**Detection of short-term cropping system-induced changes to soil bacterial communities
differs among four molecular characterization methods**

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Abstract

Perennial grass-based agroecosystems are under consideration as sustainable sources of bioenergy feedstocks. Establishing these systems on land previously used for conventional agricultural production is expected to dramatically alter the composition and functional capacity of their associated soil bacterial communities, but the rate at which these changes will occur is unclear. Methods for characterizing bacterial communities are both varied and useful for documenting different aspects of the soil microbiota and their dynamics during this transition. Here, we studied the soil-associated bacterial communities of continuous corn and restored prairies systems within a cropping systems experiment 2 to 4 years after establishment using 1) phospholipid fatty acid (PLFA) profiling, 2) shotgun metagenomic sequencing, 3) amplicon sequencing of the 16S rRNA gene and 4) sequencing of the nitrogen-cycling gene *nosZ*. All characterization methods discriminated the bacterial communities between the two cropping systems, but the largest differences were observed with PLFA profiling. Differences between the two cropping systems did not significantly increase during the study period. The community compositions described by sequence-based methods were mutually correlated, but were only weakly correlated to the composition described by PLFA profiling. Shotgun metagenomics detected a much higher abundance of Actinobacteria than amplicon sequencing and revealed more consistent changes between cropping systems over time. Cropping system and interannual effects on the ratios of biomarkers associated with Gram-negative and Gram-positive bacteria were entirely different for PLFAs, rRNA amplicons, and shotgun-sequenced 16S rRNA. Our findings highlight how soil bacterial community characterization methods differ in their detection of microbial community composition as a result of recent land use change.

Highlights

- 41 • Soil bacterial communities diverge rapidly between corn and prairie cropping systems
- 42 • Community differences do not increase from 2 to 4 years after divergence
- 43 • The extent of observed change varies among community characterization methods

44 **Keywords**

45 microbial community composition; phospholipid fatty acids; metagenomic sequencing; 16S
46 rRNA; *nosZ*; cellulosic bioenergy

1. Introduction

The interaction between soil microbial communities and their environment is a major driver of terrestrial agroecosystem dynamics. Microbial communities directly influence agroecosystem productivity and plant community composition (Callaway et al., 2004; Kardol et al., 2007), soil carbon dynamics (Bardgett et al., 2008), and major nutrient cycles (Hawkes et al., 2005; Nannipieri et al., 2003). Agroecosystems in turn influence the composition and function of soil microbial communities (Oehl et al., 2010; Sayer et al., 2013). The move to develop cropping systems that can produce biomass feedstocks for bioenergy and bioproducts in a sustainable manner has motivated increased interest in the ecological effects of introducing diverse perennial systems onto land previously used for agriculture (Robertson et al., 2008), including the effect on microbial communities (Li et al., 2015; Mao et al., 2013). There is substantial evidence that the composition and functional capabilities of a soil microbial community change when transitioning between conventional agricultural and less intensively managed perennial systems (Allison et al., 2005; Liang et al., 2012; Xue et al., 2013). However, the timing of microbial community change following such a transition remains unclear.

Soil microbial communities shift on a decadal timescale following establishment of perennial systems on soils with a previous history of annual cropping (Allison et al., 2005). Long-term shifts are not monotonic, with systems passing through transitional states as they continue to mature (Jangid et al., 2010); short-term dynamics are less well characterized. Soil microbial communities exhibit less temporal variation than those in other environments (Shade et al., 2013) possibly because of the prevalence of dormancy in soil microbiota (Lennon and Jones, 2011), and microbial community compositions may reflect land uses from decades in the past (Jangid et al., 2011). This apparent resistance to change suggests transitions following land use

change should occur in consistent, progressive steps toward major transitional or terminal states. At the same time, there is evidence that soil microbial communities are susceptible to external perturbation (Allison and Martiny, 2008) and that community succession can occur over a single growing season (Schmidt et al., 2007). From this perspective, microbial community transitions could be uneven in the short-term, exhibiting expected patterns only over longer periods of time. Given the linkage between microbial community composition and function (Frey et al., 2004; Reed and Martiny, 2007; Wakelin et al., 2008), the consistency with which soil microbial communities change following land use conversion could be important for conditioning expectations for microbially-mediated functions in during the establishment phase of perennial cropping systems.

