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REGULAR ARTICLE

Long-term fertilizer and crop-rotation treatments differentially affect soil bacterial community structure

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Abstract
Background and aims Soil microbial communities influence nutrient cycling, chemistry and structure of soil, and plant productivity. In turn, agronomic practices such as fertilization and crop rotation alter soil physical and chemical properties and consequently soil microbiomes. Understanding the long-term effects of agronomic practices on soil microbiomes is essential for improving agronomic practices to optimize these microbial communities for agricultural sustainability. We examine the composition and substrate-utilization profiles of microbial communities at the Morrow Plots in Illinois.
Methods Microbial community composition is assessed with 16S rRNA gene sequencing and subsequent bioinformatic analyses. Community- level substrate utilization is characterized with the BIOLOG EcoPlate.
Results Fertilizer and rotation treatments significantly affected microbial community structure, while substrate utilization was affected by fertilizer, but not crop-rotation treatments. Differences in relative abundance and occurrence of bacterial taxa found in fertilizer treatments can explain the observed differences in community level substrate utilization.
Conclusion Long-term fertilization and crop-rotation treatments affect soil microbial community composition and physiology, specifically through chronic nutrient limitation, long-term influx of microbes and organic matter via manure application, as well as through changes in soil chemistry. Relatively greater abundance of Koribacteraceae and Solibacterales taxa in soils might prove useful as indicators of soil degradation.

Keywords Fertilization · Crop-rotation · Carbon source utilization patterns · Indicator species · Microbial community composition · 16S ribosomal- RNA gene sequencing

Introduction

Long-term agricultural productivity and sustainability relies on positive interactions between three key components with which crops interact – soils, microbial communities in the soils, and agricultural management practices. The soil environment plays a role in selecting the resident microbial community. In turn, microbial communities in soils drive biogeochemical cycles of critical nutrients such as nitrogen and carbon and can have positive or negative interactions with crops. Agricultural management practices affect the chemical and physical characteristics of soils over the long term and can also directly influence the composition of the microbial communities in these soils. Agricultural soils are

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a critical but constrained resource that is under increasing pressure due to climate change and population growth, as well as susceptible to deterioration through suboptimal agronomic management practices (Doran and Smith 1987; Greenland 1981; Tilman et al. 2002). Agronomic practices, including fertilizer treatments and crop rotations, can positively or negatively affect properties of soils that influence agricultural productivity (Angers et al. 1993a; Aref and Wander 1997; Dick 1992; Havlin et al. 1990; Khan et al. 2007; Lupwayi et al. 1998; Mitchell et al. 1991; Mulvaney et al. 2009; Six et al. 2006; van Diepeningen et al. 2006). In contrast to processes like soil erosion and compaction that can occur rapidly, changes in the chemical properties of soils (e.g. organic matter content, capacity to retain moisture, aggregate structure, etc.), often manifest themselves far more slowly – on the order of decades – even under sharply contrasting management practices (Aref and Wander 1997; Darmody and Norton 1993; Khan et al. 2007; Mitchell et al. 1991; Mulvaney et al. 2009; Nafziger and Dunker 2011). While changes in the amount and chemical forms of key plant nutrients as well as pH can be altered by fertilization in the short term without causing substantive changes in the soils' chemical and physical properties, these properties can change with agronomic management over decades (Rivard et al. 2015; Velde and Peck 2002). Therefore, it is likely that the feedback effects between the soil microbiome and soil chemistry also manifest on similarly long time scales. Well-documented and consistently maintained long-term agricultural research plots can provide important information about the long-term effects of agronomic practices on agricultural soils (Enwall et al. 2007; Hallin et al. 2009; Neumann et al. 2013; Powlson et al. 2014; Widmer et al. 2006). The Morrow Plots at University of Illinois were established in 1876 with continuous-corn treatment and unfertilized treatments that date back to establishment. The current suite of modern fertilizer and crop-rotation treatment combinations have been in place since 1968. Therefore, the Morrow Plots are a unique and valuable resource for investigating the long-term effects of agronomic management on soil microbiomes and soil quality.

Microbial communities exhibit immense diversity in terms of structure (i.e. community composition) as well as function (i.e. physiology of all species combined) that vary from place to place as well as over time, but the interactions between microbial communities in the soil and their environment remain poorly understood (Giller et al. 1997; Horner-Devine et al. 2004; McGuire and Treseder 2010). This is in part due to the fact that the vast majority of the highly diverse microbes in soils cannot be cultured and characterized individually (Kirk et al. 2004). As complex microbial communities in soils are a critical component of the agricultural ecosystem, we must improve our understanding of how they interact with agronomic practices, soil, crops, and the environment. This knowledge is critical because microbial communities drive the biogeochemical processes that affect nutrient and organic matter composition of the soil (Balser and Firestone 2005; Jackson et al. 2003; Nannipieri et al. 2002) as well as the soils' environmental functions. Improved knowledge of microbial communities could help reduce nitrate runoff and emission of greenhouse gases (Cavigelli and Robertson 2000; Kramer et al. 2006). Characterizing the structure and functions of microbial communities from long-term agronomic research plots can provide insights into the mechanisms underlying the effects of agronomic practices on soil properties (Chu et al. 2007b; Jackson et al. 2003; Neumann et al. 2013; Wessén et al. 2010b) and may also lead to development of indicators of soil health, environmental function, and agricultural productivity (Franchini et al. 2007; Mummey et al. 2002; Otto et al. 2005; Pascual et al. 2000; Powlson et al. 1987).

