 cm), and became more homogeneous (with less variation) at the 50–100 cm depth (Fig. S4B).

3.4. Taxon abundances

OTUs were classified to 10 major phyla (each more than 1% of the total sequences, Table 3) that together accounted for 81.0% of the total sequences, and 22 minor phyla accounting for an additional 3.7%. A total of 14.2% of the sequences were unclassified at the phylum level (unclassified_Bacteria, Table 3). Relative abundances of individual phyla were highly variable with depth and among cropping systems, and were significantly correlated with soil properties (Table 3, Fig. 5, Tables S3 and S4). *Proteobacteria*, *Actinobacteria*, *Planctomycetes*, *Bacteroidetes*, and candidate WPS-2 decreased from the surface to deeper soils, while *Firmicutes* and unclassified *Bacteria* increased. The most abundant phylum,

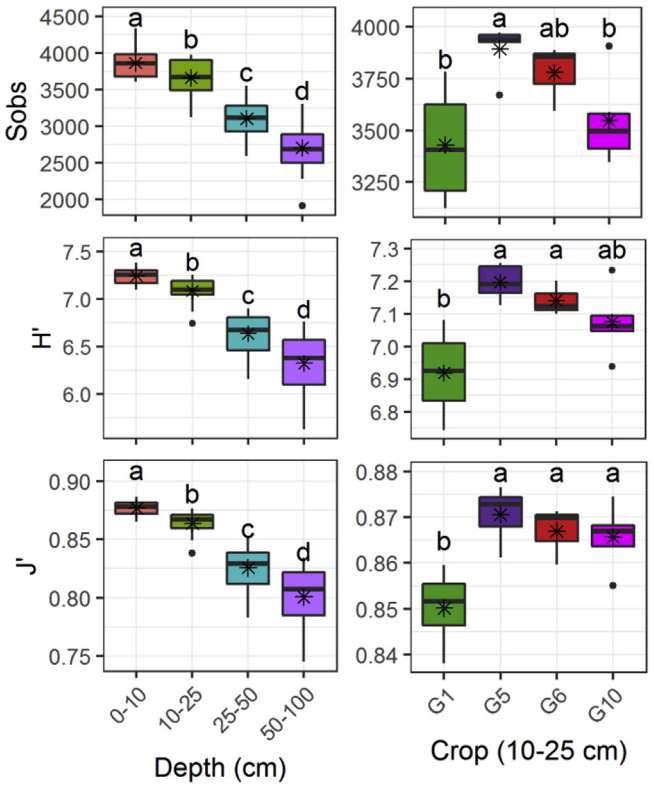

Fig. 2. Comparisons of bacterial diversity indexes among soil depths and among crops at the depth of 10–25 cm. The four crop abbreviations are defined in Table 1. Letters indicate the ANOVA grouping among depths and crops ($\alpha = 0.05$). Explanation of boxes and stars as for Fig. 1. S_{obs}, observed number of OTUs; H′, Shannon diversity index; J′ Pielou's evenness index.

Table 2
Effect of soil depth, crop, and their interactions on bacterial community structures based on PERMANOVA and generalized UniFrac distances ($\alpha = 0.5$).

	Depth		Crop		Depth × Crop	
	F	R^2	F	R^2	F	R^2
0-10 cm, 10 crops	–	–	1.64^{***}	0.28	–	–
4 depths × 4 crops	19.51^{***}	0.42	2.43^{**}	0.05	1.47^{*}	0.10
0-10 cm, 4 crops			2.14^{***}	0.31		
10-25 cm, 4 crops	–	–	1.39^{**}	0.24	–	–
25-50 cm, 4 crops	–	–	2.20^{***}	0.29	–	–
50-100 cm, 4 crops	–	–	1.24^{ns}	0.19	–	–

Significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Acidobacteria, as well as *Verrucomicrobia* and *Chloroflexi* were generally more abundant in the middle layers. The relative abundance of *Thaumarchaeota*, the most abundant *Archaea*, showed a bimodal distribution with depth. Among cropping systems, corn-related soils harbored more *Proteobacteria* (especially *Alpha-*) and *Chloroflexi*, but less *Acidobacteria* than perennial crop soils at 0–10 cm. Less abundant *Actinobacteria* and *Planctomycetes* but more unclassified *Bacteria* were present in corn soils at 10–25 cm. Switchgrass soils contained more *Bacteroidetes* in layers from 25 to 100 cm, and more unclassified *Bacteria* but less *Thaumarchaeota* at 25–50 cm as well as less *Spartobacteria* but more subdivision 3 of the *Verrucomicrobia* than the other three crops.

Although the predominant classes of *Acidobacteria* (Gp6, Gp4, Gp16, Gp17) displayed similar trends as the phylum with soil depth, some classes (Gp7, Gp1, Gp2) were proportionally more abundant with soil depth, while Gp3 remained quite stable with depth (Fig. 6 and Table S5). These contrasting patterns of classes within the same phyla with soil depth were also observed in *Verrumicrobia* (*Spartobacteria* vs Subdivision3) and *Choroflexi* (*Ktedonobacteria* vs *Caldilineae*) and even in the consistently-increasing phylum *Firmicutes* (*Clostridia* vs *Bacilli*) (Table S5), but were not detected in classes of consistently-decreasing phyla *Proteobacteria* and *Bacteroidetes*. Similarly, corn-related soils had lower abundances of Gp6 and Gp17 but greater abundances of Gp1 and Gp3 at 0–10 cm. Switchgrass soils accumulated more *Delta-* and *Gamma-* but less *Alpha-proteobacteria* at 25–50 cm, as well as higher percentage of subdivision 3 and *Verrucomicrobiae* but lower *Spartobacteria* of *Verrucomicrobia* (Fig. 5 and Table S6).

3.5. Indicators

Indicator analysis identified 271 genera significantly ($q < 0.1$) associated with a specific depth or a combination of depths (Table S7), of which 91 genera were clearly classified and had relative abundances over 0.005% (Fig. 7). Using the same analysis, 20, 5, and 7 indicator genera were characteristic ($q < 0.1$) of one crop or a combination of crops at 0–10 cm, 10–25 cm, and 25–50 cm, respectively (Table S8). Of these, 27 genera were unique and clearly classified (Fig. 8). Indicator genera characteristic of 0–10 cm and 10–25 cm were relatively similar (more shared), but different from those at the two deeper layers that were similar to each other as well. Corn-related soils were characterized by indicators different from those associated with perennial crops at 0–10 cm and 10–25 cm, while switchgrass soils had more distinctive indicators at 25–50 cm. Overall, most of indicator genera belonged to *Proteobacteria* and *Actinobacteria*, followed by *Bacteroidetes* and *Acidobacteria*.

4. Discussion

In this study, we observed significant differences in soil prokaryotic communities and soil properties among four soil depths (down to 100 cm) under four different biofuel crop systems and among 10 crops in near surface soils after 6 crop growing seasons. As a proxy for changing soil edaphic factors, especially total C and N, soil depth was a major driver of bacterial community structure among the four cropping systems examined over depth, whereas crop effects were secondary and significantly correlated with several soil variables at 0–10 cm (Fig. 3). Moreover, our indicator analyses identified exclusive and shared genera characteristic of soils in different layers and soils under different crops at the same depth, which suggests patterns to be tested more broadly as well as for consistency of their driving ecological features.