Modern efforts to track changes in soil microbial communities typically employ culture-free characterization, particularly sequencing-based methods that interrogate the metagenome (Hirsch et al., 2010). These metagenomes are dominated by bacterial sequences (Fierer et al., 2012), effectively causing most recent work in soil microbiology to primarily reflect bacterial communities and dynamics. Many characterization methods focus on distinct aspects of a community, such as the taxonomic makeup of individuals or the relative abundance of functional genes, which could potentially differ in their responsiveness to land use change.

We previously used phospholipid fatty acid (PLFA) profiling to examine the soil microbial community of continuous corn and sown tallgrass prairie cropping systems during the two to four years following their establishment on historically agricultural soil (Herzberger et al., 2014). In the present study, we reexamine these soils, focusing on their bacterial component. To better resolve the phylogenetic and functional changes undergone by this community, we supplemented the bacterial PLFA data with amplicon sequencing of 16S rRNA and *nosZ*, a gene

that identifies denitrifiers, as well as functional genes and 16S rRNA sequences derived from shotgun metagenomics sequencing (SMG). Our goals were to compare how these four distinct methods characterized the divergence between the soil bacterial communities of recently-established corn (*Zea mays* L.) and sown tallgrass prairie, and evaluate whether yearly increases in this divergence were visible despite interannual variability in community composition.

2. Methods

2.1 Site description and soil sampling

This experiment was conducted on the Bioenergy Cropping Systems Experiment (BCSE) at Arlington Agricultural Research Station (Arlington, Wisconsin, USA, 43°18'10 N, 89°20'40 W). The BCSE consisted of eight cropping systems, including continuous corn and sown tallgrass prairie, in a randomized complete block design with five replicates (full details are given in Sanford et al., 2016). Prior to 2005, the site was used for agronomic trials and production, with corn, soybean, and alfalfa as the primary crops. From 2005 to 2007, the site was under a hayfield mix of alfalfa (*Medicago sativa* L.) and orchardgrass (*Dactylis glomerata* L.) (blocks 1 to 3) or corn (blocks 4 and 5). All treatments were established in spring 2008. The experiment is situated on a highly productive Plano silt loam (Fine-silty, Mixed, Superactive, Mesic Typic Arguidolls). These are deep (>1 m), well-drained soils with little relief, formed under tallgrass prairie vegetation in loess deposits over calcareous glacial till. Mean annual minimum and maximum air temperatures are -14.6 and 27.6 °C, respectively, with 869 mm mean annual precipitation.

Soils were sampled in late August in 2010, 2011, and 2012. For each plot, 5 cores (3.7 cm diameter, 15 cm depth) were collected in a staggered transect. Cores were immediately placed on wet ice, sieved to 2 mm within 24 h of sampling, and frozen at -20 °C within 48 h of

sampling. Frozen soils were lyophilized for storage prior to lipid and DNA extraction. All molecular characterization methods were performed on each individual sample.

2.2 PLFA extraction and quantification

We employed a combined phospholipid fatty acid (PLFA) and fatty acid methyl ester (FAME) extraction method, as described in Herzberger et al. (2014). Briefly, soils were extracted with 1:2 CHCl₃:CH₃OH with the extracted lipids saponified with NaOH and methylated with HCl. Lipids were analyzed with a Hewlett-Packard Agilent 6890A gas chromatograph (Agilent Tech. Co., Santa Clara, CA) equipped with a 25-m×0.2-mm×0.33-μm Agilent Ultra-2 (5% phenyl)-methylpolysiloxane capillary column (Hewlett Packard, Palo Alto, CA) and flame ionization detector. Fatty acids were identified using MIDI's EUKARY method database. We restricted our analysis to PLFAs frequently associated with bacteria, as described in Table S1 (Balser and Firestone, 2005; Frostegård et al., 1993; Hill et al., 2000; Wilkinson, 1988; Zelles et al., 1992).