Fertilizer treatments affect microbial communities through direct influence on soil nutrient content and chemistry (Peacock et al. 2001; Zhong et al. 2010). Crop rotations can affect soil microbial communities through differences in plant-microbe interactions (Larkin 2008; Navarro-Noya et al. 2013). Both fertilizer treatments and crop-rotations also influence soil microbial communities through differences in the quantity and composition of root exudates (Baudoin et al. 2003; el Zahar Haichar et al. 2008), rhizodeposits and above-ground crop residues (Bending et al. 2002; Benizri et al. 2002; Butler et al. 2003; Miller et al. 2008; Navarro-Noya et al. 2013; Nelson and Mele 2006; Paterson et al. 2007). While modern techniques have been employed in numerous studies to understand the effects of medium to long-term (10 years or more) fertilizer treatments on soil microbiota (Börjesson et al. 2012; Chu et al. 2007a; Clark and Hirsch 2008; Hirsch et al. 2010; Kamaa et al. 2011; Neumann et al. 2013; Ogilvie et al. 2008; Wessén et al. 2010a; Wessén et al. 2010b; Widmer et al. 2006; Wu et al. 2011), the effect of medium-term or long-term combined fertilizer and crop-rotations treatments on

microbial communities appears to have been studied with modern methods in very few instances (Navarro-Noya et al. 2013; Souza et al. 2015; Xuan et al. 2012; Yin et al. 2010) primarily because long-term experiments with crop-rotation and fertilizer treatment combinations are extremely rare. To improve agronomic practices for enhancing long-term sustainability, it is therefore essential to be able to predict the potential long-term effects of existing and improved practices on agricultural soils and their microbial ecosystems. Extant, well-maintained long-term field-experiments with a range of crop-rotation and fertilizer treatments are therefore a valuable resource for building our understanding of long-term effects of agronomic management.

In this study, we characterize the composition, diversity, and richness as well as community-level substrate utilization of soil microbiomes under contrasting fertilizer and crop-rotation treatments. While chemical and structural characteristics of arable soils under long-term management treatments have been previously characterized (Aref and Wander 1997; Darmody and Norton 1993; Khan et al. 2007; Mitchell et al. 1991; Mulvaney et al. 2009; Nafziger and Dunker 2011), understanding the long-term effects of agricultural management on the structure and diversity in microbial community function is a critical knowledge gap. Characterizing soil microbial communities under contrasting management practices will enable us to discover the drivers of differences between the microbial communities as well as the potential long-term effects of divergent microbial communities on the productivity and sustainability of agricultural soils.

Materials and methods

Research site

The Morrow Plots, located on the campus of the University of Illinois at Urbana-Champaign, are the oldest continuously maintained agricultural research plots in the United States. The plots were established in 1876 with the intention of conducting experiments useful to farmers. While the plots have generated valuable crop-yields and soil chemistry data for over 130 years, microbial communities in the plots have not yet been systematically examined. The fertilizer and crop rotations have changed gradually since establishment of the plots. The current basic rotations and fertility treatments have been in place since 1968; small modifications have been made to tillage (with conversion from moldboard plowing to chisel plowing), planting density, crop variety, and herbicide choice, during that ~50 year period. Briefly, the current experiment (Fig. S1) consists of three blocks of crop rotation treatments – one block each of continuous corn (*Zea mays*) (C), a two-year corn and soybean rotation (CS), and a three-year corn-oats-alfalfa rotation (COA), with an 8 ft margin between blocks. It is important to note that the continuous corn and unfertilized treatments date back to 1876, making these the oldest continuously managed agricultural research plots in the western hemisphere. Even though the rotation blocks are not replicated, they are one of the best resource available for understanding the effects of very long-term agronomic treatments on soils and the microbial communities in them. Each crop rotation block is split into eight plots comprising replicated fertilizer treatments: unfertilized (UF); inorganic fertilizers (IN) with nitrogen (as urea), phosphorus (as P_2O_5), potassium (as K_2O), and limestone; and organic fertilization (OR) with cattle manure, limestone, and phosphorus. Corn yields from fertilized plots and from blocks with crop rotations have been consistently higher (Fig. S2). For a comprehensive description of the treatment history, site conditions, and yields, please refer to the description by Aref and Wander (Aref and Wander 1997) and Odell et al. (Odell et al. 1982).

Sample collection and processing

Soil samples were collected on a single day late in the growing season in 2009 – a year when all the plots were planted in corn. Sampling during the all-corn year controlled for transient effects of plant-microbe interactions from the different crops, enabling the investigation of long-term effects of crop-rotation on the microbial communities. Each sample consisted of five cores (1.9 cm dia × 12 cm deep). All cores were collected at least 3 m from the edge of the plot in order to minimize edge effects. The samples represent bulk soil, not rhizosphere, as the cores were collected in the planting rows, but away from the root zone. The cores from each plot were placed in sealed plastic bags on ice while in the field and transported back to the lab and processed within two hours of collection. The cores from each plot were composited and homogenized with a 2 mm sieve. Subsamples from each the composited, homogenized soil sample were processed as appropriate for chemical analyses, community level physiological profiling with

BIOLOG, and DNA extraction for bacterial community bioinformatic analyses as described below.

Microbial community physiological profiling

Substrate utilization-based fingerprints of the soil microbial communities were generated with the BIOLOG assay using EcoPlates™ (Biolog®, Hayward, CA, USA) – a 96 well plate with three replicates of 31 individual substrates and one control (no substrate). A suspension of the soil microbial community was created by vortexing 0.5 g fresh soil with glass beads in 2 mL sterile PBS for 2 min and then centrifuging the mixture at 4 °C and 750 x g for 6 min to precipitate large soil particles and debris. The supernatant was diluted 1:25 with sterile PBS and 150 μL of the diluted supernatant was added to each well of the EcoPlate™. The plates were incubated in the dark at room temperature. Metabolic response of the community to available substrate in each well was measured by recording the absorbance at 590 nm daily for 6 days.