The significant decrease in alpha diversity with depth is mirrored in previous studies using pyrosequencing (Will et al., 2010; Eilers et al., 2012; Tsitko et al., 2014), clone libraries (Hansel et al., 2008), and fingerprinting approaches (Fierer et al., 2003; Agnelli et al., 2004). Eilers et al. (2012) observed that microbial communities based on weighted UniFrac distances among sites were most variable in the near-surface horizons, relatively uniform at intermediate depths, and again highly variable at deep layers. Indeed, in our study sample variability within the same layer based on generalized UniFrac distances was highest in the surface and deepest soils for most cases (Fig. S4B). The soil depth associated with community change appears to extend to approximately 50 cm, with communities becoming less similar (greater phylogenetic distance) with increasing physical distance. Indicator analyses revealed many specific genera for soil depths with diverse putative functions: for example, biodegradation/decomposition (*Actinoplanes* (Goodfellow and Williams, 1983), *Cellvibrio* (Mergaert et al., 2003)) and nitrogen-fixation (*Microvirga* (Ardley et al., 2012),

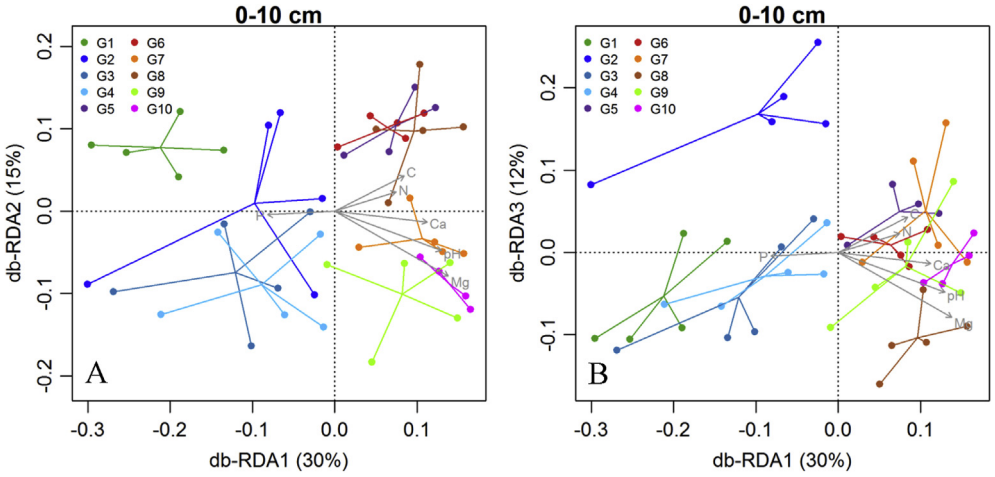

Fig. 3. Differences in soil bacterial community structures among crops for the 0–10 cm layer. db-RDA constrained on crops was fitted with significantly correlated soil properties.

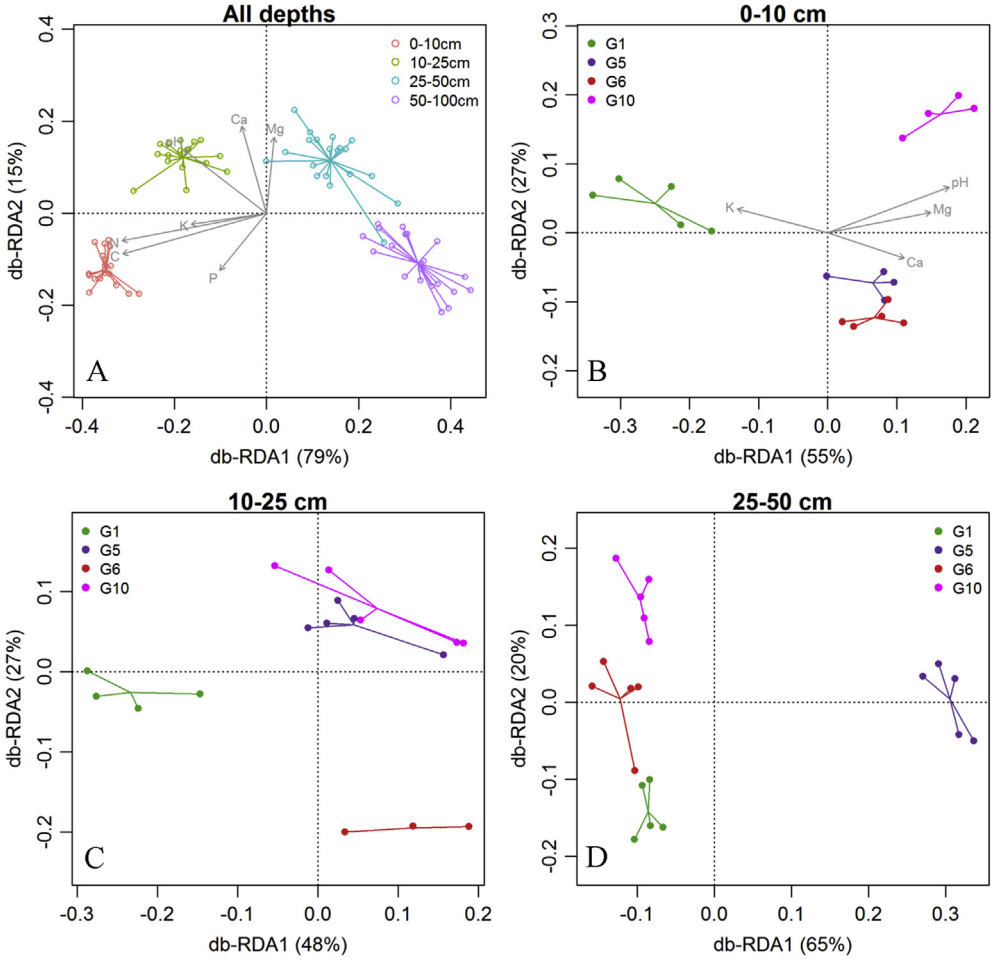

Fig. 4. Differences in soil bacterial community structures among depths (A) and under crops at the same depth (B–D). The variations explained by depths or crops and statistics from permutation tests are given in Table 2 db-RDAs constrained by depths or crops were then fitted with significantly correlated soil properties. There is no plot for the 50–100 cm layer because there were no significant differences in soil bacterial communities among crops (Table 2). Data were based on the 4 crops with depth.

Azoarcus (Reinhold-Hurek et al., 1993)) in shallow soils, as well as denitrification (*Microvirgula* (Patureau et al., 1998)) and utilization of recalcitrant compounds (*Rubrobacter* (Carreto et al., 1996)) in deeper layers. While C and N were highly correlated to microbial community changes, other soil properties such as aggregate size, water holding capacity, and redox status can vary among soil horizons and also impact which species thrive or senesce at each depth. Past studies in grasslands (Will et al., 2010) and peat (Tsitko et al., 2014) have also documented a strong correlation to soil total C and N. Microbial biomass is well known to decline with depth

Table 3
Relative abundances of major phyla (>1%) and their changes with soil depth and correlations to soil properties. More details in Table S3.

Phylum	Abundance	F-value	Changes with depth	Correlation to soil variables						
				C	N	pH	P	K	Ca	Mg
Acidobacteria	28.68±4.57	24.21***		0.47***	0.43***	0.59***	-0.09	0.09	0.26*	0.15
Proteobacteria	14.28±3.82	118.24***	↓	0.89***	0.88***	0.38***	0.44***	056***	-0.01	-0.161
unclassified_Bacteria	14.24±4.34	106.22***	↑	-0.89***	-0.88***	-0.30**	-0.32***	-0.51***	-0.13	-0.04
Verrucomicrobia	9.49±2.01	17.08***		-0.46***	-0.49***	-0.37***	-0.27*	-0.09	0.22	0.39***
Actinobacteria	6.86±1.85	9.53***		0.45***	0.47***	0.31**	0.35**	0.27*	0.03	-0.04
Firmicutes	5.42±4.57	75.10***	↑	-0.73***	-0.70***	-0.53***	-0.17	-0.35**	-0.21	-0.02
Chloroflexi	4.91±2.39	27.32***		-0.60***	-0.65***	-0.42***	-0.24*	-0.14	0.31**	0.36**
Planctomycetes	4.58±1.18	46.49***		0.74***	0.77***	0.33**	0.33**	0.38***	0.09	-0.15
Bacteroidetes	2.90±1.84	60.72***	↓	0.81***	0.81***	0.38***	0.26*	0.48***	0.01	-0.07
Thaumarchaeota	2.26±0.92	25.41***		0.50***	0.55***	-0.02	0.32**	0.15	-0.36**	-0.40***
candidate WPS-2	1.64±0.93	248.30***	↓	0.90***	0.91***	0.40***	0.25*	0.46***	0.12	-0.08

Significance: * P<0.05, ** P<0.01, *** P<0.001.