2.3 DNA extraction

DNA was extracted from the soil using an adaptation of a method developed by Stevenson and Weimer (2007). Lyophilized soil was ground with liquid nitrogen in a ceramic mortar to disrupt aggregates. We prepared 2 tubes per sample, with each tube receiving 0.5 g ground soil (1 g total), 0.5 g 0.1-mm silica-zirconia beads (BioSpec Products, Bartlesville, OK), 1 ml extraction buffer (EB; 100 mM Tris-HCl, 10 mM EDTA, 0.15 M NaCl, pH 8.0), 50 μl 20% SDS, and 500 μl cold phenol (buffer equilibrated to pH 7.9). Tubes were bead beaten for 10 min on a converted paint shaker, incubated 10 min at 60 °C, then beaten an additional 10 min. Samples were separated by centrifugation (16,000 × g, 10 min) and the aqueous layer was washed successively with 500 μl phenol, 500 μl 1:1 phenol:chloroform, and 500 μl chloroform, with centrifugation (16,000 × g, 10 min) at each step and the aqueous layer brought to 1 ml volume with EB. To

precipitate, 900 μ l of aqueous layer was combined with 100 μ l 3 M Na acetate and 600 μ l isopropanol, incubated for 30 min at 4 °C and centrifuged (16,000 $\times$ g, 20 min). The DNA pellet was washed once with 70% ethanol, then both tubes from a sample were recombined in 150 μ l 1/10 TE (10 mM Tris pH8, 0.1 mM EDTA). These samples were subsequently cleaned with a Power Soil Cleanup Kit (Mo Bio Laboratories, Carlsbad, CA) following manufacturer instructions.

2.4 DNA sequencing and classification

We characterized the composition of the overall and denitrifying bacterial communities using 16S rRNA and *nosZ* amplicons. We amplified the V6-V8 variable region of the bacterial 16S rRNA gene using primers described by de Oliveira et al. (2013) and a region of the catalytic subunit of *nosZ* using *nosZF* (Kloos et al., 2001) and *nosZ1622R* (Throbäck et al., 2004) primers. Primer sequences are given in Table S2. Segments were amplified using 5-ng template DNA and 0.5 μ M of each primer in 25 μ l volume using Platinum Blue PCR SuperMix (Life Technologies, Carlsbad, CA). Polymerase chain reaction (PCR) was performed following de Oliveria et al (2013): denaturation for 2 min at 94 °C; 30 cycles of 30 s at 94 °C, 45 s at 50 °C, and 1.75 min at 68 °C; and a final extension for 10 min at 68 °C. We limited our amplification to 30 cycles to allow for detection of low-copy template and to ensure the overwhelming majority of amplicons would be created in the 15-25 cycle range (Lee et al., 1996). PCR products were size-selected by electrophoresis on a 1% AquaP ϕ r LM low-melt agarose (National Diagnostics, Atlanta, GA) TAE gel and recovered with a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA). Sequencing was carried out on a Roche 454 GS Junior Titanium sequencer following manufacturer protocols and using Lib-L emulsion PCR kits (Roche Life Sciences, Indianapolis, IN) with ~0.8 DNA molecules per bead.

The program *mothur* v. 1.33.3 (Schloss et al., 2009) was used to process 16S rRNA amplicon sequences. Reads were denoised using *shhh.flows*. Quality filtration removed sequences under 200 nucleotides or with >6 nucleotide homopolymers, >2 primer mismatches, or any barcode mismatches. Alignment was done against the SILVA reference database (Pruesse et al., 2007), removing sequences of eukaryotic origin. Chimeric sequences were removed using *chimera.uchime*. Operational Taxonomic Units (OTUs) were defined at 95% sequence identity and a representative sequence for each OTU was taxonomically classified with the Ribosomal Database Project Classifier (Wang et al., 2007) using an assignment cutoff of 0.8.