DNA extraction

DNA from the microbial community of the Morrow Plots soil samples was extracted using FastDNA for Soil kit from MP Biomedicals (Solon, OH, USA). The extracted DNA was purified using CTAB (cetyl trimethyl ammonium bromide) purification process to remove humic contaminants (Sambrook et al. 1989). Extracted DNA was stored at −20 °C until used in DNA-based analyses described below.

Bacterial community 16S rRNA gene sequencing

Bacterial community composition was assessed by sequencing the V4 – V5 region of 16S rRNA gene using the PCR primers 515F and 926R(Baker et al. 2003; Dojka et al. 1998; Haas et al. 2011). Primers were modified with adapter sequences required for Illumina sequencing (Caporaso et al. 2011) and a unique dual-index barcode was assigned to each sample (Hamady et al. 2008). To prepare the templates for sequencing, 50 μl PCR reactions with following composition were conducted: 25uL 2X KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Woburn, MA, USA), 500 μM of each primer and 50 ng DNA. Thermal cycling was carried out with an initial denaturation at 98 °C for 45 s. followed by 25 cycles of 98 °C for 15 s., 65 °C for 30 s., 72 °C for 30 s., and ended with a final extension step at 72 °C for 2 min. Amplicons from each sample were cleaned with 0.8 X volume of AMPure® XP beads (Agencourt Bioscience, Beverly, MA, USA) and then quantified on the Qubit® Fluorometer (Invitrogen-Molecular Probes, Eugene, OR, USA) using the Quant-iT™ dsDNA HS Assay kit (Invitrogen, Carlsbad, CA, USA). Equal concentrations of amplicons from each sample were pooled for sequencing. The amplicon pool was sequenced at W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign (Urbana, IL, USA) on Illumina MiSeq platform with a 2 × 250 bp reads configuration using the MiSeq V2 Nano flowcell (Illumina, San Diego, CA, USA).

Each sequence was assigned to its original sample according to the barcode. After de-multiplexing, the forward and reverse read of each paired-end sequence were merged using software FLASH (Fast Length Adjustment of SHort reads) (Magoč and Salzberg 2011). Sequences with more than 10 % bases with quality score lower than 30 and sequences containing ambiguous bases "N" were removed from downstream processing. Filtered sequences were clustered into operational taxonomic units (OTUs) using USEARCH (Edgar 2010). USEARCH was used to (1) de-replicate sequences and remove singletons to reduce the data size and calculating time; (2) remove chimeras contained in the sequences using GOLD (Reddy et al. 2014) as a reference database (http://drive5.com/uchime/uchime_download.html); (3) form clusters of 97 % identity sequences and represent each OTU by consensus sequences (representative sequences). The cluster file was converted into an OTU table using a customized script derived from QIIME (Kuczynski et al. 2012). These consensus sequences were used as representative sequences in each OTU and the taxonomic attribution of filtered sequences was assigned in QIIME with uclust using 97 % similarity score and 51 % consensus against the August 2013 Greengenes database. Fewer than 1 % of sequences across all samples received no taxonomic assignments and were removed from further analyses. The OTU table was subsampled to compensate for differences in total copies sequenced between samples. The OTU table was rarefied to equal abundance (6800 reads/sample) for further analyses. The sample from one of the OR plots from the COA block exhibited very low reads and was excluded from further analyses.

Chemical analysis

Soil chemical parameters, including pH, total nitrogen, NO_3-nitrogen, NH_4-nitrogen, total carbon, and total organic matter were quantified at the Iowa State Soil and Plant Analysis Laboratory (Ames, IA). Soil moisture was quantified using gravimetric soil moisture analysis.

Data analysis

Data were analyzed using multivariate correlational and ordination methods in the R statistical environment (R Core Team 2016), using R packages vegan (version 2.0–10) (Oksanen et al. 2015) and labdsv (version 1.6–1) (Roberts 2012).

The effect size and statistical significance of rotation and fertility treatments on each of the experimental analyses were determined with Permutational Multivariate Analysis of Variance (PerMANOVA, function adonis) (Anderson 2005). In addition, since fertilization and crop-rotation can affect microbial communities independently, treatment effects on microbial communities were visualized with partial ordination analyses that controlled for the effect of the other treatment category - i.e. the effect of rotation treatments was visualized while controlling for the effect of fertility treatments and vice versa. Differences in community structure (16S rRNA gene sequencing) were also visualized with partial correspondence analysis (pCA) (Legendre and Legendre 2012). Where necessary, the ranges of the ordination axes were manually set to exclude an extreme outlier. Correlation of soil chemical parameters with each of the ordinations was quantified with the function envfit and plotted on the pCA plots.

The taxonomic information obtained from 16S rRNA gene sequencing was used to quantify Shannon-Weaver diversity index (Hill et al. 2003) for each community (function diversity) and to estimate community richness (function estimateR) with Abundance-based coverage estimate (ACE) and the chao-1 estimate (Chao et al. 2005).

Indicator Value Analysis (function indval in R package labdsv) (Dufrêne and Legendre 1997) was employed to identify microbial taxa that were indicators of a specific group of samples, and hence, the treatments that these samples represented. Indicator value analysis assesses differences in relative abundance and frequency of presence of microbial OTUs between sets of samples. We focused this analysis on the high-abundance, or top 19.24 % (586 out of 3045) of the operational taxonomic units with assigned taxonomy, which constituted 81.53 % of all fragment reads. Each of these OTUs comprised a total of at least 50 sequence reads across the rarified (i.e. equal-abundance) sample pool. Indicator value analysis was iterated 10,000 times and OTUs that cleared the significance threshold of 0.05 were accepted as indicators of a treatment. OTUs were assessed for indicator status of one of the fertilizer treatments, controlling for the effects of crop-rotation treatments and vice versa. Indicator value analysis was primarily conducted for higher abundance OTUs for two reasons. First, because high-abundance OTUs are likely to be responsible for the bulk of the functional attributes of microbial communities, examining abundance and frequency differences among higher abundance OTUs is more relevant than those of rarer taxa for investigating functional differences between microbial communities. Second, indicator value analysis can overestimate the indicator status of lower abundance OTUs since their low total reads across all samples heightens the probability of large differences in abundance between sample classes. However, in order to examine the effects of agronomic treatments on rare taxa we also present indicator value analysis of all OTUs in the supplementary materials (Fig. S3, Tables S6 and S7).