(Agnelli et al., 2004; Eilers et al., 2012), consistent with our low DNA extraction yield, especially at 50–100 cm. Overall, the effects of depth (0–10 cm vs. 10–25 cm) had a greater influence on bacterial communities than selective pressures from different cropping systems at 0–10 cm. Similarly, it was reported that the depth effects on microbial communities, even as little as 10–20 cm within the same core, can reach a similar extent as that of surface soils separated by thousands of kilometers (Eilers et al., 2012). Together, these results suggest that soil depth integrates environmental gradients along soil profiles and forms a strong ecological filter for selecting/shaping microbial communities within different depths, as proposed by Eilers et al. (2012).

In addition to the overwhelming depth influences, we also identified significant effects of crops on soil microbial communities. These crop effects were constrained within the top 50 cm, presumably due to rhizospheric influences such as plant root exudates, root cap sloughing, and root turnover (Acosta-Martinez et al., 2008). Perennial plants are known to produce more abundant microbial resources through rhizodeposition and root turnover and can thus accommodate a larger population of more diverse microorganisms (Liang et al., 2012b). In addition, crop rotation, tillage (including annual planting), and other management influences (such as fertilization and herbicides) serve to differentiate perennial from annual crops. The distant grouping of continuous corn away from the other three corn-based systems and the separation of perennial monocultures from multispecies grasses can infer these impacts (Fig. 4). Specifically, corn soils harbored the most distinct communities, compared to the perennial biofuel crops in the top 25 cm, similar to results comparing corn, switchgrass, and *Miscanthus* surface soils (down to 24 cm depth) (Liang et al., 2012b; Mao et al., 2013; Jesus et al., 2016). This microbial pattern under different crops was also reflected in indicator genera. For example, continuous corn soils at 0–10 cm, which were lower in pH than other crop soils (Table 1), were characterized by *Rhodanobacter* that is capable of denitrification and dominate at low pH (Green et al., 2012; Kostka et al., 2012). In contrast, perennial switchgrass and *Miscanthus* soils were strongly associated with lignin-degrading *Sphingobacterium* (Wang et al., 2013), while switchgrass soils were also characterized by lignin-degrading *Novosphingobium* (Chen et al., 2012b) at 10–50 cm. The plant growth-promoting *Acinetobacter*, an indicator at lower depth layers, especially in switchgrass soils might provide benefits by fixing nitrogen, solubilizing P, producing siderophores and indole-3-acetic acid, and promoting root-growth (Huddedar et al., 2002; Sachdev et al., 2010; Shi et al., 2011).

Differences in microbial community structure among crops have also been attributed to the perennial nature and rooting systems of the crops. Corn has a shorter photosynthetic life cycle, shallower rooting depth, lower root densities, and less root biomass than perennial grasslands, including switchgrass (Jackson et al., 1996; Tufekcioglu et al., 1999; Culman et al., 2010). Indeed, we identified abundant roots in switchgrass soil down to 100 cm, but almost no roots in corn soil deeper than 25 cm. Furthermore, a recent study demonstrated that switchgrass root length densities and root dry weight (both peaking at 30–45 cm) are much higher than *Miscanthus* (peaking at 0–10 cm, 90% concentrating in the top 35 cm) in relatively deeper soil (Monti and Zatta, 2009), although we have not observed such differences here (Sprunger et al., unpublished). Together, these observations suggest that switchgrass may possess the most unique root structure and/or quality and quantity of root exudates and hence support the highest uniqueness of the associated soil microbial communities at the 25–50 cm depth as seen in this study. Switchgrass accumulates greater microbial biomass than corn and this influence of resource availability can move deeper within the soil over time (to 30 cm after 9 years) (Stewart et al., 2015). We detected significantly higher diversity in switchgrass than continuous corn at 10–25 cm after 6 cropping years. Therefore, given the inherently different rooting systems, we predict that switchgrass will eventually significantly shape microbial communities as deep as 100 cm over time through root-mediated C allocation.

The dominances of *Acidobacteria*, *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Firmicutes*, and *Chloroflexi* observed in this study have been detected in various soil types, biomes, and depths (Jesus et al., 2010; Will et al., 2010; Eilers et al., 2012; Liang et al., 2012b; Tsitko et al., 2014), which indicates the ubiquity and important roles of these phyla. However, the relative abundances of these taxa changed distinctly with soil depth and among cropping systems. The most striking of these changes was associated with *Proteobacteria*, whose relative abundance decreased with depth, a trend observed in other studies as well (Eilers et al., 2012; Tsitko et al., 2014). Given the strong correlation to soil C (Table 3), the decrease of *proteobacterial* abundances with depth is likely due to their lifestyle, as they are more abundant in soil with higher C availability (Fierer et al., 2007; Eilers et al., 2010; Goldfarb et al., 2011). This decreasing trend was consistent for all four *proteobacterial* classes. Crop type, possibly in conjunction with sampling time, also influenced *Proteobacteria*, with *Alphaproteobacteria* and