Processing of *nosZ* amplicons was similarly carried out in *mothur* through the quality filtration step. Further processing was conducted through the Functional Gene Pipeline (Fish et al., 2013). Unique sequences were translated with Framebot (Wang et al., 2013) with a minimum length of 66 amino acids and an identity cutoff of 0.3. These translations were aligned with Aligner (Cole et al., 2014), then clustered using *mcClust* (Fish et al., 2013). *NosZ* OTUs were classified at 90% amino acid sequence similarity, following Mao et al. (2013).

Shotgun sequencing was carried out at the Department of Energy Joint Genome Institute using the Illumina HiSeq platform (Illumina, San Diego, CA), multiplexing 12 samples per lane. Trimmed and screened reads were assembled using SOAPdenovo (v1.05). Gene calling was done with FragGenScan (v1.16), prokaryotic GeneMark.hmm (v2.8), Metagenome Annotator (v1.0), and Prodigal (v2.50). Reads identified as belonging to the 16S rRNA genes were taxonomically classified in the same fashion as the 16S rRNA amplicons. Reads were annotated using the updated clusters of orthologous groups (COGs) database (Galperin et al., 2015; Tatusov et al., 2000).

2.5 Statistical analysis

Data were analyzed in the R statistical package and environment (v3.1.1, R Core Team, 2014). Multivariate analyses used the ‘vegan’ package (Oksanen et al., 2013). Prior to multivariate analysis, individual PLFAs, functional genes, or OTUs were removed from the data if not present in at least three out of five blocks and two out of three years. We did not relativize PLFA values by sample, as PLFA extractions are quantitative (Liang et al., 2012). COG abundances were relativized following He et al. (2015). COG read counts were first relativized by the length of their consensus sequence to neutralize the overrepresentation of longer sequences. We then used the mean abundances of 37 COGs present in a single copy in nearly all prokaryotic genomes (Table S3) to relativize across samples. Amplicon read counts were relativized to the total number of reads in the sample. Euclidian distances were used for PLFA distance matrices, while Bray-Curtis distances were used in all other cases.

Standard statistical comparisons used linear mixed effects models from the ‘lme4’ package (Bates et al., 2015) using block as a random term. Means testing was done with the ‘lsmeans’ package (Lenth, 2013).

2.6 Accession numbers

PLFA data are available on the Dryad database (<http://datadryad.org>) under doi: 10.5061/dryad.rk384. Amplicon sequences were deposited in the NCBI Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) as BioProject 279094. Shotgun metagenomes are in the Joint Genome Institute Genomes Online Database (<https://gold.jgi.doe.gov>) under Study ID Gs0095510. Accession numbers for individual samples are given in Table S4.

3. Results

3.1 Sequencing overview

Shotgun metagenomic (SMG) sequencing generated 1.3 to 2.0 Gbp per sample (48.3 Gbp total), with 2.4 to 4.0×10^6 functional gene reads of which 2,151 to 8,135 reads per sample mapped to the 16S rRNA. Diversity and coverage statistics for individual samples are given in Table S4. With amplicon sequencing, we generated 2,382 to 6,206 reads per sample for 16S rRNA and 1,116 to 6,681 reads per sample for *nosZ*. We found 3,717 operational taxonomic units (OTUs) using 16S rRNA amplicon sequencing (490 to 750 OTUs per sample), and 1,072 *nosZ* OTUs (150 to 290 per sample). Good's coverage for 16S rRNA amplicons (89 to 95%, 98.5% globally) was lower than for *nosZ* amplicons (93 to 99%, 99.6% globally). Shannon's diversity indices for 16S rRNA OTUs were lower for 2010 corn than for all other crop-year combinations, which were not different from each other, while Shannon's diversity indices for *nosZ* OTUs were the same for all crop-year combinations (Table S5). Read number was correlated to unique OTUs for 16S rRNA amplicons ($r = 0.79$, $P < 0.0001$), but not for *nosZ* amplicons ($r = 0.14$, $P = 0.22$). Within samples, unique OTUs for both amplicon types were correlated ($r = 0.77$, $P < 0.0001$), but total read numbers were not ($r = 0.21$, $P = 0.13$).