Differences between substrate consumption profiles (BIOLOG assay) of the microbial communities were visualized using partial Principal Components Analysis (PCA) (Härdle and Simar 2012). Where necessary, the ranges of the ordination axes were manually set to exclude an extreme outlier. Average well-color development (AWCD) from the BIOLOG assay was calculated for all substrates as well as for the subsets of substrates containing nitrogen and/or phosphorus following conventional procedures (Garland and Mills 1991). This allowed us to test the availability of carbon, nitrogen, and phosphorus as drivers of community-level microbial physiology, i.e. consumption of all substrates, or substrates containing nitrogen, or substrates containing nitrogen and phosphorus.

Similarities between community diversity, community richness, and BIOLOG AWCD data from pairs of treatments were examined using Tukey's Honest Significant Difference test to correct for multiple comparisons. A significance threshold of $p \leq 0.05$ was used to determine significance for all analyses.

Results

Bacterial community composition

3045 bacterial OTUs with two or more 16S rRNA gene reads were assigned taxonomic classification, amounting to a total of over 156,000 reads. The broad metrics of total-community diversity and richness, with adjustments for multiple comparisons, of the communities were generally not significantly different ($p \leq 0.05$), with only the communities under OR treatment being richer (ACE) than those under IN among fertilizer treatments (Fig. 1). Shannon diversity and chao1 richness estimates were not significantly different between communities under either the fertilizer or rotation treatments. (Table S1).

However, microbial community structures inferred from 16S rRNA gene sequences were significantly dissimilar between the rotation treatments as well as fertility treatments, with PerMANOVA $R^2 = 0.27$, $p < 0.0001$ for comparisons among fertility treatments, and $R^2 = 0.16$, $p = 0.0007$ for comparisons among rotation treatments. The interaction of rotation and fertilizer treatments also significantly affected community composition ($R^2 = 0.17$, $p = 0.04$). Partial correspondence analysis clearly demonstrates that communities obtained from the same treatment are similar to each other and distinct from communities under other treatments (Fig. 2) reinforcing the finding based on community diversity metrics that long-term fertilizer and crop-rotation treatments are associated with distinct microbial communities.

Indicator value of OTUs by treatments

Among high abundance microbial OTUs, differences in relative abundance and frequency of microbial taxa were observed to a greater extent among the fertilizer treatments (a total of 179 OTUs, with 74 in UF, 63 in OR, and 42 in IN) than among crop-rotation treatments (a total of 75 OTUs, with 32 in COA, 30 in C, and 13 in CS) (Fig. 3). Between fertilizer treatments, more OTUs belonging to Actinobacteria and Planctomycetes were indicators of UF treatment than OR or IN treatments, while fewer Acidobacteria taxa were indicators of IN treatment compared to UF and OR treatments. Among crop-rotation treatments, more taxa belonging to Actinobacteria and Proteobacteria were indicators of communities under COA rotation, while a greater number of taxa belonging to Acidobacteria and Bacteroidetes were indicators of communities in the continuous corn (C) treatment.

Groups of taxonomically related and functionally notable OTUs that were found to be indicators of the treatments are described below and listed in Tables S4-

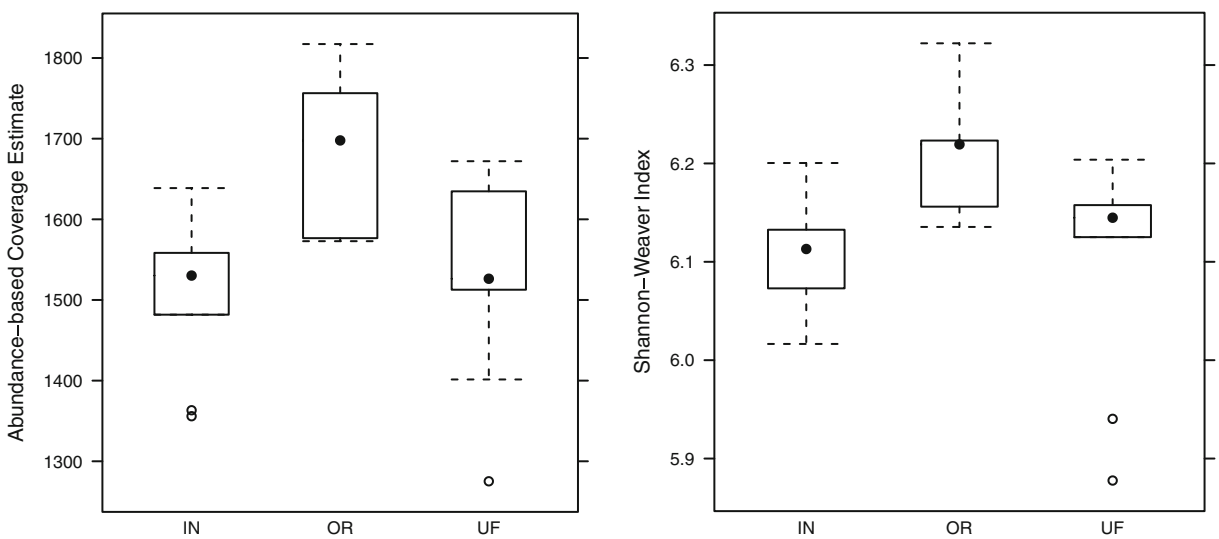

Fig. 1 Bacterial communities from OR plots exhibit significantly greater diversity (left panel) compared to communities from IN ($p = 0.028$) or UF ($p = 0.052$) plots as well as richness (right panel) compared to communities from IN ($p = 0.016$) or UF ($p = 0.048$) plots. Differences in community diversity or richness between the crop-rotation treatments were not statistically significant. Box-plots are plotted with the usual convention - with boxes demarcating the middle two quartiles and filled circles denoting the median datum. Whiskers extend to the farthest data-points still within 1.5 times inter-quartile range (IQR). Outliers beyond 1.5 IQR are shown as empty circles. (IN: inorganic fertilizer treatment, OR: manure fertilizer treatment, UF: unfertilized treatment)