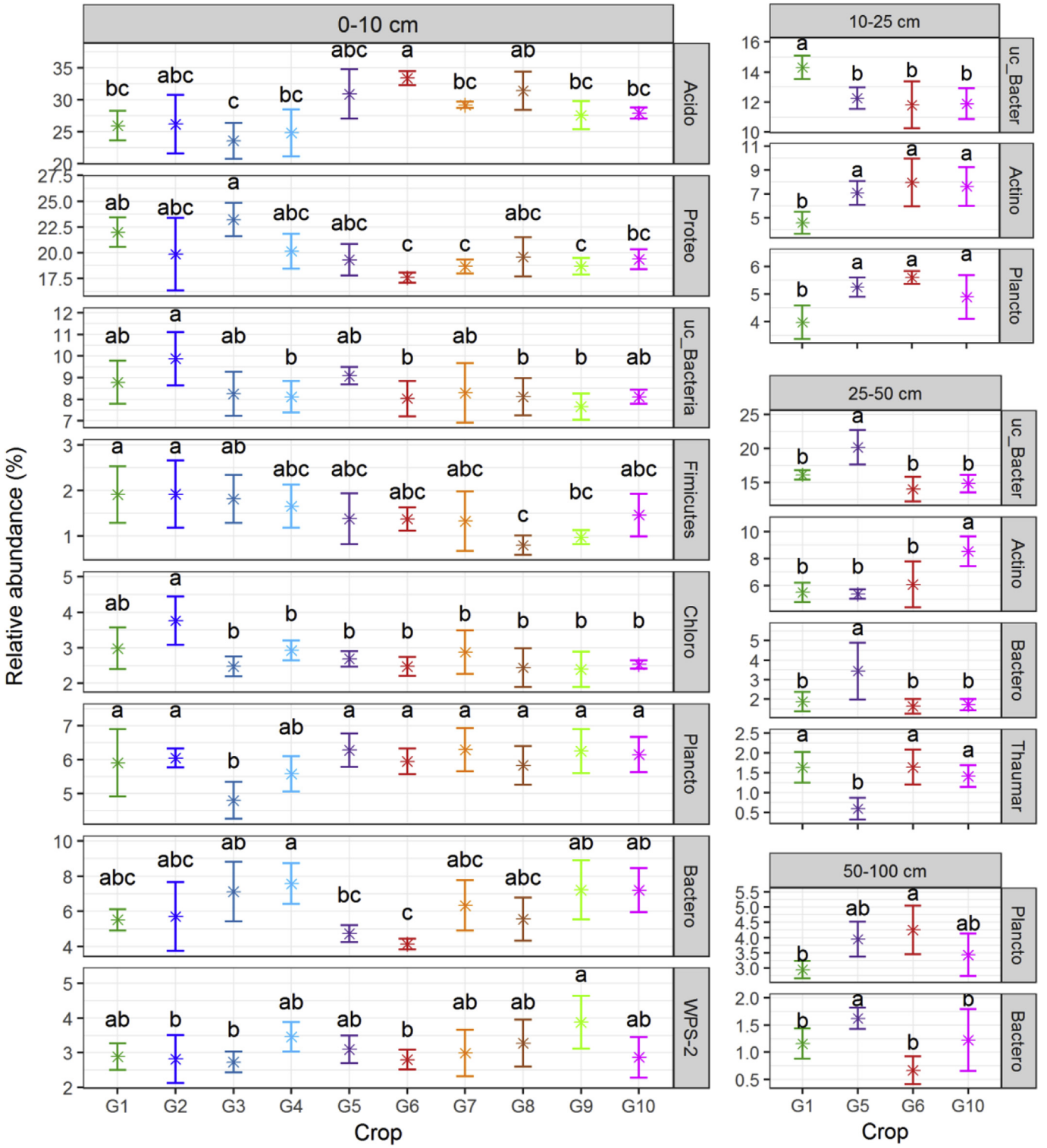

Fig. 5. Differences in relative abundances of major phyla (>1%) under crops at the same depths. Stars and error bars are the means and standard deviations, respectively. First letters of the phylum names were used to save space. Letters indicate the ANOVA grouping among depths and crops. More details and more phyla as well as their correlations to soil properties are in Table S4.

Gammaproteobacteria more abundant in corn-based soil at 0–10 cm. This may reflect more labile organic C in the surface soil, perhaps due to the degradation of dead corn roots, as the sampling time was approximately one month after harvest. More dead roots in corn soils were also supported by the observed indicator *Granulicella* at 0–10 cm, which hydrolyzes a wide range of sugars and complex polysaccharides (Rawat et al., 2013). Additionally, this ecological lifestyle-based hypothesis explains the declines of *Actinobacteria* and *Bacteroidetes* (Fierer et al., 2007) with depth. *Planctomycetes* (Ivanova and Dedysh, 2012) and candidate division

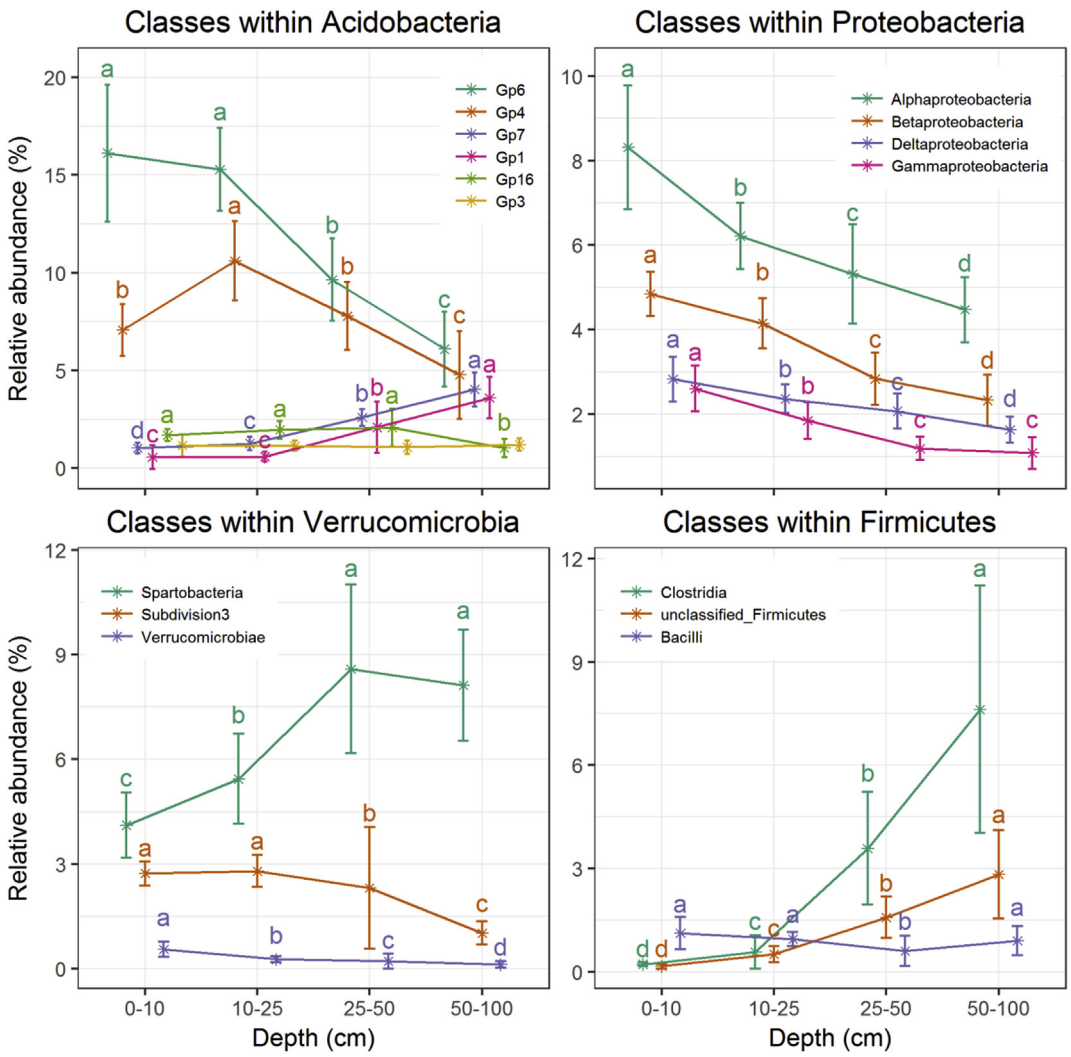

Fig. 6. Relative abundances of bacterial classes within the same phylum with soil depth. Stars and error bars are the means and standard deviations, respectively. Letters indicate the ANOVA grouping among depths and crops. More details and more classes as well as their correlations to soil properties are in Table S5.

WPS-2 (Tsitko et al., 2014) have also been reported to decrease with depth, consistent with our observation. Recently, *Planctomycetes* were shown to be capable of aerobic degradation of plant saccharides (Erbilgin et al., 2014), which agrees with their decreasing abundances with depth.