3.2 Factors determining microbial community composition

We used *adonis*, a permutational analogue to multivariate analysis of variance, to quantify treatment effects on overall community composition (Table 1). All characterization methods found statistically significant cropping system and interannual effects, although interannual effects were only weakly significant in the PLFA and *nosZ* data. The amount of variation these factors explained differed among methods. Much of the variation in the PLFA data existed between cropping systems, while interannual effects were less important. Conversely, more of the variance in the SMG COG and rRNA amplicon data existed among years than between systems. These were the only methods to have significant interactions between years and

cropping systems, likely driven by the 2010 continuous corn samples (Fig. 1B, C). Soil carbon and nitrogen concentrations did not change between 2009 and 2013 and were not different between the two cropping systems (Table S5). Soil bulk density did not differ between cropping systems in 2009, but was lower overall in 2013, more so in the sown tallgrass prairie than in the continuous corn system (Table S5).

3.3 Ordination of community compositions

Two-dimensional NMDS ordination adequately represented the community compositions found by all four characterization methods (Fig. 1), although two-dimensional stress was greater for the two amplicon sequencing datasets (Table 2). PLFA profiles of the continuous corn and restored prairie bacterial communities were clearly distinct, with greater variability within the prairie communities (Fig. 1A). By contrast, in both rRNA amplicon and SMG COG profiles there was more variability in the corn system than the prairie system, largely stemming from the differentiation of 2010 corn-associated soil from all other systems (Fig. 1 B, C). Despite overall separation of the bacterial communities from both cropping systems, there was no indication that this separation changed over the study period. In the *nosZ* amplicon data, both corn and prairie communities shifted in the same direction in 2011 before reversing that transition in 2012 (Fig. 1 D).

The three DNA-based characterization methods were correlated both in the Bray-Curtis distances between pairs of samples and in the distances among samples following NMDS ordination (Table 2). The SMG COG and 16S rRNA amplicon data were particularly well correlated. Inter-sample distance correlations between PLFA and the DNA-based data were significant, but generally weaker (for exact values, see Table 2).

3.4 Taxonomic profiles from amplicon and short read sequencing

We obtained 16S rRNA sequences from amplicons and SMG data, with roughly equal numbers of reads generated from both methods (Table S4). At the phylum level, both datasets were dominated by Actinobacteria, Acidobacteria, and Proteobacteria (Fig. 2, Table S6). In both datasets, Verrucomicrobia abundance increased over time and was higher in sown tallgrass prairie than corn cultivation. Similarly, both datasets observed high γ -Proteobacteria abundance in the corn system in 2010.

Beyond these similarities, the taxonomic profiles generated by amplicon and SMG sequencing contained substantial differences. The proportions of reads assigned to a given phylum differed significantly between the two sequencing approaches for all common phyla (Table S6, common phyla defined as accounting for >5% of all reads from at least one of the systems). Actinobacteria were twice as abundant in the SMG sequences as in the amplicon sequences. The SMG sequence data also contained cropping system and temporal trends that were not present in the amplicon sequence data. In the SMG data, Firmicutes and Bacteroidetes were significantly more abundant in sown tallgrass prairie than continuous corn cultivation. SMG reads from major Gram-positive phyla (Actinobacteria and Firmicutes) became rarer over time, while reads from rare or unidentified taxa became more abundant.