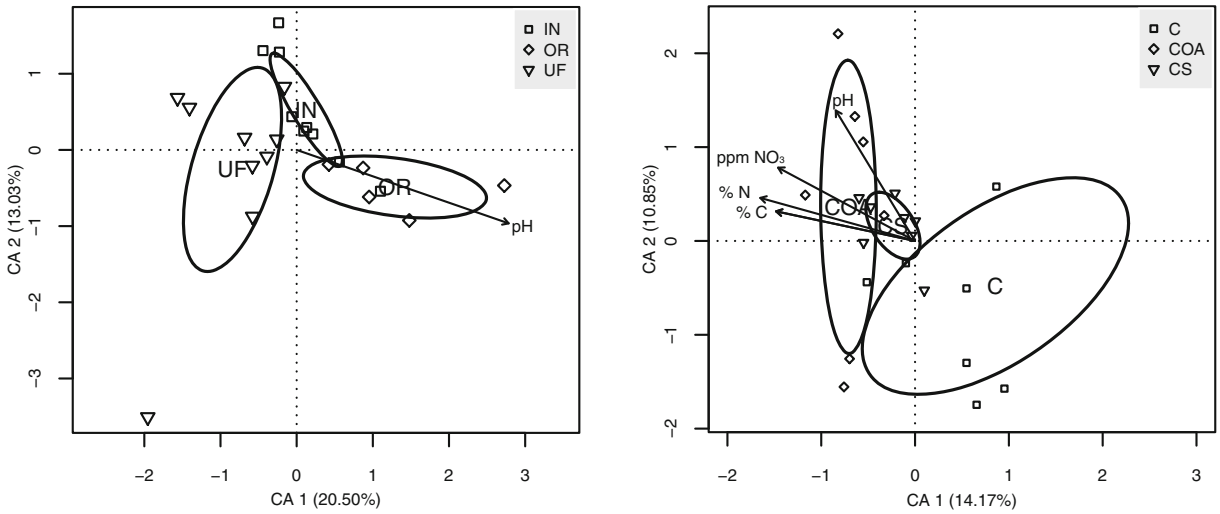

Fig. 2 Partial correspondence analysis ordinations of the microbial community diversity as revealed by 16S rRNA gene sequencing. Community structure was affected by both fertilizer (left panel, PerMANOVA $R^2 = 0.27$, $p < 0.0001$) controlling for the effect of rotation and by rotation (right panel, $R^2 = 0.16$, $p = 0.0007$) controlling for the effect of fertilizer treatments. Soil chemical parameters significantly correlated with each treatment are plotted on each ordination including pH, percent carbon (% C), total percent nitrogen (% N), and parts per million nitrates (μg/g NO_3 N). Greater values of soil chemical parameters are significantly correlated with longer rotation and organic amendment. Ellipses circumscribe the 99 % confidence limit around the centroid of each treatment group. In the left panel, CA 2 range is set manually to exclude an extreme outlier UF sample, while in the right panel, CA 1 range is set manually to exclude an extreme outlier C sample. (IN: inorganic fertilizer treatment, OR: manure fertilizer treatment, UF: unfertilized treatment, COA: corn-oats-alfalfa rotation, CS: corn-soybean rotation, C: continuous corn rotation)

S7. The OTUs known through sequencing and culture-based studies to be adapted to low-nutrient conditions (Eichorst et al. 2011; Kanokratana et al. 2010; Ward et al. 2009), including eleven OTUs belonging to the family Koribacteraceae, and three OTUs of the genus *Candidatus Koribacter*, were indicators of UF communities and were also the most highly abundant OTUs in these communities. In addition, five OTUs of the order Solibacterales, including two OTUs belonging to the genus *Candidatus Solibacter* were indicators of UF communities. All of these bacteria belonged to the Acidobacteria phylum.

The OTUs of the order Syntrophobacterales, which are obligate anaerobic chemolithotrophs (Kuever 2014),

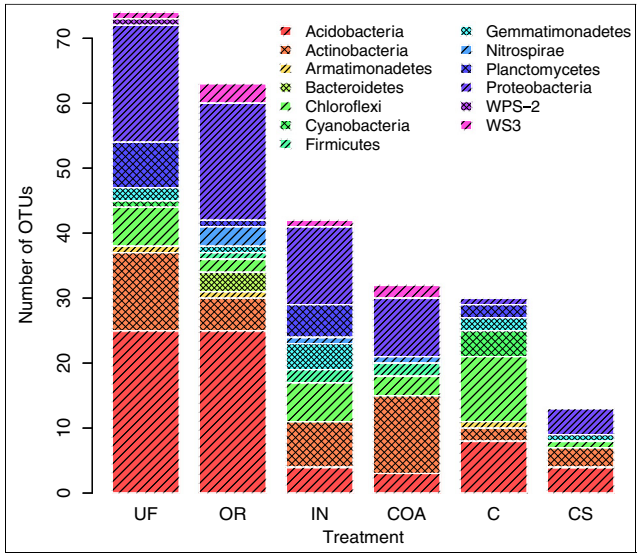

Fig. 3 Abundant (top 80 % of sequenced 16S rRNA gene fragment reads) bacterial OTUs that are indicators of each treatment, binned together by phylum. The greatest number of OTUs were significant indicators of UF communities, followed by OR communities and IN communities. (Figure S3 displays indicator taxa across all OTUs.) (IN: inorganic fertilizer treatment, OR: manure fertilizer treatment, UF: unfertilized treatment, COA: corn-oats-alfalfa rotation, CS: corn-soybean rotation, C: continuous corn rotation)

were indicators of OR communities (3 OTUs), UF communities (2 OTUs) and COA communities (1 OTU).