Other bacterial lineages increased with depth or peaked in the middle layers. *Acidobacteria* (the most abundant), *Verrucomicrobia*, and *Chloroflexi* peaked at mid-layers, while *Firmicutes* and unclassified *Bacteria* consistently increased with depth. *Acidobacteria* are well known to be widely distributed along the soil profiles (Hansel et al., 2008; Eilers et al., 2012), and are considered to be oligotrophs with higher abundances associated with lesser C resources (Fierer et al., 2007). This may explain increases in relative abundances from the surface to the mid-layer (higher C to lower C). However, it is not clear what drives a decrease in relative abundance in the deeper layers. Although this is an overall trend at the coarse phylum level, subgroup populations may possess varying physiologies (Fierer et al., 2007) that drive variations with soil depth and edaphic properties. For example, most subgroups (e.g. Gp7, Gp1, and Gp2) increased with depth, reflecting oligotrophic status. In contrast, the most abundant Gp6 decreased with depth, appearing to exhibit a more copiotrophic-like physiology. However, a simple C-based impact on Gp6 abundance is unlikely as they were less abundant in the four corn systems than in the perennial ones, suggesting that possibly C quality, soil properties (e.g. significantly positive correlation to pH, Table S6), or other management practices may impact their status. Moreover, acidobacterial abundances have been found to be negatively correlated with pH (Hartman et al., 2008; Jones et al., 2009). This correlation was not observed at the phylum level in this study. Rather we found some classes (especially Gp1 and Gp2) were negatively correlated to pH while some (e.g. Gp6, Gp4) were positively correlated with pH, similar to earlier findings (Jones et al., 2009). Distinct genera within *Acidobacteria* identified as indicators associated with different depths can also be explained by their various physiologies.

The peak in *Verrucomicrobia* abundance in the middle depths was also shown previously, and was suggested to be partially linked to their oligotrophic classification (Eilers et al., 2012). Similar to the *Acidobacteria*, we also observed variations in trends between the two major classes, *Spartobacteria* and Subdivison 3, with the latter more abundant in switchgrass soil at 25–50 cm. The *Firmicutes* were predominately represented by the obligately anaerobic *Clostridia* in this study. Their increasing proportions with depth were not expected since these are well-drained, sandy soils, and limited oxygen would not be expected. However, the ability of *Clostridia* to sporulate as well as restricted metabolism due to substrate

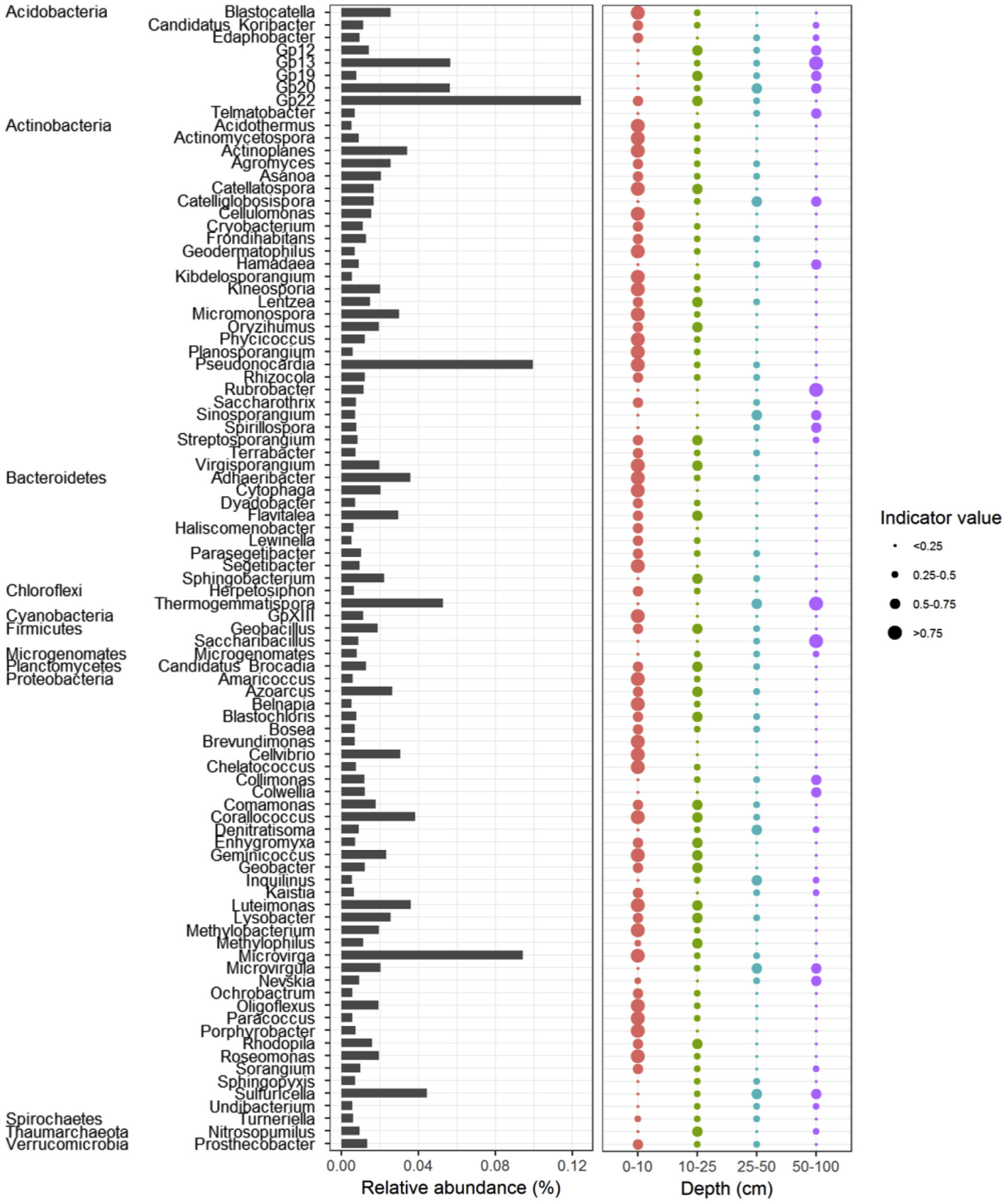

Fig. 7. Indicator genera significantly ($q < 0.1$) associated with one soil depth or a combination of soil depths. The bar indicates the relative abundance of each indicator genus, while the size of each circle represents the indicator value (association strength) of a specific genus with the different soil depths: 0–0.25, not characteristic; 0.25–0.5, weakly characteristic; 0.5–0.75, characteristic; and 0.75–1.0, strongly characteristic. Taxonomic information, indicator values, P-values, and q-values of all indicator genera are given in Table S7. Data were based on 4 crops with depth.