3.5 Functional group biomass and gene abundance

We compared ratios of PLFAs that are frequently associated with either Gram-positive or Gram-negative bacteria (Table S1) to ratios of SMG and amplicon 16S rRNA reads grouped by phylum-level Gram-staining properties (Table S6). Interannual and cropping system effects on these ratios differed among the three community characterization methods (Fig. 3). PLFA biomass associated with Gram-negative taxa increased in the prairie system, but did not change over time. In contrast, SMG 16S rRNA reads belonging to non-staining phyla were not different

between cropping systems but increased in relative abundance over time. There were no interannual or cropping system effects in the 16S rRNA amplicons.

4. Discussion

4.1 Characterization methods differed in their detection of cropping system divergence

In this study, we used four methods to characterize change over time in the bacterial communities of continuous corn and sown tallgrass restored prairie cropping systems. All methods revealed significant differences between systems, but varied in the extent of the differences they showed. The greatest cropping system effects were observed using bacterial PLFA profiles, which produced similar results to those previously observed using PLFAs from the entire microbial community (Herzberger et al., 2014). PLFAs associated with Gram-negative bacteria were more abundant in the prairie, matching the difference observed between corn and prairie sites throughout south-central Wisconsin (Liang et al., 2012). Differences between cropping systems were considerably smaller, but still significant, for the DNA-based methods.

Biomass-based microbial community measures, such as PLFA, may be more responsive to environmental change than those based on DNA. Prior studies have observed responses to seasonal variability (Jangid et al., 2011, 2010) and vegetation composition (Ritz et al., 2004) in lipid profiles, but not in 16S rRNA data. The apparently limited responsiveness of DNA-based characterization methods may in part stem from the prevalence of dormant organisms (Lennon and Jones, 2011) as well as the potential persistence of DNA from nonviable organisms (Córdova-Kreylos et al., 2006), both of which could delay detection of changes in the microbial community. Our results highlight the continued value of lipid profiling as a valuable tool for assessing microbial community change and a useful complement to metagenomic methods.

We expected that the sown tallgrass restored prairie system would gradually and progressively shift its associated bacterial community toward compositions typically found under restored prairies in this region (Allison et al., 2005; Liang et al., 2012). Progressive divergence from the corn system bordered on statistical significance in the PLFA data, similar to increasing trends in AMF, Gram-negative, and total microbial biomass we had previously reported (Herzberger et al., 2014), and suggested that continued measurement of the systems would have captured increasing divergence. For DNA-based methods, however, we saw no indication of increasing differentiation during the study period. Increases in PLFA biomass are detectable in the top 5 cm of soil shortly after prairie restoration, but are only visible at greater depths once the restoration has been in place for considerably longer (Allison et al., 2007). By sampling from the top 15 cm of soil, we may thus have diluted the more rapidly shifting near-surface microbial community. This dilution may not have been as impactful for PLFA profiles, which should reflect the concentration of microbial biomass in the top 5 cm of soil (Fierer et al., 2003), as it would be for DNA-based methods, potentially explaining the apparent difference in their responsiveness. At the same time, we observed considerable interannual variability in bacterial communities, suggesting our observations were not driven solely by a slower response to land use change by bacteria deeper in the soil profile. The dramatic shift in the composition of corn associated bacteria between 2010 and 2011, observed in both the 16S rRNA and SMG data, best exemplifies this. Soil microbial communities may exhibit considerable seasonal variability (Schmidt et al., 2007). Some of the interannual variability we observed may have been due to slight differences in the seasonal timing of our sampling. Nonetheless, all methods detected differences between the two cropping systems only two years after their establishment. The change in disturbance regimes may have spurred the initial rapid differentiation (Allison et al.,

2005; Jangid et al., 2010), with broader cropping-system differences driving more gradual shifts in the community.