Five OTUs of the order Xanthomonadales, including one of the genus *Lysobacter*, were indicators of OR communities, IN communities (2 OTUs) and UF and CS communities (1 OTU each).

When low-abundance taxa – i.e. the bottom 80 % of OTUs (2459 out of 3045) that constituted only 20 % of sequencing reads – are included in the analysis, the general trends described above for the abundant OTUs persist - with the exception of far more OTUs being indicators of OR communities (310 OTUs for OR communities, compared to 162 of UF communities) (Fig. S3). Conducting indicator value analysis for all taxa highlights the unique rare taxa in the communities of OR plots. In all, 310 OTUs were found to be indicators of OR treatment, 162 of UF treatment, and 69 of IN treatment. Relatively fewer were found to be indicators of each of the crop-rotation treatments, with 98 of COA, 76 of C, and 35 of CS.

Community level physiological profiles

The ability of the microbial communities to utilize substrates, as assessed by BIOLOG substrate utilization assays, was affected by fertility treatments but not by crop rotation treatments. PerMANOVA analysis showed a stronger and statistically significant effect from fertility treatments on substrate utilization (R^2 = 0.20, p = 0.0002) while the effect of rotation treatments was not statistically significant (R^2 = 0.10, p = 0.13). The interaction of rotation and fertility treatments was also statistically non-significant (R^2 = 0.12, p = 0.76). The dissimilarity in physiological profiles between unfertilized treatments compared to the organic and inorganic fertility treatments is notable and apparent from the PCA ordination (Fig. 4).

Utilization of nitrogen-containing substrates varied among fertilizer treatments to a greater extent than utilization of the complete substrate panel (Fig. S4). Communities from unfertilized treatments and inorganic fertility treatments differed the most, though the effect was less than statistically significant (AWCD 1.02 vs. 0.94; p = 0.16). Similarly, utilization of nitrogen-containing substrates differed somewhat among communities from unfertilized and organic fertilized plots (AWCD 1.02 vs. 0.94; p = 0.23). Notably, communities from inorganic and organic fertilizer treatment plots showed statistically similar (p = 0.99) consumption of nitrogen-containing substrates (Table S2).

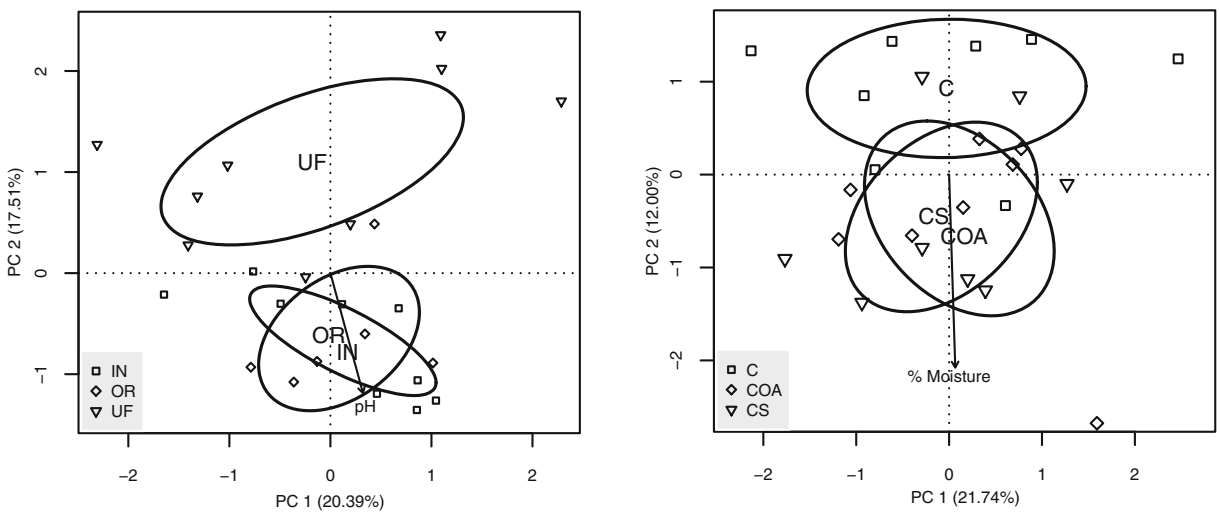

Fig. 4 Partial principle components analysis ordinations of BIOLOG profiles. Substrate consumption activity of communities was affected by fertilizer treatments (controlling for effect of rotation) (left panel, PerMANOVA R^2 = 0.20, p = 0.0002) with communities from UF plots substantially dissimilar to communities from OR and IN plots. Rotation treatments (controlling for the effect of fertilizer treatments) did not significantly affect substrate consumption profiles (right panel, R^2 = 0.10, p = 0.13). Soil chemical parameters significantly correlated with each treatment are plotted on each ordination including pH and percent soil moisture. Ellipses circumscribe the 99 % confidence limit around the centroid of each treatment group. (IN: inorganic fertilizer treatment, OR: manure fertilizer treatment, UF: unfertilized treatment, COA: corn-oats-alfalfa rotation, CS: corn-soybean rotation, C: continuous corn rotation)