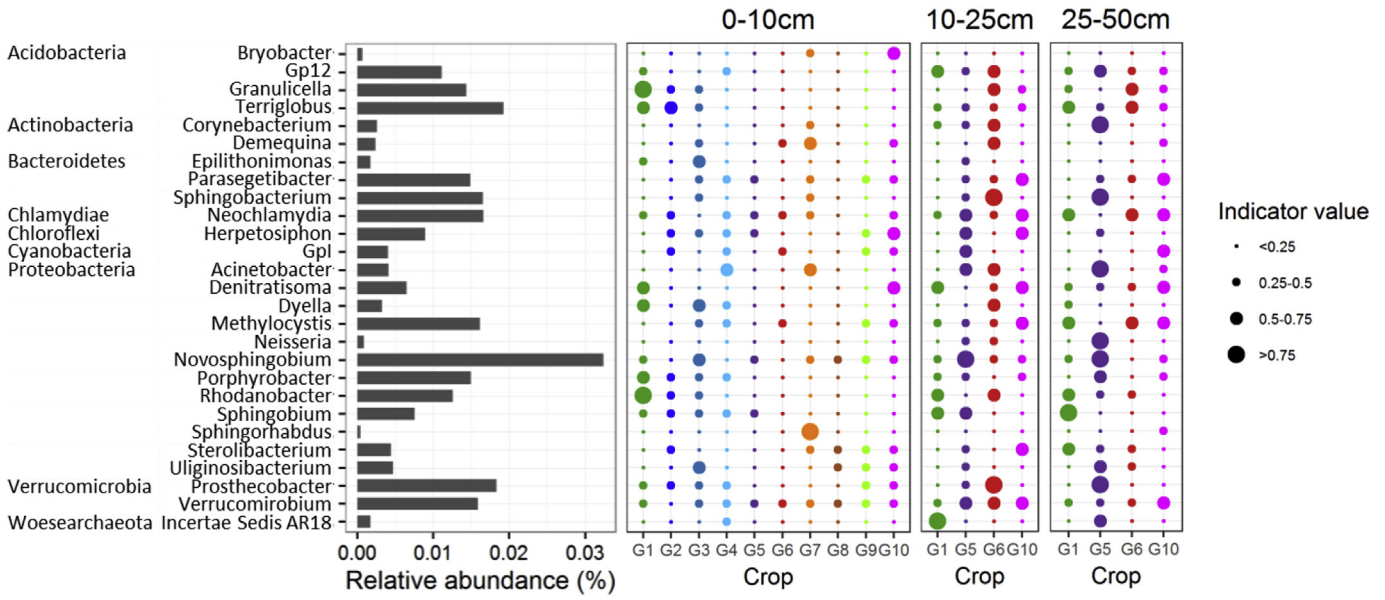

Fig. 8. Indicator genera significantly ($q < 0.1$) associated with soils under one crop or a combination of crops at the each depth. The bar indicates the relative abundance of each indicator genus, while the size of each circle represents the indicator value (association strength) of a specific genus with the different crops: 0–0.25, not characteristic; 0.25–0.5, weakly characteristic; 0.5–0.75, characteristic; and 0.75–1.0, strongly characteristic. Taxonomic information, indicator values, P-values, and q-values of all indicator genera are given in Table S8.

limitation, thereby preventing oxygen damage, could explain the results.

Thaumarchaeota was the most abundant archaeal phylum found in our soil profiles. It has been proposed that *Thaumarchaeota* are able to compete with diverse bacteria in soil (Hansel et al., 2008). The ammonia oxidizer *Nitrososphaera* (Hatzenpichler et al., 2008; Tourna et al., 2011) constituted the majority of the *Thaumarchaeota* in our study, which suggests an important role in N cycling, especially at depth.

Organic carbon arriving from aboveground litter or root exudates/metabolites is dependent on root allocation and surface area as well as bioturbation (Rumpel and Kogel-Knabner, 2011). As such, we anticipated that a greater amount of C and N is retained in perennial crop soils throughout the soil profiles. However, only poplar had significantly higher C and N than continuous corn at 0–10 cm. This may be due to the large quantity of poplar leaf litter and root residues retained in the system since poplar is harvested every 6 years, while recent studies also found that the C gained in the poplar system may be decomposed/respired during early regrowth of poplar (Syswerda et al., 2011; Gelfand et al., 2013). A methodological consideration is that visible roots were removed from the soil during sampling, whereas these roots would substantially contribute to the in-field soil organic carbon. Regardless, we did detect relatively higher (although not statistically significant) C and N in all other systems, compared to continuous corn at 0–10 cm. The lack of significant differences here after 6 years and especially in the deeper soils may be simply related to temporal factors. Culman et al. (2010) reported that perennial grasslands and pastures contained higher SOM, SOC and TOC down to 60 cm than annual systems after 75 years of cultivation. Likewise, Stewart et al. (2015) showed that switchgrass rooting systems penetrated deeper, from 0 to 5 cm in 3 years to 0–30 cm in 9 years, thus adding C to subsurface soils. As such, we expect that the trends observed here, in regards to C storage, would become more apparent in the future.

In conclusion, we found that soil depth, as a proxy for changes in soil biogeochemical, edaphic, and structural properties, was a strong factor in shaping bacterial community structure under a wide range of cropping systems. Crop type was a secondary driver and was significant at soil depths above 50 cm after 6 years, with switchgrass' effects deeper. Distribution patterns of individual bacterial phyla and classes along the depth gradient and under different crops reflected their ecological niches, with C and N availability, pH, and other potential environmental factors likely driving relative abundances. Root penetration depth and density are believed to play an important role in differentiating corn-based from perennial systems, although unresolved management effects likely have impacts as well. Overall, sustainable agriculture requires that we increase C sequestration within soils, or, at the very least, conserve the C already present. Here we find that poplar soils, followed by perennial crops had higher (though not always significant) C content after 6 years. Evidence suggests that, over time, these differences will become more apparent and will vary in deeper soils as perennial roots penetrate and add labile C. While we now observe striking differences in bacterial communities with depth, we know little about how microbial community functions will change with increased rooting depth, or what physiological traits result in proportional shifts in taxa.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.04.019.

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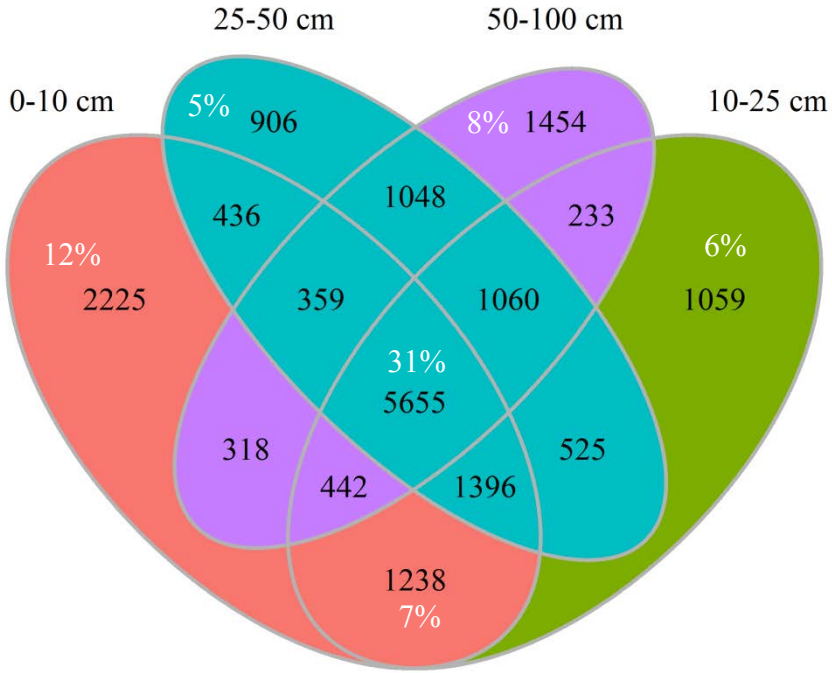

Figure S1 Venn plot of unique OTUs shared by and exclusive to the four soil depths, based on samples from G1, G5 , G6, and G10. Corresponding percentages are noted for some overlaps.