A variable not directly addressed in our work is the impact of storage on sample quality. There are varying views on the appropriate time a soil may be stored unfrozen prior to microbial analyses (Bloem et al., 2005). Although it is generally accepted that freezing and extraction should happen as soon after sampling as possible, some guidelines suggest soils may be stored under refrigeration for several weeks prior to PLFA extraction (Palojärvi, 2005) and in well-drained soils no changes in PLFA biomass were observed over 30 days of refrigeration (Wang et al., 2014), although other studies report changes within shorter time periods (Wu et al., 2009). Soils from our two cropping system might have responded differently to storage. Agricultural soils may be less perturbed by storage than grassland or forest soils (Cui et al., 2014), possibly because microbial communities in the latter systems typically receive more labile carbon (Gonzalez-Quiñones et al., 2009). Although no differences in total soil carbon developed during our study period (section 3.2), it is possible that a difference in the pool of available carbon could have gone unnoticed. If so, the sown tallgrass prairie, with its more active rhizosphere, could have lost more biomass during storage than the corn-associated soils, causing us to underestimate divergence between the two systems.

4.2 DNA-based methods identified similar community compositions

Soil bacterial community composition was well correlated among DNA-based methods. Fierer et al. (2012) reported high correlation between taxonomic composition and functional gene abundance over an ecological gradient that included arctic tundra, desert, and tropical forests. Our findings suggest this relationship may hold within a gradient of ecologically similar systems. Although many functional genes in bacteria are not restricted to a narrow set of taxa (Ragan and

Beiko, 2009), there are likely linkages between taxonomic and functional composition at the community level (Langille et al., 2013).

We observed limited *nosZ* diversity, dominated by a small number of taxa, similar to previously published results (Ruiz-Rueda et al., 2009). It is also worth noting that the *nosZ* primers we used do not capture ‘atypical’ *nosZ* genes (Orellana et al., 2014; Sanford et al., 2012), indicating our analyses exclude a sizeable component of the denitrifying community. It is, however, interesting that the number of unique *nosZ* types we observed in a sample correlated strongly with the number of unique 16S rRNA OTUs. This suggests that the subset of the *nosZ*-containing community we sampled largely responded to broad drivers of bacterial diversity, even though *nosZ* community composition did not display the same patterns as compositions based on taxonomic or functional gene abundances.

4.3 Characterization methods detected different abundances of specific groups

The abundance of specific bacterial groups was more influenced by interannual and cropping system effects in the SMG sequences than the 16S rRNA amplicon sequences. There were phylum-level abundance differences between the cropping systems in the SMG data that were not present in the amplicon data, but the inverse was not true. SMG-derived 16S rRNA sequences have several advantages over amplicon sequences, notably in the avoidance of primer biases (Logares et al., 2013). Primer biases were likely behind the underrepresentation of Actinobacteria in our 16S rRNA amplicon data. Fierer et al. (2012) found a similar underrepresentation of Actinobacteria in amplicon data, albeit using a different 16S rRNA region. Although abundance of Bacteroidetes and Firmicutes did not differ markedly between methods, only SMG data detected cropping system differences in their abundance. The increased abundance of γ -Proteobacteria in the 2010 continuous corn soils stands as an interesting

counterpoint, as this dynamic was present in both datasets. It seems reasonable that γ -Proteobacteria were similarly well detected by both methodologies, and further implies that many responses to cropping system and interannual effects occur in bacterial groups that were less well detected by our amplicon sequencing.

The interpretation of certain PLFAs as indicative of Gram-positive or Gram-negative bacteria is problematic, both because PLFAs are not strict phylogenetic markers (Frostegård et al., 2011), and because Gram staining is of increasingly limited utility as a framework for classifying bacteria. Nonetheless, this terminology remains common in soil microbiology, possibly because these sets of lipids frequently exhibit coherent and interesting ecological behaviors. Rhizosphere-extracted PLFAs associated with Gram-negative bacteria are enriched in carbon from plant exudates (Butler et al., 2003), while both sets of PLFAs can correlate to distinct nitrogen cycling processes in the soil (Balser and Firestone, 2005). In our earlier study, we found that PLFAs associated with Gram-negative organisms increased in the sown tallgrass prairie while PLFAs associated with Gram-positive organisms were similar for both systems (Herzberger et al., 2014). In this study, we tested whether these PLFA-based patterns behaved similarly to DNA from phyla that could be classified as Gram-positive or Gram-negative, finding that the two methods had entirely different dynamics. This may indicate a mismatch between classification methods, microbial activity vs abundance, or be another instance of the differences in how PLFAs and DNA respond to environmental influences (section 4.1). Our findings provide further evidence that comparison of this set of fatty acids can be an ecologically informative aspect of PLFA profiles. We hope this further highlights the need to revisit the use of the Gram-negative/-positive framework for interpreting these fatty acids and the potential utility of identifying the taxa that these biomarkers actually represent.