Soil chemical parameters

The majority of soil fertility parameters were highest in manure-amended plots and/or under the longest rotation (Corn-Oats-Alfalfa). Total carbon, total nitrogen, organic matter content, and soil moisture were affected by both rotation and fertilizer treatments, with highest values under OR and COA treatments (Table S3). Soil pH and ammonia-N content were significantly affected by fertilizer treatments but not by rotation treatments. Soil pH was highest in plots under organic fertilizer treatment and lowest in unfertilized plots. Soil ammonium was highest in plots receiving inorganic fertilizer and lowest in plots receiving organic fertilizer. Soil nitrate content was significantly affected by rotation treatments, being highest in plots under the corn-oats-alfalfa rotation, and lowest in plots under continuous-corn rotation. This likely reflects differences in soil moisture content, which follow the same order and are a by-product structural enhancement associated with soil organic carbon. Soil nitrate content, which was extremely low (~3.7 μg/g), did not differ among fertilizer treatments, which is not unexpected given that sampling was carried out late in the growing season and the soil was quite dry (gravimetric moisture content ~22 %) so nitrification was suppressed (NH_4 values were ~4.3 μg/g).

Discussion

The results presented above demonstrate that the structure and function of microbial communities can be differentially affected by fertilizer and rotation treatments. Long-term fertilizer treatments are correlated with differences in both composition and community-level physiological profile of soil microbial communities. On the other hand, while long-term crop rotation treatments are correlated with soil microbial communities that were structurally distinct from each other, the community-level dissimilarities arising from rotation treatments did not translate to significant differences in their substrate-utilization profiles.

While the effects of long-term fertilizer treatments on bacterial community substrate-induced respiration and structure have been elucidated previously (Enwall et al. 2007; Widmer et al. 2006), the effect of crop-rotation treatments on community structure, but not on substrate utilization, is one of the notable results of the present study. This difference between the effect of crop-rotations on community structure and substrate utilization profiles suggests that functional redundancy plays a role in how management practices affect community structure and function in agricultural soil microbiomes. Microbial functions related to substrate utilization may be redundant across the taxa whose relative abundance is affected by crop-rotations. Conversely, between fertilizer treatments, microbial functional diversity related to substrate-utilization may play a greater role in shaping microbial community structure. Indicator value analysis of microbial OTUs, which assesses the differential relative abundance and frequency of presence of microbial taxa between communities, offers a plausible mechanism for this disconnect between microbial community structure and physiology. At the Morrow Plots, the long-term chronic nutrient deficit in the UF plots appears to have resulted in an enrichment of taxa adapted for low-nutrient conditions in microbial communities of these plots. These taxa are capable of acquiring and utilizing available substrates more effectively (Eichorst et al. 2011; Kanokratana et al. 2010; Ward et al. 2009), and are less abundant in communities from plots that received inorganic or manure fertilizer. The oligotrophic taxa may be particularly efficient utilizers of nitrogen-containing substrates, as reflected in the somewhat greater AWCD of N-containing substrates for UF communities compared to for IN and OR communities (Fig. S3, Table S2).

These differences in the abundance as well as physiology of key taxa between communities may have manifested in the observed differences in community-level physiological profiles of the microbial communities under the fertilizer treatments. Conversely, taxa adapted to nutrient-depletion exhibit smaller differences between crop-rotation treatments. The smaller contribution of oligotrophic taxa to the differences in community structure under crop-rotation treatments compared to communities under fertilizer treatments is congruent with the distinct substrate utilization profile of UF communities compared to IN and OR communities (Fig. 4, left panel) and the not-statistically distinct substrate utilization profiles of the C, CS, and COA communities. It is also worth noting that communities associated with the continuous-Corn (C) rotation have a substantial number of oligotrophic indicator taxa (Tables S5 and S7) and their substrate utilization profiles appears somewhat distinct from those of CS and COA communities (Fig. 4, right panel).

OTUs adapted for nutrient depleted conditions

One of the strongest patterns of indicator species observed was that OTUs adapted to nutrient-depleted conditions were indicators of communities from low-nutrient plots. Strains of *Candidatus Koribacter* and *Candidatus Solibacter* have been characterized with genomic sequencing, culture-based studies, and community sequencing, and are known to be adapted for low nutrient conditions (Eichorst et al. 2011; Kanokratana et al. 2010; Ward et al. 2009). The low-nutrient adaptations of these taxa is congruent with their greater relative abundance in UF communities which are subjected to low organic-matter, carbon, and nitrogen content of the UF soils. The greater relative abundance of these OTUs in UF communities may also have contributed to the distinct BIOLOG activity of the UF communities relative to IN and OR communities. These taxa happen to belong to the phylum Acidobacteria.

Koribacteraceae and Solibacterales OTUs were also indicators of communities under the continuous-Corn treatment suggesting that regardless of the fertilizer treatment, the high nutrient requirement of corn depletes soil nutrients, as is also evident from the soil chemistry measurements presented. The higher relative abundance of taxa adapted to nutrient-depleted environment in unfertilized and continuous-Corn treatments reinforces previous observations that these practices degrade soil quality (Havlin et al. 1990; Karlen et al. 2006; Ketcheson 1980).

Finally, Solibacterales OTUs were found to be indicators of IN communities, suggesting that while inorganic fertilizers provide sufficient plant nutrients to enable high agricultural yields, they may promote conditions that result in deterioration of the soil over the long-term. This is supported by the observed soil chemistry of IN soils – which had lower organic-matter contents and pH than OR plots but values were similar to those of UF plots. The indicator status of such taxa in the synthetically fertilized IN plots suggests that conventional fertilizer practices should be carefully managed to reduce deleterious effects on arable soils as previously suggested by others (Khan et al. 2007; Mulvaney et al. 2009).