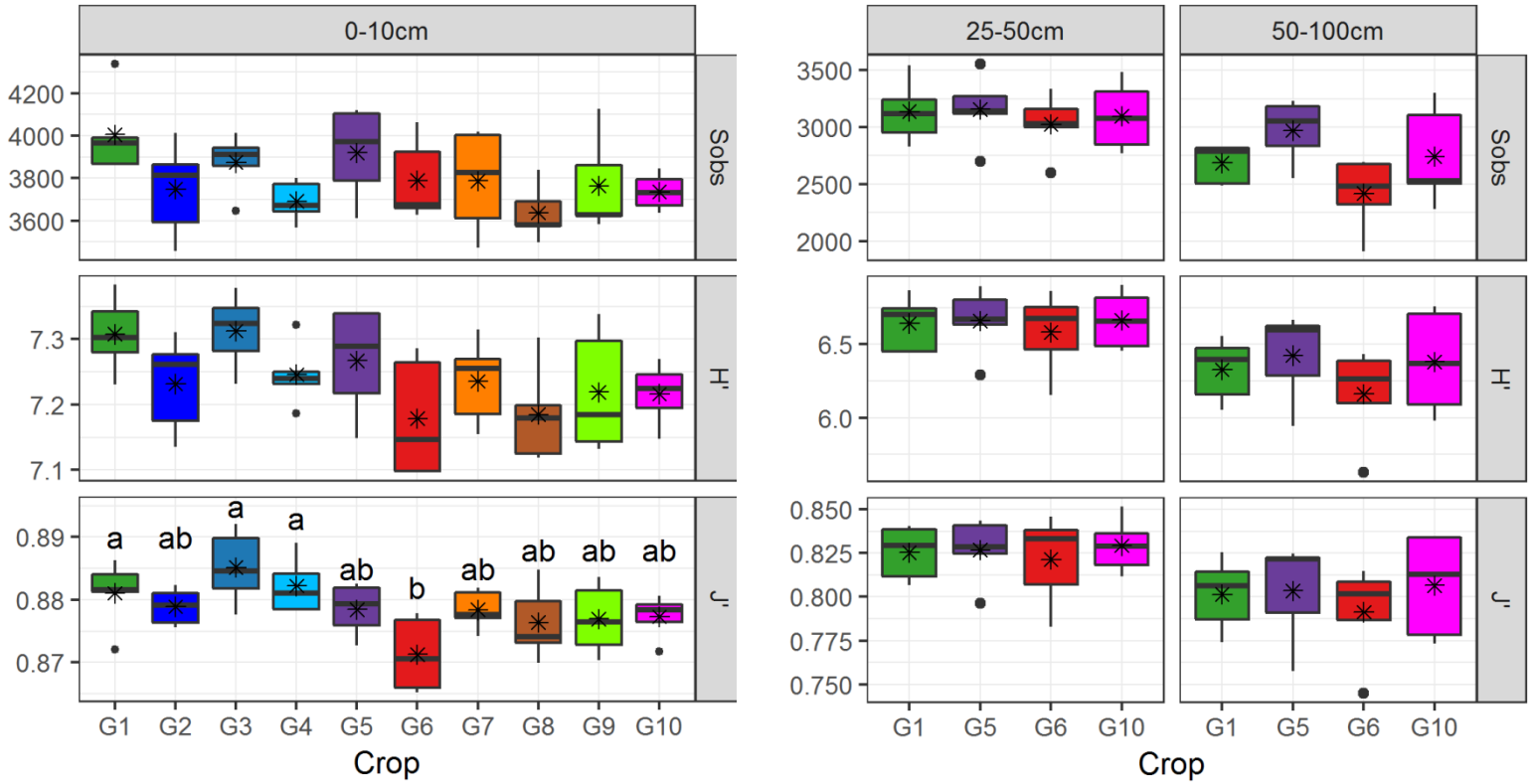

Figure S2 Comparisons of bacterial diversity indexes among soil under crops at depths of 0-10 cm, 25-50 cm, and 50-100 cm. The crop abbreviations are defined in Table 1. Stars represent the mean values. Letters indicate the ANOVA groupings ($\alpha <= 0.05$) among evenness estimates (J') for the crops. Significant differences among crops were found only for J' in the 0-10 cm layer.

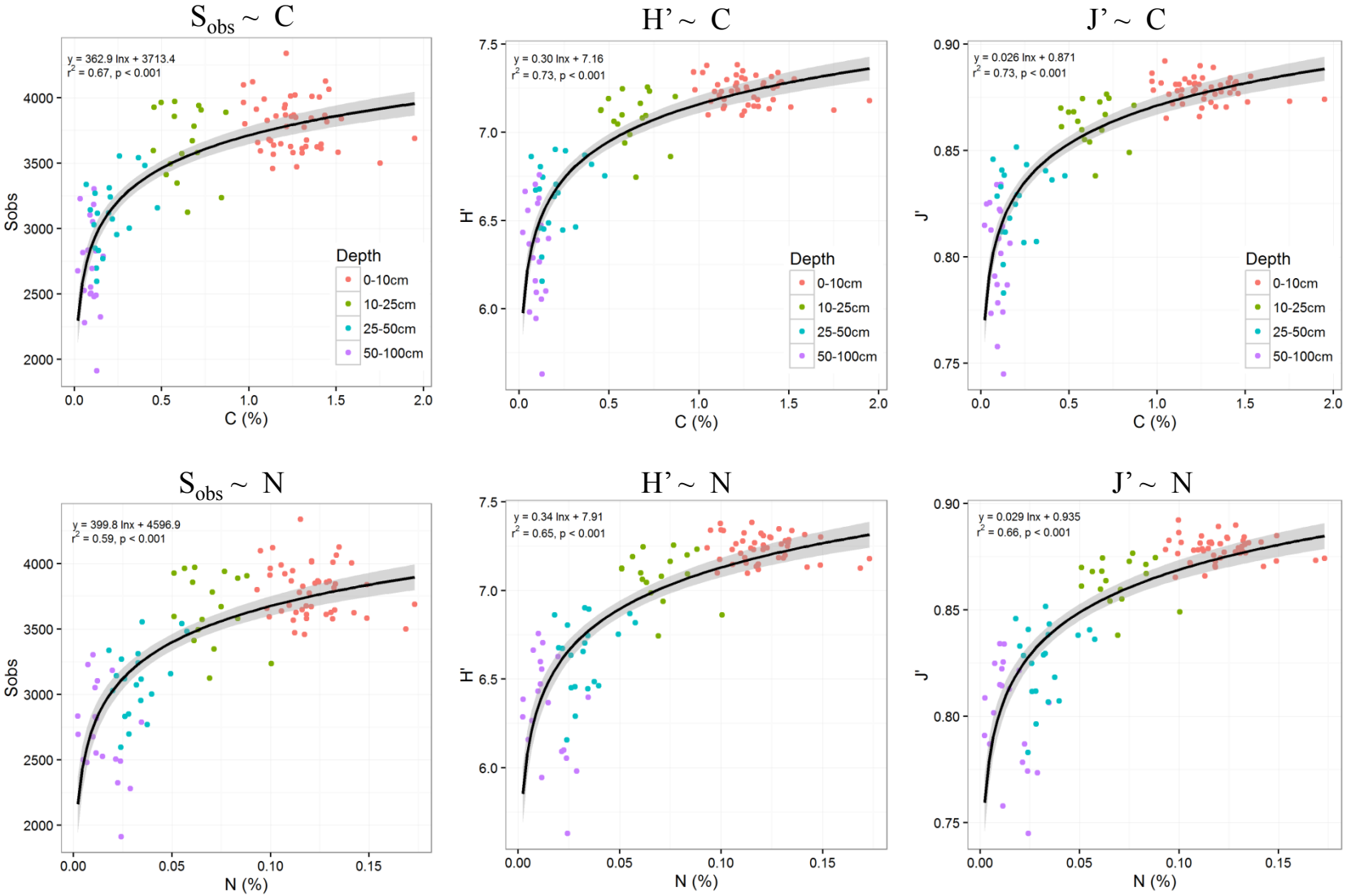

Figure S3 Relationships between diversity indexes (S_{obs}, H', and J') and soil C and N contents. The shadow is the 95% confidence interval of the regression.