5. Conclusions

The detected soil bacterial community composition of continuous corn and restored tallgrass restored prairie cropping systems were found to be different by all four characterization methods we employed. The systems differed more in the composition of their bacterial community biomass, as detected by PLFA profiling, than in their functional gene or taxonomic compositions as detected by DNA amplicon sequencing. Differences between the two cropping systems did not increase over the three years that we sampled the soils. All characterization methods identified some effect of prairie establishment, but with DNA-based methods interannual variability appeared to have an equivalent impact. Overall community compositions detected by the alternative methods were fairly well correlated, although abundance estimates for specific taxa could vary substantially among methods. We found that soil microbial communities could change considerably from year to year, but that these changes did not contribute toward a progressive divergence or convergence between two very different land uses.

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Figure 1. NMDS ordinations of soil microbial communities based on A) PLFA profiling (see Table S1 for list of PLFAs); B) shotgun sequenced functional gene abundances; C) 16S rRNA amplicon sequence OTUs; and D) *nosZ* amplicon clusters. Line patterns link individual plots sampled over successive years. Grey areas represent ordination hulls for the two cropping systems.

Figure 2. Proportion of reads assigned to select bacterial phyla and classes detected in 16S rRNA sequences from S) shotgun metagenomic and A) amplicon sequence data. Bars represent arithmetic means of five independent samples. Rare taxa are defined as contributing < 5% of the total reads for both sequencing types.

Figure 3. Ratios of biomarkers associated with Gram-negative and Gram-positive phyla. The PLFA ratio is based on indicator lipid biomass (see Table S1 for list of PLFAs); DNA-based ratios are derived from phylum-level assignments of 16S rRNA gene reads from either shotgun metagenomic (SMG) or amplicon sequencing (phylum details in Table S6). Values are arithmetic means (± 1 standard error) of $\log_2$ -transformed ratios. Within a method, groups sharing a letter are not significantly different ($P > 0.05$).

Table 1 Factors accounting for multivariate variance from four methods assessing microbial community change over time between corn and prairie cropping systems

Factor	<i>df</i>	PLFA			SMG functional genes			16S rRNA amplicon OTUs			<i>nosZ</i> amplicon OTUs		
		SS	R ²	<i>P</i>	SS	R ²	<i>P</i>	SS	R ²	<i>P</i>	SS	R ²	<i>P</i>
Cropping system	1	0.071	0.62	<0.01	0.001	0.13	<0.01	0.173	0.09	<0.01	0.352	0.15	<0.01
Year	2	0.008	0.07	0.04	0.003	0.23	<0.01	0.361	0.20	<0.01	0.225	0.10	0.05
System × Year	2	0.006	0.06	0.07	0.001	0.08	0.04	0.181	0.10	<0.01	0.069	0.03	0.99
Residuals	24	0.029	0.25		0.007	0.56		1.121	0.61		1.682	0.72	
Total	29	0.114			0.012	1.00		1.836			2.327		

PLFA: bacterial phospholipid fatty acids; SMG: shotgun metagenomic sequencing; OTU: operational taxonomic unit; SS: sum of squares. Values estimated using *adonis*. *P*-values based on 9999 data permutations.

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Figure 1

2010 2011 2012

Continuous corn ● → ● → ●
Restored prairie ■ → ■ → ■

Figure 2

Figure 3