In contrast, no Koribacteraceae or Solibacterales OTUs or other OTUs with known nutrient depletion adaptations were indicators of OR, CS, and COA communities, in agreement with the generally higher values of soil chemical parameters measured from these plots. The lack of competitive advantage to oligotrophic taxa in these plots is also expected from studies that have shown improved soil quality from manure fertilization and crop rotation treatments (Edmeades 2003; Karlen et al. 2006; Manna et al. 2005). The greater relative abundance of Koribacteraceae or Solibacterales OTUs in UF and IN soils compared to OF soils containing higher OM levels suggests that the relative proportion and abundance of these and other functionally similar taxa might be used as markers for soil deterioration and soil quality.

OTUs that are obligate anaerobes

Syntrophobacterales taxa, which are obligate anaerobic chemolithotrophs (Kuever 2014), were indicators of OR and COA communities - suggesting a greater prevalence of anaerobic microsites in the treatments. Anaerobic sites are known to occur more frequently in larger soil aggregates (Sexstone et al. 1985) which have been previously characterized at the Morrow Plots in the CS and COA plots (Darmody and Norton 1993). Larger soil aggregates with greater water-stability and erosion-resistance are also known to be associated with greater soil organic matter content (Tisdall and Oades 1982) and conservation agriculture practices such as no-till, manure application, and crop-rotation (Angers et al., 1993b; Beare et al. 1994; Mikha and Rice 2004; Wortmann and Shapiro 2008). Obligate anaerobes may therefore be useful biomarkers of beneficial soil structures in agricultural soils.

Disease suppressive OTUs

Xanthomonadales OTUs, including one of the genus *Lysobacter*, which are known to be are abundant in disease suppressive soils (Islam et al. 2005; Mendes et al. 2011; Postma et al. 2010; Rosenzweig et al. 2012) were found to be indicators of OR communities. While many Xanthomonadales taxa are known to be plant pathogens, phenology and yield data at the Morrow Plots provide no indication that the prevalent Xanthomonadales taxa in the Morrow Plots were interacting pathogenically with the plants. This suggests that manure application may increase the abundance of potential plant-beneficial bacteria in agricultural soils.

Indicator taxa including rare OTUs

While the discussion above has focused on relative enrichment among higher abundance OTUs, it is worth

noting that the OR communities contain a highly distinctive rare microbiome. This heightened diversity in the community more than four months after manure application indicates that the presence of these taxa is not a short-term effect of inputs, but reflects a long-term shift in the community structure. This shift may be driven by the greater diversity of niches provided by recalcitrant substrates introduced via the manure compared to synthetic fertilizers, as well as the influx of microbial taxa through the manure (Doan et al. 2014; Nair and Ngouajio 2012; Zhen et al. 2014). The distinctive and higher-diversity rare-microbiome in OR communities as well as their overall greater richness and diversity may confer benefits on the microbial communities in soils receiving manure-based amendment, including greater resistance to disturbances as well as functional stability and resilience (Allison and Martiny 2008; Girvan et al. 2005; Griffiths and Philippot 2013; Lehman et al. 2015; Shade et al. 2014).

The differential abundance of OTUs with known functional or habitat characteristics among contrasting soil environments and treatments thus appear to have the potential to serve as microbial biomarkers of soil health, nutrient cycling, and agricultural productivity in conjunction with differences in soil-chemistry as well as management history.

Effects of crop rotation on community structure

The differences in enriched OTUs between the higher-diversity COA rotation and the continuous corn rotation indicates that soil microbial community structure retains certain legacy effects from the long-term crop rotation treatment. The relatively fewer indicator OTUs between crop-rotations compared to fertilizer treatments may be in part due to the all-corn sampling year, which presented a uniform rhizodeposit and exudate environment for the microbial community. The lack of statistically significant differences in the diversity of the microbial community subjected to the different crop-rotation treatments at the Morrow Plots is in contrast to the finding by Lupwayi et al. (1998) of greater diversity in communities under diversified crop-rotations compared to continuous cropping of wheat. While the substantial differences between these two experiments complicate direct comparison of the two data sets, it may also be true that the effects of crop-rotation treatments on soil microbial communities reported here may not be easily generalizable, perhaps in part due to the legacy design of the Morrow Plots experiment. Additionally, comparing the crop-rotations at the Morrow Plots necessitates the integration of nutrient-exhausted and fully-fertilized treatments within each rotation. These fertilization treatments each have significantly different plant residue returns to the soil, which are as notable as differences in carbon flows resulting from the different cropping regimes. By contrast, while the experimental plots studied by Lupwayi et al. are better replicated, they were sampled just three to four years post-establishment and the fertility treatments did not include a no-fertilization treatment.

Conclusions

Long-term fertilizer and crop-rotation treatments have led to differences in structure and function of the soil microbial communities at the Morrow Plots, and are also reflected in differential abundance of taxa adapted to conditions generated by the specific treatments. The greater community level substrate utilization in unfertilized communities compared to those subjected to either inorganic or manure fertilizers suggests that chronic nutrient limitation is a more significant force than type of fertilizer in determining the substrate consumption activity of microbial communities. However, higher-diversity rare microbiome, and greater relative abundance of potential plant-beneficial bacteria in manure-fertilized soils suggests that the long-term influx of organic matter and microbial biodiversity through manure application may serve to enhance beneficial microbial diversity in agricultural soils in addition to improved soil fertility parameters. A more complete understanding of the long-term interactions and feedback between agronomic practices, agricultural soil, and structure and function of microbial communities derived from and building on these results is essential for enabling the design of optimal agronomic practices that improve agricultural sustainability. Finally, it is important to note that the differences in community structure were not broadly reflected in metrics such as Shannon diversity and community richness estimation, suggesting that these metrics are not always sufficient for understanding the differences between microbial communities. Conversely, the use of indicator taxa as biomarkers may have the potential to enable assessment of wide variety of soil characteristics, agricultural productivity potential, and environmental impacts of

agricultural soils with a single and increasing affordable modern analytical tool.

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