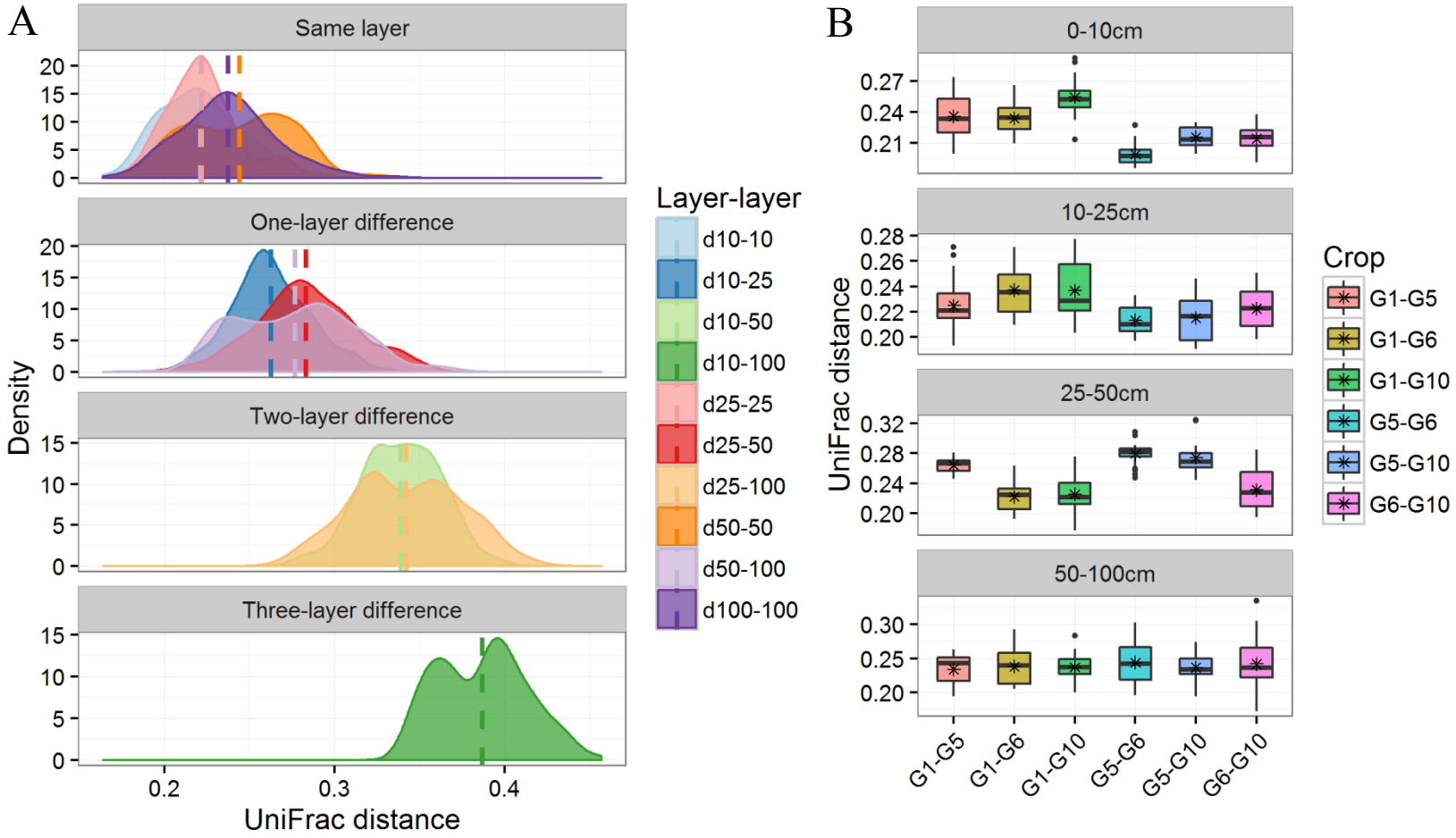

Figure S4 Pairwise generalized UniFrac distances between each two soil layers (A) and between two crops within the same layers (B), both based on G1, G5, G6,and G10. Panel A shows larger phylogenetic distances with larger spatial distance. Legend indicates the two layers compared. For example, "d10-10" means pairwise generalized Unifrac distance between samples from 0-10cm and 0-10cm. Vertical dashed lines indicate the averaged distance of each comparison. Panel B shows that bacteria under corn had larger phylogenetic distance from other crops in top 25cm, while switchgrass had the larger differences at 25-50cm. Crop abbreviations in legend are as in Table 1.

Table S1 Crop species and cropping histories of the ten biofuel systems. All aboveground biomass was typically removed at harvest (both grain and stover for corn-related systems, biomass taller than 10 cm for other crops).

Cropping systems	Crops at sampling time (2013)	Species	Cropping history	
			2008-2011	2012-
G1	Corn (no till)	*Zea mays*	Continuous corn (since 2008)	
G2	Corn + cover crop (no till)	*Zea mays* + *Pisum sativum* (Austrian winter pea) and *Secale cereale* (rye)	Corn-soybean-canola	Continuous corn + cover crop
G3	Soybean + cover crop (no till)	*Glycine max*+ *Pisum sativum* (Austrian winter pea) and *Secale cereale* (rye)	Soybean-canola-corn	Corn-soybean + cover crop
G4	Corn+ cover crop (no till)	*Zea mays*+ *Pisum sativum* (Austrian winter pea) and *Secale cereale* (rye)	Canola-corn-soybean	Soybean-corn + cover crop
G5	Switchgrass	*Panicum virgatum*	Switchgrass (since 2008)	
G6	*Miscanthus*	Hybrid *Miscanthus* x *giganteus*	Miscanthus (since 2008)	
G7	Native grass mix	*Panicum virgatum* (switchgrass) *Elymus canadensis* (Canada wildrye) *Andropogon gerardii* (big bluestem) *Schizachyrium scoparium* (little bluestem) *Sorghastrum nutans* (Indiangrass)	Native grass mix (since 2008)	
G8	Poplar	Hybrid *Populus nigra* x *P. maximowiczii 'NM6'*	Poplar (since 2008)	
G9	Early successional	*Solidago canadensis* (Canada goldenrod) *Setaria faberi* (giant foxtail) *Conyza canadensis* (horseweed) *Rumex obtusifolius* (broad-leaved dock) *Ambrosia artemisiifolia* (common ragweed)	Early successional (since 2008)	
G10	Restored prairie	*Panicum virgatum* (switchgrass) *Elymus canadensis* (Canada wildrye) *Andropogon gerardii* (big bluestem) *Schizachyrium scoparium* (little bluestem) *Sorghastrum nutans* (Indiangrass) *Koeleria cristata* (prairie Junegrass) *Desmodium canadense* (showy ticktrefoil) *Lespedeza capitata* Michx (roundhead lespedeza) *Baptisia leucantha* (white false indigo) *Rudbeckia hirta* (blackeyed Susan) *Anemone canadensis* (Canadian anemone) *Asclepias tuberose* (butterfly milkweed) *Silphium perfoliatum* (cup plant) *Monarda fistulosa* (wild bergamot) *Ratibida pinnata* (pinnate prairie coneflower) *Solidago rigida* (rigid goldenrod) *Solidago speciosa* (showy goldenrod) *Aster novae-angliae* (New England aster)	Restored prairie (since 2008)	

Cover crop is planted after harvesting corn or soybean.
Canola: *Brassica napus*
G9 species are composed entirely of whatever is in the seed bank, and the most common species were listed here.

Table S2 Correlation coefficients (r^2) and significances (P) of soil properties fitted onto db-RDA of Generalized UniFrac distance constrained by depth (G1, G5, G6, and G10) or by crop (all 10 crops) at 0-10cm.

Properties	Fitted to db-RDA by depth		Fitted to db-RDA by crops at 0-10cm	
	r^2	P	r^2	p
C	0.91	0.001	0.21	0.003
N	0.88	0.001	0.14	0.025
pH	0.44	0.001	0.45	0.001
P	0.22	0.001	0.16	0.018
K	0.24	0.001	0.08	0.178
Ca	0.33	0.001	0.30	0.001
Mg	0.24	0.001	0.59	0.001